Supporting Information

Antisense oligonucleotide silencing of a glycosyltransferase, *Poglut1*, improves the liver phenotypes in mouse models of Alagille syndrome

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Supporting Materials and Methods

Mouse breeding

Jag1$^{+/-}$ animals were crossed with *Albumin-Cre* animals to generate *Jag1*$^{+/-}$*;Albumin-Cre* progeny. *Sox9*$^{flox/flox}$ animals were crossed with *Jag1*$^{+/-}$*;Albumin-Cre* animals to generate *Jag1*$^{+/-}$*;Albumin-Cre;Sox9*$^{+/flox}$ (*Jag1*$^{+/-}$*;Sox9*$^{+/\Delta}$) mice. *Jag1*$^{+/-}$*; Sox9*$^{+/\Delta}$ animals were crossed to *Sox9*$^{flox/flox}$ animals to generate *Jag1* heterozygous, *Sox9* conditional knockout progeny (*Jag1*$^{+/-}$*; Sox9*$^{\Delta/\Delta}$). *Poglut1*$^{+/-}$ mice were used for the generation of *Poglut1*$^{+/-}$ MEFs. We have previously reported that ~21% of *Jag1*$^{+/-}$ animals exhibit malocclusion, compared to 2.5% of the WT C57BL/6 mice.[1] Given the potential effects of malocclusion on the liver phenotypes, we excluded all animals with malocclusion from our studies.

Mouse liver staining

For paraffin embedding, livers were harvested from animals and fixed overnight in 4% PFA at 4°C. The following day, livers were washed in 70% ethanol, followed by 2X washes in 95% ethanol, followed by 2X washes in 100% ethanol, each wash was for 1 hour at 4°C. Next, samples were incubated in HistoChoice® Clearing Agent (VWR, H103-4L) for three washes, each one for 0.5 hour at room temperature, followed by embedding the liver samples in paraffin. Five-µm sections were prepared such that they cover a majority of the liver from a similar depth and used for staining. After antigen retrieval (Vector laboratories H-3301), slides were incubated with the following primary antibodies overnight at 4°C: rat anti-CK8 (DHSB, TROMA-I, 1:20), rat anti-CK19 (DHSB, TROMA-III, 1:20), and mouse anti-αSMA (Sigma-Aldrich, A2547, clone 1A4, 1:200). For some antigens, fixed frozen 10-µm sections embedded in O.C.T. (Tissue-Tek®) were incubated with primary antibodies [rabbit anti-ASO (Ionis, 1:5,000), mouse anti-αSMA (Abcam, ab7817, 1:600), goat anti-OPN (R&D Systems, AF808, 1:100)]. Anti-ASO antibody staining was followed by Biotin-SP AffiniPure Donkey anti-rabbit (Jackson ImmunoResearch 711-065-152, 1:1,000) and then Streptavidin-Cy3 (Jackson ImmunoResearch 016-160-084, 1:300). Staining with other primary antibodies was followed by appropriate fluorescently conjugated secondary antibodies (Jackson ImmunoResearch 705-545-147, 1:300; 715-605-150, 1:300; 715-175-151, 1:200; Invitrogen, A-21208, 1:200) each 4 hours at room temperature or overnight at 4°C diluted either in 10% normal donkey serum (Jackson ImmunoResearch) in PBS or 2% bovine serum albumin with 0.2% milk in PBS. Images were acquired using a Nikon AXR confocal on a Ti-2 inverted microscope, or a Leica DMI6000 B Inverted Fluorescence Motorized Phase Contrast Microscope. To

generate the ASO staining images, 20 optical sections from each confocal dataset were subjected to average intensity projection in Fiji ImageJ.

Quantification of the BD/PV ratio

Liver paraffin or frozen blocks were sectioned so that most if not all liver lobes were present on a given slide, followed by staining with anti-CK8, anti-CK19 and anti-αSMA as described above. The slides, when possible, were blinded for genotypes and treatments. Imaging was performed under Leica DMI6000 B Inverted Fluorescence Motorized Phase Contrast Microscope at 20X magnification. All liver lobes were analyzed, and each portal vein was documented along with any surrounding fully patent bile ducts. The data were tabulated and an average bile duct to portal vein ratio was determined for each liver.

Fibrosis quantification

Slides were stained with Sirius Red (Sigma-Aldrich, 36-554-8 and P-6744, concentrated at 5 g per 500 mL saturated picric acid) and imaged on a Nikon ECLIPSE Ti2 inverted microscope with approximately 15-20 images per slide at 2.5X. Images were imported into ImageJ software and once a threshold was set for Sirius Red staining, the area of staining was quantified and divided by the total area of the liver on the same slide to determine fibrosis percentage.

Hematoxylin and eosin staining and necrosis quantification

Five-micron sections were stained with hematoxylin and eosin (H&E) and imaged using Nikon DS-Fi2 camera mounted on an Olympus BX41 microscope. For necrosis quantification, H&E slides were scanned to create a single image with Nikon elements software using a Nikon DS-Ri1 camera and a Nikon Eclipse Ni microscope. The visible individual areas of necrotic tissue were manually determined and marked using the ImageJ software. The sum of the necrotic areas on each slide was divided by the entire tissue surface to calculate the percentage of total tissue area that is necrotic.

RNAscope

Fixed frozen 10-µm sections were briefly dried, washed in PBS and baked. Sections were then re-fixed in chilled 4% paraformaldehyde, dehydrated and air dried prior to treating with Target Retrieval (ACDbio, 322000) and Hydrogen Peroxide (ACDbio, PN322381), with dehydration and air dying

between and after each step prior to Protease Plus (ACDbio, PN322381). The probe mixture was prepared using 50X volume Mm-Sox9 probe (ACDbio, 401051), 1X volume Mm-Poglut1-C2 probe (ACDbio, 552431-C2) and 1X volume Mm-Jag1-C3 probe (ACDbio, 412831-C3). Opal 520 (Akoya Biosciences, OP-001001), Opal 620 (Akoya Biosciences, OP-001004) and Opal 690 (Akoya Biosciences, OP-001006) diluted 1:1000 in TSA buffer (ACDbio, 322809) were used. Sections were stained with DAPI (Invitrogen D1306) and mounted in Prolong gold antifade (ThermoFisher P36934). Images were acquired using a Nikon AXR confocal on a Ti-2 inverted microscope.

Images were analyzed using Nikon Elements. Images were first denoised using Denoise.ai algorithm. The middle 20 optical sections were manually selected. 241x214 pixel parenchyma regions were manually cropped from 60X images, and 60X with 2.5X zoom images were used for quantification of PV regions. PV regions were manually size-matched according to BD size. Pixel area of *Poglut1* staining was determined using an NIS Elements General Analysis program with identical manual set intensity threshold for all samples. Two separate programs were used for PV and parenchyma regions. Background subtraction was individually calculated using an intensity threshold general analysis in the DAPI channel. Calculations were performed using Microsoft Excel. GraphPad Prism was used to graph and perform t-tests with Welch's correction.

Images were merged using Fiji ImageJ. A single DAPI frame was used for better visualization. Maximum intensity images were used for *Sox9*, *Poglut1* and *Jag1* RNAscope. Brightness and contrast were adjusted to better visualize RNA transcript puncta.

Mouse muscle staining and quantification of PAX7$^+$ cells

Quadriceps muscles of P30 mice were harvested and fixed with 4% paraformaldehyde for 24 hours at 4°C. Cryosections of samples were collected and antigen retrieval was performed in pH 6.0 citrate buffer for 6 minutes. The samples were then permeabilized with 0.2% Triton-X 100 for 15 minutes and blocked with 2% BSA and Mouse on Mouse solution for 1 hour. Primary antibodies used were mouse monoclonal anti-PAX7 (DSHB, PAX7, 1:2.5) and rabbit anti-laminin (Sigma, L9393, 1:200). The following secondary antibodies were used: Goat anti-mouse IgG (H+L) Highly Cross Absorbed Secondary Antibody Alexa Fluor 555 (Invitrogen, A-21424, 1:500) and Goat anti-rabbit IgG (H+L) Highly Cross Absorbed Secondary Antibody Alexa Fluor 488 (Invitrogen, A-11008, 1:500). Fluorescent images were obtained using Leica DFC365 FX and processed with the ImageJ software. Images of at

least five random fields of each sample were taken and the number of $PAX7^+$ cells in each field of view was counted manually.

Serum biochemistry

Serum was collected from animals retro-orbitally under euthanasia and was analyzed by Ionis Pharmaceuticals for total bilirubin, ALT and AST.

RNA extraction and qRT-PCR

RNA was extracted from liver and quadriceps muscle tissues from animals at P12 and P30, using Direct-zol RNA Kits (Zymo Research Cat# R2050). RNA quality and quantity was verified using a NanoDrop (ThermoFisher). qRT-PCR was performed using 100 ng of total RNA per well and TaqMan One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA). *Poglut1* mRNA was detected using a commercially available assay (Mm00552419_m1; Applied Biosystems). Relative mRNA level was compared using the ΔΔCt method, with 18S as control (4319413E; Applied Biosystems). For *JAG1* overexpression studies, mRNA was isolated from stable MEFs expressing *JAG1* and quantified on a NanoDrop (ThermoFisher). cDNA was produced from the resultant mRNA. For qPCR, 100 ng of cDNA was used per reaction using the amfiSure PCR Premix (genDepot). qPCR primers can be found in Supporting Table S1.

Generation of JAG1 overexpression lentivirus constructs

Gateway cloning was used to generate the JAG1 expression lentivirus. A 3.7-kb human *JAG1* cDNA was PCR amplified to add attB sites (Supporting Table S1) and inserted into pDONR^{TM} 223 (Invitrogen) by BP reaction (Invitrogen). The *JAG1* cDNA product was then inserted into pCW57.1 plasmid (Addgene plasmid#41393; gift from Dr. David Root) by recombination using LR recombinase (Invitrogen) to generate inducible the pCW57-JAG1 lentiviral expression construct.

Lentivirus production

To produce lentivirus, the lentiviral plasmid pCW57-JAG1, together with the pCMV-VSV-G envelope construct (Addgene #8454; gift from Dr. Bob Weinberg) and the packaging psPAX2 construct (Addgene #12260; gift from Dr. Didier Trono) were co-transfected into HEK293T cells. HEK293T cells used for lentivirus production were cultured in DMEM supplemented with 10% FBS (GIBCO) without antibiotic

prior to packaging. Cells were cultured at 37°C and 5% CO_2. For each 10 cm plate of cells, 3.9 μg lentiviral plasmid, 2.6 μg of envelope plasmid, and 3.8 μg of packaging plasmid were mixed and co-transfected using HD-FuGene (Promega). The media were collected and replaced 48 hours and 72 hours after the transfection. The virus-containing media were filtered through 0.45 μm filters (Millipore) and concentrated using Lenti-X Concentrator (Takara) according to the manufacturer's instructions. The virus pellet was resuspended in PBS and stored at −80°C as 10X stocks.

Site-direct mutagenesis

Site-directed mutagenesis was performed using NEB Q5 Site-Directed Mutagenesis Kit (E0554) according to the manufacturer's instructions. The following mutations (individual or combined) were introduced into the human *JAG1* expression vector described above: S342A (EGF4), S493A (EGF8), S635A (EGF11), S826A (EGF16). The list of primers used for mutagenesis is provided in Supporting Table S1. Lentiviral particles for the mutant versions of human JAG1 were generated as described above.

Cell culture

Primary Mouse embryonic fibroblasts (MEFs) were isolated from WT and *Poglut1*$^{+/-}$ animals as previously described,[2] and were immortalized using SV40 plasmid (Addgene #21826; gift from Dr. David Ron). MEFs, COS7 or HEK293T cells were cultured in a standard 5% CO_2 incubator. All cell lines were maintained in Dulbecco's-MEM (genDepot) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies).

Generation of transgenic JAG1 doxycycline inducible cell lines

Stable *tet-JAG1* MEFs and HEK293T cells were generated by transducing cells with pCW57-JAG1 (WT or EGF mutant) lentivirus in the presence of 7 μg/mL Polybrene (Sigma). Twenty-four hours after infection, cells were selected with 2 μg/mL puromycin for 3-4 days until all the non-infected control cells were dead. For maintenance, the stable cells were kept under 1 μg/ml puromycin selection. To induce JAG1 expression, cells were treated with 1 μg/mL doxycycline.

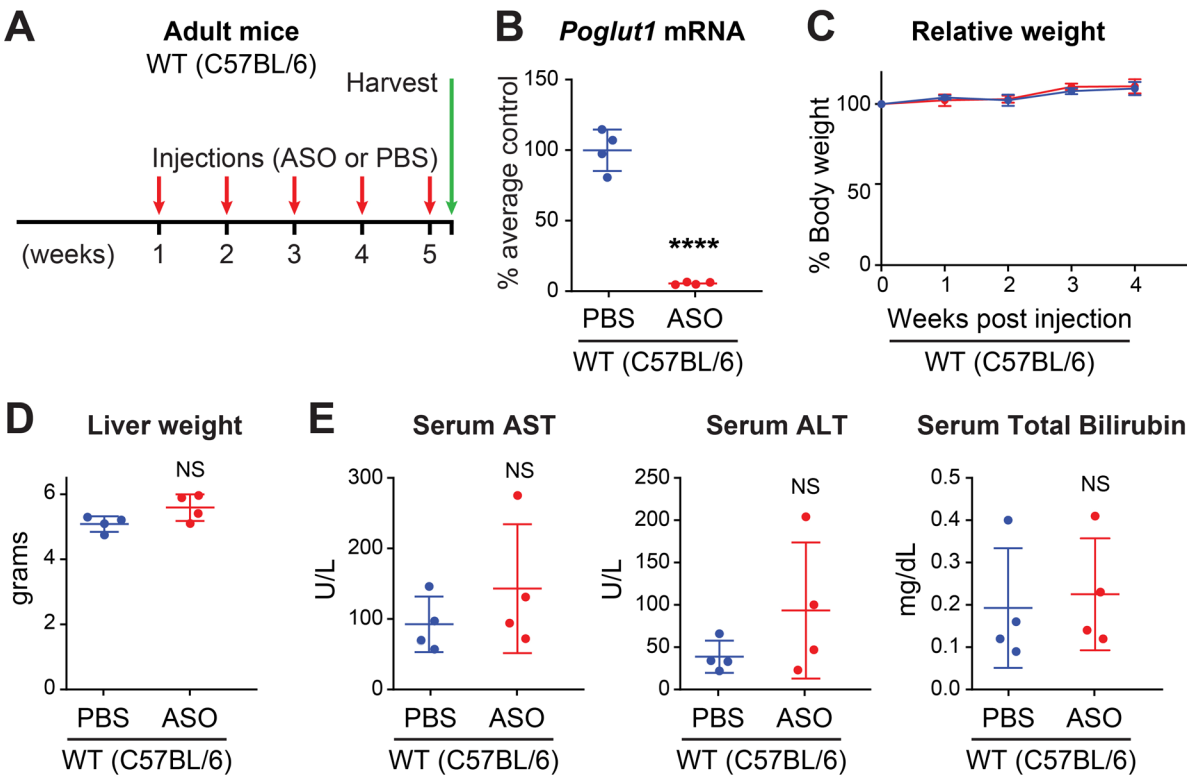
Western blotting

Cells were harvested in RIPA buffer supplemented with protease inhibitors (Promega). Total proteins were quantified using Pierce BCA Protein Assay (BioRad) and bovine serum albumin was used as standard. Protein extracts (20 µg per well) were separated on a 4-15% SDS-PAGE mini-PROTEAN TGX Precast gradient gel (Bio-Rad). Proteins were transferred onto polyvinylidene difluoride membranes (BioRad). Membranes were blocked in 5% skim milk powder dissolved in 0.1% TBS-T at room temperature for 1 hour. The following primary antibodies were used: mouse anti-JAG1 (Santa Cruz, sc-390177, 1:1,000), mouse anti-βActin-HRP (Santa Cruz, sc-47778 HRP, 1:1,000) and rabbit anti-cleaved NOTCH1 (Cell Signaling, 4147, 1:1,000). The antibodies were diluted in blocking buffer and incubated with the membrane overnight at 4°C. For anti-JAG1 and anti-cleaved NOTCH1 antibodies, HRP-linked anti-mouse (Jackson ImmunoResearch, 115-035-003, 1:10,000) and anti-rabbit (Jackson ImmunoResearch, 111-035-003, 1:10,000) secondary antibodies were used, respectively, followed by ECL detection. For each western blot on cell lysates, 3-4 independent experiments were performed.

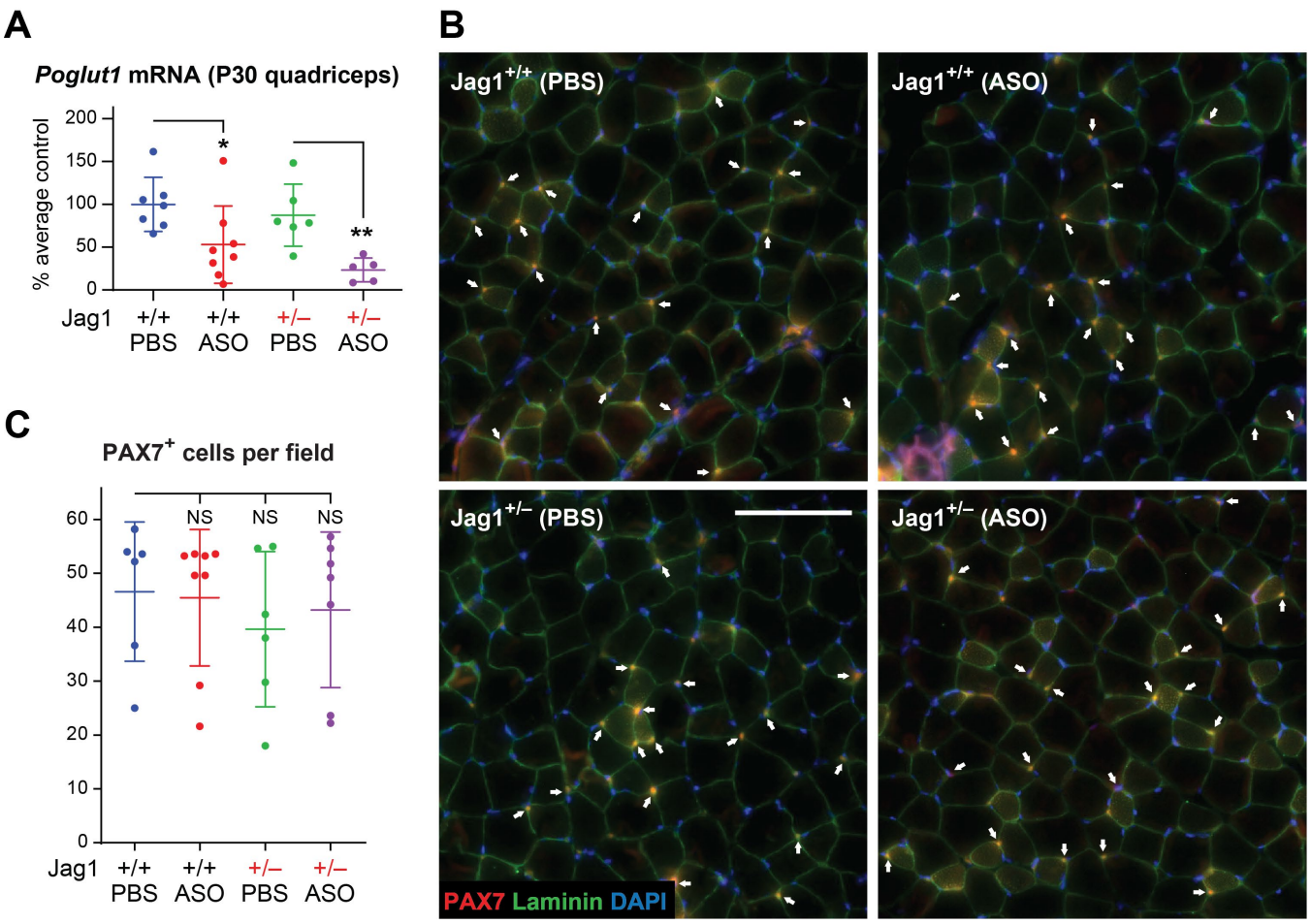
References

1. Adams JM, Huppert KA, Castro EC, Lopez MF, Niknejad N, Subramanian S, et al. Sox9 is a modifier of the liver disease severity in a mouse model of Alagille syndrome. Hepatology 2020;71:1331-1349.

2. Durkin ME, Qian X, Popescu NC, Lowy DR. Isolation of Mouse Embryo Fibroblasts. Bio Protoc 2013;3:e908.



Supporting Fig. S1. Subcutaneous injection of ASO-1 can efficiently knockdown *Poglut1* in the adult mouse liver with no liver toxicity. (**A**) Injection regimen of ASO-1 and PBS in adult WT animals (50 mg/kg/dose). (**B**) qRT-PCR assays for *Poglut1* mRNA levels in the livers of adult WT animals injected with ASO or PBS based on the regimen shown in **A**. (**C**) Relative body weight of the animals shown in **B** over the course of ASO and PBS injections. The weight of each animal was set as 100% on the day of the first injection (0 weeks post injection in the graph). On the day of each subsequent injection, the weight of each animal was calculated as a percentage of the weight of the same animal in the beginning of the injection regimen. Each circle shows the average weight of the four animals with the same genotype/condition on that day (WT-PBS in blue, WT-ASO in red). Error bars represent ± SD for each group. (**D, E**) Liver weight and serum AST, ALT and total bilirubin are shown for each animal at the end of the injection regimen (72 hours after the fifth injection). In **B**, **D**, **E**, each circle represents an animal and horizontal lines show mean ± SD. NS: not significant, ****P<0.0001, using unpaired t-test.



Supporting Fig. S2. The ASO induces significant *Poglut1* KD in the muscle but does not reduce the number of muscle stem cells. (**A**) qRT-PCR assays for *Poglut1* mRNA levels in quadriceps muscles of WT and *Jag1*$^{+/-}$ P30 mice injected with two doses of ASO or PBS at P1 and P7. The results were normalized to WT-PBS muscles. Each circle represents an animal and horizontal lines show mean ± SD. **P*<0.05, ***P*<0.01, using unpaired t-tests between WT-PBS versus WT-ASO and *Jag1*$^{+/-}$-PBS versus *Jag1*$^{+/-}$-ASO. (**B**) Shown are immunofluorescent staining of P30 quadriceps muscle sections from the indicated genotypes/conditions with DAPI and antibodies against PAX7 and laminin. Arrows mark satellite cells. Scale bar is 100 μm. (**C**) Shown is the average number of PAX7^{+} cells (satellite cells) per field of view quantified in P30 quadriceps muscle sections from the indicated genotypes/conditions. Each circle represents an animal and horizontal lines show mean ± SD. NS: not significant, using one-way ANOVA with Tukey's multiple comparisons test.

Supporting Table S1. List of the primers used in this study

JAG1 EGF4_F	5'-CGCCTGCCTCGCTGATCCCTG-3'
hJAG1 EGF4_R	5'-TGCTCAGCAATTTCACAGTTGG-3'
hJAG1 EGF8_F	5'-TGAATGTGCCGCCAACCCCTGTTTG-3'
hJAG1 EGF8_R	5'-TCGATGTCTCTCTCACAG-3'
hJAG1 EGF11_F	5'-TGACTGTGAGGCCAACCCTTGTAG-3'
hJAG1 EGF11_R	5'-TTAATATTTTCATGGCAGTATG-3'
hJAG1 EGF16_F	5'-TGAATGCCAGGCTTCACCTTG-3'
hJAG1 EGF16_R	5'-TTGATGTTTATTCTGCAGTCG-3'
hJAG1 attB-F	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCGTTCCCCACGGAC-3'
hJAG1 attB-R	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCTATACGATGTACTCCATTCGGTTTAAGC-3'
hJAG1 qPCR-F	5'-GTCCTAAGCATGGGTCTTGC-3'
hJAG1 qPCR-R	5'-CCCAGTTGGTCTCACAGAGG-3'
18S rRNA qPCR-F	5'-GTAACCCGTTGAACCCCATT-3'
18S rRNA qPCR-R	5'-CCATCCAATCGGTAGTAGCG -3'

Supporting Table S2. List of the antibodies used in this study

Antibody	Host	Company	Catalogue No.	Dilution
CK8	Rat	DSHB	TROMA-I	1:20 (IF)
CK19	Rat	DSHB	TROMA-III	1:20 (IF)
αSMA	Mouse	Sigma-Aldrich	A2547, clone 1A4	1:200 (IF)
αSMA	Mouse	Abcam	ab7817	1:600 (IF)
OPN	Goat	R&D Systems	AF808	1:100 (IF)
Tubulin	Mouse	Santa Cruz	sc-8035	1:1,000 (WB)
ASO	Rabbit	Ionis Pharmaceuticals	N/A	1:5,000 (IF)
Cleaved NOTCH1 (Val1744)	Rabbit	Cell Signaling	4147	1:1,000 (WB)
PAX7	Mouse	DSHB	PAX7	1:2.5 (IF)
Laminin	Rabbit	Sigma	L9393	1:200 (IF)
βActin-HRP	Mouse	Santa Cruz	sc-47778 HRP	1:1,000 (WB)
JAG1	Mouse	Santa Cruz	sc-390177	1:1,000 (WB)
Anti-rabbit Alexa Fluor 488	Goat	Invitrogen	A-11008	1:500 (IF)
Anti-mouse Alexa Fluor 555	Goat	Invitrogen	A-21424	1:500 (IF)
Anti-rat Alexa Fluor 488	Donkey	Invitrogen	A-21208	1:200 – 1:500 (IF)
Anti-rabbit Biotin-SP	Donkey	Jackson ImmunoResearch	711-065-152	1:1,000 (IF)
Anti-rabbit IgG-HRP	Goat	Jackson ImmunoResearch	111-035-003	1:10,000 (WB)
Anti-mouse IgG-HRP	Goat	Jackson ImmunoResearch	115-035-003	1:10,000 (WB)
Anti-mouse Alexa Fluor 647	Donkey	Jackson ImmunoResearch	715-605-150	1:300 (IF)
Anti-goat Alexa Fluor 488	Donkey	Jackson ImmunoResearch	705-545-147	1:300 (IF)
Anti-mouse Cy5	Donkey	Jackson ImmunoResearch	715-175-151	1:200 (IF)