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ZBP-89 negatively regulates self-renewal of liver cancer stem cells via suppression of Notch1 signaling pathway

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Abstract

Liver cancer stem cells (LCSCs) initiate hepatocellular carcinoma (HCC) and contribute to its recurrence and treatment resistance. Studies have suggested ZBP-89 as a candidate tumor suppressor in HCC. We explored the role of ZBP-89 in the regulation of LCSCs. This study was

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Author Contributions

NW, PBSL and GGC conceived the study and designed the experiments. NW, ML, YL, JR, ZZ, SW, SY, S-IY and BH performed experiments and acquired data. NW, ML, YL and GGC analysed and interpreted data. NW drafted the manuscript. JLM, PBSL and GGC critically revised the manuscript. NW and ML did statistical analysis. CCNC, JLM and PBSL provided administrative, technical, or material supports. PBSL and GGC supervised the project.

Supplementary Material

Supplemental methods and data can be found online.

Conflicts of interest

The authors declare no conflicts of interest.

performed in liver tissue samples from 104 HCC patients, 2 cell lines and mouse tumor models. We demonstrated that ZBP-89 was weakly expressed in LCSCs. Patients with high expression of LCSC markers displayed reduced survivals and higher recurrence rates after curative surgical operation. The expression of ZBP-89 was predictive for decreased recurrence. LCSC markers were negatively correlated with ZBP-89 in HCC tissues and in enriched liver tumor spheres. The exogenous expression of ZBP-89 attenuated the tumor-sphere formation and secondary colony formation capabilities of LCSCs *in vitro* and tumorigenicity *in vivo*. Furthermore, the negative effect of ZBP-89 on cancer stemness was Notch1-dependent. Localized with Notch1 intracellular domain (NICD1) in the nucleus, ZBP-89 repressed the Notch1 signaling pathway by competitive binding to NICD1 with MAML1. Collectively, ZBP-89 negatively regulates HCC stemness via inhibiting the Notch1 signaling.

Keywords

hepatocellular carcinoma; liver cancer stemness; recurrence; Notch1; ZBP-89

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and ranks as the third leading cause of cancer death [1]. Although the curative resection of HCC results in excellent survival, high recurrence remains as one of the major obstacles to achieve long-term cure. The 5-year survival rate after resection is approximately 70%, but the cumulative recurrent rate is up to 100% [2]. Studies have suggested that tumor recurrence can be explained by the function of cancer stem cells (CSCs) which are a subset of cells with stem/progenitor cell features [3]. In fact, CSCs have shown remarkable characters of self-renewal, differentiation and tumor initiation, and are believed to lead to metastasis, chemo-resistance and relapse of various cancers [3]. It has also been found that CSCs can be identified based on the expression of surface markers. OCT4, SOX2 and c-Myc are basic transcription factors (TFs) that are expressed in both CSCs and embryonic stem cells [4–6]. In the case of liver cancer, the well-known specific CSC surface markers are epithelial cell adhesion molecule (EpCAM), CD13, CD90, CD133, and CD44 [7].

Accumulating evidence has indicated that CSCs share the same regulatory genes and signaling pathways with embryonic or tissue stem cells [4]. Notch, Hedgehog, Wnt and other signaling pathways have been acknowledged as mediators of cancer stemness [8]. Notch signaling modulates multiple aspects of tumor progression and acts as a cell-fate-determination pathway [9]. Activation of Notch receptors by canonical Notch ligands and Jagged ligands results in the release of intracellular Notch (NICD) domain into the nucleus. Also, this intracellular fragment can interact with nuclear factors to regulate the transcription of target genes [10]. However, details of the modulation of target genes by Notch are still largely unknown. Therefore, the identification of novel modulators involved in regulating target gene expression is crucial to understand the Notch signaling pathway and has a potential for developing novel therapeutic strategies to target liver CSCs (LCSCs).

ZBP-89, also called as ZNF148, is a Krüppel-type (Cys2-His2-type) zinc-finger protein. ZBP-89 primarily functions as a transcription factor which binds to gene promoters that contain GC-rich sequences. It either activates genes like Bak, or represses genes such as vimentin, gastrin, and p16, which are involved in cell growth and apoptosis [11]. Previous reports indicated that ZBP-89 was differentially expressed in HCC and that the expression of ZBP-89 was positively correlated with better survival rates among HCC patients, suggesting that ZBP-89 is a candidate of tumor suppressor in HCC [12]. However, an association between ZBP-89 expression and HCC recurrence rate remains poorly defined. A recent study reported that ZBP-89 cooperates with the Wnt/ β -catenin pathway to promote carcinogenesis in colorectal cancer [13]. It has also been demonstrated that the deletion of ZBP-89 can result in a decreased expression of Notch1 in colon stem cells [14]. Taken together, these data suggested that ZBP-89 may participate in the modulation of CSCs. In our study, we show that ZBP-89 is negatively correlated with HCC recurrence. ZBP-89 down-regulates the self-renewal of LCSCs by suppressing the Notch1 signaling pathway.

2. Materials and Methods

2.1 HCC patient samples.

A total of 104 patients with histologically confirmed diagnosis of HCC underwent tumor resection in the Prince of Wales Hospital between 2007 and 2011 were enrolled in the study. All subjects provided their written informed consent for specimen collection prior to surgery. Human ethics approval was obtained from the joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Management committee. Complete clinicopathologic and follow-up data were collected. The specimens were immediately stored in liquid nitrogen after surgery.

2.2 Statistical analysis.

GEO datasets were downloaded from NCBI. Heatmaps were created in R language after normalization. Statistical tests for *in vitro* or *in vivo* experiments were analyzed by Graph Pad Prism version 7.00. Experiments were conducted at least three times. One-way ANOVA followed by multiple mean comparisons by Student's t-test were performed to obtain p-values. Clinical characteristics were analyzed by Pearson's chi-squared test or Fisher's exact test by SPSS version 16.0. Kaplan-Meier plots and log-rank test were used for survival analysis. $p < 0.05$ was considered as statically significant.

3. RESULTS

3.1 ZBP-89 is weakly expressed in LCSCs and negatively correlated with CSC markers in HCC patients.

It has been reported that embryonic stem cells (ESCs) share multiple transcriptional programs with CSCs [4]. Essential inducers for induced pluripotent stem cell (iPSC), such as SOX2, OCT4 and c-Myc, are also known as CSC markers [4–6]. Thus, studying the TFs which are differentially expressed in ESCs/iPSC and hepatic differentiated cells would provide us a strategy to identify other TFs that may potentially regulate LCSCs. GEO database GSE14897 is the only published database which records gene expression profiles

involved in the hepatocyte differentiation from iPSC and ESCs. In order to identify TFs that may potentially regulate LCSCs, we analyzed a total of 225 TFs gene expression profiles from GSE14897. We previously reported that the expression of ZBP-89 was positively correlated with better survival rates among HCC patients, which suggested ZBP-89 as a candidate tumor suppressor in HCC [12]. And ZBP-89 was among the top five TFs that were weakly expressed in ESCs and iPSCs but highly expressed in hepatic differentiated cells (Fig.1A). We also found positive correlations between the expression of ZBP-89 and 25 representative genes that were reported as fingerprints of the differentiated hepatic phenotype with GSE14897 (Supplementary Fig.S1) [15]. To further identify the possible role of ZBP-89 in LCSCs, we conducted a series of analyses with our HCC patient samples. A negative correlation was presented between ZBP-89 and EpCAM/CD44 mRNA levels in 30 HCC samples by RT-qPCR (Fig.1B). Immunoblotting was performed to detect their protein levels in 5 randomly selected paired HCC tissues (Fig.1C). Furthermore, we measured the expression of ZBP-89 and LCSC markers in 104 HCC samples through the immunohistochemical approach (Fig.1D). By performing the Spearman correlation nonparametric test, we found that ZBP-89 was negatively correlated with the levels of CSC markers, including EpCAM, CD44 and SOX2 (Fig.1E). The demographic information of these clinical samples is shown in Supplementary Table S1.

Previous studies have shown that the high level of ZBP-89 predicts better HCC survival rates [12]. In our study, Kaplan-Meier analysis indicated that patients with the higher expression of CSC markers including EpCAM, SOX2 and CD44 displayed reduced overall and disease-free survival after surgical operation (Supplementary Fig.S2). Interestingly, when we took the expression of ZBP-89 and CSC markers together into consideration, the variation of survivals was shown. ZBP-89 expression correlated with better overall and disease-free survivals when EpCAM was low expressed or CD44 was highly expressed (Supplementary Fig.S3). For other panels including CD44^{low}, SOX^{low}/SOX^{high} and EpCAM^{high}, although ZBP-89 was shown to have positive influences on the survival rates, the statistical results were not significant. CSCs are thought to be responsible for cancer recurrence after anti-cancer treatment [16]. Clinical investigations suggested that patients with HCC recurrence after surgical resection displayed the higher expression of SOX2 and CD44. In addition, ZBP-89 expression was correlated with a lower risk of HCC recurrence (Fig.1F). Collectively, ZBP-89 is negatively associated with the expression of LCSC markers and clinical outcomes including survival rates and recurrence in HCC.

3.2 ZBP-89 overexpression decreases the survival of CSCs and the expression of stem cell markers

To determine whether ZBP-89 plays a critical role in regulating the proliferation and self-renewal of LCSCs, we stably overexpressed ZBP-89 in HCC cell lines with lentiviral vectors. First, the proliferation assay showed significantly reduced cell growths in Huh7 and Hep3B cells when ZBP-89 was overexpressed (Fig.2A). Second, we observed that ZBP-89-overexpressing Huh7 and Hep3B cells formed fewer tumor spheres and less densely packed, suggesting an impaired tumor sphere forming efficiency (Supplementary Fig.S4A). Third, we utilized the colony-forming assay to measure the self-renewal capacity of CSCs and found that the overexpression of ZBP-89 in LCSCs was negatively associated with the

colony formation (Fig.2B). Therefore, ZBP-89 was considered to reduce the capability of self-renewal in HCC cells.

To address the significance of ZBP-89 in the negative regulation of LCSCs, we examined the effect of ZBP-89 on the expression of CSC markers. Our results indicated that the overexpression of ZBP-89 strongly down-regulated the mRNA and protein expression of LCSC markers such as EpCAM, SOX2, c-Myc, CD133 and CD44 in our cultured tumor spheres (Fig.2CD). Moreover, tumor spheres with ZBP-89 overexpression showed a decreased expression of EpCAM, SOX2 and CD13 (Fig.2E). These results demonstrated a potential role of ZBP-89 in suppressing cancer stemness in HCC.

We next investigated whether the overexpression of ZBP-89 in HCC cells inhibited tumorigenicity. We established a xenograft model to evaluate the role of ZBP-89 in tumor initiation *in vivo*. Huh7 cells were injected subcutaneously into the back of nude mice and the growth of tumors was monitored. We observed that all mice bearing tumors from ZBP-89-overexpressing Huh7 cells showed reduced tumor growth rate with smaller tumor sizes (Fig.2F, Supplementary Fig.S4B). IHC staining showed that the expression of EpCAM and SOX2 was reduced in xenograft tumors derived from ZBP-89-overexpressing Huh7 cells compared with tumors from control cells (Supplementary Fig.S4C). RT-qPCR analysis also indicated that the overexpression of ZBP-89 reduced the expression of EpCAM, SOX2 and CD44 compared with the control tumors (Supplementary Fig.S4D). These results suggested that the overexpression of ZBP-89 attenuated tumorigenicity and cancer stemness of HCC cells *in vivo*.

3.3 ZBP-89 knockdown promotes self-renewal of LCSCs and the expression of stem cell markers

Next, we examined whether the knockdown of ZBP-89 could lead to enhanced cancer stemness. We silenced ZBP-89 in Huh7 and Hep3B cells using lentivirus shRNA plasmids. Two shRNAs targeting ZBP-89 were designed and they could strongly reduce ZBP-89 mRNA and protein expression (Fig.3A). Compared to control cells, the MTT assay revealed that the knockdown of ZBP-89 significantly increased the growth of Huh7 and Hep3B cells (Fig.3B). To study the self-renewal property of CSCs, we observed larger sizes and greater numbers of tumor spheres and secondary colonies in the ZBP-89 deficient Huh7 and Hep3B cells, indicating that ZBP-89 depletion could remarkably promote tumor sphere formation and CSC colony formation (Fig.3CD). In addition, ZBP-89 depletion enhanced the expression of CSC markers including EpCAM, CD133, SOX2 and c-Myc in freshly generated Huh7 and Hep3B tumor spheres by immunoblotting (Fig.3E). These data clearly support that ZBP-89 can negatively regulate LCSCs.

3.4 ZBP-89 suppresses the self-renewal of LCSCs via Notch1 signaling

Cancer stemness was governed by intricate molecular signaling pathways, such as Hedgehog, Wnt and Notch pathways. To elucidate the underlying mechanism of how ZBP-89 suppressed liver cancer stemness, we measured mRNA levels of representative genes involved in the three major self-renewal pathways in ZBP-89-overexpressing cells versus control Huh7 cells [17]. It was found that ZBP-89 overexpression substantially down-

regulated the mRNA levels of Notch target genes including HES1, HEY1, HES6 and NRARP (Fig.4A). These results suggested that the ZBP-89-mediated negative regulation on cancer stemness might depend on the Notch signaling in LCSCs. Immunoblotting analysis further demonstrated that the overexpression of ZBP-89 reduced the expression of HES1 protein in tumor spheres (Fig.4B). In addition, this result was verified by analyzing the levels between ZBP-89 and HES1 derived from published GEO database (GSE14520). The analysis of the dataset showed that the expression of ZBP-89 had a negative correlation with HES1 in both adjacent non-tumor and HCC tissues (Fig.4C). Similarly, by analysis of GSE9843 data we found that the expression of ZBP-89 was negatively correlated with another Notch target gene NRARP (Fig.4D).

Among the Notch family members, Notch1, 2, 3 have been recognized as pivotal regulators in LCSCs [17–19]. Thus, it is important to identify which Notch member(s) is predominantly involved in ZBP-89-mediated negative regulation on cancer stemness. Among three Notch members, Notch1 was most significantly down-regulated by ZBP-89 in both Huh7 and Hep3B tumor spheres (Fig.4E). We also noticed that the expression of Notch1 was down-regulated in ZBP-89-overexpressing tumor spheres (Fig.4F). However, such a negative correlation was not found in parental HCC cells (Supplementary Figure S5AB), suggesting that ZBP-89 may not be able to inhibit the expression of Notch1 directly in HCC cells. The positive association between Notch1 and liver cancer stemness was evidenced by the fact that Notch1 deficiency led to reduced tumor sphere and secondary colony formation of Huh7 and Hep3B cells (Supplementary Figure S6AB). To further demonstrate that Notch1 is indispensable in ZBP-89-mediated maintenance of liver cancer stem cells, we knocked down Notch1 in ZBP-89-deficient Huh 7 and Hep3B cells with shRNA. Notch1 silencing prevented sphere formation induced by ZBP-89 depletion, demonstrating that Notch1 and ZBP-89 had opposite effects on LCSCs (Fig.5A). The knockdown of Notch1 also abrogated the secondary colony-forming activities mediated by ZBP-89 (Fig.5B). These results suggested that ZBP-89 and Notch1 regulated cancer stemness through the same pathway. Furthermore, we found that simultaneous silencing of ZBP-89 and Notch1 decreased the expression of Notch1 target genes and LCSC markers induced by the deficiency of ZBP-89 alone (Fig.5C). We also noticed that the knockdown of Notch1 alone led to the upregulation of ZBP-89 (Fig.5D). These results have suggested that ZBP-89 suppresses liver cancer stemness through inhibiting the Notch1 signaling pathway.

3.5 ZBP-89 competitively binds to NICD1 and interferes with the interaction between NICD1 and MAML1

The receptor and ligand binding results in the proteolytic cleavage of Notch1 by γ -secretase and the generation of Notch1 intracellular domain (NICD1) which is released and translocated into the nucleus. Activated NICD1 binds to the transcription factor CSL and recruits coactivators such as MAML1 to trigger the expression of its target genes [20]. Notch1 target genes, such as HES1, HEY1 and NRARP, were positively associated with the expression of EpCAM and CD133 in HCC tissues [17]. Thus, we queried whether there were direct transcript interactions between NICD1 and LCSC markers such as EpCAM and CD44. To this end, we performed dual-luciferase assays with EpCAM and CD44 reporters in Huh7 and Hep3B cells. The result demonstrated that NICD1 and MAML1 potentially

triggered the transcription of EpCAM and CD44 in HCC cells. We also observed that the increase of EpCAM and CD44 promoter activities induced by NICD1 and MAML1 was blocked by ZBP-89 in a dose-dependent manner in Huh7 (Fig.6A) and Hep3B (Fig.6B), suggesting that ZBP-89 inhibited NICD1-mediated transcriptional activation by competing with MAML1.

As shown in Fig.6C, the localization patterns captured by the confocal microscope demonstrated that ZBP-89 and NICD1 localized to the nucleus of HCC cells, increasing the likelihood that ZBP-89 interacted with the nuclear components of Notch1 signaling directly. Co-immunoprecipitations were carried out to identify the potential binding site between ZBP-89 and NICD1. We found that ZBP-89 formed a protein complex with NICD1 in the nucleus (Fig.6D). Furthermore, the N-terminal (amino acids 6–180) of ZBP-89 was essential for this binding interaction by domain mapping (Fig.6E). It is well-recognized that NICD1 and MAML1 form a protein complex as co-activators in the nucleus to trigger the Notch1 signaling. Given the direct binding interaction between ZBP-89 and NICD1, as well as the opposite effects performed by these two proteins, we queried whether ZBP-89 interrupted the binding activity between NICD1 and MAML1. We demonstrated that anti-MAML1 antibody precipitated NICD1 in HEK-293T cells, but failed to pulldown ZBP-89 directly. The absence of direct interaction between ZBP-89 and MAML1 was also confirmed by co-immunoprecipitation with ZBP-89 and MAML1 co-transfections (Supplementary Figure S7). Moreover, we also found that the amount of NICD1 that precipitated with MAML1 antibody decreased when ZBP-89 was co-transfected. As expected, the amount of MAML1 precipitated with anti-NICD1 antibody was diminished with the co-expression of ZBP-89 (Fig.6F). Altogether, ZBP-89 interfered with the formation of NICD1-MAML1 complex in the nucleus and acted as a suppressor of the Notch1 signaling pathway (Supplementary Figure S8AB).

4. DISCUSSION

ZBP-89 was initially identified by screening an expression library with a GC-rich epidermal growth factor (EGF) response element of gastrin gene derived from a rat pituitary adenoma cell line [21]. ZBP-89 protein levels have been shown to be elevated in gastric and colorectal cancer [22, 23]. In HCC, studies have revealed that the expression of ZBP-89 protein is elevated at the early stage (I), but its expression decreased at the more advanced stages (II–IV). Importantly, the high expression of ZBP-89 is believed to be associated with better survival in HCC patients [12]. Although, the reason why ZBP-89 is increased in the early stage of HCC remains uncertain, it has been found that ZBP-89 induces apoptosis of HCC through targeting Bak promoter region and stimulating its transcriptional activity, suggesting that patients may benefit from the upregulation of ZBP-89 [24]. ZBP-89 has been shown to regulate telomerase and plays a role in cell differentiation, providing an opportunity to increase the sensitivity to chemotherapy in HCC [25, 26]. Data mining of the published GEO database indicated that ZBP-89 was highly expressed in hepatic cells compared to iPSCs and ESC. Our results demonstrated that ZBP-89 was negatively associated with the expression of LCSC markers in HCC tumor tissues. Consistent with previous reports, we showed that the high levels of CSC markers including EpCAM, CD44 and SOX2 were associated with poor survivals [27–29]. Furthermore, we found that ZBP-89 expression was

related to better survivals when EpCAM was low expressed or CD44 was highly expressed. However, limited by the size of available clinical tissue/data and the grouping complexity, it failed to provide enough evidence to indicate the influence of ZBP-89 on survival rates in CD44^{low}, SOX^{low}/SOX^{high} and EpCAM^{high} subgroups. We also found that the strong expression of CD44 and SOX2 was associated with HCC recurrence after surgical resection, while ZBP-89 had a negative association with recurrence.

We showed that the overexpression of ZBP-89 reversed the self-renewal capability of LCSCs as evidenced by the tumor sphere formation assay and the secondary colony formation assay. And weak tumorigenicity and reduced cancer stemness were further confirmed in xenograft models. Moreover, ZBP-89 suppressed the mRNA or/and protein expression of LCSC markers including EpCAM, CD133, c-Myc, SOX2, CD44 and CD13 in cultured HCC tumor spheres. Consistently, we demonstrated that the knockdown of ZBP-89 increased the expression of multiple CSC markers and the initiation of tumor spheres with more and larger spheres. These results suggest that there is a reverse association between the expression of ZBP-89 and CSC markers and that ZBP-89 negatively regulates cancer stemness in HCC.

Identification of signaling pathways involved in ZBP-89-mediated cancer stemness regulation is important for the understanding of LCSC biology and the development of novel anti-cancer therapies. ZBP-89 has been implicated as an activator of Wnt/ β -catenin in colorectal cancer via directly binding to CTNNB1 promoter [13]. However, another study has been revealed that although the expression of ZBP-89 was relatively higher in primary colorectal tumors when compared with adjacent non-tumors, its expression was inversely associated with malignant phenotypes [23]. In our study, we did not observe significant changes of selective target genes in Wnt pathway when ZBP-89 was overexpressed in LCSCs. These results can be explained by various genetic backgrounds among different cancer types or characteristics between parental tumor cells and CSCs. HES and HEY family members and NRARP are target genes of the Notch signaling. It has been reported that the deletion of HEY1 and NRARP impairs the cancer stemness of LCSCs [17]. Our results showed that the overexpression of ZBP-89 resulted in the downregulation of the Notch target gene expression. Furthermore, we have also found that among the Notch family, Notch1 plays a key role in ZBP-89-mediated regulation on cancer stemness in HCC. ZBP-89 has been shown to down-regulate the expression of Notch1 in enriched tumor spheres. But this effect failed to be found in parental HCC cells, suggesting that the regulatory mechanism of Notch1 was different between LCSCs and non-CSC HCC cells. Though we did not explore the relevant mechanism responsible for the difference, it was possible that ZBP-89 would target Notch1 via relevant CSC molecules since ZBP-89 had a profound inhibitory effect on CSC biomarkers. Nevertheless, our finding is in line with a positive role of Notch1 in the promotion of CSCs in HCC [30]. We have also noticed that ZBP-89 could be upregulated when Notch1 was inhibited. This finding suggests that the interaction between ZBP-89 and Notch1 is likely a two-way system in which Notch1 may also negatively regulate ZBP-89. Such a negative feedback loop is usually in favor of the cancer growth, as a compensation mechanism of cancer cells to maintain the survival in a disadvantaged micro-environment [31,32].

The Notch1 signaling has been implicated as a major pathway in maintaining the function of CSCs. Higher Notch1 expression was detected in advanced HCC patients and associated with tumor sizes, tumor stages, metastasis and invasion [33]. To elucidate the complex regulatory network in the Notch1 signaling pathway, especially the identification of proteins that can interrupt the dynamic interactions in CSCs, is pivotal for the discovery of potential treatment against HCC. The Notch1 receptor is cleaved into extracellular Notch1 (NECD1) and NICD1 by metalloprotease and γ -secretase once it binds with Delta or Jagged ligands [34]. NICD1 translocates into the nucleus and associates with DNA-binding protein CSL, transforming CSL from a transcriptional repressor to an activator [35]. The NICD1-CSL complex further recruits MAML1 as an activator to stabilize the complex, triggering the transcription of Notch pathway target genes [36]. However, it is largely unknown if other transcription factors participate in this process. Since both ZBP-89 and NICD1 were localized in the nucleus, we determined if these two proteins had direct interactions by binding. Our results illustrated that the N-terminus of ZBP-89 bound to NICD1 directly, competitively impeding the binding interaction between NICD1 and MAML1. Recently, studies have shown that the NICD transcriptional activator complex activates the transcription of a substantial number of genes far beyond the classical HES/HEY proteins [37]. Here we reported that NICD1 and MAML1 synergistically activated the transcription of EpCAM and CD44 as evidenced by the dual-luciferase assay. The overexpression of ZBP-89 impairs the transcriptional activity of EpCAM and CD44 induced by NICD1-MAML1 interaction in a dose-dependent manner. It suggests that ZBP-89 acts as a repressor of the Notch1 signaling.

In conclusion, we have shown that ZBP-89 impedes the formation of NICD1-MAML1 complex to inactivate the transcription of NICD1 target genes, resulting in negative regulatory effects on liver cancer stemness. Notch1 may also inhibit ZBP-89, forming a negative feedback loop to maintain the survival of cancer cells. Our study establishes a previous unidentified link among ZBP-89, Notch1 signaling cascade and cancer stemness in HCC. These results have advanced the current understanding of the ZBP-89 function as a tumor suppressor in HCC. Our data support the upregulation of ZBP-89 as a potential therapeutic strategy for the treatment of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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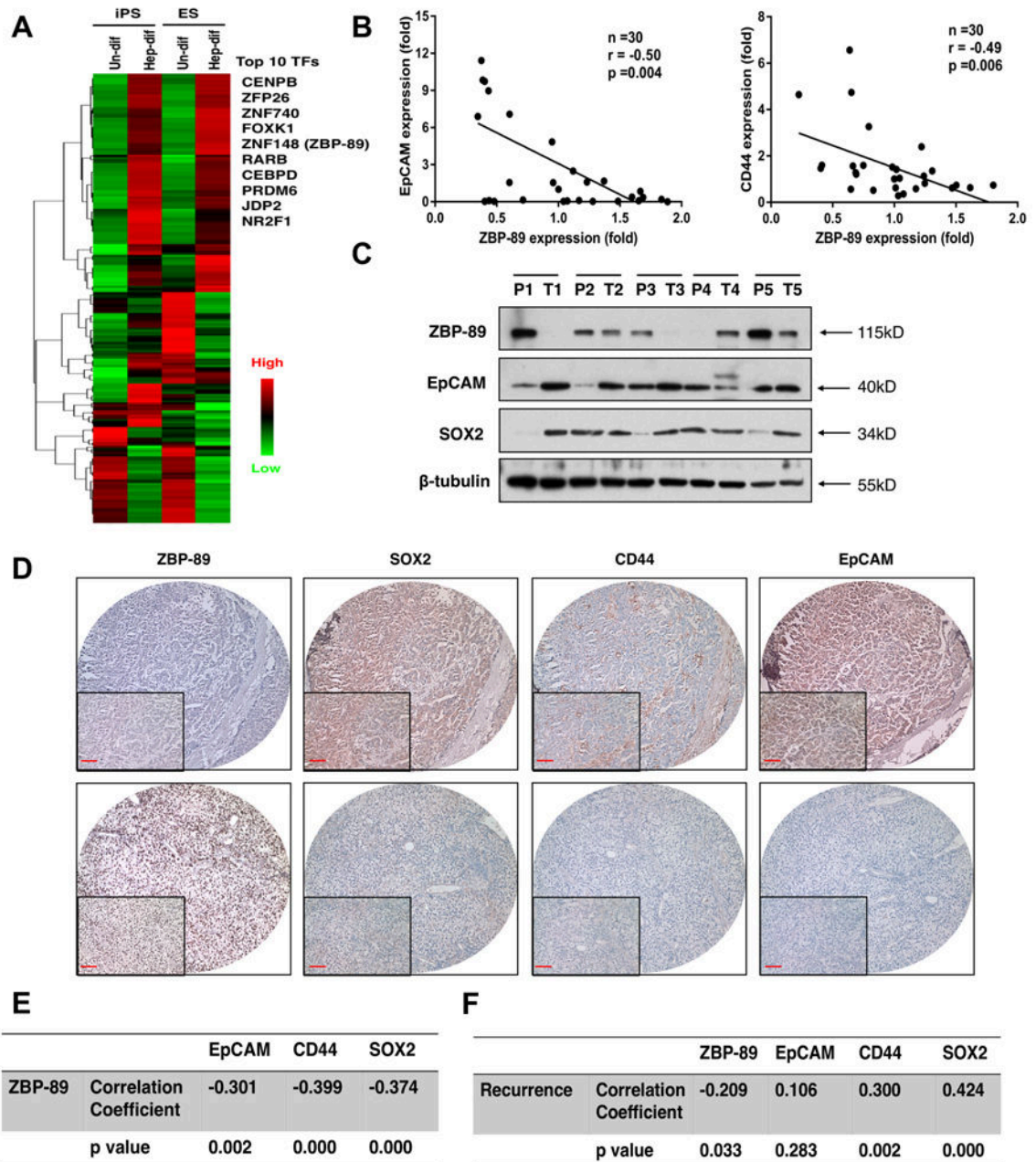


Fig. 1. ZBP-89 is negatively correlated with CSC markers in HCC patients.

(A) Using R language, we analyzed 225 TFs gene expression in undifferentiated/hepatic-differentiated ESCs and iPSCs provided by GEO database (GSE14897). ZBP-89 (ZNF148) was among the top 5 TFs which were weakly expressed in ESCs and iPSCs but highly expressed in hepatic-differentiated cells. (B) The mRNA levels of EpCAM, CD44 and ZBP-89 were analyzed in 30 HCC samples by RT-qPCR. The expression of ZBP-89 was negatively correlated with EpCAM ($r = -0.50$, $p = 0.004$) and CD44 ($r = -0.49$, $p = 0.006$). (C) The expression of ZBP-89 and CSC markers was verified by immunoblotting. P: peri-tumor;

T: tumor. (D, E) The negative correlations between ZBP-89 and CSC markers of 104 HCC tissue samples were analyzed by immunohistochemistry. Scale bars: 50 μ m. The expression of ZBP-89 and CSC markers was scored by IRS method and analyzed by Spearman correlation nonparametric test. (F) The Spearman coefficient was utilized to evaluate correlations between gene expression patterns and HCC recurrence. ZBP-89 had a negative correlation with HCC recurrence rate ($r = -0.209$, $p = 0.033$). CD44 ($r = 0.300$, $p = 0.002$) and SOX2 ($r = 0.424$, $p = 0.000$) were positively associated with cases of recurrence.

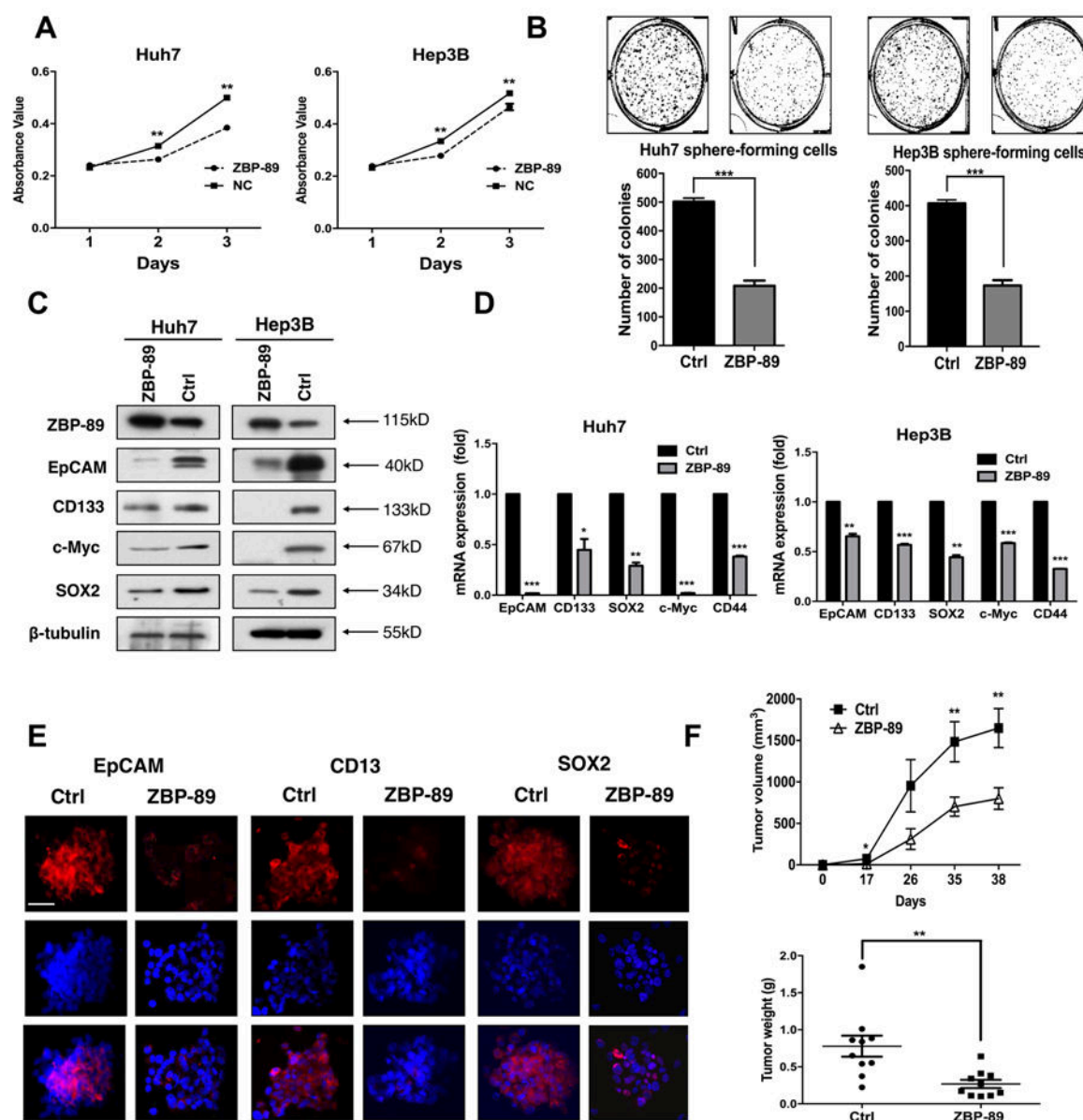


Fig. 2. Overexpression of ZBP-89 reduces cancer stemness in Huh7 and Hep3B HCC cells. (A) Growth curves for Huh7 and Hep3B cells were measured by MTT after lentivirus transduction encoded with ZBP-89 overexpression. ZBP-89 overexpression reduced the proliferation of HCC cells (**p < 0.01). (B) ZBP-89-overexpressing and control Huh7 and Hep3B cells were cultured for tumor-spheres and trypsinized into single cells with CSC traits and then seeded into 6 well plates for colony formation. The forced expression of ZBP-89 reduced the secondary colony-forming capability of HCC CSCs (***p < 0.001). (C) The overexpression of ZBP-89 diminished the expression of CSC markers including

EpCAM, CD133, c-Myc and SOX2 in freshly enriched Huh7 (left panel) and Hep3B (right panel) tumor spheres by immunoblotting. (D) CSC markers including EpCAM, CD133, c-Myc, SOX2 and CD44 were down-regulated in enriched ZBP-89-overexpressing Huh7 and Hep3B tumor spheres by RT-qPCR. (E) Enriched ZBP-89-overexpressing and control Hep3B tumor spheres were stained with anti-EpCAM, anti-CD13 and anti-SOX2 and observed under immunofluorescence microscopy. Scale bars: 20 μ m. (F) ZBP-89-overexpressing and control Huh7 cells (5×10^6) were subcutaneously injected into nude mice (n=10). The growth of tumors was monitored. ZBP-89 overexpression attenuated tumor-initiating ability in xenograft model with reduced tumor volume and weight.

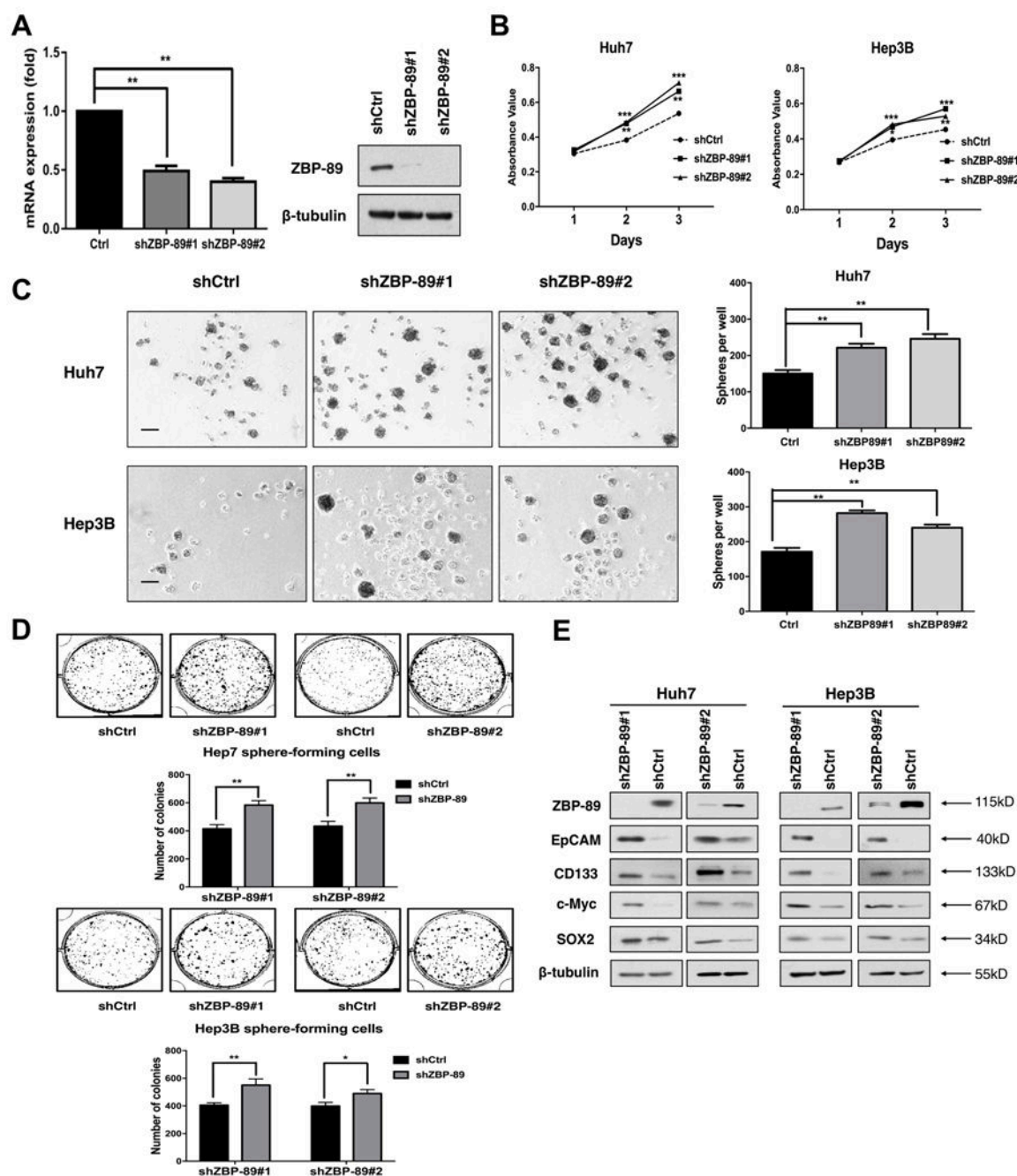


Fig. 3. ZBP-89 knockdown promotes self-renewal of LCSCs and the expression of stem cell markers.

(A) Two stable ZBP-89 knockdown Huh7 cell lines were generated with two different shRNA constructs against ZBP-89 (shZBP-89#1, shZBP-89#2) using the lentivirus system. Knockdown efficiency was evaluated by RT-qPCR (** $p < 0.01$) and immunoblotting. (B) Growth curves for Huh7 and Hep3B were measured by MTT after the stable knockdown of ZBP-89. ZBP-89 deficiency promoted the proliferation of HCC cells (** $p < 0.001$). (C) Two ZBP-89 deficient and control cell lines for Huh7 and Hep3B were cultured for tumor spheres for 7 days. ZBP-89 deficiency promoted the tumor-sphere formation of HCC cells

(** $p < 0.01$). Scale bar: 300 μ m. (D) Knockdown of ZBP-89 enhanced the secondary colony formation of Huh7 (** $p < 0.01$) and Hep3B (* $p < 0.05$) CSCs. (E) Loss of ZBP-89 enhanced the expression of CSC markers in freshly enriched Huh7 (left panel) and Hep3B (right panel) tumor spheres analyzed by immunoblotting.

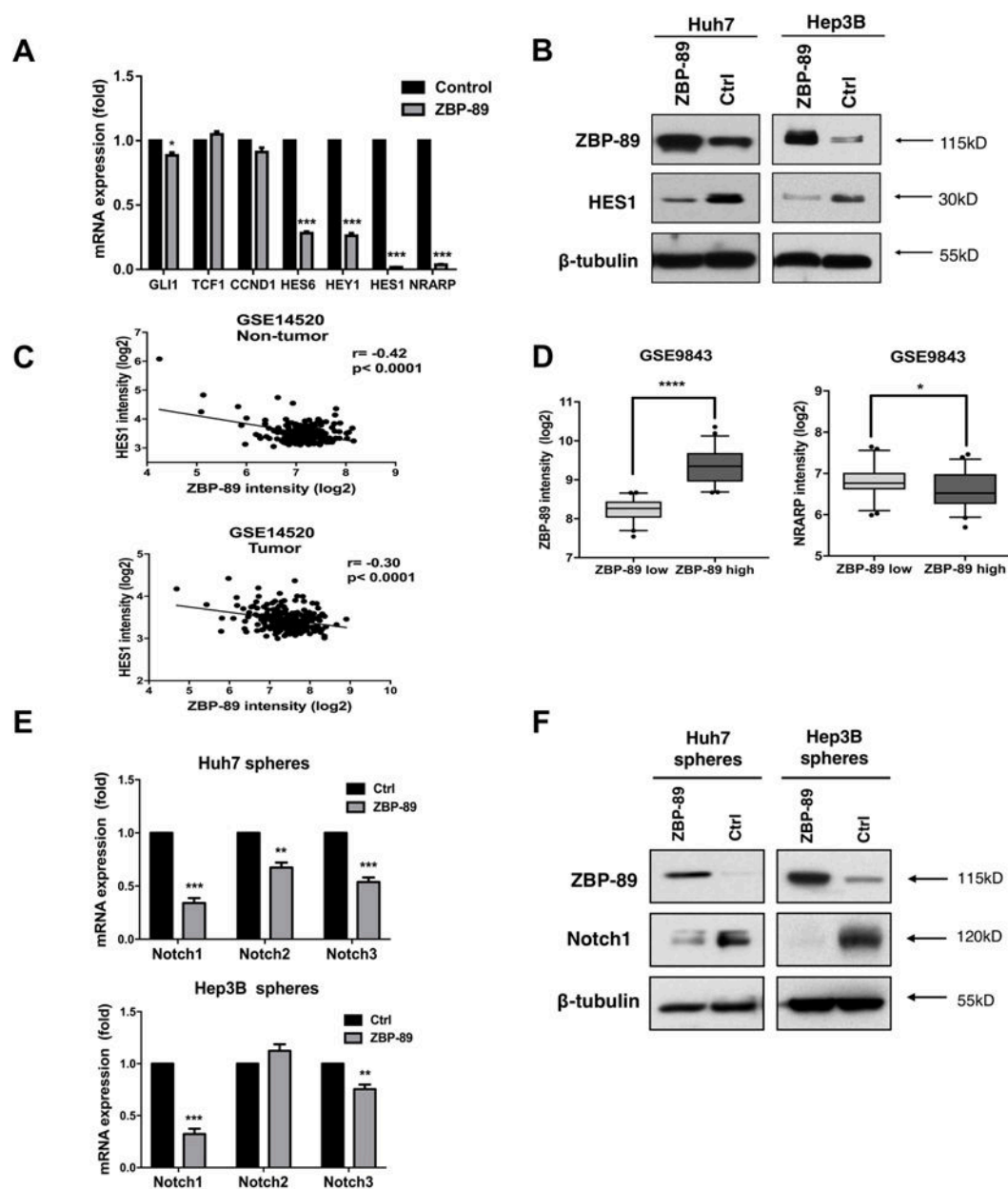
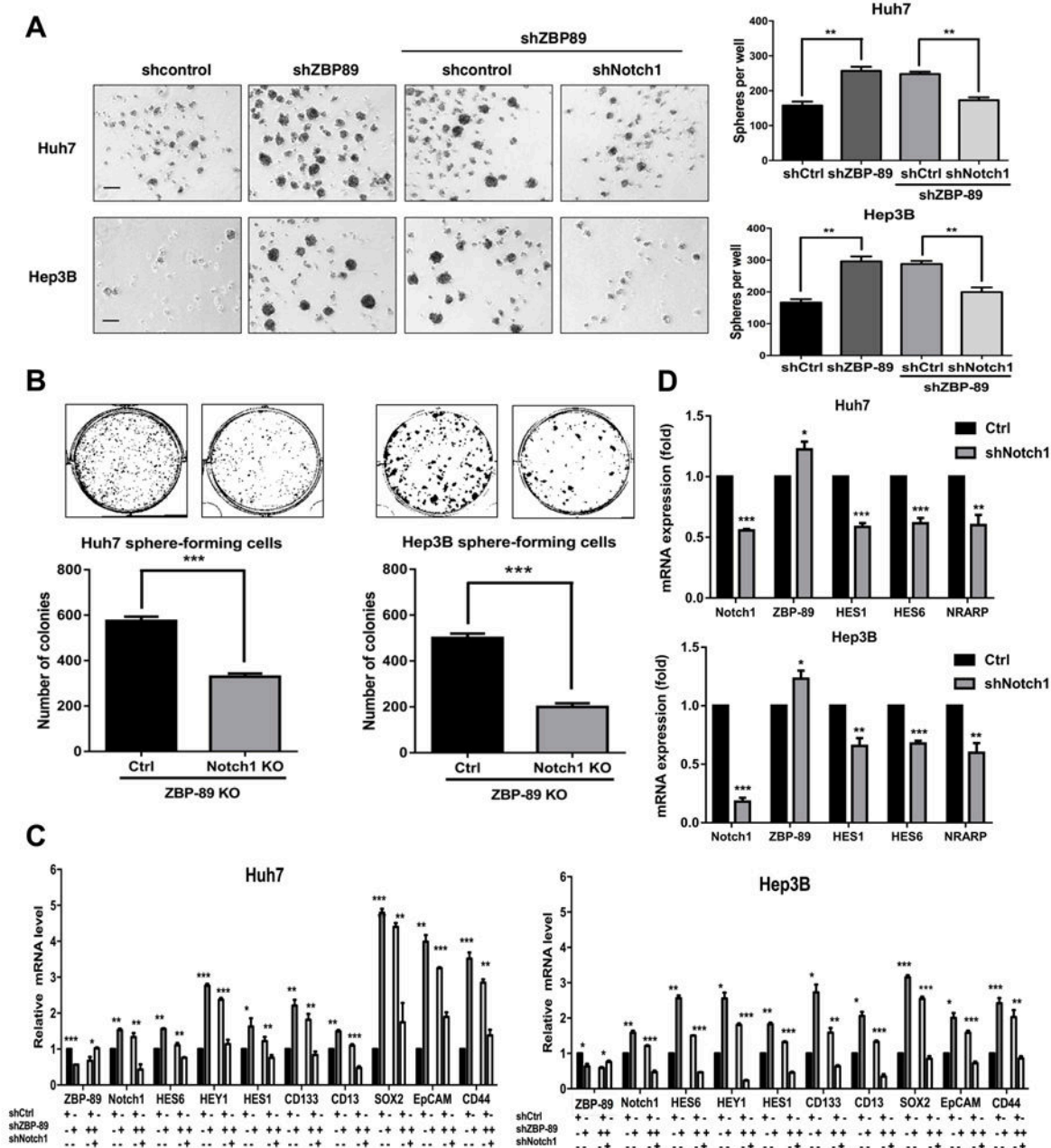


Fig. 4. ZBP-89 inhibits the expression of Notch target genes.

(A) Target genes involved in major stemness signaling pathways were examined in ZBP-89-overexpressing and control Huh7 enriched tumor spheres. ZBP-89 overexpression suppressed the Notch signaling pathway. (B) Immunoblotting further confirmed that HES1 was weakly expressed in tumor spheres generated by ZBP-89-overexpressing Huh7 (left panel) and Hep3B (right panel) cells. (C) HES1 expression was negatively correlated with the expression of ZBP-89 in both non-tumor ($r = -0.42$, $p < 0.0001$) and tumor ($r = -0.30$, $p < 0.0001$) samples by analyzing GSE14520. (D) The expression of ZBP-89 and NRARP

was analyzed in GEO database (GSE9843). HCC samples were divided into two subgroups according to their expression of ZBP-89. NRARP was negatively correlated with the expression of ZBP-89 (* $p < 0.05$). (E) The mRNA levels of Notch family members were measured in generated Huh7 and Hep3B tumor spheres. Notch1 mRNA levels were significantly decreased in both Huh7 and Hep3B tumor spheres when ZBP-89 was overexpressed (** $p < 0.001$). (F) The expression of Notch1 protein in Huh7 and Hep3B tumor spheres with ZBP-89 modification was analyzed by immunoblotting.

**Fig. 5.**

Notch1 is indispensable in ZBP-89-mediated regulation of liver cancer stemness. (A) Notch1 was stably knocked down in ZBP-89-silenced Huh7 and Hep3B cell lines using the lentivirus system. Notch1 deficiency suppressed the enhanced tumor-sphere forming capability induced by ZBP-89 depletion in Huh7 (** $p < 0.01$) and Hep3B (** $p < 0.01$) cells. (B) Secondary colony forming capabilities were inhibited by Notch1 depletion in ZBP-89-silenced Huh7 (** $p < 0.001$) and Hep3B (** $p < 0.001$) cells. (C) The expression of CSC markers and Notch1 target genes were analyzed by RT-qPCR in ZBP-89-knockdown and

control Huh7 and Hep3B enriched tumor spheres, along with Notch1-silenced and control cells with ZBP-89 deficiency. (D) The mRNA levels of ZBP-89 and Notch1 target genes were analyzed in Notch1-depleted HCC tumor spheres. ZBP-89 mRNA levels were significantly increased in both Huh7 and Hep3B tumor spheres when Notch1 was knocked down (* $p < 0.05$).

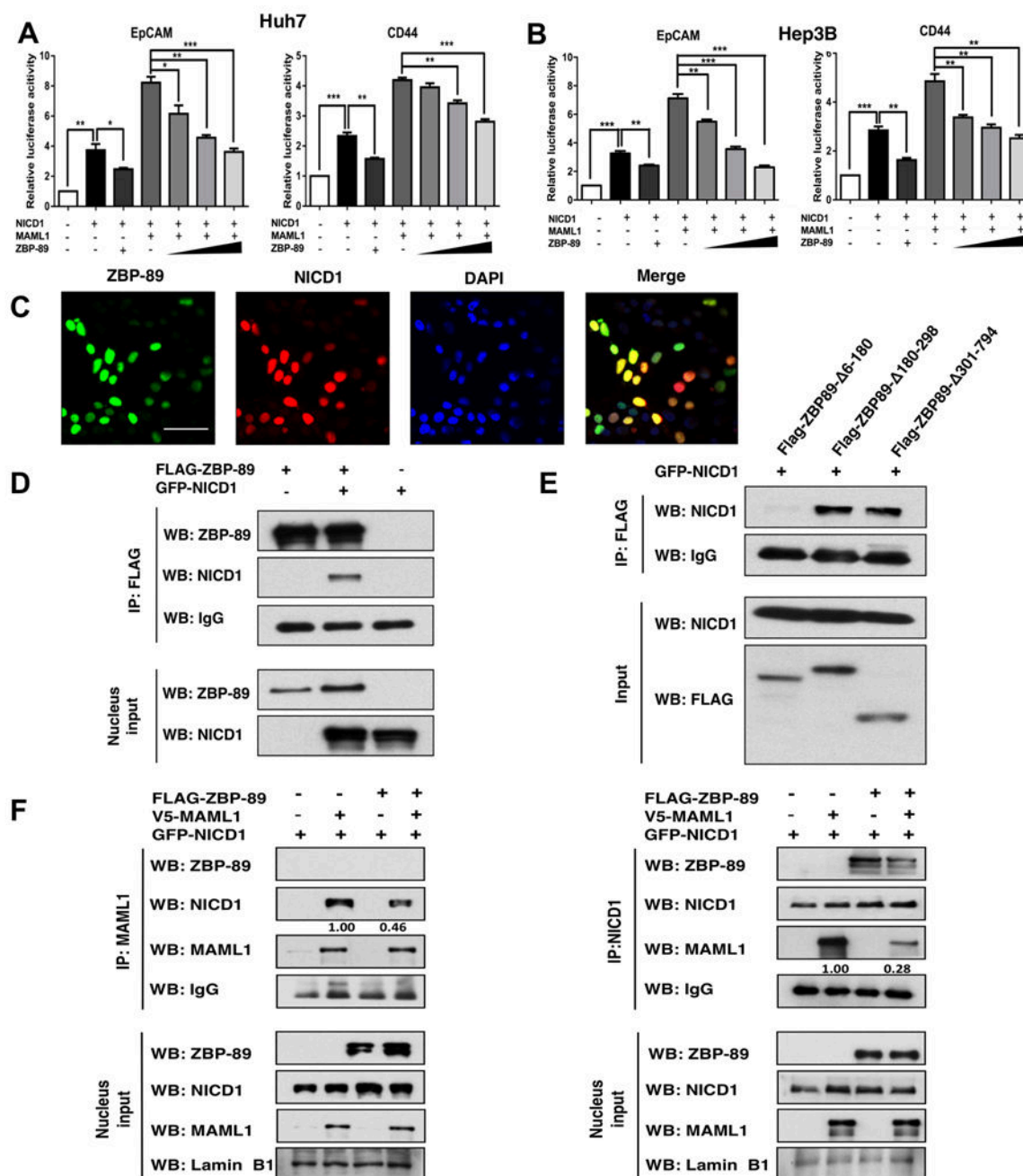


Fig. 6. ZBP-89 competitively binds to NICD1 and impedes the interaction between NICD1 and MAML1.

(A, B) Huh7 and Hep3B cells were transfected with ZBP-89, NICD1, MAML1 expression vectors and EpCAM or CD44 promoter reporters for 48h and analyzed for dual luciferase activity. ZBP-89 expression blocked the transcriptional activities of EpCAM and CD44 induced by NICD1 expression. The upregulation of ZBP-89 decreased the transcriptional activities of EpCAM and CD44 induced by NICD1 and MAML1 in a dose-dependent manner. (C) Representative images of ZBP-89 and NICD1 localizations in the nucleus. Huh7 cells transfected with ZBP-89 and NICD1 were stained with anti-ZBP-89 and anti-

NICD1 antibodies and observed by confocal microscopy. Scale bars: 40µm. (D) ZBP-89 bound to NICD1 directly in the nucleus. Co-immunoprecipitation showed the interaction between ZBP-89 and NICD1 in HEK-293T cells transfected with FLAG-ZBP-89 and NICD1. (E) Amino acids 6–180 of ZBP-89 were essential for the interaction between ZBP-89 and NICD1. Various deletions of ZBP-89 amino acids were co-transfected with NICD1 for co-immunoprecipitation. (F) ZBP-89 blocked the interaction between NICD1 and MAML1. HEK-293T cells were co-transfected with ZBP-89, NICD1 and MAML1 for co-immunoprecipitation. Co-IP results were quantified by ImageJ software. Lamin B1 was used as the nuclear loading control.