Hepatocyte Wnts are dispensable during diethylnitrosamine and carbon tetrachloride-induced injury and hepatocellular cancer

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ABSTRACT

Activation of the Wnt/β-catenin signaling is reported in large subsets of hepatocellular carcinoma (HCC). Upregulation of Wnt genes is one contributing mechanism. In the current study, we sought to address the role of hepatocyte-derived Wnts in a model of hepatic injury, fibrosis and carcinogenesis. We subjected hepatocyte-specific Wntless knockout mice (HP-KO), unable to secrete Wnts from hepatocytes, and littermate controls (HP-CON), to diethylnitrosamine and carbon tetrachloride (DEN/CCl₄) and harvested at 3, 5, and 6 months for histological and molecular analysis. Analysis at 5 months displayed increased hepatic expression of several Wnts and upregulation of some but not all β-catenin targets, without mutations in Ctnnb1. At 5 months, HP-CON and HP-KO had comparable tumor burden and injury, however HP-KO uniquely showed small CK19-positive foci within tumors. At 6 months both groups were moribund with comparable tumor burden and CK19-positivity. While HCC histology was indistinguishable between the groups, HP-KO exhibited increased active-β-catenin, and decreased c-Myc, Brd4, E-Cadherin, and others. Hepatic injury, inflammation and fibrosis were also indistinguishable at 3 months between both groups. Thus, lack of Wnt secretion from hepatocytes did not affect overall injury, fibrosis or HCC burden although there were protein expression differences in the tumors occurring in the two groups.
INTRODUCTION

Hepatocellular carcinoma (HCC) is the 6th commonest malignancy worldwide, with a median survival of 11 months and increasing incidence rates\(^1\). While liver transplant is a promising treatment for a subset of patients, factors including lack of donor organs and failure to meet Milan criteria make transplant an unlikely option. FDA-approved therapies Sorafenib and Regorafenib, while helpful and limit HCC progression, extend patient survival by 3 months\(^2,3\). Further, HCC develops in cirrhotic livers in 70-90% of cases, resulting from chronic liver injuries of all etiologies\(^4\). Due to increasing rates of liver diseases leading to fibrosis and tumorigenesis, the need for improved therapies to target and prevent HCC is growing.

\(\beta\)-Catenin signaling is upregulated in 20-90% of HCC patients\(^5\). \(\beta\)-Catenin is part of the Wnt signaling pathway with many roles in liver pathophysiology\(^6\). \(\beta\)-Catenin activation in HCC can result from mutations in Ctnnb1, the gene encoding for \(\beta\)-catenin, or other mechanisms including overexpression of Wnt and its receptor Frizzled (Fzd)\(^7\). In fact, overexpression of many Wnts and Fzds have been implicated in different cancers including HCC\(^7,8\) and several HCC cell lines\(^9\). These modifications result in increased nuclear accumulation of \(\beta\)-catenin, leading to transcriptional upregulation of target genes and promotion of tumorigenesis\(^9\). When Wnt antagonist fusion proteins are injected into an orthotopic HCC model harboring wildtype \(\beta\)-catenin, animal survival increases, and tumor volume, \(\beta\)-catenin activity, and angiogenesis decreases, suggesting that Wnts may be a promising therapeutic target\(^11\). There are Wnt inhibitors in phase I clinical trials for solid tumors which may be effective for HCC patients with wildtype \(\beta\)-catenin (NCT01351103), (NCT01608867). Furthermore, patients with
activated wild-type β-catenin resulting from Wnt or Fzd activation have more aggressive, dedifferentiated tumors and poorer prognosis than those with mutated β-catenin, yet less is known about the mechanism of wild type β-catenin in tumor progression.8

The main goal of our study was to assess hepatocytes as a source of Wnts which may be essential in chronic liver injury, fibrosis and HCC. We first confirmed a model of chronic injury (diethylnitrosamine and carbon tetrachloride, or DEN/CCl₄) induced HCC and simultaneously induced transcriptional upregulation of Wnts, β-catenin activation, but not Ctnnb1 mutations, consistent with previous reports12. We next assessed whether Wnts from hepatocytes may be required for tumorigenesis after DEN/CCl₄. We utilized hepatocyte-specific Wntless knockout mice unable to secrete Wnts from hepatocytes (HP-KO)13. We subjected HP-KO and littermate controls (HP-CON) to DEN/CCl₄ until 5 months of age. Comparable tumors were observed in HP-KO and HP-CON, although we observed small CK19-positive foci in HP-KO. Overall, HCC behaved analogously in HP-KO and HP-CON up to 6 months despite increased activated β-catenin expression and decreased c-Myc, Brd4, Erk1/2, Bax, and E-cadherin expression in HP-KO. We also assessed HP-CON and HP-KO at an early time-point, but did not detect any differences in the injury microenvironment. Thus, our data suggests that hepatocyte-specific Wnts do not contribute to injury and fibrosis, but do participate in HCC development and their loss leads to comparable tumor burden and histology in response to DEN/CCl₄.
MATERIALS AND METHODS

Animals. Animal work was performed in accordance with the Institutional Animal Care and Use Committee at the University of Pittsburgh. Albumin-Cre Wntless knockout mice were generated as described previously\(^\text{13}\). Male pups (HP-KO, HP-CON) were injected with 25mg/kg diethylnitrosamine (Sigma) at 14-16 days prepared in sterile 0.9% saline. Animals were transferred to a BSL2+ facility and injected with 0.5ml/kg carbon tetrachloride (CCl\(_4\)) twice per week from week 8 to 22, prepared in corn oil. Three days after the last injection, animals were sacrificed (n=4) and livers were harvested. Livers were also harvested from a pre-cancer time-point of 3 months (n=3) and advanced-cancer time-point of 6 months (n=4). Animals were monitored daily for signs of morbidity. At time of sacrifice, liver weights (LW) and body weights (BW) were assessed for LW/BW and differences tested for significance by Student’s T Test with p<0.05 to be considered significant. Blood was collected from the *inferior vena cava* and serum was sent to the University of Pittsburgh Medical Center Clinical Laboratory for ALT, AST, and ALP testing.

Immunohistochemistry. Paraffin sections were processed as described elsewhere\(^\text{14}\). Sirius Red, Ki67, H&E, CD45, CK19 and GS staining was performed as also described in detail previously\(^\text{14}\).

Protein isolation and analyses. Protein was extracted in RIPA buffer, quantified, and 30µg was loaded onto a precast BioRad SDS gel. Gels were transferred using BioRad semi-dry transfer system. Antibody information can be found in Table 1. Horseradish peroxidase conjugated secondary antibodies (Invitrogen) were used. Ponceau or Gapdh was used as a loading control. Densitometric analysis was performed with ImageJ and
normalized to housekeeping control. Statistics were calculated using One-way ANOVA with multiple comparisons and P values less than 0.05 were considered significant.

For reverse phase protein array (RPPA) analysis, samples were prepared via instructions from the MD Anderson Cancer Center RPPA Core Facility, and sent for analysis. Data was analyzed via Student’s t-test, and select proteins with statistically significant differences were validated by western blot.

**RNA isolation and QPCR.** RNA was extracted from DEN/CCl₄ treated control livers and untreated livers (n=3) using Trizol (Thermo Fisher), and after DNAse treatment (Thermo Fisher) 2µg of RNA was reverse transcribed using Superscript III (Thermo Fisher). Samples were pooled together and Sybr Green (Thermo Fisher) was used for qPCR. Ct values were normalized to GAPDH, and primer sequences are included in Table 2.

**DNA extraction and Ctnnb1 sequencing.** gDNA was isolated from livers (XNAT, Sigma), and primers flanking intron-exon junctions of Ctnnb1 exon 2 described previously¹⁵ were used in PCR. Product was gel purified and sequenced by the Genomics Research Core at University of Pittsburgh. Sequences were validated using ApE Software. Tumors in HP-CON and HP-KO at 5 and 6 months (N=3) were sequenced.

**Online supplement available at:**

https://drive.google.com/open?id=1zbEhnTG20BouorVTHpxAb8RdmmJoTBTS
RESULTS

**DEN/CCl₄ leads to wildtype Ctnnb1 and upregulation of several Wnts.** To develop an injury-based carcinogenesis model, we adapted the DEN/CCl₄ protocol used to induce inflammation, fibrosis, and tumors¹⁶,¹⁷. This model utilizes one injection of diethylnitrosamine at day 15, and biweekly injections of carbon tetrachloride from week 8 to week 22 (DEN/CCl₄) (Figure 1A). DEN/CCl₄ shows 100% penetrance at 5 months for HCC in conjunction with fibrosis and inflammation, thereby representing what is seen in patients (Figure 1B, 2E).

This model is known to cause HCC without β-catenin mutations as confirmed through sequencing and supported by lack of GS staining¹², which is a surrogate, albeit debated, for mutated β-catenin¹⁸,¹⁹. Via immunohistochemistry, all tumors were GS-negative (Figure 1C). Further, tumors were sequenced for mutations in exon 2 of Ctnnb1, analogous to exon 3 in humans. This is home to GSK3β phosphorylation sites and the most frequent activating mutations in HCC¹⁵,²⁰. Wildtype Ctnnb1 was identified in all tumors (Figure 1D). This was also corroborated using microarray data obtained from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE33446, comparing data from DEN/CCl₄ treated animals to untreated animals¹⁶. We queried the data and noted no change in expression of Glul, gene encoding for GS, after DEN/CCl₄. However, several β-catenin targets were upregulated as listed in Table 3 suggesting that modest activation of the pathway does occur despite absent Ctnnb1 mutations.

Next, we assessed whether Wnts were overexpressed after DEN/CCl₄, and tested mRNA expression of select Wnts in livers after DEN/CCl₄ compared to untreated
livers. Wnt1, Wnt6, Wnt10a, Wnt10b, Wnt11, and Wnt16 showed upregulation of at least two-fold in DEN/CCl₄ livers versus control livers (Figure 1E). We hypothesized that at least some of these may be originating from hepatocytes and hence interrogated tumorigenesis in mice lacking ability to secrete all Wnts from hepatocytes owing to Wntless loss.

**HP-KO and HP-CON have comparable tumor burden at 5 months, although HP-KO have more CK19+ nodules after DEN/CCl₄.** To assess the role of Wnts from hepatocytes, we subjected previously described hepatocyte-specific Wntless KO referred henceforth as HP-KO and littermate controls (HP-CON) to DEN/CCl₄. Mice sacrificed at 5 months exhibited comparable tumor burden reflected by similar LW/BW between the two groups (Figure 2A, 2B). Serum analysis revealed comparable and modestly elevated ALT, AST, and ALP in HP-CON and HP-KO (Figure 2C-2E). H&E staining verified these tumors to be similar HCCs as reflected by well-circumscribed lesions composed of hepatocytes with basophilic cytoplasm, some pleomorphic nuclei, and mitotic figures in HP-CON and HP-KO (Figure 2F). Further, we noted no differences in fibrosis via Sirius red or α-SMA (Figure 2F and data not shown), or inflammation, vascularization, or proliferation, via assessment of CD45, CD31, and Ki67, respectively (Figure 2F and data not shown). We also validated wildtype Ctnnb1 in HP-CON and HP-KO (Figure 2G).

To further characterize the tumor phenotype, we queried the differentiation status and assessed the dedifferentiation marker CK19, as it positively correlates with poor prognosis, increased tumor size and invasiveness. Immunohistochemistry revealed small CK19-positive foci in all HP-KO mice, while expression was restricted to
bile ducts in HP-CON mice (Figure 3A). Not all nodules in HP-KO were CK19-positive, however large tumors frequently contained smaller foci of heterogeneous CK-19-positive cells. These overall findings indicated that HCC occurring in absence of Wnts from hepatocytes were comparable to controls, other than the occurrence of small CK19-positive areas within tumors.

**No difference in tumor burden in HP-KO versus HP-CON at advanced stages.** We predicted CK19-positive foci in HP-KO to develop into more aggressive tumors than HP-CON. To test this, we followed an independent cohort for an additional timeframe. However, by 6 months, all mice became moribund and required euthanasia. Grossly, livers from both groups were excessively stiff and showed sizable tumor burden (Figure 3B). LW/BW at 6 months failed to show significant differences between the two groups and both groups showed increased ratios over the 5-month time-point (Figure 3C). Mutations in Ctnnb1 were still absent at 6 months (Figure 3D). Intriguingly, at 6 months, both HP-CON and HP-KO displayed comparable, diffuse, and broader staining for CK19 within tumors (Figure 3E). Similar to 5-month time-point, comparable fibrosis, inflammation and proliferation was observed as seen by Sirius red (Figure 3E), CD45, and Ki67 staining, respectively (data not shown). Thus, there were no phenotypic differences in HCC occurring in HP-CON and HP-KO at 6 months after DEN/CCl$_4$.

**HP-CON and HP-KO after DEN/CCl$_4$ show distinct temporal protein expression.** Despite comparable tumor burden and histology in HP-CON and HP-KO at 5 and 6 months, we queried whether there are differences in protein expression, likely correlating with altered signaling patterns, as a result of absent Wnt secretion from hepatocytes. First, we assessed proliferation markers and relevant β-catenin activation
markers via western blot (Figure 4A, 4C). Hypo-phosphorylated β-catenin, suggesting activation, was comparable at 5 months and significantly upregulated in HP-KO at 6 months. GS was variable, while Cyp2e1, a β-catenin target altered by CCl₄, was lower in HP-KO at 5 months, although levels in both HP-CON and HP-KO were comparably reduced at 6 months. Total levels of β-catenin were comparably reduced at 6 months compared to 5 months. Cyclin-D1 was increased at 6 months compared to 5 months in both groups. PCNA remained comparably high at all times in both groups. Intriguingly, c-Myc was reduced in HP-KO at both 5 and 6 months. Thus, overall hepatocyte-specific loss of Wnts appears to promote β-catenin hypo-phosphorylation and decrease c-Myc levels after DEN/CCl₄.

We sought a holistic approach to assess protein levels, and performed Reverse Phase Protein Array to assess expression of over 240 proteins involved in cancer as described in Methods. Several proteins had significant differences between HP-CON and HP-KO in the 5 month or 6 month time-point, and select proteins were confirmed by western blot (Figure 4B, 4D). Intriguingly, at 5 months HP-KO had significantly less expression of ERK1/2, Bax, Brd4, and E-cadherin than HP-CON. By 6 months, HP-KO had increased ERK1/2 and decreased Brd4, and comparable Bax and E-cadherin. Expression of Wee1, Yap, and Cdc25 were variable, and while PKAα expression was only evident at 6 months, there was no difference between HP-CON and HP-KO (Figure 4B, 4D). Taken together, this suggests HP-specific Wnts temporally regulate expression of oncogenes including ERK1/2, Bax, E-cadherin, and Brd4.

**Comparable onset and progression of injury in response to DEN-CCl₄ in HP-KO and HP-CON.** Lastly, we asked whether hepatocyte-Wnts contribute to DEN/CCl₄-
induced injury and hence the pre-tumoral microenvironment. We subjected HP-CON and HP-KO to this insult until 3 months of age, and noted no gross hepatic differences (Online Supplement: Fig.1A). Histology showed comparable cell death, hepatocyte ballooning, inflammation, and scarring in HP-CON and HP-KO (Online Supplement: Fig.1B). Further analysis revealed similar fibrosis, inflammation, and proliferation via Sirius red, number of CD45+ cells, and number of Ki67-positive hepatocytes in HP-CON and HP-KO (Online Supplement: Fig.1C). We therefore concluded removing Wntless from HP insignificantly contributes to hepatic injury during DEN/CCl₄ treatments.

**DISCUSSION**

Activation of Wnt/β-catenin signaling in HCC is due to various mechanisms⁵,²². Overexpression of Wnt and Fzd genes have been implicated as a contributor to β-catenin activation in a subset of HCC cases⁷. In fact, patients with intratumoral overexpression of Wnt and/or Fzd have more dedifferentiated tumors correlating with aggressiveness⁸. Wnt upregulation has also been reported in HCC cell lines including Hep3B cells¹⁰. To address the role of hepatocyte-derived Wnts in hepatocarcinogenesis in a relevant model, we examined injury and tumorigenesis in control mice and mice lacking Wntless in hepatocytes, which disallows secretion of all Wnts from these cells due to loss of this cargo receptor specific and essential for Wnts¹³,²³,²⁴.

We studied the DEN/CCl₄ model of tumorigenesis, as it leads to HCC following chronic injury and fibrosis, mimicking patient progression¹⁷. More importantly, we confirmed DEN/CCl₄-induced HCC provides evidence of β-catenin activation without
mutations in Ctnnb1. Indeed, microarray data available publicly via Gene Expression Omnibus (GSE33446), confirms β-catenin target genes including CD44, Cyclin D1, EGFR, and Survivin are induced in this model whereas β-catenin targets like GS and Regucalcin, which are associated with more sustained β-catenin activation through mutations are unchanged. Further, when mRNA expression of several Wnts was assessed, Wnt1, Wnt6, Wnt10a, Wnt10b, Wnt11, and Wnt16 were upregulated at least 2-fold in comparison to control livers. While in the current study, we addressed the loss of all hepatocyte-derived Wnts, studying the relevance of individual Wnts will be interesting, especially since Wnt6 and Wnt11 can act non-canonical, independent of β-catenin.

Upon challenging HP-CON and HP-KO with DEN/CCl₄, comparable tumor burden was observed at 5 months. We noted similar injury, fibrosis, inflammation, and proliferation. However, in assessing tumor differentiation, we observed the presence of small but frequent CK19-positive foci within larger CK19-negative tumors, in HP-KO. Presence of CK19 in HCC is suggested to correlate with a more dedifferentiated phenotype, overall poor prognosis and worse outcome after surgery. Finding CK19-positive foci in HP-KO was surprising, since a positive correlation between Wnt/β-catenin activation and stem markers including CK19 has been previously reported. However, since secretion of all Wnts is being disrupted, it is likely that the loss of non-canonical Wnts may be allowing for increased CK19 positivity. Indeed, hepatocytes and transformed hepatocytes are known to be a source of non-canonical Wnts that suppress β-catenin activity. Intriguingly, the increased numbers of CK19-positive foci did not lead to an overall aggressive HCC in HP-KO at 6 months and both groups of mice
succumbed to excessive tumor burden, and CK19 staining was comparable at this time-point. These data suggest that the DEN/CCl4 model is too robust leading to an aggressive disease and a model with an indolent course may be more suitable to address the overall role of hepatocyte-derived Wnts.

We also noted a reduction in c-Myc in HP-KO at 5 months and 6 months. Wnt/β-Catenin activates c-Myc, particularly during hepatocarcinogenesis30. Therefore, a reduction in c-Myc would likely suggest a reduction in β-catenin activity. While β-catenin target gene Cyp2e1 was reduced in HP-KO at 5 months, target gene GS and hypo-phosphorylated and total β-catenin levels were unchanged. Although at 6 months a reduced c-Myc in HP-KO correlated with increased hypo-phosphorylated (and thus activated) β-catenin. c-Myc can be upregulated in HCC independent of β-catenin, for example by amplification31. The mechanism by which c-Myc is downregulated in HP-KO remains unclear and will be elucidated in the future. Overall, decreased c-Myc does not seem to be altering the overall tumor biology in HP-KO since comparable tumor burden is evident in HP-KO and HP-CON. However, c-Myc upregulation in HP-CON does appear to be partially Wnt/β-catenin dependent as shown previously30,32.

RPPA analysis further revealed altered protein expression in HP-KO versus HP-CON. While the relevance of these changes remains unclear, there are several intriguing relationships among these data. Bromodomain 4 (Brd4) is a transcription coactivator involved in many cancers. In HCC, Brd4 is overactive and its suppression correlates with cMyc suppression33. At 5 month and 6 month timepoints, cMyc and Brd4 were both reduced in HP-KO. Whether HP-Wnts regulate cMyc through Brd4 remains a plausible mechanism to assess. E-cadherin inversely correlates with aggressive HCC,
as loss leads to increased epithelial to mesenchymal transition and invasiveness\textsuperscript{34}. Reduction of E-cadherin in HP-KO at 5 months, then comparable expression to HP-CON at 6 months, supports the findings that at 5 months HP-KO tumors began to dedifferentiate, but all differences are ablated by 6 months. This likely reaffirms the model is too hepatotoxic to appreciate contributions of HP Wnts, as other pathways are able to compensate. E-cadherin can activate ERK signaling in other cancers\textsuperscript{35}, which may explain why reduced E-cadherin correlates with reduced ERK1/2 at 5 months, however further studies are required to confirm pathway activation. Finally, the role of HP-Wnts in Bax expression is unclear, despite HP-KO having reduced Bax at 5 months. Overall, we conclude HP-Wnts contribute to protein expression, which likely corresponds to signaling changes but are insignificant to the overall tumor phenotype.

Wnt signaling from hepatocytes also appears to not influence the overall pre-tumor environment. At 3 months, comparable injury, inflammation, and fibrosis was observed between HP-CON and HP KO. This negative data is novel and important, as we provide \textit{in vivo} evidence that targeting hepatocyte-specific Wnts will not be an effective clinical therapy, despite literature demonstrating increased Wnts in HCC and transformed hepatocytes \textit{in vitro}. It will be of interest to assess Wnt contributions from additional cell types, including macrophages and endothelial cells, to identify roles to injury, fibrosis, and carcinogenesis.
Table 1: List of antibodies used in the study

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Table 2: List of qPCR primers used in the study

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Table 3: List of β-catenin target genes altered in the DEN/CCl₄ model of injury, fibrosis and HCC. The data was obtained from microarray data available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE33446 and values presented below is ratio of the expression of genes in DEN/CCl₄ treated versus untreated mice\(^6\).

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* References are to the studies showing the gene as being target of the Wnt/β-catenin signaling pathway.
REFERENCES


FIGURE LEGENDS

Figure 1: DEN/CCl\textsubscript{4} model of HCC shows Wnt upregulation but non-mutated β-catenin (A) Schematic of DEN/CCl\textsubscript{4} injection protocol, beginning at day of birth and concluding at 5 months of age. (B) High magnification (200x) Hematoxylin and Eosin stain (H&E) highlighting intratumoral dysplastic hepatocytes. (C) Assessing GS staining in tumors in DEN/CCl\textsubscript{4} model of HCC. No GS-positive tumors were observed, suggesting wildtype β-catenin. (D) Representative sequencing of β-catenin exon 2, focusing on GSK3β phosphorylation motifs, displays non-mutated and identical sequence between tumors (Tu.) and wildtype (WT). (E) RT-PCR analysis showing upregulation in the transcript levels of several Wnts at 5 months of age after continued DEN/CCl\textsubscript{4} treatment in mice as compared to untreated controls.

Figure 2: HP-CON and HP-KO have comparable tumor burden after 5 months of DEN/CCl\textsubscript{4}. (A) Representative gross images of HP-CON and HP-KO at 5 months. (B) HP-CON and HP-KO have comparable liver weight to body weight ratios after DEN/CCl\textsubscript{4}. (C-E) Serum analysis reveals comparable Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Alkaline Phosphatase (ALP) in HP-CON and HP-KO. (F) Representative images of hematoxylin and eosin (200x), CD45, Sirius Red, and Ki67 (50x) reveal comparable tumors and injury burden in HP-CON and HP-KO. (G) Representative sequencing shown for HP-CON and HP-KO tumors, which are both CTNNB1 wildtype.

Figure 3: HP-KO have CK19-positive foci absent in HP-CON at 5 months, however HCC is comparable in HP-CON and HP-KO at 6 months after DEN/CCl\textsubscript{4}. (A) CK19 staining is positive in biliary epithelial cells in HP-CON and HP KO, but is evident in
small tumor foci within larger tumors in HP-KO at 5 months. (B) Gross images show advanced HCC in both HP-CON and HP-KO at 6 months after DEN/CCl₄. (C) Liver weight to body weight ratios are comparable at 6 months after DEN/CCl₄. (D) At 6 months, HP-CON and HP-KO have wildtype CTNNB1. (E) Representative H&E (200x), Sirius Red (50x), and CK19 (50x) suggest similar tumor composition, fibrosis, and CK19 pattern, respectively, in HP-CON and HP-KO at 6 months.

**Figure 4: HP-CON and HP-KO have divergent protein expression patterns at 5 and 6 months.** (A) Western blot assessing glutamine synthetase (GS), cMyc, hypophosphorylated β-catenin, Cyclin D1, PCNA, Cyp2e1, and total β-catenin. Ponceau shows comparable loading. (B) Western blot assessing select proteins based off RPPA analysis including Wee1, Yap, Erk1/2, Bax, Cdc25, Brd4, E-cadherin, PKAa. Gapdh shows comparable loading. (C) Densitometry of western blot from figure A highlights significant changes between HP-CON and HP-KO at 5 and 6 months. Relative expression was normalized to Ponceau. (D) Densitometry analysis of western blot from figure B, normalizing values to Gapdh. Statistics performed with one-way ANOVA using multiple comparisons. *P<0.05, **P<0.01.
Figure 1
Figure 2
Figure 3
Figure 4