

Studying synthetic lethal interactions in the zebrafish system: insight into disease genes and mechanisms

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The post-genomic era is marked by a pressing need to functionally characterize genes through understanding gene-gene interactions, as well as interactions between biological pathways. Exploiting a phenomenon known as synthetic lethality, in which simultaneous loss of two interacting genes leads to loss of viability, aids in the investigation of these interactions. Although synthetic lethal screening is a powerful technique that has been used with great success in many model organisms, including *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans*, this approach has not yet been applied in the zebrafish, *Danio rerio*. Recently, the zebrafish has emerged as a valuable system to model many human disease conditions; thus, the ability to conduct synthetic lethal screening using zebrafish should help to uncover many unknown disease-gene interactions. In this article, we discuss the concept of synthetic lethality and provide examples of its use in other model systems. We further discuss experimental approaches by which the concept of synthetic lethality can be applied to the zebrafish to understand the functions of specific genes.

Introduction

“Death hath so many doors to let out life” – Fletcher and Massinger, *The Customs of the Country*.

The 100th anniversary of T. H. Morgan’s landmark papers in *Science*, in which he used *Drosophila* genetics to investigate the physical nature of the gene and the basis of heredity (Morgan, 1911), has recently passed. In the intervening years, model organisms have continued to make great contributions to biology. Genetic studies in yeast, worms, flies, mice and other organisms have helped to

clarify not only mechanisms of inheritance, but have also fundamentally altered our understanding of cellular and molecular physiology, embryonic development, epigenetics, sex determination and evolutionary biology, among other areas. These studies have used a variety of genetic techniques to query large sets of genes in an effort to understand their function. Among the most widely applied and most powerful of these techniques is forward genetic screening, in which (usually random) alterations are made in the genome of an organism, and the resulting phenotypes are studied to divine the function of the altered genes. In many instances, the results of these screens have provided valuable clues to the basis of human disease. Genetic models in the lab have, in turn, been enriched by human genetic studies from the clinic, with each venue providing to the other both a source of candidate disease alleles and a means of validating those candidates – a ‘bench-to bedside and back’ paradigm.

Now, a century removed from Morgan’s seminal experiments, we are firmly in the post-genomic era. In some senses, model organisms are more important than ever, because the sheer wealth of data generated by deep sequencing approaches creates unique challenges. With vast numbers of potentially pathogenic genomic alterations being uncovered, experimental systems to test the functional significance of these changes, and to place them in the context of gene networks and biochemical pathways, become even more essential (Lamitina, 2006; Suter et al., 2006; Kabashi et al., 2010; Roy et al., 2010). Model organisms are invaluable for these functional genomic studies, offering the possibility of rapid, in vivo validation of genomic data. Among model organisms, zebrafish (*Danio rerio*) are well suited for disease modeling because, as vertebrates, they share with humans a highly conserved anatomy, physiology and disease susceptibility. But simple gain- or loss-of-function assays cannot recapitulate the complexity of the genetic landscape revealed by genome-wide association studies (GWAS) and deep sequencing. This is a particularly important issue for disease modeling, because susceptibility to specific diseases, response to therapies and vulnerability to medication side effects can all arise from the interactions of multiple alleles. In fish, flies, worms, yeast and other models, genetic approaches, including enhancer-suppressor and small-molecule screens, have been developed to discover genes and drugs that interact directly or indirectly with specific disease alleles. ‘Synthetic lethal’ screening is a specialized form of enhancer screen that identifies pairs of interacting genes through the lethal phenotype that results when both genes are defective. Although this technique has proved very powerful in many different model organisms, synthetic lethal screening has not been widely applied in the zebrafish system. In

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this Commentary, we describe the concept of synthetic lethality and provide examples of its use. We further suggest ways in which this technique could be applied to the zebrafish system in order to better understand the functions of specific genes.

Synthetic lethality: definitions

Synthetic lethality is a cellular condition in which two non-essential, non-allelic genes, each dispensable for viability, have a lethal phenotype when co-depleted. Synthetic lethal screens are an essential component of a geneticist's toolbox, serving as a starting point to identify gene products with similar or overlapping functions, and pathways with parallel or compensatory

mechanisms. In Fig. 1, we consider four different scenarios in which synthetic lethality can arise. In Case I, two non-lethal mutations, when present in the same linear essential pathway, lead to loss of viability. Case II is a variant scenario in which two functionally redundant paralogs function in the same pathway, and synthetic lethality occurs upon inactivation or loss of both paralogs. This case is particularly relevant in zebrafish, which have undergone genome duplication during evolution (Postlethwait et al., 1998). Case III considers two parallel pathways, either one of which can supply an essential product; simultaneous impairment of both pathways is lethal. A variation of this scenario is depicted in Case IV for independent, parallel survival pathways wherein one serves as a compensatory pathway in the absence of the other to mediate a common, essential function. Although the examples given above concern loss-of-function mutations, alternative situations can also fit the definition of synthetic lethality; for example, a gain-of-function mutation might be lethal only in the presence of a simultaneous second-site mutation, or a drug treatment that selectively kills cells carrying a specific genetic alteration. Also, combinations of mutations that fall short of killing the organism, but render it incapacitated in some way – so-called 'synthetic sick' interactions – can also reveal important information about gene function. Finally, it is important to note that, although we consider here the relatively simple case of digenic interactions, more complicated interactions involving multiple different mutations can also give rise to synthetic lethality.

Lessons from model systems

Large-scale genetic and genomic screens conducted in model systems have laid the foundation for understanding synthetic lethality in a cellular and developmental context. Working in the yeast *Saccharomyces*, Bender and Pringle conducted one of the first synthetic lethal screens in the early 90s, opening the door to this approach (Bender and Pringle, 1991). Later, Hartman, Garvik and Hartwell envisioned a comprehensive approach to the study of gene interaction networks (Hartman et al., 2001). Subsequent large-scale analyses of synthetic lethal interactions in yeast have allowed geneticists to map genetically compensatory pathways and identify redundant gene pathway pairs (Ma et al., 2008; Costanzo et al., 2010). Edgar and co-workers performed a clonal screen in the *Drosophila* eye to identify a mutation in peptidyl prolyl isomerase (PPIase) that was lethal only in cells deficient for the tumor suppressor protein retinoblastoma (Rb) (Edgar et al., 2005). Because nearly half of all cancers are deficient in Rb, this work suggests that PPIase inhibitors could be used as anti-neoplastics. In *Caenorhabditis elegans*, an RNA interference (RNAi)-based synthetic lethal screen was used to show interactions between genes associated with the *bubr-1* (*mad-3*) spindle checkpoint and the centromere protein F (*CENP-F*) homolog *hcp-1*. This approach shed light on the roles of these proteins in development and cell division, and further elucidated unique and redundant roles of *hcp-1* and *hcp-2* in nematode development (Hajeri et al., 2008). Systematic use of RNAi in worms allowed Lehner and co-workers to test 65,000 gene pairs for genetic interaction (Lehner et al., 2006). Similar genome-scale synthetic lethal screens have been conducted using innovative techniques such as synthetic genetic array (SGA) analysis and synthetic lethality analyzed by microarray (dSLAM) methodologies in yeast, and have established functional

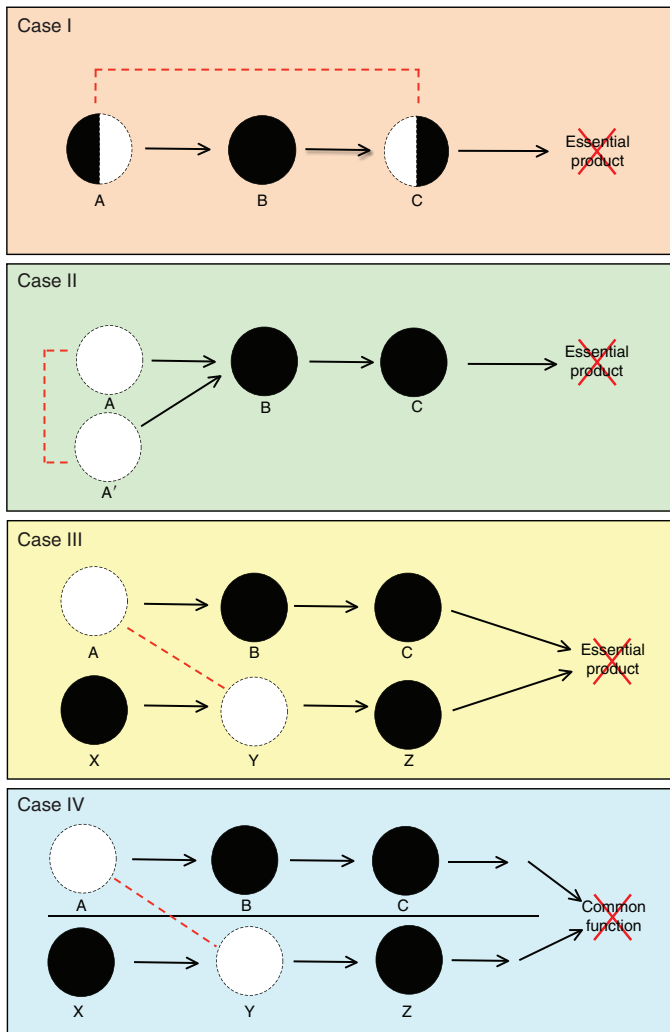


Fig. 1. Four possible scenarios of synthetic lethality that can be revealed by screens. Black indicates normal function; white indicates deficiency. Red broken line indicates the synthetic lethal interaction between two genes. Case I: partial reduction in function of two non-essential, synthetic lethal genes in a common pathway results in loss of an essential product. Case II: synthetic lethal interaction between two redundant genes that are present in a common pathway. Simultaneous loss, or decrease in function, of both redundant genes compromises cell viability. Case III: synthetic lethality between genes located in parallel pathways leading to the synthesis of a common essential product. Case IV: synthetic lethal interaction between two genes that are present in independent parallel survival pathways.

relationships between genes and produced a global map of gene function (Tong et al., 2001; Ooi et al., 2003; Pan et al., 2007; Baryshnikova et al., 2010). The availability of comprehensive collections of mutant alleles and tools such as RNAi, along with robust methods for high-throughput phenotypic analysis, underlies the success of these approaches (Dixon et al., 2009).

More recently, the ability to conduct genome-wide RNAi studies in human cell culture has allowed systematic investigation of the synthetic lethal relationships of combinations of genes (Luo et al., 2009). In this way, synthetic lethal approaches can reveal mechanisms by which human genetic variation contributes to disease by identifying interacting partners for specific normal and disease alleles. From a pharmacogenomics standpoint, synthetic lethality can be a powerful tool to understand differential responses of patients to identical drugs (Evans and Relling, 2004; Davies, 2006). Synthetic lethality has been particularly influential in forming a framework for designing small-molecule cancer-specific cytotoxic drugs that can selectively target cancer cells while sparing normal cells. This concept is based on approaches such as screening for chemicals that are synthetic lethal with oncogenes, or screening for chemicals that are synthetic lethal with tumor suppressor gene deficiency (Canaani, 2009; Kaelin, 2009; Shangary and Wang, 2009).

Zebrafish disease models

Although a relative newcomer to the model organism scene, the zebrafish has proven to be a valuable model to understand the pathogenesis of human diseases at the cellular and molecular level. Recent reviews highlight specific features of the model that have made these advances possible (Patton and Zon, 2001; Guyon et al., 2007; Feitsma and Cuppen, 2008; Milan and Macrae, 2008; Payne and Look, 2009), which we briefly summarize as follows. First, zebrafish are genetically tractable, exhibiting high fecundity (200 eggs per clutch), rapid development to adulthood (3 months) and small size at maturation (3-4 cm long as an adult), making it practical to work with large numbers of organisms quickly and economically. Second, embryonic development is rapid and experimentally accessible because the transparent embryos develop external to the mother fish. Third, zebrafish have conserved vertebrate anatomy and physiology: the zebrafish genome encodes orthologs of nearly 90% of human genes, increasing their relevance as a disease model. It is also important to note some weaknesses of the system. Although efficient techniques of chemical or viral mutagenesis are in routine use, screens in zebrafish cannot typically approach the saturation coverage of the genome achieved in organisms such as *Drosophila*, yeast and *C. elegans*. The zebrafish field lacks a comprehensive collection of knockouts or transposon insertion lines. In addition, although recent progress in the use of zinc-finger nucleases for site-specific mutagenesis is encouraging, gene targeting by homologous recombination is not currently available in the fish system. Translation- or splice-blocking morpholino oligonucleotides are extremely useful for knocking down gene expression in early embryos, but the cost of these reagents makes them impractical for genome-wide approaches.

Despite these limitations, zebrafish models of many human diseases have successfully been developed, including for melanoma (Patton et al., 2005; Ceol et al., 2011), Li-Fraumeni syndrome (p53 deficiency) (Parant et al., 2010), hematopoietic disorders (Danilova

et al., 2010; Ellett and Lieschke, 2010), heart disease (Sehnert and Stainier, 2002), glaucoma (Veth et al., 2011), inflammatory bowel disease (Oehlers et al., 2010), Duchenne muscular dystrophy (Berger et al., 2010) and neuromuscular diseases, including amyotrophic lateral sclerosis (Kabashi et al., 2010; Olson et al., 2010; Ramesh et al., 2010). Nearly all of these models have been constructed via mutation or knockdown of a single gene, or by heterologous expression of a transgene. This work demonstrates the utility of the zebrafish as a model, and also paves the way for 'second-generation' screens to identify interacting genes and pathways.

Modifier screens in fish: setting the stage for synthetic lethal approaches

Building on the success of zebrafish disease models, several groups have described genetic or chemical screens to identify components of a pathway or parallel pathways that modify a disease phenotype, or new drugs that rescue the disease phenotype. For example, Peterson and colleagues conducted a small-molecule-based screen using the zebrafish *gridlock* mutant to suppress the phenotype of malformed aorta (aortic coarctation) (Peterson et al., 2004). They identified a class of compounds that, by upregulating VEGF, a protein involved in specification and migration of angioblasts, suppressed the *gridlock* phenotype and allowed survival to adulthood. Similarly, Stern et al. conducted an embryo-based suppressor screen to identify persynthamide, a small molecule that suppressed the *bmyb*-dependent mitotic defects in the *crash&burn* mutant, providing evidence that chemical suppressor screening is able to identify pathways that interact with specific cell cycle phenotypes (Stern et al., 2005). Recently, Bai and colleagues conducted an ENU-based screen to identify suppressors of defective erythropoiesis resulting from a mutation in the gene encoding transcriptional intermediary factor 1- γ (TIF1 γ ; also known as TRIM33). By identifying suppressor mutations in PolII-associated factors, they discovered an unidentified role for transcriptional elongation in the control of cell fate (Bai et al., 2010).

In contrast to the above examples of suppressor screens, synthetic lethality is actually an extreme example of an enhancer screen, with the enhanced phenotype being death. Enhancer screens conducted using zebrafish cancer models would provide an opportunity to identify traditional recessive or haploinsufficient tumor suppressors, mutations of which would act as an enhancer of cancer affecting the latency of tumor onset, tissue specificity, or rate of disease progression or metastasis. Performing enhancer screens that worsen a disease phenotype can pose a challenge because many fish models of disease have a 'sensitized' phenotype, making them sick and unable to reach sexual maturity. These challenges can be circumvented using alternative approaches, such as driving gene expression with a conditional promoter or by creating lines of fish with temperature-sensitive alleles. Zebrafish expressing the *Myc* oncogene under control of the lymphocyte-specific *rag2* promoter develop T-cell acute lymphoblastic leukemia (T-ALL), but most animals die of the disease before reaching reproductive age (Langenau et al., 2003). Using the *Cre-loxP* system and a conditional allele of *rag2-Myc*, Langenau, Feng and colleagues generated a zebrafish model of T-ALL with increased latency (Langenau et al., 2005). These transgenic fish can now be utilized to conduct enhancer screens to gain a better understanding

of the molecular mechanisms regulating the multi-step pathogenesis of leukemia. Alternatively, a drug-targeted small-molecule screen can be conducted using fish with a sensitized genetic background to identify modifiers. For example, Hultman and colleagues conducted a small-molecule-based drug screen to identify modifiers of melanocyte migration defect using the temperature-sensitive allele of Kit receptor tyrosine kinase (*kit^{ts}*). Using this approach, the authors were able to identify enhancers and suppressors of the *kit^{ts}* mutant melanocyte migration phenotype (Hultman et al., 2008). These efforts not only further the goal of using fish disease models for translation to the clinic, but also provide proof-of-principle that synthetic lethal screens will be fruitful in the zebrafish system.

The synthetic lethal approach in zebrafish

With these examples at hand, one can imagine several potential applications of synthetic lethal screens in zebrafish. The first, which has already been achieved by several groups, uses loss-of-function approaches to reveal redundant function of paralogous genes (Case II in Fig. 1). Owing to a genome duplication event early in the evolutionary history of teleosts, many zebrafish genes are duplicated (Postlethwait et al., 1998), and the redundant functions encoded by these genes can mask the effects of mutations. For example, loss-of-function mutations in either copy of the tumor suppressor PTEN are viable, but *ptena^{-/-};ptenb^{-/-}* double mutant larvae die with multiple pleiotropic defects, indicating that the two paralogs play at least partly redundant roles in early development (Faucherre et al., 2008). The phenotype of embryos with severe developmental defects due to loss of redundant gene functions can be difficult to interpret. In this context, it is important to note that 'lethality' can apply not only to the whole organism, but also to a specific tissue. Several groups have used combinations of genetic mutants or morpholino knockdown approaches to demonstrate redundant gene function. For example, simultaneous depletion of the transcription factors Gata4 and Gata6, or Gata4 and Gata5, causes defects in cardiac development, but simultaneous loss of Gata5 and Gata6 causes impaired cardiomyocyte specification and absence of the heart – a sort of synthetic lethality for heart tissue (Holtzinger and Evans, 2007). Redundant gene functions have been reported by other groups, either for true paralogs or for related members of a gene family (Phillips et al., 2001; Cermenati et al., 2008; Gjini et al., 2010). Although these studies have typically examined candidate genes, the approach could easily be broadened to include forward genetic screens. In this scenario, a mutant or transgenic with a non-lethal phenotype would be subjected to mutagenesis, and second-site mutations that were synthetic lethal with the original mutation could be identified, owing to the types of interactions outlined in Fig. 1. The eventual development for zebrafish of FLP-FRT lines to support somatic mitotic recombination, on a par with *Drosophila*, would make possible rapid, tissue-specific synthetic lethal screens.

For many disease models, repair or restoration of tissue function, not lethality, is the translational goal. In these cases, synthetic lethal screening is more relevant for clarifying interacting networks of genes. In the case of cancer, however, the aim is to cause death or quiescence of the tumor while sparing the normal host tissue. Synthetic lethal screens performed on zebrafish cancer models can thus identify direct targets for therapy of specific malignancies by

revealing genes and pathways necessary for the survival of tumor cells. This information can be crucial, because many pathogenic mutations – for example, mutated tumor suppressor genes – are not directly susceptible to targeted therapies. Perhaps the most exciting opportunity in this area is the growing use of small-molecule screening in zebrafish, which takes advantage of the small size and rapid development of the fish and the ease of adding compounds to the water (Peal et al., 2010). Recently, screens carried out on human cell lines successfully identified small molecules that are synthetic lethal with known mutations or with established chemotherapy drugs. In one example, cells bearing activated Ras^{V12} were screened against chemical libraries, leading to the identification of several novel drugs that are selectively toxic to RAS-transformed cells (Dolma et al., 2003; Yang and Stockwell, 2008). Another exciting advance was the discovery that *BRCA1*- and *BRCA2*-deficient cells, which require PARP-1-dependent breakpoint excision repair activity, are selectively susceptible to treatment with PARP-1 inhibitors (Bryant et al., 2005; Farmer et al., 2005). Similarly, synthetic lethal drug screens in fish cancer models could directly identify promising lead compounds for targeted therapy of cancer. Performing these screens in zebrafish could have additional added value; for example, by providing the opportunity to test whether a drug is metabolically activated in the liver or the yolk, and also to assess toxicity in the context of a whole animal.

Conclusion

Since the early efforts of Bender, Pringle and others two decades ago, synthetic lethal screens have come of age. Now, with techniques for whole-genome sequencing, knockdown and small-molecule screening becoming more widely available, the importance of this unique genetic method will only increase. The elegant and powerful synthetic lethal screens that have been carried out in yeast, worms and flies can serve as an inspiration to zebrafish researchers contemplating a similar approach. Admittedly, the zebrafish system has some distance to go in terms of genetic tools and infrastructure before it can match these other models. However, the complementary strengths of the zebrafish system, especially for modeling human disease, mean that fish synthetic lethal screens are poised to make an immediate impact for translational medicine. An exciting era of discovery awaits!

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COMPETING INTERESTS

The authors declare that they have no competing or financial interests.

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