

# INPP5F translocates into cytoplasm and interacts with ASPH to promote tumor growth in hepatocellular carcinoma

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## Research

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# Abstract

## Background

Increasing evidence has suggested inositol polyphosphate 5-phosphatase family contributes to tumorigenesis and tumor progression. However, the role of INPP5F in hepatocellular carcinoma (HCC) and its underlying mechanisms is unclear.

## Methods

The expression of INPP5F in HCC was analyzed in public databases and our clinical specimens. The biological functions of INPP5F were investigated *in vitro* and *in vivo*. The molecular mechanism of INPP5F in regulating tumor growth were studied by transcriptome-sequencing analysis, mass spectrometry analysis, immunoprecipitation assay and immunofluorescence assay.

## Results

High expression of INPP5F was found in HCC tissues and was associated with poor prognosis in HCC patients. Overexpression of INPP5F promoted HCC cell proliferation, and vice versa. Knockdown of INPP5F suppressed tumor growth *in vivo*. Results from transcriptome-sequencing analysis showed INPP5F not only regulated a series of cell cycle related genes expression (c-MYC and cyclin E1), but also promoted many aerobic glycolysis related genes expression. Further study confirmed that INPP5F could enhance lactate production and glucose consumption in HCC cells. Mechanistically, INPP5F interacted with ASPH through which INPP5F activated Notch signaling and subsequently promoted the expression of c-MYC and cyclin E1. Interestingly, INPP5F was commonly nuclear-located in cells of adjacent tissues, while cytoplasmic-located was more common in HCC cells. LMB (nuclear export inhibitor) treatment restricted INPP5F in nucleus and was associated with inhibition of Notch signaling and cell proliferation. Furthermore, sequence of nuclear localization signals (NLSs) and nuclear export signals (NESs) in INPP5F aminoacidic sequence were identified. Alteration of the NLSs or NESs influenced the localization of INPP5F and the expression of its downstream molecules.

## Conclusion

These findings indicate that INPP5F functions as an oncogene in HCC via a translocation mechanism and activating ASPH-mediated Notch signaling pathway. INPP5F may serve as a potential therapeutic target for HCC patients.

## Background

Hepatocellular carcinoma (HCC) is the sixth-most common cancer worldwide and the third-leading cause of cancer-related deaths<sup>1</sup>. The incidence and mortality rates for HCC are still increasing<sup>2</sup>. However, the effect of current therapeutic approaches remain to be satisfied. Therefore, understanding the molecular

mechanisms of HCC tumorigenesis and progression may help to improve the therapeutic outcomes for HCC patients.

Previous studies have revealed that deregulation of phosphatases contributes to tumorigenesis and progression of HCC<sup>3-4</sup>. Because of the relative feasibility of developing small molecule inhibitors to block phosphatases<sup>5</sup>, studies focus on the role of such phosphatases may provide clinical significance for the management of HCC. Our previous studies have reported a series of phosphatases that play important roles in HCC. For instance, we found PRL-1 promotes HCC cells migration and invasion through endothelial-mesenchymal transition induction<sup>6</sup>. On the other hand, PRL-3 facilitates HCC progression by co-amplifying with FAK on chromosome 8q24.3 and enhancing FAK phosphorylation through upregulating TGFB1 expression(unpublished).

Inositol polyphosphate 5-phosphatases are another kind of phosphatases, which are involved in regulating phosphorylation of phosphoinositide and associated with in a series of human pathologies such as the Lowe syndrome, the Joubert and MORM syndromes and several type of cancers<sup>7-11</sup>. Inositol polyphosphate 5-phosphatases contain 10 different isoenzymes and several splice variants in the human genome. Inositol polyphosphate-5-phosphatase F (INPP5F, also known as Sac 2) is a member of inositol polyphosphate 5-phosphatases, which has been demonstrated to hydrolyze different type of phosphoinositide and exhibit different functions, such as regulating endocytic recycling and attenuating heart hypertrophy<sup>12-13</sup>. Recently, INPP5F is also reported to play an important role in the occurrence and progression of malignant tumor, although the role is inconsistent. In gliomas, INPP5F is a potential tumor suppressor. Downregulation of INPP5F may lead to gliomagenesis<sup>14</sup>. While in chronic lymphocytic leukemia, overexpression of INPP5F is associated with chemoresistance<sup>15-16</sup>.

So far, the clinical significance of INPP5F and its role in HCC is unclear. In this study, we sought to assess the expression and clinical significance of INPP5F in HCC patients, as well as explore the underlying mechanisms of how INPP5F functions in HCC.

## Methods And Materials

### Patients and clinical samples

Eighty-eight HCC samples were collected for determination of mRNA levels of INPP5F from Sun Yat-Sen Memorial hospital (SYSMH). The samples were obtained from the HCC patients who undergone hepatic resection between Mar 2015 and Feb 2016. These sample were frozen and stored in liquid nitrogen until further analysis. In addition, a cohort of 232 paraffin-embedded HCC cases diagnosed between Jan 2010 to Dec 2013 at SYSMH was recruited. The HCC and para-tumor tissue of these patients were collected immediately after surgery, stored in formalin and then made into tissue microarray. The patients' clinical and prognostic data were acquired from the specimen library of Department of hepatobiliary surgery (SYSMH). There was no relation between RNA samples and paraffin-embedded cases. Histological examination was used to confirm the diagnosis in all patients. None of the patients had received

radiotherapy or chemotherapy before surgery. The study protocol was approved by the Ethics Committee of SYSMH. Informed consent was obtained from each patient.

### Immunohistochemistry (IHC)

The tissue microarray was subjected to deparaffinization and dehydration. After antigen retrieval, H<sub>2</sub>O<sub>2</sub> treatment and non-specific antigens blocking, the slides were incubated with monoclonal mouse anti-human INPP5F (1:200, Abcam, ab236391) at 4 °C. After overnight incubation, the slides were incubated with secondary antibody, followed by DAB staining. The expression levels were scored as previously describe<sup>17</sup>. The total scoring of the tissue microarray was independently completed by two pathologists who had no knowledge of the patients' clinical data. We defined the case as high expression if the total score was greater than 4 points, otherwise defined it as low expression.

### Glucose consumption and lactate production assays.

Cells were cultured to 40% confluency and then changed with fresh culture medium. After 24 h, the culture medium was collected, and measurement of glucose consumption and lactate production was performed using kits from Biovision (USA, catalogue nos. ab136955 and ab65331) according to the manufacturer's instructions.

### Animal Model

For xenograft model,  $5 \times 10^6$  MHCC-97H cells stably expressing luciferase and control or INPP5F shRNA and were subcutaneously injected into the left flank of male BALB/c nude mice aged 3–4 weeks. Four weeks later, mice were monitored by bioluminescence with the IVIS imaging system (Xenogen, MA, USA). And then the mice were sacrificed, tumor weight and size were measured. Volumes were calculated using the following formula: Volume (mm<sup>3</sup>) = [width<sup>2</sup> (mm<sup>2</sup>) × length (mm)]/2. To further detect the effect of INPP5F on tumor growth, negative control or INPP5F shRNA MHCC-97H cells ( $2 \times 10^6$ ) were orthotopically injected under the liver capsular of NOD/SCID mice. Mice were monitored using the IVIS200 imaging system. All animal procedures were in accordance with the National Institutes of Health guide for the care and use of laboratory animals and approved by the Animal Ethical and Welfare Committee of Sun Yat-Sen University.

### Co-immunoprecipitation Assay And Mass Spectrometry

FLAG-INPP5F and HA-ASPH plasmids were transfected into Huh7 cells. Crude cell lysate was prepared 72 h after transfection. The protein complex interacting with FLAG-INPP5F was obtained using Pierce™ Coimmunoprecipitation Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Mass spectrometry analysis of immunoprecipitant was performed by the Medical Research Center of SYSMH. Co-immunoprecipitation and western blot were used for validating the interacted protein identified by mass spectrometry analysis.

### Construction Of Mutation And Truncations

NLSs and NESs in INPP5F aminoacidic sequence were predicted by bioinformatic tools (NLS: [http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS\\_Mapper\\_form.cgi](http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), NES: <http://www.cbs.dtu.dk/services/NetNES> ). When constructing the NESs mutation, we changed the leucine in the predicted NESs to alanine in order to make INPP5F lose the nuclear export ability. The three truncations were constructed through deletion of different lengths of aminoacidic sequence in the predicted NLSs to facilitate INPP5F transport out of the nuclear. The mutation and truncation sequences were cloned into the plasmids pcDNA3.1 (Igebio company, Guangzhou, China).

## Statistical Analysis

All data analysis was performed using SPSS version 25.0. Student's t test and Chi-square test were used to analyze quantitative data and categorical data, respectively. Kaplan–Meier analyses and log-rank test were used for survival analysis. The cox proportional hazards regression model was used to verify the independent risk factors based on the variables selected in univariate and multivariate analysis. P value < 0.05 were considered to be statistically significant.

# Results

## Increased INPP5F expression predicts poor clinical outcome in HCC patients

To investigate the potential role of INPP5F in human HCC pathogenesis, we firstly carried out an analysis of Oncomine database. We found that the mRNA expression of INPP5F was upregulated in HCC tissues (Fig. 1A). This result was validated by TCGA-LIHC cohort (Fig. 1B). Moreover, Kaplan-Meier analysis using the TCGA-LIHC cohort revealed that patients with higher INPP5F expression exhibited a relatively poorer overall survival (Fig. 1B). We further validated these results using data from our center. QRT-PCR results revealed that mRNA level of INPP5F was frequently upregulated in HCC tissues compared with the corresponding para-tumor tissues (Fig. 1C). IHC staining in 232 pairs of HCC specimens showed consistent results (Fig. 1D). Further analysis showed that high expression of INPP5F was associated with large tumor size, poor tumor differentiation and cirrhosis (Table S1). Patients with high INPP5F expression had shorter overall survival than those with low INPP5F expression (Fig. 1E). Multivariate analyses using Cox regression model revealed INPP5F as an independent prognostic factor for overall survival in HCC patients (Table S2). Similarly, the expression of INPP5F was also significantly higher in HCC cell lines than immortalize hepatocyte LO2 cells (Fig. 1F and G). Taken together, our data suggest an oncogenic role of INPP5F in HCC.

## INPP5F promotes HCC cell proliferation in vitro and in vivo

The biological function of INPP5F in HCC was next investigated. Because the above results showed significantly high expression of INPP5F in HCC with large tumor size, we first focused on the association between INPP5F and the proliferation ability of HCC cells. INPP5F expression was knocked down in SK-Hep1 and MHCC-97H cells, and overexpressed in Huh7 cells (Fig. 2A and B, Fig. S1A and B). INPP5F knockdown reduced the EdU positive cells, while overexpression of INPP5F led to an increased effect

(Fig. 2C, Fig. S1C). Consistent results were observed from colony formation assays (Fig. 2D). Moreover, INPP5F downregulation arrested HCC cells at G1 phase, while INPP5F overexpression reduced G1 phase (Fig. 2E, Fig. S1D). We also explored the effect of INPP5F on HCC cells migration and invasion. However, transwell assays and wound-healing assay all showed that INPP5F had no significant impact on tumor migration and invasion (Fig. S1E-G). These findings indicate that INPP5F is a proliferation-promoting factor in HCC and this effect may through affecting G1/S phase transition.

To confirm the above in vitro findings, we first employed subcutaneous xenograft model using MHCC-97H cells with stable INPP5F knockdown (Fig. 3A). The volume and weight of tumors formed in INPP5F knockdown group were significantly decreased compared with those formed in control group (Fig. 3B-D). H&E and IHC staining showed less necrosis and Ki67 expression in INPP5F knockdown group (Fig. 3E). We then established orthotopic tumor models by orthotopically injecting indicated cells under the liver capsular of mice. Consistently, INPP5F downregulation effectively inhibited the growth of orthotopic xenograft tumors in mice livers (Fig. 3F). Taken together, these results suggest that INPP5F exerted its oncogenic role probably by promoting proliferation of HCC cells.

### INPP5F upregulates the expression of c-MYC and cyclin E1 in HCC

Given the findings above, we sought to explore the molecular mechanism underlying INPP5F-mediated tumor growth. Firstly, we compared whole-genome transcriptome between SK-Hep1-Ctrl and SK-Hep1-shINPP5F through RNA-seq, and obtained Differentially Expressed Genes (DEGs) via statistical analysis. By using 1.5-fold change as the cut-off point, we found 200 upregulated DEGs and 218 downregulated DEGs (Fig. S2A and B). Gene Ontology (GO) analysis showed that there were 59 DEGs related to cell growth, including 29 genes associated with cell cycle (Fig. 4A). Since the above findings suggested that INPP5F may affect cell proliferation by regulating G1/S phase transition, we focused on the G1/S-related genes. Among the G1/S-related gene list, c-MYC and cyclin E1 are well-known genes tightly correlated with G1/S phase transition<sup>18-19</sup>. Accordingly, INPP5F knockdown significantly decreased the mRNA expression of c-MYC and cyclin E1 in SK-Hep1 and MHCC-97H, whereas overexpression of INPP5F increased both genes expression in Huh7 cells (Fig. 4B). Results from western blot further supported the regulation of c-MYC and cyclin E1 by INPP5F (Fig. 4C). Furthermore, knockdown of c-MYC and cyclin E1 significantly inhibited the INPP5F-enhanced cell proliferation (Fig. 4D). Thus, our data suggest that INPP5F regulates HCC cells G1/S phase transition and proliferation through c-MYC and cyclin E1.

### INPP5F Enhances Aerobic Glycolysis Of HCC Cells

Besides proliferation-related genes, many genes previously reported to be associated with aerobic glycolysis were also found in the DEGs list (Fig. 4A). We selected hexokinase 2 (HK2), Hypoxia-inducible factor 1-alpha (HIF1A), glycolytic enzymes glucose transporter 1 (GLUT1) as well as GLUT3 for further validation. INPP5F knockdown decreased the expression of these genes (Fig S2C). In contrast, overexpression of INPP5F upregulated these gene' expression (Fig. S2C). We speculated that INPP5F is also associated with aerobic glycolysis in HCC. Indeed, we observed the cell culture media of INPP5F

knockdown cells was redder than the control cells (Fig. S2D), suggesting there may be lower lactate concentration in the media of INPP5F knockdown cells. We further detected the lactate production and glucose consumption in INPP5F overexpression and knockdown cells. The lactate production and glucose consumption were reduced after INPP5F was knocked down, but increased when INPP5F was overexpressed (Fig. S2E and F). Collectively, these preliminary data suggest that INPP5F enhanced aerobic glycolysis of HCC cells.

### INPP5F Activates Notch Signaling Pathway Via Interacting With ASPH

We then designed to identify how INPP5F regulates the expression of its downstream molecules in HCC. Results from immunofluorescence indicated that INPP5F was mainly located in cytoplasm of HCC cells (Fig. S3A). Cytoplasmic signaling such as AKT-mTOR and STAT3 pathway have been reported to be downstream of INPP5F<sup>20</sup>. However, we found that INPP5F did not regulate the activation of AKT-mTOR and STAT3 signaling in HCC cells (Fig. S3B). We therefore employed immunoprecipitation combined with mass spectrometry to explore interacting proteins potentially mediating the biological function of INPP5F in HCC. Results showed that aspartate- $\beta$ -hydroxylase (ASPH) was a potential interacting protein of INPP5F (Fig. 5A). The interaction between INPP5F and ASPH was confirmed by co-immunoprecipitation (Fig. 5B). Further study showed that INPP5F did not alter the expression of ASPH at both mRNA and protein levels (Fig. S3C and D), suggesting INPP5F may affect the function of ASPH. Previous reports showed that the Notch pathway is downstream signaling of ASPH<sup>21-22</sup>. Coincidentally, c-MYC and cyclin E1 are important targets of the Notch signaling pathway<sup>23-24</sup>. Thus, the effect of INPP5F on the activation of Notch signaling was determined. Western blot showed that the expression of Notch 1 intracellular domain (NICD) as well as its downstream HES1 and HEY1 was decreased in HCC cells with INPP5F knockdown, whereas increased in cells with INPP5F overexpression (Fig. 5C). Moreover, upon treatment of ASPH siRNA, INPP5F-mediated Notch pathway activation and c-MYC and cyclin E1 upregulation were dramatically suppressed (Fig. 5D and E). Functionally, knockdown of ASPH significantly attenuated INPP5F-enhanced cell proliferation, G1/S phase transition (Fig. 5F and G) and aerobic glycolysis (Fig. S3E and F). Together, these data suggest that INPP5F activates Notch signaling pathway in HCC via interacting with ASPH, leading to cell proliferation and aerobic glycolysis.

### INPP5F Translocates Into Cytoplasm To Exhibit Its Oncogenic Activity

When evaluating the IHC staining of INPP5F, we surprisingly found that INPP5F was commonly nuclear-located in cells of adjacent tissues, while in HCC tissues, cytoplasmic staining was more common (Fig. 6A and S4A). In addition, nuclear-positive staining of INPP5F in adjacent tissues was associated with better prognosis in HCC patients (Fig. S4B). The different sub-cellular localization of INPP5F between HCC and adjacent tissues prompted us to hypothesize that cytoplasmic translocation is important for INPP5F to display its oncogenic function. To explore this hypothesis, we firstly detected whether translocation of INPP5F existed in HCC cells. We used leptomycin B (LMB) to inhibit nuclear export. Both immunofluorescence and western blot indicated that INPP5F was restricted in nucleus after LMB treatment (Fig. 6B and C). Moreover, the nuclear restriction of INPP5F by LMB was associated with

inhibition of Notch signaling and cell proliferation (Fig. 6D and Fig. S4C). We then employed bioinformatic tools to predict nuclear localization signals (NLSs) and nuclear export signals (NESs) in INPP5F aminoacidic sequence. According to the prediction, one mutant with the potential NESs mutation (retained the NLSs) and three truncations with the potential NLSs deletion (retained the NESs) were constructed (Fig. 6E). Immunofluorescence and western blot showed that the NESs mutant restricted INPP5F in nucleus, while truncation 2 and 3 but not truncation 1 limited INPP5F in cytoplasm (Fig. 6F and G), suggesting the NESs sequence is responsible for the nuclear export of INPP5F and NLSs, probably located in 83 to 113 of the INPP5F aminoacidic sequence, is related to the nuclear import of INPP5F. We found that compared with the NESs mutant, all the truncations significantly upregulated the colony formation, lactate production and glucose consumption of HCC cells, especially truncation 2 and 3 (Fig. S4D-F). Furthermore, results from both western blot (Fig. 6H) and QRT-PCR (Fig. S4G) showed that the expression of NICD, HES1, HEY1, c-MYC and cyclin E1 was significantly upregulated by truncation 2 and 3, while NESs mutant had no effect on the expression of these molecules. Taken together, our data suggested there exists a translocation mechanism of INPP5F in HCC cells. Cytoplasmic translocation is important for INPP5F to display its oncogenic function in HCC.

## Discussion

Inositol polyphosphate 5-phosphatases are a group of phosphatases involved in regulating phosphorylation of phosphoinositide. Increasing evidence has suggested inositol polyphosphate 5-phosphatases contribute to tumorigenesis and tumor progression. For instance, depletion of INPP5J reduces cell migration and invasion by regulating AKT1 signaling in breast cancer<sup>9</sup>. Loss of INPP5A expression predicts poor overall survival in recurrent and metastatic disease of cutaneous squamous cell carcinoma<sup>10</sup>. INPP5E is reported to promote Sonic Hedgehog medulloblastoma progression via a phosphoinositide signaling axis at cilia<sup>11</sup>. In this study, we focused on INPP5F. We found that INPP5F is overexpressed in HCC tissues and cell lines. High expression of INPP5F predicts poor prognosis in patients with HCC. Mechanically, we identified ASPH is an interacting protein of INPP5F. INPP5F promotes HCC cell proliferation, aerobic glycolysis and activating Notch-c-MYC/cyclin E1 pathway through ASPH. Furthermore, we found that the oncogenic function of INPP5F in HCC is dependent on its cytoplasmic translocation (Fig. 7). Thus, our data indicate that INPP5F is an oncogene in HCC. However, whether the oncogenic mechanism of INPP5F in HCC is link to its inositol-phosphatase activity still needs further investigation in future.

ASPH is a member of the  $\alpha$ -ketoglutarate-dependent dioxygenase family. It can catalyze the hydroxylation of aspartyl and asparaginy residues in the EGF-like domains of various proteins, such as Notch1 and JAG2, leading to activation of Notch signaling pathway<sup>25-26</sup>. Overexpression of ASPH has been reported in more than 20 tumor types<sup>27</sup>. Indeed, recent study has revealed that ASPH is highly expressed in HCC and is one of the major activators of Notch pathway, playing an important role in HCC progression<sup>22, 28</sup>. In this study, we identified INPP5F as an interactor of ASPH in HCC. INPP5F activates Notch pathway and enhances cell growth and aerobic glycolysis through ASPH. Besides, knockdown of INPP5F inhibited

Notch signaling without affecting ASPH expression. These data suggest INPP5F may also be necessary for ASPH to activate Notch pathway. Our results not only explore the oncogenic role of INPP5F in HCC, but also improve our understanding of how ASPH functions in HCC.

Our data showed that INPP5F not only promotes cell proliferation, but also enhances aerobic glycolysis of HCC cells. Aerobic glycolysis, also known as the Warburg effect, is a general feature of glucose metabolism in cancer cells<sup>29</sup>. Unlike normal cells, cancer cells prefer glycolysis for ATP production, even under aerobic conditions<sup>30</sup>. Along with ATP production, aerobic glycolysis also generates various of metabolic intermediates which are essential for the rapid growth of tumor cells<sup>31</sup>. Thus, the enhancement of aerobic glycolysis by INPP5F could subsequently provide substrates for anabolic pathways and finally promotes cell proliferation. Furthermore, either Notch pathway or c-Myc alone have been reported to enhance the aerobic glycolysis of cancer cells<sup>32-33</sup>. Hence, there may exist an INPP5F-dependent network connecting cell proliferation and aerobic glycolysis in HCC cells, leading to HCC progression.

Another interesting finding of our study is the diverse sub-cellular localization of INPP5F between adjacent tissues and HCC tissues. Shuttling of specific proteins out of nucleus is essential for the regulation of intracellular signaling and can influence the biological function of tumor cells<sup>34</sup>. Actually, nuclear-cytoplasmic shuttling is a targetable process<sup>35</sup>. XPO1 is the main mediator of nuclear export in many cell types. Regulating the sub-cellular localization of NESs and NLSs-containing oncogenes is the major function of XPO1 in malignant tumors<sup>36</sup>. Recently, one of XPO1 inhibitors, selinexor, has been approved by FDA for multiple myeloma patients with a refractory disease<sup>37</sup>. In the current study, we noticed the nuclear-cytoplasmic shuttling of INPP5F could be impaired by LMB, a small molecule inhibitor of XPO1, accompanied by inhibition of Notch pathway and cell proliferation, suggesting XPO1 inhibitor may also perform its anti-tumor effect through regulating INPP5F in HCC. In addition, our data showed that NESs mutation affected nuclear export and led to nuclear accumulation of INPP5F as LMB treatment did. Meanwhile, NLSs deletion led to cytoplasmic localization of INPP5F, providing an opportunity to bind to ASPH and thereby activate the Notch pathway to exert its cancer-promoting function. Thus, mutating the NESs and/or retaining the NLSs of INPP5F may be another strategy for targeting INPP5F in HCC. Future mechanistic studies may shed light on additional functions of the cytoplasmic and nuclear INPP5F in oncogenic transformation, making it a more attractive target for anti-tumor therapies.

## Conclusions

In summary, the present study identifies a novel oncogenic role of INPP5F in HCC. The upregulation of INPP5F predicts poor prognosis in HCC patients. Mechanically, we found that INPP5F connects HCC cell proliferation and aerobic glycolysis through translocating into cytoplasm and activating ASPH-mediated Notch signaling. Suppressing the cytoplasmic translocation of INPP5F in HCC cells inhibit its oncogenic function. Thus, our data indicated that INPP5F, as a newly identified nuclear-cytoplasmic shuttling protein, may be a potential therapeutic target for HCC.

# Abbreviations

INPP5F: Inositol polyphosphate-5-phosphatase F; ASPH: Aspartate- $\beta$ -hydroxylase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HE: Hematoxylin-eosin; DAB: Diaminobenzidine; DMEM: Dulbecco Modified Eagle Medium; PVDF: polyvinylidene fluoride; PBS: phosphate buffer saline; BSA: bovine serum albumin; NOD/SCID: non-obese diabetic/severe combined immune deficiency; DAPI: 4',6-diamidino-2-phenylindole;

# Declarations

## Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Sun Yat-Sen Memorial hospital (SYSMH). Informed consent was obtained from each patient. All experimental procedures involving animals were in accordance with the National Institutes of Health guide for the care and use of laboratory animals and approved by the Animal Ethical and Welfare Committee of SYSU (DD-17-0906).

## Consent for publication

Not applicable.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

## Competing interests

All authors read and approved the final version of the manuscript. The authors declare that they have no competing interests

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## Authors' contributions

The final version of the manuscript has been read and approved by all authors. Q.Z. carried out most of the experiments and drafted the manuscript. Q.L. and J.L. performed the bioinformatics analysis,

analyzed the experiment data and revised the manuscript. Y.Y., W.Y. and R.C. performed the Tumorigenesis assay and metastasis assay in nude mice. C.H., J.W. and J.Z. collected the clinical samples. Q.Z. and Z.X. collected the clinical data and managed the experimental design. K.M., Z.Z. and Z.X. reviewed and revised the manuscript, and provided funding support for the experiments.

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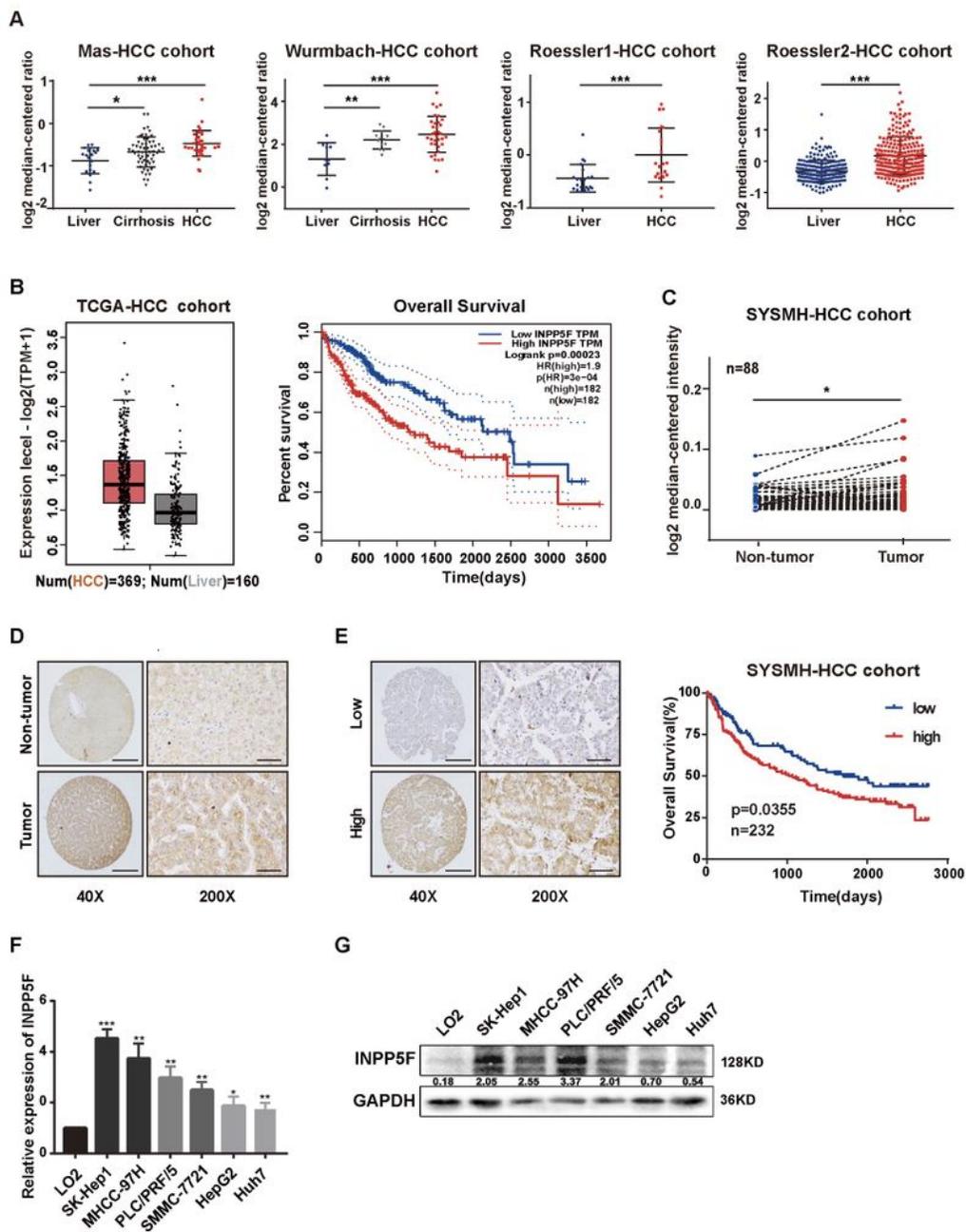
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## Figures



**Figure 1**

INPP5F expression is elevated in HCC and associated with poor clinical outcome. (A) HCC datasets from Oncomine database (<https://www.oncomine.org>) showed increase of INPP5F mRNA in HCC samples. (B) Analysis of TCGA-HCC dataset (<http://gepia.cancer-pku.cn>) confirmed the increase of INPP5F mRNA in HCC samples (left). Kaplan-Meier analysis of the overall survival using the TCGA-LIHC cohort according to INPP5F expression (right). (C) The mRNA levels of INPP5F in 88 pairs of HCC and adjacent non-tumor

tissues were measured by QRT-PCR. (D) The expression of INPP5F in 232 pairs of HCC and adjacent non-tumor tissues was detected by IHC. (E) The clinical significance of INPP5F expression in overall survival was confirmed in SYSMH-HCC cohort by Kaplan-Meier survival analysis. (F) QRT-PCR and (G) western blot compared INPP5F expression levels in different HCC cell lines and immortalized hepatocyte LO2 cells. ALL \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. Scale bar: 100  $\mu$ m.

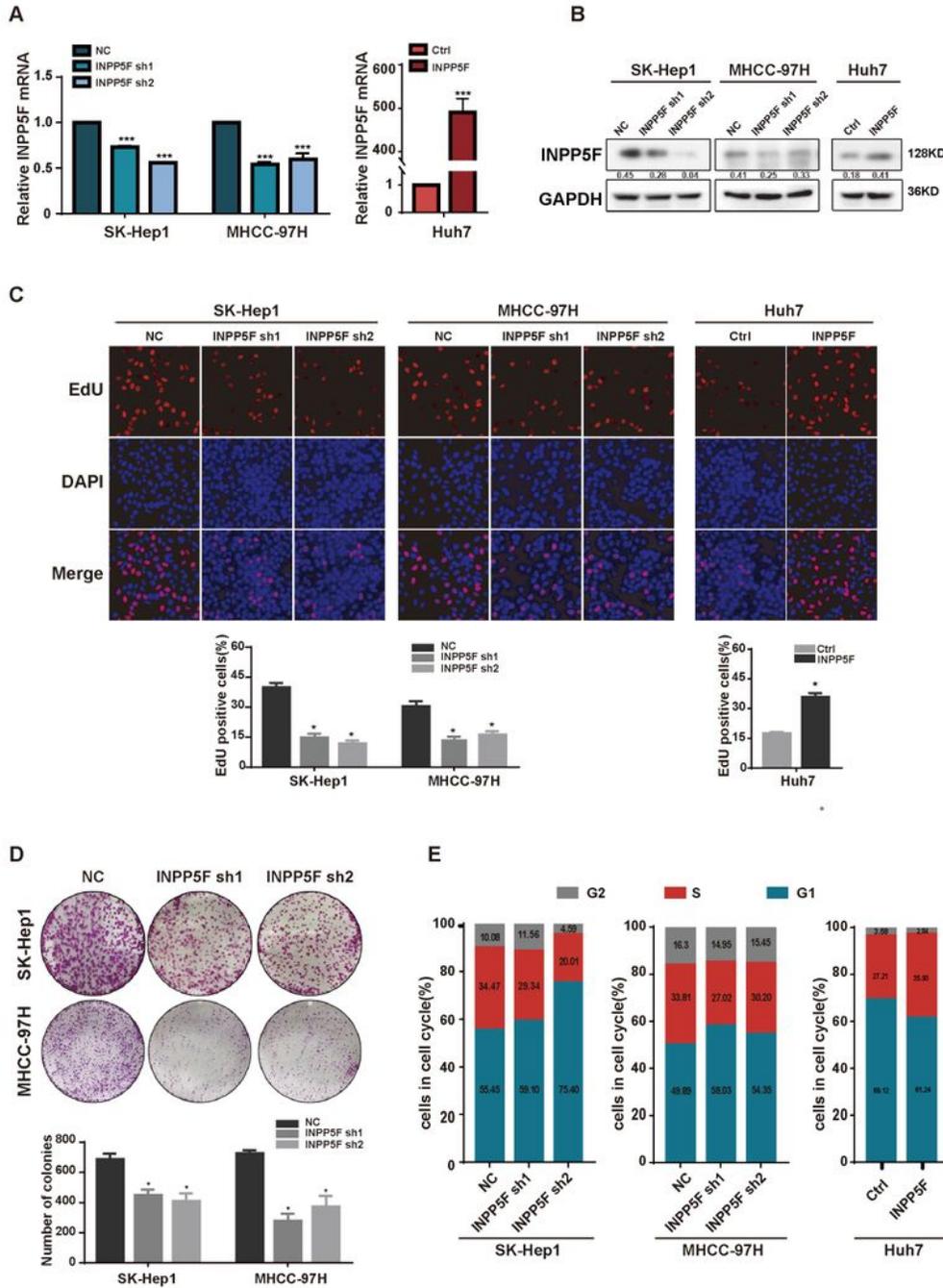


Figure 2

INPP5F promotes HCC cell proliferation in vitro. (A) QRT-PCR and (B) western blot confirmed the efficiencies of INPP5F knockdown in SK-Hep1 as well as MHCC-97H and overexpression in Huh7, respectively. (C) The effect of INPP5F on cell proliferation was determined by EdU assays. The representative images and the percentage of EdU positive cells are shown. (D) Colony formation was performed to validate the impact of INPP5F on cell proliferation. (E) The cell cycle analysis was performed in cells with INPP5F knockdown or overexpression. The percentage of cells in G1, S and G2 phase was indicated. Data is presented as means  $\pm$  standard error for three independent experiments, \*P < 0.05, \*\*\*P < 0.001.

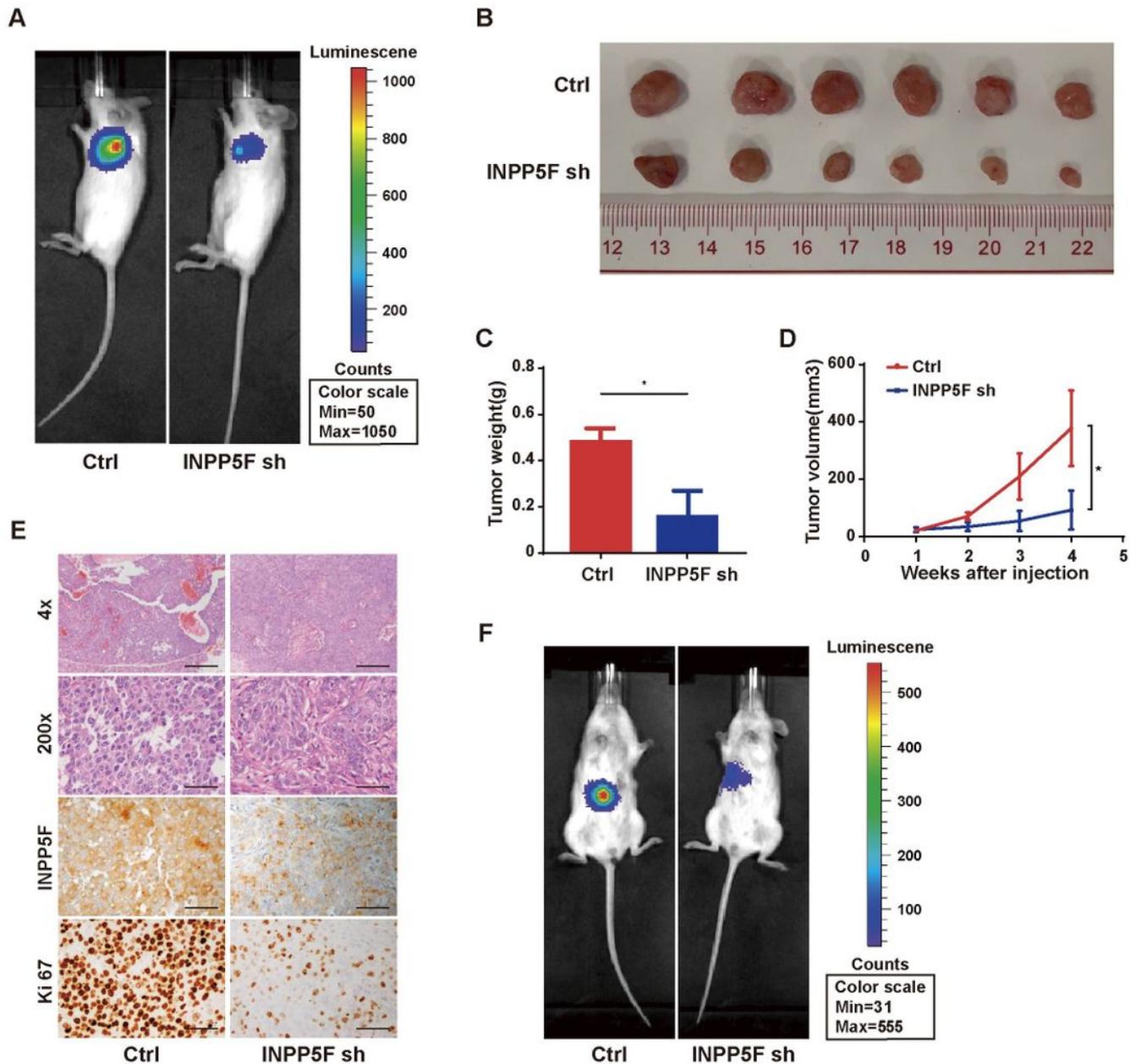
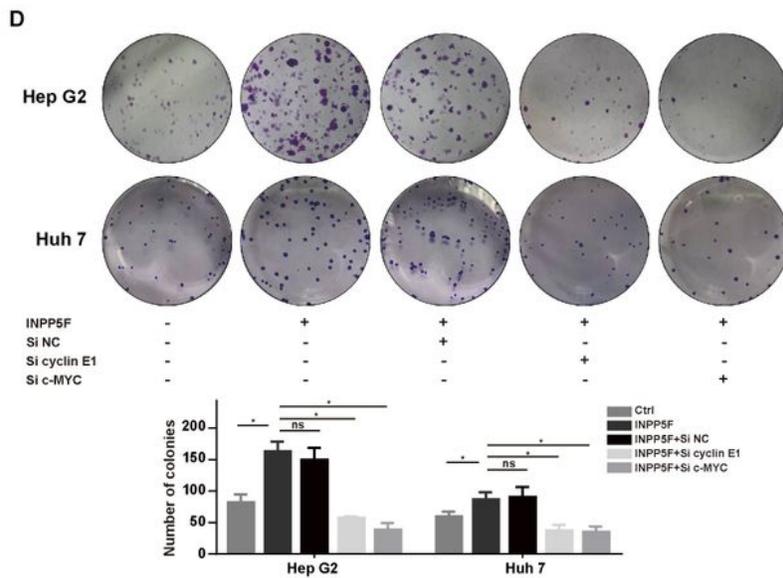
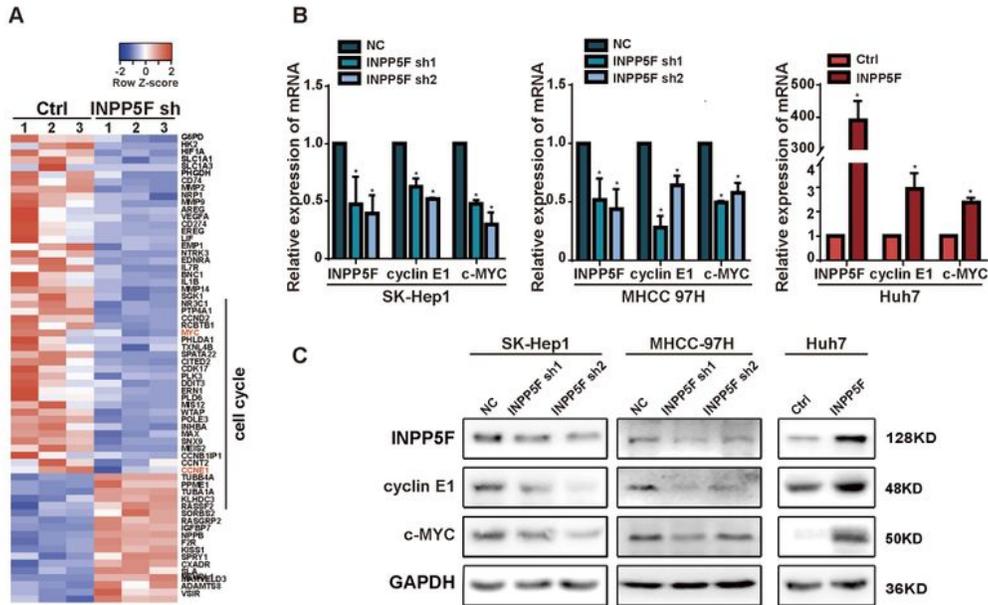


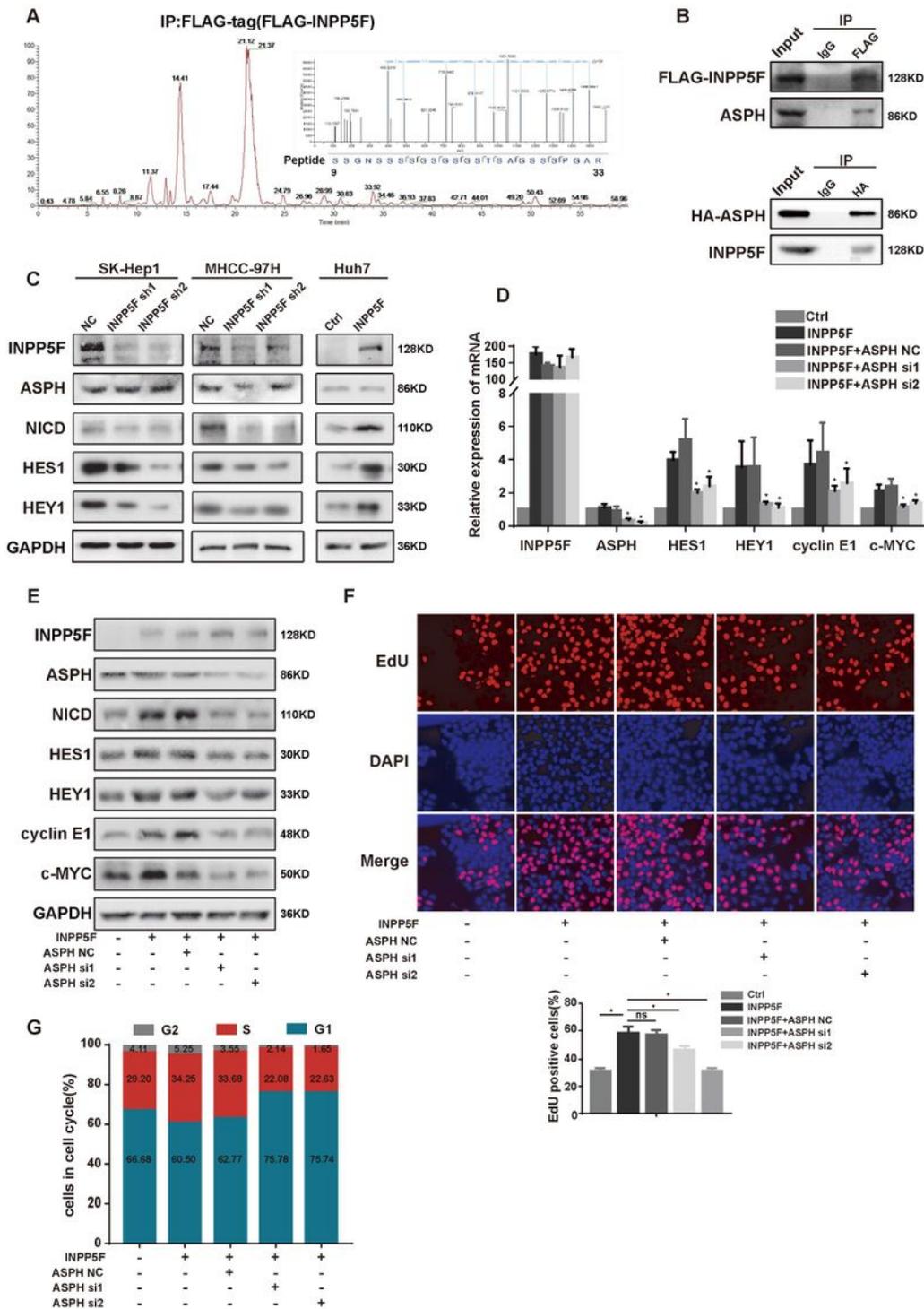
Figure 3

INPP5F facilitates HCC tumor growth in vivo. (A) Representative images of subcutaneous xenograft model using INPP5F stable knockdown MHCC-97H and its control cells. (B) Tumors derived from the subcutaneous xenograft model in both groups. (C) The tumor weight in both groups was compared. (D) The tumor volumes were measured every week and indicated by curves. (E) H&E, INPP5F and Ki67 staining were conducted in serial sections of tumors from both groups. (F) Orthotopic xenograft model was performed to further evaluate the effect of INPP5F on cell growth in vivo. All \* $P < 0.05$ . Scale bar: 100  $\mu\text{m}$ .



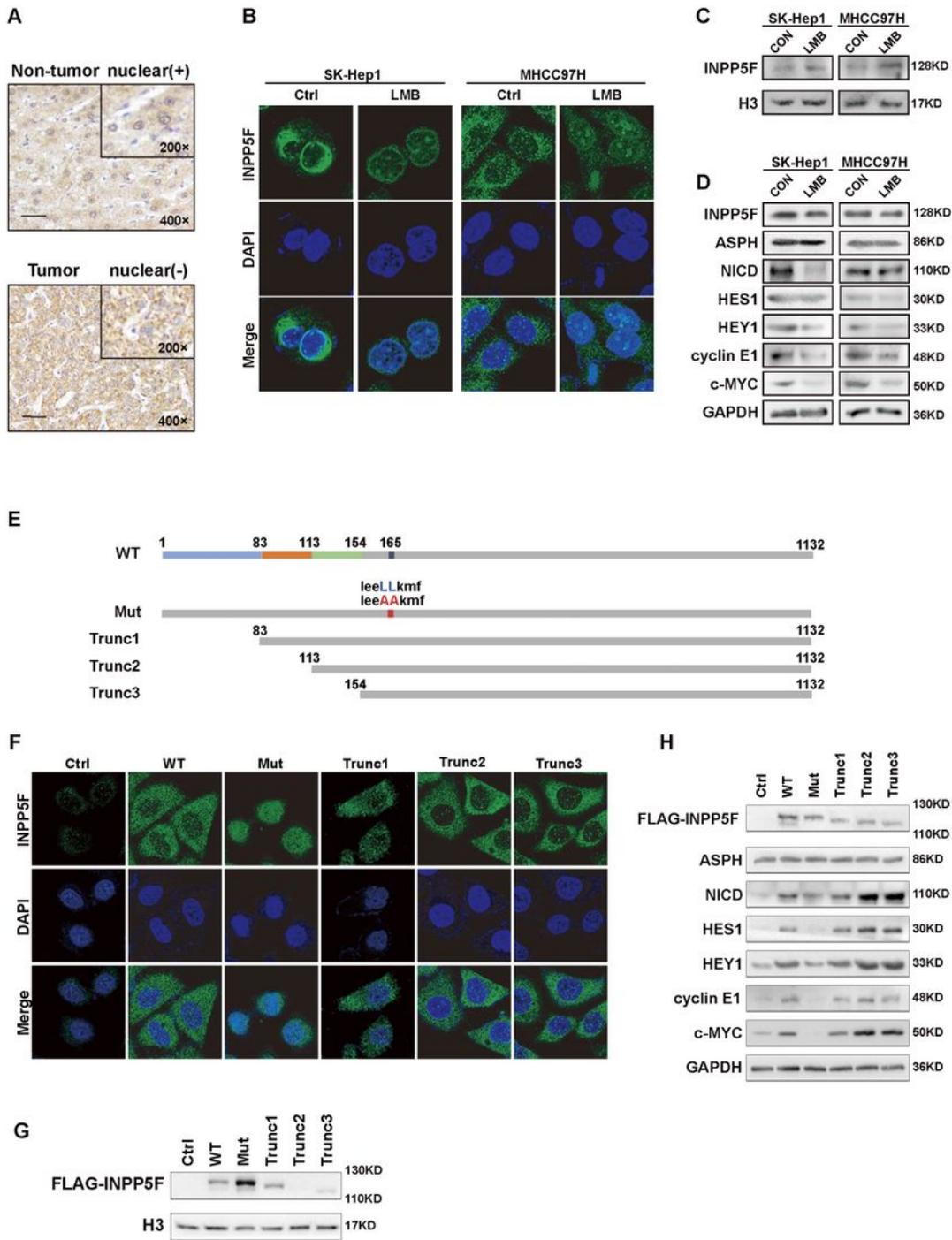
**Figure 4**

INPP5F upregulates the expression of c-MYC and cyclin E1 in HCC. (A) Heat map of DEGs associated with cell growth and aerobic glycolysis. The DEGs were obtained from SK-Hep1-Ctrl and SK-Hep1-shINPP5F through RNA-seq. (B) The mRNA and (C) protein expression of c-MYC and cyclin E1 in HCC cell lines with INPP5F knockdown or overexpression. (D) The impact of c-MYC and cyclin E1 knockdown on INPP5F-mediated cell proliferation was determined by colony formation assays. Data is presented as means  $\pm$  standard error for three independent experiments, \*P < 0.05, ns: not significant.



## Figure 5

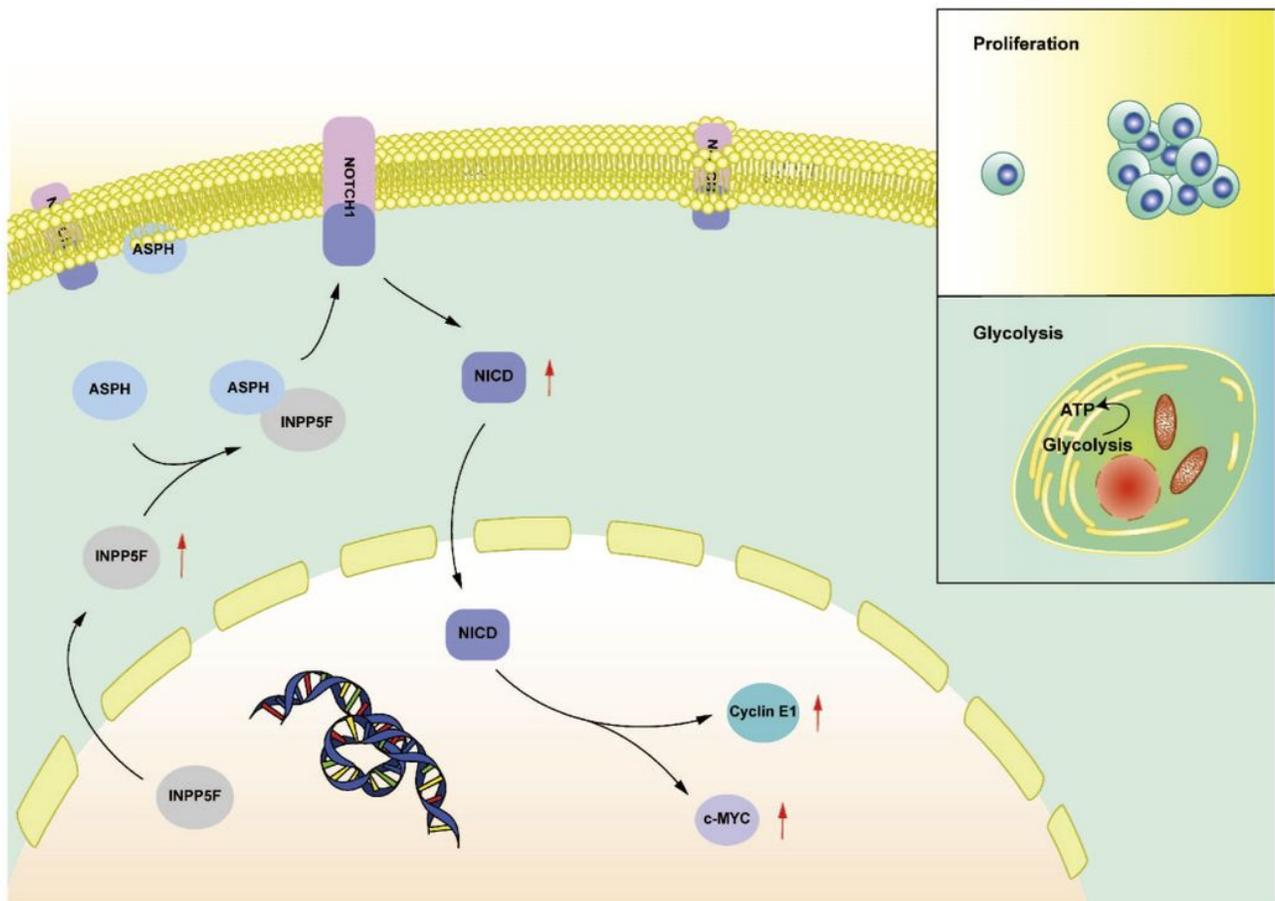
INPP5F activates Notch signaling pathway through interacting with ASPH. (A) The base-peak plot of mass spectrometry analysis of protein complex immunoprecipitated by anti-FLAG antibody in Huh7 overexpressing FLAG-INPP5F. The INPP5F pulled-down peptide of ASPH was indicated. (B) Lysates of Huh7 transiently overexpressing FLAG-INPP5F or HA-ASPH were immunoprecipitated for FLAG or HA and immunoblotted for ASPH or INPP5F, respectively. (C) Western blot was performed to investigate the influence of INPP5F on the expression of ASPH, NICD, HES1 and HEY1 in SK-Hep1, MHCC-97H and Huh7 cells. (D-G) Huh7 overexpressing INPP5F were transfected with ASPH siRNA for 24 h, and then were subjected to (D) QRT-PCR, (E) western blot, (F) EdU assay, and (G) cell cycle analysis. Knockdown of ASPH significantly attenuated INPP5F-enhanced expression of HES1, HEY1, cyclin E1 and c-MYC, as well as cell proliferation and G1/S phase transition. Data is presented as means  $\pm$  standard error for three independent experiments, \*P < 0.05, ns: not significant.



**Figure 6**

INPP5F translocates into cytoplasm to exhibit its oncogenic activity. (A) INPP5F was commonly nuclear staining in cells of adjacent tissues, and cytoplasmic staining in HCC cells. (B-D) Indicated cells were treated with LMB (SK-Hep1: 10 ng/ml and MHCC 97H: 40 ng/ml) for 6 h, and then were subjected to (B) immunofluorescence for detection of sub-cellular localization of INPP5F, (C) western blot for detection of INPP5F protein expression in nuclear, and (D) western blot for detection of the expression of INPP5F-

related downstream molecules. (E) Schema of the NESs mutant and the three truncations (Trunc1, Trunc2 and Trunc3) of INPP5F. The prediction of NLSs was obtained from [http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS\\_Mapper\\_form.cgi](http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), and the prediction of NESs was obtained from <http://www.cbs.dtu.dk/services/NetNES/>. (F-H) Cells were transfected with wild type (WT), NESs mutant or the three truncations. (F) Sub-cellular localization of different INPP5F was detected by immunofluorescence. (G) The nuclear localization of different INPP5F was validated by western blot. (H) The expression of INPP5F-related downstream molecules was detected by western blot. Scale bar: 100 um.



**Figure 7**

Model for the mechanism of INPP5F in facilitating HCC tumor growth. INPP5F translocates into cytoplasm and activates Notch-c-MYC/cyclin E1 pathway through interacting with ASPH, which finally enhances HCC cells proliferation and aerobic glycolysis.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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