

SPOTLIGHT

The zebrafish issue: 25 years on

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ABSTRACT

In the 1990s, labs on both sides of the Atlantic performed the largest genetic mutagenesis screen at that time using an emerging model organism: the zebrafish. Led by Christiane Nüsslein-Volhard in Tübingen, Germany, and Wolfgang Driever in Boston, USA, these colossal screens culminated in 1996 with the publication of 37 articles in a special issue of *Development*, which remains the journal's largest issue to this day. To celebrate the anniversary of the zebrafish issue and reflect on the 25 years since its publication, five zebrafish researchers share what the issue means to them, how it has contributed to their career and its impact on the zebrafish community.


The big zebrafish screens: from then to now, 25 years later!

Mary C. Mullins

It has been 25 years since the 'zebrafish screen' papers were published, thanks to Chris Wylie, Editor-in-Chief at the time, who embraced reviewing and publishing the forward genetic mutant screen papers in a special zebrafish issue of *Development* in 1996. The 481-page volume contains 37 papers from four labs, describing hundreds of different mutants with developmental defects in almost every organ or tissue of the embryo or larva. As a new vertebrate genetic model, highlighting these immense mutant screens was exactly what this burgeoning field needed.

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George Streisinger (University of Oregon, USA) chose the zebrafish as a vertebrate genetic model and, by 1990, Charles Kimmel, Monte Westerfield and Judith Eisen had already published some fascinating developmental mutants in the zebrafish. Following Streisinger's untimely death, it became the vision of Christiane 'Janni' Nüsslein-Volhard (Max Planck Institute, Tübingen, Germany) to repeat her and Eric Weischaus's Nobel prize-winning *Drosophila* saturation mutant screens for pattern formation mutants in the zebrafish. I became part of that vision, as the first postdoc in her lab to work with zebrafish.

In 1990, a new graduate student, Matthias Hammerschmidt, and I worked to establish a DNA mutagenesis method to generate high-frequency point mutations in spermatogonial cells of adult fish, step 1 of establishing a large-scale screen. Step 2 was pragmatic: establishing an efficient aquarium system to house the thousands of fish lines needed. Janni drove the design of aquarium prototypes, we tested them and AquaSchwarz built them. These recirculating aquarium systems remain the standard in the field.

One year later, postdoc Pascal Haffter joined us, and together we performed a pilot F2 zygotic screen, step 3. We isolated over 100 mutant lines, two-thirds comprising broad pleiotropic phenotypes that we postulated were mutant genes in housekeeping processes (Mullins et al., 1994). It was important to identify these phenotypes, so that we could focus on the lines with specific developmental defects, discarding these others.

A new building was constructed for performing the large-scale screen, the Fish House, which opened in 1992. Pascal and I had generated thousands of F1 mutagenized fish ready to cross to make the F2 families that would be screened for F3 mutant embryos. An additional nine postdocs and graduate students joined the lab to embark on the screen (Box 1).

Although team science is normal today, this team work was different; working together every minute of every day was a most unusual endeavor for individualistic academic scientists

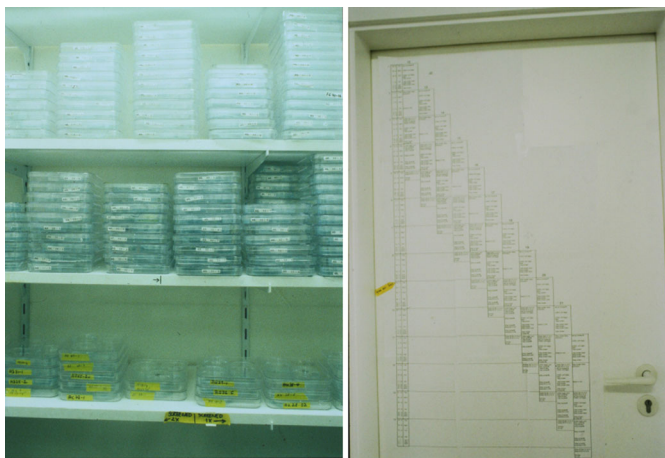
Box 1. The Tübingen screen team

The Tübingen screen team was made up of Michael Brand, Fredericus van Eeden, Makoto Furutani-Seiki, Michael Granato, Pascal Haffter, Matthias Hammerschmidt, Carl-Philipp Heisenberg, Yun-Jin Jiang, Donald Kane, Robert Kelsh, Mary Mullins and Jörg Odenthal. (Incidentally, I was the only woman.)

The large-scale screen was performed as a team effort in Tübingen. We worked side-by-side 5-7 days a week, setting up 500-1000 crosses weekly, collecting, sorting and screening together for over a year, beginning at 09:00 and ending when the work was done, sometimes after midnight. Logistics and organization were key; Janni printed a schedule about 1.5 meters long, but with an endpoint! She kept us on task. Although team science is normal today, this team work was different; working together every minute of every day was a most unusual endeavor for individualistic academic scientists. While sometimes grueling, it was successful, producing the largest number of developmental mutants in a vertebrate at that time, almost 1200. It was exciting too; almost every day, one or more new cool mutants would be discovered and shared. Many phenotypes were novel and unexpected, giving much to contemplate during the hours of screening.

By early 1994, the mutants and team were split into four groups, each reidentifying mutant carriers, performing complementation tests and preserving the lines. In late 1994 to early 1995, we finally had time to analyze the mutants and write the papers. Wolfgang Driever's lab [Massachusetts General Hospital (MGH), Boston, USA] carried out a similar screen in parallel, led by Lila Solnica-Krezel. Wolfgang was a former graduate student of Janni's, making coordinating the paper submissions straightforward. Papers from Janni's and Wolfgang's labs, along with those from Mark Fishman's lab (MGH) and Frederick Bonhoeffer's lab (Tübingen), were submitted and published together in 1996.

These large-scale screens had the desired effect, fueling the expansion of the field. Janni's vision was fulfilled, and my goal achieved, as I had bet my career on it. The National Institutes of Health and the Sanger Center joined the effort and the field took off. What good fortune it all turned out to be! This initially small field had the advantage of everyone knowing everyone, leading to many



Slides at the Tübingen lab. Left: top two shelves in the warm room closet contained F3 embryo dishes to be screened. Lower shelf contains retest crosses of previous mutants found. Right: Janni's printed schedule for the screen. Note the yellow tab 'You are here' midway on left.

collaborations and openness in exchanging new advances. As the new vertebrate model on the block, there was curiosity and skepticism about zebrafish. I remember my ten faculty job interviews as largely questioning sessions about how to do this, that or the other in zebrafish; there was still much to work out. We all wanted this new zebrafish field to succeed, which I believe contributed to the amazing camaraderie and collegiality that remains today.

Over the years, zebrafish research has expanded far beyond developmental biology to disease modeling, regeneration, behavior, physiology, etc. The field has leaped further by technical advances, from the Sanger genome sequence to CRISPR genome editing. Scientists from other disciplines now take advantage of this versatile, inexpensive, vertebrate genetic model with wonderful live imaging. I look forward to what the next 25 years brings!

Oh, the places you'll go, little fish!**Joaquín Navajas Acedo (he/him)**

Zebrafish is an outstanding model organism: fish produce hundreds of eggs per clutch, their embryos develop rapidly outside the mother, they are transparent and amenable to genetic and pharmacological manipulations, and they share physiological and genetic similarities with humans.

This sentence will probably be familiar to many people that have read a publication or grant that uses zebrafish to tackle a biological problem. It summarizes the reason why zebrafish first won George Streisinger's attention and why zebrafish keeps being a popular organism in research.

The truly ground-breaking work achieved in Tübingen and Boston pioneered the path for those of us that were born too late to participate in it. In my case, at least, I have been lucky and privileged to be a member of laboratories whose work is rooted in both places: first, in Tatjana Piotrowski's lab as a PhD student; and now as a postdoc in Alex Schier's lab. Every time I attend a zebrafish meeting, I still find it a great experience putting a face to people whose work I have read and I deeply respect, and I still remember how warm and welcoming the zebrafish community was when I joined.

25 years have passed since the *Development* zebrafish issue, which is full of treasures that I always love reading, and I recommend that everybody takes a look. I think it is full of fantastic final visionary statements such as:

We expect that once the genes identified by mutations are mapped and eventually cloned, a number of novel mechanisms will be discovered, and important insights into previously untractable [sic] processes will be achieved (Haffter et al., 1996)

and:

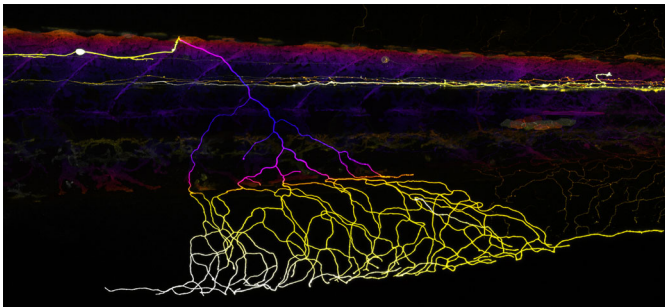
efficient ways to screen genomic regions...for sequences expressed in the affected structures of the embryo by in situ hybridization, cloning of mutant genes will hopefully soon be a routine procedure similar to those in invertebrate genetic model systems (Driever et al., 1996)

From the time when the zebrafish issue was published, the zebrafish genome has been sequenced, and old and new tools, such as *in situ* hybridization, microinjection of mRNAs and morpholinos, cell transplants, fluorescent transgenics or genome editing (ZFNs, TALENs and CRISPR/Cas9), have become our allies for interrogating the invisible. Since then, the zebrafish system has undoubtedly contributed to our understanding of embryogenesis, patterning, morphogenesis, cell differentiation, regeneration or complex behaviors in health and disease. We can now achieve

unprecedented genomic, molecular, cellular and organismal resolution to study development in space and time, and I truly believe it is an exciting time to be a developmental biologist that uses zebrafish in their research. New fluorescent proteins, faster reporters of cellular processes, more precise lineage tracers and genome editors, high-throughput transcriptional and chromatin assays, bioinformatic tools, biophysical theories and modeling – and people with new and innovative backgrounds – open a new era in zebrafish and biology as a whole.

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I will always remember the first time I took a time-lapse video of a fluorescent zebrafish or how I spent an entire day watching somites pop up one after each other, and how exciting and beautiful it was. As a developmental biologist who's now been working with zebrafish for 8 years, I can't help but feel a privilege of being present relatively close to the beginning and to wonder what marvels the future will bring (dear reader: some mutants of the screen have not even been mapped yet!). I hope I can contribute intellectually, play a part in making everybody feel welcome and help other people feel as excited as I am about this little fish.



Anamniote Rohon-Beard somatosensory neurons. These neurons detect peripheral stimuli, such as pain, touch or temperature, using their elaborated sensory arborizations. The image depicts a clonally labeled neuron in a 5-day-old zebrafish larva using a *neurog1:GFP-CAAX* construct (a kind gift from Anand Chandrasekhar, Bond Life Sciences Center, University of Missouri, USA) color coded with a depth look-up table (LUT).

The rise of zebrafish

Rashmi Priya

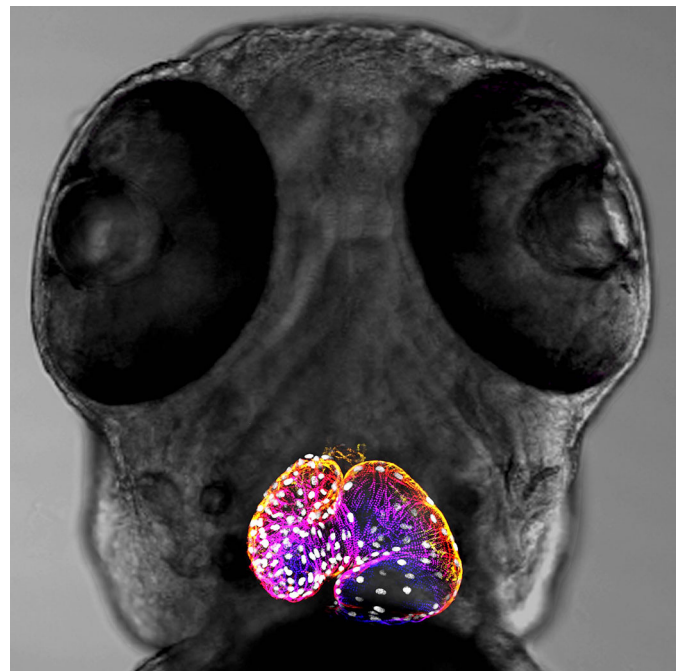
It's quite a remarkable tale how zebrafish, a favorite pet in home aquariums, emerged as one of the prominent model organisms in developmental biology. They were first described in the early 1800s by Francis Hamilton as beautiful fish with blue and silver stripes found in the gangetic planes of north-east India – where I was born (Parichy, 2015; Hamilton, 1822). In the late 1960s, George Streisinger brought zebrafish from commercial suppliers to his lab in Oregon to study the genetics of vertebrate neuronal development. His landmark paper describing genetic methods to generate homozygous diploid clones of zebrafish was published in 1981 (Streisinger, et al., 1981), and this was the birth of 'zebrafish genetics'. Fast forward to 1993, the beginning of 'The Big Screen', a tour de force effort led by Christiane Nüsslein-Volhard in Tübingen, Germany, and Wolfgang Driever and Mark Fishman in Boston, USA. Three years later, their monumental efforts culminated in 37 papers, describing genetic mutations affecting almost every developmental

aspect of zebrafish, which were published in a dedicated issue of journal *Development* (December 1996). These papers changed the course of developmental biology forever and the humble zebrafish rose to prominence as a developmental model system. Since then, the field of zebrafish research has come a long way, with now nearly 1000 labs taking advantage of this organism to discover the fundamental principles of health and disease.

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My tryst with zebrafish is a recent one. In the autumn of 2016, I moved to Didier Stainier's lab to start my postdoc. I remember being completely overwhelmed, because I had no prior experience with zebrafish. So, in one of the meetings I asked Didier, where should I start? He suggested I look at *The Zebrafish Book* and *The Zebrafish Information Network* (ZFIN; <https://zfin.org/>), and said 'just start looking at the embryos under the microscope'. And, as much of a cliché as it might sound, I could watch those 22-somite embryos twitching inside their chorions for hours. I have continued this practice in my lab. My PhD student, Christopher Chan, has just started and we are simply observing zebrafish embryos growing under a microscope every day. I am reliving the excitement of autumn 2016!

For a long time, zebrafish have served as an excellent model system for genetics research and have revolutionized our understanding of the developmental mechanisms. However, in recent years, we have started to realize its potential for studying *in vivo* cell biology and complex morphogenetic problems. Zebrafish embryos are transparent, accessible and have fully formed functional organs by



Heart of a 3 days post fertilization zebrafish embryo. This *myl7:MYL9-mScarlet*; *myl7:H2B-GFP* transgenic zebrafish line labels cardiomyocyte cytoskeleton (*myl7:MYL9-mScarlet*, depth color-coded) and cardiomyocyte nuclei (*myl7:H2B-GFP*, gray).

5 days post-fertilization. One can actually visualize finer anatomical details such as valve or trabecular layer using a relatively simple compound microscope (Haffter, et al., 1996; Stainier, et al., 1996). With the advent of state of the art microscopic techniques, precision genetic tools to generate fluorescent reporters and biophysical manipulations/measurements, we can perform 4D whole-organ imaging at single-cell resolution, manipulate molecular signals in a controlled manner, and quantify subcellular forces and dynamics in a living functional organ (Bornhorst, et al., 2019; Cayuso, et al., 2016; Fukui, et al., 2021; Gebala, et al., 2016; Gunawan, et al., 2019; Mickoleit, et al., 2014; Munjal et al., 2020 preprint; Priya, et al., 2020; Yang, et al., 2018). Thus, zebrafish is bridging the gap between developmental genetics analysis and quantitative cell biology. We are at a turning point with a suite of modern quantitative tools and powerful theoretical approaches available to us. Thus, I would argue, now is the best time to use zebrafish and study the multi-scale complexity of tissue and organ morphogenesis, in its native state, thus bringing this powerful model system closer to its full potential.

The awesome power of forward genetics: from fruit fly to zebrafish

Lilianna Solnica-Krezel

The 1980 Nature reprint by Christiane Nüsslein-Volhard and Eric Wieschaus, reporting systematic searches for embryonic lethal mutations that revealed the genetic logic of fruit fly embryogenesis (Nüsslein-Volhard and Wieschaus, 1980), reached me in Poland. For an undergraduate genetics enthusiast at Warsaw University, the ability of forward genetics to unravel an interacting set of genes that create an animal body plan was captivating. This work inspired my imagination and set my scientific course.

In 1991, my confidence in the power of forward genetics to dissect vertebrate gastrulation brought me to the nascent Wolfgang Driever lab in Boston to establish efficient methods for germline mutagenesis and conduct a systematic search for zygotic embryonic lethal mutations in zebrafish. As the first mutagenized lines were growing, the ‘screeners’ wondered about prospective phenotypes.

After all, such a large-scale screen had not been performed in vertebrates. What would embryonic lethal phenotypes look like? Would mutants phenocopy microsurgical removal of embryonic organizers? Could mutations separate embryonic patterning and morphogenesis? After innumerable crosses, microscopic inspection of millions of embryos and complementation testing by fantastic collaborative efforts of Nüsslein-Volhard (Box 1) and Driever (Box 2) lab trainees, the Tübingen and Boston screens identified thousands of mutants in hundreds of genes, and the answers to our prescreen questions were reported in the 1996 zebrafish issue of Development.

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The typical embryonic lethal phenotype quickly became apparent with almost every mutagenized family yielding progeny exhibiting massive cell death in Mendelian proportions. The earliest onset was during segmentation – degeneration first appeared in the brain and spread caudally. Degeneration phenotypes constituted about 20% of mutants. It also became clear that the zygotic mutations predominantly affected organogenesis: heart, blood, kidney, eye and ear, warranting individual manuscripts. Vertebrate-specific developmental phenotypes, such as notochord anomalies, were also frequent, featuring absent, twisted or incompletely differentiated notochords.

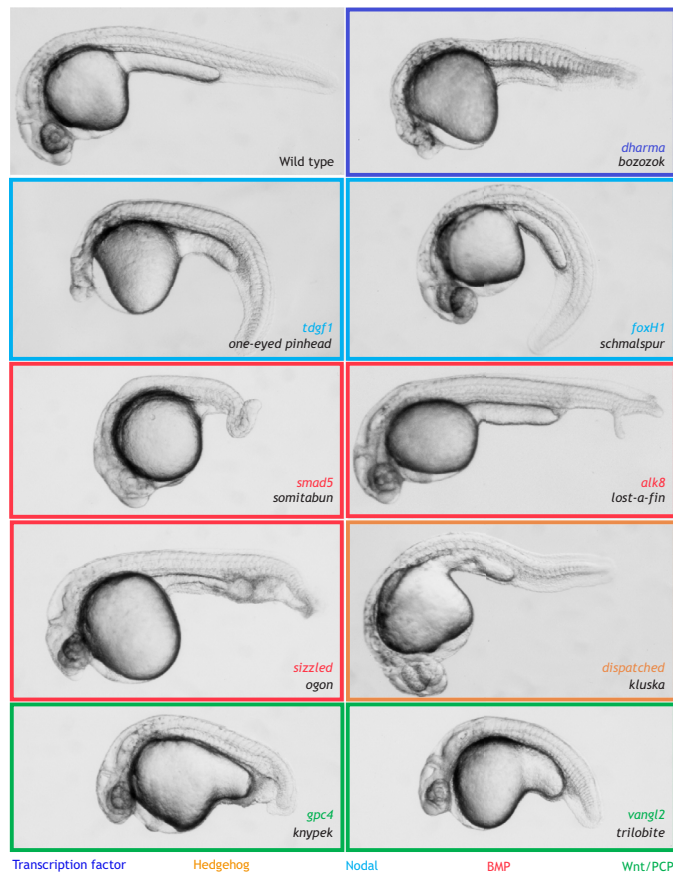
Although less frequent, gastrulation mutants were highly informative (Hammerschmidt et al., 1996a,b; Kane et al., 1996; Mullins et al., 1994; Solnica-Krezel et al., 1996). The most severe *bozozok* mutants lacked notochord, prechordal plate, eyes and telencephalon, thus resembling embryos after extirpation of the zebrafish Spemann-Mangold organizer. Accordingly, *bozozok* was later shown to encode a transcriptional repressor, essential for the organizer formation. Mutations in several genes, including *one eyed-pinhead* and *schmalspur*, caused synophthalmia or cyclopia, and prechordal plate, endoderm and floor plate deficiencies, were shown to affect Nodal signaling components. Another phenotypic class, including *ogon* and *chordino*, exhibited excess posterior and ventral tissues (tail, blood), at the expense of dorsoanterior fates, and had rounder gastrula shapes. Conversely, dorsalized mutants presented with an elongated gastrula shape and deficiencies of ventroposterior tissues. These ventralized and dorsalized mutants identified bone morphogenetic protein (BMP) signaling components, and, intriguingly, affected both patterning and gastrulation morphogenesis, raising the question of how these processes are coordinated. Another phenotypic class of ventrally curved mutants, defined by *kluska*, affects Hedgehog signaling. Each pathway provided part of the system instructing the vertebrate body plan.

Yet some mutations appeared to affect gastrulation movements without overt patterning and cell specification defects. In *half-baked* and *volcano* mutants, epiboly, the spreading of embryonic tissues around the yolk cell, was halted, followed by embryo dissociation, which was later linked to inactivation of E-cadherin cell-adhesion molecule. *trilobite* and *knypek* mutants exhibited normally patterned, but shorter and broader, bodies, owing to impaired

Box 2. The Boston screen team – Wolfgang Driever’s laboratory

From the left: Jarema Malicki, Fried Zwartkruis, Jane Belak, Stephan Neuhaus, Derek Stemple, Alex Schier, Wolfgang Driever (sitting), Jeanine Downing, Eliza Mountcastle-Shah, Lila Solnica-Krezel and Michelle Harvey. Not in the picture: Salim Abdulillah Seyfried, Colin Boggs and Zehava Rangini, as well as Michael Pack and Didier Stainier from Mark Fishman’s lab, who screened for intestinal and cardiovascular phenotypes, respectively.





Phenotypic classes of gastrulation mutants found in the Boston screen.
From Solnica-Krezel et al. (1996).

convergence and extension gastrulation movements. Mutations in this class, we learned, inactivate Wnt/planar cell polarity (PCP) pathway components. Therefore, the systematic searches for chemically induced embryonic lethal mutations in zebrafish identified phenotypic classes, which defined the major signaling pathways regulating vertebrate embryo patterning and morphogenesis. The zebrafish screens rewarded the risks taken by the screen team members – I left Boston with a suitcase of my favorite gastrulation mutants to start the next adventure of learning what these mutants can teach us about mechanisms of gastrulation and embryogenesis in my independent laboratory. The unprecedented mutant collection from the Tübingen and Boston screens advanced the pioneering efforts of George Streisinger, Charles Kimmel, Monte Westerfield and Judith Eisen at the University of Oregon, and fueled rapid expansion of the zebrafish research around the world.

25 years ago – surely not! Stephen W. Wilson

So, it seems that 25 years have passed since *Development* published 37 papers describing mutants from the Tübingen and Boston screens. Back then, I was one of the relatively few zebrafish researchers who was not involved in either screen, nor had I worked at the University of Oregon, where zebrafish were first established as a laboratory model animal. Rather, a few years before the screens started, I moved from London to the University of Michigan to join Steve Easter's lab to study eye development in zebrafish. For my first experiment, I inadvertently pushed a tracer injection needle a

little too deep and labelled neurons in the brain – this set the course for my entire future career. Among the wonderful friends I made at that time were Ajay Chitnis and Hitoshi Okamoto, who were both working on fish in John Kuwada's lab next door. Ajay later joined Wolfgang Driever's lab in Boston to participate in a follow up screen to identify mutations affecting neurogenesis. I moved back to London in 1991 to set up my own lab around the same time that I started to hear exciting rumors about screens starting in Janni's and Wolfgang's labs.

Our research needed mutants! We had a couple of good ones, but not enough. In the early 1990s, the first central nervous system patterning genes were being identified and whole-mount *in situ* hybridization was an exciting new technique. Consequently, most of our experiments around that time involved asking what the expression of our latest new gene looked like in *cyclops*, *notail* and *spadetail* mutant embryos. As only one of these mutants affected the brain, we weren't making a whole lot of headway into using mutants to understand forebrain development.

I can still remember the excitement when Chris Wylie sent manuscripts destined for the 1996 special issue to Nigel Holder and me to review – at no time since has the simultaneous arrival of multiple reviewing tasks elicited such elation! Yes, dear authors, I was the oh-so-fussy 'reviewer 2' on a bunch of those papers. In an instant, I could see the research field opening up – zebrafish were about to 'make a splash', as Judith Eisen would say in her review of the issue (Eisen, 1996). It was an amazing time to be in the field. As Mary points out in her commentary, we all knew each other and, more than that, for a brief time, we all knew about pretty much all the research that was being carried out across the entire burgeoning zebrafish research community. How that has changed!

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My decision to work with zebrafish was based on the beautiful papers coming from the Oregon labs in the 1980s, but the success of my career has been in no small part due to the 'big' screens and those who participated in them – friends and colleagues from that day to this.

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Competing interests

The authors declare no competing or financial interests.

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