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Gata5 and Gata6 are functionally redundant in zebrafish for specification of cardiomyocytes

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Abstract

An outstanding problem in vertebrate development has been to define the genetic program that specifies the cardiomyocyte lineage. It has been a challenge to define the transcription factors that control specification, since candidate gene knockouts typically cause rather complex morphogenetic defects. In contrast, *Drosophila* genetics identified single transcription factors that are essential for specification of cardiomyocytes from uncommitted mesoderm. For those vertebrate orthologs, it has been considered that paralogous family members might compensate for the loss-of-function of individual genes. However, this hypothesis had not been formally tested. In zebrafish, defects in *gata5* can lead to a loss of myocardial tissue, but most embryos depleted for any single vertebrate Gata4/5/6 transcription factor develop a cardiac morphogenetic defect, and cardiomyocytes are specified and differentiate. Here we show that in zebrafish the *gata5* and *gata6* genes are redundant for specification of cardiomyocytes. Embryos depleted of these two gene products are heartless. Restoring either gene product is sufficient to rescue cardiomyocyte specification. In contrast, embryos depleted of Gata4 and Gata6, or Gata4 and Gata5, develop defective heart tubes. Our study identifies a specific pair of vertebrate transcription factor paralogs that is essential for cardiomyocyte specification.

Keywords

cardiogenesis; myocardium; GATA factors; mesoderm

Introduction

Defining the transcriptional program that specifies cardiomyocyte fate from uncommitted progenitors is an important goal, since this could impact cellular strategies for treating cardiomyopathies and heart disease. A number of transcription factors, including those from the GATA, NKX2, MEF2, and TBX gene families, are established as key regulators of normal heart development (Brand, 2003; Frasch, 1999). Orthologous genes are essential for cardiomyocyte specification in *Drosophila*, exemplified by the ‘heartless’ phenotypes of *pannier* and *tinman*, mutants for Gata and Nkx2 orthologues, respectively. However, genetic experiments in vertebrate animal models have not revealed essential genes that specify cardiomyocytes. Rather, mouse knockouts and zebrafish mutants for these genes show morphological disruptions and in some cases chamber-specific developmental defects. This is generally interpreted as reflecting genetic compensation by related family members in the

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context of the more complex vertebrate heart (Xin et al., 2006). Some evidence for this is provided by forced expression of potential dominant-negative isoforms that might block family member function (Fu et al., 1998; Horb and Thomsen, 1999). However, the concept has not yet been demonstrated formally; a challenge is that some of these candidate specification genes have additional early requirements, for example in extra-embryonic tissues.

GATA proteins comprise a small family of zinc finger transcription factors that regulate diverse functions during embryogenesis and in the adult (Patient and McGhee, 2002). The *gata4*, *gata5* and *gata6* genes are each implicated in the development of the intestinal and cardiovascular systems (Peterkin et al., 2003). Since they share both spatial and temporal expression patterns throughout embryogenesis, each gene may be required for specification of distinct cell types, or for a different step of cardiac morphogenesis. Alternatively, a total specific amount of GATA factor in particular cells might be needed to direct expression of a specific program. Zebrafish studies provide evidence that each factor fulfills a different stage-specific requirement during cardiogenesis. The *gata5* gene appears to have the earliest function for zebrafish cardiogenesis, as revealed by analysis of the *faust* mutant (Reiter et al., 1999; Reiter et al., 2001; Trinh et al., 2005). The phenotype of *faust* is variable in expressivity, and while some mutant embryos show a significant loss of myocardium (suggesting a key role in specification or proliferation), in most sibling mutants the cardiac progenitors fail to fuse at the midline of the embryo, leading to a *cardia bifida* phenotype. Thus, *gata5* plays a role in the migration of cardiac progenitors from the anterior lateral plate mesoderm towards the midline of the embryo. Also required for cardiac morphogenesis is *gata6*, essential for proper cardiac tube formation. Embryos depleted of Gata6 show variable cardiac phenotypes including *cardia bifida*, partially fused tube, and fused but non-looping tube (Peterkin et al., 2003). There is also an essential role for *gata4*, required for a relatively late “jogging” and growth step of heart tube morphogenesis. In *gata4* morphants, the atrium fails to expand or migrate rostrally, causing defects in heart tube looping (Holtzinger and Evans, 2005).

Gain-of-function experiments suggest that individual vertebrate GATA factors encode activity sufficient to enhance cardiogenesis, for example shown by forced expression of Gata4 in *Xenopus* embryos (Jiang and Evans, 1996) or P19 embryonal carcinoma cells (Grepin et al., 1997), or Gata5 in zebrafish (Reiter et al., 1999). Gata4 over-expression is sufficient to induce cardiomyocyte differentiation in *Xenopus* ectodermal explants, suggesting the ability to specify cell fate (Latinkic et al., 2003). However, it is not clear if these effects relate to cell specification, progenitor proliferation, or are non-physiological phenotypes induced by over-expression. If GATA factors are an essential component of the cardiomyocyte specification program, loss-of-function for *gata4/5/6* gene activity should result in a heartless phenotype, analogous to *pannier* (Sorrentino et al., 2005). Here we test this hypothesis using the zebrafish system, which facilitates targeted knockdown of multiple genes. Our results show that *gata5* and *gata6* function together to specify the cardiomyocyte lineage.

Materials and Methods

Zebrafish Strains and Morpholino Microinjections

Zebrafish embryos were maintained and staged as described (Westerfield, 1993). The morpholino specific to *gata4* is a translation blocker (G4 MO) and was described (Holtzinger and Evans, 2005). A morpholino specific to *gata5* that blocks a splice site (ssG5 MO) was described (Trinh et al., 2005). To confirm specificity, we also designed a translation blocker (tbG5 MO; 5'-AAGATAAAGCCAGGCTCGAATACAT-3'). The morpholino specific to the “long form” of *gata6* (G6 MO) was also described previously (Peterkin et al., 2003). Using individual morpholinos each embryo was injected with: 10ng G4 MO; 20 ng ssG5 MO, 16 ng tbG5 MO, or 2.5 ng G6 MO. Under these conditions the *gata5* morpholinos phenocopy the *faust* mutant, while the *gata4* and *gata6* morpholinos block completely detectable GFP in

gata4:gfp and *gata6:gfp* transgenic reporter fish, respectively (Holtzinger and Evans, 2005). However, when injected with combinations including 2.5 ng of the *gata6* morpholino, embryos did not survive. Therefore, for combinatorial injections, 1.25 ng of the *gata6* morpholino was used. This was still sufficient to eliminate GFP in the *gata6:gfp* reporter fish, and to generate identical *gata6* single-morphant phenotypes. The *cmlc2:gfp* strain was generated originally by H.J. Tsai (Huang et al., 1997), and we generated equivalent lines independently. For rescue experiments, batches of embryos were injected with morpholinos, and injected independently in sibling groups (either before or after) with various amounts of *in vitro* generated and purified mRNA.

Gene Expression Analysis

Whole-mount *in situ* hybridization was performed essentially as described (Reiter et al., 1999). Briefly, embryos were treated with 0.003% phenylthiourea (PTU) to prevent pigmentation. After fixation, embryos older than 28 hours were treated with 10 µg/ml proteinase K. Hybridization was performed at 70°C, in 60% formamide buffer with digoxigenin-labeled RNA anti-sense probes. The probes used for *in situ* hybridization were prepared and used as described, for *gata4/5/6* (Heicklen-Klein and Evans, 2004), *nkx2.5*, *cmlc2*, *vmhc*, and *amhc* (Reiter et al., 1999), *pax2.1* (Krauss et al., 1991), *lmo2* (Zhu et al., 2005), and *no tail* (Schulte-Merker et al., 1992). For quantitative RT-PCR, embryos were either uninjected or injected with G4+ssG5 MOs; G4+G6 MOs; ssG5+G6 MOs or tbG5+G6 MO. An equal number of embryos was harvested for each sample and total RNA was isolated (TRI REAGENT, Molecular Research Center, Cincinnati, OH). First-strand cDNA synthesis was performed (Superscript III First-Strand Synthesis System for RT-PCR, Invitrogen) and the cDNA subjected to quantitative RT-PCR (Opticon DNA Engine 2, MJ Research, Watertown, MA) with data analysis as described (Livak and Schmittgen, 2001). Statistical analysis was based on three independent experiments each comprised of triplicate samples. Primers were: *beta actin*, F: 5'-CAACGGAAACGCTCATTGC, R: 5'-CGCGCAGGAGATGGGAACC; *cmlc2*, F: 5'-AGACCCAGAGGAAACCATCC, R: 5'-TTGGGTCATTAGCAGCCTCT.

Results and Discussion

The *faust* (*gata5*) mutant was described as demonstrating variable expressivity with 0 – 45% of the mutant embryos in a given clutch showing abnormal hearts (Reiter et al., 1999; Reiter et al., 2001). This could be explained by partial to full compensation from other co-expressed GATA factors. Indeed, previous work revealed redundancy between *gata4* and *gata6* for liver bud formation (Holtzinger and Evans, 2005). Moreover, evidence for cooperation between *Gata4* and *Gata6* during cardiogenesis was revealed by examining trans-heterozygote mutant mouse embryos (Xin et al., 2006). The *faust* mutant represents in principle a good starting point for targeting GATA factors, since these embryos express low levels of *gata5* mRNA (Reiter et al., 1999). However, analyzing phenotypes in the context of the *faust* background is complicated for several reasons. First, the mutations are not null, and the remaining levels might contribute to the variable expressivity. Second, the molecular basis for the stronger *fau^{s26}* allele is not known, which complicates genotyping required to “prove” that any individual embryo is a *gata5* mutant. Third, only 25% of the embryos derived from heterozygous *faust* parents are mutant, limiting our ability to analyze statistically the results of manipulating several different gene combinations in the *faust* background. For these reasons, we used a morpholino-based approach for depleting combinations of cardiogenic GATA factors. The *gata4* and *gata6* transcripts were targeted using previously characterized and validated morpholinos (Holtzinger and Evans, 2005; Peterkin et al., 2003).

However, we first sought to confirm that a morpholino approach was valid for use in place of the *faust* mutant. A *gata5*-specific morpholino was shown previously to phenocopy the *faust*

mutant (Trinh et al., 2005). Since this morpholino targets a *gata5* splice site, its effectiveness can be evaluated by whole-mount *in situ* hybridization for *gata5* transcript levels. Indeed, embryos derived from fertilized eggs injected with this splice site *gata5* (ssG5) morpholino show little if any detectable *gata5* transcripts, confirming the ability of ssG5 to efficiently inhibit *gata5* expression (Fig. 1A,B). We also tested a translation blocker (tbG5 MO) because this morpholino will inhibit also expression from maternal *gata5* transcripts. Embryos derived from fertilized eggs injected with either the ssG5 or the tbG5 morpholino were compared to embryos derived from crossing heterozygous *fau^{s26}* adults, by analyzing transcripts for the cardiac progenitor marker *nkx2.5* by whole-mount *in situ* hybridization. Compared to control (wild-type) embryos (Fig. 1C) all embryos injected with the tbG5 morpholino display a bifid heart (Fig. 1D,E; Table 1). The phenotype of embryos injected with the ssG5 morpholino was overall more variable, consistent with the failure to block translation of maternal-derived *gata5* transcripts. While the majority of the embryos still display a *cardia bifida* (61%), the phenotypes vary primarily between this and a fused or partially fused heart tube (Fig. 1F–H; Table 1). In a low percentage of morphant embryos, *nkx2.5* transcripts are largely depleted (Fig. 1I; Table 1). The progeny of a cross between two heterozygous *fau^{s26}* adults shows the same range of cardiac phenotypes as seen with the ssG5 morphants (Fig. 1, J–M). Considering that only 25% are homozygous *fau^{s26}*, the majority of embryos displaying a relatively normal heart tube are presumed to be wild-type or heterozygous (Table 1). Of the remaining abnormal embryos, the large majority of *fau^{s26}* embryos display a *cardia bifida* phenotype (Table 1), although again a very small percentage show a significant loss of *nkx2.5* transcripts (Fig. 1M, Table 1), consistent with results reported previously (Reiter et al., 1999). Therefore, while loss of Gata5 can cause depletion of myocardial progenitors, in most embryos this is not the case and myocardial cells are specified and development proceeds, albeit with *cardia bifida*. Therefore, the specificity and efficiency of two independent *gata5* morpholinos was validated and they consistently generate embryos that replicate the phenotype of *faust* mutants. Therefore, we next sought to test for compensation in the context of the *gata5* morphant.

Gata5 and Gata6 are together essential for cardiomyocyte development

In order to test specific requirements for GATA factors in myocardium, different combinations of morpholinos were injected into fertilized eggs derived from a transgenic reporter strain expressing GFP in cardiomyocytes (*cmlc2:gfp*). Heart development was analyzed throughout embryogenesis by examining GFP expression in each morphant. The *gata4* gene has a relatively late role in heart tube morphogenesis and GFP expression is relatively unaffected in the *gata4* morphants (data not shown), while the GFP expression patterns for the *gata5* or *gata6* morphants reflect heart tube fusion defects (Fig. 2A–G). Embryos injected with morpholinos that target both *gata4* and *gata5* (*gata4+5* morphants) show the same cardiac phenotypes as those injected with the *gata5* morpholino alone, primarily *cardia bifida* (data not shown). Likewise, *gata4+6* double morphants display a fused or partially fused heart tube, comparable to the *gata6* morphant phenotype (data not shown). In a similar manner, we generated *gata5+6* double morphants. In this case we observed a complete lack of GFP expression (Fig. 2H). These observations were reproduced in many experiments analyzing hundreds of embryos for each combination. GFP expression was also absent from *gata4+5+6* triple morphants (data not shown).

The specificity of the cardiomyocyte phenotype was demonstrated in rescue experiments by injecting *gata5* and/or *gata6* mRNA to restore Gata5 and/or Gata6 proteins to the double morphant embryo. This experiment is complicated because forced expression of Gata5 or (to a lesser extent) Gata6 causes abnormal embryogenesis. This is likely caused by an inappropriate expression pattern since it occurs regardless of whether morpholinos are included, and it is not possible for technical reasons to deliver accurately ectopic RNA back to the endogenous pattern. However, by titrating the amount of exogenous RNA, cardiomyocyte specification is

rescued in the double morphant background. For these experiments we used the ssG5 morpholino that does not inhibit expression from the injected *in vitro* transcribed *gata5* mRNA. Morpholinos were injected in embryos derived from *cmlc2:gfp* reporter fish, and mRNA was injected independently. Over the course of many experiments, control injections lacking RNA yielded embryos that showed no or few GFP+ cardiomyocytes. In a representative set of experiments, 6/81 embryos showed only sparse GFP+ cells (7%). In contrast, GFP+ cardiomyocytes were readily found in 30/47 (64%), 49/79 (62%), 31/75 (41%), or 12/23 (52%) embryos injected with 12.5 pg, 25 pg, 50 pg, or 100 pg of *gata5* and *gata6* mRNA, respectively. Embryos injected with this RNA were often abnormal, particularly at higher doses, but they did generate cardiomyocytes in the expected embryonic region. In multiple other experiments, similar numbers of embryos (approximately 50%) were rescued using only *gata5* or *gata6* mRNA, and in some cases cardiac tube formation was restored. Overall, we did not find a statistical difference between rescue using *gata5* mRNA, *gata6* mRNA, or a combination of both mRNAs.

Since these experiments were done in the context of a transgenic reporter, we also carried out quantitative RT-PCR experiments to analyze at 32 hours post fertilization (hpf) the expression of the endogenous *cmlc2* gene (Fig. 2I). The levels of *cmlc2* transcripts as measured in different morphants confirmed quantitatively the qualitative observations made in the transgenic reporter fish. There is no significant change in *cmlc2* expression in batches of *gata4+5* or *gata4+6* morphants, although the variability within these two samples likely reflects the phenotypic variations, indicating that some individual morphants have reduced *cmlc2* expression. For the analysis of *gata5+6* morphants, embryos were injected with the *gata6* morpholino in combination with either the ssG5 or tbG5 morpholino. Both combinations showed a striking loss in relative levels of endogenous *cmlc2* transcripts (98% and 99% decreased, compared to wild-type, respectively).

Loss of Gata5 and Gata6 causes a heartless phenotype

To more completely evaluate myocardial development we documented at 32 hpf the expression patterns for the atrial differentiation marker Atrial Myosin Heavy Chain (*amhc*, Fig. 3). The *gata4+5* morphants show the same patterns of *amhc* transcripts as seen in the *gata5* morphants, using either the ssG5 or tbG5 morpholino. The phenotypic variability seen in *gata6* morphants was similarly observed in the prospective atrium of 32 hpf *gata4+6* morphant embryos, and all of the embryos showed *amhc* transcripts in the abnormal heart tube. In striking contrast, none of the *gata5+6* morphants expressed *amhc* transcripts.

Similarly, we evaluated in morphants the relative expression pattern of the ventricular differentiation marker Ventricular Myosin Heavy Chain (*vmhc*, Fig. 4). As was observed for *amhc*, the *gata4+5* morphants at 32 hpf display a range of cardiac phenotypes identical to the single *gata5* morphants. In *gata4+6* morphants, the presumptive ventricular portion of the heart appears less affected than the atrium, and *vmhc* transcripts are similar to the single *gata6* morphant. Again in contrast, *vmhc* transcripts are not detected in any of the *gata5+6* morphants. Importantly, all the embryos, including the *gata5+6* morphants, express apparently normal patterns of *vmhc* transcripts in the somitic mesoderm, showing that the phenotype is specific to the heart. The *gata5+6* double morphants are therefore heartless, lacking cells that express myocardial markers.

One possibility is that in *gata5+6* morphants the cardiogenic program initiates normally, but is not maintained, leading to a failure in differentiation. To evaluate the initiation of the cardiogenic program, injected embryos were analyzed by whole mount *in situ* hybridization for transcripts encoding the early cardiogenic marker *nkx2.5*. The *gata4+5* morphants show expression of *nkx2.5* transcripts at 17 somites in a *cardia bifid* pattern comparable to *gata5* morphants; in the *gata4+5* morphants there also appears to be a modest decrease in the levels

of *nkx2.5* transcripts (Fig. 5A,C,E). The *nkx2.5*-positive cells are also observed at 12 somites (not shown), indicating that cardiac progenitors are specified. Therefore, *gata4* and *gata5* are not essential for the initiation of the cardiac program and *gata6* alone is sufficient to initiate cardiomyocyte specification. At 17 somites *gata4+6* double morphants show normal or slightly reduced levels of *nkx2.5* transcripts, similar to *gata6* single morphants (Fig. 5A,B,D,F), and also seen in 12-somite embryos (not shown). Therefore, *gata5* alone is also sufficient to initiate the cardiogenic program. However, every *gata5+6* double morphant fails to express detectable levels of *nkx2.5* transcripts at 17 somites (Fig. 5G) or 12 somites (see below). The *gata5* and *gata6* genes are therefore redundant for cardiomyocyte specification.

The mesodermal defect in *gata5+6* morphants is specific to myocardium

The *gata5+6* morphants lack mesoderm-derived myocardium, so we tested if this is specific to the heart, or if mesoderm derivatives are more widely deficient in double morphants. We first tested the effect of *gata5+6* depletion by analyzing the intermediate mesoderm using *in situ* hybridization to detect transcripts for the pronephric marker *pax2.1*. While *gata5+6* morphants lack *nkx2.5*-expressing cardiac progenitors at 12 somites (Fig. 6A,B), the *pax2.1* expression pattern is unaffected at the same stage (Fig. 6C,D). A medial mesoderm marker, *no tail*, was analyzed in 20-somite embryos, and displayed a normal expression pattern in the tail and the notochord in both wild-type and *gata5+6* morphants (Fig. 6E,F). Finally, we analyzed the expression of the lateral plate mesoderm marker *lmo2*, and found that this pattern is also not affected in *gata5+6* morphants (Fig. 6G,H). This is consistent with the normal pattern of trunk endothelium seen in *gata5+6* morphants using the *fli:gfp* reporter line that marks vascular endothelium (data not shown). Therefore, the mesoderm defect caused by loss of *gata5* and *gata6* is specific to the heart. Moreover, there is no obvious expansion of patterns for other mesoderm markers, for example *lmo2*, in the anterior lateral plate mesoderm. This suggests that prospective myocardium in *gata5+6* morphants fails to be specified, rather than being trans-fated into other mesoderm derivatives.

Gata4 alone is insufficient to rescue cardiogenesis

Since depletion of *gata5* and *gata6* together is sufficient to block myocardial development, this implies that the *gata4* gene is unable to compensate for their loss. However, as our previous work suggested, it is possible that GATA factors regulate the expression of each other (Holtzinger and Evans, 2005). This could complicate the interpretation, for example if *gata4* expression is itself dependent on Gata5 and/or Gata6 protein. Therefore, we examined by *in situ* hybridization at 12 somites the transcript levels of each factor, in every morphant and double morphant combination and failed to find a significant change in the levels of *gata4* transcripts in the *gata5+6* morphants (not shown). Thus, the heartless phenotype observed in the *gata5+6* morphants is due specifically to the lack of Gata5 and Gata6, and does not require the loss of all three factors. We have thus far not found forced expression of Gata4, by mRNA injection, to rescue the *gata5+6* morphant phenotype, with the caveat that this is a negative result.

GATA factors and cardiogenesis

Our study defines a specific pair of transcription factor paralogs that is essential in zebrafish for specification of cardiomyocyte fate from mesoderm progenitors. Similar to *Drosophila pannier* or *tinman* embryos that lack expression of a Gata or Nkx2 orthologue, respectively, the *gata5+6* morphants are completely heartless. While our study is focused on the cardiomyocyte population, we also found no evidence for endocardium or epicardium in these morphants (our unpublished data). This is not surprising since functions for GATA factors in these tissues are also documented. However, whether the specification of progenitors for endocardium and epicardium are also dependent on one or more specific Gata-4/5/6 genes is

not addressed by our experiments. These tissues develop relatively late compared to the myocardial progenitors, and their differentiation and morphogenesis is likely dependent on normal development of the primitive myocardial tube. Further clarification of this issue awaits definition of more definitive specification markers analogous to Nkx2.5 for myocardium. GATA factors also have important functions in endoderm and its derivatives, and so the heartless phenotype could in principle be due to a non-cell-autonomous defect for induction of cardiomyocytes. However, previous studies, for example with the endoderm defective mutant *casanova*, suggest that endoderm is not essential for cardiomyocyte specification. In addition, the endoderm-derived organ defects seen in *gata* mutants and morphants are relatively late compared to this step of cardiogenesis.

We note that using a morphant approach the targeted gene levels are reduced but may not be equivalent to a null mutant. Currently there are no null mutants for GATA factors in zebrafish. Therefore, it is possible that complete genetic ablation of either *gata5* or *gata6* might be sufficient to generate the heartless phenotype, suggested for example by the fact that a small number of *faust* or *gata5* morphant embryos show a significant reduction in myocardium. However, this caveat is less relevant to the issue of defining functional redundancy. We show that the phenotype caused by depletion of Gata5 or Gata6 alone is dramatically distinct from depletion of both genes, showing that they are functionally redundant. We analyzed many hundreds of embryos and essentially 100% of the double morphants lack cardiomyocytes, which is rarely (*gata5*) or never (*gata6*) seen with single morphants or mutants. Importantly, this demonstrates formally a concept that has for some time been hypothesized generally for cardiac transcription factor subfamilies in vertebrates.

The shared function for *gata5* and *gata6* shown here raises the question of whether this functional redundancy is conserved in mammals. We observed that among the key conserved amino acids within the DNA-binding domain that are diagnostic for the three family members from frog, chick, and mammalian species, not all are conserved in fish (Heicklen-Klein et al., 2005). Indeed, the literature has generally considered that the roles of zebrafish and mammalian GATA factors might differ. For example, the mouse *Gata4* knockout phenotype shows abnormal cardiac morphogenesis resulting in *cardia bifida* (Kuo et al., 1997; Molkenin et al., 1997). Since the zebrafish *gata5* (*faust*) mutant also displays a *cardia bifida*, it was considered that mouse *Gata4* might be the ortholog of the zebrafish *gata5* gene (Reiter et al., 1999). Consistent with this idea, *cardia bifida* is not seen in the zebrafish *gata4* morphant or the mouse *Gata5* knockout. However, the mouse *Gata4* knockout phenotype is indirect, due to a primary defect in extra-embryonic endoderm (Morrisey et al., 1998; Narita et al., 1997; Soudais et al., 1995). Rescue of the extra-embryonic defect by tetraploid complementation showed that GATA4 depletion in the mouse embryo proper leads to a cardiac morphogenetic defect (Watt et al., 2004) remarkably similar to that observed in the zebrafish *gata4* morphant (Holtzinger and Evans, 2005). The *cardia bifida* caused in zebrafish by loss of *gata5* was not described in the mouse *Gata5* knockout (Molkenin et al., 2000). However, the targeted mouse *Gata5* mutation deleted only the first exon, leaving an in-frame ATG in exon 2, and so it may not be a null mutant. Unlike in zebrafish (Peterkin et al., 2003), a role for *Gata6* in cardiogenesis has not been reported in the mouse knockout (Koutsourakis et al., 1999). However, the redundant function of *gata6* with *gata4* for liver morphogenesis is conserved between mouse and zebrafish (Holtzinger and Evans, 2005; Zhao et al., 2005). Overall, there is more evidence for conserved functions of GATA factors than for ortholog switching, and the literature thus far does not rule out a redundant function for *Gata5* and *Gata6* for mammalian cardiogenesis. Our results demonstrate that the function of vertebrate GATA factors for cardiomyocyte specification is conserved with the *Drosophila* gene *pannier*, suggesting that similar heartless phenotypes may be revealed by specific combinatorial knockdown of other paralogs, for example from the NKX2, MEF2, and TBX gene families.

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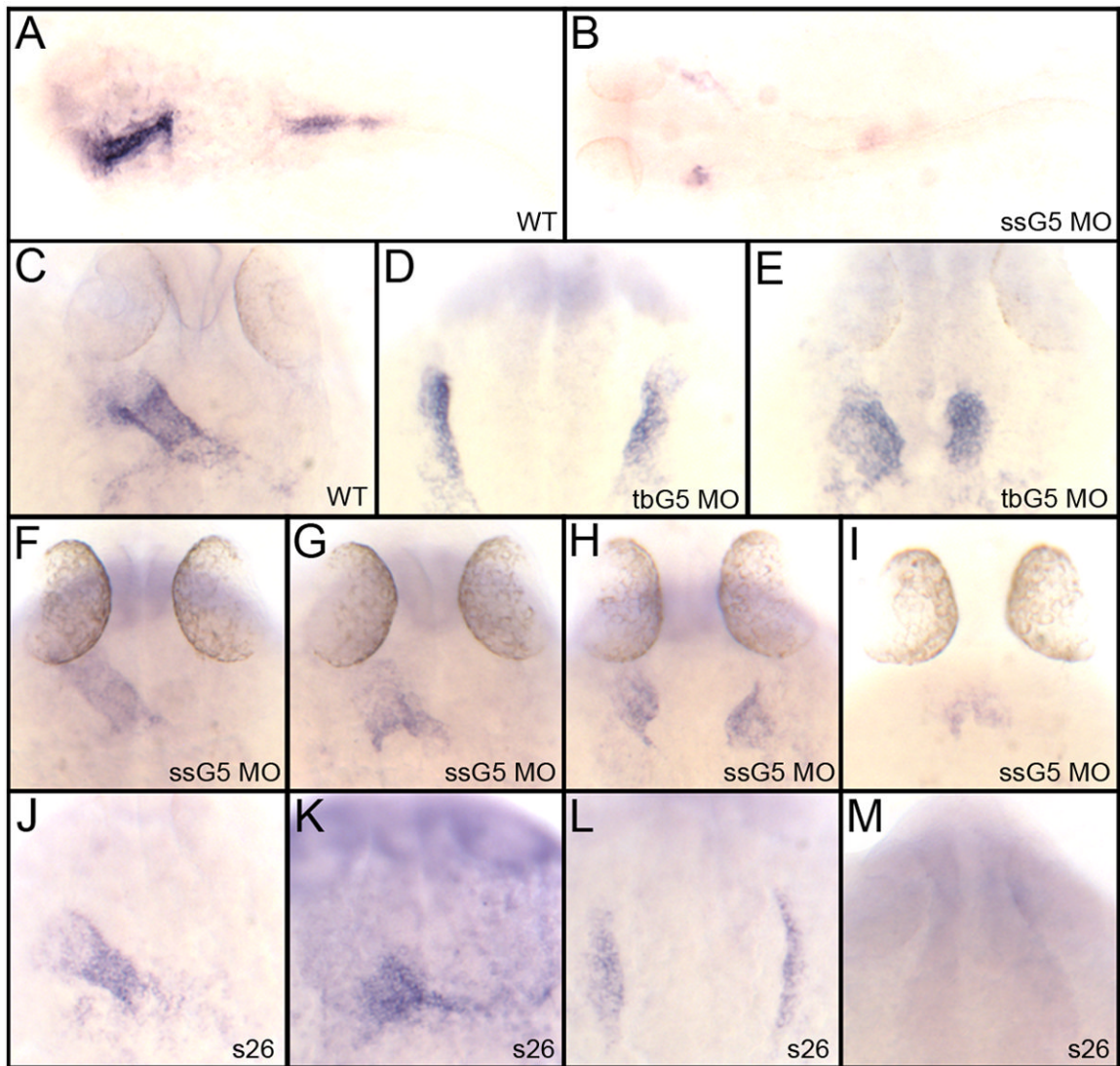


Fig. 1. The *gata5* morpholinos can be used to phenotype the *faust* mutant

A, B: Shown are representative embryos following *in situ* hybridization using a *gata5*-specific probe at 24 hpf analyzing (A) wild type embryos or (B) embryos injected with a *gata5* morpholino that targets a splice site (ssG5 MO). Injection of ssG5 MO efficiently blocks accumulation of mature *gata5* mRNA. While most embryos lack detectable message, the embryo shown in B represents an example of one that demonstrates a low level of remaining transcripts in a bifid pattern (arrows). C–M: Shown are representative embryos following whole mount *in situ* hybridization to detect transcripts for the cardiac marker *nkx2.5* at 24 hpf. Samples represent results with (C) wild-type embryos (D, E) embryos injected with a translation-blocker morpholino specific to *gata5* (tbG5), (F–I) ssG5 morphants, and (J–M) embryos derived from crossing two adult fish heterozygous for the *faus*^{s26} allele. The ssG5 morphants display the same variable expressivity phenotype as the *faus*^{s26} mutants, including embryos with a fused heart tube (F,J), a partially fused tube (G,K), *cardia bifida* (H,L) or a significant reduction of cardiac progenitors (I,M). The tbG5 morpholino more reproducibly generates *cardia bifida*. See Table 1 for all statistics. In A and B, embryos were flat mounted, views are dorsal, with anterior to the left. All other views are dorsal, with anterior to the top.

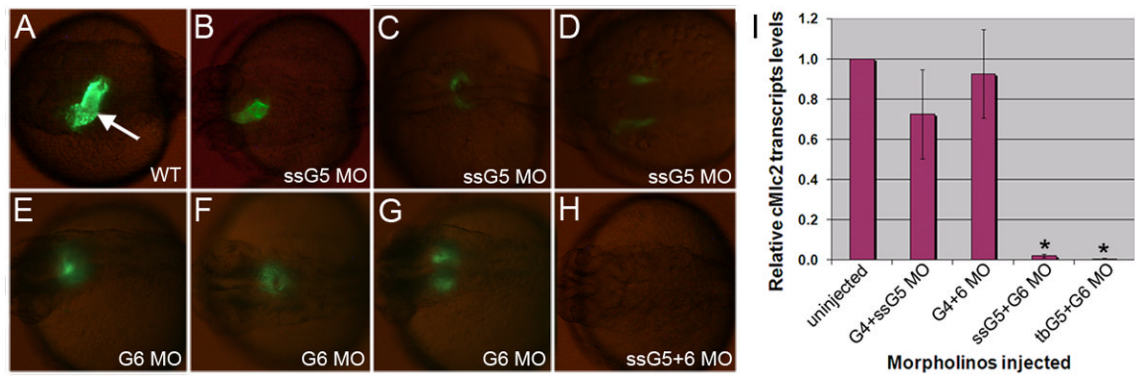


Fig. 2. Embryos targeted for *gata5* and *gata6* fail to express GFP in a *cmlc2:gfp* reporter line (A) A typical *cmlc2:gfp* embryo at 30 hpf shows GFP+ cardiomyocytes forming a heart tube (arrow). (B–G) Examples are shown representing cardiac phenotypes generated by injection of the ssG5 MO (B–D) and G6 MO (E–G). Co-injection of ssG5 MO and G6 MO results in an absence of GFP+ cardiomyocytes (H). Brightness of GFP appears lower in the reproduced images of B–G compared to A, because the injected embryos are slightly delayed, but also because the signal is more diffuse in the defective heart tubes. I: Quantitative real time PCR for *cmlc2* transcripts. Endogenous *cmlc2* transcript levels are reduced to approximately 1% the normal level in ssG5+6 and tbG5+6 double morphants ($p < 0.01$).

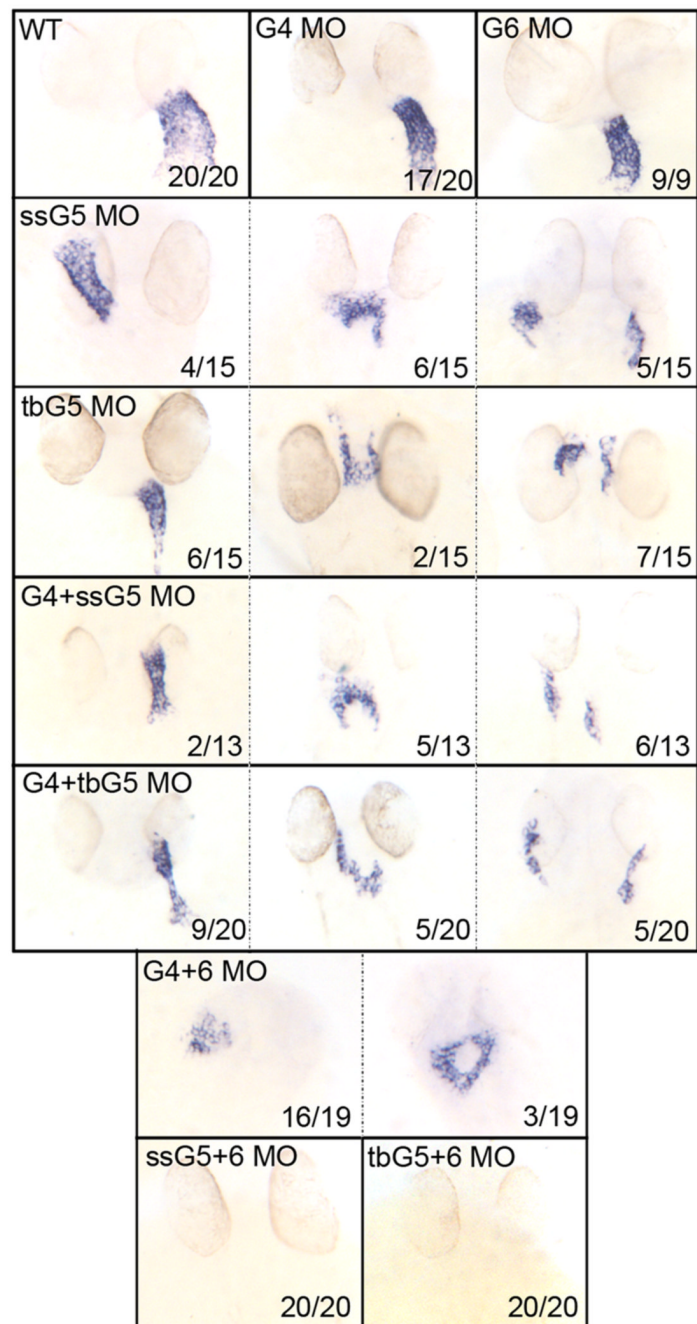


Fig. 3. The *gata5* and *gata6* genes are functionally redundant for atrial cardiomyocyte differentiation

Shown in each panel is a typical representative embryo following processing by whole mount *in situ* hybridization to detect transcripts for the atrial cardiomyocyte marker *amhc* at 32 hpf. Samples represent: wild-type embryos (WT), *gata4* morphants (G4 MO), *gata6* morphants (G6 MO), *gata5* morphants (ssG5 MO or tbG5 MO), *gata4+5* double morphants (G4+ssG5 MO or G4+tbG5 MO), *gata4+6* double morphants (G4+6 MO), and *gata5+6* double morphants (ssG5+6 MO and tbG5+6 MO). Despite morphogenetic defects, the atrium is specified in both *gata4+6* and *gata4+5* double morphants. However, *amhc* transcripts are not detectable in *gata5+6* double morphants. While these results were reproduced in multiple

independent experiments, the number of embryos from the experiment represented in this figure by the phenotype (x) for a given number of embryos (n) is shown in each panel (x/n). Views are either ventral or dorsal, depending on the heart tube position, with anterior to the top.

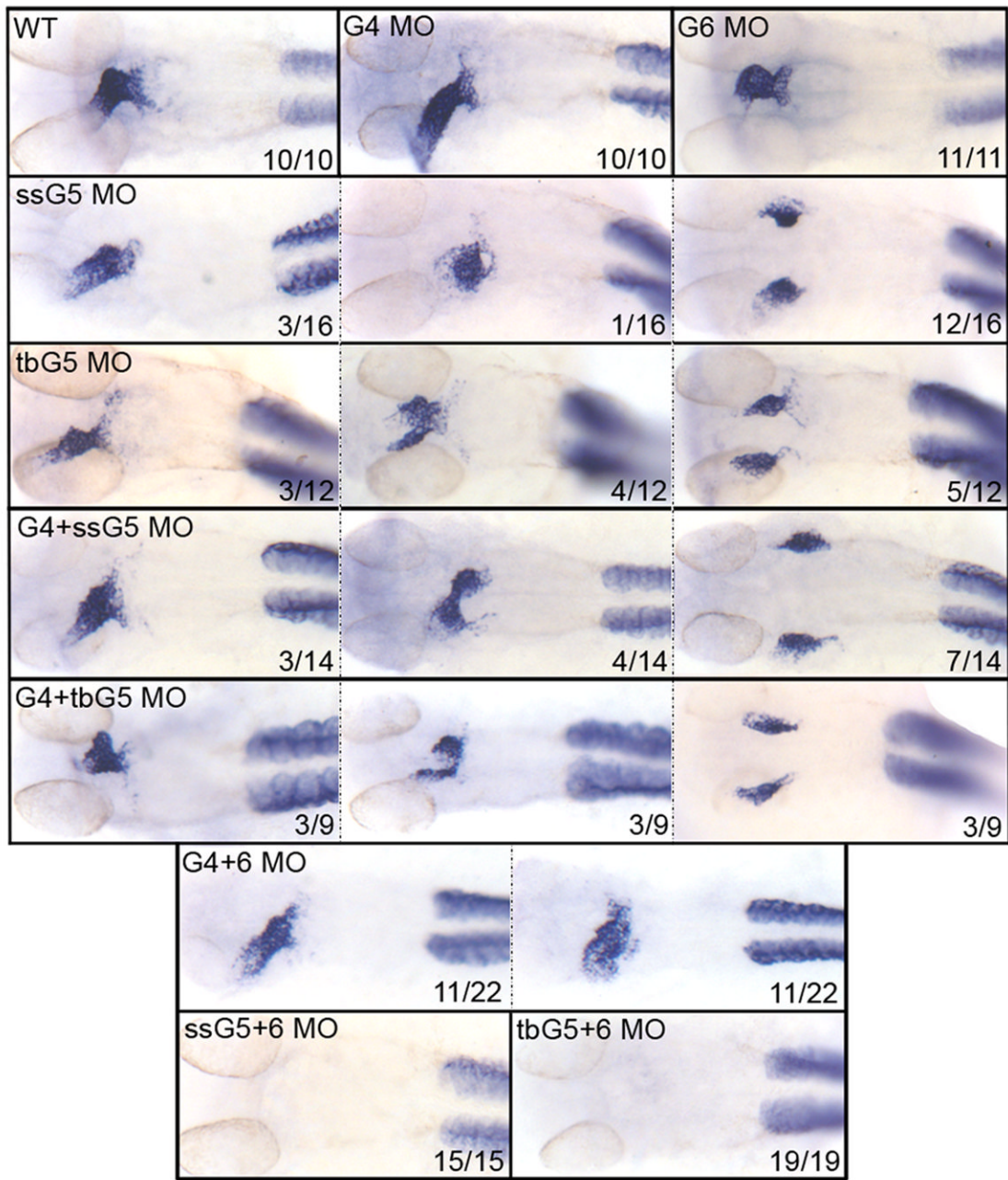


Fig. 4. The *gata5* and *gata6* genes are functionally redundant for ventricular cardiomyocyte differentiation

Shown in each panel is a typical representative embryo following processing by whole mount *in situ* hybridization to detect transcripts for the ventricular cardiomyocyte marker *vmhc* at 32 hpf. Samples represent: wild-type embryos (WT), *gata4* morphants (G4 MO), *gata6* morphants (G6 MO), *gata5* morphants (ssG5 MO or tbG5 MO), *gata4+5* double morphants (G4+ssG5 MO or G4+tbG5 MO), *gata4+6* double morphants (G4+6 MO), and *gata5+6* double morphants (ssG5+6 MO and tbG5+6 MO). Despite morphogenetic defects, the ventricle is specified in both *gata4+6* and *gata4+5* double morphants. However, *vmhc* transcripts are not detectable in *gata5+6* double morphants. Note that staining is unperturbed in the somites, showing that the defect is specific to the heart. While these results were reproduced in multiple

independent experiments, the number of embryos from the experiment represented in this figure by the phenotype (x) for a given number of embryos (n) is shown in each panel (x/n). Views are ventral, with anterior to the top.

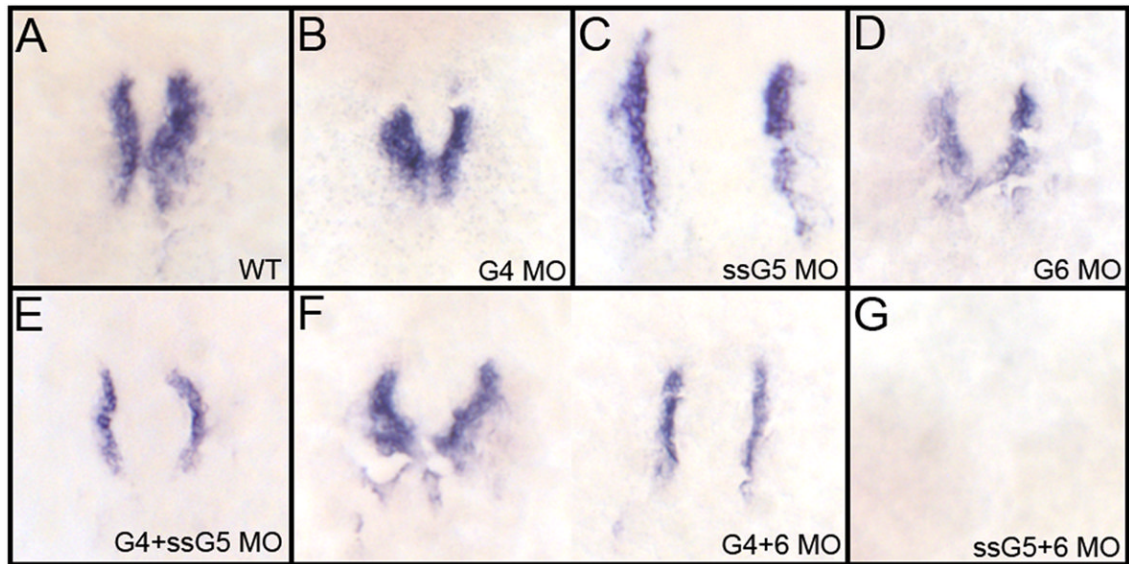


Fig. 5. The *gata5* and *gata6* genes are functionally redundant for cardiomyocyte specification
 Shown in each panel is a typical representative embryo following processing by whole mount *in situ* hybridization to detect transcripts for the cardiomyocyte progenitor marker *nkx2.5* at the 17 somite stage. (A) wild-type embryos, (B) *gata4* morphants, (C) *gata5* (ssG5) morphants, (D) *gata6* morphants, (E) *gata4+5* (ssG5) double morphants, (F) *Gata4+6* double morphants and (G) *Gata5*(ssG5)+6 double morphants. The cardiogenic program is initiated and cardiomyocytes are specified in both *gata4+6* and *gata4+5* double morphants. However, the early cardiac progenitors are missing in *gata5+6* morphants. Embryos were flat mounted; views are dorsal, with anterior to the top. Reproduced in multiple independent experiments, the phenotypes/number of embryos from this experiment represent A:17/17; B: 20/20; C+D combined: 26/26; E: 29/29; F: 25/25; G: 25/25.

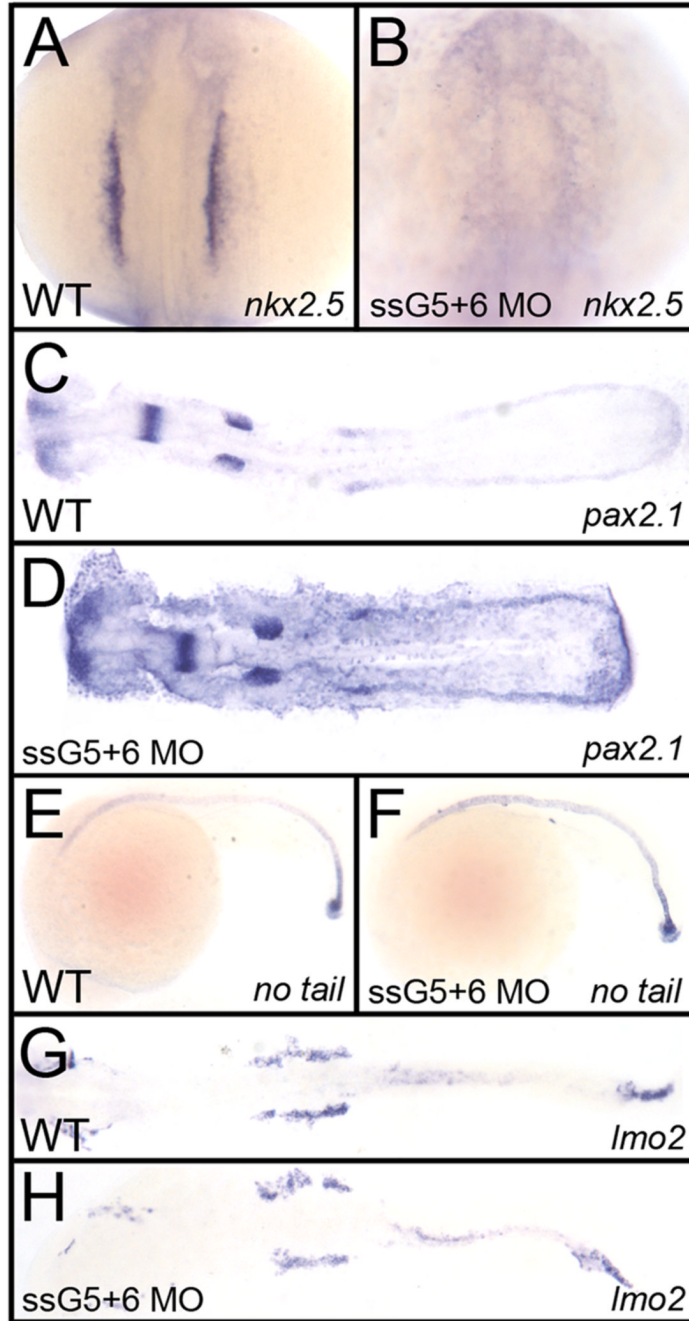


Fig. 6. The defect in *gata5+6* morphants is specific to cardiac mesoderm

Shown in each panel is a typical representative embryo following processing by whole mount *in situ* hybridization. Embryos were either wild-type (A, C, E, G) or *gata5* (*ssG5*) +6 double morphants (B, D, F, H). (A, B) The cardiomyocyte progenitor marker *nkx2.5* at the 12 somite stage, shows the lack of cardiac progenitors in *gata5+6* morphants. (C,D) The pronephric marker *pax2.1* shows that the intermediate mesoderm was not affected in morphants. (E,F) The axial mesoderm marker *no tail* shows that *gata5+6* morphants develop a normal notochord. (G, H) The lateral plate mesoderm, marked by the *lmo2* probe, is also not altered in *gata5+6* morphants. A,B: views are dorsal, with anterior to the top. C,D,G,H: embryos were flat mounted, views are dorsal, with anterior to the left. E,F: Views are from the left side, with

anterior to the left. These panels represent patterns seen in A: 39/39; B: 38/38; C: 10/10; D: 23/23; E: 26/26; F: 45/45; G: 28/28; H: 42/42.

Table 1

Comparison of phenotypes in embryos derived from crossing *fau*^{s26/+} adults, with those embryos derived from wildtype adults, but injected either with the splice site *ssG5* MO or translation-blocker (*tbG5* MO) morpholinos targeting *gata5*. The percentage of embryos is shown for each of 4 characteristic phenotypes (fairly normal heart tube, partially fused heart tube, bifid heart, or significant loss of cardiac tissue).

	Heart tube	Partially fused tube	Cardia bifida	Dramatic decrease or absence of cardiac progenitors
ssG5 MO (51 embryos)	12%	24%	61%	4%
tbG5 MO (31 embryos)	0%	0%	100%	0%
Embryos from s26 hets crossed (68 embryos)	66% (assumed to be wt and +/s26)	4%	27%	3%