Learning to Fish with Genetics: A Primer on the Vertebrate Model *Danio rerio*

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ABSTRACT In the last 30 years, the zebrafish has become a widely used model organism for research on vertebrate development and disease. Through a powerful combination of genetics and experimental embryology, significant inroads have been made into the regulation of embryonic axis formation, organogenesis, and the development of neural networks. Research with this model has also expanded into other areas, including the genetic regulation of aging, regeneration, and animal behavior. Zebrafish are a popular model because of the ease with which they can be maintained, their small size and low cost, the ability to obtain hundreds of embryos on a daily basis, and the accessibility, translucency, and rapidity of early developmental stages. This primer describes the swift progress of genetic approaches in zebrafish and highlights recent advances that have led to new insights into vertebrate biology.

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10.1534/genetics.116.190843
Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.190843/-/DC1.
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Natural History

Danio rerio (initially designated *Brachydanio rerio*) were described in a book about fishes of the Ganges River by English physician Sir Francis Hamilton (Hamilton 1822). Hamilton identified 10 species; now there are 45 known species in the *Danio* genus. *D. rerio* are a monophyletic species in the Cyprinidae family, characterized by a bilobate caudal fin, and are members of the ray-finned fishes in the infraclass of Teleosti, which includes over 26,000 extant species (Fang *et al.* 2009). The large number of species is likely due to adaptive radiation following genome duplication in a shared ancestor during the evolution of ray-finned fish over 300 million years ago (Figure 1) (Taylor *et al.* 2003).

In the wild, *D. rerio* are a tropical freshwater fish living in small rivers, streams, paddy fields, and channels in South Asia, including India, Myanmar, Bangladesh, and Nepal (Engeszer *et al.* 2007; Spence *et al.* 2008; Arunachalam *et al.* 2013). Zebrafish prefer low-flow waters with vegetative overhangs that tend to have few predators (McClure *et al.* 2006; Spence *et al.* 2006; Engeszer *et al.* 2007; Arunachalam *et al.* 2013), though they are occasionally found in deeper running streams, usually in inlet regions (Arunachalam *et al.* 2013). Larger streams are possible avenues for movement of fish between isolated populations, thus increasing genetic diversity. Potential predators include snakeheads and freshwater needlefish, as well as predatory birds such as the Indian pond heron and the common kingfisher (Spence *et al.* 2008).

The natural environments for zebrafish breeding are ponds that form during monsoons. Typically, these ponds are still and shallow with pebble, sand, or silt substrata that likely protects the clear eggs from predation. The breeding season correlates best to the onset of the monsoon season, although mature ova have been observed during the dry season. Thus, breeding is more likely to correspond to the more abundant availability of food during the monsoon season (Spence *et al.* 2006).

In the Laboratory

Zebrafish are hardy fish that lend themselves well to a laboratory environment. Successful husbandry relies on many of the properties of the natural habitat. Zebrafish thrive in clear, alkaline (pH ~8.0) water with temperatures ranging from 20 to 33° (Engeszer *et al.* 2007; Lawrence 2011). Water in

Box 1: *golden* mutants and skin pigmentation

An unexpected connection between zebrafish and human genetics came from study of the *golden* mutant, identified by Streisinger *et al.* (1981), which lacks pigmentation in the skin. Keith Cheng's laboratory and collaborators found that a mutation in the *slc24a* gene, which encodes a cation exchanger important for Ca^{2+} cycling (Altimimi and Schnetkamp 2007), was responsible for the lighter pigmentation in zebrafish *golden* mutants and in humans of European decent (Lamason *et al.* 2005; Sturm 2006). Slc24a protein is normally found in an intracellular compartment, suggesting that it regulates calcium in the melanosome, an intracellular organelle that contains the pigment melanin. In humans, a single-amino-acid change in the Slc24a protein is associated with differences in skin pigmentation: those of European descent have a threonine at position 111, whereas those of African, East Asian, and Native American descent have an alanine (Figure B1).

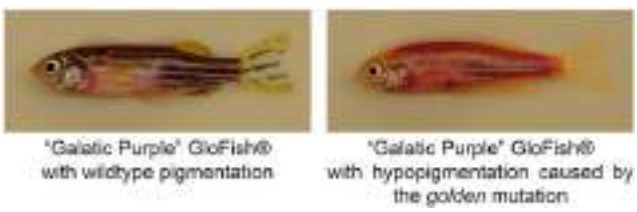

Figure B1 Lack of pigment in *golden* mutants. Commercially available GloFish® lack pigment because they are homozygous for the *golden* mutation (compare *golden* mutant in right panel to normally pigmented fish in left panel) (http://www.glofish.com). Anterior to the left and dorsal to the top. Images courtesy of Sooji (Katie) Jo.

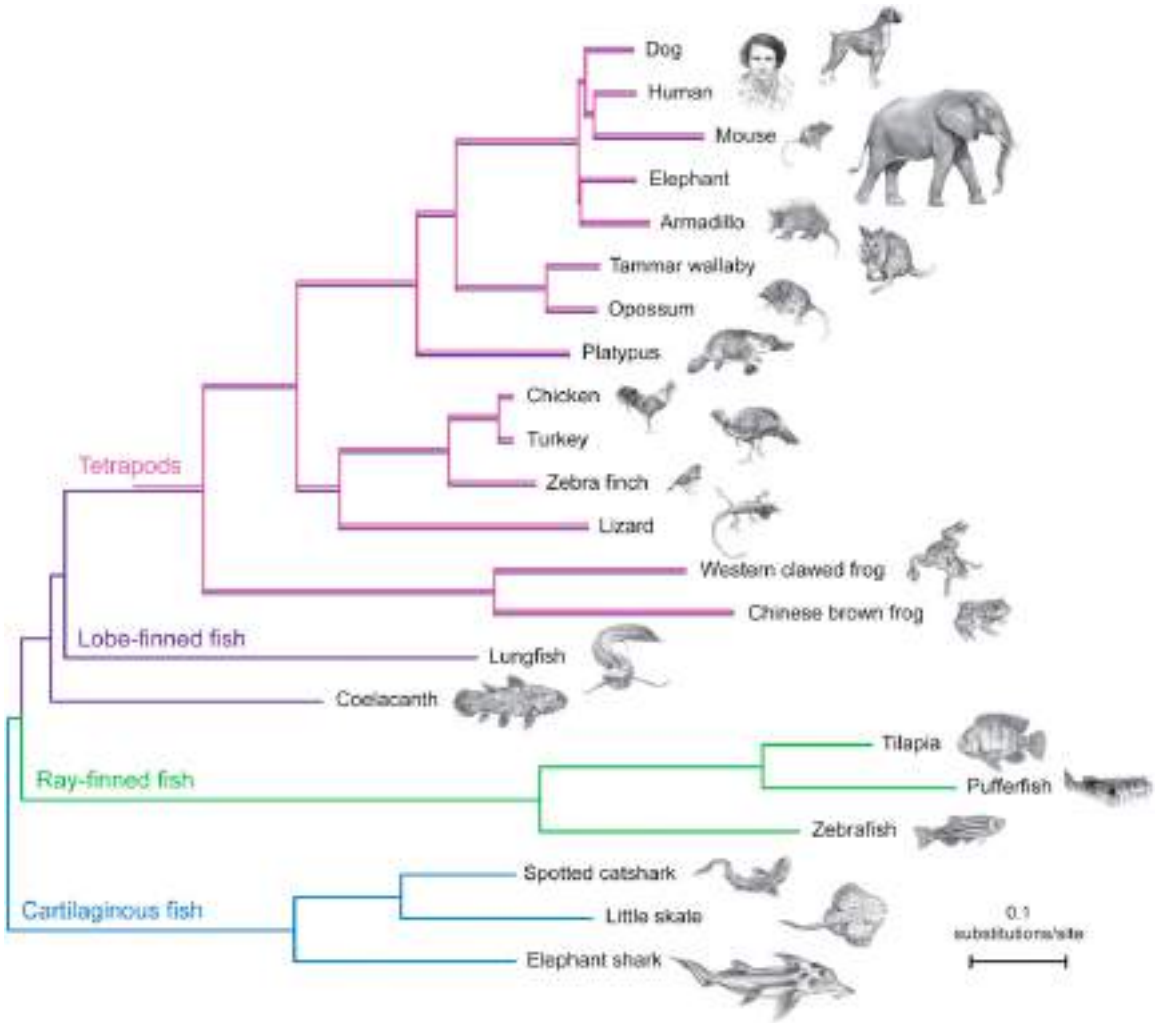

Figure 1 Phylogenetic tree of vertebrate evolution. The phylogenetic tree shows the relationship of zebrafish to a broad range of jawed vertebrates and was constructed from sequence alignments of 251 genes and rooted on cartilaginous fish. Reprinted by permission from Macmillan Publishers Ltd: Nature (Amemiya *et al.*, 2012).

laboratory facilities is typically maintained at 28.5° (Westerfield 2000). Their diet in the wild consists mainly of insects, insect larvae, nematodes, and crustaceans. In the laboratory, artificial food is typically supplemented with live food such as brine shrimp or mealworms for a more balanced diet (McClure *et al.* 2006; Spence *et al.* 2008). Because adult zebrafish average <3.5 cm in length, many thousands can be kept in a confined laboratory space.

Many wild-type (WT), mutant, and transgenic strains of zebrafish are available through the Zebrafish International Resource Center in Eugene, Oregon (http://zebrafish.org/home/guide.php) (Sprague *et al.* 2003). Other sources include zebrafish research laboratories, fish suppliers, and pet stores. Fish from outside sources either should be obtained as bleached eggs treated to kill surface pathogens or should be kept in quarantine because of the possibility of disease contamination. Excellent resources are available to guide a new zebrafish researcher, including *The Zebrafish Book* (Westerfield 2000), *Zebrafish: A Practical Approach* (Nusslein-Volhard and Dahm 2002), the Zebrafish Model Organism Database (http://zfin.org), and a comprehensive review on zebrafish husbandry by Lawrence (2011).

Zebrafish are prolific breeders, producing transparent embryos that allow researchers to study early developmental events in detail. Males are distinguished by their yellow coloring and larger anal fins (Figure 2). Breeding pairs spawn within the first 2 hr after dawn or just before dusk (Legault 1958; Hisaoka and Firlit 1962; Darrow and Harris 2004; Liang *et al.* 2011a), producing clutch sizes as large as several hundred eggs. The timing of spawning is controlled by a molecular circadian clock and will persist for several days in the absence of environmental cues (Blanco-Vives and Sanchez-Vazquez 2009). Because the chorion and embryo are clear, zebrafish are particularly amenable to live-cell imaging to characterize cell morphology and cell division and migration patterns. Embryos develop rapidly, starting with synchronous divisions that subdivide the single blastomere, which sits on a yolk ball, into several thousand cells (Kimmel

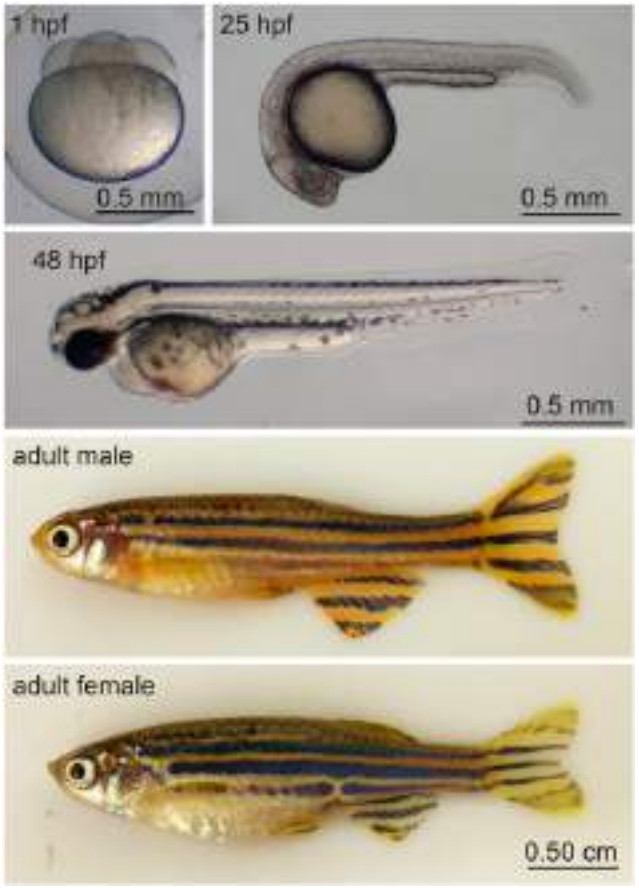

Figure 2 Life stages of zebrafish. A zebrafish embryo at the four-cell stage [1 hr postfertilization (hpf)] has four cells at the animal pole that sit atop a single yolk cell and that are formed through discoidal cleavage. By 25 hpf, the body axis of the embryo has formed. Embryos at 48 hpf have rudiments of most major organs (*e.g.*, note the heart tube just ventral to the eyes). Zebrafish reach adulthood at 3 months postfertilization. Adult males have a streamlined shape and a yellow hue, while adult females are larger and have a whiter hue. Lateral views with animal pole to the top (four-cell-stage embryo) or anterior to the left and dorsal to the top (all other images).

et al. 1995). By 24 hr, the embryos have a defined body axis and rudimentary organs, including a contractile heart (Figure 2). Zebrafish have a rapid generation time, reaching adulthood in approximately 3 months with an average lifespan of 2–3 years.

Adoption of Zebrafish as a Genetic Model

By the 1920s, fish were being recommended as a potential genetic and embryologic research model with an emphasis on the striped zebrafish (Goodrich 1929), and techniques for using zebrafish in embryologic research were outlined by Creaser (1934). George Streisinger (Figure 3) was the first researcher to use zebrafish as a genetic model. After focusing his research on the genetics of bacteriophages T2 and T4, he set his sights on a more complex organism in which to study the nervous system and behavior. To accomplish this, Streisinger felt it was important to work on a genetically tractable vertebrate model (Stahl 1995; Grunwald and Eisen 2002).

Streisinger devised approaches to identify mutations in zebrafish without extensive breeding that became the framework for the first genetic screens (Figure 4). Haploid embryos were generated by fertilizing oocytes with UV-irradiated sperm that activated cleavage but did not contribute any paternal DNA. The resulting haploid embryos were viable for 3–5 days postfertilization (dpf) and thus could be screened for early morphologic phenotypes (Streisinger *et al.* 1981).

Strategies to produce homozygous gynogenetic diploid embryos also use UV-irradiated sperm to activate development in the absence of a genetic contribution from the male (Figure 4B). In one approach, a short heat shock prevents the first mitotic cleavage of the gamete, generating embryos that have a diploid genome that is homozygous for every locus (Streisinger *et al.* 1981). In the second strategy, gynogenetic diploid embryos are generated by preventing expulsion of the second polar body during meiosis II. This is accomplished by briefly putting the early embryos under 8000 lb/in^2 of pressure (Streisinger *et al.* 1986). The early-pressure method has two significant advantages over haploid screens: (1) a large number of the embryos are viable to adulthood, producing both males and females, and (2) gynogenetic diploids are homozygous at all loci except for those that were separated by meiosis I crossing-over events. Thus, mutated genes can be mapped to chromosomal positions via the calculation of recombination frequencies based on recovered homozygotes. These techniques laid the foundation for genetic screens and the creation of a genetic map.

Forward Genetic Screening Approaches

In 1984, Streisinger, in collaboration with Charles Kimmel, took the next important step in establishing zebrafish as a genetic model: the first systematic genetic screen. Their screen was designed to find mutants with defects in neural patterning or behavior. Unfortunately, Streisinger died in 1984 and never knew the impact of his work. Charles Kimmel, Judith Eisen, and Monte Westerfield continued Streisinger's work and defined the key stages of embryogenesis and characterized the first zebrafish mutants. A large selection of guides to zebrafish development, aimed at everyone from experienced researchers to K–12 students, is now available (Table 1).

The first haploid and early-pressure diploid screens focused on identifying mutants with abnormal morphology (Table 2). Gamma irradiation was used to induce chromosomal rearrangements, such as small and large deletions, inversions, and translocations, in the zebrafish genome (Chakrabarti *et al.* 1983; Walker and Streisinger 1983; Streisinger *et al.* 1986). These screens generated novel mutants with defects in neural tube patterning (*cyclops/ndr2*, *neural degeneration 1*), mesoderm development (*no tail/ta*), and cell movements during gastrulation (*spadetail/tbx16*) (Grunwald *et al.* 1988; Ho and Kane 1990; Molven *et al.* 1990; Hatta *et al.* 1991). The fortuitous identification of the *no tail* mutant

George Streisinger

Figure 3 Dedication to Dr. George Streisinger in *The Zebrafish Book*, a guide for using zebrafish in the laboratory (Westerfield 2000). Page reprinted with permission.

in Oregon (Halpern *et al.* 1993) and the cloning and expression analysis of the zebrafish homolog of the mouse *Brachyury* gene in Germany (Schulte-Merker *et al.* 1992) led to a candidate approach making the first match between a zebrafish mutant and the affected gene (Schulte-Merker *et al.* 1994). Recovery of these initial mutants demonstrated the value of zebrafish as a model organism whose strength was in the combination of classic embryology and developmental genetics.

Other laboratories subsequently initiated screens for particular developmental defects, taking advantage of the ease of whole-mount RNA *in situ* hybridization and antibody staining. For instance, parthenogenic diploid embryos were screened with antibodies to identify mutants with defects in neural crest cells and their derivatives (Henion *et al.* 1996) or in motor axons (Beattie 2000). Alteration in gene expression detected by *in situ* hybridization was the basis for a haploid screen to identify mutants with altered hindbrain segmentation (Moens *et al.* 1996).

Scaling up to large screens

By the late 1980s, word of the value of zebrafish as a model organism had spread. Christiane Nüsslein-Volhard initiated a large-scale screen to identify zebrafish point mutations, following a similar plan that she and others had carried out in *Drosophila* (Figure 4C) (Mullins *et al.* 1994; Haffter *et al.* 1996). A parallel mutant screen was initiated in the United States by Wolfgang Driever and Marc Fishman (Driever *et al.* 1996; Grunwald and Eisen 2002). These "big screens" resulted in the discovery of approximately 1500 mutants. Because they used the chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU), most of the mutants had single-base-pair changes that fell into

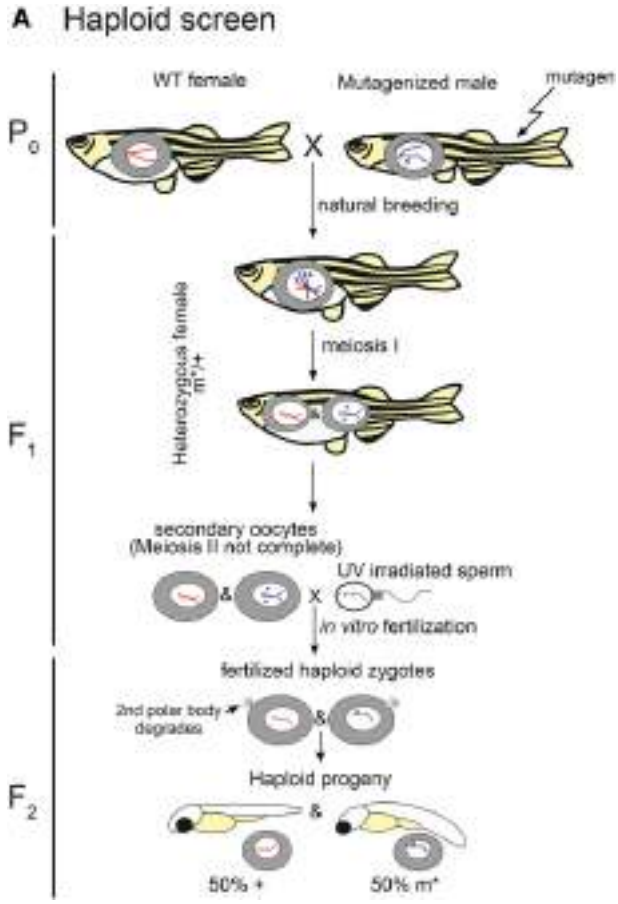

Figure 4 Forward genetic screens. (A) In haploid screens, the parental generation (P_0) and first filial generation (F_1) are produced by natural breeding, but the second filial generation (F_2) is produced by *in vitro* fertilization with UV-inactivated sperm to generate haploid embryos. (B) Homozygous F_1 gynogenetic diploid screens are similar to the haploid screens except the resulting embryos are 2*n* because they are exposed either to heat shock (HS) or to early pressure (EP) to inhibit the second meiotic division of the oocyte or the first mitotic division of the zygote, respectively. (C) Large-scale screens in Tubingen and Boston used an additional generation, and so were F_3 screens. The symbols inside the cells (circles) indicate genotypes of germ cells in different stages of meiosis, with the exception of the P_0 fish and the F_1 fish in C, where they indicate the genotype of the fish. For simplicity, genotype at only one (A, B) or two chromosomes (C) is indicated. The * and # symbols indicate two different mutations induced in the P_0 males.

over 300 loci. These results were presented in 37 papers published in a special issue of *Development* [Vol. 123, No. 1 (1996)] (Figure 5). Identification of the genetic lesions in these mutants, and those that followed, has illuminated the genetic pathways that regulate developmental processes.

Maternal- and paternal-effect screens

The first developmental events in vertebrate embryos are controlled by maternal or paternal factors loaded into the oocyte or sperm and then activated at fertilization. For instance, in zebrafish, the onset of zygotic transcription begins at the midblastula transition (MBT), when the embryo has

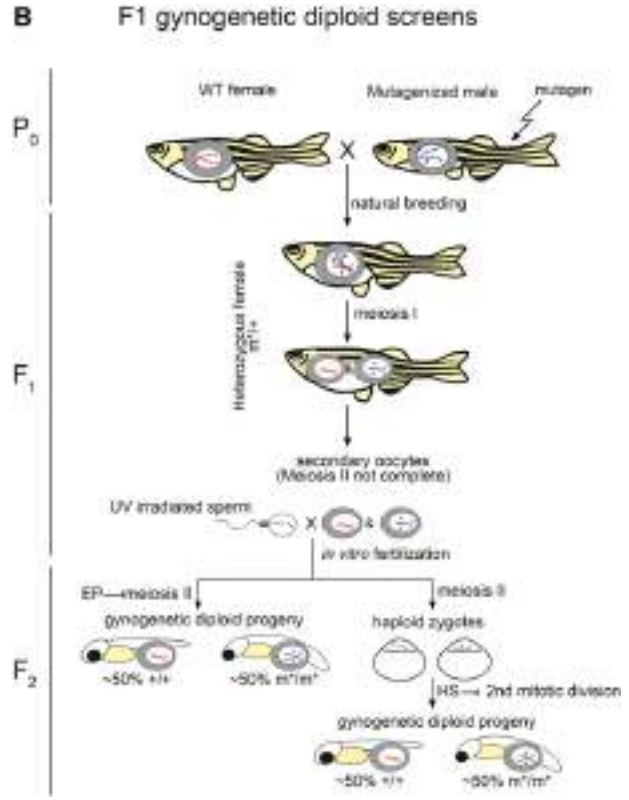

Figure 4 *Continued.*

512 cells (Kane and Kimmel 1993), whereas in mice it begins at the two-cell stage (Stern and Downs 2012). A wealth of new maternal and paternal factors involved in gamete and embryonic development was identified with specially designed screens (Pelegri *et al.* 2004; Pelegri and Mullins 2011). Recessive maternal-effect mutations were identified in an innovative screen for mutants in which F_2 gynogenetic diploids were raised to adulthood and bred: F_3 females were crossed to WT males, and the F_4 progeny were screened for mutant phenotypes. In another approach, the F_3 generation of an F_2 diploid screen was raised to adulthood, and the males and females were intercrossed or outcrossed to WT fish. Crosses between two polymorphic strains of fish were used to facilitate mapping of the mutated genes (Dosch *et al.* 2004; Wagner *et al.* 2004). Together these approaches identified a suite of new mutations affecting oogenesis, egg activation following fertilization, early cell or nuclear divisions, and embryo polarity.

Screens for maternal- or paternal-effect mutations provided several unexpected outcomes. Interestingly, some phenotypes became apparent only after the MBT, suggesting that the parental factors either persisted past the onset of zygotic transcription or were needed to initiate a cascade of events subsequent to MBT. Additionally, the identification of only a small number of paternal-effect mutants supports the specialized function of the sperm in the fertilization process. Phenotypes of paternal mutants are likely caused by defective centrosomes or chromosomes contributed by the sperm during fertilization (Wagner *et al.* 2004).

Many developmentally important genes are expressed from maternally loaded messenger RNAs (mRNAs) as well as the zygotic genome following MBT. Germ-line replacement provides a technique to test the maternal role of genes with an existing mutant fish line. In germ-line replacement, the host embryo is injected with an antisense morpholino that inhibits primordial germ-cell development (Ciruna *et al.* 2002). The pool of donor embryos (a mix of homozygous WT, heterozygous mutant, and homozygous mutant embryos) is labeled with fluorescent markers for all cells or for primordial germ cells only. Successful germ-line replacement is scored by fluorescent cells in gonadal mesoderm. Once generated, fish carrying homozygous mutant germ lines can be bred to produce progeny with neither maternal nor zygotic mRNA. Females can also be mated to WT males to produce clutches of embryos without maternal gene products. Germ-line replacement has now been used on a wide variety of fish species and even in cross-species transplants, opening up additional opportunities for research on gamete development (Saito *et al.* 2008; Shimada and Takeda 2008; Goto *et al.* 2012).

Functional Screens

The increase in molecular tools available in zebrafish has enabled targeted genetic and chemical screens that rely on tissue-specific markers. Several laboratories have characterized the temporal and spatial expression patterns of large numbers of zebrafish genes, resulting in searchable databases of gene expression patterns (http://zfin.org/). Researchers have also used functional assays in screens to identify mutants affecting fin and heart regeneration, vision, and behaviors ranging from spontaneous movement to prepulse inhibition (Brockerhoff *et al.* 1997; Orger *et al.* 2004; Hoptak-Solga *et al.* 2008; Dickover *et al.* 2013).

Temperature-sensitive fin-regeneration screens

Adult zebrafish are an excellent vertebrate model to study regeneration because both the fin and the heart regenerate after lesioning. Johnson and Weston (1995) conducted the first temperature-sensitive screen in zebrafish to isolate mutations perturbing fin regeneration. Fish were reared to adulthood at a permissive temperature, and then tail fins were challenged to regenerate at a restrictive temperature. An alternative approach (Johnson and Bennett 1999) was to screen for recessive mutations in F_2 generation adults created using the parthenogenesis techniques described earlier. Conditional mutations identified in this manner include the cell-cycle regulator *mps1/ttk protein kinase*, the growth factor *fibroblast growth factor20a* (*fgf20a*), and the regulator of cellular trafficking *sec1 family domain containing 1* (*scfd1/sly1*) (Poss *et al.* 2002; Nechiporuk *et al.* 2003; Whitehead *et al.* 2005). Each of these genes is critical for the establishment and function of the fin blastema, a compartment of proliferating cells required for continued outgrowth during fin regeneration.

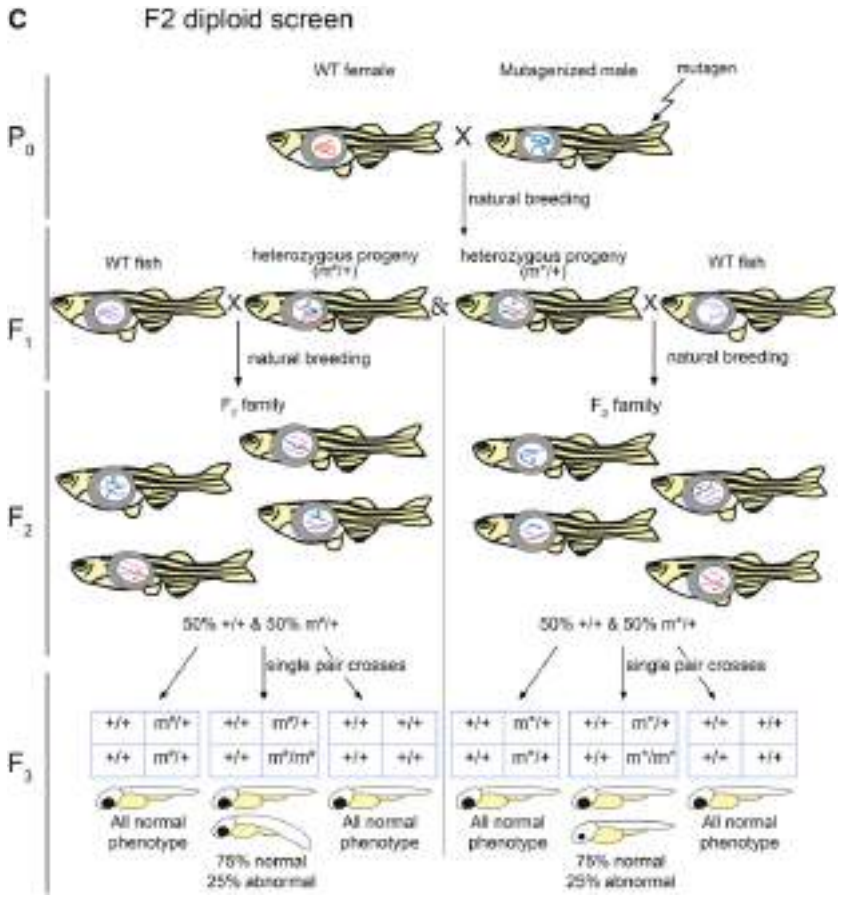

Figure 4 *Continued.*

Behavioral screens

Zebrafish, especially at larval stages, have many characteristics that make them amenable to forward genetic approaches aimed at discovering genes that underlie development of the nervous system and formation of a complex network of neural circuits (Fleisch and Neuhauss 2006; Renninger *et al.* 2011; Wolman and Granato 2012). As stated by Wolman and Granato (2012), "in a mere 5 days, fertilized zygotes have become free swimming and self-feeding larvae with a rich repertoire of stereotyped motor behaviors that operate on a simple blueprint of a vertebrate nervous system." Such behaviors include swimming and turning, the light startle response, phototaxis, the optokinetic response, the escape response, and many others. Here we will discuss just two examples of screens that have taken advantage of the robust sensorimotor behavior of larval zebrafish.

Larvae use the optokinetic response (OKR) to track the movement of objects in their environment. Several screens have taken advantage of this behavior to identify mutants in the visual system (Brockerhoff *et al.* 1995; Baier *et al.* 1996; Neuhauss *et al.* 1999; Muto *et al.* 2005). Zebrafish larvae are immobilized and placed in the center of a rotating drum with alternating white and black vertical stripes. Larvae with normal vision track the rotation of the stripes by moving their eyes smoothly in the same direction. When the object is out of the field of vision, eye position is reset by saccadic (fast and jerky) movements (Neuhauss 2003). Most mutants identified through OKR screening have impairments in the visual system, while *belladonna* mutants have defects in the ipsilateral projection of the optic nerve due to disruption in axon guidance (Neuhauss *et al.* 1999). A related screen for dominant mutations in ENU mutagenized fish used a visual escape response to a threat. It identified *night blind a* (*nba*) mutants, which show a slow degradation of their retinas (Li and Dowling 1997). These and other visual assays have provided powerful approaches to uncover novel genes and neural connections relevant to our understanding of human retinal function and degeneration (Morris 2011).

Another robust behavior of larval zebrafish used in genetic screens is the startle or escape response. In response to a strong stimulus, such as a loud noise, zebrafish make a rapid turn called a *C-bend* followed by a less severe bend in the opposite direction before swimming rapidly away (Kimmel *et al.* 1974). Burgess and Granato (2007) carried out a screen to identify genes that modulate the startle response through prepulse inhibition. Such approaches demonstrate the power of the zebrafish model to find genes involved in specific complex behaviors. Defects in prepulse inhibition are associated with several human psychiatric disorders, including schizophrenia and

Table 1 Guides to zebrafish embryonic and larval development

Kimmel *et al.* (1995)	Guide to staging zebrafish embryos and larva. Includes images of live embryos and larva as well as detailed line drawings of major structures. Text description of major events at each stage. Images from this manuscript as a poster: http://homepages.wmich.edu/~dkane1/Pubs/Zebrafish%20Embryonic%20Staging%20Poster.pdf.
Zebrafish K–12 website	Annotated guides to zebrafish development and anatomy using the line drawings from Kimmel *et al.* (1995): http://www.uoneuro.uoregon.edu/k12/zfk12.html.
Karlstrom and Kane (1996)	Flipbook movie assembled from a series of still photographs of early zebrafish development, shown in lateral view, from the two-cell stage to about the 16-somite-cell stage: http://www.bio.umass.edu/biology/karlstrom/Movies/ZFFlipBookMovie.Big.mov.
Appendix 2: "Atlas of Embryonic Stages of Development in the Zebrafish," in *Zebrafish: A Practical Approach* (Nusslein-Volhard and Dahm 2002)	Information on staging zebrafish embryos as in Kimmel *et al.* (1995) with the addition of camera lucida drawings of internal and external anatomic structures.
The Exploratorium (www.exploratorium.edu)	A movie of zebrafish development from the one-cell stage to 48 hpf. This embryo is still in its chorion and free to move: http://www.exploratorium.edu/imaging-station/students/zebrafish_dev.html. This movie is a close up of a beating heart in WT and mutant zebrafish http://www.exploratorium.edu/imaging-station/research/zebrafish/story_zebrafish4.php.
Searchable database on ZFIN (Sprague *et al.* 2003)	Search for anatomic features present at different stages of development: http://zfin.org/action/ontology/ontology-search.
Zebrafish in the Classroom website (Liang *et al.* 2011b)	Virtual challenge to identify the stages of embryos pictured in a number of images: http://www.zfic.org/virtual%20experiments/stage2stagingbackground2.html.
Aoki (2009)	Contains a figure that can be printed and made into a flipbook illustrating zebrafish embryonic development.
Parichy *et al.* (2009); Singleman and Holtzman (2014)	Guides to staging postembryonic zebrafish (>3 dpf) using external markers in live fish.

This table lists many of the useful guides available for observing the development and anatomy of unstained, live zebrafish embryos and larvae.

Tourette syndrome, suggesting that research on these zebrafish mutants ultimately may have an impact on human health.

Insertional Mutagenesis

Neither chemical nor radiation methods of mutagenesis mark the gene of interest; thus, recombination mapping and positional cloning are required to identify the mutations. As an alternative approach, the zebrafish genome can be modified through injection of DNA into the one-cell-stage embryo. Injected DNA, in the form of a retrovirus, linearized plasmid, or transposon, incorporates into the genome to cause heritable change. Of these, retroviruses may have the most potential as effective mutagenic agents.

Retroviral DNA injected into blastula-stage embryos yields founders carrying multiple integrations that are intercrossed to generate F_1 families (Figure 6). Southern blotting identifies F_1 fish with a high number of inserts, which are bred to generate F_2 families. The impact of homozygous insertions is assessed after intercrosses between F_2 family

Box 2: Left-right brain asymmetry

The brains of many vertebrate species have functional and/or morphologic differences between the left and right sides. Research in zebrafish has identified genes that control directional asymmetry of the brain. Expression of genes in the left forebrain ultimately leads to dorsal-ventral differences in innervation of the midbrain interpeduncular nucleus, which influences behavior (Halpern *et al.* 2003; Barth *et al.* 2005; Facchin *et al.* 2009; Dadda *et al.* 2010; Roussigne *et al.* 2012) (Figure B2)

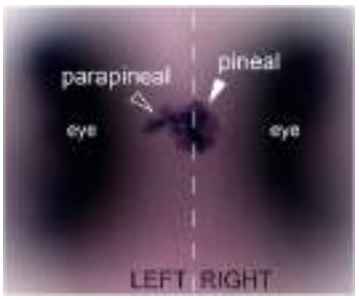

Figure B2 Left-right asymmetry in the dorsal forebrain. One left-right asymmetry in the zebrafish embryo is in the pineal complex, composed of a centrally located pineal organ and a parapineal typically positioned on its left. Dorsal view of the head of an ~30 hr postfertilization (hpf) embryo, with the eyes, pineal, and parapineal stained purple using whole-mount *in situ* hybridization for the gene *otx5*.

Table 2 Timeline of advances in zebrafish genetics

Year	Event	Citations
1960s	George Streisinger begins work on zebrafish.	Reviewed in Grunwald and Eisen (2002)
1972	George Streisinger produces haploid embryos.	Reviewed in Grunwald and Eisen (2002)
1981	Clones of homozygous zebrafish produced through gynogenetic approaches.	Streisinger *et al.* (1981)
1983	Methods for inducing mutations by gamma irradiation described.	Chakrabarti *et al.* (1983); Walker and Streisinger (1983)
1988	Publication of the first induced embryonic lethal mutation (*neural degeneration 1*).	Grunwald *et al.* 1988
1990	First meeting on zebrafish held in Eugene, OR.	Reviewed in Grunwald and Eisen (2002)
1993	Large-scale genetic screens initiated in Tubingen, Germany, and Boston, MA.	Reviewed in Grunwald and Eisen (2002)
1994	First Cold Spring Harbor conference on zebrafish genetics and development.	Reviewed in Grunwald and Eisen (2002)
1994	Identification of a gene affected in a zebrafish mutant (*no tail*) using a candidate approach.	Schulte-Merker *et al.* (1994)
1994	Publication of first inherited retrovirus integration.	Lin *et al.* (1994)
1994	Creation of the Zebrafish Information Network (ZFIN).	http://www.zfin.org; Westerfield *et al.* (1997)
1994–1999	Publication of genetic linkage maps for zebrafish.	Postlethwait *et al.* (1994); Johnson *et al.* (1996); Knapik *et al.* (1998); Shimoda *et al.* (1999)
1996	Publication of results of large-scale genetic screens.	Reviewed in Driever *et al.* (1996) and Haffter and Nusslein-Volhard (1996)
1997	Trans-NIH Zebrafish Initiative established.	http://www.nih.gov/science/models/zebrafish/
1998	Identification of a gene affected in a zebrafish mutant (*one eyed pinhead*) using positional cloning.	Zhang *et al.* (1998)
1999	Genetic screen using retroviral-mediated insertional mutagenesis.	Gaiano *et al.* (1996)
2000	Demonstration of antisense morpholinos to knock down function of specific genes.	Nasevicius and Ekker (2000)
2001	Whole-genome sequencing initiated by the Wellcome Trust Sanger Institute.	Howe *et al.* (2013)
2001	Zebrafish International Resource Center (ZIRC) is founded.	
2002	Gene targeting using TILLING.	Wienholds *et al.* (2003)
2004	Gene and enhancer trap screens using Tol2 transposition.	Kawakami *et al.* (2004); Parinov *et al.* (2004)
2008	Genome editing using ZFNs.	Doyon *et al.* (2008); Meng *et al.* (2008)
2011	Genome editing using TALENs.	Huang *et al.* (2011); Sander *et al.* (2011)
2013–2014	Genome editing using CRISPR/Cas9.	Gonzales and Yeh (2014)
2014	Simultaneous targeting of multiple genes using CRISPR/Cas9.	Ota *et al.* (2014)

members. F_3 progeny are then screened to identify morphologic mutant phenotypes, and the affected gene can be identified through inverse PCR or linker-mediated PCR (Huang *et al.* 2012). Insertional mutants have been used to conduct a variety of phenotypic screens, including screens focused on the visual system (Gross *et al.* 2005), cancer (Amsterdam *et al.* 2004b), and development (Amsterdam *et al.* 2004a; Nissen *et al.* 2006; Barresi *et al.* 2010).

The retroviral method was adapted for the purpose of saturation mutagenesis of the zebrafish genome to identify all protein-coding genes. Proof of principle came from a small-scale screen in which about 20% of all insertions caused reduced mRNA levels (Wang *et al.* 2007). Insertion sites were identified by direct sequencing of genomic DNA of F_1 generation adult males carrying heterozygous insertions (Varshney *et al.* 2013). An important drawback to this approach is that each individual in the screen contains multiple insertion sites. Therefore, multiple outcrosses are necessary to ensure that the phenotype of interest is due to the integration event in the gene of interest. Fish lines containing insertional mutations are available to the community from the Zebrafish International Resource Center (ZIRC) (http://zebrafish.org/zirc/home/guide.php).

Transgenesis

The ability to modify the genome by insertion of transgenes made it possible for investigators to generate stable transgenic lines expressing fluorescent proteins and use them for developmental studies as well as innovative genetic screens. The adoption of transposons as a means of readily generating transgenic lines has been a significant advance for the study of gene functions and cell behavior. Both the Tol2 transposon from Medaka (*Oryzias latipes*) and the Tc1/mariner-type synthetic transposon Sleeping Beauty were adapted for use in zebrafish. To make the Tol2 system amenable for genomic integration in zebrafish, the transposon ends required for integration were isolated, and genes of interest were cloned between them. When Tol2 vectors were co-injected into one-cell-stage embryos with a source of Tol2 transposase, the exogenous DNA was randomly inserted in the zebrafish genome (Kawakami

Box 3: Zebrafish help solve mysteries of cancer

The ability to follow disease in live fish facilitates studying the process and genetics of cancer, including one of the least curable: pancreatic cancer. Human pancreatic ductal adenocarcinomas currently result in over 80% lethality. Mutations in the *KRAS* viral oncogene are detected in over 90% of adenocarcinomas and precancerous pancreatic lesions. Mutation of *KRAS* leads to other cancer-related changes, including dysregulation of signaling, resistance to apoptosis, and increased cell division (Bardeesy and DePinho 2002; Ryan *et al.* 2014). The same mutations in *KRAS* cause pancreatic cancer in both humans and zebrafish, and similar downstream changes are induced as cancer progresses. $KRAS^{mut}$ transgenic zebrafish are thus attractive models for identifying new cancer treatments.

The GAL4/UAS system has been used to make a series of transgenic zebrafish lines expressing oncogenic *KRAS* in the pancreas. These lines offer an unprecedented opportunity to dissect disease progression *in vivo*. In one study, transgenic fish expressing oncogenic $KRAS^{G12D}$ fused to GFP (green fluorescence protein) were crossed to lines that report the activity of different signaling pathways through expression of the red fluorescent reporter *mCherry*. Researchers demonstrated that several signaling pathways associated with human cancer were activated in the transgenic zebrafish, including the TGFβ, Sonic Hedgehog, and Notch pathways (Schiavone *et al.* 2014) (Figure B3).

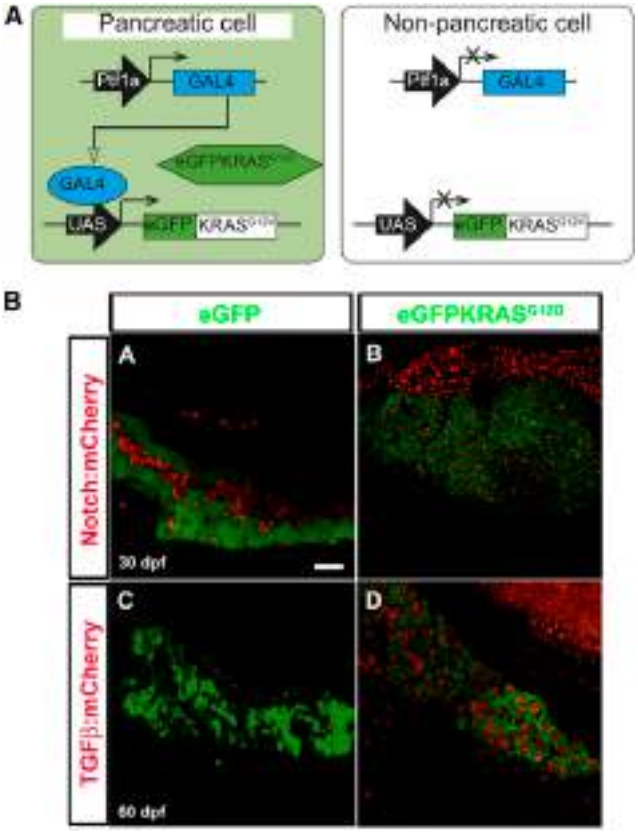

Figure B3 Following signaling pathway activation in a pancreatic cancer transgenic line. (A) In pancreatic cells, the *ptf1a* promoter is activated, causing transcription and translation of GAL4, which, in turn, activates UAS-regulated transcription and translation of the eGFPKRAS^{G12V} fusion protein and GFP labeling of pancreatic cells. (B, D) In control fish, there is no overlap between the pancreatic cells (green) and (B) TGFβ-induced or (D) Notch-induced *mCherry* expression (red), demonstrating that these pathways are not activated in pancreatic cells. (C, E) In fish expressing the eGFPKRAS^{G12V} fusion protein, the TGFβ and Notch pathways are activated in cells expressing the oncogenic form of *KRAS*. This is indicated by the overlap between the GFP^{+} pancreatic cells that are also positive for (B) TGFβ or (D) Notch signaling–induced *mCherry*. Images are full confocal Zstacks from (B, D) 30 and (C, E) 60 dpf fish. Panel A is adapted from Liu and Leach (2011), and panel B is reprinted with permission from Schiavone *et al.* 2014).

2007). The Tol2 system significantly increases the efficiency of genomic integration and therefore expedites creation of new transgenic lines. Development of the "Tol2-kit" further simplified the generation of new transgenic lines by providing building blocks for efficient construction of transgenes (http://tol2kit.genetics.utah.edu) (Kwan *et al.* 2007).

Screens using fluorescent transgenic lines

Using tissue-specific promoters that drive expression of fluorescent proteins to label developing tissues and organs has permitted screens that focus on dynamic processes that are hard to visualize (Table 3 and Table 4). For example, Xiao *et al.* (2005) used the *brnc3* promoter to drive expression

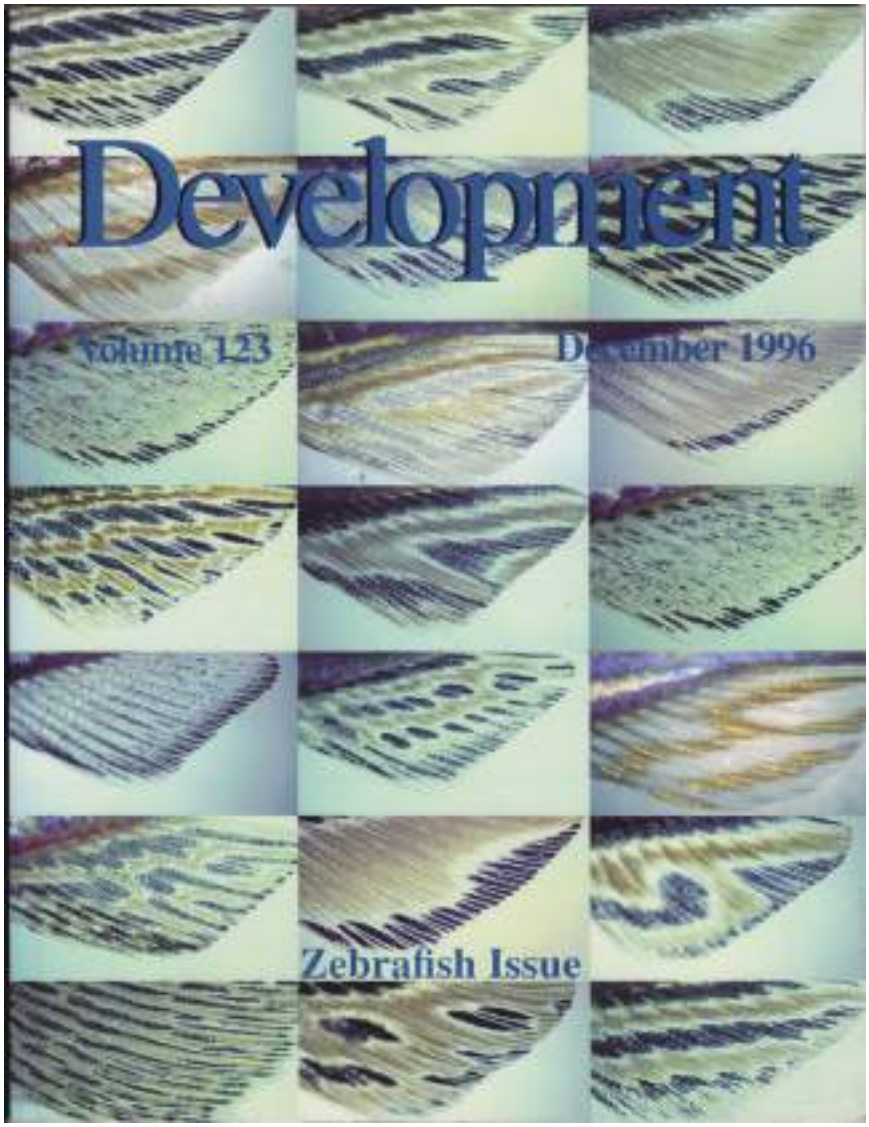

Figure 5 Cover page of the December 1996 special "Zebrafish Issue" of the journal *Development*. This issue contained the results of the genetic screens carried out in Tubingen, Germany, and Boston, MA. In total, over 1500 different zebrafish mutants were identified, and the scientific impact of this project was large enough to justify a special issue given its own volume number, a rare occurrence [*Development* 123(1) (1996): http://dev.biologists.org/content/123/1.toc). The images on the cover illustrate the pigment patterns in the anal fins of different adult mutant zebrafish. Reprinted with permission.

of GFP in retinal ganglion cells and their axons, enabling visualization of the major connections between the retina and the brain. Their forward genetic screen using this transgenic line identified new mutants with defective architecture or temporal development of the retinotectal tract.

Fluorescent transgenic lines are also used widely in chemical screens aimed at drug discovery and toxin identification. Importantly, transgenic zebrafish larvae can be used in high-throughput automated approaches that quickly identify changes in the shape, size, and organization of a cell or tissue type. For instance, larvae with GFP-labeled neutrophils were used to identify compounds that modulate neutrophil migration, a step that is important in regulating the inflammatory response (Robertson *et al.* 2014; Wang *et al.* 2014). A screen of Food and Drug Administration (FDA)–approved drugs in fish with labeled islet cells identified chemicals that induce insulin-producing β-cells within the developing pancreas (Rovira *et al.* 2011). Fluorescent vasculature in zebrafish (Arbiser *et al.* 2007; Tran *et al.* 2007; Lam *et al.* 2008; Crawford *et al.* 2011) was used to identify small molecules that inhibit angiogenesis. Because angiogenesis is required for many cancers, this research could lead to new anticancer drugs. The consequence of toxin exposure on heart development has been examined extensively using a number of cardiac-specific fluorescent transgenes, taking advantage of the ease with which cardiac morphology can be assessed *in vivo* (Supplemental Material, File S1) (Grimes *et al.* 2008; Wen *et al.* 2012; Incardona *et al.* 2013). In addition, inducible fluorescent transgenic reporter lines provide *in vivo* reports of signaling pathway modulation and even activation of signaling pathways by environmental toxins, thus providing a way to use zebrafish as a biosensor.

Enhancer-trap screens

A variation of transgenesis takes advantage of the transparent zebrafish embryo by inserting DNA for a transgene containing a basal promoter and the open reading frame of a fluorescent protein. These transgenes can then "report" proximity to an

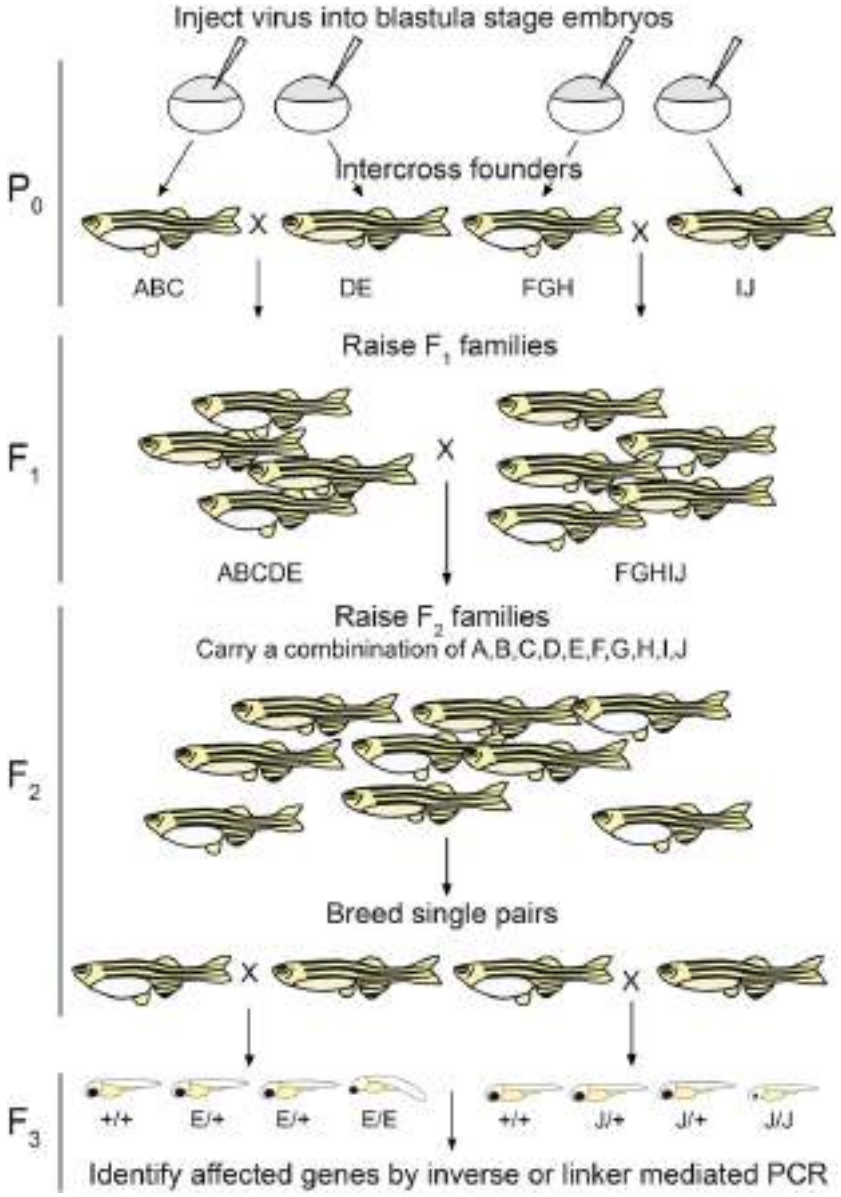

Figure 6 Breeding scheme for retroviral-mediated insertional mutagenesis. Retrovirus is injected in embryos during blastula stages, when they have between 512 and 2048 cells, and the embryos are raised to produce the P_0 generation. Each founder fish will have a mosaic germ line, with each germ-line stem cell containing a different set of retroviral insertions. Single-pair crosses between founders will produce F_1 progeny with different combinations of insertions (capital letters represent unique insertion sites). To increase the number of insertions per genome, F_1 fish are mated to each other. The results are F_2 families, each with unique insertions present in 50% of the F_2 fish. Each F_2 family member is crossed with six siblings so that each insertion is homozygosed in at least one cross. The F_3 fish are screened for morphologic defects. The gene affected by the insertion is identified by inverse or linker-mediated PCR, which amplifies genomic DNA flanking the insertion. Figure based on Amsterdam (2003) and Huang *et al.* (2012).

endogenous enhancer element and are therefore called *enhancer traps*. Insertion of the transgene containing the reporter is mediated by either retroviruses or transposons (Korzh 2007; Jao *et al.* 2008). Enhancer-trap screens have been used to identify tissue-specific gene expression patterns as well as developmentally regulated enhancers.

Several groups have combined Tol2 with the Gal4/UAS binary system for gene/enhancer-trap screens to target specific cell populations with toxins, as well as to induce truncation alleles. In zebrafish and other organisms, the GAL4/UAS system uses the Gal4 transcriptional activator from yeast to drive expression from promoters containing UAS elements (Gal4-binding sites). In one creative application, Asakawa *et al.* (2008) fused the UAS to the gene encoding tetanus toxin, which inhibits release of synaptic vesicles and disrupts neuronal activity. Tol2 transposition introduced the Gal4 gene near enhancers that drive expression in specific neuronal populations, which led to activation of the UAS:tetanus toxin transgene. Depending on the affected neuronal population expressing the tetanus toxin, different behavioral defects were observed.

The combined Tol2 Gal4/UAS system has also been used to induce truncation alleles in screens referred to as *gene traps*. Gene-trap constructs typically include a "splice acceptor" site upstream of the Gal4 sequence. When the transgene is integrated into the sense strand of a gene via Tol2-mediated transgenesis, the new splice acceptor site can generate a truncation allele, thereby diminishing gene function. Because the Gal4 gene trap is still expressed based on the local enhancers, a UAS-fluorescent reporter identifies cells where gene function is lost.

Continued modification of this system has enabled development of a system for protein-trap mutagenesis, in which a

Table 3 Examples of genetic screens using fluorescent transgenic strains

Transgene	Transgene expression	Genetic screen	References
Tg(fli1a:EGFP)^{y1}	GFP expressed in all blood vessels throughout embryogenesis	Haploid screen for putative disruptors of vascular endothelial growth factor (VEGF) signaling, which is required for blood vessel formation	Lawson and Weinstein (2002); Covassin *et al.* (2009)
Tg(Brn3c:mGFP)	GFP expressed in the membranes of retinal ganglion cells	F_2 screen to identify defects in retinotectal projections, which are made up by the axons of retinal ganglion cells	Xiao *et al.* (2005)
Tγ(α1 tubulin:GFP)	GFP expressed in developing neurons	F_2 screen to identify mutants with defects in neurogenesis and axon elaboration	Gulati-Leekha and Goldman (2006)
Tg(P20-rh/P:GFP)	GFP expression in the pineal gland and in retinal rod photoreceptors	Dominant screen to identify genes that regulate the transcription of pineal genes.	Kojima *et al.* (2008)
Tg(lck:GFP)	GFP expression in T lymphocytes	F_1 dominant screen for fish with ectopic GFP expression and ultimately for T-cell-based malignancies	Kim *et al.* (2006); Frazer *et al.* (2009)

Box 4: Sex determination in zebrafish

Understanding sex determination in zebrafish has been surprisingly challenging. Researchers working on the laboratory strains AB or TU have long struggled with the variability of sex ratios in the laboratory. Stress factors such as high density, low food availability, hypoxia, and high temperatures all lead to increased production of males, supporting a role for environmental cues (Walker-Durchanek 1980; Shang *et al.* 2006; Villamizar *et al.* 2012). This notion is further supported by the ability of zebrafish to switch sexes; fertile adult females will transform into fertile males after oocyte depletion (Dranow *et al.* 2013).

Karyotypes of domesticated zebrafish strains have not revealed gender-specific chromosomes, but a small set of sex-linked loci have been identified on several autosomes (Bradley *et al.* 2011; Liew and Orban 2014). Yet a study of wild-caught zebrafish in India defined a female-specific chromosome, indicating that females are the heterogametic sex (Sharma *et al.* 1998).

Recent work reconciles these early data and suggests that wild zebrafish possess a sex-determining gene that was lost during domestication. The Postlethwait laboratory identified sex-associated SNPs in six different wild and domesticated zebrafish strains. Surprisingly, a single locus, highly correlated with sex, was identified in all four wild strains, indicating that wild populations have a female-*WZ*/male-*ZZ* sex-determining system in which *ZZ* fish are always male, while most *WZ* fish are female (Wilson *et al.* 2014). Thus, zebrafish seem to have secondary genetic and/or environmental sex-determination mechanisms that function in sex-determination in laboratory strains and in some wild individuals (Figure B4).

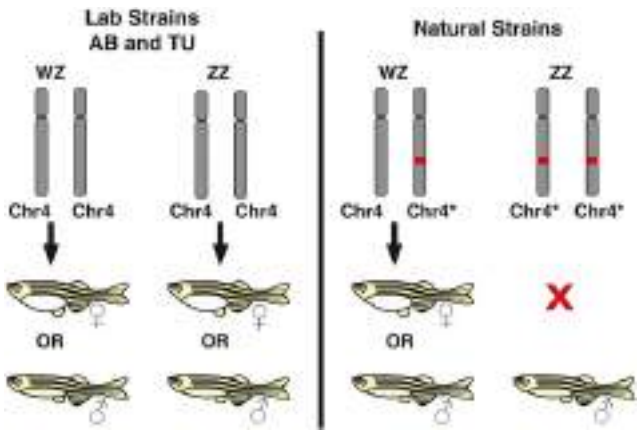

Figure B4 Genetic sex determination in zebrafish. The zebrafish laboratory strains AB and TU have lost a locus on chromosome *4* (red band) that is associated with genetic sex determination and can become either male or female. In natural strains, fish homozygous for the sex-determination locus on chromosome *4* (*Chr4**/*Chr4**) become males, while the majority of the fish heterozygous at this locus (*Chr4**/*Chr4*) become females.

Table 4 Systems used for recombinase-based genome editing

System	Source of recombinase	Pioneering citations
Cre/lox	Recombinase from P1 bacteriophage	Dong and Stuart (2004); Langenau *et al.* (2005); Thummel *et al.* (2005)
Dre/rox	Recombinase from D6 bacteriophage	Park and Leach (2013)
phiC31 integrase	Integrase from phiC31 bacteriophage	Lister (2010); Hu *et al.* (2011); Lu *et al.* (2011)
Flp/FRT	Flippase from *Saccharomyces cerevisiae*	Wong *et al.* (2011)

truncated protein is tagged with a reporter to simultaneously disrupt gene function and report the expression pattern of the gene (Clark *et al.* 2011, 2012). This system was made reversible by flanking the transgene with loxP recombination sites. When Cre recombinase was added, the transgene was excised, and the gene was converted back to its original, nonmutated state. These methods, in addition to other advanced ways to use the Tol2 and Gal4/UAS system to assay gene expression and function, have been reviewed recently (Trinh and Fraser 2013).

From Genetic Map to Whole-Genome Sequence

The zebrafish genetic map enabled identification of mutations responsible for mutant phenotypes. A genetic map provides molecular landmarks in the form of polymorphisms, or markers, distributed along each chromosome. The first genetic map for zebrafish was created with random amplified polymorphic DNA (RAPD) markers. DNA primers were used to amplify random segments of zebrafish genomic DNA by PCR. Each primer pair yielded 6–12 amplicons,

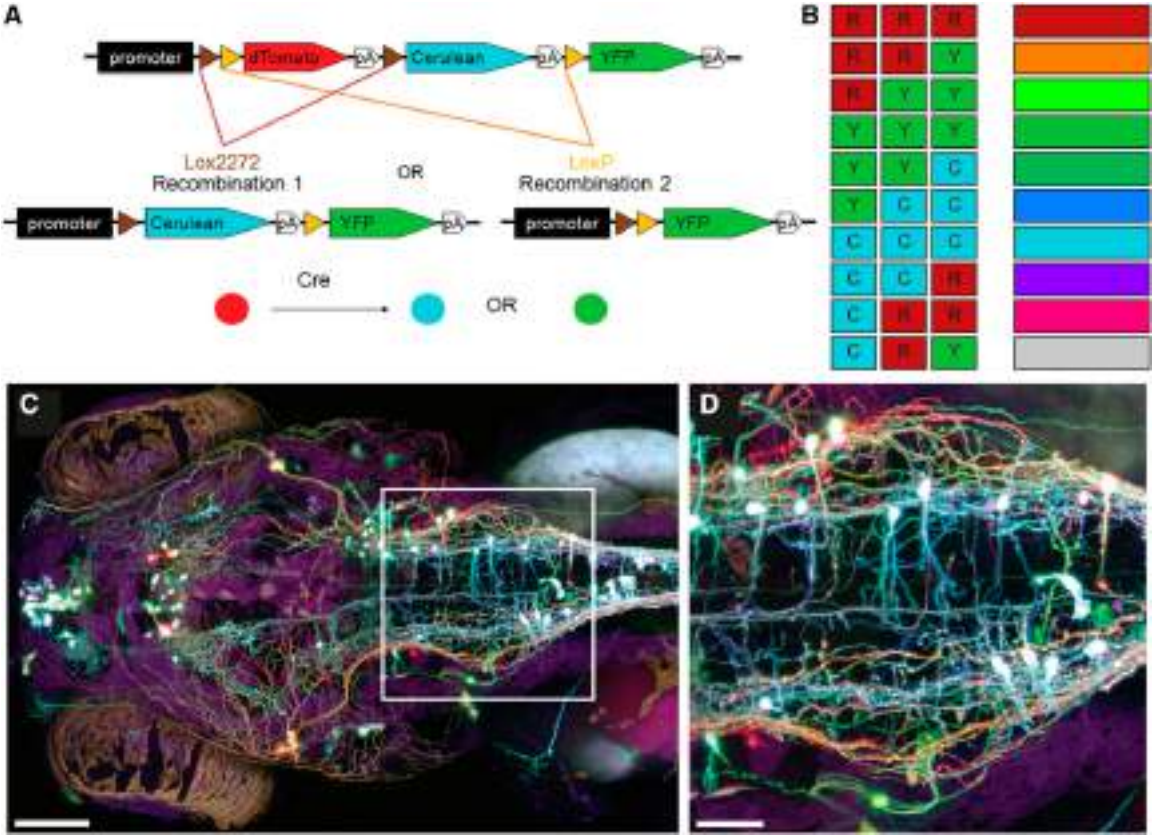

Figure 7 Brainbow zebrafish. Neurons in a Brainbow zebrafish express different ratios of red, yellow, and blue fluorescent proteins, making it possible to follow the axonal and dendritic projections of neighboring cells and to begin building the zebrafish connectome. (A) The Brainbow transgene is composed of the neuron-specific islet1 promoter followed by the coding regions for three fluorescent proteins, dTomato, which emits red fluorescence; Yellow Fluorescent Protein (YFP), which emits yellow-green fluorescence; and Cerulean, which emits blue fluorescence. The coding regions are flanked by two pairs of lox sites (Lox2272, brown; and LoxP, yellow). On expression of Cre recombinase, three events can occur. The cassette can remain unrecombined, resulting in red fluorescence. The lox2272 sites (brown) can recombine, resulting in blue fluorescence, or the loxP sites (yellow) can recombine, resulting in green fluorescence. (B) Because transgenes typically insert as large tandem arrays, each cell will express a slightly different combination of red, green, and blue, resulting in a slightly different color to each cell. This example illustrates what colors could occur if there were three copies of the Brainbow transgene in the array. (C) Dorsal view of a 120-hpf larval islet1-Brainbow zebrafish showing neurons with many different colors of fluorescence. (D) High-magnification image of the boxed region in C. Scale bars, 50 μm (C) and 20 μm (D). Panels A and B adapted from Lichtman *et al.* (2008) and Panels C and D reprinted with permission from Pan *et al.* (2011).

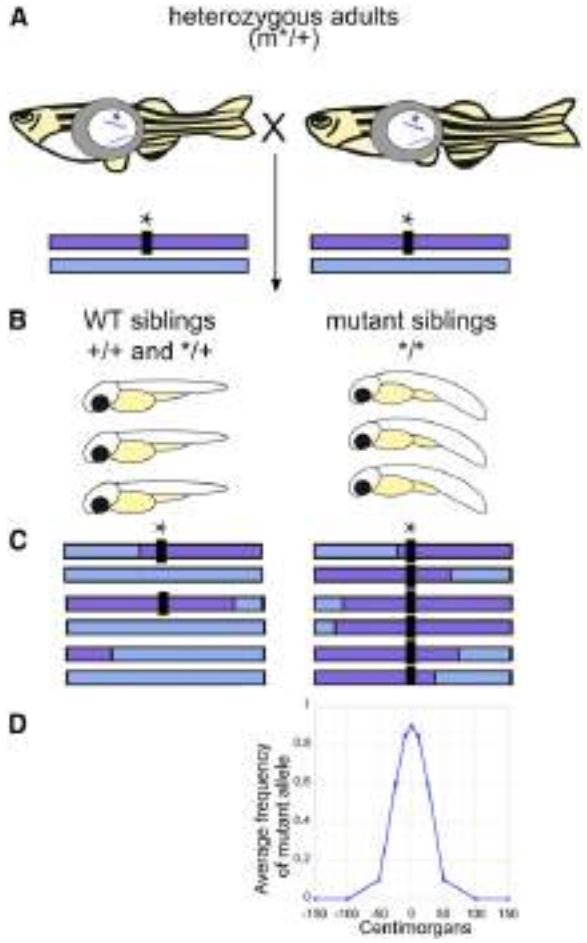

Figure 8 Using next-generation RNA sequencing to identify genetic lesions in zebrafish mutants. (A) Adult fish heterozygous for the mutation (*) are crossed. (B) Mutant progeny and their WT siblings are separately pooled shortly after the appearance of the phenotype. (C) RNA is extracted using standard methods, and three replicates of each mutant and WT pool are sequenced using RNA-seq. The sequences are aligned with the zebrafish genome, and SNPs are identified within the sequenced regions. (D) For the mutant mRNAs, the frequency of homozygosity for SNPs at or near the mutation will approach 1, or 100%, while the level of homozygosity will gradually increase as the SNPs map further from the mutation. Thus, the region containing the causative mutation will correspond to the peak in homozygosity. The peak contained between 1 and 25 candidate genes in initial trials of this method (Hill *et al.* 2013; Miller *et al.* 2013).

which varied in size. To generate the zebrafish genomic map, fish from two independently isolated strains that were partially inbred, AB and Darjeeling, were crossed. Each strain yielded a different pattern of amplicons from a specific RAPD marker and thus could be used for genetic mapping. To simplify the approach, individual haploid progeny from the AB/Darjeeling cross were evaluated for strain-specific polymorphisms at 401 RAPD markers. Markers were assigned to 29 different "linkage groups" based on their patterns of cosegregation (Johnson *et al.* 1994; Postlethwait *et al.* 1994).

Refining the genetic map

The 29 linkage groups were assigned to the 25 zebrafish chromosomes using markers linked to the centromeres of specific chromosomes (Johnson *et al.* 1996). This was greatly facilitated by the use of half-tetrad embryos in which embryos contain chromosomes resulting from the first meiotic division. Markers closer to the centromere are homozygous at a higher rate than markers further from the centromere. Thus, frequency of homozygosity is used to calculate the distance between centromere and marker. Centromere-linkage analysis had the added advantage of increasing the number of markers on the genetic map.

Microsatellite markers, readily detectable repeats of di-, tri-, or tetranucleotide sequences found throughout most genomes, were used to refine the genetic map even further. Mapping of microsatellites greatly increased the level of resolution of the zebrafish genetic map, to 1.2 cM, sufficient to allow chromosomal walking to identify mutations of interest. The Zebrafish Information Network (ZFIN) (Westerfield *et al.* 1999) is the essential repository for integration of mapping data, gene expression and protein information, description of mutant and transgenic phenotypes, and the genomic positions and physical properties of mutant alleles. The genetic map was critical for success of the Zebrafish Genome Sequencing Project (Howe *et al.* 2013).

Synteny and the zebrafish genome

In addition to aiding mutation mapping, comparison of syntenic regions among different species provides insights into vertebrate evolution. For instance, the *Hox* genes, involved in setting up the body plan, are found in multigene arrays. Zebrafish genomes, similar to those of other teleosts, have seven *hox* gene clusters compared to the four clusters present in the mouse genome. Phylogenetic analyses revealed that the additional teleost *hox* clusters represent duplications of single mammalian *hox* clusters (Amores *et al.* 1998). This two-to-one ratio of zebrafish genes to mouse genes was found in other syntenic regions (Postlethwait *et al.* 1998; Howe *et al.* 2013). These and other studies provided strong evidence for whole-genome duplication in the ray-finned fishes after the divergence of tetrapods (Figure 1) (Taylor *et al.* 2003; Catchen *et al.* 2011; Braasch *et al.* 2014). Genome duplication offers opportunities for duplicated genes to adopt new properties. A duplicate copy of a gene may (1) become a nonfunctional pseudogene, (2) gain a new function (neofunctionalize), or (3) take on part of the original gene's function (subfunctionalize) (Force *et al.* 1999). Following subfunctionalization, overlapping functions of the duplicate genes provide redundancy in zebrafish that is not present in mammals.

Targeted Gene Inactivation

A limitation of the zebrafish model for many years was the lack of a method to disrupt specific genes. Happily, many

reverse genetic techniques now allow researchers to uncover the functions of genes of interest.

Morpholinos

In 2000, single-stranded oligonucleotides, called *morpholinos*, were first injected into one- to two-cell-stage zebrafish embryos and found to knock down protein function. Morpholinos are designed to base pair either to the translation start site, thereby preventing translation, or to a splice site of a target RNA, thereby preventing processing of pre-mRNA (Nasevicius and Ekker 2000; Draper *et al.* 2001). Morpholinos provide for efficient loss-of-function experiments without multigenerational genetic screens. Additionally, injection of morpholinos into mutant lines or simultaneous injection of multiple morpholinos enables studies of genetic interactions. However, morpholinos do not always completely prevent protein production; thus their use is a "knockdown," not a "knockout," approach. Morpholinos also become diluted as cells divide and therefore are typically only effective for the first few days of development. Off-target effects due to an upregulation of the p53 apoptotic pathway may occur and confound the phenotype (Bill *et al.* 2009). Finally, a subset of morpholino-induced phenotypes is not found in the corresponding mutants, emphasizing the need for verification with rigorous control experiments or complementary methods (Kok *et al.* 2015; Stainier *et al.* 2015).

Targeting induced local lesions in genomes

Targeting induced local lesions in genomes (TILLING), initially developed for use in *Arabidopsis*, can be used to generate heritable gene deletions in zebrafish. TILLING has been used in large-scale screens designed to search for mutations in many genes simultaneously. A panel of heavily ENU mutagenized genomes is maintained as live fish or frozen sperm with the corresponding DNA screened by PCR. If a mutation has occurred, denaturation and renaturation of the PCR products cause heteroduplexes to form, which are recognized by sequencing or digestion with the endonuclease *CEL1*. F_2 fish are generated from the sperm or living fish, and carriers of the desired mutation are subsequently identified via sequencing or detection of a restriction-length polymorphism or allele-specific primers called *dCAPS* (Neff *et al.* 2002). The F_2 fish are then intercrossed to confirm that the resulting phenotype segregates with the identified mutation and are outcrossed to eliminate any background mutations.

Genome editing with targeted nucleases

A number of strategies enable researchers to generate mutations in specific loci with relative ease. A nuclease is targeted to a specific site in the genome and makes a double- or single-stranded break in the chromosome. When the break is repaired, changes in the sequence often occur. Zinc-finger nucleases (ZFNs) are engineered proteins that contain the backbone of the zinc-finger transcription factor DNA binding domain and the bacterial endonuclease Fok1 DNA cleavage domain. ZFNs act as dimers, and their target can be specified via use of a code that matches protein sequence with the DNA sequence it binds. The ZFN dimer binds to the targeted sequence, and the endonuclease makes a double-strand break between the binding sites (Bibikova *et al.* 2003). The double-strand break is then repaired by nonhomologous end joining, usually resulting in indels (Urnov *et al.* 2010; Huang *et al.* 2012). Alterations in the zinc-finger DNA binding domain are made to target the desired target sequence in the genome.

A related approach uses transcription activator-like effector nucleases (TALENS) (Hisano *et al.* 2013). The DNA binding domain for TALENS, derived from the plant pathogen *Xanthomonas* TALE proteins, is also connected with the catalytic domain of Fok1 endonuclease. DNA recognition is mediated by TALE repeats that have more consistent rules of sequence recognition than do ZFNs, making it easier and more efficient to target a specific sequence.

The CRISPR/Cas9 system (Hwang *et al.* 2013), based on a defense system that evolved in bacteria and archaea to target and degrade foreign viral DNA (Levin *et al.* 2013), is comprised of two elements: the Cas9 protein and a *trans*-activating CRISPR RNA (crRNA). The crRNA and Cas9 protein form a complex, with the crRNA targeting a specific DNA sequence and the Cas9 protein catalyzing a double-strand break at the target site (Auer *et al.* 2014). The ease and relative low cost of CRISPR tools have made editing of the zebrafish genome accessible to many laboratories.

Future Directions

The connectome

The biological sciences have become full of "-omes" (*e.g.*, transcriptomes, proteomes, metabalomes, etc.), a suffix that reflects the growing ability of geneticists and cell biologists to take a systems approach to biology. One of the most challenging of these is the *connectome*, a complete map of the nervous system, including all the neurons and their connections. Zebrafish larvae are likely to be one of the first vertebrate animals with an extensively mapped connectome. Because larvae are relatively small and transparent, the entire nervous system is accessible using confocal microscopy or via serial reconstructions by three-dimensional electron microscopy (3D-EM). Sophisticated transgenic techniques allow labeling of neuronal subpopulations and tracing of their connections (Figure 7). However, studies in zebrafish can go beyond a wiring diagram to a dissection of the *in vivo* functions of connections. Using multiphoton calcium imaging in fish carrying calcium sensors, it is possible to record neuronal activity simultaneously throughout the brain (Friedrich *et al.* 2013; Renninger and Orger 2013; Fosque *et al.* 2015). Neural networks involved in visual activity, hunting behavior, and navigation have already been defined (Ahrens *et al.* 2013; Muto *et al.* 2013; Bianco and Engert 2015; Romano *et al.* 2015). *In vivo* experiments combined with mathematical modeling are beginning to explain how neural circuits function

(Stobb *et al.* 2012; De Vico Fallani *et al.* 2014; Freeman *et al.* 2014; Portugues *et al.* 2015). Thus, the zebrafish larva is emerging as a valuable model to link genes, neuronal networks, and behavior.

Zebrafish models for human disease

Zebrafish are poised to provide advances in our understanding of the genetics and physiology of human disease (Patton *et al.* 2014). Because cellular changes can be followed in live animals, zebrafish are particularly useful for identifying the etiology and pathology of diseases that affect multiple tissues and organ systems, such as cancer, diabetes, atherosclerosis, and obesity. Orthologs for 82% of known human disease genes have been identified (Howe *et al.* 2013). Several projects are underway to generate mutations in every zebrafish protein-encoding gene (Figure 8), and genes in specific tissues can be activated or inactivated at specific times (Ni *et al.* 2012). The combination of mutant strains and inducible, reversible transgenes enables genetic approaches that closely mimic levels of gene expression characteristic of human disease. Zebrafish are already having an impact on diseases such as melanoma and other cancers, tuberculosis, autism, and cardiovascular disease (Phillips and Westerfield 2014).

Acknowledgments

We thank Elizabeth De Stasio and Marnie Halpern for guidance during the writing of this manuscript; Sooji (Katie) Jo, Alanna Leung, and Corinna Singleman for contributing images and movies; and Alicia Coughlin for proofreading. In addition, we thank Monte Westerfield for providing the photograph of George Streisinger.

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Communicating editor: E. A. De Stasio

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.190843/-/DC1

Learning to Fish with Genetics: A Primer on the Vertebrate Model *Danio rerio*

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DOI: 10.1534/genetics.116.190843

File S1. Movie of circulating blood in the trunk of a ~2 day old embryo. Individual blood cells can be seen flowing across the notochord and along its length. The movement of the blood cells occurs in waves, reflecting contraction of the heart. Movie recorded at 240 frames per second. Courtesy of Alanna Leung. (.mov, 20,965 KB)

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