MATR3 disruption in human and mouse associated with bicuspid aortic valve, aortic coarctation and patent ductus arteriosus

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Abstract

Cardiac left ventricular outflow tract (LVOT) defects represent a common but heterogeneous subset of congenital heart disease for which gene identification has been difficult. We describe a 46,XY,t(1;5)(p36.11;q31.2)dn translocation carrier with pervasive...
Introduction

Congenital heart defects (CHDs) are the most common human birth defects, affecting nearly 1 per 100 liveborn infants, and a leading cause of infant mortality [reviewed in (1,2)]. The etiology for most of these defects is unknown, but both environmental and genetic factors are likely to be contributory (3,4). Complex genetics, combined with the traditional classification of cardiac malformations by physiology and anatomy, have made investigation of the genetic component of CHD challenging.

Left ventricular outflow tract obstruction (LVOTO) malformations comprise ~14% of CHD and consist of anatomically varied defects with a wide spectrum of clinical severity, including bicuspid aortic valve (BAV), coarctation of the aorta (CoA), hypoplastic left heart (HLH) and interrupted aortic arch (IAA) type A. BAV is the most common form of LVOTO and of CHD (5). Although genetically related, as both phenotypes often co-occur and may result from a common mechanism involving neural crest perturbation (28). Besides the genes mentioned earlier, Hey2 (29), TBL1Y (30) and MCTP2 (31) have been associated with a predominant CoA phenotype. Patent ductus arteriosus (PDA) is frequently associated with other cardiac anomalies [reviewed in (32)], but in mouse and human, only TFAF28 mutations are associated with PDA as the predominant cardiac phenotype (33–35).

To identify additional genes responsible for LVOTO defects, we utilized the Developmental Genome Anatomy Project (DGAP), which uses balanced chromosomal rearrangement breakpoints to identify potential candidate genes (36). We identified and analyzed a subject, designated DGAP105, with a balanced translocation 46,XY,t(1;5)(p36.11;q31.2)dn. This proband exhibited a global delay in development with a marked delay in speech and was also given a clinical diagnosis of Noonan-like syndrome with BAV, CoA and PDA; these and other cardiac defects are present in ~50% of Noonan syndrome (NS) cases (37). However, we were unable to identify mutations in PTPN11, KRAS and SOS1 that, collectively, account for the majority of NS (37). Therefore, we used the DGAP105 translocation breakpoints to gain insight into the proband’s phenotype and identified disruptions in two genes, MATR3 and AHDC1, which together account for many features of the proband’s phenotype. Recent work (38) indicates that AHDC1 loss-of-function is sufficient to account for the impaired cognitive development and pervasive developmental disorder of the DGAP105 proband. Furthermore, we find that MATR3 transcripts are disturbed and that Matrin 3 protein expression is affected in DGAP105 cells and in affected heterozygous Matr3+/-mutant mice. In addition, both human and mouse MATR3 mutants exhibit similar BAV, CoA and PDA phenotypes. Collectively, these results support a role for Matrin 3 in the formation of the cardiac outflow valves and great vessels and indicate that subtle alterations in human MATR3 can cause medically important cardiac LVOT phenotypes.
Results

DGAP105 cardiac phenotype

At birth, the proband exhibited BAV, CoA and PDA (Figure 1A–D). An echocardiogram at 2 days of age showed CoA with a posterior shelf just distal to the left subclavian artery (Fig. 1C), with turbulent flow in the descending aorta. Doppler measurements revealed a 30 mmHg peak gradient across the CoA. The transverse aortic arch between the carotid and left subclavian arteries also appeared hypoplastic and narrow with a 4.4-mm diameter for the transverse aorta compared with 5.7 mm for the ascending aorta (Fig. 1D). A BAV was present, with normal flow velocity across it. The intercoronary commissure was fused between right and left (R–L) coronary cusps, with non-fused commissures between right and non-coronary (R–N) and between left and non-coronary (L–N) cusps. Mild-to-moderate tricuspid insufficiency was also noted. The mitral valve orifice appeared small in the short axis view, but mitral inflow velocity (98 cm/s) and pressure halftime (61 ms) were normal, and the papillary muscles intact. The right ventricle and pulmonary artery appeared dilated, but a normal flow velocity was measured in the pulmonary artery. Left ventricular function and pulmonary vein drainage into the left atrium were normal. A PDA (Fig. 1C) with predominant left-to-right shunting and a small left-to-right shunt across a patent foramen ovale were present. An ECG revealed sinus rhythm with left axis deviation. The CoA was corrected surgically via end-to-end anastomosis at 3 weeks of age, and subsequent echocardiograms to 17 years of age show no evidence of aortic re-narrowing or of regurgitant flow across the BAV. Other clinical findings, including impaired development of intellectual, speech

Figure 1. Clinical features, cardiac phenotype and translocation breakpoint structure in DGAP105. (A) Facial features at 4 years of age included mild hypertelorism, bilateral epicanthal folds, downslanting palpebral fissures, strabismus and a broad nose with a smooth philtrum and thin vermilion border. (B) Ideograms depicting 46,XY,t(1;5)(p36.11;q31.2)dn. Arrows mark locations of AHDC1 and MATR3 breakpoints. (C and D) Echocardiograms at age of 2 days. (C) Ductal view showing distal aortic arch, CoA just distal to the left subclavian artery (LSGA), accompanied by a prominent posterior unfolding (‘posterior shelf’) and PDA. (D) Aortic arch view showing ascending aorta (5.7-mm diameter), hypoplastic aortic arch (4.4-mm diameter) and CoA posterior shelf. (E) Summary of the 1p36.11 and 5q31.2 breakpoints in DGAP105. The 1p36.11 breakpoint disrupts AHDC1 intron 1, whereas the 5q31.2 breakpoint disrupts MATR3 exon 15 in the 3’ UTR. BACs used in FISH analyses are indicated.
and motor skills, are summarized in Supplementary Material under ‘DGAP105 clinical history’.

Disruption of MATR3 and AHDC1 in DGAP105

Chromosomal analysis revealed a de novo balanced translocation that was assigned as 46,XY,t(1;5)(p36.11;q31.2)dn based on FISH mapping (Fig. 1B; Supplementary Material, Figs S1 and S2). Further FISH and Southern blot analysis localized the breakpoint region to intron 1 of AHDC1, encoding AT hook, DNA-binding motif containing-1 protein (Fig. 1E; Supplementary Material, Fig. S2). Amplification of the der(1) junction fragment by suppression PCR retrieved the 5q31.2 breakpoint, which was confirmed by FISH (Fig. 1E; Supplementary Material, Fig. S2). Sequence analysis of the junction fragment from the der(1) revealed an intronic 13-bp deletion of chromosome 1, whereas sequence of the fragment cloned from the der(5) showed a 3-bp insertion (Supplementary Material, Fig. S3). The 5q31.2 breakpoint occurred 667 bp downstream of the stop codon and in the 3' UTR of MATR3 exon 15. Thus, two distinct genes, AHDC1 at chromosome 1p36.11 and MATR3 at 5q31.2, are disrupted by the reciprocal translocation breakpoints in DGAP105. Array CGH at ∼9-kb resolution did not detect gain or loss of genomic sequence at the breakpoints or in the other regions of the genome.

RT–PCR showed that both AHDC1 and MATR3 are expressed in lymphoblasts. To further characterize the 3' UTR of MATR3 and the consequences of its disruption by the translocation, we performed 3' RACE (Fig. 2A and B). 3' RACE on control human lymphoblast and human fetal heart RNA revealed two MATR3 3' RACE products of 1589 and 963 bp, each using a different AAUAAA polyadenylation signal. Lymphoblast MATR3 transcripts predominantly employ a distal AAUAAA signal in exon 15 located 988 bp downstream of the MATR3 stop codon (Fig. 2B). However, in human fetal heart, the predominant transcripts employ a proximal AAUAAA, located 362 bp downstream of the stop codon (Fig. 2B). This pattern of differential polyadenylation was supported by northern blot analysis of adult human tissues (Fig. 2C), which detected a 3.5-kb transcript, consistent with use of the distal polyadenylation signal in all tissues examined except for heart and skeletal muscle. In these two tissues, a shorter 2.9-kb transcript predominates, consistent with the use of the proximal polyadenylation signal (Fig. 2C).

Based on these results, the translocation breakpoint should disrupt the longer MATR3 transcripts that employ the distal polyadenylation signal, but not the shorter transcripts that use the proximal polyadenylation signal. To test this hypothesis, we performed 3’ RACE on RNA from DGAP105 and control lymphoblasts (Fig. 2B). As expected, we observed a reduction in the levels of the longer 1589-bp product in DGAP105 lymphoblasts. Notably, however, we also observed up-regulated expression in DGAP105 lymphoblasts of the shorter 963-bp product that is normally present in fetal heart, but only at very low levels in control lymphoblasts (Fig. 2B). Thus, DGAP105 lymphoblasts exhibit reduced expression of distal polyadenylated MATR3 transcripts but increased expression of proximal polyadenylated MATR3 transcripts that normally predominate in human fetal heart.

These results suggest that alterations in the levels of the two MATR3 transcript classes may be causally linked to the LVOT defects observed in the DGAP105 proband. To assess the potential impact on Matrin 3 protein levels, we performed western blot analyses on control and DGAP105 lymphoblasts (Fig. 2D and E). Using a Matrin 3-specific antibody, we detected a statistically

**Figure 2.** Analysis of human MATR3 transcripts and protein expression in control and DGAP105 lymphoblasts. (A) Schematic of the MATR3 exon 13–15 region with the chromosomal translocation breakpoint in patient DGAP105 marked by dotted line. The proximal AAUAAA polyadenylation signal and the distal AAUAAA polyadenylation signal site are shown flanking the breakpoint in the 3' UTR. TAA denotes the stop codon in exon 15. (B) MATR3 3' RACE products in human control (Lane 1) and DGAP105 lymphoblast (Lanes 2, 3), and control human fetal heart tissue (Lanes 4, 5). RT ‘+’ or ‘−’ denote inclusion or omission of reverse transcriptase in the cDNA synthesis. The large product (1589 bp, arrow) uses the distal polyadenylation signal and predominates in control human lymphoblasts (Lane 1). In contrast, the short product (963 bp, arrow) predominates in DGAP105 lymphoblasts (Lane 2) and in control human fetal heart (Lane 4) and represents MATR3 transcripts that use the proximal polyadenylation signal. (C) Northern blot analysis of adult human tissues shows MATR3 transcripts of ~3.5 and ~2.9 kb. In heart and skeletal muscle, the 2.9-kb transcript predominates and likely corresponds to the 3’ RACE product using the proximal polyadenylation signal. In brain and other tissues, the 3.5-kb transcript predominates and corresponds to the 3’ RACE product using the distal polyadenylation signal. (D) Western blot analysis of protein isolated from DGAP105 and three control lymphoblast lines, showing up-regulation of Matrin 3 in DGAP105 compared with controls. Gapdh was used as loading control. (E) Quantification of Matrin 3 protein expression in D. Bars represent the mean fold expression of four independent experiments ± SEM, corrected for loading, and normalized to Control 2, *P < 0.05 between DGAP105 and mean of the three control lines via paired Student’s t-test.
significant 2.7 ± 0.1-fold (mean ± SEM) up-regulation in Matrin 3 protein levels in DGAP105 cells compared with mean Matrin 3 level in three control lymphoblastoid lines (P<0.05; n = 4 experiments; t-test).

Developmental expression of mouse MATR3 and AHDC1 homologs

To further assess the roles of MATR3 and AHDC1 in the DGAP105 heart phenotype, we analyzed the spatial and temporal expression of their mouse homologs in heart, limb and brain at E11.5, E16.5 and newborn stages by semi-quantitative RT-PCR (Fig. 3A). Mouse Matr3 was strongly expressed in embryonic heart, limb and brain at E11.5 and E16.5 but down-regulated in newborn heart and limb. In contrast, Ahdc1 expression was present only weakly in E11.5 and E16.5 limb and brain, and either undetectable or expressed at very low levels in developing heart at all stages examined (Fig. 3A). Ahdc1 expression was readily detected in adult non-cardiac tissues, excluding concerns about detection (data not shown).

To localize Matr3 expression in the developing heart and to exclude further significant Ahdc1 expression, we performed section in situ hybridization for Matr3 and Ahdc1 in mouse embryos at E11.5 (Fig. 3B). These experiments revealed Matr3 expression in the heart, the CNS, pharyngeal arch mesenchyme and limb bud. In contrast, Ahdc1 expression was detected only weakly in the embryo and not in the heart. Thus, in contrast to Ahdc1, Matr3 is strongly expressed in the developing mouse heart at developmental times that correlate with genesis of the cardiac abnormalities in DGAP105.

Matrin 3<sup>GT-ex13</sup> homoygotes are early embryonic lethal

We next prepared Matr3<sup>GT-ex13</sup> mutant mice (see Materials and Methods, and Fig. 4A) to assess whether the MATR3 disruption could be causal for the DGAP105 BAV, CoA and PDA phenotypes. Genotype analysis of 14 litters of newborn mice (n = 121) and 12 litters of E8.5–18.5 embryos (n = 92) from Matr3<sup>GT-ex13</sup> heterozygous crosses revealed no homozygotes, indicating a prenatal homozygous lethal phenotype (Table 1). To determine whether Matr3<sup>GT-ex13</sup> homozygotes die before implantation, we collected five litters of E1.5–3.5 embryos (n = 55 total) from heterozygous crosses; homozygous genotypes were detected in only 6 of the 55 embryos (cf. ∼14 expected; P<0.05, chi-square test) (Table 1). Thus, Matr3 plays an essential role in early mouse embryonic survival, with significant demise in Matr3<sup>GT-ex13</sup> homozygous embryos occurring before E4.5 and the demise of all remaining embryos occurring between the E4.5 implantation and E8.5 neural fold stages. Proper levels of Matr3 function are therefore required for embryonic viability both prior to the blastocyst stage and between that time and the neural fold stage.

Functional properties of the Matr3<sup>GT-ex13</sup> allele

Because early embryonic lethality precluded a cardiac-specific analysis of Matr3<sup>GT-ex13</sup> homozygotes, we turned to Matr3<sup>GT-ex13</sup> heterozygotes. We first sought to determine the mechanism of the Matr3<sup>GT-ex13</sup> mutation (Fig. 4A and B). To do so, we analyzed Matr3 transcript and Matrin 3 protein expression in wild-type and Matr3<sup>GT-ex13</sup> heterozygotes by 3′ RACE and western blot (Fig. 4C–E).

3′ RACE was performed for mouse Matr3 using E14.5 heart and brain RNA. Analogous to human MATR3, these results confirmed the usage of two AAUAAA signals located 977 and 355 bp downstream of the mouse Matr3 stop codon. A longer 3′ RACE product of 1647 bp was detected in both tissues, but similar to human, a shorter albeit minor product of 1025 bp was detected only in developing heart RNA (Fig. 4C). Thus, mouse Matr3 and human MATR3 are strongly expressed in developing heart, and in both species, there are two distinct polyadenylation signals.

As expected, given the location of the Matr3<sup>GT-ex13</sup> gene trap in exon 13, 5′ to both polyadenylation signals, 3′ RACE showed reductions in the long Matr3 transcript in Matr3<sup>GT-ex13</sup> heterozygous

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Figure 3. Analysis of mouse Matr3 and Ahdc1 transcripts expression in developing heart. (A) RT-PCR analyses of Matr3 and Ahdc1. (a) Semi-quantitative RT-PCR analyses show strong Matr3 expression in developing mouse heart, limb and brain at E11.5, 16.5 stages, (b) with down-regulation at the newborn (NB) and adult stages. In contrast, Ahdc1 expression is only weakly detected in limb and brain at E11.5 and 16.5. (B) Section in situ hybridizations at E11.5 for mouse Matr3 and Ahdc1. Matr3 is expressed in CNS, pharyngeal arches, limb buds and in the developing heart (enlarged section), whereas Ahdc1 expression was undetectable in heart (enlarged section). Sense controls (not shown) showed no expression.
Table 1. Early embryonic lethality in Matr3\textsuperscript{GT-ex13} homozygous mouse embryos

<table>
<thead>
<tr>
<th>Age (total litters)</th>
<th>n</th>
<th>Matr3\textsuperscript{+/-}</th>
<th>Matr3\textsuperscript{GT-ex13/+}</th>
<th>Matr3\textsuperscript{GT-ex13/GT-ex13}</th>
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<tr>
<td>E1.5–4.5 (5)</td>
<td>55</td>
<td>12</td>
<td>37</td>
<td>6\textsuperscript{a}</td>
</tr>
<tr>
<td>E8.5–18.5 (12)</td>
<td>92</td>
<td>22</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>Newborn (14)</td>
<td>121</td>
<td>31</td>
<td>90</td>
<td>0</td>
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</table>

\textsuperscript{a}Note striking deficiency of Matr3 homozygous embryos and newborns. The under-representation of homozygous embryos at E1.5–4.5 is statistically significant (\(\chi^2 = 8.56; P = 0.014\)). Of the six E1.5–4.5 homozygous embryo recovered, three were identified at the blastocyst stage; one, cultured for an additional day, hatched successfully. Additional litters collected between E7.5 and E9.5 contained five dead embryos. These may have represented homozygous embryos that died at or before the E8.5 neural-fold stage.

Cardiac expression of Matrin 3

As 3’ RACE and western blot experiments established that Matrin 3\textsuperscript{β-Geo} fusion transcripts and protein are expressed from the Matr3\textsuperscript{GT-ex13} gene trap allele, we sought to use the β-gal reporter feature of the β-geo gene trap in Matr3\textsuperscript{GT-ex13} heterozygotes to more precisely assay endogenous Matr3 expression. We therefore wished to establish the fidelity of β-gal reporter for Matrin 3 at the cellular and subcellular levels. Double-label immunofluorescence for Matrin 3 and β-galactosidase confirmed a high degree of co-localization within E13.5 and E15.5 cardiomyocyte nuclei (Supplementary Material, Fig. S4) and in embryonic kidney and brain (not shown).

We next used X-Gal staining to examine the cardiac expression of the Matrin 3\textsuperscript{β-Geo} fusion protein in detail. Prominent X-Gal staining was present from E8.5 to term in the developing heart (Fig. 5). Expression starts as early as E8.5 with expression in the right and left ventricular precursors, the bulbus cordis and the common ventricular chamber (Fig. 5B and C). At E11.5, X-Gal staining was present throughout the endocardium and the myocardium (Fig. 5D and E). In newborn mice, X-Gal staining extends throughout all cardiomyocytes in the ventricular, atrial and septal regions (Fig. 5F, and data not shown). We also used Matrin 3 immunochemistry to confirm that Matrin 3 protein is concordantly expressed in the newborn mouse heart (Fig. 6A–C). Immunofluorescent detection showed strong Matrin 3 protein expression in the nuclei of newborn mouse.
ventricular and interventricular septal cardiomyocytes and in the nuclei of cardiac valve interstitial cells and endocardial cells (Fig. 6C).

Matrin 3 is also present in large arterial and venous vascular endothelial cells and in arterial smooth muscle cells as revealed by Matrin 3 and PECAM-1 (platelet-endothelial cell adhesion molecule) double immunostaining (Fig. 6D–I). In large arteries, Matrin 3 is expressed in smooth muscle cell nuclei, external to the expression domain of the endothelial marker PECAM-1 (39); Matrin 3 is also expressed in vascular endothelial cell nuclei, internal to the PECAM-1 expression domain. Endothelial Matrin 3 expression is prominent in large veins, which lack significant smooth muscle (Fig. 6G–I). Thus, in addition to the expression in non-cardiac tissues (Supplementary Material, Fig. S5), Matrin 3 is expressed in developing murine cardiac tissues, including endocardium, endocardial cushions, outflow valves and vascular
endothelium that correspond to the cardiac tissues affected in DGAP105.

**Matr3<sup>GT-ex13</sup> heterozygotes exhibit subaortic VSD and DORV**

Newborn Matr3<sup>GT-ex13</sup> heterozygotes frequently exhibited abnormal shape and position of the cardiac apex with a characteristic ‘boot shape’ (Fig. 7A and C, insets). Subsequently, 17 wild-type and 36 heterozygous specimens in 2 different mixed genetic backgrounds (C57BL x 129 and C57BL x FVB) at E16.5, E18.5 and newborn stages were collected and analyzed by histology (Fig. 7, Table 2). No cardiac defects were noted in the wild-type littermates. Of the 36 Matr3<sup>GT-ex13</sup> heterozygotes, 11 (30%) exhibited VSDs that were typically subaortic in location (Fig. 7C–F, Table 2). Specifically, in most cases, the aortic valve overrode the right ventricle with 50% or greater overlap and the VSD was closely aligned with the aortic valve, thereby satisfying the definition of DORV with subaortic VSD. In addition, muscular and subpulmonic VSD phenotypes were also observed (Table 2). The collective incidence of VSD in Matr3<sup>GT-ex13</sup> heterozygotes (11 of 36) is statistically significant (P < 0.05; Fisher exact test).

**Aortic and outflow valve abnormalities in Matr3<sup>GT-ex13</sup> heterozygotes**

In addition to VSD, aortic and pulmonary valve defects were also present. BAV was noted in 3 of 26 Matr3<sup>GT-ex13</sup> heterozygotes (15%), and in 1 case bicuspid pulmonic valve was observed (Fig. 8, Table 2). Although this incidence is not statistically significant, incompletely penetrant BAV phenotypes are also observed in Gata5<sup>−/−</sup>, eNos<sup>−/−</sup> and Nkx2-5<sup>HDneo/+</sup> mouse models, and BAV occurs only rarely in wild-type mice (15,16,40). Particularly striking abnormalities were present in the aortic arch and descending aorta, with severe CoA and PDA phenotypes (Fig. 9, Table 2). A total of 43 Matr3<sup>GT-ex13</sup> newborn heterozygotes were analyzed by corrosion cast analysis after injection of red polymer into the left ventricle, and in some cases, of blue polymer into the left ventricle, and in some cases, of blue polymer into the...

**Figure 7.** Subaortic VSD and DORV phenotypes in Matr3<sup>GT-ex13</sup> heterozygotes. Transverse serial sections through the hearts of E18.5 wild type (A and B), and two different representative Matr3<sup>GT-ex13</sup> heterozygotes (embryo 1 in C and D and embryo 2 in E and F), each sectioned at a cranial and caudal level, illustrating the DORV with subaortic VSD phenotype in Matr3<sup>GT-ex13</sup> heterozygotes (see Table 2). (A and B) Wild-type sections show left and right ventricles separated by the interventricular septum, the two atrioventricular (tricuspid, mitral) valves and the aortic valve (pulmonic valve not seen in this view). Heterozygous sections do not closely resemble wild-type sections in overall cardiac configuration because, in addition to specific cardiac anomalies, affected newborn Matr3<sup>GT-ex13</sup> heterozygote hearts are frequently maloriented and exhibit an abnormal ‘boot shape’, with the cardiac apex pointing horizontally to the animal’s left (see insets, A and C). (C) Subaortic VSD is directly inferior to and aligned with the aortic valve. (D) The aortic valve significantly overrides the right ventricle, which together with the normal communication of right ventricle to pulmonary artery (data not shown), establishes DORV. (E) In this specimen, an unusually close continuity exists between the tricuspid and aortic valves. (F) Subaortic VSD and DORV are shown. AoV, aortic valve; LA and LV, left atrium and ventricle; MV, mitral valve; RA and RV, right atrium and ventricle; TV, tricuspid valve; VSD, ventricular septal defect. Scale bar: 500 μm.
right ventricle. A range of aortic phenotypes was identified, including CoA, IAA and hypoplastic aortic arch (HAA) (overall, 16%). When present, the CoA phenotype occurred at a juxtaductal position, just distal to the left subclavian artery. In addition, a FDA phenotype was noted in 5 of 43 newborn Matr3<sup>GT-ex13</sup> heterozygotes (12%), in some cases in association with VSD and IAA, whereas no overt examples of ductus patency were observed in 13 newborn wild-type controls by corrosion cast analysis. The presence of 12 total aortic arch defects including FDA in 43

Matr3<sup>GT-ex13</sup> newborns is statistically significant (P < 0.05; Fisher exact test). Thus, appropriate levels of Matr3 function are required for normal cardiac development including formation of the subaortic interventricular septum, morphogenesis of the aorta and, very likely, cuspal development of the aortic and pulmonic valves.

### Discussion

AHDC1 haploinsufficiency accounts for impaired cognitive and speech development

The DGAP105 translocation disrupts MATR3 and AHDC1 at 5q and 1p breakpoints, respectively. To assign causality for individual components of the proband’s phenotype to either or both of these genes, we pursued two approaches: (1) identification of intragenic mutations in independent but phenotypically similar cases and (2) recapitulation of a homologous phenotype by targeted knockout in mouse. For AHDC1, a recent report describes de novo truncating mutations in four independent subjects with a syndrome defined by intellectual disability, global developmental delay (especially of speech and motor milestones), hypotonia, obstructive sleep apnea, mild facial dysmorphism and mild brain abnormalities by MRI (38). The co-occurrence of de novo truncating mutations in AHDC1 in four independent cases with concordant phenotypes is prima facie evidence for causation of this syndrome by AHDC1 loss-of-function, with dominant-negative and haploinsufficiency mechanisms suggested as potential explanations for this disorder (38).

DGAP105 shares a high degree of phenotypic and molecular similarity with the four AHDC1 truncation index cases. Phenotypes shared in common include impaired intellectual, speech and motor development, facial dysmorphism and respiratory and sleep disturbances. Moreover, cardiac defects were not reported in the four index cases, two of whom had normal echocardiograms. In DGAP105, the 1p breakpoint occurs in AHDC1 intron 1 and would disrupt the 5’ UTR. Furthermore, AHDC1 expression is reduced to ∼50% of wild type in DGAP105 lymphoblastoid cells (Supplementary Material, Fig. S6). Therefore, AHDC1 haploinsufficiency is the most likely cause of the developmental delay and

### Table 2. Congenital heart defects in Matr3<sup>GT-ex13</sup> embryonic and newborn heterozygotes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Matr3&lt;sup&gt;GT-ex13&lt;/sup&gt;/+</th>
<th>Matr3&lt;sup&gt;+/+&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>No. affected/</td>
<td>No. affected/</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td>1. VSD (histology) (30% penetrance)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscular VSD</td>
<td>1/36</td>
<td>0/17</td>
</tr>
<tr>
<td>Membranous VSD</td>
<td>3/36</td>
<td>0/17</td>
</tr>
<tr>
<td>DORV with Subaortic VSD</td>
<td>6/36</td>
<td>0/17</td>
</tr>
<tr>
<td>DORV with Subpulmonic VSD</td>
<td>1/36</td>
<td>0/17</td>
</tr>
<tr>
<td>VSD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Aortic arch phenotype (corrosion cast) (27% penetrance)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoA</td>
<td>3/43</td>
<td>0/13</td>
</tr>
<tr>
<td>IAA</td>
<td>2/43</td>
<td>0/13</td>
</tr>
<tr>
<td>HAA</td>
<td>2/43</td>
<td>0/13</td>
</tr>
<tr>
<td>PDA</td>
<td>5/43</td>
<td>0/13</td>
</tr>
<tr>
<td>3. Heart outflow valve phenotype (microdissection) (15% penetrance)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicuspid aortic valve</td>
<td>3/26</td>
<td>0/10</td>
</tr>
<tr>
<td>Bicuspid pulmonic valve</td>
<td>1/26</td>
<td>0/10</td>
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Analyses were conducted in C57BL/6J × 129/SvJ and C57BL/6J × FVB/N backgrounds. VSD analyses were conducted at E16.5, E18.5 and newborn stages. Aortic arch phenotypes (CoA, FDA) were analyzed at the newborn stage. Although the ductus does not normally close until after birth, we did not observe FDA in controls at this stage by corrosion casting.<sup>a</sup>

<sup>a</sup>Incidence of VSDs in heterozygotes is statistically significant (P < 0.05; Fisher exact test).

<sup>b</sup>Although PDAs occurred in mice with IAA (and VSD), the total number of aortic arch defects in heterozygotes is statistically significant (P < 0.05; Fisher exact test).

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**Figure 8.** Semilunar heart valve defects in Matr3<sup>GT-ex13</sup> heterozygotes. (A) Diagram of ascending aorta, aortic arch and descending aorta, showing plane of section for aortic valve analysis. Note left and right coronary ostia (openings) below the fibrous annulus (ring) that demarcates the valve (Cleveland Clinic Foundation (CCF), with permission). (B) Wild-type newborn aortic valve (AoV) showing tri-leaflet (†) morphology in open configuration. Commissural attachments to the annulus are marked (arrows). (C) Matr3<sup>GT-ex13</sup> heterozygote newborn bicuspid AoV (BAV) showing two leaflets in closed configuration. (D) Wild-type newborn pulmonic valve (PuV) showing tri-leaflet morphology in open configuration. (E) Matr3<sup>GT-ex13</sup> heterozygote newborn bicuspid PuV (BPV) showing two leaflets in closed configuration.
intellectual disability phenotypes in DGAP105 and is unlikely to cause the LVOT defects.

Assignment of human cardiac outflow tract phenotypes to MATR3 disruption

To confirm our impression that the disruption of AHDC1 was an unlikely cause of the congenital cardiac disease in DGAP105, we turned to the mouse and assessed expression of Ahdc1 and Matr3 during stages of mouse embryonic development that correspond to times at which the relevant developmental timing and tissue interactions for LVOT development occur. Ahdc1 expression was low or undetectable by RT–PCR and whole-mount in situ hybridization in the developing heart and cardiac outflow vessels. In contrast, Matr3 RNA and protein were both strongly expressed in the developing heart, cardiac outflow tract valves and aorta. Taken with the absence of cardiac phenotypes associated with the previously described AHDC1 truncation mutations, these results make a major role for AHDC1 in human or mouse cardiac development unlikely and allowed us to prioritize Matr3 for gene targeting in mouse.

The presence of distinct embryonic lethal phenotypes in homozygous and LVOT phenotypes in heterozygous Matr3Gt-ex13 mice indicates a sensitive dependence on Matr3 gene dosage for different developmental processes. Significantly, Matr3Gt-ex13 heterozygotes exhibited incompletely penetrant BAV, CoA and PDA phenotypes that closely resemble those in DGAP105. We conclude that the cardiac anomalies in Matr3Gt-ex13 heterozygotes result from subtle perturbation in the level or function of Matr3 transcripts and proteins. In potentially analogous fashion, disruption of the MATR3 3′ UTR and slight alterations in Matrin 3 protein levels appear to account for the cardiac findings in DGAP105.

Potential mutational mechanisms involving MATR3

In contrast to the AHDC1 haploinsufficiency observed in DGAP105, a different mutational mechanism for MATR3 seems to apply that involves subtle alterations in the MATR3 transcripts and protein levels. We initially anticipated that the disruption of MATR3 3′ UTR by the DGAP105 translocation breakpoint should interfere with the proper polyadenylation of MATR3 transcripts and lead to their destabilization, resulting in loss-of-function. Confounding this simple interpretation, 3′ RACE experiments in human lymphoblasts and fetal heart tissue revealed tissue-specific usage of two different polyadenylation signals, located 362 and 988 bp downstream of the stop codon in exon 15 of MATR3. The DGAP105 translocation breakpoint falls at position 667 bp downstream of the stop codon and, therefore, separates the distal but not the proximal polyadenylation signal from the MATR3 transcription unit. As expected, MATR3 transcripts in DGAP105 lymphoblasts show a significant but incomplete reduction in

Figure 9. Aortic arch abnormalities in Matr3Gt-ex13 heterozygotes. (A and B) Newborn wild-type aortic arch vasculature, showing pre- (A) and post-corrosion (B) cast analysis. (C–L) Matr3 heterozygous newborns with various outflow tract defects. (C and D) Tubular hypoplasia and CoA. The deformed aortic arch is uniformly narrowed (segment between arrowheads), and a CoA (arrow) lies distal to the LSA near a closed DAO. (E and F) CoA (arrow) just distal to the LSA and at the level of the closed DAO also called a ‘juxaductal CoA’. (G and H) Interrupted aortic arch just distal to the LSA, with a strand of tissue joining the two segments (‘atrial aortic arch’); arrow. AVSD with left to right shunting is also present, as evident by red polymer in both ventricles. A large PDA (arrowhead) is the sole source of blood to the lower half of the body. (I and J) A wide PDA (arrowhead) and VSD are present. Following LV injection, both ventricles and the PT contain red polymer; the PT is connected to the PDA that joins the DAO. (K and L) Dual-color corrosion casting shows admixture of red (injected into LV) and blue polymers (injected into RV) in both ventricles, confirming the presence of a VSD (arrow, K). Both polymers are also present in the pulmonary trunk and aorta. A small PDA is present (arrowheads, K and L). AAo, ascending aorta; BA, brachiocephalic artery; DAO, descending aorta; IAA, interrupted aortic arch; LV, left ventricle; PT, pulmonary trunk; RCC/LCC, right/left common carotid arteries; RSA/LSA, right/left subclavian arteries; RV, right ventricle; VSD, ventricular septal defect.
distal polyadenylated transcripts, consistent with residual expression from the wild-type allele.

Interestingly, however, this reduction in longer, distal polyadenylated MATR3 transcripts is accompanied by up-regulation of shorter proximal polyadenylated transcripts, the form that predominates in fetal heart. As both the longer and shorter transcripts appear to encode the same Matrin 3 protein, and the relative stabilities and translation efficiencies of both transcripts in the developing heart are unknown, the 3’ disruption of MATR3 could theoretically produce either an up-regulation or a down-regulation of Matrin 3 protein levels. Consistent with the former possibility, western blot analyses revealed a modest but statistically significant up-regulation of Matrin 3 protein of DGAP105 lymphoblasts.

To further clarify the mutational mechanism involved in the MATR3 disruption in DGAP105, we generated and investigated a mouse Matr3 gene trap model in which the gene trap insertion in exon 13 toward the 3’ end of mouse Matr3 approximates the human MATR3 translocation disruption in exon 15. In this case, owing to its more 5’ location in exon 13, the gene trap insertion results in a reduction in both proximal and distal polyadenylated Matr3 transcripts. However, in this case, these reductions are accompanied by significant expression of Matrin 3-β-Geo fusion transcript and protein, and the latter largely co-localizes to the same subnuclear domains as endogenous Matrin 3. Although the functional activity of Matrin 3-β-Geo fusion protein relative to wild-type Matrin 3 is unknown, the mouse Matr3<sup>GT-<ins>ex-15</ins></sup> allele may act similarly to DGAP105 in producing a net up-regulation of Matrin 3 levels or activity and therefore constitute a gain-of-function state. Because we cannot know Matrin 3 levels or activity in the developing DGAP105 heart, or the activity of the Matrin 3-β-Geo fusion protein in the mouse, we cannot categorically distinguish between gain- and loss-of-function models for the mouse or human MATR3 mutations. However, both mutants share disruptions of the 3’ end of MATR3 that very likely produce subtle alterations in the level of Matrin 3 activity—quantitatively in the case of human MATR3 as reflected by increased protein levels, or qualitatively in the case of mouse Matr3, as reflected by the presence of the Matrin 3-β-Geo fusion protein in embryonic heart. Interestingly, in a MATR3 sequence analysis of 48 patients with BAV and CoA and in some cases VSD, although no overtly pathogenic coding region mutations (e.g. frameshift or nonsense variants) were observed, we did identify two unique non-coding deletion variants (c.2235-15_16delGT and c.3086_3089delTTAG; NCBI RefSeq: NM_001194955.1). The first variant represents a 2-bp deletion at the –15 and –16 positions in the exon 12 splice acceptor, whereas the second represents a 4-bp deletion in the MATR3 3’ UTR. Further experiments are underway to determine whether these variants influence Matrin 3 expression levels.

We attribute the difference in mutational mechanisms for AHDC1 and MATR3 to the fact that the DGAP105 translocation breakpoint disrupts the 5’ end of AHDC1, in intron 1, but the 3’ end of MATR3, in exon 15. It is therefore perhaps not surprising that the DGAP105 translocation breakpoint in AHDC1 produces a loss-of-function allele, whereas the situation for MATR3 is significantly more complex, involving subtle alterations in gene and protein expression levels. An attractive molecular model for the latter could involve the loss of distal MATR3 3’ UTR microRNA (miR) binding sites. Owing to the absence of miR binding sites that may negatively regulate the translation of distal polyadenylated transcripts, the proximal polyadenylated MATR3 transcripts might be more efficient in supporting the translation of Matrin 3.

Relation to Matrin 3 S85C mutation
Recently, an autosomal dominant neurological disorder, originally described as ‘vocal cord and pharyngeal weakness with distal myopathy’, or VCPDM (OMIM #606070), was reclassified as a slowly progressive form of amyotrophic lateral sclerosis, or ALS21 (41-44). VCPDM was originally attributed to a heterogeneous S85C missense variant in human Matrin 3 (41,42). However, the mutational mechanism associated with S85C or with other missense variants recently reported in this form of familial ALS remains unclear (44). One clear difference between the S85C VCPDM/ALS phenotype and the cardiac phenotype reported here is that the dominant familial ALS phenotype linked to S85C reflects a missense mutation, whereas the cardiac LVOT phenotypes described here result from 3’ UTR disruptions that affect MATR3 transcripts that differ at their 3’ ends, thereby influencing protein expression. Thus, the two sets of results are not necessarily inconsistent and appear to reflect distinct functions of MATR3 in development and disease pathogenesis. We conclude that MATR3 functions in normal cardiac outflow tract formation and that modest perturbations in Matrin 3 expression in human and mouse are associated with similar LVOTO malformations. Further studies will be required to determine the precise tissue location and nature of the molecular functions attributed to Matrin3 during cardiogenesis.

Materials and Methods

Human genetic analyses

Clinical data
Non-cardiac clinical information for the DGAP105 subject is provided in Supplementary Material under ‘DGAP105 clinical history’. Cardiac clinical information is presented in the manuscript under ‘Results’. All human studies were conducted under an approved IRB protocol.

Cytogenetics and fluorescence in situ hybridization
Lymphocyte cell transformation was performed at the MGH Center for Human Genetic Research Genomics Core Facility (Boston, MA, USA). DNA samples were prepared from normal individuals (as controls) and from individuals with phenotypes similar to those found in the proband. Metaphase chromosomes were prepared from lymphoblastoid cell lines according to routine protocols. Chromosomes were GTG banded at a resolution of ≥550 bands using standard methods and at least 10 metaphase spreads were examined. The DGAP105 karyotype was initially reported as 46,XY,t(1;5)(p36.11;q31.2)dn after molecular cytogenetic analysis. BACs for chromosome rearrangement breakpoint mapping were selected using the UCSC Genome Browser and the NCBI Human Genome Browser and labeled directly with either SpectrumOrange- or SpectrumGreen-conjugated dUTP using a nick translation kit (Vysis, Downers Grove, IL, USA). BAC RP11-288L9 and PAC RP1-159A19 were obtained from CHORI (Children’s Hospital Oakland Research Institute, Oakland, CA, USA), and BACs CTC-315N8 and CTB-43P18 from Invitrogen (Carlsbad, CA, USA). Metaphase chromosome preparations from DGAP105 were prepared on glass slides following standard hypotonic lysis and fixation, and dehydration in a series of ethanol washes as previously described (45). Array CGH was conducted using an Agilent (Santa Clara, CA, USA) 244K human CGH microarray (~8.9 kb of overall resolution, ~7.4 kb for RefSeq genes).
**Breakpoint cloning**

Southern blotting was performed by standard methods. BAC sequences from breakpoint critical regions were examined using RepeatMasker to identify regions from which unique probes could be generated. Genomic DNAs were digested with DraI, EcoRI, HindIII, RsaI, Scal and probed with a 505-bp fragment amplified from RP1-159A19. Rearrangement breakpoints were cloned using modifications of the suppression PCR protocol (45). Cloned junction fragments were sequenced by standard methods.

3' rapid amplification of cDNA ends (3' RACE) analysis (human and mouse)

For quantitative real-time RT–PCR, Taqman assays were employed with the LightCycler® 480 SYBR Green I Master Kit (Roche, Nutley, NJ, USA). On a LightCycler® 480 Real Time PCR System (Roche). The PCR reaction consisted of 10 μl of Master Mix 2× concentrate, 0.35 μM of each forward and reverse primer, template cDNA and PCR grade water to a final volume of 20 μl in the LightCycler 480 96-well plate. An initial denaturation step at 95°C for 10 min was followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 10 s. A single fluorescence measurement was conducted at the end of the 72°C extension segment. After amplification, melting curve analysis was performed by heating the sample to 95°C for 5 s, then cooling it to 65°C for 1 min, followed by a linear temperature increase to 95°C at a rate of 0.11°C/s, while continuously monitoring the fluorescent signal. All primer sets were confirmed by agarose gel electrophoresis to produce a single band of expected size that had the expected melting curve. Data analysis was performed with LightCycler® 480 Relative Quantification software (Roche) (46). Multiple primer sets were selected for AHDC1 to enable analysis of transcripts from either the wild-type allele only (by designing primer pairs that flank the breakpoint), e.g. AHDC1 exons 1–2 flanking the intron 1 site of the breakpoint CTCCACAGCTCTCTCCTCTCCA + AATCAGGGAAGGT CCCCAC, or from wild-type plus translocated alleles, e.g. AHDC1 exon 3: ACCCTGAGAGAAGTTGAGGAG + TCCAGACACTCGGT AGAGAG; AHDC1 exon 6: GAGCTCATCTCCGACGACCG + GCTCCA TCATCGATTCTCACA. 22M (β2 microglobulin: CGTCTACGAGCA GAG + GCAAAGTCTGAATGCTCC) was used as an internal control for normalization. All assays were performed in triplicate and included marker-positive and marker-negative controls and reagent with no template controls.

Western blot analyses (human and mouse)

For human Matrin 3 analyses, lymphoblastoid cell lines were grown in suspension in T25 flasks in RPMI 1640 culture medium supplemented with 10% FBS, 1% Pen/Strep and 1% L-glutamine. For suspension human lymphoblast cells, RIPA buffer was added after the cell pellets were washed with 1× cold PBS. Cells were disrupted by repeated aspiration through a 21-gauge needle. Mouse heart or brain tissues from E14.5 embryos were homogenized with RIPA buffer on ice. Samples were centrifuged for 10 min at 4°C. For western blots, 40 μg of protein was diluted with 2× protein-loading buffer to 1× final concentration and heated at 95°C for 10 min. Proteins were resolved on NuPAGE 4–12% Bis-Tris Gel (Life Technologies). Western blots were treated with a rabbit anti-Matrin 3 antibody (Cat. no. ab70336, Abcam). This Matrin 3 antibody recognizes a region between residue 475 and 500 of human Matrin 3. Protein loading controls were detected with an anti-Gapdh antibody from Millipore. Band intensity was quantified using ImageJ.

For mouse Matrin 3 analyses, total protein from newborn Mat3C5–ex11 mouse tissues was isolated using a NEP3229 Barocycler (Pressure BioSciences, West Bridgewater, MA, USA) according to the manufacturer’s standard protocol. For western blots, 40 μg of protein was diluted with 6× protein loading buffer to 1× final concentration and heated at 95°C for 20 min. Proteins were resolved on 8% SDS–PAGE. Western blots employed a rabbit anti-Matrin 3 antibody (Cat. no. ab70336, Abcam; cat. no. A300-591A, Bethyl Laboratories, Montgomery, TX, USA). This Matrin 3 antibody recognizes a region between residue 800 and the C-terminus (residue 847) of human Matrin 3.

**Mutation screening**

As noted, because of phenotypic overlap between DGAP105 and NS, we screened DGAP105 for exonic mutations in PTPN11,
and Eosin staining and histological analyses were performed using standard procedures.

**X-Gal staining and whole-mount in situ hybridization**

X-Gal staining to detect β-galactosidase activity was performed according to standard protocols. Briefly, embryos were fixed for 30–90 min in fixative consisting of 1% formaldehyde and 0.2% glutaraldehyde in PBS. Whole-mount or OCT-embedded frozen sections were stained with a solution containing 1 mg/ml X-Gal for 3-6 h. Whole-mount in situ hybridization experiments were performed as previously described (48) using a Matr3 1.3-kb anti-sense and Ahdc1 564-bp anti-sense probes.

**Vascular analyses by corrosion casting**

Newborn Matr3Gt-ex13 heterozygotes and wild-type littermate controls were anesthetized with Avertin and the heart exposed by thoracic incision and rib removal. Batson no. 17 methacrylate plastic (cat. No. 07349, Polysciences, Inc., Warrington, PA, USA) was injected into the beating left ventricle after inclusion of red pigment (for arterial analysis) or into the right ventricle with blue pigment (for venous analysis), according to the manufacturer’s instructions. After hardening, tissue was removed with maceration solution (Polysciences) at 50°C for 72 h, and the resulting corrosion casts were photographed. Outflow tract cardiac valve morphology was assessed by microdissection, in some cases after light staining with Toluidine blue.

**Note Added in Proof**

The AHDC1 disruption described here has now been assigned as OMIM #615829, Xia-Gibbs Syndrome.

**Supplementary Material**

Supplementary Material is available at HMG online.

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**Conflicts of Interest statement.** None declared.

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Web Resources
BayGenomics database (now part of International Gene Trap Consortium: http://www.genetrap.org/)
Developmental Genome Anatomy Project (DGAP) (http://www.bwpathology.org/dgap/)
Ensembl (http://www.ensembl.org/Homo_sapiens/)
NCBI Human Genome Browser (http://www.ncbi.nlm.nih.gov/)
Repeat Masker (http://www.repeatmasker.org)
UCSC Genome Browser (http://genome.ucsc.edu)

References


