FBXL6 governs c-MYC to promote hepatocellular carcinoma through ubiquitination and stabilization of HSP90AA1

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Abstract

**BACKGROUND:** Heat shot protein 90 (HSP90) AA1 functions as an onco-protein to regulate the assembly, manipulation, folding and degradation of its client proteins, including c-MYC. However, the mechanisms underlying the regulation of HSP90AA1 are poorly understood.

**METHODS:** Transcriptome RNA-sequencing data of Liver hepatocellular carcinoma (LIHC) samples were used to detect the mRNA expression of FBXL6. Immunoprecipitation/Mass Spectrum (IP/MS) method was used to identify the interacting proteins of FBXL6. The co-immunoprecipitation assay was used to determine the interaction between FBXL6 and HSP90AA1. The in vivo ubiquitination assay was performed to determine the regulation of HSP90AA1 by FBXL6. Luciferase reporter and chromatin immunoprecipitation assays were used to determine the transcriptional regulation of FBXL6 by c-MYC. Cell counting and colony formation assays were implemented to detect the biological effects of FBXL6 on the growth of HCC cells in vitro. The effect of FBXL6 on HCC tumor growth in vivo was studied in a tumor xenograft model in mice.

**RESULTS:** Here, we identified the orphan F-box protein FBXL6, a substrate recognition subunit of an SCF (Skp1-Cul1-F-box protein) complex, as the ubiquitin ligase for HSP90AA1. FBXL6 promoted K63-dependent ubiquitination of HSP90AA1 to stabilize it. Through analysis of TCGA dataset, we found that FBXL6 was significantly increased in liver cancer tissues and positively correlated with c-MYC pathway. FBXL6 accumulation in liver cancers causes the stabilization and activation of c-MYC by preventing HSP90AA1 degradation. Activated c-MYC, which in turn directly bound to the promoter region of FBXL6 to induce its mRNA expression.

**CONCLUSION:** Collectively, our data revealed an unknown FBXL6-HSP90AA1-c-MYC axis which might contribute to the oncogenesis of liver cancer, and we propose that inhibition of FBXL6 might represent an effective therapeutic strategy for liver cancer treatment.

Background

Hepatocellular carcinoma (HCC) is one of the most common cancer in the world and the second malignant tumor of global cancer mortality, and its morbidity and mortality are gradually increasing [1]. The pathogenesis of HCC is extremely complicate and the discovery of new molecular drug
targets will benefit HCC treatment [2].

The ubiquitin (Ub)-proteasome system (UPS) plays a prominent role in a variety of cellular activities, including cell cycle control, apoptosis, DNA damage repair, immune response and tumorigenesis [3]. Ubiquitination is catalyzed by a three-enzyme cascade consisting of the E1 Ub-activating enzyme, the E2 Ub-conjugating enzyme, and the E3 Ub-protein ligase [4]. In UPS, Ub modifies protein substrates mostly in the form of a K48-or K11-linked polyUb chain, which serves as a signal for proteasome-dependent degradation [5]. However, K63-linked polyUb chain is not associated with proteasome degradation of the substrate protein [6].

The selectivity of Ub-mediated proteolysis is determined by the E3 ligases which could be grouped into two classes based on their structural features: the RING (really interesting new gene) E3s and the HECT (homologous to the E6AP carboxyl terminus) E3s. The RING E3s constitute the largest E3 ligases family with more than 600 documented members, which directly catalyze the transfer of ubiquitin from an E2 to a substrate [7]. The substrates-recruiting and catalytic modules could be found in a single polypeptide or in different subunits of a E3 complex, such as the anaphase-promoting complex (APC) and the Cullin–RING ligases (CRLs) [8]. In mammals, there are approximately 200 CRLs has been reported. Each of CRLs contains a different Cullin subunit that using its carboxyl terminus to bind to the E2 enzyme and N terminus to bind to the substrate recognition factors. The CRL1 ligases, better known the SCF (Skp1-Cul1-F-box protein) complex, are the best characterized. SCF is a four-protein complex consisting of the constant Cullin1, RBX1, SKP1 and one of ~70 various F-box proteins [9]. Early studies have demonstrated that F-box proteins play indispensable roles in cell cycle regulation [10-12], and in recent years more and more F-box proteins have been reported to be closely related to tumorigenesis [13, 14]. However, given the larger number of F-box proteins, only a few F-box proteins have identified substrates and functions.

Heat shock proteins (HSPs) are a class of highly conserved proteins during biological evolution and widely found in prokaryotic and eukaryotic organisms [15]. HSPs could be induced under diverse stress conditions (virus infection, hypoxia, ultraviolet radiation, etc.). HSPs are both biomarkers of cellular stress response and also important molecular chaperone proteins in cells [15]. HSPs
participate in maintaining the correct folding of the client’s protein, enabling the protein to form the conformation required for physiological functions, thereby playing an important role in regulating the balance of protein synthesis/degradation and protein localization [16]. HSPs are mainly divided into five families: HSP90 family (83-90 kD), HSP70 family (66-78 kD), HSP60 family, small molecule smHSP family (15-30 kD), and macromolecular HSPs with molecular weights ranging from 100 to 110 kD. Among them, HSP90 is abundant in cells, accounting for 1% to 2% of total cellular protein [17]. In humans, there are four Hsp90 isoforms including the cytoplasmic Hsp90α and Hsp90β, as well as the endoplasmic reticulum isoform Grp94 and mitochondrial isoform TRAP1, respectively [18]. Hsp90α (Hsp90AA1), encoded by the HSP90aa1 gene, is composed of three major domains: the N-terminal domain, the intermediate domain, and the C-terminal domain. These three domains work together to play the molecular chaperone function of HSP90AA1, which is dependent on the binding of ATP to the ATPase domain at the N-terminus [19]. The binding and hydrolysis of ATP produces a conformational transition that regulates the assembly of the multi-subunit complexes involved. HSP90AA1 plays an important role in the assembly, manipulation, folding and degradation of its client proteins. Numerous studies have shown that inhibition of HSP90AA1 function can lead to degradation of its client protein through the ubiquitin-protease pathway [20]. Many of the client proteins regulated by HSP90AA1 are proto-oncogene products (such as c-MYC) or important signal transduction factors during tumor pathogenesis, which are closely related to the occurrence and development of tumors [21, 22]. Thus, inhibition of HSP90AA1 might affect cancer cells growth and survival from multiple pathways, making HSP90AA1 a promising anti-tumor drug target.

Here, through analysis of TCGA dataset, we found that the mRNA expression of an orphan F-box protein FBXL6 was significantly increased in HCC compared with normal ones and positively correlated with c-MYC expression. We further showed that FBXL6 forms a classical SCF E3 ligase complex to exert its oncogenic roles by stabilizing HSP90AA1 to activate c-MYC, which in turn directly bound to the promoter region of FBXL6 to induce its expression. Thus, our data revealed an unknown positive feedback axis of FBXL6-HSP90AA1-c-MYC, whose abnormal activation might contribute to the oncogenesis of HCC.
Material And Methods

Clinical samples and data acquisition

Transcriptome RNA-sequencing data of hepatocellular carcinoma (HCC) samples were downloaded from the TCGA data portal (https://cancergenome.nih.gov/), which contained data from 374 primary HCC and 50 non-tumor tissues. Raw count data was downloaded for further analyses. To selected genes involved in the onset of HCC, differentially expressed genes between HCC and non-tumor tissues were screened via the R software Linear Models for Microarray and RNA-Seq Data (Limma) package (http://bioconductor.org/packages/Limma/). We performed differential gene analysis of all transcriptional data, setting a log2 |fold change| > 1 and a false discovery rate (FDR) < 0.05 as the cutoff values. The Wilcox-test was used for analyses. 87 HCC clinical samples were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, sectioned and stained with haematoxylin and eosin. Immunohistochemical staining of the paraffin-embedded tumor tissues was performed using anti-FBXL6 and anti-HSP90AA1 antibodies.

Cell culture and reagents

HEK293T cells and hepatocellular carcinoma cell lines SMMC-7721 and Hep3B cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), supplemented with 10% FBS (Gibco), 100 units/mL penicillin, and 100 mg/mL streptomycin (Gibco). MG132 and Cycloheximide (CHX) was purchased from Sigma.

Plasmids

F-box proteins were amplified from 293T or SMMC-7721 cells by polymerase chain reaction and cloned into pbabe-Flag vector. pCherry.90 alpha was a gift from Didier Picard (Addgene plasmid # 108222 ; http://n2t.net/addgene:108222 ; RRID:Addgene_108222). c-myc-PT3EF1a was a gift from Xin Chen (Addgene plasmid # 92046 ; http://n2t.net/addgene:92046 ; RRID:Addgene_92046). pRK5-HA-Ubiquitin-K63 was a gift from Ted Dawson (Addgene plasmid # 17606 ; http://n2t.net/addgene:17606 ; RRID:Addgene_17606). All plasmids were completely sequenced and transfected into cells by using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions.

RNA interference, RNA isolation and real-time PCR
The Lentiviral Human FBXL6 shRNA was purchased from Merck and the target sequences for short hairpin RNA (sh-RNA)-expressing plasmids were the following: FBXL6-shRNA1: CACCGGCATCAACCGTAATAG; FBXL6-shRNA2: TGGAGTGGCTTATGCCCAATC. Total RNA of cell lysate was extracted by using TRIzol reagent (Invitrogen, Shanghai). Oligo dT was used to prime cDNA synthesis. Real-time PCR was then performed by using a SYBR Green Premix Ex Taq (TaKaRa) on Light Cycler480 (Roche, Switzerland). GAPDH was used as internal control. Differences in gene expression were calculated using 2-\(\Delta\Delta C_t\) method. Primers used for qPCR analysis were list as follows: FBXL6 forward, 5’- GGAGACCGCATTCCCTTG-3’; reverse, 5’- AAAACCGATGGCATAAGCC-3’. HSP90AA1 forward, 5’- AGGAGGTGAGACGTTCGC-3’; reverse, 5’- AGAGTTCGATCTTGTTTGTCCG-3’. GAPDH forward, 5’- TGTGGGCATCAATGGATTTGG-3’; reverse, 5’- ACACCATGTATTCCGGGTCAAT -3’.

**CRISPR/Cas9 knock out (KO) cell lines.**

SMMC-7721 cells were transfected with FBXL6 CRISPR/Cas9 KO (h) KO plasmid (sc-408853, Santa Cruz Biotechnology) using Lipofectamine2000 following the manufacturer’s instructions. Cells were selected with 1 µg/ml puromycin two weeks. Single clones were then selected and the knockout efficiency was verified by western blot assay.

**Western blotting and antibodies**

Cells were lysed with lysis buffer (100 mM Tris-HCl, pH 6.8, 100 mM DTT, 1% SDS, 10% glycerol). Proteins were separated by 10-12% SDS-PAGE, and transferred to NC membrane. Membranes were blocked in 5% non-fat milk in phosphate-buffered saline (PBS) for 1 h before incubation with primary antibody overnight at 4 °C. Membranes were washed with and incubated with secondary antibody for 1 h. Primary antibodies used as indicated: anti-Flag M2 (1:4,000 dilution, F1804, Sigma), anti-HSP90AA1 (1:1,000 dilution, 13171-1-AP, Protein tech), anti-FBXL6 (1:1,000 dilution, SAB1407299, Sigma), anti-Cul1 (1:1000 dilution, sc-17775, Santa cruz, U.S.A), anti-SKP1 (1:2,000 dilution, #12248, Cell Signaling Technology, U.S.A), anti-c-Myc (1:1,000 dilution, #18583, Cell Signaling Technology, U.S.A), and anti-GAPDH (1:5,000 dilution, #5174, Cell Signaling Technology, U.S.A).

**Immunoprecipitation (IP) and Mass spectrometry (MS)**

Cells were lysed with IP buffer (100 mM NaCl, 20 mM Tris-cl PH8.0, 0.5 mM EDTA, 0.5% (v/v) Nonidet
P-40) with protease inhibitor cocktail and phosphorylate inhibitor for 30 min on ice. Cells were sonicated and the lysates were centrifuged. The supernatant was incubated with appropriate antibodies and protein A/G beads overnight at 4 °C in a rotating wheel. Immunoprecipitates were washed with IP buffer. SDS loading buffer was then added and proteins were eluted by boiling at 95°C for five minutes. For mass spectrometry assay, lysates from 293T cells transfected with Flag-con or Flag-FBXL6 were cleared by centrifugation at 15,000 g for 20 minutes at 4°C to remove cell debris. The resulting lysates were subjected to IP with Flag M2 beads overnight at 4°C. Bound proteins were eluted by boiling, resolved by SDS-PAGE and stained with coomassie blue staining, followed by mass spectrometry analysis.

**In vivo Ubiquitination assay**

Cells co-transfected His-K63-Ubiquitin with EV or Flag-FBXL6 plasmids were sonicated in IP buffer containing 8M urea and 10mM imidazole. His-K63-Ubiquitin-conjugated proteins were recovered with Ni-NTA resin (Qiagen), washed eight times in urea lysis buffer containing 20mM imidazole, and eluted with IP buffer containing 5% SDS and 200mM imidazole. The boiled samples were separated by 10% SDS-PAGE and subjected to western blot with antibodies as indicated. For endogenous ubiquitinated protein accumulation, Tandem Ubiquitin Binding Entity 2 (TUBE2) resin (LifeSensors) was used. Cells were lysed with IP buffer with protease inhibitor cocktail and phosphorylate inhibitor for 30 min on ice. Cells were sonicated and the lysates were centrifuged. The supernatant was incubated with TUBE2 resin overnight at 4 °C in a rotating wheel. The resin was then washed with IP buffer and boiled in SDS loading buffer. Boiled samples were separated by 10% SDS-PAGE and subjected to western blot with antibodies as indicated.

**Colony formation analysis**

Cells were seeded in a six-well plate at a density of 1000/well and then cultured for 2 weeks. The numbers of colonies containing more than 50 cells were counted by crystal purple staining.

**Apoptosis analysis**

Cells were seeded into 6 well plates. Apoptosis cells were determined using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit according to the
manufacturer’s instruction. Cell apoptosis was then analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Apoptosis was also determined by measuring the activity of the caspases 3 and 7 using a luminescent substrate (Caspase-Glo 3/7; Promega) according to manufacturer’s instructions.

**Luciferase reporter and chromatin immunoprecipitation assays**

The promoter region of FBXL6 gene was amplified from the human genomic DNA and inserted into pGL4.15 vector (Promega, Madison, Wisconsin, USA). For the luciferase reporter assays, HEK293T cells were seeded in 24-well plates and transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) for 36 hours. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). The firefly luciferase luminescence data were normalized by the Renilla luciferase luminescence data. A chromatin immunoprecipitation (ChIP) assay kit (Upstate, Billerica, MA) was used according to manufacturer instructions. Briefly, cells were fixed with formaldehyde and DNA was sheared to fragments at 200-1,000 bp by repeated sonication. Chromatin was then incubated and precipitated with antibodies against c-Myc or IgG. Primers for GAPDH were used as negative control.

**Xenograft assays**

Animal study was approved by Animal Care and Use Committee of Fudan University Shanghai Cancer Center. 8-week-old male BALB/cA nude mice were purchased from National Rodent Laboratory Animal Resources (Shanghai, China). All mice were kept in a specific pathogen-free facility and housed at 21 °C ± 1 °C with humidity of 55 ± 10%, fed with sterilized food and water, and kept on a 12 h light/dark cycle. FBXL6+/+ and FBXL6−/− SMMC-7721 cells at a density of 1×10⁷ were suspended in 50 µl of DMEM medium, mixed 1:1 with Matrigel (Corning) and injected into the flanks of male nude mice. Tumor sizes were measured by a caliper and calculated using the formula length × width 2 × 1/2. Tumor weights were measured after mice were sacrificed.

**Statistical analyses**

All experiments were at least repeated three times. Data are presented as mean ± standard deviation (SD). Statistical analysis was performed with GraphPad Prism 7.0 software. The differences between
groups were calculated using the Student's t-test or one-way ANOVA using a Tukey post-hoc test. P values of <0.05 were considered statistically significant. Statistical significance is displayed as * P < 0.05, ** P < 0.01, and *** P < 0.001, respectively.

Results

**FBXL6 is highly expressed in liver cancer tissues and associated with the c-MYC pathway**

To identify key genes involved in the tumorigenesis of HCC, transcriptome RNA-sequencing data of 374 primary HCC samples and 50 non-tumor tissues were downloaded from the TCGA data portal (https://cancergenome.nih.gov/). The Limma R package identified 7,667 differentially expressed genes, 7,273 up-regulated and 394 down-regulated (Figure 1A-B). The output of the whole differentially expressed genes was provide in the supplementary table 1. Among those up-regulated genes, we focused on FBXL6, the expression of which was significantly increased in HCC (P =2.75E-25) (Figure 1C). In 374 HCC samples, the expression correlation coefficients of FBXL6 and all other genes were calculated using R, and the Gene Set Enrichment Analysis (GSEA) enrichment analysis was performed using the GSEABase package. We identified many pathways that were significantly enriched, such as MYC-target, bile acid metabolism, fatty acid metabolism and UV response (Figure 1D), suggesting that FBXL6 might play a role in these pathways. Notably, given the critical role of c-MYC oncogene in the tumorigenesis of HCC, the enrichment of MYC-target signature suggested a potential regulation of FBXL6 by c-MYC in HCC (Figure 1E). In supporting with this notion, we found that the c-MYC and FBXL6 mRNAs have a notable correlation in liver cancer samples (R=0.27, P =1.3e-0.7) (Figure 1F) [23]. Moreover, the expression of FBXL6 was also correlated with many c-MYC target genes (Supplementary Table S2). Together, these data suggested FBXL6 is highly expressed in HCC sample and associated with the c-MYC pathway.

**FBXL6 exhibits tumor-promoting ability in HCC**

To examine the roles of FBXL6 in growth control, we firstly used two small hairpin RNA (shRNA) constructs to reduce the expression of FBXL6 in Hep3B cells (Figure 2A). Silencing the expression of FBXL6 caused delayed cell growth and colony formation ability (Figure 2B-C). Flow cytometry assay showed that the spontaneous apoptosis rate of FBXL6-depeltaed Hep3B cells was higher than that of
control cells (Figure 2D), and the activities of caspase3 and caspase7 were also enhanced in the absence of FBXL6 (Figure 2E). To avoid selection of HCC lines that may not accurately reflect the effects of FBXL6 deletion, we also constructed FBXL6 knock out (KO) SMMC-7721 cell line using CRISPR-Cas9 technology, and found that FBXL6 deficiency decreased proliferation and colony formation compared with the control wild type (WT) cells (Figure 2F-G). Furthermore, we also used nude mice model to investigate whether FBXL6 affected HCC cells proliferation in vivo. Four weeks old BALB/c nude mice were subcutaneously injected with $1 \times 10^7$ WT or FBXL6 KO SMMC-7721 cells. We found that knock out of FBXL6 significantly decreased tumor volume and tumor weight compared with WT cells (Figure 2H). Thus, these data indicated that FBXL6 played a critical role in liver cancer cells proliferation both in vitro and in vivo.

**HSP90AA1 is associated with FBXL6**

To investigate the molecule mechanism underline the tumor-promoting role of FBXL6, we used the immunoprecipitation/Mass Spectrum (IP/MS) method to identify the interacting proteins of FBXL6. Flag-FBXL6 or Flag-Con plasmids were transfected into 293T cells and the cell lysates of these cells were subjected to MS analysis after purification by Flag M2 beads. As expected, our MS data analysis found that the known FBXL6 interacting proteins such as Cullin1 and SKP1, suggesting FBXL6 forms a classical SCF E3 ligase complex with both proteins. Importantly, we also identified several unknown new interacting proteins such as HSP90AA1 (Figure 3A). We then performed western blot assay to confirm our MS data. We found that Cullin1, SKP1 and HSP90AA1 could only be detected in Flag-FBXL6 immunoprecipitate (Figure 3B). To demonstrate the specificity of this binding, we screened 9 human F-box protein family members. Flag-tagged F-box proteins were expressed into 293T cells and then immunoprecipitated to evaluate their interaction with endogenous HSP90AA1 protein. Although each F-box protein binds to SKP1, the only F-box protein that binds to HSP90AA1 is FBXL6 (Figure 3C). The endogenous interaction between FBXL6 and HSP90AA1 was also verified in Hep3B and SMMC-7721 cells (Figure 3D-E). FBXL6 is composed of an N-terminal F-box domain and multiple leucine-rich repeat sequences. By protein interaction domain mapping assay, we found that FBXL6 bound to HSP90AA1 via its leucine-rich repeat sequences at its C-terminus (Figure 3F). Taken together, these
data indicated that FBXL6 specifically associated with HSP90AA1.

**FBXL6 stabilizes HSP90AA1 protein by promoting its K63-ubiquitination**

Typically, F-box proteins usually ubiquitinate substrate proteins and promote their proteasomal degradation [24]. In order to investigate whether FBXL6 can promote the degradation of HSP90AA1, we first overexpressed FBXL6 in SMMC-7721 cells. Interestingly, overexpression of FBXL6 did not reduce HSP90AA1 expression. Instead, it significantly induced the expression of HSP90AA1 protein, without affecting its mRNA level (Figure 4A). The same phenomenon was also observed in Hep3B cells (Figure 4B). On the contrary, silencing the expression of FBXL6 by shRNAs significantly reduced HSP90AA1 expression (Figure 4C). In agreement, the protein level, but not the mRNA level, of HSP90AA1 was significantly decreased in FBXL6 KO cells compared with WT cells (Figure 4D).

Furthermore, overexpression of an F-box domain-deleted FBXL6 (FBXL6ΔF-box) mutant failed to regulate HSP90AA1 expression (Figure 4E), suggesting that FBXL6-induced HSP90AA1 expression required its E3 ligase activity. To test this possibility, we compared the half-life of HSP90AA1 in HCC cells with or without FBXL6, and found that it was significantly reduced in FBXL6 KO cells relative to WT counterparts (Figure 4F). In agreement, treatment with the protease inhibitor MG132 restored the expression of HSP90AA1 in FBXL6 KO cells (Figure 4G). However, overexpression of FBXL6 increased the global ubiquitination form of HSP90AA1, suggesting that FBXL6 might promote HSP90AA1 ubiquitination to prevent its degradation (Figure 4H). Indeed, co-transfected with a His-ubiquitin-K63 plasmid, which coding ubiquitin with only K63 and other lysines were mutated to arginines, showed that FBXL6 significantly promoted HSP90AA1 K63-dependent ubiquitination (Figure 4I). Because K63-dependent ubiquitination modifications are usually not involved in protein degradation, our data suggest that FBXL6 might promote K63-dependent ubiquitination of HSP90AA1 to stabilize it.

Moreover, we performed immunohistochemical analysis to evaluate the potential association between FBXL6 and HSP90AA1 in 87 human HCC specimens using anti-FBXL6 and anti-HSP90AA1 antibodies (Supplementary Figure 1A). We observed a positive correlation between FBXL6 and HSP90AA1 (Supplementary Figure 1B, $X^2=19.24$, $P<0.001$). Altogether, our results demonstrated that FBXL6 was positively correlated with HSP90AA1 in HCC.
**FBXL6 stabilizes c-MYC via HSP90AA1**

HSP90AA1 can exert its tumor-promoting effect by stabilizing c-MYC protein in several cancer types [22, 25]. We found that overexpression of HSP90AA1 in SMMC-7721 and Hep3B also up-regulated c-MYC protein, suggesting c-MYC might be a critical client protein of HSP90AA1 in liver cancer (Figure 5A). Moreover, overexpression of FBXL6 promoted the expression of c-MYC in a dose-dependent manner in Hep3B cells (Figure 5B). However, overexpression of FBXL6ΔF-box mutant could not affect c-MYC expression, suggesting this regulation required the E3 ligase activity of FBXL6 (Figure 5C). Consistent with this, the expression of c-MYC in FBXL6 KO cells was significantly reduced (Figure 5D), with reduced half-life of c-MYC protein and increased ubiquitination form (Figure 5E-F). As expected, there is no evidence to support the directly binding between FBXL6 and c-MYC proteins, suggesting the indirect regulation of FBXL6 towards c-MYC. Indeed, overexpression of HSP90AA1 in FBXL6 KO cells partially reversed FBXL6-induced c-MYC expression (Figure 5G). Therefore, these data indicated that FBXL6 stabilized c-MYC protein via HSP90AA1.

**c-MYC transcriptional activates FBXL6 in HCC**

Bioinformatics analysis revealed that c-MYC and FBXL6 mRNAs have a significant correlation in liver cancer samples (Figure 1D). Since FBXL6 is an ubiquitin E3 ligase and c-MYC is a classical transcription factor, we hypothesized that c-MYC may regulate the transcription of FBXL6 in liver cancer. Indeed, we found that overexpression of c-MYC induced the mRNA expression of FBXL6 in both SMMC-7721 and Hep3B cells (Figure 6A). On the contrast, knockdown of c-MYC by siRNAs inhibited the mRNA expression of FBXL6 (Figure 6B). The C terminus of c-MYC contains a HLH-LZ domain, which is known to bind to the canonical E-box (CACGTG) to regulate downstream genes expression [26]. We next performed Chromatin immunoprecipitation (CHIP) experiments in Hep3B cells to determine whether c-MYC directly binds to the genomic locus of FBXL6. 3KB of sequence of the FBXL6 promoter region was then examined for putative c-MYC-binding sites. We identified one potential E-box in the FBXL6 promoter region by Jaspar database (http://jaspar.genereg.net/) (Figure 6C). ChIP assay revealed that anti-c-MYC antibody efficiently immunoprecipitated −2500 bp to −2300 bp upstream from the transcription start site (TSS) of FBXL6 gene in Hep3B cells (Figure 6D),
suggesting c-MYC directly binds to the promoter region of FBXL6. We also constructed a luciferase reporter vector containing the FBXL6 promoter region of this E-box. The luciferase reporter assay found that c-MYC increased the promoter activity in cells transfected with E-box WT vectors but not in cells with E-box mutant vectors (Figure 6E). Therefore, our data indicate that FBXL6 is a downstream target gene of c-MYC.

Discussion

Here, we report that FBXL6 stabilizes HSP90AA1 expression in HCC cells and that FBXL6 expression is correlated with c-MYC expression in HCC tissues, of which a great ratio expresses higher mRNA levels of FBXL6. In line with these observations, we show that genetic inhibition of FBXL6 eliminates HCC cells proliferation in vitro, and thus tumor progression in subcutaneously transplanted HCC mice, indicating a critical role of FBXL6 in the pathogenesis of liver cancer.

By using IP/MS assay, we identified HSP90AA1 was a FBXL6 interacting protein. We further showed that FBXL6 directly interacted with HSP90AA1 via its C-terminus leucine-rich repeat sequences. During the preparation of this manuscript, another group also found that HSP90AA1 was an interacting protein of FBXL6 by high throughput assay [27]. However, the relationship between FBXL6 and HSP90AA1 was still undetermined. Interestingly, unlike the canonical degradation-promoting function of most F-box proteins, we found that FBXL6 ubiquitinates HSP90AA1 to counteract its degradation. Our data further suggested FBXL6 promoted K63-dependent ubiquitination of HSP90AA1, although we could not exclude the possibility that FBXL6 could also stimulate other types of ubiquitination that might help to explain this discrepancy. It is possible that FBXL6-mediated HSP90AA1 K63-dependent ubiquitination counteract its K48-dependent ubiquitination of other E3 ligases. Indeed, another F-box protein FBXL21 has shown to ubiquitinate cryptochromes to stabilize these proteins [28, 29], suggesting the shared common underline mechanisms of both F-box proteins. However, whether FBXL21 also promoted K63-dependent ubiquitination of cryptochromes is still unknown. Thus, further investigation will be still required to understand the detail mechanism of how FBXL6 stabilized HSP90AA1 protein.

The correlation expression of the mRNA levels of FBXL6 and c-MYC promotes us to determine whether
FBXL6 is transcriptional regulated by c-MYC. By using CHIP and luciferase assays, we found that c-MYC directly bound to the E-box of FBXL6 promoter region to promote the mRNA expression of FBXL6, suggesting that FBXL6 is a downstream target gene of c-MYC in liver cancer.

Conclusions
Thus, our data revealed an unknown positive feedback loop of FBXL6-HSP90AA1-c-MYC axis, the aberrant regulation of which might contribute to the oncogenesis of liver cancer, and suggesting that reagents targeting FBXL6 would be beneficial for inhibiting liver cancer.

Declarations

Ethics approval and consent to participate
Animal study was approved by Animal Care and Use Committee of Fudan University Shanghai Cancer Center.

Consent for publication
All listed authors have actively participated in the study and have read and approved the submitted manuscript.

Availability of data and material
Please contact corresponding author for data requests.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
WS and ZM collaborated to design the study. WS, LF, SD, ZN, YH and LL were responsible for experiments. WS, ZC and ZM analyzed the data. WS and ZM wrote the paper. All authors collaborated to interpret results and develop the manuscript. All authors read and approved the final version of the manuscript.

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Abbreviations
APC: anaphase-promoting complex, CRLs: Cullin-based ubiquitin E3 ligases, HCC: hepatocellular carcinoma, HECT: homologous to the E6AP carboxyl terminus, HSPs: heat shock proteins, IP: immunoprecipitation, LIHC: Liver hepatocellular carcinoma, Limma RING: Linear Models for Microarray and RNA-Seq Data, really interesting new gene, SCF: Skp1/Cullin1/F-box protein, UPS: ubiquitin (Ub)-proteasome system.

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Figures
FBXL6 is highly expressed in liver cancer tissues and associated with the c-MYC pathway. A. Heatmap demonstrated differentially expressed genes between 374 HCC and 50 non-tumor tissues. B. Volcano plot demonstrated differentially expressed genes between 374 HCC and 50 non-tumor tissues. Blue dots represent down-regulated genes and red dots represent up-regulated genes. C. The mRNA expression of FBXL6 between 374 HCC and 50 non-tumor tissues. D. the GSEA enrichment analysis showed the expression correlation coefficients of FBXL6 and all other genes in 374 HCC samples. E. MYC-target enrichment plots in the expression correlation coefficients of FBXL6 and all other genes in 374 HCC samples. F. The correlation of the mRNAs of FBXL6 and c-MYC in GEPIA website.
Figure 2

FBXL6 exhibits tumor-promoting ability in HCC. A. Western blot analysis of the whole cell lysate (WCL) derived from Hep3B cells infected with the indicated shRNA lentiviruses. B. The cell growth curve of Hep3B cells in (A). C. Clonogenic assay of Hep3B cells in (A). D. Hep3B cells infected with the indicated shRNA lentiviruses were analyzed by FACS with Annexin V-PI assay. The graph represents the percentage of Annexin V positive cells. E. Caspase3 and Caspase7 activity was measured in Hep3B cells infected with the indicated shRNA lentiviruses. The y axis indicates the caspase3 and caspase7 activity over cell number. The value given for the caspase activity in control-infected cells was set as 100. F. FBXL6-/- SMMC-7721(KO) cells were generated by CRISPR assay and detected by western blot. G. The cell growth curve of FBXL6+/+ and FBXL6-/- SMMC-7721 cells. H. Clonogenic assay of FBXL6+/+ and FBXL6-/- SMMC-7721 cells. I. Each nude mouse was subcutaneously injected with 1 x 10^7 FBXL6+/+ or FBXL6-/- SMMC-7721 cells for about three weeks. Tumour growth was measured using a caliper at the indicated times after injection. n=8 for each group. *** P<0.001. J. The image shows representative tumor-bearing mice for each group. K. Tumor
weights were measured after mice were sacrificed. ** P<0.01.

| Protein   | Peptide | Reported Interaction |
|-----------|---------|----------------------|
| Cullin1   | 15      | YES                  |
| SKP1      | 12      | YES                  |
| HSP90AA1  | 10      | NO                   |
| PRIM1     | 7       | NO                   |
| KRT16C    | 6       | NO                   |
| RAIB1     | 2       | NO                   |
| PXO1      | 2       | NO                   |

Figure 3

HSP90AA1 is associated with FBXL6 A. A partial list of proteins identified by mass spectrometry analysis. 293T cells transfected with Flag-FBXL6 or Flag-Con plasmids were subjected to Flag M2 resin purification. Bound proteins were resolved by SDS-PAGE and stained with Coomassie blue staining, followed by mass spectrometry. B. 293T cells transfected with Flag-FBXL6 or Flag-Con plasmids were subjected to Flag M2 purification. Bound proteins were analyzed by immunoblot with indicated antibodies. C. 293T cells were infected with retroviruses encoding the indicated FLAG-tagged F-box proteins (FBPs) and subjected to Flag M2 resin purification. The immunocomplexes were probed with HSP90AA1, SKP1 and Flag antibodies. Lane 1 shows a whole cell lysate from cells infected with an empty virus (EV). D. The cell lysate of Hep3B cells were subjected to immunoprecipitation with anti- HSP90AA1 antibody or IgG. The bound proteins were detected by immunoblotting with antibodies against HSP90AA1 and FBXL6. E. The cell lysate of SMMC-7721 cells were subjected to immunoprecipitation with anti-FBXL6 antibody or IgG. The bound proteins were detected by immunoblotting with antibody against HSP90AA1 and FBXL6. F. 293T cells were
transfected with the indicated Flag-BRAT1 WT or deletion constructs for 36 hours. Cells were harvested and lysed. The WCL was immunoprecipitated by anti-Flag M2 resin and immunoblotted with antibody against HSP90AA1 and Flag.

FBXL6 stabilizes HSP90AA1 protein by promoting its K63-ubiquitination A. The protein and mRNA levels of HSP90AA1 from SMMC-7721 cells transfected with Flag-Con (EV) or Flag-FBXL6 indicated plasmids were detected by immunoblot and Real-time PCR, respectively. B. The protein and mRNA levels of HSP90AA1 from Hep3B cells transfected with indicated plasmids were detected by immunoblot and Real-time PCR, respectively. C. Western blot analysis of the WCL derived from SMMC-7721 cells or Hep3B cells infected with the indicated shRNA lentiviruses. D. The protein and mRNA levels of HSP90AA1 from FBXL6+/+ and FBXL6−/− SMMC-7721 cells were detected by immunoblot and Real-time PCR, respectively. E. Western blot analysis of the WCL derived from SMMC-7721 cells transfected with EV or Flag-FBXL6ΔF-box plasmids. F. FBXL6+/+ and FBXL6−/− SMMC-7721 cells were
treated with 20 μM cycloheximide (CHX) for the indicated time. The whole cell lysate was
immunoblotted with anti-HSP90AA1 antibody. The quantification plot was based on scanning
densitometry analysis using the Image J software. Relative protein levels were normalized to
FBXL6-/- control group. G. Western blot analysis of the WCL derived from FBXL6+/+ and
FBXL6-/- SMMC-7721 cells treated with 10 μM MG132 for 6 hours. H. SMMC-7721 cells
transfected with EV or Flag-FBXL6 plasmids were harvested and lysed. WCL were
immunoprecipitated by Tandem Ubiquitin Binding Entity 2 (TUBE2) resin for ubiquitinated
proteins enrichment and immunoblotted as indicated. I. SMMC-7721 cells were co-
transfected Flag-FBXL6 and his-K63-ub for 36 hours, cell lysate was subjected to
immunoprecipitation by Ni+ purification. Immunoprecipitate was detected by immunoblot
using indicated antibodies.
FBXL6 stabilizes c-MYC via HSP90AA1. A. Western blot analysis of the WCL derived from SMMC-7721 cells or Hep3B cells transfected with EV or HSP90AA1 plasmids. B. Western blot analysis of the WCL derived from Hep3B transfected with increased dose of Flag-FBXL6. C. Western blot analysis of the WCL derived from SMMC-7721 cells transfected with EV or Flag-FBXL6ΔF-box plasmids. D. The protein levels of c-Myc from FBXL6+/+ and FBXL6−/− SMMC-7721 cells were detected by Western blot. E. FBXL6+/+ and FBXL6−/− SMMC-7721 cells were treated with 20 μM cycloheximide (CHX) for the indicated time. The whole cell lysate was immunoblotted with anti-c-Myc antibody. The quantification plot was based on scanning densitometry analysis using the Image J software. Relative protein levels were normalized to FBXL6−/− control group. F. The WCL from FBXL6+/+ and FBXL6−/− SMMC-7721 cells were immunoprecipitated by Tandem Ubiquitin Binding Entity 2 (TUBE2) resin for ubiquitinated proteins enrichment and immunoblotted as indicated. G. FBXL6+/+ and FBXL6−/− SMMC-7721 cells transfected with vector control or HSP90AA1 plasmids were subjected to western blot assay with indicated antibodies.
c-MYC transcriptional activates FBXL6 in HCC. A. The mRNA levels and protein levels of FBXL6 from SMMC-7721 cells or Hep3B cells transfected with EV or c-Myc plasmids were detected by Real-time PCR and immunoblot, respectively. B. The mRNA levels and protein levels of FBXL6 from SMMC-7721 cells or Hep3B cells infected with the indicated shRNA lentiviruses were detected by Real-time PCR and immunoblot, respectively. C. Proximal promoter region of human FBXL6 gene contains a potential binding site of c-Myc. D. Chromatin immunoprecipitation (CHIP) assays showing representative c-Myc binding to the FBXL6 promoter in Hep3B Cells. Cells were subjected to ChIP assays with anti-IgG or c-Myc antibodies. The promoter of GAPDH was used as negative control. E. Luciferase reporter assays. HEK293T cells were co-transfected EV or c-Myc plasmids with luciferase reporter plasmids containing wild-type (WT-Luc) or mutant (Mut-Luc) binding site of c-Myc.

Supplementary Files
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