Expression of the type 3 $InsP_3$ receptor is a final common event in the development of hepatocellular carcinoma

Supplementary Figures Legends

Supplementary Figure 1. ITPR3 is expressed in human liver cancer cell lines but not in human hepatocytes. ITPR3 expression is detected in four different human liver cancer cell lines: SKHep1, HepG2, HuH7 and HLE. Normal human hepatocytes express ITPR1 and ITPR2 but not ITPR3, similar to what has been reported in rodent hepatocytes [8]. Positive controls: mouse cerebellum, AR42J cells and RINm5F cells for ITPR1, ITPR2 and ITPR3, respectively. GAPDH was used as a loading control.

Supplementary figure 2. ITPR3 expression precedes ALD-related tumor. (A) Immunohistochemistry for ITPR3 and 5mC in cirrhosis (n=20) and tumor (n=20) related to alcoholic liver disease (ALD). (B) Histological score of ITPR3 and 5mC staining in human biopsies shows the inverse correlation between these two markers. GSD (n=10) was used as control specimens. Scale bar, 50 μm.

Supplementary Figure 3. ITPR3 expression precedes tumor development in a mouse model of HCC and absence of ITPR3 reduces tumor burden in xenograft tumors. (A) Immunohistochemistry for ITPR3 in mice at different time points after treatment with a single dose of diethylnitrosamine (DEN). After 6 months, no macroscopic tumor is present and ITPR3 is not detected in parenchymal cells. After 9 months, ITPR3 staining is present, but no tumor is observed. At 12 months, there is both ITPR3 staining and well-developed tumor

nodules. Images are representative of what was observed in n=4 animals per time point. (B) Tumor volumes of WT and ITPR3KO HepG2 cells implanted in nude mice. WT tumors (652.6 ± 229.6 mm^3, n=6) were significantly larger that ITPR3KO tumors (142.7 ± 125.7 mm^3, n=8, $p<0.05$ by Mann-Whitney test).

Supplementary Figure 4. DNA methylation levels in human *ITPR1* and *ITPR2* promoter regions. (A) The eight regions in the promoter region of *ITPR1* gene, analyzed by bioinformatics using GEO data bank (control) and TCGA data bank (HCC patients). (B) DNA methylation levels in the promoter region of *ITPR1* gene are similar between patients with HCC (black dots) and control individuals represented by white dots (**$p<0.05$). (C) Diagram of the six regions in the promoter domain of *ITPR2* gene, analyzed by bioinformatics. (D) As with ITPR1, DNA methylation levels of *ITPR2* gene are similar in patients with HCC (black dots) and controls (white dots, **$p<0.05$); n=23 in control group and 40 patients with HCC.

Supplementary Figure 5. 5'-aza treatment does not cause liver damage or other nonspecific effects. (A) Body weight of mice during 5'-aza treatment. (B) Blood cell count after 5'-aza treatment. (C) Serum level of alkaline phosphatase (ALP). (D) Serum level of alanine aminotransferase (ALT). (E) Serum level of aspartate aminotransferase (AST). (F) Serum level of gamma glutamyl transferase (GGT). (G) H&E staining of a representative mouse liver sections shows no difference in the liver morphology between the control and 5'aza-treated groups. Scale bar, 100 μm (n=5 animals per group).

Supplementary figure 6. Strategy for quantification of nuclear calcium *in vivo*. Low power image of a representative liver lobule loaded with the fluorescent Ca^{2+} dye Fluo4 (left panel). Magnified regions (Box 1 and 2) were used to identified individual hepatocytes (middle panel). Further magnification (blue and red framed images) allow for the identification of regions of interest, negative spaces within single hepatocytes, corresponding to the nucleus of individual hepatocytes.

Supplementary figure 7. Calcium signaling in isolated hepatocytes. (A) Representative pseudo-colored confocal fluorescence images of isolated hepatocytes from control and 5'-aza treated animals. (B) Representative time course of nuclear Ca^{2+} signal after vasopressin (AVP – 100 nM) stimuli. (C) Representative time course of cytosolic Ca^{2+} signal in hepatocytes after AVP stimuli. (D) Quantification of Ca^{2+} signal in the hepatocytes. No significant difference was observed in calcium signaling, either nuclear or cytosolic, between control hepatocytes and hepatocytes from mice treated with 5'-aza.

Supplementary figure 8. Expression of ITPR1 and ITPR2 after partial hepatectomy. (A) Representative immunoblot for ITPR1 and ITPR2 after 24, 48 and 72 hours of partial hepatectomy (PH). (B) Quantification of ITPR1 expression in the liver after the PH. (C) Quantification of ITPR2 expression in the liver after PH. Expression of both ITPR1 and ITPR2 were similar in all time points.

Supplementary figure 9. Liver regeneration in liver specific ITPR3 knockout mice. (A) Liver-to-body weight ratio after 48 hours of PH in control and ITPR3 liver specific knockout (LSKO) mice treated with 5'-aza showed increased liver regeneration in LSKO mice (*$p<0.05$). (B) Quantification of PCNA positive cell 48 hours after PH. The percentage of PCNA-positive cells was decreased in LSKO3 livers as compared to control R3Floxed. (n=3 animals/group, $p<0.05$ by Student t-test. (C) Representative images of ITPR3 expression in control and LSKO3 liver slices. ITPR3-positive cells are indicated by the arrowheads. Scale bar is 50 μm.