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GATA6 regulates aortic valve remodeling and its haploinsufficiency leads to RL-type Bicuspid Aortic Valve

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Abstract

Background—Bicuspid aortic valve (BAV), the most common congenital heart defect affecting 1–2% of the population, is a major risk factor for premature aortic valve disease and accounts for the majority of valve replacement. The genetic basis and the mechanisms of BAV etiology and pathogenesis remain largely undefined.

Methods—Cardiac structure and function was assessed in mice lacking a *Gata6* allele. Human *GATA6* gene variants were analyzed in 452 BAV cases from the BAV consortium and 1849 controls from the Framingham GWAS study. GATA6 expression was determined in mice and human tissues using qRT-PCR and immunohistochemistry. Mechanistic studies were carried out in cultured cells.

Results—*Gata6* heterozygous mice have highly penetrant RL type BAV, the most frequent type in human. GATA6 transcript levels are lower in human BAV as compared to normal tricuspid valves. Mechanistically, *Gata6* haploinsufficiency disrupts valve remodeling and extracellular matrix composition through dysregulation of important signaling molecules including matrix metalloproteinase 9. Cell-specific inactivation of *Gata6* reveals an essential role for GATA6 in secondary heart field myocytes as loss of one *Gata6* allele from *Isl-1* positive cells, but not from endothelial or neural crest cells-recapitulates the phenotype of *Gata6* heterozygous mice.

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Disclosures

None

Conclusion—The data identify a new cellular and molecular mechanism underlying BAV. The availability of an animal model for the most frequent human BAV opens the way for the elucidation of BAV pathogenesis and the development of much needed therapies.

Keywords

Bicuspid Aortic Valve; GATA proteins; Extracellular Matrix; MMP9; GWAS

Introduction

Aortic valve disease (AVD) is a major cause of morbidity and mortality worldwide and represents a significant health and socio-economic burden. From 2000 to 2012, hospitalization for AVD increased by 59% and in 2015, the cost to the healthcare system was estimated at $3.207 billion in the USA alone[1]. Despite intense efforts, the etiology and pathophysiology of AVD remain incompletely elucidated which impedes to the development of effective preventive and therapeutic regimens. At present, surgical approaches including valve replacement, the second leading cardiac surgery in North America, are the only effective treatment options. Cardiovascular diseases such as atherosclerosis and hypertension as well as congenital malformations like bicuspid aortic valve (BAV) and Marfan Syndrome are risk factors for premature valve deterioration and aortopathy[2]. BAV is the most common heart defect affecting 1–2% of the population with a higher male prevalence. It features the presence of two usually-asymmetric instead of the normal three symmetrical leaflets. Individuals with BAV are at increased risk of valve deterioration and account for the majority of valve replacements particularly in patients under 65 years of age. They are also at increased risk of aortic dilatation and dissection and many develop serious cardiovascular complications 10 years earlier than individuals with a tricuspid aortic valve (TAV)[2,3]. As such, BAV has the greatest health burden of all other congenital heart diseases. The molecular mechanisms underlying the etiology and pathophysiology of BAV and BAV related valvulo-aortopathy remain largely undefined.

BAV is an autosomal dominant trait with variable expressivity and incomplete penetrance suggestive of genetic and environmental modifiers. Human genetic studies have provided evidence for linkage of 3 loci, 18q, 5q and 13q[4] with BAV with *MATRIN3* as the candidate 5q gene[5]. Mutations in *NOTCH1* and in *GATA5* –a regulator of the Notch pathway, have also been identified in some BAV patients[6,7]. Loss of function mutations in genetically engineered mice confirmed a causal relationship between the *Notch1* and *Gata5* genes and BAV[8]. However, mutations in these genes account for only a small percentage of human BAV and the genetic basis for the majority of BAVs remains undetermined.

Additionally, we lack mechanistic understanding of BAV pathophysiology. For example, it is unclear why BAV is associated with accelerated valve calcification and deterioration, or with aorthopathy. Why different BAV subtypes are associated with different clinical outcomes supports the genetic hypothesis. In human, BAVs are classified according to which leaflets – right (R), left (L), and non-coronary (N), are fused together. The RL type (right and left leaflet fusion), is the most frequent in human, accounting for 59% of BAV whereas the RN type accounts for 37%[9]. Retrospective analysis suggests that BAV morphology is of

prognostic relevance with RN BAV being associated with a greater degree of valve dysfunction and a shorter time to valve intervention[10,11]. BAV morphology also results in different types of aortopathy and distinct hemodynamics across the ascending aorta[12]. Elucidating the molecular mechanisms underlying BAV associated aortopathy has been hampered by the paucity of BAV animal models. *Gata5* null mice provides a model for RN type BAV[8]. In the case of RL-BAV, an inbred line of Syrian hamsters for which the genetic basis remains unknown is available[13]. Both RL and RN type BAVs were reported in mice lacking *Jag1* in cardiac cells or *Notch1* in endothelial cells; these mice have additional cardiac defects and compromised postnatal survival[14]. We now report that mice heterozygous for a mutated *Gata6* allele have highly penetrant RL-type BAV. Mechanistically, defective valve remodeling due to dysregulated extracellular matrix (ECM) degradation and decreased cell death are the underlying cause of BAV formation. In human, we found that GATA6 transcripts and protein levels are lower in the valves and aorta of individuals with BAV as compared to those with TAV and that three *GATA6* gene variants associate with BAV in a cohort of European ancestry. Together, the data suggests that *GATA6* may be a novel BAV causing gene. The study also provides a well-defined animal model of the most frequent type of BAV in human, opening the way for molecular dissection of BAV associated aorthopathy.

Methods

The data, analytic methods, and study materials will be available to other researchers for purposes of reproducing the results or replicating the procedure upon request.

Animals

Mouse handling and experimentation were performed in accordance with institutional guidelines. Protocols were approved by the institutional Animal Care committee. *Gata6* heterozygous (*Gata6*$^{+/-}$, C57/B6) mice were previously described[15]. Cell-specific knockouts were generated by crossing *Gata6* floxed mice with mice harboring Cre recombinase. The *Tie-cre* (C57/B6) expressing mouse line was previously described[8]. *Wnt1-cre* (129S4) and *Isl1-cre* lines (C57/B6) were obtained from Jackson laboratories (USA). Unless otherwise specified, mice were put on regular chow (Harlan 2018). For high fat diet experiment, mice were put on high fat/high carb chow for a period of 4 months (bioserv High Fat/High Carb Diet (F3282, 5kg box)).

Echocardiography

Transthoracic echocardiography was performed using a visual sonics Vevo 770 ultrasound system with a RMV 707 30-MHz transducer as previously described[16]. M-mode imaging was obtained from 150–220 days old mice (n=11–14 mice per group).

Histology

Mouse tissues were fixed with 4% paraformaldehyde in PBS, paraffin embedded, sectioned at 4-μm intervals, and processed. Masson trichrome, Alcian Blue and Movat Pentachrome stainings were performed by the histology facility at the University of Ottawa.

Immunohistochemistry and immunofluorescence

Immunohistochemical studies were performed as described previously[8,17]. The GATA6 antibody[18] was used at 1/1000 dilution. Goat polyclonal IgG GATA4 (C20) antibody was purchased from SANTA CRUZ (SC-1237X; dilution 1/600). The following antibodies were purchased from ABCAM: Semaphorin3C (ab 135842; dilution 1/350), SOX9 (ab3697, dilution 1/100), Periostin (POSTN) (ab14041, dilution 1/1000), alpha smooth muscle actin (ab5694, dilution 1/750), total and cleaved Versican (ab19345 and ab177480, dilution 1/250 each), and NCID (ab8925, dilution 1/500). The Anti-phospho-Histone H3 (Ser10) antibody was from MILLIPORE (06-570, dilution 1/750). The biotinylated anti-Goat IgG and anti-Rabbit antibodies were from Vector Laboratories (BA5000) and Jackson (Cederlane) (711-065-152) respectively. Streptavidin-HRP conjugate was from Perkin Elmer (NEL 750000 1EA). Immunofluorescence was carried out using Anti-HA (Santa Cruz, Santa Cruz, CA, USA, sc-805) and Alexa Fluor 546 Goat Anti-Rabbit IgG (Life Technologies, Carlsbad, CA, USA, A-11035) at a dilution of 1/500 respectively. Image acquisition was completed using the Zeiss AxioObserver D1 microscope (Oberkochen, Germany).

Cell count and TUNEL

Image J software was used to count the number of mesenchymal and endocardial cells in AV and OFT cushions in 3 different sections of 3–4 hearts per genotype. Terminal deoxynucleotidyl transferase-mediated dUTP end labeling (TUNEL) assays were carried out using Apoptag kit according to the manufacturer's instructions (Intergen, Purchase, NY, USA).

qRT-PCR

Total RNA was isolated from snap-frozen hearts with TRIzol reagent (Life technologies, 15596018) using FastPrep beads (MP-Bio, 6913-100); cDNAs were generated using the Omniscript RT kit (Qiagen, 205113). Oligonucleotide sequences are available on request.

Luciferase assay

Transfections were carried out as previously described[17]. Total amount of DNA was maintained constant by adding appropriate amounts of empty DNA vector. The BMP4-Luc and GATA6 constructs were previously described[17]. Full length MMP9-Luc reporter construct[19] was a kind gift from Prof. Wolfgang Eberhardt. Site-directed mutagenesis was used to generate mutant constructs which were verified by sequencing.

Western blot and Electrophoretic mobility shift assays

Nuclear and cytoplasmic extracts from AD293 cell line overexpressing GATA6 WT and mutant proteins were used. Anti-HA (Santa Cruz, Santa Cruz, CA, USA, sc-805), anti-GAPDH (ABCAM, ab8245) and anti-Nucleolin (Cell Signaling, D4C70, 14574) were used at a dilution of 1/2000. Secondary anti-mouse (Jackson, Cedarlane, 715-035-151) and anti-rabbit (Jackson, Cedarlane, 711-035-152) antibodies were used at 1/40000 dilution. DNA binding activity of GATA6 proteins was assessed using nuclear extracts and the proximal GATA site from the BMP4 and MMP9 promoters as described previously[18].

Human subjects

Written informed consent was received from participants prior to inclusion in all the studies. Microarray analysis: study was approved by the ethics committee of the “Institut Universitaire de Cardiologie et de Pneumologie de Québec” (IUCPQ). Human genetic study: The study was approved by the Partners HealthCare Human Research Committee. Human aorta staining: the study was approved by the University of Ottawa Heart Institute Research Ethics Board.

Microarray analysis

Gene expression was obtained from 12 aortic valves in each group and measured with the HumanHT-12 v4 Expression BeadChip. Bicuspid aortic valves (BAV) were obtained from male patients who underwent aortic valve replacement surgery. Normal tricuspid aortic valves (TAVn) were obtained from male patients who underwent heart transplantation. Gene expression differences between groups of valves were tested using t-test.

Human aortas staining

Patients from the University of Ottawa Heart Institute undergoing surgical intervention for aortic valve and/or aortic disease were included in this study. Aortic wall tissue specimens were obtained and fixed for 24 hours in 10% buffered formalin. Six sections were taken horizontally across the excised aortic segment and paraffin embedded; 5μm sections were prepared.

Human genetic studies

480 Caucasian BAV cases genotyped with the Omni2.5 chip were used yielding 2,379,855 genetic markers from BAV-Consortium database, and 2,477 Caucasian controls genotyped using the HumanOmni5.0 bead chip with 4,271,233 genetic markers from dbGaP (FHS*). Quality control (QC) of the genotype data from both cohorts was performed using Genome Studio and PLINK (Supplementary Figure 1). We considered markers with a MAF>1% and performed extensive principal components-based filtering for population stratification. After merging cases and controls and further QC, we used 452 BAV cases and 1,849 Caucasian controls with a common set of 1,355,128 single nucleotide polymorphisms (SNPs). An additive logistic regression model was performed for association analysis adjusted for gender and race using PLINK[20].

Statistics

For echocardiography and gene expression analysis, values are presented as means ± standard error of the mean (SEM). P values were generated using Student’s 2-tailed t test. For statistical analysis of phenotype-genotype association, Fisher Exact Test (2×2 contingency table) was used. For luciferase assay, statistical analysis was done by one-way ANOVA followed by Dunnett’s multiple comparison post-hoc analysis. In all cases, P values < 0.05 were considered as an index of statistical significance.

Results

Gata6 haploinsufficiency leads to RL-type BAV

Previously, we reported that transcription factor *Gata5* is involved in valvulogenesis and that its loss leads to BAV[8]. Genetic studies aimed at determining modifiers of *Gata5* revealed a strong interaction with *Gata6*. Whereas *Gata5* heterozygous mice have no detectable cardiac phenotype, *Gata5*$^{+/-}$ *Gata6*$^{+/-}$ compound heterozygous embryos have disrupted valvulogenesis and die perinatally due to severe defects in outflow track (OFT) formation[15].Similarly, *Gata4*$^{+/-}$ *Gata6*$^{+/-}$ mice die embryonically at E13.5 due to vascular and OFT defects[21]. Functional cardiac analysis of adult *Gata6* heterozygous mice using echocardiography revealed a significant decrease in fractional shortening (FS) vs their control littermates (Figure 1A). Additionally, large percentage of *Gata6*$^{+/-}$ mice had significantly elevated aortic valve gradient, with no changes in the aortic root diameter, suggestive of AVD (Figure 1B, 1C and 1D). Consistent with the presence of cardiac stress, qRT-PCR performed on adult ventricular tissues revealed increased levels of stress markers (ANF, HIF1alpha) and cardiac remodeling genes (FGFR1/3, CTGF), and decreased levels of sarcoplasmic calcium-ATPase (SERCA). GATA4 levels were unchanged (Figure 1E).

Analysis of cardiac structure revealed the presence of BAV in 56% of *Gata6*$^{+/-}$ males and in 27% of *Gata6*$^{+/-}$ females (32 out of 57 males and 7 out of 26 females) (Figure 1F). BAV frequency was assessed by the Fisher exact test for both male ($p<0.0001$) and female ($p=0.0043$) groups. Interestingly, all BAVs were of the RL-type, the most frequently occurring type in human (Figure 1F and1G). Thickening of aortic valve leaflets was also observed in *Gata6*$^{+/-}$ mice (Figures 1H and 2A, left panels a–c). This could be reflective of postnatal remodeling caused by vascular or myocardial disease. Defective valvulogenesis could also be the underlying cause of the valve phenotype. Masson Trichrome staining of newborn *Gata6*$^{+/-}$ mouse heart sections (P0) showed significant aortic valve thickening in 7 out of 11 *Gata6*$^{+/-}$ mice but not in their *Gata6*$^{+/+}$ littermates (n=6) (Figure1I, left panels a–c). No thickening was observed in the mitral or tricuspid valves (data not shown) suggesting defective OFT but normal AV cushion formation. Movat pentachrome staining revealed thickened valves of unequal size with an increase in ECM deposition in *Gata6*$^{+/-}$ (blue color) when compared to controls. Increased collagen fiber content (yellow color) was also evident especially in the cushion-like aortic valves (Figure 1I, right panels d–f). These results suggest the presence of abnormal ECM content in the aortic valves of *Gata6*$^{+/-}$ mice. Similarly, Alcian blue staining of adult *Gata6*$^{+/-}$ sections revealed greater glycosaminoglycan content in the valves of *Gata6*$^{+/-}$ mice, indicative of abnormal matrix composition (Figure 2A, right panels d–f).

BAV is a risk factor for premature AVD including valve calcification and sclerosis (presence of accelerated fibrosis). To determine whether *Gata6*$^{+/-}$ are prone to AVD, we analyzed adult Ao valves using Masson trichrome staining which revealed increased total collagen content in some *Gata6*$^{+/-}$ BAV and TAV, indicative of valve sclerosis (Figure 2B). We also analyzed the levels of several transcripts in dissected aortic valves. As shown in Figure 2C, significant gene expression changes were noted in *Gata6*$^{+/-}$ valves including decreased levels of TIMP1 (Tissue inhibitor of Metalloproteinase 1), FBN1 (Fibrillin 1), and VCAN (Versican). These

changes are consistent with altered ECM integrity and valve elasticity. Reduced level of SOX9 transcripts in *Gata6*$^{+/-}$ valves is also noteworthy since decreased expression of SOX9 promotes heart valve calcification[22] (Figure 2D, panels a–b). Similarly, decreased levels of periostin (POSTN) causes de-repression of the osteogenic potential of the mesenchymal cells within the OFT and calcium deposition within the aortic valve[23]. Immunohistochemistry showed that expression of POSTN is downregulated in *Gata6*$^{+/-}$ valves compared to wildtype littermates (Figure 2E, panels a–c). Interestingly, levels of SOX9 and periostin were differentially regulated in mice fed a normal or a high fat diet (HFD). In *Gata6*$^{+/+}$ valves, SOX9 levels were increased in mice on the HFD whereas POSTN levels were decreased. In contrast, POSTN levels were upregulated in *Gata6*$^{+/-}$ mice on HFD and SOX9 levels remained unchanged (Figure 2D and 2E, lower panels). Thus, *Gata6* haploinsufficiency promotes a pro-osteogenic state in the aortic valves and disrupts genetic reprogramming in response to pro-osteogenic stimuli.

Abnormal ECM and valve remodeling underlie *GATA6*-dependent BAV

Defects in Epithelial to Mesenchymal transformation (EMT)-a critical stage during valvulogenesis, can lead to BAV[24]. To determine whether cell number and composition of the OFT cushions are changed in *Gata6*$^{+/-}$, we performed Alcian blue staining and cell counts on E11-11.5 cushions. Cell number was similar in *Gata6*$^{+/-}$ and *Gata6*$^{+/+}$ mice (Supplementary Figure 1A and C) and staining for Phosphohistone H3 (PHH3) revealed no changes in proliferation, neither in OFT nor in AV cushions (Supplementary Figure 1D). Smooth muscle alpha actin staining was also similar in both groups suggesting normal differentiation (Supplementary Figure 1B). Next, cardiac neural crest cell (CNCC) dysregulation was examined. qRT-PCR revealed that expressions of SEMA3C, a secreted class 3 semaphorin present in and adjacent to migrating CNCC, and PLXNA2 (Plexin A2), a semaphorin receptor expressed on CNCC-[25] were similar in *Gata6*$^{+/-}$ and *Gata6*$^{+/+}$ littermates (Supplementary Figure 1E, right panel). Immunohistochemistry revealed a similar SEMA3C expression pattern at the level of the cells surrounding the branchial arch arteries and in the left lateral wall of the conus in E11-11.5 from both genotypes (Supplementary Figure 1E, left panels). These results suggest that the defect observed in *Gata6*$^{+/-}$ is not due to defective CNCC migration to the OFT cushions. Thus, the formation of BAV in *Gata6*$^{+/-}$ does not appear to be caused by defective cell proliferation, migration or differentiation processes.

Abnormal septation and valve thickening could be the result of either excessive proliferation in the valves or defective remodeling which involves cell apoptosis. Valve remodeling was examined in transverse sections of E14.5 embryos using TUNEL or PHH3 staining. As shown in Figure 3A and B, cell death was significantly lower in *Gata6*$^{+/-}$ valves whereas cell proliferation was modestly increased. This result suggests abnormal apoptosis and valve remodeling in *Gata6*$^{+/-}$ mice which could result from an abnormal synthesis and/or breakdown of the ECM, a regulator of cell survival, migration and proliferation[26]. Matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of MMPs (TIMPs), play an important role in the ECM degradation process. Expression of mRNA levels of MMP9, TIMP1, MMP2 and TIMP2 was assessed in *Gata6*$^{+/-}$ E11.5 hearts. At this stage, a significant decrease in MMP9 levels along with a trend of decrease in MMP2 was

observed (Figure 3D), which could lead to dysregulated ECM degradation. Consistent with this, immunohistochemistry revealed lower levels of cleaved versican in E14.5 *Gata6*$^{+/-}$ valves (Figure 3C). Expression of genes known to be involved in OFT formation and valve remodeling was also examined. BMP4, a direct target for GATA6, whose deletion from the anterior heart field (AHF) alters OFT septation[18,27], was significantly decreased (48%) in *Gata6*$^{+/-}$ hearts (Figure 3D).

We tested whether MMP9 is transcriptionally regulated by GATA6. In silico analysis of the MMP9 promoter revealed the presence of conserved GATA binding sites (Figure 3E). Co-transfection in NIH3T3 cells of GATA6 expressing vector and MMP9 luciferase constructs showed significant GATA6 activation of the MMP9 promoter that is dependent on the presence of the proximal GATA site (Figure 3E and 4B). Mutations in human *GATA6* have been reported in congenital heart disease associated with OFT defects (Figure 4A and supplementary Table 1)[28]. The 2nd zinc finger (ZnF) in GATA6 mediates most protein-protein interactions as well as binding to DNA and is the site of many human mutations. We examined the effect of several GATA6 mutant proteins on transcriptional activation. All mutants showed reduced transcriptional activation of MMP9 and BMP4 promoters (Figure 4B and data not shown). Interestingly, most mutations (except for T452A) showed reduced nuclear accumulation and increased cytoplasmic localization (Supplementary Figure 2A and B). Additionally, all GATA6 mutants tested were unable to bind GATA elements (Supplementary Figure 2C). The results suggest that functional *GATA6* haploinsufficiency resulting from these mutations may cause human CHD.

Loss of one *Gata6* allele in Isl1+ cells recapitulates the aortic valve phenotype of *Gata6* heterozygous mice

Endothelial, neural crest and second heart field myocytes contribute to OFT formation. Endothelial cells within and lining the interior of the OFT vessels undergo EMT, giving rise to the endocardial cushions[29]. Cardiac neural crest cells (CNCC) that arise from the dorsal neural tube and migrate as mesenchymal cells to populate the OFT, are known to contribute to the formation of the endocardial cushion and the septum separating the aortic and pulmonary trunks[30]. Recent studies have suggested that secondary heart field (SHF) precursors lying in the ventral pharynx are able to interact with CNCC migrating to the OFT cushion; together they control ECM development and apoptosis during valve remodeling[31]. Later on, invading CNCC merge with the endocardial cushions cells and SHF mesenchymal cells to ensure the elongation and proper septation of the OFT. Since GATA6 is present in all these lineages[18,32,33], we used mouse genetics to test which cell types are responsible for BAV formation. *Gata6* was deleted from endothelial, neural crest and SHF cells by crossing *Gata6*$^{Fl/Fl}$ mice with *Tie2cre, Wnt1cre* and *Isl1cre* mice respectively. No BAVs were found in mice with a deleted *Gata6* allele in endothelial (*Tie2cre*+ *G6*$^{Wt/Fl}$) or neural crest (*Wnt1cre*+ *G6*$^{Wt/Fl}$) cells. However, removal of one copy of *Gata6* from *Isl1*+ cells resulted in BAV in 44% of *Isl1cre*+ *G6*$^{Wt/Fl}$ mice and recapitulated the valve phenotype of *Gata6*$^{+/-}$ mice (Figure 5A, B and C). The BAV frequency in *Isl1cre*+ *G6*$^{Wt/Fl}$ mice was significant as assessed by the Fisher Exact Test (p=0.0021). Thus, GATA6 appears to be essential in SHF cells for proper aortic valve formation.

GATA6 expression and variants in Human BAV

We investigated whether variants within the *GATA6* gene associate with human BAV using 452 sporadic BAV cases and 1849 controls. After quality control (detailed in supplementary Figure 3), an additive logistic regression model revealed nominal association of several variants (Figures 6A and B and supplementary Table 2 and 3). However, none reached the locus wide significance (0.00014). We also analyzed GATA6 expression in human tissues of individuals with BAV and TAV. In human aortic valve tissues, GATA6 transcripts were significantly lower in the BAV vs TAV specimens (Figure 6C and supplementary Table 4). Similarly, GATA6 immunoreactivity was lower in aortic tissue sections from BAV vs TAV patients who underwent aortic repair surgery (Figure 6D and supplementary Table 5)). Interestingly, this decrease was more pronounced in tissues from patients with RL vs RN type BAV. Immunostaining for activated Notch intracellular domain (NCID) and smooth muscle actin were used as controls. Together the data suggests a potential role for GATA6 in human aortic valve disease.

Discussion

Bicuspid aortic valve is the most common congenital heart defect in human and a risk factor for aortic valve disease. The genetic basis of BAV formation and aortopathy in the majority of individuals remains unknown. The data presented identify *GATA6* as a potential BAV causing gene and offers a unique animal model to study the pathogenesis of the most frequent type of human BAV.

GATA6 regulation of aortic valve formation

BAV is a genetically heterogeneous defect. Mutations in *GATA5* and *NOTCH1* have been reported in some BAV patients. In addition, 5 distinct loci on human chromosomes 5q, 9q, 13q, 17q and 18q, have been associated with BAV in families[4,34,35]. *MATR3*, the gene encoding MATRIN3, is located on 5q; it is reportedly linked to human BAV and its deletion in mice leads to partially penetrant BAV[5]. The identity of the causative genes within the other linked loci remains undetermined. The *GATA6* gene is located on 18q11.2 and mutations in *GATA6* as well as a microdeletion including *GATA6* have been reported in patients with congenital heart disease[36,37]. The present study shows that GATA6 expression is decreased in the valves and aortas of individuals with a BAV vs TAV. In the present study, we did not find any common (MAF>1%) variants at the *GATA6* locus associated with BAV, after accounting for multiple testing. Further studies using whole exome sequencing or targeted gene sequencing would be required to determine if there are rare mutations in the *GATA6* gene, associated with BAV.

GATA6 belongs to the GATA family of zinc finger transcription factors and is predominantly expressed in the heart and gut[18]. Its role within the heart is not fully elucidated and often overlaps with that of another GATA protein, GATA4. *Gata6* null mice are embryonic lethal at implantation but cell-specific loss of *Gata6* from myocytes, smooth muscle, or neural crest cells affects cell proliferation and hypertrophic growth and is associated with structural cardiac defects[38,39]. No gross defects were reported in *Gata6* heterozygote mice. The data presented unravels a new role for GATA6 in aortic valve formation and points to its essential

function in second heart field (SHF)-derived cells therein. Loss of one *Gata6* allele from *Isl-1*+ cells recapitulates the highly penetrant BAV and thickened valve phenotypes seen in *Gata6* heterozygote mice. Both phenotypes reflect remodeling defects caused, at least in part, by changes in ECM degradation and dysregulated cellular apoptosis. These can result from decreased levels of GATA6 regulated genes such as BMP4 and MMP9. BMP4 is a known GATA6 target[18] and this study identifies MMP9 as a new GATA6 downstream target.

Regulation of ECM plays an important role in normal and pathogenic development and is a key feature of many diseases such as congenital heart disease, cancer and inflammatory disorders. Interestingly, in colorectal cancers, GATA6 expression correlates with increased invasion and metastasis while in vitro gain and loss of function indicate that GATA6 levels regulate cell migration and invasion[40]. Human *GATA6* mutations have been reported in a wide spectrum of CHD (ASD, VSD, PDA, PTA, TOF) all of which implicate defective ECM due to alteration in expression of several metalloproteinases. For example, increased MMP2 and MMP9 activities were linked with the pathogenesis of VSD in 96 children with peri-membranous VSD[41]. Similarly, loss of metalloproteinase Tolloid –like (TTL-1) leads to incomplete formation of the interventricular septum in *Tll-1* knockout mice. An insertion mutation in the exon 10 of TLL-1 was found in patients with ASD, VSD and PDA[42]. Another metalloproteinase, CCN1, plays a role in heart development as *Ccn1*-null mice have impaired cardiac valvulo-septal morphogenesis resulting in severe atrioventricular septal defect (AVSD); impaired gelatinase activity and apoptosis may underlie the phenotype[43]. It is now well established that matrix metalloproteases regulate cell survival, proliferation and differentiation as well as cell adhesion and migration. More specifically, it is reported that MMP9 can lead to increased apoptosis during development. In fact *Mmp9* null mice display a delay in hypertrophic chondrocyte apoptosis in addition to delayed vascularization and ossification[44]. Together with our findings, this raises the intriguing possibility for a broader role for GATA6 as ECM regulator in development and disease.

Development of an animal model of RL-type BAV

BAV is a major risk for premature onset of potentially fatal aortic disease. The heterogeneity of BAV and associated aortopathy combined with the paucity of corresponding animal models constitute a formidable challenge for the development of predictive tools for patient management. Identifying patients who could benefit from prophylactic reparative surgery or other interventions is presently an unachieved goal. Evidence is mounting that BAV aortopathy is not similar to that of genetic connective tissue disorders, like Marfan Syndrome, yet the clinical guidelines for BAV are extrapolated from those of Marfan Syndrome in which the molecular mechanisms of disease are well delineated[45]. Recent reviews of knowledge gaps have emphasized the critical need of deciphering the molecular pathology of BAV in order to identify markers of complications and targets for therapies[2]. As mentioned earlier, very few mouse models of BAV have been reported and in most cases, the BAV orientation is exclusively RN (*Gata5*$^{-/-}$ and *Nos3*$^{-/-}$ mice)[8,46]. Loss of the Notch ligand *Jag1* from cardiac cells results in a polyvalvular phenotype with 47% BAV of both RN and RL orientations; these mice have VSD and significantly reduced late gestational/perinatal viability[14]. The development of an animal model of RL type BAV, the most common type in human, represents a step forward towards understanding the etiology and

pathophysiology of BAV including the role of genetics in BAV associated aortopathy. Among others, the presence of BAV in only 50% of *Gata6*$^{+/-}$ mice will make it possible to address the unanswered question of the contribution of genetics vs hemodynamics to BAV aortopathy and ultimately to developing much needed biomarkers. Indeed, the influence of cusp orientation on the 3D flow patterns across the ascending aorta remains controversial[12] and genetically controlled studies in human patients have proved to be challenging. Similarly, retrospective analyses have associated valve configuration with distinct aortopathy risks and long term outcome of BAV repair[47]. However, the lack of molecular knowledge prevents development of much needed predictive tools.

In human, BAV is twice as common in male as in female but the reason for this gender bias is unclear. Remarkably, the prevalence of BAV is greater in male *Gata6*$^{+/-}$ mice vs female. BAV presentation is also heterogeneous in human even within each subtype (RL, RN, LN). Variations include presence/ absence of raphe and leaflet thickness and size. This heterogeneity is also observed in the *Gata6*$^{+/-}$ mice, with variable valve thickness and functionality. The availability of a mouse model that faithfully recapitulates features of the human disease will help unravel the genetic and environmental modifiers of BAV and BAV aortopathy. For example, the work presented indicates profound gene expression changes in BAV as well as TAV from *Gata6*$^{+/-}$ mice (Figure 2). In both cases, we observed increased evidence of sclerosis as well as increased markers of the pro-osteogenic program. In addition, gene expression changes in response to a pro-inflammatory/pro-calcific stimulus (HFD) were disrupted in *Gata6*$^{+/-}$ mice irrespective of the valve orientation. Together, these observations suggest that genetics may play a critical role in progression of aortic valve disease. The availability of mice with bicuspid or tricuspid aortic valves on the same genetic background opens the way for molecular dissection of the etiology and pathophysiology of aortic valve disease which will ultimately result in the identification of biomarkers and therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical perspective

1- What is new?

- Bicuspid Aortic Valve (BAV) is a major risk factor for premature aortic valve disease but its etiology and pathophysiology remain largely elusive.
- Our data suggest that haploinsufficiency of transcription factor GATA6 leads to BAV.
- Our study characterizes a mouse model (*Gata6*$^{+/-}$) for the most frequent type of BAV in human opening the way for pathophysiological investigations.
- Our results indicate that abnormal differentiation of Secondary heart field myocytes lead to BAV.

2- What are the clinical implications?

- The identification of a potential new gene associated with BAV is relevant for the elucidation of the genetic basis of human aortic valve disease.
- Studies in *Gata6* heterozygote mice suggest a role for genetics in valve deterioration and offer unique tools for the study of BAV-related aortopathy.
- Insight into the cellular and molecular basis of normal and pathological aortic valve development may lead to preventative and therapeutic approaches to valve degeneration.
- The work has implications for the diagnosis and follow up of individuals with aortic valve disease.

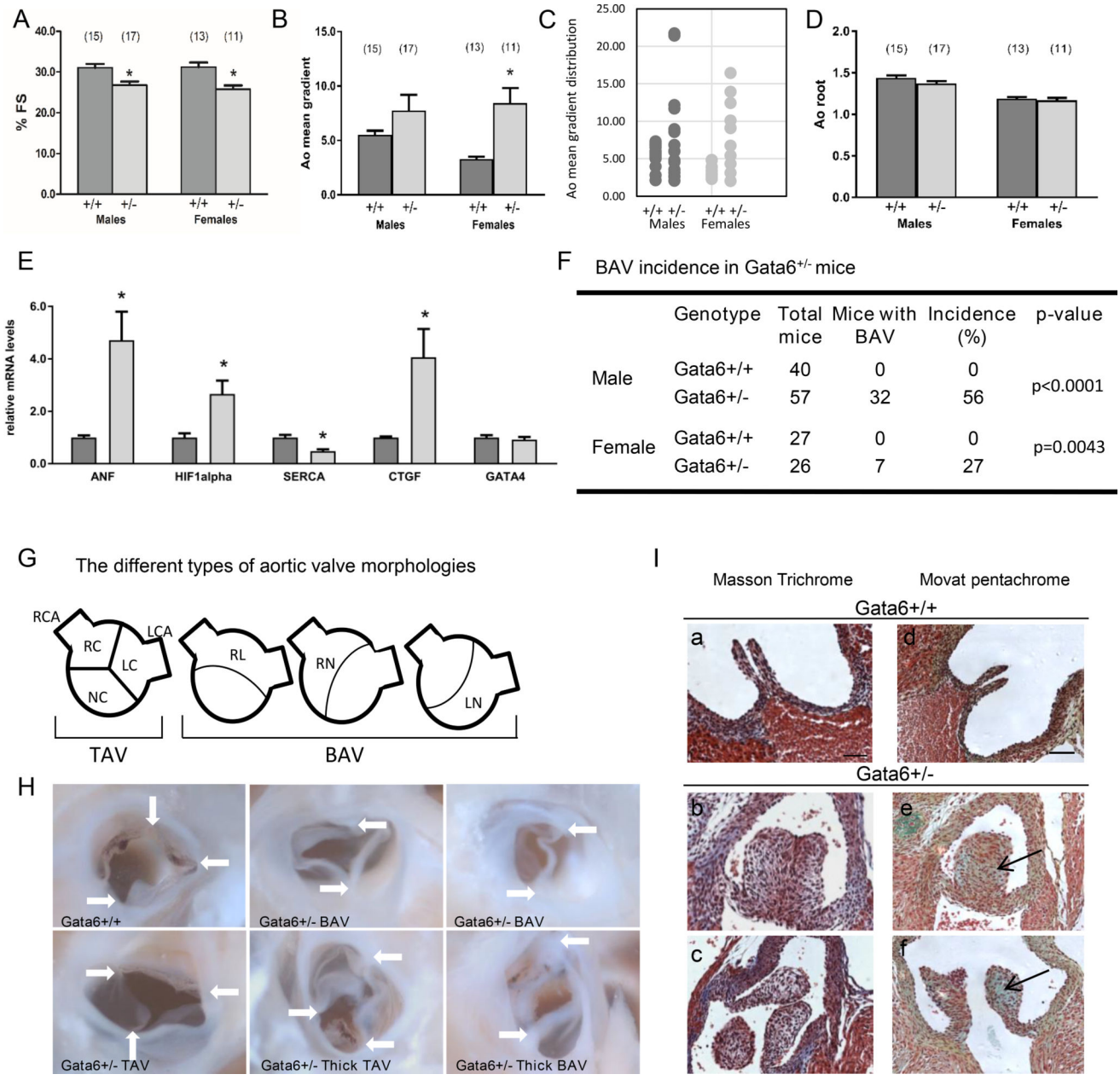


Figure 1. Cardiac dysfunction in *Gata6*$^{+/-}$ mice

Echocardiography analysis of *Gata6*$^{+/+}$ and *Gata6*$^{+/-}$ mice at 150–220 days of age (n = 15–17 per male group and n=11–13 per female group) showing **A)** decreased Fractional shortening (FS) in *Gata6*$^{+/-}$ groups, **B)** and **C)** increased Aortic mean gradient (AoMG), **D)** no changes in Aortic root area when compared to control littermates. Two groups are shown in **A–D**: group1: *Gata6*+/+, group 2: *Gata6*+/−. B) t-test shows significant difference between female *Gata6*+/+ and *Gata6*+/− (p<0.05). **C)** The scattered plot shows the distribution of aortic mean gradient in both groups, with normal (2–7) and above normal (>7) aortic mean gradient shown in group 2. Fisher test was performed to assess the prevalence of elevated Ao gradient: Males: *Gata6*$^{+/-}$ 6 out of 17 (p=0.01918) and Females: *Gata6*$^{+/-}$ 5 out of 11 (p=0.01087). **(E)** Enhanced expression of stress and remodeling

markers in *Gata6*$^{+/-}$ ventricles as revealed by qRT-PCR of same mice (n = 5–8 per group) (corrected to RPS16). **(F)** BAV incidence in *Gata6*$^{+/-}$ mice. **(G)** The different types of aortic valve morphologies. TAV: Tricuspid Aortic Valve. BAV: Bicuspid Aortic Valve. RL: Right-left. RN: Right-noncoronary. LN: Left-noncoronary. LCA: left coronary artery. RCA: right coronary artery. **(H)** Anatomical analysis of *Gata6*$^{+/-}$ mice revealing the presence of TAV and BAV with multiple presentations (thick and thin valves). **(I)** Trichrome staining of P0 valves from frontal heart sections showing thick aortic valves in *Gata6*$^{+/-}$ when compared to *Gata6*$^{+/+}$ littermates. Movat pentachrome staining showing abnormal ECM in P0 *Gata6*$^{+/-}$ Ao valves marked by increased blue staining within the leaflets of the Ao valves. Scale bar: 400μm. Values are mean + SEM. *P < 0.05.

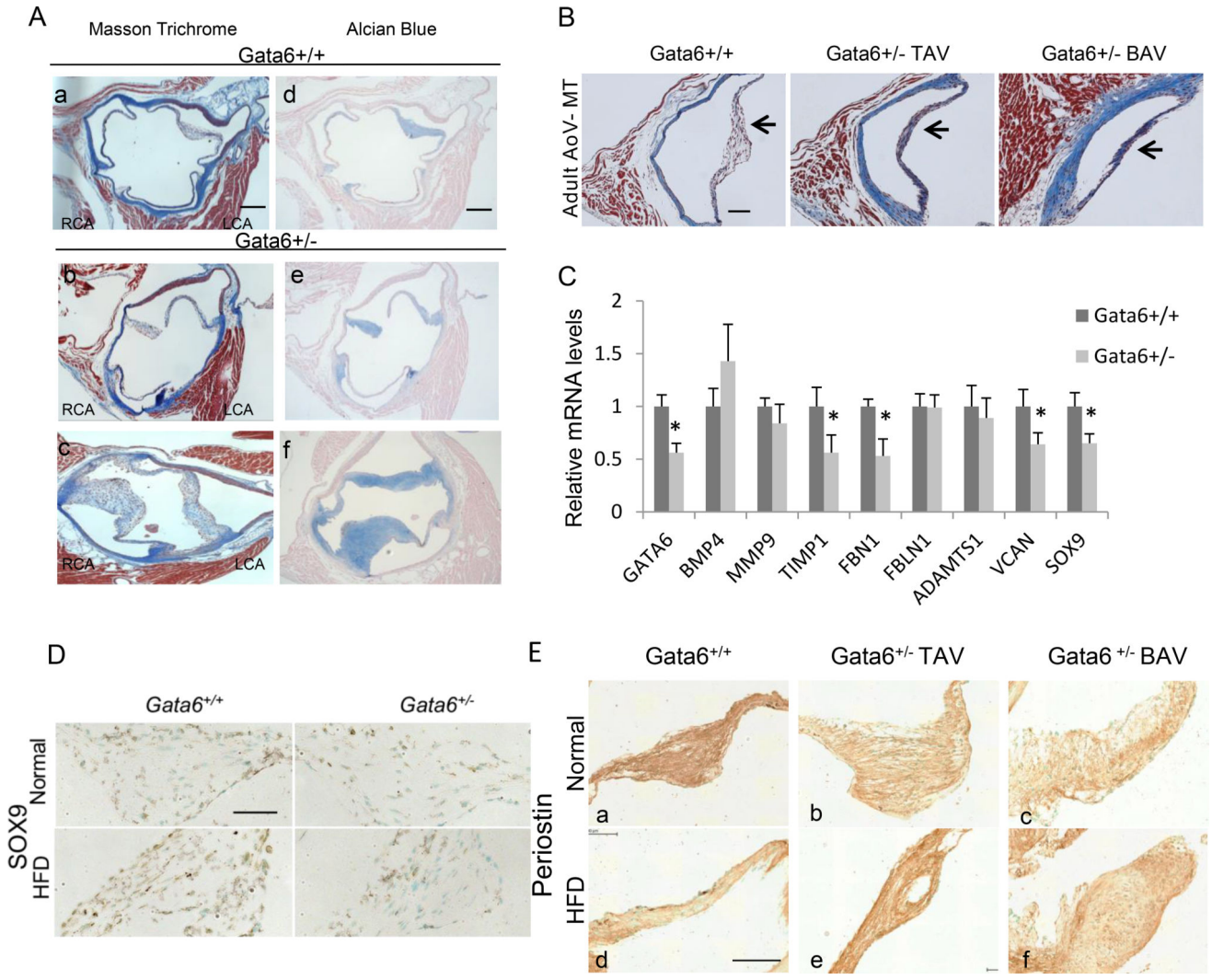


Figure 2. Analysis of valve structure and gene expression

(A) Masson Trichrome staining of transverse sections of adult aortic valve showing the presence of 2 instead of 3 aortic leaflets in *Gata6*$^{+/-}$ mice along with the presence of the RL-type BAV (left panel). Alcian blue staining of adult aortic valves transverse sections showing abnormal glycosaminoglycan composition (increased blue staining) within the aortic leaflets of *Gata6*$^{+/-}$ when compared to control mice (right panel). Scale bars: 200 μm. **(B)** Masson Trichrome stain showing total collagen (blue) content in bicuspid and tricuspid leaflets of *Gata6*$^{+/-}$ compared to their wildtype littermates. Scale bar: 100 μm. **(C)** qRT-PCR on RNA extracted from dissected adult aortic valves showing altered expression of important ECM components. (corrected to RPS16). Values are mean + SEM. **P* < 0.05. **(D and E)** Sox9 and Periostin staining of adult Ao valves from mice on normal diet and from mice that were fed with a high fat diet for 4 months. HFD: high fat diet. (n=4–7 in each group) Scale bar: **(D)** 50μm, **(E)** 100μm.

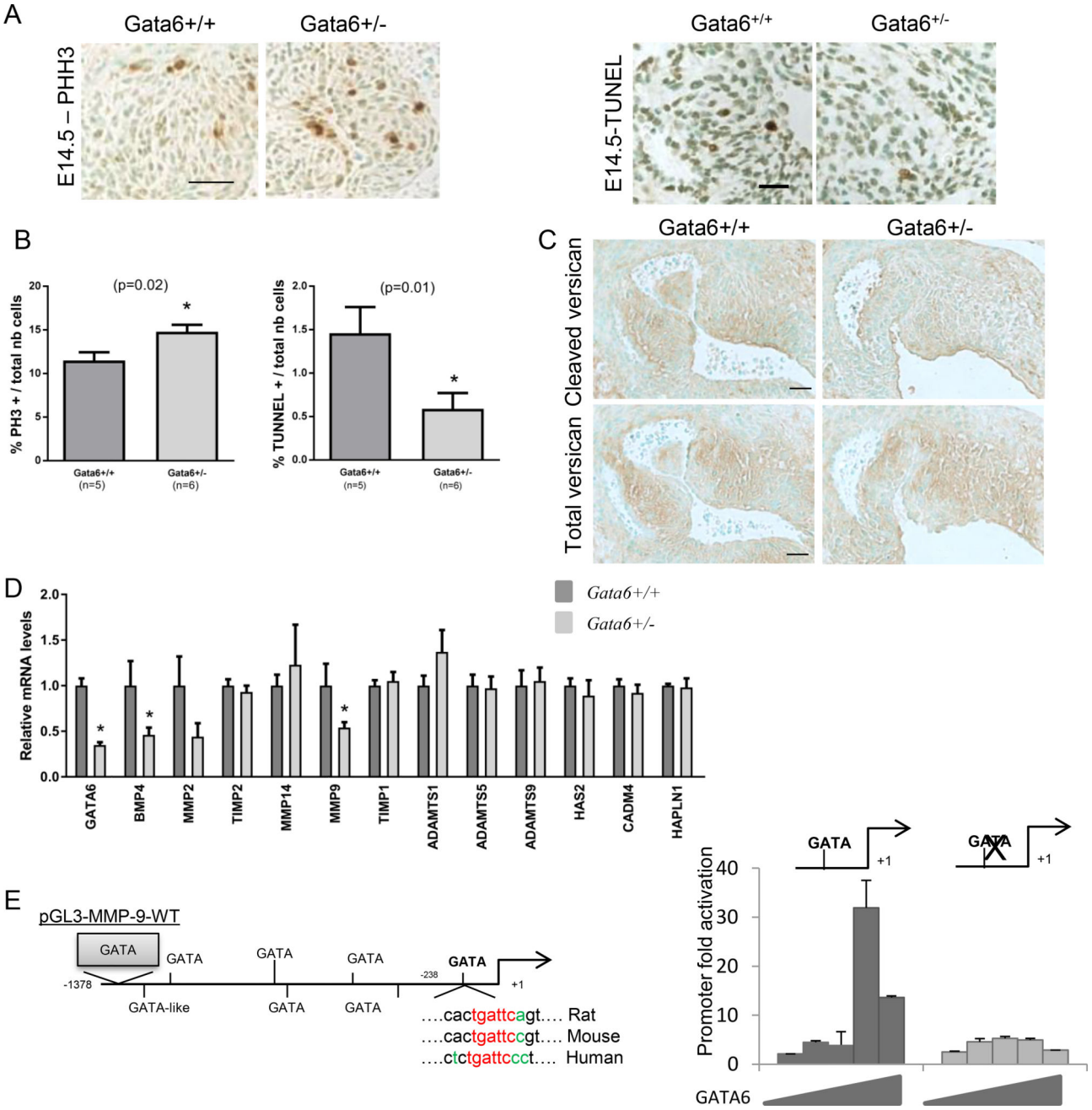


Figure 3. Dysregulation of matrix remodeling in *Gata6*$^{+/-}$ mice

(A) Representative images of Phosphohistone H3 and TUNEL positive sections of aortic valve from *Gata6*$^{+/-}$ E14.5 embryos (transverse sections). Scale bar: 40μm. **(B)** Quantification graphs of Phosphohistone H3 and TUNEL positive cells showing the increased cell proliferation and decreased cell death in aortic valves of *Gata6*$^{+/-}$ mice. **(C)** Staining for total and cleaved versican reveals decreased presence of cleaved versican (Neo-VCAN) within the ECM of Ao valves in *Gata6*$^{+/-}$ BAV mice when compared to control group. (n=5–6 in each group). Scale bar: 40μm. **(D)** qRT-PCR on RNA extracted from E11.5 hearts showing altered expression of important septation regulators and ECM components;

note the significant decrease in the expression of matrix metalloprotease 9 (MMP9), BMP4 and GATA6 (corrected to RPS16, n=5–8 per group). **(E)** Schematic representation of GATA sites on the MMP9 promoter. pGL3-MMP9-One GATA site is a 5' deletion leaving only one GATA site and p-GL3-MMP9-No GATA site promoter has a mutation in this site. Increasing amounts of GATA6 expression vector are transiently cotransfected with the indicated luciferase reporters in NIH3T3 cells (25, 50, 100, 250 and 500 ng of expression vector). Relative luciferase activities are represented as fold changes. The data is a representative of 3 independent experiments done in duplicates. Values are. $*P < 0.05$.

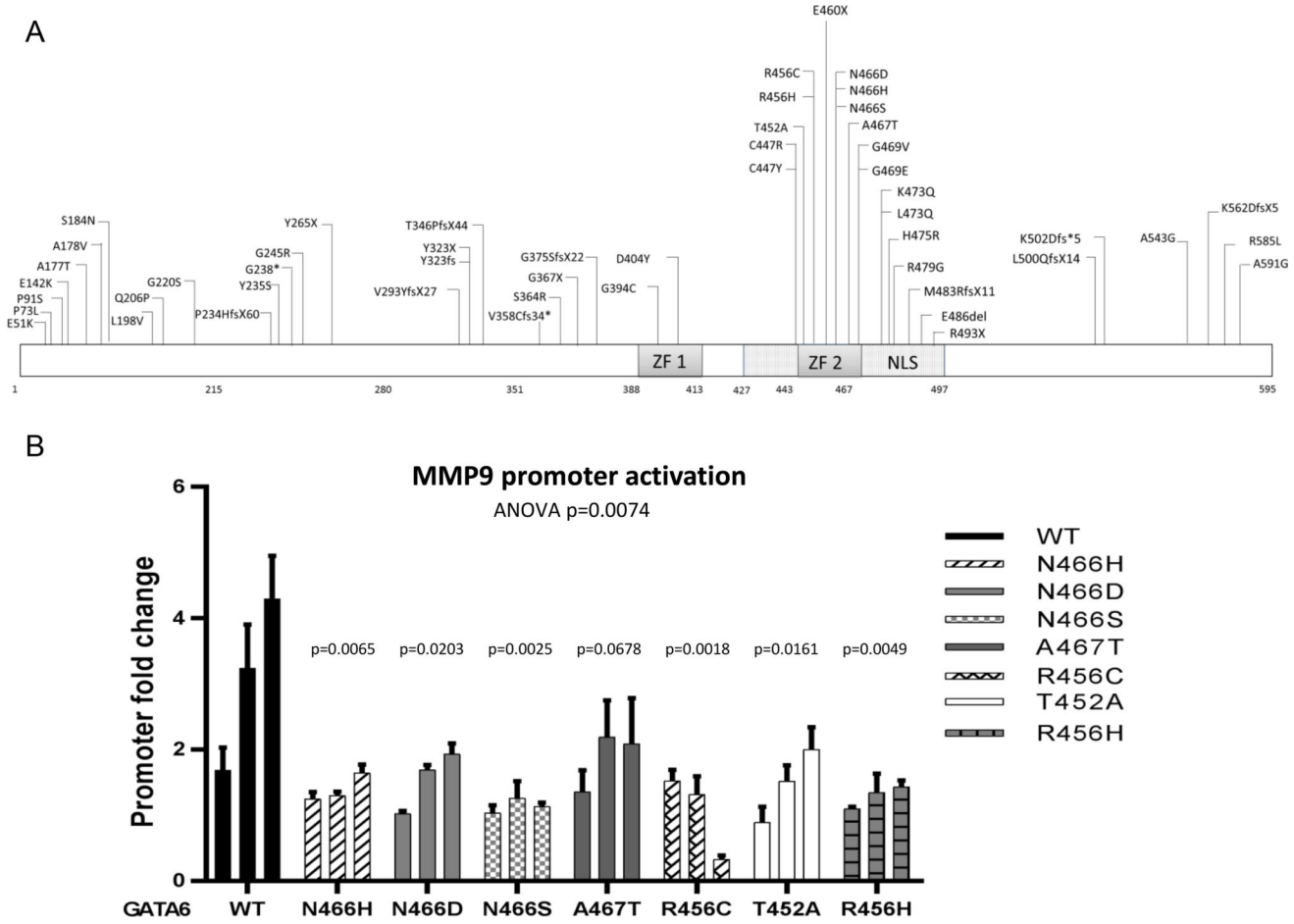


Figure 4. Effect of *GATA6* human mutations on transcriptional activity

(A) Schematic representation of reported human *GATA6* mutations. **(B)** Transcriptional activity of WT and mutant GATA6 proteins on full length MMP9 promoter. Transient cotransfection was carried out in NIH3T3 cells using 70, 125 and 500 ng of GATA6 expression vector. Relative luciferase activities are represented as fold changes vs empty vector control. The data are the mean ± SEM of 3 independent experiments done in duplicates. Note that all mutants had significantly lower activity vs WT GATA6. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison post-hoc analysis.

A

Mouse Genotype	Total mice	Mice with BAV	BAV incidence (%)	p-value
Gata6+/+	49	0	0	
Gata6+/-	83	39	47	p<0.0001
Cre-G6Wt/Fl	27	0	0	
Tie2cre+G6Wt/Fl	10	0	0	
Wnt1cre+G6Wt/Fl	13	0	0	
Isl1cre+G6Wt/Fl	9	4	44	p=0.0021

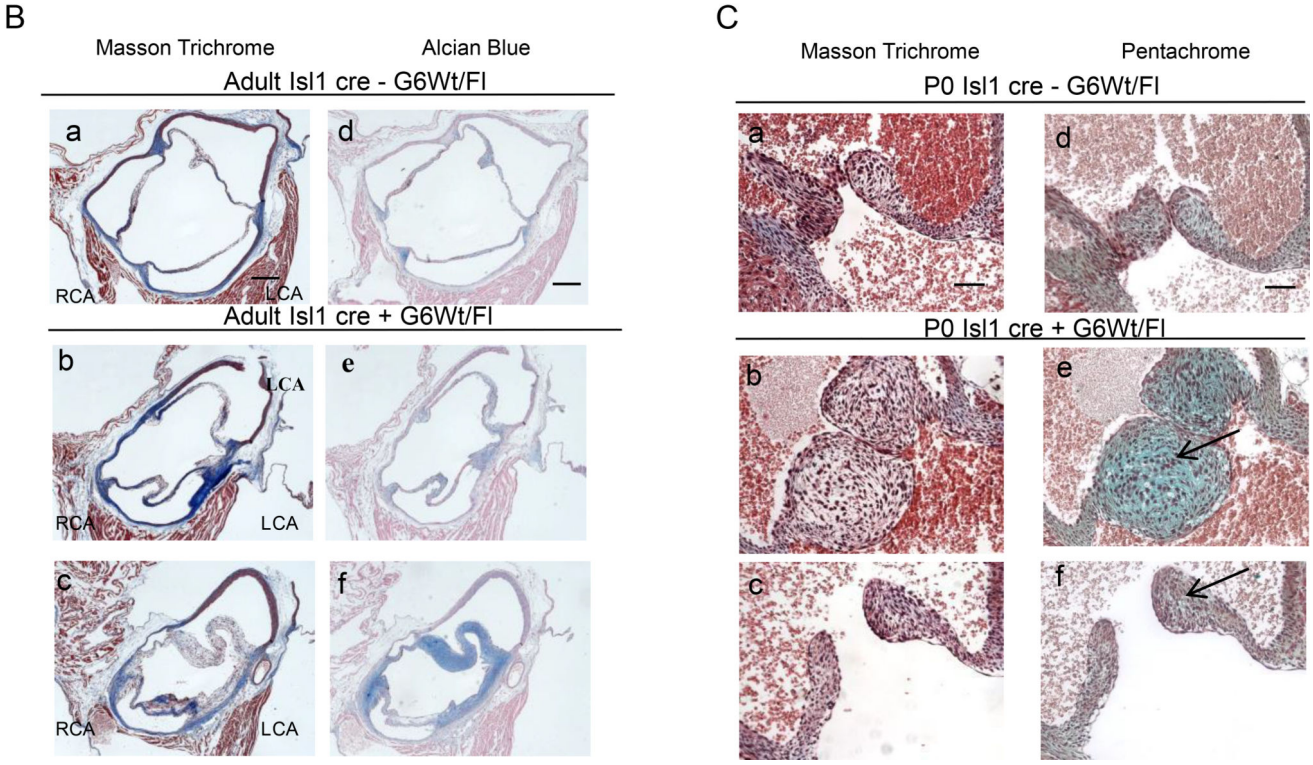
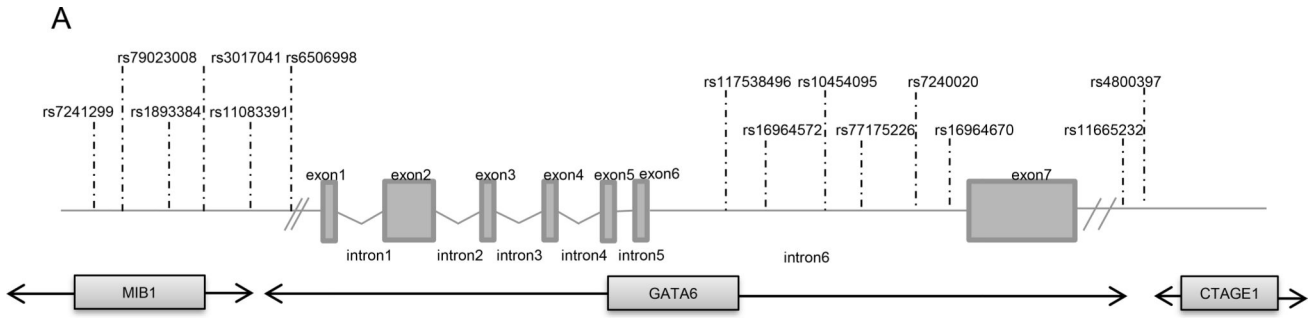


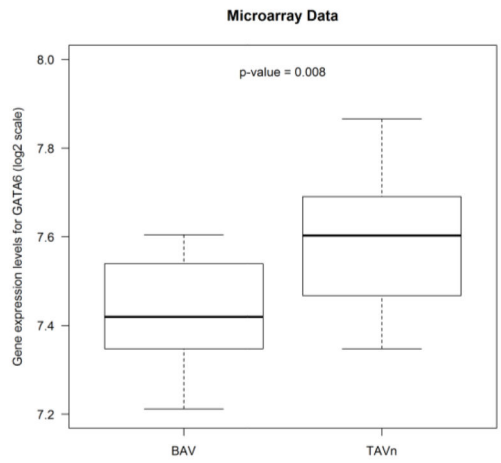
Figure 5. ***Isl1cre*$^{+}$ *G6*$^{Wt/Fl}$ mice recapitulate the phenotype of *Gata6*$^{+/-}$**

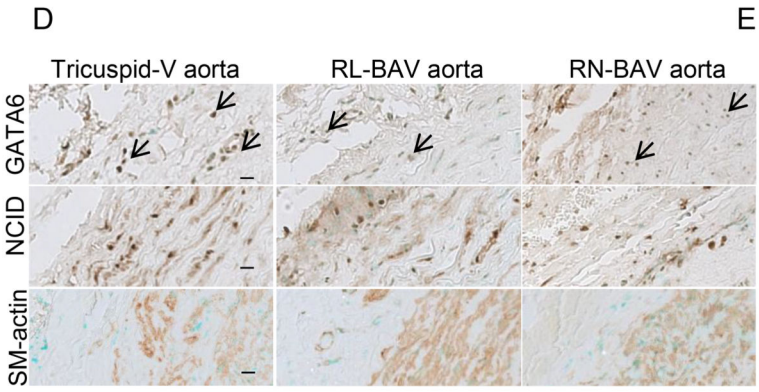
(A) Percentage of BAV in the different mouse lines. The incidence in *Gata6*$^{+/-}$ was compared to *Gata6*$^{+/+}$ littermates; *Isl1cre*+*Gata6*$^{Wt/Fl}$ was compared to *cre*−*Gata6*$^{Wt/Fl}$. **(B)** Trichrome and Alcian Blue staining of transverse sections of adult aortic valve from *Isl1cre*−*Gata6*$^{Wt/Fl}$ *and Isl1cre*+*Gata6*$^{Wt/Fl}$ showing the presence of RL type BAV and increased deposition of glycosaminoglycan within the leaflets of the aortic valve, recapitulating the phenotype observed in *Gata6*$^{+/-}$ mice. Scale bar: 200μm. **(C)** Trichrome staining of P0 valves from frontal heart sections showing thick aortic valves in *Isl1cre*+*Gata6*$^{Wt/Fl}$ when compared to *Isl1cre*−*Gata6*$^{Wt/Fl}$ littermates. Movat pentachrome staining showing abnormal ECM in P0 *Isl1cre*+*Gata6*$^{Wt/Fl}$ Ao valves marked by increased blue staining within the leaflets of the Ao valves (n=6–9 per group). Scale bar: 400μm.



B

Nb	SNP ID	Odds Ratio (OR)	P-value
1	rs7241299	1.478	0.00283
2	rs79023008	1.492	0.00236
3	rs1893384	1.314	0.00095
4	rs3017041	1.271	0.00366
5	rs11083391	1.457	0.00401
6	rs6506998	0.720	0.00683
7	rs117538496	0.786	0.273
8	rs16964572	0.563	0.142
9	rs10454095	0.967	0.685
10	rs67205814	1.062	0.598
11	rs7240020	1.025	0.762
12	rs16964670	1.036	0.759
13	rs11665232	1.256	0.00323
14	rs4800397	0.793	0.00404





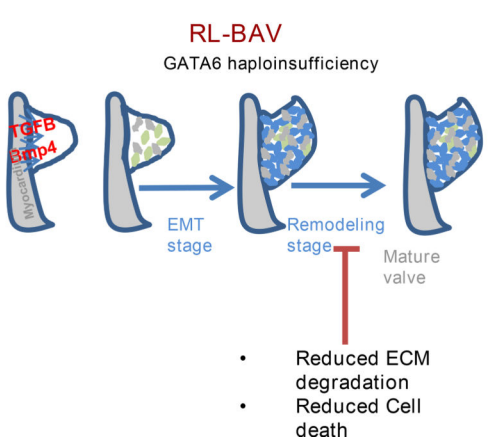


Figure 6. GATA6 expression and variants in human BAV

(A and B) Genotyping of 452 BAV cases from the Framingham GWAS study (with 1849 controls) revealed several SNPs in and around *GATA6* (chromosome 18). Schematic representation of the identified SNPs on *GATA6* is shown. **(C)** Microarray analysis of human aortic valve samples. Boxplot of gene expression levels in human aortic valves for GATA6 according to the two groups of aortic valves (calcified BAV and non-calcified TAV controls). The y-axis presents the mRNA expression levels for GATA6 on a log 2 scale. Gene expression was obtained from 12 aortic valves in each group and measured with the HumanHT-12 v4 Expression BeadChip. Values are mean +/− SEM. **(D)**

Immunohistochemical staining of GATA6, NCID, and Smooth muscle actin on samples of dilated aortas from patients with normal TAV (nTAV), RL, and RN, type BAVs showing markedly lower expression of GATA6 in tissues from RL-BAV patients. The data are representative of n=6 for each group. Scale bar: 20 μm. **(E)** Representative summary model of BAV etiology in *Gata6*$^{+/-}$ mice.