

Showcase on Research

Regulation of Development in the Zebrafish

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The problem of development

A developing embryo undergoes coordinated changes in cell size, number and type. The mechanisms regulating these changes must be sufficiently sophisticated to create the embryo's astonishing cellular complexity. Nevertheless, they must be robust enough to resist perturbation by fluctuations in environmental conditions.

The molecular genetic analysis of developmental control mechanisms began with the systematic search by Nüsslein-Volhard and Wieschaus for mutations affecting the development of *Drosophila melanogaster* embryos (1). The subsequent molecular characterisation of these mutations led to the discovery of numerous genes encoding, predominantly, transcription factors and proteins involved in intercellular signalling.

Searches for homologous genes in organisms of greater or lesser complexity indicated that many of these genes have been conserved during animal evolution. Conservation of the developmental mechanisms they control has been confirmed experimentally by mutation screens in the nematode, *Caenorhabditis elegans*, and by targeted mutation in mice.

Vertebrates such as ourselves are far more complex than *Drosophila*. Vertebrate genomes possess three to four times as many genes (2) and vertebrate bodies have a greater variety of cell types than those of arthropods. Some tissues, such as the neural crest, are unique to vertebrates. Thus, while many developmental control mechanisms show conservation, these mechanisms must be more sophisticated in vertebrates and novel mechanisms may exist. How can we detect and analyse them?

The zebrafish solution

Gene knockout technology in mice allows analysis of the function of putative developmental control genes. However, even if one were to knockout every gene in the mouse genome (and Lexicon Genetics Inc. in Texas, USA is attempting

this) there are other obstacles to the analysis of embryo development in mice. Mouse embryos that die due to lethal developmental mutations are rapidly

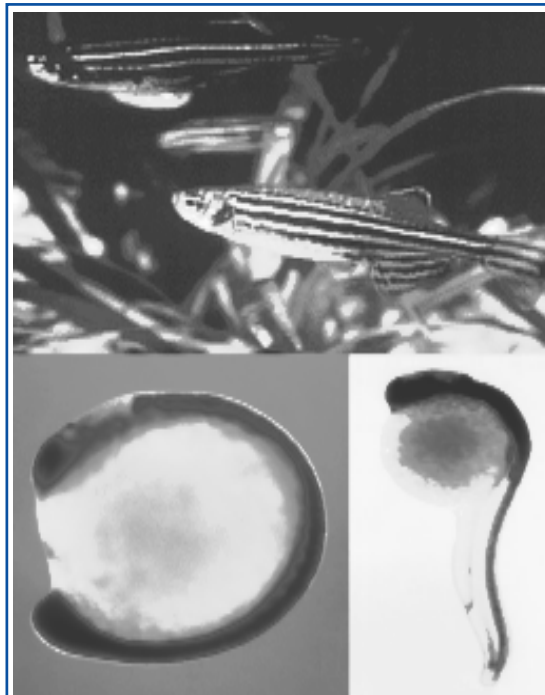


Fig. 1. Zebrafish adults and embryos. Top: Adult zebrafish. Bottom left: Zebrafish embryo 15.5 hours post fertilisation (hpf) stained to reveal *notch1a* gene transcription (dark areas). Bottom right: Zebrafish embryo at 24 hpf stained to reveal *notch5* gene transcription. Top image reprinted with permission from the Zebrafish Information Network.

resorbed *in utero*. Also, the mother must be killed in order to access the embryo and hence cannot be bred to perpetuate the mutation!

Following her success with the screen for mutations affecting development in *Drosophila*, Christiana Nüsslein-Volhard wished to repeat the exercise in a vertebrate system. To do this she needed an organism combining the best aspects of *Drosophila* embryology – rapid development of large clutches of easily observed embryos external to the mother – with a vertebrate body plan and genome. A small, hardy freshwater fish from northern India, the zebrafish *Danio rerio*, fitted these criteria. Its short generation time of three months made mutation screening a viable proposition.

Adult zebrafish (**Fig. 1**) are two to three centimetres in length, gregarious and tolerate high stocking densities. One female fish can release tens of thousands of eggs in its fertile life span of approximately 6 months. Once fertilised, an egg develops into an advanced embryo that has completed somite (vertebra/muscle precursor) formation and possesses a functioning nervous system within 24 hours! (In comparison, mice reach an equivalent stage after 10 days). The embryos are fertilised and develop external to the mother. They are completely transparent allowing excellent analysis of cell fate by dye or green fluorescent protein (GFP) transgene labelling (3).

Importantly whole mount techniques for observing three-dimensional patterns of gene transcript and protein expression can be performed at both very early and late stages of development (**Fig. 1**). Embryos that fail to develop normally due to mutation or other factors (eg. teratogens, experimental manipulation) cannot be resorbed.

A great catch!

During the first half of the 1990's, Nüsslein-Volhard in Tübingen and Wolfgang Driver in Boston conducted large scale screens for mutations affecting zebrafish embryo development.

They screened for mutations affecting developmental events such as early cell cleavage and migration, gastrulation (the creation of specialised layers of cells in an embryo), segmentation, neural development (including neurogenesis, brain regionalisation and axon pathfinding), haematopoiesis, ear, eye and fin development and the generation of pigment patterns.

The results of this work were published in a special "Zebrafish Issue" of *Development* in December 1996. These researchers identified thousands of mutations affecting development and these mapped to several hundred separate loci. These screens were not "saturating", however, and many hundreds more loci

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doubtless await discovery.

Currently, the study of zebrafish development and genomics is expanding exponentially. Techniques for transgenesis, transplantation, tissue culture and *in vivo* cell labelling are being refined. An EST project is under way and it is only a matter of time before large scale sequencing of the entire genome is initiated. Radiation hybrid panels for the rapid mapping of cloned genes (4) are freely available. A large number of highly innovative mutation screens have either been conducted or are under way to analyse phenomena such as regeneration (5) and photo-periodicity (6). Space restrictions permit me to describe only a small part of this research below.

Like-minded

A unique characteristic of vertebrates is their complex brain structure. The early development of the brain is the same in all vertebrates. An initial cylinder of neural precursors lying in the midline of the embryo is divided into particular regions that adopt specialised forms and functions. All vertebrates possess a spinal cord, hindbrain, midbrain and forebrain derivatives. In humans the forebrain is grossly expanded to form the cerebral cortex. In zebrafish, this region is quite small while the visual cortex derived from the midbrain is expanded to process the input from the fish's large eyes.

The mechanism by which the central nervous system is divided into regions along its length is a classical question in developmental biology. A number of mutations in zebrafish affect this process. The *no isthmus* and *acerebellar* mutations disturb the formation of the boundary between the midbrain and hindbrain and, consequently, the cerebellum that develops from this region (7).

These mutations affect the genes *pax2.1* and *Fgf8* (7,8), which had previously been cloned by homology with genes in other vertebrates. Analysis of their transcription patterns indicated early restriction to the midbrain-hindbrain boundary (mhb) precursor region, suggesting a role for them in mhb formation. There are at least 24 other mutant loci that affect brain morphology/regionalisation (9) and their characterisation will give us great insights into this process.

Crossed lines

The small size of developing zebrafish and their availability in large numbers lends them to analysis by array techniques. Living zebrafish larvae (newly hatched fry) arrayed into microtitre plates and observed by sophisticated video analysis software are being screened for dominant mutations affecting circadian rhythm (6).

Friedrich Bonhoeffer arrayed over 100,000 fixed mutant larvae in agarose gels and used a robotic injection device to dye-label retinal ganglion cells and their axons (10). In this way he was able to identify 19 loci required for normal projection of these axons from the eye to the optic tectum on the opposite side of the brain.

In *belladonna* mutants for example, the retinal ganglion cell axons are unable to cross the midline but, instead, project along the diencephalon on the same side of the embryo. The defective function appears to lie in the axons since the embryo does not appear to have any midline defects (11).

Pairs rule

The large number of zebrafish researchers studying the developmental neurobiology of this fish is unsurprising considering that neural tissue comprises over half of the cells of the embryo for much of its early development. However, other developmental processes are not being ignored. Another fundamental question in developmental biology is how particular tissues in an embryo become divided into units of equal size or segments. In vertebrates the hindbrain, the limbs and the ribs/vertebrae are all examples of segmented structures.

Insects and vertebrates are both segmented but is this due to a common evolutionary inheritance or parallel evolution? The dramatic differences in segment formation between *Drosophila* and vertebrates support the latter idea. However, the short germband insects such as the red flour beetle, *Tribolium castaneum*, produce segments sequentially like vertebrates, not simultaneously as in *Drosophila*. One of the most astonishing results in vertebrate developmental genetics has come from the cloning of zebrafish homologues of the *Drosophila hairy* pair rule gene that controls the formation of seg-

ment boundaries (and hence the formation of segments). As in *Drosophila*, the zebrafish *hairy* homologue *her1* is expressed in a pattern of stripes with two segment periodicity in the precursor tissue of the somites (the presomitic mesoderm) that later forms the segmental vertebrae and associated musculature (12). This suggests that fundamentally similar genetic mechanisms control segmentation in arthropods and vertebrates.

What does it take to gastrulate?

Before gastrulation an animal embryo is a fairly simple sheet of pluripotent cells. After gastrulation it is a complex, trilayered structure with separate layers ("derms") forming skin and nervous system (ectoderm), muscle and bone (mesoderm) and the digestive system (endoderm). To gastrulate, cells must migrate and make morphological changes in a complex pattern. Mutations at more than 14 loci have been found to affect these processes (13).

One of these mutants, *one-eyed pinhead* (*oep*), lacks some forebrain structures and has a single cyclopic eye. When this mutant locus was cloned positionally using bulk segregant analysis with randomly amplified polymorphic DNA (RAPD) markers (14, 15) it was shown to encode a protein related to *cripto*, a human protein resembling EGF that is an autocrine growth factor for mammary tissue and that contributes to cancer when over-expressed (16). This demonstrates a central theme in developmental genetics – that developmental control genes are frequently oncogenic when inappropriately activated.

Mutate or perish?

Zebrafish have come to prominence for their utility in mutation screens. However, the characteristics of their embryos provide significant advantages that allow alternative strategies for investigating vertebrate developmental genetics. Since zebrafish embryos are transparent, small, do not change size greatly during development and are available in large numbers, they allow large scale screening of monoclonal antibody or cDNA sets to identify proteins or genes expressed in restricted patterns during embryogenesis. A number of lines of monoclonal antibodies marking specific cells and tis-

