C-terminal truncated HBx protein activates caveolin-1/LRP6/β-catenin/FRMD5 axis in promoting hepatocarcinogenesis

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Abstract

Hepatitis B virus X protein mutants, particularly truncated at C-terminal (HBxΔC), generated during random viral integration, are frequently detected in hepatocellular carcinoma (HCC) and exert a more potent oncogenic effect than full-length form (FL). Here, we showed that caveolin-1 (Cav1), a robust metastasis promoter, is transcriptionally upregulated by HBxΔC but not by FL HBx. Promoting effect of HBxΔC in HCC cell aggressiveness is abolished when Cav1 is suppressed. Expression profiling identified FERM domain containing 5 (FRMD5) protein as a downstream target of Cav1. In accordance with the regulation of Cav1, HBxΔC upregulates FRMD5. Knockdown of FRMD5 in HBxΔC cells recapitulated the functional effect of Cav1 knockdown in HBxΔC cells. The regulation of FRMD5 by HBxΔC-induced Cav1 is mediated by the protein stablilization of LRP6 leading to the activation of β-catenin. Expression of a constitutively active β-catenin in Cav1 knockdown cells rescued FRMD5 expression and HCC tumorigenesis and metastasis. Clinical relevance of HBxΔC/Cav1/LRP6/FRMD5 pathway is demonstrated by the significant correlation of Cav1, LRP6 and FRMD5 expressions in HCC. The findings of this study uncover a novel HBxΔC-regulated molecular pathway which has profound implications in HCC therapeutics.

Highlights

- HBxΔC enhances HCC cell aggressiveness through upregulation of Cav1
- Cav1 stabilizes LRP6 protein leading to the enhancement of β-catenin-mediated transcription of FRMD5
- FRMD5 promotes HCC tumorigenesis and metastasis

Keywords

Metastasis, MicroRNA, Therapeutics, Biomarker, Protein stability

List of abbreviations

HCC, Hepatocellualr carcinoma; HBV, hepatitis B virus; HBx, HBV x protein; HBxΔC, C-terminal truncated HBx; Cav1, caveolin-1; FRMD5, FERM domain containing 5; LRP6, low density lipoprotein receptor related protein 6; EMT, epithelial-mesenchymal-transition; qPCR, quantitative polymerase chain reaction.

1. Introduction

Liver cancer is the second most common cause of cancer death worldwide, accounting for 9.1% of the total cancer mortality (World Health Organization, *Cancer: Fact Sheet*, 2012). Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. Chronic hepatitis B virus (HBV) infection accounts for half of the total HCC cases in which Asia has the highest incidence [1]. Although HBV is well established to be one of the major etiological factors of HCC, however, the development of HBV-associated HCC remains elusive. It is evident that the smallest protein, HBV x protein (HBx), encoded by HBV plays a crucial role. HBx is imperative in HBV infection and modulates multifaceted oncogenic pathways during HCC pathogenesis [2, 3].

Our previous study has shown that random HBV genome integration frequently results in 3'-deletion in HBx gene leading to the formation of C-terminal truncated HBx (HBxΔC).[4] In HBV-infected HCC, high prevalent of HBxΔC is found in 46-80% of the cases [5-7]. Functionally, HBxΔC has been shown to be more potent than full-length HBx (FL-HBx) to transform an immortalized normal liver cell line [6]. In *in vivo*, diethylnitrosamine-treated HBxΔC-transgenic mice displayed a more rapid onset of HCC than FL-HBx-transgenic mice under the same treatment [8]. These studies suggest that HBxΔC probably plays a more crucial role than full-length form in HCC development.

Caveolin-1 (Cav1) is the major scaffolding protein of caveolae which is involved in cellular transport. Apart from localizing at caveolae, Cav1 has been found in diverse cellular compartments and exerts caveolae-independent functions [9]. Compelling evidence has shown the tight association between Cav1 and human cancers. Cav1 is regarded as a tumor suppressor

in multiple malignancies [10]. Conversely, Cav1 acts an oncogene in advanced-stage cancers and is found to be highly expressed in metastatic tissues [11]. Indeed, Cav1 overexpression is associated with higher tumor stage, metastasis and worse prognosis in various carcinomas.[12] We and others demonstrated the crucial role of Cav1 in HCC metastasis and its clinical correlation with venous invasion [13, 14]. From the same perspective, other groups showed that Cav1 overexpression is significantly correlated with metastasis and worse prognosis in HCC [15, 16]. Cell motility is one of the critical components in metastasis. Cancer cell has to undergo sequential morphological changes in order to migrate. The crucial role of Cav1 in focal adhesion dynamics, cell adhesion, migration and invasion via diverse signalling cascades has been shown in different cellular contexts [17, 18]. Apart from cancer cell motility, Cav1 has been shown to modulate the apoptotic response, chemoresistance, angiogenesis and metabolism in different cancers [19-22].

Various studies have demonstrated the pivotal role of Cav1 in HCC, yet the understanding about the regulation of Cav1 expression is limited. In previous study, we found that intratumoral hypoxia in HCC elevates level of Cav1 in a hypoxia-inducible factor dependent manner and augments its ability to drive metastasis [23]. Here, we report another transcriptional regulation of Cav1 by HBxΔC. Significant clinical correlation between expressions of Cav1 and HBxΔC is observed. We also demonstrate the oncogenic activity of HBxΔC in HCC tumorigenesis and metastasis is indeed driven by Cav1. Mechanistically, the enhanced level of Cav1 results in the activation of Wnt/β-catenin pathway leading to the increased level and activity of FERM domain containing 5 (FRMD5). Knockdown of FRMD5 significantly diminishes the oncogenic capacity of Cav1 induced by HBxΔC. Although Cav1 has been reported to regulate epithelial-mesenchymal-transition (EMT) in HCC cells by inducing nuclear translocation of β-catenin [24], the mechanism by which Cav1 potentiates β-catenin activity remains unknown.

In this study, we show that Cav1 increases the protein stability of low density lipoprotein receptor related protein 6 (LRP6) leading to the accumulation and increased transactivating activity of β-catenin. Mutations in *CTNNB1*, *AXIN1* and *APC* are recurrently reported in HCC contributing to the activation of Wnt/β-catenin pathway [25]. Our study about the regulation of LRP6/β-catenin by Cav1 unravels a new regulatory mechanism in maintaining the aberrant activity of Wnt/β-catenin pathway in HCC. Based on the high incidence of HBxΔC in HCC and significant contribution of Wnt/β-catenin pathway in hepatocarcinogenesis, targeting HBxΔC-induced Cav1/LRP6/β-catenin/FRMD5 signaling pathway will have profound therapeutic implications in HCC.

2. Materials and Methods

2.1. Clinical samples

Clinical samples of HCC comprising primary tumor and the corresponding non-tumorous liver tissues were obtained at the time of surgical resection from patients at Queen Mary Hospital, Hong Kong. Fifty cases were randomly selected for the study. All specimens were snap-frozen in liquid nitrogen and kept at -80°C until use. The samples were provided by the Department of Surgery, The University of Hong Kong. The clinicopathological parameters are listed in Supplementary Table S1. The use of human samples was approved by the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB). Detailed Materials and Methods are available in the Supplementary Information.

2.2. Subcutaneous injection and orthotopic liver implantation

Stable clones were analysed for tumorigenicity by subcutaneous injection. For each stable clone, 1.5×10^6 cells were inoculated into the right flank of 4-week-old male BALB/c nude mice. Tumor size was monitored and estimated according to the following formula: volume = 1/2 (largest diameter) × (smallest diameter)^2. To obtain a tumor seed for orthotopic implantation, a 100-μl cell suspension (containing 5×10^6 cells) in PBS was subcutaneously injected into the flanks of male BALB/C nude mice at the age of 6 weeks. Mice were sacrificed two weeks later and the tumors were cut into approximately 1 mm^3 for successive orthotopic implantation into 6 week old male BALB/c nude mice. Mice were anesthetized and laparotomy was performed to expose the liver, and the tumor cube was inserted into a small hole formed by mechanical injury on the liver capsule using a needle. The tumor cube was then secured by suture. The abdominal wall was closed by suture. For tumors that were formed from luciferase-labeled cell lines, growth of the tumor was monitored by intraperitoneal injection of D-luciferin (Xenogen) (100 mg/kg animal) followed by bioluminescence detection using IVIS 100 Imaging System (Perkin Elmer, Waltham, MA, USA). Six to eight weeks after the orthotopic implantation, the mice were sacrificed and their lungs and livers were excised. The maximum diameter was taken as the tumor size for comparison among tumors. Weight of the excised tumors was also measured. Animals (Control of Experiments) Ordinance (Hong Kong) and animal experimentation guidance from The University of Hong Kong were strictly followed for all animal work performed.

3. Results

3.1. Cav1 is preferentially expressed in HBxΔC-associated HCC and its expression is regulated by HBxΔC

Various HCC cell lines (PLC/PRF/5, SMMC7721, BEL7402, MHCC97L and MHCCLM3) and an immortalized non-tumorigenic liver cell line (MIHA) were analyzed for the presence of FL-HBx and HBxΔC by RT-PCR using primers flanking HBx (Fig. 1A). PCR product amplified by primers 66F and 154R indicates the presence of full-length HBx in the cell. On the other hand, generation of a PCR product using primers 66F and 130R but not with primers 66F and 154R suggests the presence of HBxΔC. As shown in Fig. 1B, FL-HBx was present in PLC/PRF/5 while HBxΔC was found in MHCC97L and MHCCLM3. Coincidently, Cav1 was dramatically expressed in both MHCC97L and MHCCLM3. We further determined if the correlation of HBxΔC and Cav1 expressions was physiologically relevant. Out of 50 HCC cases, 22 cases (44%) were found to express HBxΔC and higher level of Cav1 (median: 22.06) when compared to cases with the presence of FL-HBx (median: 8.89) ($P < 0.0001$) (Fig. 1C).

One major cellular function of HBx is its transcriptional activation activity. To answer whether HBxΔC transcriptionally upregulates Cav1 expression, we examined the effect of two naturally occurring HBxΔC, HBx1-130 and HBx1-119 on Cav1 expression in HCC cells. Transient expression of HBx1-130 and HBx1-119 but not FL-HBx elevated expression of Cav1 transcript level in BEL7402 and SMMC7721 (Fig. 1D). The upregulation of Cav1 protein by HBx1-119 was well correlated with the transcriptional level (Fig. 1E). Hep3B has been reported to express HBxΔC [5], knockdown of HBx resulted in the suppression of Cav1 expression strengthened the causal relationship between HBx and Cav1 expressions (Fig. 1E).

Consistently, HBx1-130 and HBx1-119 but not FL-HBx were able to activate the promoter of Cav1 ($P < 0.01$) suggests that Cav1 is a transcriptional target of HBxΔC (Fig. 1F). Using 5' deletion mutants of Cav1 promoter, -737 to -639 region was found to be activated by HBxΔC but not FL-HBx (Supplementary Fig. S1). However, whether Cav1 is a direct or indirect target of HBxΔC needs further investigation.

3.2. HBxΔC enhances the migration, invasiveness and tumorigenicity of HCC cells

Among the two natural deletion mutants of HBx, HBx1-119 displayed a more prominent effect in elevating the expression of Cav1. Thus, we made use of doxycycline (dox)-inducible HBx119 cells to study the functional relevance of HBxΔC in SMMC7721 and PLC/PRF/5 (Fig. 2A). Dose and time dependent of inducible of HBx119 were achieved (Supplementary Fig. S2). Induced expression of HBx119 in SMMC7721 and PLC/PRF/5 significantly enhanced the cell migration ($P < 0.001$) and invasiveness ($P < 0.05$) (Fig. 2B and 2C). SMMC7721 HBx119 cells were subcutaneously injected into the flank of nude mice. Compared to the vector control group, mice injected with HBx119 cells had significant faster tumor development and formed bigger tumors ($P < 0.001$) (Fig. 2D and 2E). Furthermore, tumor development was also compared in mice treated with or without dox-containing drinking water (Fig. 2F). Mice supplied with dox-containing water formed significant bigger tumors than mice fed with water without dox ($P < 0.01$). IHC confirmed the expression of dox-induced HBx119 expression in the excised xenografts derived from HBx119 cells (Fig. 2E and 2F).

3.3. Promotion of HCC aggressiveness by HBxΔC is driven by Cav1

Our data showed that HBxΔC elevated the expression of Cav1 in HCC cells. Since Cav1 has been shown to play an imperative role in HCC tumorigenesis and metastasis [13],

we sought to determine whether HBx119-induced HCC aggressiveness was driven by the upregulation of Cav1. When Cav1 was suppressed in SMMC7721 HBx119 overexpressing cells (Fig. 3A), HBx119-induced cell migration and invasion were significantly diminished ($P < 0.001$) (Fig. 3B and 3C). In *in vivo*, HBx119-enhanced tumor formation was inhibited in Cav1 knockdown groups (HBx119/shCav1#1 and HBx119/shCav1#2) ($P < 0.001$) (Fig. 3D, left and middle panels). Suppressed Cav1 expression in dissected xenografts was confirmed by IHC (Fig. 3D). In orthotopic liver implantation model, bioluminescence imaging of animals implicated accelerated tumor development in HBx119 group when compared with vector control group. Significant reduction of luciferase signal was detected in HBx119/shCav1 groups ($P < 0.05$) (Supplementary Fig. S3). Indeed, significant bigger tumors were formed in livers of HBx119 animals when compared with the liver tumors of vector control group ($P < 0.01$) while smallest liver tumors were resulted in HBx119/shCav1 animals ($P < 0.01$) (Fig. 3E). Histological analysis showed that liver tumors of HBx119/shCav1 animals displayed bulging tumor growth front while liver tumors of vector control and HBx119 animals showed more invasive tumor contour (Fig. 3F).

3.4. Knockdown of FRMD5, a downstream target of Cav1, diminishes HCC tumorigenicity

Gene expression profiling (GEO accession number, GSE75994) identified FERM domain containing 5 (FRMD5) as one of the top listed targets of Cav1. When Cav1 was knocked down in MHCC97L in which Cav1 and HBxΔC are expressed, FRMD5 transcript and protein levels were significantly reduced ($P < 0.001$) (Fig. 4A). In other HCC cell lines, Huh7 and PLC/PRF/5, HBx119 but not FL-HBx was able to elevate the expression of FRMD5 ($P < 0.001$) (Fig. 4B). Suppression of FRMD5 in SMMC7721 HBx119 cells recapitulated the functional effect of Cav1 knockdown. Reduction of FRMD5 significantly

diminished the migratory and invasive potentials of cells ($P < 0.01$) (Fig. 4C and 4D). In animals, FRMD5 knockdown cells (HBx119/shFRMD5#1 and HBx119/shFRMD5#2) displayed significant reduced capacity to form tumors upon subcutaneous injection (Fig. 4E) as well as liver tumors in orthotopic liver implantation ($P < 0.01$) (Fig. 4F and Supplementary Fig. S3A). Similar to the effect of Cav1 knockdown in HBx119 cells, liver tumors with FRMD5 suppression displayed less invasive features as revealed by the H&E staining.

3.5. Cav1 activates canonical Wnt pathway by stabilizing LRP6 and consequently results in the elevation of FRMD5 expression

Next, we delineated the signaling pathway underlying the regulation of FRMD5 by Cav1. In SMMC7721 HBx119 cells, induced Cav1 level was detected with concomitant enhancement of low density lipoprotein receptor related protein 6 (LRP6), β-catenin and FRMD5 expressions. However, such upregulation was abrogated in HBx119/shCav1 cells (Fig. 5A). In another cell model, downregulation of LRP6, β-catenin and FRMD5 was detected when Cav1 was knocked down in MHCC97L (Fig. 5A). IHC demonstrated the corresponding downregulation of LRP6, β-catenin and FRMD5 was observed in tumors derived from Cav1 knockdown cells established in SMMC7721/HBx and MHCC97L (Fig. 5B). We then questioned how Cav1 regulates LRP6-β-catenin pathway. Co-immunoprecipitation revealed that Cav1 interacted with LRP6 (Fig. 5C). Treatment of cells using cycloheximide revealed that LRP6 was stabilized in SMMC7721 HBx119 cells. Knockdown of Cav1 partially diminished the stabilization of LRP6 suggesting Cav1 contributes to the protein stability of LRP6 (Fig. 5D). The activity of downstream effector of Wnt pathway, β-catenin was then analyzed by TOP/FOP luciferase reporter assay. The results showed that the luciferase signal was largely enhanced in HBx119 cells while decreased when Cav1 was suppressed indicating the transactivating activity of β-catenin is dependent on Cav1

($P < 0.001$) (Fig. 5E). We further delineated the potential role of β-catenin in the regulation of FRMD5 expression by a constitutively active β-catenin (Δ45β-cat) in which serine-45 was deleted [26]. This phospho-defective mutant is resistant to phosphorylation-mediated ubiquitination. Re-expression of Δ45β-cat but not WTβ-cat rescued the expression of FRMD5 at both the transcriptional and translational levels in SMMC7721 HBx119/shCav1 and MHCC97L/shCav1 cells (Fig. 5F).

3.6. Expression of β-catenin restores HCC aggressiveness caused by Cav1 knockdown in HBxΔC cells

Having demonstrated the causal relationship between β-catenin and FRMD5, we sought to examine the functional effect upon restoration of β-catenin in Cav1 knockdown cells. When Δ45β-cat was stably expressed in SMMC7721 HBx119/shCav1 cells, anchorage independent growth, cell migration and invasiveness attenuated by Cav1 knockdown were significantly rescued ($P < 0.001$) (Supplementary Fig. S5). Ability of HBx119 to drive metastasis was observed in SMMC7721 HBx119 cells (Supplementary Fig. S4B and S4C). In 1 out of 5 mice, metastatic foci were detected in lungs of the animals implanted with tumor seed of HBx119 cells. The metastatic MHCC97L was employed to study the role of HBx119-induced Cav1/β-catenin pathway in HCC tumorigenesis and metastasis. In accordance with the findings of SMMC7721 HBx119 cells, stable restoration of Δ45β-cat in MHCC97L/shCav1 cells partially rescued the anchorage independent growth ($P < 0.001$), cell migration ($P < 0.001$) and invasiveness ($P < 0.0001$) (Fig. 6A). In animals, overexpression of Δ45β-cat in MHCC97L/shCav1 cells resulted in accelerated subcutaneous and liver tumor formation (Fig. 6B, 6C and Supplementary Fig. S6) when compared to MHCC97L/shCav1 cells re-expressed with WTβ-cat or control vector. Besides restoring tumorigenesis, Δ45β-cat was able to drive distant metastasis to lungs. Bioluminescence imaging revealed

MHCC97L/HBx119 cells formed metastasis in all animals (5/5). Upon Cav1 knockdown, metastasis to lungs was completely abolished. Stable overexpression of Δ45β-cat in shCav1 cells increased incidence of metastasis to 4 of 5 mice (Fig. 6D and 6F). H&E staining of dissected liver showed that cells re-expressed with Δ45β-cat formed tumors with invasive tumor growth contour when compared to regular growth front of tumors derived from cells re-expressed with WTβ-cat and vector (Fig. 6E and 6F). Revealed by H&E staining, metastatic foci were found in lungs of shCTL control and shCav1/Δ45β-cat animals (Fig. 6D). Taken together, these results demonstrated that LRP6/β-catenin/FRMD5 axis driven by HBxΔC-induced Cav1 upregulation was critical for HCC tumorigenesis and metastasis.

3.7. Clinical relevance of Cav1/LRP6/FRMD5 signaling pathway in HCC

In the end, we examined whether Cav1-mediated LRP6/FRMD5 pathway is clinically relevant in HCC. In 19 pairs of human HCC and their corresponding nontumorous liver tissues, Cav1 was overexpressed in 12 cases. A higher percentage of tumor tissues with Cav1 overexpression showed overexpression of LRP6 than those showed neither expression nor overexpression of Cav1 ($P < 0.05$; Fig. 7B). To reveal the clinical relevant of Cav1 as a positive regulator of FRMD5, a significant positive correlation was found in HCC cases with the detection of both Cav1 and FRMD5 expressions by qPCR ($P < 0.05$; Fig. 7C). Consistently, a significant association between Cav1 and FRMD5 levels was found in TCGA data set of liver cancer ($P < 0.0001$).

4. Discussion

Independent studies have shown the high incidence of HBxΔC in HCC. Consistently, our results showed that HBxΔC was detected in 44% of our HCC cohort. HBxΔC is more prevalent in HCC than the corresponding non-tumorous tissues [5, 6, 27]. It is also significantly correlated with the presence of venous invasion and intrahepatic metastasis [5, 27]. These lines of evidence suggest that HBxΔC plays an imperative role in HCC development.

HBx protein is multi-functional, yet its functions remain elusive. Mounting evidence has shown the transactivation capacity HBxΔC contributes to its oncogenic capabilities. It acts directly by interacting with multiple transcription factors and basal transcriptional components, or indirectly by stimulating various signal transduction pathways [28]. HBxΔC suppresses expression of genes such as growth arrest-specific 2 and ubiquitin specific peptidase 16 and attenuates their activities in suppressing HCC cell proliferation and tumorigenesis [29]. HBxΔC also downregulates metastasis-suppressors results in enhancing invasion and metastasis in HCC [30]. On the contrary, HBxΔC induces the activity of wildtype matrix metalloproteinase 10 promoter with intact AP-1 binding sites to promoter HCC invasiveness [5]. It also exerts positive regulation in oncogenic miR-21 via interleukin-6 [31]. These findings support the notion that HBxΔC interacts with different proteins resulting in the formation of complex with opposing functional outcome. It is also important to note that HBxΔC and FL-HBx reveal conflicting effect in transcriptional regulation. Using miRNA profiling, it is found that HBxΔC decreases while FL-HBx increases the expression of a set of miRNAs with growth-suppressive functions [32]. Here, we show that HBxΔC but not FL-HBx upregulates expression of Cav1 and results in promoting HCC tumorigenesis and

metastasis. Our analyses reveal that Cav1 promoter region -737 to -639 is most responsive to HBxΔC activation. *Cis*-acting elements such as VBP, FKHRL, SWI/SNF are found in this region, nevertheless, whether and how HBxΔC interacts with the putative transcription factors to induced transcription of Cav1 require further investigations. Cav1 is consistently reported to be upregulated in HCC [13, 24]; contrastingly, Cav1 has been shown to be significantly downregulated in HBV-infected HCC tissues due to promoter hypermethylation. Transfection of HBx suppresses Cav1 promoter activity leading to reduced level of Cav1 [33]. However, our luciferase reporter assay did not reveal inhibition of Cav1 promoter by FL-HBx. Apart from transactivating property, C-terminal region is also required for ROS production and mitochondrial DNA damage which may play a role in HCC development.[34] Recently, HBxΔC has been reported to induce stemness genes and confer cancer and stem cell-like features in HCC [35, 36].

Aberrant activation of canonical Wnt/β-catenin pathway is one of the major drivers in HCC caused by multifaceted mechanisms [37]. Activation of Wnt/β-catenin pathway is partly contributed by mutations in *CTNNB1*, *AXIN1* and *APC* [25]. LRP6, a co-receptor of Wnt, has been reported to be upregulated in HCC by different mechanisms [38]. Downregulation of tumor suppressive miR-610 and miR-202 in HCC results in the enhanced level of LRP6 and activaiton of Wnt/β-catenin signaling [39, 40]. Overexpresison of Stearoyl-CoA desaturase is also responsible for the stablization of LRP6 mRNA in HCC [41]. Recently, LRP6 protein stability has been shown to be enhanced by its interaction with Cripto-1 or heparin-binding protein CCN2 [42, 43]. Formation of LRP6 protein complexes induce Wnt/β-catenin activation and increase stemness features of HCC. In prostate cancer cells, Cav1-LRP6 interaction stimulated by IGF-IR/IR activates integrated signaling in a β-catenin-independent manner [44]. Here, we reveal a new mechanism by which Cav1 overexpression, particularly

in metastatic tissues, contributes to the constitutive activation of canonical Wnt pathway by stabilization of LRP6 in HBxΔC related HCC.

Our study provides substantial findings about the clinical relevance and oncogenic effect of Cav1-induced enhancement of FRMD5 in HCC. We also reveal the elevation of FRMD5 is due to the activation of Wnt/β-catenin induced by stabilization of LRP6 by Cav1. In fact, there is a study revealed the transactivation of FRMD5 by β-catenin/TCF7L2 complex in colon cancer cells [45]. FRMD5 belongs to a family of FERM (4.1/ezrin/radixin/moesin) domain containing proteins [46] and its functions have not been fully defined. In lung cancer cells, knockdown of FRMD5 promotes cell migration and invasiveness and also elevates EMT markers. The same group reports the suppressive function of FRMD5 is mediated by its inhibition of ROCK1 [47, 48]. The clinical relevance of FRMD5 appeared to be ambiguous. Using available databases of gene expressions, FRMD5 is found to be downregulated in colorectal, breast and renal cancers relative to corresponding normal tissues [45, 48]. Nevertheless, FRMD5 is identified in high risk group of head and neck squamous cell carcinoma [49] and shown to be positively correlated with Cav1, a potent metastasis promoter, in HCC. Findings of FRMD5 gathered from different cancer model implicate its potential dual capacity as a suppressor and promoter in oncogenesis. It is not surprising that the cellular context, molecular basis and microenvironment in cancerous tissue orchestrate to critically determine the functional outcome of FRMD5 in different cancers.

Taken together, our findings demonstrate the clinical significance and functional role of HBxΔC in promoting tumorigenesis and metastasis in HCC through Cav1/LRP6/β-catenin/FRMD5 signaling axis (Fig. 7C). In addition, the stabilization of LRP6 induced by forming complex with Cav1 represents a new regulatory mechanism for the

activation of canonical Wnt pathway in HCC. More importantly, our study substantiates the potentiality of targeting Cav1 as a therapeutic intervention in HBxΔC-associated HCC patients.

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Conflicts of interest

The authors declare no potential conflicts of interest.

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Figure Legends

Figure 1. Correlation of HBxΔC and Cav1 in HCC cell lines and tissues. A, Schematic representation of HBx. Full-length HBx (FL-HBx) and C-terminal truncated HBx mutant (HBxΔC), HBx1-130 and HBx1-119 are shown. Locations of primer pairs flanking FL-HBx and HBxΔC used for the analysis are indicated. B, Detection of endogenous HBx in various HCC cell lines and an immortalized non-tumorigenic liver cell line MIHA by RT-PCR. Western blot analysis of endogenous Cav1 expression in HCC cell lines. MHCC97L and MHCCLM3 cells express truncated HBx showed the dramatic expression of Cav1. C, Fifty HCC clinical samples were analyzed for the presence of HBx and expression of Cav1 using qPCR. HCC clinical samples are grouped to categories of FL-HBx and HBxΔC. Cases with undetermined expression of HBx is categorized as ND. Expression of Cav1 was compared between FL-HBx and HBxΔC categories. D, HCC cells were transiently transfected with FL, 1-130 and 1-119 HBx. Expression of Cav1 of the transfected cells was analyzed by qPCR. E, Western blot analysis detected the expressions of Cav1 and HBx (Myc-tagged) of cells transfected with FL-HBx and HBxΔC (*left panel*). Hep3B was transiently transfected with siRNA against HBx (si-HBx) and subjected to western blot analysis of Cav1 expression and qPCR analysis of HBx expression (*right panel*). Cells transfected with scrambled siRNA (si-CTL) were included as control. F, HBx was coexpressed with Cav1 promoter luciferase reporter (flanking nucleotides -737 to -35 of Cav1) in HEK293 cells. Luciferase activity of Cav1 promoter was determined. $P < 0.05$ indicates statistically significant.

Figure 2. HBxΔC enhances HCC cell migration, invasiveness and tumorigenesis. A, Doxycycline (Dox)-induced expression of HBx 1-119 (Myc-tagged HBx119) in SMMC7721 and PLC/PRF/5 determined by immunoblotting. Cells induced with dox were subjected to (B)

migration assay and (C) invasion assay. Experiments were performed in triplicates. Results are expressed as mean ± SD of values. D, Subcutaneous injection (n = 5 per group) was performed with SMMC7721 dox-treated vector control and HBx119 cells. Tumor size was measured weekly during the course of experiment (*left panel*). E, Tumors formed were excised and weighed at the end of the experiment (*middle panel*). Representative IHC images of HBx expression in tumors of vector control and HBx119 cells (*right panel*). F, Subcutaneous tumor growth was compared between animals fed with or without dox-containing drinking water (n = 5 per group). At the end of experiment, tumors were excised and weighed (*left and middle panels*). IHC confirmed the expression of HBx in tumors of mice treated with dox (*right panel*). $P < 0.05$ indicates statistically significant.

Figure 3. Cav1 knockdown abolishes HBxΔC in promoting HCC cell aggressiveness. A, Cav1 expression was suppressed in SMMC7721 HBx119 by two shRNA sequences targeting Cav1. Silencing of Cav1 expression in HBx/shCav1#1 and HBx/shCav1#2 was confirmed by western blotting. Migration assay (B) and invasion assay (C) were performed on vector control, HBx119 and HBx/shCav1 cells. Experiments were performed in triplicates. Results are expressed as mean ± SD of values. D, Subcutaneous injection assay was performed (n = 5 per group) (*left panel*). Tumors were excised and weighed at the end of the experiment (*middle panel*). Representative IHC images of Cav1 expression in the excised tumors (*right panel*). E, Orthotopic liver implantation assay (n = 5 per group) was performed to reveal the growth of tumor in the liver of animals. Bioluminescence imaging of excised livers (*left panel*). Luciferase signal of liver tumors was plotted (*right panel*). F, H&E staining of liver tissues reveals the tumor growth front. Arrows indicate the invasive tumor contour. $P < 0.05$ indicates statistically significant.

Figure 4. FRMD5 is a functional effector of HBxΔC-induced Cav1. A, Cav1 was suppressed in MHCC97L in which Cav1 was highly expressed. Expression of Cav1 and FRMD5 was analyzed by qPCR (*left panel*) and western blotting (*right panel*). B, Huh7 and PLC/PRF/5 transiently transfected with vector, FL-HBx and HBx1-119 were subjected to qPCR (*left panel*) and immunoblotting analysis (*right panel*). C, FRMD5 level was suppressed in SMMC7721 HBx119 by two shRNA sequences targeting FRMD5. Reduced FRMD5 level in HBx/shFRMD5#1 and HBx/shFRMD5#2 was revealed by immunoblotting. D, Migration assay (*left panel*) and invasion assay (*right panel*) were performed on vector control, HBx119 and HBx/shFRMD5 cells. Experiments were performed in triplicates. Results are expressed as mean ± SD of values. E, Subcutaneous injection assay was performed (n = 5 per group). Tumors were excised and weighed at the end of the experiment (*left and middle panels*). Representative IHC images of suppressed FRMD5 expression in the excised tumors (*right panel*). F, Orthotopic liver implantation assay (n = 5 per group) was performed to reveal the growth of liver tumor. Bioluminescence imaging of dissected livers (*left panel*). Luciferase signal of liver tumors was plotted (*right panel*). H&E staining of liver tissues. Images show the liver-tumor boundary. Arrows indicate the invasive tumor contour. $P < 0.05$ indicates statistically significant.

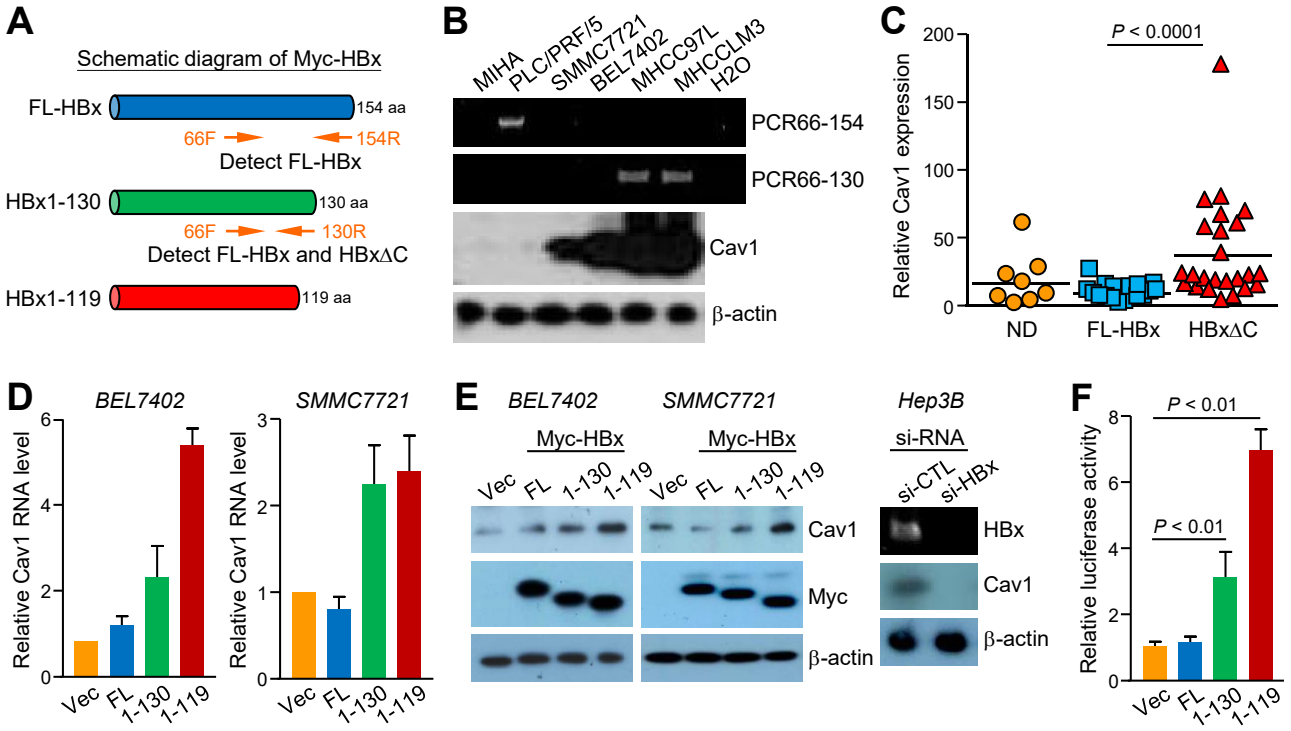
Figure 5. Cav1 stabilizes protein stability of LRP6 leading to the activation of β-catenin and expression of FRMD5. A, SMMC7721 HBx119/shCav1 (*left panel*) as well as MHCC97L shCav1 cells (*right panel*) and corresponding control cells were subjected to western blot analysis. B, IHC of tumors derived from Cav1 knockdown cells established in SMMC7721 HBx119 and MHCC97L. C, Coimmunoprecipitation revealed the interaction between Cav1 and LRP6. MHCC97L total cell lysate (TCL) was immunoprecipitated (IP) with anti-Cav1 antibody and immunoblotting with anti-LRP6 antibody. D, Cells were treated with

cycloheximide (CHX) for different time points (hr) and collected for immunoblotting of LRP6 (*left panel*). Relative expression of LRP6 in cells under CHX treatment compared to LRP6 in untreated cells was plotted (*middle panel*). E, TOP/FOP luciferase reporter was coexpressed with Renilla luciferase plasmid in SMMC7721 HBx and HBx/shCav1 cells. Luciferase activity was determined 24 hr post transfection and plotted. F, Wild-type (WTβ-cat) or constitutively active β-catenin (Δ45β-cat) was stably expressed in Cav1 knockdown cells established in SMMC7721 HBx119 and MHCC97L. Western blot analysis of stable clones revealed the expression of Cav1, β-catenin and FRMD5 (*left panel*). FRMD5 level in cells was determined by qPCR (*right panel*). Experiments were performed in triplicates. Results are expressed as mean ± SD of values. $P < 0.05$ indicates statistically significant.

Figure 6. Constitutively active β-catenin rescues HCC aggressive of Cav1 knockdown cells. A, Stable clones of wild-type (WTβ-cat) or constitutively active β-catenin (Δ45β-cat) established in MHCC97L Cav1 knockdown cells (shCav1) were subjected to soft agar assay (*left panel*), migration assay (*middle panel*) and invasion assay (*right panel*). Experiments were performed in triplicates. Results are expressed as mean ± SD of values. B, Subcutaneous injection assay was performed (n = 5 per group). Tumors were excised and weighed at the end of the experiment. C, Orthotopic liver implantation assay (n = 5 per group) was performed to reveal the growth of liver tumor. Bioluminescence imaging of dissected livers (*left panel*). Luciferase signal of liver tumors was plotted (*right panel*). D, Bioluminescence imaging of the dissected lungs. Representative images of H&E staining of lung tissues. Arrows indicate the presence of metastatic foci. E, H&E staining shows the liver-tumor boundary. Arrows indicate the invasive tumor contour. F, Summary of the aggressive features of the 4 experiment groups. $P < 0.05$ indicates statistically significant.

Figure 7. Clinical relevance of Cav1/LRP6/FRMD5 pathway in HCC. A, Expressions of Cav1 and LRP6 in human HCC (T) and corresponding non-tumorous (NT) liver tissues were determined by western blotting. Representative of HCC cases showing overexpression of Cav1 (*left panel*) and neither alteration nor expression of Cav1 (*right panel*) are shown. B, Percentage of HCC tissues with overexpression of Cav1 showing with or without LRP6 overexpression (n=19). C, In-house HCC tumorous and non-tumorous liver tissues were analyzed for Cav1 and FRMD5 expression by qPCR. Expression of Cav1 was plotted against expression of FRMD5 (*left panel*). Expressions of Cav1 and FRMD5 from TCGA dataset of liver cancer was plotted (*right panel*). D, In HCC cells, presence of HBxΔC promotes the transcription of Cav1. Enhanced Cav1 protein level contributes to the protein stability of LRP6 by forming a protein complex between Cav1 and LRP6. Stabilized LRP6 increases the level and activity of β-catenin leading to the enhanced transcription of FRMD5. FRMD5 plays critical role in HCC cell growth, motility, tumorigenesis and metastasis. $P < 0.05$ indicates statistically significant.

Figure 1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154 aa
66F
154R
Detect FL-HBx
HBx1-130
130 aa
66F
130R
Detect FL-HBx and HBxΔC
HBx1-119
119 aa
B
MIHA
PLC/PRF/5
SMMC7721
BEL7402
MHCC97L
MHCCLM3
H2O
PCR66-154
PCR66-130
Cav1
β-actin
C
P < 0.0001
Relative Cav1 expression
200
150
100
50
0
ND
FL-HBx
HBxΔC
D
BEL7402
SMMC7721
Relative Cav1 RNA level
Vec
FL
1-130
1-119
E
BEL7402
SMMC7721
Hep3B
Myc-HBx
Myc-HBx
si-RNA
Vec
FL
1-130
1-119
si-CTL
si-HBx
Cav1
Myc
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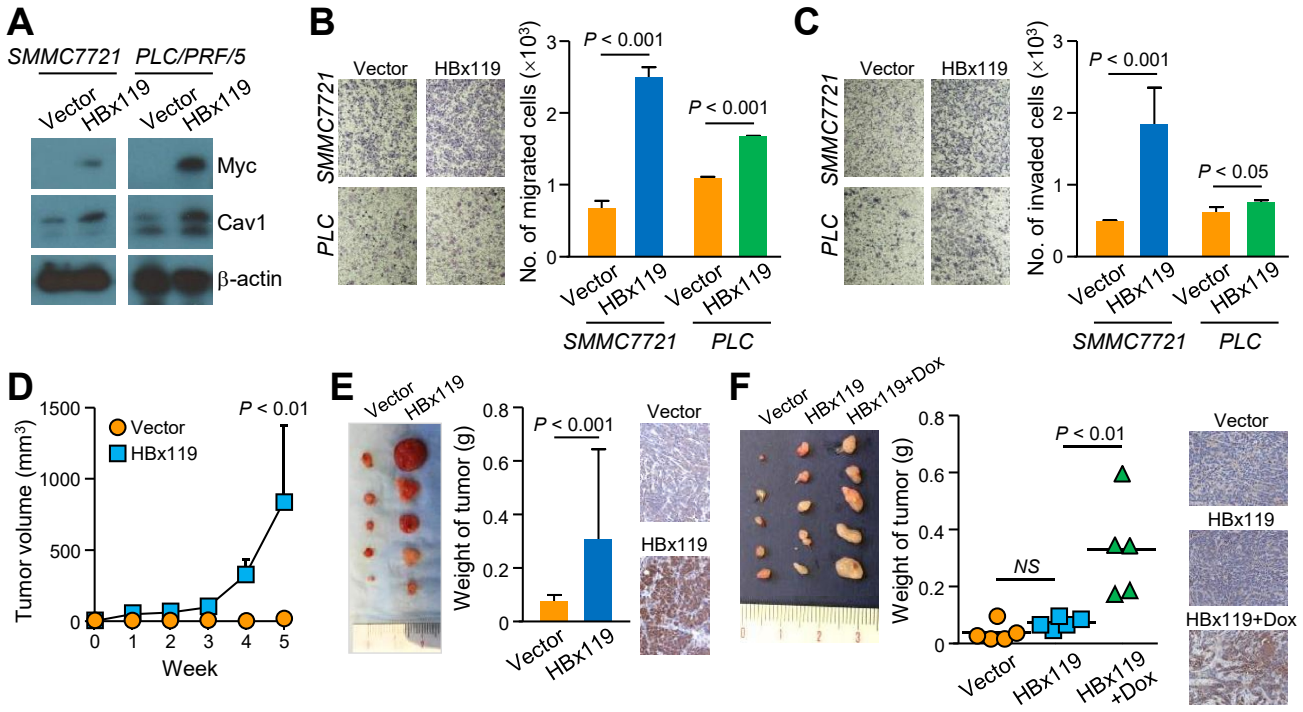
β-actin
F
P < 0.01
P < 0.01
Relative luciferase activity
Vec
FL
1-130
1-119

Figure 2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SMMC7721
PLC/PRF/5
Vector
HBx119
Vector
HBx119
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Cav1
β-actin
B
Vector
HBx119
SMMC7721
PLC
No. of migrated cells (×10³)
P < 0.001
P < 0.001
Vector
HBx119
Vector
HBx119
SMMC7721
PLC
C
Vector
HBx119
SMMC7721
PLC
No. of invaded cells (×10³)
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P < 0.05
Vector
HBx119
Vector
HBx119
SMMC7721
PLC
D
Tumor volume (mm³)
Vector
HBx119
P < 0.01
Week
E
Vector
HBx119
Weight of tumor (g)
P < 0.001
Vector
HBx119
Vector
HBx119
F
Vector
HBx119
HBx119+Dox
Weight of tumor (g)
P < 0.01
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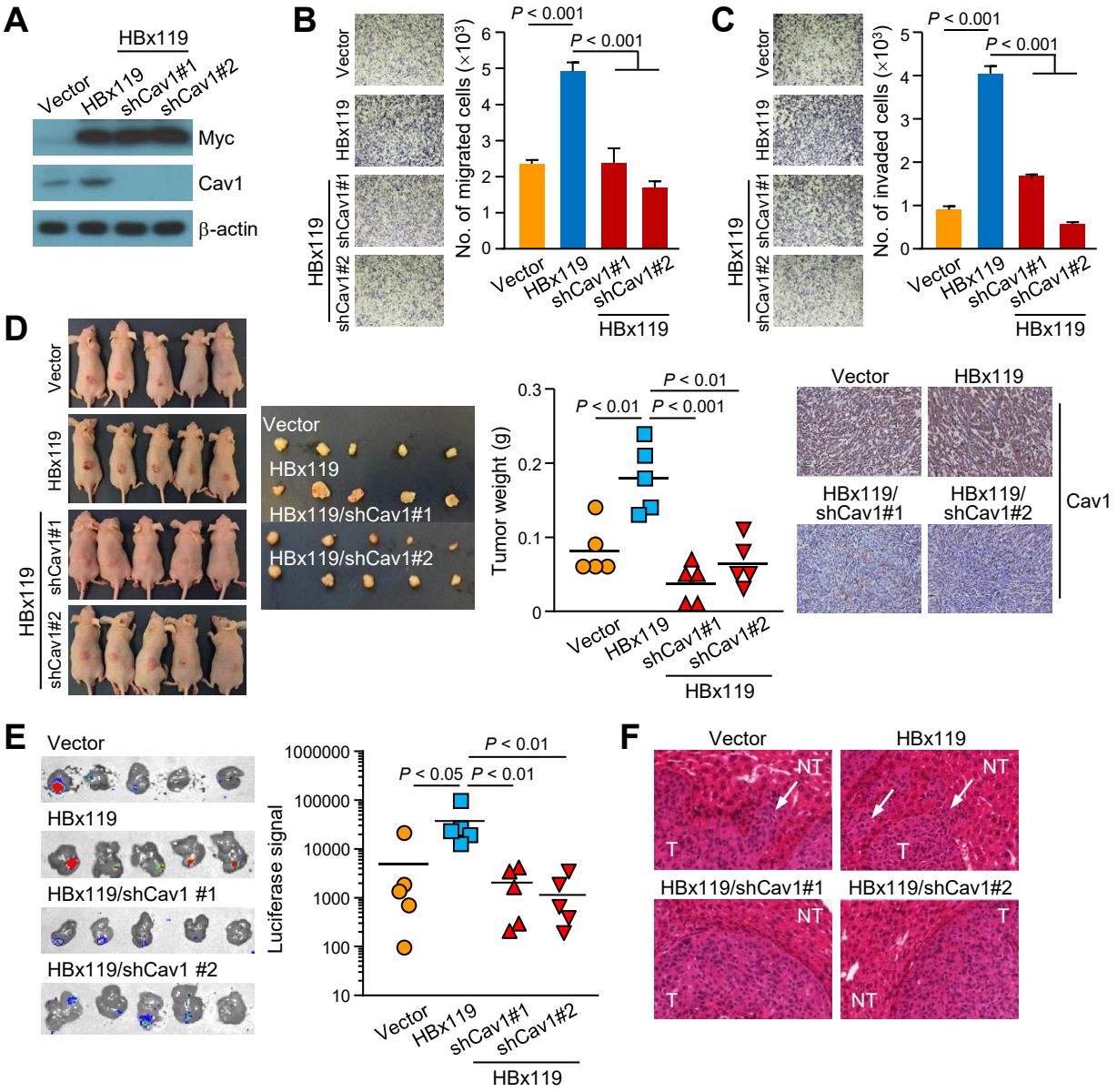
Vector
HBx119
HBx119+Dox
Vector
HBx119
HBx119+Dox

Figure 3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Cav1
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B
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shCav1#1
shCav1#2
HBx119
No. of migrated cells (×10³)
P < 0.001
P < 0.001
HBx119
C
No. of invaded cells (×10³)
P < 0.001
P < 0.001
HBx119
D
Vector
HBx119
HBx119/shCav1#1
HBx119/shCav1#2
Tumor weight (g)
P < 0.01
P < 0.01
P < 0.001
HBx119
Cav1
HBx119/ shCav1#1
HBx119/ shCav1#2
E
Vector
HBx119
HBx119/shCav1 #1
HBx119/shCav1 #2
Luciferase signal
P < 0.01
P < 0.05
P < 0.01
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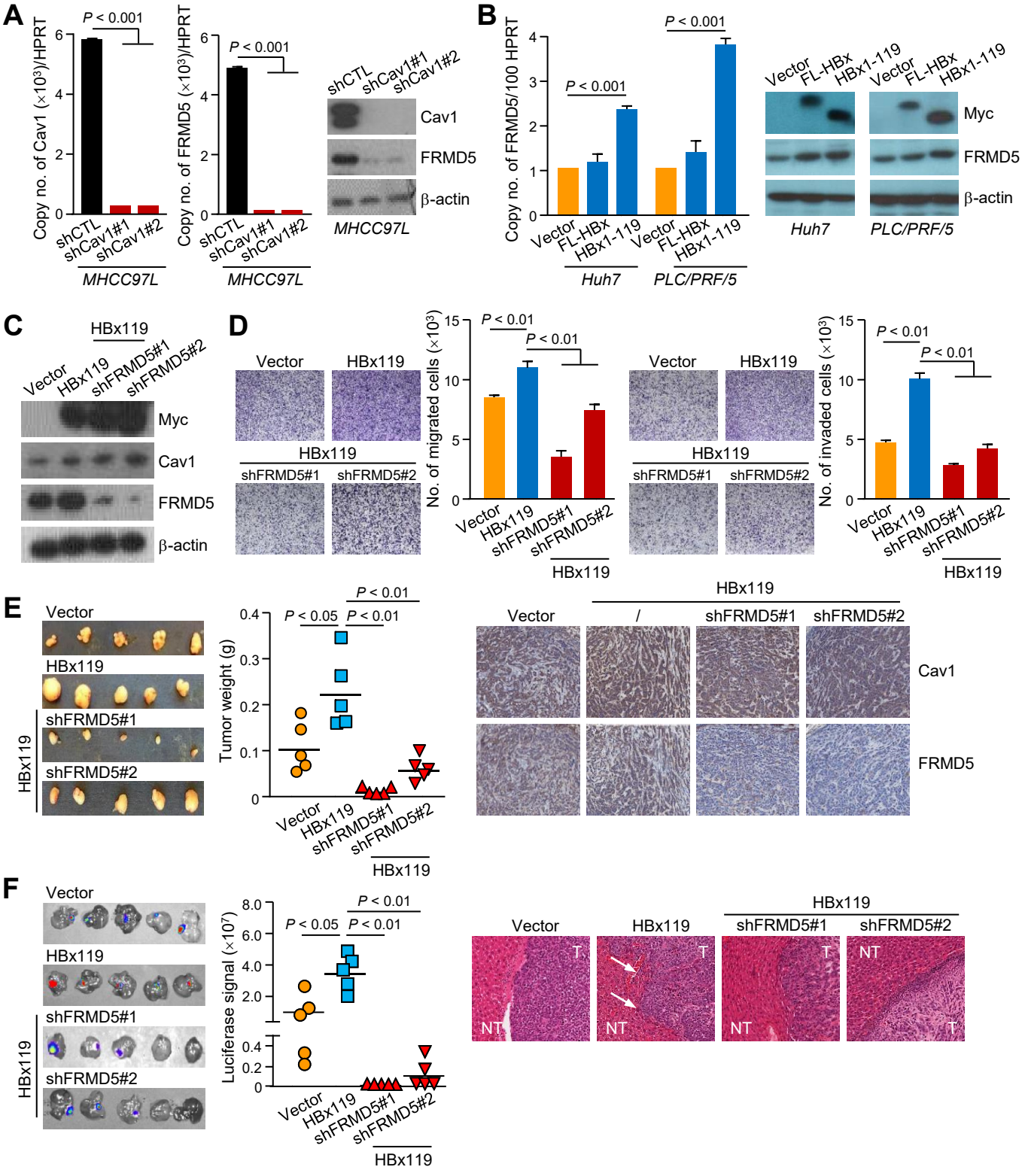
F
Vector
HBx119
NT
T
HBx119/shCav1#1
HBx119/shCav1#2

Figure 4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shCav1#1
shCav1#2
MHCC97L
shCTL
shCav1#1
shCav1#2
Cav1
FRMD5
β-actin
MHCC97L
B
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P < 0.001
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HBx1-119
Vector
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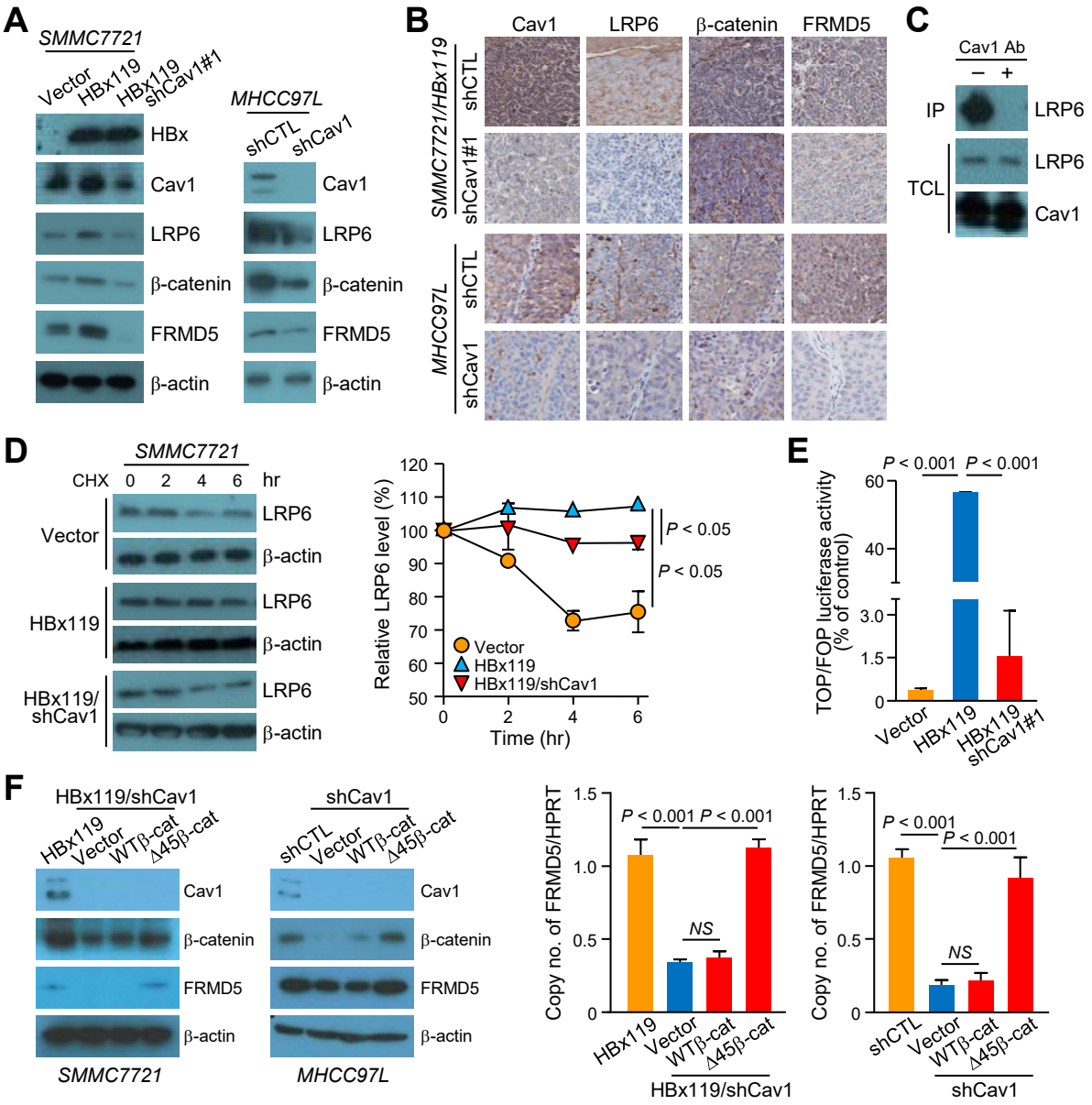
HBx1-119
Huh7
PLC/PRF/5
Vector
FL-HBx
HBx1-119
Vector
FL-HBx
HBx1-119
Myc
FRMD5
β-actin
Huh7
PLC/PRF/5
C
HBx119
Vector
HBx119
shFRMD5#1
shFRMD5#2
Myc
Cav1
FRMD5
β-actin
D
Vector
HBx119
HBx119
shFRMD5#1
shFRMD5#2
P < 0.01
P < 0.01
No. of migrated cells (×10³)
Vector
HBx119
shFRMD5#1
shFRMD5#2
HBx119
Vector
HBx119
HBx119
shFRMD5#1
shFRMD5#2
P < 0.01
P < 0.01
No. of invaded cells (×10³)
Vector
HBx119
shFRMD5#1
shFRMD5#2
HBx119
E
Vector
HBx119
shFRMD5#1
shFRMD5#2
HBx119
P < 0.01
P < 0.05
P < 0.01
Tumor weight (g)
Vector
HBx119
shFRMD5#1
shFRMD5#2
HBx119
HBx119
Vector
/
shFRMD5#1
shFRMD5#2
Cav1
FRMD5
F
Vector
HBx119
shFRMD5#1
shFRMD5#2
HBx119
P < 0.01
P < 0.05
P < 0.01
Luciferase signal (×10⁷)
Vector
HBx119
shFRMD5#1
shFRMD5#2
HBx119
HBx119
Vector
HBx119
shFRMD5#1
shFRMD5#2
T
NT

Figure 5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LRP6
β-catenin
FRMD5
β-actin
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shCTL
shCav1
Cav1
LRP6
β-catenin
FRMD5
β-actin
B
Cav1
LRP6
β-catenin
FRMD5
SMMC7721/HBx119
shCTL
shCav1#1
MHCC97L
shCTL
shCav1
C
Cav1 Ab
– +
IP
LRP6
TCL
LRP6
Cav1
D
SMMC7721
CHX 0 2 4 6 hr
Vector
LRP6
β-actin
HBx119
LRP6
β-actin
HBx119/ shCav1
LRP6
β-actin
Relative LRP6 level (%)
120
110
100
90
80
70
60
50
0 2 4 6
Time (hr)
Vector
HBx119
HBx119/shCav1
P < 0.05
P < 0.05
E
P < 0.001 P < 0.001
TOP/FOP luciferase activity (% of control)
60
40
3.0
1.5
0
Vector
HBx119
HBx119 shCav1#1
F
HBx119/shCav1
HBx119
Vector
WTβ-cat
Δ45β-cat
Cav1
β-catenin
FRMD5
β-actin
SMMC7721
shCav1
shCTL
Vector
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Δ45β-cat
Cav1
β-catenin
FRMD5
β-actin
MHCC97L
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1.0
0.5
0
P < 0.001 P < 0.001
NS
HBx119
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WTβ-cat
Δ45β-cat
HBx119/shCav1
Copy no. of FRMD5/HPRT
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1.0
0.5
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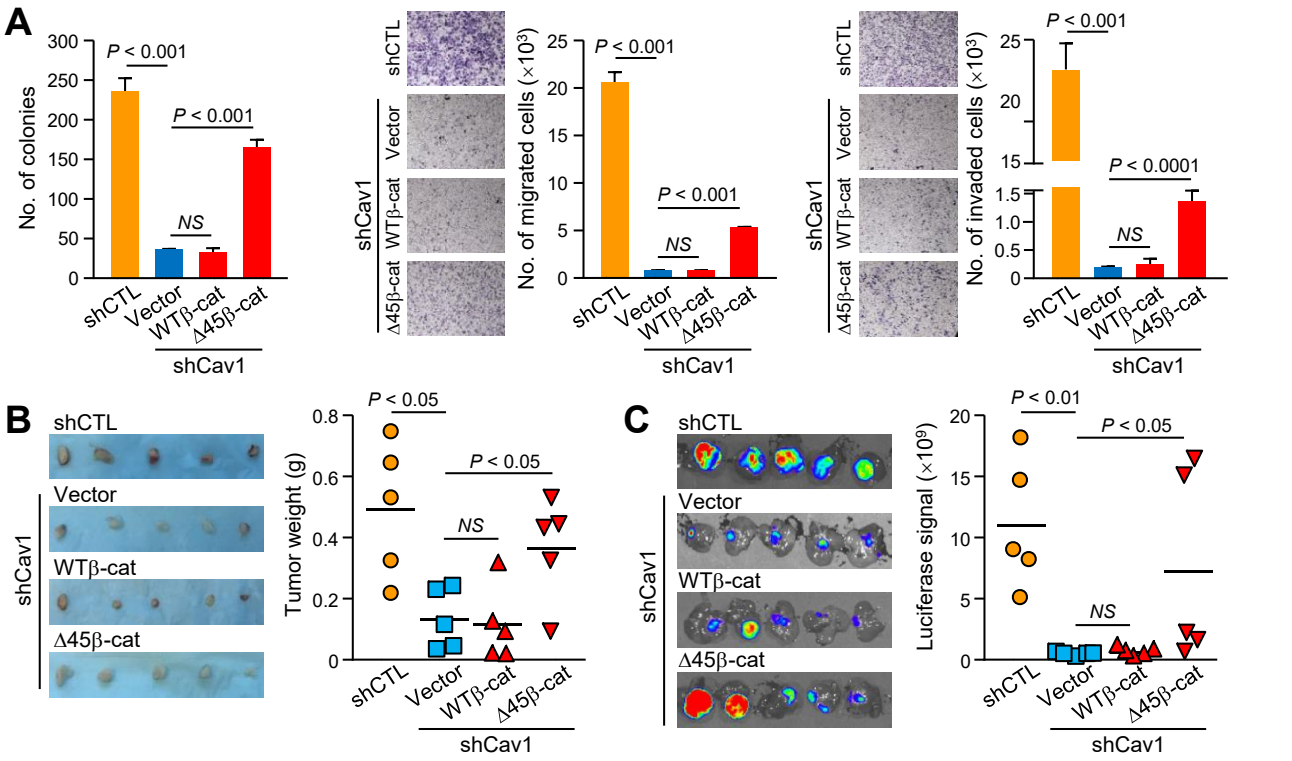
shCTL
Vector
WTβ-cat
Δ45β-cat
shCav1

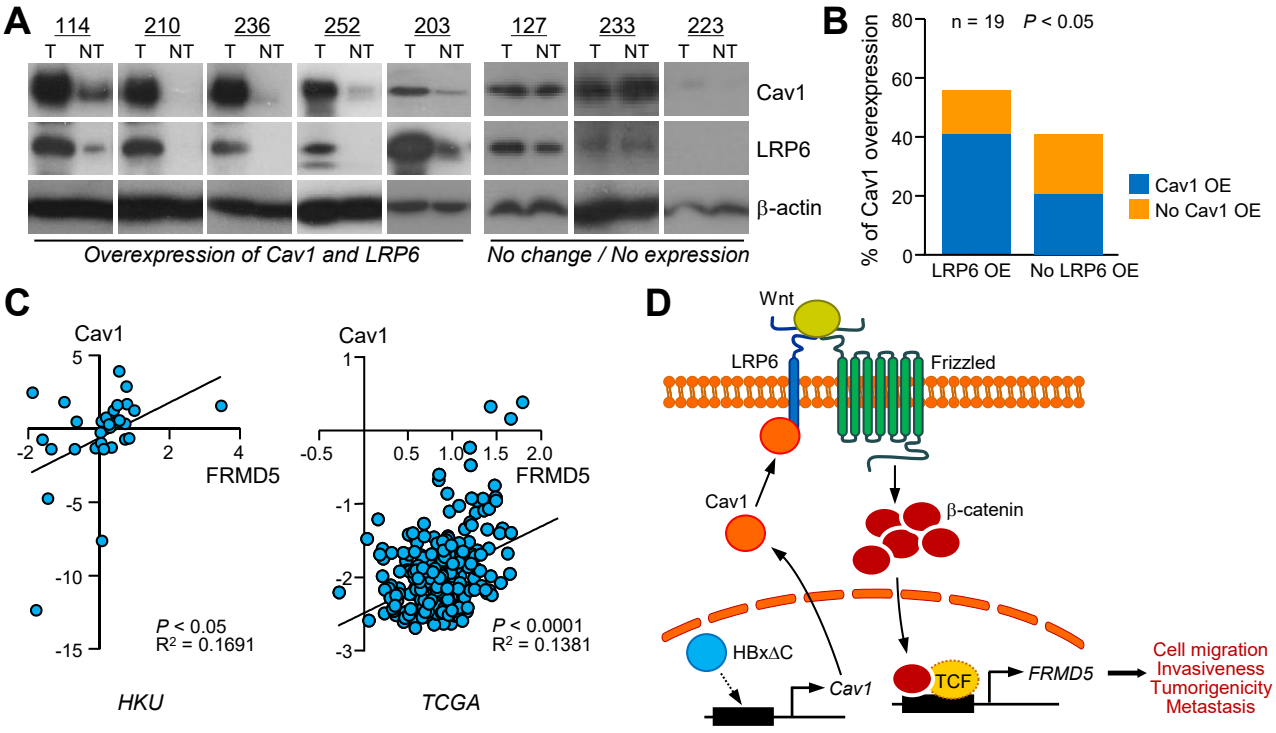
Figure 6



F

Group	Tumor growth front	Incidence of metastasis
shCTL	Irregular, invasive	5/5
shCav1/Vector	Regular, bulging	0/5
shCav1/WTβ-cat	Regular, bulging	0/5
shCav1/Δ45β-cat	Irregular, invasive	4/5

Figure 7


A
114
210
236
252
203
127
233
223
T NT
Cav1
LRP6
β-actin
Overexpression of Cav1 and LRP6
No change / No expression
B
n = 19 P < 0.05
% of Cav1 overexpression
80
60
40
20
0
LRP6 OE
No LRP6 OE
Cav1 OE
No Cav1 OE
C
Cav1
FRMD5
P < 0.05
R2 = 0.1691
HKU
P < 0.0001
R2 = 0.1381
TCGA
D
Wnt
LRP6
Frizzled
Cav1
β-catenin
HBxΔC
TCF
FRMD5
Cell migration
Invasiveness
Tumorigenicity
Metastasis