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# The zebrafish issue of *Development*

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## Summary

In December 1996, a special issue of *Development* appeared that presented in 37 papers the results of two large screens for zebrafish mutants performed in Tübingen and Boston. The papers describe about 1500 mutations in more than 400 new genes involved in a wide range of processes that govern development and organogenesis. Up to this day, the mutants provide a rich resource for many laboratories, and the issue significantly augmented the importance of zebrafish as vertebrate model organism for the study of embryogenesis, neuronal networks, regeneration and disease. This essay relates a personal account of the history of this unique endeavor.

## Introduction

I discovered zebrafish as a genetic system in 1983, when Hans Georg Frohnhöfer, the graduate student who worked on the *bicoid* mutants (Frohnhöfer and Nüsslein-Volhard, 1986), presented a paper on haploid and homozygous diploid zebrafish (Streisinger et al., 1981) in our journal club. At this time, I was junior group leader at the Friedrich-Miescher Laboratories of the Max-Planck Society in Tübingen (Germany). We had just finished a saturation screen for maternal mutants on the third chromosome of *Drosophila*, and the collection of interesting mutants with novel phenotypes almost overwhelmed us (Anderson and Nüsslein-Volhard, 1984; Nüsslein-Volhard et al., 1987). Everything seemed so incredibly complex. Could we ever resolve the puzzles without diving into positional cloning and molecular analysis of each individual of the 30 or so new genes we and others had identified? This prospect frightened me. However, when I realized that zebrafish was a system that might allow us to exploit the genetic approach in a vertebrate, I saw a fascinating challenge, particularly as genetics had worked so successfully in *Drosophila* to dissect early patterning processes (Nüsslein-Volhard and Wieschaus, 1980). I bought my first aquaria in 1984, one at home to keep beautiful pet fishes, and one with zebrafish in my office. And I visited Eugene (OR, USA) where Chuck Kimmel, Monte Westerfield and Judith Eisen secured the zebrafish legacy of the late George Streisinger. They had already, in 1985, collected a small number of zebrafish mutants with exciting phenotypes (Kimmel, 1989).

## Zebrafish mutants obviously could be obtained – why not do screens on a similar scale as in *Drosophila*?

Why was I fascinated? Zebrafish and their embryos are simply beautiful. Mutants obviously could be obtained – why not do screens on a similar scale as in *Drosophila*? We had the experience to do this. Genetic analysis might allow us to really compare vertebrate and invertebrate development. At this time, gene cloning had just started, and it was by no means obvious to what degree invertebrates and vertebrates were related – the common notion was rather not at all, as their modes of development appeared to be so different. The way embryonic development is described is dictated by the methods used for analysis. Thus, in flies, development seemed to be determined by genes (defined by mutants), whereas in frogs, enigmatic factors postulated from experimental manipulations did the job. So it was conceivable that the work on flies would not help us to understand frog (i.e. vertebrate) development. Still, I was convinced that genetics was the most successful approach to dissect and understand complex systems. The mutant and its phenotype, caused by the lack of a single gene product, is a cleaner experiment than any transplantation, constriction or centrifugation could ever be.

## From flies to fishes

Despite my early enthusiasm, the start of zebrafish work in Tübingen had to be postponed until 1988 or so, when graduate students Stefan Schulte-Merker and Matthias Hammerschmidt arrived in the lab. The molecular work on maternal *Drosophila* genes, notably the discovery of the Bicoid gradient (Driever and Nüsslein-Volhard, 1988a; Driever and Nüsslein-Volhard, 1988b) had turned out to be full of excitement and success, so there was little need, or capacity, to start a new big project. But on the side we had installed a fish facility with the help of Manfred Schartl. We bought some wild-type, long-finned and spotted zebrafish in local pet shops and tried to grow these in the lab. This proved difficult, and the Oregon recipes did not work well for us in Tübingen.

By this time, positional cloning of *Drosophila* mutants, notably the discovery of the homeobox, had led to a recognition of the high degree of homology among metazoans. Stefan cloned the mouse T-gene homolog from fish and showed it to correspond to the *no tail* mutant that had been isolated in Chuck Kimmel's group (Schulte-Merker et al., 1992; Schulte-Merker et al., 1994). Matthias cloned the fish homolog of the fly *snail* gene and showed that it was expressed, as in flies, in the invaginating mesodermal anlage (Hammerschmidt et al., 1993). Mary Mullins, together with Matthias, tested EMS (the mutagen used in flies) in fish, but this

**A *Development* classic**

The year 2012 marks 25 years since the journal *Development* was relaunched from its predecessor, the *Journal of Embryology and Experimental Morphology* (*JEEM*). In 2008, we fully digitised our *Development* and *JEEM* archives, and made them freely available online. At the same time, we took the opportunity to revisit some of the classic papers published in *JEEM*, in a series of commentaries (see Alfred and Smith, 2008). Now, to mark a quarter century of *Development*, we have been looking through our archives at some of the most influential papers published in *Development*'s pages. In this series of Spotlight articles, we have asked the authors of those articles to tell us the back-story behind their work and how the paper has influenced the development of their field. Look out for more of these Spotlight papers in the next few issues.

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was not successful and they instead developed protocols to induce mutants with ENU (the mutagen used in mice) (Mullins et al. 1994). Nancy Hopkins spent a sabbatical in the lab to learn about fish and started carrying out transfection experiments (Culp et al., 1991). And Cosima Fabian, an undergraduate student, made beautiful photographs and drawings from developing embryos.

One day I asked my graduate student Wolfgang Driever, who worked on the Bicoid gradient in *Drosophila* with incredible success (Driever and Nüsslein-Volhard, 1988a; Driever and Nüsslein-Volhard, 1988b; Nüsslein-Volhard, 2004; Driever, 2004), what he intended to do as a postdoc. It is hard to describe my amazement when he told me that he wanted to do genome-wide saturation screens in zebrafish. He had fallen in love with my best fish technician whom I had just sent to Eugene to learn various techniques and who might have influenced his decision. He explained that I had convinced him that this was the future vertebrate model organism in which genetics could be applied, and he wanted to have a share. Competition with my lab was not a concern for him – he would do insertional mutagenesis and was certain that we would eventually focus on quite different aspects of development. After a brief postdoc with Monte Westerfield in Eugene, he set up a lab at the Cardiovascular Research Centre of the Massachusetts General Hospital (MGH) in Boston (MA, USA) with the support of Mark Fishman, who was already running a small fish facility to study organ development using zebrafish mutants (Stanier and Fishman, 1994).

We managed to take advantage of this strange situation as best as we could and joined forces by exchanging experience and protocols on a regular basis. To carry out screens for mutants on a large scale, the big challenge was to develop methods to grow fish with safety, efficiency and high survival rates. In Eugene, water and space were cheap, and fish were kept in large tanks at low density with a constant flow through of fresh water. In Boston, Wolfgang and Mark developed a system in which the water was filtered and recycled. They quickly established a rather big fish facility at the MGH and started a joint ENU mutagenesis screen with a group of students and postdocs, including Lila Solnica Krezel, Alex Schier and Derek Stemple from Wolfgang's group, and Didier Stainier, Jau-Nian Chen and Michael Pack from Mark's lab (Solnica-Krezel et al., 1994).

In Tübingen, the fish house was built, starting in 1991. Together with the Schwarz company from Göttingen, we developed the two principal aquaria systems that are still used today for keeping and raising fish with recycled clean and healthy water – the mouse cages with influx and overflow systems, and the serial slit tanks (Mullins et al., 1994; Brand et al., 2002). The house hosts in six rooms 7000 aquaria of three different sizes, and huge biofilters in the basement. It was opened in August 1992, and the screening began.

## How to screen in zebrafish

What was the aim and scope of the big screen? The primary goal was to translate what we had done in *Drosophila* to vertebrates: find as many genes as possible that are involved in a specific developmental process by means of a common mutant phenotype. When Eric Wieschaus and I did the first such fly screen in Heidelberg (Germany), one of the incentives had been that there were very few genes known to affect segmentation – one of which, *engrailed* (with just one strange and unusual allele), had been described in great detail (Lawrence and Struhl, 1982) without considering that the most important information for the understanding of its function would be to see which other genes are participating in the same process. In the Heidelberg screens, we not only scored for segmentation mutants, but also isolated mutants with many different phenotypic traits, which later turned out to be immensely useful for the scientific community.

The strategy for genome-wide screens differs between flies and fishes in two important respects. First, flies have only three large chromosomes. Balancer chromosomes, conditional lethal mutants and marker mutants for each chromosome allow the convenient identification of a lethal mutant. So three 'chromosome-wide' screens had been carried out, one each for mutations on the first, second and third chromosome. The most important discoveries on segmentation in flies (Nüsslein-Volhard and Wieschaus, 1980) were based on the mutants found in the screen of the second chromosome. The results of the three screens describing a total of 120 genes were published in three back-to-back papers together with Gerd Jürgens, who had been postdoc in Eric's and my lab at the EMBL in Heidelberg (Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984; Jürgens et al., 1984). They followed the location on a chromosome, rather than a classification according to phenotype.

By contrast, zebrafish has 25 chromosomes, and no marker mutants. Therefore screens had to be done blind, with mutant carriers only identified by a test cross. The identification of a mutant therefore was based solely on the mutant phenotype, but just one big screen would suffice for a genome-wide survey.

The other fundamental difference concerned the screening itself. As ecdysozoa, fly larvae have an external skeleton, the secreted cuticle, which, if properly preserved, provides a permanent record of the mutant phenotype. Embryonic lethal individuals could be inspected whenever we had time. The cuticle structures served as a read-out to detect earlier defects in development, and allowed us to detect even very subtle deviations from normal using a compound microscope. By contrast, vertebrate embryos and larvae are composed of soft tissue that decays very quickly after death, necessitating analysis of clutches of live zebrafish embryos almost every day so as not to miss mutants with early lethal phenotypes. On the other hand, the transparency of the embryos allowed the inspection of many internal organs and tissues. The clutches of live embryos or larvae were scored under a simple stereomicroscope and recessive mutants identified based on detecting a consistent phenotype in about 25% of the embryos.

One important consideration, however, applies to both fish and fly saturation screens: the number of inbred families that had to be scored. This depends entirely on the efficiency of the mutagen, not on the number of genes in the genome or on the number of different phenotypic traits to be scored. From our pre-screens we calculated that – even though different mutagens were used (EMS in flies, ENU in fish) – hit rates were similar: about 1 hit per gene per thousand genomes (Mullins et al., 1994). This meant, in practical terms, that we needed to screen at least 3000 inbred families to approach saturation.

## The Tübingen team

This was a big project and required commitment and cooperativity to a degree that was exceptional for scientists used to working independently on their specific projects. The more experienced scientists shared with me the responsibility in organizational matters. We designed score sheets, a weekly schedule and rigid rules for how the screen should be carried out. We screened in four teams, composed in total of 12 scientists (Fig. 1): postdocs Michael Brand, Makoto Furutani-Seiki, Michael Granato, Pascal Haffter, Don Kane, Robert Kelsh and Mary Mullins; and PhD students Matthias Hammerschmidt, Carl Phillip Heisenberg, Jörg Odenthal,

**Fig. 1. The screeners in front of the Fischhaus (1993).** Top row (from left to right): Mary Mullins, Christiane Nüsslein-Volhard, Cosima Fabian, Matthias Hammerschmidt, Birgit Bochenek, Rachel Warga, Pascal Haffter, Yun-Jin Jiang, Robert Kelsh. Bottom row: Willi Ankele (animal caretaker), Don Kane, Jörg Odenthal, Freek van Eeden, Dirk Beuchle, Michael Brand, Lisa Vogelsang, and a short term helper student. Michael Granato, Carl Philipp Heisenberg, Makoto Furutani-Seiki, Ursula Schach and Hans Martin Maischein are not on the picture; they joined us a little later. Birgit left shortly after the picture was taken. Rachel did not participate in the screen.

Freek van Eeden and Yun-Jin Jiang. They were supported by four technicians: Dirk Beuchle, Hans Martin Maischein, Ursula Schach and Lisa Vogelsang. The four teams worked together to set up crosses, harvest embryos, feed fish on the weekends and to do the re-screen of putative mutants. There was a strict ban on pursuing other projects on the side, and on starting to analyze mutants that had just been found. This system worked out well, because we found so many new and exciting phenotypes. Every screener had to score all possible traits – not just the phenotypes they were most interested in. And we looked not only for mutants that were topical at the time, such as notochord, brain and gastrulation, but collected mutants with a very broad range of early and late phenotypes, including – in the group of Friedrich Bonhoeffer – retinotectal projection. With the exception of this latter screen [which was performed on fixed larvae (Baier et al., 1996)], we did not use any staining or complex microscopy, just inspection with the stereomicroscope. This required much experience and skill, and some rather subtle phenotypes tended to be overlooked by some of the screeners – but this was unavoidable.

We started the project in the spring 1992 with ENU mutagenized males crossed to wild-type females to generate F1 progeny heterozygous for a mutant genome. Single pair matings were performed among these F1 fish to establish families of about 60 fish each. We set up crosses from up to 100 families per week, and the egg clutches were scored at day 1, 2 and 5 of development. From autumn 1992 until March 1994 we screened almost 4000 families, with, on average, four crosses per family. We found a total of 4264 mutants, and after careful re-screening kept and analyzed 1163 mutants that displayed a specific phenotype.

How should we analyse and describe our harvest of mutants displaying such a richness of novel phenotypes? The mutant phenotypes covered epiboly, gastrulation, notochord, somites, brain, spinal cord, muscles, heart, circulation, blood, skin, fin, eye, ear, jaw and branchial arches, pigment pattern and formation, gut, liver, motility, and touch response. For the complementation testing and analysis period, we started a classification based on similarity of phenotypes (implying a prediction of allelism), and distributed the mutants for analysis and characterization among several groups of scientists. Each group focused on the mutants the scientists were most experienced with. Because, for some phenotypes, we did not have experts, or the people who were most interested in them had already left the lab, each group also had to take care of other classes of mutants. We also received support from Heiner Grandl, Francisco Pelegri and Tatjana Piotrowski, who joined the lab at the end of the screen and took part in the analysis. On the whole, this worked well, but required adjustments and redistributions at several points.

Right at the end of the screen, we received some requests for newly found mutants, but did not feel ready to share any mutants with other labs at that point as we were still deeply involved in the recovery, the complementation tests and the characterization. However, we invited several visiting scientists to join us in the analysis of phenotypes for which we had no expertise in the lab. Jau-Nian Chen from the Fishman lab in Boston, Corinne Houart and Tom Schilling from the Westerfield and Kimmel labs in Eugene, David Ransom from the Zon lab in Boston, and Tanya Whitfield from the Wylie lab in Cambridge (UK) spent several months in Tübingen, joining the effort and providing useful expertise. Michael Brand, Pascal Haffter, Freek van Eeden and Michael Granato organized their work and supervised undergraduate student helpers. Michael Granato set up a database, while Freek and Pascal established mutant stocks, which eventually were taken care of by Hans Georg Frohnhöfer who rejoined us as stock-keeper in 1994. Many mutants are now kept in the form of frozen sperm samples.

## The zebrafish publications

How could we publish the work of so many people and give justice to everybody? How could we prevent a race with the Boston lab, who had done a very similar thing albeit on a somewhat smaller scale? How could we avoid individual exciting stories being taken out of the common basket and published? People had worked for several years on this communal project without any publication. It had to be ensured that they received proper credit for their efforts.

**It was so important to guarantee that…nothing of value for the zebrafish community would be lost or overlooked.**

At the Cold Spring Harbor fish meeting in spring 1994, the idea of a zebrafish special issue was discussed for the first time. Such an issue would have the advantage that all papers would undergo peer review and appear back to back, and all the information would be together: good for the reader and for the authors. Most importantly, if the ban to publish prematurely was respected, people would have time for a moderately comprehensive analysis of all phenotypic classes, thus enhancing the usefulness of the mutant collection. It was so important to guarantee that, regardless of individual interests, nothing of value for the zebrafish community would be lost or overlooked. It was initially quite unclear how many papers there would be, but my vision was that each should have an interesting note, not just presenting lists and complementation data, but at least attempting to interpret and characterize the mutants for the sake of future research. Wolfgang and I did not immediately agree on this, so it took further

discussions, as well as finding a journal interested in producing such an issue of refereed papers on the results of the screens.

After some negotiations, three journals expressed interest: *Developmental Biology*, *Current Biology* and *Development*. In the spring of 1995, we finally agreed with Chris Wylie that we should prepare our papers for *Development*. The analyses were well under way: complementation tests had been carried out; in situ hybridization, double mutants and morphological description were coming. People worked hard, with the prospect of writing papers for submission in the summer. Gradually we shaped papers from the core results, attempting to tell an interesting story with each one. I well remember the time while people were writing, and I went home every night with five manuscripts or more. I became quite efficient at shaping tables, demanding cross-references to other phenotypic classes, suggesting improvements to figures and reworking boring summaries, introductions and conclusions. The writing of some papers was extra difficult because the first authors had already left the lab, or were sick. An unusual problem turned up: because everybody was writing, very few people had time to read and edit the manuscripts. The people from the Tübingen fly groups and Stefan Schulte-Merker, who had returned to Tübingen as a project leader, helped. It was really great to experience the collaborative spirit that was constantly apparent and finally led to a good ending: submission of a total of 24 papers describing a total of 372 genes, almost all of which were new, along with an overview article that I wrote (Haffter et al., 1996a). There were also three papers from the Bonhoeffer group. Each paper lists the main investigators first, followed in alphabetical order by all the screeners and technicians as co-authors.

We submitted the first nine papers together in August 1995. I almost broke down that evening: I never ever had done such a thing, but we all had worked like slaves! In the subsequent weeks, all the other papers were finished and submitted. On the last day of submission, September 9th, we had a wonderful party celebrating the successful end of an immense and unique enterprise. One month later, the Nobel prize to Eric Wieschaus and myself was announced. And 2 weeks later, on the 19th of October, the cold shower came in the form of the reviews of 16 of the papers.

Well, the result was that five papers were to be accepted after minor revisions. Two others required more data, and would be accepted after revision. In three instances, we were asked to merge two papers, and three papers were rejected, because of insufficient interest. My overview paper received a memorable comment from one of the reviewers, ‘Hence, it is difficult to decide where to place this enormous endeavor on the continuum between courage and foolishness. The feeling of awe which arises when reading the two summaries [the second overview paper came from the Driever lab (Driever et al., 1996)] mixes with an unsettling sense that the emperor has no clothes when reading…the titles and abstracts of the remaining papers’. From my letter to Chris Wylie it is quite obvious how sad and bitter I felt: ‘I have fortunately never been confronted with so much unjustice, spitefulness, lack of understanding and ignorance [and] to be accused of having maneuvered mediocre papers around the reviewers…is the very last thing I would like to be confronted with’. I wish I had kept the reviews in entirety; in retrospect it seems likely that they were more just than appeared at the time. I did acknowledge that some reviewers’ comments were very helpful indeed, and also considered the difficulty, in this emerging field, to find enough experts without a conflict of interest to review the papers. Frequently, the two reviewers wrote contradictory comments, or they did not like the results. Some complained because of the lack of data, some of overload! Often they did not respect the genetic data as interesting, being used to detail studies on single mutants. One major theme came up, and this I discussed at length with Chris: we wanted the papers to be published together, because each benefited from, or even required, the context of the others. But for some reviewers it was important that each paper be able to stand alone.

What to do? Chris was very helpful, and he must have suffered too with this mammoth endeavor! He pointed out to me the importance of having a ‘special issue’ in which all papers had undergone peer review; the possibility of a ‘supplementary issue’ that had not been refereed would not have been a good option for my collaborators – who needed reviewed papers for their careers. So I worked on putting together this special issue as I had never done before or after for any paper. We very carefully attended to the reviewers comments, added more data obtained in the meantime, and, after another round of reviewing, merged some papers. Surprisingly, one of the papers that was most difficult to get accepted was that on the somite mutants (Van Eeden et al., 1996). Of the mutants it describes, *after eight*, *deadly seven* and *beamter* later turned out to encode Delta and Notch proteins, crucial players in the vertebrate segmentation clock (Holley et al., 2000). These mutants showed that vertebrate segmentation is completely different from that of flies. The paper also described the U-shaped somite mutants that define members of the hedgehog signaling pathway, including *sonic you*, which encodes Sonic hedgehog. The reviewers were disappointed that the segmentation mutants were not lethal, and regarded the U-shaped mutants as uninteresting!

At the time (probably from sheer exhaustion) we did not exchange much information between the Tübingen and Boston groups, although mutants with similar phenotypes were crossed to test allelism, and names were adjusted according to the date of finding the first allele.


**Fig. 2. Cover of The zebrafish issue of *Development* (December 1996), displaying the color pattern of the anal fins of several adult mutant fishes.**

The zebrafish issue of *Development* appeared in December 1996. It contains 22 papers from my group, nine from the Driever group, three papers on organ development from the Fishman group, three on the analysis of the retinotectal phenotypes from the Bonhoeffer group, and a paper on the zebrafish SSLP-map from the group of Howard Jacob and the Boston labs. Finally, pictures from Don Kane's time-lapse movie were printed at the upper corner of even numbered pages, such that flipping through the pages shows the dynamics of development through the first 17 hours of the life of a zebrafish embryo. All in all, the issue, with 481 pages, contains a very rich collection of papers, providing the basis of projects in many zebrafish labs worldwide.

We had an almost entirely happy ending: we got our zebrafish issue, although somewhat later than originally planned, with a beautiful cover of adult fins from pigmentation mutants (Fig. 2). Ironically, the paper in which these adult viable mutants were described was the only one that finally was not accepted and was published elsewhere (Haffter et al., 1996b) – the referees thought that adult visible traits were not a concern of developmental biology. The mutants described in this paper are the basis for the studies now carried on in my lab: the development of structures, shape and pigment pattern of adult fishes with the aim of understanding the genetic basis of morphological evolution.

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