

How Surrogate and Chemical Genetics in Model Organisms Can Suggest Therapies for Human Genetic Diseases

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ABSTRACT Genetic diseases are both inherited and acquired. Many genetic diseases fall under the paradigm of orphan diseases, a disease found in < 1 in 2000 persons. With rapid and cost-effective genome sequencing becoming the norm, many causal mutations for genetic diseases are being rapidly determined. In this regard, model organisms are playing an important role in validating if specific mutations identified in patients drive the observed phenotype. An emerging challenge for model organism researchers is the application of genetic and chemical genetic platforms to discover drug targets and drugs/drug-like molecules for potential treatment options for patients with genetic disease. This review provides an overview of how model organisms have contributed to our understanding of genetic disease, with a focus on the roles of yeast and zebrafish in gene discovery and the identification of compounds that could potentially treat human genetic diseases.

KEYWORDS yeast; zebrafish; model organism; genetic disease; orphan disease; cancer; chemical genetics; drug discovery

GENETIC diseases can be both inherited and acquired, with cancer serving as a paradigm for an acquired genetic disease. Most inherited diseases fall under the umbrella of orphan diseases. Orphan diseases received this designation as they were traditionally “orphaned” by the academic and pharmaceutical sectors, resulting in a paucity of research into causes and treatments for these disorders (Dodge *et al.* 2011). In most jurisdictions, an orphan disease is defined as a disease that affects < 1 in 2000 persons; in the USA, an orphan disease is defined as affecting < 200,000 persons within the total USA population (Boycott *et al.* 2014).

It is predicted that there are ~7000 highly penetrant single-gene inherited disorders, with mutations in > 4000 causal genes identified to date (Online Mendelian Inheritance in Man). Although individually rare, 1 in 15 babies born worldwide has an inherited orphan disease; 75% of such diseases

affect children and 30% of these children will not reach their fifth birthday (Dodge *et al.* 2011; Boycott *et al.* 2014) (<https://www.raredisorders.ca/about-cord/>; <https://www.eurordis.org/content/what-rare-disease>). The dramatic increase in the rate of human disease gene discovery will undoubtedly increase the speed and decrease the cost of diagnosing genetic disorders, especially those that are syndromic and have a high degree of clinical heterogeneity (Bamshad *et al.* 2011; Moreau and Tranchevent 2012; Boycott *et al.* 2013; Lek *et al.* 2016). This will be especially valuable to patients that currently undergo the so-called 5–7 year “diagnostic odyssey” of sometimes expensive and invasive testing before a diagnosis is made (Boycott *et al.* 2014).

The genetic underpinnings of common adult cancers such as lung, breast, prostate, and colon, which affect large numbers of individuals, are now being subgrouped into smaller molecularly-defined cohorts, with some of these subgroups now also being categorized as orphan. Pediatric cancers, which as a group represent an orphan disease, have frequently led the way in revealing molecular markers resulting in the subclassification of various tumors (Cooper *et al.* 2017). Classic examples include *N-myc* amplification indicating poor-risk neuroblastoma (Seeger *et al.* 1985; Bosse and Maris 2016) or

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the PAX-forkhead gene fusions that have become pathognomonic of alveolar rhabdomyosarcoma (Galili *et al.* 1993; Davis *et al.* 1994; Hettmer and Wagers 2010). More recently, recognized lesions have influenced treatment strategies, such as the poorly prognostic 1p and 16q deletions in Wilms' tumor that can be mitigated with more intensive therapy (Grundy *et al.* 1994; Chagtai *et al.* 2016), and pioneering studies whereby the addition of a tyrosine kinase inhibitor targeting the BCR-Abelson1 fusion can greatly improve outcome in Philadelphia chromosome-positive acute lymphoblastic leukemia (Druker *et al.* 2001; Jeha *et al.* 2014; Desogus *et al.* 2015). Recent sequencing efforts, such as the TARGET (Therapeutically Applicable Research to Generate Effective Treatments) initiative in the USA (<https://ocg.cancer.gov/programs/target>) and the soon to be initiated PROFYLE (PRrecision Oncology For Young peopLE) project in Canada, have and will reveal detailed exome, genome, methylome, proteome, and metabolome profiles of a variety of childhood cancers, providing new information regarding disease biology and potentially targetable malignant driver lesions.

Genetic forms of human disease have mainly focused on identifying causal mutation(s) in afflicted individuals. These diagnostic efforts are important and ongoing, with model organisms having an instrumental role in validating genotype with phenotype for genetic diseases (Lehner 2013; Foley 2015). However, most genetic diseases still have no effective treatment (Dodge *et al.* 2011; Boycott *et al.* 2014). We propose that a next grand challenge for model organism researchers will be to apply genetic and chemical genetic approaches to determine novel human disease-specific genotype–phenotype correlations, with the aim of identifying novel drug targets and drugs/drug-like molecules as a starting point toward increasing treatment options for patients suffering from genetic diseases.

Model Organism Contributions to Our Overall Understanding of Genetic Diseases

Genotype–phenotype connections may be clear if the gene defect has a well-characterized function, or genes within the same pathway are known to cause the same, or a similar, disease. In cases where the genotype–phenotype connection is unknown, model organisms have served a critical role in determining if identified mutations can affect the function of the encoded protein at the subcellular, cellular, and organismal level (Lehner 2013). Linking human geneticists with model organism researchers has been integral to the rapid increase in the rate of inherited disease-causing gene discovery. A formalized matchmaking process, the Canada-led Rare Disease Models and Mechanisms Network (<http://www.rare-diseases-catalyst-network.ca/>), pairs clinicians seeking to validate novel genetic findings that may be causal for an inherited disease with model organism scientists with expertise in their gene/pathway of interest (Foley 2015). As examples, this process provided a *Drosophila* model that enabled

causality to be determined for *TMTC3* for an inherited periventricular nodular heterotopia with intellectual disability and epilepsy (Farhan *et al.* 2017), as well as zebrafish models that aided in determining that mutations in *NANS* cause an inherited infantile-onset severe developmental delay and skeletal dysplasia (van Karnebeek *et al.* 2017), and that *CEP55* mutations cause a novel multiple congenital anomaly syndrome that includes hydranencephaly (multinucleated neurons, anhydramnios, renal dysplasia, cerebellar hypoplasia, and hydranencephaly) termed MARCH (Frosk *et al.* 2017). An ultimate goal for medical research is to improve treatments. With respect to genetic diseases, we now have a large set of diseases with a known molecular cause that are readily diagnosable at a gene level, yet have poor or no treatment.

A recent analysis of over half a million human genomes identified individuals naturally resistant to several Mendelian diseases including cystic fibrosis (*CFTR*), Smith–Lemli–Opitz syndrome (*DHCR7*), familial dysautonomia (*IKBKAP*), epidermolysis bullosa simplex (*KRT14*), Pfeiffer syndrome (*FGFR1*), acampomelic campomelic dysplasia (*SOPX9*), atelosteogenesis (*SLC26A2*), and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (*AIRE*) (Chen *et al.* 2016). It has been known for some time from large pedigrees that each contain the identical disease-causing mutation and that a large variability in disease severity can exist. As an example, analysis of 32 affected family members of the childhood-blinding disorder familial exudative vitreoretinopathy, each having the same mutation in *FZD4*, noted changes in vision ranging from a slight impairment to complete blindness (Robitaille *et al.* 2002, 2011).

It is clear that genetic buffering plays an important role in genotype–phenotype outcome (Hartman *et al.* 2001; Fear *et al.* 2016). What is not clear is if this genetic buffering in patients with Mendelian diseases is due to large effects by single-gene polymorphisms/mutations or the result of the accumulation of small effects of numerous polymorphisms. There are many studies across a multitude of model organisms that have determined that second-site suppressors via a mutation at a single-gene locus can buffer against the effect of a highly penetrant mutation (Boone *et al.* 2007; Dixon *et al.* 2008; Dowell *et al.* 2010; Lehner 2013). This brings up an interesting question of whether using model organisms to search for genes whose inactivation serves to protect the organism from a deleterious mutation in a causal disease-causing gene would be a practical method that could point toward potential therapeutic targets, or drugs, for their treatment. In essence, one is looking for a mutation in a second gene, or a drug that inhibits the function of the protein encoded by that gene, which protects the organism from the disease-causing phenotype. Conversely, for cancers with a clear genetic signature, one could look for a mutation in a second gene or a drug that targets the protein product of a gene, which confers lethality specifically in the presence of gene mutations that drive cancer cell growth and viability (O'Neil *et al.* 2017).

Model organisms ranging from *Saccharomyces cerevisiae* (yeast) to *Caenorhabditis elegans* (nematode), to *Drosophila melanogaster* (fly), to *Danio rerio* (zebrafish), and to *Mus musculus* (mouse) have been important and useful in determining gene function, delineating genotype–phenotype correlations, and identifying potential therapeutic avenues for genetic diseases (Guernsey *et al.* 2009, 2011; Lehner 2013). The genomes of all of these organisms have been sequenced, making them useful in the study of genetic disease. Yeast are single-celled eukaryotes that allow for rapid genome-wide genetic and chemical genetic screens. Much of the functionality of yeast genetics is due to the ability of yeast to grow as both haploid and diploid cells, enabling second-site mutations to be introduced into diploid cells in a heterozygous state, and then testing their effects by isolating haploid cells with both the causal mutation and the second-site mutation contained in the same haploid cell (Boone *et al.* 2007; Hillenmeyer *et al.* 2008; Costanzo *et al.* 2010, 2016; Enserink 2012; van Leeuwen *et al.* 2016). However, as a single-celled organism, yeast does not offer insight into differentiated cell types and whole organs. The short life cycle and transparency of *C. elegans*, coupled with assays that rely on fluorescence and observable defects in cellular function, make it ideal for organismal screens. The fact that all 302 of its neurons have been precisely mapped is also a major attraction of this model (Lemieux and Ashrafi 2015; Hobert 2016). However, of the multi-cellular models, *C. elegans* has fewer mammalian homologs and is missing key organs present in the fly, zebrafish, and mouse models (*e.g.*, no heart and no centralized brain). *Drosophila* is an efficient, well-defined genetic model organism. Overall protein sequence homology between the fly and mammals is 40%, with 80–90% homology in conserved domains (Ugur *et al.* 2016). Furthermore, 75% of genes associated with human disease have functional orthologs in the fly. A wide range of genetic tools developed for the fly can be applied to the study of genetic disease, including the Gal4/UAS (upstream activating sequence) system, clustered regularly interspaced short palindromic repeats (CRISPR) technology, and transgenic approaches (Millburn *et al.* 2016). The fly also offers opportunities for rapid phenotypic screening such as locomotion and behavior (Boutros *et al.* 2004; Jaiswal *et al.* 2012). Zebrafish represents a powerful model system for studying genetic diseases (Howe *et al.* 2017). Zebrafish have a high level of genetic conservation with humans, 71% of human proteins have a clear zebrafish ortholog, and 82% of all human disease-causing genes have a zebrafish ortholog (Howe *et al.* 2013). Conservation extends genomically to syntenic regions that have been preserved through evolution from fish to man (Woods *et al.* 2000). Zebrafish are closer to humans in genetic homology compared to flies and have many organs similar to mammals. Importantly, for consideration of disease states, functional safeguarding of protein-coding genes has been maintained, as have their molecular networks. In addition, as a vertebrate, zebrafish provides substantial genetic complexity over simpler model organisms, and additional relevance via the

presence of conserved analogous organs. This latter feature is important for establishing functional significance, as well as for evaluating drug metabolism and toxicity. Higher-order models can serve as a critical segue between preliminary investigations performed in simpler organisms, such as yeast, and more definitive validation in murine models and eventually humans (Zon 2016).

This review will focus on two model organisms, yeast and zebrafish, with reference to their combined use in moving from gene discovery and phenotyping to the identification of pathways, processes, and compounds that could potentially ameliorate genetic diseases. We present an overview of the applicability of these models to gene and drug discovery, with exemplars used to illustrate specific concepts.

Modifying Disease Phenotypes via Genome-Wide Enhancement Genetics in Yeast

The yeast *S. cerevisiae* has been used extensively to perform near genome-wide searches for genetic suppressors of gene mutations. A mainstay of this approach is referred to as synthetic genetic array (SGA), or synthetic genetic enhancement, analysis (Tong *et al.* 2001, 2004; Boone *et al.* 2007; Dixon *et al.* 2009; Costanzo *et al.* 2010, 2016; van Leeuwen *et al.* 2016). The SGA approach was facilitated by the creation of a bar-coded yeast single-gene knockout collection, whereby every yeast gene was inactivated in a diploid cell flanked by two unique 20-bp sequences that served as strain/gene identifiers. The generation of the single-gene knockout collection itself revealed some interesting data, in that only ~20% of the yeast genome was essential for survival when yeast were grown under normal laboratory conditions (Boone *et al.* 2007; Dixon *et al.* 2009; Costanzo *et al.* 2010). This underscores the buffering capacity of the genome to deal with deleterious mutations at a single node. Essential yeast genes can be queried through numerous means, such as the generation of mRNA degron collections and the use of titratable promoters. That said, the yeast genome contains only ~6200 genes *vs.* ~21,000 in humans, thus not all pathways in human cells are represented and some genetic interactions may be missed.

Much SGA analysis conducted to date has been used to identify synthetic lethal pairs of genes, in essence two genes whose inactivation alone has very little or no phenotype but the combined inactivation of which results in a severe phenotype. This is most often measured as a defect in growth, but can be any phenotype that can be tracked, and has been used to assign phenotypes to genes of no known function based on a similar set of genetic interactions to genes of known function, and to connect what were thought to be disparate biological processes together to uncover previously unknown biochemical connections (Fairn *et al.* 2007; Curwin *et al.* 2009; Costanzo *et al.* 2010; Dowell *et al.* 2010; Gaspard and McMaster 2015; Mattiazzi Usaj *et al.* 2016). An example of using synthetic lethality in yeast to identify potential drug targets for cancer was a study on cells overexpressing *TDP1*, the expression of which is often increased in rhabdomyosarcoma, a common soft

tissue sarcoma of children (Duffy *et al.* 2016). Overexpression of *TDP1* results in chromosome instability in yeast and human cells. A synthetic lethality screen in yeast identified genes whose deletion resulted in lethality only when *TDP1* was overexpressed (Duffy *et al.* 2016). Significantly, deletion of the histone deacetylase *RPD3* gene was one of these genes. Histone deacetylase inhibitors, valproic acid or trichostatin A, both approved for use in humans, were able to selectively kill human cells overexpressing *TDP1* and human rhabdomyosarcoma cells with elevated *TDP1* levels. This study provides the impetus for future work to determine the usefulness of FDA (US Food and Drug Administration)-approved histone deacetylase inhibitors to treat rhabdomyosarcoma. In a second example, a similar line of experimentation was used to determine yeast genes, the deletion of which resulted in selective synthetic lethality upon overexpression of *MAD2* (Bian *et al.* 2014). Human *MAD2* is overexpressed in many cancer types including liver, lung, and gastric cancers, malignant lymphoma, soft tissue sarcoma, hepatocellular carcinoma, colorectal carcinoma, and osteosarcoma. Inactivation of the protein phosphatase gene *PP2A* in yeast resulted in selective lethality due to *MAD2* overexpression, as did treatment of human cancer cells overexpressing *MAD2* with the protein phosphatase 2A inhibitor cantharidin.

Contrary to gene inactivation (synthetic lethality) that prevents cell viability that can point to potential cancer treatment targets (McLornan *et al.* 2014; O'Neil *et al.* 2017), mutations that ameliorate a growth or biochemical defect associated with an inherited genetic disease may be a viable approach to identify pathways that could be targeted for their treatment. As the identification of human disease-causing genes is now rapidly expanding, it may be interesting to apply the SGA approach to query human disease-causing mutations where a similar mutation in a yeast gene, or the analogous human gene expressed in yeast, has been shown to result in a phenotype that can be monitored at a genome-wide level. Screens to identify genes whose inactivation restores the phenotype could point to potential drug targets. These screens have not been performed in depth as they require specific mutant versions of genes, and often a defect in a cell-based assay specific to the gene/process under study. Thus far, in cases where there is knowledge of the function of the disease-causing gene and the pathway in which it participates, didactic reasoning has been more common. For example, recessive mutations in human *SLC25A38* were identified to cause a common subtype of congenital sideroblastic anemia; however, the function of *SLC25A38* was not known (Guernsey *et al.* 2009). Studies on the yeast homolog *HEM25*, and human *SLC25A38* expressed in yeast, determined that *SLC25A38* is an important mitochondrial glycine importer required to provide substrate for the initiation of heme synthesis (Fernández-Murray *et al.* 2016). Knowledge of the function of *SLC25A38* was exploited to determine that high levels of exogenous glycine, or the heme biosynthetic pathway intermediate 5-aminolevulinic acid (5-Ala), were able to restore heme levels to normal in yeast cells lacking *HEM25*. These studies were then transitioned to zebrafish where, surprisingly, neither glycine nor

5-Ala could ameliorate the synthesis of hemoglobin in the *slc25a38* sideroblastic anemia zebrafish model. A major difference between yeast and zebrafish is the ability of yeast to synthesize their own folate, and mitochondrial folate and glycine metabolism are highly integrated. In zebrafish and higher eukaryotes, folate is a vitamin that must be consumed in the diet. The addition of glycine plus folate increased hemoglobin content in the zebrafish *slc25a38* congenital sideroblastic anemia model to near wild-type levels (Fernández-Murray *et al.* 2016). This example shows how one can move quickly from a gene of no known function to a potential therapy, but also notes that a direct translation of a potential treatment may have to take into account differences across genomes and functional consequences in transitioning between model organisms.

Chemical Genetics to Search for Small Molecule Modifiers of Inherited Disease Phenotypes

There has been a recent resurgence in phenotype-based (cell or organism) drug discovery, which we have termed the outside-in approach (Figure 1A), over target-based (protein) drug screening efforts, which we refer to as the inside-out approach (Figure 1B). A major driver of this renewed interest in the outside-in approach was an analysis of first-in-class approved drugs between 1999 and 2008, which revealed that 62% were discovered using phenotype-based screens, despite the fact that only a small subset of drug screens used this method (Eder *et al.* 2014; Schirle and Jenkins 2016; Moffat *et al.* 2017). Several factors are thought to contribute to the superior track record of phenotype-based screens. Phenotype-based screens take into account drug uptake by cells/organisms, and can often combine screening and counter screening (*e.g.*, against toxicity in cells or a whole organism), allowing for compounds that are both efficacious and non-toxic to be discovered using the same screen (Wagner 2016). Phenotypic screens can also discover drugs that are ameliorative in the absence of a validated drug target.

The nature of the drug or small molecule library is an important consideration when screening for potential therapeutics. There has been much interest in repurposing known drugs (Corsello *et al.* 2017; Cha *et al.* 2018). Drug repurposing focuses on identifying drugs that are approved for use in humans. On-target effects of these drugs, or off-target effects on other processes that are as yet unknown, may prove efficacious in disease treatment. The main advantage of drug repurposing is that the drug can be tested directly in the clinic for utility. For inherited orphan diseases, efficacy of a known drug in a model organism can allow for a human clinical trial in as few as 5–10 patients, with success enabling drug approval for use in humans (Bronstein and Kakkis 2016; Joppi *et al.* 2016; Ekins 2017). This is of a cost and scale that can be undertaken by academic centers and small biotechnology companies.

A major caveat to drug repurposing for genetic diseases is that many occur during childhood (Boycott *et al.* 2014; Ekins 2017), and the dosing of most drugs has not been trialed in children. Even if a drug (or metabolite, vitamin, or other

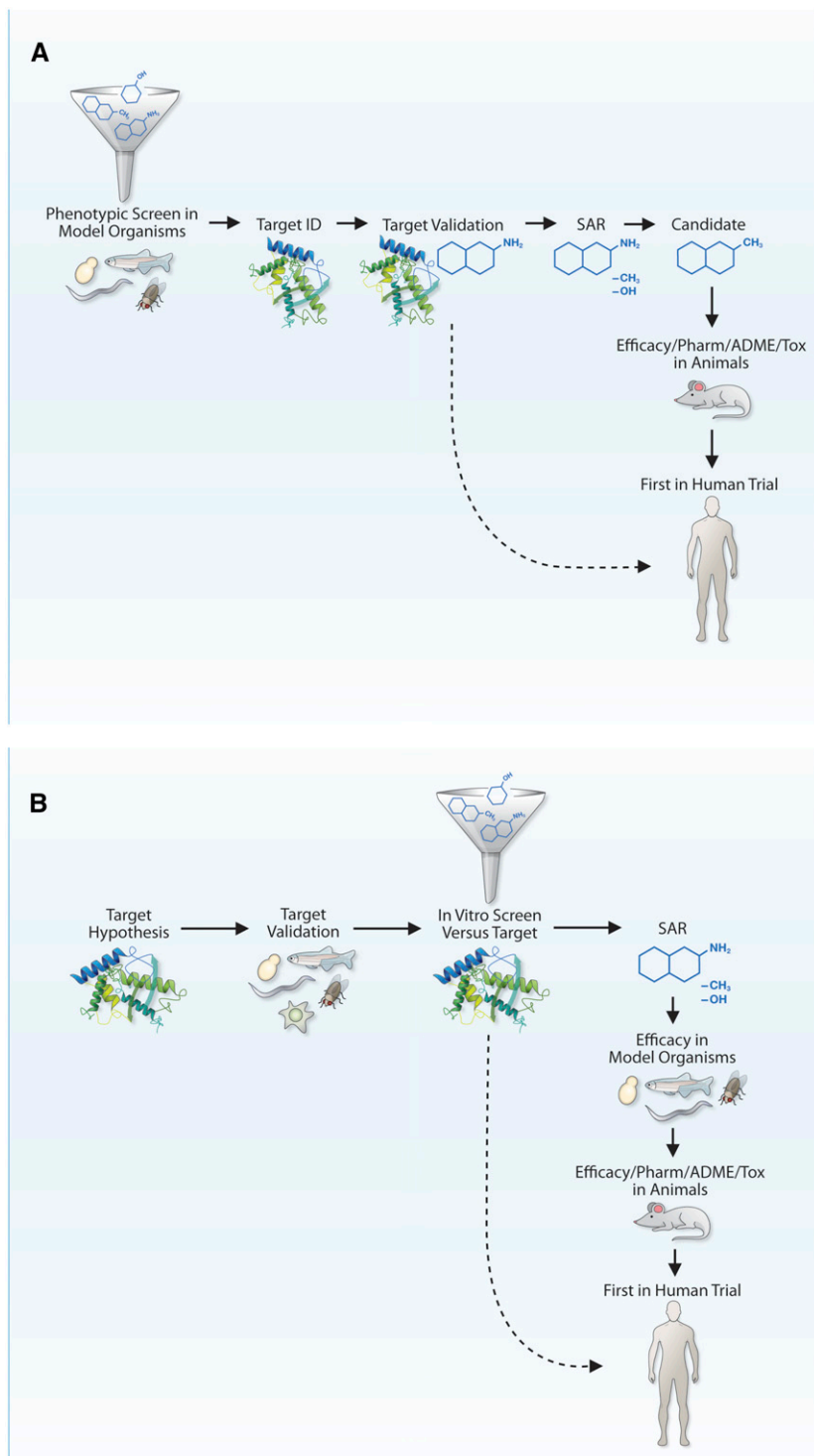


Figure 1 Outside-in and inside-out approaches to drug discovery for genetic diseases. (A) Outside-in screens start with a small-molecule screen vs. a model organism model of the genetic disease, followed by target identification (ID), validation, building an expanded library of small molecules based on structure activity relationships (SAR), and declaring a candidate drug for *in vitro*/*in vivo* pharmacokinetics (pharm)/absorption/distribution/metabolism/excretion (ADME)/toxicology (tox) studies subsequent to a first-in-human trial. (B) Inside-out screens start with inhibition (normally) of a drug target being hypothesized to be able to ameliorate the genetic disease under study, followed by target validation in model organisms, a high-throughput screen for small molecules that inhibit the drug target, building an expanded library of small molecules based on SAR, determining the efficacy of lead compounds in model organisms, declaration of a candidate drug for *in vivo* pharm/ADME/tox, and a subsequent first-in-human trial. For both approaches, if the drug screen results in a known drug being identified that has efficacy in a model organism mimic of the genetic disease, then studies may be able to proceed directly to a first-in-human trial for disease treatment.

therapy) dose is known, the effect may require a higher level than that which is known to be safe. This was likely the case when a human trial, assessing doses of oral glycine plus folate described above to ameliorate congenital sideroblastic anemia in zebrafish (Fernández-Murray *et al.* 2016), was assessed in three congenital sideroblastic anemia patients harboring the *SLC25A38* mutation at doses currently known to be safe in children (LeBlanc *et al.* 2016). Translating drug dosing in

zebrafish, and other model organisms, to humans is currently not routine or easily feasible (MacRae and Peterson 2015). If higher doses than those currently approved are required, this will likely require studies in mice and other mammalian models to determine pharmacological and toxicological profiles prior to moving into patients. Finally, there are only ~1500 FDA-approved drugs (Eder *et al.* 2014), with many in a similar chemical space. If we consider only Mendelian diseases, there

are anticipated to be ~7000 distinct diseases (Boycott *et al.* 2013, 2014). Many inherited diseases will require screening of large chemical libraries, and new chemical entity development, to find a treatment.

In yeast, similar approaches to SGA have been taken with respect to chemical genetic analysis (Fairn and McMaster 2005; Fairn *et al.* 2007; Hillenmeyer *et al.* 2008; Smith *et al.* 2010; Ho *et al.* 2011; Cong *et al.* 2012; Enserink 2012; Wohlbold *et al.* 2012; Cuesta-Marbán *et al.* 2013; Czyz *et al.* 2013; Lehner 2013; Golla *et al.* 2016; Silberberg *et al.* 2016; Wong *et al.* 2016). In chemical genetics, how a drug/compound affects the growth of the entire yeast knockout collection is assessed (Figure 2, A and B), often referred to as either drug-induced haploinsufficiency profiling (HIP) or homozygous profiling (HOP) (Giaever *et al.* 1999; Lee *et al.* 2014). With respect to the potential to discover new drugs, HIP screening vs. the diploid heterozygous yeast gene deletion set is more useful as it will often determine a direct drug target (Figure 2B). The theory is that the loss of one allele of a drug target will result in a yeast strain that is more sensitive to the drug compared to other strains within the yeast deletion collection. As an example, a HIP chemical synthetic lethality screen identified yeast *DFR1* haploinsufficient yeast, encoding dihydrofolate reductase, as being sensitive to methotrexate (Giaever *et al.* 1999, 2004). Methotrexate competitively inhibits human dihydrofolate reductase and is used to treat certain types of leukemia, as well as cancers of the breast, skin, bone, head and neck, and lung. HOP uses the haploid nonessential yeast gene deletion collection, and most often determines genes/processes that buffer against the drug (Ho *et al.* 2011). Multi-copy suppression, where individual genes are overexpressed, can also be used to identify drug targets, although this approach will also simultaneously identify genes/processes that allow for drug resistance such as decreased drug uptake, increased efflux, or increased metabolism (Ho *et al.* 2011).

Similar to synthetic genetics, synthetic chemical genetics has focused on decreased cell growth as the major phenotypic readout. This readout is amenable to finding potential new drugs for acquired genetic diseases such as cancers (Sekigawa *et al.* 2010; Albrecht *et al.* 2016; O'Neil *et al.* 2017). However, this type of analysis could be used to identify drugs that ameliorate a yeast strain containing a mimic of a genetic mutation within a human inherited disease-causing gene. These types of screens have not been routinely performed in high throughput as they require nonstandard growth conditions to be used and/or a specific yeast strain to be engineered. It will be interesting to see if the successes in chemical genetics synthetic lethality in yeast can be recapitulated for drugs/small molecules that ameliorate growth as potential treatments for inherited diseases.

Using “Humanized” Yeast to Identify Drugs to Treat Human Disease

Introducing mutations within yeast genes that correspond to the analogous regions mutated in human disease has been a

common practice in assessing if the corresponding mutation affects protein function. A second approach is to humanize yeast via expression of the human open reading frame (ORF) in a cell lacking the corresponding yeast gene and determine to what extent the human ORF can complement loss-of-function (LOF) of the analogous yeast gene (Figure 2C) (Sun *et al.* 2016). This can take place in haploid yeast cells containing a null allele of a nonessential gene, or in the case of essential genes through the use of regulatable promoters, mRNA degrons, conditional alleles (*e.g.*, temperature-sensitive strains), or through the use of a diploid heterozygous strain and subsequent isolation of haploid progeny lacking the yeast gene while expressing the human ORF (Steinmetz *et al.* 2002; Kachroo *et al.* 2015). A recent large-scale study found that 176 of 414 human ORFs could replace their essential yeast ortholog (Kachroo *et al.* 2015). An important aspect of these large-scale studies is the use of constitutive or regulatable promoters that remove both the temporal and gene dose from consideration (*e.g.*, cell cycle genes), and thus may underestimate the capacity of yeast to be successfully complemented by their human counterparts. A recent study noted that the obvious 1:1 cognate gene based on sequence similarity may not always predict the correct human complementing ORF, and that this needs to be taken into account when attempts to humanize yeast are undertaken (Hamza *et al.* 2015). A large scale “yeast humanization” project, where each potential human ORF ortholog is placed within the yeast genome at its cognate gene site such that dose and temporal issues are controlled for, would be a useful resource to assess function and complementarity of normal human ORFs, as well as how mutations in these genes could result in a phenotype(s) associated with a human disease. Rapid and extensive yeast genetic and chemical genetic screening tools are available to quickly determine potential modifier genes (drug targets) and drugs for human disease mutations.

In addition to the expression of human genes that have yeast homologs, there has also been success in learning about mechanisms of disease and potential therapies via the expression of human genes not found in yeast, but that impact pathways conserved from yeast to man. The expression of α -synuclein in yeast is one example. Parkinson's disease (PD) is linked to aggregates of α -synuclein in dopaminergic neurons (Spillantini *et al.* 1997) resulting in cell degeneration. Mutations in *SNCA* (which encodes α -synuclein) are causal for PD (Polymeropoulos *et al.* 1997; Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004). Yeast expressing wild-type and mutant (PD-causing) α -synuclein (Outeiro and Lindquist 2003; Willingham *et al.* 2003) highlight the mislocalization of mutant α -synuclein, and suggest a role for α -synuclein in vesicular trafficking and autophagy (Tenreiro *et al.* 2017). In PD patient-derived induced pluripotent stem cells (iPSCs), many of the protein trafficking phenotypes initially observed in yeast are observed (Chung *et al.* 2013). The yeast model of α -synuclein toxicity identified *N*-aryl benzimidazole analogs as compounds that can reverse the toxicity of mutant α -synuclein in both yeast and iPSCs (Tardiff *et al.* 2013).

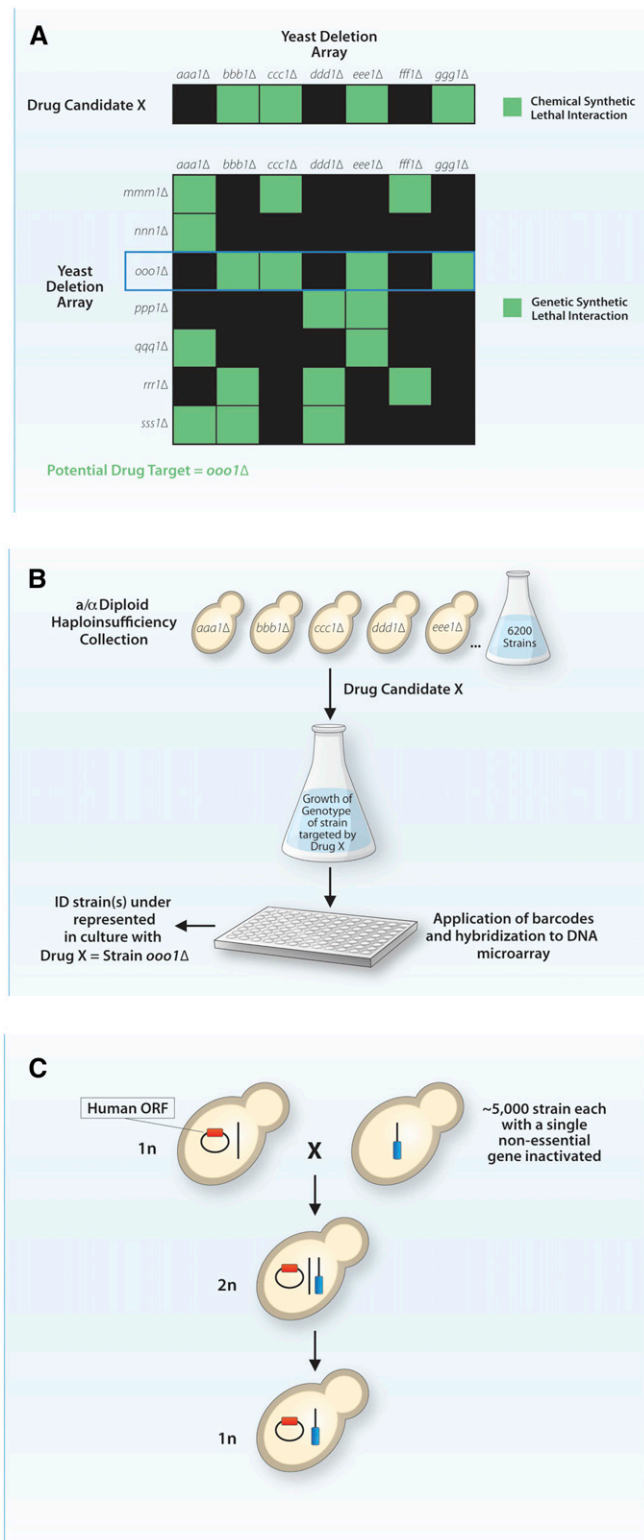


Figure 2 Application of yeast for drug discovery for genetic diseases. (A) A small molecule (drug) is screened vs. the ~5000 separate nonessential yeast gene deletion set and is compared to the known yeast deletion array synthetic lethal genetic interactions. The yeast gene deletion(s) with a similar set of interactors to the small molecule can point to the pathway or gene/protein targeted by the small molecule. (B) HIPHOP (haploinsufficiency profiling or homozygous profiling) makes use of the diploid

Although α -synuclein was the first identified inherited mutation for PD, others have been discovered. The most commonly mutated gene is *LRRK2* (Funayama *et al.* 2005; Klein and Lohmann-Hedrich 2007). Expression of a *LRRK2* fragment containing two enzymatic domains of *LRRK2* (a GTPase domain and a kinase domain) induced toxicity in yeast (Xiong *et al.* 2010). A genetic screen identified yeast genes whose deletion reversed the toxicity of *LRRK2*. Out of the seven gene deletions identified in the screen, two had human homologs, one of which was identified as ArfGAP1. ArfGAP1 is a GTPase-activating protein that accelerates *LRRK2* GTPase domain hydrolysis of GTP. Studies in cultured neurons confirmed that ArfGAP1 is required for the toxic phenotypes of the major pathogenic version of *LRRK2* (Stafa *et al.* 2012; Xiong *et al.* 2012).

Another neurodegenerative disease that has also been successfully studied in yeast is Alzheimer's disease (AD). In AD, plaques of β -amyloid are found in neurons (Chartier-Harlin *et al.* 1991; Citron *et al.* 1992; Mullan *et al.* 1992; Mawuenyega *et al.* 2010; Qiang *et al.* 2017). Amyloid precursor protein is cleaved to $A\beta$ -42, which is thought to be the form that is aggregation-prone and toxic (Tcw and Goate 2017). To create a yeast model, $A\beta$ -42 was fused with an ER-targeting signal in yeast and a genetic screen was performed looking for modifiers of toxicity (Treichel *et al.* 2011; Matlack *et al.* 2014). This screen identified the yeast homolog of the endocytic factor phosphatidylinositol-binding clathrin assembly protein (*PICALM*), as well as several endocytic factors whose relationship to $A\beta$ -42 toxicity was not known. The genes identified in yeast modified $A\beta$ -42 toxicity in glutamatergic neurons of *C. elegans* and in primary rat cortical neurons, suggesting that there was relevance of these genes to AD. The yeast model was used to perform a chemical screen with 140,000 compounds looking for drugs that reversed $A\beta$ -42 toxicity, and 30 hits were observed with half from the hydroxyquinoline family. A subset of these compounds was found to be efficacious in *C. elegans* and mouse AD models (Matlack *et al.* 2014).

haploinsufficiency collection of all yeast genes. The entire collection is exposed to a small molecule and the culture is allowed to grow. Using bar-coding technology, the yeast strain(s) whose growth is decreased compared to a no drug control can be identified (ID). The small-molecule drug target should show decreased growth in a strain that is haploinsufficient for its target compared to the other strains. (C) Several applications of synthetic genetic array technology can be used to identify drug targets. The example illustrated works on the principle that inducible overexpression of a human open reading frame (ORF) in yeast results in an easily measurable phenotype (often growth). The yeast strain containing the human ORF (uninduced) can be mated to each of the ~5000 non-essential yeast gene deletion strains and, through a series of strain selection steps, can result in the isolation of haploid yeast strains each containing the human ORF under control of an inducible promoter in each of the ~5000 individual yeast gene deletion strains. Expression of the human protein can be induced, and yeast gene deletion strains that prevent its toxicity point to potential drug targets whose inhibition could prevent the toxicity of the human protein.

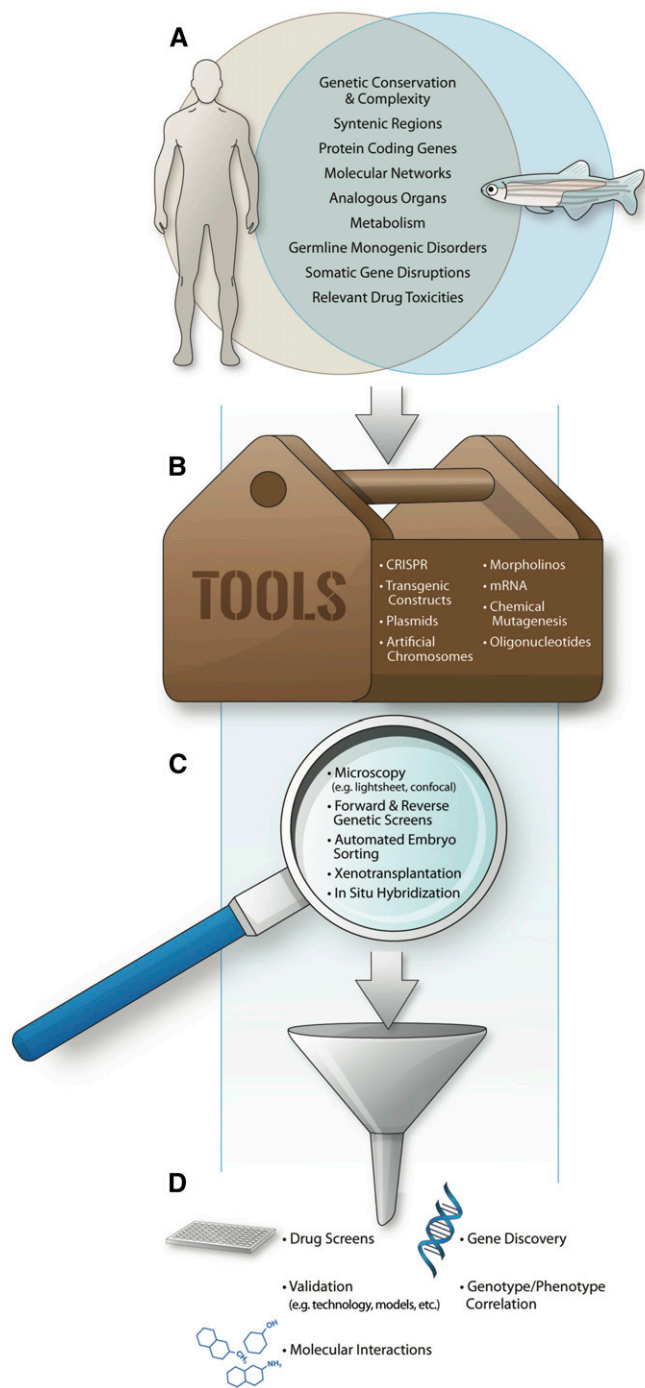


Figure 3 Applications of the zebrafish model in the study of inherited and acquired human genetic diseases. (A) Zebrafish and humans share a high degree of genetic conservation, including chromosome synteny and protein-coding genes. Molecular networks are also conserved, permitting use of zebrafish for investigating downstream pathways implicated in both inherited human diseases and somatic mutations like those found in cancer. Moreover, as a vertebrate model, the presence of analogous organs and conserved metabolism enable functional studies of the impact of genetic mutations on specific tissues and predictive readouts of therapeutic responses to drug administration. (B) The zebrafish is amenable to a number of genetic tools, including clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based genome editing and transgenic technology, to introduce specific human mutations, including inserting

Expression of human genes in yeast has also been used to identify potential cancer therapeutics using a chemical genetics approach. As an example, expression of human telomerase in yeast, a validated cancer target, decreased cell growth (Wong *et al.* 2013). A chemical genetics screen to reverse growth arrest identified three small molecules that inhibited human telomerase based on restoring yeast growth, and were found to inhibit *in vitro* telomerase activity (Wong *et al.* 2013).

The identification of gene mutations, or drugs/metabolites, in humanized yeast can serve as a jumping-off point for experiments in higher order organisms to determine if the potential drug target (inactivated gene) or drug could be of potential use in individuals with that disease-causing genetic mutation. All yeast genetic and chemical genetic interactions, and human homologs of yeast genes including functional complementation relationships, are constantly archived and updated. These can be browsed or queried through the *Saccharomyces* Genome Database (Chatr-Aryamontri *et al.* 2017) (www.yeastgenome.org).

Tools for Studying Genetic Diseases in Zebrafish

The zebrafish occupies a unique place in the parallel pipelines of gene discovery and preclinical therapeutic testing (Figure 3A). The toolbox for genetic manipulation in the zebrafish has continued to grow (Figure 3B), resulting in a variety of approaches available for both short- and long-term studies. An early whole-genome duplication event in teleost fish results in two paralogous gene equivalents in many cases (Postlethwait *et al.* 2000; Woods *et al.* 2000). This consideration is important, particularly in the context of gene knock-down studies where redundancy in function of these paralogs can mask an LOF phenotype. With this caveat in mind and appropriate mitigation (see below), gene function can be effectively and usually efficiently evaluated in the zebrafish for both germline monogenic disorders and somatic gene disruptions, such as those that occur in cancer.

oligonucleotides into the zebrafish genome. Other tools—including the facile introduction of plasmid DNA, BACs, and YACs; mRNA for transient gene overexpression studies and morpholinos for transient gene knock-down; and chemical mutagenesis—make the zebrafish a versatile model system for gene manipulation and phenotypic readouts. (C) Genetically modified zebrafish are easily surveyed using light and fluorescent microscopy, which can be enhanced with the availability of Lightsheet technology and confocal imaging. Whole-mount *in situ* hybridization provides tissue-specific data on RNA expression levels. Xenotransplantation of human cells, and in particular cancer cells, provides a transparent *in vivo* environment for real-time visualization of cell–microenvironment interactions. Embryos can be subjected to forward and reverse genetic screens. Automation of embryo sorting facilitates throughput and feasibility. (D) Translational outputs include preclinical therapeutic studies using compound libraries, dissection of molecular interactions in the context of a given genetic mutation, and novel gene discovery through phenotypic evidence of a causative mutation.

The key to these methodologies is the technical facility with which genetic material can be introduced and incorporated into the genome of the developing zebrafish. Microinjection of nucleic acid fragments, whether oligonucleotides, plasmids, or artificial chromosomes, is accomplished through the use of a needle generated from a glass capillary tube that is inserted into the yolk or cell of the early embryo and can be reproducibly repeated hundreds of times in a short time interval. Use of marker dyes and fluorescent reporters can ensure initial correct anatomic location and preserved expression of the transgene, respectively. Gain-of-function can be evaluated in transient assays using mRNA injection, which can be either zebrafish-derived, human, or mouse, given the degree of genetic conservation. Similarly, transgenic constructs driven by zebrafish promoter elements can be injected into embryos at the one-cell stage (Udvadia and Linney 2003). In both these cases, phenotypes may be evaluable in the F0 injected embryos, although these fish will be chimeras. However, in the case of somatic mutations, such as those found in cancers, a mosaic tissue distribution may well be more representative (Langenau *et al.* 2008). Germline incorporation of an injected transgene can be accomplished in a single generation time of 2–3 months, and has been greatly optimized through the inclusion of Gateway cloning vectors to facilitate precise and efficient recombination events (Villefranc *et al.* 2007). More contemporary CRISPR-based strategies (Garneau *et al.* 2010; Deltcheva *et al.* 2011; Jinek *et al.* 2012; Cong *et al.* 2013; Esvelt *et al.* 2013; Mali *et al.* 2013a,b; Sander and Joung 2014) now enable gene insertions of specific mutations in zebrafish (Hruscha *et al.* 2013; Hwang *et al.* 2013; Jao *et al.* 2013; Auer *et al.* 2014; Ota *et al.* 2014; Hisano *et al.* 2015; Varshney *et al.* 2015; Armstrong *et al.* 2016; Ceasar *et al.* 2016), which may recapitulate a human disease phenotype more accurately than was previously possible employing transgenic approaches. Transgenes driven by tissue-specific promoters may still have advantages, particularly in the somatic context, to overexpress a mutant gene in a specific cell type. This strategy has been utilized to generate zebrafish models of lymphoid and myeloid leukemia (Langenau 2003; Sabaawy *et al.* 2006; Yeh *et al.* 2007; Zhuravleva *et al.* 2008; Forrester *et al.* 2011; Gutierrez *et al.* 2014), rhabdomyosarcoma (Langenau *et al.* 2007), and neuroblastoma (Zhu *et al.* 2012). However, recent efforts have shown that Cas9 mRNA can be expressed in a tissue-dependent manner. A Tol2-based vector incorporating guide RNAs targeting the zebrafish heme biosynthetic gene *uod*, with Cas9 under the *gata1* erythrocyte promoter, was used to induce inactivation of *uod* specifically in red blood cells with a robust fluorescent readout (Ablain *et al.* 2015).

LOF mutations in zebrafish were traditionally introduced using forward genetic approaches, which included N-ethyl N-nitrosourea (ENU) mutagenesis and insertional mutagenesis screens, whereby a phenotype was observed and mapped back to a specific causative gene (Driever *et al.* 1996; Ransom *et al.* 1996; Wienholds *et al.* 2002). The use of morpholino oligonucleotides [morpholinos (MOs)] provides a “quick and dirty” strategy for gene knockdown/knockout by targeting mRNA translation start sites or, alternatively, splice sites

(Bill *et al.* 2009). Given zebrafish genome duplication, often more than one MO is needed to ensure LOF. Additionally, due to a tendency for MOs to induce p53-dependent apoptosis (Zappulla *et al.* 2005), simultaneous *tp53* knockdown may be required to ensure that phenotypes observed are specific to loss of the targeted protein (Ekker and Larson 2001; Pickart *et al.* 2006; Robu *et al.* 2007). As this approach gained in popularity, a large number of papers were published in which a host of disease phenotypes were correlated to their genetic origin through MO studies. However, recent work has called into question the accuracy of MO targeting, emphasizing the “dirty” nature of the results obtained, questioning specificity by demonstrating off-target effects, and challenging many of the phenotypes described in prior reports (Kok *et al.* 2015). The greater ease with which null mutations can now be generated in zebrafish using transcription activator-like effector nucleases (TALENs), and more recently CRISPRs, may enable some of the discrepancies in reported MO-based phenotypes to be reconciled. While TALENs and CRISPRs result in permanent germline disruption, they are laborious and have the potential for off-target consequences (Ceasar *et al.* 2016). For CRISPR, a number of *in silico* tools to aid in guide RNA creation, simultaneous targeting of duplicated genes, and determination of off-target effects have emerged (Prykhodzhiy *et al.* 2015, 2016). Evaluation of phenotypes in F0 CRISPR-injected embryos is tempting, but caution must be applied to interpretation as nonhomologous end-joining may result in broad transient gene expression outputs that are not maintained in subsequent generations following germline incorporation. Furthermore, residual wild-type gene expression has the potential to modulate the observed phenotype. While more precise and permanent genetic modifications can be established using CRISPR, gene compensation events due to the permanent nature of the genetic modification that could impact phenotypic veracity, as evidenced by differential expression profiling when compared to transient knockdown of expression using MOs, need to be kept in mind (Rossi *et al.* 2015). Parallel experimental plans incorporating both MOs and CRISPR-generated mutants may provide the most comprehensive and informative genotype–phenotype correlations (Figure 3B).

Using Zebrafish to Understand Inherited Disease

The conserved genetics between zebrafish and humans, the advancements in gene manipulation technologies, and the ease and affordability of zebrafish husbandry, have made the modeling of rare genetic disorders feasible and promising (Figure 3A). High fecundity allows for the rapid production of large cohorts of mutation-of-interest-carrying test subjects, facilitating *in vivo* disease study that would otherwise be limited to a very small population of human subjects, as is characteristic of orphan diseases.

With the identification of a putative causal gene, this genetic lesion can be introduced into the fish genome to further study phenotype, downstream effects, and identify other key molecular players. Furthermore, the model can be used to

screen for drug candidates in a high-throughput manner to optimize the selection of drugs for further investigations or a clinical trial. For example, zebrafish were a useful tool in modeling Menkes disease, a mutation of the copper Cu^{2+} transporter ATP7A, disrupting copper metabolism and resulting in hypopigmentation. Knockdown of cellular trafficking proteins in the fish caused hypopigmentation of the melanocytes in low-copper conditions. As a partner in a dual model system drug screen, yeast contributed chemical genetic interaction information, whereas zebrafish provided a phenotypic readout, facilitating the discovery of a copper metabolism pathway for a small molecule MEK kinase inhibitor (Ishizaki *et al.* 2010).

In the case of systemic mastocytosis, a rare myeloproliferative disease, recapitulating the condition in mice demonstrated variable hematopoietic phenotypes to those observed in patients (Zappulla *et al.* 2005; Gerbault *et al.* 2011; Balci *et al.* 2014). However, zebrafish sharing conservation of mast cell biological processes with humans (Dobson *et al.* 2008; Da'as *et al.* 2011, 2012) proved to be a comparable alternative. The creation of a transgenic zebrafish, expressing the *KIT*-D816V mutation, provided a phenotype representative of the human disease with elevated levels of endopeptidases and increased numbers of mast cells in the kidney marrow characteristic of the condition (Balci *et al.* 2014). Adult fish displayed a distortion of normal kidney structure and progression to a mast cell-predominant myeloproliferative neoplasm, as evidenced by expansion of PAS^+ /tryptase $^+$ cells that infiltrated into surrounding organs. Furthermore, observation of reduced neuromast numbers, resulting from downregulation of *epcam* in both embryos and adults, provided a surrogate *in vivo* platform where drug screening for a restorative phenotype could be undertaken (Balci *et al.* 2014).

A nonsense mutation of the dystrophin gene causes a premature stop codon resulting in structural characteristics of Duchenne muscular dystrophy (DMD), a rare and severe disease affecting skeletal, cardiac, and smooth muscle. This phenotype can be studied by examination of the contractile function of skeletal muscles in 5-day-old zebrafish larvae. *Sapje* mutant zebrafish that harbor a premature stop codon in the zebrafish *dmd* homolog exhibit structural muscular defects, with progressive fibrosis and muscle degeneration beginning early in embryonic development, mimicking the changes seen in human DMD patients (Bassett and Currie 2003; Berger *et al.* 2010). Ataluren (PTC124), an aminoglycoside antibiotic, can promote read-through of the premature stop codon, which should ameliorate muscle structure and function. In fact, *sapje* zebrafish treated with 0.5 μM ataluren had increased expression of dystrophin, improved muscle morphology, and better contractile function, providing biological correlative data for the effectiveness of this drug in the treatment of patients with DMD (Li *et al.* 2014). Ataluren was approved for clinical use in boys with DMD by the European Medicines Agency in Europe in 2014 (Haas *et al.* 2015).

Zebrafish can also be used to model monogenic disorders with complex multi-system phenotypes. For example, LOF

mutations in chromodomain helicase DNA-binding protein 7 (*CHD7*) are the genetic origin of CHARGE syndrome, an acronym for Coloboma, Heart Defects, Atresia of choanae, Retardation in growth, Genital abnormalities, and Ear malformations/hearing loss. MOs targeting zebrafish *chd7* result in developmentally abnormal embryos with small eyes, cardiac anomalies, and pericardial edema (Patten *et al.* 2012). Similar features were recently recapitulated in a CRISPR/Cas9-induced mutant by generating a 2-bp deletion in exon 3 of *chd7* (Prykhozhij *et al.* 2017). Conservation of organ systems in the zebrafish enables the investigation and amelioration of clinically relevant consequences of this disease, such as the impaired gastrointestinal motility that leads to tube feeding in the majority of CHARGE patients.

Thus, current genome editing approaches provide the opportunity to model human monogenic disorders in the zebrafish with a level of genetic precision not previously possible. Organ conservation in these vertebrate animals provides the additional advantage of studying functional developmental phenotypes in addition to defects in cell survival or proliferation. All of these phenotypes provide a useful readout for moderate- to high-throughput chemical screens for ameliorative compounds.

Using Zebrafish to Understand Cancer and Point to Potential Therapies

Increased understanding that cancer is a genetic disease arising from a series of genetic alterations gives credence to the application of the zebrafish for modeling human tumors. The zebrafish is well suited to forward genetic screens employing ENU chemical mutagenesis or insertional mutagenesis approaches, resulting in different phenotypes that can be evaluated and “scored” followed by genotyping to identify the underlying causative lesion. While these approaches have been exceedingly valuable in revealing key roles for developmental genes, including those that may represent novel oncogenes or tumor suppressors (Shepard *et al.* 2005; Ceol *et al.* 2011; den Hertog 2016) that potentially contribute to malignant progression, they have not been as beneficial for the generation of *a priori* cancer models. Various reverse genetic approaches, where the impact of a known human oncogenic alteration is introduced into the zebrafish, have demonstrated the versatility of this organism for modeling human cancers, particularly with a goal toward preclinical drug testing. Furthermore, the transparency of the extrauterine zebrafish embryo allows for real-time visualization of developmental perturbations, which may have particular relevance for pediatric cancers that may represent abnormalities in normal embryogenesis (Figure 3C).

Gain-of-function mutations have been introduced through the microinjection of mRNA into the one-to-eight-cell embryo, providing transient yet potentially meaningful phenotypic data over the first 2–4 days of life of the zebrafish. Genetic conservation across species permits the injection of zebrafish, mouse, or human RNA. Injection of a transgenic DNA construct,

such as a plasmid, containing the oncogene of interest may again be informative in chimeric primary injected embryos, with additional information gleaned when the construct is incorporated into the zebrafish genome and is passed on to subsequent generations through the germline (Kawakami *et al.* 2016; Rajan *et al.* 2016). The use of fluorescent reporters as components of these constructs can facilitate tracking of inheritance, while tissue-specific promoters can direct expression in the desired cellular context. The first transgenic zebrafish model of cancer incorporated the mouse oncogenic *cMyc* gene driven by a zebrafish T-cell-specific *rag2* promoter, resulting in an aggressive T-cell acute lymphoblastic leukemia (T-ALL) that consumed its host (Langenau 2003). Variations on this original theme have utilized different promoters, oncogenes, and reporters, as well as elements, to allow for inducible expression, combinations of oncogenes, and applications of systems like UAS-Gal4 in an attempt to regulate oncogene copy numbers in specific anatomic locations and developmental time points (Liu and Leach 2011; Mayrhofer and Mione 2016) (Figure 3B).

In particular, childhood tumors have been a focus of zebrafish cancer modeling, which may reflect the developmental origins of these diseases and be accurately recapitulated in the larval zebrafish (Langenau 2003; Langenau *et al.* 2007; Frazer *et al.* 2009; Leacock *et al.* 2012; Zhu *et al.* 2012). Recent transgenic zebrafish models of childhood tumors have been particularly instructive. While potential therapeutic strategies in rhabdomyosarcoma were revealed through synthetic lethality screening in yeast overexpressing *TDP1* (see earlier), new insights into the cell of origin of this disease were elucidated through a transgenic zebrafish model of rhabdomyosarcoma with differentially fluorescently labeled tumor cell populations that could be visually tracked over time. Distinctively, only *Myf5*-expressing cells were able to propagate new tumors in syngeneic host larvae (Ignatius *et al.* 2012). In neuroblastoma, crossing of *nMyc*-expressing zebrafish with *ALK*- and *LMO1*-expressing zebrafish demonstrated the greater aggressiveness of the compound tumors and shed light on the influence of these secondary lesions on sites of metastasis (Zhu *et al.* 2012, 2017).

LOF mutations, such as those that occur in tumor suppressor genes, have traditionally been more challenging to induce in zebrafish. Efforts to target the zebrafish homolog of the *TP53* tumor suppressor, mutated in > 50% of human cancers, is illustrative of the evolving paradigm for targeting LOF mutations with the emergence of new technologies. Targeting Induced Local Lesions In Genomes is a classic forward genetic strategy that was employed to generate the first zebrafish *p53* mutants. *tp53^{M214K}* fish with a mutation in the DNA-binding domains of exon 7 of zebrafish *tp53* developed malignant peripheral nerve sheath tumors in homozygous animals between 8 and 16 months of age (Berghmans *et al.* 2005). An ENU-based screen to generate *p53* mutants was subsequently applied using suppression of radiation-induced *p53*-dependent apoptosis as a readout. Both *tp53^{I66T}* homozygotes and heterozygotes developed soft tissue sarcomas beginning at 8 months of age, with loss of heterozygosity in the tumor cells reminiscent

of what occurs in Li-Fraumeni syndrome (LFS) (Parant *et al.* 2010). More recent efforts to model LFS in zebrafish have employed CRISPR-based genomic editing to induce the specific point mutations found in these patients into the zebrafish genome (S. V. Prykhodzhiy and J. N. Berman, unpublished observations). Tumor phenotypes of these fish are still being evaluated. The phenomenon of germline incorporation of oncogenic lesions in subsequent generations of both transgenically modified or CRISPR-engineered zebrafish may make this model particularly well suited for the study of cancer predisposition syndromes, which are being increasingly recognized through next-generation sequencing efforts to underlie larger proportions of pediatric tumors (Figure 3B).

Zebrafish Human Cancer Xenotransplantation

Recent successes in transplanting patient-derived tumor cells into zebrafish embryos may similarly provide a preclinical drug response readout in a shorter and more clinically actionable time frame than classic patient-derived xenograft studies in mice. Moreover, the immunopermissiveness of zebrafish larvae, lacking an adaptive immune response until 4 weeks of age, coupled with their transparency, enabling direct visualization of fluorescently labeled cancer cells, provide additional advantages of this approach (Konantz *et al.* 2012; Veinotte *et al.* 2014; Wertman *et al.* 2016). This platform was employed to reveal a novel γ -secretase inhibitor-sensitive mutation in the *NOTCH* gene in a T-ALL sample from one child, by demonstrating a selective reduction in cell proliferation to compound added to the water of zebrafish larvae transplanted with this patient's diagnostic bone marrow. In contrast, a sample from a second patient with T-ALL did not respond in this assay and was found subsequently to be *NOTCH* wild-type (Bentley *et al.* 2015).

By contrast, older zebrafish larvae possess B and T lymphocytes and likely natural killer cells (Yoder *et al.* 2001, 2004, 2010; Langenau *et al.* 2004; Page *et al.* 2013; Bentley *et al.* 2015; Weir *et al.* 2015). Antibodies are produced in response to pathogens and foreign stimuli, suggesting an as yet untapped opportunity for the zebrafish to be used to study emerging immunotherapy-based treatment strategies. The transparency of the larvae, particularly through the use of pigment mutant lines, provides an unprecedented window through which to observe the interaction of fluorescently labeled components of the microenvironment, such as inflammatory cells and cytokines with transplanted tumor cells, and test strategies to interfere with these interactions to reduce tumor burden and cancer cell migration (Figure 3C).

Generalized Usability of Zebrafish in Drug Discovery and Development

Zebrafish have recently been recognized as a relevant whole animal for initial phenotype-based drug screens. Zebrafish possess the full complement of cytochrome P450 enzymes, the

Table 1 Representative zebrafish drug discovery studies

	Condition	Representative studies	
Toxicity	Ototoxicity	Owens <i>et al.</i> (2008)	
	Cardiotoxicity	Milan <i>et al.</i> (2003)	
	Structural defects/death	Padilla <i>et al.</i> (2012), Sandoval <i>et al.</i> (2013)	
	Organophosphate toxicity	Jin <i>et al.</i> (2013)	
	Cyanide toxicity	Nath <i>et al.</i> (2013)	
Developmental	Nanoparticle toxicity	Chakraborty <i>et al.</i> (2016)	
	Mitotic defects	Stern <i>et al.</i> (2005)	
	Tissue regeneration	Mathew <i>et al.</i> (2007)	
	Hair regeneration	Namdaran <i>et al.</i> (2012), Thomas <i>et al.</i> (2015)	
	Embryogenesis	Yu <i>et al.</i> (2008), Colanesi <i>et al.</i> (2012)	
	Dorsalization	Hao <i>et al.</i> (2013)	
	Ectopic tail	Gebruers <i>et al.</i> (2013)	
	Hearing	Ou <i>et al.</i> (2010)	
	Craniofacial	Kong <i>et al.</i> (2014)	
	Behavioral	Sleep	Rihel <i>et al.</i> (2010)
Light stimuli		Kokel <i>et al.</i> (2010)	
Learning, acoustic startle		Wolman <i>et al.</i> (2011)	
Motor activity		Kokel <i>et al.</i> (2013)	
Anxiety		Collier (2017)	
Cardiovascular	Aortic coarctation	Peterson <i>et al.</i> (2004)	
	Long QT syndrome	Peal <i>et al.</i> (2011)	
	Hypertrophic cardiomyopathy	Becker <i>et al.</i> (2012), Becker and Rinkwitz (2012)	
	Cardiomyopathy	Liu and Leach (2011), Asimaki <i>et al.</i> (2014)	
Vascular	Angiogenesis	Tran <i>et al.</i> (2007), Wang <i>et al.</i> (2010), Vazão <i>et al.</i> (2017)	
Respiratory	Tuberculosis	Ordas <i>et al.</i> (2015)	
	Hyperventilation	Rahbar <i>et al.</i> (2016)	
Neurological	Notochord defects	Ishizaki <i>et al.</i> (2010)	
	Hematopoiesis	North <i>et al.</i> (2007)	
Hematopoiesis	B-cell differentiation	Rovira <i>et al.</i> (2011)	
	B-cell proliferation	Tsuji <i>et al.</i> (2014)	
	Leukocyte migration	Liu <i>et al.</i> (2013)	
	Anemia	Fernández-Murray <i>et al.</i> (2016)	
	Gastrointestinal and Metabolism	Lipid absorption	Clifton <i>et al.</i> (2010)
		Glucose homeostasis	Nath <i>et al.</i> (2015)
		Intestinal motility	de Alvarenga <i>et al.</i> (2017)
Cancer	Inflammatory bowel disease	Hanyang <i>et al.</i> (2017)	
	Leukemia	Yeh <i>et al.</i> (2009), Ridges <i>et al.</i> (2012), Gutierrez <i>et al.</i> (2014), Deveau <i>et al.</i> (2017), Hanyang <i>et al.</i> (2017)	
Renal	Melanoma	White <i>et al.</i> (2011)	
	Wnt-activated cancers	Nishiya <i>et al.</i> (2014)	
	Polycystic kidney disease	Cao <i>et al.</i> (2009)	
	Nephroprotectant	Sharma <i>et al.</i> (2014)	

major drug-metabolizing and drug–drug interaction enzymes present in humans (Goldstone *et al.* 2010). A systematic review of drugs tested in zebrafish demonstrated that not only were the therapeutic effects maintained, but also their distribution, metabolism, excretion, and allocation into specific organs, such as the brain, was conserved between zebrafish and humans (Figure 3A). For example, of 23 drugs with known cardiotoxicity in humans due to repolarization activity, 22 were also found to be cardiotoxic in zebrafish due to aberrant repolarization (MacRae and Peterson 2015). A separate study investigated 10 compounds with diverse effects across several organs and found that 8 of the 10 produced the desired effect in zebrafish to that observed in mouse models of each disease (Asnani and Peterson 2014). As an intact organism with conserved drug metabolism, zebrafish can reveal the efficacy of compounds in which downstream

metabolites are the active components, as well as provide insights into toxicities that may need to be considered in transitioning findings to clinical trials (Figure 3D).

The advent of high-throughput embryo sorting and handling has made moderate-to-high-throughput phenotypic screening in zebrafish feasible. To date, > 65 small-molecule screens have been completed in zebrafish and have identified both opportunities for drug repurposing, whereby known drugs were found to ameliorate the zebrafish model of the human disease, as well as novel small molecules (Table 1) (MacRae and Peterson 2015). FDA-approved drugs can often move directly to testing in human patients, since their dosing and safety profiles are established. In some cases, efficacy studies in mice may be warranted to establish dosing for the specific disease under study. As discussed above, small drug-like lead molecules need to undergo further testing in mice for efficacy

to move them to candidate drug status, possibly following chemistry for drug optimization and preclinical studies to establish pharmacological and safety profiles.

Summary and Future Directions

Model organisms have been, and continue to be, instrumental in genotype–phenotype correlations. This is becoming especially apparent with the speed and reduced cost of exome and genome sequencing, enabling the rapid identification of potential causal genes for genetic conditions. Model organisms across the spectrum have been a major driver of establishing genetic and chemical genetic relationships. The relevance of preclinical studies in model organisms such as yeast and zebrafish has never been more poignant than in the current era of personalized medicine. CRISPR/Cas9 genome editing can be used to induce specific patient-derived germline and somatic mutations, producing robust phenotypes that can be readily evaluated.

We propose that the time is ripe for the humanization of model organisms as part of a large-scale initiative, with a particular focus on diseases that are genetic in origin. Ultimately, this effort will permit major screening projects to identify potential drug targets through genetic screens, and potential therapies through chemical genetic screens. If model organism humanization is performed simultaneously across several models, immediate cross-validation would be possible and should accelerate the movement of potential therapies toward the clinic.

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