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Genomes and Developmental Control

GATA5 interacts with GATA4 and GATA6 in outflow tract development

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ABSTRACT

Members of the GATA family of transcription factors are critical regulators of heart development and mutations in 2 of them, *GATA4* and *GATA6* are associated with outflow tract and septal defects in human. The heart expresses 3 GATA factors, GATA4, 5 and 6 in a partially overlapping pattern. Here, we report that compound *Gata4*/*Gata5* and *Gata5*/*Gata6* mutants die embryonically or perinatally due to severe congenital heart defects. Almost all $Gata4^{+/-}Gata5^{+/-}$ mutant embryos have double outlet right ventricles (DORV), large ventricular septal defects (VSD) as well as hypertrophied mitral and tricuspid valves. Only 25% of double compound *Gata4*/*Gata5* heterozygotes survive to adulthood and these mice have aortic stenosis. Compound loss of a *Gata5* and a *Gata6* allele also leads to DORVs associated with subaortic VSDs. Expression of several transcription factors important for endocardial and myocardial cell differentiation, such as Tbx20, Mef2c, Hey1 and Hand2, was reduced in compound heterozygote embryos. These findings suggest the existence of important genetic interactions between Gata5 and the 2 other cardiac GATA factors in endocardial cushion formation and outflow tract morphogenesis. The data identify *GATA5* as a potential genetic modifier of congenital heart disease and provide insight for elucidating the genetic basis of an important class of human birth defects.

Introduction

Congenital heart disease (CHD) is estimated to occur at a prevalence of 1–2% of live births and is the leading cause of death in the first year of life (Pierpont et al., 2007). Abnormal development of the outflow tract (OFT) accounts for about 12–14% of all CHDs, leading to malformations such as persistent truncus arteriosus (PTA), tetralogy of Fallot (TOF), double outlet right ventricle (DORV) and transposition of the great arteries (TGA) (Hoffman and Kaplan, 2002). Significant efforts have been deployed over the last decade to elucidate the cell and molecular mechanisms involved in CHD. Several lines of evidence suggest that CHD is heritable (Insley, 1987), but to date, only a few human genes have been linked to CHDs. Many of those are developmental regulatory genes. For example, mutations in *NKX2.5* are associated with cases of TOF and atrial septal defects (ASD) while mutations in the *TBX5* gene cause the Holt–Oram syndrome, an autosomal dominant disease with varying cardiac defects (Basson et al., 1997; Goldmuntz et al., 2001; Li et al., 1997; Schott et al., 1998). Moreover, mutations in *GATA4* have been associated with atrial and/or ventricular septal defects, TOF and PTA (Garg et al., 2003; Nemer et al., 2006; Rajagopal et al., 2007). Interestingly, heterozygous mutations of *Gata4*, *Nkx2.5 and Tbx5* in mice recapitulate the human phenotype (Biben et al., 2000; Bruneau et al., 2001; Rajagopal et al., 2007; Winston et al., 2010). Recently, mutations in the human *GATA6* gene have been associated with PTA and TOF (Kodo et al., 2009; S.C. Lin et al., 2010; Maitra et al., 2010). What has emerged from combined human and mouse genetic studies is that mutations in different genes can lead to similar cardiac defects while mutations in the same gene can lead to varying defects. The complexity of CHD is evident at both genetic and cellular levels, as multiple lineages contribute to proper heart development. The first heart field contributes to the formation of the left ventricle, the atrioventricular canal and both atrial chambers (Buckingham et al., 2005). Whereas, the secondary heart field (SHF) contributes to the formation of the right ventricle and the OFT (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Remodeling of the OFT into the distinct vessels of the aorta and pulmonary trunk requires complex interactions between the myocardium, the endocardium and cardiac neural crest cells (NCC) (Hutson and Kirby, 2007). Consequently, impaired development of the OFT results in conotruncal defects.

Members of the GATA family of transcription factors play important roles in differentiation, proliferation and survival of different cell types. In the heart, 3 GATA factors are present in a partially overlapping pattern (Molkentin, 2000; Nemer and Nemer, 2003; Patient and McGhee, 2002). Gata4 is highly expressed in myocytes, endocardial cells and epicardial cells of the heart. Embryos lacking *Gata4* die by E8.5 because of defects in ventral migration causing cardia bifida (Kuo et al., 1997; Molkentin et al., 1997). Analysis of rescued $Gata4^{-/-}$ embryos revealed cardiac defects including disrupted heart looping, absence of endocardial cushion formation, lack of a proepicardial

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organ and hypoplastic ventricular myocardium (Watt et al., 2004). Several studies were performed to further investigate the role of Gata4 in endocardial or myocardial cell development. Inactivation of *Gata4* in endothelial cells causes embryonic lethality by E12.5 due to failure to promote endocardial cushion formation and remodeling (Rivera-Feliciano et al., 2006). Early myocardial specific deletion of *Gata4* results in myocardial thinning and hypoplastic endocardial cushions (Zeisberg et al., 2005). Moreover, haploinsufficiency of *Gata4* has been associated with cardiac defects including common atrioventricular canal, DORV and hypoplastic ventricular myocardium (Pu et al., 2004). Gata6 is expressed in myocytes but also in neural crest as well as endocardial and vascular smooth muscle cells (VSMC) (Nemer and Nemer, 2003). Inactivation of *Gata6* specifically in neural crest cells is sufficient to cause PTAs and lethality by E18.5-P2, revealing a role for GATA6 in the patterning of the OFT and aortic arch (Lepore et al., 2006). Loss of both *Gata4* and *Gata6* in mice leads to acardia, suggesting that genetic interactions between these factors are essential for the onset and/or maintenance of cardiogenesis (Zhao et al., 2008). Interestingly, mice with compound heterozygous mutation in *Gata4* and *Gata6* die embryonically around E13.5 due to vascular defects; in addition, these mice display PTA, evidence of failed OFT septation as well as myocardial thinning. These results are indicative of functional interactions between Gata4 and 6 in cardiac and vascular development (Xin et al., 2006). In contrast to GATA4 and 6, GATA5 expression is more restricted to endocardial cells and endocardial cushions of the OFT and atrioventricular canal during heart development. The dynamic expression of GATA5 in endocardial cells suggests a specific function for this transcription factor in endocardial development. Consistent with this, *faust* (which encode gata5) mutants in zebrafish have *cardia bifida* and lack endocardial cells (Reiter et al., 1999). In addition, downregulation of *Gata5* in an *in vitro* model of endocardial differentiation inhibits terminal differentiation and expression of endocardial differentiation markers (Nemer and Nemer, 2002). An important role for GATA5 in endocardial differentiation is further supported by recent findings showing that lack of *Gata5* in mice leads to bicuspid aortic valve formation (Laforest et al., 2011). Thus, all three cardiac GATA factors appear to play important functions in endocardial cushion development and/or outflow tract morphogenesis.

Because expression of the three cardiac GATA factors partially overlaps and since they can bind similar DNA regulatory elements and activate common target promoters through heterotypic interactions (Charron et al., 1999; Nemer and Nemer, 2003), we tested whether Gata5 might genetically interact with Gata4 and Gata6 in OFT development. Here we show that $Gata4^{+/-}Gata5^{+/-}$ and $Gata5^{+/-}Gata6^{+/-}$ double heterozygote mice die embryonically or perinatally due in large part to profound defects of OFT development including DORVs and VSDs. The results raise the possibility that subtle alterations in the level or activity of any 2 cardiac GATA factors might lead to human congenital heart defects.

Materials and methods

Animals

Mice handling and experimentation were performed in accordance with institutional guidelines. All protocols were approved by the institutional animal care committees.

Mice heterozygous for *Gata4*, *Gata5* and *Gata6* were generated and genotyped as previously described (Aries et al., 2004; Koutsourakis et al., 1999; Laforest et al., 2011). All lines were maintained in the C57/BL6 background. To generate double heterozygotes, mice heterozygous for *Gata4* or *Gata6* were mated to *Gata5* heterozygote mice and pregnant mothers or newborn litters were sacrificed at various embryonic timepoints and postnatal timepoints. The morning a vaginal plug was observed was defined as embryonic day (E) 0.5.

Histology

Whole embryos were fixed in 4% paraformaldehyde at 4 °C overnight, dehydrated through graded ethanol series, embedded in paraffin and sectioned at 4-μm intervals. Masson's trichrome staining was carried out on heart sections using standard procedures to visualize defects.

Cell count

Image J software was used to count the number of myocytes, endocardial cells and cushions cells in three different sections of 3–4 different heart samples for each genotype. The different cell types are easily distinguished morphologically in embryonic hearts.

Echocardiography

Transthoracic echocardiography was performed as described before (Aries et al., 2004) using a VisualSonics Vevo 770 ultrasound system with a RMV 707 30 MHz transducer. Doppler and M-mode imaging was obtained from 70 days old mice. Groups of 3–6 mice from different litters were used for the M-mode measurements and Doppler readings at 70 days.

Quantitative polymerase chain reaction (Q-PCR)

Total RNA was isolated from whole hearts at E12.5 with TRIZOL reagent (Invitrogen) according to the manufacturer's instruction. cDNAs were generated from 2 μg of total RNA using the Omniscript RT kit (Qiagen). Q-PCR was performed with cDNA diluted 1/100 using the Qiagen Q-PCR kit. Briefly, DNA template and 400 nM oligonucleotides were used at an annealing temperature of 58 °C using the Quantitect SYBR green PCR kit (QIAGEN) in an MX3500 real-time PCR machine (Stratagene, La Jolla, CA). Mean gene expression was corrected by GAPDH and calculated from wildtype and double heterozygote embryos ($n=3$–6 per group). Primers used were as previously reported (Laforest et al., 2011).

Statistical analysis

Values are presented as mean ± S.E.M. and n refers to the number of mice per group. For comparison of multiple groups, a one-way ANOVA statistical test was performed. A Student's two-tailed t-test was then performed to confirm statistical significance between two groups. Statistical significance was considered as $P<0.05$.

Results

Reduced viability of $Gata4^{+/-}Gata5^{+/-}$ embryos

Both Gata4 and Gata5 are expressed in the endocardial cushions at the same embryonic stages (between E9 and E12). We investigated possible *in vivo* interactions between them in heart development by crossing mice heterozygous for either a *Gata5* or a *Gata4* allele. According to Mendelian transmission, equal ratios of wildtypes, *Gata4* heterozygotes, *Gata5* heterozygotes and *Gata4*/*Gata5* double heterozygotes were expected. However, at weaning, $Gata4^{+/-}Gata5^{+/-}$ double heterozygotes (*G4*/*G5* hets) were obtained at far lower frequency than expected (6% *vs* 25%) (Fig. 1A). Analysis of embryos from timed matings suggested high perinatal lethality of *G4*/*G5* hets, although decreased viability was evident starting at E14.5 (Fig. 1A). Visual inspection of the $Gata4^{+/-}Gata5^{+/-}$ heterozygotes at E11.5 revealed no gross abnormalities but by E15.5, it was evident that the $Gata4^{+/-}Gata5^{+/-}$ heterozygotes were smaller (Figs. 1B–K). To determine if this phenotype was caused by growth retardation, the number of somites was calculated at E10.5; no significant changes were observed among all

A

Age	No. Genotyped	WT	*Gata4*$^{+/-}$	*Gata5*$^{+/-}$	*Gata4*$^{+/-}$*Gata5*$^{+/-}$
P30	71	28 (24%)	11 (15%)	28 (39%)	4 (6%)
E17.5-E18.5	30	8 (27%)	9 (30%)	6 (20%)	6 (20%)
E14.5-E15.5	107	28 (26%)	26 (24%)	33 (30%)	19 (18%)
E11.5-E12.5	75	14 (23%)	20 (27%)	18 (24%)	18 (24%)

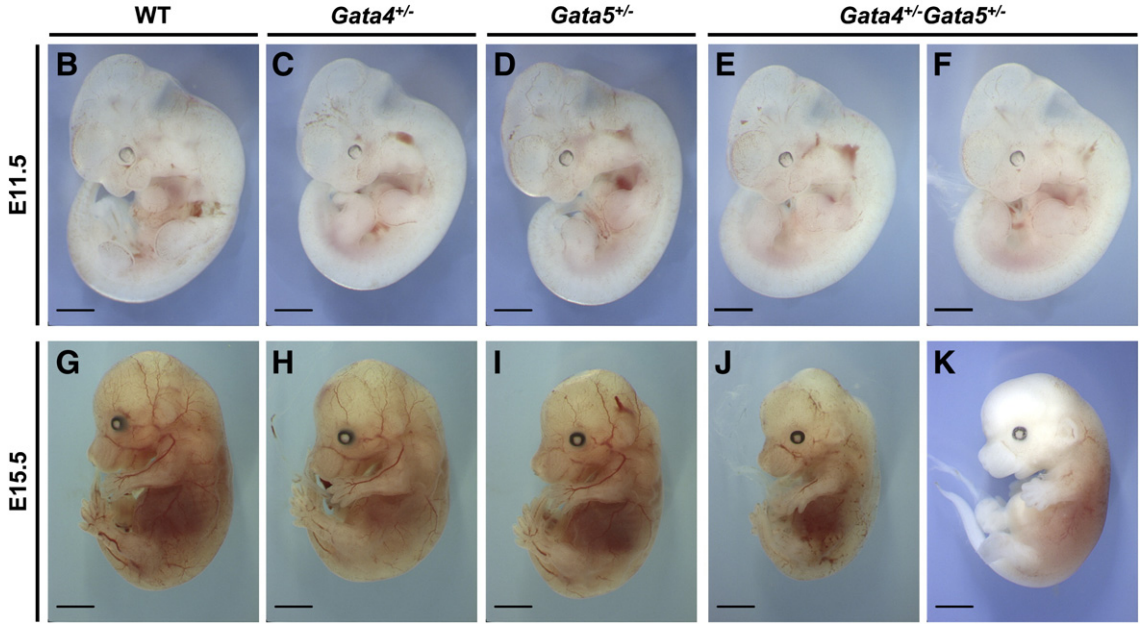


Fig. 1. Reduced viability of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes. (A) Frequency of genotypes obtained from intercrossing *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ mice. Note the reduced viability of compound *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes at weaning and the reduced frequency of genotypes (by 25%) in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ starting at E14.5; another 50% is lost perinatally. (B–K) Representative images of E11.5 (E, F) and E15.5 (J, K) in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes as compared to their littermates. Note the reduced size of double het embryos at E15.5. Embryonic lethality of 30% of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ is noted as shown in a representative embryo (K). Bars = 1000 μm (B–F) and 2000 μm (G–K).

genotypes, indicating that the *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes were not developmentally retarded. Crown–rump measurement of the embryo size at E15.5 however revealed that the *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes are significantly smaller than all their other littermates (WT, 186.77 mm ± 3.90; *Gata4*$^{+/-}$, 183.90 mm ± 3.15; *Gata5*$^{+/-}$, 180.60 mm ± 2.85 and *Gata4*$^{+/-}$*Gata5*$^{+/-}$, 166.18 mm ± 2.71, $P<0.005$). Moreover, 30% of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos were dead at E15.5 (Fig. 1K).

Gata4$^{+/-}$*Gata5*$^{+/-}$ *double heterozygotes display DORVs and VSDs*

In order to determine the cause of lethality, histological analyses were performed on E15.5 *Gata4*$^{+/-}$*Gata5*$^{+/-}$ double heterozygotes. They revealed profound structural cardiac defects. A double outlet right ventricle (*i.e.* the aortic valve opens in the right ventricle instead of the left ventricle) as well as large ventricular septal defects was evident in 86% of embryos (Figs. 2C, D, G and H). This was not observed in *Gata5*$^{+/-}$ heterozygote littermates and only 29% of *Gata4*$^{+/-}$ had a DORV (Table 1). Moreover, some *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos (2/7 embryos) had a complete atrioventricular canal defect, meaning a single atrioventricular valve associated with atrial and ventricular septal defects (Fig. 2L and Table 1). *Gata5*$^{+/-}$ heterozygote littermates did not demonstrate this defect and had two separate atrioventricular valves while 1/7 *Gata4*$^{+/-}$ embryo (only 14%) had a common atrioventricular valve (Figs. 2I–J). As both GATA4 and GATA5 are expressed in the atrioventricular (AV) cushion, we were interested to know if the valve morphology was normal. We found that the tricuspid and mitral valves of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ double heterozygotes were hypertrophied compared to *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates, a situation that could cause stenosis (Fig. 2K and Table 1). As these defects are all thought to arise from abnormal development of endocardial cushions, the results suggest that Gata4 and Gata5 genetically interact for proper EC development.

Gata4 and Gata5 cooperate in endocardial cushion formation

Histological analysis of tissue sections at E11.5 revealed smaller hypotrabeculated hearts in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ compared to single heterozygous or wildtype littermates (Figs. 3A–D). Higher magnification of the left ventricle also revealed a reduction in the left ventricular wall (Figs. 3E–H). Cell counting indicated significant decreases in the number of myocytes and endocardial cells in the left ventricle of *G4/G5* hets at E11.5 compared to *Gata5*$^{+/-}$ but not to *Gata4*$^{+/-}$ littermates (Fig. 3Q). By E15.5, 71% (5/7) *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos had thinning of the left and right ventricular compact zone (WT, 15.33 mm ± 0.78; *Gata5*$^{+/-}$, 15.91 mm ± 0.13; *Gata4*$^{+/-}$, 10.44 mm ± 0.52 and *Gata4*$^{+/-}$*Gata5*$^{+/-}$, 8.70 mm ± 1.44) (Figs. 3I–L). Of note, similar ventricular hypoplasia was observed in the *Gata4*$^{+/-}$ embryos at E11.5 and E15.5, suggesting that absence of a Gata5 allele does not appear to worsen the myocardial defects of Gata4 haploinsufficient hearts (Figs. 3F and J).

At E15.5 *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos show AV canal defects, VSDs and hypertrophied AV valves. These structures arise from endocardial

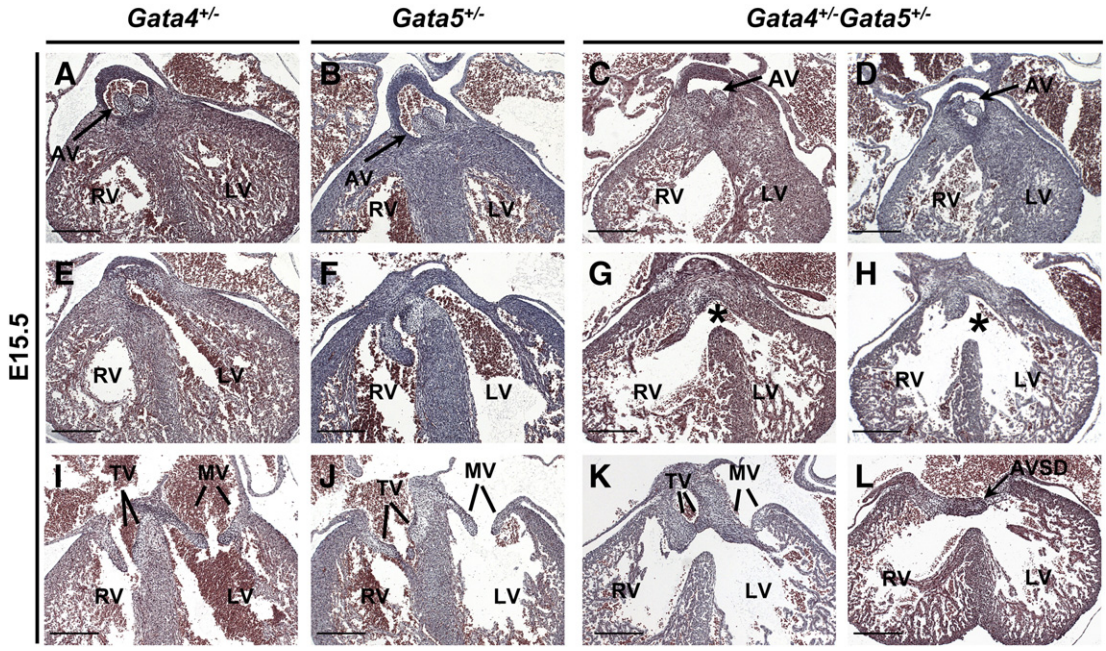


Fig. 2. Cardiac defects in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes. Trichrome staining of transverse sections of E15.5 embryos. Note how in single hets (A and B), the aortic valve opens in the left ventricle whereas in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos, the aortic valve opens in the right ventricle (C and D), a phenotype known as double outlet right ventricle (DORV). (E–H) Transverse section through the heart showing an intact ventricular septum in *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ embryos and a membranous ventricular septal defect (VSD) in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos (star in G and H). (I–L) Transverse section through the heart showing normal mitral and tricuspid valves in the single hets, whereas the majority of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos have mitral and tricuspid stenosis (K) and a minority have a common atrioventricular valve (L). Bars = 300 μm. AV, aortic valve; LV, left ventricle; RV, right ventricle.

cushion and Gata4 and Gata5 are co-expressed between E9.5 and E12.5. Consistent with a role for both factors therein, endocardial cushions of *Gata4*/*Gata5* compound heterozygote embryos were smaller but properly formed (Figs. 3M–P). *Gata4*$^{+/-}$*Gata5*$^{+/-}$ mutant embryos showed a larger reduction in the total number of cushion cells within the atrioventricular endocardial cushion compared to *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and wildtype littermates. These results indicate that GATA4 and GATA5 cooperate in endocardial cushion formation in the AV canal.

Q-PCR analysis on RNA isolated from hearts of WT and *Gata4*/*Gata5* compound mutants at E12.5 revealed that *Gata4* and *Gata5* transcripts were reduced by 50% and 60% compared to WT littermates with no change in *Gata6* levels (Figs. 4A–C). Interestingly, we noticed an upregulation of *Nppb* transcripts only in double hets, possibly indicative of early cardiac stress. Consistent with this, the level of several cardiac genes was specifically altered in the double hets as compared with control or single het littermates (Fig. 4G). *Mef2c*, *Tbx20* and *Bmp7* transcripts were significantly decreased only in *G4*/*G5* mice (Figs. 4D, F and I). Moreover, we observed more pronounced decreases in mRNA levels of components of the Notch pathway in double hets relative to *Gata4*$^{+/-}$, which already displayed changes compared to wildtype and *Gata5*$^{+/-}$ littermates. They include the Notch ligand *Jag1*, *Notch1* and the Notch target *Hey1* (Figs. 4J–L). No significant alteration in the level of other genes implicated in endocardial cushion formation like *Tgfβ1*, *Tgfβ2*, *Bmp2*, *Bmp4*, *erbb3* and *Hey2* was evident (data not shown).

Table 1
Summary of the cardiac phenotypes of the *Gata4*/*Gata5* double heterozygotes at E15.5. MV, mitral valve; TV, tricuspid valve, VSD, ventricular septal defect.

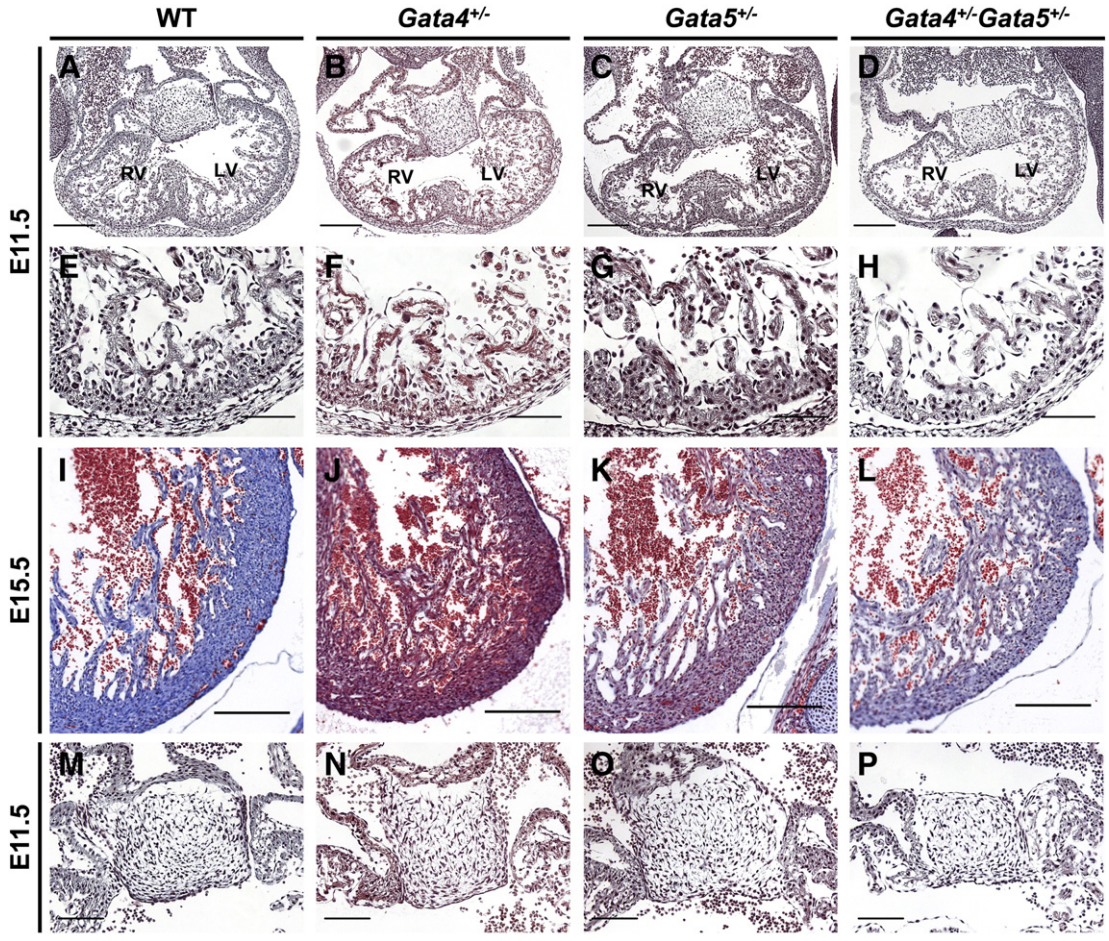
Phenotype	WT	Gata4$^{+/-}$	Gata5$^{+/-}$	Gata5$^{+/-}$Gata4$^{+/-}$
Normal	5/5	3/7	5/5	1/7
Double outlet right ventricle	0/5	2/7	0/5	6/7
Small VSD	0/5	3/7	0/6	1/7
Large membranous VSD	0/5	2/7	0/5	5/7
Atrioventricular septal defect	0/5	1/7	0/5	2/7
Thick MV and TV	0/5	2/7	0/5	4/7
Myocardial thinning	0/5	4/7	0/5	5/7

Adult Gata4/Gata5 compound mutants have aortic stenosis

Only 25% of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ double heterozygotes survive to adulthood; we were interested in assessing cardiac structure and function in this subgroup. Data obtained from echocardiographic measurements on 70 days old mice, revealed increased left ventricular mass and significantly decreased ejection fraction in adult G4/G5 hets compared to wildtype, *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates (Fig. 5A and data not shown). Hemodynamic evaluation revealed increased mean pressure gradient through the aortic valve of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ double heterozygotes (Fig. 5B). As shown on the graph, nearly half of the G4/G5 hets (3/7) had a very high pressure gradient compared to wildtype, *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates. These observations suggested the presence of aortic valve constriction. Histological analysis of these hearts confirmed the presence of left ventricular hypertrophy compared to wildtype and *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates and revealed massively hypertrophied aortic valve in adult *Gata4*$^{+/-}$*Gata5*$^{+/-}$ double heterozygotes (Fig. 5G). Together, the results are consistent with defective valve development leading to aortic stenosis.

Disrupted valvulogenesis in Gata5$^{+/-}$*Gata6*$^{+/-}$ *embryos*

The Gata5 protein also overlaps with Gata6 in outflow tract cushions. To determine if Gata6 and Gata5 genetically interact, we generated *Gata5*$^{+/-}$*Gata6*$^{+/-}$ mice. Only 1% of the expected *Gata5*$^{+/-}$*Gata6*$^{+/-}$ double heterozygotes (*G5*/*G6* hets) were present at weaning (postnatal day 30), suggesting that the combined mutations result in embryonic or perinatal lethality (Fig. 6A). Analysis of 13 post-natal litters revealed that 47% (10/21 embryos) of mice heterozygous for both *Gata5* and *Gata6* were dead at birth. Postmortem analysis of these *Gata5*$^{+/-}$*Gata6*$^{+/-}$ hearts revealed a 100% penetrance for DORV, associated with massive dilatation of the right atrium. Analysis of E15.5 and E18.5 embryos indicated reduced



Q

Number	WT	*Gata4*$^{+/-}$	*Gata5*$^{+/-}$	*Gata4*$^{+/-}$*Gata5*$^{+/-}$
Myocytes in LV	333,06 ± 17,04	209,5 ± 17,68 ***	324,92 ± 10,72	209,67 ± 7,38 ***
Endocardial cells in LV	126,5 ± 5	114,42 ± 4,88	126,25 ± 5,13	109,24 ± 2,77 *
Cushion cells in AVC	273,47 ± 23	199,33 ± 18,90 *	242,53 ± 28,53	158,5 ± 10,16 **

Fig. 3. Ventricular wall and endocardial cushion defects in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos. Trichrome staining of transverse sections of wildtype, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata4*$^{+/-}$*Gata5*$^{+/-}$ E11.5 embryos. (A–D) Note ventricular wall thinning and hypotrabeculation in double hets as well as in *Gata4* het. Bars = 200 μm. (E–H) Higher magnification of the left ventricular wall. Bars = 75 μm. (I–L) Trichrome staining of the left ventricular wall of wildtype, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata4*$^{+/-}$*Gata5*$^{+/-}$ E15.5 embryos. Note the reduced compact zone thickness in the double hets as well as in *Gata4* hets. Bars = 300 μm. (M–P) Transverse section through the AV endocardial cushions of WT, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos at E11.5. Endocardial cushions of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos are properly formed although a bit smaller. Bars = 100 μm. (Q) Quantification of the number of cells in the left ventricle or AV canal (* $P<0.05$, ** $P<0.001$, *** $P<0.0001$). Statistical significance is compared to WT controls. AVC, atrioventricular canal; LV, left ventricle.

Mendelian ratios (Figs. 6A, F–J). No growth retardation was observed at any of the stages and crown–rump measurement of the embryos (WT, 183.64 mm ± 7.75; *Gata5*$^{+/-}$, 186.02 mm ± 5.42; *Gata6*$^{+/-}$, 186.24 mm ± 2.21; *Gata5*$^{+/-}$*Gata6*$^{+/-}$, 182.86 mm ± 2.10) at E15.5 were not significantly different.

To determine the etiology of the embryonic and perinatal lethality of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos, we sectioned embryos at E15.5, when both ventricles are completely septated and the four valves of the heart have formed. Histological analysis at this stage demonstrated the presence of a DORV in 86% of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos (Fig. 7D). Similar to the *Gata4*/*Gata5* compound embryos, we also found the presence of VSDs but only at the level of the aortic valve (Fig. 7H). The VSD was present over 30–50 μm, which was much smaller than the ones in the *Gata4*/*Gata5* double heterozygotes (125–200 μm). We also noticed that a subset of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos had increased thickness of the left ventricular compact zone compared to WT, *Gata5*$^{+/-}$ and *Gata6*$^{+/-}$ littermates (data not shown). The AV and semilunar valves were formed properly and did not show signs of hypertrophy. No other cardiac defects were noted. A summary of the phenotypes is given in Table 2.

Gene expression studies were performed on RNA isolated from hearts of wildtype, *Gata5*$^{+/-}$, *Gata6*$^{+/-}$ and *Gata5*$^{+/-}$*Gata6*$^{+/-}$ mutants at E12.5. Expression of *Gata5* and *Gata6* transcripts was reduced by 40–50% in compound mutants while the expression of *Gata4* was not significantly altered (Figs. 8 A–C). In contrast to what was observed in the *G4*/*G5* hets, *Mef2c* transcripts were unchanged and *Nppb* mRNA was downregulated in *G5*/*G6* hets (Figs. 8D and G). *Tbx20* mRNA levels were more modestly but significantly decreased in these embryos (Fig. 8F). A 2-fold increase in *Raldh2* mRNA levels was noted only in the double hets, which could potentially explain the presence of DORV as excess retinoic acid signaling leads to conotruncal malformations such as TGA, DORV and TOF (Nakajima

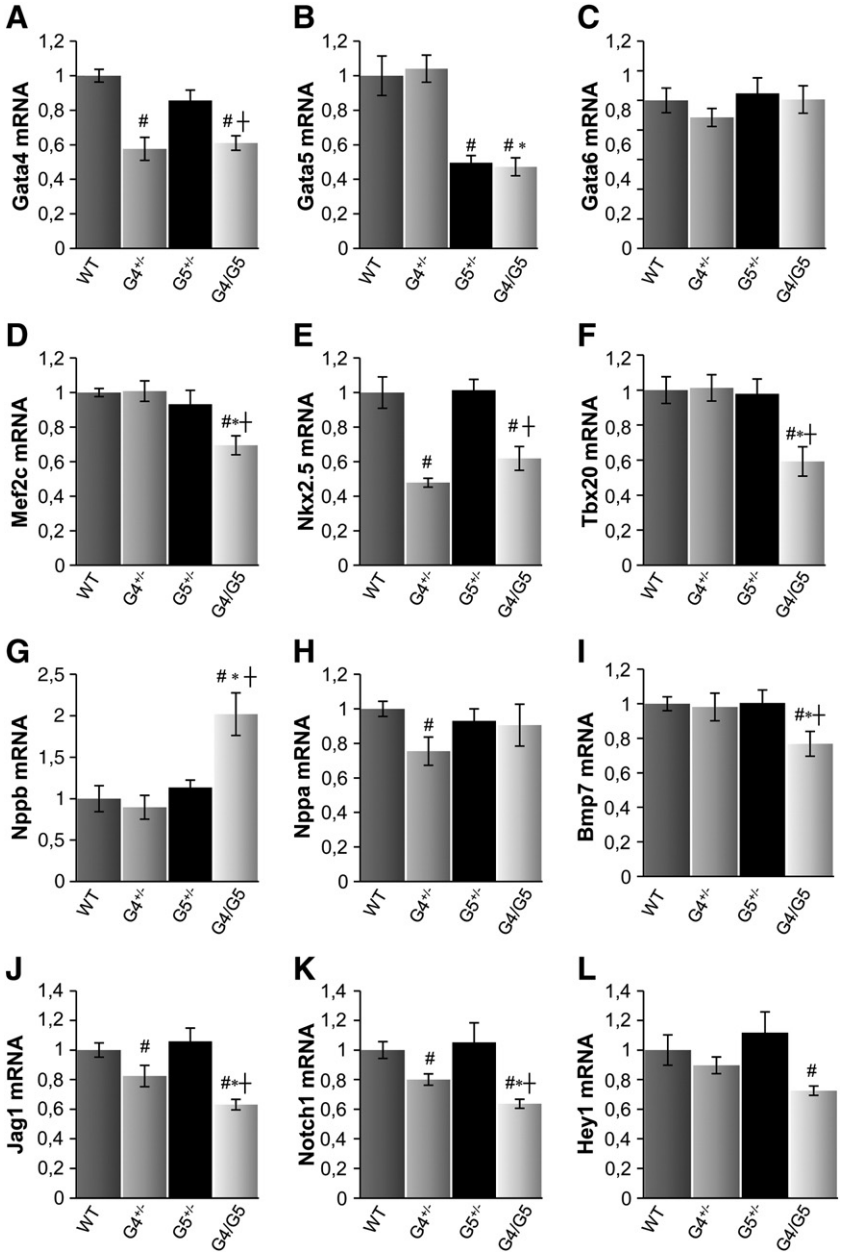


Fig. 4. Modulation of gene expression in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos. (A–L) Q-PCR analysis performed on whole hearts of E12.5 embryos. Transcript levels are normalized to GAPDH in each sample. Note the downregulation of *Mef2c*, *Nkx2.5*, *Tbx20*, *Bmp7*, *Jag1*, *Notch1* and *Hey1* transcripts and the upregulation of *Nppb* transcripts in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos compared to wildtype littermates (*, ┼, # $P<0.05$; $n=3$–6 per genotype. * Statistical significance *vs Gata4*$^{+/-}$; ┼ statistical significance *vs Gata5*$^{+/-}$; and # statistical significance *vs* WT).

et al., 1996). A significant 30% decrease of *Hand2* transcripts was also observed in *Gata5*/*Gata6* compound embryos (Fig. 8H). Together, the data reveal important genetic interactions between Gata5 and Gata6 in outflow tract development and identify potential new GATA target genes therein.

Discussion

Transcription factors Gata4, Gata5 and Gata6 are present at various stages of heart development and are important cardiac regulators (Nemer and Nemer, 2010). Within the heart, their expression pattern is unique but two or more GATA factors are often co-expressed in distinct cell types or at specific developmental stages. Most notably, expression of Gata5 overlaps with that of Gata4 in endocardial cells and with that of Gata6 in cushion cells of the outflow tract; all 3 factors are present in the secondary heart field. In the present study, we show that compound haploinsufficiency of *Gata5* and either *Gata4* or *Gata6* results in embryonic and perinatal lethality due to defective heart formation. The cardiac phenotype of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes include DORV, very large VSDs, complete AV canal defects and mitral and tricuspid stenosis. The *Gata4*/*Gata5* compound heterozygotes who survive to adulthood show signs of aortic stenosis. Similarly, compound haploinsufficiency of *Gata5* and *Gata6* leads to cardiac anomalies that include DORVs and VSDs. The defects observed in the double heterozygote embryos are either distinct or more pronounced than those found in their single heterozygote parents or littermates, indicative of a cooperative function for GATA factors during cardiac morphogenesis. The phenotype of the *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes reported here differs from the one recently published by Singh K et al. (Singh et al., 2010). In their study, Singh K et al. showed that *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos had myocardial thinning by E14.5 but were viable; however *Gata4*$^{+/-}$*Gata5*$^{-/-}$ embryos had a more severe

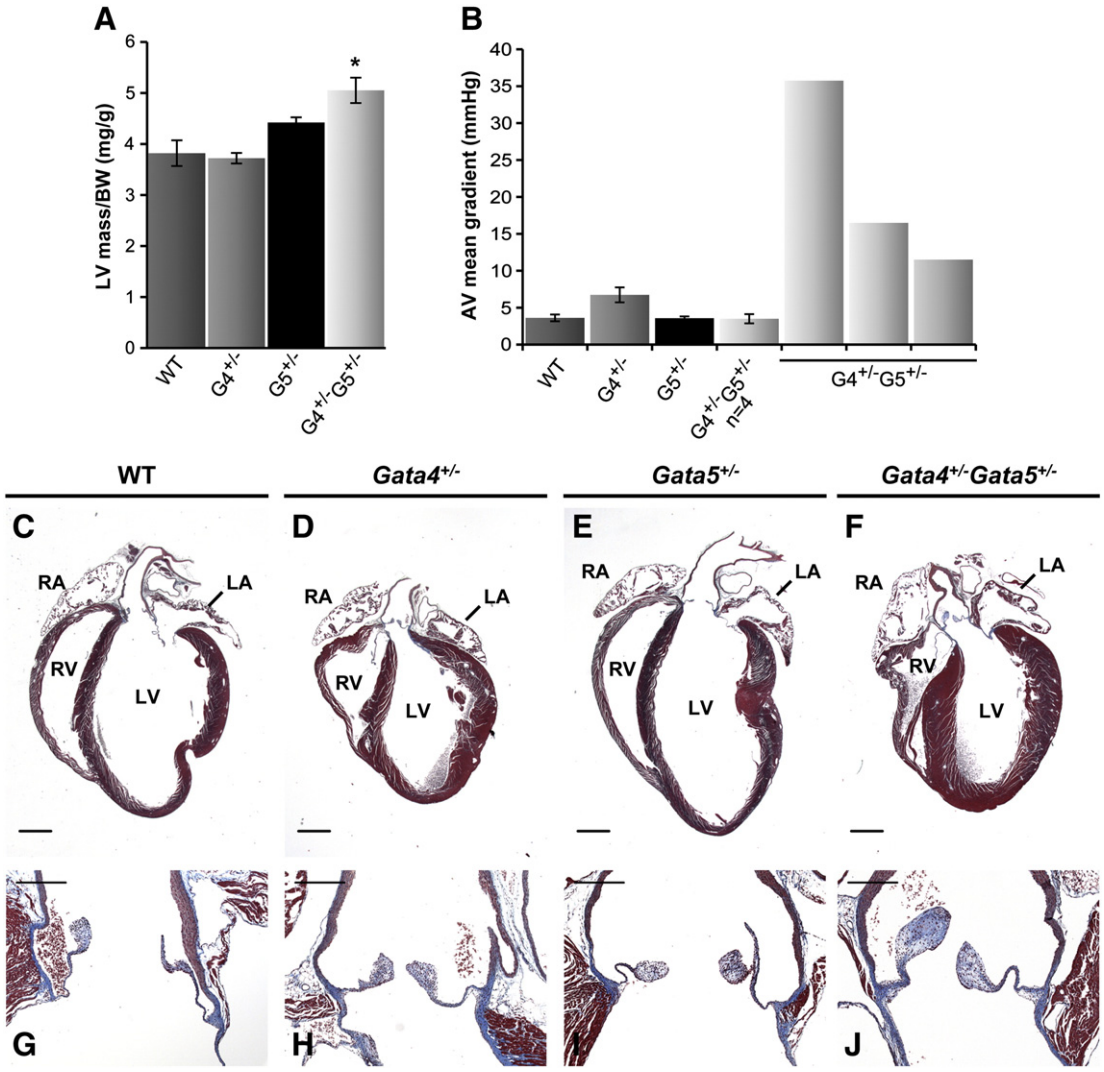


Fig. 5. Aortic stenosis in adult *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes. (A) Echocardiography of wildtype, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata4*$^{+/-}$*Gata5*$^{+/-}$ mice at 70 days (* $P<0.5$, $n=3$–6 per group). Note the increase in LV mass over body weight for *Gata4*$^{+/-}$*Gata5*$^{+/-}$ mice, suggesting left ventricular hypertrophy. (B) Echocardiography of wild-type, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata4*$^{+/-}$*Gata5*$^{+/-}$ mice at 70 showing increased pressure gradients through the aortic valve (AV) ($n=5$ for WT, *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates). Three out of seven *Gata4*/*Gata5* compound heterozygotes have very high mean pressure gradient through the aortic valve. (C–F) Trichrome staining of a frontal section from 90 days old mice. Note the increased ventricular hypertrophy of *Gata4*/*Gata5* compound heterozygotes. Bar = 1000 μm. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. (G–J) Trichrome staining of a frontal section of the aortic valve in these same mice. Note the hypertrophied aortic valve in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes compared to wildtype, *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates. Bar = 300 μm.

phenotype that included AV canal defects and myocardial thinning similar to our *G4*/*G5* hets. This indicates increased severity of the phenotype in our crosses, consistent with the heightened phenotype of our *Gata5* null mice as compared to theirs (Laforest et al., 2011). Given this and the contribution of the genetic background to cardiac defects in *Gata4*$^{+/-}$ mice (Pu et al., 2004), we hypothesize that differences in the genetic background of our lines as compared to those of Singh et al. underlie the differences in cardiac phenotype expressivity. Previously, haploinsufficiency of both *Gata4* and *Gata6* revealed an important role for these 2 GATA factors in cardiomyocyte proliferation and OFT septation (Xin et al., 2006; Zhao et al., 2008). While we had no evidence for genetic interaction between Gata5 and either of the other 2 cardiac GATA proteins in myocardial proliferation there was clear evidence of genetic interaction in cushion formation and OFT remodeling. Together with these studies, our present work underscores the exquisite sensitivity of the OFT to GATA protein dosage and reveals the importance of cooperative interactions between any 2 cardiac GATA factors in regulating various events of cardiovascular development.

Cooperative roles of GATA4, 5 and 6 in cardiac development

The majority of *Gata4*/*Gata5* and *Gata5*/*Gata6* double heterozygote embryos developed DORVs, which is thought to occur from abnormal rotation of the OFT. Results of previous work have suggested that the myocardium of the OFT and the right ventricle are derived from a population of cells located within the secondary heart field (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). According to Bajolle F et al., the myocardium at the base of the OFT is required for normal positioning of the great vessels (Bajolle et al., 2008). Germline *Gata4* and *Gata6* mutant mice die too early embryonically to be able to evaluate their function in OFT morphogenesis (Koutsourakis et al., 1999; Kuo et al., 1997; Molkentin et al., 1997). *Gata4* hypomorphs have DORVs, clearly indicating that Gata4 is required in OFT rotation (Crispino et al., 2001; Pu et al., 2004). However, this phenotype was not produced when *Gata4* was disrupted in the SHF leading the authors to suggest that Gata5 and Gata6 might compensate for Gata4 in the SHF as they were robustly expressed in the pharyngeal mesoderm (Rojas et al., 2008). Deletion of

A

Genotype	No. Genotyped	WT	*Gata5*$^{+/-}$	*Gata6*$^{+/-}$	*Gata5*$^{+/-}$*Gata6*$^{+/-}$
P30	99	26 (26%)	54 (55%)	18 (18%)	1 (1%)
P0-P1	105	37 (35%)	26 (25%)	20 (19%)	11 (10%)
E18.5	91	22 (24%)	23 (25%)	30 (32%)	14 (15%)
E14.5 - E15.5	53	13 (25%)	12 (23%)	17 (32%)	9 (17%)
E11.5-E12.5	68	14 (21%)	15 (22%)	13 (19%)	23 (33%)

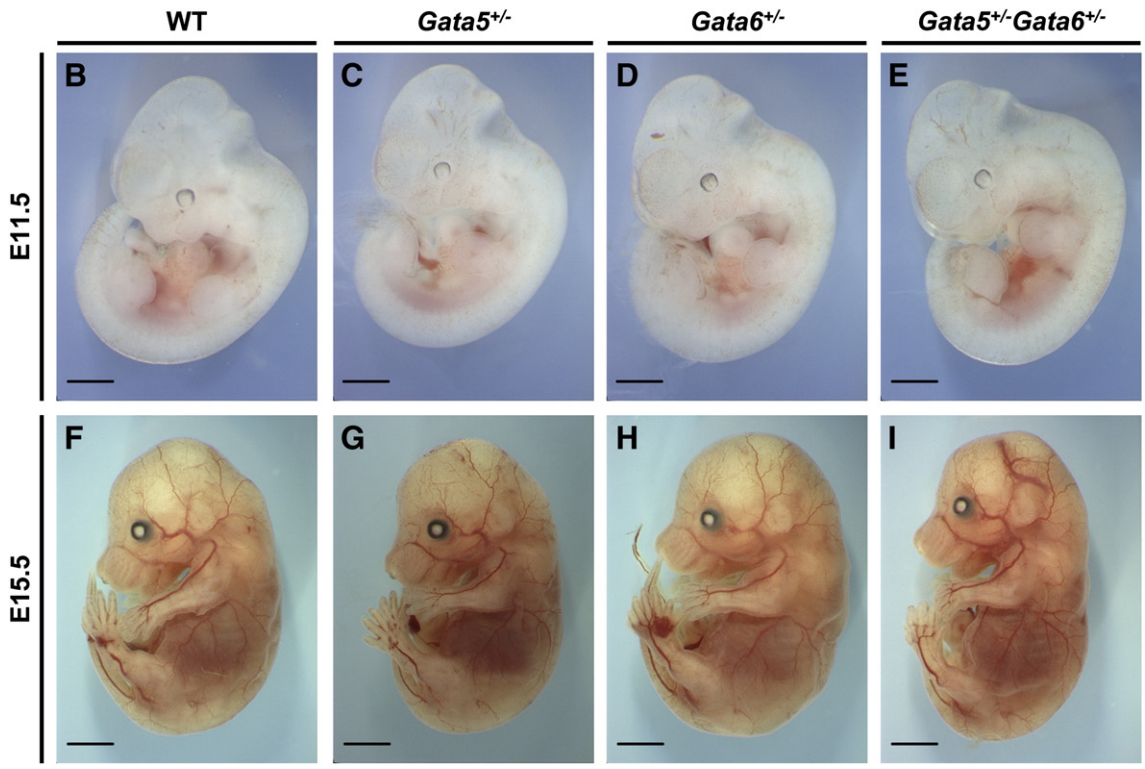


Fig. 6. Reduced viability of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes. (A) Frequency of genotypes obtained from intercrossing *Gata5*$^{+/-}$ and *Gata6*$^{+/-}$ mice. Embryonic and perinatal lethality of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes is demonstrated by the reduced frequencies of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos at various developmental stages. (B–J) Dissection and visual inspection of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes. Bars = 1000 μm (B–E), 2000 μm (F–J).

Gata6 in cardiac crest cells using *SM22*-Cre or *Wnt1*-Cre reporter mice results in the formation of PTAs and DORVs and *Gata4*/*Gata6* compound heterozygosity leads to the formation of a single OFT vessel (Lepore et al., 2006). These observations, coupled with our results, clearly indicate that OFT defects seem to be a common consequence of loss of two GATA alleles, suggesting that a threshold of GATA factors is essential in the rotation and patterning of the OFT. Whether or not this reflects action of GATA factors in the same cell type (SHF or endocardial derived) or complementation of a defective pathway arising from 2 distinct lineages remain to be defined.

Endocardial cushion defects of Gata4$^{+/-}$*Gata5*$^{+/-}$ *embryos*

Our work demonstrates that concomitant loss of one allele of *Gata4* with one allele of *Gata5* leads to VSDs, AV canal defects and AV valve hypertrophy with variable penetrance. These anomalies are thought to arise from abnormal endocardial cushion formation and are consistent with the presence and role of Gata4 and Gata5 in endocardial cell expansion and differentiation (Nemer and Nemer, 2002; Rivera-Feliciano et al., 2006). The process of endocardial cushion formation starts at E9.5 in the mouse and requires complex interactions between the endocardium and myocardium. In response to diverse signals, endocardial cells go through an epithelial-to-mesenchymal transformation (EMT) and migrate into the cushion jelly; later in development these cushions will fuse together and will be remodeled to form the valves of the hearts (Person et al., 2005). In the compound *Gata4*/*Gata5* heterozygotes, AV cushion formation appears relatively normal at E11.5. However, we noticed reduced number of cushion cells within the endocardial cushion, similar to the decreased number of mesenchymal cells in the AV cushion of *Gata5* null mice, suggestive of a role for both Gata4 and Gata5 in cushion formation (Laforest et al., 2011). Whether these interactions involve

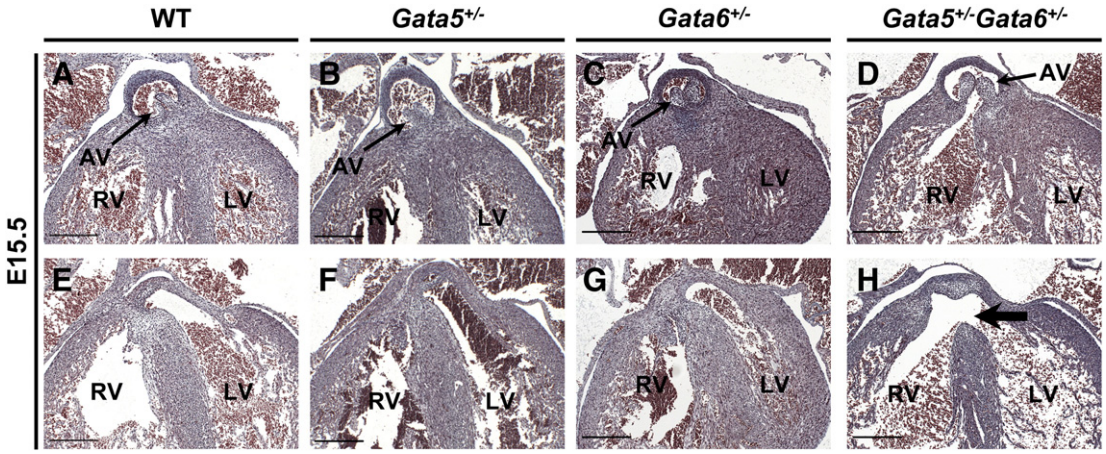


Fig. 7. Cardiac defects of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes. Trichrome staining of transverse sections of E15.5 wildtype (A, E), *Gata5*$^{+/-}$ (B, F), *Gata6*$^{+/-}$ (C, G) and *Gata5*$^{+/-}$*Gata6*$^{+/-}$ (D, H) embryos. In wildtype, *Gata5*$^{+/-}$ and *Gata6*$^{+/-}$ embryos (A–C), the aortic valve opens in the left ventricle. In *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos, the aortic valve opens in the right ventricle (D), leading to double outlet right ventricle (DORV). (E–H) Transverse section through the heart showing an intact ventricular septum in wildtype, *Gata5*$^{+/-}$ and *Gata6*$^{+/-}$ embryos. In *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos (H), a membranous ventricular septal defect (VSD) is observed, which is associated with the DORV (arrow). Bars = 300 μm (A–D) and 100 μm (E–H). AV, aortic valve; LV, left ventricle; RV, right ventricle.

myocardial–endocardial crosstalk or reflect cell autonomous function in the endocardium where both factors are expressed will need to be clarified. Generation of *Gata4*/*Gata5* double heterozygotes in which Gata4 and Gata5 are deleted only in endocardial cells will help address this question.

GATA downstream targets

The cardiac GATA factors have been shown to regulate a large number of cardiac genes including *Nppa*, *Nppb*, *Mef2c*, *Nkx2.5*, *Bmp4*, *Myh6* and *Myh7* (Charron et al., 1999; Dodou et al., 2004; Grepin et al., 1994; Nemer and Nemer, 2003). Moreover, Gata4 and Gata6 are able to heterodimerize and synergistically activate several of these promoters (Charron et al., 1999). While the phenotype of *G4*/*G5* and *G5*/*G6* double hets was very similar, distinct changes in gene expression were noted suggesting that genetic interactions between Gata5 and the other 2 Gata factors disrupt some common but also different developmental pathways. For example, haploinsufficiency of *Gata5* combined with loss of either a *Gata4* or *Gata6* allele resulted in reduced expression of *Nkx2.5*, *Tbx20* and *Hand2* but Mef2c levels were changed only in *Gata4*/*Gata5* double hets. Loss of Hand2 results in embryonic lethality and absence of right ventricle formation (Srivastava et al., 1997). Hand2 is expressed in myocardial, endocardial and neural crest lineages. Interestingly, deletion of Hand2 from neural crest cells is sufficient to produce an identical phenotype as the one reported here (*i.e.* DORVs and VSDs) possibly through regulation of proliferation and differentiation of SHF derived cells (Holler et al., 2010; Morikawa and Cserjesi, 2008). Moreover, Hand2 transcription was shown to be GATA dependent (McFadden et al., 2000). Similarly, loss of Tbx20 revealed a critical role in OFT development (Takeuchi et al., 2005). Tbx20 is expressed in both myocardial and endocardial cells where it is required for proliferation and differentiation (Shelton and Yutzey, 2008). A role for GATA factors in regulating Tbx20 has not yet been reported in the mammalian heart but in *Drosophila*, the Tbx20 ortholog, neuromancer was shown to be a downstream mediator of the GATA factor pannier in the heart (Qian and Bodmer, 2009). On the other hand, Nkx2.5 transcription is GATA dependent and loss of Nkx2.5 causes septal, valvular and myocardial dysmorphogenesis (Biben et al., 2000; Grepin et al., 1997; Lien et al., 1999). Our data reveal exquisite sensitivity of Nkx2.5 to Gata4 and Gata6 levels but not to Gata5 possibly reflecting changes in myocytes where Gata5 is not detectably expressed. Lastly, it is noteworthy that Raldh2 is upregulated only in *G5*/*G6* hets. Excess retinoic acid signaling specifically inhibit OFT cushion remodeling while *Raldh2* null mice have cardiac abnormalities due to defective SHF cell differentiation (X. Lin et al., 2010; Nakajima et al., 1996). Dysregulation of Rald2 specifically in G5/G6 hets may account for several of the cardiac phenotypes observed in the double *G5*/*G6* embryos. Interestingly, *G4*/*G5* double hets but not *G5*/*G6* double hets showed decreased expression of Bmp7 and several components of the Notch pathway, which is critical for proper cushion morphogenesis especially for outflow tract septation and valve formation (High et al., 2009; Luna-Zurita et al., 2010; Rutenberg et al., 2006; Timmerman et al., 2004). Future studies will need to determine whether this specificity reflects differential regulation of downstream GATA targets or cell specific interactions between Gata5 and the other 2 GATA proteins (in early endocardial cells Gata4 but not Gata6 is prominently expressed).

Table 2
Summary of the cardiac phenotypes of the *Gata5*/*Gata6* double heterozygotes at E15.5. VSD, ventricular septal defect.

Phenotype	WT	Gata5$^{+/-}$	Gata6$^{+/-}$	Gata5$^{+/-}$Gata6$^{+/-}$
Normal	5/5	5/5	5/5	1/7
Double outlet right ventricle	0/5	0/5	0/5	6/7
Membranous VSD	0/5	0/5	0/5	6/7
Thick myocardium	0/5	0/5	0/5	5/7

GATA4, GATA5 and GATA6 in human CHD

Heterozygous mutations in *GATA4* and *GATA6* have been linked to congenital heart defects in humans (Garg et al., 2003; Kodo et al., 2009; Nemer et al., 2006; Pehlivan et al., 1999; Zhang et al., 2008). The CHDs associated with mutations in *GATA4* are predominantly ASDs, VSDs but DORV, TOF and pulmonary stenosis have also been reported. Recently, mutations in *GATA6* in humans have been associated with PTA. To date, no *GATA5* mutations have been reported in human CHD.

Despite the significant efforts of the past decade to elucidate the molecular mechanisms and the genetic basis of congenital heart defects, the fundamental causes for the majority of CHD remain unidentified. CHD has an increased risk of recurrence within families; among affected relatives, the cardiac phenotypes are usually different and occur with variable penetrance and expressivity, likely indicating that modifying factors including genetic and environmental influence the phenotype (Gill et al., 2003). Consistent with this, influences of the genetic background on the phenotype are now well documented in experimental animal models and in humans (Bruneau et al., 2001; Rajagopal et al., 2007; Winston et al., 2010). The results of the present

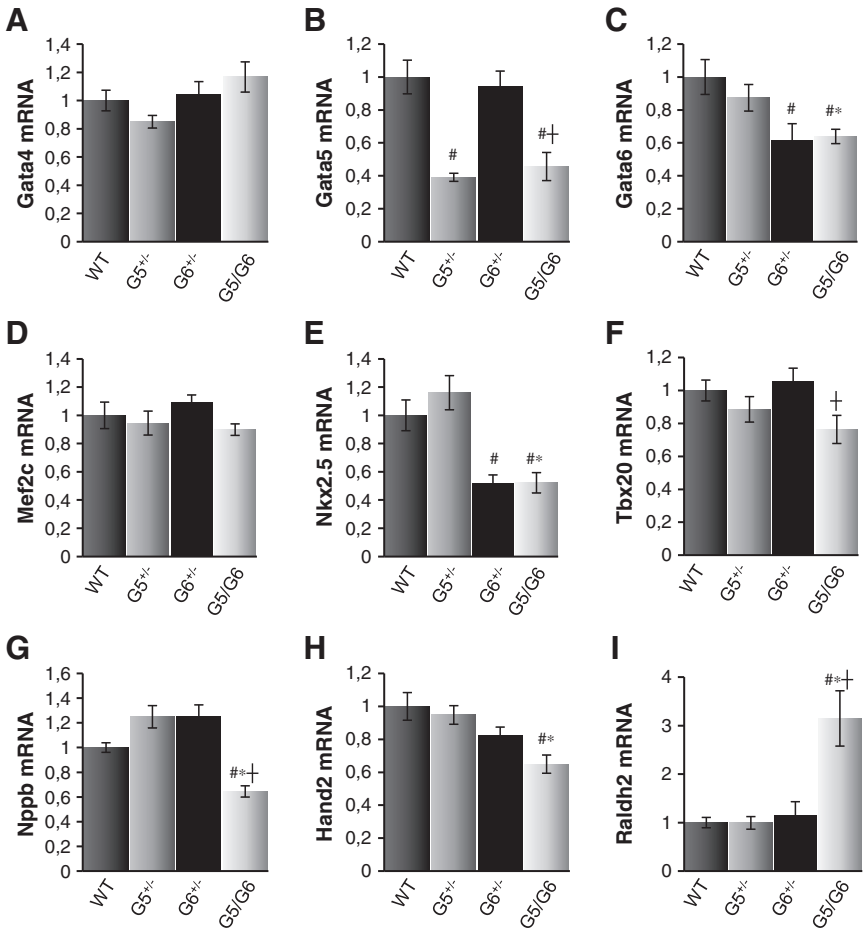


Fig. 8. Modulation of gene expression in *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos. (A–I) Q-PCR analysis performed on hearts of E12.5 embryos. Transcript levels are normalized to GAPDH in each sample. Expression of *Gata5* and *Gata6* is reduced by 40–50% and there are no significant changes in the levels of *Gata4* mRNA. Note the downregulation of *Nppb*, *Nkx2.5*, *Hand2* and *Tbx20* transcripts and the increased expression of *Raldh2* mRNA (*, +, # $P<0.05$, $n=3$–6 per genotype. * Statistical significance *vs* *Gata5*$^{+/-}$; + statistical significance *vs* *Gata6*$^{+/-}$; and # statistical significance *vs* WT).

study suggest that *GATA5* may be an important genetic modifier that could potentially contribute to human CHD and more specifically to defects of the OFT.

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