SUPPLEMENTAL INFORMATION

Supplemental Figures

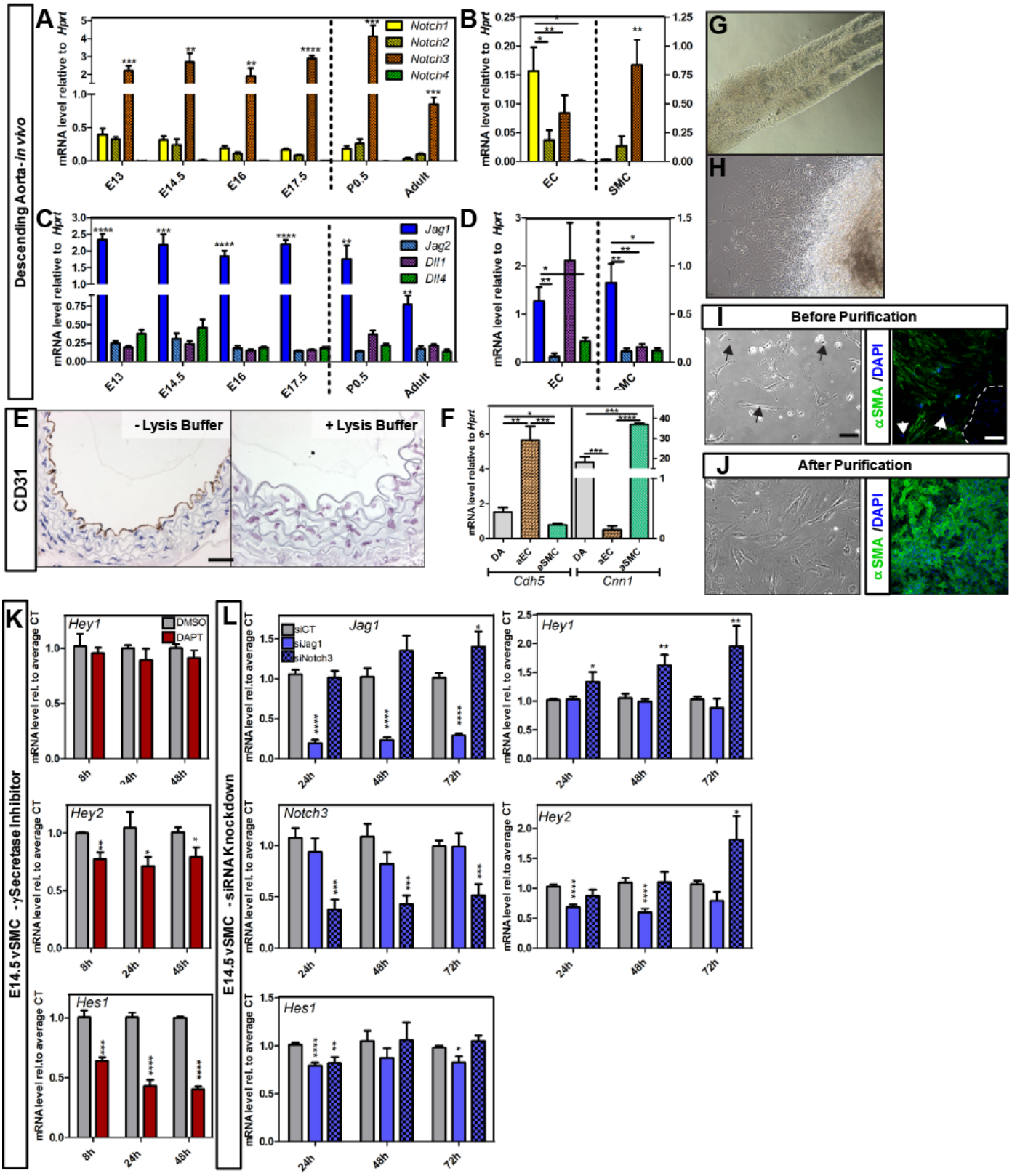
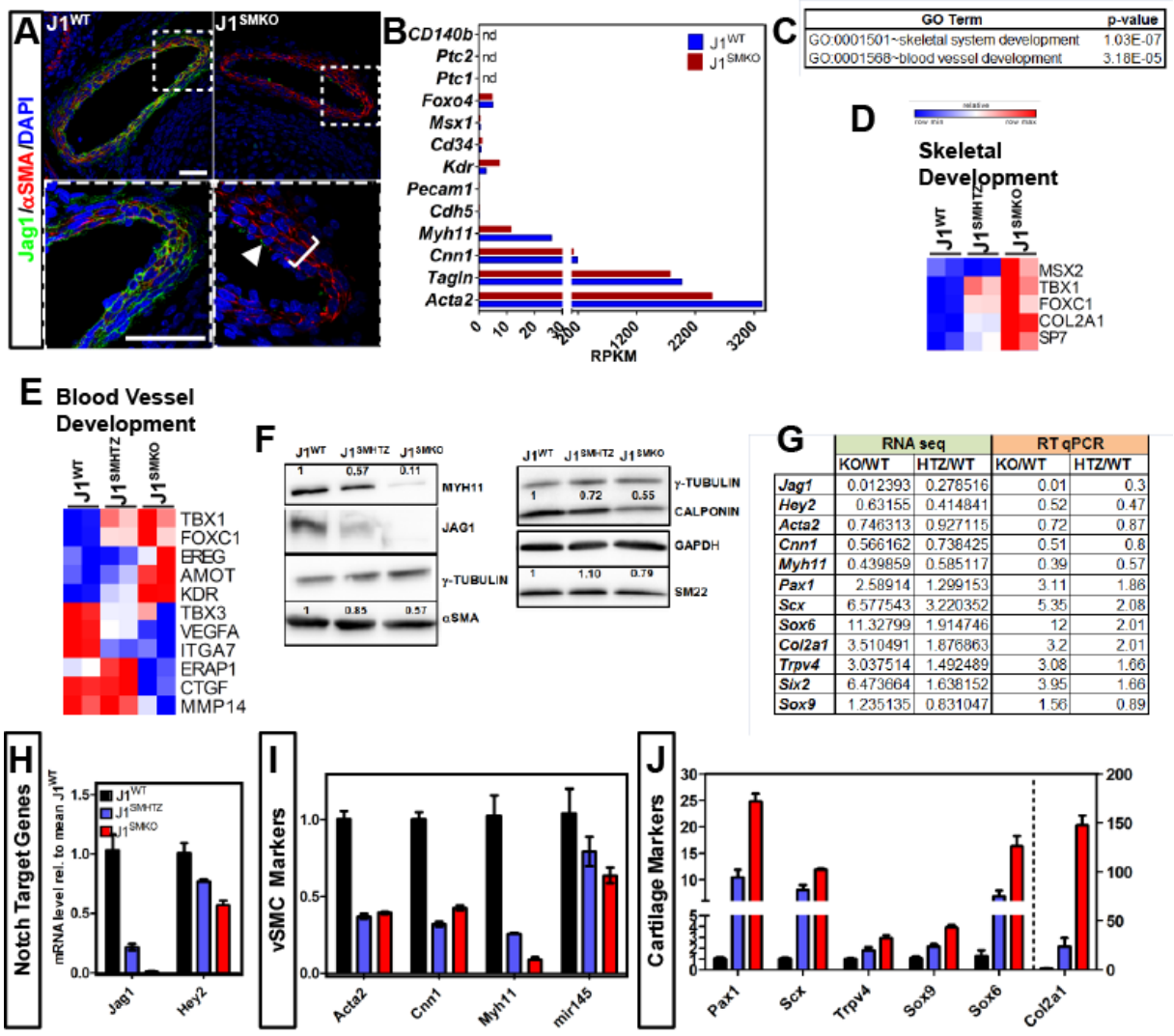


Figure S1, related to Figure 2- Expression profile of Notch receptors and ligands in the descending aorta.

A-D- Expression of Notch receptors and ligands was determined by semi quantitative RT-PCR (qRT-PCR) and normalized to *Hprt* (housekeeping gene). A, C- Transcriptional profile of total aortae from E13 to adulthood revealed that *Notch3* and *Jag1* are the predominantly expressed receptor and ligand. B, D- Transcriptional profile of adult aortic endothelial cell- (aEC) and smooth muscle cell- (aSMC) enriched fractions. E- Evaluation of relative enrichment for endothelial and smooth muscle RNA from the dorsal aorta. Essentially, RNA lysis buffer was injected in the lumen of the dorsal aorta under the microscope. Assessment of the degree of endothelial removal and effect on smooth muscle are shown. Prior to injection of lysis buffer, CD31 was homogeneously detected by immunohistochemistry in the endothelium of adults descending aorta (DA). F- After injection of lysis buffer, CD31 was no longer detected in the DA. Scale Bars: 50μm. G- qRT-PCR analysis of total DA, aortic endothelial cell (aEC) and smooth muscle cell (aSMC)-enriched fractions confirmed levels or enrichment by evaluation of *Cdh5* (VE-Cadherin) and *Cnn1* (Calponin) transcripts respectively. nE13=6; nE16.5=5; nE16=7; nE17.5=5; nP0.5=7; nAdult=5; naEC=4; naSMC=5. G-L- Embryonic vascular smooth muscle cells. G-H Phase-contrast microscopy image of DA explants after dissection from E14.5 embryos (G) and plating in tissue culture dish (H). I- Prior to purification cells positive and negative for αSMA (green) (arrows, dotted line) can be identified growing out of the explants. J- After purification, only αSMA positive vSMC were detected (green). Scale Bars: 100μm. K-L- Smooth muscle cells isolated (E14.5) from $J1^{WT}$ animals were treated with: γ-secretase inhibitor (DAPT, 50uM) or DMSO as vehicle control (K); siRNA scramble or siRNA targeting *Jag1* or *Notch3* (L). Transcriptional level of *Jag1, Notch3* and target genes was determined by qRT-PCR. nDAPT/DMSO= 5-6, nsiCT/siJag1=8, nsiNotch3=6. Data are represented as mean +/- SEM. *p<0.05; **p<0.001; ***p<0.0005; ****p<0.0001.



	RNA seq		RT qPCR	
	KO/WT	HTZ/WT	KO/WT	HTZ/WT
Jag1	0.012393	0.278516	0.01	0.3
Hey2	0.63155	0.414841	0.52	0.47
Acta2	0.746313	0.927115	0.72	0.87
Cnn1	0.566162	0.738425	0.51	0.8
Myh11	0.439859	0.585117	0.39	0.57
Pax1	2.58914	1.299153	3.11	1.86
Scx	6.577543	3.220352	5.35	2.08
Sox6	11.32799	1.914746	12	2.01
Col2a1	3.510491	1.876863	3.2	2.01
Trpv4	3.037514	1.492489	3.08	1.66
Six2	6.473664	1.638152	3.95	1.66
Sox9	1.235135	0.831047	1.56	0.89

Figure S2, related to Figure 3- Characterization of E14.5 embryonic smooth muscle cells

A- Co-immunofluorescence of Jag1 (green) and αSMA (red) in E14.5 DA demonstrated specific loss of Jag1 in vSMC in the $J1^{SMKO}$ (bracket) but retention in endothelial cells (arrowhead). Scale Bars: 25μm. B-RPKM values measured by RNA sequencing for various vascular cell type markers confirmed high enrichment in vSMC contractile markers. C-E- RNA deep sequencing data from E14.5 vSMCs submitted to ENCODE website revealed that a number of differentially expressed genes were potential targets for CTBP2. Among these genes the top two GO-BP was “Skeletal system development” (C,D) and “Blood vessel

development" (C,E). F- Western blot analysis of E14.5 vSMC confirmed deletion of Jag1 in $J1^{SMKO}$ compared to $J1^{SMHTZ}$ and $J1^{WT}$ cells. In addition smooth muscle cell markers (αSMA, CALPONIN, MYH11 and SM22) were found decreased in $J1^{SMKO}$ compared to $J1^{SMHTZ}$ and $J1^{WT}$ cells. γ-TUBULIN and GAPDH were used as loading controls. G- mRNA expression of specific genes identified by RNA sequencing were confirmed by qRT-PCR. H-J- Transcriptional analysis of Notch signaling molecules (H), smooth muscle cell markers (I) and cartilage markers (J) in vSMC *in vitro* isolated from E14.5 embryos. Data are represented as mean +/- SEM.

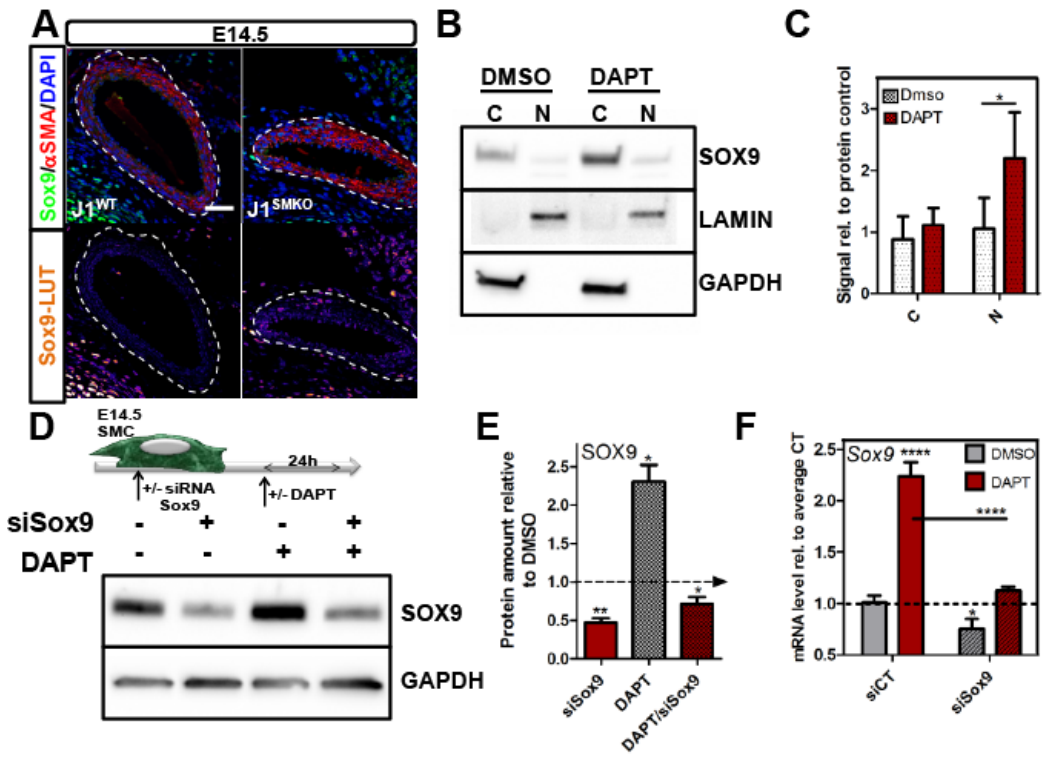


Figure S3, related to Figure 4- Sox9 in E14.5 vSMC

A-Co-immunofluorescence of Sox9 (green, LUT) and αSMA (red) in E14.5 DA shows the presence of Sox9 positive cells in the media of the $J1^{SMKO}$ whereas none were detected in $J1^{WT}$ littermates. Scale Bars: 25μm. B- Sox9 protein was determined in cytoplasmic (C) and nuclear fraction (N) of $J1^{WT}$ E14.5 vSMC treated with DAPT (50μM- 24h). C- Quantification of Sox9 protein levels from three independent experiments. D-F- vSMC from E14.5 WT mice were treated with siRNA targeting Sox9 previous to DAPT treatment (50μM- 24h). Protein (D,E; n=3- *p<0.05; **p<0.006) and mRNA (F; n=7- *p<0.05; ****p<0.0001)) levels for Sox9 were analyzed by Western blots and qRT-PCR respectively. GAPDH was used as loading control for Western blot on cytoplasmic and total lysate (B,D) and LAMIN for nuclear lysate (B). Data are represented as mean +/- SEM.

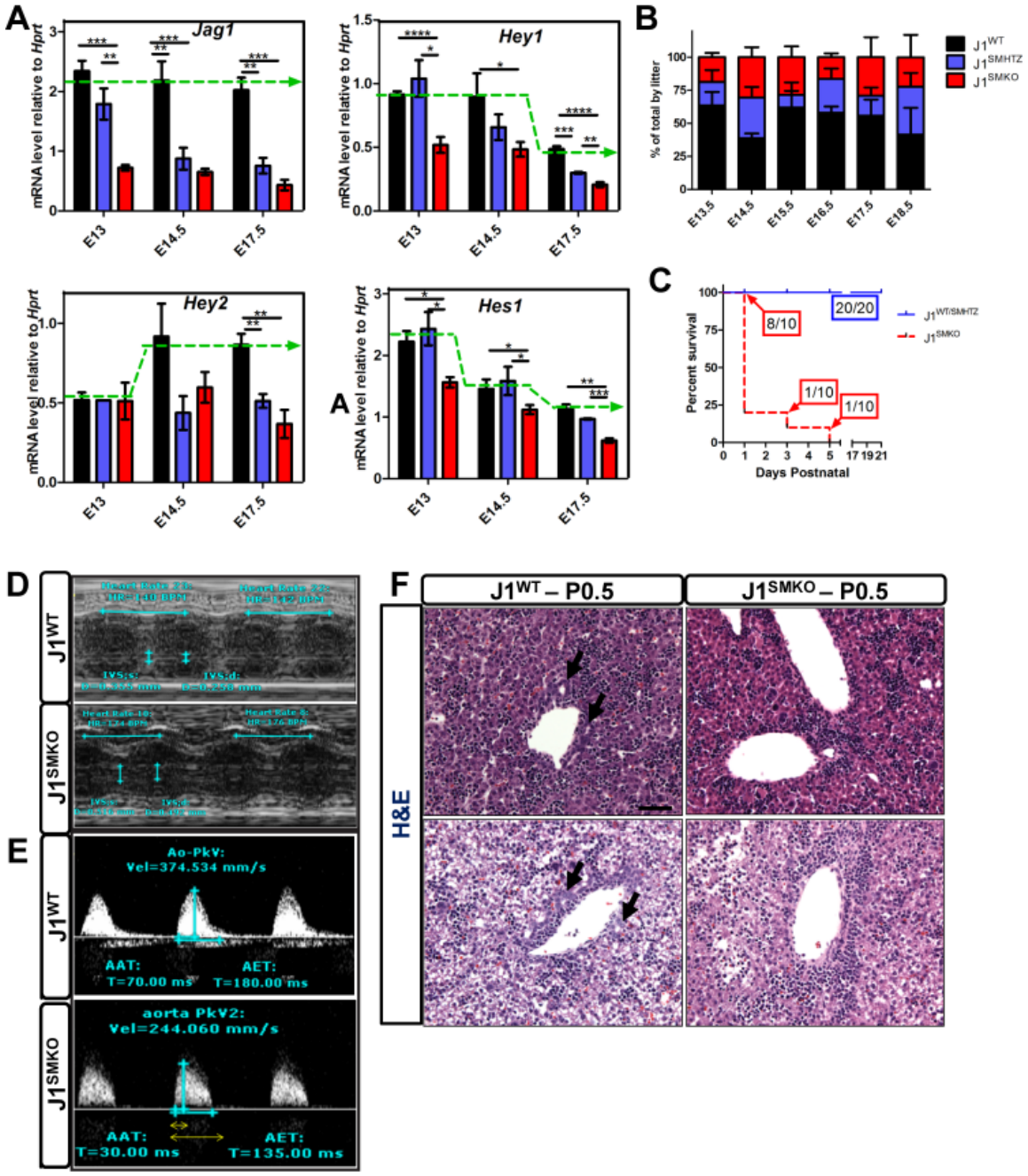


Figure S4, related to Figure 6- Characterization of J1^{SMKO} mice

A-Expression of mRNA for Jag1 and Notch target genes was determined by qRT-PCR in the developing dorsal aorta of J1^{WT} (black; n=5-9), J1^{SMHTZ} (blue; n=3-5) and J1^{SMKO} (red; n=3-5) mice. Deletion of Jag1 in SM22^{+} smooth muscle cells was associated with a global decrease in expression of Notch target genes. Transcript levels of specific genes are shown relative to *Hprt* (housekeeping control). Green dotted line represents the trend of expression of J1^{WT} over time. *p<0.05; **p<0.001; ***p<0.0005; ****p<0.0001. B- Evaluation of viability in the three

genotypes: Percentage of $J1^{WT}$ (black), $J1^{SMHTZ}$ (blue) and $J1^{SMKO}$ (red) in litters harvested during developmental stages revealed no noticeable embryonic lethality associated with deletion of Jag1. Litters analyzed: nE13.5=3, nE14.5=6, nE15.5=3, nE16.5=3, nE17.5=5, nE18.5=3. (A-B) Data are represented as mean +/- SEM. C- Deletion of Jag1 in the $Sm22^{+}$ compartment leads to early postnatal lethality (4 litters). At day 5, no $J1^{SMKO}$ remained. D-E- Prenatal Echocardiography: Hemodynamic Assessment of Fetal Cardiac Function at E18.5 in utero. D- Representative heart rate (HR) assessment based on beat-to-beat interval by using M-Mode tracing in a $J1^{WT}$ and a $J1^{SMKO}$. Compared to the wild type, $J1^{SMKO}$ mice had higher basal heart rate and thickening of inter-ventricular septum (IVS) during systole and diastole. E- Representative assessment of the aorta pulse wave velocity (PkV; Peak Velocity) by using pulse wave Doppler tracing in $J1^{WT}$ and $J1^{SMKO}$ embryos. The aorta acceleration and ejection times (AAT and AET respectively) and the aorta peak velocity (ao-Pkv) were measured as shown. Compared to $J1^{WT}$, we observed lower aorta velocity and diminished AAT/AET ratio in the mutant. These observations may reflect hemodynamic impact, secondary to structural change in the wall of the aorta. F- Histological analysis of liver cross-sections at birth confirmed that the $J1^{SMKO}$ animals lack appropriate formation of biliary ducts around the portal vein (PV) as compared to $J1^{WT}$ (arrows) in this C57Bl background as previously shown in the mixed background (Hofmann et al., 2010). Scale Bars: 50μm.

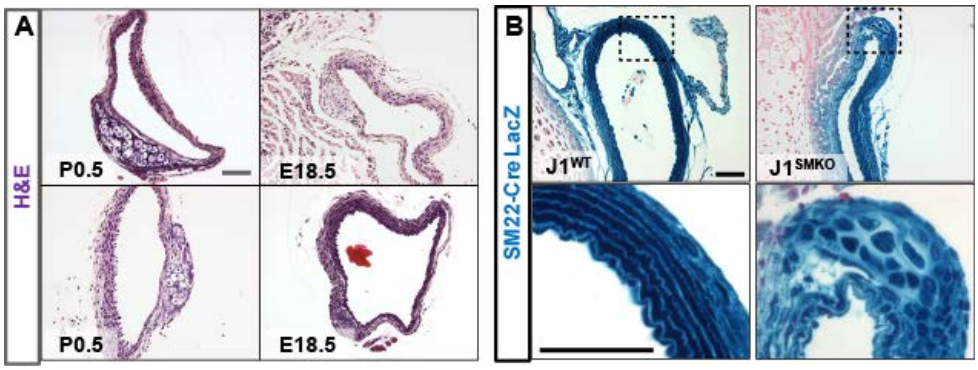


Figure S5, related to Figure 7- Loss of Jag1 in specified vSMC leads to cell fate switch and ectopic cartilage formation in the wall of the descending aorta

A- Loss of Jag1 in the vascular wall is associated with ectopic formation of cartilaginous structures as observed by H&E staining of aorta cross-sections at E18.5 and P0.5. B- Cell lineage analysis using ROSA-LacZ in the background of $J1^{SMKO}$ revealed that cartilage nodules in the DA were derived from Sm22-expressing cells. Scale Bars: 50μm.

Supplemental Movie

Movie S1, related to Figure 7. 3 dimensional reconstitution of a cartilage nodule find in the aorta media of J1^{SMKO} neonate.

3D reconstitution was generated from projection of H&E serial cross-sections using Imaris software (Bitplane Inc., South Windsor, CT).

Supplemental Experimental Procedures

Preparation of enriched fraction of endothelial cells and smooth muscle cells for transcriptional analysis

Neonatal or adult mice were perfused with sterile PBS. The descending aorta (DA) was harvested and the surrounding tissue was removed. For endothelial cells (EC) and vascular smooth muscle cells (vSMC) enriched fractions, the dissected aortae were perfused with RLT lysis buffer (QIAGEN, Valencia, CA) containing 1% β-mercaptoethanol. The perfused fraction was enriched in EC, as determine by *Cdh5* expression level. The remaining part considered as vSMC enriched also as determined by vSMC markers expression (Figure S1). Total aortae from adult mouse or vSMC enriched fraction were then homogenized in RLT lysis buffer and digest with RNA grade proteinase K (200ug, for 10 min at 55°C; Invitrogen, Carlsbad, CA) before total RNA was isolated.

Transcriptional analysis

Total RNA from cells or aortae was purified using the RNeasy micro (embryos) or mini (adult tissue and cells) kit (QIAGEN, Valencia, CA). Complementary DNA synthesis was performed with Superscript III reverse transcriptase First-Strand synthesis kit (Invitrogen, Carlsbad, CA) using oligodT primers.

Quantitative RT-PCR was performed onto DNA Engine Opticon 2 Real-Time Cycler PCR detection system (MJ Research; Biorad, Hercules, CA) using the RT^2 SYBR Green qPCR Master Mix (SABiosciences; Qiagen, Valencia, CA). Primers used are described in the table below. Results were analyzed with MJ Opticon Monitor Analysis software Version 3.1 (MJ Research; Biorad, Hercules, CA). Each reaction was run in duplicate and normalized with *Hprt* housekeeping gene or *U6* for *mir145* normalization. p-values were calculated using appropriate t-test.

Primer sequences for quantitative RT-PCR

Target cDNA	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Hprt	CTGGTTAAGCAGTACAGCCCCAA	CGAGAGGTCCTTTTCACCAGC
Notch1	CCCTTGCTCTGCCTAACGC	GGAGTCCTGGCATCGTTGG
Notch2	GGAATGGTGGCAGAGTTGAT	TCGCCTCCACATTATTGACA
Notch3	GGACAAGATGCACTGGGAAT	AGTCTCTTGGCCTCTGGACA
Notch4	TTCTCGTCCTCCAGCTCATT	CCACTCCATCCTCATCCACT
Cdh5	AAGCTGCCAGAAAACCAGAA	ATTCGGAAGAATTGGCCTCT
Jag1	CAGTGCCTCTGTGAGACCAA	AGGGGTCAGAGAGACAAGCA
Jag2	GCACCTGCACACATAACACC	TTGACGCCATCAACACAGAT
Dll1	GGCTTCTCTGGCTTCAACTG	CACCGGCACAGGTAAGAGTT
Dll4	ACCTTTGGCAATGTCTCCAC	GTTTCCTGGCGAAGTCTCTG
Hey2	TCCAGGCTACAGGGGGTAAA	AGATGAGAGACAAGGCGCAC
Hes1	ACACCGGACAAACCAAAGAC	ATGCCGGGAGCTATCTTTCT
Hey1	GAGACCATCGAGGTGGAAAA	CTTCTCGATGATGCCTCTCC
Acta2	CTGACAGAGGCACCACTGAA	CATCTCCAGAGTCCAGCACA
Tagln	GATGGAACAGGTGGCTCAAT	TTCCATCGTTTTTGGTCACA
Cnn1	GGGAACAACTTCATGGATGG	GGGAACAACTTCATGGATGG
Myh11	TCCAGGGATGAGATCTTTGC	AGCTCCTCCTTCTCCAGGTC
Mir145	GGTCCAGTTTTCCCAGG	CAGTGCGTGTCGTGGAGT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
Pax1	GGCAATGACCTTCAAACACC	GGCAGTCCGTGTAAGCTACC
Sox9	AGGAAGCTGGCAGACCAGTA	CGTTCTTCACCGACTTCCTC
Sox6	ATCTCCCACCCAGAACCTCT	CAGGGCAGGAGAGTTGAGAC
Col2a1	GGCTCCCAGAACATCACCTA	CTTGCCCCACTTACCAGTGT
Scx	GCCTCAGCAACCAGAGAAAG	GGCTCTCCGTGACTCTTCAG
Six2	GCAAGTCAGCAACTGGTTCA	CTTCTCATCCTCGGAACTGC

Trpv4	TTCACCCTCACCGCCTACTA	AGCCTCAGGTAGTCCACTGT
Eln	GCTGATCCTCTTGCTCAACC	CAATACCAGCCCCTGGATAA
Mgp	ACAGGAGAAATGCCAACACC	TGCGTTCCTGGACTCTCTTT
Runx2	GCCGGGAATGATGAGAACTA	TGGGGAGGATTTGTGAAGAC

Immunostaining and histological analysis

Tissue samples embedded in paraffin were used for histological examination. For alcian blue staining the slides were deparaffined and stained for 30 minutes with 1% Alcian Blue 8GX in 3% acetic acid solution. Counterstained was performed with a solution of 0.1% nuclear fast red for 5 minutes. For immunostaining 4μm cross-sections were deparaffined and antigen retrieval was performed with boiling citrate buffer (10mM, pH6) or Proteinase K antigen unmasking (for CD31 detection). The primary antibodies used are shown in the table below. Imaging was performed using Olympus BX40 light microscope (Olympus America, Center Valley, PA) or Zeiss LSM 710 multiphoton confocal microscope using the Zen software (Carl Zeiss, Thornwood, NY). 3D reconstitution, projection and movie were generated using Imaris software (Bitplane Inc., South Windsor, CT). For morphometric analysis we used ImageJ software.

Antibodies used for immunodetection

Target	Company
Notch1	93-4, gift from Gerry Weinmaster
Notch3	Abcam, Cambridge, MA
Collagen 2	Abcam, Cambridge, MA
Calponin	Abcam, Cambridge, MA
CD31	BD Pharmingen, San Jose, CA
Jagged1	R&D system, Minneapolis, MN

αSMA	Dako, Carpinteria, CA
Sox9	Chemicon, EMD Millipore, Billerica, MA
Osteopontin	BMA Biomedicals, Rheinstrasse, Switzerland
Laminin	Sigma-Aldrich, St.Louis, MO
TropoElastin	Abcam, Cambridge, MA

RNA sequencing and differential expression analysis

RNAseq libraries were created from 500ng or 1μg of total RNA from E14.5 purified vSMCs or ImSMC treated with DAPT (50μM, 3 days) respectively with the Truseq RNA sample prep kit (Illumina, San Diego, CA) and sequenced using the Illumina Hiseq 2000 platform. eSMC isolated from 3 independent animals (1WT, 1 HTZ, 1 KO) were multiplexed into one lane. Duplicates were sequenced on a second line. ImSMC treated with DMSO or DAPT in triplicate were multiplexed into one lane. More than 200 million 50-bp single-end reads were obtained in each lane.

After demultiplexing the data (allowing for 1 mismatch) and converting from qseq format to fastq format, the reads were aligned to the GRCm37/mm9 assembly mouse genome using the Tophat aligner (Trapnell et al., 2009) and ENSEMBL 67 annotation. Only uniquely mapped reads, and up to 2 mismatches in the read were allowed. The resulting bam file was run through HTSeq (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) to count the number of reads per gene. For the counting process, the "nonempty intersection" method was used. This matrix was run through DESeq (http://www-huber.embl.de/users/anders/DESeq/) to normalize the counts using a robust normalization process; this process linearly scales each sample's counts to minimize the square distance between them. RPKM (Reads Per Kilobase of exon per Million reads) was calculated using the total number of reads uniquely mapped, and gene length was calculated from the union of all possible exons of the gene. Finally, the p-values are calculated with

DESeq (Anders and Huber, 2010), pooling replicates together to more accurately estimate dispersion values. The data sets have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE60643.

Western blot analysis

Cells were lysed in mRIPA buffer containing 1% Triton X-100 and 10% SDS or nuclear fraction was separated using the Nuclear Complex Co-IP kit (Active Motif, Carlsbad, CA). Western blot analysis was performed using primary antibodies shown in the table below. Immuno-complexes were detected by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA) using the ChemiDoc XRS+ Molecular Imager and ImageLab Software (Bio-Rad, Hercules, CA). Quantification of bands by densitometry analysis was performed using Image J software.

Antibodies used for Western Blot analysis

Target	Company
Jagged1	Cell Signaling, Danvers, MA
Collagen 2	Abcam, Cambridge, MA
Sox9	Chemicon, EMD Millipore, Billerica, MA
Runx2	Santa-cruz Biotechnology, Santa Cruz,CA
Osteopontin	BMA Biomedicals, Rheinstrasse, Switzerland
Scleraxis(A)	ThermoFisher Scientific, Rockford, IL
Sm22	Santa-cruz Biotechnology, Santa Cruz,CA
Calponin	Abcam, Cambridge, MA
αSMA	Dako, Carpinteria, CA
Myh11	Abnova, Walnut, CA
γTubulin	Abcam, Cambridge, MA

Gapdh	EMD Millipore, Billerica, MA
Lamin A/C	Santa-cruz Biotechnology, Santa Cruz,CA

β-galactosidase staining

Tissue samples collected from mouse neonates were fixed in glutaraldehyde buffer and β–Galactosidase staining was performed as previously described (Alva et al., 2006).

Prenatal Echocardiography

At day 18.5 of gestation, pregnant females were anesthetized with Isoflurane (2% for induction and 1.5-2% throw-out imaging) maintaining the heart rate at 400–500 beats/minutes. The hair was removed from the abdomen using a chemical hair remover (Nair). After identification of the embryos, B- and M-mode ultrasound imaging was performed, using a high-resolution Vevo 2100 bio microscopy-ultrasound system with a 30 MHz transducer (Visual Sonics, Toronto, Ontario, Canada). The embryo heart rate and ventricular dimensions were measured from the short axis mid-ventricular (mid-papillary) view with 2D oriented M-mode imaging. Hemodynamic parameters were measured using Pulse wave Doppler (PWD) tracing. Aorta PkV, AAT, AET were measured from PWD tracing obtained at the aortic root.

Supplemental References

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