

1 Gata4 drives Hh-signaling for second heart field migration and outflow tract development

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30

31 **Abstract**

32 Dominant mutations of *Gata4*, an essential cardiogenic transcription factor (TF), cause  
33 outflow tract (OFT) defects in both human and mouse. We investigated the molecular  
34 mechanism underlying this requirement. *Gata4* haploinsufficiency in mice caused OFT  
35 defects including double outlet right ventricle (DORV) and conal ventricular septum  
36 defects (VSDs). We found that *Gata4* is required within Hedgehog (Hh)-receiving second  
37 heart field (SHF) progenitors for normal OFT alignment. Increased *Pten*-mediated cell-  
38 cycle transition, rescued atrial septal defects but not OFT defects in *Gata4* heterozygotes.  
39 SHF Hh-receiving cells failed to migrate properly into the proximal OFT cushion in *Gata4*  
40 heterozygote embryos. We find that Hh signaling and *Gata4* genetically interact for OFT  
41 development. *Gata4* and *Smo* double heterozygotes displayed more severe OFT  
42 abnormalities including persistent truncus arteriosus (PTA) whereas restoration of  
43 Hedgehog signaling rescued OFT defects in *Gata4*-mutant mice. In addition, enhanced  
44 expression of the *Gata6* was observed in the SHF of the *Gata4* heterozygotes. These  
45 results suggested a SHF regulatory network comprising of *Gata4*, *Gata6* and Hh-signaling  
46 for OFT development. This study indicates that *Gata4* potentiation of Hh signaling is a  
47 general feature of *Gata4*-mediated cardiac morphogenesis and provides a model for the  
48 molecular basis of CHD caused by dominant transcription factor mutations.

49

## 50 **Author Summary**

51 Gata4 is an important protein that controls the development of the heart. Human who  
52 possess a single copy of Gata4 mutation display congenital heart defects (CHD),  
53 including the double outlet right ventricle (DORV). DORV is an alignment problem in  
54 which both the Aorta and Pulmonary Artery originate from the right ventricle, instead of  
55 originating from the left and the right ventricles, respectively. To study how Gata4  
56 mutation causes DORV, we used a Gata4 mutant mouse model, which displays DORV.  
57 We showed that Gata4 is required in the cardiac precursor cells for the normal alignment  
58 of the great arteries. Although Gata4 mutation inhibits the rapid increase in number of the  
59 cardiac precursor cells, rescuing this defects does not recover the normal alignment of  
60 the great arteries. In addition, there is a movement problem of the cardiac precursor cells  
61 when migrating toward the great arteries during development. We further showed that a  
62 specific molecular signaling, Hh-signaling, is responsible to the Gata4 action in the  
63 cardiac precursor cells. Importantly, over-activating the Hh-signaling rescues the DORV  
64 in the Gata4 mutant embryos. This study provides an explanation for the ontogeny of  
65 CHD.

66

## 67 **Introduction**

68 Congenital Heart Defects (CHDs) CHDs occur in approximately 1% of live births [1]  
69 and are the most common serious birth defects in humans [2, 3]. Approximately one third  
70 of the CHDs involve malformations of the outflow tract (OFT), which leads to significant  
71 morbidity and mortality of children and adults [4]. Multiple OFT abnormalities involve the  
72 relationship of the Aorta and Pulmonary Artery to the underlying left and right ventricles.  
73 For example, double-outlet right ventricle (DORV) is an anomaly in which the Aorta and  
74 Pulmonary Artery originate from the right ventricle [4]. A key characteristic of DORV that  
75 distinguishes it from other OFT defects is that the aorta and pulmonary trunk are well  
76 separated but are improperly aligned over the right ventricle. The molecular basis of OFT  
77 misalignment in DORV has remained unclear.

78 SHF-derived cells migrate into the developing poles of the heart tube, to effect  
79 morphogenesis of the cardia inflow and outflow. The anterior SHF is essential for OFT  
80 and great artery development [5-9]. The failure of the anterior SHF-derived myocardial  
81 and endocardial contributions to the arterial pole of the heart causes a shortened OFT  
82 and arterial pole misalignment, resulting in inappropriate connections of the great arteries  
83 to the ventricular mass [10-12]. Deletion of genes responsible for SHF morphogenesis,  
84 such as *Isl1*, *Mef2c*, and *Jagged1*, leads to abnormal OFT formation including DORV [5,  
85 6, 8, 12-19]. These observations lay the groundwork for investigating the molecular  
86 pathways required for OFT development in SHF cardiac progenitor cells.

87 *Gata4*, a member of the GATA family of zinc finger transcription factors, is an essential  
88 cardiogenic transcriptional regulator implicated in many aspects of cardiac development

89 and function [20-34]. Human genetic studies have implicated haploinsufficiency of  
90 GATA4 in human CHDs, to date including atrial septal defects (ASD), ventral septal  
91 defects (VSD), and tetralogy of Fallot (TOF) [21, 35-39]. In mouse models, decreased  
92 expression of *Gata4* results in the development of common atrioventricular canal (CAVC),  
93 DORV, and hypoplastic ventricular myocardium in a large proportion of mouse embryos  
94 [27, 40]. Multiple studies have demonstrated the molecular requirement of Gata4 in the  
95 endocardium for normal cardiac valve formation [24, 30, 41]. Furthermore, we previously  
96 demonstrated that *Gata4* is required in the posterior SHF for atrial septation. Both  
97 Hedgehog (Hh) signaling and *Pten*-mediated cell-cycle progression were shown to be  
98 downstream of *Gata4* in atrial septation [42]. However, the mechanistic requirement for  
99 Gata4 in OFT development is less clear. For example, from the multiple *Gata4*  
100 transcriptional targets that have been identified in the context of heart development,  
101 including *Nppa*,  $\alpha$ -MHC,  $\alpha$ -CA, *B-type natriuretic peptide (BNP)*, *Ccnd2*, and *Cyclin D2*,  
102 and *Mef2c* [20, 23, 24, 26, 43, 44], only *Mef2c* has a functional role in OFT development  
103 [12].

104 In this study, we investigated the mechanistic requirement for *Gata4* in OFT  
105 development. We found that *Gata4* heterozygosity in SHF hedgehog (Hh)-receiving cells  
106 recapitulates the OFT misalignment observed in *Gata4* germline heterozygotes in mice.  
107 *Gata4* heterozygous embryos had decreased numbers of SHF-derived cells populating  
108 the anterior SHF and the developing OFT at E10.5. By genetic inducible fate mapping  
109 (GIFM), Hh-receiving cells fail to migrate properly into the OFT of *Gata4* mutant mice.  
110 We have previously reported that *Gata4* acts upstream of Hh-signaling for atrial septation  
111 [42]. Here we observed more severe OFT defects observed in embryos with SHF-specific

112 heterozygosity of *Gata4* and *Smo*, the obligate Hh signaling receptor. Furthermore,  
113 rescue of *Gata4*-mediated OFT misalignment by constitutive activation of Hh-signaling  
114 indicated a consistent epistatic relationship between *Gata4* and Hh signaling in OFT  
115 development. Furthermore, upregulation of *Gata6* in the *Gata4* mutant SHF may provide  
116 an explanation for the severity of OFT defects observed in *Gata4* mutant embryos. Our  
117 study thereby revealed *Gata4*-dependent pathways contributing to OFT development in  
118 *Gata4* heterozygous mouse embryos.

## 119 Results

### 120 GATA4 is required for OFT alignment

121 *Gata4* is strongly expressed in the heart, pSHF and OFT at E9.5 [27, 42, 50]. There  
122 is a gap in expression between the OFT and the pSHF at embryonic day 9.5 (Fig.1A,  
123 indicated by a “↓”). IHC staining for *Gata4* at later stages during OFT development showed  
124 strong *Gata4* expression in the heart, the developing OFT and the pSHF, but only in a  
125 limited subset of aSHF cells at E10.5 (Fig.1B, indicated by a “↓”). At E11.5, both the  
126 chamber myocardium and the developing OFT had strong *Gata4* expression, however,  
127 *Gata4* expression was absent from the cardiac neural crest (CNC)-derived distal OFT  
128 (Fig. 1C, indicated by a “↓”).

129 *Gata4* was previously reported to be required for OFT alignment [27]. To study the  
130 role of *Gata4* in OFT development, we re-examined *Gata4* heterozygotes for OFT  
131 defects. As described previously [42], *Gata4* heterozygotes were generated by crossing  
132 *Gata4<sup>fl/+</sup>* with *Ella<sup>Cre</sup>*, which drives Cre expression in the germline [51] to effect germline  
133 *Gata4* deletion. The *Gata4* germline deletion was ensured by genotyping using the  
134 embryo tail DNA. Whereas *Gata4<sup>fl/+</sup>* (n = 13) and *Ella<sup>Cre/+</sup>* (n = 12) embryos demonstrated  
135 normal heart at E14.5 (Figs.2A and A', 2B and B'), 61.1% of *Gata4<sup>+/-</sup>*; *Ella<sup>Cre/+</sup>* embryos  
136 demonstrated VSD and DORV (Figs.2C', 11/18, P=0.0004). Consistent with our prior  
137 work, we observed primum ASDs with absence of the DMP in 8/18 *Gata4<sup>+/-</sup>*; *Ella<sup>Cre/+</sup>*  
138 embryos [42] (Figs. 2C).

139 To determine the lineage requirement for *Gata4* in AV septation, we analyzed mouse  
140 embryos haploinsufficient for *Gata4* in the myocardium, CNC, endocardium or SHF. We  
141 combined *Tnt: Cre* [52] with *Gata4<sup>fl/+</sup>* to create *Gata4* haploinsufficiency in the

142 myocardium. Normal OFT alignment was observed in all *Tnt<sup>Cre/+</sup>; Gata4<sup>fl/+</sup>* (12/12) and  
143 littermate control *Gata4<sup>fl/+</sup>* embryos (9/9) at E13.5 (P=1) (Figs. 2E and E' vs. 2D and D',  
144 P=1). We combined *Wnt1: Cre* [53, 54] with *Gata4<sup>fl/+</sup>* create *Gata4* haploinsufficiency in  
145 the CNC. Normal OFT alignment was observed in all *Wnt1<sup>Cre/+</sup>; Gata4<sup>fl/+</sup>* mutant embryos  
146 (24/24) and littermate control *Gata4<sup>fl/+</sup>* embryos (16/16) at E13.5 (Figs. 2F and F' vs. 2D  
147 and D', P=1). We combined *Nfat1c: Cre* [53, 54] with *Gata4<sup>fl/+</sup>* create *Gata4*  
148 haploinsufficiency in the endocardium. Normal OFT alignment was observed in nearly all  
149 *Nfatc1<sup>Cre/+</sup>; Gata4<sup>fl/+</sup>* mutant embryos (14/15) and littermate control *Gata4<sup>fl/+</sup>* embryos  
150 (10/10) at E13.5 (Figs. 2G and G' vs. 2D and D', P=1). These results demonstrated that  
151 *Gata4* haploinsufficiency in the myocardium, CNC or endocardium supported normal OFT  
152 alignment.

153 ***Gata4* is required in the SHF Hedgehog (Hh) signal-receiving progenitors for OFT**  
154 **alignment.**

155 We hypothesized that *Gata4* is required in the aSHF for OFT alignment in aSHF-  
156 specific *Gata4* heterozygous mice. We tested this hypothesis by combining *Mef2cAHF:*  
157 *Cre* with *Gata4<sup>fl/+</sup>*. Surprisingly, OFT misalignment with DORV was only observed in 1 out  
158 of 22 embryos and none in the littermate controls (Fig. 2I and I' vs. 2H and H', P=1). We  
159 next tested if *Gata4* is required in the pSHF for OFT alignment in in pSHF-  
160 specific *Gata4* heterozygous mice by crossing *Osr1<sup>CreERT2/+</sup>* [46, 47] with *Gata4<sup>fl/+</sup>*.  
161 Similarly, neither *Gata4<sup>fl/+</sup>; Osr1<sup>CreERT2/+</sup>* embryos (0/5) nor littermate  
162 control *Gata4<sup>fl/+</sup>* embryos (0/6) demonstrated OFT misalignments at E14.5 (Fig. 2J and J'  
163 vs. 2H and H', P=1). These results demonstrated that *Gata4* haploinsufficiency in either  
164 aSHF or pSHF supported normal OFT alignment.



165 Previous studies have shown that SHF Hh signal-receiving progenitors localized in  
166 both the aSHF and the pSHF, and regulated the migration of SHF toward the OFT and  
167 inflow tract (IFT) to form the pulmonary artery and the atrial septum separately [45, 55,  
168 56]. We combined *Gli1<sup>Cre-ERT2</sup>* with *Gata4<sup>fl/+</sup>* to create *Gata4* haploinsufficiency in SHF Hh  
169 signal-receiving progenitors. CreERT2 was activated by tamoxifen (TM) administration at  
170 E7.5 and E8.5 in *Gli1<sup>Cre-ERT2</sup>; Gata4<sup>fl/+</sup>* embryos. With TM administration at E7.5 and E8.5,  
171 66.7% of *Gli1<sup>Cre-ERT2</sup>; Gata4<sup>fl/+</sup>* embryos displayed DORV, while the littermate control  
172 *Gata4<sup>fl/+</sup>* embryos displayed normal OFT alignment (Figure 2K and K' vs. 2H, 2H', 8/12  
173 vs. 0/15, P=0.0002). We concluded that *Gata4* is required in the SHF Hedgehog (Hh)  
174 signal-receiving progenitors for OFT alignment.

#### 175 ***Gata6* was overexpressed in the SHF of the *Gata4* heterozygotes**

176 *Gata4* and *Gata6* double mutant embryos display PTA [40]. We examined *Gata6*  
177 expression in *Gata4* mutants. *Gata6* was expressed in the heart, the OFT and strongly  
178 in the splanchnic mesoderm (Fig. 3A, arrow), but not neural crest cell derivatives (Fig.  
179 3A, arrowhead) of the *Gata4<sup>fl/+</sup>* embryo at E9.5. In *Gata4* knockdown embryos specifically  
180 in the Hh-receiving cells, *Gata6* expression domain was strongly enhanced in the OFT  
181 and the splanchnic mesoderm. Consistently enhanced expression of *Gata6* in the OFT  
182 and the SHF of the *Gata4<sup>fl/fl</sup>; Gli1<sup>Cre-ERT2/+</sup>* was further confirmed by the real-time PCR at  
183 the mRNA level (Fig.3B). The *Gata4* expression in the SHF of *Gata4<sup>fl/fl</sup>; Gli1<sup>Cre-ERT2/+</sup>*  
184 mouse embryo was 2.7-fold that observed in control *Gata4<sup>fl/+</sup>* embryos (P<0.05). *Gata6*  
185 expression in the OFT of the *Gata4<sup>fl/fl</sup>; Gli1<sup>Cre-ERT2/+</sup>* mouse embryo was 4.4-fold that of  
186 the littermate control (P<0.01). Our results suggested a negative association between the  
187 expression of *Gata4* and *Gata6* in the SHF and developing OFT.

188

### 189 ***Gata4* regulates cell proliferation in the OFT conal cushion**

190 We wonder if *Gata4* is required for proliferation during the OFT cushion  
191 development. Cell proliferation was examined by BrdU incorporation at E11.5. *Gata4<sup>fl/+</sup>*;  
192 *Gli1<sup>Cre-ERT2/+</sup>* embryos demonstrated 17% fewer BrdU-positive SHF cells in the OFT conal  
193 cushion (Fig. 4C vs. 4A and 4E;  $P = 0.0134$ ), but not the OFT truncal cushion (Fig. 4D vs.  
194 4B and 4F;  $P = 0.1998$ ), compared to the littermate *Gata4<sup>fl/+</sup>* embryos at E11.5. This result  
195 demonstrate that *Gata4* is required for normal cell proliferation in OFT conal cushion  
196 development. We assessed cell death by TUNEL staining and observed no differences  
197 in either the conal or truncal cushion between *Gata4<sup>fl/+</sup>*; *Gli1<sup>Cre-ERT2/+</sup>* and the  
198 *Gata4<sup>fl/+</sup>* embryos (Fig. 4G - 4J). Together, these findings define a requirement  
199 for *Gata4* in the proliferation but not in the survival of OFT conal cushion cells.

### 200 **Rescue of SHF proliferation by disruption of *Pten* does not rescue DORV in *Gata4*** 201 **mutant embryos**

202 Our previous study demonstrated that *Gata4* regulates the cell cycle progression in  
203 posterior SHF cardiac precursors and that genetically targeted disruption of *Pten* rescued  
204 the proliferation defects in SHF of the *Gata4* heterozygotes [57]. Hence, we examined  
205 whether proliferation rescue in SHF, by *Pten* downregulation (TMX at E7.5 and E8.5),  
206 could rescue DORV in Hh-receiving cell-specific *Gata4* heterozygotes. We observed that  
207 decreased *Pten* dose caused only one DORV, but no ASD, in 20 embryos (Fig. 5A-C).  
208 Consistent with our previous report, although the ASD in *Gli1<sup>Cre-ERT2/+</sup>*; *Gata4<sup>fl/+</sup>* embryos  
209 was rescued by *Pten* downregulation (Fig. 5C vs. 5B, 1/20 in *Gli1<sup>Cre-ERT2/+</sup>*; *Gata4<sup>fl/+</sup>*; *Pten<sup>fl/+</sup>*  
210 vs. 14/29 in *Gli1<sup>Cre-ERT2/+</sup>*; *Gata4<sup>fl/+</sup>*,  $P = 0.0013$ ), *Gli1<sup>Cre-ERT2/+</sup>*; *Gata4<sup>fl/+</sup>*; *Pten<sup>fl/+</sup>* embryos still

211 displayed DORV with an incidence rate unchanged from *Gli1<sup>Cre-ERT2/+</sup>;Gata4<sup>fl/+</sup>* embryos  
212 (Fig. 5E vs. 5F, 12/29 vs. 6/20, Table 1, P = 0.5495). This data suggested to us that  
213 correction of the SHF proliferation defects was not able to rescue the OFT misalignment  
214 of the *Gata4* mutant embryos.

### 215 ***Gata4* acts upstream of Hh signaling in OFT development.**

216 We have previously reported that *Gata4* acts upstream of Hh-signaling for atrial  
217 septation [42]. The requirement of *Gata4* in *Hh*-receiving cells for OFT alignment  
218 suggested that *Gata4* and *Hh* signaling may interact genetically in the SHF for OFT  
219 development. We tested this hypothesis in the *Gata4* and *Smo* compound heterozygotes  
220 (*Gata4<sup>fl/+</sup>;Smo<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>*) versus littermate controls (*Gata4<sup>fl/+</sup>; Gli1<sup>Cre-ERT2/+</sup>* or  
221 *Smo<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>*). Consistent OFT defects were observed in compound *Gata4; Smo*  
222 embryos (*Gata4<sup>fl/+</sup>;Smo<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>*) (5/9, Fig 6C - 6E) whereas no OFT defects were  
223 observed in *Smo<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>* embryos (0/7, Fig 6B and B'; P= 0.0337). The total  
224 incidence of OFT defects occurred in the *Gata4<sup>fl/+</sup>;Smo<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>* was not different  
225 than in the *Gata4<sup>fl/+</sup>; Gli1<sup>Cre-ERT2/+</sup>* embryos (Fig6C-E, 5/9 vs. 4/6, P= 0.7326). However,  
226 more severe range of OFT defects was observed in *Gata4<sup>fl/+</sup>;Smo<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>*  
227 embryos, including DORV (3 out of 5, Figs. 6C and C'), OA (1 out of 5, Figs. 6D and D')  
228 and persistent truncus arteriosus (PTA) (1 out of 5, Figs. 6E and E'). PTA, caused by a  
229 combined defect of alignment and separation, was only observed in  
230 *Gata4<sup>fl/+</sup>;Smo<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>*. This result suggest an interaction between *Gata4* and Hh-  
231 signaling in OFT development.

232 We tested the hypothesis that *Gata4* actis upstream of Hh-signaling for OFT  
233 development using a genetic epistasis study. We tested whether increased Hh-signaling

234 via a constitutively activated Smo mutant, *SmoM2* [58], could rescue the OFT  
235 misalignment in *Gata4*-heterozygotes. DORV was observed in 28.6% of littermate  
236 control *Gli1<sup>Cre-ERT2/+</sup>;R26-SmoM2<sup>fl/+</sup>* embryos (2/7) (Fig. 6G and G') and 58.3% of littermate  
237 control *Gli1<sup>Cre-ERT2/+</sup>;Gata4<sup>fl/+</sup>* embryos at E14.5 (7/12) (Fig. 6H and H'). In contrast, none  
238 of *Gata4<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>;R26-SmoM2<sup>fl/+</sup>* embryos showed DORV (Fig. 6I and I'), indicating  
239 significant rescue by *R26-SmoM2<sup>fl/+</sup>*, *Gli1<sup>Cre-ERT2/+</sup>* (Fig. 6I vs Fig. 6H, P = 0.0071, Table 1).  
240 This results demonstrated rescue of DORV in *Gata4*-mutant embryos by constitutive Hh  
241 signaling.

#### 242 ***Gata4* is required for the contribution of Hh-receiving cells to the OFT.**

243 Hh signaling has been reported to regulate the migration of SHF Hh-receiving cells  
244 toward the arterial pole of the heart [45]. We therefore hypothesized that *Gata4* is required  
245 for the SHF Hh-receiving cells migration toward the developing OFT. We tested this  
246 hypothesis using genetic inducible fate mapping (GIFM) [59]. The Hh-receiving lineage  
247 cells were marked in *R26R<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>* embryos by TM administration at E7.5 and E8.5  
248 and *β-gal* expression was evaluated at E10.5 in *Gata4* heterozygotes. The total number  
249 of *β-gal* positive cells was obtained by counting those on each individual sections and  
250 adding up all through the SHF and the OFT. We have previously reported decreased  
251 number of Hh-receiving cells in the pSHF at E9.5 associated with developing defects of  
252 DMP in the *Gata4<sup>fl/+</sup>;R26R<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>* embryos [57]. We observed that there were also  
253 significantly less Hh-receiving cells within the aSHF region (Fig. 7A vs. 7D and Fig. 7G,  
254  $334.0 \pm 1.4$  vs.  $186.7 \pm 4.9$ , P=0.009) of the *Gata4<sup>fl/+</sup>;R26R<sup>fl/+</sup>;Gli1<sup>Cre-ERT2/+</sup>* embryos. The  
255 cells of Hh-receiving lineage were observed in the developing OFT at this stage. By  
256 counting the number of *β*-galactosidase-expressing cells in the proximal half (Fig. 7B vs.

257 7E and 7H,  $49.7 \pm 9.6$  vs.  $26.7 \pm 6.7$ ,  $P=0.097$ ) and the distal half of the OFT (Fig.7C vs.  
258 7F and 7I,  $91.7 \pm 9.2$  vs.  $57.0 \pm 1.4$ ,  $P=0.0362$ ), we found that both of the regions of the  
259 *Gata4* heterozygotes had less  $\beta$ -galactosidase-expressing cells than the littermate  
260 controls (Figs. 7E and 7F).

261 To examine if *Gata4* haploinsufficiency influenced the SHF cell recruitment within  
262 the proximal OFT, we analyzed the fate map of SHF lineage cells in the OFT of the *Gata4*  
263 heterozygotes. Defined by *Mef2cAHF:Cre* expression:  $\beta$ -galactosidase-expressing cells,  
264 the total number of the SHF lineage cells within the proximal half and the distal half of the  
265 OFT were compared between the *Mef2cAHF::Cre;Gata4<sup>fl/+</sup>; R24R<sup>fl/+</sup>* and the  
266 *Mef2cAHF::Cre;R24R<sup>fl/+</sup>* embryos at E10. The number of SHF lineage cells populating the  
267 proximal OFT of the *Mef2cAHF::Cre;Gata4<sup>+/-</sup>; R24R<sup>fl/+</sup>* embryos was significantly less  
268 than that those in control *Mef2cAHF::Cre; R24R<sup>fl/+</sup>* embryos (Fig. 7J vs. 7M ); however,  
269 this decrement was not observed in the distal OFT (Fig. 7K vs. 7N). The distribution  
270 pattern of the SHF lineage was not different in the *Mef2cAHF::Cre;Gata4<sup>+/-</sup>; R24R<sup>fl/+</sup>* and  
271 the *Mef2cAHF::Cre;R24R<sup>fl/+</sup>* embryos (Figs. 7L vs. 7O). AS a control, we observed fewer  
272 cells populating the developing dorsal mesocardium protrusion (DMP) in  
273 *Mef2cAHF::Cre;Gata4<sup>+/-</sup>; R24R<sup>fl/+</sup>* (red arrow, Fig.7L vs. 7O), consistent with our previous  
274 report that *Gata4* is required in the SHF for the DMP [42]. These results demonstrated  
275 the requirement of *Gata4* for the SHF lineage cells populating in the developing OFT.

276

277

## 278 Discussion

279 The requirement of Gata4 for OFT development has been reported in mice and  
280 human, and mouse Gata4 mutations cause DORV [22, 27, 40]. Here we demonstrate  
281 that Gata4 is required in the SHF Hh-receiving cells for OFT alignment in the SHF. Our  
282 previous study has demonstrated that Gata4 is required for Hh signaling in the SHF for  
283 cell proliferation. However, the current study suggested that the cell proliferation defects  
284 in the SHF caused by Gata4 mutation may not directly associate with the OFT  
285 misalignment; instead, the migration defects of the SHF cells is. And the migration defects  
286 were associated with disrupted Hh-signaling, because the OFT misalignment was  
287 rescued by over-activating of Hh-signaling. In addition, our data suggested breaking down  
288 the threshold of GATA including *Gata4* and *Gata6*, and Hh signaling tone might be  
289 associated with the severity of OFT defects.

290 The SHF was initially described as a progenitor field for the cardiac OFT and a rich  
291 literature has established the requirement of anterior SHF contributions for OFT  
292 development [5, 10-19, 60-63]. More recently, the contribution of posterior SHF cardiac  
293 progenitors to the OFT and the future subpulmonary myocardium has been reported,  
294 however, the mechanistic requirement for this contribution is not well understood [45, 64-  
295 66]. The cell lineage in which Gata4 is required for OFT development has not been  
296 reported. Gata4 is expressed in both the aSHF and pSHF, although its expression is  
297 much stronger in the pSHF than in the aSHF [57]. The decreased number of *Mef2C*-  
298 *AHF::Cre* positive cells in the proximal OFT cushion of E10.5 *Gata4*<sup>-/+</sup> embryos  
299 demonstrated that Gata4 plays a role in adding the SHF progenitor cells to the developing  
300 OFT. However, surprisingly, OFT defects were not observed in either aSHF-specific or  
301 pSHF-specific Gata4 haploinsufficiency. Instead, we found that OFT defects severity

302 and incidence rate in embryos with *Gata4* haploinsufficiency in *Hh*-receiving cells were  
303 identical to those in *Gata4*<sup>-/+</sup> embryos. Because Hh-receiving cells are located throughout  
304 the SHF, these observations suggest *Gata4* is required in both pSHF and aSHF  
305 progenitor cells for OFT alignment.

306 We provided evidence that *Gata4* acts upstream of Hh-signaling in the SHF for OFT  
307 development. The *Gata4*<sup>-/+</sup> embryos have combined phenotypes of ASD and DORV [57].  
308 We previously reported the *Gata4*-Hh-signaling regulation in atrial septation and identified  
309 *Gli1* as the direct target of GATA4 [42]. Here, our data of less percentage of BrdU+ cells in  
310 the conal cushion of the OFT at E11.5 of the *Gata4*<sup>fl/+</sup>; *Gli1*<sup>Cre-ERT2/+</sup> embryos, suggesting  
311 a role of *Gata4* in regulating the OFT cushion cell proliferation. In the posterior SHF,  
312 *Gata4*-Hh-signaling controls cell cycle progression and thereby the proliferation of the  
313 cardiac progenitors. Diminished *Gata4*-Hh signaling causes a failure of development of  
314 the DMP, the anlage of the atrial septum, resulting in ASDs [57]. The effect of this pathway  
315 on the cell cycle is balanced by *Pten* via transcriptional inhibition of Cyclin D4 and Cdk4  
316 [20, 57], as DMP hypoplasia and SHF cell cycle defects are rescued by *Pten* knockdown  
317 [57]. In the current study, *Pten* knockdown was unable to rescue DORV or OA defects in  
318 *Gata4* heterozygous mutants. This observation suggests that correction of SHF cell  
319 proliferation is not sufficient to support a normal OFT development in *Gata4* mutants, and  
320 that *Gata4* plays a distinct role in the anterior SHF.

321 Endodermal Hh signaling is required for the survival of the pharyngeal endoderm,  
322 which cell non-autonomously affects SHF survival and OFT lengthening [55]. In our study,  
323 increased apoptosis was not observed in the SHF of *Gata4* heterozygote mutant embryos  
324 [57]. However, fate mapping of the SHF using either *Mef2c::Cre* or the *Gli1Cre:ERT2*

325 disclosed less SHF-derived cells in the distal OFT in *Gata4* mutant embryos. Specifically,  
326 there was decreased number of SHF Hh-receiving cells throughout the migration route  
327 from the SHF into the OFT: from the dorsal mesocardium through the rostral splanchnic  
328 mesoderm, past the distal OFT to the proximal OFT. Hh-receiving progenitors have been  
329 found to migrate from the aSHF to populate the pulmonary trunk between E9.5 to E11.5  
330 [45], suggesting that Hh-signaling is required for SHF cell migration. The observation that  
331 DORV in *Gata4* mutant embryos can be rescued by constitutive Hh-signaling implies  
332 correction of both the proliferation and the migration defects of the SHF cardiac  
333 progenitors, not proliferation defects only. Overall, here we provide cellular, molecular  
334 and genetic evidence that *Gata4*-Hh signaling hierarchy is required in OFT alignment,  
335 with specific regulation of both proliferation and migration of SHF progenitors.

336 Although important *Gata4* transcriptional targets in the heart have been identified  
337 [20, 26, 44], *Gata4*-dependent molecular pathways required for OFT development have  
338 remained unknown. We previously identified *Gli1* as a downstream target of *Gata4* in the  
339 posterior SHF for atrial septation [42]. In the current study we further demonstrated that  
340 *Gata4* regulated Hh-signaling via transcriptional regulation through *Gli1* in the anterior  
341 SHF for cell migration and OFT alignment. In addition, we provide evidence that *Gata6*  
342 expression is negatively regulated by *Gata4* in the OFT. Enhanced *Gata6* expression in  
343 *Gata4* mutants might illustrate a compensatory feedback loop, given that *Gata6* and  
344 *Gata4* are redundant for cardiac myocyte differentiation [67, 68]. *Gata4/Gata6* compound  
345 heterozygotes displayed persistent truncus arteriosus (PTA), a severe OFT defect caused  
346 by combined alignment and OFT septation defects (40). Here we find that *Gata4/Smo*  
347 compound heterozygotes show a similar phenotype. *Gata4* heterozygotes alone do not



348 display PTA, which might be due to the partial recovery of GATA function from enhanced  
349 *Gata6* expression. Together with previous study [40], these data suggest a threshold of  
350 *Gata4*, *Gata6*, and *Hh* signaling and that is required for OFT development. This suggests  
351 that GATA TFs may be essential for the quantitative regulation of Hh signaling, and that  
352 strongly diminished GATA function or diminished GATA and Hh signaling together may  
353 cause worse OFT defects through regulation of OFT Hh signaling. Future studies will  
354 focus on the quantitative relationship between GATA tone and Hh signaling tone and on  
355 the *Gata4* dependent gene regulatory network (GRN) [69] for OFT development.

356

357

## 358 **Materials and methods**

### 359 **Mouse lines**

360 All mouse experiments were performed in a mixed B6/129/SvEv background. *Gata4<sup>fl/+</sup>*,  
361 *Gli1<sup>CreERT2/+</sup>*, *Mef2cAHF::Cre*, *Tie2<sup>Cre/+</sup>*, *Smo<sup>fl/+</sup>* mouse lines were kind gifts from Dr. Ivan  
362 Moskowitz lab (University of Chicago, Chicago). *TnT<sup>Cre/+</sup>* mouse line was from Dr. Yiping  
363 Chen lab (Tulane University, New Orleans). *Nfat1c<sup>Cre/+</sup>* mouse line was from Dr. Bin Zhou  
364 lab (Albert Einstein College of Medicine, Bronx, NY). The *SmoM2<sup>fl/+</sup>*, *Osr1<sup>Cre-ERT2/+</sup>* and  
365 *Ella<sup>cre/+</sup>* mouse lines were purchased from the Jackson Laboratory. Mouse experiments  
366 were completed according to a protocol reviewed and approved by the Institutional Animal  
367 Care and Use Committee of the Texas A&M University and the University of North  
368 Dakota, in compliance with the USA Public Health Service Policy on Humane Care and  
369 Use of Laboratory Animals.

### 370 **Tamoxifen administration and X-gal staining**

371 Tamoxifen (TM) -induced activation of *CreERT2* was accomplished by oral gavage with  
372 two doses of 75 mg/kg TM at E7.5 and E8.5 [45, 46]. X-gal staining of embryos was  
373 performed as described [45].

### 374 **BrdU incorporation and Immunohistochemistry Staining (IHC)**

375 Standard procedures were used for histology and IHC. IHC was performed using the  
376 following antibodies: anti-Gata4 (Abcam), anti-Gata6 (Abcam). For BrdU incorporation,  
377 pregnant mice were given 100mg BrdU per kg bodyweight at 10mg/mL concentration  
378 solutions at E11.25 with two doses, 3 hours and 6 hours before sacrifice, respectively.  
379 The BrdU staining was performed using a BrdU In-Situ detection kit (EMD Millipore). For

380 TUNNEL staining, an ApopTag plus peroxidase In-Situ apoptosis detection kit was used  
381 (EMD Millipore).

### 382 **Micro-dissection of pSHF and RNA extraction**

383 To obtain the pSHF splanchnic mesoderm for use in quantitative realtime-PCR, E9.5  
384 embryos were dissected as described before [47, 48]. The heart, aSHF, and pSHF were  
385 collected separately in RNA-later, and then stored at  $-20^{\circ}\text{C}$  until genotyping was  
386 completed.

### 387 **Realtime-PCR**

388 Total RNA was extracted from the PSHF regions of mouse embryos hearts using RNeasy  
389 Mini Kit (QIAGEN), according to the manufacturer's instructions. Two hundred ng of total  
390 RNA was reverse transcribed using a SuperScript™ III Reverse Transcriptase kit from  
391 Invitrogen. qPCR was performed using a POWER SYBER Green PCR mater mix from  
392 Applied Biosystems. Results were analyzed using the delta-delta Ct method with *GAPDH*  
393 as a normalization control [49].

394

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396



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400

402 **FIGURE LEGEND**

403 **Figure 1. Gata4 is strongly expressed in the developing heart, the OFT and the**  
404 **pSHF.** Gata4 expression was detected in *wildtype* mouse embryos by IHC at (A) E9.5,  
405 (B) E10.5 and (C) E11.5. Red arrows indicate anterior second heart field at E9.5 or E10.5  
406 (A and B), and proximal outflow tract at E11.5 (C).

407 Magnificence: A: 40X; B and C: 100X

408 **Figure 2. Gata4 is required in Hh-receiving cells for OFT development.**

409 (A-G') Histology of Gata4 transgenic mouse embryo heart at E14.5. Statistics were  
410 summarized in table 1. Histology of Gata4 transgenic mouse embryo heart at E13.5. . LV,  
411 left ventrium; RV, right ventrium; ao, aorta artery, PT, pulmonary trunk. Magnificence: 40X

412 (H-K') Histology of Gata4 transgenic mouse embryo heart at E14.5. Histology of Gata4  
413 transgenic mouse embryo heart at E13.5. LV, left ventrium; RV, right ventrium; ao, aorta  
414 artery, PT, pulmonary trunk.

415 **Figure 3. Gata6 was overexpressed in the OFT and the SHF of the Gata4 mutant**  
416 **embryos at E9.5.**

417 (A) IHC of the Gata6 in *Gata4<sup>fl/+</sup>* and *Gata4<sup>fl/+</sup>; Gli1<sup>Cre-ERT2/+</sup>* embryos at E9.5. the  
418 arrowhead indicated the NCCs-derived cells and the arrow indicates the splanchnic  
419 mesoderm. Magnificence: 200X.

420 (B) Gata6 was measured by realtime-PCR in the micro-dissected SHF and the OFT  
421 of the *Gata4<sup>fl/+</sup>* and *Gata4<sup>fl/fl</sup>; Gli1<sup>Cre-ERT2/+</sup>* embryos at E9.5. \*p<0.1, \*\*p<0.05, n=3

422

423 **Figure 4. Gata4 regulates cell proliferation in conal OFT.**

424 (A-D) BrdU staining in conal OFT and truncal OFT in *Gata4<sup>fl/+</sup>*; *Gli1<sup>Cre-ERT2/+</sup>* embryos and  
425 control embryos at E10.5. Magnificence: 400X.

426 (E and F) Quantification of BrdU labelled cells. Data is presented as mean±SE, \*p<0.05,  
427 n=3, One-way ANOVA.

428 (G-J) TUNEL staining in both *Gata4<sup>fl/+</sup>*; *Gli1<sup>Cre-ERT2/+</sup>* embryos and control embryos at  
429 E10.5. Magnificence: 100X

430 **Figure 5. Genetically targeted ablation of Pten rescues atrioventricular septal**  
431 **defect.**

432 (A-I) Histology of Gata4 transgenic mouse embryo heart at E13.5. . LV, left ventrium; RV,  
433 right ventrium; ao, aorta artery, PT, pulmonary trunk. Magnificence: 40X.

434 **Figure 6. Gata4 acts upstream of Hh signaling pathway.**

435 (A-I') Histology of Gata4 transgenic mouse embryo heart at E14.5. LV, left ventrium; RV,  
436 right ventrium; ao, aorta artery, PT, pulmonary trunk; CAT, common artery trunk.  
437 Magnificence: 40X.

438 **Figure 7. Gata4 is required for the contribution of Hh-receiving cells to the OFT.**

439 (A-F) LacZ staining of Gli1-expressing cells in Gata4 transgenic mouse embryos at E10.5  
440 focusing on aSHF (E and H), dOFT (F, I) and pOFT (G, J).

441 (G-I) Quantification of stained cells within selected regions. Data is presented as  
442 mean±SE, \*p<0.05, \*\* p<0.1, n=3, One-way ANOVA.

443 (J-O) LacZ staining of cells with Mef2cAHF:Cre expression in Gata4 transgenic mouse

444 embryos at E10.5. The red arrow indicated a developing DMP region.

445 Magnificence: A-D and A'-D' 40X; E-J: 100X; N-S: 100X

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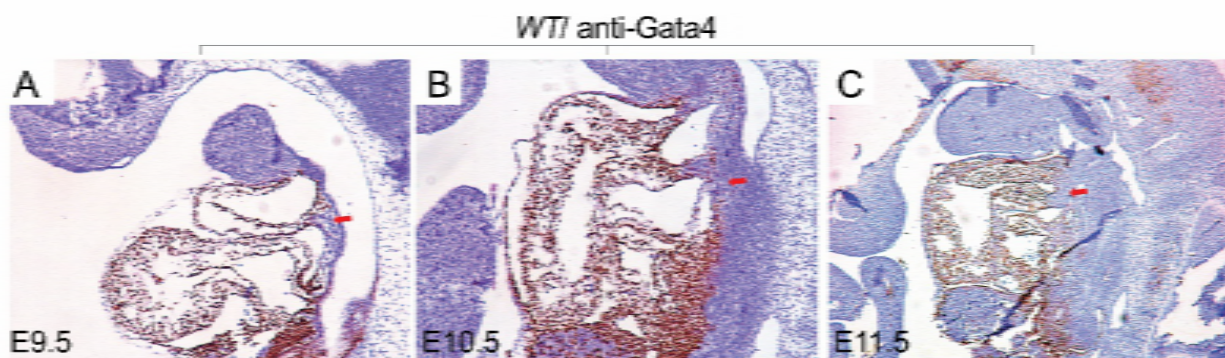
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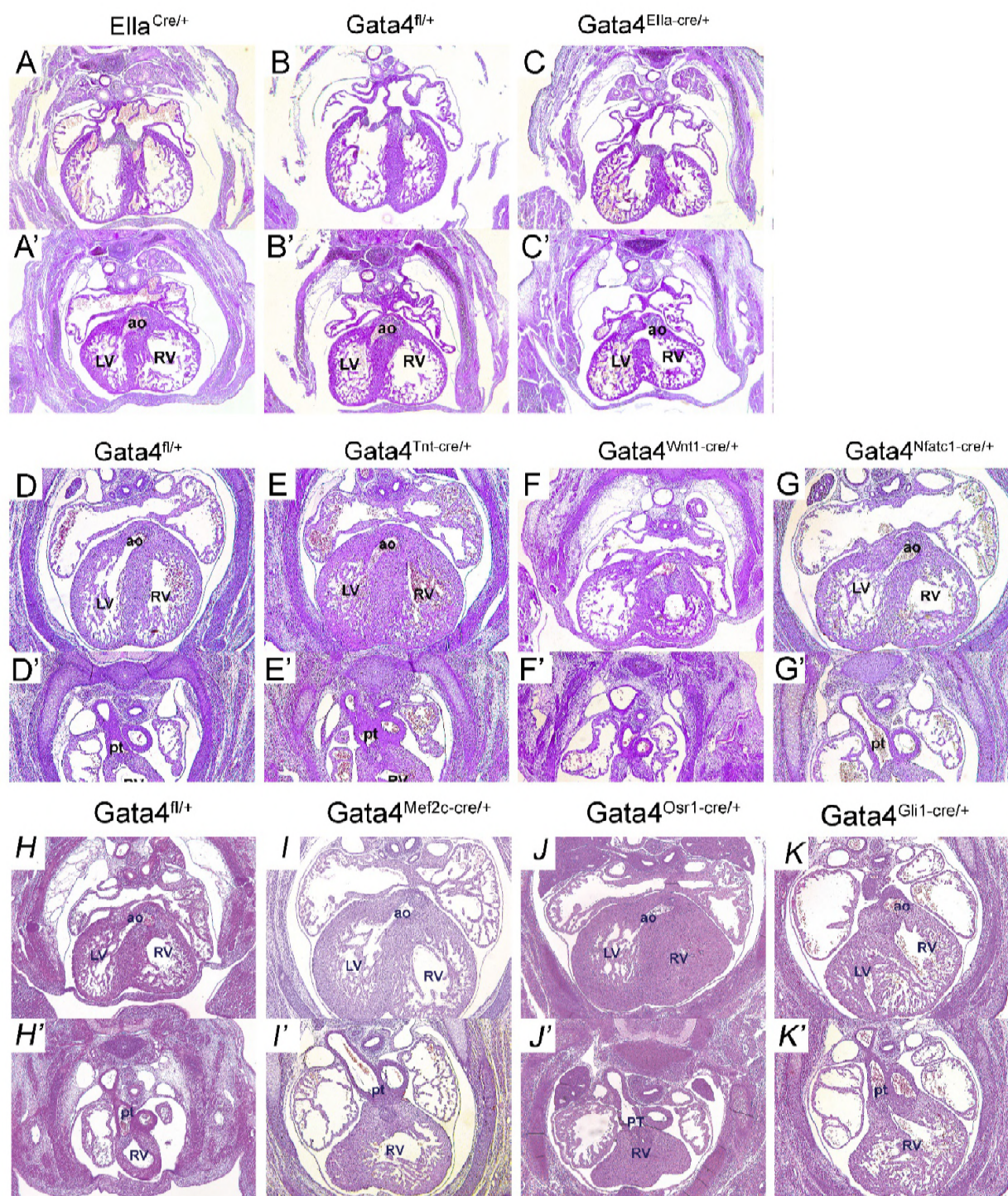
688 Figure 1.



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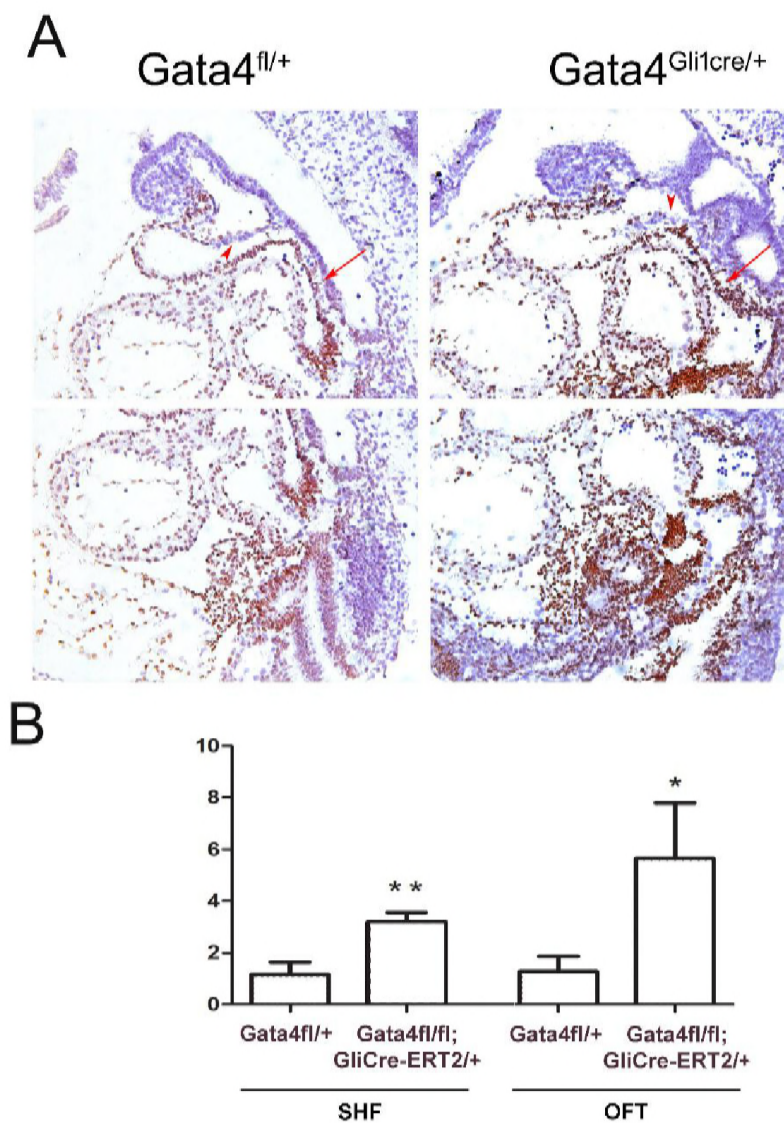
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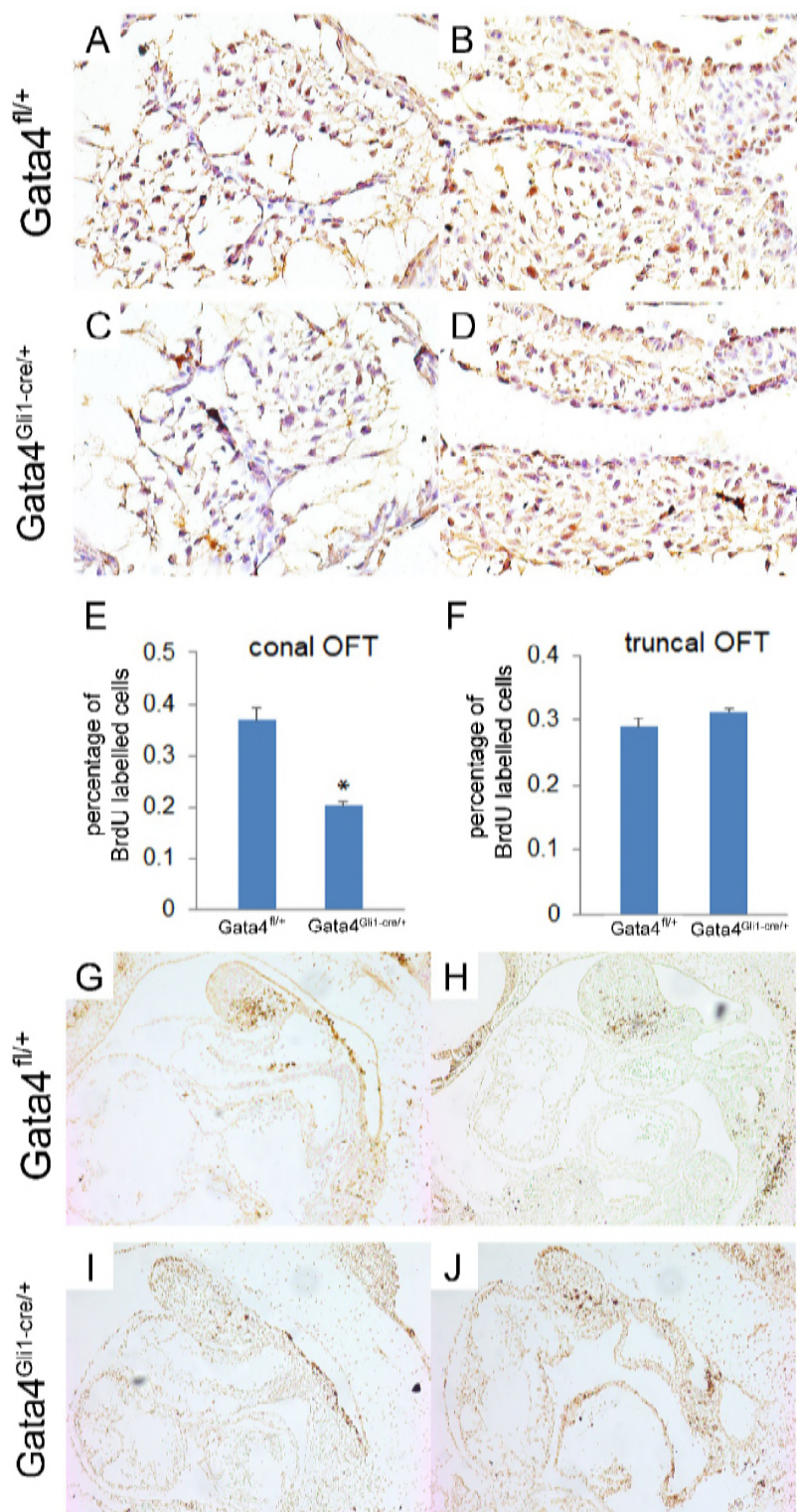
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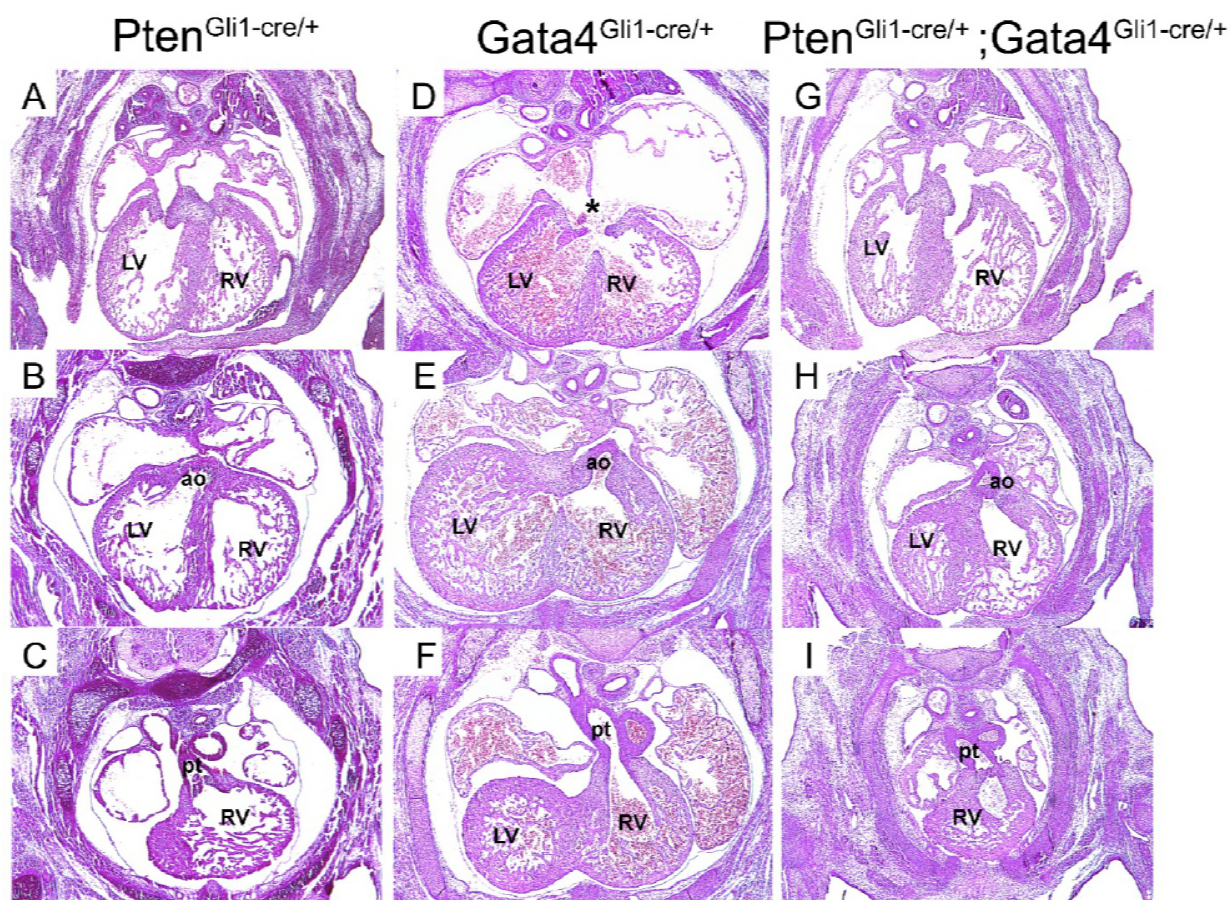
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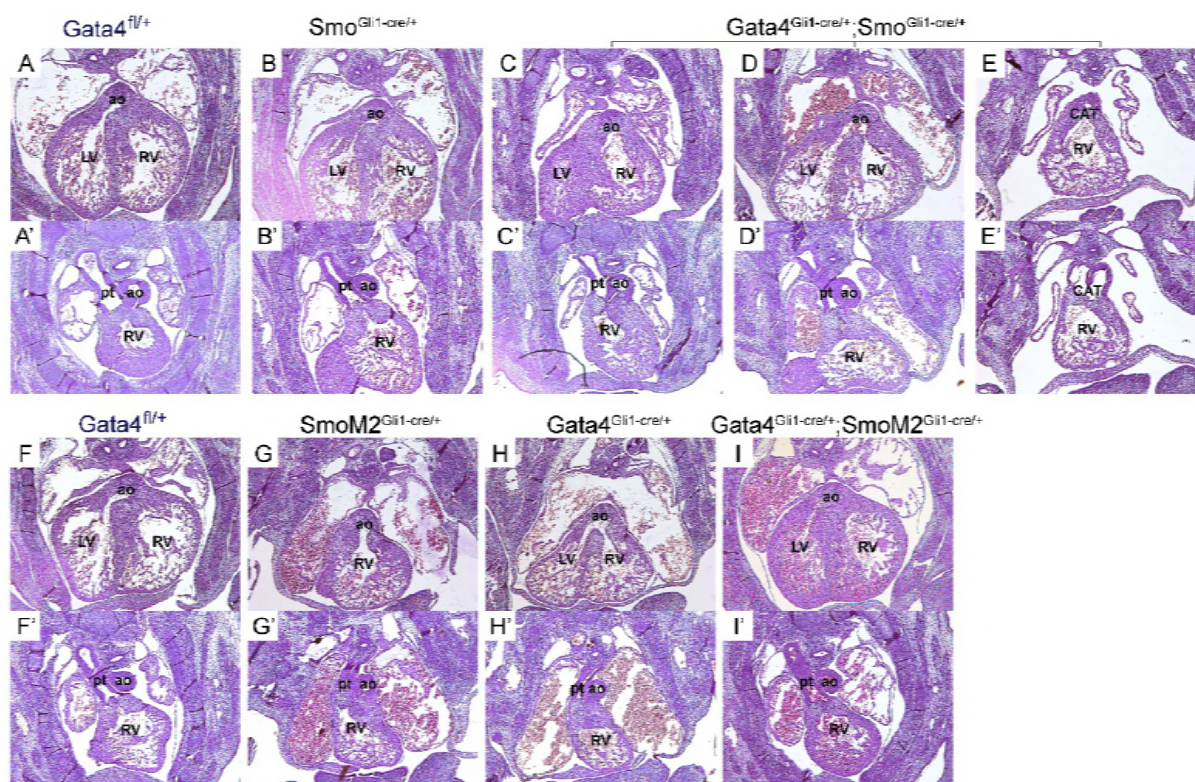


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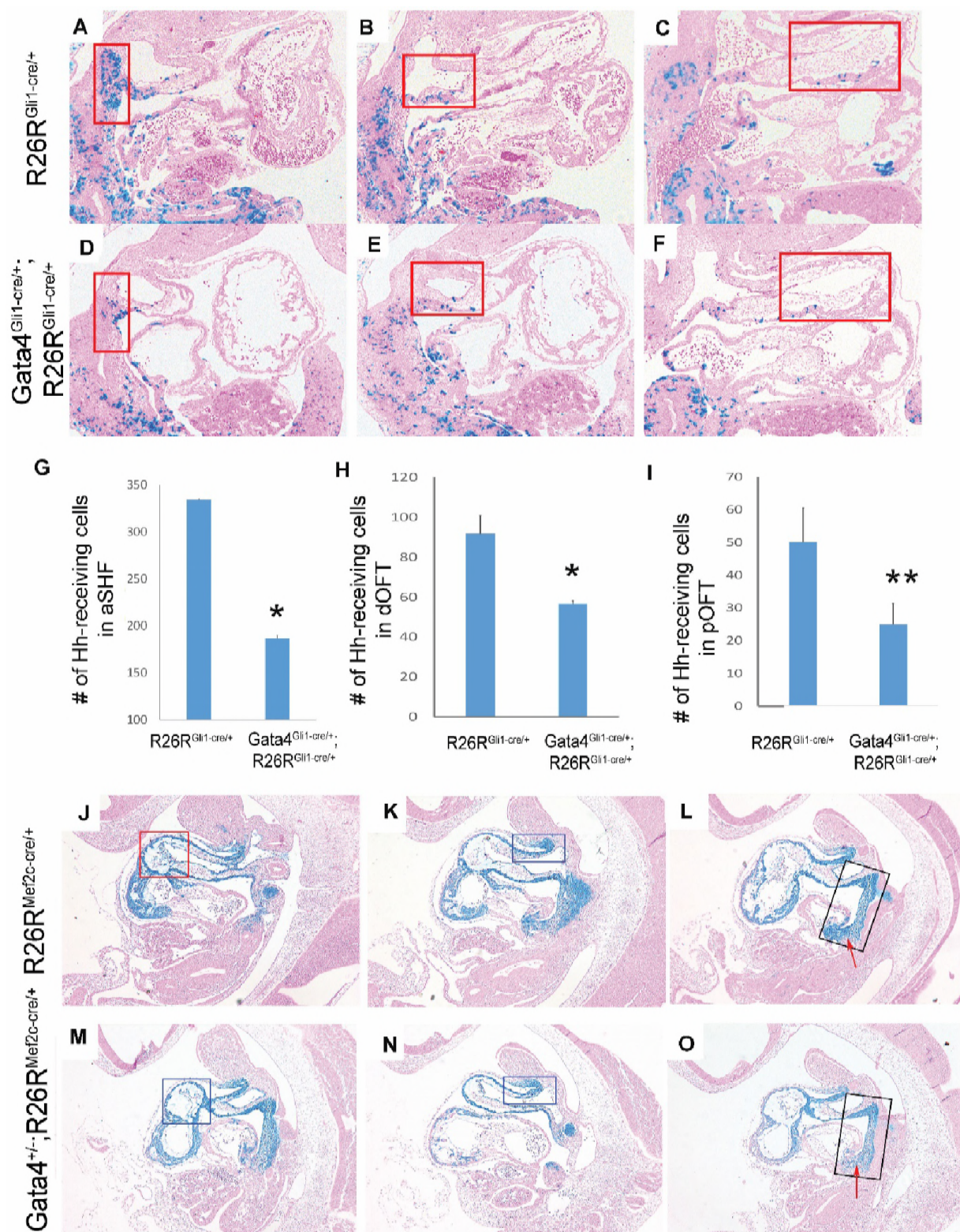
703 Figure 6.



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706 Figure 7.



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709 **Table 1. Incidence of OFT defect in *Gata4* mutant embryos**

Genotype	OFT defect	Total	Type	vs. control	p value
Conditional <i>Gata4</i> mutant embryos					
<i>Gata4</i> <sup>+/-</sup> ; <i>Ella</i> <sup>cre/+</sup>	11	18	DORV, OA	<i>Gata4</i> <sup>fl/+</sup> (0/13)	0.0004
<i>Gata4</i> <sup>fl/+</sup> ; <i>Tnt</i> <sup>cre/+</sup>	0	12	—	<i>Gata4</i> <sup>fl/+</sup> (0/9)	1
<i>Gata4</i> <sup>fl/+</sup> ; <i>Mef2c</i> <sup>cre/+</sup>	1	22	—	<i>Gata4</i> <sup>fl/+</sup> (0/15)	1
<i>Gata4</i> <sup>fl/fl</sup> ; <i>Mef2c</i> <sup>cre/+</sup>	13	13	DORV, OA	<i>Gata4</i> <sup>fl/+</sup> ; <i>Mef2c</i> <sup>cre/+</sup> (1/7)	0.0002
<i>Gata4</i> <sup>fl/+</sup> ; <i>Wnt1</i> <sup>cre/+</sup>	0	24	—	<i>Gata4</i> <sup>fl/+</sup> (0/16)	1
<i>Gata4</i> <sup>fl/+</sup> ; <i>Osrf1</i> <sup>cre/+</sup>	0	5	—	<i>Gata4</i> <sup>fl/+</sup> (0/6)	1
<i>Gata4</i> <sup>fl/+</sup> ; <i>Nfatc1</i> <sup>cre/+</sup>	1	15	DORV	<i>Gata4</i> <sup>fl/+</sup> (0/10)	1
<i>Gata4</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup> (TMX E7.5+8.5)	8	12	DORV, OA	<i>Gata4</i> <sup>fl/+</sup> (0/15)	0.0002
<i>Gata4</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup> (TMX E8.5+9.5)	0	9	—	<i>Gata4</i> <sup>fl/+</sup> (0/9)	1
<i>Tbx5</i> - <i>Gata4</i> compound mutant embryos					
<i>Gata4</i> <sup>+/-</sup> ; <i>Tbx5</i> <sup>+/-</sup>	7	10	DORV, OA	<i>Tbx5</i> <sup>+/-</sup> (1/15)	0.0017
				<i>Gata4</i> <sup>+/-</sup> (4/8)	0.6305
<i>Gata4</i> <sup>fl/+</sup> ; <i>Tbx5</i> <sup>fl/+</sup> ; <i>Mef2c</i> <sup>cre/+</sup>	4	9	DORV	<i>Tbx5</i> <sup>fl/+</sup> ; <i>Mef2c</i> <sup>cre/+</sup> (0/10)	0.0325
				<i>Gata4</i> <sup>fl/+</sup> ; <i>Mef2c</i> <sup>cre/+</sup> (0/13)	0.0172
<i>Pten</i> - <i>Gata4</i> compound mutant embryos					
<i>Gata4</i> <sup>fl/+</sup> ; <i>Pten</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup>	6	20	DORV	<i>Pten</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup> (1/20)	0.0915
				<i>Gata4</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup> (12/29)	0.5495
<i>Smo</i> - <i>Gata4</i> compound mutant embryos					
<i>Gata4</i> <sup>fl/+</sup> ; <i>Smo</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup>	5	9	DORV, OA, PTA	<i>Smo</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup> (0/7)	0.0337
				<i>Gata4</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup> (4/6)	1
<i>Gata4</i> <sup>fl/+</sup> ; <i>SmoM2</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup>	0	9	—	<i>SmoM2</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup> (2/7)	0.1750
				<i>Gata4</i> <sup>fl/+</sup> ; <i>Gli1</i> <sup>cre/+</sup> (7/12)	0.0071
<i>Gata4</i> <sup>fl/+</sup> ; <i>Smo</i> <sup>fl/+</sup> ; <i>Mef2c</i> <sup>cre/+</sup>	3	15	DORV	<i>Smo</i> <sup>fl/+</sup> ; <i>Mef2c</i> <sup>cre/+</sup> (0/12)	0.2308
				<i>Gata4</i> <sup>fl/+</sup> ; <i>Mef2c</i> <sup>cre/+</sup> (0/14)	0.2241
<i>Gata4</i> <sup>+/-</sup> ; <i>Smo</i> <sup>+/-</sup>	5	7	DORV, OA	<i>Gata4</i> <sup>+/-</sup> (1/5)	0.2424
				<i>Smo</i> <sup>+/-</sup> (0/4)	0.0606

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