Supplementary Materials and Methods

Reagents

Culture media, Dulbecco modified Eagle's minimal essential medium, Ham's F12, a-MEM, fetal bovine serum, minimal essential medium nonessential amino acid solution, minimal essential medium vitamin solutions, glyceryl monostearate, chemically defined lipid concentrate, soybean trypsin inhibitor, penicillin/streptomycin, gentamycin, and glutamine were purchased from Invitrogen (Carlsbad, CA). Epidermal growth factor, dexamethasone, triiodothyronine, EDTA, collagenase IV, forskolin, DNAase, pronase, direct-red-80, dibutyl cAMP were purchased by Sigma-Aldrich (St Louis, MO). HEPES and TritonX-100 were purchased by American Bioanalytical (Natik, MA). The protease and phosphatase inhibitor cocktail was purchased by Biovision (Milpitas, CA). The NE-PER nuclear and cytoplasmic extraction reagent, total cell lysis reagent, ultra-V block and the Coomassie reagent for protein measurement were purchased from Thermo Scientific. STAT3 Inhibitor (STATIC V), JAK inhibitor I, NLRP3 inflammasome inhibitor (MCC950) were purchased by Millipore (Billerica,MA). β-Catenin inhibitor (ICG-001) was purchased by Selleckchem (Houston, TX). NF-kB Inhibitor (ACHP), CXCR3 antagonist (AMG-487) and PKA inhibitor (14-22 Amide) were purchased by Tocris (Minneapolis, MN). Rac1 Inhibitor (NSC 23766) and PKA inhibitor was purchased from Cayman (Minneapolis, MN). IL-1β was purchased by R&D (Minneapolis, MN). Cytofix/cytoperm kit, Perm/Wash Buffer, Streptavidin-Pacific blue, DAPI were purchased by BD Bioscience (San José, CA), ACK Lysing Buffer was purchased by Thermo Scientific (Waltham, MA). The Dual-Luciferase® Reporter (DLR™) Assay System for the measurement of NF-kB transcriptional activity was purchased by Promega. The rat anti-mouse K19 antibody (TROMA-III) developed by Kemler R. was obtained from the Developmental Studies Hybridoma Bank developed under the

auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Western blot

Samples of nuclear, cytoplasmic or whole-cell extracts were used for Western blot. In detail, total cell lysates were extracted using a lysate buffer (50 mM Tris-HCl, 1% NP40, 0.1% SDS, 0.1% Deoxycholic acid, 0.1 mM EDTA, 0.1 mM EGTA) containing fresh protease and phosphatase inhibitor cocktails (Sigma, St Louis, CA). Nuclear and cytosolic fractions were isolated using the NE-PER Kit (Pierce, Rockford, IL), per the manufacturer's instructions. Protein concentration was measured using the Coomassie protein assay reagent (Pierce, Rockford, IL). Equal amounts of total lysate 20 μg were applied to a 4-12% NuPAGE® Novex Bis-Tris gel (Invitrogen, Carlsbad, CA) and electrophoresed. Proteins were transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA). Membranes were blocked with 5% non-fat dry milk (Bio-Rad Laboratories) in phosphate-buffered saline containing 0.1% Tween-20 (PBST) for 1h and then incubated with specific primary antibodies overnight. The following antibodies were used: anti-mouse β-catenin, p675-β-catenin, pSTAT3(Tyr705), STAT3, actin, lamin-B, PKM, caspase-1. Nitrocellulose membranes were washed three times with PBST and then incubated with horseradish peroxidase conjugated secondary antibodies for 1h. Proteins were visualized by enhanced chemiluminescence (ECL Plus kit; Amersham Biosciences, Piscataway, NJ, USA). The intensity of the bands was determined by scanning video densitometry using the Total lab Tl120DM software (Nonlinear USA Inc, Durham NC).

Isolation and culture of Cholangiocytes. Mouse cholangiocytes were isolated from *Pkhd1*$^{del4/del4}$ and WT mice at 3 months of age as previously described (1-6). The cells were cultured in 6 wells plate in medium containing [DMEM/F12 (1:1), P/S 1%, Gentamicyn 0.2%, glutamine 2mM, Insulin-transferrin-selenium, forskolin, dexamethasone, thrihydothyronine, FBS 10%, trypsin inhibitor, vitamins, non-essential amino acids]. Before the treatments, the cells were serum starved with quiescent media (0.5% FBS) for 24 hours. Cells were treated for 24 hours with dibutyl cAMP (D0627, Sigma, 100 μM), JAK inhibitor (Pan-JAK-Tyk2 inhibitor, Calbiochem CAS 457081-03-7, 10 μM), STA3 Inhibitor (Stattic V, Millipore, 573099, 10 μM), NF-kB Inhibitor (ACHP, TOCRIS, 4547, 1 μM), PKA inhibitor (14-22 Amide, Millipore, 1 μM), Rac1 Inhibitor (NSC 23766, Cayman 50 μM), NLRP3 inflammasome inhibitor (MCC950, Selleckchem, S7809, 10 μM) and recombinant mouse IL-1β (401-ML-005, R&D, 5 ng/ml) or their combinations. At the end of the treatments, the supernatant was collected and RNA and protein were isolated. The amount of IL-1β and CXCL10 in the supernatants were quantified by ELISA using a kit for IL-1β (Abcam, ab197742) and for CXCL10 (Thermo scientific, EMCXCL10) respectively, and following the manufacturer's protocols. The results were normalized to the total protein concentration measured with the Coomassie reagent.

Immunohistochemistry: Liver tissue sections obtained from both *Pkhd1*$^{del4/del4}$ and WT mice were deparaffinized in xylene and rehydrated in water. The staining was performed as detailed in the supplementary material. Hydrogen peroxide blocking was performed with 3% H_2O_2 in methanol for 30 minutes. Next depending on the kind of antibody, antigen retrieval was performed in a steamer for 20 minutes. The slides were blocked with Ultra V Block for 10 min and O/N

staining was performed with the following antibodies: K19, CD45, IL-1β, CXCL10. Quantification of K19 and CD45 cells by immunohistochemistry were made as previously described (2).

Immunocytochemistry Cells grown on 6-wells plates, were fixed on cold methanol for 10 minutes at -20°C. Cells were permeabilized with 0.2% triton X-100 in PBS (PBS/t) and then unspecific binding sites were blocked by incubation with 3% BSA in PBS for 1h at room temperature. A polyclonal rabbit anti-mouse -pSTAT3, β-Catenin and NF-kB (Ser276), Ser (536) antibody was applied to the cells and incubated overnight in 1% BSA in PBS/t. The primary antibody was replaced by the secondary antibody Alexa Fluor 555 goat anti-rabbit (Invitrogen, Carlsbad, CA; 1:200) for 1h at room temperature. Nuclei were counterstained using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Liver Cell Isolation and Fluorescence-Activated Cell Sorting Analysis. Fluorescence-activated cell sorting analysis was performed to characterize the different cell subsets contributing to the portal inflammatory cell infiltrate and to characterize the cell populations expressing pSTAT3 and CXCR3, as previously described (2, 7). In detail, liver cells were isolated from *Pkhd1*$^{del4/del4}$ and WT mice at different ages in the Cell Isolation Core of the Yale Liver Center. The liver was perfused with collagenase type IV and the non-parenchymal cells (NPC) and the biliary tree were isolated. The biliary tree was cut in small pieces and it was digested in a small flask T-25 with 30 ml of digestion media (3% FBS, 1% BSA, 0.06% Collagenase type IV, 0.3% Pronase, 0.06% DNAase in a-MEM) for 30 min at 37^{O}C under agitation. The solution was filtered with 70 μm cell strainer and after centrifugation for 10 min at 1500 RPM, 8˚C, the biliary tree pellet was combined with the NPC pellet and red blood cell lysis was performed with ACK Lysing Buffer (A1049201, Thermo scientific). After washing with PBS, the cells were resuspended in FACS buffer (1XPBS,

3% FBS and 0.05% sodium azide). 0.5-1x10^6 cells/ml were used for each staining. For the surface staining the cells were blocked with Fc blocker (CD16/32, 2.4G2) for 20 min on ice and then the primary monoclonal rat anti-mouse conjugated-antibodies (EpCAM-Brilliant Violet 421, CD19-FITC, CD11b-PE/Cy7, CD11b-APC, CD45-Pacific blue, CD45-PerCP, NK1.1-APC, CXCR3-APC, CD4-APC, CD8-FITC, Gr-1-PE, F4/80-FITC) or their corresponding isotype controls were applied for 30 min on ice. For intracellular staining, cells were permeabilized using a cytofix/cytoperm kit (561651, BD Biosciences) for 20 min on ice. Then the primary anti-mouse unconjugated antibody of pSTAT3(Tyr705), monoclonal conjugated anti-mouse antibodies of Egr2-APC, NOS2-PE, K19-PE and Collagen-biotin, diluted in perm/wash were added to the corresponding wells. Following incubation for at least 30 min at 4°C in the dark, the cells were washed with perm/wash buffer 2 times (CRF at 1500 rpm for 5 min). The fluorochrome-labeled secondary antibody diluted 1:1000 or Streptavidin-Pacific blue diluted 1:1000 in perm/wash buffer was then added and incubated with the cells for at least 20-30 minutes at 4ºC in the dark. The cells were washed 3 times with PBS filtered. The pellet was resuspended in PBS (500 ul) in FACS tube and were analyzed on a BD LSRII Flow Cytometer (BD, NJ). Data were analyzed with FlowJo. Gating strategy used to identify myeloid-cell subsets in the liver and to characterize the cell populations expressing pSTAT3(Tyr705) and CXCR3. Cells were isolated from enzymatically digested mouse livers, and after doublets and debris were excluded the immune cells, were identified based on CD45 staining following by sequential gating strategy for cells expressing specific markers: macrophages (MΦ) ($CD11b^+$ $F4/80^+$), granulocytes ($CD11b^+$ Ly-6G/Ly-$6C^+F4/80^-$), NK1.1 Cells ($CD11b^{hi}$-$NK\text{-}1.1^+$), T-CD4 cells ($CD11b^-$ $CD4^+$), T-CD8 cells ($CD11b^-$ $CD8^+$), B Cells ($CD11b^-$ $CD19^+$). For M1 and M2 macrophages after gating the CD45 cells, M1 were the $NOS2^+$ $F4/80^+$ cells and the M2 were the $Erg2^+$ $F4/80^+$(8). For the cultured

cholangiocytes, intracellular staining for pSTAT3 was performed as described above for the liver cells. In cholangiocytes silenced for β-Catenin, nuclei were stained with DAPI for 5 min before the measurement.

PLA (Proximity ligation assay): Cells grown on 6-wells plates, were fixed on PFA 4% for 10 minutes at RT. Cells were permeabilized with 0.2% triton X-100 in PBS (PBS/t) and then unspecific binding sites were blocked by incubation with blocking solution (Olink Bioscience) for 30 min at room temperature. A monoclonal mouse anti-mouse -pSTAT3 and rabbit anti-mouse β-Catenin was applied to the cells and incubated for 1 hour in 1% BSA in PBS/t. Secondary antibodies linked to PLA oligonucleotide probes PLUS and MINUS (Olink Bioscience) were added and bound to the primary antibodies after incubation for 1 h at 37 °C. Each of the PLA probes has a unique short DNA strand attached to it. If the PLA probes are in close proximity (that is, if the two original proteins of interest are in close proximity, or part of a protein complex, as shown in the figures), the DNA strands can participate in rolling circle DNA synthesis when appropriate substrates and enzymes are added. The DNA synthesis reaction results in several-hundredfold amplification of the DNA circle. Next, coverslips were incubated with the amplification polymerase 20 solution for 100 min at 37 °C to amplify hybridized oligonucleotides and fluorescently label (Alexa Fluor 488) the amplification products. Cells were then stained with Rhodamine conjugate Phalloidin for 20 minutes and coverslips were mounted on slides with Duolink. In Situ Mounting Medium with DAPI. Imaging was done using a Zeiss LSM 710 Duo confocal microscope.

β-Catenin silencing: Pre-designed custom short-interfering RNA (siRNA) from Dharmacon were used for β-catenin silencing, 4 specific siRNAs were used. Their target sequences were: 1: GUGAAAUUCUUGGCUAUUA,2:GCGCUUGGCUGAACCAUCA,3:AGCAAAUCAUGCGCCUU, 4:AAGCUGACCUGAUGGAGUU. The cells were plated at confluency 15-75% in 24 wells plates after splitting and were grow overnight. The next day the media was changed 1 hour before the transfection. The specific β-catenin siRNAs were incubated with lipofectamine 2000 for 20 min and then were added to the cells. The media was changed with the quiescent media 24 hours post transfection and 48 hours post transfection the supernatants were collected and protein and RNA were isolated.

Supplementary Figure Legends

Supplementary Figure 1: CXCR3 expression in liver cells in WT and *Pkhd1*$^{del4/del4}$ mice. A-C) Gene expression of CXCL-9, CXCL-10 and CXCL-11 shows upregulation of only CXCL-10 in the liver of *Pkhd1*$^{del4/del4}$ mice compared to WT liver (n=5). **D-I)** FACS analysis of whole liver of 3 months old WT and *Pkhd1*$^{del4/del4}$ mice showed that CXCR3 is expressed in **D, E)** Fibroblasts (Collagen 1^{+} cells), **F, G)** Macrophages (F4/80^{+} Cells) and **H, I)** Cholangiocytes (K19^{+}Cells) of *Pkhd1*$^{del4/del4}$ mice (n=4). The FACS plots are created after the gating of live single total liver cells and the bar graphs showing the quantification of FACS analysis results. The results are presented as mean(SEM). (*p<0.05; unpaired Students Ttest). K19: Cytokeratin-19.

Supplementary Figure 2: Phosphorylated STAT3 at Tyr705 was overexpressed in liver samples from *Pkhd1*$^{del4/del4}$ mice with respect to WT. IHC analysis for pSTAT3(Tyr705) in liver specimens from 3-months-old WT or *Pkhd1*$^{del4/del4}$ mice showed increased expression of pSTAT3(Tyr705) in FPC-defective liver cysts compared to the WT bile ducts. FPC: Fibrocystin.

Supplementary Figure 3: β-catenin silencing or its inhibition affects pSTAT3(Tyr705) nuclear translocation only in FPC-defective cholangiocytes and not in WT cells. A) Representative image from Immunocytochemistry for pSTAT3(Tyr705) (red) in WT/FPC-defective cholangiocytes (FPC^{-}) treated for 1 hour with β-Catenin inhibitor (ICG-001) or with its vehicle (untreated) (n=3). Nuclei were stained with DAPI (blue). **B, C)** Representative plot of FACS analysis for pSTAT3(Tyr705) showing reduced nuclear pSTAT3(Tyr705) expression upon β-Catenin silencing only in FPC-defective cholangiocytes but not in WT cells. The Q1 is the nuclear part positive for pSTAT3 (DAPI^{+}pSTAT3^{+}) and the Q2 is the cytoplasmic part positive for pSTAT3 (DAPI^{-}pSTAT3^{+}). The results are presented as mean(SEM), (n=3). FPC: Fibrocystin.

Supplementary Figure 4: IL-1β precedes the expression of CXCL10 in FPC-defective cholangiocytes. A) Immunofluorescence for IL-1β and CXCL10 in liver specimens from 1 and 3 months old WT/ *Pkhd1*$^{del4/del4}$ mice, shows that cysts from *Pkhd1*$^{del4/del4}$ mice that express IL-1β (red) were negative for CXCL10 (green) whereas in the liver specimens from 3 months old mice CXCL10 expression was increased in *Pkhd1*$^{del4/del4}$ mice as compared to WT mice and was colocalized with IL-1β.

Supplementary Figure 5: β-catenin silencing didn't affect pSTAT1(Tyr701) nuclear translocation in FPC-defective cholangiocytes. STAT1 and STAT5 are not involved in the IL-1β mediated CXCL10 production A, B) Western blot analysis for pSTAT1(Tyr701), STAT1 and actin in nuclear and cytoplasmic extracts of FPC-defective cholangiocytes and the respective quantification shows no reduction of nuclear pSTAT3(Tyr705) upon β-Catenin silencing (n=5). **C)** IL-1β induced CXCL10 gene expression was not affected upon inhibition of STAT1 and STAT5 for 24 hours. The results are presented as mean(SEM), (n=3). FPC: Fibrocystin.

Supplementary Figure 6: NF-kB transcriptional activity is β-Catenin dependent and regulates IL-1β production and secretion. A) Immunocytochemistry analysis for phosphorylated p65 of NFkB at Ser276/Ser536 (red) of FPC-defective (FPC$^-$) and WT cholangiocytes (n=3) showed increased phosphorylated p65 at Ser 276/Ser 536 in the nucleus of FPC-defective as compared to WT cholangiocytes. Nuclei were stained with DAPI (blue). **B)** NF-kB transcriptional activity was increased in FPC-defective as compared to WT cholangiocytes (n=4). **C)** *Pro-IL-1β* gene expression was reduced only in FPC-defective but not in WT cholangiocytes treated with the NF-kB inhibitor for 24 hours (ACHP) (n=8). **D)** IL-1β protein levels measured with ELISA in cell supernatant were reduced only in FPC-defective but not in WT cholangiocytes with the same treatment (n=5). **E)** NF-kB transcriptional activity was reduced

upon β-Catenin silencing or upon **F)** STAT3 inhibition in FPC-defective cholangiocytes compared to the relative controls (n=4). **G)** *Pro-IL-1β* gene expression was reduced only in FPC-defective cholangiocytes treated for 48 hours with β-Catenin siRNAs as compared to the same cells treated with the scrambled siRNAs or the untrasfected ones (untreated) (n=6). **H)** IL-1β protein levels measured with ELISA in cell supernatant were reduced only in FPC-defective cholangiocytes treated for 48 hours with β-Catenin siRNAs as compared to the same cells treated with the scrambled siRNAs or the untrasfected ones (untreated) (n=6). The results are presented as mean(SEM) and the statistics were performed with the one sample Ttest. (*$p<0.05$ denotes statistical significant using unpaired Students Ttest or ANOVA with post hoc corrections). FPC: Fibrocystin.

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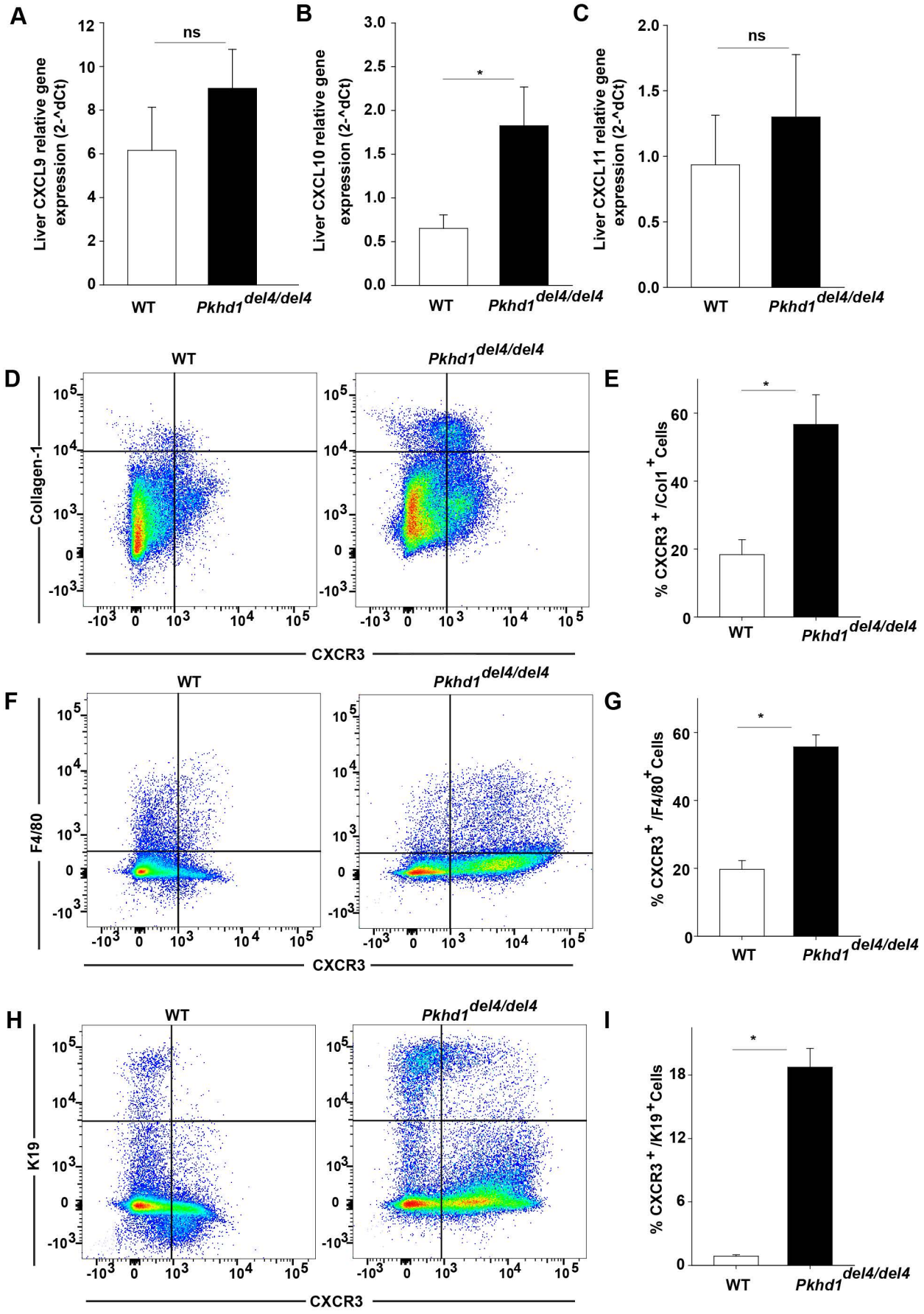
The bold letters indicate equal authorship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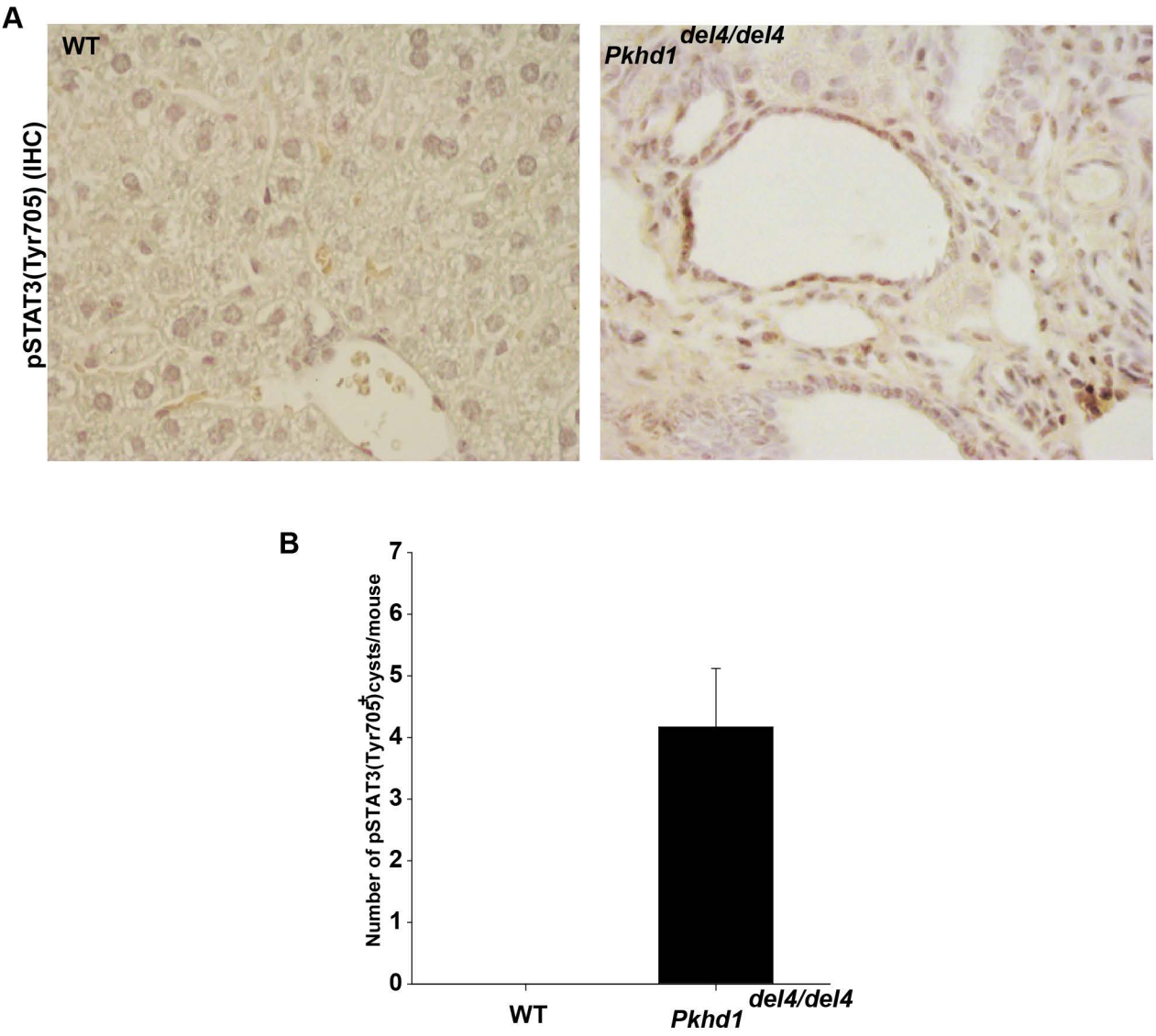
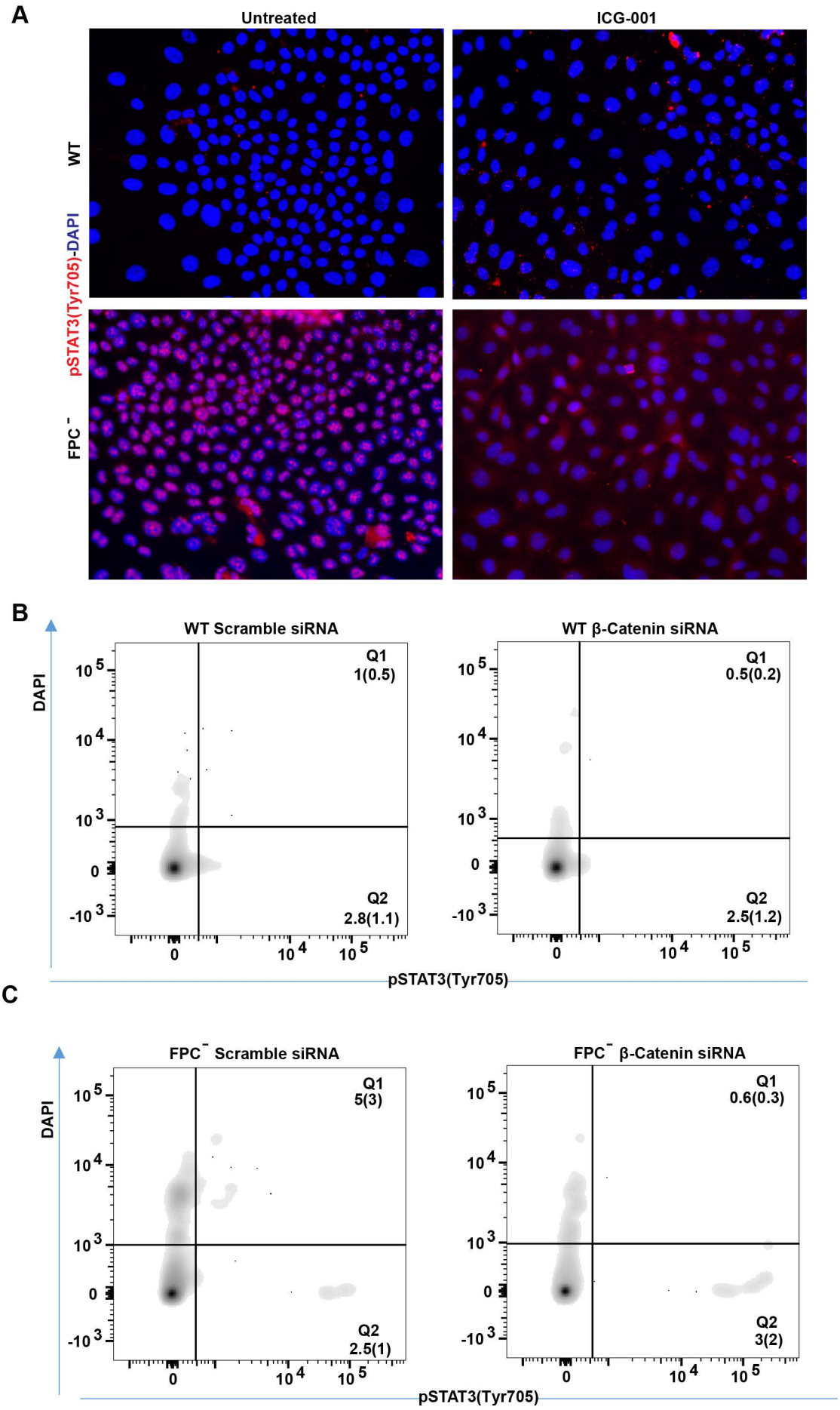
Liver CXCL9 relative gene expression (2-^dCt)
ns
WT
Pkhd1del4/del4
B
Liver CXCL10 relative gene expression (2-^dCt)
*
C
Liver CXCL11 relative gene expression (2-^dCt)
ns
D
WT
Pkhd1del4/del4
Collagen-1
CXCR3
E
% CXCR3+/Col1+Cells
F
F4/80
G
% CXCR3+/F4/80+Cells
H
K19
I
% CXCR3+/K19+Cells


A
WT
Pkhd1 del4/del4
pSTAT3(Tyr705) (IHC)
B
Number of pSTAT3(Tyr705)+cysts/mouse
0
1
2
3
4
5
6
7
WT
Pkhd1 del4/del4

Supplementary Figure 3



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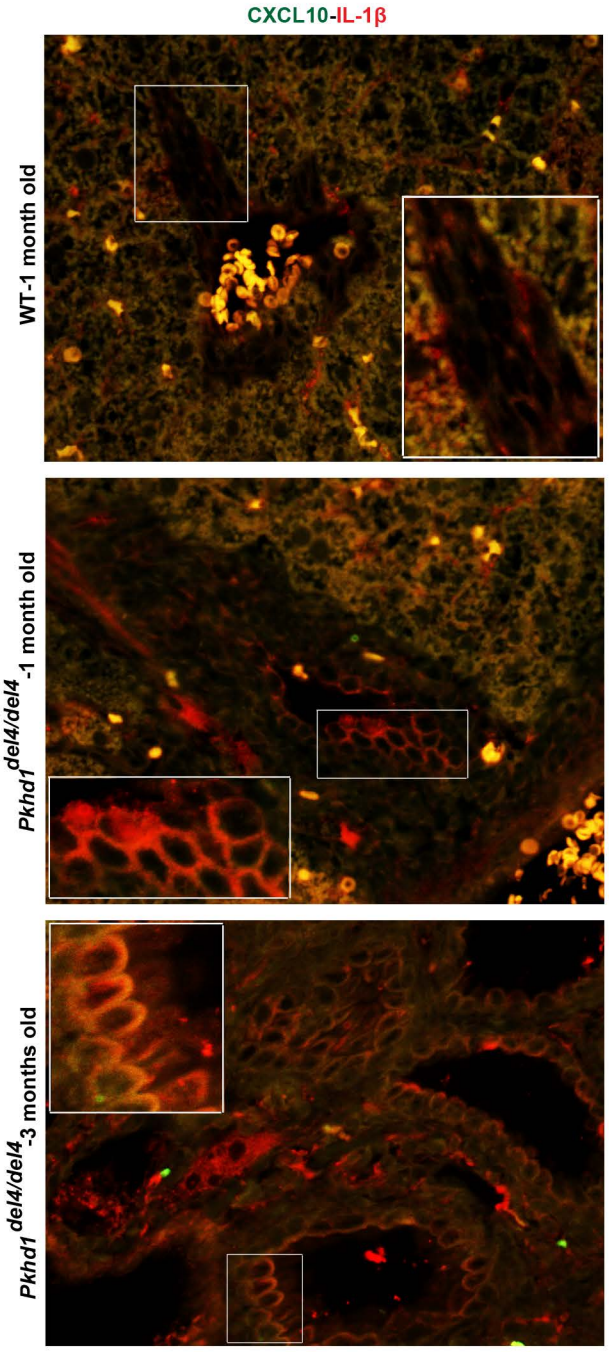
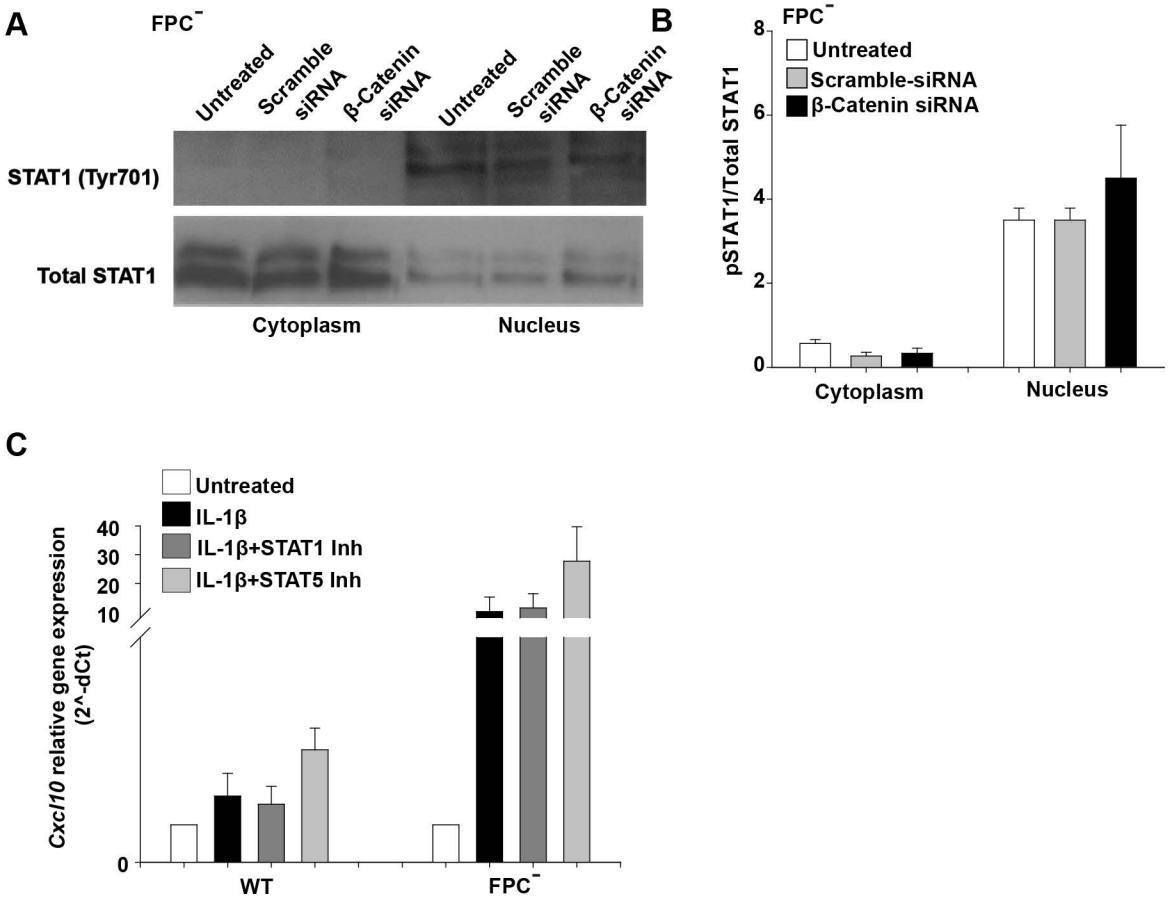
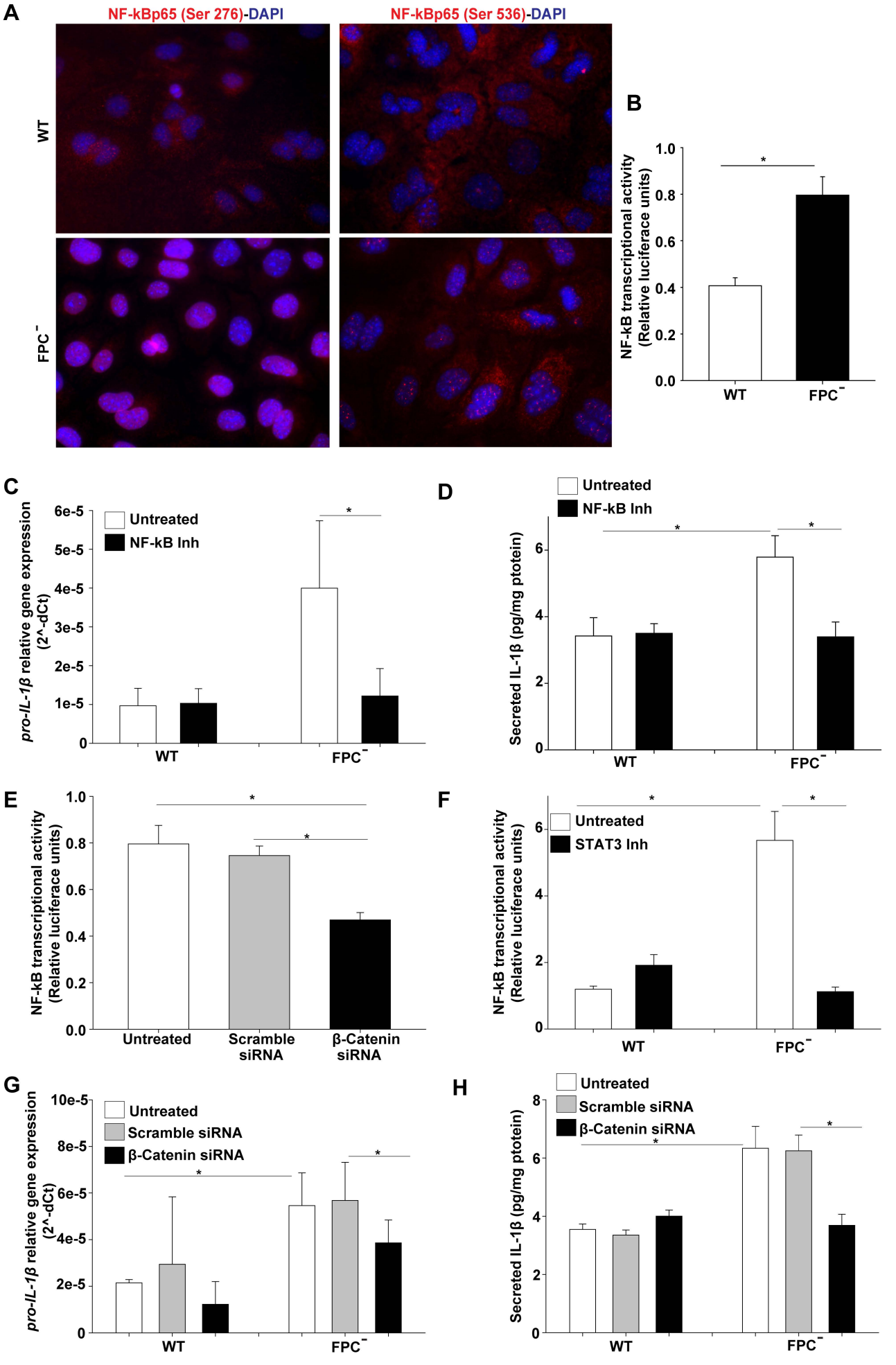

CXCL10-IL-1β
WT-1 month old
Pkhd1del4/del4-1 month old
Pkhd1del4/del4-3 months old


A
FPC⁻
Untreated
Scramble siRNA
β-Catenin siRNA
Untreated
Scramble siRNA
β-Catenin siRNA
STAT1 (Tyr701)
Total STAT1
Cytoplasm
Nucleus
B
FPC⁻
Untreated
Scramble-siRNA
β-Catenin siRNA
pSTAT1/Total STAT1
0
2
4
6
8
Cytoplasm
Nucleus
C
Untreated
IL-1β
IL-1β+STAT1 Inh
IL-1β+STAT5 Inh
Cxcl10 relative gene expression (2^-dCt)
0
10
20
30
40
WT
FPC⁻


A
NF-kBp65 (Ser 276)-DAPI
NF-kBp65 (Ser 536)-DAPI
WT
FPC-
B
NF-kB transcriptional activity (Relative luciferace units)
1.0
0.8
0.6
0.4
0.2
0.0
*
WT
FPC-
C
pro-IL-1β relative gene expression (2^-dCt)
6e-5
5e-5
4e-5
3e-5
2e-5
1e-5
0
Untreated
NF-kB Inh
*
WT
FPC-
D
Untreated
NF-kB Inh
Secreted IL-1β (pg/mg ptotein)
6
4
2
0
*
*
WT
FPC-
E
NF-kB transcriptional activity (Relative luciferace units)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Untreated
Scramble siRNA
β-Catenin siRNA
F
NF-kB transcriptional activity (Relative luciferace units)
Untreated
STAT3 Inh
6
4
2
0
*
*
WT
FPC-
G
pro-IL-1β relative gene expression (2^-dCt)
10e-5
8e-5
6e-5
4e-5
2e-5
0
Untreated
Scramble siRNA
β-Catenin siRNA
*
*
WT
FPC-
H
Untreated
Scramble siRNA
β-Catenin siRNA
Secreted IL-1β (pg/mg ptotein)
8
6
4
2
0
*
*
WT
FPC-

Supplementary Table 1. Antibodies used and suppliers

Antibodies	Code	Company
Actin	A4700	Sigma, S. Louis MO
β-Catenin	9562	Cell Signaling
Caspase-1 (p10)	sc-514	Santa cruz
CD11b-PE-Cy7	101215	Bio legend
CD11b-APC	101211	BD Bioscience
CD16/32, 2.4G2	553141	BD Pharmingen™
CD19-FITC	557398	BD Bioscience
CD45	550539	BD Pharmigen,
CD45-Pacific blue	103125	Bio legend
CD45-PerCP	103129	Bio legend
CD4-APC	100411	Bio legend
CD8-FITC	100726	Bio legend
Collagen 1-biotin	600-406-103	Rockland
CXCL10	Bs-1502R	Bioss
CXCR3-APC	FAB1685A	R&D
Egr2-APC (clone erongr2)	17-6691-80	E Bioscience
EpCAM-Brilliant Violet 421	118225	Bio legend
F4/80-FITC	123108	Bio legend
Gr-1-PE	108407	Bio legend
IL-1β	15F01-MM	InvivoGen
K19		Troma-III
K19-PE	SPM561	Novus
Lamin B	ab133741	Abcam
NFkB (Ser276)	PA5-37718	Thermo Scientific
NFkB (Ser536)	ab86299	Abcam
NK-1.1 APC	108710	Bio legend
NOS2-PE	12-5920-80	E Bioscience
PKM	3190T	Cell signaling
pSTAT3(Tyr705) (D3A7)	9145	Cell Signaling
pSTAT3(Tyr705) (Y705)	4113S	Cell Signaling
Total STAT3	12640	Cell Signaling