

# Perspectives Series: Molecular Medicine in Genetically Engineered Animals

## The Zebrafish: Heritable Disorders in Transparent Embryos

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### *Promise of the zebrafish*

The rise of the zebrafish, *Danio rerio*, as a genetic system, is based on the emerging evidence that its genetics will reveal the molecular control of vertebrate development. The embryo is transparent and the fish is amenable to large-scale mutagenesis. Thus, genetic screens in the zebrafish could provide a "phenotype first" approach to gene discovery, complementary to "genes first" techniques of differential display, subtraction cloning, or random partial cDNA sequencing. The two first screens have been completed. Already the relevance to medicine appears to be great, providing clues, for example, as to how single genes regulate the assembly of organs, and how they might fail, causing both congenital and degenerative disorders. At the current time the position of the zebrafish in the genetic pantheon is less established than the mouse or fly or worm, but there is enough of an international following to ensure rapid generation of the missing pieces of the genetic armamentarium.

Why isn't knowledge of *Drosophila* sufficient for an understanding of development, especially if complemented by the

elegant embryological work on early development of frog or chick, and targeted gene mutation in mice? It is true that similarities abound between *Drosophila* and human. Despite separation from a presumptive common ancestor over the last 400 million years, there is conservation of genetic programs which direct gastrulation, axial determination, and metameric regionalization. However, several new features characterize the vertebrate which are not present in invertebrates. For example, the notochord, the transient embryonic backbone which defines chordates, is not only a support system but also generates signals which are required for the differentiation of adjacent nervous system, bone, and muscle. The neural crest, the migratory cells from the nervous system which contribute to the face, pharyngeal arches, heart, peripheral nervous system, and melanocytes, also appears to be a vertebrate invention. The vertebrate heart, vasculature, kidney, and gut derivatives such as pancreas, have no clear *Drosophila* cognates, although elements may have emerged in invertebrates by convergent evolution. Therefore, it is important to turn to a vertebrate, and to one established as a genetic system. The mouse is ideal in this regard for study of late-acting genes, but the opacity of mother and uterus makes early development less accessible, and large-scale screens are prohibitively expensive.

### *Status of the zebrafish*

Fifteen years ago George Streisinger introduced as a genetic organism the zebrafish, a fresh water tropical cyprinid fish (1).

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He noted that the embryos of the zebrafish are transparent, and that fertilization is external, so that all stages of development are accessible. Development is rapid, with a heart beating by the end of the first day and most organs, or at least their primordia, in place by five days after fertilization (2). The fish are ~ 3 cm long, can be raised in large numbers, and lay hundreds of eggs at weekly intervals. Streisinger also introduced methods to produce homozygous fish, by using genetically incompetent sperm to induce the maternal chromosomes of the egg to complete meiosis II, while transiently applying pressure to prevent the first cell division (1). Before his untimely death, Streisinger had hopes to use the zebrafish especially to unravel the development of the nervous system and behavior.

Two types of groundwork, embryological and genetic, were needed thereafter. Kimmel, Westerfield, Eisen and their colleagues in Eugene, Oregon provided much of the outline now in place for early embryonic cell fate, lineage, and patterning, and for nervous system development (3–5). In terms of genomics, the size of the zebrafish genome is about 2900 centiMorgans and  $1.7 \times 10^9$  bp, about half the physical size of the human. Still needed is a dense genomic map and large insert genomic libraries. The former has been initiated by Postlethwait in Oregon (6, 7) and by us, in collaboration with H. Jacob, in Boston (8); the latter are under construction. Transgenesis is readily accomplished, although expression in the injected embryos is invariably mosaic and expression in subsequent generations depends strongly on the site of genomic integration (9, 10). Whether transgenesis can be rendered efficient enough for insertional mutagenesis is currently under study, especially by N. Hopkins at MIT (11, 12). Rescue of mutant phenotypes and/or interference by anti-sense RNA or ribozymes are methodologies which remain to be established for the zebrafish.

Although arduous, these components of the infrastructure will likely come along in short order. The real issue is, after screens are done, will we have learned anything new about the nature of regulation of vertebrate development.

#### *The genetic screens*

The approach of a genome-wide saturation mutagenesis screen is new to vertebrates. Its premise is that mutation of single genes may produce phenotypes which are informative with regard to steps of development. Its postulate is that it will be easier to solve a puzzle once crucial pieces have been revealed. Here the puzzle is the genetic control of vertebrate development, and the pieces are all the genes involved. Traditionally, vertebrate biologists have searched, using specific assays, for single, specific entry points into genetic circuits. In contrast, the new screens are genome-wide. Saturation mutagenesis screening has previously been applied only to the study of invertebrates, *Drosophila* and *C. elegans*, and the plant, *Arabidopsis* (13–15). The large numbers of animals needed for saturation screens make them prohibitively expensive for any current vertebrate model organism, except for the zebrafish.

The first step towards the screens was the identification of efficient conditions for mutagenesis. The mutagen of choice needed to induce single intragenic gene defects, so that the phenotypes would reflect the effect of single gene mutations, and the mutations could thereafter be used with confidence as single genes in further genetic analysis. Therefore, the focus was on chemical mutagens, which can cause point mutations, rather than irradiation, which induces large multigenic lesions. Work in mice had revealed ethyl-nitrosourea (ENU) as a most

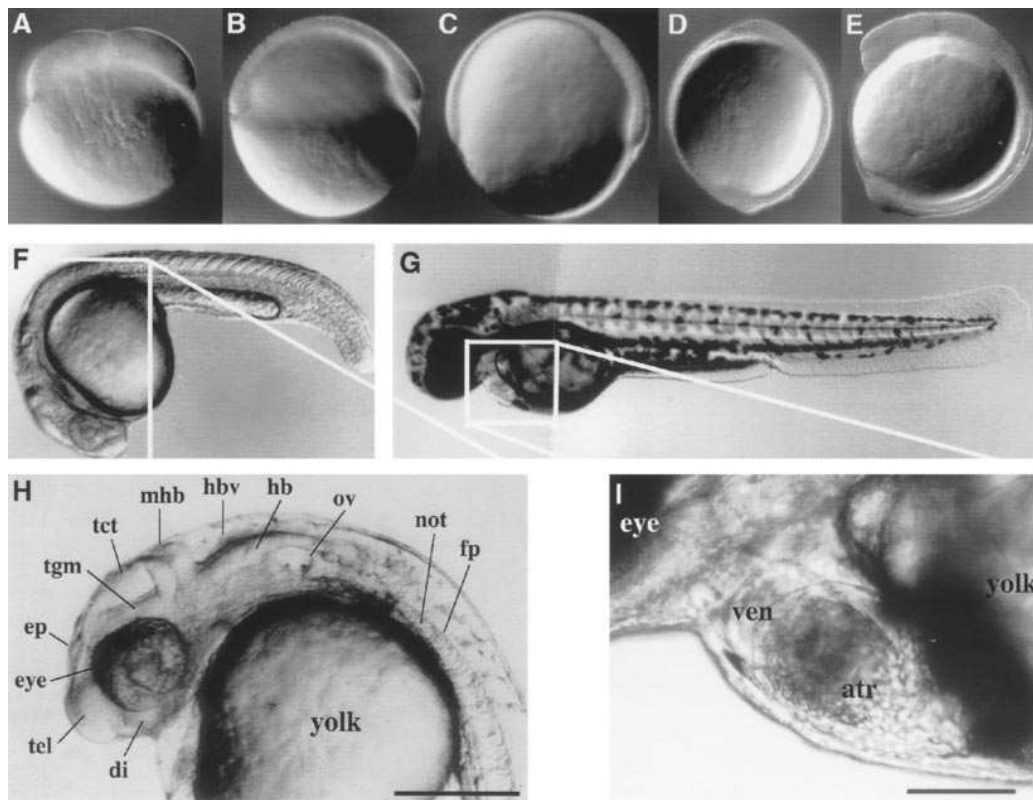
efficient germline mutagen (16), and systematic tests in zebrafish revealed a dose of ENU which induced new mutations at defined “tester” pigmentation loci at rates between one in 300 and one in 2,000 mutagenized genomes (17, 18). Distinct genes are of quite different mutability, but the average rates for induction of mutations reported are better than one in 1,000. This figure is important, since it was the basis for the design of the screens at the MGH in Boston (19) and at the Max-Planck Institute in Tübingen (20, 21). The projection was that a screen of three or four times the number of genomes determined by the specific locus rate should recover three to four alleles per locus, and alleles in > 90% of all genes. Both screens used similar mutagenesis conditions, and bred to homozygosity in a three generation scheme. While the generation of gynogenetic progeny is a potential screening short-cut, the chosen classical breeding scheme had three important advantages (22): (a) it has a much lower background of nongenetic developmental abnormalities; (b) it is not biased against certain gene classes, compared to gynogenetic half tetrad analysis, where mutations in genes close to the telomeres are underrepresented because of recombination away from the centromere; and, most importantly, (c) zygotic recessive mutations can be defined by appearance in one quarter of the egg clutches, and independent mutations can be distinguished and segregation determined, which are not possible for gynogenetic fish. The large number of embryos (50–100) in an egg clutch is important because the ratio of mutant embryos helps to distinguish mutations from nongenetic abnormalities.

The main tool for identification of mutant phenotypes was detailed visual inspection under the dissecting microscope. Zebrafish embryos and larvae are transparent, such that a visual screen, with the help of a good dissecting microscope at 50–100× magnification, can be used to assay small organs, or blood cells flowing through the vessels. In detailed screening protocols, more than a million embryos each were analyzed by dedicated scientific teams for developmental abnormalities at five stages of development, some of which are shown in Figure 1: gastrulation at about 6 h post fertilization (hpf), CNS development and overall body patterning at 24 hpf, cardiovascular development at 48 hpf, early organogenesis and body patterning at 72 hpf, and later tissue differentiation and late organogenesis at 120 hpf. Both labs also included some specialty screens: The Tübingen lab, in collaboration with Bonhoeffer's group, devised a screen to identify mutations affecting retino-tectal projection by dye filling of nasal and temporal neurons (23). The Boston lab performed a small pilot screen for mutations affecting optokinetic behavior (24).

What is the harvest of these screens? Combined together, the two screens identified 6647 mutations that lead to morphological defects during the developmental period investigated. About 2,000 of these mutations were deemed interesting, due to region or organ-specific defects, and were maintained for further analysis. The genetic and phenotypic analysis of about 1,200 mutations are presented in a series of papers in *Development*. While complementation analysis has not been completed between the two labs, the data presented indicate that more than 500 genes of developmental importance might be defined by these mutations (19, 20).

#### *What can the mutations teach?*

Mutations are informative in teaching us the logic of development. In particular we would like to learn about vertebrate-



**Figure 1.** Zebrafish embryos are transparent and develop rapidly. (A) About 45 min after fertilization, two cells are on top of the large yolk cell. (B) The early gastrula consists of about 4000 cells at 6 h post fertilization (hpf), which have spread half-way over the yolk cell. The embryonic shield, equivalent to the *Xenopus* organizer region, forms on the dorsal side (B, right). (C) Gastrulation proceeds with formation of the germ layers (8 hpf). (D) At about 10 hpf, cells cover the yolk cell completely. (E) Cells converge toward the dorsal side (E, right), to form the embryo proper. The domains of the brain can already be distinguished at this time, and the first somites are forming. (F) By 24 hpf, many organ rudiments have been formed, the heart beats, and the tail moves. (G) At 48 hpf, the

larvae has hatched from its chorion, pigmentation has developed, and larvae respond to touch by an escape reflex. (H) The transparent nature of the larvae allows a detailed structural analysis of the domains of the central nervous system (26 hpf; scale bar 200  $\mu$ m; from: Schier et al., 1996), and (I) heart and other internal organs, like the heart (48 hpf; bar, 100  $\mu$ m). *di*, diencephalon; *tel*, telencephalon; *ep*, epiphysis; *tgm*, tegmentum; *tct*, tectum; *mhb*, midbrain-hindbrain border; *hbv*, hindbrain ventricle; *hb*, hindbrain; *ov*, otic vesicle; *not*, notochord; *fp*, floor plate; *ven*, ventricle; *atr*, atrium. (A–F) Living embryos removed from chorion and imaged using Nomarski optics; animal pole is top, dorsal to the right (F–I) Living embryos photographed in transmitted light; anterior to left, dorsal up.

specific development. How is vertebrate form generated? What is the role of the notochord, the primitive and characteristic vertebrate embryonic backbone? What are the binary decisions that generate organs? Are there genes for organ size, characteristic organotypic structures, for seamless integration between tubular tissues? If there are, can they be discovered by a screen. It could have been that the temporally and spatially repetitive use of genes during development would mean that only the earliest decisions could be found by screens and all later actions, such as organogenesis, would be buried in pleiotropic secondary effects. Alternatively, as targeted gene mutation has shown, loss of presumably critical genes may be tolerated without evident effect.

Fortunately, neither caveat turned out to be a significant impediment. We focus here upon mutations discovered in these first screens that affect a few systems, chosen as examples because they are vertebrate-specific, have clinical relevance, and already hint at new paradigms for development.

#### Heart and vasculature

The zebrafish embryonic heart resembles that of the three week post-implantation human embryo (25–28). It is subdivided into chambers and lined by endocardium throughout. The form evolves by growth and hypertrophy of the myocardium, especially of the ventricle, generation of cushions between the chambers, and looping to the right side. The heart

beat is evident by 22 h after fertilization, first as a peristaltic wave and then as sequential synchronous contractions of the atrium and the ventricle. Only late in cardiac development does the heart of mammals diverge from fish, as they generate the atrial and ventricular septa to separate the pulmonary circulation, while fish retain a single atrium and ventricle.

Mutations affect many of these steps, and do so with remarkably discrete and informative action (29, 30). Some affect the size of the heart. For example, the heart in *heart and soul* is diminutive and in *santa* is huge, nearly four times normal. Other mutations disrupt one step of organogenesis. For example, *miles apart* blocks fusion of the left and right primitive heart tubes, such that two relatively normal appearing cardiac structures are formed on either side of the midline. In *jekyll*, the heart valves never form, although chamber demarcation and function are retained. *pandora* selectively ablates the ventricle. In *cloche* the heart forms and is divided into chambers, but lacks endocardium and cushions. In addition, there are essentially no endothelial or blood precursors. This indicates that *cloche* affects a common blood-endothelial precursor, referred to as a “hemangioblast” (31). One fascinating attribute of these mutations is the apparent precision of effect. This suggests that it will be possible to genetically dissect pathways leading to the formation of one chamber, or of the endothelium, or of the heart valves. They also may reveal interactions between tissues otherwise inextricably linked. For example,

the ventricular myocardium of *cloche* beats weakly, suggesting that the endocardium may function to regulate contractility of the myocardium.

Vascular form and integrity are compromised in several mutations (29). For example, there is localized hemorrhage in the brain in *bubble head* and in the pericardium in *leaky heart*. *gridlock* selectively prevents the assembly of a branch point where the two dorsal aortae fuse to generate the single dorsal aorta. The embryo may survive, and does so by generation of collateral blood flow. In location and effects of the lesion, *gridlock* resembles the congenital disorder, coarctation of the aorta (32). It is the first gene found to pattern a region of the embryonic vasculature. Furthermore, it is of interest because branch points set up patterns of flow which are retained throughout life, and become vascular sites with a predisposition to atherosclerosis.

The principal phenotypes of several mutations are cardiac dysfunction (29, 30). Poor contractility may affect the atrium, as in *passive aggressive*, the ventricle, as in *hal*, or both, as in *pickwick*. Some are accompanied by chamber dilatation. In others the chambers pump poorly, and appear to be stiff and to restrict filling. In these regards these resemble, respectively, dilated and restrictive cardiomyopathies. Rhythmicity mutants affect rate, rhythm, and conduction. *slomo* is bradycardic, *tremblor* mutants have chaotic activity resembling fibrillation, *reggae* mutants appear to have problems with initiation or exit of the impulse from the sinus region, and *ginger* mutants have second degree atrio-ventricular block. These are of interest because it has not been straightforward to extrapolate from single gene function to the complexities of global cardiac dysfunction, such as understanding when and how single ion channel defects might lead to arrhythmias. In addition, although lethal in these mutants, in partially expressive form in humans these genes could be candidates for those predisposing to cardiomyopathies and to common arrhythmias.

#### Gastrointestinal tract

The three day embryonic zebrafish intestine evidences visible peristaltic contractions, and is microscopically differentiated, with a polarized epithelium bearing the characteristic absorptive, endocrine, and goblet cells of higher vertebrates. At the anterior end of the gastrointestinal tract is an esophagus and at the posterior is a short region believed to be a homologue of the colon. One bud off the gut forms the liver, composed of cords of hepatocytes and bile ducts, and another, the pancreas, with an insulin-generating islet surrounded by exocrine cells.

Signals which regulate regional epithelial differentiation and renewal are poorly understood. Some of the mutations speak to this issue (30, 33). For example, maturation of the anterior gut is arrested in *slim jim*, and is followed by degeneration. In *meltdown*, the posterior gut is disorganized and bears an expanded mesenchyme. The most evident defect in *beef-eater* is hepatic degeneration, again occurring after the initial processes of differentiation, suggesting an interference with trophic support. Pancreatic exocrine cells degenerate in *slim jim*, but the adjacent endocrine cells are unaffected, of additional interest because of the debate as the nature of interactions and lineages between the two pancreatic cell types. Obviously, these genes may be relevant not only to congenital disorders of morphogenesis, but also to repair of cirrhosis, diabetes, cystic fibrosis, and chronic colitis, all of which could be

approached therapeutically with an eye to the capture of precursor cells and their trophic support systems.

#### Hematopoiesis

The early zebrafish embryo is particularly advantageous for study of blood because, unlike mammals, it can survive in the absence of a circulation without secondary deterioration. Teleosts have erythrocytes, monocytes, granulocytes, and thrombocytes. The screens to date focused upon the very earliest blood cell generation (34, 35). This is of especial interest because, although much is known about blood cell generation in bone marrow or other definitive sites, the earliest sources of blood in the embryo are poorly defined. In the zebrafish, which lacks yolk sac hematopoiesis, the first intraembryonic blood is believed to arise in the mesoderm, near the aorta. The first wave of blood is primarily the red cell lineage. Since the embryos survive for several days without circulating blood, mutations which affect these "primitive" cells are more accessible than they would be in mammals.

Some of the mutants, such as *vampire*, generate no red blood cells (34). Cell transplantation may help reveal whether these mutations affect extrinsic trophic supporting factors or intrinsic regulators of development. Others interfere with maturation of the red blood cells, with changes in the membrane such that the lentiform shape is not acquired and the cells adhere abnormally to the endothelium. Some, such as *dracula*, resemble the human porphyrias in causing cells to be photosensitive, such that ambient light causes hemolysis.

#### CNS development and neuronal survival

The CNS is undoubtedly the most complex organ in the vertebrate. While there are similarities between *Drosophila* and vertebrate (e.g., in that homologous genes may control primary neurogenesis, differentiation of neural cell types, anterior-posterior patterning, and axonal pathfinding processes) (36–38), there is only very fragmentary understanding of the genetic control of most aspects of CNS development in vertebrates. The molecules that establish initial anterior-posterior pattern in the neural plate are as unknown as are the genes that regulate neural identity and survival within the global neural network (39). Zebrafish brain has structures analogous to many components of the mammalian brain, although lacking some of the later evolutionary additions, such as the cortex. Several hundred mutations affecting the CNS were isolated. Interestingly, a large portion affect dorso-ventral patterning, a process also well studied in other vertebrate systems. The ventral fore-, mid-, and hindbrain as well as floor plate are deleted in *one-eyed pinhead*, *uncle freddy*, and *cyclops*, and severely affected in *bashful*, *sleepy*, and *grumpy* (40–43). The first three mutations also delete *sonic hedgehog* expression in the ventral neurectoderm. The *masterblind* mutation is deficient in the telencephalon, which can be interpreted as a defect in dorsalization of the forebrain (44). Surprisingly few mutations perturb anterior-posterior patterning in the CNS. The most prominent mutations in this category, *no isthmus*, *acerebellar*, and *spiel ohne grenzen*, all affect formation of the midbrain–hindbrain border (41, 45). It is possible that many additional mutations remained undetected, since subtle defects would have escaped this visually based screen. In contrast, a large number of mutations affecting morphology of the brain, especially the ventricles, were recovered (41, 46). Preliminary studies of these mutations indicate that many may be secondary to cardiovascular

defects, and due to a lack of cerebrospinal fluid pressure. Only a small number of the brain ventricle mutations produce visible phenotypes before the onset of circulation, and may primarily affect ventricle formation. A choroid plexus has not been localized in early zebrafish larvae so far.

One mutation, *mind bomb*, results in an increased number of primary neurons, reminiscent of neurogenic mutations in *Drosophila* (41, 46, 47). A large number of mutations with neural degeneration phenotypes were isolated, acting as early as somitogenesis stages, and falling into several phenotypic categories. Some had widespread and others local effects. Some transiently deleted domains of the CNS. Study of these genes should reveal some of the mechanisms that control neuronal cell number and neuronal survival in vertebrates (48, 49).

Mutations have also been recovered which affect functional aspects of neuronal circuitry. For example, screening for touch response and optokinetic nystagmus revealed mutations affecting motility, escape reflexes, and the visual system (24, 50, 51).

In a beautiful set of experiments, a large number of genes were identified to affect specific axonal pathways (23). The retinotectal projection screen in Bonhoeffer's lab produced mutations affecting all choice points during axonal pathfinding to proper tectal positions. *belladonna* embryos project to the wrong tectal lobe, while in *detour* mutants each eye projects ipsi- or bilaterally, to both left and right tectum. In *bashful*, axons often don't find their way out of the eye (52). In addition, a number of mutations project to the proper tectal half, but don't project to the proper regions within the tectum (53).

#### Notochord

The notochord, derived from the "Organizer" region of the zebrafish early gastrula and axial mesoderm, plays a prominent role in establishment of body pattern, and is a vertebrate-specific evolutionary invention (54–56). A genetic dissection of notochord formation and its signaling activities are essential to understanding the generation of body form during embryogenesis. Signals derived from the notochord pattern the ventral central nervous system as well as the somitic mesoderm. The mutations *floating head* and *bozozok* delete the notochordal precursor cells (55, 57, 58). Differentiation of chordamesoderm is regulated by six genes isolated during the screen, among them *no tail*, the zebrafish homologue of the murine *T/Brachyury* gene (59–61). A further set of nine genes are required for maturation of the notochord (42, 61). The availability of this series of mutations makes it possible to dissect the temporal order of signaling activities of chordamesoderm and notochord. Further, the various mutations differentially affect the expression of chondrogenic markers, which will help to understand the late development of the notochord as a chondrogenic organ.

#### Craniofacial development

The formation of the vertebrate craniofacial skeleton is a topologically complex process with a strong contribution by neural crest cells, an evolutionary "new" invention of vertebrates, for which no genetic models exist in invertebrates. While the screens were not sensitive enough to detect changes in peripheral derivatives of neural crest, a rich plethora of phenotypes affecting pigment pattern and head skeleton were discovered (62–66).

Mutations appear to affect three aspect of craniofacial de-

velopment. Mutations such as *quadro* and *nocyrano* are characterized by absence or displacement of individual elements of the pharyngeal skeleton. Since all the pharyngeal arches are initially formed, it is likely that most of the mutations affect the subsequent differentiation of the chondrogenic neural crest in a region-specific manner. Some of these genes might be downstream of the *Hox* genes that are involved in the patterning of this region of the vertebrate body.

In a group of mutations, including *crusher* and *zhivago*, all elements of the head skeleton are initially laid down but differentiation into cartilage bars does not proceed normally. It is likely that most of these mutations define crucial steps in chondrogenesis, rather than initial patterning. This may include components of the extracellular matrix. In this regard, they may resemble the mouse *cartilage matrix deficient* (*CMD*) mutation, which deletes the aggrecan gene.

A third group of mutations develop a normal pattern, and, on a gross anatomical level, skeletal elements differentiate normally. However, individual elements are bent to various degrees, and normal spatial relationships distorted. In mutations like *brak*, malformations may be explained by reduced mechanical stability and bending of cartilage bars. Mutations like *pelican*, which express a gaping jaw phenotype, may be involved in the orchestration of skeletal elements, tendons and muscle into a functional cranium.

#### Future directions

The two completed large-scale zebrafish screens were based upon a visually evident phenotype. This is a type of screen sensitive for the heart and circulation, but less so for organs buried deep within the animal, such as the gut, or for assays of cell populations which are dispersed or defined only biochemically. New screens will highlight such tissues by use of specific probes (67). Also possible could be a screening approach to integrative behaviors, such as learning and memory, but these would depend upon the generation of robust assays for modifiable behavior in young embryos. Positional cloning awaits completion of a dense map and large insert libraries, likely within a couple of years, as well as tools for genetic rescue.

It may be that the power of the zebrafish, in terms of generation of new paradigms for development, is precisely with regard to new vertebrate features, such as organ assembly. Of course, its contributions can only be enhanced, once the genes are cloned, by extrapolation to mouse, by targeted gene ablation, and to human, by assessment of linkage to common disorders.

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#### References

1. Streisinger, G., C. Walker, N. Dower, D. Knauber, and F. Singer. 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature (Lond.)* 291:293–296.
2. Kimmel, C.B., W.W. Ballard, S.R. Kimmel, B. Ullmann, and T.F. Schilling. 1995. Stages of embryonic development of the zebrafish. *Dev. Dyn.* 20: 253–310.
3. Kimmel, C.B., T.F. Schilling, and K. Hatta. 1991. Patterning of body seg-

ments of the zebrafish embryo. *Curr. Topics Dev. Biol.* 25:77–110.

4. Eisen, J.S. 1994. Development of Motoneuronal Phenotype. *Annu. Rev. Neurosci.* 17:1–30.
5. Westerfield, M., F. Wegner, B.G. Jegalian, E.M. DeRobertis, and A.W. Puschel. 1992. Specific activation of mammalian Hox promoters in mosaic transgenic zebrafish. *Genes & Dev.* 6:591–598.
6. Postlethwait, J.H., S.L. Johnson, C.N. Midson, W.S. Talbot, M. Gates, E.W. Ballinger, D. Africa, R. Andrews, T. Carl, J.S. Eisen, S. Horne, C.B. Kimmel, M. Hutchinson, M. Johnson, and A. Rodriguez. 1994. A genetic linkage map for the zebrafish. *Science (Wash. DC)*. 264:699–703.
7. Johnson, S.L., M.A. Gates, M. Johnson, W.S. Talbot, S. Horne, K. Baik, S. Rude, J.R. Wong, and J.H. Postlethwait. 1996. Half-Tetrad Analysis in zebrafish II: centromere-linkage Analysis and Consolidation of the Zebrafish Genetic Map. *Genetics*. In press.
8. Knapik, E.W., A. Goodmann, S. Atkinson, C.T. Roberts, M. Shiozawa, C.U. Sim, S. Weksler-Zangen, M. Trolliet, C. Futrell, B.A. Innes et al. 1996. A Reference Cross for Zebrafish (*Danio rerio*). *Development*. In press.
9. Stuart, G.W., J.V. McMurray, and M. Westerfield. 1988. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development*. 103:403–412.
10. Lin, S., S. Yang, and N. Hopkins. 1994. lacZ expression in germline transgenic zebrafish can be detected in living embryos. *Dev. Biol.* 161:77–83.
11. Lin, S., N. Gaiano, P. Culp, J.C. Burns, T. Friedmann, J.K. Yee, and N. Hopkins. 1994. Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. *Science (Wash. DC)*. 265:666–669.
12. Rossant, J., and N. Hopkins. 1992. Of fin and fur: mutational analysis of vertebrate embryonic development. [Review]. *Genes & Dev.* 6:1–13.
13. Mayer, U., A.T. Ruiz, T. Berleth, S. Misera, and G. Jurgens. 1991. Mutations affecting body organization in the *Arabidopsis* embryo. *Nature (Lond.)*. 353:402–407.
14. Nüsslein-Volhard, C., and E. Wieschaus. 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature (Lond.)*. 287:795–801.
15. Hirsh, D., and R. Vanderslice. 1976. Temperature sensitive developmental mutants of *Caenorhabditis elegans*. *Dev. Biol.* 49:220–235.
16. Shedlovsky, A., T. King, and W. Dove. 1988. Saturation germ line mutagenesis of the murine *t* region including a lethal allele at the quaking locus. *Proc. Natl. Acad. Sci. USA*. 85:180–184.
17. Mullins, M.C., M. Hammerschmidt, P. Haffter, and C. Nüsslein-Volhard. 1994. Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr. Biol.* 4:189–202.
18. Solnica-Krezel, L., A.F. Schier, and W. Driever. 1994. Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics*. 136:1401–1420.
19. Driever, W., L. Solnica-Krezel, A.F. Schier, S.C.F. Neuhauss, J. Malicki, D.L. Stemple, D.Y.R. Stainier, F. Zwartkruis, S. Abdelilah, Z. Rangini, J. Belak, and C. Boggs. 1996. A genetic screen for mutations affecting embryogenesis in zebrafish. *Development*. In press.
20. Haffter, P., M. Granato, M. Brand, M.C. Mullins, M. Hammerschmidt, D.A. Kane, J. Odenthal, F.J.M. van-Eeden, Y.-J. Jiang, C.-P. Heisenberg et al. 1996. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development*. In press.
21. Nüsslein-Volhard, C. 1994. Of flies and fishes. *Science (Wash. DC)*. 266:572–574.
22. Driever, W., D. Stemple, A. Schier, and L. Solnica-Krezel. 1994. Zebrafish: genetic tools for studying vertebrate development. *Trends Genet.* 10:152–159.
23. Baier, H., S. Klostermann, T. Trowe, R.O. Karlstrom, C. Nüsslein-Volhard, and F. Bonhoeffer. 1996. Genetic dissection of the retinotectal projection. *Development*. In press.
24. Brouwerhoff, S.E., J.B. Hurley, W. Driever, S. Neuhauss, and J.E. Dowling. 1995. A behavioral screen for isolating zebrafish mutants with visual system defects. *Proc. Natl. Acad. Sci. USA*. 92:10545–10549.
25. Stainier, D.Y., and M.C. Fishman. 1992. Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. *Dev. Biol.* 153:91–101.
26. Stainier, D.Y., R.K. Lee, and M.C. Fishman. 1993. Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. *Development*. 119:31–40.
27. Stainier, D.Y.R., and M.C. Fishman. 1994. The zebrafish as a model system to study cardiovascular development. *Trends Cardiovascular Med. Trend Cardiovasc. Med.* 4:207–212.
28. Lee, R.K., D.Y. Stainier, B.M. Weinstein, and M.C. Fishman. 1994. Cardiovascular development in the zebrafish. II. Endocardial progenitors are sequestered within the heart field. *Development*. 120:3361–3366.
29. Stainier, D.Y.R., B. Fouquet, J.N. Chen, K. Warren, B. Weinstein, S. Meiler, M.M. Pallithotangal, S.C.F. Neuhauss, L. Solnica-Krezel, A.F. Schier, F. Zwartkruis, D. Stemple, J. Malicki, W. Driever, and M.C. Fishman. 1996. Mutations which affect cardiovascular form and function in zebrafish embryos. *Development*. In press.
30. Chen, J.-N., P. Haffter, J. Odenthal, E. Vogelsang, M. Brand, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt, C.-P. Heisenberg et al. 1996. Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development*. In press.
31. Stainier, D.Y.R., B.M. Weinstein, H.W. Detrich III, L.I. Zon, and M.C. Fishman. 1995. cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development*. In press.
32. Weinstein, B., D.L. Stemple, W. Driever and M.C. Fishman. 1995. gridlock, a localized heritable vascular patterning defect in the zebrafish. *Nat. Med.* 1:1143–1147.
33. Pack, M., L. Solnica-Krezel, J. Malicki, S.C.F. Neuhauss, A.F. Schier, D.L. Stemple, W. Driever, and M. Fishman. 1996. Mutations which disrupt development of digestive organs in zebrafish embryos. *Development*. In press.
34. Weinstein, B., A.F. Schier, S. Abdelilah, J. Malicki, S. Solnica-Krezel, D.L. Stemple, D.Y.R. Stainier, F. Zwartkruis, W. Driever, and M.C. Fishman. 1996. Hematopoietic mutations in zebrafish. *Development*. In press.
35. Ransom, D., P. Haffter, J. Odenthal, A. Brownlie, E. Vogelsang, M. Brand, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt, C.-P. Heisenberg, Y.-J. Jiang, D.A. Kane, R.N. Kelsh, M.C. Mullins, and C. Nüsslein-Volhard. 1996. Characterization of zebrafish mutations with defects in embryonic hematopoiesis. *Development*. In press.
36. Beddington, R.S.P. and J.C. Smith. 1993. Control of vertebrate gastrulation: inducing signal and responding genes. *Curr. Opin. Genet. Dev.* 3:655–661.
37. Chitnis, A., D. Henrique, J. Lewis, D. Ish-Horowitz, and C. Kintner. 1995. Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene Delta. *Nature (Lond.)*. 375:761–766.
38. Krumlauf, R. 1994. Hox Genes in Vertebrate Development. *Cell*. 78:191–201.
39. Altamir, A. 1994. Pattern formation in the vertebrate neural plate. *TINS*. 17:233–243.
40. Hatta, K., C.B. Kimmel, R.K. Ho, and C. Walker. 1991. The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature (Lond.)*. 350:339–341.
41. Schier, A.F., S.C.F. Neuhauss, M. Harvey, J. Malicki, L. Solnica-Krezel, D.Y.R. Stainier, F. Zwartkruis, S. Abdelilah, D.L. Stemple, Z. Rangini, H. Yang, and W. Driever. 1996. Mutations affecting the development of the embryonic zebrafish brain. *Development*. In press.
42. Haffter, P., J. Odenthal, Y.-J. Jiang, R.O. Karlstrom, E. Vogelsang, M. Brand, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt et al. 1996. Mutations in the zebrafish affecting notochord differentiation. *Development*. In press.
43. Brand, M., C.-P. Heisenberg, D. Beuchle, Y.J. Jiang, R.O. Karlstrom, R.M. Warga, F. Pelegri, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato et al. 1996. Mutations affecting development of the spinal chord and general body shape during zebrafish embryogenesis. *Development*. In press.
44. Heisenberg, C.P., M. Brand, Y.-J. Jiang, R.M. Warga, D. Beuchle, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato, P. Haffter, M. Hammerschmidt et al. 1996. Genes involved in forebrain development in the zebrafish, *Danio rerio*. *Development*. In press.
45. Brand, M., C.P. Heisenberg, Y.-J. Jiang, D. Beuchle, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato, P. Haffter, M. Hammerschmidt, D.A. Kane, R.N. Kelsh, M.C. Mullins, J. Odenthal, and C. Nüsslein-Volhard. 1996. Mutations in two zebrafish genes affect the formation of the boundary between mid-brain and hindbrain. *Development*. In press.
46. Jiang, Y.-J., M. Brand, C.-P. Heisenberg, D. Beuchle, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato, P. Haffter, M. Hammerschmidt, D.A. Kane et al. 1996. Genes involved in early neurogenesis, development of the hindbrain, and formation of the brain ventricles in the zebrafish, *Danio rerio*. *Development*. In press.
47. Campos-Ortega, J.A. 1994. Mechanisms of neurogenesis in *Drosophila melanogaster*. *Advances in Developmental Biology*. 3:1–40.
48. Abdelilah, S., E. Mountcastle-Shah, M. Harvey, L. Solnica-Krezel, A.F. Schier, D.L. Stemple, J. Malicki, S.C.F. Neuhauss, F. Zwartkruis, D.Y.R. Stainier, Z. Rangini, and W. Driever. 1996. Mutations affecting neural survival in zebrafish. *Development*. In press.
49. Furutani-Seiki, M., Y.-J. Jiang, M. Brand, C.P. Heisenberg, C. Houart, D. Beuchle, F.J.M. van-Eeden, M. Granato, P. Haffter, M. Hammerschmidt et al. 1996. Neural degeneration mutants in the zebrafish *Danio rerio*. *Development*. In press.
50. Granato, M., F.J.M. van-Eeden, U. Schach, T. Trowe, M. Brand, M. Furutani-Seiki, P. Haffter, M. Hammerschmidt, C.-P. Heisenberg, Y.-J. Jiang et al. 1996. Genes controlling and mediating locomotion behavior of the zebrafish embryo. *Development*. In press.
51. Malicki, J., S.C.F. Neuhauss, A.F. Schier, L. Solnica-Krezel, D.Y.R. Stainier, D.L. Stemple, S. Abdelilah, and W. Driever. 1996a. Mutations affecting development of the zebrafish retina. *Development*. In press.
52. Karlstrom, R.O., T. Trowe, M. Brand, H. Baier, A. Crawford, B. Grunewald, P. Haffter, M. Hammerschmidt, H. Hoffman, S. Klostermann et al. 1996. Zebrafish mutations affecting retinotectal axon pathfinding. *Development*. In press.
53. Trowe, T., S. Klostermann, H. Baier, B. Grunewald, H. Hoffmann, M. Granato, P. Haffter, M. Hammerschmidt, F.J.M. van-Eeden, E. Vogelsang et al. 1996. Mutations disrupting the ordering and topographic mapping of axons in the retinotectal projection of the zebrafish, *Danio rerio*. *Development*. In press.
54. Pourquie, O., M. Coltey, M.A. Teillet, C. Ordahl, and N.M. Le Douarin.

1993. Control of dorsoventral patterning of somitic derivatives by notochord and floor plate. *Proc. Natl. Acad. Sci. USA*. 90:5242-5246.
55. Talbot, W.S., B. Trevarrow, M.E. Halpern, A.E. Melby, G. Far, J.H. Postlethwait, T. Jovett, C.B. Kimmel, and D. Kimelman. 1995. A homeobox gene essential for zebrafish notochord development. *Nature (Lond.)*. 378:150-157.
56. Yamada, T., and Pfaff. 1993. Control of Cell pattern in the neural tube-motor neuron induction by diffusible factors from notochord and floor plate. *Cell*. 73:673-686.
57. Solnica-Krezel, L., D.L. Stemple, E. Mountcastle-Shah, Z. Rangini, S.C.F. Neuhauss, J. Malicki, A. Schier, D.Y.R. Stainier, F. Zwartkruis, S. Abdelilah, and W. Driever. 1996. Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish. *Development*. In press.
58. Odenthal, J., P. Haffter, E. Vogelsang, M. Brand, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt, C.-P. Heisenberg, Y.-J. Jiang et al. 1996. Mutations affecting the formation of the notochord in the zebrafish, *Danio rerio*. *Development*. In press.
59. Halpern, M.E., R.K. Ho, C. Walker and C.B. Kimmel. 1993. Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. *Cell*. 75:99-111.
60. Schulte-Merker, S., F.J.M. Van-Eeden, M.E. Halpern, C.B. Kimmel and C. Nusslein-Volhard. 1994. No Tail (Ntl) Is the Zebrafish Homologue of the Mouse T (Brachyury) Gene. *Development*. 120:1009-1015.
61. Stemple, D.L., L. Solnica-Krezel, F. Zwartkruis, S.C.F. Neuhauss, A.F. Schier, J. Malicki, D.Y.R. Stainier, S. Abdelilah, Z. Rangini, E. Mountcastle-Shah, and W. Driever. 1996. Mutations affecting development of the notochord in zebrafish. *Development*. In press.
62. Neuhauss, C.F., L. Solnica-Krezel, A.F. Schier, F. Zwartkruis, D.L. Stemple, J. Malicki, S. Abdelilah, D.Y.R. Stainier, and W. Driever. 1996. Mutations affecting craniofacial development in zebrafish. *Development*. In press.
63. Schilling, T.F., T. Piotrowski, H. Grandl, M. Brand, Y.J. Jiang, C.P. Heisenberg, D. Beuchle, F.J.M. vanEeden, M. Furutani-Seiki, M. Granato et al. 1996. Mutations affecting the development of the jaw and branchial arch I: Gill arches. *Development*. In press.
64. Piotrowski, T., T.F. Schilling, H. Grandl, M. Brand, Y.J. Jiang, C.P. Heisenberg, D. Beuchle, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato et al. 1996. Mutations affecting the development of the jaw and branchial arch II: Jaw. *Development*. In press.
65. Kelsh, R.N., M. Brand, Y.-J. Jiang, C.-P. Heisenberg, S. Lin, P. Haffter, J. Odenthal, M.C. Mullins, F.J.M. van-Eeden, M. Furutani-Seiki et al. 1996. Analysis of neural crest development using zebrafish pigmentation mutations. *Development*. In press.
66. Odenthal, J., K. Rossmagel, P. Haffter, R.N. Kelsh, E. Vogelsang, M. Brand, F.J.M. van-Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt et al. 1996. Genes required for Xanthopore pigmentation in the zebrafish, *Danio rerio*. *Development*. In press.
67. Henion, P.D., D.W. Raible, C.E. Beattie, K.L. Stoesser, J.A. Weston, and J.S. Eisen. 1996. A screen for mutations affecting development of zebrafish neural crest. *Developmental Genetics*. In press.