PART II

Chemical Screens

CHAPTER 21

Chemical Screening in Zebrafish for Novel Biological and Therapeutic Discovery

Justin L. Tan and Leonard I. Zon

Stem Cell Program and Division of Hematology and Oncology, Children's Hospital Boston, Dana-Farber Cancer Institute, Howard Hughes Medical Institute and Harvard Medical School, 1 Blackfan Circle, Boston, Massachusetts, USA

Abstract

Zebrafish chemical screening allows for an *in vivo* assessment of small molecule modulation of biological processes. Compound toxicities, chemical alterations by metabolism, pharmacokinetic and pharmacodynamic properties, and modulation of cell niches can be studied with this method. Furthermore, zebrafish screening is straightforward and cost-effective. Zebrafish provide an invaluable platform for novel therapeutic discovery through chemical screening.

METHODS IN CELL BIOLOGY, VOL 105

0091-679X/10 $35.00
DOI 10.1016/B978-0-12-381320-6.00021-7

I. Introduction

In the past decade, many successful therapeutics have been efficiently discovered by cell-based and biochemical drug screening. However, these screening methods do not consider *in vivo* small molecule activity. Potential therapeutics from such screens often do not pass *in vivo* testing in live organisms such as mice, since they have inherent toxicity and poor pharmaco-properties undetectable by the screening process. Also, small molecules may act differently in whole organisms due to their complex biology, as compared to more straightforward biology in cell cultures and purified proteins. Such screens are encountering problems with proteins that are difficult to target, such as transcription factors. These proteins are termed "undruggable," since they are inept in binding small molecules and often carry out their functions through protein–protein or protein–DNA/RNA interactions.

Zebrafish chemical screening can address the problems inherent in cell-based and biochemical screens (Fig. 1). Screening in a whole organism context means drug toxicity and *in vivo* drug effects are addressed concurrently. Whole organism screening has the advantage of being less targeted than cell-based and biochemical screens, allowing the drug to interact with any biological pathway. The readout is an alteration of a whole organism phenotype that relates well to disease. In contrast, protein–compound binding or cell-based reporters give little indication of disease phenotype modulation. Furthermore, technological advances have made zebrafish screens straightforward and cost-effective. It has been about a decade since the first zebrafish screen was attempted, and already, a number of potential therapeutics have been discovered that target processes ranging from hematopoiesis to cancer (Table I). Zebrafish screening might also provide the ability to discover therapeutic modulators of "undruggable" processes, as it explores biology to a complexity unseen in cell-based or biochemical screens. Overall, zebrafish screening is a convenient and ideal technology for novel therapeutic discovery.

II. Rationale

Zebrafish screening allows for high-throughput chemical genetics *in vivo*. This is its greatest advantage over cell-based and biochemical screening. Screening chemicals in the context of the whole organism allows for unique phenotypes to be screened for, other than the traditional alteration of cell state in cell-based assays or target identification in protein-binding biochemical assays. Furthermore, small molecules are screened in the context of the complex biology of the whole organism. This allows for assessment of (1) compound toxicity, (2) chemical alteration by metabolism, (3) drug pharmacokinetics and pharmacodynamics,

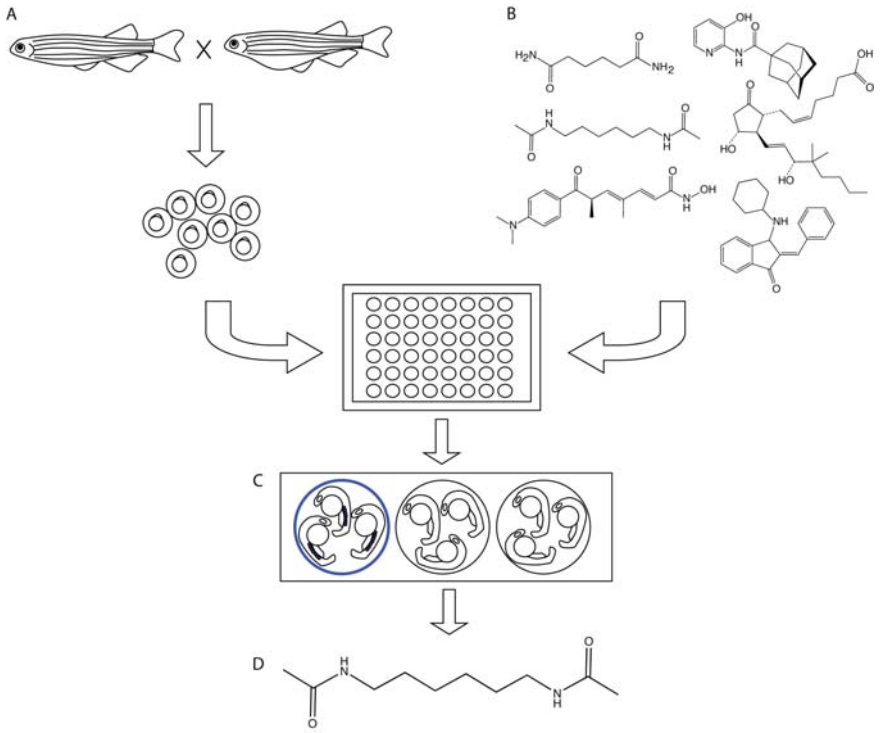

Fig. 1 Chemical screening workflow. (A) Zebrafish are crossed to generate embryos that are distributed by hand into the required plate format. (B) Chemical libraries are then added to the wells. (C) After the required incubation time, individual wells are scored for the appropriate phenotype. (D) This identifies the small molecule(s) that elicit(s) the phenotypic change. (For color version of this figure, the reader is referred to the web version of this book.)

and (4) drug modulation of cell niches (Wheeler and Brändli, 2009; Zon and Peterson, 2005).

In addition, zebrafish embryonic screening is reasonably cost-effective, straightforward, and biologically relevant (Wheeler and Brändli, 2009; Zon and Peterson, 2005). Firstly, fish husbandry requirements are straightforward and embryos are easily obtained in large numbers of 200–300 per mating pair. Secondly, embryos develop *ex utero* so their development can be monitored easily. Thirdly, embryos are more easily manipulated under a microscope. Fourthly, embryos can be screened at stages with no pigment, so phenotypes are easily observed. Fifthly, drug targets between humans and zebrafish are conserved. Sixthly, *in vivo* toxicity is observed, eliminating hits that are poor drug candidates. With the feasibility of high-throughput screening and the advantages associated with *in vivo* drug assessment, the zebrafish are an ideal organism for whole organism-based therapeutic drug discovery.

Table I
Screening phenotypes

	Reference	Phenotype	Fish
Morphology (specific)	Alvarez *et al.*	Angiogenesis	*Tg(fli1:EGFP)*
	Cao *et al.*	Polycystic Kidney Disease	PKD mutants
	Hong *et al.*	Coarctation	*gridlock* mutant
	Kitambi *et al.*	Angiogenesis	*Tg(fli1:EGFP)*
	Mathews *et al.*	Fin regeneration	Wildtype
	Milan *et al.*	Heart rate	Wildtype
	Oppedal *et al.*	Fin regeneration	Wildtype
	Owens *et al.*	Hair cells	Wildtype
	Peterson *et al.*	Coarctation	*gridlock* mutant
	Shafizadeh *et al.*	Hematopoiesis	*Tg(gata1:EGFP)*
	Tran *et al.*	Angiogenesis	*Tg(VEGFR:GRCFP)*
	Wang *et al.*	Angiogenesis	*Tg(flk1:EGFP)*
	Xu *et al.*	Angiogenesis	*Tg(fli1:EGFP)*[a]
	Yu *et al.*	Dorsal-ventral axis	Wildtype
Morphology (nonspecific)	Das *et al.*	Body axis/cardiac defects	Wildtype
	Jung *et al.*	Pigmentation	Wildtype
	Khersonsky *et al.*	Brain and eye	Wildtype
	Moon *et al.*	Microtubule disruption	Wildtype
	Peterson *et al.*	Multiple organs	Wildtype
	Sachidanandan *et al.*	Multiple organs	Wildtype
	Spring *et al.*	Multiple organs	Wildtype
	Sternson *et al.*	Notochord	Wildtype
	Torregroza *et al.*	Body axis/cardiac defects	Wildtype
	Wong *et al.*	Cardiac defects	Wildtype
Behavior	Kokel *et al.*	Photomotor response	Wildtype
	Rihel *et al.*	Rest/wake	Wildtype
Cell state	Molina *et al.*	Dusp6 expression	*Tg(dusp6:EGFP)*
	Murphey *et al.*	Cell Cycle	*crb* mutant
	North *et al.*	Hematopoiesis	Wildtype
	Paik *et al.*	Hematopoiesis	*cdx4* mutant
	Stern *et al.*	Cell Cycle	*crb* mutant
	Yeh *et al.*	Leukemia (AML1-ETO)	*Tg(hsp:AML1-ETO)*

[a] Personal communication from respective authors.

III. Materials and Methods

A. Zebrafish Screen Scoring Phenotypes

There are many phenotypes that can be scored in an *in vivo* system. Zebrafish are easily manipulated to produce a number of different readouts in chemical screens (Table I). Each readout is suited for its specific study goal. Three types of phenotypic

scoring are available in zebrafish screening: developmental morphology (specific and nonspecific), behavioral and cell state scoring.

1. Developmental Morphology Scoring – Specific

A variety of observable developmental phenotypes have been characterized in zebrafish due to genetic or chemical perturbation. Specific morphology screens are conducted to generate hypotheses on a specific biological question. These screens are scored based on a chosen morphology change of interest. The aim of these screens is to discover specific chemical modifiers of disease or biological pathways (Alvarez *et al.*, 2009; Cao *et al.*, 2009; Hong *et al.*, 2006; Kitambi *et al.*, 2009; Mathew *et al.*, 2007; Milan *et al.*, 2003; Oppedal and Goldsmith, 2010; Owens *et al.*, 2008; Peterson *et al.*, 2004; Shafizadeh *et al.*, 2004; Tran *et al.*, 2007; Wang *et al.*, 2010; Xu *et al.*, 2010; Yu *et al.*, 2007). Often, morphological changes are scored by eye but certain morphologies can be scored by automation. Automation is possible because investigators are looking for a specific phenotype, rather than a range of developmental defects. For example, in a chemical suppressor screen for inhibitors of polycystic kidney disease (PKD) zebrafish models, Cao *et al.* designed a computer algorithm that could identify modulation of laterality and curvature in embryos. The error rate was low at 2.2%, suggesting that automating morphology scoring is highly possible (Cao *et al.*, 2009). The screen identified histone deacetylase inhibitors as suppressors of the PKD phenotype, eliciting viable drug candidates for treating PKD. As illustrated by this example, investigating specific morphology changes focuses on one or a few disease pathways, and allows for a more directed and automated screening approach.

2. Developmental Morphology Scoring – Nonspecific

Less frequently conducted are nonspecific morphology screens that score any morphological change observed. These have been carried out to determine compound bioactivity in broad terms; the readouts being any observable perturbation of development (Das *et al.*, 2010; Jung *et al.*, 2005; Khersonsky *et al.*, 2003; Moon *et al.*, 2002; Peterson *et al.*, 2000; Sachidanandan *et al.*, 2008; Spring *et al.*, 2002; Sternson *et al.*, 2001; Torregroza *et al.*, 2009; Wong *et al.*, 2004). Often, in-house synthetic libraries containing one specific pharmacophore are screened on wildtype zebrafish embryos. Phenotypes are scored and characterized by eye. For example, Das *et al.* conducted a screen on wildtype embryos using a synthetic retinoid analogue library. Their aim was to discover novel retinoids that showed bioactivity *in vivo*. Hence, they nonspecifically scored any developmental defect they observed. This led to the discovery of BT10 which caused cardiovascular defects in fish, and which bound specifically to RAR receptors. This example highlights that undirected-phenotype screens are typically conducted with pharmacophore analogues, in order to discover

better tools or drugs in pathways already known to be modulated by small molecules.

3. Behavioral Scoring

Zebrafish movement, in response to stimuli, can be characterized. Changes in such movements can be scored for alteration by chemical perturbation. Currently, zebrafish have been screened for psychotropic and neuroactive drugs by characterizing changes in their photomotor response (PMR) (Kokel *et al.*, 2010) or rest/wake behavior (Rihel *et al.*, 2010). This type of screen showcases the robustness of the zebrafish in identifying drugs that target complex pathways *in vivo*. Such drug discovery is impossible in *in vitro* screens which cannot recapitulate the biology of an entire organism. Phenotypes are scored by camera recordings and computer analyses. A behavioral screen conducted by Kokel *et al.* overcame the inability of cell-based and biochemical chemical screening to identify compounds that modulate the central nervous system (CNS). CNS biology manifests itself in organism behavior, so a convincing study involves the intact whole organism. Kokel *et al.* discovered that light-stimulating zebrafish embryos resulted in a PMR that could be easily barcoded. The PMR was recorded by a camera and barcoding was performed by custom computer scripts. A diverse collection of libraries, including neurotransmitters and ion channel binders were screened and scored for perturbation of the PMR. This study discovered novel neuroactive compounds at a highly efficient rate, illustrating the usefulness of the zebrafish in neuroactive and psychotropic drug discovery.

4. Cell State Scoring

Cell state is defined here as a molecular phenotype not evident to the naked eye. Examples include mRNA expression levels, protein phosphorylation, and cell mitotic state. When scoring a change in cell state, one requires a consequent secondary assay after chemical screening. The three secondary assays that have been applied to zebrafish cell state screens are (1) *in situ* hybridization (North *et al.*, 2007; Paik *et al.*, 2010; Yeh *et al.*, 2009), (2) immunohistochemistry (Murphey *et al.*, 2006; Stern *et al.*, 2005), and (3) fluorescent protein reporter expression (Molina *et al.*, 2009).

In situ hybridization involves hybridizing an mRNA specific probe to expressed mRNA transcripts in fixed embryos. A color reaction with the probe localizes expressed transcripts to specific tissues. In addition, color intensity provides a semiquantitative assessment of transcript levels in the tissue(s) of interest. North *et al.* utilized *in situ* hybridization to assess the expression levels of *cmyb* and *runx1*, two genes required for hematopoietic stem cell (HSC) development. They sought to discover modulators of HSC formation in their chemical screen. Thirty-five and forty-seven compounds increased or decreased *cmyb/runx1* expression respectively

in the screen. This resulted in the discovery that compounds that modulate prostaglandin E2 levels modulate overall HSC homeostasis.

Immunohistochemistry can be used to identify levels of modified proteins via specific antibodies. Two screens have been carried out with immunohistochemical readouts to serine-10-phosphrylated histone H3 protein (Murphey *et al.*, 2006; Stern *et al.*, 2005). Histone H3 serine-10 phosphorylation occurs in late G2 to early M phase and is dephosphorylated in anaphase (Hendzel *et al.*, 1997). Both screens were conducted on a *bmyb* zebrafish mutant to identify chemical suppressors of the *bmyb* phenotype. The *bmyb* mutant phenotype entails decreased cyclin B1, mitotic arrest, and genomic instability (Shepard *et al.*, 2005). Mitotic arrest in *bmyb* mutants results in an accumulation of antibody-detectable histone H3 phosphorylation (Murphey *et al.*, 2006; Stern *et al.*, 2005). The screen by Stern *et al.* identified a small molecule, persynthamide, which reduced histone H3 phosphorylation to wildtype levels, suppressing the *bmyb* phenotype.

It is also possible to screen transgenic zebrafish with a fluorescent reporter for compounds that modulate a pathway of interest. Molina *et al.* designed a screen based on *Tg(dusp6:EGFP)*^{pt6} embryos and looked for changes in EGFP fluorescence. This transgenic line reports on the fibroblast growth factor (FGF) signaling pathway, since Dusp6 is involved in feedback attenuation of this pathway (Thisse and Thisse, 2005; Tsang and Dawid, 2004). Molina *et al.* discovered a small molecule, BCI, which increased EGFP fluorescence in the embryos and further characterized BCI as a Dusp6 inhibitor.

Zebrafish cell state screening is very versatile, since different readouts can be scored, not limited to those mentioned above. This scoring type may also allow for automation since the readout is straightforward. There are potentially many more cellular pathways that can be efficiently studied with cell state screening.

B. Choice of Small Molecule Library

Three broad categories of small molecule libraries are available: commercial vendor libraries, natural product libraries, and synthetic libraries. The majority of zebrafish screens have utilized commercial libraries, specifically the subcategory of bioactive, annotated small molecules. A small number of screens have used personalized synthetic libraries to address specific issues (Table II). A description of each library is as follows.

1. Commercial Libraries

These libraries consist of small molecules adhering to Lipinski's rules. These rules describe chemical aspects of small molecules that give them good pharmacokinetics and dynamics. Compounds have low molecular weight, partition coefficient values that afford efficient membrane absorption, and a total number of hydrogen bond

Table II
Compound libraries

	Reference	Compound libraries
Morphology (specific)	Alvarez *et al.*	Regulators of angiogenesis
	Cao *et al.*	Bioactives (Sigma-Aldrich, Calbiochem, Biomol, and Cayman)
	Hong *et al.*	DIVERSet E (Chembridge)
	Kitambi *et al.*	Spectrum Collection (Microsource)
	Mathews *et al.*	Bioactives (Microsource)
	Milan *et al.*	Custom bioactives
	Oppedal *et al.*	Lopac 1280 (Sigma-Aldrich)
	Owens *et al.*	DIVERSet E (Chembridge)
	Peterson *et al.*	DIVERSet E (Chembridge)
	Shafizadeh *et al.*	DIVERSet E (Chembridge)
	Tran *et al.*	Lopac 1280 (Sigma-Aldrich)
	Wang *et al.*	Spectrum Collection (Microsource)
	Xu *et al.*	Synthetic xyloketals
	Yu *et al.*	DIVERSet E (Chembridge), Bioactives (Microsource), Others (Sigma-Aldrich)
Morphology (nonspecific)	Das *et al.*	Synthetic retinoids
	Jung *et al.*	Tagged triazines
	Khersonsky *et al.*	Trisubstituted triazines
	Moon *et al.*	Tagged triazines
	Peterson *et al.*	DIVERSet E (Chembridge)
	Sachidanandan *et al.*	DIVERSet E (Chembridge)
	Spring *et al.*	Synthetic biaryl-containing medium rings
	Sternson *et al.*	1,3-dioxanes
	Torregroza *et al.*	Synthetic chromenes
	Wong *et al.*	Synthetic 1,3-dioxanes
Behavior	Kokel *et al.*	DIVERSet (Chembridge), Spectrum Collection (Microsource), Prestwick (Prestwick), Neurotransmitter/Ion Channel/Orphan Ligand (Biomol)
	Rihel *et al.*	LOPAC 1280 (Sigma-Aldrich), Spectrum Collection (Microsource), Prestwick (Prestwick), Neurotransmitter/Ion Channel/Orphan Ligand (Biomol)
Cell state	Molina *et al.*	NCI diversity set, Natural Products (Microsource), Phosphatase-targeted (ChemDiv)
	Murphey *et al.*	DIVERSet E (Chembridge)
	North *et al.*	NINDS Custom, Spec Plus, ICCB bioactives (Biomol)
	Paik *et al.*	ICCB bioactives
	Stern *et al.*	DIVERSet E (Chembridge)
	Yeh *et al.*	Spectrum Collection (Microsource)

donors and acceptors within appropriate limits. These properties predict good bioavailability in organisms (Lipinski *et al.*, 2001).

2. Bioactive Libraries

These libraries are a subset of commercial libraries that are annotated with known protein targets, drug-like molecules, and bioactivity. These libraries are extremely useful in identifying small molecule targets after screening, since the target or pathway is already known. Also, screening such libraries can yield valuable information on multiple pathways in a disease phenotype. Bioactive libraries used in zebrafish screening include the DIVERSet E (Chembridge), the ICCB Known Bioactives (Biomol), the LOPAC1280 (Sigma-Aldrich), the NINDS Custom Collection (NIH/National Institute of Neurological Disease and Stroke), and the Spectrum Collection (MicroSource).

3. In-House Synthetic Libraries

Some labs synthesize their own compounds depending on their specific goals. In-house libraries are advantageous in (1) discovering novel bioactive small molecules and (2) straightforward target identification. Such libraries are based on known pharmacophores, so one can shortlist candidates of possible targets. The outcome of such a library screen is usually the discovery of novel bioactivity of an analogue of a known pharmacophore. An example of such a study is a zebrafish screen carried out with novel retinoid analogues that discovered a novel retinoid with retinoid receptor specificity. This lead compound is useful for probing the biology of the retinoic acid signaling pathways (Das *et al.*, 2010). A second aspect of a synthetic library is that compounds can be designed with tags, allowing for target identification through protein pull-down. Tagged compounds are screened in the zebrafish to confirm that the tag does not interfere with the bioactivity of the molecule. One such screen with a tagged-triazine library identified a novel inhibitor of mitochondrial ATPase, which induces pigmentation in early zebrafish embryos (Jung *et al.*, 2005). The pull-down protocol was straightforward since the tagged compounds were chemically ideal for binding resin. Also, the tag was already confirmed as noninterfering with target binding by the screen.

4. Diversity-Oriented Synthesis (DOS) Libraries

DOS libraries expand the boundaries of chemical space by the synthesis of novel pharmacophores (Schreiber, 2000). Such libraries encompass chemical space that is not covered by commercial libraries, hence providing greater potential for novel modulation of "undruggable" pathways and targets if screened. The application of DOS compounds in zebrafish screening has yet to be explored.

5. Natural Product Libraries

These libraries consist of compounds extracted from nature (Clardy and Walsh, 2004). Famous examples of therapeutics derived from natural products are cancer drugs like taxol from the Pacific Yew tree or antibiotics like penicillin from *Penicillium* fungi. Like DOS compounds, natural product libraries increase the potential of novel discovery in screening.

C. Chemical Screening Platform

The actual screen is performed in a specific order: First, a small-scale optimization screen is conducted to determine the appropriate screening parameters. Second, sufficient zebrafish embryos are generated. Third, the screen is carried out either by hand, for a small screen, or by automation for high-throughput. Fourth, hits are rescreened for validation.

The optimization screen is performed with a small number of embryos and compounds to determine optimal parameters such as desired plate format, compound concentrations, number of embryos per well, and embryonic stage. It is helpful if a compound that causes a positive phenotype in the assay is available. This would provide an ideal positive control and allow for compound concentrations to be fine-tuned, generating an obvious scoring phenotype without causing embryonic lethality. Determining the developmental stage at which embryos are screened is also important since this can affect the phenotypic readout.

Large numbers of zebrafish embryos are needed for high-throughput screens. Traditionally, these are generated by setting up large numbers of mating pairs in multiple tanks. This method takes up a lot of space, makes embryo collection tedious, and may not yield synchronized embryos. Recently, this bottleneck in zebrafish screening has been solved by the introduction of the zebrafish spawning vessel technology (Fig. 2). The zebrafish spawning vessel allows for over 200 fish of any given strain to be spawned simultaneously. This allows for collection of a maximum of 10,500 highly synchronized embryos with a typical spawning time of 10 min. In addition, the apparatus has a small footprint, saving lab space (Adatto *et al.*, in prep). Obtaining large numbers of synchronized embryos is now efficient and no longer limits the scale of chemical screening.

In addition to improving embryo collection, advances in technology have also made the handling of large compound libraries easy. Liquid handling robots, such as the TECAN robot (Tecan, Durham, NC), are used to distribute media and chemicals into plate wells rapidly and accurately. These robots are easily calibrated to operate for a range of compound volumes and plate formats. Unfortunately, there are currently no robots designed to distribute embryos accurately into plate wells. This step is performed by hand. However, since this task is highly analogous to liquid transferring, building an embryo-handling robot is a definite possibility in the near future.

Fig. 2 Zebrafish spawning vessel. More than 200 fish can be spawned simultaneously in the vessel, giving rise to ~10,000 highly synchronized embryos in a short time period. With its slim design, the apparatus takes up a small fingerprint. (For color version of this figure, the reader is referred to the web version of this book.)

Depending on the aim of the study, the mechanics of the screen can be conducted in a number of ways (Table III). There exists variation in the plate format, the number of embryos per well, and the compound concentration in each well. Embryos are distributed into a variety of plate sizes, ranging from 12-well up to 384-well transparent plates, with the common practice being three embryos per 96-well plate. Such plates are amenable to liquid handling robotics for compound library addition as well as automated image recording for high-throughput phenotypic readouts. Compound libraries are added to plates with well concentrations ranging from 1 to 100 μM, the common practice being 10 to 20 μM. Present library sizes range from 10 to 16,320. The size and compound composition frequently depend on the respective study goals.

Table III
Screening formats

	Reference	Embryos/well	Plate format	Compound concentration
Morphology (specific)	Alvarez *et al.*	5	48-well[a]	Varies
	Cao *et al.*	25	12-well	10 × IC50
	Hong *et al.*	3	96-well	2.5 μM
	Kitambi *et al.*	3[a]	96-well	5 μM
	Mathews *et al.*	2	96-well	25 μM
	Milan *et al.*	3–5	96-well	1, 10, 100 μg/mL
	Oppedal *et al.*	2	250 mL cups	10 μM
	Owens *et al.*	1–2	96-well	10 μM
	Peterson *et al.*	3	96-well	2 μg/mL
	Shafizadeh *et al.*	3–6	96-well	2 μg/mL
	Tran *et al.*	1	384-well	30 μM
	Wang *et al.*	5	96-well	10 μM
	Xu *et al.*	3	96-well	0.1–100 μM
	Yu *et al.*	3	96-well	5–10 μM
Morphology (nonspecific)	Das *et al.*	3–10	12-well	10 μM
	Jung *et al.*	3	96-well	10 μM
	Khersonsky *et al.*	3	96-well	50 μM
	Moon *et al.*	3	96-well	10 μM
	Peterson *et al.*	3	96-well	1 μM
	Sachidanandan *et al.*	3[a]	96-well	20 μM
	Spring *et al.*	3	96-well	10 μM
	Sternson *et al.*	Not available	Not available	60 μM
	Torregroza *et al.*	3–10	12-well	10, 100 μM
	Wong *et al.*	3	96-well	5–10 μM
Behavior	Kokel *et al.*	8–10	96-well	10–100 μM
	Rihel *et al.*	1	96-well	10–30 μM
Cell state	Molina *et al.*	5	96-well	10 μM
	Murphey *et al.*	20[a]	48-well	20 μM
	North *et al.*	5	48-well	10, 20, 50 μM
	Paik *et al.*	20	48-well	30 μM[a]
	Stern *et al.*	20	48-well	20 μM
	Yeh *et al.*	5	96-well	20 μM

[a] Personal communication from respective authors.

D. Target Identification (Target ID)

In order to develop effective therapeutics, a near complete assessment of the drug candidate is required. This involves identifying the compound's toxicity, pharmacokinetic and dynamics, and very importantly, its molecular target(s). A range of target ID methods have been applied to zebrafish chemical screens (Table IV). These include the traditional techniques of protein pull-down, cell-based assays, *in vitro* biochemical assays, and computer docking simulations. However, the most

Table IV
Target identification methods

	Reference	Target ID
Morphology (specific)	Alvarez *et al.*	Known targets
	Cao *et al.*	Known targets
	Hong *et al.*	Candidate-based
	Kitambi *et al.*	Known targets
	Mathews *et al.*	Known targets
	Milan *et al.*	Known targets
	Oppedal *et al.*	Candidate-based
	Owens *et al.*	Candidate-based
	Peterson *et al.*	Candidate-based
	Shafizadeh *et al.*	Candidate-based
	Tran *et al.*	Candidate-based
	Wang *et al.*	Known targets
	Xu *et al.*	Nil
	Yu *et al.*	Candidate-based, cell-based assay
Morphology (nonspecific)	Das *et al.*	Candidate-based, cell-based assay
	Jung *et al.*	Protein pull-down
	Khersonsky *et al.*	Protein pull-down
	Moon *et al.*	Candidate-based, biochemical assay
	Peterson *et al.*	Candidate-based
	Sachidanandan *et al.*	Candidate-based, cell-based assay
	Spring *et al.*	Nil
	Sternson *et al.*	Small molecule arrays
	Torregroza *et al.*	Candidate-based
	Wong *et al.*	Nil
Behavior	Kokel *et al.*	Known targets
	Rihel *et al.*	Known targets
Cell state	Molina *et al.*	Candidate-based, computational analysis, biochemical assay
	Murphey *et al.*	Known targets
	North *et al.*	Known targets
	Paik *et al.*	Known targets
	Stern *et al.*	Candidate-based
	Yeh *et al.*	Known targets

commonly used target ID method in zebrafish screens is candidate-based ID, which utilizes (1) annotated bioactive libraries, (2) chemoinformatics, and/or (3) genetics to elicit the target.

1. Candidate-Based Identification

Most screens address a specific biological question, such as perturbations to the hematopoietic system, pigmentation, or cardiovascular system. This allows one to

narrow down the list of possible pathways modulated by the small molecules screened. However, pinpointing the exact protein being targeted is difficult in the context of a whole organism, since traditional biochemical and cell-based target ID methods are not feasible due to the complexity of the organism. Candidate-based ID is achieved by using drug libraries with known targets, chemoinformatic analysis to infer targets, and/or genetic experimentation to infer modulated pathways. One or a combination of these methods is used to achieve a more complete understanding of the detailed biological effect(s) exerted by the drug candidate in question.

a. Annotated Bioactive Libraries

Annotated bioactive libraries are often used in chemical screens. These libraries contain therapeutic compounds with known targets and/or drug-like compounds. With these libraries, compound effects and targets are already known or can be easily predicted. This allows for straightforward target identification upon hit confirmation.

b. Chemoinformatics

When a hit with unknown targets is identified, it can be subjected to chemoinformatic analysis to predict its possible target(s). Often, from a chemical screen with diverse compound libraries, one finds interesting hit compounds that are either not well-annotated or unannotated. The simplest way to hypothesize the target pathway or protein of such compounds is to find well-annotated compounds with similar structural features. Classes of compounds with similar chemical structures typically target similar pathways. Chemoinformatics can compile structural similarity information for the hit compound of interest, allowing the researcher to use pre-existing structure–activity information from other similar molecules to hypothesize the possible activity of the compound of interest.

Chemoinformatic analysis can be applied by utilizing the many chemical databases with integrated search options. These databases compile useful information such as structural information, bioactivity, 3D molecular models, literature and patent links, material safety data sheets, and commercial availability. Some examples are PubChem, ChemBank, and CrossFire Beilstein. Searches can be performed with the chemical structure of interest to find analogous chemicals with known bioactivity. This might elicit related structures such as known pharmacophores or reactivity groups with biochemical activity. Also, hits obtained from screens performed by others can be compared for similar compound activity. In this way, the bioactivity of the hit compound can be predicted, so that the appropriate validation experiments can be conducted (Brown, 2005; Parker and Schreyer, 2004; Trompouki and Zon, 2010).

Chemical structures of search compounds can be entered into chemoinformatics tools in a variety of ways. The most common molecular format that gives detailed structural information in a highly simplified manner is the Simplified Molecular

Input Line Entry Specification (SMILES) format. The SMILES format involves a simple textual representation of chemical features such as bonds, aromaticity, stereochemistry, branching, and isotopes without the use of complicated chemical drawing software. This format allows for rapid data interpretation by computers with little ambiguity in the chemical structure. Other chemical formats used are the Chemical Markup Language (CML), GROMACS, CHARMM, the Chemical File Format, and the SYBYL Line Notation. To convert between the formats, one can use open source tools such as OpenBabel and JOELib. Integrated applets for drawing traditional two-dimensional chemical structures into the search engine are also available (Trompouki and Zon, 2010).

There are different search algorithms one can use to search chemical databases. Examples include the commonly used Tanimoto similarity scoring, the SEA algorithm, or the Tversky similarity algorithm. Some databases also use proprietary algorithms unique to their services. Different algorithms often lead to varying results so one might need to test more than one algorithm should the first one prove unsuccessful. Although not always consistent with each other, these algorithms are all equally important in hypothesis generation through the compilation of structurally similar compounds to the small molecule of interest. This information allows the researcher to test if the shortlisted compounds can phenocopy the compound of interest, thus narrowing down the activity of the compound of interest (Brown, 2005; Parker and Schreyer, 2004; Trompouki and Zon, 2010).

One particularly powerful online chemoinformatics tool is DiscoveryGate (http://www.discoverygate.com). DiscoveryGate uses a proprietary algorithm to search its compound databases, which encompass many sources such as journal articles, patent information, and commercial and proprietary chemical databases. One can perform structural or text-based searches on DiscoveryGate to obtain vast amounts of pharmacological and biological information on chemicals related to the search compound. This tool requires a flat fee for usage (Trompouki and Zon, 2010).

Chemoinformatics was used for target ID by Hong *et al.* In their chemical screen, Hong *et al.* obtained a hit compound GS4898 that rescued tail and trunk circulation in zebrafish *gridlock* mutant embryos. GS4898 had not been previously characterized so Hong *et al.* used chemoinformatics to predict that GS4898 might be a protein kinase inhibitor, since the molecule was structurally related to flavone kinase inhibitors. They then tested structurally related flavone kinase inhibitors and found that a specific phosphatidylinositol-3 kinase (PI3K) inhibitor phenocopies the rescue of the *gridlock* mutation by GS4898. This allowed Hong *et al.* to further validate that GS4898 did indeed inhibit PI3K.

c. Genetics

If the small molecule of interest is poorly annotated and chemoinformatics does not provide a viable hypothesis, one can perform genetic studies to predict the mode of action of the small molecule. Target ID via genetics first involves identifying a candidate pathway or pathways most likely modulated by the chemical. Secondly,

the expression levels of genes in each pathway are analyzed to see if they are perturbed by the chemical. Thirdly, if chemical inhibitors of various steps of the pathway are available, these can be used to see if known inhibitor treatment phenocopies the effect of the chemical of interest. If inhibitors are not available, gene knockdowns can be performed to try and phenocopy the effect of the chemical of interest. Genetic studies give evidence for what pathway the chemical of interest is modulating and serves as the basis for other follow-up biochemical and cell-based experiments directed at a specific pathway.

Gene expression changes can be measured in a number of ways. Microarrays are used to generate a large data-set for gene expression changes encompassing a myriad of pathways. This is useful when either (1) a significant number of pathways are responsible for the phenotypic change or (2) the pathways responsible are unclear. In cases where a small number of candidate pathways can be shortlisted, real-time PCR is used to evaluate the expression changes of individual genes in the pathways of interest. *In situ* hybridization, which allows for visual observation of gene expression changes in an intact zebrafish, allows for assessment of spatial changes in gene expression if present.

Yu *et al.* took a multistep genetics approach to identify the target of dorsomorphin, a chemical they obtained from their screen that looked for small molecule effectors of zebrafish embryo dorsalization. The dorsoventral axis is established by bone morphogenic protein (BMP) signaling gradients, and excess BMP signaling causes ventralization while reduced BMP signaling causes dorsalization (Fürthauer *et al.*, 1999; Mintzer *et al.*, 2001; Mullins *et al.*, 1996; Nguyen *et al.*, 1998). The screen by Yu *et al.* was based on the hypothesis that BMP signaling antagonists would cause dorsalization in zebrafish. To identify the target of their chosen molecule, dorsomorphin, they first performed *in situ* hybridization to investigate the effects of dorsomorphin on dorsal and ventral gene markers. They noticed that the level of ventral marker *eve1* was reduced while dorsal markers such as *egr2b* and *pax2a* underwent lateral expansion during dorsomorphin treatment. Since dorsomorphin phenocopies BMP antagonism in fish, the second experiment they performed was to use dorsomorphin treatment to rescue zebrafish that were deficient in the endogenous BMP antagonist, chordin. Dorsomorphin was able to rescue the phenotype of *chordin* morphants, thus validating that dorsomorphin is indeed a BMP antagonist. From this genetic evidence, Yu *et al.* proceeded with biochemical and cell-based assays to show that dorsomorphin inhibits SMAD-dependent BMP signals and BMP type I receptor function.

2. General Target ID Methods Applied to Zebrafish Screens

a. Protein Pull-Down

This method is relatively straightforward for target ID. It involves immobilizing a compound of interest to resin, by means of a chemical linker group, and incubating the resin with cell lysates. This allows intracellular binders to associate tightly with

the immobilized compound, pulling it out from suspension. The resin is then washed to remove nonspecific binders, and the bound proteins are analyzed by mass spectrometry to identify them. Protein pull-down is often used with synthetic libraries, which are screened with a linker group already present on the compounds. This ensures that *in vivo* activity is unaffected by the linker. Compounds from other libraries are more difficult to modify for resin linkage.

b. Cell-Based Assays

Cell-based assays are used to confirm that drug candidates can bind their target *in vivo*. Such an assay would involve addressing a specific target of interest in a cellular environment. For example, if the hit compound is hypothesized to inhibit an enzyme that phosphorylates a certain substrate, then one can design an experiment that indicates that compound addition inhibits substrate phosphorylation. Cell-based assays are used as a confirmation for target ID, rather than for broad spectra target discovery.

c. *In vitro* Biochemical Assays

Similar to cell-based assays, *in vitro* biochemical assays are more suited to confirm targets than for discovering them. Biochemical assays are nearly identical to cell-based assays, but do not involve complex cell biology. These assays require purification of the target of interest and if necessary, the target's substrate. The ability of the target to carry out its function on its substrate is then assessed under *in vitro* conditions with and without the small molecule.

d. Computer Docking Simulations

If crystal structures of targets are available, computer modeling can be performed to study the possibility of small molecules binding to their targets. Molina *et al.* use this method in their analysis of Dusp6 inhibition by BCI. Since the crystal structures of Dusp catalytic sites exist (Almo *et al.*, 2007; Jeong *et al.*, 2006, 2007; Stewart *et al.*, 1999), Molina *et al.* could ascertain the probable binding site of BCI to Dusp6 with a program called ORCHESTRAR (Tripos).

IV. Discussion/Caveats

A. Biological Relevance of Zebrafish Screening

Multiple phenotypes can be observed in zebrafish chemical screening. One can observe behavioral changes such as sleep/wake patterns or a movement response to light. In addition, one can also observe changes in gene expression either overall or in specific tissues due to chemical action. Such observations are not

possible in cell-based or biochemical screening platforms. Zebrafish screening allows for exploration into the behavioral effects of small molecules, something only whole organism-screens can achieve. In addition, gene expression changes are observed *in vivo* so this reflects accurately the biologically relevant action of the molecules. In zebrafish screening, there is no doubt that small molecules exert an effect in the context of a multicellular organism with active metabolism. This cannot be said for cell-based or biochemical platforms where further *in vivo* testing is required to confirm biological relevance. Compound toxicity or side effects are also not readily apparent in cell-based screens, unlike in zebrafish screens (Zon and Peterson, 2005).

Zebrafish chemical screening accounts for the biological response of cell niches. This allows one to conclude that phenotypic changes resulting from the chemical are relevant in a multicellular environment. Conversely, traditional biochemical and cell-based screens only indicate chemical activity on a specific target or cell type, ignoring the interactions of the cellular niche and the metabolic activity of the whole organism. Some chemicals undoubtedly exert phenotype change due to modulation of the surrounding cells, rather than the cell type in question. Also, phenotypic change can occur by modulation of a wide range of cell types. *In vivo* screening can elicit hits that *in vitro* screens cannot pick up. One might therefore observe different sets of hits and/or unexpected outcomes when comparing *in vivo* and *in vitro* screens. Hits from the zebrafish screen are more biologically relevant since phenotypes are due to chemical action on the whole organism instead of on one protein or one cell type.

Drug effects in humans are largely conserved in zebrafish, so zebrafish are ideal for human therapeutic discovery. Data from previous zebrafish chemical screens has shown a high degree of conservation between mammals in zebrafish, in terms of drug effects and toxicities. Cardiotoxicity screening has shown a high degree of correlation between humans and zebrafish (Milan *et al.*, 2003). In addition, screens to discover novel neuroactive and psychotropic drugs detected known human drugs with similar effects on zebrafish (Kokel *et al.*, 2010; Rihel *et al.*, 2010). Furthermore, 50–70% of chemicals in a zebrafish cell cycle screen show similar effects when tested in a mammalian cell culture assay (Zon and Peterson, 2005). As such, zebrafish screening is very relevant when applied to novel human therapeutic discovery.

B. Screening Technology Caveats

Compound concentrations are fixed per screen, so some compounds that should be classified as hits may be left out because they are not effective at the screening concentration. Others might cause toxicity at the screening concentration and are also ignored, even if they might be nontoxic and effective at lower doses.

In addition, certain phenotypes are not amenable to high-throughput screens due to specific scoring requirements. One example is fin regeneration, where workflow dictates that only small libraries of less than 2000 compounds are feasible for

screening (Mathew *et al.*, 2007; Oppedal and Goldsmith, 2010). In these studies, juvenile/adult fish require a tail clip before compounds are added. Scoring requires anesthetizing of individual fish for observation of tail regeneration. This workflow is difficult to automate, so screening larger chemical libraries is prohibited.

C. The Feasibility of Juvenile/Adult Chemical Screens

Nearly all chemical screens have been performed on embryos at various stages of development while juvenile/adult screens are rare. There are a number of caveats associated with screening fish at later developmental stages. Firstly, juvenile/adult fish screens require embryos to fully develop, adding time to the screen. More resources are thus needed to maintain the fish while they develop. Secondly, juvenile/adult fish are less easily manipulated as they are more mobile, but this can be solved by anesthetics like tricaine. Thirdly, juvenile/adult fish are pigmented so observation of phenotypes is more challenging than unpigmented embryos. However, this can be overcome by using the transparent fish line *casper*, where pigmentation is ablated (White *et al.*, 2008). Fourthly, suppressor screening in fish with a mutation that is embryonic lethal is impossible in juveniles/adults. Significant technological advancement is required for high-throughput adult zebrafish screening to become feasible.

D. Hit Detection Caveats

Hit rates from all zebrafish studies range from $<1\%$ to 70% (Table V). This can be influenced by scoring system and zebrafish biology. As mentioned previously, there are three different scoring types: morphological, behavioral, or cell state alteration. Using a different scoring type can lead to vastly different results. Also, human judgment plays an important role in what is scored as a hit. Since it is difficult to find two or more individuals with identical interpretation of scoring phenotype, human error introduces more variability into the screen.

In addition, variability in screening results is observed when similar chemicals are applied at different stages of development. There are a number of possible entry sights for small molecules into the fish. In embryos, chemicals permeate through the chorion and uptake can occur through the epidermal layer or through the digestive system. In the larval and adult stage, entry points are similar with the inclusion of uptake through gills. The epidermal layer of the larvae is much less permeable than that in developing embryos, so one can surmise that the distribution of compound uptake by each of the aforementioned means is different depending on the stage of development. This distribution affects the action of small molecules since differing chemical modifications occur when compounds are subjected to varying cellular environments. Compounds that enter the digestive tract are subject to first-pass metabolism that often alters their chemical properties. Compounds that permeate through the epidermis or that are administered intravenously typically undergo little

Table V
Hit rates

	Reference	Number of compounds	Number of hits	Hit rate (%)
Morphology (specific)	Alvarez *et al.*	11	1	9
	Cao *et al.*	115	17	15
	Hong *et al.*	7000	4	0.06
	Kitambi *et al.*	2000	5	0.3
	Mathews *et al.*	2000	17	0.9
	Milan *et al.*	100	22	22
	Oppedal *et al.*	520	2	0.4
	Owens *et al.*	10,960	2	0.02
	Peterson *et al.*	5000	2	0.04
	Shafizadeh *et al.*	5000	4	0.08
	Tran *et al.*	1280	3	0.2
	Wang *et al.*	2000	7	0.35
	Xu *et al.*	22	14	64
	Yu *et al.*	7570	1	0.01
Morphology (nonspecific)	Das *et al.*	22	3	14
	Jung *et al.*	1536	1	0.07
	Khersonsky *et al.*	1536	1	0.07
	Moon *et al.*	109	3	2.8
	Peterson *et al.*	1100	11	1
	Sachidanandan *et al.*	5760	175	3
	Spring *et al.*	1412	1	0.07
	Sternson *et al.*	1300	1	0.08
	Torregroza *et al.*	10	7	70
	Wong *et al.*	384	1	0.3
Behavior	Kokel *et al.*	14,000	982	7
	Rihel *et al.*	5648	547	10
Cell state	Molina *et al.*	5220[a]	1	0.02
	Murphey *et al.*	16,320	29	0.2
	North *et al.*	2480	82	3
	Paik *et al.*	2640	2	0.08
	Stern *et al.*	16,320	30	0.2
	Yeh *et al.*	2000	15	0.8

[a] Personal communication from respective authors.

chemical change. The biological activity of certain chemicals requires activation of these compounds by chemical modification *in vivo*. On the other hand, the biological activity of certain other chemicals is abolished by *in vivo* chemical modification. The influence of the whole organism biological system can alter screening results and lead to inconsistencies that require further study.

Biological organism screening variability is also affected by penetrance. The genetic background of the fish exerts a considerable influence on screening results, and can affect confirmation assays. Even in wildtype strains, genetic variability

leads to different levels of drug penetrance in different clutches. Often, one can look for consistent penetrance percentages to determine true hits.

V. Summary

Zebrafish chemical screening is very useful for therapeutic and bioprobe discovery. It provides a medium- to high-throughput manner of assessing the phenotypic effects of small molecule libraries on an *in vivo* system. This allows for toxicity, pharmaco-properties, and effects of compounds to be studied in a complex biological system, taking into account metabolism and cell–cell interactions. Also, zebrafish provide a wide variety of scoring phenotypes that can be adapted to specific study aims. In addition, compound library choices are abundant and although the largest library used so far was ~16,000 compounds, technological advances can potentially bring this to the 50,000 to 100,000 level. Scaling up embryo generation to a larger scale should also not be problematic.

However, there are some caveats to note when screening zebrafish. Using juvenile/adult fish restricts the throughput since fish at this stage take longer to accumulate in large numbers. Also, juvenile/adult manipulation is more challenging. Variable hit rates are observed in screens due to compound libraries used, human error, scoring type, genetic penetrance, and fish stage. Target ID is another challenge in zebrafish screening. The complexity of the whole organism means that traditional target ID methods are not ideal, and the main target ID method is candidate-based inference.

Overall, zebrafish chemical screening is an indispensable tool in therapeutic discovery. The "low hanging fruit" of drug discovery has already been taken and the focus is now on "undruggable" targets such as transcription factors and protein–protein interactions. Traditional cell-based and biochemical drug discovery screens are no longer efficient in finding therapeutics to "undruggable" targets. Also, these methods do not consider *in vivo* drug interactions, which could result in unwanted side effects. Zebrafish screening has the added advantages of assessing drug toxicity at an early stage of drug development (Zon and Peterson, 2005). Also, any drug processing by metabolism is taken into consideration. Furthermore, pharmacokinetics and pharmacodynamics can be studied in the fish. Screening in zebrafish can also discover drugs that modulate the cell niche, rather than the target cell-type directly. This type of drug target would not be detected in cell-based and biochemical screens, which focus on a specific cell-type or protein target. With the aforementioned advantages, whole organism screens are undoubtedly the next step forward in chemical screening for therapeutic discovery.

Acknowledgments

The authors thank Isaac Adatto and Christian Lawrence for their input on the zebrafish spawning vessel technology and Richard White for his input on the chemoinformatics section. Justin L. Tan is sponsored by

the Agency for Science, Technology & Research (A*STAR). Leonard I. Zon is an investigator of the Howard Hughes Medical Institute. Leonard I. Zon is a founder and stockholder of Fate, Inc. and a scientific advisor for Stemgent.

References

Almo, S. C., Bonanno, J. B., Sauder, J. M., Emtage, S., Dilorenzo, T. P., Malashkevich, V., Wasserman, S. R., Swaminathan, S., Eswaramoorthy, S., and Agarwal, R., *et al*. (2007). Structural genomics of protein phosphatases. *J. Struct. Funct. Genomics* **8**, 121–140.

Alvarez, Y., Astudillo, O., Jensen, L., Reynolds, A. L., Waghorne, N., Brazil, D. P., Cao, Y., O'Connor, J. J., and Kennedy, B. N. (2009). Selective inhibition of retinal angiogenesis by targeting PI3 kinase. *PLoS ONE* **4**, e7867.

Brown, F. (2005). Editorial opinion: Chemoinformatics – a ten year update. *Curr. Opin. Drug Discov. Dev.* **8**, 298–302.

Cao, Y., Semanchik, N., Lee, S. H., Somlo, S., Barbano, P. E., Coifman, R., and Sun, Z. (2009). Chemical modifier screen identifies HDAC inhibitors as suppressors of PKD models. *Proc. Natl. Acad. Sci.* **106**, 21819–21824.

Clardy, J., and Walsh, C. (2004). Lessons from natural molecules. *Nature* **432**, 829–837.

Das, B. C., McCartin, K., Liu, T., Peterson, R. T., and Evans, T. (2010). A forward chemical screen in zebrafish identifies a retinoic acid derivative with receptor specificity. *PLoS ONE* **5**, e10004.

Fürthauer, M., Thisse, B., and Thisse, C. (1999). Three different noggin genes antagonize the activity of bone morphogenetic proteins in the zebrafish embryo. *Dev. Biol.* **214**, 181–196.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P., and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348–360.

Hong, C. C., Peterson, Q. P., Hong, J., and Peterson, R. T. (2006). Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. *Curr. Biol.* **16**, 1366–1372.

Jeong, D. G., Cho, Y. H., Yoon, T., Kim, J. H., Ryu, S. E., and Kim, S. J. (2007). Crystal structure of the catalytic domain of human DUSP5, a dual specificity MAP kinase protein phosphatase. *Proteins* **66**, 253–258.

Jeong, D. G., Yoon, T., Kim, J. H., Shim, M. Y., Jung, S., Son, J. H., Ryu, S. E., and Kim, S. J. (2006). Crystal structure of the catalytic domain of human MAP kinase phosphatase 5: Structural insight into constitutively active phosphatase. *J. Mol. Biol.* **360**, 946–955.

Jung, D., Williams, D., Khersonsky, S. M., Kang, T., Heidary, N., Chang, Y., and Orlow, S. J. (2005). Identification of the F1F0 mitochondrial ATPase as a target for modulating skin pigmentation by screening a tagged triazine library in zebrafish. *Mol. Biosyst.* **1**, 85.

Khersonsky, S. M., Jung, D., Kang, T., Walsh, D. P., Moon, H., Jo, H., Jacobson, E. M., Shetty, V., Neubert, T. A., and Chang, Y. (2003). Facilitated forward chemical genetics using a tagged triazine library and zebrafish embryo screening. *J. Am. Chem. Soc.* **125**, 11804–11805.

Kitambi, S. S., McCulloch, K. J., Peterson, R. T., and Malicki, J. J. (2009). Small molecule screen for compounds that affect vascular development in the zebrafish retina. *Mech. Dev.* **126**, 464–477.

Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C. Y. J., Mateus, R., Healey, D., Kim, S., Werdich, A. A., and Haggarty, S. J., *et al*. (2010). Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat. Chem. Biol.* **6**, 231–237.

Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* **46**, 3–26.

Mathew, L. K., Sengupta, S., Kawakami, A., Andreasen, E. A., Lohr, C. V., Loynes, C. A., Renshaw, S. A., Peterson, R. T., and Tanguay, R. L. (2007). Unraveling tissue regeneration pathways using chemical genetics. *J. Biol. Chem.* **282**, 35202–35210.

Milan, D. J., Peterson, T. A., Ruskin, J. N., Peterson, R. T., and MacRae, C. A. (2003). Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation* **107**, 1355–1358.

Mintzer, K. A., Lee, M. A., Runke, G., Trout, J., Whitman, M., and Mullins, M. C. (2001). Lost-a-fin encodes a type I BMP receptor, Alk8, acting maternally and zygotically in dorsoventral pattern formation. *Development* **128**, 859–869.

Molina, G., Vogt, A., Bakan, A., Dai, W., de Oliveira, P. Q., Znosko, W., Smithgall, T. E., Bahar, I., Lazo, J. S., and Day, B. W., *et al*. (2009). Zebrafish chemical screening reveals an inhibitor of Dusp6 that expands cardiac cell lineages. *Nat. Chem. Biol.* **5**, 680–687.

Moon, H., Jacobson, E. M., Khersonsky, S. M., Luzung, M. R., Walsh, D. P., Xiong, W., Lee, J. W., Parikh, P. B., Lam, J. C., and Kang, T., *et al*. (2002). A novel microtubule destabilizing entity from orthogonal synthesis of triazine library and zebrafish embryo screening. *J. Am. Chem. Soc.* **124**, 11608–11609.

Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., and Heisenberg, C. P., *et al*. (1996). Genes establishing dorsoventral pattern formation in the zebrafish embryo: The ventral specifying genes. *Development* **123**, 81–93.

Murphey, R. D., Stern, H. M., Straub, C. T., and Zon, L. I. (2006). A chemical genetic screen for cell cycle inhibitors in zebrafish embryos. *Chem. Biol. Drug Design* **68**, 213–219.

Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M., and Mullins, M. C. (1998). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. *Dev. Biol.* **199**, 93–110.

North, T. E., Goessling, W., Walkley, C. R., Lengerke, C., Kopani, K. R., Lord, A. M., Weber, G. J., Bowman, T. V., Jang, I., and Grosser, T., *et al*. (2007). Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature* **447**, 1007–1011.

Oppedal, D., and Goldsmith, M. I. (2010). A chemical screen to identify novel inhibitors of fin regeneration in zebrafish. *Zebrafish* **7**, 53–60.

Owens, K. N., Santos, F., Roberts, B., Linbo, T., Coffin, A. B., Knisely, A. J., Simon, J. A., Rubel, E. W., and Raible, D. W. (2008). Identification of genetic and chemical modulators of zebrafish mechanosensory hair cell death. *PLoS Genet.* **4**, e1000020.

Paik, E. J., de Jong, J. L. O., Pugach, E., Opara, P., and Zon, L. I. (2010). A chemical genetic screen in zebrafish for pathways interacting with cdx4 in primitive hematopoiesis. *Zebrafish* **7**, 61–68.

Parker, C. N., and Schreyer, S. K. (2004). Application of chemoinformatics to high-throughput screening: Practical considerations. *Methods Mol. Biol.* **275**, 85–110.

Peterson, R. T., Link, B. A., Dowling, J. E., and Schreiber, S. L. (2000). Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc. Natl. Acad. Sci. USA* **97**, 12965–12969.

Peterson, R. T., Shaw, S. Y., Peterson, T. A., Milan, D. J., Zhong, T. P., Schreiber, S. L., MacRae, C. A., and Fishman, M. C. (2004). Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation. *Nat. Biotechnol.* **22**, 595–599.

Rihel, J., Prober, D. A., Arvanites, A., Lam, K., Zimmerman, S., Jang, S., Haggarty, S. J., Kokel, D., Rubin, L. L., and Peterson, R. T., *et al*. (2010). Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. *Science* **327**, 348–351.

Sachidanandan, C., Yeh, J. J., Peterson, Q. P., and Peterson, R. T. (2008). Identification of a novel retinoid by small molecule screening with zebrafish embryos. *PLoS ONE* **3**, e1947.

Schreiber, S. L. (2000). Target-oriented and diversity-oriented organic synthesis in drug discovery. *Science* **287**, 1964–1969.

Shafizadeh, E., Peterson, R. T., and Lin, S. (2004). Induction of reversible hemolytic anemia in living zebrafish using a novel small molecule. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **138**, 245–249.

Shepard, J. L., Amatruda, J. F., Stern, H. M., Subramanian, A., Finkelstein, D., Ziai, J., Finley, K. R., Pfaff, K. L., Hersey, C., and Zhou, Y., *et al*. (2005). A zebrafish bmyb mutation causes genome instability and increased cancer susceptibility. *Proc. Natl. Acad. Sci. USA* **102**, 13194–13199.

Spring, D. R., Krishnan, S., Blackwell, H. E., and Schreiber, S. L. (2002). Diversity-oriented synthesis of biaryl-containing medium rings using a one bead/one stock solution platform. *J. Am. Chem. Soc.* **124**, 1354–1363.

Stern, H. M., Murphey, R. D., Shepard, J. L., Amatruda, J. F., Straub, C. T., Pfaff, K. L., Weber, G., Tallarico, J. A., King, R. W., and Zon, L. I. (2005). Small molecules that delay S phase suppress a zebrafish bmyb mutant. *Nat. Chem. Biol.* **1**, 366–370.

Sternson, S. M., Louca, J. B., Wong, J. C., and Schreiber, S. L. (2001). Split-pool synthesis of 1,3-dioxanes leading to arrayed stock solutions of single compounds sufficient for multiple phenotypic and protein-binding assays. *J. Am. Chem. Soc.* **123**, 1740–1747.

Stewart, A. E., Dowd, S., Keyse, S. M., and McDonald, N. Q. (1999). Crystal structure of the MAPK phosphatase Pyst1 catalytic domain and implications for regulated activation. *Nat. Struct. Biol.* **6**, 174–181.

Thisse, B., and Thisse, C. (2005). Functions and regulations of fibroblast growth factor signaling during embryonic development. *Dev. Biol.* **287**, 390–402.

Torregroza, I., Evans, T., and Das, B. C. (2009). A forward chemical screen using zebrafish embryos with novel 2-substituted 2H-chromene derivatives. *Chem. Biol. Drug Design* **73**, 339–345.

Tran, T. C., Sneed, B., Haider, J., Blavo, D., White, A., Aiyejorun, T., Baranowski, T. C., Rubinstein, A. L., Doan, T. N., and Dingledine, R., *et al*. (2007). Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer Res.* **67**, 11386–11392.

Trompouki, E., and Zon, L. I. (2010). Small molecule screen in zebrafish and HSC expansion. *Methods Mol. Biol.* **636**, 301–316.

Tsang, M., and Dawid, I. B. (2004). Promotion and attenuation of FGF signaling through the Ras-MAPK pathway. *Sci. STKE* **2004**, pe17.

Wang, C., Tao, W., Wang, Y., Bikow, J., Lu, B., Keating, A., Verma, S., Parker, T. G., Han, R., and Wen, X. (2010). Rosuvastatin, identified from a zebrafish chemical genetic screen for antiangiogenic compounds, suppresses the growth of prostate cancer. *Eur. Urol.* **58**, 418–426.

Wheeler, G. N., and Brändli, A. W. (2009). Simple vertebrate models for chemical genetics and drug discovery screens: Lessons from zebrafish and *Xenopus*. *Dev. Dyn.* **238**, 1287–1308.

White, R. M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., Bourque, C., Dovey, M., Goessling, W., and Burns, C. E., *et al*. (2008). Transparent adult zebrafish as a tool for *in vivo* transplantation analysis. *Cell Stem Cell* **2**, 183–189.

Wong, J. C., Sternson, S. M., Louca, J. B., Hong, R., and Schreiber, S. L. (2004). Modular synthesis and preliminary biological evaluation of stereochemically diverse 1,3-dioxanes. *Chem. Biol.* **11**, 1279–1291.

Xu, Z., Li, Y., Xiang, Q., Pei, Z., Liu, X., Lu, B., Chen, L., Wang, G., Pang, J., and Lin, Y. (2010). Design and synthesis of novel xyloketal derivatives and their vasorelaxing activities in rat thoracic aorta and angiogenic activities in zebrafish angiogenesis screen. *J. Med. Chem.* **53**, 4642–4653.

Yeh, J. J., Munson, K. M., Elagib, K. E., Goldfarb, A. N., Sweetser, D. A., and Peterson, R. T. (2009). Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. *Nat. Chem. Biol.* **5**, 236–243.

Yu, P. B., Hong, C. C., Sac/hidanandan, C., Babitt, J. L., Deng, D. Y., Hoyng, S. A., Lin, H. Y., Bloch, K. D., and Peterson, R. T. (2007). Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat. Chem. Biol.* **4**, 33–41.

Zon, L. I., and Peterson, R. T. (2005). *In vivo* drug discovery in the zebrafish. *Nat. Rev. Drug Discov.* **4**, 35–44.