Regulation of left-right patterning in mice by growth/differentiation factor-1

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The transforming growth factor-β (TGF-β) superfamily encompasses a large group of structurally related polypeptides that are capable of regulating cell growth and differentiation in a wide range of embryonic and adult tissues[1]. Growth/differentiation factor-1 (Gdf-1, encoded by *Gdf1*) is a TGF-β family member of unknown function that was originally isolated from an early mouse embryo cDNA library[2] and is expressed specifically in the nervous system in late-stage embryos and adult mice[3]. Here we show that at early stages of mouse development, *Gdf1* is expressed initially throughout the embryo proper and then most prominently in the primitive node, ventral neural tube, and intermediate and lateral plate mesoderm. To examine its biological function, we generated a mouse line carrying a targeted mutation in *Gdf1*. *Gdf1*$^{-/-}$ mice exhibited a spectrum of defects related to left-right axis formation, including visceral *situs inversus*, right pulmonary isomerism and a range of cardiac anomalies. In most *Gdf1*$^{-/-}$ embryos, the expression of *Ebaf* (formerly *lefty-1*) in the left side of the floor plate and *Leftb* (formerly *lefty-2*), *nodal* and *Pitx2* in the left lateral plate mesoderm was absent, suggesting that *Gdf1* acts upstream of these genes either directly or indirectly to activate their expression. Our findings suggest that *Gdf1* acts early in the pathway of gene activation that leads to the establishment of left-right asymmetry.

Northern-blot analysis of whole embryo RNA using a *Gdf1* probe detected two developmentally regulated transcripts, a 1.4-kb transcript containing the *Gdf1* coding region, which was expressed at early embryonic stages, and a bi-cistronic 3.0-kb transcript, which was expressed in later embryonic stages and in the nervous system of adult mice[3] (Fig. 1*a*). To examine the distribution of the 1.4-kb *Gdf1* transcript, we performed whole-mount *in situ* hybridization experiments on early stage mouse embryos. At 7.5 days post-coitum (d.p.c.), we detected *Gdf1* mRNA uniformly throughout the embryo proper, but not in extra-embryonic structures (Fig. 1*b*,*c*). By 8.0–8.5 d.p.c., we saw *Gdf1* mRNA in many tissues including the crown of the primitive node, ventral neural ectoderm and paraxial, intermediate and lateral plate mesoderm (Fig. 1*d*–*g*). At all stages examined, the expression pattern of *Gdf1* appeared to be bilaterally symmetric with respect to the left-right axis.

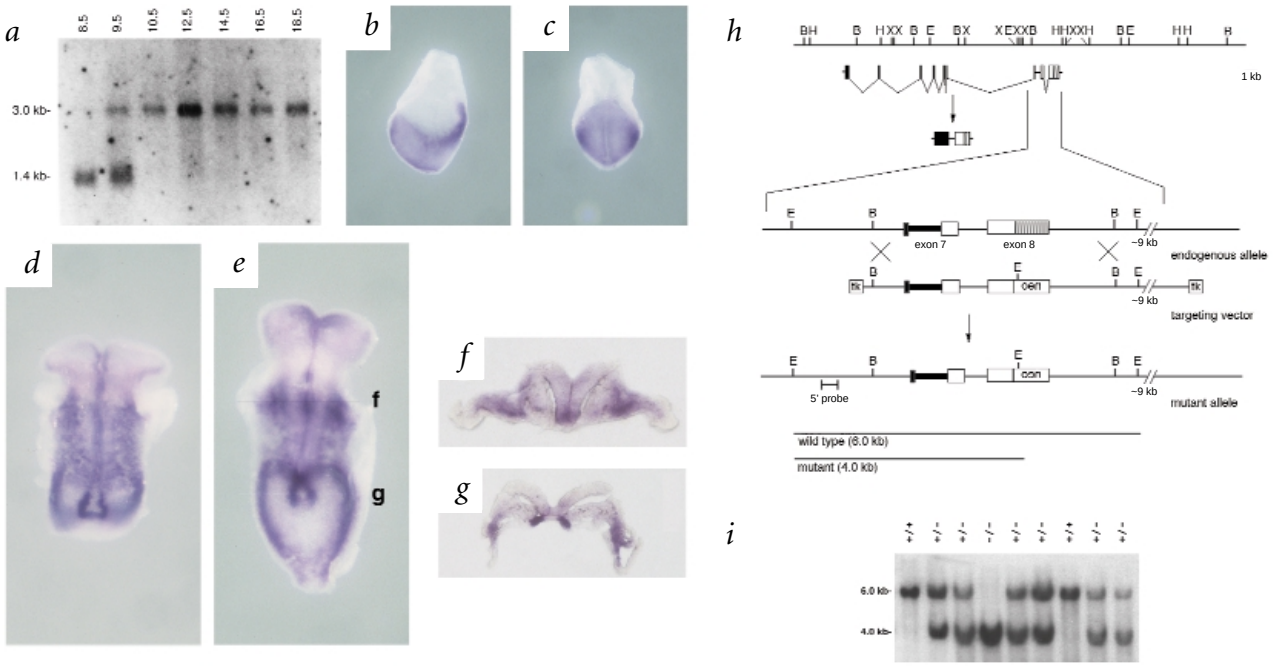

Fig. 1 *Gdf1* expression during early mouse development. ***a***, Northern-blot analysis of whole embryo RNA. Poly(A)$^+$-selected mRNAs prepared from CD-1 mouse embryos at the indicated d.p.c. were electrophoresed, blotted and probed with the entire *Gdf1* coding sequence. *b*–*g*, Whole-mount *in situ* hybridization analysis of 7.5 d.p.c. (***b***, lateral view; ***c***, anterior view), 8.0 d.p.c. (***d***) and 8.5 d.p.c. (***e***) mouse embryos. Expression is symmetric at all stages examined. ***f***,***g***, Sections through a 8.5 d.p.c. embryo at the levels shown in (*e*). ***h***, Genomic map of the *Gdf1* locus and targeting strategy. Filled boxes represent the coding sequence for UOG-1 (ref. 3). Open and shaded boxes represent the coding sequences for the pro- and carboxy-terminal regions of Gdf-1, respectively. A probe derived from genomic sequence upstream of the targeting construct hybridizes to a 6-kb *Eco*RI fragment in wild-type *Gdf1* and a 4-kb *Eco*RI fragment in a homologously targeted *Gdf1* allele. ***i***, Genomic Southern blot of *Eco*RI-digested liver DNA prepared from newborn offspring of a mating of heterozygous mice.

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Table 1 • Defects in *Gdf1*$^{-/-}$ newborn mice

		A	B	C	D	E	F	G	H	I
Heart malformation		✓	✓	✓	✓	✓	✓	✓	✓	✓
Right pulmonary isomerism		✓	✓	✓	✓	✓	✓	✓	✓	✓
GI tract, spleen and pancreas	normal	✓	✓	✓	✓	✓	–	–	–	–
	reversed	–	–	–	–	–	✓	✓	✓	✓
Liver	normal	✓	✓	–	–	–	–	–	–	–
	reversed	–	–	–	–	–	✓	✓	–	–
	symmetric	–	–	✓	✓	✓	–	–	✓	✓
Kidneys	normal	✓	–	✓	–	–	–	–	–	–
	reversed	–	–	–	✓	–	✓	–	✓	–
	symmetric	–	✓	–	–	✓	–	✓	–	✓
Total		10	2	2	2	1	9	1	4	3

Newborn *Gdf1*$^{-/-}$ mice were classified into groups on the basis of their combinations of anatomical *situs* defects.

To investigate the biological function of *Gdf1*, we generated mice in which the entire region encoding the mature Gdf-1 peptide was deleted by gene targeting (Fig. 1*h*). Among 481 offspring examined from *Gdf1*$^{+/-}$ intercrosses, only one homozygous mutant survived to adulthood. *Gdf1*$^{-/-}$ embryos appeared to be viable up to approximately 14.5 d.p.c. Only two-thirds of *Gdf1*$^{-/-}$ embryos survived until birth, however, and nearly all of these died within the first 48 hours after birth. The cause of death was likely to be related to the presence of extensive cardiac defects, although we have not ruled out the possibility that other abnormalities in these mutants might also have contributed to their severely reduced viability.

Homozygous mutant mice exhibited a complex spectrum of abnormalities related to improper establishment of left-right asymmetry (Table 1 and Fig. 2). The most obvious manifestations were the placement of the abdominal organs, which appeared to be randomized in *Gdf1*$^{-/-}$ mutants with respect to the left-right axis. Visceral *situs inversus* was most readily apparent with respect to the stomach, which is normally positioned on the left side (Fig. 2*a–c*). In 50% of *Gdf1*$^{-/-}$ mutants the stomach was positioned on the right side. In *Gdf1*$^{-/-}$ mice with right-sided stomachs, the direction of rotation of the small and large intestines was also reversed (Fig. 2*d,e*, and data not shown). Hence, the gastrointestinal tract appeared to have a mirror-image configuration relative to the left-right axis in approximately 50% of *Gdf1*$^{-/-}$ mice.

Abnormalities in left-right axis formation were evident in other abdominal organs as well. For example, the positions of the pancreas and spleen, which are normally left-sided (Fig. 2*b*), paralleled that of the stomach in *Gdf1*$^{-/-}$ mutants (Fig. 2*c*). The defects in the development of these organs, however, were more complex, as many *Gdf1*$^{-/-}$ mutants had an annular pancreas, and all had severely malformed spleens. Mutation of *Gdf1* also altered the relative positioning of the kidneys and adrenal glands, which are normally displaced caudally on the left side (Fig. 2*f*). In *Gdf1*$^{-/-}$ mice, the left kidney and adrenal were positioned either more cranially than the right kidney and adrenal (Fig. 2*g*) or at the same rostral-caudal level. Similarly, the normal asymmetric arrangement of the liver was also disrupted in *Gdf1*$^{-/-}$ mice. In wild-type mice, the liver consists of a large left lateral lobe, left and right medial lobes, and three smaller lobes on the right side. In *Gdf1*$^{-/-}$ mice, the liver lobes appeared to be reversed with respect to the left-right axis or bilaterally symmetric, with two equally sized lateral lobes beneath a fused medial lobe.

We also saw aberrant left-right patterning of the heart and lungs in homozygous mutants, but the nature of the defects in the thoracic organs appeared to be independent of whether or not the abdominal organs exhibited *situs inversus*. Wild-type mice have five lung lobes, a single large lobe on the left side and four smaller lobes on the right side (Fig. 2*h*). In contrast, *Gdf1*$^{-/-}$ mice had eight lung lobes that were symmetrically distributed with respect to the left-right axis (Fig. 2*i*). The duplication of the right-sided pattern, or right pulmonary isomerism, was observed in all homozygous mutants. The heart defects in *Gdf1*$^{-/-}$ mice were much more complex and variable. In wild-type mice, the apex of the heart points towards the left side of the animal (Fig. 3*d–f*). In *Gdf1*$^{-/-}$ mice, however, the position of the apex was randomized (Fig. 3*g–l*). All mutant hearts also showed abnormal positioning of the great vessels (Fig. 3*a–c*). The pulmonary artery (blue) normally exits the heart ventrally and to the right of the aorta (yellow). In contrast, the pulmonary artery in *Gdf1*$^{-/-}$ mice was positioned more dorsally than the aorta,

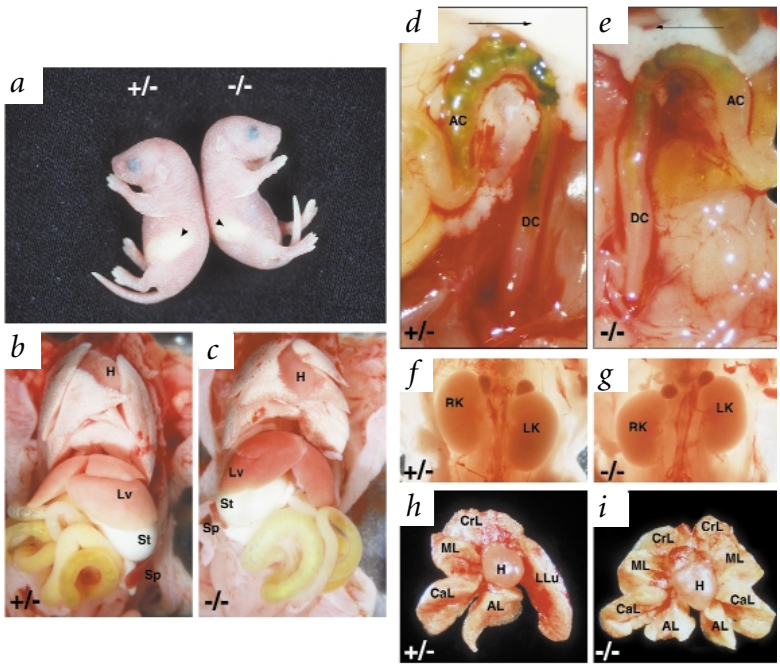

Fig. 2 Analysis of *situs* defects in *Gdf1*$^{-/-}$ mice. ***a***, *Gdf1*$^{+/-}$ and *Gdf1*$^{-/-}$ newborn mice with stomachs (arrowheads) on the left and right sides, respectively. Ventral views of tissues from newborn *Gdf1*$^{+/-}$ (*b,d,f,h*) and *Gdf1*$^{-/-}$ (*c,e,g,i*) mice are shown. ***b,c***, Reversal of the orientation of the abdominal organs in *Gdf1*$^{-/-}$ mice. Note also the streak-like appearance of the spleen and the abnormally shaped medial lobe of the liver. ***d,e***, Reversal of the direction of rotation of the colon in *Gdf1*$^{-/-}$ mice. ***f,g***, Reversal of the rostral-caudal arrangement of the kidneys in *Gdf1*$^{-/-}$ mice. ***h,i***, Right pulmonary isomerism in *Gdf1*$^{-/-}$ mice. H, heart; Lv, liver; St, stomach; Sp, spleen; AC, ascending colon; DC, descending colon; RK, right kidney; LK, left kidney; CrL, cranial lung lobe; ML, medial lung lobe; CaL, caudal lung lobe; AL, accessory lung lobe; LLu, left lung lobe.

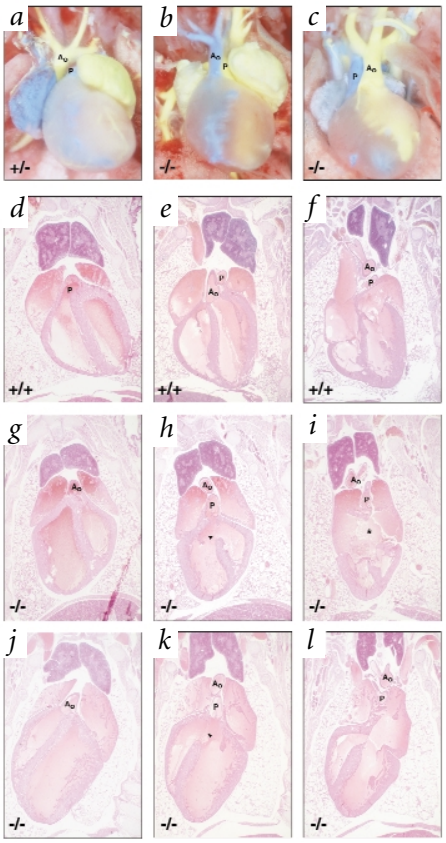

Fig. 3 Heart defects in *Gdf1*$^{-/-}$ mice. *a–c*, Dye injections of *Gdf1*$^{+/-}$ (***a***) and *Gdf1*$^{-/-}$ (***b***,***c***) mice. Yellow dye was injected into the ventricle positioned on the left side, and blue dye was injected into the ventricle on the right side. Note that the relative dorsal/ventral and or left/right relationships between the positions of the aorta and pulmonary artery are reversed in *Gdf1*$^{-/-}$ mice. Frontal sections of wild-type (***d***,***e***,***f***) and two *Gdf1*$^{-/-}$ (***g***,***h***,***i***, mesocardia; ***j***,***k***,***l***, dextrocardia) hearts are shown. For each, three sections progressing from the ventral to the dorsal side are shown. In wild-type heart, the pulmonary artery exits the heart more ventrally than does the aorta. In *Gdf1*$^{-/-}$ mice, the aorta exits more ventrally. Ventricular septal defects (*h*,*k*,*l*, arrowheads) and atrial septal defects (*i*, asterisk) were also seen in *Gdf1*$^{-/-}$ mice.

although the left-right relationship of these arteries was randomized. In these mutants, the abnormal positioning of the aorta and pulmonary artery reflected a transposition of the great vessels. Histological examination of serial sections of five *Gdf1*$^{-/-}$ hearts revealed additional defects as well, including atrial and ventricular septal defects, common atrioventricular canal and persistent left vena cava (Fig. 3*g*, and data not shown).

To examine the relationship of *Gdf1* with other genes implicated in left-right determination, we carried out whole-mount *in situ* hybridization on *Gdf1*$^{-/-}$ embryos with probes directed against *Ebaf*, *Leftb* and *nodal*, encoding TGF-β family members, as well as *Pitx2*, encoding a transcription factor (Table 2). In wild-type embryos at the headfold stage (~4–6 somites), *Ebaf* is predominantly expressed on the left side of the presumptive floor plate, and *Leftb* is mainly expressed in the left lateral plate mesoderm[4] (LPM; Fig. 4*a*). Using a probe that detects both *Ebaf* and *Leftb* transcripts[5], we were unable to detect expression of these genes in most (6/7) headfold-stage *Gdf1*$^{-/-}$ embryos examined (Fig. 4*b*). Expression of *nodal* in headfold-stage wild-type embryos is normally seen in the node and left LPM (Fig. 4*c*; refs 6,7). Although the expression of *nodal* in the node remained unchanged, the expression of *nodal* in the LPM was absent in all mutant embryos examined (Fig. 4*d*). *Pitx2*, which is normally present in the left LPM of wild-type embryos[8,9] (Fig. 4*e*), was also downregulated in 8 of 11 *Gdf1*$^{-/-}$ embryos (Fig. 4*f*). These results suggest that *Gdf1* is necessary for the induction or maintenance of the asymmetric expression of *Ebaf*, *Leftb*, *nodal* and *Pitx2* in the early embryo.

Our findings indicate that *Gdf1* is essential for proper establishment of the left-right axis in mice. Although a number of other secreted proteins, including other members of the TGF-β superfamily, have been implicated as important regulators of left-right axis determination in vertebrates[10], Gdf-1 is unusual in that loss of Gdf-1 function leads to complete visceral *situs inversus* in a large percentage of mutant animals. In this regard, the *Gdf1*$^{-/-}$ phenotype more closely resembles that of mice carrying the *Dnahc11*^{iv}

Table 2 • Gene expression in *Gdf1*$^{-/-}$ embryos

			Floor plate				Lateral plate mesoderm				
Gene	Genotype	Somites	left	right	bilateral	absent	left	right	bilateral	absent	Total
Ebaf/*Leftb*	+/+	0–4	1	–	–	3	–	–	–	4	4
		4–6	5	–	–	1	6	–	–	–	6
		6–8	–	–	–	1	1	–	–	–	1
	+/–	0–4	5	–	–	4	–	–	–	9	9
		4–6	10	–	–	1	9	–	–	2	11
		6–8	2	–	–	6	8	–	–		8
	–/–	0–4	–	–	–	4				4	4
		4–6	1	–	–	6	1			6	7
		6–8	–	–	–	6	1			5	6

			Node		Lateral plate mesoderm				
			normal	absent	left	right	bilateral	absent	Total
nodal	+/+	0–4	6	–	–	–	–	6	6
		4–6	8	–	8	–	–		8
		6–10	3	1	–	–	–	4	4
	+/–	0–4	9	–	–	–	–	9	9
		4–6	12	–	12	–	–	–	12
		6–10	6	2	1	–	–	7	8
	–/–	0–4	6	–	–	–	–	6	6
		4–6	3	–	–	–	–	3	3
		6–10	7	2	1	–	–	8	9

			Lateral plate mesoderm				
			left	right	bilateral	absent	Total
Pitx2	+/+	6–10	5	–	–	–	5
	+/–	6–10	23	–	–	5	28
	–/–	6–10	3	–	–	8	11

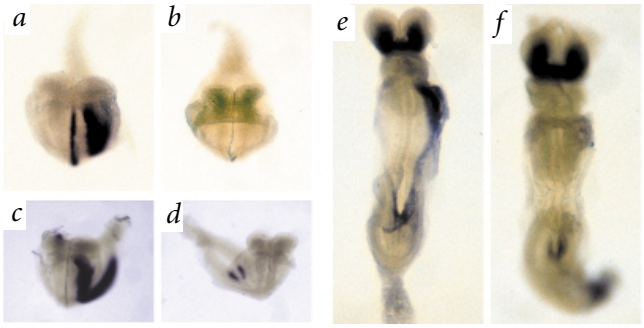

Fig. 4 Asymmetric gene expression in *Gdf1*$^{-/-}$ mice. Embryos in (*a*–*d*) are viewed from the anterior; (*e*) and (*f*) are seen from the ventral side. ***a***, Normal *Ebaf* and *Leftb* expression in the left LPM and floor plate. The expression of *Ebaf* and *Leftb* in the floor plate was verified by examining sections of these embryos. ***b***, Absence of visible *Ebaf* and *Leftb* expression in *Gdf1*$^{-/-}$ embryos. The midline staining in (*b*) represents background hybridization that did not correspond to the floor plate based on an analysis of sections of these embryos. ***c***, Normal expression of *nodal* in the left LPM (node staining is obscured by the LPM staining). ***d***, Absence of *nodal* expression in the LPM of *Gdf1*$^{-/-}$ embryos. Note the normal expression of *nodal* in the node. ***e***, Normal expression of *Pitx2* in the head mesenchyme and left LPM. ***f***, Absence of *Pitx2* expression in the LPM of *Gdf1*$^{-/-}$ embryos, whereas expression in the head mesenchyme remains normal.

allele[11,12] and that of humans with certain asplenia syndromes that are characterized by *situs inversus*[13,14]. *Gdf1* is also unusual in that its expression pattern during development appears to be symmetric with respect to the left-right axis. Although we cannot rule out the possibility that there is some asymmetric expression of *Gdf1* that is either very transient or below the level of detection in our experiments, our data suggest that the mechanism by which *Gdf1* influences left-right asymmetry must involve other molecules that are asymmetrically expressed, such as those responsible for generating biologically active Gdf-1 protein or for transducing the Gdf-1 signal. Alternatively, Gdf-1 protein may be synthesized in a symmetric pattern, but become asymmetrically distributed by some other mechanism. In this regard, it has been hypothesized that a leftward flow of fluid generated by cilia present in the node may be responsible for the directional movement of a morphogen produced by the node[15,16]. Given the intense expression of *Gdf1* in the node and the finding that *Gdf1* acts upstream of *Ebaf*, *Leftb*, *nodal* and *Pitx2*, Gdf-1 may be a possible candidate for this morphogen. An elucidation of the mechanism of action of Gdf-1 will require a careful examination of the distribution of the mature Gdf-1 protein and the identification of molecules directly involved in Gdf-1 signalling, including the Gdf-1 receptor.

Methods

***In situ* hybridization and northern-blot analysis.** We carried out hybridizations using a probe corresponding to the entire *Gdf1* coding region[2]. We prepared the *Ebaf*, *Leftb* (ref. 5) and *nodal* (ref. 17) probes as described. The *Pitx2* probe was provided by M. Blum and M.R. Kuehn. Embryos were isolated from timed matings of CD-1 mice. We carried out northern-blot analysis using poly(A)-selected RNA (2 μg) as described[2]. Whole-mount *in situ* hybridization analysis was carried out as described[18,19], except that 20% heat-inactivated sheep serum was used for antibody blocking and incubation steps. Sections (10–12 μm) of stained embryos were prepared using a cryostat.

Construction and analysis of *Gdf1*-null mice. The structure of *Gdf1* was deduced from restriction mapping and partial sequencing of phage clones isolated from a mouse 129 Sv/J genomic library. Vectors for preparing the targeting construct were provided by P. Soriano and K. Thomas. RI embryonic stem cells (provided by A. Nagy, R. Nagy and W. Abramow-Newerly) were transfected with linearized targeting construct (50 μg) and selected with gancyclovir (2 μM) and G418 (200 μg/ml). Following electroporation of the targeting construct into embryonic stem cells, we identified homologous targeting by Southern-blot analysis in 4 of 90 clones resistant to both G418 and gancyclovir. Examination of the *Gdf1*-mutant phenotype was carried out using offspring on a C57Bl/6/129SV/J hybrid background from a chimaera derived from blastocyst injection of one of these clones. Genomic Southern blots were carried out as described[20]. We genotyped embryos by PCR using genomic DNA isolated from extra-embryonic membranes[21]. Primers for genotyping were as follows: *Gdf1* wild-type allele, 5′–GTTGCG GCTGGAGGCTGAGAG–3′ and 5′–CCCACTGGACCAACTTCTACC–3′; *Gdf1* targeted allele, 5′–CCACTGCAGCCTGTGGGCGC–3′ and 5′–GGAA GACAATAGCAGGCATGCTGG–3′.

For analysis of heart morphology, we performed latex dye injections as described[22]. Casting dyes (Connecticut Valley Biological Supply) were injected into the ventricles of the heart using a pulled capillary glass pipette. For histological analysis of *Gdf1*-mutant hearts, we killed newborn mice and infused them with Bouin's fixative through the trachea. The entire animal was then fixed by immersion in Bouin's fixative for at least 24 h. Mouse chests were step-sectioned (5 μM sections, 10 μM between each section) from the exit of the aorta or pulmonary artery to their respective branch points dorsally. For analysis, we stained sections with haematoxylin and eosin.

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