# Endothelial Notch1 Is Required for Proper Development of the Semilunar Valves and Cardiac Outflow Tract

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**Background**—Congenital heart disease is the most common type of birth defect, affecting ≈2% of the population. Malformations involving the cardiac outflow tract and semilunar valves account for >50% of these cases predominantly because of a bicuspid aortic valve, which has an estimated prevalence of 1% in the population. We previously reported that mutations in *NOTCH1* were a cause of bicuspid aortic valve in nonsyndromic autosomal-dominant human pedigrees. Subsequently, we described a highly penetrant mouse model of aortic valve disease, consisting of a bicuspid aortic valve with thickened cusps and associated stenosis and regurgitation, in *Notch1*-haploinsufficient adult mice backcrossed into a *Nos3*-null background.

**Methods and Results**—Here, we described the congenital cardiac abnormalities in $Notch1^{+/-};Nos3^{-/-}$ embryos that led to ≈65% lethality by postnatal day 10. Although expected Mendelian ratios of $Notch1^{+/-};Nos3^{-/-}$ embryos were found at embryonic day 18.5, histological examination revealed thickened, malformed semilunar valve leaflets accompanied by additional anomalies of the cardiac outflow tract including ventricular septal defects and overriding aorta. The aortic valve leaflets of $Notch1^{+/-};Nos3^{-/-}$ embryos at embryonic day 15.5 were significantly thicker than controls, consistent with a defect in remodeling of the semilunar valve cushions. In addition, we generated mice haploinsufficient for *Notch1* specifically in endothelial and endothelial-derived cells in a *Nos3*-null background and found that $Notch1^{fl/+};Tie2\text{-}Cre^{+/-};Nos3^{-/-}$ mice recapitulate the congenital cardiac phenotype of $Notch1^{+/-};Nos3^{-/-}$ embryos.

**Conclusions**—Our data demonstrate the role of endothelial Notch1 in the proper development of the semilunar valves and cardiac outflow tract. (*J Am Heart Assoc*. 2016;5:e003075 doi: 10.1161/JAHA.115.003075)

**Key Words:** bicuspid aortic valve • cardiovascular genetics • congenital heart defect • conotruncal heart defects • Notch1 signaling

Bicuspid aortic valve (BAV) is the most common congenital anomaly, with an estimated incidence of 1.3% in the population.[1] Along with BAV, developmental anomalies of the semilunar valves and cardiac outflow tract (OFT) account for a significant proportion of congenital heart defects and result in a substantial clinical burden for affected patients.[2–4] Multiple cell lineages are responsible for the development of the cardiac OFT and semilunar valves.[5] During early heart development, cells from the anterior second heart field (SHF) contribute to formation of the cardiac OFT, also known as the conotruncus.[6–8] In addition, a subpopulation of endothelial cells that line the OFT undergo endothelial-to-mesenchymal transition to form the OFT cushions. These mesenchymal cells are joined by the migrating cardiac neural crest cells, which are required for septation of the aorta and pulmonary artery.[9–11] Proper migration and signaling of all 3 cell lineages, the SHF, cardiac neural crest and endothelial-derived mesenchymal cells are required for normal OFT cushion development and semilunar valve remodeling.

The Notch signaling pathway has been shown to be important for multiple aspects of heart development, from endocardial cushion formation to myocardial development, and mutations in this pathway have been linked to a spectrum of congenital heart defects in humans.[12] The Notch family is composed of 4 receptors responsible for various cell-fate decisions and for vascular development and disease.[12,13] These receptors are transmembrane signaling proteins that contain a ligand-binding extracellular domain and an
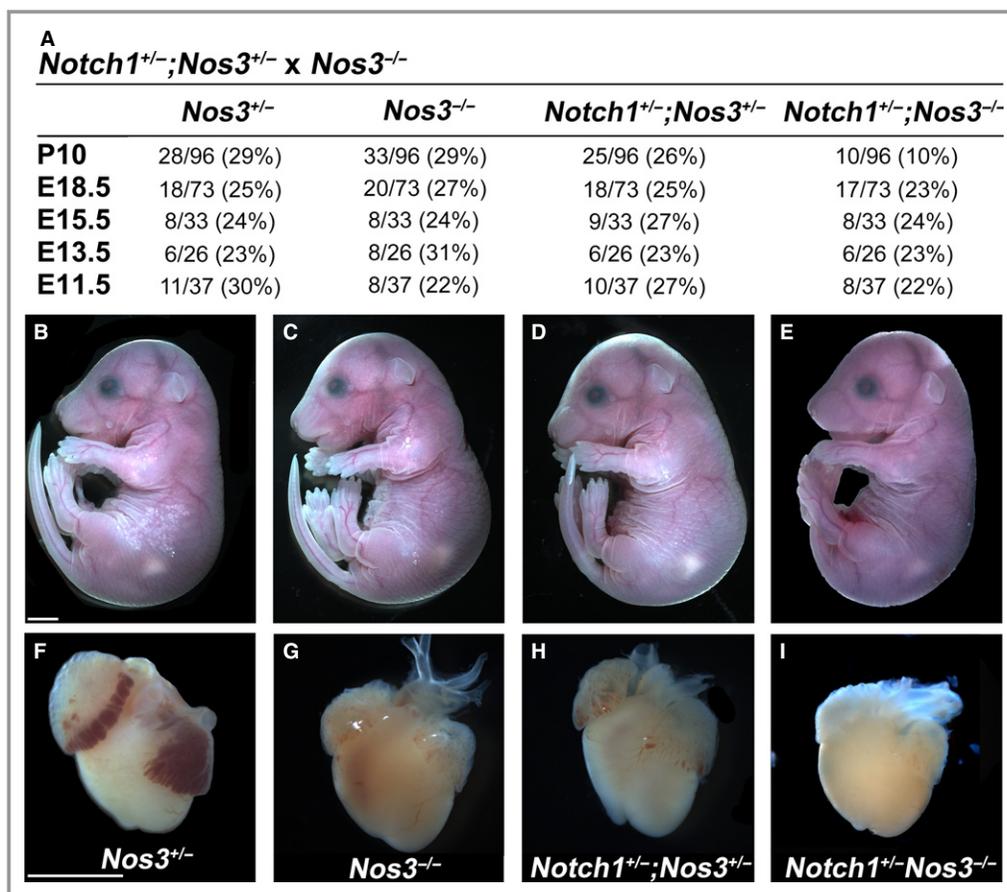
**Figure 1.** *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ mice display perinatal lethality. Compound mutant mice suffered ≈65% lethality by P10, as shown in (A). Nevertheless, compared with *Nos3*$^{+/-}$ (n=18) (B), *Nos3*$^{-/-}$ (n=20) (C), and *Nos3*$^{+/-}$;*Notch1*$^{+/-}$ (n=18) (D) littermates, *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ mice did not display any embryonic lethality (A) or growth retardation at E18.5 (n=17) (E). Compared with controls (F through H), examination of E18.5 hearts revealed right ventricle enlargement in compound mutants (I). Scale bars, 2 mm. E indicates embryonic day; P, postnatal day.

intracellular domain. On binding with its ligand, a series of cleavage events release the Notch intracellular domain, which translocates to the nucleus and binds with its cofactors mastermind-like protein and RBPJκ to regulate expression of target genes.[14] Specifically, the Notch signaling family has been shown to play a critical role in cardiac OFT development because loss of the Notch signaling pathway, specifically in the SHF via a dominant-negative truncated form of mastermind-like protein or loss of Jagged1, results in a spectrum of OFT defects including aortic valve abnormalities.[15] Furthermore, mutations in *NOTCH2* and the Notch ligand *JAGGED1* are responsible for Alagille syndrome, which is characterized by pulmonary stenosis, ventricular septal defects, coarctation of the aorta, and tetralogy of Fallot, among other developmental defects.[16,17] Notch1 is expressed in the endothelial cells lining the cardiac OFT during development, and mutations in *NOTCH1* have been linked primarily to human BAV and other left-sided cardiac malformations.[18,19] Although

these studies indicate the important role for Notch signaling in the development of the cardiac OFT and aortic valve, the underlying mechanisms and the cell lineages in which Notch1 is required have not yet been elucidated.

We previously described reduced survival to adulthood in *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ mice, suggesting a potential embryonic phenotype.[20] To further investigate the cause of this lethality, we bred *Notch1*$^{+/-}$;*Nos3*$^{+/-}$ female mice with *Nos3*$^{-/-}$ male mice and examined the resultant litters. We observed 65% neonatal lethality in *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ mice and found that compound mutant embryos displayed a spectrum of congenital cardiac malformations, including thickened semilunar valves, ventricular septal defects, and overriding aorta. Using a conditional gene deletion approach (Cre/LoxP), we found that loss of endothelial Notch1 was responsible for the cardiac phenotypes observed in the *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ mice. Our results indicate a novel role for endothelial Notch1 in the development of the semilunar valves and cardiac OFT.

## Methods

### Mice

Animal use was approved and monitored by the institutional animal care and use committee at the Research Institute at Nationwide Children's Hospital. $Nos3^{-/-}$ and $Notch1^{+/-}$; $Nos3^{+/-}$ mice were bred to obtain $Notch1^{+/-};Nos3^{-/-}$ mice (n=49) and littermate controls (n=216) and were genotyped, as described previously.[20] For lineage-specific deletions of Notch1 (using Tie2-Cre,[21] Mef2C-Cre,[22] Wnt1-Cre[23]), $Nos3^{-/-}$; $Cre^{+/-}$ male mice were bred with $Notch1^{flox/wt};Nos3^{+/-}$ female mice to obtain $Notch1^{flox/wt};Tie2\text{-}Cre^{+/-};Nos3^{-/-}$ (n=6) mice, $Notch1^{flox/wt};Mef2C\text{-}Cre^{+/-};Nos3^{-/-}$ (n=7), $Notch1^{flox/wt};Wnt1\text{-}Cre^{+/-};Nos3^{-/-}$ (n=4), and control littermates (n=4, n=4, n=4, respectively). SHF lineage tracing was completed by breeding $Mef2C\text{-}Cre^{+/-}$ male mice with $ROSA26^{mT/mG}$ female mice.[24]

### Tissue Fixation and Histology

Embryos were harvested at the indicated time points and fixed in 10% formalin at 4°C overnight. Sections (6 μm) were stained with hematoxylin and eosin and imaged at ×50. Valve area was determined by the average area across 3 sections of each leaflet using AxioVision software (Zeiss). Cell density was determined by dividing the number of nuclei in each valve leaflet by the measured area. Valve excavation, as described by Dupuis et al,[25] was determined by the ratio of space across the valve by sections en face and calculated by ImageJ (National Institutes of Health). A minimum of 3 sections of each valve were performed. Immunofluorescence was performed using anti–green fluorescent protein (ab290, 1:1000; Abcam) and anti-PECAM1 (sc-1506, 1:50; Santa Cruz Biotechnology) and was counterstained with Vector Laboratories Hardset Mounting Medium with DAPI (H-1500).

### Statistics

Statistical analysis was performed on quantitative graphs using the Mann–Whitney test because of the small number of mice used and the lack of normality, with median and 25th and 75th percentiles reported. For categorical data, the Fisher exact test was used. $P<0.05$ was considered significant.

## Results

### Neonatal Lethality in $Notch1^{+/-};Nos3^{-/-}$ Mice

To determine the embryonic phenotype of $Notch1^{+/-};Nos3^{-/-}$ mice, we bred $Notch1^{+/-};Nos3^{+/-}$ and $Nos3^{-/-}$ mice and examined the resultant litters at postnatal day 10. We found ≈65% lethality in $Notch1^{+/-};Nos3^{-/-}$ pups at postnatal day 10, whereas no lethality was observed in littermate controls (Figure 1A). Interestingly, this was contrasted by expected Mendelian ratios for all genotypes between embryonic day (E) 11.5 and E18.5 (Figure 1A). Examination of $Notch1^{+/-};Nos3^{-/-}$ embryos at E18.5 revealed no gross abnormalities or growth retardation compared with littermates (Figure 1B through 1E). Gross examination of embryonic hearts at E18.5 revealed abnormal cardiac morphology with an enlarged right ventricle in the $Notch1^{+/-};Nos3^{-/-}$ embryos compared with control littermates, suggesting that a congenital cardiac malformation
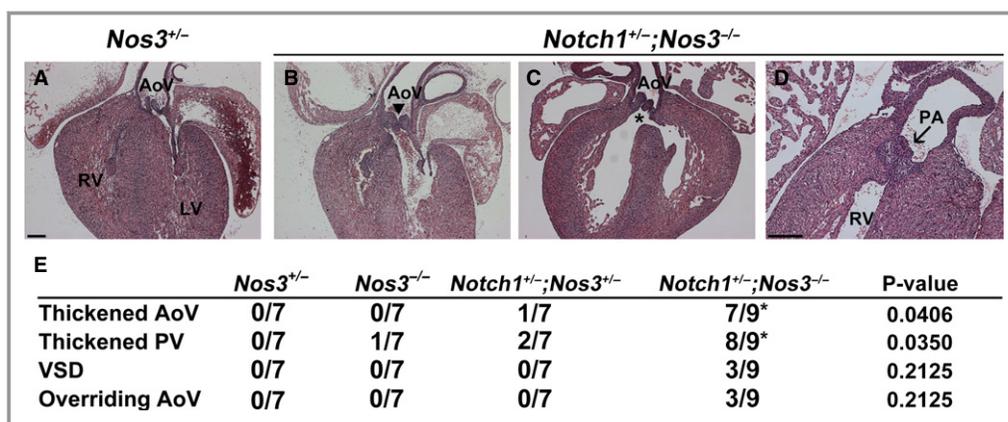


| E | $Nos3^{+/-}$ | $Nos3^{-/-}$ | $Notch1^{+/-};Nos3^{+/-}$ | $Notch1^{+/-};Nos3^{-/-}$ | P-value |
|---|---|---|---|---|---|
| Thickened AoV | 0/7 | 0/7 | 1/7 | 7/9* | 0.0406 |
| Thickened PV | 0/7 | 1/7 | 2/7 | 8/9* | 0.0350 |
| VSD | 0/7 | 0/7 | 0/7 | 3/9 | 0.2125 |
| Overriding AoV | 0/7 | 0/7 | 0/7 | 3/9 | 0.2125 |

**Figure 2.** $Notch1^{+/-};Nos3^{-/-}$ embryos display cardiac outflow tract and semilunar valve malformations. Compared with $Nos3^{+/-}$ control mice (A), compound mutant mice (B through D) displayed AoV thickening (B, arrowhead), VSD with overriding Ao (C, asterisk), and PV thickening (D, arrow), as summarized in the table (E). $Nos3^{+/-}$, n=7; $Nos3^{-/-}$, n=7; $Notch1^{+/-};Nos3^{+/-}$, n=7; $Notch1^{+/-};Nos3^{-/-}$, n=9. Scale bars=200 μm. *$P<0.05$. Ao indicates aorta; AoV, aortic valve; LV, left ventricle; PA, pulmonary artery; PV, pulmonary valve; RV, right ventricle; VSD, ventricular septal defect.

was contributing to their neonatal lethality (Figure 1F through 1I).

## Notch1$^{+/-}$;Nos3$^{-/-}$ Mice Display Congenital Heart Malformations and Thickened Semilunar Valves

To determine whether congenital cardiac malformations were present in the compound mutant animals, histological examination of hearts at E18.5 was performed. *Notch1$^{+/-}$; Nos3$^{-/-}$* hearts were found to have a spectrum of cardiac malformations, including thickened aortic and pulmonary valves, ventricular septal defects, and overriding aorta (Figure 2B through 2E), compared with control littermate hearts (Figure 2A and 2E). Although a small subset of *Notch1$^{+/-}$; Nos3$^{+/-}$* animals demonstrated mildly thickened semilunar valve leaflets at E18.5, we did not observe any other malformations in the *Nos3$^{+/-}$*, *Nos3$^{-/-}$* or *Notch1$^{+/-}$; Nos3$^{+/-}$* embryos (Figure 2E).

Further examination of the aortic valves at E18.5 demonstrated that the *Notch1$^{+/-}$;Nos3$^{-/-}$* mutant valves (Figure 3D) were significantly larger than *Nos3$^{+/-}$*, *Nos3$^{-/-}$* or *Notch1$^{+/-}$;Nos3$^{+/-}$* valves (Figure 3A through 3C and 3K). Although the valve leaflets of compound mutant animals were larger, they contained fewer nuclei per unit area (Figure 3I, 3J, and 3L). Remodeling of the aortic valve cushions occurs during the later stages of gestation; therefore, examination of *Notch1$^{+/-}$;Nos3$^{-/-}$* embryos at E15.5 was performed. Compound mutant hearts demonstrated thickened valve leaflets (Figure 3H) compared with control animals (Figure 3E through 3G) and were accompanied by a reduction in aortic valve excavation (Figure 3M).[25] These results suggest that Notch1 signaling is required for normal development of the cardiac OFT and for proper remodeling of the semilunar valves.
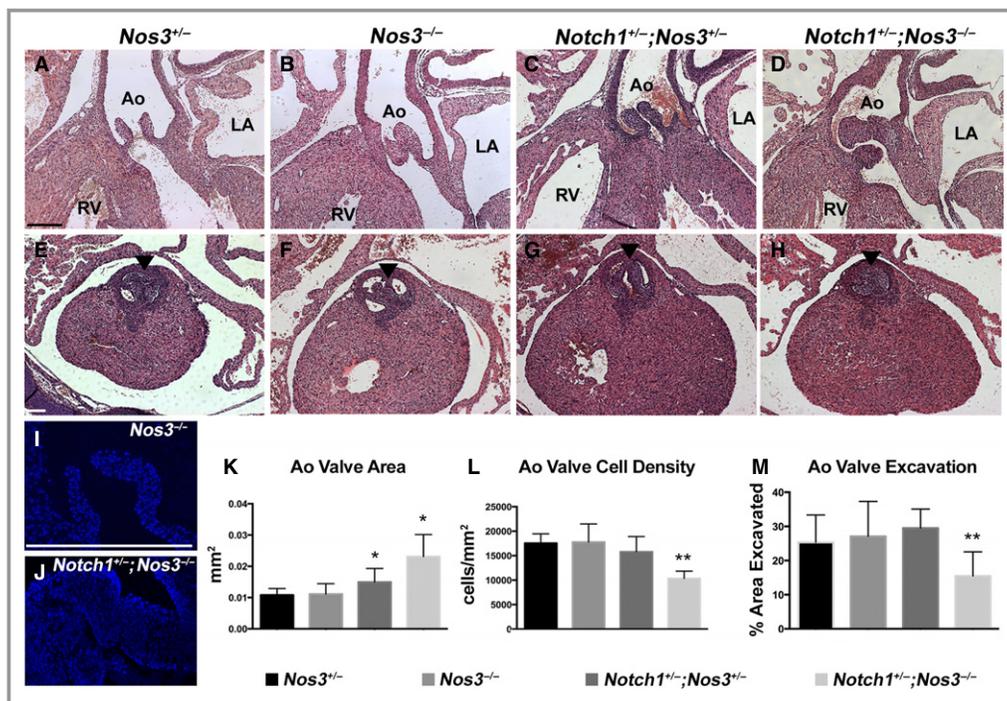


**Figure 3.** *Notch1$^{+/-}$;Nos3$^{-/-}$* embryos display abnormal aortic valve remodeling. Compared with *Nos3$^{+/-}$* (A), *Nos3$^{-/-}$* (B), and *Nos3$^{+/-}$;Notch1$^{+/-}$* (C) animals at embryonic day 18.5, compound mutant mice demonstrated thickened aortic valves (D), as summarized in (K) (*Nos3$^{+/-}$*: n=5, median 0.01 [IQR 0.01–0.01125]; *Nos3$^{-/-}$*: n=5, median 0.01 [IQR 0.01–0.01]; *Notch1$^{+/-}$;Nos3$^{+/-}$*: n=5, median 0.015 [IQR 0.01–0.02]; *Notch1$^{+/-}$;Nos3$^{-/-}$*: n=5, median 0.0225 [IQR 0.02–0.03]). *Notch1$^{+/-}$;Nos3$^{-/-}$* animals also display a reduced number of nuclei per unit area (J and L) compared with *Nos3$^{-/-}$* animals (I) and controls (L) (*Nos3$^{+/-}$*: n=5, median 17 200 [IQR 15 675–19 750]; *Nos3$^{-/-}$*: n=5, median 18 000 [IQR 14 975–20 375]; *Notch1$^{+/-}$;Nos3$^{+/-}$*: n=5, median 14 600 [IQR 14 350–16 950]; *Notch1$^{+/-}$; Nos3$^{-/-}$*: n=5, median 10 400 [IQR 9000–11 767]). *Notch1$^{+/-}$;Nos3$^{-/-}$* mice were found to have thickened aortic valve leaflets (arrowhead) at embryonic day 15.5 (H) compared with *Nos3$^{+/-}$* (E), *Nos3$^{-/-}$* (F) and *Notch1$^{+/-}$;Nos3$^{+/-}$* (G) animals, accompanied by a decrease in aortic valve excavation (M) (*Nos3$^{+/-}$*: n=4, median 23.90 [IQR 18.80–33.60]; *Nos3$^{-/-}$*: n=4, median 25.10 [IQR 17.30–37.80]; *Notch1$^{+/-}$;Nos3$^{+/-}$*: n=4, median 29.80 [IQR 24.00–32.80]; *Notch1$^{+/-}$;Nos3$^{-/-}$*: n=4, median 16.80 [IQR 10.40,– 20.05]). Scale bars=200 μm. *$P<0.05$; **$P<0.01$. IQR shows the 25th and 75th percentiles. Ao indicates aorta; IQR, interquartile range; LA, left atria; RV, right ventricle.

## Notch1 Is Required Within the Endothelial Cell Lineage for Proper Morphogenesis of the Aortic Valve

The mesenchymal cells of the OFT cushions are derived from multiple sources including the cardiac neural crest, the SHF, and endothelial-derived cells. To determine the specific cell lineage in which loss of Notch1 contributes to the aortic valve phenotype in $Notch1^{+/-};Nos3^{-/-}$ mice, we used mice containing a $Notch1$-floxed allele.[26] Because Notch1 is expressed throughout the developing valve mesenchyme in all of the aforementioned lineages, we used Cre-specific drivers for each cell lineage. To test whether the endothelium may be critical in the role of Notch1 in valve development, we bred $Notch1^{fl/wt};Nos3^{+/-}$ animals to $Nos3^{-/-}$ mice harboring the endothelial-specific driver $Tie2$-$Cre$.[21] We discovered that $Notch1^{fl/wt};Tie2$-$Cre^{+/-};Nos3^{-/-}$ mice recapitulated the phenotypes seen in our $Notch1^{+/-};Nos3^{-/-}$ animals, as summarized in Figure 4E. $Notch1^{fl/wt};Tie2$-$Cre^{+/-};Nos3^{-/-}$ mice displayed thickened aortic (Figure 4C) and pulmonary valves (Figure 4D) compared with littermate controls (Figure 4A and 4B), and the aortic valves were also found to have reduced cell density (Figure 4F) similar to that of $Notch1^{+/-};Nos3^{-/-}$ mice (Figure 3L). To determine whether Notch1 was required in other cell lineages, we used the SHF-specific driver $Mef2C$-$Cre$.[22] $Notch1^{fl/wt};Mef2C$-$Cre^{+/-};Nos3^{-/-}$ animals also displayed thickened aortic and pulmonary leaflets compared with littermate controls, although to a lesser extent than the $Notch1^{fl/wt};Tie2$-$Cre^{+/-};Nos3^{-/-}$ animals (Figure 5A through 5F). SHF lineage tracing with $ROSA26^{mT/mG24}$ mice showed that a subset of endothelial cells within the aorta and aortic valves were derived from the SHF (Figure 5G and 5H). SHF-derived endothelial cells are also known to populate the pulmonary trunk and pulmonary valve, suggesting that the effect of Notch1 may still be limited to the endothelial cell layer.[22] Because other publications have implicated the role of neural crest cells in OFT formation, we obtained neural crest–specific $Wnt1$-$Cre$ mice to ascertain the role of Notch1 specifically in the neural crest.[23] We did not observe any cardiac phenotypes in $Notch1^{fl/wt};Wnt1$-$Cre^{+/-};Nos3^{-/-}$ mice. This finding suggests that Notch1 in the neural crest is not responsible for the cardiovascular and semilunar valve phenotypes observed in the $Notch1^{+/-};Nos3^{-/-}$ mice (Figure 6). Although our results do not preclude the possibility of SHF cells requiring Notch1, they demonstrate a critical role of Notch1 within the endothelial cells for the proper development of the semilunar valves and cardiac OFT.

## Discussion

We have described the cardiac phenotype in $Notch1$-haploinsufficient embryos backcrossed into a $Nos3$-null background. Our studies demonstrate that although adult survivors display isolated aortic valve anomalies, mutant embryos have a spectrum of cardiac phenotypes including thickened semilunar valve leaflets, overriding aorta, and ventricular septal defects. In addition, we found that loss of Notch1 in endothelial and
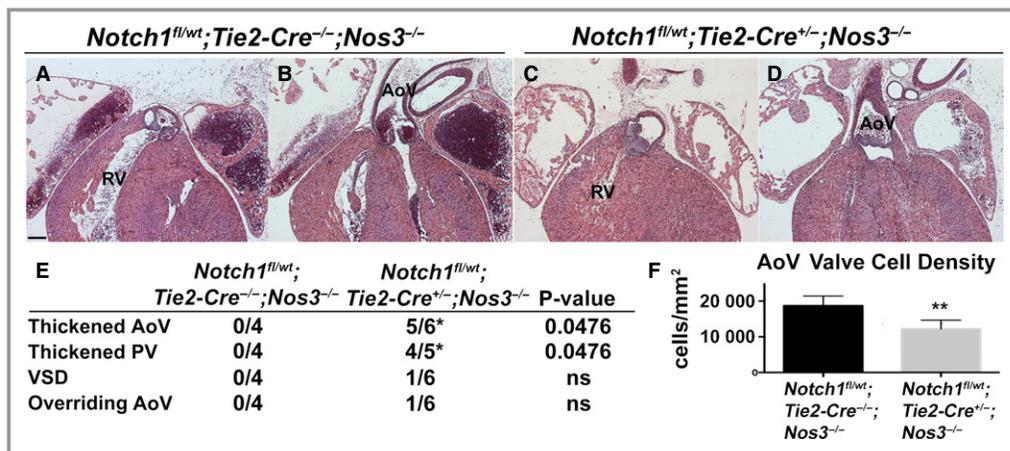


**Figure 4.** Endothelial-specific *Notch1* haploinsufficiency in a *Nos3*-null background recapitulates the $Notch1^{+/-};Nos3^{-/-}$ cardiac phenotype. $Notch1^{fl/wt};Tie2$-$Cre^{+/-};Nos3^{-/-}$ embryos (n=6) were found to have thickened aortic and pulmonary valve leaflets (C and D) at embryonic day 18.5 compared with $Notch1^{fl/wt};Tie2$-$Cre^{-/-};Nos3^{-/-}$ animals (n=4) (A and B). E, Table showing cardiac phenotypes in $Notch1^{fl/wt};Tie2$-$Cre^{+/-};Nos3^{-/-}$ embryos, which also displayed VSD and overriding Ao. F, Reduced AoV cell density in $Notch1^{fl/wt};Tie2$-$Cre^{+/-};Nos3^{-/-}$ embryos (n=5, median 12 196 [IQR 9769–14 446]) compared with $Notch1^{fl/wt};Tie2$-$Cre^{-/-};Nos3^{-/-}$ embryos (n=4, median 17 100 [IQR 16 745–21 473]). Scale bar=200 μm. *$P$<0.05; **$P$<0.01. IQR shows the 25th and 75th percentiles. Ao indicates aorta; AoV, aortic valve; IQR, interquartile range; ns, not significant ($P$=1.00); PV, pulmonary valve; RV, right ventricle; VSD, ventricular septal defect.
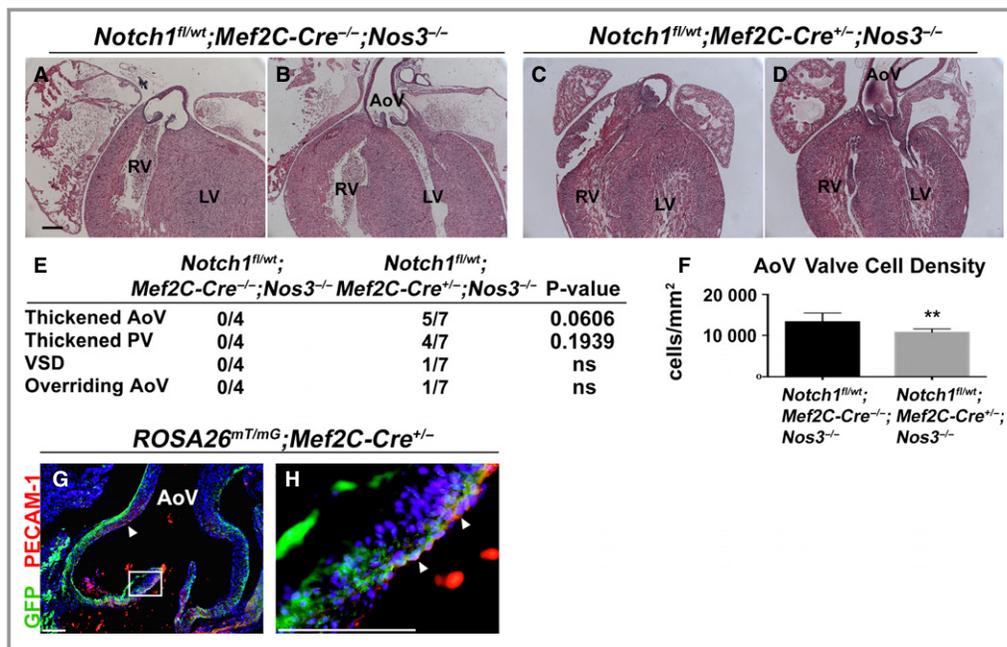
**Figure 5.** SHF-specific *Notch1* haploinsufficiency in a *Nos3*-null background partially recapitulates the *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ cardiac phenotype. *Notch1*$^{fl/wt}$;*Mef2C-Cre*$^{+/-}$;*Nos3*$^{-/-}$ embryos (n=7) were found to have thickened AoV and PV leaflets (C and D) at embryonic day 18.5 compared with *Notch1*$^{fl/wt}$;*Mef2C-Cre*$^{-/-}$;*Nos3*$^{-/-}$ animals (n=4) (A and B). *Notch1*$^{fl/wt}$; *Mef2C-Cre*$^{+/-}$;*Nos3*$^{-/-}$ mice also displayed VSDs and overriding Ao (E), although to a lesser extent than what is seen in the *Notch1*$^{+/-}$;*Nos3*$^{-/-}$ mice, which is also observed by a reduction in AoV cell density in *Notch1*$^{fl/wt}$;*Mef2C-Cre*$^{+/-}$;*Nos3*$^{-/-}$ embryos (n=3, median 10 455 [IQR 10 144–11 442]) compared with *Notch1*$^{fl/wt}$;*Mef2C-Cre*$^{-/-}$;*Nos3*$^{-/-}$ animals (n=3, median 13 258 [IQR 11 271–14 512]) (F). Lineage tracing with *ROSA26*$^{mT/mG}$;*Mef2C-Cre*$^{+/-}$ mice (n=4) revealed SHF-derived endothelial cells (arrowheads) lining the AoV and aortic root (G and H). GFP indicates SHF-derived cells; PECAM1 indicates endothelial cells. Scale bars=100 μm. **$P<0.01$. IQR shows the 25th and 75th percentiles. Ao indicates aorta; AoV, aortic valve; GFP, green fluorescent protein; IQR, interquartile range; ns, not significant ($P$=1.00); LV, left ventricle; PV, pulmonary valve; RV, right ventricle; SHF, second heart field; VSD, ventricular septal defect.

endothelial-derived cells resulted in a spectrum of cardiac OFT defects and semilunar valve anomalies in our model. In summary, these findings highlight the importance of endothelial Notch1 in multiple aspects of cardiac OFT development including semilunar valve remodeling.

Notch1 is a transmembrane signaling receptor responsible for many developmental processes.[14] The Notch signaling family proteins are expressed in multiple cardiac lineages and during different stages of development and have the ability to act in a noncell autonomous fashion, resulting in signal transduction to multiple cell types.[12,15] Our results indicate that Notch1 is required within the endothelial cell lineage for proper OFT development and semilunar valve remodeling; however, the cell lineages with which Notch1 communicates have not been well defined. Similar to Notch1, deletion of Gata5 or Alk2 in the OFT endothelial (endocardial) and endothelial-derived mesenchymal cells is sufficient to cause BAV.[27–29] In addition, the cardiac neural crest is critical for the development of the cardiac OFT because loss of BMP signaling via the receptor

ALK2 has been shown to cause persistent truncus arteriosus, improper cardiac neural crest migration causes OFT defects, and the loss of Rho kinase signaling within neural crest cells gives rise to BAV.[30–32] We did not observe any cardiac malformations using a neural crest–specific Cre driver to delete Notch1; however, this does not exclude the possibility that Notch1 in endothelial-derived cells may be signaling to neural crest cells in the cardiac OFT. Neural crest cells have been shown to direct mesenchymal cell fate decisions within the developing cardiac OFT, and it is possible that Notch1 in endothelial and endothelial-derived cells has the capacity to act in a similar role via the neural crest.[33] Our data also demonstrate a role for Notch1 in SHF-derived cells because use of *Mef2C-Cre* mice resulted in a partial recapitulation of our observed cardiac phenotype. We reason that this is likely because of deletion of Notch1 in SHF-derived endothelial cells but cannot exclude a potential role for Notch1 in other SHF-derived cells.

The human aortic valve is composed of 3 leaflets called the right coronary (R), left coronary (L), and noncoronary (NC)
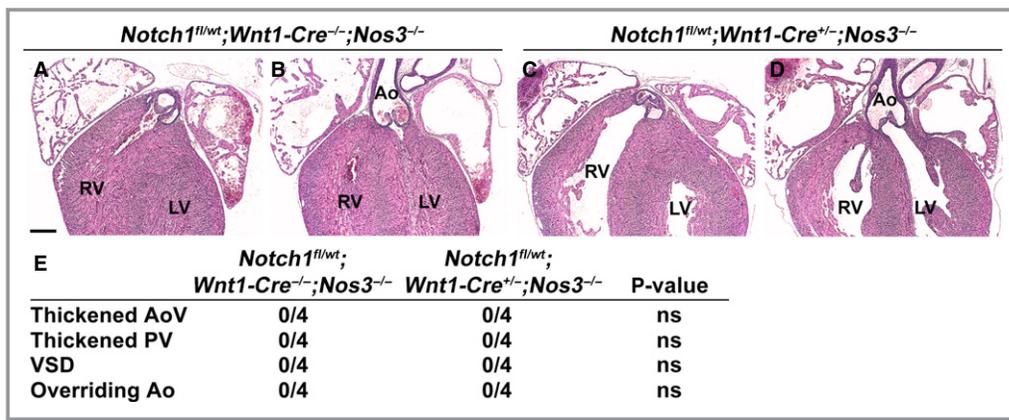
**Figure 6.** Neural crest–specific *Notch1* haploinsufficiency in a *Nos3*-null background does not recapitulate the cardiac phenotype of the *Notch1$^{+/-}$;Nos3$^{-/-}$* mice. *Notch1$^{fl/wt}$;Nos3$^{+/-}$* mice were bred to *Notch1$^{wt/wt}$;Wnt1-Cre$^{+/-}$;Nos3$^{-/-}$* male mice, creating neural crest–specific *Notch1* haploinsufficiency in a *Nos3$^{-/-}$* background. *Notch1$^{fl/wt}$;Wnt1-Cre$^{+/-}$;Nos3$^{-/-}$* embryos (n=4) were found to have normal AoV and PV leaflets (A through D) at embryonic day 18.5 compared with *Notch1$^{fl/wt}$;Wnt1-Cre$^{-/-}$;Nos3$^{-/-}$* animals (n=4) (E). There was no evidence of VSDs and overriding Ao in *Notch1$^{fl/wt}$; Wnt1-Cre$^{+/-}$;Nos3$^{-/-}$* mice (E). Scale bar=200 μm. Ao indicates aorta; AoV, aortic valve; ns, not significant (*P*=1.00); LV, left ventricle; PV, pulmonary valve; RV, right ventricle; VSD, ventricular septal defect.

leaflets, so named for their spatial arrangement to the coronary arteries. BAVs may result from the fusion of any leaflet, although in humans, right–left fusion is most common.[34] The process of leaflet fusion is currently not well understood because leaflet fusion may occur early in cushion development or later during valve remodeling. *Gata5* knockout mice display BAV, which is caused by an early fusion without leaflet thickening, unlike that of ALK2 mutant mice, which demonstrate thickened valve leaflets at early stages.[27,30] Some studies have concluded that BAV subtypes are a result of specific genetic etiologies because *Gata5$^{-/-}$* mice and *Nos3$^{-/-}$* mice display BAVs with right–noncoronary fusion.[27,35] Further research using genetic sequencing in humans has also suggested this because human BAV patients harboring rare *GATA5* mutations have a higher incidence of right–left and right–noncoronary BAVs.[36] Nevertheless, a study of 1849 inbred Syrian hamsters with a high probability of homozygosity found variable aortic valve morphology and suggested that environmental factors, rather than genetics, account for BAV morphology.[37,38] Gene–environment interactions may also play a role in the development of BAV because mutations in the Notch signaling pathway combined with environmental effectors have been demonstrated to increase the penetrance of disease states.[39] Accordingly, we examined the BAV morphology in the *Notch1$^{+/-}$;Nos31$^{-/-}$* adult mice and identified a variable morphology, with left–right, right–noncoronary, and left–noncoronary fusion all observed (n=4, data not shown).

In summary, our work demonstrates the role for Notch1 in OFT and semilunar valve development. Interestingly,

examination of the families reported in the original publication linking *NOTCH1* mutations and congenital heart defects demonstrates the presence of an individual with tetralogy of Fallot, a phenotype consistent with the phenotype observed in the *Notch1$^{+/-}$;Nos3$^{-/-}$* embryos.[18] This potential link was further supported by the identification of a microdeletion encompassing *NOTCH1* in a patient with tetralogy of Fallot by chromosomal microarray.[18,40] Further recent evidence supporting a link between *NOTCH1* mutations and right-sided congenital heart defect is the identification of mutations in *NOTCH1* in 2 additional families with malformations of right-sided cardiac structures.[41,42] Further investigation is required to determine whether mutations in *NOTCH1* are found in children with malformations affecting both the right (pulmonary) and left (aortic) sides of the developing OFT.

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## Disclosures

None.

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