



Patterning during organogenesis: genetic analysis of cardiac chamber formation

Deborah Yelon and Didier Y.R. Stainier*

A classical genetic approach, in which mutagenized organisms are screened for phenotypes of interest, is appealing for the analysis of developmental processes. Here, we describe the advantages of zebrafish genetics for the study of heart development. As an example of the utility of this strategy, we discuss its potential to illuminate the molecular mechanisms of cardiac chamber formation. The signals that specify ventricular and atrial lineages and the differentiation pathways that produce distinct chambers are poorly understood. Recently identified zebrafish mutations that disrupt ventricular or atrial development promise to reveal genes essential for these processes.

Key words: atrium / development / genetics / ventricle / zebrafish

© 1999 Academic Press

A genetic approach to the study of cardiac development

DEVELOPMENT OF THE VERTEBRATE heart is a complex process, requiring the integration of multiple cell types into a specific arrangement that is essential for cardiac function. Despite a longstanding fascination with these processes, our knowledge of the molecular mechanisms that control cardiac development remains extremely limited. Given that we are familiar with so few of the key molecules, a classical genetic approach, in which mutagenized organisms are screened for phenotypes of interest, is appealing. In contrast to targeted mutagenesis, a genetic approach

does not require prior knowledge of specific genes nor make assumptions about their roles; these aspects of gene targeting can be time-consuming and risky, especially considering the likelihood of functional overlap within the vertebrate genome.

The zebrafish (*Danio rerio*) presents opportunities for genetic analyses unique among vertebrates, primarily due to its small size, fecundity, and brief generation time. Of course, such practical benefits are inconsequential without the ability to identify mutants with cardiac defects easily; here the aesthetic advantages of the transparent zebrafish embryo come into play. The developing zebrafish heart is constantly visible,¹ in contrast to the mammalian heart's relative inaccessibility. Additionally, unlike the mammalian embryo, the zebrafish embryo does not require a functioning cardiovascular system for survival during embryogenesis;² diffusion of oxygen from the surrounding medium is sufficient. This feature allows continued study of mutant hearts and also provides an opportunity to analyze additional effects of specific mutations on other embryonic organs.

Here, we illustrate the utility of zebrafish genetics in the investigation of a specific aspect of cardiac development—the formation of cardiac chambers. We review our current understanding of this process, highlighting unanswered questions, and then describe recent efforts to employ a genetic approach to these problems.

Chamber formation creates a simple anterior-posterior pattern within the embryonic vertebrate heart

In all vertebrates, bilateral precardiac regions of the lateral plate mesoderm migrate toward the embryonic midline where they fuse to form the definitive heart tube (Figure 1).³ Further differentiation of the heart tube, including constriction and valve forma-

*From the *Department of Biochemistry and Biophysics and Programs in Human Genetics and Developmental Biology, University of California, San Francisco, San Francisco, CA 94143-0448, USA*

©1999 Academic Press

1084-9521/99/010093+06 \$30.00/0

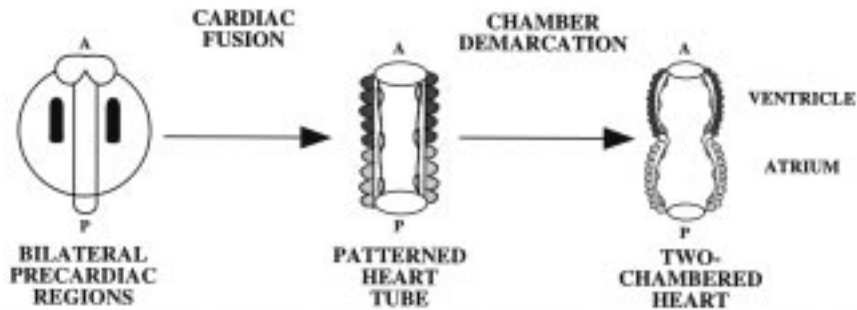


Figure 1. Stages of zebrafish cardiac development after the completion of gastrulation, dorsal views with anterior at top. These stages are morphologically similar in all vertebrates.³ Bilateral precardiic regions within the lateral plate mesoderm are depicted as black ovals. These regions migrate toward the embryonic midline, where they fuse to form a myocardial tube with an endocardial lining. Distinct preventricular and preatrial lineages exist within the heart tube. Here, the preventricular myocardial cells within the heart tube are shown in dark gray, and the preatrial myocardial cells within the heart tube are shown in light gray. Further differentiation of the heart tube produces two morphologically obvious chambers, an anterior ventricle (dark gray) and a posterior atrium (light gray).

tion at the atrioventricular boundary, divides it into two major chambers, an anterior ventricle and a posterior atrium (Figure 1). This simple anterior-posterior (A-P) pattern is also evident in the histological and physiological differences between ventricular and atrial tissue. For example, each chamber contains specific isoforms of contractile proteins (reviewed in ref 4), and each chamber has a distinct rate of contraction.⁵ Many chamber-specific characteristics persist through adulthood (Figure 2).

Conceptually, at least, cardiac chamber formation can be separated into two phases—the initial specification of separate preventricular and preatrial lineages and the subsequent differentiation events that produce distinct ventricular and atrial chambers. The signals that initiate chamber specification, the receptors that transduce these signals, and the factors that control chamber-specific differentiation all remain mysterious.

Mapping the origins of ventricular and atrial cells

We do not know precisely when the specification of preventricular and preatrial lineages occurs. Fate mapping studies suggest that these lineages may originate very early, even prior to gastrulation. For instance, in zebrafish, a single precardiic cell labelled at the midblastula stage gives rise to labelled progeny in either the ventricle or the atrium, but never in both chambers.⁷ In the chick, precardiic cells that

ingress through the rostral portion of the primitive streak end up in the ventricle, while cells that ingress through more caudal regions end up in the atrium.⁸ Taken together, these studies suggest the existence of physically separate preventricular and preatrial fields within the blastula, the relative positions of which are maintained during gastrulation and cardiac fusion.

Molecular evidence for distinct lineages within the heart tube

Fate mapping experiments cannot establish when preventricular and preatrial cells become distinct from each other on a molecular level. This may occur shortly after gastrulation is complete: at this stage, explants of subregions of chick bilateral precardiic mesoderm exhibit restricted developmental potential.⁹ However, overt chamber-specific differentiation has not been detected until cardiac fusion is underway. As the heart tube forms, the restricted expression of chamber-specific genes defines separate preventricular and preatrial regions (Figure 1). For instance, in chick and zebrafish, an atrial myosin heavy chain isoform is expressed only at the preatrial end of the forming heart tube;^{6,10} in mouse, the expression of a ventricular myosin light chain isoform is restricted to the preventricular end of the forming heart tube.¹¹ Thus, preventricular and preatrial cells have different characteristics prior to chamber demarcation, at least by the time of cardiac fusion, and probably earlier.

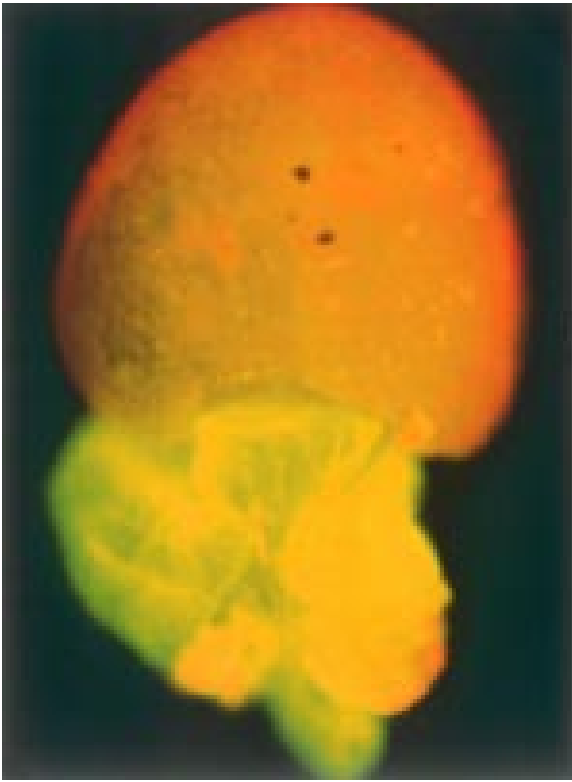


Figure 2. Ventricular and atrial chambers are histologically and physiologically distinct. An adult zebrafish heart was immunostained with the MF20 (anti-myosin heavy chain) and S46 (anti-atrial-specific myosin heavy chain) monoclonal antibodies.⁶ Red fluorescence indicates MF20 (TRITC) staining of ventricular myocardium and yellow fluorescence indicates the overlap of MF20 (TRITC) and S46 (FITC) staining of atrial myocardium. Thus, morphological differences between the two chambers are readily visible; furthermore, chamber-specific gene expression programs are evident.

The role of retinoic acid in atrial development

The data in favor of early separation of preventricular and preatrial lineages do little to clarify the relevant molecular players; most efforts in this regard have focused on the potential roles of retinoids. In particular, retinoic acid (RA)—often implicated in the A-P patterning of segmented structures, such as digits, hindbrain rhombomeres, and vertebrae (reviewed in ref 12)—may play a similar role in cardiac A-P patterning. Exogenous RA treatments can cause anterior segments of various tissues to assume more posterior identities;¹² similarly, in zebrafish and chick, exogenous RA treatments inhibit the development of ventricular cells.^{6,9,10} The effectiveness of exogenous RA suggests that endogenous RA signalling may en-

courage atrial development at the expense of ventricular development.

Recent studies of RA synthesis and activity within the embryonic mouse heart further support the importance of endogenous RA for cardiac A-P patterning.¹³ As the murine heart tube forms, the RA-synthesizing enzyme RALDH2 is only present in preatrial regions. Furthermore, transgenic mice carrying a reporter gene under the control of a RA-responsive promoter element demonstrate that retinoid signalling is restricted to the same preatrial locations. Taken together with the effects of exogenous RA treatment, these data suggest that cell-autonomous retinoid signalling within the myocardium controls atrial differentiation.

The targets of retinoid signalling in preatrial cells are not yet known. Posterior transformations of anterior digits, rhombomeres, or vertebrae by RA are thought to be controlled by RA-mediated shifts in Hox gene expression.¹² It is not clear what roles Hox genes may play in cardiac A-P patterning. However, several chick Hox genes are more highly expressed in preatrial regions than in preventricular regions, and some of these Hox genes are upregulated in RA-treated precardiac explants.¹⁴

A nascent model of cardiac chamber formation

Based on the data described above, we can begin to sketch a plausible scenario for cardiac chamber formation. In the early embryo, perhaps even the blastula, preventricular and preatrial specification occur in response to unidentified signals emanating from an unidentified source. Recent studies indicate that A-P patterning of the mouse embryo is influenced prior to gastrulation by an A-P prepattern within extraembryonic tissues;¹⁵ similarly, the transfer of information from extraembryonic sources to the embryo may establish the cardiac A-P prepattern. Alternatively, intraembryonic signals may be responsible for specification of preventricular and preatrial lineages. The orientation of the precardiac field relative to an embryonic signalling center, such as the dorsal organizer, may result in differential reception of critical signals within the field, thereby producing a pattern. In any case, transduction of specification signals triggers the expression of chamber-specific transcription factors that initiate chamber-specific gene expression programs. In preatrial cells, these factors regulate RALDH2 production, RA synthesis begins, and subsequent RA signalling controls further

atrial differentiation. The control of the ventricular program is more mysterious; either a lack of RA signalling is sufficient to initiate ventricular differentiation, or other unknown signals are involved. Finally, as differentiation proceeds, the bilateral pairs of pre-ventricular and pre-atrial fields join together, forming a patterned heart tube.

While this model is reasonable, we have yet to grasp the actual molecular basis for the specification and differentiation of cardiac chambers. It is now essential to identify the specific genes involved; unfortunately, no extant mouse mutation directly does so. A few mouse mutations do cause notable ventricular defects; for instance, mutations in the genes encoding the transcription factors MEF2C¹⁶ and dHAND¹⁷ and the protein core of the proteoglycan versican^{18,19} all cause hypoplasia of the right ventricle. Since the right ventricle develops from the most anterior portion of the heart tube,²⁰ these defects may result from improper cardiac A-P patterning. However, it has not yet been established whether these errors occur during ventricular specification or during downstream steps of ventricular differentiation. In the absence of any clear candidates for regulators of cardiac chamber formation, a genetic analysis in the zebrafish is likely to be a productive strategy to identify relevant genes.

Screening for mutations affecting the zebrafish embryo

Motivated by the potential of the zebrafish for genetic analyses, groups in Boston and Tübingen recently conducted large-scale screens aimed at identifying mutations critical for numerous aspects of zebrafish embryogenesis.^{21,22} In general, these screens involved evaluating the gross morphology of mutant embryos, including the form and function of the heart. Overall, these efforts were enormously successful; together, the Boston and Tübingen screens identified nearly 2000 different mutations, representing between 400 and 1000 different genes, each affecting specific developmental processes. More than 100 of these mutations, representing over 60 different genes, cause specific defects in cardiovascular development.^{23,24} For example, mutations in six distinct genes block cardiac fusion, and mutations in 26 genes disrupt cardiac contractility. Only two genes identified in the Boston and Tübingen screens—*pandora* (*pan*) and *lonely atrium* (*loa*)—specifically affect cardiac chamber formation.

Pandora and *lonely atrium*

Both the *pan* and *loa* mutations affect ventricular development; the hearts in these mutant embryos have only one major chamber—an atrium, with little if any ventricular tissue attached. In *pan* mutants, a small stub of ventricle-like tissue is attached to the anterior end of the dilated atrium;²³ furthermore, *pan* mutants exhibit runted tails, reduced pigmentation, ear defects, and malformations of the ventral retina, indicating that the affected gene plays a role in multiple processes of patterning and morphogenesis. In contrast, the phenotype of *loa* mutants appears to be cardiac-specific;²⁴ the ventricle is missing, while the rest of the embryo seems normal. It will be interesting to learn more about the nature of the *pan* and *loa* mutations. In particular, it will be important to determine whether these ventricular defects are cell-autonomous, whether the developmental errors occur during ventricular specification or differentiation, and what exactly goes wrong on a molecular and cellular level. Finally, it will be exciting to determine the identity of the affected genes and understand their roles in the normal process of cardiac A-P patterning.

Expanding the collection of mutations

As intriguing as *pan* and *loa* are, achieving a thorough understanding of cardiac chamber formation will require the identification of additional relevant genes. The large-scale Boston and Tübingen screens did not attain saturation,^{21,22} suggesting that further screening could uncover additional useful mutations; we have recently completed a screen directed toward this goal.²⁵ In contrast to the previous screens, which examined cardiac morphology in live embryos, we employed molecular criteria in order to enhance the detection of more subtle mutations. Specifically, for the inspection of cardiac chamber formation, we stained the heart tubes of fixed embryos with antibodies that distinguish ventricular and atrial myosin heavy chain isoforms.⁶ Furthermore, in order to expedite the identification of relevant mutations, we employed a novel screening strategy, in which we analyzed the haploid progeny of 340 mosaic F1 females, each estimated to carry ten ENU-induced embryonic lethal mutations.²⁵ Altogether, we identified 20 mutations affecting ventricular development and one mutation affecting atrial development (Figure 3).

The relative ease of identifying zebrafish mutations

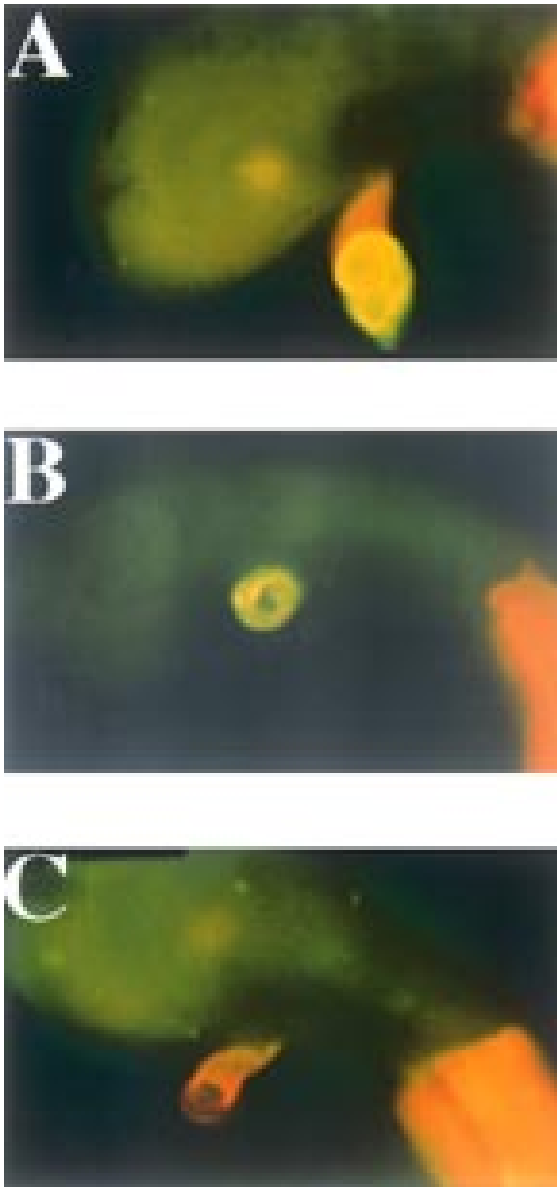


Figure 3. Zebrafish mutations disrupt cardiac chamber formation.²⁵ Wild-type and homozygous mutant embryos were immunostained with the MF20 (anti-myosin heavy chain) and S46 (anti-atrial-specific myosin heavy chain) monoclonal antibodies at 48 h post-fertilisation.⁶ Red fluorescence indicates MF20 (TRITC) staining of ventricular myocardium and yellow fluorescence indicates the overlap of MF20 (TRITC) and S46 (FITC) staining of atrial myocardium. Some skeletal muscle (red) within the somites is also visible. Panels (A)-(C) show lateral views, head to the left. Panel (A) shows the ventricle (red) and atrium (yellow) of a wild-type embryo. Panel (B) depicts a mutant embryo with a defect in ventricle development, such that only an atrium (yellow) is visible. Panel (C) depicts a mutant embryo with a defect in atrial development, such that only a ventricle (red) is visible.

that disturb ventricular development, in comparison to atrial development, suggests that the specification of preventricular cells may be more sensitive to disruption than the specification of preatrial cells. Perhaps the process of cardiac A-P patterning is primarily a matter of inducing ventricular cell fates via suppression of RA synthesis. Alternatively, the bias toward detection of mutations affecting ventricular development may not reflect the complexity of ventricular specification, but instead the complexity of ventricular differentiation, an idea supported by the relative frequency of mouse mutations that cause ventricular hypoplasia.

Using the mutations to investigate cardiac chamber formation

As described above for *pan* and *loa*, further characterisation of these additional mutations will reveal the precise nature of each defect. Mutations that cause cell-autonomous defects in ventricular development may help to identify the receptors and downstream molecules that initiate and enact ventricular differentiation, while those mutations that cause cell non-autonomous defects may help to identify the tissues responsible for ventricular specification or differentiation and the signals that these tissues produce. The sole mutation affecting the atrium may help to identify a player in the RA-mediated signalling that influences atrial development or may reveal another pathway involved in atrial specification or differentiation. This collection of mutations is an excellent resource not only for identifying novel genes, but also for clarifying their precise roles and identifying interacting genes as well as downstream targets. Such work will be greatly aided by recent improvements in zebrafish genomic resources,^{26,27} positional cloning techniques,²⁸ and insertional mutagenesis strategies.²⁹⁻³¹

Over time, analyses of these zebrafish mutations will lead to the assembly of a genetic pathway responsible for cardiac A-P patterning, particularly for ventricular specification and differentiation. The genetic analysis of cardiac chamber formation in the zebrafish promises to add substantially to our understanding of this specific process. Importantly, this work may also illuminate the mechanisms of patterning during organogenesis in general. Similarly, future applications of zebrafish genetics to the study of additional elements of cardiac development are likely

to provide a broader comprehension of many aspects of vertebrate organogenesis.

Acknowledgements

We would like to thank Jonathan Alexander, our collaborator on the haploid screen; we are also grateful for Leslie Reeves' contribution to Figure 2. Additionally, we appreciate the constructive comments of Jonathan Alexander, Emily Walsh, and David Chu. D.Y. is an Amgen fellow of the Life Sciences Research Foundation. This work was supported in part by the AHA, the NIH, and the Packard and March of Dimes Foundations.

References

1. Stainier DYR, Fishman MC (1994) The zebrafish as a model system to study cardiovascular development. *Trends Cardiovasc Med* 4:207–212
2. Pelster B, Burggren WW (1996) Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebrafish (*Danio rerio*). *Circulation Research* 79:358–362
3. Fishman MC, Chien KR (1997) Fashioning the vertebrate heart: earliest embryonic decisions. *Development* 124:2099–2117
4. Lyons GE (1994) *In situ* analysis of the cardiac muscle gene program during embryogenesis. *Trends Cardiovasc Med* 3:184–190
5. Satin J, Fujii S, DeHaan RL (1988) Development of cardiac beat rate in early chick embryos is regulated by regional cues. *Dev Biol* 129:103–113
6. Stainier DYR, Fishman MC (1992) Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. *Dev Biol* 153:91–101
7. Stainier DYR, Lee RK, Fishman MC (1993) Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. *Development* 119:31–40
8. Garcia-Martinez V, Schoenwolf GC (1993) Primitive-streak origin of the cardiovascular system in avian embryos. *Dev Biol* 159:706–719
9. Yutzey KE, Gannon M, Bader D (1995) Diversification of cardiomyogenic cell lineages *in vitro*. *Dev Biol* 170:531–541
10. Yutzey KE, Rhee JT, Bader D (1994) Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. *Development* 120:871–883
11. O'Brien TX, Lee KJ, Chien KR (1993) Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proc Natl Acad Sci USA* 90:5157–5161
12. Langston AW, Gudas LJ (1994) Retinoic acid and homeobox gene regulation. *Curr Opin in Genetics and Development* 4:550–555
13. Moss JB, Xavier-Neto J, Shapiro MD, Nayeem SM, McCaffery P, Drager UC, Rosenthal N (1998) Dynamic patterns of retinoic acid synthesis and response in the developing mammalian heart. *Dev Biol* 199:55–71
14. Searcy RD, Yutzey KE (1998) Analysis of Hox gene expression during early avian heart development. *Dev Dyn* 213:82–91
15. Beddington RSP, Robertson EJ (1998) Anterior patterning in mouse. *Trends in Genetics* 14:277–284
16. Lin Q, Schwarz J, Bucana C, Olson EN (1997) Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* 276:1404–1407
17. Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN (1997) Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. *Nat Genet* 16:154–160
18. Yamamura H, Zhang M, Markwald RR, Mjaatvedt CH (1997) A heart segmental defect in the anterior-posterior axis of a transgenic mutant mouse. *Dev Biol* 186:58–72
19. Mjaatvedt CH, Yamamura H, Capehart AA, Turner D, Markwald RR (1998) The *Cspg2* gene, disrupted in the *hdf* mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol* 199:56–66
20. Stalsberg H, DeHaan RL (1969) The precardiac areas and formation of the tubular heart in the chick embryo. *Dev Biol* 19:128–159
21. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DYR, Zwartkruis F, Abdelilah S, Rangini Z, Belak J, Boggs C (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 123:37–46
22. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nusslein-Volhard C (1996) The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* 123:1–36
23. Stainier DYR, Fouquet B, Chen JN, Warren KS, Weinstein BM, Meiler SE, Mohideen MA, Neuhauss SC, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple DL, Malicki J, Driever W, Fishman MC (1996) Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* 123:285–292
24. Chen J-N, Haffter P, Odenthal J, Vogelsang E, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang Y-J, Kane DA, Kelsh RN, Mullins MC, Nusslein-Volhard C (1996) Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development* 123:293–302.
25. Alexander J, Stainier DYR, Yelon D (1998) Screening mosaic F1 females for mutations affecting zebrafish heart induction and patterning. *Developmental Genetics* 22:288–299
26. Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, Brownlie A, Donovan A, Egan ES, Force A, Gong Z, Goutel C, Fritz A, Kelsh R, Knapik E, Liao E, Paw B, Ransom D, Singer A, Thomson M, Abduljabbar TS, Yelick P, Beier D, Joly J-S, Larhammar D, Rosa F, Westerfield M, Zon LI, Johnson SL, Talbot WS (1998) Vertebrate genome evolution and the zebrafish gene map. *Nat Genet* 18:345–349
27. Knapik EW, Goodman A, Ekker M, Chevrette M, Delgado J, Neuhauss S, Shimoda N, Driever W, Fishman MC, Jacob HJ (1998) A microsatellite genetic linkage map for zebrafish. *Nat Genet* 18:338–343
28. Zhang J, Talbot WS, Schier AF (1998) Positional cloning identifies zebrafish one-eyed pinead as a permissive EGF-related ligand required during gastrulation. *Cell* 92:241–251
29. Gaiano N, Amsterdam A, Kawakami K, Allende M, Becker T, Hopkins N (1996) Insertional mutagenesis and rapid cloning of essential genes in zebrafish. *Nature* 383:829–832
30. Raz E, van Luenen HG, Schaerlinger B, Plasterk RHA, Driever W (1998) Transposition of the nematode *Caenorhabditis elegans* *Te3* element in the zebrafish *Danio rerio*. *Curr Biol* 8:82–88
31. Ivics Z, Hackett PB, Plasterk RH, Izsvak Z (1997) Molecular reconstruction of sleeping beauty, a *Tc1*-like transposon from fish, and its transposition in human cells. *Cell* 91:501–510