

Mutations affecting the cardiovascular system and other internal organs in zebrafish

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SUMMARY

In a screen for early developmental mutants of the zebrafish, we have identified mutations specifically affecting the internal organs. We identified 53 mutations affecting the cardiovascular system. Nine of them affect specific landmarks of heart morphogenesis. Mutations in four genes cause a failure in the fusion of the bilateral heart primordia, resulting in *cardia bifida*. In *lonely atrium*, no heart ventricle is visible and the atrium is directly fused to the outflow tract. In the *overlooped* mutant, the relative position of the two heart chambers is distorted. The heart is enormously enlarged in the *santa* mutant. In two mutants, *scotch tape* and *superglue*, the cardiac jelly between the two layers of the heart is significantly reduced. We also identified a number of mutations affecting the function of the heart. The mutations affecting heart function can be subdivided into two groups, one affecting heart contraction and another affecting the rhythm of the

heart beat. Among the contractility group of mutants are 5 with no heart beat at all and 15 with a reduced heart beat of one or both chambers. 6 mutations are in the rhythmicity group and specifically affect the beating pattern of the heart. Mutations in two genes, *bypass* and *kurzschluss*, cause specific defects in the circulatory system. In addition to the heart mutants, we identified 23 mutations affecting the integrity of the liver, the intestine or the kidney. In this report, we demonstrate that it is feasible to screen for genes specific for the patterning or function of certain internal organs in the zebrafish. The mutations presented here could serve as an entrypoint to the establishment of a genetic hierarchy underlying organogenesis.

Key words: heart, circulation, cardiovascular system, intestine, liver, kidney, zebrafish

INTRODUCTION

During organogenesis, cells from different germ layers are incorporated into the body organs. The heart is the first organ formed in the developing embryo. In vertebrates, the heart precursors from both sides of the embryo move medially during gastrulation and reside on either side of the midline as part of the lateral mesoderm. The bilateral heart primordia then fuse at the midline and form the primitive heart tube, consisting of two concentric tubes. The outer layer, the myocardium, will form the heart muscles and the inner layer, the endocardium, will become the inner lining of the heart (DeHaan, 1965; DeRuiter et al., 1992; Stainier and Fishman, 1994). The heart starts beating regularly soon after the fusion, and loops to the right hand side of the embryos, eventually placing the atrium on top of the ventricle. Four distinct chambers, sinus venosus,

atrium, ventricle and bulbus arteriosus (outflow tract), become detectable in the heart tube.

All vertebrates have a closed circulation system in which the blood cells circulate through the whole body within vessels, return to the atrium via the sinus venosus and are pumped predominantly by the ventricle through the outflow tract to the whole body. As the pacemaker, the sinus venosus is the first to contract. A wave of muscle contraction is then propagated up to the ventricle through the atrium. To maintain the forward blood flow, the atrium does not relax until the ventricle contracts. The valves develop at the boundaries of chambers to prevent backflow.

The molecular mechanisms governing the development of the cardiovascular system are poorly understood. In recent years, a few genes expressed in the developing heart and vascular system have been identified. Their relevance in the

developing cardiovascular system has been tested in the mouse by the embryonic stem cell (ES cell) based gene knock-out technique. For some genes, the knock-out phenotype is consistent with the expression pattern. For example, *N-myc* and *Neurofibromatosis type 1 (NF-1)* are highly expressed in the developing ventricles at the time that massive growth of the ventricular musculature is observed. The knock out mice of *N-myc* and *NF-1* have severe defects in the growth of the ventricular musculature (Charron et al., 1992; Moens et al., 1993; Stanton et al., 1992; Brannan et al., 1994). Four receptor tyrosine kinases (RTKs), *flk-1*, *flt-1*, *tie-1* and *tie-2* are expressed during embryonic vasculogenesis and angiogenesis. The knock-out mice show different defects in the vasculature, demonstrating that these genes play important but distinct roles in the developing vasculature (Shalaby et al., 1995; Fong et al., 1995; Sato et al., 1995; Dumont et al., 1994). For other genes expressed in the developing cardiovascular system, however, the knock-out mice do not show a cardiovascular phenotype (Kern et al., 1995), perhaps due to redundancy. In fact, the disruption of the retinoic acid receptors (RAR) α , β or γ individually do not result in any obvious phenotypes, but the compound mutations show severe defects in various organs, including the cardiovascular system (Mendelsohn et al., 1994).

Drosophila has been a great source of information about genes important for vertebrate development. The early events in heart formation seem to be conserved between the fly and the vertebrates. As in the vertebrate, the *Drosophila* heart precursors move from either side of the embryo and fuse at the midline to form a primitive heart tube. A gene, *tinman*, involved in the cell fate commitment of the heart precursors has been isolated from the fly (Frasch, 1995; Staehling-Hampton et al., 1994). The lack of the biological function encoded by *tinman* leads to the complete absence of the heart (Bodmer, 1993). The vertebrate homologues of *tinman* have been isolated from *Xenopus* and mouse and their expression patterns correlate with the heart development (Lints et al., 1993; Tonissen et al., 1994). The knock-out mouse homologue, *Nkx-2.5*, of *tinman* has significant cardiac malformations (Lyons et al., 1995). These findings suggest that the *Drosophila* system could be applied to studies on early heart morphogenic events. The fly and vertebrate heart development diverge soon after the primitive heart tube is formed. In contrast to the vertebrate, the fly heart has no endocardium, no valves and no chamber demarcation. Furthermore, the circulation is an open system in which the internal tissues are soaked in hemolymph. Therefore, it is not likely to find genes responsible for these later events in *Drosophila*.

Without a vertebrate genetic system, studies on cardiovascular development have been restricted to biochemical analysis and the already cloned genes such as the *Drosophila* homologues. A systematic scanning through the vertebrate genome could isolate novel genes involved in organogenesis. We and others have shown that such a screen is feasible in the zebrafish (Mullins et al., 1994; Solnica-Krezel et al., 1994). The zebrafish system is especially suitable for studying the cardiovascular system. The fish heart is the prototype of the vertebrate heart. The heart formation processes are the same in fish and other vertebrates. Only because the lungs are used as the gas exchanging organ, intrachamber septa are developed in birds and mammals to separate the deoxygenated and oxygenated blood. The heart is placed prominently on the ventral

side of the zebrafish embryos and can be observed under the dissecting microscope soon after the heart tube is formed. The myocardial and endocardial layers of the heart are distinguishable. The heart starts beating regularly at about 24 hours postfertilization (hpf) and the circulation is strong in the trunk and head by 36 hpf. Therefore, mutations with defects in the cardiovascular system can be easily scored.

Because of the transparency of the embryo, the other internal organs can also be easily observed under the dissecting microscope. Therefore, it is feasible to study the development of all the internal organs in zebrafish. Here, we describe mutations affecting the cardiovascular system, liver, intestine and kidney of the zebrafish. The phenotypes and the numbers of alleles of these mutations are summarized in Tables 1 and 2.

MATERIALS AND METHODS

Fish were raised as described by Mullins et al. (1994). The embryos were staged as described by Kimmel et al. (1995). Complementation analysis was done by pairwise crossing of two heterozygotes mutants (Haffter et al., 1996). Groups of mutants were assigned based on the mutant phenotypes and the allelisms were tested within these groups. The embryos were fixed and sectioned for histology as described by Stainier et al. (1993). For whole-mount immunohistochemistry, the embryos were fixed and stained as described by Dent et al. (1989). S46, anti-atrial-specific myosin heavy chain, was a kind gift from Dr Jeff Miller, Massachusetts General Hospital.

RESULTS

Heart

We identified 40 mutations affecting either heart pattern formation or the maintenance of heart function. These mutants are categorized into two subclasses, morphogenic and functional. There are 11 mutations in the morphogenic group which bear obvious defects in the patterning of the heart, whereas the 29 mutations in the functional group show defects in the heart function before the abnormal heart shape becomes obvious.

Morphogenic mutants

Cardia bifida

As in all vertebrates, the zebrafish heart is the result of the fusion of the bilateral heart primordia, which takes place between the 21-somite and 26-somite stages (Stainier et al., 1993). Mutants with two hearts, one on either side of the midline, known as cardia bifida phenotype, were isolated. The mutants bear two swollen pericardial sacs with a beating structure in each one of them (Fig. 1B). With an antibody against the atrial-specific myosin heavy chain, S46, two hearts residing on either side of the midline were clearly visualized (Fig. 1C). Histological sections suggest that these mutants have two relatively complete hearts. Both chambers, atrium and ventricle, are distinguishable (Fig. 1D). Although the heart beats, no circulation has ever been observed in homozygotes of these mutants.

Four loci responsible for the cardia bifida phenotype were identified in the screen. Each one bears additional defects which can be used to distinguish them from each other. Embryos mutant for *miles apart (mil)* have blisters at the tip of the tail which is obvious by 36 hpf (Fig. 1F). One allele of *mil* was also

Table 1. The summary of the mutants that show the cardiovascular phenotypes

Gene name	Allele name	Cardiovascular phenotype	Other phenotype	Reference
Heart morphogenesis				
Cardia bifida				
<i>miles apart (mil)</i>	<i>te273, m93</i>	Two hearts	Blisters in the tail	a
<i>casanova (cas)</i>	<i>ta56</i>	Two hearts	Gradually losing motility	
<i>faust (fau)</i>	<i>tm236a</i>	Two hearts, fused in a fraction of the embryos		
<i>natter (nat)</i>	<i>ta219, tl43c</i>	Two hearts in 10% of the embryos	Flat hindbrain	b
Chamber				
<i>lonely atrium (loa)</i>	<i>tu29d</i>	No ventricle detectable		
Chamber positioning				
<i>overlooped (olp)</i>	<i>ts33b</i>	Ventricle and atrium are placed at a 90 degree angle relative to each other		
Size				
<i>santa (san)</i>	<i>ty219c, m775</i>	Enlarged heart chambers		a
Thin matrix				
<i>scotch tape (sco)</i>	<i>te382, tt239</i>	Myocardium and endocardium attach to each other	Disorganized endothelia	
<i>superglue (sug)</i>	<i>te352</i>	Myocardium and endocardium attach to each other		
Heart function				
Contractility				
<i>silent heart (sih)</i>	<i>tc300b, b109</i>	No heart beat		c
<i>still heart (sth)</i>	<i>to241a, tm123a, b212</i>	No heart beat	Severely reduced motility	c
Unresolved	<i>tw212e</i>	No heart beat	Severely reduced motility	
<i>viper (vip)</i>	<i>ta52e</i>	No heart beat	Retarded brain development	b
<i>pipe heart (pip)</i>	<i>tq286e</i>	Weak contraction of the ventricle		
<i>tango (tan)</i>	<i>tg299c, to216b</i>	Weak contraction of the ventricle		
<i>weak atrium (wea)</i>	<i>tw220a, m58, m229, m118</i>	Nearly silent atrium		a
<i>polka (plk)</i>	<i>tg290, ty105b</i>	Isolated cell contractions in the heart		
<i>weak beat (web)</i>	<i>tc288b</i>	Weak contraction of both chambers		
<i>quiet heart (quh)</i>	<i>tq268</i>	Nearly silent heart, weak contraction of both chambers		
<i>stretched (str)</i>	<i>tu255b</i>	Weak contraction of both chambers, heart is pulled into a string-like structure		
<i>herzschlag (hel)</i>	<i>tg287</i>	Weak contraction of the heart	Immotile	d
<i>heart atact (hat)</i>	<i>te313</i>	Weak contraction of the heart	Immotile	d
Unresolved	<i>tk34b</i>	Weak contraction of the heart	Immotile	d
<i>slop (slp)</i>	<i>tq235c</i>	Weak contraction of the heart	Reduced motility, reduced muscle striation	d
<i>jam (jam)</i>	<i>tr254a</i>	Weak contraction of the heart	Reduced motility, reduced muscle striation	d
<i>slinky (sky)</i>	<i>ts254</i>	Weak contraction of the heart	Reduced motility, reduced muscle striation	d
Rhythmicity				
<i>breakdance (bre)</i>	<i>tb218</i>	Beating rate of atrium: ventricle = 2:1		
<i>hiphop (hip)</i>	<i>tx218</i>	Beating rate of atrium: ventricle = 3:1		
<i>tremblor (tre)</i>	<i>tc318d, te381b, m116, m139, m158, m276, m736</i>	Fibrillating heart		a
<i>legong (leg)</i>	<i>tj201</i>	Heart initially beats weakly and becomes fibrillating later		
<i>slip jig (sli)</i>	<i>tm117c</i>	Heart initially beats weakly and becomes fibrillating later		
Transient				
Unresolved	<i>tr206d</i>	Weak contraction and edema at embryonic day 2, but recovered later		
Unresolved	<i>ts206</i>	Weak contraction and edema at embryonic day 2, but recovered later		
Unresolved	<i>tm96</i>	Weak contraction and edema at embryonic day 2, but recovered later		
Circulation				
<i>bypass (byp)</i>	<i>tu244c, tm209</i>	Blood drains into the heart more anteriorly	Posterior position of the yolk	
<i>kurzschluss (kus)</i>	<i>tr12</i>	No circulation in the trunk		
<i>sonic-you (syu)</i>	<i>tq252</i>	No or delayed circulation	No horizontal myoseptum	e
<i>you (you)</i>	<i>tm146c, ty97, tz310b</i>	Delayed circulation	No horizontal myoseptum	e
<i>you-too (yot)</i>	<i>ty17a, ty119</i>	No circulation in the trunk	No horizontal myoseptum	e
<i>chameleon (con)</i>	<i>tf18b, th6, tm15a, ty60</i>	Reduced circulation	No horizontal myoseptum	e

Unresolved: complementation testing not complete.

The t alleles were isolated in the Tübingen screen. The m alleles were from the Boston screen. The b alleles were isolated from Oregon.

References: a, Stainier et al., 1996; b, Jiang et al., 1996; c, Kimmel et al., personal communication; d, Granato et al., 1996; e, van Eeden et al., 1996.

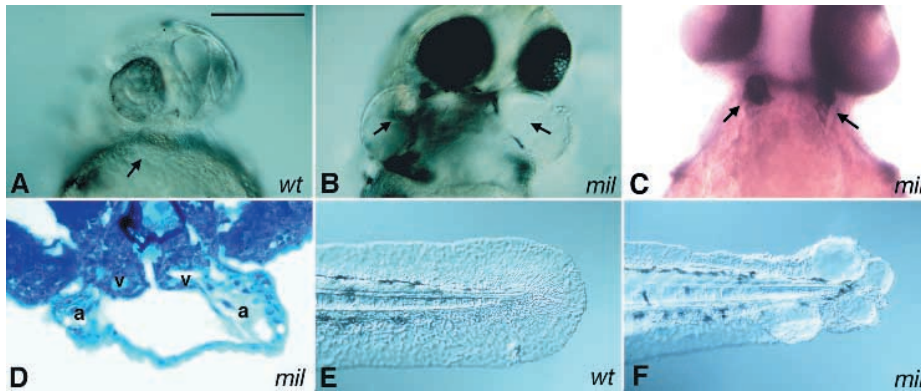


Fig. 1. Cardia bifida mutants. Two hearts are located at either side of the embryo. A ventral view of 1-day old embryos shows two swollen pericardial sac in *mil^{e273}* (B) but not in the wild-type sibling (A). Two hearts can be clearly visualized either by immunostaining or in histological sections. (C) A 2-day old *mil^{e273}* embryo stained with the antibody S46, which labels the atria. (D) A transverse section of a 2-day old *mil^{e273}* embryo. The hearts are indicated by arrows; a, atrium; v, ventricle. Images of tails of live 1-day old wild-type and *mil^{e273}* embryos are shown in E and F, respectively. Scale bar, 100 μ m.

isolated in the Boston screen (Stainier et al., 1996). A mutation in *casanova* (*cas*) causes reduced motility. 1-day old *cas* embryos are as motile as the wild-type siblings. The motility then gradually reduces, and after 2 days of development, *cas* embryos are immotile. The cardia bifida phenotype in *faust* (*fau*) is somewhat variable. The majority of the *fau* embryos clearly have two hearts, while in a small fraction of them, the heart tubes eventually fuse (the fraction varies from cross to cross). Embryos mutant for *natter* (*nat*) show abnormalities in the subdivision of the brain (Jiang et al., 1996). However, heart tubes fail to fuse in only 10% of the *nat* embryos.

Heart chambers

In the zebrafish embryo, the atrium and the ventricle can be clearly distinguished by 36 hpf (Fishman and Stainier, 1994). In embryos mutant for *lonely atrium* (*loa*), the heart is significantly enlarged at 30 hpf. After 2 days of development, a tube-like structure, morphologically resembling the outflow tract, is connected to an enlarged chamber, morphologically resembling the atrium. No obvious structure resembling the ventricle can be observed in *loa* embryos either microscopically or histologically (Fig. 2B,D). Whether this is the result of a missing ventricle or a failure in chamber demarcation is not clear yet.

Chamber positioning

A mutation in the gene *overlooped* (*olp*) results in an abnormal positioning of the heart chambers. In 2-day old wild type

embryos, the ventricle and atrium are usually parallel to each other with the atrium on the left and the ventricle on the right side of the embryo. In the *olp* mutant, the ventricle is placed on top of the atrium at a 90 degree angle (Fig. 3). After 3 days of development, the outflow tract of *olp* mutants becomes slightly enlarged. Despite the abnormal positioning of the heart chambers and the enlarged outflow tract, the contractility of the heart and the circulation in *olp* embryos are relatively normal when compared to the wild-type siblings. Homozygotes are currently being raised and presumably will survive to the adulthood.

Size of the heart

Mutations in the gene *santa* (*san*) result in an enormously big heart. The enlargement of the heart becomes evident after 36 hours of development. In 2-day old *san* embryos, the heart looks like three big connected bubbles with the atrium located posteriorly, the ventricle in the middle and the outflow tract at the anterior of the heart (Fig. 4B). The phenotype of *san* seems to be restricted to the heart. No obvious overgrown tissues other than the heart were observed in *san* embryos. Both myocardium and endocardium are present (Fig. 4D). The ventricular myocardium in *san* embryo stays as a single cell layer after 3 days of development, whereas multiple layers are present in the wild-type siblings. The heart contracts vigorously, but no or very weak circulation is observed in *san* embryos. Another allele of *san* was identified in the Boston screen (Stainier et al., 1996).

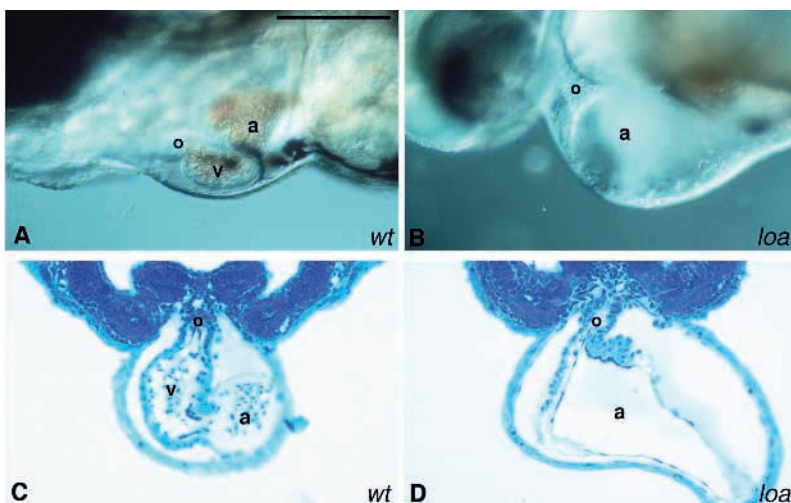


Fig. 2. Chamber demarcation mutant. Images of the hearts of live 2-day old wild-type (A) and *loa^{tu29d}* (B) embryos are shown. The atrium (a) is significantly enlarged in *loa^{tu29d}*. No obvious structure representing the ventricle (v) is seen between the atrium and the outflow tract (o). The atrium, ventricle and outflow tract are present in a transverse section of the heart in a 2-day old wild-type embryo (C). However, no obvious structure representing the ventricle is seen in a transverse section of the heart of the 2-day old *loa^{tu29d}* embryo (D). The embryos are oriented with the anterior to the left and dorsal to the top. Scale bar, 100 μ m.

Table 2. The summary of the mutants that show the internal organ phenotypes

Gene name	Allele name	Phenotype	Reference
Intestine			
<i>flotte lotte (flo)</i>	<i>ti262c</i>	Thin intestinal epithelium, no folding in the intestine	
<i>stuffed (stf)</i>	<i>tz259</i>	Thin intestinal epithelium, grey material in the gut	
Liver			
<i>lumpazi (lum)</i>	<i>th209b</i>	Liver necrosis	
<i>gammmler (gam)</i>	<i>tf207d</i>	Liver necrosis	
<i>tramp (trp)</i>	<i>tm117d</i>	Liver necrosis	
<i>tippelbruder (tip)</i>	<i>th203</i>	Accumulation of blood in the liver	
Kidney			
<i>no isthmus (noi)</i>	<i>tb21, th44, tm243a, tu29a, ty22b, ty31z</i>	No pronephros, no midbrain-hindbrain boundary	a
<i>locke (lok)</i>	<i>tj8, tl215, tm138a to237b, ts277</i>	Cyst at the location of the pronephros, curly tail down	a
Unresolved	<i>tf214a, tf206, tg238a, tm304, tp49d, tz214</i>	Cyst at the location of the pronephros, curly tail down	a

Unresolved, complementation testing not complete; a, Brand et al., 1996b.

Thin matrix

The heart consists of two concentric tubes with the outer myocardial cell layer and the inner endocardial cell layer. An extracellular matrix (cardiac jelly) is located between the two layers. In the mutants *scotch tape (sco)* and *superglue (sug)*, the atrium is slightly enlarged and the endocardial cell layer is not visible under the compound microscope. However, the endocardium can be observed in histological sections, which reveals that the space between the myocardium and endocardium is very narrow. Especially in the atrium, these two layers seem to attach to each other, leaving no space in between. We therefore conclude, that the cardiac jelly is significantly reduced in these mutants. The heart contracts powerfully, but the circulation is significantly reduced in both

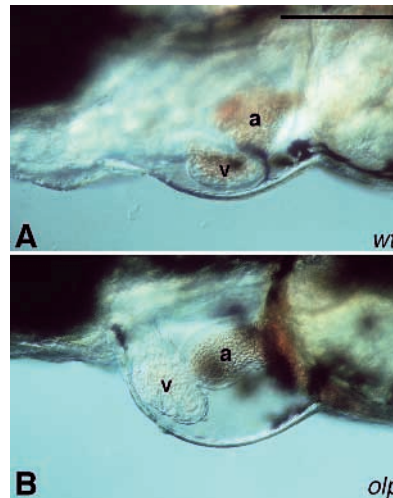


Fig. 3. Chamber positioning mutant. Images of the heart of live 2-day old wild-type (A) and *olp* (B) embryos. The ventricle (v) is placed on top of the atrium (a) at a 90 degree angle in the wild-type embryo (A). Scale bar, 100 µm.

mutants. No backflow is observed. Consistent with that, a pair of well-formed valves are located at the atrial-ventricular junction as shown in the histological section (Fig. 5A,B).

In addition to the attached endocardium-myocardium phenotype, the organization of endothelia in the tail fin seem to be disrupted in *sco*. In 2-day old wild-type embryos, the endothelial cells in the ventral fin are well organized. The blood cells go through this path and return to the axial vein. In *sco* embryos, the endothelial cells are disorganized leaving empty spaces in the ventral fin. The blood cells are, therefore, stuck in the ventral fin (Fig. 5D). In severe cases, the ventral tail fin is completely missing. We isolated two alleles, *sco^{te382}* and *sco^{tt239}*. Both alleles are homozygous viable and no obvious abnormality can be detected in the adult homozygotes.

Functional mutants

We found 29 mutants in which the heart does not function properly. No obvious abnormalities in the heart morphology of these mutants can be observed before the functional defect appears. However, the gradually failing heart eventually leads to distortions in the shape of the heart. Fig. 6 shows a few examples of functional heart mutants. The functional heart

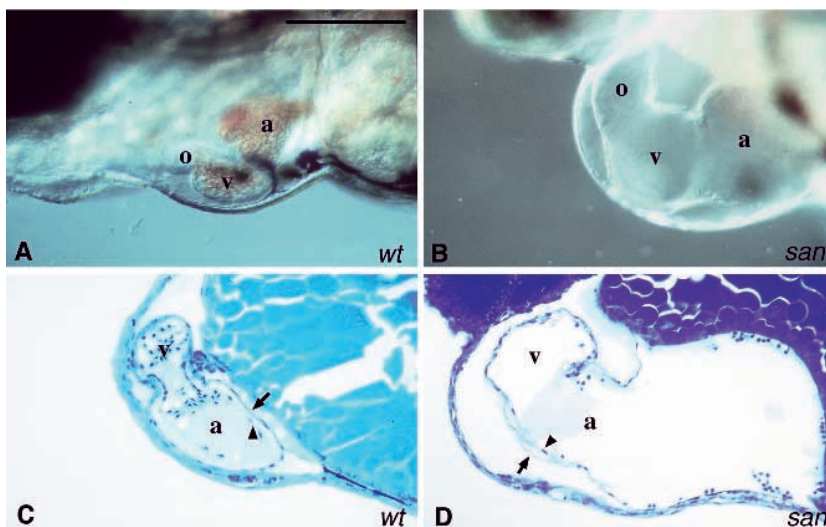


Fig. 4. Mutant with an enlarged heart. Images of the hearts of live embryos. (A) Wild-type embryo. As shown, the atrium (a), ventricle (v) and the outflow tract (o) are located on the ventral side of the embryo. Looping places the atrium to the left side of the embryo. The circulation is strong and the heart is full of blood cells. (B) The heart of a 2-day old *san^{ty219c}* embryo. The atrium (a), ventricle (v) and the outflow tract (o) are significantly enlarged in this mutant. The absence of circulation results in a lack of blood cells in the heart chambers. Sagittal sections of the hearts of normal 2-day old wild-type (C) and *san^{ty219c}* (D) embryos. Because of the looping the chambers are placed in different focal planes so only the ventricle and the atrium are shown in these sections. The myocardium (arrow) and endocardium (arrowhead) are clearly distinguishable. The space between the myocardial and endocardial cell layers in the mutant atrium is much thinner than in the normal heart. Scale bar, 100 µm.

mutations can be divided into two subgroups, one in which the contractility of the heart is affected and another one, in which the rhythm of the heart beat is abnormal.

Contractility mutants

Mutations affecting the contractility of the heart can be further subdivided into five phenotypic subgroups. 5 mutants do not show any heart beat, 9 mutants have a weak heart beat of both chambers, in 3 mutants the ventricle beats weakly, in one mutant the atrium beats weakly, and 2 mutants only show single cell contractions in the heart.

Silent heart

In the silent heart group, no contraction of the heart has ever been observed. At 24 hpf, the morphology of the heart is quite normal but gradually falls apart. The endocardium peels away from the myocardium and the sinus venosus and the ventricle often collapse after 3 days of development (Fig. 6B).

We isolated five mutations that fall into the silent heart group. In addition to lacking contractility of the heart, they suffer different degrees of motility defects. *sih^{tc300b}* is allelic to *silent heart* (*sih^{b109}*) which was isolated in a screen after γ -ray irradiation (Kimmel et al., personal communication). The contractility defect seems to be restricted to the heart. No obvious motility defect is observed in this mutant, and the mutant embryos respond to touch just as well as their wild-type siblings. Three alleles of *still heart* (*sth*) were isolated, including *sth^{b212}* from the Oregon screen. In contrast to *sih*, motility is severely reduced in *sth* embryos. *sth* mutant embryos do not swim away after being touched. However, they do respond to touch by twitching. Histological studies reveal that the organization of the musculature in the somites is severely disrupted (data not shown). Based on these results, it is conceivable that a myofibril component present in both the cardiac muscle and the skeletal muscle, is affected in these mutants. One recently discovered mutant, *tw212e*, suffers the most severe motility defect in the silent heart group and is completely immotile. The allelisms of *tw212e* with *sih* and *sth* have not been tested yet. A mutation in the gene *viper* (*vip*) causes defects in the subdivisions of the brain similar to *nat* (Jiang et al., 1996). In addition to the brain phenotype, *vip* also has a silent heart.

Weak contractility of both chambers

In this class, both chambers of the mutant heart contract with much less power than the wild type siblings. Mutations in three genes were found in this class. In *weak beat* (*web*) mutants, both chambers are slightly enlarged (Fig. 6C). The contractions seem to be slightly weaker than that of the wild-type siblings and circulation is significantly reduced. The heart beat of *quiet heart* (*quh*) is nearly silent, however, the morphology of the heart is relatively normal. In the mutant, *stretched* (*str*), the heart beats but no circulation occurs. Because of the failure of the heart, *str* homozygotes suffer severe edema and the hearts are pulled into a string-like structure (Fig. 6D). Furthermore, a group of mutants with motility defects also suffers reduced contractility in the heart (Table 1). These mutants are described by Granato et al. (1996). Complementation analysis between these motility mutants and the heart mutants with motility defects has not been performed.

Weak ventricle

Mutations in two genes were found to specifically affect the contractility of the ventricle. At about 36 hpf, when the circulation becomes evident, these mutants can be easily distinguished from wild type by the reduced beat of the ventricle and the significantly reduced circulation. In 2-day old mutant embryos of this class, the circulation ceases and the morphology of the heart becomes abnormal. Hearts of *pipe heart* (*pip*) embryos turn into an elongated pipe-like structure (Fig. 6E), whereas in mutants of *tango* (*tan*) the ventricle collapses and the atrium enlarges. In addition to the morphological defect, the rhythm of the heart beat seems abnormal in *tan^{tg299c}* embryos. In some cases, a 3:2 ratio of atrial to ventricular beat rate is observed. We also identified a weak allele, *tan^{to216b}*, which causes a much milder phenotype. The contractility of the ventricle is slightly weaker than the wild-type siblings. The overall heart morphology in *tan^{to216b}* embryos is quite normal, and the circulation is only slightly reduced. Most *tan^{to216b}* homozygotes survive to adulthood and are fertile.

Weak atrium

The *weak atrium* (*wea*) mutants show a specific deficiency in atrial contractility. In 1-day old *wea* mutant embryos, the atrium contracts weakly and gradually becomes nearly silent. At this stage, the circulation is significantly reduced. In *wea* mutant embryos that are 2-days old, only the cells near the atrial-ventricular boundary are still contracting. In 3-day old *wea* embryos the atrium is silent. The circulation is significantly reduced, although the ventricle is pumping powerfully. In the Boston screen, three alleles of *wea* were found (Stainier et al., 1996).

Isolated cell contraction

The hearts of *polka* (*plk*) embryos are nearly silent. Uncoordinated single cell contraction can be observed under the dissecting microscope. However, these never trigger the contraction of adjacent cells. We identified two alleles of *plk* in our screen. Three mutations in *island beat* (*isl*) which cause the same phenotype were isolated in the Boston screen (Stainier et al., 1996), but allelism between *plk* and *isl* has not been tested yet.

Rhythmicity

The normal heart beats regularly and coordinately with a 1:1 ratio of atrial to ventricular beat. In order to prevent backflow, the atrium does not relax until the contraction of the ventricle starts. In the rhythmicity group, two mutants show an abnormal beating pattern of the heart. In *breakdance* (*bre*) mutant embryos, a 2:1 ratio is observed, such that the ventricle contracts once after the atrium contracts twice. A 3:1 pattern is observed in embryos mutant for *hiphop* (*hip*) such that the ventricle contracts once after the atrium contracts three times. In *hip* mutant embryos, the heart is stiff (Fig. 6F) and sometimes stays in the contracted state for almost one second before it returns to the 3:1 pattern.

A group of mutants in which the heart contracts in a chaotic and irregular fashion, known as fibrillation, have been found in the screen. In embryos mutant for *legong* (*leg*) or *slip jig* (*slj*) the heart contracts only weakly during the first 2 days of development. By the time these mutants are 2 days old the hearts begin to fibrillate. In another mutant called *tremblor*

(*tre*) the heart fibrillates as soon as it becomes visible under the dissecting microscope. *tre* seems to be an easily accessible locus. We found two alleles of *tre* in our screen and another five alleles were found in the Boston screen (Stainier et al., 1996).

Transient phenotypes

Three mutations, *tr206d*, *ts206* and *tm96*, show mild heart defects. 2-day old embryos of these mutants have a pericardial edema, the heart is slightly enlarged and the circulation is slightly reduced. However 1 day later, most of these mutants can hardly be distinguished from their wild-type siblings.

Circulation

In zebrafish, the circulation starts at approximately 24 hpf. Initially, the circulation consists of a single loop initiated at the ventral aorta (Rieb, 1973). After the ventral aorta, blood flows through the first aortic arches into the aorta and then continues into the caudal artery in the tail. Venous blood returns through the axial vein, emptying laterally through the ducts of Cuvier onto the yolk sac. From there it flows down across the yolk sac before reentering the heart at the sinus venosus. Cranial circulation begins shortly thereafter, with blood flowing through the internal carotid arteries and anterior cardinal veins. By 36 hpf, the circulation is strong and easy to observe. Two mutants with a normal heart but with defects in circulation were found in the screen. In the *kurzschluss* (*kus*) mutant, circulation is restricted to the heart and the head. Little or no circulation is observed in the trunk and tail. Another mutant, *gridlock* (*gdl*), which was found in the Boston screen, shows a very similar phenotype (Stainier et al., 1996). Complementation between *kus* and *gdl* has not been tested yet. In embryos mutant for *bypass* (*byp*), the yolk is positioned more posteriorly. The returning blood flow drains into the heart more anteriorly across the yolk rather than laterally as in the wild-type siblings.

Four groups of mutants lacking the horizontal myoseptum also have defects in circulation (van Eeden et al., 1996). In *chamaeleon* (*con*) and *you-too* (*yot*), the circulation is restricted to the head. In *you* (*you*), however, circulation appears a few hours later than in the wild-type siblings. In embryos mutant for *sonic-you* (*syu*), the circulation defect is variable. It either appears at a much later developmental stage than normal or not at all. Further description of this group of mutants can be found in van Eeden et al. (1996).

Intestine

In wild type embryos that are 3-days old, the intestine is visible under the dissecting microscope. The two major components of the intestine are the smooth muscles and the intestinal epithelium. The smooth muscles are responsible for the peristalsis of the intestine. The intestinal epithelium forms deep folds with microvilli facing the lumen, which is important for nutrient absorption.

We isolated two mutants with specific defects in the intestine, *flotte lotte* (*flo*) and *stuffed* (*stf*). These mutants can be distinguished from their wild-type siblings by the thin and unfolded intestine by the age of 4 days (Fig. 7B,C). No peristalsis can be observed in either mutants. In addition, grey material starts to accumulate in the intestinal lumen of 5-day old *stf* embryos (Fig. 7C). We do not know whether the unfolded intestine wall phenotype is due to a lack of prolifer-

ation or differentiation of the intestinal epithelium or whether this defect is due to degeneration of the epithelium and smooth muscle. Detailed histological studies will be necessary to address this question.

Liver

Mutations in three loci, *lumpazi* (*lum*), *gammaler* (*gam*) and *tramp* (*trp*), give specific defects in the liver with similar phenotypes. In embryos of these mutants that are 3-days old, the liver becomes abnormally enlarged compared to wild type and accumulates grey granules (Fig. 7E). In embryos mutant for *tippelbruder* (*tip*), red blood cells start to accumulate in the liver by 4 days of development (Fig. 7F).

Kidney

The kidneys are paired, longitudinal structures ventral to the dorsal aorta, used to excrete the large amounts of water which enter the fish body through the gills. Two basic anatomical types of kidneys are recognized in fishes, pronephric and mesonephric. The pronephros is anterior to the mesonephros. In most fish, the pronephros is transitional in that it functions in early life and later its function is taken over by the mesonephros. Embryos mutant for *no isthmus* (*noi*) lack a pronephros in addition to missing the midbrain-hindbrain boundary. 5 alleles of *noi* were found in the screen (Brand et al., 1996a). 11 potential kidney mutants with a cyst at the pronephric duct were found. Five of these mutants fall into one complementation group termed *locke* (*lok*). All these potential kidney mutants have a curved body shape. Further description of these mutants is found in Brand et al., 1996b.

DISCUSSION

In our screen, mutations eliminating an entire organ have not been found. This could be due to redundancy in the vertebrate, allowing related genes to compensate for mutations in other genes. It is also possible that the crucial genes needed for the development of certain organs are also required for early events during embryogenesis. Therefore, mutations in these genes might lead to early developmental defects. The late effects in organogenesis are therefore hard to interpret.

The total number of the internal organ mutants is relatively small and most of the genes have only one allele. It is very likely that we are still far from saturation for genes involved in organogenesis or in the maintenance of internal organ functions. The major reason perhaps is the criteria we set for keeping mutants during the screen. In such a large scale screen, it is not possible to keep all the mutants isolated. We have decided to discard mutants with general edema, retardation, or necrosis and only keep mutants with distinct phenotypes (Haffter et al., 1996). Mutations affecting organogenesis of internal organs may share some phenotypic properties with mutants in the general class that were discarded. It is likely, that several mutations affecting internal organs were not kept for that reason. Mutations in genes that are required for a wide range of cell types or during a broad time span in embryogenesis might generate multiple defects, which therefore might be discarded. Secondly, defects of the internal organs are often subtle. Since we did not focus on looking for internal organ abnormalities, it is very likely that many of the mutants were

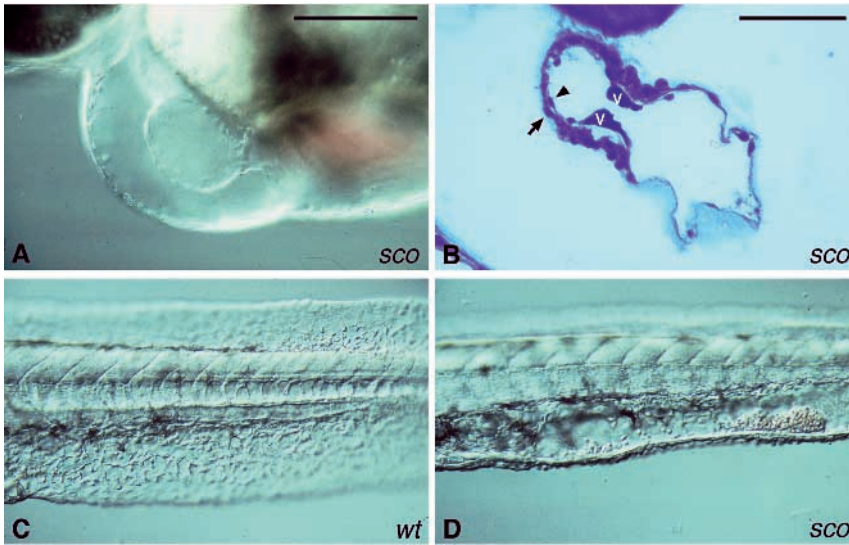


Fig. 5. Mutants with reduced cardiac jelly.

(A) Image of the heart of a live 2-day old *sco^{te382}* embryo. Only the myocardial layer is visible in the atrium but not the endocardial layer. The low circulation results in a lack of blood cells in the heart. (B) A sagittal section of the heart of a 2-day old *sco^{te382}* embryo. Both, the myocardium (arrow) and endocardium (arrowhead) are present in the heart. However, the matrix between the two cell layers is very thin. The endocardium seems to attach to the myocardium, especially in the atrium. Valves (v) are formed and placed at the atrial-ventricular junction. In addition, the endothelial cells in the ventral fin of 2-day old *sco^{te382}* embryos (D) are not as organized as in the wild type siblings (C), and blood cells accumulate in the empty space in the ventral tail fin. Scale bar, (A,C and D) 100 μ m; (B) 50 μ m.

overlooked during the screen. The phenotypes of the cardiovascular mutations are often prominent compared to mutations affecting other internal organs. This is probably the reason why we still have a relatively good collection of heart mutants.

The advantage of studying development with a genetic approach is that the roles of specific genes in these processes are judged by the mutant phenotypes. Therefore, it is not restricted to any preconceived genes. Building a genetic hierarchy helps in dissecting the pathways involved in these processes. In the case of heart, we have isolated mutations affecting most if not all the landmarks of heart morphogenesis. It has been shown that the fusion of the bilateral heart primordia can be blocked by the application of pressure at the embryonic midline, by causing a wound at the midline, or by the insertion of tissue between the two heart primordia (Goss, 1935; DeHaan, 1959; Rosenquist, 1970). Extracellular molecules, such as fibronectin, can promote cell adhesion, spreading and migration. Mice lacking fibronectin die early in gastrulation with severe defects including the failure to fuse the two heart tubes (George et al., 1993). In a certain percentage of the *fau* and *nat* homozygotes, the two heart tubes do fuse, as seen in the fibronectin null mutation. In *mil* homozygotes, although two hearts are always present, the relative position of the two hearts is variable. In some cases, the two hearts are far apart, whereas in others, they are adjacent to each

other, suggesting that they do attempt to fuse. Studying these mutations might reveal the molecular nature underlying this fusion process.

The ventricle and the atrium not only bear significant morphological differences, but also differential expression of subsets of myofibril genes that are specific for the two heart chambers. This differential gene expression is crucial for maintaining normal function of the heart and takes place before the morphological distinction of the chambers (Litvin et al., 1992). Little is known about how the two heart chambers are separated. The lineage study in zebrafish suggests that the atrial and ventricular lineages separate by late blastula stage (Stainier et al., 1993). Whether the differentiation of the heart tube is crucial for chamber demarcation or whether particular molecules set the border of the chambers is still an open question. Studying the mutant *loa*, which lacks any obvious structure resembling the ventricle, might lead us to further understanding of the mechanisms underlying chamber demarcation or the distinction of ventricular and atrial cell lineages.

In zebrafish embryos that are 2 days old cardiac looping has occurred, and the ventricular myocardium begins a massive cell proliferation-differentiation process resulting in multiple cell layers in the ventricle, which is a crucial event for the pumping activity (Stainier and Fishman, 1994). In the *san* mutant, the heart is not only significantly enlarged, but it also

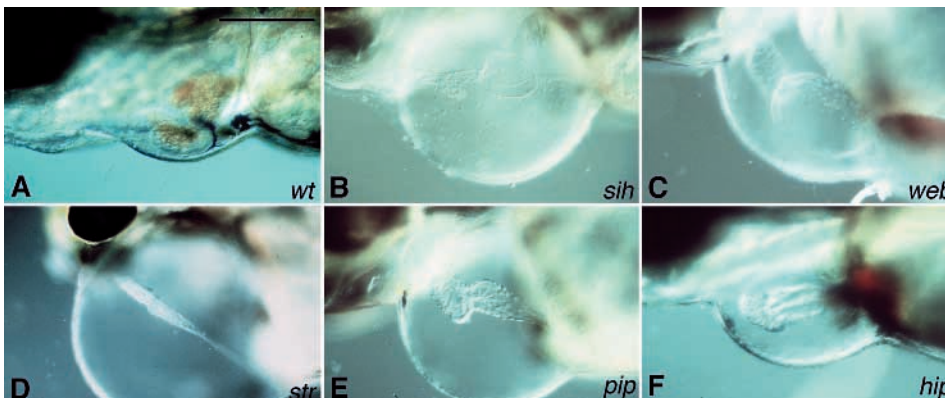


Fig. 6. Abnormal heart morphology as a result of heart failure. The shape of the heart in the mutant embryos is often distorted as the result of the failing function. Images of the hearts of live 2-day old wild-type (A), *sih^{te300b}* (B), *web^{te288b}* (C), *str^{tu255b}* (D), *pip^{tq286e}* (E) and *hip^{tx218}* (F) embryos. Scale bar, 100 μ m.

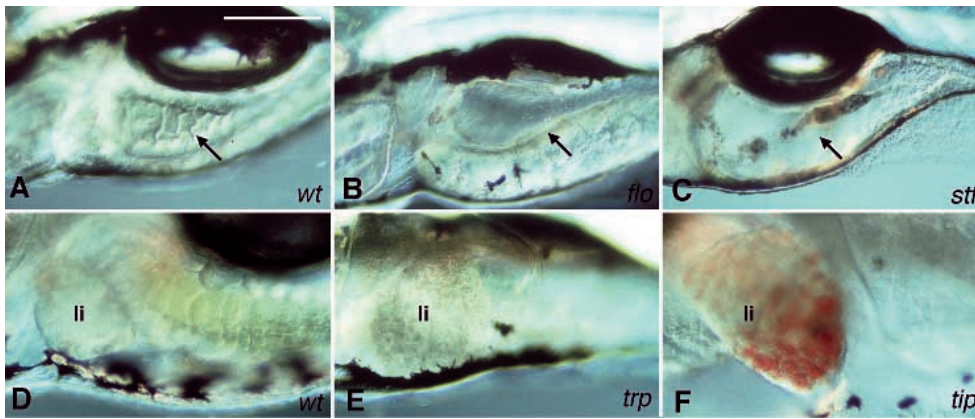


Fig. 7. Intestine and liver mutants. (A-C) Images of the intestines of live 5-day old embryos. A thick epithelial cell layer with folds is the characteristic feature of the intestine in wild-type (A). Mutants of *flo*^{ti262c} and *stf*^{z259} have a thin epithelial cell layer and lack the characteristic folds of the intestine (B,C). In addition, there is grey material in the gut of 5-day old *stf*^{z259} embryos (C). The arrows point to the intestine. (D-F) Images of the livers (li) of live 5-day old

embryos. Mutants in the necrosing liver class have grey granules in the liver. The liver of *trp*^{tm117d} embryos is shown in E. In *tip*^{th203} embryos, the liver accumulates red blood cells (F). Scale bar, 100 μ m.

fails to loop and the myocardial layer of the ventricle remains as a single cell layer. No valve formation is observed in *san* homozygotes. It is conceivable that the 'sensor' which is responsible for differentiation is disrupted in *san* homozygotes. Whether or not this is the case will be an exciting question to pursue.

The valves at the chamber boundaries are required to prevent backflow. In zebrafish, the valve precursors delaminate from the endocardium near the chamber boundaries and undergo an epithelial-mesenchymal transition starting at about 36 hpf (Stainier and Fishman, 1994). The extracellular matrix (cardiac jelly) located between the myocardial and the endocardial layers is thought to be an important factor in the induction of the endothelial cell delamination (Crossin and Hoffman, 1991; Markwald et al., 1977; Runyan and Markwald, 1983; Spence et al., 1992). Surprisingly, embryos that are mutant for *sco* and *sug* develop valves even though they have a very thin matrix separating the two cell layers of the heart. Extensive studies on these mutants might lead to further understanding about the role of cardiac jelly in valve formation.

We have identified a number of mutations affecting the normal functioning of the heart. In the mammalian system, gradual effects causing heart failure in the embryo cannot easily be followed because of the intrauterine development. Since the heart is the crucial organ supplying nutrient and oxygen, embryos die quickly as a result of heart failure. Zebrafish embryos are transparent and the heart is placed prominently on the ventral side of the embryo, which makes it easy to observe. Because of the relatively small size of the embryo, oxygen can diffuse through the skin, so that the zebrafish embryos can survive at least for one week without having a functional heart (Stainier and Fishman, 1993). Therefore, mutants with gradual failure of the heart can be followed for quite a while. We identified 29 mutations in at least 21 complementation groups affecting the normal function of the heart such as the contractility and the rhythmicity of the heart. Studying these mutations might provide more insight into the gradual effects of congenital cardiovascular diseases.

Although saturation has not been reached, we are encouraged by the fact that mutants with specific defects in certain internal organs can be isolated by such a screen. It is conceivable that with a screen designed for specific aspects of organo-

genesis, more mutations can be isolated and a genetic hierarchy can be established. The mutations we present here could serve as entrypoints into such developmental pathways. We are optimistic about the prospect of using the zebrafish as a genetic model system for the cardiovascular system of vertebrates. With the advantages of both, embryology and genetics, we should be able to study certain aspects of organogenesis in the zebrafish at the molecular level in the near future.

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