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Heat Shock Protein 90α–Dependent B-Cell-2–Associated Transcription Factor 1 Promotes Hepatocellular Carcinoma Proliferation by Regulating MYC Proto-Oncogene c-MYC mRNA Stability

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B-cell lymphoma 2 (Bcl-2)-associated transcription factor 1 (Bclaf1) is known to be involved in diverse biological processes, but, to date, there has been no evidence for any functional role of Bclaf1 in hepatocellular carcinoma (HCC) progression. Here, we demonstrate that Bclaf1 is frequently up-regulated in HCC and that Bclaf1 up-regulation is associated with Edmondson grade, lower overall survival rates, and poor prognosis. Overexpression of Bclaf1 in HCC cell lines HepG2 and Huh7 promoted proliferation considerably, whereas Bclaf1 knockdown had the opposite effect. Xenograft tumors grown from Bclaf1 knockdown Huh7 cells had smaller tumor volumes than tumors grown from control cells. Furthermore, our study describes MYC proto-oncogene (c-Myc) as a downstream target of Bclaf1, given that Bclaf1 regulates c-MYC expression posttranscriptionally by its RS domain. To exert this function, Bclaf1 must interact with the molecular chaperone, heat shock protein 90 alpha (Hsp90α). In HCC tissue samples, Hsp90α levels were also increased significantly and Hsp90α-Bclaf1 interaction was enhanced. Bclaf1 interacts with the C-terminal domain of Hsp90α, and this interaction is disrupted by the C-terminal domain inhibitor, novobiocin (NB), resulting in proteasome-dependent degradation of Bclaf1. Moreover, NB-induced disruption of Hsp90α-Bclaf1 interaction dampened the production of mature c-MYC mRNA and attenuated tumor cell growth *in vitro* and *in vivo*. *Conclusion:* Our findings suggest that Bclaf1 affects HCC progression by manipulating c-MYC mRNA stability and that the Hsp90α/Bclaf1/c-Myc axis might be a potential target for therapeutic intervention in HCC. (Hepatology 2019;69:1564-1581).

Hepatocellular carcinoma (HCC) is the fifth-most common tumor worldwide and the third-leading cause of cancer mortality, causing 746,000 cancer deaths annually.[1] Over the past two decades, incidence of HCC has tripled, whereas the 5-year survival rate has remained less than 12%.[2]

Abbreviations: 17-DMAG, 17-dimethylamino-ethylamino-17-demethoxygeldanamycin; ActD, actinomycin D; Bcl-2, B-cell lymphoma 2; Bclaf1, Bcl-2-associated transcription factor 1; CCK-8, Cell Counting Kit-8; CHX, cycloheximide; Co-IP, co-immunoprecipitation; CTD, C-terminal dimerization domain; c-MYC, MYC proto-oncogene; EV, empty vector; GO, gene ontology; HA, hemagglutinin; HCC, hepatocellular carcinoma; Hsp90, heat shock protein 90; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; MD, middle domain; NB, novobiocin; NBD, N-terminal nucleotide binding domain; shRNA, short hairpin RNA; SILAC, stable isotope labeling with amino acids in cell culture; siRNA, small interfering RNA; SRSF1, serine/arginine-rich splicing factor 1; WT, wild type.

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Most patients are diagnosed at an advanced stage, when curative treatments, including ablation, resection, and liver transplantation, are no longer available.[3] Thus, to discover new effective therapeutic treatments and to reduce HCC mortality, it will be necessary to better understand the molecular mechanisms of HCC.

HCC is largely caused by genomic catastrophes that result in the inactivation of tumor suppressor genes and/or activation of proto-oncogenes. One of the most commonly activated oncogenes associated with the pathogenesis of HCC is MYC proto-oncogene (c-MYC). Animal models revealed that overexpression of c-Myc can induce HCC,[4,5] whereas inhibition of c-Myc expression results in a loss of the carcinoma's neoplastic properties. In addition to genetic aberrations, regulators of c-MYC gene expression may control HCC proliferation and contribute to tumorigenesis. In this study, we found evidence for a link between the molecular chaperone, heat shock protein 90 alpha (Hsp90α), B-cell lymphoma 2 (Bcl-2)-associated transcription factor 1 (Bclaf1/Btf), c-MYC gene expression, and HCC progression.

The ATP-dependent 90-kDa heat shock proteins (Hsp90) are evolutionarily highly conserved molecular chaperones that assist maturation of an array of client proteins (https://www.picard.ch/downloads/Hsp90interactors.pdf), in particular overexpressed and mutationally activated oncogenic kinases and receptors. Hsp90s consist of an N-terminal nucleotide binding domain (NBD), a middle domain (MD), and a C-terminal dimerization domain (CTD), and all three domains were shown to interact with clients.[6] Natural products were discovered that specifically inhibit Hsp90, binding to the ATP binding pocket in the NBD (e.g., 17-dimethylamino-ethylamino-17-demethoxygeldanamycin [17-DMAG]) and to a second binding site in the CTD (e.g., novobiocin [NB]).[7] The specific molecular chaperone function makes Hsp90 a powerful tool to search for new, critical oncoproteins that might be lowly abundant, but vital for tumor progression. With a combination of co-immunoprecipitation (Co-IP) and stable isotope labeling with amino acids in cell culture (SILAC), we studied the interaction of Bclaf1 and Hsp90 in an HCC cell line and further explore the biological function of Bclaf1 in HCC.

Bclaf1 was originally identified as an interaction partner of the adenoviral, antiapoptotic Bcl-2-related protein, E1B, and suggested to promote apoptosis.[8] Meanwhile, there is ample evidence that Bclaf1 has a much broader function and serves in such diverse processes as lung development, T-cell activation, proliferation of Kaposi's sarcoma-associated herpesvirus, and human cytomegalovirus infection, processes not generally associated with Bcl-2 family members.[9,10] Bclaf1 contains an arginine-serine–rich (RS) domain typically found in proteins playing a role in mRNA processing or pre-mRNA splicing.[11] *In vitro* studies also implicated Bclaf1 in pathways linking transcriptional events to cell death, DNA damage, or autophagy.[9,12,13] Although most studies suggest that Bclaf1 acts as a tumor suppressor, more-recent studies provided evidence for an oncogenic function of Bclaf1 in bladder and colon cancer.[14,15] Thus, the function

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of Bclaf1 in carcinogenesis is controversial and still needs to be further explored in different tumor tissues.

Here, we show that Bclaf1 mRNA and protein levels are up-regulated in human HCC as compared to normal liver and that BCLAF1 mRNA levels correlate with c-MYC mRNA levels. Bclaf1 depletion in HCC cells inhibits proliferation, consistent with down-regulation of c-Myc mRNA and protein levels. The molecular chaperone, Hsp90α, is required for this function of Bclaf1. Hsp90α inhibition destabilizes Bclaf1 and reduces levels of mature c-MYC mRNA. Importantly, a stable reduction of Bclaf1 in Huh7 cells impairs tumor growth in a murine xenograft model. Therefore, our study suggests Bclaf1 as a potential target in HCC treatment.

Materials and Methods

HUMAN TISSUES

HCC samples were collected with the informed consent of patients, and the ethics committee of Taizhou Hospital (Zhejiang, China) approved all experiments. The human tissues are detailed in the Supporting Information.

OTHER METHODS

The following methods are described in the Supporting Information, including tissue microarrays and immunohistochemistry (IHC), cell culture and reagents, cell proliferation and colony-survival assay, western blotting and immunoprecipitation, reverse-transcription and semiquantity PCR, transfection of small interfering RNA (siRNA) and plasmids, apoptosis assay, soft agar colony formation assay, SILAC labeling, RNA sequencing (RNA-Seq), mouse xenotransplantation experiments, and immunofluorescent and immunostaining.

STATISTICAL ANALYSIS

All experiments were repeated three times. Data are described as mean ± SD. Survival curves were calculated using Kaplan-Meier and log-rank tests. Effects of variables on survival were determined by uni- and multivariate Cox proportional hazards modeling. The Mann-Whitney U test was used to analyze the relationship between Bclaf1 expression and clinicopathological characteristics. Statistical P values were analyzed by a two-tailed Student t test. SPSS software (version 16.0; SPSS, Inc., Chicago, IL) was used for statistical analysis, and significance was set at $P < 0.05$.

Results

HSP90α INTERACTS WITH BCLAF1 IN HCC

Hsp90α was found to be associated with many human cancers, including HCC, and pharmacological inhibition of Hsp90 in HCC significantly reduced viability in cancer cell lines, but much less so in nonmalignant cells.[16-18] At the outset of our study, we analyzed the link between Hsp90α protein levels in HCC tissues and survival rates (Fig. 1A-C) and found Hsp90α to be a prognostic marker for HCC progression ($P < 0.05$), consistent with a recent publication.[19] To better understand the molecular mechanism for why HCC seems to be addicted to Hsp90α, we wanted to find clients of Hsp90α important for HCC growth. Because a large number of Hsp90 clients are already known,[18] we wanted to search for clients somewhat off the beaten path. Reasoning that because of hypoxic conditions and nutrients depletion tumor cells are constantly under stress conditions in their microenvironment, we searched for proteins that are more prominently bound to Hsp90α under stress conditions (30 minutes and 4 hours heat shock at 42°C) than under control conditions, using SILAC proteomics (Supporting Fig. S1A,B; Supporting Table S1).

Out of the significantly enriched Hsp90α interactors, Bclaf1 caught our attention because its function was controversial, being described as a tumor suppressor in some studies and an oncoprotein in others. To verify Bclaf1 as an Hsp90α interactor, we performed a series of experiments. Co-IP from HCC cell lines indicated that endogenous Hsp90α immunoprecipitated with endogenous Bclaf1 (Fig. 1D,E). Immunofluorescence staining, in combination with confocal microscopy, revealed intense staining of Bclaf1 in nuclei. Hsp90α was found throughout the cytoplasm and nucleus, and colocalized with Bclaf1 within nuclei (Supporting Fig. S1C). To evaluate the situation in liver tissue, Co-IP assays were performed using tissue extracts derived from three pairs of freshly collected HCC and adjacent normal tissues (Fig. 1F). Results showed that binding of Hsp90α with Bclaf1 was robustly enhanced in HCC.

REGULATION OF BCLAF1 PROTEIN STABILITY BY HSP90α

Given that our data demonstrated an association between Hsp90α and Bclaf1, we wondered whether

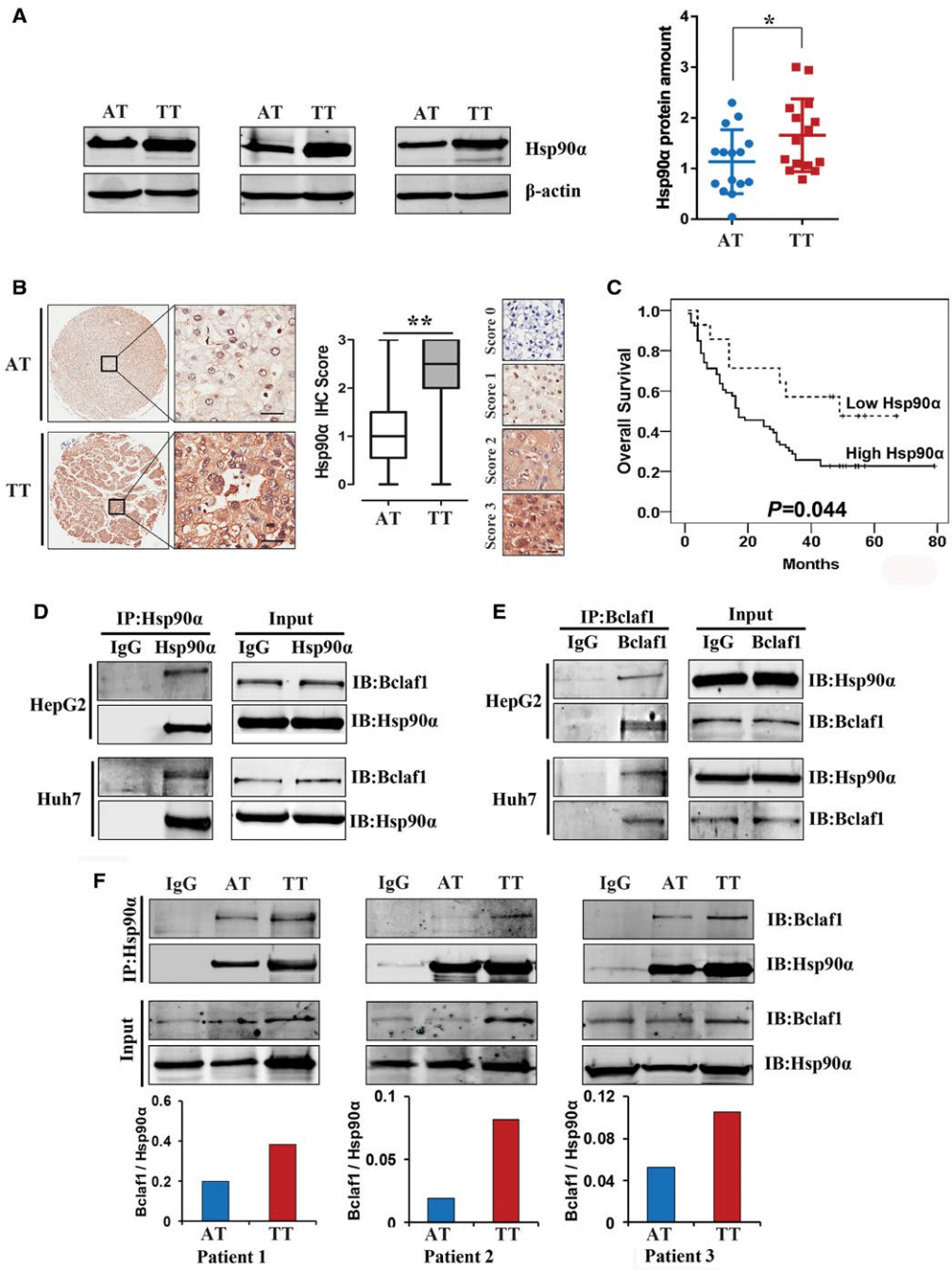

FIG. 1. Hsp90α interacts with Bclaf1 in liver cancer cell lines. (A) Protein levels of Hsp90α were determined by immunoblotting in liver tissue form patients with HCC (tumor tissue; TT) compared to adjacent nontumor liver tissue (adjacent tissue; AT) in a total of 15 HCC cases. Values were expressed as mean ± SD; $^{*}P < 0.05$. (B) Representative images of IHC staining of Hsp90α in tissue specimens; scale is 50 μm (left panel). Quantification of Hsp90α levels according to IHC scores in adjacent and tumor tissue, respectively; $^{**}P < 0.01$ (middle panel). Scores indicate Hsp90α levels in representative tumor tissue samples; scale is 50 μm (right panel). (C) Kaplan-Meier survival analysis of HCC patients based on Hsp90α levels. Low Hsp90α, IHC score 0-1; high Hsp90α, IHC score 2-3. (D,E) Identification of the Hsp90α-Bclaf1 interaction in HCC cells by Co-IP. Endogenous Bclaf1 was pulled down with anti-Hsp90α, compared with IgG and vice versa, and detected by immunoblotting. (F) Immunoprecipitation of Hsp90α was performed with lysates of adjacent and tumor tissue samples. Precipitated Hsp90α and Bclaf1 were analyzssed by western blotting. Abbreviations: IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation.

Bclaf1 stability requires Hsp90α. Down-regulation of Hsp90α by siRNA transfection reduced Bclaf1 levels (Fig. 2A, left panel, and Supporting Fig. S1D). However, molecular silencing of Hsp90β, which shares 86% sequence identity and 93% similarity with Hsp90α, had no impact on expression of Bclaf1 (Fig. 2A, right panel). These data indicated that Bclaf1 protein levels depend specifically on Hsp90α. Next, we generated an Hsp90α$^{-/-}$ HepG2 cell line by CRISPR-Cas9 (knockout; KO). Strikingly, Bclaf1 protein levels were significantly reduced in KO cells as compared to the parental Hsp90α-WT (wild-type) HepG2 cells, but assessment of BCLAF1 mRNA levels revealed no difference between WT and Hsp90α KO cells (Fig. 2B). Together, these data further confirmed Bclaf1 as a putative Hsp90α client in HCC cells and suggest that Bclaf1 is regulated by Hsp90α at the protein level.

Because all three domains of Hsp90α (NBD, MD, and CTD) have been implicated in client binding, we wanted to elucidate which of the Hsp90α domain(s) interacts with Bclaf1 and contributes to its stability. Therefore, we transiently expressed genes encoding NBD-MD, MD, and MD-CTD constructs as hemagglutinin (HA) fusions (Fig. 2C, left panel). The MD-CTD construct contained a C-terminal coiled-coil domain to guarantee stable homodimerization with itself and prevent heterodimerization with the endogenous Hsp90α full-length protein.[20] Co-IP results of HEK293T transfected with the various HA-Hsp90α domain constructs revealed that Bclaf1 associated with the MD-CTD construct of Hsp90α (Fig. 2C, right panel). Then, HCC cells treated with the CTD binding inhibitor, NB, revealed a clear decrease of Bclaf1 in a dose- and time-dependent manner (Fig. 2D), indicating that a functional C-terminal domain of Hsp90α was vital for the stability of Bclaf1. In contrast, inhibitors like 17-DMAG and STA-9090, known to bind to the NBD in a nucleotide-competitive manner, did not cause any decrease in Bclaf1 protein levels but reduced levels of an established Hsp90 client: the protein kinase, Akt (protein kinase B; Supporting Fig. S2A,B).[21] Moreover, exogenous expression of the ATPase-deficient Hsp90α-E47A variant[22] decreased levels of Akt by 61%, but had little effect on Bclaf1 levels (Supporting Fig. S2C).

To determine whether reduced levels of Bclaf1 in NB-treated HCC were caused by changes at the level of gene transcription, RT-PCR was performed 24 hours posttreatment. As expected, the abundance of BCLAF1 mRNA transcript was not significantly changed (Supporting Fig. S2D), indicating that NB acted on Bclaf1 through a transcription-independent mechanism. Consistently, examination of the abundance of Bclaf1 in control or NB-treated Huh7 cells after incubation with cycloheximide (CHX) showed that Hsp90α inhibition accelerated Bclaf1 disappearance (Supporting Fig. S2E), suggesting that Hsp90α prevents Bclaf1 from degradation. We next characterized the mechanism of Bclaf1 degradation after treatment with NB. Pretreatment of Huh7 cells with 26S proteasome inhibitor MG-132, followed by incubation with NB, rescued Bclaf1 levels to the baseline level of the normal control (Fig. 2E). Consistent with its degradation by the proteasomal pathway, Bclaf1 was more extensively ubiquitinated in Huh7 cells after treatment with NB in combination with MG-132 (Fig. 2F). Taken together, these findings indicated that inhibition of Hsp90 by the CTD inhibitor, NB, promoted Bclaf1 ubiquitination and degradation by the proteasome.

HIGH LEVEL OF BCLAF1 PARALLELS WITH POOR PROGNOSIS IN HCC PATIENTS

To investigate the function of Bclaf1 in HCC progression, we first explored whether Bclaf1 levels are increased in clinical HCC tissue samples. Western blotting analysis demonstrated that Bclaf1 protein levels in 15 cases of human HCC tissue samples were much higher than those in adjacent nontumor liver tissues ($P < 0.05$; Fig. 3A). Microarray-based IHC studies, which involved 85 paraffin-embedded HCC specimens, also confirmed that levels of Bclaf1 were increased in HCC as compared to matched adjacent nontumor tissue and suggested that Bclaf1 was predominantly accumulated in nuclei of HCC tissues ($P < 0.01$; Fig. 3B). These data strongly implicate that Bclaf1 is up-regulated in HCC.

To determine whether Bclaf1 levels are related to HCC progression, we analyzed the association between Bclaf1 and clinical pathological status in HCC patients (Supporting Table S2). Statistical analysis represented a strong correlation between Bclaf1 levels and Edmondson grade and cirrhosis ($P < 0.05$), respectively (Supporting Table S3). Furthermore, the IHC score of both Hsp90α and Bclaf1 increased stepwise with pathological grade ($P < 0.05$; Fig. 3D,E), and a Kaplan-Meier and log-rank test analysis revealed a positive correlation between high Bclaf1 levels and significantly reduced overall survival rates ($P < 0.05$; Fig. 3C). Finally, multivariate analysis confirmed that a

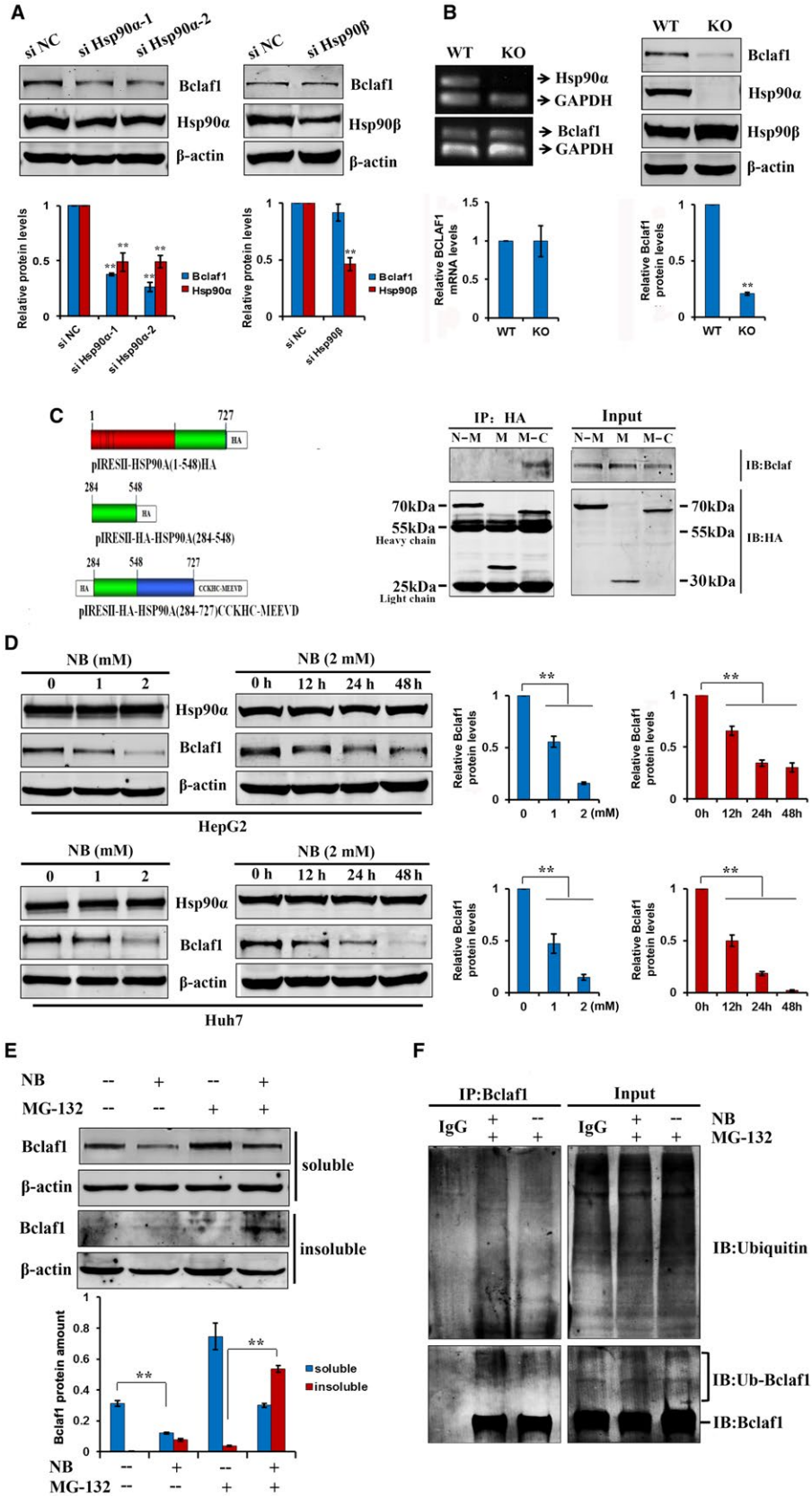
A
si NC
si Hsp90α-1
si Hsp90α-2
si NC
si Hsp90β
Bclaf1
Hsp90α
β-actin
Hsp90β
Relative protein levels
B
WT
KO
Hsp90α
GAPDH
Bclaf1
Hsp90β
Relative BCLAF1 mRNA levels
Relative Bclaf1 protein levels
C
pIRESII-HSP90A(1-548)HA
pIRESII-HA-HSP90A(284-548)
pIRESII-HA-HSP90A(284-727)CCKHC-MEEVD
IP: HA
Input
N-M
M
M-C
IB:Bclaf
IB:HA
70kDa
55kDa
Heavy chain
25kDa
Light chain
30kDa
D
NB (mM)
NB (2 mM)
0 h
12 h
24 h
48 h
HepG2
Huh7
E
NB
MG-132
soluble
insoluble
Bclaf1 protein amount
F
IP:Bclaf1
Input
IgG
IB:Ubiquitin
IB:Ub-Bclaf1
IB:Bclaf1

FIG. 2. Stability of Bclaf1 depends on Hsp90α. (A) Protein levels of Hsp90α, Hsp90β, and Bclaf1 were determined in HuH7 cell transfection with a nontargeting control siRNA (siNC) or siRNAs targeting HSP90AA1 (Hsp90α) and HSP90AB1 (Hsp90β) mRNA, respectively. Values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$, versus si NC. (B) Reverse-transcription and semiquantitative PCR were performed to detect the amount of HSP90AA1 and BCLAF1 transcripts presented in HepG2 WT and KO (HSP90AA1$^{-/-}$) cells (left panel). Total protein of HepG2 WT and KO cells were subjected to western blotting. Membranes were probed with Hsp90α-, Hsp90β-, and Bclaf1-specific antisera (right panel). Values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$, versus WT. (C) Hsp90α domain constructs used for the interaction study (left panel). HEK293T cells were transiently transfected with plasmids encoding HA-Hsp90α domains, and immunoprecipitation was performed with a mouse monoclonal anti-HA. Membranes were probed with anti-HA and anti-Bclaf1 antisera (right panel). (D) HepG2 (upper panels) and Huh7 (lower panels) cells were incubated with increasing amounts of NB, as indicated for 24 hours and with 2 mM of NB for the indicated time points. Western blotting analysis was conducted with antisera against Hsp90α, Bclaf1, and β-actin. Values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$. (E) Huh7 cells were treated with 1 μM of MG-132 and 2 mM of NB, as indicated, and cell lysates were subjected to western blotting with anti-Bclaf1 and anti-β-actin antisera. Values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$. (F) Huh7 cells were treated as described in (F), and Bclaf1 was immunoprecipitated with a specific antiserum. Membranes were probed with Ubiquitin and Bclaf1-specific antisera. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IB, immunoblotting; IgG, immunoglobulin G; IRES, internal ribosome entry site.

high Bclaf1 level in HCC tissues was an independent predictor for reduced overall survival of HCC patients ($P < 0.01$; Table 1). These data revealed that up-regulation of Bclaf1 is significantly associated with poor prognosis of HCC.

BCLAF1 PROMOTES CELL PROLIFERATION IN HCC CELLS

The above data suggest that Bclaf1 may play an important role in HCC progression. Accordingly, we evaluated functions of Bclaf1 in HCC cells using both *in vitro* and *in vivo* assays.

To elucidate the effects of Bclaf1 on cell proliferation *in vitro*, we first investigated endogenous Bclaf1 levels of different liver cells and modulated their Bclaf1 levels using siRNA and a plasmid expressing the full-length Bclaf1-encoding gene (FL). We found significantly increased Bclaf1 levels in HCC cells (HepG2, Huh7) compared to normal liver cells (LO2). Bclaf1 depletion or overexpression (FL) had no significant effects on cell growth and colony formation in LO2 cells (Supporting Figs. S3 and). However, a considerable inhibitory effect on proliferation was observed in the Bclaf1-siRNA group in HCC cells (Fig. 4B,C), and this was associated with induction of apoptosis as indicated by enhanced poly (ADP-ribose) polymerase (PARP) cleavage and flow cytometry analysis (Supporting Fig. S5). Conversely, Bclaf1 overexpression dramatically enhanced the proliferative capacity of HCC cells (Fig. 4B,C) and decreased levels of cleaved PARP as well as the percentage of apoptotic cells (Supporting Fig. S5). To gain more insights into the importance of Bclaf1 for proliferation, we generated an Huh7 cell line stably expressing a short hairpin RNA (shRNA) for BCLAF1 knockdown. Visible single colonies expressing green fluorescence were observed within 15 and 30 days; these colonies grew in a time-dependent manner. However, compared to the control group, the size of the colonies observed in the BCLAF1-shRNA–transfected group was much smaller (Supporting Fig. S6).

To confirm this finding, we further measured the role of Bclaf1 for tumor growth *in vivo*. Huh7 cells stably transfected of shBCLAF1 or negative control cells were injected into flanks of nude mice to establish a xenograft tumor model. Consistent with the cell model, knockdown of Bclaf1 considerably decreased tumor size and tumor growth rate (Fig. 4E), demonstrating that Bclaf1 also promoted tumor growth *in vivo*. To determine the underlying mechanism responsible for reduced tumor growth, we examined the impact of Bclaf1 knockdown on cell proliferation *in vivo*. In accord with our *in vitro* observations, proliferation indicator Ki67 staining was much lower in xenografts derived from shBCLAF1 cells than in control xenografts (Fig. 4F). Taken together, these *in vitro* and *in vivo* gain- and loss-of-functional studies demonstrate that Bclaf1 plays an important role in promoting HCC growth.

BCLAF1 REGULATES C-MYC RNA PROCESSING

To explore the underlying mechanisms of the protumorigenic effect of Bclaf1 in HCC, transcriptomes of BCLAF1-shRNA knockdown Huh7 cells and Huh7 cells transfected with shNC were analyzed by RNA-Seq. Intriguingly, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

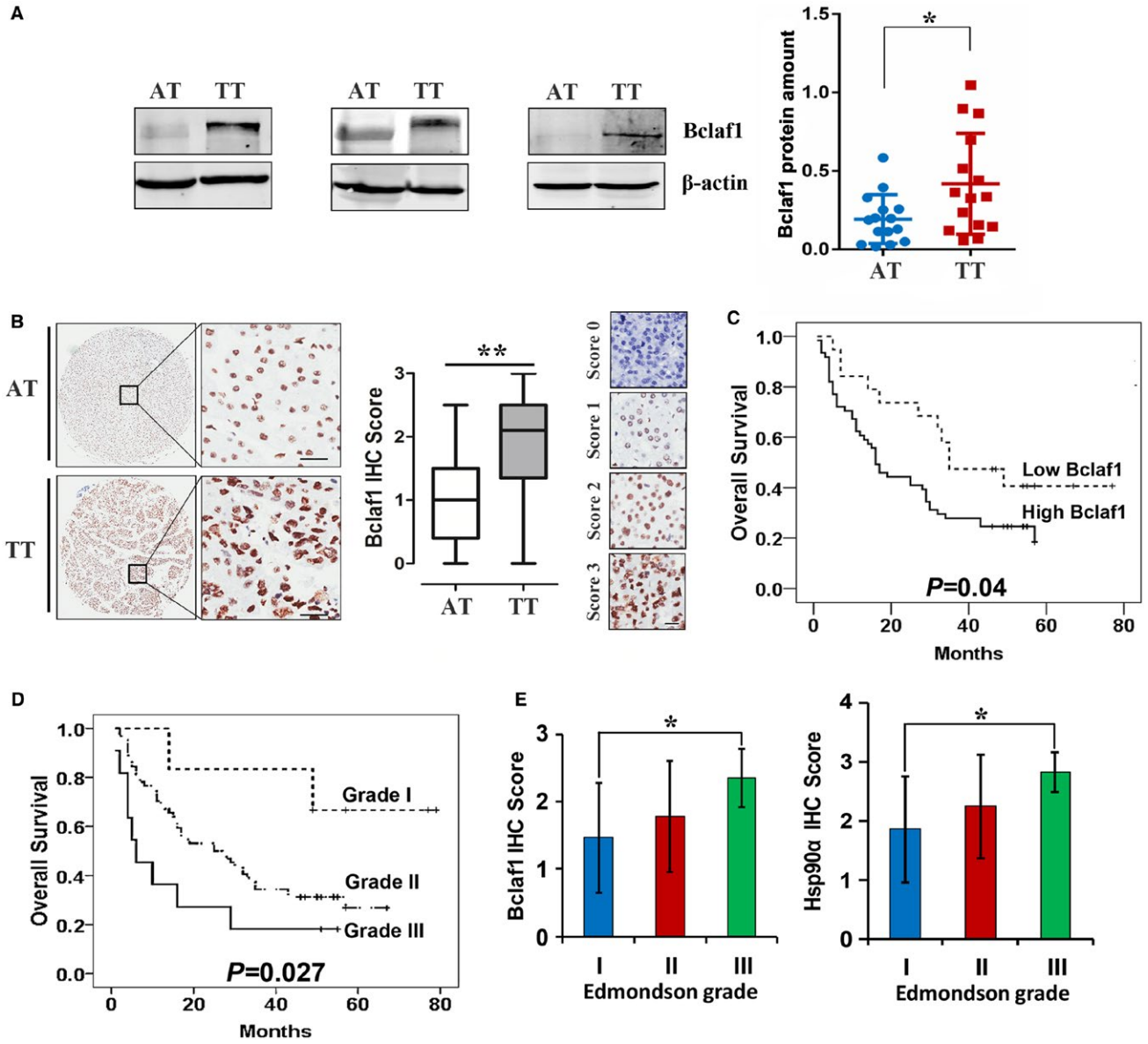

FIG. 3. Increased levels of Bclaf1 correlate with poor prognosis of HCC. (A) Protein levels of Bclaf1 were determined in liver tissue specimens form patients with HCC (tumor tissue; TT) compared to adjacent nontumor liver tissue (adjacent tissue; AT) in a total of 15 HCC cases by western blotting. Values are expressed as mean ± SD; $^{*}P < 0.05$. (B) Representative images of IHC staining of Bclaf1 in tissue specimens; scale is 50 μm (left panel). Quantification of Bclaf1 levels according to IHC scores in adjacent and tumor tissue, respectively; $^{**}P < 0.01$ (middle panel). Scores indicate Bclaf1 levels in representative tumor tissue samples; scale is 50 μm (right panel). (C) Kaplan-Meier survival analysis of HCC patients based on Bclaf1 levels. Low Bclaf1, IHC score 0-1; high Bclaf1, IHC score 2-3. (D) Kaplan-Meier survival analysis of HCC patients based on Edmondson tumor grade. (E) Mean ± SD of Bclaf1 scores (left panel) and Hsp90α scores (right panel) within each Edmondson grade. Edmondson grade I (n = 6), Edmondson grade II (n = 64), and Edmondson grade III (n = 11). $^{*}P < 0.05$.

analysis indicated that the genes down-regulated by knocking down Bclaf1 were associated with cellular process, cell proliferation, cell growth, and death (Supporting Fig. S7), supporting the role of Bclaf1 in HCC proliferation. Among the down-regulated genes, a significant decrease was observed in expression of oncogenes, such as c-MYC, RAS, and MET (Supporting Fig. S8A), which are often up-regulated in HCC.[23,24] c-Myc represented the most interesting target because of its established role as regulator of proliferation and apoptosis in

TABLE 1. Uni- and Multivariate Analysis of HCC Patients by Cox Regression Analysis

	Univariate Analysis			Multivariate Analysis		
Variables	Hazard Ratio	95% CI	*P* Value	Hazard Ratio	95% CI	*P* Value
Age	0.808	0.458-1.428	0.464			
Sex	0.849	0.311-2.315	0.749			
Edmondson grade	0.487	0.143-1.661	0.251			
Cirrhosis	0.151	0.360-1.172	0.649			
AJCC stage	2.282	0.781-6.666	0.131			
Tumor size	2.615	1.342-5.097	0.005*	2.102	1.204-3.670	0.009*
Bclaf1 levels	2.250	1.047-4.835	0.038*	2.092	1.076-4.067	0.030*

**P* values <0.05 were considered statistically significant.
Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence interval.

diverse human cancers, including HCC.[25] Thus, we focused on the role of Bclaf1 in modulating c-MYC expression.

First, we validated the expression of c-Myc after Bclaf1 depletion in Huh7 cells. mRNA and protein of c-Myc were dramatically down-regulated upon Bclaf1 knockdown by shRNA, indicating that c-Myc is a downstream target of Bclaf1 (Fig. 5A). We next studied whether BCLAF1-directed enhancement of c-MYC expression was also detectable in human HCC. We found that BCLAF1 and c-MYC mRNA were both induced significantly in HCC compared to adjacent normal tissues (Supporting Fig. S8B). In addition, consistent with a BCLAF1-dependent control of c-MYC expression *in vitro*, we observed a modest, but significant, positive correlation between mRNA expression of BCLAF1 and c-MYC in 100 HCC patients in a publicly available database (GSE62043; Fig. 5B).[26]

The Bclaf1 protein contains a basic zipper homologous region and an Myb DNA-binding domain, suggesting a potential function as a DNA-binding protein. Consistently, Bclaf1 was shown to bind to DNA and function as a regulator of transcription.[8] We therefore tried to confirm whether its effect on c-MYC was primarily through transcriptional regulation. Using primer pairs specifically designed to amplify the prespliced and postspliced mRNA of c-MYC, we found that depletion of Bclaf1 had no effect on levels of prespliced c-MYC mRNA, but caused a significant decrease in postspliced c-MYC mRNA in both HepG2 and Huh7 cells, resulting in a change in protein levels of c-Myc (Fig. 5C,D). These results indicate that the Bclaf1-induced effect on c-MYC expression is primarily posttranscriptional. To substantiate this hypothesis, we examined the effect of inhibiting transcription using actinomycin D (ActD) with or without Bclaf1 knockdown. Analysis of the change of the spliced c-MYC mRNA revealed a significant decrease in mature c-MYC mRNA when combined with Bclaf1 knockdown, suggesting that Bclaf1 protects mature c-MYC mRNA from degradation (Fig. 5E). Taken together, these data suggest Bclaf1 to be a critical post-slicing regulator of c-MYC mRNA stability.

BCLAF1 CONTROLS C-MYC EXPRESSION THROUGH ITS RS DOMAIN

Sequence analysis suggests that Bclaf1 is an RS-domain–containing protein, which is typically linked with pre-mRNA biogenesis and mRNA processing. Therefore, we hypothesized that Bclaf1 may regulate c-MYC expression at the posttranscriptional/postsplicing level by its RS domain. To test this hypothesis, we prepared vectors expressing FLAG fusion constructs of different domains of Bclaf1, encompassing the sequences for full-length Bclaf1 (FL), for a Bclaf1 truncation variant containing only the RS domain (RS), for an RS domain deleted variant (ΔRS), for an Myb domain deleted variant (ΔMyb), and the empty vector (EV) as control (Fig. 6A,B). RT-PCR results of HepG2 and Huh7 cells transfected with the different vectors indicated that RS and ΔMyb led to the same effects as those observed after overexpression of full-length Bclaf1, that is, an obvious up-regulation in mature c-MYC mRNA (Fig. 6C). This trend was also observed in c-Myc protein levels (Fig. 6D). Consistent with our previous results (Fig. 5C,D), no effect of Bclaf1 domains was observed on unspliced c-MYC mRNA. These results suggest that the RS-rich domain of Bclaf1 is required for protecting c-MYC mRNA from degradation.

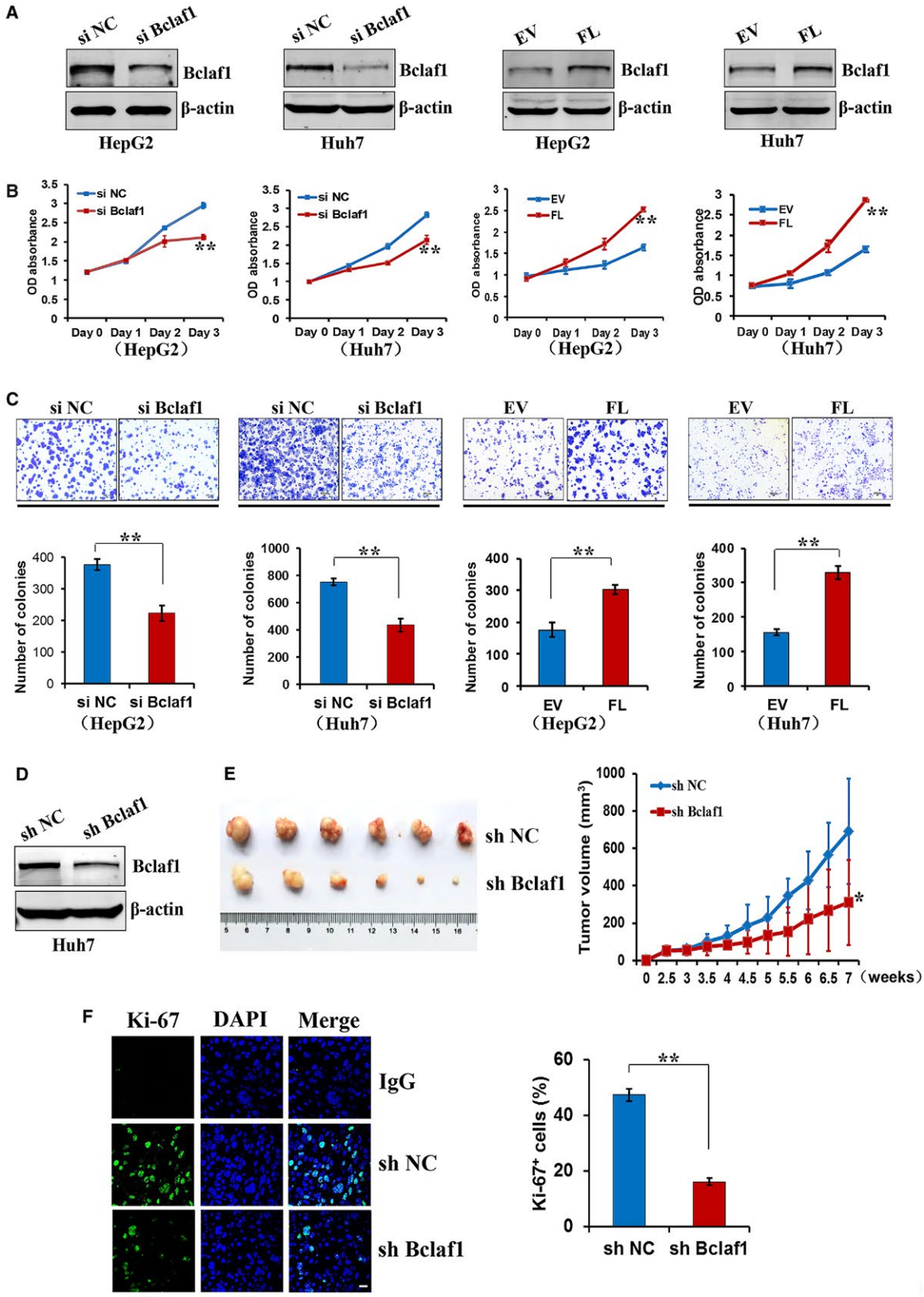
A
si NC
si Bclaf1
Bclaf1
β-actin
HepG2
Huh7
EV
FL
B
OD absorbance
Day 0 Day 1 Day 2 Day 3
(HepG2)
(Huh7)
**
C
Number of colonies
D
sh NC
sh Bclaf1
E
Tumor volume (mm3)
(weeks)
*
F
Ki-67
DAPI
Merge
IgG
Ki-67+ cells (%)

FIG. 4. Bclaf1 promotes HCC cell proliferation and tumor growth. (A) Confirmation of Bclaf1 knockdown and overexpression (FL) in HCC cell lines. (B) Cell proliferation of HCC cells transfected, as described, was determined by the CCK-8 assay. Values were expressed as mean ± SD; n = 3; $^{**}P < 0.01$. (C) HCC cells were treated, as described, and colonies were assessed by crystal violet staining. Scale is 100 μm. Values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$. (D) Huh7 cells were stably transfected with plasmids expressing a control small hairpin RNA (shNC) and a BCLAF1-specific shRNA (shBCLAF1), and knockdown efficiency was confirmed by western blotting. (E) Photographs of tumors that were surgically extracted 7 weeks after transplanting shBCLAF1 and shNC cells into nude mice (left panel). Stable Huh7/shBclaf1 and shNC cells were injected into nude mice, and tumor volumes were measured every 3-4 days. Each point represents mean volume ± SD of tumor volumes; n = 6; $^{*}P < 0.05$ (right panel). (F) Tumors were formalin-fixed, paraffin-embedded, and sliced for Ki-67 staining. Ki-67 (Green) stains the nucleus region of proliferating cells, and DAPI (Blue) stains for nuclei of all cells. Scale is 30 μm. Values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; IgG, immunoglobulin G; OD, optical density.

Given that the RS domain of RNA processing factors is known to mediate protein-protein interactions,[27] elucidation of proteins associated with Bclaf1 will provide further understand into its mechanistic pathway in regulating c-MYC expression. First, we tested whether Bclaf1 interacts with serine/arginine-rich splicing factor 1 (SRSF1), which has been reported to colocalize with Bclaf1 in the nucleus to engage in posttranscriptional events.[28,29] Endogenous Bclaf1 co-immunoprecipitated with endogenous SRSF1, indicating that Bclaf1 binds to SRSF1 (Fig. 6E). Given that, according to our data, Bclaf1 regulates c-MYC expression, we wondered whether SRSF1 is involved in this regulatory process. siRNA-mediated depletion of SRSF1 led to a decrease in c-Myc protein levels, and simultaneous depletion of SRSF1 and Bclaf1 had no additive effects, indicating that these two proteins function in the same pathway, most likely in a cooperating complex (Fig. 6F).

NB DISRUPTED HSP90α-BCLAF1 INTERACTION AND SUPPRESSES BCLAF1-MEDIATED C-MYC EXPRESSION

Because we have described above (Fig. 2) that Bclaf1 stability depends on Hsp90α, we next assessed the impact of Hsp90α on c-Myc expression. When Hsp90α expression was reduced by siRNA-mediated knockdown, c-Myc levels were decreased simultaneously with reduced levels of Bclaf1, an effect that was reversed by overproducing full-length Bclaf1 (Fig. 7A,B). These data strongly suggest that Hsp90α is required for Bclaf1-mediated c-Myc expression.

It has been suggested that NB forces Hsp90 into a conformation that releases client proteins.[7] As expected, our results revealed that NB disrupted the interaction of Hsp90α with Bclaf1 (Fig. 7C). Next, we determined the impact of NB on c-Myc expression. We found that, similar to Hsp90α knockdown, NB decreased the production of mature c-MYC mRNA and c-Myc protein (Fig. 7D,E), in parallel with a significantly reduced proliferation as measured by Cell Counting Kit-8 (CCK-8; Fig. 7F). Overexpression of the FL form of Bclaf1was able to partially rescue c-Myc mRNA and protein levels, resulting in restored cell growth of Huh7 cells (Fig. 7D-F). A similar change was obtained in HepG2 cells (Supporting Fig. S9). These findings are in line with our previous finding that Bclaf1-mediated c-MYC expression plays a major role in tumor cell growth, and suggest that these properties can be counteracted by Hsp90 inhibition-mediated Bclaf1 depletion.

Finally, we tested whether NB affects tumor growth by injecting 1×10^7 Huh7 cells into flanks of nude mice. In line with the findings *in vitro*, administration of NB resulted in substantially decreased tumor growth, suppressed Hsp90α-Bclaf1 complex formation, and reduced steady-state levels of Bclaf1 and c-MYC mRNA (Fig. 8). These results suggest a strong link between NB-induced loss of cell proliferation, and NB-caused disruption of the Hsp90α-Bclaf1 complex, leading to a significantly reduced c-MYC expression.

Discussion

In this study, we gained four insights into the molecular basis of HCC progression.[1] Bclaf1 regulates c-MYC mRNA stability by its RS domain. Thus, expression of the driver oncogene, c-MYC, depends on Bclaf1.[2] Bclaf1 overproduction favors HCC progression and is a prognostic marker for survival rates. Thus, in the case of HCC, Bclaf1 acts as an oncoprotein.[3] Bclaf1 stability and function depend on interaction with Hsp90α, but not Hsp90β. Thus, Bclaf1 is a *bona fide* Hsp90α client and provides one rational for the addiction of HCC to Hsp90α.[4] Interaction of Bclaf1

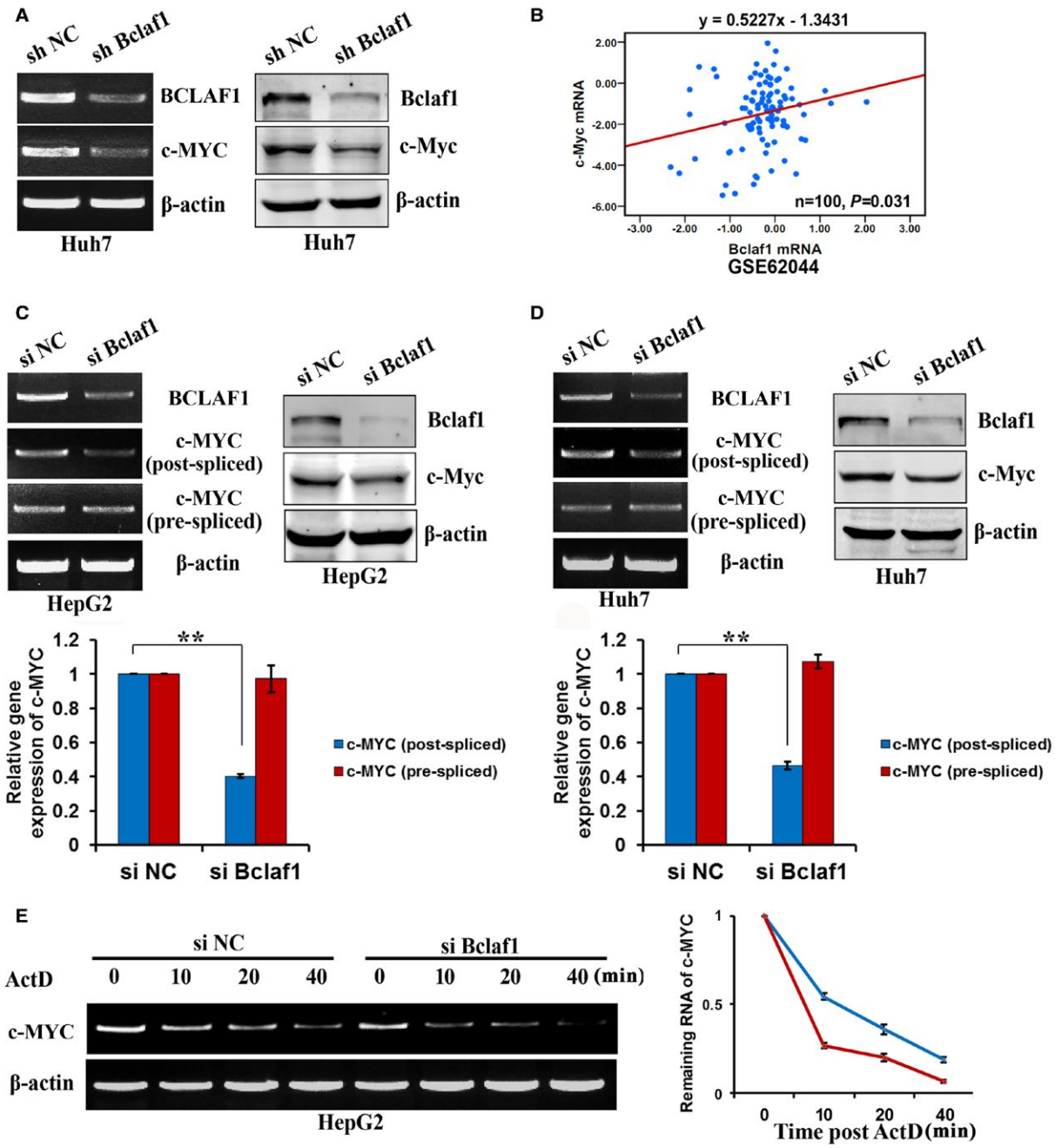

FIG. 5. Bclaf1 is required for the co-transcriptional stability of c-Myc mRNA. (A) Assessment of mRNA levels (left panel) by RT-PCR. Assessment of protein levels (right panel) was performed by immunoblotting analysis using antisera directed against Bclaf1 and c-Myc. (B) Correlation between c-MYC mRNA and BCLAF1 mRNA expression in a clinical HCC data set (GSE62043). Scale, $\log_2$ ratio representing gene expression. Relationships between these two variables were determined by Pearson's correlation coefficients. The correlation was analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL). (C,D) HepG2 and Huh7 cells were treated with a nontargeting control siRNA (siNC) or siRNAs targeting BCLAF1. Relative levels of unspliced and spliced RNAs were then assessed by RT-PCR (left panel). Values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$ (lower panel). The unspliced transcript is detected using primers within exon 2 and intron 2, and the spliced transcript is detected using primers situated within exon 1 and exon 2. Western blotting analysis (right panel) was performed using antisera directed against Bclaf1 and c-Myc. (E) HepG2 cells were transfected with control or Bclaf1-targeting siRNA, then treated with 10 μg/mL of ActD before RNA extraction at the time points indicated. Transcript levels were then analyzed by RT-PCR. Values are expressed as mean ± SD; n = 3.

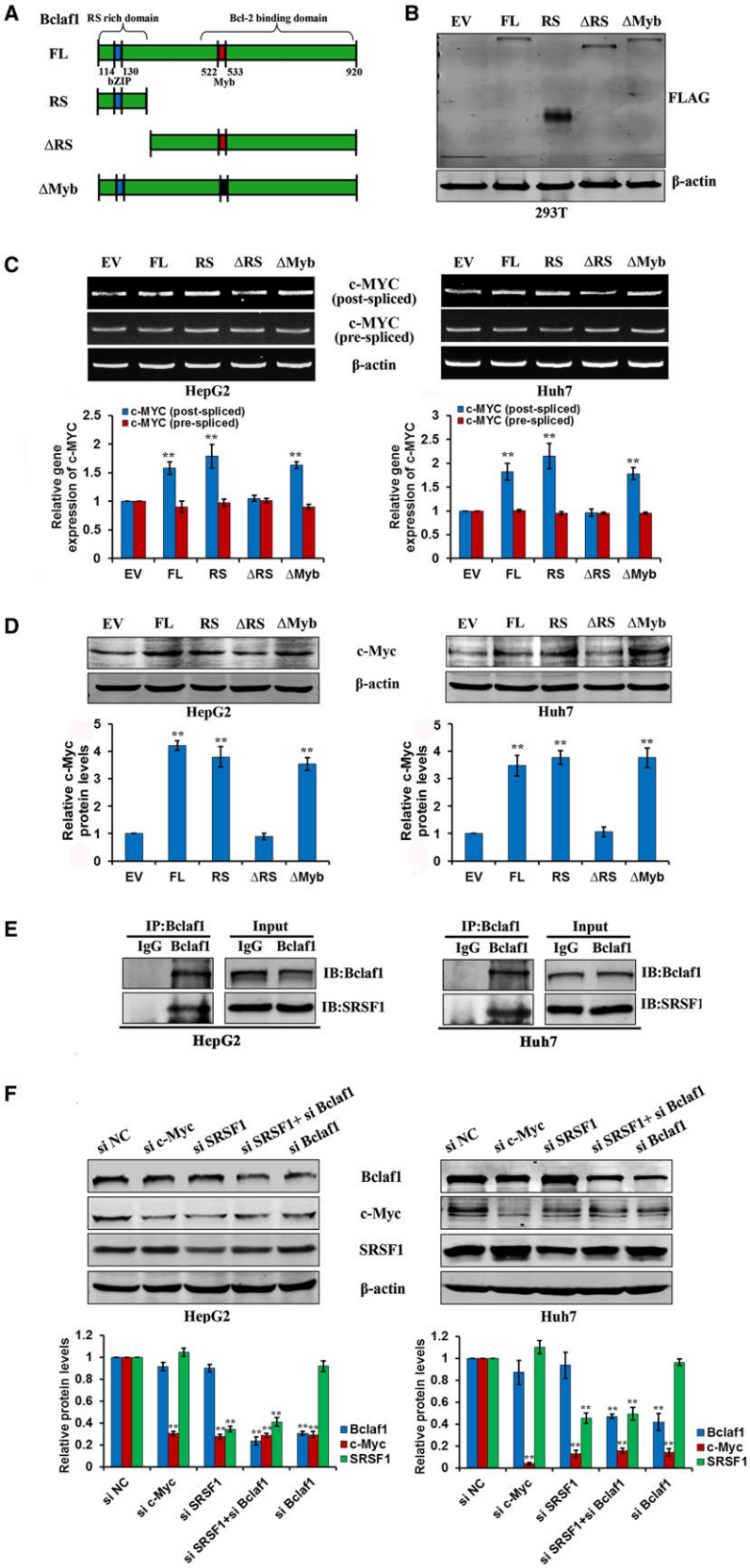
A
Bclaf1
RS rich domain
Bcl-2 binding domain
FL
114 130
bZIP
522 533
Myb
920
RS
ΔRS
ΔMyb
B
EV FL RS ΔRS ΔMyb
FLAG
β-actin
293T
C
EV FL RS ΔRS ΔMyb
c-MYC (post-spliced)
c-MYC (pre-spliced)
β-actin
HepG2
Huh7
c-MYC (post-spliced)
c-MYC (pre-spliced)
Relative gene expression of c-MYC
D
c-Myc
β-actin
Relative c-Myc protein levels
E
IP:Bclaf1
Input
IgG Bclaf1
IB:Bclaf1
IB:SRSF1
F
si NC
si c-Myc
si SRSF1
si SRSF1+ si Bclaf1
si Bclaf1
Bclaf1
c-Myc
SRSF1
β-actin
Relative protein levels

FIG. 6. Bclaf1 interacts with SRSF1 to modulate c-Myc mRNA stability. (A) Schematic structure of Bclaf1 domains. (B) 293T cells were transfected with expression plasmids encoding Bclaf1 domains, and transfection efficiency was confirmed by western blotting. (C) HepG2 (left panel) and Huh7 (right panel) cells were transfected with the different Bclaf1-encoding plasmids, and transcript levels were analyzed by RT-PCR. Values are expressed as mean ± SD (lower panel); n = 3; $^{**}P < 0.01$. (D) HepG2 (left panel) and Huh7 (right panel) cells were transfected with the different Bclaf1-encoding plasmids, and protein levels were assayed by immunoblotting analysis using c-Myc- and β-actin–specific antisera. Values are expressed as mean ± SD (lower panels); n = 3; $^{**}P < 0.01$, versus EV. (E) Endogenous SRSF1 was co-precipitated with anti-Bclaf1 antisera, but not with unspecific IgG, and detected by immunoblotting. (F) HepG2 (left panel) and Huh7 (right panel) cells were transfected with siRNAs, as indicated, and cell extracts were subjected to western blotting analysis using Bclaf1-, c-Myc–, and β-actin–specific antisera. Values are expressed as mean ± SD (lower panels); n = 3; $^{**}P < 0.01$, versus si NC. Abbreviations: IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation.

with Hsp90α depends on Hsp90's C-terminal domain and is prevented by an Hsp90 inhibitor that binds to the C-terminal domain, NB, but not a nucleotide competitive inhibitor, providing one explanation for the antitumor growth effect of NB.

Bclaf1 is a nuclear protein that was initially identified in yeast and was proposed to interact with members of the Bcl-2 family of proteins and promote apoptosis.[8] Subsequently, it was described as being induced in radiation-exposed cells and as a γ-H2AX-interacting tumor suppressor.[30] More recently, Bclaf1 has attracted attention because of its oncogenic features in some human tumors[14,15] and diverse functions have been assigned to it. For example, in bladder cancer cells, Bclaf1 activates autophagy and promotes cancer progression; in colon cancer cells, it functions as an important modulator for cytoskeletal architecture and cell adhesion. These differences suggest that the specific mode of action of Bclaf1 in tumor progression may depend on the cellular context. Our study revealed that Bclaf1 regulates c-MYC mRNA expression to promote HCC proliferation. It is well known that the c-Myc oncoprotein is a key player associated with malignant transformation by regulating cell-cycle progression, cell growth, apoptosis, differentiation, and other tumor traits.[31] In our study, we found that down-regulation of c-Myc phenocopies the cellular response to Bclaf1 depletion (Supporting Fig. S10), spurring the speculation that Bclaf1 supports HCC progression through regulation of c-Myc.

Regulation of mRNA metabolism (processing, export, stabilization, etc.) contributes more relevantly than previously suspected to the genesis of a wide range of diseases, including cancer.[32] It is a remarkable fact that regulation of mRNA stability is an important mechanism for modulating the level of gene expression during cell growth and differentiation. c-Myc abundance is controlled at the level of protein and mRNA stability. Deregulation of the c-Myc-encoding gene, resulting in an inappropriate increase of gene product, can contribute to cancer formation. One of the ways in which expression of the c-MYC gene can be deregulated is by stabilization of the labile c-MYC mRNA.[33,34] For instance, p62/SQSTM1 mediates stabilization of c-MYC mRNA to enhance breast cancer stem-cell–like properties.[35] In this study, inhibiting transcription, using ActD, followed by analysis of c-MYC mRNA degradation, indicated a posttranscriptional role for Bclaf1. In addition, given that unspliced c-MYC mRNA does not accumulate upon Bclaf1 depletion, splicing of the primary RNA transcript itself is unlikely to be the stage at which Bclaf1 is required. In agreement with this hypothesis, a correlation between BCLAF1 and c-MYC mRNA abundance was observed in clinical HCC tissues, further supporting the role of Bclaf1 in manipulating c-MYC mRNA stability.

Bclaf1 lacks RNA recognition motifs that are typically found in splicing factors.[28] However, Bclaf1 contains an RS domain and phosphorylated RS domains of splicing factors were shown to mediate protein-protein interactions.[27] In keeping with this, mass-spectrometry studies found more than 30 phosphorylation sites within Bclaf1.[36] We found that c-MYC mRNA stability depends on the RS domain of Bclaf1, that the splicing factor SRSF1 can be co-precipitated with Bclaf1, that SRSF1 knock-down has the same effect on c-MYC mRNA as Bclaf1 knockdown, and that there are no additive effects on c-MYC mRNA when both SRSF1 and Bclaf1 are down-regulated. Our data are therefore consistent with Bclaf1 stabilizing c-MYC mRNA by interaction with SRSF1. Interestingly, the RNA-Binding Protein DataBase (RBPDB; https://rbpdb.ccbr.utoronto.ca/) provides evidence for direct binding of SRSF1 to c-MYC mRNA, further supporting our results. Although we found that Bclaf1 increases the amount of spliced c-MYC mRNA and c-Myc protein, the mechanism through which this occurs requires further investigation. It may be that SRSF1, like the splicing

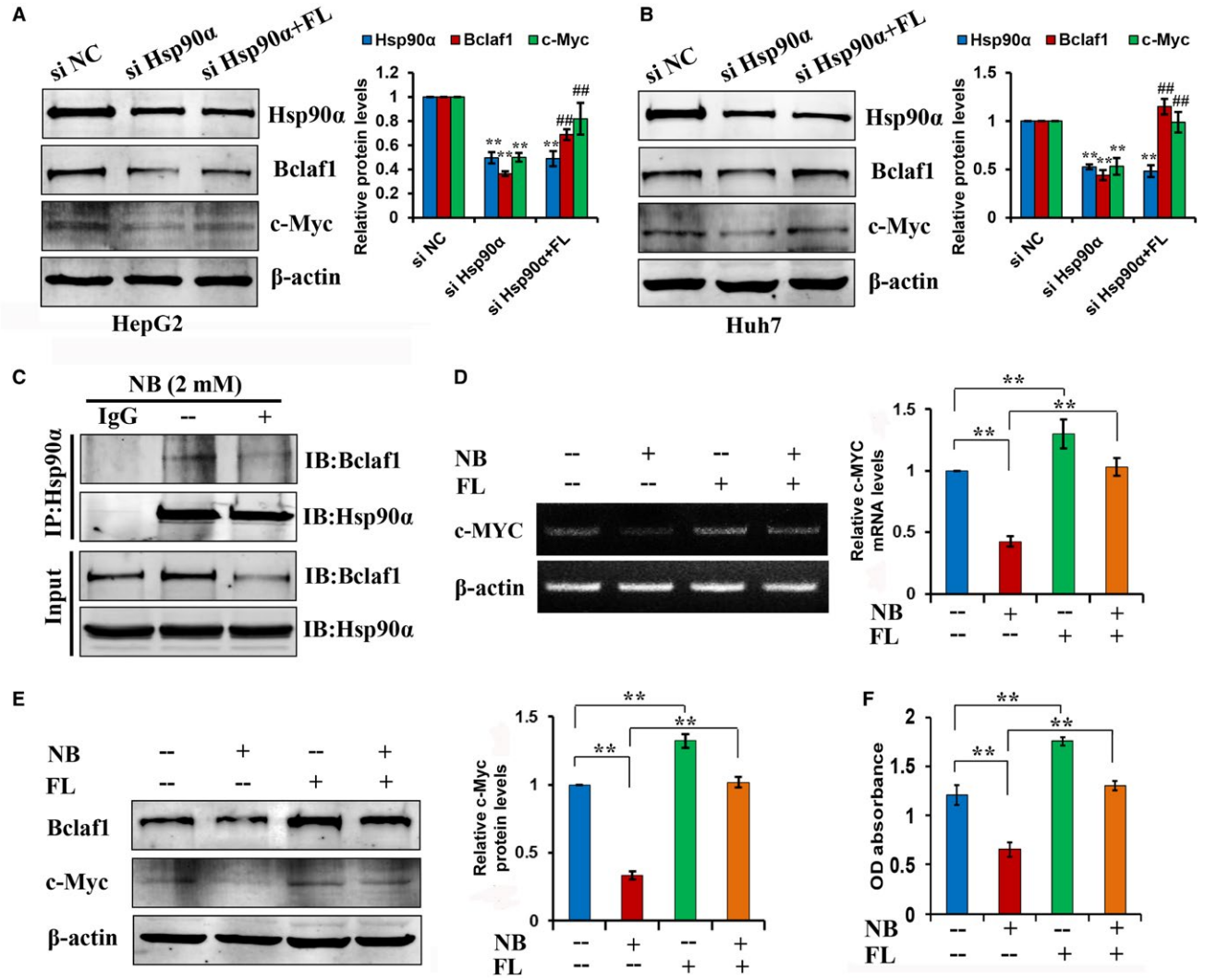

FIG. 7. Ectopic Bclaf1 restores c-Myc expression after Hsp90α knockdown and inhibition. (A,B) HCC cells were transfected with control siRNA (siNC) or siRNA targeting Hsp90AA1 mRNA without or with a plasmid expressing Bclaf1 (FL). Hsp90α, Bclaf1, and c-Myc levels were detected by western blotting. Values are expressed as mean ± SD (right panel); n = 3; $^{**}P < 0.01$, versus si NC; $^{\#\#}P < 0.01$, versus si Hsp90α. (C) Huh7 cells were treated with 2 mM of NB for 24 hours, and immunoprecipitation analysis was performed with an Hsp90α-specific antiserum. Membranes were probed with Hsp90α- and Bclaf1-specific antisera. (D) Huh7 cells, transfected with a Bclaf1-expressing vector (FL) or EV, were incubated with or without NB, and the amounts of c-Myc transcripts were detected by reverse-transcription and semiquantitative PCR. Values are expressed as mean ± SD (right panel); n = 3; $^{**}P < 0.01$. (E) Cell extracts of Huh7 cells, transiently transfected with a vector overexpressing Bclaf1 (FL) or an EV and cultured in the presence or absence of NB, were subjected to western blotting analysis with Bclaf1-, c-Myc–, and β-actin–specific antisera. Values are expressed as mean ± SD (right panel); n = 3; $^{**}P < 0.01$. (F) Proliferation of HCC cells, transiently transfected with a vector overexpression B Bclaf1 (FL) or an EV and cultured in the presence or absence of NB, was determined by the CCK-8 assay. Values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$. Abbreviations: IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation; OD, optical density.

factor, ASF/SF2, regulates other aspects of RNA metabolism, such as nuclear export, mRNA stability, and association with translating ribosomes, and may also stimulate the translation of mRNAs.[37,38] We found that Bclaf1 localizes to nuclear speckles, a compartment to which many splicing factors (SR proteins) predominantly localize and which are believed to be sites for storage of splicing factors, RNA modification, and RNA metabolism.[39] SRSF1 and other SR proteins shuttle between the nucleus and cytoplasm. Our data do not rule out that Bclaf1 also shuttles in and out of the nucleus and associates with other

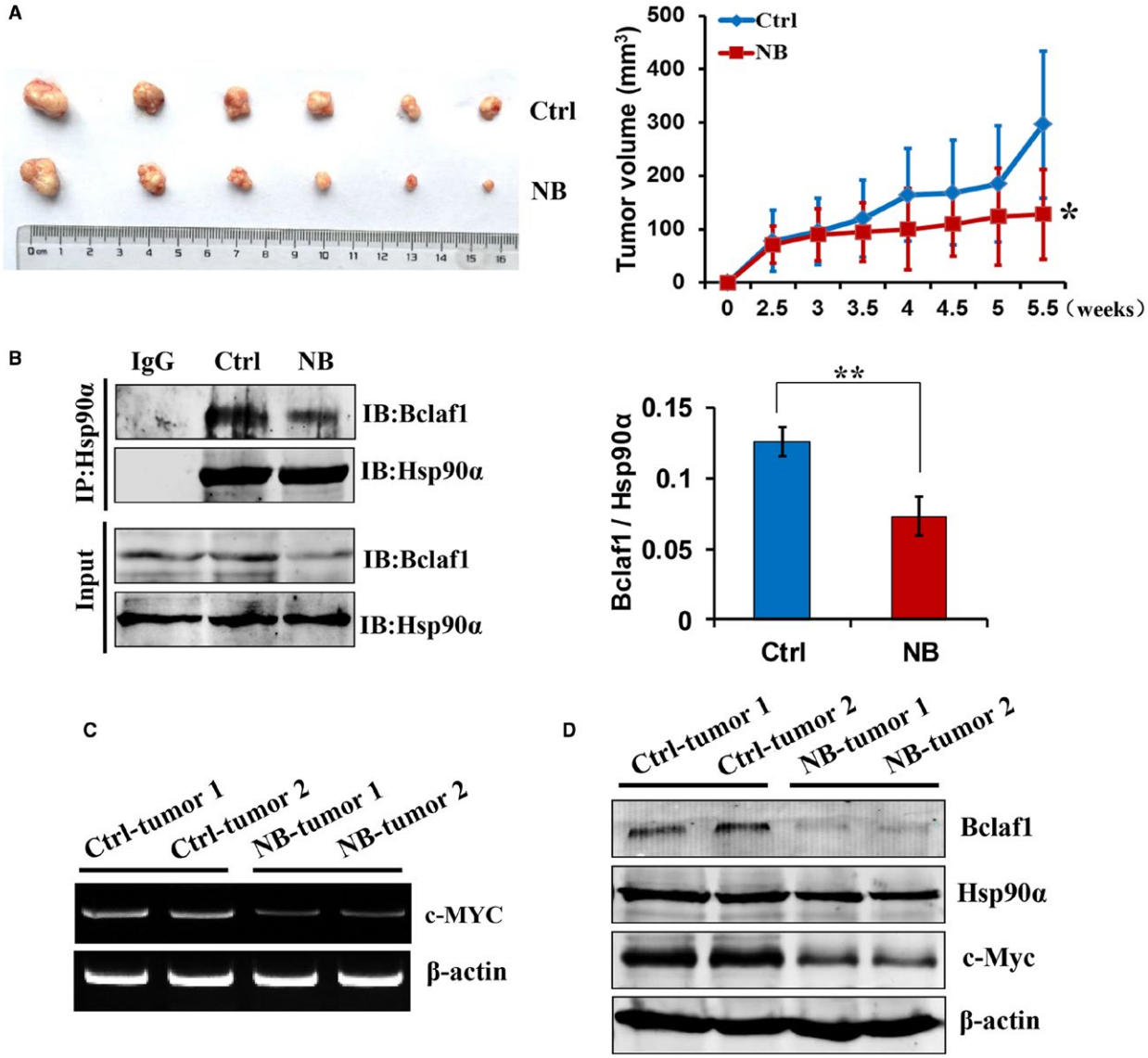

FIG. 8. NB suppresses Bclaf1-mediated c-MYC expression and tumor growth. (A) Huh7 cells (1×10^7) were injected subcutaneously into nude mice. Two and a half weeks later, mice in the NB group were treated 150 mg of NB/kg three times a week. Photographs of tumors were taken after 5.5 weeks (left panel). Tumor volumes were measured every 3-4 days. Each point represents the mean volume ± SD of tumor volumes; n = 6; $^*P < 0.05$ (right panel). (B) Immunoprecipitation of Hsp90α was performed with lysates of the tumors. Hsp90α and Bclaf1 levels were determined by western blotting. Bands intensities were quantified and values are expressed as mean ± SD; n = 3; $^{**}P < 0.01$. (C) mRNA levels of c-Myc were detected in tumors by RT-PCR. Tumor tissues from 2 mice are pooled and loaded onto a single lane because of the small amount of tumor tissue. (D) Protein levels of Hsp90α, Bclaf1, and c-Myc was determined in tumors by western blotting. Tumor tissues from 2 mice are pooled per lane. Abbreviations: Ctrl, control; IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation.

RNA processing factors to participate in regulation of c-MYC expression. Perhaps Bclaf1 assembles into the mRNP complex in order to co-regulate postsplicing events, such as mRNA export or stability.[28] However, more work will be required to understand this complex process.

Within this study, we suggest that Bclaf1 is a client protein of Hsp90α. Most Hsp90 clients depend on ATP binding, and hydrolysis by Hsp90's NBD and Hsp90's ATPase cycle regulates binding and release of these clients. Recent studies suggest that clients could also bind to the CTD, which contains a binding

site for certain Hsp90 inhibitors.[7] Here, we suggest that neither inhibition of Hsp90α with the ATP-competitive inhibitors, 17-DMAG and STA-9090, nor mutational disruption of Hsp90α's ATPase activity impacts the steady-state levels of Bclaf1. In contrast, a drug binding to Hsp90α's CTD reduced the half-life of Bclaf1 without altering the level of Bclaf1-encoding mRNA. Moreover, we illustrate that the interaction site for Bclaf1 in Hsp90α is in the C-terminal region. Consistent with this hypothesis, NB treatment increased Bclaf1 ubiquitination and degradation by the proteasome pathway, a common fate for most clients deprived of interaction with Hsp90.[40] We also suggest that c-MYC expression can be modulated by the molecular chaperone, Hsp90α, most likely by its ability to maintain the stability of Bclaf1. Hsp90α knockdown or pharmacological inhibition result in a decrease in mature c-MYC mRNA, phenocopying the effect of Bclaf1 knockdown. More important, this effect can be rescued by transfection with an expression plasmid encoding full-length Bclaf1. Our data suggest that Hsp90α affects c-MYC expression directly through Bclaf1 and not through an indirect path involving other Hsp90α clients.

Given that many oncoproteins, proteins involved in all hallmarks of cancer, rely heavily on Hsp90 to chaperone their conformation, Hsp90 inhibitors could play a unique role in cancer treatment. A caveat to this scheme is that NBD binding inhibitors of Hsp90, which are the most potent currently available inhibitors, induce heat shock response through activation of heat shock transcription factor 1 HSF1) and thus induce an increase of prosurvival factors, like Hsp70 and Hsp27.[41,42] This is currently the major problem in cancer treatment using ATP-competitive Hsp90 inhibitors. One of the potential benefits associated with some of the Hsp90 inhibitors binding to the CTD is that these inhibitors do not activate HSF1. This property allows targeting Hsp90's cancer-cell–protective and antiapoptotic functions, while avoiding the unwanted feedback effect caused by the N-domain inhibitors.[43] Corroborating our findings with the amino coumarin, NB, silibinin, a structurally unrelated flavonolignan, which also binds to the C-terminal region of Hsp90, shows pharmacological similarity to NB and also induced the degradation of Bclaf1 (Supporting Fig. S1E). Inhibition of tumor growth by NB (Fig. 8A) appears to be more efficient than Bclaf1 knockdown (Fig. 4E). This observation could have several reasons. Bclaf1 levels might be more significantly reduced in the presence of NB than upon expression of the BCLAF1 shRNA. There is also accumulating evidence that NB interferes with the interaction of Hsp90 with other clients, notably ErbB2, Raf1, and v-Src, which have been reported to play a critical regulatory role in HCC progression.[44,45]

In summary, our study of the Hsp90α/Bclaf1/c-Myc dependence pathway that facilitates HCC proliferation might suggest a means for treating HCC using C-terminal inhibitors of Hsp90α or combinations of inhibitors of Hsp90α and Bclaf1 to achieve treatment efficacy with lower toxic side effects. Our study also suggests that Bclaf1 expression may represent a suitable biomarker in HCC and perhaps in other cancers. Given that high Bclaf1 expression correlates with low survival rates, further clinical investigation seems warranted to evaluate Hsp90 inhibition as a treatment strategy in HCC patients stratified for Bclaf1 expression.

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REFERENCES

1) Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533-543.
2) El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011;365:1118-1127.
3) Villanueva A, Hernandez-Gea V, Llovet JM. Medical therapies for hepatocellular carcinoma: a critical view of the evidence. Nat Rev Gastroenterol Hepatol 2013;10:34-42.
4) Liu P, Ge M, Hu J, Li X, Che L, Sun K, et al. A functional mammalian target of rapamycin complex 1 signaling is indispensable for c-Myc-driven hepatocarcinogenesis. Hepatology 2017;66:167-181.
5) Shachaf CM, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, et al. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. Nature 2004;431:1112-1117.
6) Mayer MP, Le Breton L. Hsp90: breaking the symmetry. Mol Cell 2015;58:8-20.
7) Marcu MG, Schulte TW, Neckers L. Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. J Natl Cancer Inst 2000;92:242-248.
8) Kasof GM, Goyal L, White E. Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. Mol Cell Biol 1999;19:4390-4404.
9) Sarras H, Alizadeh AS, McPherson JP. In search of a function for BCLAF1. Sci World J 2010;10:1450-1461.
10) Ziegelbauer JM, Sullivan CS, Ganem D. Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. Nat Genet 2009;41:130-134.

11) Haraguchi T, Holaska JM, Yamane M, Koujin T, Hashiguchi N, Mori C, et al. Emerin binding to Btf, a death-promoting transcriptional repressor, is disrupted by a missense mutation that causes Emery-Dreifuss muscular dystrophy. Eur J Biochem 2004;271:1035-1045.
12) Lamy L, Ngo VN, Emre NC, Shaffer AR, Yang Y, Tian E, et al. Control of autophagic cell death by caspase-10 in multiple myeloma. Cancer Cell 2013;23:435-449.
13) Vohhodina J, Barros EM, Savage AL, Liberante FG, Manti L, Bankhead P, et al. The RNA processing factors THRAP3 and BCLAF1 promote the DNA damage response through selective mRNA splicing and nuclear export. Nucleic Acids Res 2017;45:12816-12833.
14) Zhou X, Li X, Cheng Y, Wu W, Xie Z, Xi Q, et al. BCLAF1 and its splicing regulator SRSF10 regulate the tumorigenic potential of colon cancer cells. Nat Commun 2014;5:4581.
15) Shen B, Tan M, Mu X, Qin Y, Zhang F, Liu Y, et al. Upregulated SMYD3 promotes bladder cancer progression by targeting BCLAF1 and activating autophagy. Tumour Biol 2016;37:7371-7381.
16) Jarosz D. Hsp90: A Global regulator of the genotype-to-phenotype map in cancers. Adv Cancer Res 2016;129:225-247.
17) Breinig M, Caldas-Lopes E, Goeppert B, Malz M, Rieker R, Bergmann F, et al. Targeting heat shock protein 90 with non-quinone inhibitors: a novel chemotherapeutic approach in human hepatocellular carcinoma. Hepatology 2009;50:102-112.
18) Vartholomaiou E, Echeverria PC, Picard D. Unusual suspects in the twilight zone between the Hsp90 interactome and carcinogenesis. Adv Cancer Res 2016;129:1-30.
19) Zhou Y, Deng X, Zang N, Li H, Li G, Li C, et al. Transcriptomic and proteomic investigation of HSP90A as a potential biomarker for HCC. Med Sci Monit 2015;21:4039-4049.
20) Ratzke C, Nguyen MN, Mayer MP, Hugel T. From a ratchet mechanism to random fluctuations evolution of Hsp90's mechanochemical cycle. J Mol Biol 2012;423:462-471.
21) Ory B, Baud'Huin M, Verrecchia F, Royer BB, Quillard T, Amiaud J, et al. Blocking HSP90 addiction inhibits tumor cell proliferation, metastasis development, and synergistically acts with zoledronic acid to delay osteosarcoma progression. Clin Cancer Res 2016;22:2520-2533.
22) Obermann WM, Sondermann H, Russo AA, Pavletich NP, Hartl FU. In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. J Cell Biol 1998;143:901-910.
23) Breuhahn K, Longerich T, Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. Oncogene 2006;25:3787-3800.
24) Shiraha H, Yamamoto K, Namba M. Human hepatocyte carcinogenesis (review). Int J Oncol 2013;42:1133-1138.
25) Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer 2008;8:976-990.
26) Thurnherr T, Mah WC, Lei Z, Jin Y, Rozen SG, Lee CG. Differentially expressed miRNAs in hepatocellular carcinoma target genes in the genetic information processing and metabolism pathways. Sci Rep 2016;6:20065.
27) Chen M, Manley JL. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. Nat Rev Mol Cell Biol 2009;10:741-754.
28) Varia S, Potabathula D, Deng Z, Bubulya A, Bubulya PA. Btf and TRAP150 have distinct roles in regulating subcellular mRNA distribution. Nucleus-Phila 2013;4:229-240.
29) Sun S, Zhang Z, Sinha R, Karni R, Krainer AR. SF2/ASF autoregulation involves multiple layers of post-transcriptional and translational control. Nat Struct Mol Biol 2010;17:306-312.
30) Lee YY, Yu YB, Gunawardena HP, Xie L, Chen X. BCLAF1 is a radiation-induced H2AX-interacting partner involved in gamma H2AX-mediated regulation of apoptosis and DNA repair. Cell Death Dis 2012;3:e359.
31) Dang CV. MYC on the path to cancer. Cell 2012;149:22-35.
32) Audic Y, Hartley RS. Post-transcriptional regulation in cancer. Biol Cell 2004;96:479-498.
33) Iaccarino I. lncRNAs and MYC: an intricate relationship. Int J Mol Sci 2017;18:1497.
34) Laird-Offringa IA. What determines the instability of c-myc proto-oncogene mRNA? Bioessays 1992;14:119-124.
35) Xu LZ, Li SS, Zhou W, Kang ZJ, Zhang QX, Kamran M, et al. p62/SQSTM1 enhances breast cancer stem-like properties by stabilizing MYC mRNA. Oncogene 2017;36:304-317.
36) **Diella F, Cameron S**, Gemund C, Linding R, Via A, Kuster B, et al. Phospho.ELM: a database of experimentally verified phosphorylation sites in eukaryotic proteins. Bmc Bioinformatics 2004;5: 79.
37) Sanford JR, Gray NK, Beckmann K, Caceres JF. A novel role for shuttling SR proteins in mRNA translation. Genes Dev 2004;18:755-768.
38) Lemaire R, Prasad J, Kashima T, Gustafson J, Manley JL, Lafyatis R. Stability of a PKCI-1-related mRNA is controlled by the splicing factor ASF/SF2: a novel function for SR proteins. Genes Dev 2002;16:594-607.
39) Galganski L, Urbanek MO, Krzyzosiak WJ. Nuclear speckles: molecular organization, biological function and role in disease. Nucleic Acids Res 2017;45:10350-10368.
40) Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature 2011;475:324-332.
41) Bagatell R, Paine-Murrieta GD, Taylor CW, Pulcini EJ, Akinaga S, Benjamin IJ, et al. Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of hsp90-binding agents. Clin Cancer Res 2000;6:3312-3318.
42) McCollum AK, Teneyck CJ, Sauer BM, Toft DO, Erlichman C. Up-regulation of heat shock protein 27 induces resistance to 17-allylamino-demethoxygeldanamycin through a glutathione-mediated mechanism. Cancer Res 2006;66:10967-10975.
43) Zuehlke AD, Beebe K, Neckers L, Prince T. Regulation and function of the human HSP90AA1 gene. Gene 2015;570:8-16.
44) Ikeda S, Tsigelny IF, Skjevik ÅA, Kono Y, Mendler M, Kuo A, et al. Next-generation sequencing of circulating tumor DNA reveals frequent alterations in advanced hepatocellular carcinoma. Oncologist 2018;23:586-593.
45) **Bera R, Chiou CY**, Yu MC, Peng JM, He CR, Hsu CY, et al. Functional genomics identified a novel protein tyrosine phosphatase receptor type F-mediated growth inhibition in hepatocarcinogenesis. Hepatology 2014;59:2238-2250.

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Supporting Information

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