Notch Activation of Jagged1 Contributes to the Assembly of the Arterial Wall

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Background—Notch signaling in vascular smooth muscle precursors is required for smooth muscle differentiation. Jagged1 expression on endothelium activates Notch in vascular smooth muscle precursors including those of neural crest origin to initiate the formation of a smooth muscle layer in a maturing blood vessel.

Methods and Results—Here, we show that Jagged1 is a direct Notch target in smooth muscle, resulting in a positive feedback loop and lateral induction that propagates a wave of smooth muscle differentiation during aortic arch artery development. In vivo, we show that Notch inhibition in cardiac neural crest impairs Jagged1 messenger RNA expression and results in deficient smooth muscle differentiation and resultant aortic arch artery defects. Ex vivo, Jagged1 ligand activates Notch in neural crest explants and results in activation of Jagged1 messenger RNA, a response that is blocked by Notch inhibition. We examine 15 evolutionary conserved regions within the Jagged1 genomic locus and identify a single Notch response element within the second intron. This element contains a functional Rbp-J binding site demonstrated by luciferase reporter and chromatin immunoprecipitation assays and is sufficient to recapitulate aortic arch artery expression of Jagged1 in transgenic mice. Loss of Jagged1 in neural crest impairs vascular smooth muscle differentiation and results in aortic arch artery defects.

Conclusions—Taken together, these results provide a mechanism for lateral induction that allows for a multilayered smooth muscle wall to form around a nascent arterial endothelial tube and identify Jagged1 as a direct Notch target. (Circulation. 2012;125:314-323.)

Key Words: muscle, smooth ■ heart defects, congenital ■ vasculature

Vascular smooth muscle is derived from multiple embryonic sources including neural crest and lateral plate mesoderm.\(^1\)\(^-\)\(^2\) Notch signaling in vascular smooth muscle precursors is required for smooth muscle differentiation. The Notch ligand, Jagged1, is expressed by endothelium and activates Notch in vascular smooth muscle precursors to initiate the formation of a smooth muscle layer in a maturing blood vessel.\(^3\) Thus, inhibition of Notch signaling in neural crest leads to impaired smooth muscle differentiation and aortic arch artery defects although neural crest migration is unaffected.\(^4\) In vitro studies suggest that Notch directly regulates smooth muscle \(\alpha\)-actin expression in vascular smooth muscle cells.\(^5\)\(^,\)\(^6\)

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The importance of the Notch signaling in the endothelium and vascular smooth muscle is further highlighted by the spectrum of cardiovascular defects associated with mutations of Notch ligands or receptors. Mutations in NOTCH3, encoding a Notch receptor found in vascular smooth muscle, cause the autosomal dominant disorder CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy).\(^7\)\(^,\)\(^8\) Notch3 knockout mice, though viable, show diminished expression of some vascular smooth muscle markers in a subset of arteries, indicating that Notch may promote some aspects of smooth muscle differentiation or maturation in vivo.\(^9\) Mutations in NOTCH2 or the Notch ligand JAGGED1 are associated with Alagille syndrome (AGS).\(^10\)\(^,\)\(^11\) Alagille syndrome is a multifaceted disorder including congenital heart defects and vascular pathologies.\(^10\)\(^-\)\(^12\) Mice lacking Jagged1 die early in development because of defective remodeling of both the embryonic and yolk sac vasculature.\(^13\) Similar vascular defects are observed when Jagged1 is specifically deleted in the endothelium.\(^3\) Jagged1 is also expressed by smooth muscle, and deletion using the smooth muscle protein 22-alpha promoter driven Cre (SM22-Cre) resulted in the absence of intrahepatic bile ducts,\(^14\) a feature of AGS. Deletion of Jagged1 in murine smooth muscle, again utilizing SM22-Cre, is also associated
with early postnatal mortality due to patent ductus arteriosus, a common human congenital heart defect. Normally, the ductus arteriosus closes at birth, but defective smooth muscle differentiation after Jagged1 deletion prevents proper vessel remodeling.

Notch is a highly conserved signaling pathway. In mammals, association of 1 of 4 Notch receptors (Notch 1–4) with 1 of 5 Notch ligands (Jagged1, Jagged2, Delta-like 1 [Dll1], Delta-like 3 [Dll3] and Delta-like 4 [Dll4]) initiates juxtacrine signaling. After ligand-receptor association, proteolytic cleavage releases the Notch intracellular domain (NICD), which translocates to the nucleus. Notch intracellular domain then forms an active transcriptional complex with the DNA-binding protein Rbp-J and the coactivator Mastermind-like (MAML), and target genes are transcribed. Classically, Notch signaling has been thought to function through lateral inhibition in which a stochastic decision by 1 cell prevents adjacent cells from adopting the same cell fate. This asymmetry in cell fate is typically associated with a decrease of Notch ligand expression in neighboring cells, imparting a selective advantage to the single differentiating cell.

Alternatively, Notch can function as part of a positive feedback loop in which Notch receptor activation promotes Notch ligand expression in surrounding cells thus relaying a signal, a process known as lateral induction.

Lateral induction of Notch signaling has been documented in diverse physiological systems. In the developing inner ear, for example, overexpression of Notch both induces Jagged1 expression and sensory specification from nonsensory epithelium. Additional examples of Notch/Jagged lateral induction can be found both in macrophages and the ocular lens. In studies most relevant to vascular development, endothelial Jagged1 expression activates Notch3 in mural cells resulting in increased Jagged1 expression, and Jagged1 protein is decreased in retinal blood vessels of Notch3 knockout animals.

In this report, we demonstrate that smooth muscle precursors derived from neural crest upregulate Jagged1 messenger RNA (mRNA) on Notch activation. We show that Jagged1 is a direct Notch target and we identify a Notch response element in the Jagged1 genomic locus that mediates vascular smooth muscle lateral induction. These results help to explain how a multilayered vascular smooth muscle wall forms around a nascent endothelial tube.

**Methods**

**Mice**

All mice were maintained on a mixed genetic background. Pax3CreERT2;24 dominant negative MAML gene (DNMAML),25 and Jagged1flox/+;26 mice were genotyped as previously described. The newly created stable mouse line, containing ECR6 5′ of the heat shock protein 68 promoter directing lacZ gene expression (ECR6-hsp68-LacZ) was genotyped using the following primers:

ECR6Tg Forward: 5′-CGGACCTACAACACTAAGACAG-3′

ECR6Tg Reverse: 5′-GGTAAACGCCAGGTTTCCCCAGTC-3′

All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

**Histology, Immunohistochemistry, and In Situ Hybridization**

Samples were harvested, fixed overnight in 4% paraformaldehyde, and subsequently dehydrated through an ethanol series. All samples were then paraffin embedded and sectioned. Antibodies used for immunohistochemistry were anti-Jag1 rabbit polyclonal H-114 (Sigma-Aldrich). The Jagged1 and hairy and enhancer of split–related transcription factor 1 (HRT1) in situ probes used were previously published.4 Radioactive in situ hybridizations were completed as detailed in a previous protocol.27

**Primary Vascular Smooth Muscle Cell Culture**

To isolate neural crest–derived smooth muscle, the aortic arch to the origin of the left subclavian artery and the proximal regions of the major arch vessels were dissected from E17.5 or E18.5 embryos. Primary smooth muscle cell cultures were performed according to previously published protocols for aortic vascular smooth muscle cells.28 Jagged1-Fc and control-Fc constructs were kindly provided by Tom Kadesch, PhD, and have been described elsewhere.29 Smooth muscle cells were stimulated by plating on Jagged1-Fc or control-Fc coated plates at a density of 2.5×10⁴ per 10-cm plate. Cells were harvested 24 hours after stimulation, and RNA was extracted using Trizol reagent (Invitrogen). Semiquantitative real-time polymerase chain reaction (PCR) was performed using standard protocols and the following primers:

Jagged1 forward: 5′-GCTTCCACTGCACTGTAGTTTC-3′

Jagged1 reverse: 5′-TGGTCACTAAATTCCCCCCTC-3′

GAPDH forward: 5′-ACACAGCTCATGCCATCAC-3′

GAPDH reverse: 5′-GAATGCACAGGAGAAGAAGTC-3′

**Sequence Analysis**

A comparative sequence alignment was completed using the National Center for Biotechnology DCODE resource (http://www.dcode.org) to ascertain evolutionarily conserved regions (ECRs) within and surrounding the Jagged1 genomic locus. This analysis encompassed ~30 kb upstream, all intronic regions (~40 kb) and 10 kb downstream of the Jagged1 locus on mouse chromosome 2. The ECRs were identified as elements of at least 200 bp bearing a minimum sequence identity of 70% among mouse, human, dog, and opposum. This analysis identified 15 ECRs. All conserved regions were amplified with primers containing a 5′ attB1 site and a 3′ attB2 site to allow for Gateway recombination into pDONR221 (Invitrogen). Genomic locations of all ECRs are listed in online-only Data Supplement Table I whereas primer sequences can be found in online-only Data Supplement Table II. All ECRs were sequence verified after insertion into pDONR221. Conserved elements were then recombined into a Gateway-compatible pGL3-promoter vector for use in luciferase assays (Promega, vector courtesy of R. Addis, PhD, and J. Gearhart, PhD, University of Pennsylvania, Philadelphia, PA).

**Luciferase Assay**

Twenty-four hours before transfection, HeLa cells were plated in 12-well plates. Similarly, in experiments using the Jagged1-Fc and control-Fc constructs, HeLa cells were plated 24 hours before transfection. HeLa cells were maintained at 37°C with 5% CO₂ in DMEM medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Transfections were completed using FuGene6 (Roche) with 300 ng of the ECRx-pGL3-promoter DNA, 300 ng of mouse NICD (mNICD) or empty vector, and 1.5 ng CMV-renilla luciferase (Promega). In luciferase experiments using the Jagged1-Fc and control-Fc constructs, no mNICD was transfected. Cellular extracts were collected 48 hours after transfection for use in a dual-luciferase assay (Promega). Twenty μL of cellular extract was
used to assess first flye luciferase activity, followed immediately by the measurement of renilla luciferase activity. Firefly luciferase activity from the ECRx-pGL3-promoter vector was normalized to the renilla luciferase activity. All experiments were performed in duplicate on at least 3 separate occasions. Statistical differences between conditions were analyzed using a 2-tailed, paired t test.

Deletion Series
ECR6 deletion constructs were PCR amplified from full-length ECR6-pGL3-promoter. All deletion constructs were amplified with primers containing a 5′ attB1 site and a 3′ attB2 site to allow for Gateway recombination into pDONR221 (Invitrogen), and all primer sequences can be found in online-only Data Supplement Table II. All ECR6 deletions were sequence verified after insertion into pDONR221. Deletion constructs were then recombined into a Gateway-compatible pGL3-promoter vector for use in a luciferase assay.

ECR Mutagenesis
The predicted Rbp-J binding site within ECR6 was mutated using QuikChange Mutagenesis (Stratagene). All mutagenic primer sequences can be found in online-only Data Supplement Table II with underlined nucleotides indicating mutagenic sequences. M1 (***) primers modify the wild-type Rbp-J site sequence from 5′-TTTCCCACAGT-3′ to 5′-AATCCCAAGT-3′. M2 (**) primers modify the wild-type Rbp-J site sequence from 5′-TTTCCCACAGT-3′ to 5′-TTTCCGGACAGT-3′. M3 (XXXXX) primers modify the wild-type Rbp-J site sequence from 5′-TTTCCCACAGT-3′ to 5′-TGACAGACAGT-3′. The mutations were created in ECR6-pDONR221 vector and were fully sequenced to verify the presence of the intended mutations and exclude other polymerase errors. ECR6 mutants were then recombined into a Gateway-compatible pGL3-promoter vector for use in luciferase assays.

Generation of Transgenic Mice
Wild-type and mutant (M3) ECR6 were inserted upstream of the lacZ gene with an hsv68 minimal promoter. The mutation in ECR6 was identical to “M3” used for luciferase assays and described above. Transgenic mice were generated by injection of linearized DNA into the male pronucleus of C57BL6xSJL-F1/J zygotes. Embryos were harvested at E11.5 for analysis of β-galactosidase expression and genotyping. Genotyping for lacZ was performed as previously described. Embryos were fixed with 2% paraformaldehyde in PBS for 20 minutes at 4°C. After fixation, samples were washed twice for 10 minutes in PBS at 4°C. Embryos were stained in 1 mg/mL X-gal, 5 mmol/L K3Fe(CN)6, 5 mmol/L K4Fe(CN)6, 2 mmol/L MgCl2, 0.01% NP-40, and 0.01% sodium deoxycholate in PBS. Staining reactions were incubated overnight at 37°C. For optical projection tomography analysis, embryos were embedded in 1% low-melt agarose, dehydrated in methanol, and then cleared in 1:2 (v/v) benzyl alcohol and benzyl benzoate. Embryos were scanned using the Bioptons Optical Projection Tomography Scanner (3001 M). Image stacks were reconstructed using OsiriX software.

Chromatin Immunoprecipitation
Twenty-four hours before transfection, 293T or HeLa cells were plated in 100-mm plates. Three 100-mm plates were prepared per condition. Cells were maintained at 37°C with 5% CO2 in DMEM medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Transfections for endogenous chromatin immunoprecipitation (ChIP) assays used FuGene6 (Roche) with 1 μg of a N-terminal c-myc-tagged Rbp-J. Transfections for transient ChIP assays used FuGene6 (Roche) with 800 ng of the ECR6-pGL3-promoter DNA (wild-type or M3), 100 ng of a C-terminal FLAG-tagged NICD, and 100 ng of a N-terminal c-myc-tagged Rbp-J. Both the NICD-FLAG and cmyc-Rbp-J complementary DNAs were fully sequence verified to exclude any polymerase errors. Forty-eight hours after transfection, cells were washed twice in cold PBS, cross-linked with 1% formaldehyde (Sigma) for 10 minutes at room temperature, and then incubated in 0.14 mol/L glycine for 5 minutes at room temperature to quench the cross-linking reaction. Cells were collected in cold PBS, pelleted by centrifugation and resuspended in SDS lysis buffer (10 mm Tris-HCl, pH 8.0, 10 mmol/L NaCl, 3 mmol/L MgCl2, 1% NP-40, 1% SDS, and 0.5% deoxycholic acid and protease inhibitors). Chromatin was sonicated using the BioRuptor (Diagenode) on high for 5 minutes, cycling 30 seconds on/30 seconds off. Before immunoprecipitation, samples were precleared. Chromatin samples were diluted in dilution buffer (16.7 mmol/L Tris-HCl, pH 8.1, 167 mmol/L NaCl, 0.01% SDS, 1.1% Triton-X 100), protease inhibitors, protein-G agarose (Invitrogen), and control immunoglobulin G (rabbit: Santa Cruz, mouse: Calbiochem) were added, and then samples were incubated at 4°C for 1 hour while rotating. After preclearance, the lysate was divided and immunoprecipitated with 0.5 μg of antiamyc (Millipore), normal-rabbit immunoglobulin G (Santa Cruz), anti-FLAG (Sigma), or normal-mouse immunoglobulin G (Calbiochem) for 4 hours at 4°C. Protein-G agarose beads were then added to each reaction for 1 hour at 4°C. The agarose beads were then washed 2× in low-salt buffer for 30 minutes (20 mmol/L Tris-HCl, pH 8.1, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.1% SDS, 1% Triton X-100), 2× in high-salt buffer for 15 minutes (20 mmol/L Tris-HCl, pH 8.1, 500 mmol/L NaCl, 2 mmol/L EDTA, 0.1% SDS, and 1% Triton X-100), 1× in LiCl buffer for 10 minutes (10 mmol/L Tris-HCl, pH 8.1, 0.25 mol/L LiCl, 1 mmol/L EDTA, 1% NP-40, and 1% deoxycholic acid), and 1× with TE buffer for 5 minutes (10 mmol/L Tris-HCl, pH 8.1, 1 mmol/L EDTA), all at 4°C. Complexes were eluted in 200 μL elution buffer (1% SDS, 0.1 mol/L NaHCO3) at room temperature for 15 minutes. Five mol/L NaCl was added to each sample and incubated overnight at 65°C to reverse chromatin cross-linking. Proteins were degraded after 1-hour incubation at 45°C in the presence of proteinase K (Roche), and remaining DNA was purified with the QIagen PCR Purification Kit (Qiagen). Samples were then PCR amplified using JumpStart PCR mix (Sigma). Primers to the endogenous Jagged1 genomic loci:

ECR1-Forward: 5′-TCCCCAGCTCATGTATCTTTGCTTGC-3′
ECR1-Reverse: 5′-GGAAGAATGCAGATCAAAGCGAAGTCT-3′
ECR6-Forward: 5′-CTCAACACACTAACAGGAGGACG-3′
ECR6-Reverse: 5′-GCTTCACTATTTACAGGACGG-3′

U primers to Full-Length ECR6 Vector Sequence:
ChIP-Forward: 5′-GGCACCTTCTTTGCAGTGGT-3′
ChIP-Reverse: 5′-AGCCATGAGAAAAAGCGAGGAGT-3′

Results
Inhibition of Notch in Neural Crest Impairs Jagged1 Expression
In order to determine if Jagged1 expression is regulated by Notch, we examined Jagged1 mRNA and protein expression in animals in which Notch was inhibited by expression of a previously well-characterized DNAMAML in neural crest. The dominant negative MAML protein inhibits function of all Notch receptors by binding to NICD and preventing recruitment of coactivators such as p300,25,32 R26 DNAMAML mice allow for Cre-mediated activation of DNAMAML expression in a tissue-specific manner, and we crossed R26 DNAMAML with Pax3Cre/+ mice to inhibit Notch in neural crest.

Inhibition of Notch signaling in neural crest with DNAMAML resulted in diminished Jagged1 mRNA surrounding the
 paired aortic arch arteries that are populated by neural crest derivatives (Figure 1A and 1E). Jagged1 protein expression was also diminished (Figure 1B, 1C, 1F, and 1G), and this correlated with impaired smooth muscle differentiation as assessed by expression of smooth muscle actin (Figure 1D and 1H). The decrease in Jagged1 expression and smooth muscle differentiation was not a result of absence of neural crest cells from the forming arterial wall. Fate-mapping experiments using a Cre-inducible GFP reporter, Z/EG, demonstrated similar quantities of neural crest cells in the region of the forming aortic arch arteries in the presence or absence of the DNMAML allele (online-only Data Supplement Figure I). Similar decreases in Jagged1 protein expression and smooth muscle differentiation were also observed when DNMAML was activated by Wnt1-Cre (online-only Data Supplement Figure II), which, like Pax3\textsuperscript{Cre+}, is expressed by premigratory neural crest. Jagged1 protein expression was quantified in the regions surrounding each of the aortic arch arteries. Notch-inhibited animals exhibited a significant decrease in the area of Jagged1 protein expression compared with control littermates, with nearly 3-fold less Jagged1 protein detected overall (online-only Data Supplement Figure IIIA). Expression was significantly diminished in each aortic arch artery except the right VI arch, which has largely regressed by E12.5 (online-only Data Supplement Figure IIIB).\textsuperscript{33} Jagged1 expression was not affected in the descending aorta where vascular smooth muscle is not derived from neural crest precursors, and DNMAML was not expressed (online-only Data Supplement Figure IV).\textsuperscript{34,35}

**Figure 1.** Inhibition of Notch in neural crest impairs Jagged1 expression. A through D, Frontal sections of E12.5 control embryos. E through H, Frontal sections of E12.5 Pax3\textsuperscript{Cre+} DNMAML embryos. A and E, In situ hybridization of Jagged1 in control (A) and Notch-inhibited mutants (E). Pax3\textsuperscript{Cre+} DNMAML embryos exhibit decreased Jagged1 mRNA expression surrounding each of the aortic arch arteries (arrowheads). B, C, F, and G, Immunostaining of Jagged1 in control (B and C) and Notch-inhibited mutants (F and G). The left sixth-arch arteries (arrowheads B and F) are shown in higher magnification (G and H, respectively). D and H, Smooth muscle actinstained sections adjacent to those shown in C and G, respectively. Scale bars: 100 μm. I, Semiquantitative real-time PCR from primary vascular smooth muscle cells stimulated with Fc fragments (cntl) or Fc-Jagged1 (Jag1). Wild-type smooth muscle (DNMAML-) cells induce Jagged1 expression after stimulation whereas induction is absent in Pax3\textsuperscript{Cre+} DNMAML cells (DNMAML+). GAPDH is used as a loading control. E11.5 whole embryo complementary DNA and H2O were used as positive and negative controls, respectively. DNMAML indicates dominant negative MAML gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Notch Activation in Primary Vascular Smooth Muscle Induces Jagged1 Expression**

In order to test whether Notch activation of vascular smooth muscle is sufficient to induce Jagged1 expression, we cultured primary vascular smooth muscle from the aortic arches of control or Pax3\textsuperscript{Cre+}, DNMAML E17.5 embryos and cultured these cells on plates coated with control-Fc fragments or with Jagged1-Fc fragments that functionally activate Notch receptors on cultured cells. This method previously demonstrated that control cells exposed to Jagged1-Fc, but not control-Fc, upregulate the Notch targets Hrt1, Hrt2, and Hrt3, as determined by real-time PCR, and that this response is blocked by expression of DNMAML.\textsuperscript{4} Interestingly, control cells exposed to Jagged1-Fc also upregulate Jagged1 message (Figure II), and this response is also blocked by DNMAML. These results, consistent with previous studies, suggest that Jagged1 is a downstream target of Notch signaling in vascular smooth muscle.\textsuperscript{15,23}

**Identification of a Notch-Responsive Enhancer in the Jagged1 Genomic Locus**

We sought to determine if Jagged1 is a direct Notch target in vascular smooth muscle. Comparative sequence analysis of the Jagged1 genomic locus, including 30 kb upstream of the transcription start site, introns, and 10 kb downstream of the last exon, identified 15 ECRs of at least 200 bp bearing a minimum sequence identity of 70% between mouse, human, dog, and opposum (Figure 2A, arrow heads). Nine of the 15 ECRs contain putative Rbp-J
Figure 2. Identification of a Notch-responsive enhancer in the Jagged1 genomic locus. A, Schematic representation of the Jagged1 genomic locus. White rectangles represent exons; gray rectangles represent 5′ and 3′ untranslated regions. Fifteen ECRs are indicated by black arrowheads. The 9 predicted Rbp-J binding sites (§) are shown. B, Results of dual luciferase reporter assays in HeLa cells with each of the 15 ECRs (numbered 1–15) or with negative control luciferase vector (empty) or with positive control Hes1-luciferase reporter shown. Activity with cotransfected mNICD1 (white bars) was compared to control expression vector (black bars). Luciferase activity was normalized for transfection efficiency. All experiments were performed in duplicate on at least 3 separate occasions and data shown are the mean ± SEM. C, ECR6 sequence alignment of the conserved Rbp-J site and proximal 5′ and 3′ regions across human, mouse, dog, opossum, and chicken. The Rbp-J binding site is in bold; * indicates conservation across species. The genomic locations used in the alignment are human: hg18: chr20: 10593999 to 10594050; mouse: mm9: chr2: 136933564 to 136933614; dog: canFam2 chr24: 14663338 to 14663389; opossum: monDom4: 14408520 to 14408570; and chicken: galGal3 chr3: 614433896 to 614433948; and chicken. The Rbp-J binding site is near-identical sequence between mouse, human, dog, opossum, and chicken (Figure 2C). Further luciferase assays were completed to determine if endogenous Notch signaling could activate ECR6. Cells were plated on either control-Fc fragments or Jagged1-Fc fragments and then transfected with empty vector, Hes1-luciferase reporter, or ECR6 luciferase. Parallel experiments examined luciferase activity after no additional treatment, vehicle (DMSO) exposure, or incubation with a γ secretase inhibitor (10 μmol/L DAPT). In both the nontreated and vehicle-treated samples, cells cultured on Jagged1-Fc fragments activated both Hes1 and ECR6 (Figure 2D). This activation was not apparent after culture on control-Fc fragments. The presence of DAPT was sufficient to prevent Hes1 and ECR6 luciferase activity after culture on Jagged1-Fc fragments, suggesting that the observed activation was indeed in response to endogenous Notch signaling (Figure 2D). Furthermore, epitope-tagged Rbp-J can occupy the predicted Rbp-J binding site within ECR6 as determined by ChIP (Figure 2E). This occupancy is specific for the ECR6 genomic location, as Rbp-J was not bound to a distant ECR, ECR1, which was predicted to contain a Rbp-J binding site, but was not Notch responsive in the luciferase reporter assay (Figure 2A).

Successive deletions of up to 305 bp of the 5′ end of ECR6 failed to impair Notch responsiveness in luciferase assays (Figure 3A and 3B). These deletions left intact the predicted Rbp-J binding site. However, deletion of 308 bp from the 3′ end, which removed the Rbp-J site, abolished Notch activation. Three independent mutations of the predicted Rbp-J binding site of ECR6 (see Methods; each mutation changed 2–4 bp) were independently tested, and each abolished Notch-mediated transactivation (Figure 3B). Furthermore, in transfected HEK293T cells, epitope-tagged Rbp-J and NICD can each occupy the putative Rbp-J binding site as determined by ChIP (Figure 3C and 3D). NICD occupancy is not observed when the Rbp-J site is mutated (Figure 3E).
These data suggest that Notch activation of Jagged1 transcription is mediated directly via Rbp-J and NICD binding to ECR6.

**ECR6 Directs Transgenic Expression to Vascular Smooth Muscle In Vivo**

To determine if ECR6 can mediate expression that recapitulates that of Jagged1 in smooth muscle of the aortic arch arteries, transient transgenic animals were created. Transgenic mice containing ECR6 upstream of a minimal hsp68 promoter and LacZ were examined at E11.5. Whole-mount X-gal staining revealed strong transgenic expression in the distal cardiac outflow tract and great vessels (Figure 4A and 4B). This pattern was observed in 5/7 transient transgenic embryos. Optical projection tomography of a genotype-positive transgenic animal further validated the robust outflow tract–specific expression (online-only Data Supplement Movie I). On sectioning, transgene expression was evident in the aortic arch arteries (Figure 4C and 4D) coincident with smooth muscle actin (SMA; Figure 4E) and Jagged1 (Figure 4J and 4K) expression. Aortic arch artery expression was eliminated in 10/10 genotype-positive transient transgenic embryos.

**Figure 4.** Evolutionarily conserved region 6 directs transgenic expression to vascular smooth muscle in vivo. A through E, E11.5 transient transgenic ECR6-lacZ embryos. Whole-mount X-gal–stained embryos reveal robust expression in the distal cardiac outflow tract and great vessels (Figure 4A and 4B). This pattern was observed in 5/7 transient transgenic embryos. Optical projection tomography of a genotype-positive transgenic animal further validated the robust outflow tract–specific expression (online-only Data Supplement Movie I). On sectioning, transgene expression was evident in the aortic arch arteries (Figure 4C and 4D) coincident with smooth muscle actin (SMA; Figure 4E) and Jagged1 (Figure 4J and 4K) expression. Aortic arch artery expression was eliminated in 10/10 genotype-positive transient transgenic embryos.
embryos derived from a modified construct in which the Rbp-J site within ECR6 was mutated (equivalent to “mutation 3” used for luciferase assays described above; Figure 4F and 4I). Thus, ECR6 is sufficient to mediate reporter gene expression in vascular smooth muscle in the aortic arch arteries and this expression pattern requires an intact Rbp-J binding site.

To more fully characterize ECR6 expression throughout development, a stable ECR6-hsp68-LacZ mouse line was created (Figure 5). Time course analysis revealed that ECR6 was not active at E9.5 (5A through 5C), but X-gal activity was readily detectable by E10.5 (5D–5F). At E11.5, robust X-gal activity was observed surrounding aortic arch arteries iii, iv, and vi (5G–5I). Transgenic expression is specific for the 3 aforementioned arch arteries, which are populated by neural crest–derived smooth muscle, as transgenic expression was absent at the second arch arteries (Figure 5J). Further, expression was also restricted to neural crest–derived vascular smooth muscle, as no transgenic expression was observed surrounding the descending aorta (Figure 5K).

Inactivation of Jagged1 in Neural Crest Results in Aortic Arch Remodeling Defects

Notch inhibition in neural crest induces aortic arch remodeling defects and congenital heart disease,4 and our present data suggest that Notch activation in neural crest induces Jagged1 expression. Hence, we examined the effects of Jagged1 gene inactivation in neural crest to determine if aortic arch remodeling was affected. We crossed Pax3Cre+/H11001; Jagged1flx/flx mice to Jagged1flx/flx mice and observed a decrease in smooth muscle actin expression in Pax3Cre+/H11001; Jagged1flx/flx embryos, indicative of deficient smooth muscle differentiation (Figure 6A and 6B). A similar decrease in smooth muscle differentiation was also observed with staining for an earlier marker of differentiation, SM-22α (online-only Data Supplement Figure V). Further, Hrt1, a prototypical Notch target gene,4,37,38 was downregulated in Pax3Cre+/H11001; Jagged1flx/flx embryos compared with controls (Figure 6C and 6D). We analyzed aortic arch patterning in 29 Pax3Cre+/H11001; Jagged1flx/flx neonates (see online-only Data Supplement Tables III and IV) and identified abnormal aortic arch patterning in 23. Defects included short or absent brachiocephalic artery, common carotid trunk, and retroesophageal right subclavian artery (Figure 6E–6H).

Discussion

Notch signaling is critical in neural crest–derived smooth muscle differentiation and for proper remodeling of the aortic arch arteries and cardiac outflow tract. Inhibition of MAML-dependent Notch signaling in neural crest impairs the formation of the smooth muscle layer of the aortic arch arteries though the nascent endothelial tubes form normally.4 In this study, we identify a Notch-responsive control element within the Jagged1 genomic locus that is capable of directing...
Jagged1 expression to the smooth muscle layer of the aortic arch arteries. Identification of this Notch-responsive ECR in Jagged1 helps to provide a mechanism to explain how a multilayered smooth muscle wall might form around an endothelial tube to produce a mature artery. We propose that Jagged1 expression by endothelial cells engages Notch receptors on neighboring mesenchyme, including neural crest-derived mesenchyme in the case of the aortic arch arteries. Notch activation in mesenchyme triggers smooth muscle differentiation, resulting in a single layer of smooth muscle around the endothelial tube, and simultaneously directly activates Jagged1 expression via ECR6 (and perhaps additional enhancers in various arterial distributions). This positive feedback, or lateral induction, mechanism results in Notch activation of progressively distant mesenchyme and reiterated smooth muscle differentiation events that produce a multilayered smooth muscle wall. Eventually, the signal decays or is terminated by opposing morphogens that have not yet been identified.

This model is supported by significant experimental data. Jagged1 expression by endothelium is required for smooth muscle differentiation of adjacent mesenchyme, and Jagged1 is expressed by both endothelium and vascular smooth muscle. Inhibition of Notch by DNMAML in neural crest impairs Jagged1 expression, both in vivo and in explant cultures, as we show in this report. Also, in accord with this model, deletion of Jagged1 in neural crest results in only relative impairment of smooth muscle differentiation because endothelial Jagged1, and perhaps other redundant Notch receptors, are sufficient to induce some vascular smooth muscle. This results in relatively mild though penetrant aortic arch remodeling defects.

The description of Jagged1 as a direct Notch target is in agreement with published data that support positive feedback between Notch and Jagged1. For example, NIH3T3 cells display Notch-mediated activation of Jagged1, and Jagged1 transcript and protein expression decreases after short hairpin RNA-mediated knock down of either Notch3 or Rbp-J in ovarian cancer cell lines. Notch activation in the basal epidermis causes upregulation of Notch signaling and Jagged1 expression in both epidermis and dermis. In secondary fiber cells of the rat lens, Notch2 is required for fibroblast growth factor–mediated Jagged1 induction and lens fiber cell differentiation. Finally, Feng et al have proposed a lateral induction mechanism involving Notch and Jagged1 to explain patent ductus arteriosus in mice lacking Jagged1 in smooth muscle.

The identification of a functional role for ECR6 may be relevant for genetic testing in patients with AGS, congenital heart disease, or other Notch-related disorders. A recent report predicted that nearly 1/4 of human disease-causing mutations may be located within intronic sequences. Given the tissue-specificity of ECR6 in transgenic mice, interesting patient populations to screen for mutations within this conserved region might include those with AGS, patent ductus arteriosus, bicuspid aortic valve, or aortic arch abnormalities.
In summary, we identify a conserved enhancer in the Jagged1 genomic locus that directs expression to the outflow tract and smooth muscle layer of the forming aortic arch arteries. We provide evidence for Notch/Jagged1 lateral induction and for direct regulation of Jagged1 by Notch. Finally, we provide a model to explain how a multilayered smooth muscle wall may form around a primitive endothelial tube.

Acknowledgments

We thank Klaus Kaestner and Kathy Loomes for the use of floxed Jagged1 mice, and Warren Pear for R26 DNAMAML mice. We are grateful to the late Tom Kadesch for Fc-Jagged1.

Sources of Funding

This work was supported by National Institutes of Health grants U01 HL100405 and R01 HL095634, the American Heart Association John Holden DeHaan Myogenesis Center, the W.W. Smith Professorship, and the Spain Cardiovascular Research Fund.

Disclosures

None.

References

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**CLINICAL PERSPECTIVE**

Notch signaling is an evolutionarily conserved pathway that has been implicated in several forms of congenital heart disease and vascular pathologies. Alagille syndrome, a multisystem disorder associated with mutations in the Notch2 receptor or the Notch ligand Jagged1, results in both cardiac defects such as pulmonary artery stenosis and tetralogy of Fallot and a variety of vascular lesions. These vascular lesions may be the result of defective smooth muscle development. The vascular smooth muscle of the aortic arch arteries provides for the eventual smooth muscle of much of the mature cardiac outflow tract and is derived from neural crest cells. In this article, we demonstrate a critical role for an autoamplification of Notch signaling in cardiac neural crest during vascular smooth muscle development. Notch activation, mediated by endothelial Jagged1, stimulates smooth muscle differentiation of undifferentiated mesenchyme to become vascular smooth muscle. This smooth muscle differentiation is accompanied by an upregulation of Jagged1 expression that activates Notch in subsequent neural crest cells. This lateral induction is mediated by a newly identified enhancer within the Jagged1 genomic locus, demonstrating that Jagged1 is a direct transcriptional target of Notch in vascular smooth muscle. Interruption of this signaling loop, through inhibition of Notch signaling or deletion of the Jagged1 receptor in the neural crest, results in reproducible forms of congenital heart disease involving the outflow tract of the heart. The elucidation of Jagged1 and Notch regulation during smooth muscle development will aid our understanding of congenital abnormalities and vascular defects associated with Jagged1/Notch deficiencies.
Notch Activation of Jagged1 Contributes to the Assembly of the Arterial Wall
Lauren J. Manderfield, Frances A. High, Kurt A. Engleka, Feiyuan Liu, Li Li, Stacey Rentschler
and Jonathan A. Epstein

Circulation. 2012;125:314-323; originally published online December 6, 2011;
doi: 10.1161/CIRCULATIONAHA.111.047159
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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World Wide Web at:
http://circ.ahajournals.org/content/125/2/314

Data Supplement (unedited) at:
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Supplemental Methods

Mice

All mice were maintained on a mixed genetic background. Wnt1-Cre\textsuperscript{1} and Z/EG\textsuperscript{2} mice were genotyped as previously described. All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Histology and immunohistochemistry

Samples were harvested, fixed overnight in 4% paraformaldehyde and subsequently dehydrated through an ethanol series. All samples were then paraffin embedded and sectioned. The antibody used for SM-22 alpha immunohistochemistry was anti-SM-22 goat polyclonal (Ab10135, Abcam).

Supplemental Tables

Supplemental Table 1: Genomic Location of Jagged1 ECRs

<table>
<thead>
<tr>
<th>ECR #</th>
<th>Chromosome2 (chr2) Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECR1</td>
<td>chr2:136942017-136942473</td>
</tr>
<tr>
<td>ECR2</td>
<td>chr2:136961395-136961782</td>
</tr>
<tr>
<td>ECR3</td>
<td>chr2:136962589-136963124</td>
</tr>
<tr>
<td>ECR4</td>
<td>chr2:136936138-136936622</td>
</tr>
<tr>
<td>ECR5</td>
<td>chr2:136935754-136935968</td>
</tr>
<tr>
<td>ECR6</td>
<td>chr2:136933719-136934335</td>
</tr>
<tr>
<td>ECR</td>
<td>chr2:136932948-136933314</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>ECR8</td>
<td>chr2:136925118-136925510</td>
</tr>
<tr>
<td>ECR9</td>
<td>chr2:136924094-136924282</td>
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<td>ECR10</td>
<td>chr2:136917659-136918032</td>
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<td>ECR11</td>
<td>chr2:136913540-136914031</td>
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<tr>
<td>ECR12</td>
<td>chr2:136904329-136904753</td>
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<tr>
<td>ECR13</td>
<td>chr2:136903925-136904234</td>
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<tr>
<td>ECR14</td>
<td>chr2:136899727-136900004</td>
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<tr>
<td>ECR15</td>
<td>chr2:136899238-136899515</td>
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Supplemental Table 2. Jagged1 ECR, ECR6 deletions and ECR6 mutagenesis Primers

<table>
<thead>
<tr>
<th>Evolutionarily Conserved Region Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer Name</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>ECR1-Forward</td>
</tr>
<tr>
<td>ECR1-Reverse</td>
</tr>
<tr>
<td>ECR2-Forward</td>
</tr>
<tr>
<td>ECR2-Reverse</td>
</tr>
<tr>
<td>ECR3-Forward</td>
</tr>
<tr>
<td>ECR3-Reverse</td>
</tr>
<tr>
<td>ECR4-Forward</td>
</tr>
<tr>
<td>ECR4-Reverse</td>
</tr>
<tr>
<td>ECR5-Forward</td>
</tr>
<tr>
<td>ECR5-Reverse</td>
</tr>
<tr>
<td>ECR6-Forward</td>
</tr>
<tr>
<td>ECR6-Reverse</td>
</tr>
</tbody>
</table>

Evolutionarily Conserved Region Primers
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECR7-Forward</td>
<td>GGGGACAAAGTTTGTAACAAAAAACGAGGCTACCCCATGGGTGTGGGTGCC</td>
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<tr>
<td>ECR7-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
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<tr>
<td>ECR8-Forward</td>
<td>GGGGACAAGTTTGTAACAAAAAACGAGGCTTATGGGCCATAGCTGGCATCT</td>
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<tr>
<td>ECR8-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
</tr>
<tr>
<td>ECR9-Forward</td>
<td>GGGGACAAGTTTGTAACAAAAAACGAGGCTTACGCTGACTGTCATCAGAAT</td>
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<tr>
<td>ECR9-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
</tr>
<tr>
<td>ECR10-Forward</td>
<td>GGGGACAAGTTTGTAACAAAAAACGAGGCTTACGCTGACTGTCATCAGAAT</td>
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<tr>
<td>ECR10-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
</tr>
<tr>
<td>ECR11-Forward</td>
<td>GGGGACAAGTTTGTAACAAAAAACGAGGCTTACGCTGACTGTCATCAGAAT</td>
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<tr>
<td>ECR11-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
</tr>
<tr>
<td>ECR12-Forward</td>
<td>GGGGACAAGTTTGTAACAAAAAACGAGGCTTACGCTGACTGTCATCAGAAT</td>
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<td>ECR12-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
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<tr>
<td>ECR13-Forward</td>
<td>GGGGACAAGTTTGTAACAAAAAACGAGGCTTACGCTGACTGTCATCAGAAT</td>
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<tr>
<td>ECR13-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
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<tr>
<td>ECR14-Forward</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
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<td>ECR14-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
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<tr>
<td>ECR15-Forward</td>
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<tr>
<td>ECR15-Reverse</td>
<td>GGGGACAAGTTTGTAACAAAAAACGAGGCTTACGCTGACTGTCATCAGAAT</td>
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**ECR6 Deletion Series Primers**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5' to 3')</th>
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<tbody>
<tr>
<td>D1-Forward</td>
<td>GGGGACAAAGTTTGTAACAAAAAACGAGGCTTACCCCATGGGTGTGGGTGCC</td>
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<tr>
<td>D1-Reverse</td>
<td>GGGGACCACCTTGTACAAAGAAGCTGGGTGACATATTAGTTTCCCAAAAC</td>
</tr>
<tr>
<td>D2-Forward</td>
<td>GGGGACAAAGTTTGTAACAAAAAACGAGGCTTACCCCATGGGTGTGGGTGCC</td>
</tr>
<tr>
<td>D2-Reverse</td>
<td>Same as Deletion1 Reverse</td>
</tr>
<tr>
<td>D3-Forward</td>
<td>GGGGACAAAGTTTGTAACAAAAAACGAGGCTTACCCCATGGGTGTGGGTGCC</td>
</tr>
<tr>
<td>D3-Reverse</td>
<td>Same as Deletion1 Reverse</td>
</tr>
</tbody>
</table>
Supplemental Table 3: Genotype distribution resulting from $Pax3^{Cre/+}$; $Jag1^{flox/+} \times Jag1^{flox/flox}$

<table>
<thead>
<tr>
<th>Age</th>
<th>$	ext{Jag1}^{flox/+}$</th>
<th>$	ext{Jag1}^{flox/flox}$</th>
<th>$Pax3^{Cre/+}$</th>
<th>$Pax3^{Cre/+}$</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$	ext{Jag1}^{flox/+}$</td>
<td>$	ext{Jag1}^{flox/flox}$</td>
<td>$Pax3^{Cre/+}$</td>
<td>$Pax3^{Cre/+}$</td>
<td></td>
</tr>
<tr>
<td>E9.5-14.5</td>
<td>25</td>
<td>23</td>
<td>21</td>
<td>14</td>
<td>83</td>
</tr>
<tr>
<td>P0</td>
<td>31</td>
<td>35</td>
<td>34</td>
<td>38</td>
<td>138</td>
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<tr>
<td>P10-P12</td>
<td>14*</td>
<td>12*</td>
<td>11*</td>
<td>14*</td>
<td>51*</td>
</tr>
<tr>
<td>3 weeks</td>
<td>14*</td>
<td>12*</td>
<td>11*</td>
<td>5*</td>
<td>42*</td>
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</tbody>
</table>
* These offspring (51 total) were followed from the time of genotyping (P10-P12) until 4 weeks of age to determine post-natal lethality of $Pax3^{Cre/+}$ $Jag1^{flox/flox}$ mutants compared with their littermates.

Supplemental Table 4: Aortic arch phenotypes in $Pax3^{Cre/+}$; $Jag1^{flox/flox}$ neonates and littermates

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wildtype</th>
<th>$Jag1^{flox/+}$</th>
<th>$Jag1^{flox/flox}$</th>
<th>$Pax3^{Cre/+}$</th>
<th>$Pax3^{Cre/+}$ $Jag1^{flox/+}$</th>
<th>$Pax3^{Cre/+}$ $Jag1^{flox/flox}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total analyzed</strong></td>
<td>6</td>
<td>32</td>
<td>27</td>
<td>8</td>
<td>36</td>
<td>29</td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>31</td>
<td>26</td>
<td>8</td>
<td>29</td>
<td>6</td>
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<tr>
<td>Short or absent brachiocephalic artery</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Common carotid trunk</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Aberrant right subclavian artery</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>
Supplemental Figure S1

$Pax3^{Cre/+}$ Z/EG

$Pax3^{Cre/+}$ DNMAML Z/EG
Supplemental Figure S2
Supplemental Figure S3
Supplemental Figure S4
Control

Pax3\textsuperscript{Cre/+};\textit{Jagged1}\textsuperscript{flox/flox}

SM-22\textalpha

Supplemental Figure S5
Supplemental Figure Legends

Supplemental Figure S1. Inhibition of Notch in the neural crest does not alter neural crest cell number. (A-B) Frontal sections of E12.5 Pax3^{Cre/+} Z/EG embryos. (C-D) Frontal sections of E12.5 Pax3^{Cre/+} DNMAML Z/EG embryos. (A-D) Immunostaining of GFP in control (A-B) and Notch inhibited mutants (C-D). No alteration of neural crest cell localization or number was observed in animals expressing the DNMAML allele. Scale bars: 100\(\mu\)m.

Supplemental Figure S2. Inhibition of Notch in neural crest impairs Jagged1 expression. (A-C) Frontal sections of E12.5 control embryos. (D-F) Frontal sections of E12.5 Wnt1-Cre DNMAML embryos. (A, B, D and E) Immunostaining of Jagged1 in control (A and B) and Notch inhibited mutants (D and E). Notch inhibited animals display decreased Jagged1 protein expression. The most marked decrease in Jagged1 protein is observed at the left sixth arch artery (arrowheads in A, D and shown in higher magnification in B, E, respectively). (C, F) Adjacent sections to those shown in B, E, respectively, stained for smooth muscle actin (SMA). Wnt1-Cre DNMAML embryos display a decrease in SMA expression (F) indicating decreased smooth muscle differentiation as compared to control embryos (C). Scale bars: 100\(\mu\)m.

Supplemental Figure S3. Quantification of Jagged1 expression following Inhibition of Notch in neural crest. (A) Overall Jagged1 expression surrounding the six aortic arch arteries in control and Pax3^{Cre/+} DNMAML embryos. Jagged1 protein expression area was calculated using ImageJ software (National Institutes of Health, Bethesda, MD,
USA, http://imagej.nih.gov/ij/, 1997-2011). Jagged1 positive area was determined based upon the presence of brown stain at the cell membranes. Seven pairs of control and $Pax3^{Cre/+} \text{DNMAML}$ embryos from 3 independent litters were included in the analysis. From each embryo, 4 non-consecutive sections were stained for Jagged1 and areas were calculated covering ~50$\mu$m of each artery. Overall Jagged1 protein expression is significantly decreased in $Pax3^{Cre/+} \text{DNMAML}$ embryos. Data represent the mean + standard error of the mean (SEM). (B) Jagged1 protein expression area at each individual arch artery is indicated. Jagged1 protein expression is significantly decreased at all arch arteries, except the right sixth. Data represent the mean + standard error of the mean (SEM). Asterisks denote statistically significant differences between the wildtype and $Pax3^{Cre/+} \text{DNMAML}$ embryos, as assessed a two-tailed, paired $t$ test.

**Supplemental Figure S4.** Jagged1 expression is not affected in all vascular smooth muscle when Notch is inhibited in the neural crest. (A-D) Frontal sections of E12.5 embryos. Examination of Jagged1 protein and smooth muscle actin expression along the descending aorta in control and $Pax3^{Cre/+} \text{DNMAML}$ embryos. The vascular smooth muscle surrounding the descending aorta is not derived from neural crest and is not affected by inhibition of Notch by DNMAML in neural crest indicating that smooth muscle defects are cell autonomous and not generalized in these embryos. Scale bars: 100$\mu$m.

**Supplemental Figure S5.** Inactivation of Jagged1 in neural crest results in decreased smooth muscle differentiation. (A-B) Frontal sections of E12.5 mouse embryos. (A, B)
Left sixth aortic arch arteries of control (A) and $Pax3^{Cre/+}$; $Jagged1^{flox/flox}$ (B) embryos stained for SM-22 alpha (SM-22α). Scale bars: 100µm.

**Supplemental References**


**Supplemental Movie 1.** Optical projection tomography of E11.5 ECR6-lacZ embryo shows extent of X-gal staining.