THE ART AND DESIGN OF GENETIC SCREENS: ZEBRAFISH

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Inventive genetic screens in zebrafish are revealing new genetic pathways that control vertebrate development, disease and behaviour. By exploiting the versatility of zebrafish, biological processes that had been previously obscured can be visualized and many of the responsible genes can be isolated. Coupled with gene knockdown and overexpression technologies, and small-molecule-induced phenotypes, genetic screens in zebrafish provide a powerful system by which to dissect vertebrate gene function and gene networks.

GYNOGENESIS
Development of an organism derived from the genetic material of the female gamete.

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Twenty years ago, George Streisinger's pioneering research revealed the potential of the zebrafish as a vertebrate organism that was suitable for forward genetic screening[1]. A relatively small fish (3–4 cm long as an adult), zebrafish can be easily managed in large numbers in the laboratory environment. The ability to combine embryological and genetic methodology has established the zebrafish as a powerful research tool. External development of transparent embryos allows fundamental vertebrate developmental processes — from gastrulation to organogenesis — to be visualized and studied; in addition, the heart beats and blood circulation of the embryo are readily observed. Touch, sight and behavioural responses can also be monitored in live embryos under the dissecting microscope. Several features, such as a short generation time of 3–4 months, mean that zebrafish are particularly suitable for genetic studies. In addition, mutations can be induced with high frequency in zebrafish, and recessive mutations can be recovered within two generations[2,3]. Large progeny sizes (females lay about 100–200 eggs) facilitate large-scale genetic screening and mutation analysis in a Mendelian fashion.

Here, we review the methods of genetic screening in zebrafish, and include some examples of the resulting mutants and genes discovered from diploid, haploid and GYNOGENETIC diploid screens. We follow with a sampling of the recent, resourceful genetic screening techniques now carried out in zebrafish to show the potential and versatility of the zebrafish genetic system.

First large-scale vertebrate genetic screens

Systematic genome-wide screens for mutations in worms, flies and plants have successfully identified many genes that define embryological pathways. Smaller collections of mammalian mutants provide valuable insights into developmental processes. However, identifying large numbers of mutations in the mammalian system is problematic because of intrauterine development and expensive supporting laboratory facilities. The remarkable characteristics of the zebrafish, along with the initial success of the first zebrafish genetic screens[4], inspired two groups of scientists in Boston[5] and Tübingen[6] to undertake the first large-scale genetic screens in a vertebrate organism. The Boston and Tübingen screens identified mutant embryonic phenotypes in the F_3 generation (FIG. 1). Some of the ~2,000 mutated developmental genes that were identified in these two screens have been cloned, which assists in the dissection of the gene networks that control early development. For example, the genes that are mutated in the endoderm mutants *casanova* (*cas*), *bonnie and clyde* (*bon*), and *faust* (*fau*) can be assembled into a genetic pathway, and have been shown to encode transcription factors that are necessary for endoderm formation[7–12]. Embryo transparency, as well as conspicuous heart, blood and blood vessels in the zebrafish have also made it possible to identify cardiovascular system mutants in a manner that is unprecedented in other animal systems. As an example, the gene *jekyll* (*jek*) is necessary for formation of the

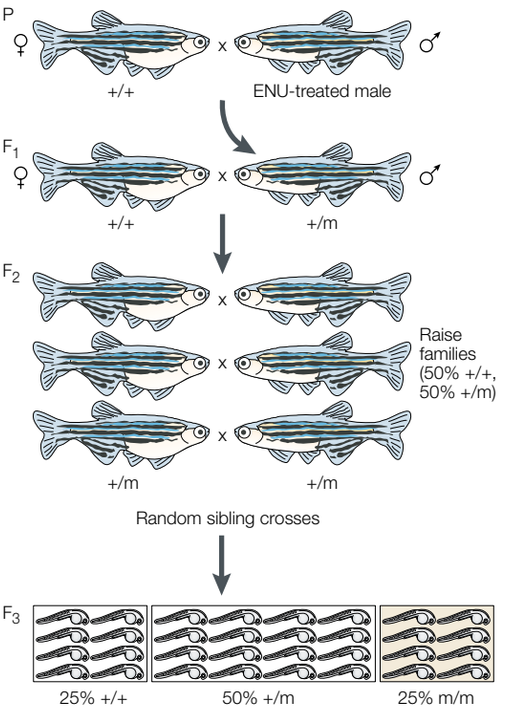


Figure 1 | **Outline of large-scale F_2 genetic screens.** In F_2 screens, a mutagen, such as ethylnitrosourea (ENU), is used to generate hundreds of point mutations in the male pre-meiotic germ cells (spermatogonia). ENU-treated males are crossed to wild-type females to produce the F_1 heterozygous progeny. F_1 fish are then crossed to siblings to create F_2 families, half of which are genotypically heterozygous for a specific mutation (m), whereas the other half are wild type. F_2 siblings are crossed, and the resulting F_3 progeny are 25% wild type (+/+), 50% heterozygous (+/m) and 25% homozygous (m/m) for a recessive mutation. Together, the Boston and Tübingen screens, starting from about 300 ENU founder males, involved raising more than 5,000 F_2 families, analysing more than 6,000 mutagenized genomes and selecting more than 2,000 new developmental mutants for characterization.

cardiac valve, which prevents retrograde blood flow between the atrium and ventricle[13]. The *jek* gene encodes the enzyme UDP(uridine 5′-diphosphate)-glucose dehydrogenase, which is required for the production of (among other products) glycosaminoglycans[14]. Valve formation therefore seems to require Jekyll function in a new glycosaminoglycan-dependent signalling pathway.

Several of the mutant phenotypes identified in the screens resemble human genetic disease conditions, and some of the genes cloned have homologues in other animal systems. Anaemic mutants (scored by their low blood-cell counts) can be models for human blood diseases[15–17]. Positional cloning of the *weissherbst* (*weh*) mutant identified a mutation in a novel iron exporter, Ferroportin 1 (REF. 18; FIG. 2b). Blood mutants that are analogous to erythropoietic porphyria syndromes have also been found, such as the *dracula* (*drc*) and *yquem* (*yqe*) mutants, which have a rapid, light-dependent lysis of red blood cells[15,16]. The *drc* gene encodes Ferrochelatase, which, when disrupted in humans, leads to erythropoietic protoporphyria owing to the accumulation of light-sensitive, toxic intermediates of the haem biosynthetic pathway[19]. The *yqe* gene encodes uroporphyrinogen decarboxylase (UROD), and as in the fish, homozygous mutations in this enzyme lead to hepatoerythropoietic porphyria in humans[20]. The wealth of knowledge gained from just a sampling of the phenotypic groups of mutants identified in the Boston and Tübingen screens illustrates the power and potential of zebrafish genetics, and validates the use of zebrafish as a model for vertebrate biological processes.

Haploid and homozygous diploid screens. Phenotypic detection of a recessive allele within an F_3 generation requires many crosses in F_2 families (FIG. 1). Streisinger *et al.* attempted to eliminate this 'cumbersome' step by creating haploid and PARTHENOGENETIC diploid animals that were suitable for use in genetic screens[1] (BOX 1). A genetically convenient feature of zebrafish is that they can live up to 3 days post-fertilization as haploid organisms[1]. Recessive alleles in F_1 fish are therefore exposed within a generation, streamlining the screen for both size and time (BOX 1, figure panel a). Haploids have the same body plan as diploids, but are shorter, contain more and smaller cells than diploids, and can have other morphological defects that complicate screening (BOX 1, figure panel b). Nevertheless, molecular markers that are essentially normal in wild-type, haploid embryos can be used successfully in haploid screens to identify genes that are crucial for the early stages of zebrafish embryogenesis. For example, a haploid screen successfully uncovered genes that were involved in brain patterning[21]. Haploid embryos from F_1 females were screened simultaneously for the expression pattern of six genes in the brain by RNA *in situ* hybridization[21]. Three *valentino* (*val*) alleles had atypical *krox20* expression, as well as abnormally smooth and unsegmented hindbrains. *val* is required for the subdivision and expansion of the hindbrain region that normally gives rise to RHOMBOMERES (r) 5 and 6, and is a homologue of the mouse segmentation-factor-encoding gene *Mafb* (kreisler)[22]. The *lazarus* (*lzr*) mutant is defective for *krox20* expression in r3 and r5, but then recovers expression in r5 at 15 hours post-fertilization (hpf)[23]. *lzr* encodes a novel Pbx factor, which together with the Hox segmentation factors orchestrates the vertebrate body plan[23].

Manipulation of the normal egg cell cycle, with heat shock (HS) or early hydrostatic pressure (EP), contributes to the creation of gynogenetic diploid embryos[24] (BOX 1, figure panel c). EP has been used successfully in a screen for primary motor-neuron mutants, which can be visualized with anti-motor-neuron antibodies at 24 hpf (REFS 24–26). Primary motor neurons are the earliest developing spinal motor neurons, and have distinct axon projections and pathways. The mutant *stumpy* (*sty*) extends the Caudal Primary motor neuron (CaP) along common but not CaP-neuron-specific pathways[24,25]. EP-treated clutches from F_1 females that are heterozygous for *sty* show Mendelian inheritance (50% wild type, 50% *sty*) and, accordingly, the *sty* mutation maps near to the

PARTHENOGENESIS
Development of an organism derived from an unfertilized gamete.

RHOMBOMERE
Each of seven neuroepithelial segments found in the embryonic hindbrain that adopt distinct molecular and cellular properties, restrictions in cell mixing and ordered domains of gene expression.

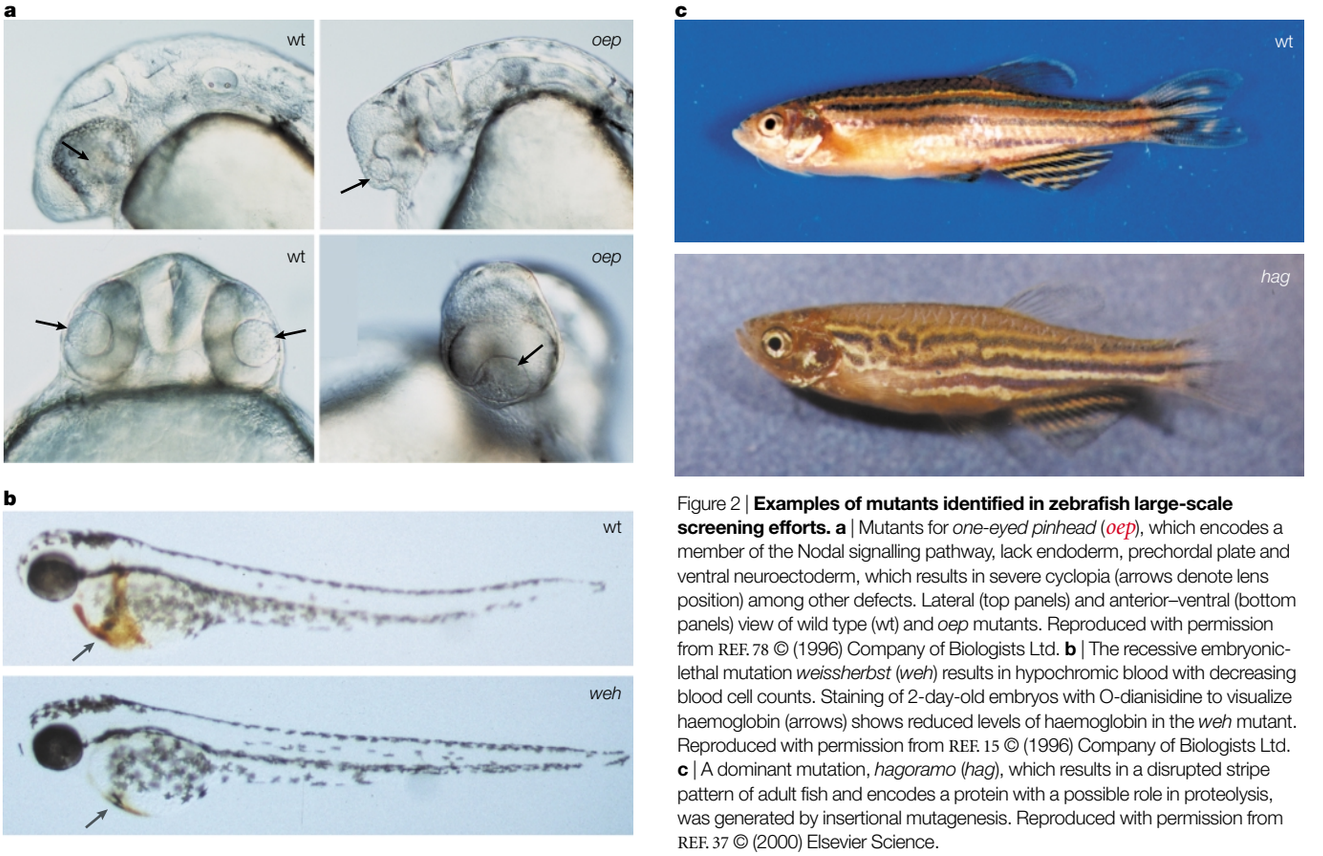


Figure 2 | **Examples of mutants identified in zebrafish large-scale screening efforts. a** | Mutants for *one-eyed pinhead* (*oep*), which encodes a member of the Nodal signalling pathway, lack endoderm, prechordal plate and ventral neuroectoderm, which results in severe cyclopia (arrows denote lens position) among other defects. Lateral (top panels) and anterior–ventral (bottom panels) view of wild type (wt) and *oep* mutants. Reproduced with permission from REF. 78 © (1996) Company of Biologists Ltd. **b** | The recessive embryonic-lethal mutation *weissherbst* (*weh*) results in hypochromic blood with decreasing blood cell counts. Staining of 2-day-old embryos with O-dianisidine to visualize haemoglobin (arrows) shows reduced levels of haemoglobin in the *weh* mutant. Reproduced with permission from REF. 15 © (1996) Company of Biologists Ltd. **c** | A dominant mutation, *hagoramo* (*hag*), which results in a disrupted stripe pattern of adult fish and encodes a protein with a possible role in proteolysis, was generated by insertional mutagenesis. Reproduced with permission from REF. 37 © (2000) Elsevier Science.

centromere[25] (BOX 1). A second motor-neuron mutant was identified that also has defects in somite formation; its phenotype was caused by a new allele of the fused-somite *deadly seven* (*des*) mutation[26]. Other somite mutants also have neuronal hyperplasia, but nervous system abnormalities had not previously been described for *des*[27]. By altering the search criteria from somite formation to motor-neuron projections, this new allele of *des* has coupled anterior–posterior (AP) patterning of the somites and the myotome with nervous system development[26]. So, although mutant representation varies in an EP-treated clutch, EP screens provide an efficient way to uncover new mutant phenotypes in specific genetic pathways.

Mosaic screens. Haploid and diploid screens can be altered to increase the frequency of mutation events of interest[28–30]. Standard ethylnitrosourea (ENU) mutagenesis involves mutagenizing pre-meiotic germ cells in adult males, then breeding for several weeks to fix the mutation through several rounds of DNA replication before the development of mature sperm cells[2,3]. As ENU alkylates the bases on only one DNA strand, mutations in the ENU-treated post-meiotic germ cells become fixed only during cell divisions after fertilization[2,3]. The resulting F_1 generation is genetically mosaic for a specific mutation. To increase the efficiency of identifying specific mutations, breeding the ENU-mutagenized males for a brief period after mutagenesis will generate F_1 generations that are mosaic for an estimated tenfold greater mutational load[28]. In theory, mosaic fish are able to carry this mutational burden because wild-type cells in all tissues compensate for the heterozygous mutant cells[28]. By screening F_2 haploids that are derived from mosaic F_1 mothers, numerous heart mutations have been successfully identified, including many new mutations that affect cardiac induction and AP patterning[30]. This screen shows the value of mosaic screening; however, several mutations in haploid embryos and the unpredictable phenotypic representation within a clutch can complicate screening and allele recovery. Nonetheless, mosaic screening has proven to be a robust and rapid method to identify cardiac induction and AP-patterning mutations, a phenotypic class under-represented in the Boston and Tübingen screens[5,6,30].

Mutagen selection. The mutagen selected to generate the parental fish affects the number and types of mutation that are passed on to subsequent generations[31]. ENU induces mainly point mutations, and was effectively used in the Boston and Tübingen screens[2,3,5,6]. ENU efficiently generates mutations in most genes, although the mutation frequency can vary widely between loci. Ethyl methanesulphonate (EMS), although a potent mutagen in *Drosophila*, is much less potent in zebrafish[2,3]. Ionizing radiation, such as γ-rays and X-rays, also induce genetic alterations, which can

CHIASMA INTERFERENCE
The inhibition of crossover events during meiosis such that there is generally only one crossover event per chromosome arm.

vary from point mutations through large genomic deletions to translocation events[32,33]. Large deletions and translocations can alter more than one gene and generate several phenotypes in a clutch of embryos. The chemical mutagen trimethylpsoralen includes small deletions (100 bp to 15 kb), and might circumvent the polygenic burden of radiation-induced deletions[34]. Positional cloning of mutations generated with the above mutagens has been highly successful, and completion of the zebrafish genome in 2002 will greatly facilitate positional cloning efforts[35]. Nonetheless, it remains labour intensive and expensive.

Box 1 | Haploid and homozygous diploid screens

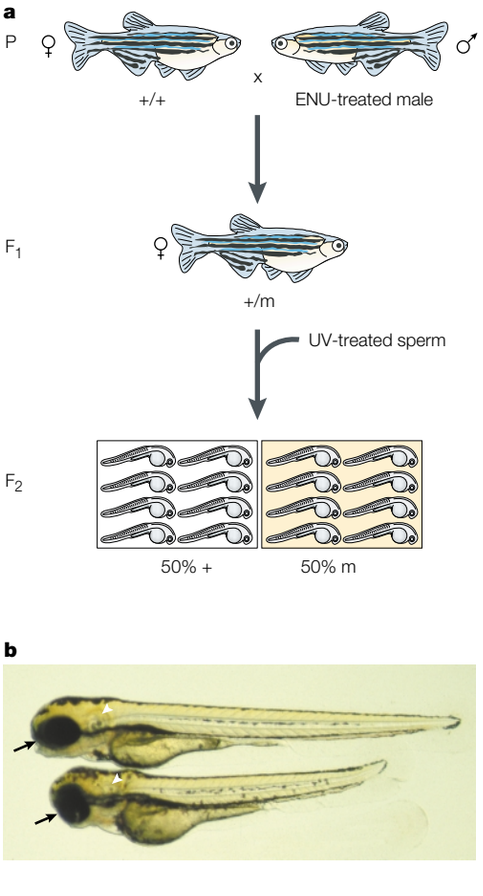
To avoid the cumbersome step of screening thousands of progeny for recessive mutations in conventional F_2 screens (FIG. 1), methods have been devised to uncover recessive alleles in a single generation by exploiting the ability to create haploid or homozygous diploid embryos.

Haploid screens

Recessive mutations can be revealed more quickly in zebrafish by taking advantage of their ability to survive for several days as haploid organisms[77]. In a haploid screen (a), female F_1 fish (derived from the cross between a wild-type female and an ethylnitrosourea (ENU)-mutagenized male) are squeezed gently to release their eggs, which are then fertilized with ultraviolet (UV)-treated sperm to generate haploid embryos. UV treatment destroys the parental DNA, without affecting its ability to activate the egg. A haploid clutch derived from a heterozygous female will contain 50% mutant and 50% wild-type embryos. Panel b shows 3-day-old diploid (top) and haploid (bottom) zebrafish embryos. Note that diploid and haploid embryos share a similar overall morphology, but haploid embryos are visibly shorter with abnormal eye (arrow) and otic vesicle (arrowhead) development.

Homozygous diploid screens: methods to induce gynogenesis

Eggs extracted from a female have completed meiosis I (the separation of homologous chromosomes) during ovulation, and initiate meiosis II (the separation of sister chromatids) on fertilization[24]. Early pressure (EP) applied to embryos during the first few minutes post-fertilization breaks down the meiotic spindle, and the egg maintains both sister chromatids (c, left). Subsequently, eggs undergo their first mitosis as diploids, with two sets of maternal chromosomes. By contrast, heat-shock treatment (HS; c, right) inhibits the first mitotic division, and eggs activated with UV-treated sperm enter the first mitotic division as haploids, abort mitosis and directly enter the second mitotic division as diploids. In meiosis, recombination and CHIASMA INTERFERENCE occur between homologous chromosomes when aligned as tetrads, so that there is, on average, a single crossover event per chromosome arm. Therefore, embryos that are derived from EP treatment will be homozygous for loci that are proximal to the crossover event that occurred at meiosis I (allele 'a' in the figure) and heterozygous for loci that are distal to it. Similar to haploid clutches, a gynogenetic diploid clutch that is derived from a heterozygous female and generated by HS will contain 50% mutant and 50% wild-type embryos. As embryos generated by HS are homozygous at all loci, they would be preferable to embryos generated by EP for use in genetic screens, except for their reportedly poor viability (10–20%).



SWIM BLADDER
An internal fish organ filled with gases, which are regulated to allow the fish to rise and fall. In some teleosts, the swim bladder can have a role in respiration and sound production and reception.

SOMITE
Paired cubical paraxial mesodermal segment, which is often used as a staging index during embryogenesis.

INVERSE PCR
A technique for amplifying DNA by PCR that uses primers that initiate replication in opposite directions to each other, as compared with standard PCR, which uses primers that initiate replication towards one another.

As an alternative to chemical- or radiation-induced mutations, insertional mutagenesis with a retrovirus is mutagenic and allows for rapid cloning of the gene[36–38]. Large-scale screening for recessive developmental mutations with retroviral insertions is now being done by Nancy Hopkins and colleagues[36] (in Cambridge, Massachusetts, USA; FIG. 3). Similar to the Boston and Tübingen screens, the Cambridge screen has identified genetic mutations with both specific and non-specific phenotypes[36]. Non-specific defects generally cause death and are predicted to be mutations in genes that are necessary for cell survival or growth. Some examples of highly specific mutations include those that affect the pigment, fin, brain, circulation, body size and SWIM BLADDER formation. Among these, the *smoothened* (*smo*) mutant has phenotypic characteristics that are similar to Hedgehog (Hh) pathway mutants, including U-shaped SOMITES, mild cyclopia and lack of pectoral fin outgrowth[38]. Analysis of *smo* shows that, as in *Drosophila*, it is required for Hh signalling, which provides new insight into the conserved and divergent roles of *smo* in vertebrates[38]. Two dominant alleles of *hagoramo* (Japanese for a dress for a goddess, *hag*; FIG. 2c) were also identified by their disrupted body-stripe pattern in the adult F_1 generation[36,37]. The *hag* gene encodes an F-box/WD40 repeat protein, which are often involved in regulating ubiquitin-mediated proteolysis[37]. Although insertional mutagenesis is only one-ninth as effective at generating mutations as ENU, disrupted genes are rapidly and easily cloned by INVERSE PCR methods. Overall, the relatively facile cloning method of insertional mutagenesis compensates for its labour intensivity, making this a powerful screening instrument in zebrafish.

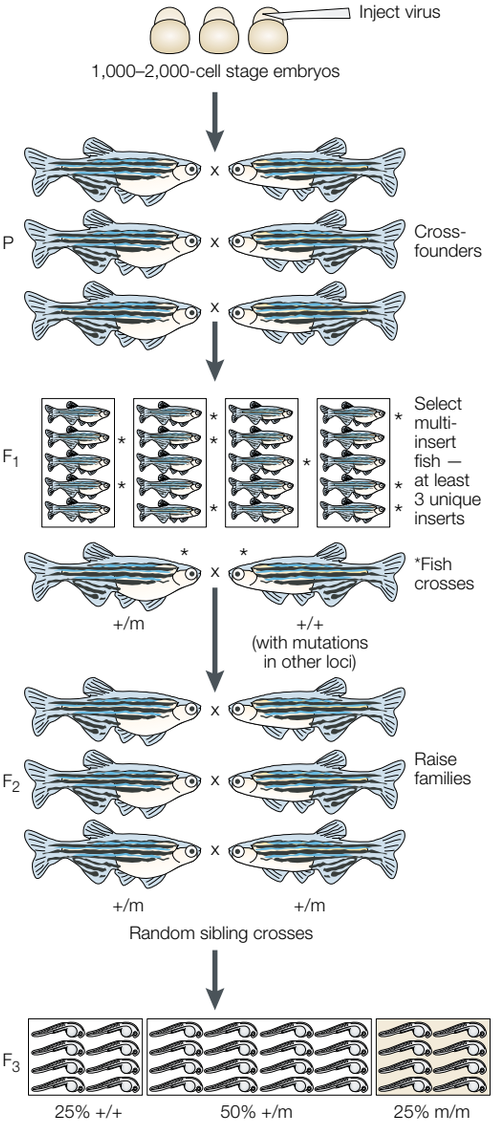


Figure 3 | **Outline of insertional mutagenesis screen.** The goal of the Cambridge screen is to identify ~1,000 genes involved in embryogenesis[36]. The protocol for large-scale insertional mutagenesis screening involves injecting virus into 250,000 embryos at the 1,000–2,000-cell stage[36]. The virus infects many cells, several times, among them the primordial germ cells. Approximately 36,000 embryos are raised (the founder fish; P), mated and several insertions transmitted to the F_1 generations. More than 10,000 F_1 families are raised, and multi-insert F_1 fish are selected by carrying out real-time PCR and Southern blot analysis of DNA isolated from tail biopsies. Multi-insert F_1 fish are crossed to each other and 10,000 F_2 families are raised. More than six sibling matings for each F_2 family are carried out to identify ~1,000 homozygous mutations in the F_3 generation. m, mutagenized chromosome.

Inventive screening techniques

The optical clarity of the zebrafish reveals developmental processes that are obscured in other organisms, such as *Drosophila*[5,6]. Molecular markers that highlight processes of interest, including RNA or protein staining, fluorescence and precise measurements of organ or tissue function, have unveiled zebrafish mutations that are normally not visible to the eye. Creative, behaviour-based assays have also been designed in zebrafish to begin dissecting the complex workings of animal behaviour.

Fluorescent reporters. Similar to mammals, zebrafish larvae process lipids throughout the intestine and hepatobiliary system, and are sensitive to drugs that block cholesterol synthesis. Farber *et al.* exploited this homology to design a screen that would identify mutants that are unable to correctly process phospholipids and cholesterol[39]. As a reporter for lipid processing, an engineered, quenched fluorescent moiety was placed at a phospholipase A_2 (PLA_2) cleavage site that is normally targeted by a phospholipid (PED6), so that cleavage resulted in unquenching and visual fluorescence. Normal PLA_2 cleavage of the quenched lipid (and hence lipid metabolism) resulted in immediate fluorescence of the gall bladder and the intestinal lumen in living larvae. Direct testing of pancreatic and intestinal mutants — *slim jim* (*slj*) and *piebald* (*pie*) — had reduced gall-bladder fluorescence, which shows that the differences in fluorescence could be detected in mutants that are abnormal for digestive organ morphology[39].

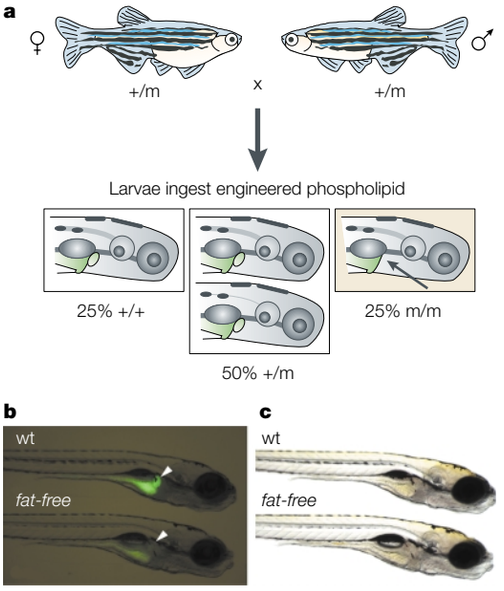


Figure 4 | **Fluorescent reporter screens. a** | Fluorescent reporters can be used to screen for mutations in specific enzymatic processes in live embryos[39]. For example, larvae derived from crossing two fish that are heterozygous for a specific digestive tract mutation (for example, *fat free*, see part **b**) ingest phospholipids that are engineered to fluoresce during normal lipid processing. One-quarter of the larvae are homozygous for a recessive, digestive-tract mutation and lack gall-bladder fluorescence (arrow), which indicates a defect in normal lipid processing. **b,c** | The larval digestive mutant *fat free* is defective for lipid processing. Wild type (wt) and *fat free* mutants were bathed in a type of phospholipid (PED6) that was engineered to fluoresce when cleaved by the phospholipase (PLA_2) enzyme (for details, see REF. 39). Wild-type day 5 larvae show intense gall-bladder fluorescence (arrowhead in **b**), whereas *fat free* day 5 larvae show severely reduced gall-bladder fluorescence. Note that under normal lighting conditions (**c**), the digestive tract appears normal in *fat free* mutant larvae. m, mutation. Panel **b** is reproduced with permission from REF. 39 © (2000) American Association for the Advancement of Science.

In a pilot screen, new mutants that were defective for digestive organ function were revealed using the fluorescent lipid reporter[39] (FIG. 4). F_3 embryos that arose from crosses in F_2 families were bathed in PED6 and screened for fluorescence. One mutation, called *fat free*, has normal digestive organ morphology, but with greatly reduced gall-bladder fluorescence (FIG. 4b). Although several other digestive mutants were found with reduced fluorescence, *fat free* was the only mutation that was defective for specific organ function but not morphology, and therefore would not have been identified in a screen based on organ morphology alone. Assays to measure the enzymatic activity of PLA_2 determined that, although there was less overall PLA_2 activity, PLA_2 remained active in *fat free* animals. Using another fluorescent lipid — a cholesterol analogue that requires biliary emulsification for absorption — *fat free* showed very weak fluorescence in the gall bladder and digestive tract, which indicates a possible defect in bile synthesis or secretion. Visualizing enzymatic activity in a genetic screen of live larvae therefore identified a mutant with normal organ morphology but with a specific digestive defect in bile synthesis or secretion.

Similarity between fish and humans in lipid processing is further underscored by the finding that the drug atorvastatin — an inhibitor of cholesterol synthesis in humans — blocked PED6 labelling of the gall bladder in zebrafish[39]. Future screens for mutants that bypass or exacerbate drug function, or screens for drugs that alter mutant function, will help to probe the mechanistic basis of digestive physiology and possibly human digestive disorders.

Other fluorescent reporters have been used for screening and to directly visualize specific processes. Fluorescent dyes injected into the eyes of day 5 larvae label the entire length of the retinal axons, which can be directly visualized through the skin. Using fluorescent dyes as a marker, a screen of F_3 embryos for defects in the retinotectal pathfinding of retinal ganglion cells (RGCs) recovered the viable, recessive mutant *ast* (*ast*)[40,41]. Eye transplantation experiments, coupled with fluorescent monitoring of retinal axons, showed *ast* activity to be required in the eye and not in the brain[42]. Interestingly, *ast* mutants show no visual-behaviour defects[43] (see below). The *ast* phenotype is caused by a mutation in the zebrafish *roundabout* (*robo2*) gene, which is expressed in the extending axons of RGCs[42]. Roundabout proteins belong to a family of axon-guidance receptors that bind specific ligands to guide axons to their destinations through a complex environment. So, the use of fluorescent dyes has uncovered an eye-autonomous gene that is required for RGC pathfinding and has provided new insight into the molecular basis of vertebrate visual projection.

By linking green fluorescent protein (GFP) to genes or promoters of interest, it is possible to visualize normally obscured processes. Spatial and temporal expression patterns, and the development of embryonic haematopoiesis, can be visualized in living zebrafish by the expression of GFP from the promoter of the haematopoietic transcription factor *gata1* (REF. 44). Other tissue-specific promoters that have been linked to GFP include the skin-specific type II cytokeratin (CK) promoter and the muscle-specific muscle creatine kinase (mck) promoter[45]. These, and other fluorescent-based tools, provide important markers for monitoring the development of specific tissues and will be valuable reagents when screening for mutants that perturb normal gene expression, protein abundance and tissue development.

Behaviour screens. Genetic screens in zebrafish can be designed to reveal mutations not only in developmental and physiological processes, but also in behavioural activities and movement. Normal 2–3-day-old larvae are not very active, but will swim rapidly away from a stimulus (such as a needle) applied to the tail. As part of the first, large-scale zebrafish screens, Granato *et al.*[46] selected embryos that were defective in the touch

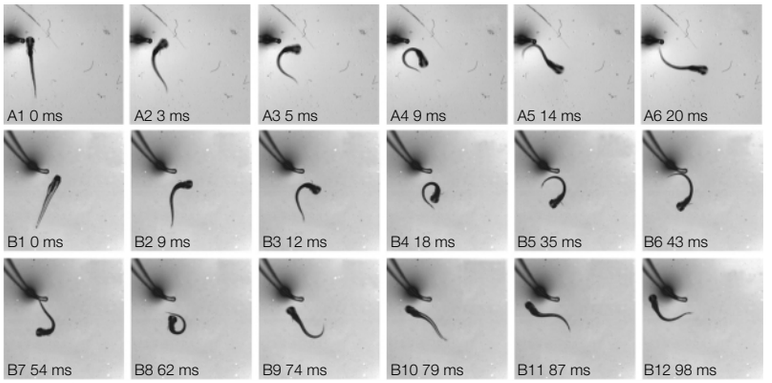


Figure 5 | **The *space cadet* locomotion mutant.** The *space cadet* mutant shows abnormal swimming behaviours during a stimulus-induced escape response. High-speed camera images capture the C-shaped bend of a wild-type larva as it rapidly moves its head away from the stimulus source (A1–A4; the timing of each frame is given, in milliseconds (ms)). The larva then makes a less powerful counterturn (A5, A6), before rapidly swimming away from the source (not shown). In response to a stimulus, the *space cadet* larva makes a C-shaped bend away from the stimulus in a similar manner to wild-type larvae (B1–B4), but has a poor counterturn (B5, B6). It then initiates a second turn towards the same side (B7–B9), before swimming away using a series of fast, bilateral tail flexures. A movie of the swimming defects of *space cadet* can be viewed at http://dev.biologists.org/cgi/content/full/128/11/2131/DC1 and http://www.nature.com/nsu/010607/010607-1.html. Modified with permission from REF. 47

response. One mutant, *nevermind* (*nev*), rotates around its long body axis and displays a corkscrew swimming pattern. Although the muscle morphology is normal, the dorsal retinotectal axon projections in *nev* are abnormal, terminating on both the dorsal and ventral side of the TECTUM[40,46]. Another mutant, *space cadet* (*spc*) shows specific abnormalities in the fast-turning escape response, and will also spontaneously undergo escape-response turning while swimming[46,47] (FIG. 5). The *spc* gene is required for the axonal pathfinding of a subset of commissural axon trajectories that control locomotive behaviour[47]. So, a simple touch test has identified complex mutant phenotypes that link locomotion with neuronal development defects.

More recently, screens have been developed to monitor behaviour in response to a visual stimulus, revealing genes that control vertebrate visual-behaviour responses. Neuhauss *et al.* conducted a 'shelf screen' and re-examined more than 400 zebrafish developmental mutants from the Tübingen screen for abnormal behavioural responses to visual stimuli[43,48]. To monitor their optokinetic response (OKR), a black and white drum was rotated slowly around zebrafish larvae that had been partially immobilized in a Petri dish, and their eye movements were recorded[43,48]. Normal fish eyes will smoothly scan in the direction of the stripes (clockwise or counter-clockwise) and, then, in a rapid eye movement, reset to the midline before following the stripes again[48]. The optomotor response (OMR) was measured by placing day 5 larvae at one end of transparent, long, thin chambers, that were placed on top of, and at right angles to, black and white stripes that move across the computer screen[43]. Wild-type fish swim in the direction of the motion and accumulate at the opposite end of the channel. Mutants with an abnormal OKR, or who failed the OMR test by not accumulating at one end of the channel, were re-examined by electroretinography and by histology[43,48]. These secondary assays allowed the authors to distinguish between sight-specific and sight-non-specific mutations that might cause a fish to fail one or both of the tests[43] (such as a locomotive defect).

By using previously identified developmental mutants, this screen questioned if the genes that are required for development might overlap with those required for visual-behaviour responses. At least 25 developmental mutants were found to have defects in the normal visual-behaviour response to stimuli[43]. Interestingly, mutants that failed the OKR and OMR tests were not restricted to defects in a specific developmental process. Small- or large-eye mutants, *microps* (*mic*) and *blowout* (*blw*) respectively, passed the OKR and OMR tests, indicating that eye morphology might be controlled by a different set of genes than visual performance[43]. Interestingly, darkly pigmented fish frequently failed the visual-behaviour response tests. In a light environment, melanin in the melanophore aggregates and the animal appears pale, whereas in darker surroundings melanin is dispersed and the animal darkens. Thirteen mutants with dispersed pigment were also defective for both the OKR and OMR, probably because they were unable to visually process their surrounding environment[43]. As this adaptation response is controlled by a projection from the retina to the hypothalamus and is a neuroendocrine process, the dark fish might be black as a secondary response to being blind. For example, the dark fish *lakritz* (*lak*) (German for liquorice) failed both the OKR and OMR, has a reduced number of RGCs and has a markedly smaller optic nerve[43]. Some of the zebrafish mutants resemble human disease phenotypes and 13 of the sight mutants involved retinal degeneration — the most common cause of hereditary blindness in humans. Fish might share genetic similarity with humans in eye development and prove a valuable model for studying human eye disorders.

Using a behavioural assay based on the zebrafish escape response (FIG. 6) in an F_1 screen for dominant defects in visual sensitivity to light, revealed an unusual mutation called *night blindness b* (*nbb*)[50]. Subsequent experiments showed that an increased visual threshold in $nbb^{+/-}$ fish is related to a reduction of DOPAMINERGIC INTERPLEXIFORM CELLS (DA-IPCs) in the inner retina[50]. Supporting this conclusion, infusion of the dopamine inhibitor 6-hydroxydopamine (6-OHDA), which kills DA-IPCs, phenocopies the $nbb^{+/-}$ phenotype[50,51]. Importantly, $nbb^{+/-}$ animals carry an unexpected defect in the neural retina connections with the centrifugal fibres originating from the terminal nerves of the olfactory bulb[50]. Underscoring the biological significance of this neuronal connection defect, the $nbb^{+/-}$ phenotype can be mimicked by excising the olfactory epithelium and bulb. Innervation of the retina with olfactory centrifugal fibre connections might therefore be an important DA-IPC regulatory mechanism, and therefore control dark-adapted visual sensitivity. So, a subtle assay for visual adaptation mutants has shown unexpected connections between distinct neurological pathways.

TECTUM
The dorsal portion of the midbrain (mesencephalon) that mediates reflexive responses to visual and auditory stimuli.

DOPAMINERGIC INTERPLEXIFORM CELL (DI-IPC)
Residing in the inner nuclear layer of the retina, this type of cell releases dopamine to regulate light adaptation in the retina.

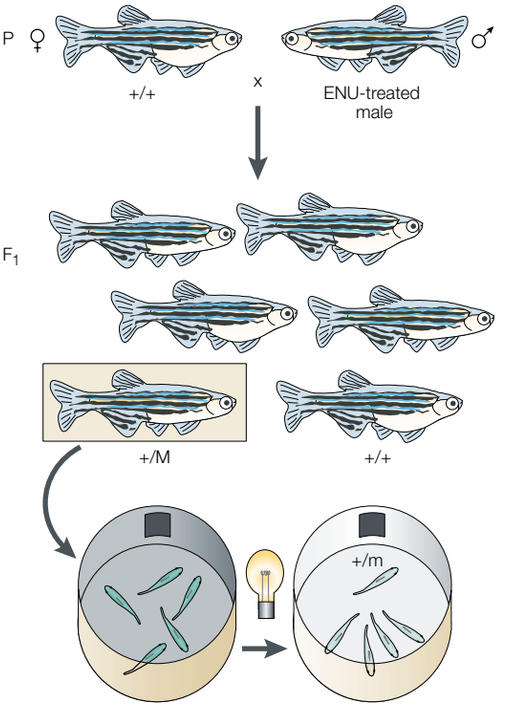


Figure 6 | **Behaviour screens: visual adaptation mutants.** A behavioural test can be a measure of visual sensitivity and be used to screen for subtle, eye-specific mutations in adult zebrafish. Adult F_1-generation fish derived from ethylnitrosourea (ENU)-mutagenized founders are placed in a transparent container that is surrounded by a rotating drum marked with a black square that represents a threatening object[49,50]. After initial dark adaptation, normal zebrafish rapidly 'escape' the threatening object in light above (but not below) their visual threshold (right drum). By contrast, fish that are heterozygous for the mutation *night blindness b* show the escape response at a visual threshold that is 2–3 log units above the average[50]. M, dominant mutation on an ENU-mutagenized chromosome.

Zebrafish show many other behavioural responses that are amenable to genetic screening, including complex social behaviours, such as schooling and territorial responses (see REF. 52 and references therein). In response to low levels of alcohol, aggressive and locomotive behaviours are increased, whereas higher levels of alcohol have inhibitory effects; screening is already underway for mutations that alter the behavioural effects of alcohol on zebrafish[52]. Cocaine also alters zebrafish behaviour and zebrafish will show a preference for a chamber supplemented with cocaine over an adjoining chamber without cocaine[53] (called conditioned place preference, CPP). The genetics of cocaine addiction have been probed in an F_2-generation screen for zebrafish with altered, cocaine-induced CPP. Three dominant mutants, *dumbfish* (*dum*), *jumpy* (*jpy*) and *goody-two-shoes* (*gts*), are insensitive to cocaine and could provide insight into the normal dopaminergic signalling in the brain and addiction-related behaviours[53].

TELEOST
Ray-finned bony fishes.

TURBIDOMETRY
A way to measure the solution turbidity, this can be used to assay for the formation of fibrin in the form of visible clotting in plasma.

Biochemical blood screens. In mammals, coagulation requires the activation of factor VII to initiate a cascade of proteases that eventually lead to the cleavage of fibrinogen and to fibrin clotting. Similarities between mammals and TELEOSTS have recently been reported to extend to the blood coagulation pathway with the identification of factor VII in zebrafish[54]. Using new biochemical assays, adult F_2-generation EP-generated diploids were screened for clotting defects in zebrafish, by clipping the tail of individual fish (the tail regenerates within a few weeks), collecting small amounts of blood and testing for the conversion of human fibrinogen to fibrin by TURBIDOMETRY[55]. Eight mutants were found that had prolonged clot formation. To further characterize which part of the coagulation pathway was defective, reconstitution assays for the extrinsic, intrinsic and common pathways were developed[55]. It is expected that these biochemical advances will provide a fruitful genetic means by which to explore the homology between zebrafish and human coagulation pathways.

Screening at high magnification. During retinal development, unspecified neuroepithelium progresses to a highly organized, laminated structure of differentiated retinal cells. The transition from multipotent precursor cells to fully mature retinal cells requires cells first to withdraw from the cell cycle and undergo specification (marked by migration to the appropriate laminar position and distinct gene expression), followed by differentiation and maturation (marked by morphological and biochemical changes). Lamination, a hallmark of normal retinal development, can be clearly viewed at high resolution. Using a magnification of ×200, differential interference contrast (DIC) microscopy was used to screen F_3-generation day 4 embryos derived from ENU-mutagenized founders for defects in retinal lamination[56,57]. Two *young* (*yng*) alleles were identified that were blocked for the transition from specification to differentiation[56]. Two mutants, *perplexed* (*plx*) and *confused* (*cfs*), were unable to exit the cell cycle and to become post-mitotic retinal cells[57]. Although *yng*, *plx* and *cfs* are seen to have slightly smaller eyes later in development, screening with microscopy for retinal development defects has identified subtle mutants that are defective for key transitions in retinal histogenesis. Finally, high-resolution DIC time-lapse video has also been reportedly used to visualize details of nuclear movement in live embryos[58], which is suggestive of a new screening tool in zebrafish.

Dissecting genetic pathways in zebrafish

Altering an initial phenotype, through genetic or synthetic means, can provide information about gene function and gene order in pathways. Genetic screens for secondary mutations that suppress or enhance a phenotype are an exciting next step in zebrafish genetics, and some are already underway. A serendipitous recessive enhancer of *cyclops* (*cyc*), called *squint* (*sqt*), was identified in specific genetic backgrounds that enhanced the severity of mesoendoderm formation[59]. *sqt*, like *cyc*, encodes a Nodal-related signalling protein[59]. Enhancer mutations in

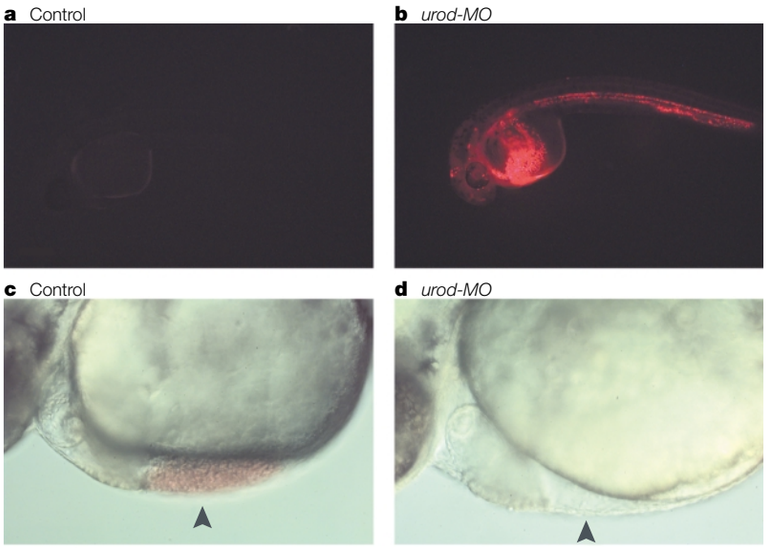


Figure 7 | **Morpholino-induced antisense phenotypes.** An example of morpholino (MO) targeting of the *uroprophyrinogen decarboxylase* (*urod*) gene phenocopies the *urod* loss-of-function mutant, called *yquem* (*yqe*). **b, d** | As in the *yqe* mutant, the *urod-MO* causes porphyria phenotypes similar to those seen in humans, including red-blood-cell autofluorescence in ultraviolet light (**b**), and rapid lysis when exposed to light (**d**). Panels **a** and **c** show sibling embryos to those shown in (**b**, **d**), injected with a control MO. Reproduced with permission from REF. 62 © (2000) Macmillan Magazines Ltd.

zebrafish can therefore identify genes that are involved in the same genetic process, and this example validates the use of zebrafish for suppressor/enhancer-based screens.

Allele specificity can also assist in dissecting a genetic pathway. Stronger or weaker alleles can be generated by crossing an F_1 generation from an ENU-treated founder with a heterozygous mutant, and by screening for non-complementing alleles. This has been successfully done to generate stronger alleles of *sonic you* (*syu*), *you-too* (*yot*), *fused somites* (*fss*) and *unplugged* (*unp*), simultaneously. Van Eeden *et al.*[60] crossed fish that were heterozygous for *syu*, *yot*, *fss* and *unp* to the F_1 generation of ENU-mutagenized fish. To ensure that the F_1 ENU fish were not mixed up with the heterozygous fish, the F_1 ENU fish carried homozygous *leopard* (*leo*) mutations that cause spotted pigmentation, and a dominant *long fin* (*lof*) mutation that causes long fins, thereby allowing easy separation of fish after mating[60]. One application of this technique might be to generate non-lethal hypomorphic alleles of embryonic-lethal mutants that could be subsequently used in suppressor/enhancer screens.

Temperature-sensitive (ts) alleles can be used to reveal stage-specific gene function. For example, an EP screen for adult fish with ts defects in fin regeneration identified several *regeneration* (*reg*) mutants, including *reg6* — a mutant that is defective in the early outgrowth stage of regeneration[61]. At the permissive temperature, *reg6* mutants regenerate fins normally, in contrast to the non-permissive temperature, at which *reg6* mutants develop misshapen fin rays and dysmorphic blood-filled growths on the regenerating fins. *reg6* embryos raised at the non-permissive temperature result in severe embryonic defects and death, indicating that non-conditional alleles of *reg6* might have defects in normal embryogenesis[61].

Zebrafish geneticists have a range of tools that can be used in conjunction with genetic mutations to confirm and explore gene function, to study maternal versus zygotic gene usage and to link genetic pathways. Morpholino (MOs) gene-knockdown technology has been recently developed in zebrafish[62]. MOs are antisense, chemically modified oligonucleotides that inhibit translation in a specific manner, and have been used to show genetic interactions[62]. For example, sonic hedgehog (*shh*) mutations cause HOLOPROSENCEPHALY in humans[63], but zebrafish *shh* does not have dorsal–ventral patterning defects[64]. MOs of both *shh* and *tiggy-winkle hedgehog* (*twhh*), a *shh* homologue that has no MO phenotype, act synergistically to produce strong dorsal–ventral patterning defects, indicating that *shh* and *twhh* in zebrafish might function together to carry out the function of *shh* in mammals[62]. The strength of this technique is underscored by the number of genes that have been subject to MOs, including blood and pigment genes, and genes that are involved in embryonic patterning, angiogenesis and brain development[65] (FIG. 7).

Ectopic gene-expression methods have been used to place genes in genetic pathways. Notably, forced expression (by mRNA injection) of the haematopoietic stem-cell transcription factor gene *scl*, or the haematopoietically expressed homeobox gene *hhex*, can partially rescue the blood and endothelial deficiencies of *cloche* (*clo*), placing *scl* and *hhex* downstream of *clo*[66,67]. Other overexpression techniques include RNA CAGING and the GAL4/UAS SYSTEM, which allow for specific control over the time and tissue in which a gene is expressed[68–70]. Control of gene expression in individual cells has also been achieved by exploiting the optical clarity of the embryo to focus a laser microbeam or beam of light onto specific cells, thereby activating expression of a transgene or uncaging RNA molecules, respectively[68,71].

Another way to identify and clone genes, and to place them into functional pathways, relies on looking for genes with informative expression patterns. This technique, called whole-mount *in situ* hybridization is described in BOX 2.

Integration of Tc3, a member of the Tc1/mariner/Sleeping Beauty family of transposons is another advancing technology that provides the basis for transposon-mediated genetic transformation and inducible removal of DNA from integrated constructs[72]. In addition, the recent production of germ-line chimaeras from zebrafish embryo cell cultures is progress towards achieving targeted gene inactivation in the zebrafish[73]. Among the many useful applications of such technology is a reverse genetic approach to screening.

Small molecules can also generate specific phenotypes in zebrafish. The zebrafish CHORION is permeable to small molecules, and is amenable to high-throughput screening for chemicals that specifically inhibit developmental processes and mimic genetic mutations. Unlike gene disruptions, chemicals can be

HOLOPROSENCEPHALY
Failure of the forebrain (prosencephalon) to divide into hemispheres or lobes, often accompanied by a deficit in midline facial development.

RNA CAGING
RNA inactivation through the covalent attachment of a photo-removable synthetic compound called the caging group. RNA is reactivated by photo-illumination with a specific light wavelength.

GAL4/UAS SYSTEM
A genetic system for controlling the induction of gene expression. An activator line that expresses the yeast transcriptional activator *GAL4* gene under the control of the heat-shock 70 promoter (*hsp70*) or a tissue-specific promoter is crossed to an effector line that carries the DNA-binding motif of Gal4 (UAS) fused to the gene of interest. As a result, the progeny of this cross expresses the gene of interest in an activator-specific manner.

Box 2 | **Complementing the genetic screen: *in situ*-based screens**

Whole-mount *in situ* hybridization (WISH)-based screens reveal expression patterns for new genes and known genes and can be a complement to the genetic screen (C. Thisse and B. Thisse, personal communication). Using cDNA libraries from organ- or stage-specific embryos, WISH reveals genes with similar expression patterns that can be grouped as potentially working in the same genetic pathway. Genes with interesting expression patterns are directly sequenced, and mapped, and their map position compared with the locations of ethylnitrosourea-generated mutants. This technique has been used to clone *casanova*[7], and *chardonnay* (*cdy*) a haematopoietic divalent metal iron transporter (*DMT1*; A. Donovan, L.Z., C. Thisse and B. Thisse, personal communication).

applied during specific windows of time in development, bypassing effects in early development that might lead to gross abnormalities or death. Chemicals can also potentially interfere with one or more targets, which circumvents problems with genetic redundancy in a particular pathway. One molecule, called 31N3, disrupts OTOLITH formation in the ear in a temporally specific manner[74]. 31N3 is potent even at very low doses, and related compounds with only minor chemical modifications have no effect on otolith formation[74]. Another molecule, called concentramide, mimics a *heart and soul* (*has*) mutant that is defective in heart-chamber morphogenesis[75]. Both the *has* mutation and concentramide allow for chamber formation, but the ventricle is abnormally formed in the atrium. Interestingly, *has* mutations and concentramide seem to operate in different molecular pathways: *has* encodes PKCλ (protein kinase Cλ), which is required for cell polarity, epithelial layer integrity and spindle orientation[75,76], and concentramide disrupts AP patterning in the heart[75]. So, a combined genetic and chemical approach has identified a pivotal, previously uncharacterized, developmental step in heart development. Future chemical-based screens could identify other compounds that might suppress, enhance or otherwise alter specific mutant phenotypes, laying the foundation for future drug design.

CHORION
An extraembryonic membrane that surrounds the zebrafish embryo during the first 2 days of development.

OTOLITH
One of the small particles of calcium carbonate in the sacculus of the inner ear. Pressure of the otoliths on the hair cells of the macula (the most sensitive area of the ear) provide sensory inputs about acceleration and gravity.

Practical considerations and conclusion

Designing a genetic screen in zebrafish involves balancing the screen parameters that best allow mutant detection with those that are manageable in individual laboratories. Zebrafish genetic screens are also labour intensive, requiring many hours of fish husbandry, preparation, screen processing and mutant re-identification. Owing to the large space and time requirements that are necessary for large-scale screening of F_3 diploids, our laboratory has carried out large-scale screening for blood mutants in collaboration with the Nüsslein-Volhard lab. By comparison, in our own fish facility, we have carried out several small-scale haploid and EP screens simultaneously, which are managed by individuals or small teams of investigators. The Boston and Tübingen screens discovered thousands of mutants, including alleles of previously identified mutants, but even so, did not reach saturation. From the first large-scale screen, members from our lab have contributed to finding 32 blood mutants, which formed 17 complementation groups[15], whereas in our small-scale screens (covering ~600 genomes), we recover up to ten mutants, most being single alleles. We generally invest 1 year for our small-scale screens: about 6 weeks to 2 months to generate ENU-mutagenized males (founders), ~3–5 months for F_1 ENU females to be primed for egg extraction, and at least 3 months to initiate several sibling crosses to re-identify mutants in the F_2 generations.

With the high homology between zebrafish and human genes, and conserved gene order (synteny), genetic screens in zebrafish provide a powerful means to elucidate the complexities of development and disease. Genetic and radiation hybrid maps, and the soon-to-be-completed zebrafish genome sequence, are continually advancing the ease of cloning mutated genes of interest. Although practical considerations shape screen design, as the screens described here show, imaginative screens can, and will continue to divulge gene functions and illuminate the intricacies of gene networks.

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