**ROBO4** variants predispose individuals to bicuspid aortic valve and thoracic aortic aneurysm

Russell A. Gould¹²,³¹, Hamza Aziz¹²,³¹, Courtney E. Woods¹³,³¹, Manuel Alejandro Seman-Senderos³, Elizabeth Sparks¹, Christoph Preuss³⁴, Florian Wünnewann³, Djahida Bedja³⁶, Cassandra R. Moats⁵⁷, Sarah A. McClymont⁶, Rebecca Rose⁷, Nara Sobreira¹, Hua Ling⁸, Gretchen MacCarrick⁹, Ajay Anand Kumar⁹, Ilse Luyckx⁹, Elyssa Cannaerts⁹, Aline Verstraeten⁹, Hanna M. Björk¹⁰, Ann-Cathrin Lehssau¹¹, Vinod Jaskula-Ranga¹², Henrik Lauridsen¹³, Asad A. Shah¹⁴, Christopher L. Bennett¹⁻², Patrick T. Ellinor¹⁵,¹⁶, Honghuang Lin¹⁷, Eric M. Isselbacher¹⁸, Christian Lacks Lino Cardenas¹⁹, Jonathan T. Butcher¹³, G. Chad Hughes²⁰, Mark E. Lindsay²¹, Baylor-Hopkins Center for Mendelian Genomics²², MIBAVA Leducq Consortium²², Luc Mertens²³, Anders Franco-Cereceda²⁴, Judith M. A. Verhagen²⁵, Marja Wessels²⁶, Salah A. Mohamed¹¹, Per Eriksson¹⁰, Seema Mital²⁶, Rut Van Laer²⁷, Bart L. Loeys²⁷, Gregor Andelfinger²⁸, Andrew S. McCallion¹⁵,²⁹,³²*, and Harry C. Dietz¹²,²⁹,³⁰,³²*

Bicuspid aortic valve (BAV) is a common congenital heart defect (population incidence, 1–2%)¹⁻³ that frequently presents with ascending aortic aneurysm (AscAA).² BAV/AscAA shows autosomal dominant inheritance with incomplete penetrance and male predominance. Causative gene mutations show autosomal dominant inheritance with incomplete penetrance (skewed towards females), isolated BAV, isolated AscAA, and variable location of the site of ascending aortic aneurysm (BAV/AscAA), there is often wide variation in cardiovascular manifestations between affected individuals, including complete non-penetrance (skewed towards females), isolated BAV, isolated AscAA, and variable location of the site of ascending aortic dilatation, including isolated aortic root aneurysm (AoRA).

Targeted silencing of **ROBO4** or mutant **ROBO4** expression in endothelial cell lines results in impaired barrier function and a synthetic repertoire suggestive of endothelial-to-mesenchymal transition. This is consistent with BAV/AscAA-associated findings in patients and in animal models deficient for **ROBO4**. These data identify a novel endothelial etiology for this common human disease phenotype.

In kindreds segregating bicuspid aortic valve with ascending aortic aneurysm (BAV/AscAA), there is often wide variation in cardiovascular manifestations between affected individuals, including complete non-penetrance (skewed towards females), isolated BAV, isolated AscAA, and variable location of the site of ascending aortic dilatation, including isolated aortic root aneurysm (AoRA);
Fig. 1 | Identification of ROBO4 variants segregating in families with BAV and aortic aneurysms. a, WES showed segregation of a heterozygous obligate splice-site mutation (g.124757628C>A, c.2056+1G>T) in a multigenerational family. b, cDNA amplicons spanning exons 11-14 were analyzed from patient (proband 1.II:4) and control fibroblasts. Sanger sequencing confirmed skipping of exon 13 (108 bp). c, A missense variant (p.Arg64Cys) was observed in a small family. The altered amino acid resides at the immunoglobulin (Ig)-like C2-type 1 extracellular domain. d, WES showed segregation of a heterozygous obligate splice-site mutation (g.124757628C>A, c.2056+1G>T) in an affected individual. e, Exon 12 Exon 13

at the sinuses of Valsalva) or more distal ascending aortic aneurysm (DAscAA; collectively AscAA)10–12. This observation suggests that BAV and AscAA are both variably penetrant primary manifestations of the same underlying gene defect (or defects). Although enrichment for BAV can be seen in syndromic presentations of thoracic aortic aneurysm (for example, Loeys–Dietz syndrome caused by mutations in genes encoding primary effectors of the transforming growth factor-β (TGFβ) signaling pathway), isolated DAscAA is exceedingly rare in these conditions, suggesting a mechanistic distinction for nonsyndromic BAV/AscAA13–15. The genetic etiology and molecular pathogenesis of nonsyndromic BAV/AscAA remains largely elusive despite intensive effort. Probable obstacles to progress include extreme locus heterogeneity and the confounding influence of incomplete penetrance, sex bias, and environmental and/or genetic modification of disease onset and severity. In this study, we sought to identify genes responsible for nonsyndromic BAV/AscAA using whole-exome sequencing (WES) and a familial segregation approach.

A total of nine patient families (Fig. 1a,c and Supplementary Fig. 1), 286 individual probands, and 193 unrelated controls without structural heart disease were enrolled in our WES initiative. In one large family (family 1), eight individuals showed AoRA with or without DAscAA, and two had associated BAV (Fig. 1a).

Initial sequencing of a panel of genes implicated in familial thoracic aortic aneurysm (ACTA2, FBN1, MYH11, MYLK, SMAD3, TGFBR1, TGFBR2)16–21 identified no mutations. WES in five affected individuals of family 1 identified a novel heterozygous mutation in the human genome and complement DNA and complementary DNA, respectively; NC_000011.9 and NM_019055.5 (Fig. 1a and Supplementary Fig. 2a). We confirmed that all eight affected family members carried the mutation. Interestingly, seven of eight affected individuals were male, including both with BAV. Two clinically unafflicted female family members were heterozygous for the ROBO4 splice-site mutation. Pathology from one family member with BAV showed valve thickening in association with a pronounced fibroproliferative process (Fig. 1b). Amplification and sequencing of complementary DNA derived from patient and control fibroblasts showed that the splice-site mutation results in the skipping of the 108-bp encoding exon 13 of ROBO4, resulting in an in-frame transcript encoding a protein isoform missing 36 amino acids from the intracellular domain (Fig. 1b). In family 2, WES identified a heterozygous ROBO4 missense variant (c.190C>T) in a woman with atrial septal defect (ASD) and aortic valve stenosis (AVS) that required surgery, and her son with ASD, BAV, and significant AVS (Fig. 1c and Supplementary Fig. 2c). This variant is exceedingly rare (Exome Aggregation Consortium (ExAC) frequency: 19 out of 98,998), predicted to be deleterious by Polymorphism Phenotyping (Polyphen) and SIFT (Sorting Intolerant From Tolerant v6.2.1), and predicted to be among the top 1% of most deleterious substitutions possible in the human genome by the Combined Annotation Dependent Depletion (CADD v1.0) algorithm (score 21.6). The mutation substitutes an evolutionarily conserved residue in the immunoglobulin-like C2-type 1 extracellular domain of ROBO4. Of the additional 286 individual probands evaluated, four variants (p.Ala95Thr, p.Thr232Met, p.His411Gln, and p.Arg568Ter) met our a priori filter, restricting our focus to exceedingly rare (minor allele frequency (MAF) < 0.01%) or rare and predicted highly deleterious (MAF < 0.1% and CADD > 20) variants (Fig. 1d and Table 1). All four variants were confirmed by Sanger sequencing.

We then sequenced an additional 441 probands with BAV/AscAA and 183 unrelated controls, with echocardiogram-confirmed absence of structural cardiovascular disease, for ROBO4...
mutant endothelial lining of the vasculature and a described role in establishing uniquely showed infiltration of ROBO4 α-actin (SMA, encoded by ACTA2), a vascular smooth muscle cell mesenchymal marker (Fig. 2b). However, the aneurysm tissue uniquely showed infiltration of ROBO4+ cells into the aortic media with co-staining for α-SMA. Given ROBO4 expression in the endothelial lining of the vasculature22 and a described role in establishing endothelial barrier function23,24, we hypothesized enhanced vascular permeability in the ascending aorta of patients with BAV/AscAA. In keeping with this hypothesis, immunostaining for albumin was restricted to the endothelial surface in control aorta, but substantially increased cellularity and the accumulation of collagen upon Masson’s trichrome staining. Furthermore, decreased elastin content, and fragmentation and disarray of elastic fibers in the superficial media were observed upon Verhoeff–Van Gieson (VVG) staining. Collectively, these data suggest that ROBO4 variants disrupt endothelial cellular performance and barrier function, contributing to pathological remodeling of the aortic media.

To further address the impact of ROBO4 alterations on endothelial cell performance, cultured human aortic endothelial cells (HAECs) were transiently transfected with short interfering RNA (siRNA) against ROBO4 or with constructs overexpressing either wild-type ROBO4, a form lacking exon 13, or a form with the p.Arg64Cys missense variant. Furthermore, to assess the individual performance of the ROBO4 variant seen in family 1, HAECs with silenced endogenous ROBO4 expression (siRNA targeting sequence in exon 13) were transfected with the expression construct lacking exon 13 (termed SS-Alone). The expression of wild-type and mutant ROBO4 mRNA was assessed by quantitative RT–PCR (PCR with reverse transcription) (Fig. 3a).

ROBO4 silencing or expression of ROBO4 variants resulted in loss of endothelial barrier function in a dextran permeability assay (Fig. 3b). Loss of barrier function was associated with downregulation of expression of TJPI and VE-cadherin mRNA and protein, indicative of loss of tight- and adherens-junction integrity, respectively (Fig. 3c). Other phenotypes that were seen included induction of expression of α-SMA and Snail1 mRNA, and cellular elongation and invasion. Assessment of expression of prototypical target genes suggested a modest decline in bone morphogenetic protein (BMP) and Notch activity without altered TGFβ responses upon ROBO4

### Table 1 | ROBO4 variants are more frequent in cases than in controls

| WES families | Variant | Verification | Domain | Fusion pattern | Family history | ExAC | CADD |
|--------------|---------|-------------|--------|---------------|---------------|------|------|
| BAV/AscAA g.124757628C>T | Ac.2056+1G>T | Sanger | Depth allele 1 | Depth allele 2 | N/A | R-N | Yes | Absent | 17.6 |
| BAV/AscAA c.190C>T | Arg64Cys | Sanger | IgL1 | L-R | Yes | 19/98,998 | 21.6 |
| BAV/AscAA c.283G>A | Arg95Thr | 7 | 6 | IgL1 | R-N | No | 5/117,304 | 6.42 |
| BAV/AscAA c.695C>T | Thr232Met | 42 | 36 | N/A | R-L | Yes | 10/121,068 | 19.56 |
| BAV/AscAA c.1233T>A | His411Gln | 31 | 30 | FN2 | R-N | Yes | 0.002 |
| BAV/AscAA c.1702C>T | Arg568Ter | 43 | 49 | N/A | R-L | No | 12/120,920 | N/A |
| BAV/AscAA c.190C>T | Arg64Cys | 63 | 69 | IgL1 | L-R | Yes | 19/98,998 | 21.6 |
| BAV/AscAA c.740T>C | Val247Ala | 1,204 | 1,187 | N/A | L-R | N/A | 1/121,370 | 10.5 |
| BAV/AscAA c.839A>G | Tyr280Ser | 590 | 469 | FN1 | R-N | N/A | 5/117,546 | 22 |
| BAV/AscAA c.1601_1614delGly534Glufs*49 | 124 | 114 | N/A | Yes | Absent | N/A |
| BAV/AscAA c.1864G>C | Asp622His | 767 | 721 | N/A | L-R | N/A | 4/110,004 | 21.3 |
| BAV/AscAA c.1864G>C | Asp622His | 1,300 | 1,118 | N/A | L-R | No | 4/110,004 | 21.3 |
| BAV/AscAA c.2245_2246delinsCT | Ala749Leu | 195 | 173 | N/A | R-N | Absent | 16.7 |
| Control c.1529A>T | P.Val510Val | 69 | 51 | N/A | N/A | N/A | Absent | 11.6 |

| ExAC | CADD |
|------|------|
| 21.6 | 17.6 |
| 19.56 | 6.42 |
| 10.5 | 22 |
| 21.3 | 21.3 |
| 16.7 | 11.6 |

| Variant | Depth allele 1 | Depth allele 2 | Domain | Fusion pattern | Family history | ExAC | CADD |
|---------|---------------|---------------|--------|---------------|---------------|------|------|
| WES families | BAV/AscAA g.124757628C>T | Ac.2056+1G>T | Sanger | Depth allele 1 | Depth allele 2 | N/A | R-N | Yes | Absent | 17.6 |
| BAV/AscAA c.190C>T | Arg64Cys | Sanger | IgL1 | L-R | Yes | 19/98,998 | 21.6 |
| BAV/AscAA c.283G>A | Arg95Thr | 7 | 6 | IgL1 | R-N | No | 5/117,304 | 6.42 |
| BAV/AscAA c.695C>T | Thr232Met | 42 | 36 | N/A | R-L | Yes | 10/121,068 | 19.56 |
| BAV/AscAA c.1233T>A | His411Gln | 31 | 30 | FN2 | R-N | Yes | 0.002 |
| BAV/AscAA c.1702C>T | Arg568Ter | 43 | 49 | N/A | R-L | No | 12/120,920 | N/A |
| BAV/AscAA c.190C>T | Arg64Cys | 63 | 69 | IgL1 | L-R | Yes | 19/98,998 | 21.6 |
| BAV/AscAA c.740T>C | Val247Ala | 1,204 | 1,187 | N/A | L-R | N/A | 1/121,370 | 10.5 |
| BAV/AscAA c.839A>G | Tyr280Ser | 590 | 469 | FN1 | R-N | N/A | 5/117,546 | 22 |
| BAV/AscAA c.1601_1614delGly534Glufs*49 | 124 | 114 | N/A | Yes | Absent | N/A |
| BAV/AscAA c.1864G>C | Asp622His | 767 | 721 | N/A | L-R | N/A | 4/110,004 | 21.3 |
| BAV/AscAA c.1864G>C | Asp622His | 1,300 | 1,118 | N/A | L-R | No | 4/110,004 | 21.3 |
| BAV/AscAA c.2245_2246delinsCT | Ala749Leu | 195 | 173 | N/A | R-N | Absent | 16.7 |
| Control c.1529A>T | P.Val510Val | 69 | 51 | N/A | N/A | N/A | Absent | 11.6 |

igL1, immunoglobulin-like 1. FN1 and FN2, fibronectin type III-like domain 1 and 2. For the fusion patterns, R-L, right-left coronary cusp fusion; L-R, left-right coronary cusp fusion; R-N, right-noncoronary cusp fusion; N/A, no data available.
silencing (Supplementary Fig. 3). Together, these results suggest that reduction or structural disruption of ROBO4 compromises endothelial identity and performance, as well as its capacity to suppress transition to a mesenchymal invasive character and synthetic repertoire. These data suggest that loss of function (by functional haploinsufficiency or the antimorphic effects of overexpressed mutant protein) is the relevant pathogenic mechanism. The distribution of missense variants along the full length of the gene and protein argues against particular relevance for any domain-specific function such as homo- or heterodimerization, interaction with other binding partners, or recruitment of SLIT ligands.

ROBO1, ROBO2, SLIT2, and SLIT3 are expressed in or adjacent to the endocardial cushions and heart valves during mouse development7. Targeted homozygous disruption of both Robo1 and Robo2 or Slit3 resulted in highly penetrant septal defects with variably penetrant valve anomalies, including BAV. We observed that mouse ROBO4 was expressed in the endocardial layer of the cushions and delamination zones at embryonic day 11.5 (E11.5; Supplementary

---

**Fig. 2** | Evaluation of ascending aortic aneurysm tissue resected from patient 1.II:1, compared to an age- and sex-matched control.  
**a.** Immunofluorescence staining of CD31, ROBO4, and DAPI at the intima–media interface. Arrows indicate ROBO4+ cells deep within the aortic media.  
**b.** Immunofluorescence staining of α-SMA, ROBO4, and DAPI at the intima–media interface.  
**c.** Immunofluorescence staining of CD31, ROBO4, and DAPI at the endothelial layer.  
**d.** Immunofluorescence staining of albumin and DAPI at the luminal surface.  
**e.** Histological staining (H&E, Masson’s, and VVG) of patient and control ascending aortic tissue.
Fig. 4a). By E17, ROBO4 was detected in both the endothelial and interstitial cells of the developing aortic valve and endothelial cells of the proximal aorta (Supplementary Fig. 4a,b). At 5 weeks after birth, ROBO4 was localized to the endothelial layer of the ascending aorta and persisted throughout postnatal development (Supplementary Fig. 4b). In normal adult human ascending aorta, ROBO4 is expressed in endothelial and intimal cells (Fig. 2 and Supplementary Fig. 4c). Similarly, robo4 is known to be expressed in the developing vasculature of zebrafish26. Together, these results confirm the expression of ROBO4 in cells relevant to the pathogenesis of BAV/AscAA.

Next, we established a mutant zebrafish (Danio rerio) line harboring a 7-bp deletion in exon 6 of robo4 (robo4∆7; Supplementary Fig. 5a). This frameshift mutation leads to a low steady-state abundance of robo4 mRNA due to nonsense-mediated mRNA decay (Supplementary Fig. 5b). We observed no gross defect in robo4−mutant larvae (Supplementary Fig. 6). Among the adult mutant zebrafish assayed by echocardiography, 7 out of 26 (26.9%) robo4∆7/+ and 4 out of 15 (26.7%) robo4∆7/∆7 zebrafish displayed regurgitation and/or turbulence across the ventriculo-bulbar valve compared to 4 out of 45 (8.9%) of the wild-type controls (P < 0.05; Supplementary Fig. 5c–e and Supplementary Table 2). Thus, targeted
disruption of robo4 in zebrafish perturbs outflow tract function. Although overt vascular enlargement was not observed, it is unclear whether the zebrafish system is ideal for assessing this specific aspect of the phenotype given substantive anatomic and physiologic differences from mammals and the limited duration of observation.

We next examined the effect of ROBO4 loss of function in mice using the Robo4<sup>tm1Lex</sup> model, which lacks exons 1 to 3. Robo4 transcripts were not detected in homozygous Robo4<sup>tm1Lex/tm1Lex</sup> mice via qRT–PCR (Supplementary Fig. 7a). None of the evaluated heterozygotes (Robo4<sup>tm1Lex/+</sup>) presented with any structural or functional abnormality. Homozygous Robo4<sup>tm1Lex/tm1Lex</sup> knockout mice exhibited a complex cardiovascular phenotype that includes a combination of aortic valve thickening with or without BAV, aortic valve stenosis and/or regurgitation and/or AscAA. In general, these phenotypes were observed with low penetrance and male predominance. Overall, at 20 weeks of age, 5 out of 28 (17.9%) male and 2 out of 18 (11.1%) female Robo4<sup>tm1Lex/tm1Lex</sup> mice were affected compared to 0 out of 22 (0%) and 0 out of 19 (0%) Robo4<sup>+/+</sup> males and females, respectively (P < 0.01; Fig. 4). In addition, one mouse with aortic regurgitation presented with a quadricuspid aortic valve (Supplementary Fig. 7b).

We next sought to evaluate the in vivo consequence of the splice mutation (g.124757628C>A, c.2056+1G>T) that we had observed in family 1 by establishing a knock-in mouse line harboring a mutation (Robo4<sup>skip13</sup>) at the splice donor site in intron 13 of Robo4<sup>+</sup> mice (Supplementary Fig. 8a). Sanger sequencing of cDNA amplicons spanning exons 11–14, using mRNA derived from

|                      | Robo4<sup>+/+</sup> | Robo4<sup>tm1Lex/</sup> | Robo4<sup>tm1Lex/tm1Lex</sup> | WT vs KO |
|----------------------|--------------------|--------------------------|-----------------------------|----------|
| Regurgitation        | 0/22               | 0/19                     | 4/28                        | 1/18     | P = 0.037 |
| Flow >600 mm s<sup>-1</sup> | 0/22               | 0/14                     | 5/28                        | 1/18     | P = 0.019 |
| AscAA (Z > 2)        | 0/22               | 0/14                     | 3/28                        | 0/18     | P = 0.143 |
| AoRA (Z > 2)         | 0/22               | 0/14                     | 2/28                        | 0/18     | P = 0.277 |
| Total affected       | 0/22               | 0/14                     | 5/28                        | 2/18     | P = 0.009 |
| AoV defect<sup>*</sup> | 0/3                | N/A                      | 5/5                         | 2/2      | P = 0.008 |
| Pure BAV<sup>**</sup> | 0/25               | N/A                      | 1/41                        | N/A      | P = 0.627 |

Fig. 4 | Robo4 knockout causes aortic valve defects and aortic aneurysm in mice. a, The cardiovascular phenotype was determined for wild-type (Robo4<sup>+/+</sup>), heterozygous (Robo4<sup>+/tm1Lex</sup>), and knockout (Robo4<sup>tm1Lex/tm1 Lex</sup>) mice 20 weeks after birth. Aortic dilatation was defined as a Z-score greater than two when compared to wild-type male mice by echocardiography. Statistical differences were assessed using one-tailed Fisher’s exact test (n = 112 total male and female mice, P < 0.05 was considered statistically significant). Aortic valve (AoV) defect: abnormal aortic valve morphology, including BAV, distal commissure fusion, leaflet thickening, or quadricuspid aortic valve among mutant mice with flow abnormalities by echocardiogram. Comparison is made to a representative sample of wild-type mice. Pure BAV: pure bicommissural aortic valve that was definitively evident by gross inspection. N/A, not assessed. See Supplementary Table 3 for raw data. b, Representative parasternal long axis and pulsed-wave Doppler images. Red arrows indicate a dilated segment of the ascending aorta. The yellow arrow shows increased velocity of outflow across the aortic valve during systole, while the orange arrow shows regurgitant flow during diastole in a Robo4<sup>tm1Lex/tm1Lex</sup> mouse (similar patterns were seen in the seven affected mice). c, Gross and histological examination of the aortic valve. Numbers refer to individual commissures.
the ascending aorta of targeted animals, showed either in-frame skipping of exon 13 (exons 12/14) or inclusion of exon 13 with activation of a cryptic splice donor in intron 13 (Supplementary Fig. 8b,c). Using TaqMan probes specific to individual exons or splice junctions, we determined that wild-type mice show 100% normal splicing (exons 12/13/14). Representation of exons 12/13/14, exons 12/13 + intron 13, and exons 12/14 transcripts was approximately 50%, 28%, and 22%, or approximately 0%, 55%, and 45% in Robo4+/−/Skip13 or Robo4Skip13/Skip13 animals, respectively (Supplementary Fig. 8d). We conclude that the Robo4Skip13 mutation alters premRNA splicing with significant representation of mature mRNA lacking exon 13.

Like the knockout mice, Robo4Skip13 mice presented with an incompletely penetrant complex cardiovascular phenotype that included aortic valve defects and aortic valve dysfunction and AscAA. At 20 weeks, 1 out of 31 (3.2%) Robo4+/−/Skip13 and 4 out of 35 (11.4%) Robo4Skip13/Skip13 male mice were affected compared to 0 out of 18 Robo4+/+ mice (P < 0.05; Fig. 5).
We demonstrate that heterozygous mutations in ROBO4 are sufficient to cause a nonsyndromic presentation of BAV/AscAA. The distribution of expression of ROBO4 in both the developing valve and the ascending aorta is consistent with the view that BAV and aortic aneurysm present as independent primary manifestations of the same underlying gene defect, a concept further supported by the presence of either BAV/AscAA, isolated BAV, or isolated AscAA in family members segregating the same ROBO4 variant. This study highlights many obstacles in the elucidation of etiologies for BAV/AscAA. Such obstacles include a relatively low frequency for involvement of any specific disease gene (also evident for NOTCH1 and SMAD6), apparent extreme locus heterogeneity, functional redundancy within relevant pathways, low penetrance with sex bias, and the lack of signatures for loss-of-function intolerance that is likely to be related to both the penetrance issue and to later onset of the phenotype and hence minimal, if any, impact on reproductive fitness. This study focuses attention on endothelial cell biology in BAV/AscAA, with impairment of barrier function and/or dysregulation of mesenchymal transition potentially contributing to disease expression. Prior work has suggested that abnormal plasma protein (for example plasminogen) trafficking in the vessel wall might contribute to aneurysm phenotypes by enhancing proteolytic activity, TGFβ bioavailability, or other mechanisms12–15. Other studies have suggested an association between BAV/AscAA and an endothelial-to-mesenchymal (EnMT)-like phenotype in the aortic wall16 and decreased mesenchymal potential of endothelial cells derived from such patients17, but neither study had defined the etiology or mechanism for these findings. Although our study of isolated endothelial cells did not identify a predominant pathway driving apparent EnMT in the context of ROBO4 deficiency, a confluence of data provides further incentive to interrogate altered mesenchymal transition as a pathogenetic driver and a therapeutic target in BAV/AscAA using model systems that mimic the anatomic and physiologic complexity of the left ventricular outflow tract and ascending aorta.

URLs. Circularity in ImageJ, https://imagej.nih.gov/ij/plugins/circularity.html; CRISPR design tool, http://crispr.technology.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41588-018-0265-y.

Received: 1 June 2017; Accepted: 26 September 2018; Published online: 19 November 2018

References
1. Fedak, P. W. M. et al. Clinical and pathophysiological implications of a bicuspid aortic valve. Circulation 106, 900–904 (2002).
2. Mack, G. & Silverbach, M. Aortic and pulmonary stenosis. Pediatr. Rev. 21, 79–85 (2000).
3. Ward, C. Clinical significance of the bicuspid aortic valve. Heart 83, 81–85 (2000).
4. Tadros, T. M., Klein, M. D. & Shapira, O. M. Ascending aortic dilatation associated with bicuspid aortic valve: pathophysiology, molecular biology, and clinical implications. Circulation 119, 880–890 (2009).
5. Cripe, L., Andelfinger, G., Martin, L. J., Shooner, K. & Benson, D. W. Bicuspid aortic valve is heritable. J. Am. Coll. Cardiol. 44, 138–143 (2004).
6. Gavriely, E. et al. Mutations in NOTCH1 cause aortic valve disease. Nature 437, 270–274 (2005).
7. McKechnie, S. H. et al. Novel NOTCH1 mutations in patients with bicuspid aortic valve disease and thoracic aortic aneurysms. J. Thorac. Cardiovasc. Surg. 134, 290–296 (2007).
8. Tan, H. L. et al. Nonsynonymous variants in the SMAD6 gene predispose to congenital cardiovascular malformation. Hum. Mutat. 33, 720–727 (2012).
9. Clementi, M., Notari, L., Borghi, A. & Tenconi, R. Familial congenital bicuspid aortic valve: a disorder of uncertain inheritance. Am. J. Med. Genet. 62, 336–338 (1996).
10. Huntington, K., Hunter, A. G. W. & Chan, K. L. A prospective study to assess the frequency of familial clustering of congenital bicuspid aortic valve. J. Am. Coll. Cardiol. 30, 1809–1812 (1997).
11. Mckusick, V. A., Logue, R. B. & Bahnsen, H. T. Association of aortic valvular disease and cystic medial necrosis of the ascending aorta; report of four instances. Circulation 16, 188–194 (1957).
12. Loscalzo, M. L. et al. Familial thoracic aortic dilation and bicommissural aortic valve: a prospective analysis of natural history and inheritance. Am. J. Med. Genet. A 143, 1960–1967 (2007).
13. Juselbacher, E. M. Thoracic and abdominal aortic aneurysms. Circulation 111, 816–828 (2005).
14. Williams, J. A. et al. Early surgical experience with Loeys-Dietz: a new syndrome of aggressive thoracic aortic aneurysm disease. Ann. Thorac. Surg. 83, 5785–5790 (2007).
15. Van Hemelrijck, C., Renard, M. & Loeys, B. The Loeys-Dietz syndrome: an update for the clinician. Curr. Opin. Cardiol. 25, 546–551 (2010).
16. Guo, D.-C. et al. Mutations in smooth muscle alpha-actin (ACTA2) lead to thoracic aortic aneurysms and dissections. Nat. Genet. 39, 1488–1493 (2007).
17. Pereira, L. et al. Targeting of the gene encoding fibrillin-1 recapitulates the vascular aspect of Marfan syndrome. Nat. Genet. 17, 218–222 (1997).
18. Zhu, L. et al. Mutations in myosin heavy chain 11 cause a syndrome associated with thoracic aortic dilation and dissection and patent ductus arteriosus. Nat. Genet. 38, 343–349 (2006).
19. Wang, L. et al. Mutations in myosin light chain kinase cause familial aortic dissections. Am. J. Hum. Genet. 87, 701–707 (2010).
20. van de Laar, I. M. B. H. et al. Phenotypic spectrum of the SMAD3-related aneurysms–osteochondritis syndrome. J. Med. Genet. 49, 47–57 (2012).
21. Loeys, B. L. et al. Aneurysm syndromes caused by mutations in the TGF-β receptor. N. Engl. J. Med. 355, 788–798 (2006).
22. Park, K. W. et al. Robo4 is a vascular-specific receptor that inhibits endothelial migration. Dev. Biol. 261, 251–267 (2003).
23. Jones, C. A. et al. Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. Nat. Med. 14, 448–453 (2008).
24. Cai, H. et al. Roundabout 4 regulates blood–tumor barrier permeability through the modulation of ZO-1, occludin, and claudin-5 expression. J. Neurophilathol. Exp. Neurrol. 74, 35–37 (2015).
25. Mommersteeg, M. T. M., Yeh, M. L., Park, A. J. G. & Andrews, W. D. Disrupted Slit-Robo signalling results in membranous vascular septum defects and bicuspid aortic valves. Cardiovasc. Res. 106, 55–66 (2015).
26. Bedell, V. M. et al. Roundabout4 is essential for angiogenesis in vivo. Proc. Natl Acad. Sci. USA 102, 6373–6378 (2005).
27. Carmeliet, P. et al. Urokinase-generated plasmin activates matrix metalloprotease-2 and -9 in tissues of aggressive thoracic aortic aneurysms-osteoarthritis syndrome. Genet. 17, 439–444 (1997).
28. Borges, L. F. et al. Fibrinolytic activity is associated with presence of cystic medial degeneration in aneurysms of the ascending aorta. Histopathology 57, 917–932 (2010).
29. Maleki, S. et al. Mesenchymal state of internal cells may explain higher propensity to ascending aortic aneurysm in bicuspid aortic valves. Sci. Rep. 6, 35712 (2016).
30. Kostina, A. S. et al. Notch-dependent EMT is attenuated in patients with aortic aneurysm and bicuspid aortic valve. Biochim. Biophys. Acta 1862, 733–740 (2016).

Acknowledgements
We gratefully acknowledge support from the Leukemia Foundation to A.S.M. and H.C.D., from the National Human Genome Research Institute (NHGRI) (1U54HG006542) to D.V. and J.L., from the National Heart, Lung, and Blood Institute (NHLBI) (1HL110328, 1HL128745) and the NIH (S10OD012287) to J.T.B. We also thank the American Philosophical Society for support of H.A. through the Daland Fellowship. In addition, we thank Johns Hopkins University School of Medicine, McKusick Nathans Institute of Genetic Medicine Center for Functional Investigation in Zebrafish (FINZ) for their technical support and Corinne Boehm for her assistance in depositing variant information to ClinVar. B.L.L. is senior clinical investigator of the Fund for Scientific Research, Flanders, and holds a starting grant from the European Research Council (ERC-StG-2012-30972-BRAVE). A.V. is a postdoctoral researcher supported by the Fund for Scientific Research Flanders. L.L. is supported by a PhD grant from the Agency for Innovation by Science and Technology Research (IWT). M.E.L. is supported by the Tooney Fund for Aortic Dissection Research and the Friedman Research Fund in Aortic Disease. G.A. is a FQRS Senior Clinical Research Fellow.

Author contributions
H.C.D., B.L.L., G.A., E.S., G.M., and the MIBAVA Leducq Consortium recruited participants for the study. A.S.M., H.C.D., G.A., and B.L.L. were instrumental
in the experimental design and interpretation of the data. C.P., N.S., H.Ling, A.A.K., I.L., E.C., A.V., H.M.B., A.-C.L., V.J.-R., H.J., A.A.S., C.L.B., P.T.E., H.Lin, E.M.I., C.L.C., J.T.B., G.C.H., M.E.L., Baylor-Hopkins Center for Mendelian Genomics, I.M., A.F.-C., J.M.A.V., M.W., S.M., P.E., S.A.Mohamed., L.V.L., and F.W. were instrumental in analyses of portions of the sequencing data and clinical descriptions. R.A.G. and M.A.S.-S. performed in vitro experiments, and R.A.G. performed mouse experiments with assistance from D.B. under the supervision of H.C.D. C.E.W. performed all zebrafish experiments with assistance from C.R.M., R.R., and S.A.McClymont under the supervision of A.S.M. The initial mouse studies, genetic analysis, and identification of the gene of interest were performed by H.A. under the supervision of H.C.D. H.A., A.S.M., C.E.W., R.A.G., H.A., and M.A.S.-S. wrote the manuscript with contributions from all remaining authors.

Baylor-Hopkins Center for Mendelian Genomics

David Valle¹, Hua Ling⁸ and James Lupski³³

MIBAVA Leducq Consortium

Harry C. Dietz¹,²,²⁹,³⁰, Andrew S. McCallion¹⁵,²⁹, Gregor Andelfinger⁴,²⁸, Bart L. Loeys⁹,²⁷, Lut Van Laer⁹, Per Eriksson¹⁰, Salah A. Mohamed¹¹, Luc Mertens²³, Anders Franco-Cereceda²⁴ and Seema Mital²⁶

¹³Baylor College of Medicine, Houston, TX, USA.
Methods

Study participants. Individuals were recruited from the Connective Tissue Clinic at Johns Hopkins Hospital (H.C.D.), Duke University (G.C.H.), Radboud University Hospital/Antwerp University Hospital (B.L.L.), Centre Hospitalier Universitaire Sainte-Justine (G.A.), Karolinska University Hospital (P.E.), University of Luxembourg (S.A.Mohamed), SickKids Hospital (S.M.), and Erasmus University Medical Center (M.W.) in strict compliance with all relevant ethical regulations. All skin biopsies and research protocols were collected in compliance with the Institutional Review Board at each respective institution after informed consent was obtained. Echocardiograms were performed and interpreted as described previously.23

Whole-exome sequencing. Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. DNA fragmentation was performed using a Covaris S2 system, and exon capture was performed using the Agilent SureSelect Human All Exon Enrichment kit. DNA sequencing was performed on an Illumina Genome Analyzer IIx instrument with using standard protocols for 75-bp paired-end runs.

Bioinformatics analysis. Reads were mapped to the human reference genome (UCSC hg19) using the Burrows–Wheeler Aligner (BWA) and a variant list created as described previously.31

Targeted sequencing. ROBO4 Purification kit (Qiagen). Cycle sequencing was performed using the BigDye Terminator v3.1 kit and an ABI 3730xl DNA Analyzer in accordance with the manufacturer's instructions for each primer set (Thermo Scientific). Sanger DNA sequencing assays were performed using primers designed 60–120 bp from the variants or intron–exon boundaries to confirm candidate variants or sequence candidate genes. PCR was performed using a DNA Engine Dyad thermal cycler (Bio-Rad). Phusion Flash High Fidelity PCR Master Mix was used in accordance with the manufacturer's instructions for each primer set (Thermo Scientific). Cycle sequencing was performed using the BigDye Terminator v3.1 kit and an ABI 3730xl DNA Analyzer in accordance with the manufacturer's instructions (Life Technologies). Samples were purified using the QIAquick PCR Purification kit (Qiagen).

Targeted sequencing. Enrichment for all exons of ROBO4, including ±10 bp of adjacent intronic sequences, was performed with a custom Haloplex target enrichment panel, following instructions of the manufacturer (Agilent Technologies). Probe design covered a theoretical 99.7% of the complete target region. Pooled samples were sequenced either on a HiSeq 2500 (Illumina) with 2×150 bp reads or on a HiSeq 1500 (Illumina) with 2×100 bp reads.

Cell culture and transfection. Primary human dermal fibroblasts were derived from forearm skin biopsies from one control individual, a control cell line (ATCC), or a single proband. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) in the presence of antibiotics and were passaged from forearm skin biopsies from one control individual, a control cell line (ATCC), or a single proband. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) in the presence of antibiotics and were passaged. DNA fragmentation was performed using a Covaris S2 system, and exon capture was performed using the Agilent SureSelect Human All Exon Enrichment kit. DNA sequencing was performed on an Illumina Genome Analyzer IIx instrument with using standard protocols for 75-bp paired-end runs.

Mutation validation and Sanger sequencing of candidate genes. Bidirectional Sanger DNA sequencing assays were performed using primers designed 60–120 bp from the variants or intron–exon boundaries to confirm candidate variants or sequence candidate genes. PCR was performed using a DNA Engine Dyad thermal cycler (Bio-Rad). Phusion Flash High Fidelity PCR Master Mix was used in accordance with the manufacturer's instructions for each primer set (Thermo Scientific). Cycle sequencing was performed using the BigDye Terminator v3.1 kit and an ABI 3730xl DNA Analyzer in accordance with the manufacturer's instructions (Life Technologies). Samples were purified using the QIAquick PCR Purification kit (Qiagen).

Targeted sequencing. Enrichment for all exons of ROBO4, including ±10 bp of adjacent intronic sequences, was performed with a custom Haloplex target enrichment panel, following instructions of the manufacturer (Agilent Technologies). Probe design covered a theoretical 99.7% of the complete target region. Pooled samples were sequenced either on a HiSeq 2500 (Illumina) with 2×150 bp reads or on a HiSeq 1500 (Illumina) with 2×100 bp reads.

Cell culture and transfection. Primary human dermal fibroblasts were derived from forearm skin biopsies from one control individual, a control cell line (ATCC), or a single proband. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) in the presence of antibiotics and were passaged. DNA fragmentation was performed using a Covaris S2 system, and exon capture was performed using the Agilent SureSelect Human All Exon Enrichment kit. DNA sequencing was performed on an Illumina Genome Analyzer IIx instrument with using standard protocols for 75-bp paired-end runs.

RNA isolation and qRT–PCR. For zebrafish, RNA was isolated from dissected whole embryos at 24 h. HAEC media was then replaced and allowed to culture for an additional 48 h.

Antibody staining. Samples were fixed in 4% paraformaldehyde overnight at 4°C. Samples were then washed for 15 min on a rocker three times with PBS buffer, permeabilized with 0.2% Triton-X 100 (VWR International) for 10 min, and washed another three times with PBS. Samples were incubated in 4% BSA in a 1% BSA (Rockland Immunochemicals) blocking solution followed by another 4°C overnight incubation with antibodies against ROBO4 (Abcam, ab1203674), ROBO4 (Santa Cruz, sc-46497), CD31 (Biocare Medical, CM303A), PECAM1 (Santa Cruz, sc-1506), CDH5 (Santa Cruz, sc-28644), TJP1 (Santa Cruz, sc-8146), ACTA2 (Abcam, ab78177), or ALB (Santa Cruz, sc-6293). After three washes for 15 min with PBS, samples were exposed to Alexa Fluor 488 or 568 conjugated (Invitrogen) species-specific secondary antibodies at 1:100 in 1% BSA for 2 h at room temperature. Three more washes with PBS for 15 min were followed by incubation with either DRAQ5 (far-red) nuclear stain or DAPI (UV) nuclear stain (Enzo Life Sciences) at 1:1,000.

Permeability assay. Permeability assays were performed as described previously.24 HAEcs were utilized at or before passage six. Cells were detached using 0.05% trypsin-EDTA (Thermo Fisher Scientific), pelleted, then re-suspended in a growth medium to a concentration of 0.5–1×106 cells ml−1. Dry collagen pre-coated porous inserts were rehydrated for 15 min in a growth medium prior to cell seeding (1.0–μm pores, EMD Millipore). HAEcs were seeded in 200μl of growth medium per 24-well insert. 500μl were added to each receiver plate well. HAEcs were cultured in a tissue culture incubator at 37°C with 5% CO2 for 72 h to allow for a monolayer formation. Following endothelial monolayer formation, growth medium was carefully removed with a pipette. The inserts were rehydrated with different ROBO4 constructs (see Supplementary Table 5). Samples were exposed to 37°C incubation for 24 h. Afterwards, the treatment medium was carefully removed. A high molecular weight FITC-dextran solution (40kDa) was created by 1:40 dilution in a growth medium and added at 150μl. Growth medium was added to each receiver plate well at 500μl (24-well plate). The FITC-dextran permeated the monolayers for 2 h. The inserts were removed from the receiver wells to stop permeation. The medium in the receiver wells was then mixed thoroughly and 100μl was removed from each well to a black 96-well opaque plate for fluorescence measurement. Permeability was quantified on a Synergy II fluorescent plate reader (Biotek) via fluorescence at 485 nm excitation/535 nm emission wavelengths (4 h, 5 min exposure count time). The endothelial monolayer was stained after completion of fluorescein isothiocyanate (FITC)–dye permeability testing for subsequent confocal imaging.

Cell morphology assay. Cell shape was calculated as described previously, using Circularity in ImageJ (see URIs). Cell shape changes were tracked automatically over subsequent images, and quantified as a cell circularity index (CI = 4 π x (area/perimeter²)). A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon.

Endothelial aggregate invasion assay. For invasion assays, cells were re-suspended in culture media and allowed to aggregate overnight in hanging drop culture (2000–50000 cells). The aggregates were then placed on the surface of neutralized type I collagen hydrogels (1.5 mg ml−1), as described previously and allowed to adhere for 2 h before adding treatments. Cultures were then transferred for 24 h with the ROBO4 constructs (see Supplementary Table 5) and maintained for another 72 h. Cell invasion was quantified manually at a 50-μm depth into the gel and normalized to control.

Proliferation assay. 5-Bromo-2′-deoxyuridine (BrdU) incorporation was used to detect proliferating cells, as described previously. Cultures were transfected for 24 h with the ROBO4 constructs (see Supplementary Table 5). BrdU labeling reagent (Invitrogen) was added to the culture media for 12 h after treatment. After 24 h, cells were fixed and targeted with a monoclonal Anti-BrdU Alexa Fluor 488 conjugate (PRB-1, Invitrogen) while total DNA was counterstained using a DAPI dye. Positive fluorescent areas for each cell were measured using ImageJ and normalized by cell nuclei.

Scratch assay. Migratory ability was tested as described previously. In brief, cells were cultured to confluency and then transfected for 24 h with the ROBO4 constructs. A scratch was then introduced using a 200-μl pipette tip. The cells were then incubated at 37°C and observed at 24 h. Differences in filling in the scratch were observed to establish the capability of cellular migration.

Zebrafish and mouse maintenance. Adult AB and robo4−/− mutant zebrafish lines were maintained in system water according to standard methods.25 Mouse were housed in the Johns Hopkins University School of Medicine East Baltimore campus. All strains were maintained on a mixed C57BL/6J and F129/SVE background. All upkeep and experimental procedures for zebrafish and mice were in accordance with the ethical guidelines set by the Johns Hopkins Institutional Animal Care and Use Committee.

Generation of a robo4 knockout zebrafish line by CRISPR–Cas9. We designed a single guide RNA (gRNA, see sequences in Supplementary Table 6) to target the
Robo4 gene (gRNA

 Cas9 RNA was synthesized from linearized pCS2-nls-zCas9-nls (AddGene, no. 47929)\textsuperscript{46}. We injected 100 ng ml\textsuperscript{-1} of gRNA and 300 ng of Cas9 in a 5-µl solution into one-cell wild-type (AB) zebrafish embryos and raised them to maturity (F\textsubscript{0}). To identify fish capable of germline transmission, we crossed F\textsubscript{0} males with AB females. Germline transmission was confirmed by Surveyor Assay (IDT) in pooled F\textsubscript{1} embryos (n = 50) followed by Sanger sequencing. Heterozygous F\textsubscript{1} adults with the same 7-bp deletion were inbred to establish the line (Robo4\textsuperscript{−/−}). All subsequent generations were genotyped using primer set F2 and R2 (see Supplementary Table 6).

Robo4 knockout mice and generation of Robo4 knock-in mice. Robo4\textsuperscript{−/−} knockout mice were purchased through the mutant mouse resource and research centers supported by the National Institutes of Health (NIH) (MG\textsubscript{E}:5007309, Lexicon Pharmaceuticals). The mice were bred in a heterozygous state on a mixed background of 129S5/SvEvBrd and C57BL6/J. To generate Robo4 knock-in mice, we targeted the Robo4 locus using CRISPR–Cas9 (clustered, regularly interspaced palindromic repeat–CRISPR-associated protein 9). Two single-guide RNA (sgRNA) sequences (see Supplementary Table 7) were designed to target exon 13 of Robo4 (NC_000074.5) using a gRNA CRISPR design tool (see URLs)\textsuperscript{47}. The gRNAs were not predicted to have any off-target effects. These oligonucleotides were cloned into a pX459 plasmid\textsuperscript{48} (Addgene, no. 48139) and appended with a T7 promoter. The sgRNA was transcribed in vitro. The homology directed repair (HDR) template was purchased as a 4-nmol Ultramer (IDT, see Supplementary Table 6). The sgRNA, Cas9 (TriLink Biotechnologies), and HDR were co-injected into C57BL/6J zygotes (Johns Hopkins University Transgenic Core). Pups (n = 41) were screened for locus editing by Sanger sequencing (IDT, see Supplementary Table 7). Mice (Robo4\textsuperscript{−/+}) were subsequently genotyped by Sanger sequencing (271-bp amplicon). Mice were then crossed to a mixed background (129S5/SvEvBrd and C57BL6).

Zebrafish and mouse echocardiography. For zebrafish, we used the Vevo 2100 Imaging System equipped with a 70-MHz ultrasound transducer (VisualSonics). The zebrafish were anesthetized by placing the fish into a plastic cup filled with 100 ml of 168 µg ml\textsuperscript{-1} MS (Tricaine methanesulfonate, Fluka Analytical). For mice, echocardiograms were undertaken on awake, unsedated mice using the Vevo 2100 Imaging System (VisualSonics) and a 40-MHz transducer. Echocardiographic recordings for the mice were taken using a parasternal long-axis or sagittal short-axis view; three independent measurements of the maximal internal dimension at the sinus of Valsalva and ascending aorta were made and averaged. All data acquisition and measurements for mice and zebrafish were performed blinded to the genotype.

Statistics and reproducibility. For experiments including multiple comparisons, P values refer to one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. For experiments including only one comparison, P values refer to the unpaired two-tailed Student’s t-test, two-tailed Welch’s t-test, or two- or one-tailed Fisher’s exact test. P values of <0.05 were considered significant. P values are provided as exact figures wherever possible, and otherwise reported as a range when a large amount of comparisons were performed in a single figure or panel. Data are shown as mean ± s.e.m. or s.d.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. ROBO4 variants have been submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and have the following accession codes: SCV000804228, SCV000804229, SCV000804230, SCV000804231, SCV000804232, SCV000804233, SCV000804234, SCV000804235, SCV000804236, SCV000804237, SCV000804238, SCV000804239. Exome sequencing data are not publicly available owing to consent restrictions.

References
31. Brooke, B. S. et al. Angiotensin II blockade and aortic-root dilation in Marfan’s syndrome. N. Engl. J. Med. 358, 2787–2795 (2008).
32. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760 (2009).
33. Li, H. et al. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).
34. Wang, K., Li, M. & Hakonarson, H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 38, e164 (2010).
35. McKenna, A. et al. The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303 (2010).
36. DePristo, M. A. et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat. Genet. 43, 491–498 (2011).
37. Robinson, J. T. et al. Integrative genomics viewer. Nat. Biotechnol. 29, 24–26 (2011).
38. Andeler, J., Ma, J. & Armstrong, L. Improved assays for quantification of in vitro vascular permeability (https://www.nature.com/appNotes/nmeth/2012/121007/pdf/an8623.pdf).
39. Gould, R. A. et al. Multi-scale biomechanical remodeling in aging and genetic mutant murine mitral valve leaflets: insights into Marfan syndrome. PLoS ONE 7, e44639 (2012).
40. Chiu, Y.-N., Norris, R. A., Mahler, G., Recknagel, A. & Butcher, J. T. Transforming growth factor β, bone morphogenetic protein, and vascular endothelial growth factor mediate phenotype maturation and tissue remodeling by embryonic valve progenitor cells: relevance for heart valve tissue engineering. Tissue. Eng. Part A 16, 3375–3383 (2010).
41. Gould, R. A. et al. Cyclic strain anisotropy regulates valvular interstitial cell phenotype and tissue remodeling in three-dimensional culture. Acta Biomater. 8, 1710–1719 (2012).
42. Liang, C.-C. C., Park, A. Y. & Guan, J.-L. L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat. Protoc. 2, 329–333 (2007).
43. Westerfield, M. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). 5th edn, (Univ. Oregon Press, Eugene, 2007).
44. Sander, J. D., Zaback, P., Joung, J. K., Voytas, D. F. & Dobbs, D. Zinc Finger Targeter (ZiFiT): an engineered zinc finger/target site design tool. Nucleic Acids Res. 35, W599–W605 (2007).
45. Sander, J. D. et al. ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool. Nucleic Acids Res. 38, W462–W468 (2010).
46. Jiao, L.-E., Wenz, S. R. & Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proc. Natl Acad. Sci. USA 110, 13904–13909 (2013).
47. Jaksula-Ranga, V. & Zack, D. J. grID: A CRISPR-Cas9 guide RNA database and resource for genome-editing. Preprint at bioRxiv https://doi.org/10.1101/09.352 (2016).
48. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308 (2013).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
|     | The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement |
|     | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
|     | The statistical test(s) used AND whether they are one- or two-sided |
|     | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
|     | A description of all covariates tested |
|     | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
|     | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
|     | For null hypothesis testing, the test statistic (e.g. \(F, t, r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted |
|     | Give \(P\) values as exact values whenever suitable. |
|     | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
|     | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
|     | Estimates of effect sizes (e.g. Cohen's \(d\), Pearson's \(r\)), indicating how they were calculated |
|     | Clearly defined error bars |
|     | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

- Data collection: Vevo 2100 Imaging Suite
- Data analysis: Burrows-Wheeler Aligner, Genome Analysis Toolkit, Integrated Genome Viewer, Graphpad, SAS, Excel and Image J

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

ROBO4 variants were submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and have the following accession codes: SCV000804228, SCV000804229, SCV000804230, SCV000804231, SCV000804232, SCV000804233, SCV000804234, SCV000804235, SCV000804236, SCV000804237, SCV000804238, SCV000804239.
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size          | For in-vitro studies, experiments were conducted with 3 biological replicates, each with 2 technical replicates. For mouse studies, we achieved >10 (and often >20) mice per genotype and/or condition. This was based on Mendelian segregation and not any imposed cutoffs. |
|----------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions      | No data were excluded                                                                                                                                                                             |
| Replication          | Experimental findings were reliably reproduced. All experiments and data points were included in the analyses.                                                                                  |
| Randomization        | In vitro samples were allocated based on experimental conditions. Organisms were allocated based on genotype and sex. Human patients were allocated based on phenotype.                                |
| Blinding             | Echocardiographic measurements and phenotypic assessments were all performed by observers blinded to genotype. Genotype was not revealed until all analyses were complete.                                   |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | ☑ Unique biological materials |
| ☑   | Antibodies |
| ☑   | Eukaryotic cell lines |
|     | Palaeontology |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq |
|     | Flow cytometry |
| ☑   | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

The ROBO4 mouse knock-in lines developed and described in the manuscript can be readily obtained through Jax Laboratories

Antibodies

Antibodies used

ROBO4 (Abcam, ab103674), ROBO4 (Santa Cruz, sc-46497), CD31 (Biocare Medical, CM303A), PECAM1 (Santa Cruz, sc-1506), CDH5 (Santa Cruz, sc-28644), TJP1 (Santa Cruz, sc-8146), ACTA2 (Abcam, ab7817), or ALB (Santa Cruz, sc-46293).

Validation

All antibodies were commercially available and used for their intended application (assay and species). All antibodies identified epitopes with the expected subcellular localization and validated in part by correlating with mRNA levels in cell culture experiments.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

PromoCell-Human Aortic Endothelial Cells (HAoEC)-C-12271
### Authentication
Confirmed from the supplier. vWF+, CD31+, Dil-Ac-LDL uptake+

### Mycoplasma contamination
Confirmed mycoplasma free by supplier.

### Commonly misidentified lines
(See ICLAC register)
No commonly misidentified cell lines were used.

### Animals and other organisms

#### Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**
Adult AB and robo4Δ7 mutant zebrafish lines were maintained in system water according to standard methods. Mice (Mus musculus) were housed in the Johns Hopkins University School of Medicine East Baltimore campus. All strains were maintained on a mixed C57BL/6J and F129/SVE background, with 24 week-old male and female mice used for echocardiography measurements and littermates used for comparisons. All zebrafish and mice upkeep and experimental procedures were in accordance with the ethical permits set by the Johns Hopkins Institutional Animal Care and Use Committee.

**Wild animals**
Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.

**Field-collected samples**
For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

### Human research participants

#### Policy information about studies involving human research participants

**Population characteristics**
Affected individuals were recruited from Johns Hopkins Hospital (H.C.D.), Duke University (G.C.H.), Radboud University Hospital/Antwerp University Hospital (B.L.L.), Centre Hospitalier Universitaire Sainte-Justine (G.A.), Karolinska University Hospital (P.E.), University of Luebeck (S.A.M.), Sickkids Hospital (S.M.), and Erasmus University Medical Center (M.W.).

**Recruitment**
All patients were being seen for the clinical indication of cardiovascular disease and specifically bicuspid aortic valve and/or ascending aortic aneurysm. No ascertainment or recruitment bias was imposed based on gender, ethnic origin, or any other demographic parameter. All skin biopsies and research protocols were collected in compliance with the Institutional Review Board at each respective institution after informed consent was obtained.