Forward and Reverse Genetic Approaches for the Analysis of Vertebrate Development in the Zebrafish

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The development of facile forward and reverse genetic approaches has propelled the deconvolution of gene function in biology. While the origins of these techniques reside in the study of single-cell or invertebrate organisms, in many cases these approaches have been applied to vertebrate model systems to gain powerful insights into gene function during embryonic development. This perspective provides a summary of the major forward and reverse genetic approaches that have contributed to the study of vertebrate gene function in zebrafish, which has become an established model for the study of animal development.

Introduction

Historically, geneticists have relied on the identification of mutant phenotypes to define and dissect a particular pathway or process without a priori knowledge of the genes involved. Indeed, early application of this forward genetic approach in bacteriophage, bacteria, and other microorganisms established the relationship between genes and enzymes, defined gene structure and helped to decipher the triplet genetic code (for examples see Beadle and Tatum, 1941; Brenner et al., 1965). Subsequent application of these approaches to multicellular animals allowed the systematic dissection of developmental processes in both *Caenorhabditis elegans* and *Drosophila* (Brenner, 1974; Nüsslein-Volhard and Wieschaus, 1980). In principle, these forward genetic approaches could be applied to a vertebrate organism to elucidate the genes required for embryonic development. However, since such genetic studies require detailed morphological observations of embryos, widely used mammalian models, such as the mouse, provided a challenging system for such an endeavor due to their internal development. As an alternative, Streisinger et al. initiated studies that demonstrated the feasibility of utilizing the zebrafish, *Danio rerio*, as a model for the genetic analysis of vertebrate development (Chakrabarti et al., 1983; Grunwald et al., 1988; Streisinger et al., 1986; Streisinger et al., 1981; Walker and Streisinger, 1983). The zebrafish proved ideal as egg clutch size per mating pair was much larger (>100) and its externally fertilized embryos developed synchronously ex vivo and more rapidly than other related fish models (e.g., Medaka). Most importantly, zebrafish embryos remain transparent through much of their early embryonic stages, permitting detailed serial microscopic observations throughout early development. As an added benefit, zebrafish are relatively small and generally hardy and consequently can be maintained at much higher densities and at much lower costs than mammalian model systems.

The first large-scale forward genetic screens in zebrafish provided the basis for the discovery of a multitude of new genes and pathways fundamental to vertebrate development (Driever et al., 1996; Haffter et al., 1996). Subsequently, the recent explosion of genomic resources has precipitated the need for complementary reverse genetic approaches to directly interrogate genes and pathways of interest. As with other models founded largely on their amenability to forward genetics (e.g., *C. elegans*, *Drosophila*), developing reverse genetic approaches for zebrafish initially proved challenging. Fortunately, a number of techniques applied to other model systems have now been successfully adapted to the zebrafish to enable relatively straightforward reverse genetic manipulation of genes of interest. In this review, we provide an overview of major forward and reverse genetic approaches utilized in the zebrafish with a particular emphasis on the technical advantages and shortcomings of each method. We also highlight cases where these approaches have provided new insights into particular aspects of embryonic development.

Forward Genetic Approaches

Forward genetic approaches seek to identify genes involved in a biological pathway or process through the screening of populations of animals that contain random modifications throughout the genome that can alter gene function. Carriers of interesting modified alleles are identified by the observation of particular displayed phenotypes, and subsequent mapping of the allele within the genome reveals genes that are associated with the observed biological process. The potential to apply forward genetic approaches for comprehensive genetic dissection of vertebrate development was the initial attraction for researchers to utilize the zebrafish as a model system. Following is an overview of the major forward genetic approaches employing the zebrafish, along with examples that have provided fundamental new insights into vertebrate development.

Mutagens

The first step in any forward genetic approach is the creation of heritable mutagenic lesions that can be screened for phenotypic effects. In zebrafish, several different agents have been applied for this purpose. Initial work utilized gamma irradiation to induce

chromosomal breaks, leading to embryonic mutant phenotypes (Chakrabarti et al., 1983; Walker and Streisinger, 1983). However, the resulting lesions were often large deletions, translocations, or other gross chromosomal aberrations, making it difficult to accurately identify the causative genes responsible for a mutant phenotype. Subsequent efforts applied alkylating agents, in particular N-ethyl-N-nitrosourea (ENU), which can achieve high mutagenic loads in zebrafish premeiotic germ cells (roughly one visible mutant per genome scored), where the induced phenotypes can be discretely linked to lesions in one gene (Mullins et al., 1994; Solnica-Krezel et al., 1994).

ENU is currently the standard choice for chemical mutagenesis in both forward and reverse genetic screening (see below), as it can be easily applied to adult male zebrafish by simply exposing them to water containing the compound. However, the primary drawback to this mutagenesis method is the difficulty in subsequently identifying the induced lesions that are responsible for an observed mutant phenotype (see below). To address this limitation, several groups have applied replication-deficient pseudotyped retroviruses or transposons as insertional mutagens (Gaiano et al., 1996; Nagayoshi et al., 2008; Sivasubbu et al., 2006). For these applications, parental founders are created by injection of the mutagenic agent into either one-cell (transposon) or 1000-cell stage embryos (retrovirus). However, retroviruses and transposons typically achieve a much lower overall mutagenic frequency than ENU (approximately one-ninth the efficiency; Amsterdam et al., 1999), requiring significantly larger libraries of mutagenized fish to be generated for screening in comparison to chemical mutagenesis. This extra effort is partially compensated for by the fact that the targeted locus can be readily identified by inverse or ligation-mediated PCR using the insertional element as an amplification tag (Amsterdam et al., 2004a; Golling et al., 2002).

Breeding Schemes

Following mutagenesis, several different breeding schemes can be utilized in a forward genetic screen. The choice of scheme depends on both experimental factors, such as the phenotypes that will be assayed, and logistical considerations, such as available space, personnel, and overall cost. The first large-scale screens performed in the Driever (Boston) and Nüsslein-Volhard (Tubingen) laboratories focused on the broad identification of multiple embryonic phenotypes at different developmental stages using simple microscopic observation. In these screens, both groups observed phenotypes associated with homozygous recessive mutations in diploid F3 embryos, requiring a two-generation breeding scheme (Driever et al., 1996; Haffter et al., 1996; Mullins et al., 1994). In this case, ENU-treated males are out-crossed to generate a large population of F1 individuals (Figure 1A). Following standard ENU regimens, each F1 individual typically harbors approximately one mutagenic lesion that will cause an embryonic phenotype (Driever et al., 1996; Haffter et al., 1996). F1 progeny are grown to adulthood and used to generate F2 families of heterozygous carriers (Figure 1A). F1 adults can be in-crossed at this point to increase the number of mutagenized genomes screened per F2 family. Alternatively, F1 adults can be out-crossed to a genetically distinct wild-type line to incorporate polymorphic genetic markers in the F2 family that will enable subsequent linkage analysis in F3 progeny.
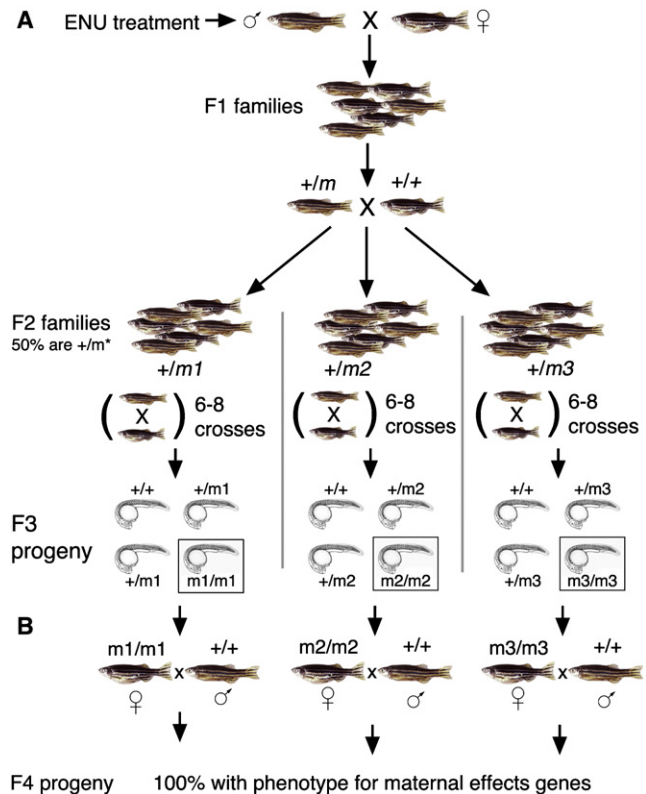

Figure 1. Overview of Breeding Scheme for ENU-Based Mutagenesis
(A) Germline mutations are generated in ENU-treated males, which are out-crossed to generate F1 families. Members of these families are subsequently out-crossed to generate F2 families, whose members are in-crossed to generate F3 progeny for phenotypic analysis.
(B) Phenotypically "normal" F3 females can be out-crossed to detect maternal effect mutations. Camera lucida images of embryos in all figures were modified with permission from Kimmel et al. (1995).

Embryonic phenotypes are subsequently identified in F3 progeny from multiple individual in-crosses between F2 family members (Figure 1A). Using this approach, the Boston and Tubingen groups initially identified over 6000 mutant phenotypes, from an approximately equal number of screened genomes, of which about one-third led to specific defects in pattern formation, differentiation, or organogenesis (Driever et al., 1996; Haffter et al., 1996). Most of the remaining phenotypes were general necrosis or those similar to the "*minute*" phenotypes (general growth retardation and developmental delay) observed in other model organisms, such as *Drosophila* (Schultz, 1929). Subsequent analysis of a select number of mutants with more specific phenotypes identified nearly 600 discrete loci required for gastrulation, embryonic patterning, and organogenesis.

Insertional mutagenesis (retroviral or transposon) has also been applied in the context of two-generation F3 screens. While similar, the use of insertional mutagens requires some alteration to standard ENU-based schemes. After generating a population of founders by injection of the mutagenic agent, these animals are subsequently crossed to generate an F1 population for screening (Figure 2). Individual F1 fish are then prescreened for high insert number per genome (Amsterdam et al., 1999) or
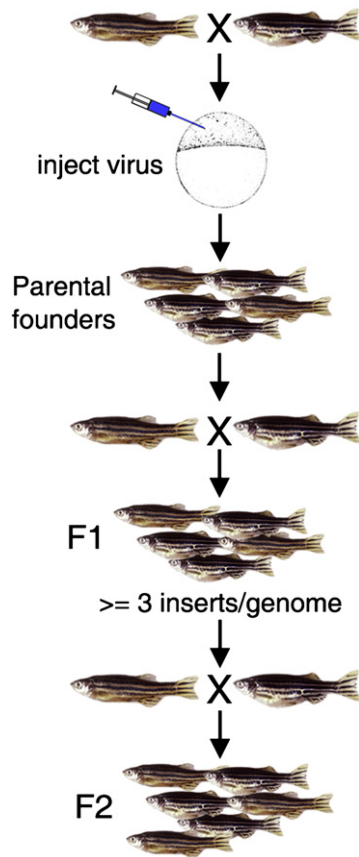
Figure 2. Overview of Breeding Scheme for Retroviral-Based Insertional Mutagenesis
Pseudotyped virus is injected into 1000-cell stage embryos to create a population of founders for subsequent breeding and mutant analysis. These F2 families are screened as in ENU-based mutagenesis (Figure 1).

favorable expression patterns from an incorporated trapping system (Nagayoshi et al., 2008) to increase the mutational frequency and optimize screening. Animals bearing favorable insertional loads are then used to make F2 families (Figure 2) from which F3 progeny are obtained and screened as in ENU-based screens (Figure 1). The use of insertional mutagens thus requires much more hands-on work prior to actual screening and the analysis of larger libraries of mutagenized fish due to the lower mutagenic rate, but it subsequently allows rapid identification of the disrupted allele.

The design of most large-scale F3 screens has been biased toward the identification of homozygous recessive mutations that affect zygotic gene function. To investigate maternal or paternal gene function, early zygotic phenotypes can be rescued with mRNA injections to permit survival to adulthood (Gritsman et al., 1999), although successful rescue is typically limited to genes that are required only during early embryonic development. Alternatively, cell transplantation can be applied to transfer germ cells from mutant embryos into those with wild-type somatic cells to establish adults bearing homozygous null germ cells (Ciruna et al., 2002). To more broadly identify maternal effects genes in the context of a genetic screen, it is possible to apply a three-generation breeding scheme (Figure 1B). The

crossing scheme is identical to an F3 screen, except that viable F3 progeny are grown to adulthood. Putative homozygous mutant females are then crossed to wild-type males to generate F4 progeny, which are subsequently screened for defects in early embryonic patterning. In this case, a fully penetrant maternal mutation will result in all of the F4 embryos exhibiting a mutant phenotype. This approach has led to identification of maternal loci required for early egg formation and activation, as well as later steps of development, such as gastrulation and pattern formation (Dosch et al., 2004; Wagner et al., 2004). These F4 screens also revealed abnormal phenotypes in some of the adult F3 fish, implying that screens could be directed at identifying mutants affecting postembryonic developmental stages. Indeed, adult F3 screens have successfully identified mutants affecting pigment patterning (Parichy and Turner, 2003), formation of skeletal structures (Andreeva et al., 2011; Harris et al., 2008), and fin regeneration (Johnson and Weston, 1995). In this latter case, Johnson and Weston (1995) relied on the identification of conditional temperature sensitive mutants to permit screening of postembryonic phenotypes. While this approach has proven valuable for uncovering gene function at postembryonic stages, especially in cases where a null allele causes severe early developmental defects (Tian et al., 2003), identification of temperature-sensitive alleles is still underutilized in zebrafish compared to other model systems (e.g., *C. elegans* and *Drosophila*).

Despite the success of large-scale F3 screens, this approach requires an enormous investment of time due to the relatively long maturation time of the zebrafish (approximately three months). Likewise, the large numbers of F2 individuals needed in an F3 screen can require a great deal of personnel and tank space for their care and maintenance. Fortunately, it is possible to generate haploid embryos from zebrafish females by treatment of eggs with UV-inactivated sperm (Streisinger et al., 1981). Consequently, by screening haploid embryos from F1 females for mutant phenotypes (Figure 3), the total amount of time from mutagenesis to screening can be significantly reduced. This approach also permits screening at a much higher rate (usually 4 to 5 times more genomes per week) than in an F3 screen. Since a single-generation breeding screen also occupies much less tank space and requires less personnel, its application is more amenable to smaller research groups. However, haploid embryos arrest after several days of development and often display aberrant morphology, allowing identification of only overt embryonic defects. These problems can be alleviated by treating haploid embryos with early pressure or heat shock, which suppresses the second meiotic division, leading to formation of gynogenetic diploid embryos that are normal and can be grown to adulthood (Streisinger et al., 1981). In these embryos, the frequency of mutant phenotypes is inversely proportional to the distance of the associated gene to the centrosome, resulting in a much lower penetrance of phenotypes than observed in haploid embryos and loss of mutants that would have a distal chromosome locus (Streisinger et al., 1986). Additionally, generation of haploid and gynogenetic diploid embryos is technically challenging. Despite these drawbacks, gynogenetic diploid screening has proven valuable by streamlining otherwise cumbersome breeding schemes, such as in maternal effects screens (Pelegri et al., 1999).
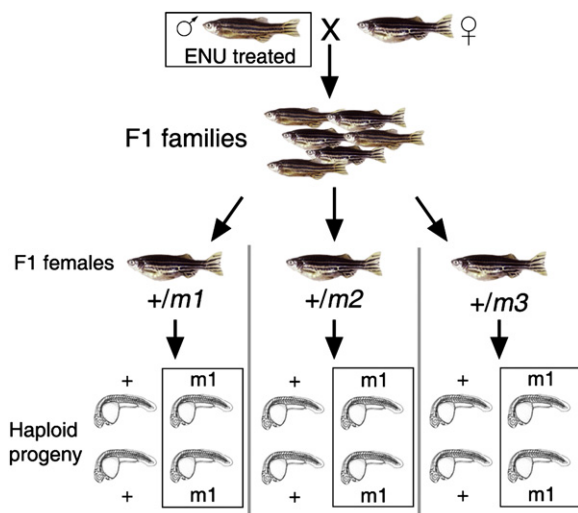
Figure 3. Overview of Haploid Breeding Scheme for ENU-Based Mutagenesis

Mutagenesis and generation of F1 families are performed as in the standard ENU-based diploid screen. However, to simplify the screening process, eggs are obtained from F1 females and treated with UV-inactivated sperm to generate haploid progeny that can be screened for early embryonic phenotypes.

Modifier Screens

A powerful aspect of forward genetic analysis in other model systems (e.g., yeast, *Drosophila*, and *C. elegans*) is the ability to perform screens using a sensitized background in order to concentrate on a particular pathway or biological process. Given the limited focus of such studies, they often require screening many more genomes, making them more difficult to apply, especially in vertebrate models. Not surprisingly, there have been only a few reports of successful modifier screens using the zebrafish.

Kramer et al. were among the first to demonstrate the utility of such an approach in the zebrafish model by performing a dominant enhancer screen for mutants affecting dorsoventral patterning (Kramer et al., 2002). This was accomplished by crossing mutagenized males against females that were heterozygous for a mutation in the *chordin* gene, which normally leads to ventralization in homozygous mutant embryos. The resulting F1 fish were screened for ventral tail fin defects, leading to the identification of seven enhancing mutations. Although each of the associated genes had been previously implicated in dorsoventral patterning, the recovered alleles validated the principle of the screen. Importantly, these enhancing mutations were identified from only 824 F1 genomes, where the analysis is both straightforward and relatively rapid, suggesting that additional screening could yield novel mutants required for dorsoventral patterning.

Genetic suppressor screens have also been successfully employed in zebrafish. For example, Bai et al. utilized a haploid screening strategy to identify genetic suppressors of a mutation in the *moonshine* (*mon*) gene, which encodes Transcriptional intermediary factor 1 gamma (*tif1γ*, Bai et al., 2010). Since loss of *mon* normally causes anemia and larval lethality, they first generated a transgenic line carrying the wild-type *moonshine* gene, along with a ubiquitous GFP marker, on a bacterial artificial

chromosome (BAC) capable of rescuing the mutant phenotype. Homozygous *mon* mutant males bearing the transgene were mutagenized and crossed to transgene-rescued *mon* females to generate F1 female carriers. Eggs from these animals were treated with UV-irradiated sperm and GFP-negative haploid embryos, which should exhibit the *mon* phenotype, were screened for *globin* expression by in situ hybridization. This led to the identification of the *sunrise* mutation, which fully restored *globin* expression in *mon* mutants. Subsequent analysis demonstrated that *sun* encodes the *cdc73* gene, a component of the Pol II transcriptional elongation complex (Shi et al., 1997), revealing a regulatory interaction between *tif1γ* and transcriptional elongation during erythroid development.

A central challenge to performing modifier screens in zebrafish is the availability of an appropriate genetic background. This usually requires a viable adult phenotype or the parallel generation of transgenic backgrounds that can conditionally rescue the mutant phenotype. While temperature-sensitive mutants could provide valuable genetic backgrounds for this application, there is a paucity of such lines available. An intriguing alternative approach is the utilization of small-molecule modifiers to mimic a sensitized genetic background. This can be especially powerful since compounds can be used to either activate or inhibit a specific pathway in a temporally restricted manner, thus providing a conditional sensitized background. This approach has been successfully applied by Milan et al. to identify genes involved in cardiac function (Milan et al., 2009). They treated embryos derived from a collection of approximately 300 retroviral insertion mutants with dofetilide, which normally causes defects in myocardial repolarization in zebrafish embryos, and subsequently identified 15 that displayed either enhanced or suppressed atrioventricular block. About half of these mutants displayed normal cardiac morphology, suggesting a specific defect in cardiac function. Interestingly, one of these genes mapped to an interval associated with defective myocardial repolarization in humans, demonstrating the utility of sensitized screens in zebrafish embryos for the elucidation of human disease processes.

Phenotyping

The first large-scale screens relied on simply observing live embryos serially over several days for a variety of morphological or simple behavioral defects. This approach enabled the identification of mutations that affected a myriad of developmental processes, including dorsoventral patterning, gastrulation, and the formation of various organ systems (Driever et al., 1996; Haffter et al., 1996). Since that time, zebrafish researchers have greatly expanded the spectrum of phenotypes identified in mutant screens, including more detailed analysis of morphological defects as well as functional assays.

A simple way to achieve more detailed phenotypic screening is through the use of molecular markers for a given cell or tissue type. This approach has been successfully implemented through the application of whole mount in situ hybridization or immunostaining. For example, the *valentino* mutation, which causes subtle defects in hindbrain pattering, was identified in a haploid screen using whole mount in situ hybridization with a *krox20* riboprobe, which specifically labels rhombomeres 3 and 5 in the hindbrain (Moens et al., 1996). While *val* mutations also

display morphological defects that could be detected in live embryos, many cell types can only be observed using molecular markers. For example, mutations affecting the development of lymphoid cells and the thymus, which would be nearly impossible to detect based on simple morphological criteria, have been identified using whole mount in situ hybridization with a *rag1* probe (Trede et al., 2008). Thus, the use of molecular probes allows the identification of subtle defects in tissues, thereby increasing the spectrum of phenotypes that can be identified through a forward genetic screen.

Despite these benefits, whole mount in situ or immunostaining methods require more hands-on time and effort and require sample fixation, eliminating the ability to perform serial observations. These problems can be alleviated in many cases by employing a tissue-specific transgenic line, in which a particular cell lineage is stably marked by fluorescent protein expression, for phenotypic screening. In some cases, these approaches have been used as secondary screens after an initial morphology-based screen (Chi et al., 2008; Jin et al., 2007), while in others, transgenic markers have been used as the primary screening tool (Covassin et al., 2009; Gulati-Leekha and Goldman, 2006) (e.g., to identify defects in vascular development in haploid embryos). These latter examples, as with the identification of the *val* mutant, highlight the particular benefit of using molecular probes or transgenic lines to more easily identify defects in haploid embryos, which often display overt general developmental defects.

Researchers have also exploited the zebrafish in genetic screens to identify mutants that affect organ physiology. An early example of this approach was the identification of zebrafish larvae lacking an appropriate optokinetic response (Brockerhoff et al., 1995). In a small-scale F3 screen, several mutants were identified that failed to exhibit tracking eye movement in response to a moving visual stimulus. Importantly, two of these mutants were otherwise morphologically normal, indicating that screens designed to assay embryonic organ function can reveal phenotypes that would otherwise go unnoticed by a standard visual screen. A more recent example of such an approach is the use of fluorescently labeled phospholipids to identify mutants affecting digestive tract function. In this case, Farber et al. designed phospholipid probes that, when fed to larvae, exhibited robust fluorescence within the gut upon cleavage by phospholipase A2 (Farber et al., 2001). The application of this probe in the context of a small-scale F3 screen led to identification of the *fat free* mutation. As with the optokinetic mutants, *ffr* mutants did not otherwise exhibit obvious morphological defects in gut development. Subsequent cloning of the *ffr* gene revealed an ADP-ribosylation factor that was required for Golgi-trafficking and lipid absorption in the digestive tract (Ho et al., 2006).

Zebrafish researchers continue to develop new phenotype-based assays to screen for novel mutations affecting a variety of different developmental and physiological processes. Notable examples include the application of X-ray analysis to identify defects in skeletal patterning in adult zebrafish (Fisher et al., 2003) and behavior-based screens to identify mutants affecting locomotion and nicotine responses (Granato et al., 1996; Nicolson et al., 1998; Petzold et al., 2009). Screens have also been applied to identify genes important in cancer susceptibility (Frazer et al., 2009), as well as those that play a role in host response to infectious disease (Tobin et al., 2010). In this latter case, identification of the *lta4h* gene, which modulates the zebrafish response to *Mycobacterium marinum* infection, revealed an important role for the orthologous gene in tuberculosis and leprosy susceptibility in humans. This particular study, which capitalized on the ability of the zebrafish embryo to mount an appropriate innate immune response as a screening assay, underscores the powerful translational aspects of forward genetic screening in the zebrafish embryo.

Identifying Causative Mutations

A mutant allele affecting a given developmental process is an invaluable tool for a variety of studies. However, the ultimate goal is to identify the causative genetic lesion responsible for the observed phenotype. A number of efforts have enabled the positional and candidate cloning of zebrafish mutants identified in forward genetic screens using chemical mutagens. These include the establishment of reliable physical and genetic linkage maps (Gates et al., 1999; Geisler et al., 1999; Kelly et al., 2000; Woods et al., 2005), along with development of a large panel of known polymorphic microsatellite repeat markers that can be used for linkage mapping (Shimoda et al., 1999). This panel is typically used for initial bulk segregant mapping on wild-type and mutant embryos to establish the genetic interval in which a mutation lies. Given the current density of available markers, this genetic interval can often easily be reduced to a centimorgan or less. In the past, additional markers and candidate genes within an interval could then be identified using physical maps generated through radiation hybrid mapping or by isolating physical clones in BACs, or other large-insert constructs. With the advent of a nearly complete zebrafish genome sequence, as well as the annotation of known single-nucleotide polymorphisms (SNPs) between strains (Flicek et al., 2011), it is now possible to subsequently narrow an interval without the need for generating a physical map. The available genome assembly also allows the rapid identification of candidate genes within an interval. Once candidate genes are identified, it is possible to capitalize on available expression pattern and microarray data to further narrow the list of genes of interest, followed by sequencing to identify the genetic lesion and rescue for validation.

With the decreasing cost and wider accessibility of deep sequencing, it is possible to apply this technique to rapidly identify candidate genes. Whole genome sequencing has already been successfully applied in *C. elegans* to identify EMS-induced lesions (Sarin et al., 2008). While the zebrafish genome is more than an order of magnitude larger than that of *C. elegans* (1.5×10^9 bp versus 9.7×10^7 bp), modified sequencing approaches have begun to be applied with great effect. For example, Gupta et al. have successfully applied targeted massively parallel sequencing of a mutant locus to identify the causative mutation in the zebrafish *magellan* allele, which perturbs normal oocyte polarity (Gupta et al., 2010b). In this instance, after identifying a candidate interval by bulk segregant analysis and fine mapping, the authors captured genomic DNA fragments spanning several hundred kilobases of the candidate locus for deep sequencing, leading to the identification of a 31 bp deletion in the *microtubule actin crosslinking factor 1* (*macf1*) coding sequence. While this approach was limited to a relatively

small region of the genome, it is conceivable that larger intervals can be similarly captured and sequenced. As the number and length of deep sequencing reads continues to increase with technological improvements, this methodology will likely enable straightforward whole genome or exome sequencing to identify mutant lesions in the near future.

As noted above, insertional mutagens (retroviruses or transposons) allow rapid identification of disrupted genes linked to an observed phenotype. The use of a pseudotyped murine retrovirus as a mutagen in zebrafish for a large-scale screen was pioneered by Nancy Hopkins. In an F3 screen, the Hopkins group identified over 500 mutant phenotypes using similar morphological criteria employed in the original large-scale ENU-based screens (Allende et al., 1996; Amsterdam et al., 1999; Amsterdam et al., 2004a; Gaiano et al., 1996; Golling et al., 2002). However, unlike these ENU-based screens, all insertional mutants displaying embryonic phenotypes were maintained, including those with general defects, such as necrosis and developmental delay, with the expectation that there might be genes of interest in this later phenotypic class. This has indeed proven to be the case. The most striking examples are mutants within the ribosomal genes, most of which resulted in *minute* phenotypes in homozygous mutant embryos (Amsterdam et al., 2004b). Interestingly, closer inspection of adult heterozygous carriers of these alleles indicated a role for ribosomal proteins as haploinsufficient tumor suppressors. In all, the Hopkins group identified the causative genes for over 300 mutant embryonic phenotypes covering approximately 25% of the embryonic-essential genes in zebrafish. Despite the ease of identifying modified alleles generated through insertional mutagenesis, a major drawback for forward genetic screening is that the mutagenesis frequency is several times lower than that of ENU. Not surprisingly, there have been few subsequent attempts at large-scale forward screens using retroviral mutagenesis, although several smaller-scale efforts have been applied using similar approaches, such as transposable elements (Nagayoshi et al., 2008; Petzold et al., 2009).

Forward Genetics—Perspective and Future Directions

Nearly 30 years ago, George Streisinger initiated forward genetic studies in zebrafish by assessing the ability of gamma rays to induce germline mutations that resulted in distinct developmental phenotypes (Chakrabarti et al., 1983; Grunwald et al., 1988). Subsequent large-scale screening efforts, and innumerable smaller-scale screens, have revealed a broad spectrum of previously unknown genes required for multiple aspects of vertebrate development. These genes span diverse biological functions, from those required for gastrulation and mesoderm induction, cilia formation, and cardiovascular development (for examples, see Bakkers, 2011; Sun et al., 2004; and Zhang et al., 1998), to those required for infectious disease progression, lipid metabolism, and regeneration (see above). These mutants have helped to define entirely new developmental pathways required for organogenesis, including those required for early endoderm specification and heart development (Bakkers, 2011; Ober et al., 2003). In many of these cases, the zebrafish has revealed conserved pathways and new genes that are responsible for human disease. Despite the significant effort required to perform forward screens in zebrafish, researchers

continue to develop and apply novel screening strategies to explore vertebrate development in greater breadth and depth.

Reverse Genetic Approaches

As described above, unbiased forward genetic screens using the zebrafish have revealed novel genes and pathways required for vertebrate development. However, given the large size of the zebrafish genome, the space and personnel required for forward screens, and the redundancy inherent due to the additional genome duplication in teleost fish, it is likely impossible to identify all relevant developmental genes using forward screening. Additionally, a number of approaches over the past decade, including the sequencing and assembly of the zebrafish genome (Flicek et al., 2011; Vogel, 2000) and the extensive expression analyses, mostly through large-scale whole mount in situ hybridization screening (Thisse and Thisse, 2008), have revealed thousands of intriguing candidate genes that are likely playing roles in development. However, it is only recently that definitive reverse genetic approaches have been established to interrogate the function of these genes. These new tools and resources, some of which are highlighted below, portend an exciting new period in zebrafish research where the combination of a suite of forward and reverse genetic approaches will allow the zebrafish model to be fully exploited for the systems level analyses of developmental and disease processes.

Morpholino-Mediated Gene Knockdown

RNAi-based approaches have previously proven problematic in zebrafish (Skromne and Prince, 2008), although recent studies in *Xenopus* embryos suggest that codelivery of Ago2 may overcome this problem (Lund et al., 2011). As a consequence, antisense gene knockdown via Morpholino oligonucleotides has been widely adopted due to the ease with which these reagents can be administered into the zebrafish embryo and their efficacy. Although this approach is not truly a genetic manipulation, Morpholinos have proven to be a valuable tool for assessing gene function in development, as first demonstrated in *Xenopus* (Heasman et al., 2000), particularly in systems where alternate reverse genetic strategies were not readily available. Therefore, we present a discussion of their use in the context of reverse genetic approaches.

Morpholinos (MOs), which employ a neutral, nonribose backbone displaying the four DNA bases (Summerton, 1999; Summerton and Weller, 1997), stably basepair with RNA but are resistant to degradation, resulting in penetrant gene knockdown effects (Bill et al., 2009). MOs function by forming heteroduplexes with the target transcript to interfere with protein synthesis or splicing. Ekker and colleagues were the first to demonstrate the efficacy of MOs in zebrafish by targeting the translation start site for a number of developmental genes, where treated embryos mimicked phenotypes observed in corresponding mutant alleles (Nasevicius and Ekker, 2000). By targeting splice site junctions within the pre-mRNA, it is possible to selectively interfere with zygotic transcripts through exon skipping (Draper et al., 2001), which can result in abrogation of gene expression. Disruption of splicing has the advantage that the efficiency of gene knockdown can be verified by RT-PCR analysis of the RNA target, whereas assessing the effectiveness of a MO that targets a translation start site requires a specific antibody

to evaluate changes in protein expression level (Draper et al., 2001). By targeting unique splice sites, isoform-specific knockdown can also be achieved (Mably et al., 2003; Xu et al., 2002). MO-based inhibition of target genes is not limited merely to protein-coding RNAs, but can also be used to suppress miRNA function, either by interfering with miRNA maturation (Flynt et al., 2007; Kloosterman et al., 2007) or miRNA-target 3′ UTR interactions (Choi et al., 2007). Due to their efficacy, MOs have become the method of choice for the rapid analysis of gene function in early development—for example, playing important roles in understanding primordial germ cell migration (Doitsidou et al., 2002), the genesis of hematopoietic stem cells (North et al., 2009), and the formation of left-right asymmetry (Neugebauer et al., 2009). MOs have also been employed in moderate-scale functional genomic screens to investigate gene function at a systems level (Eckfeldt et al., 2005; Pickart et al., 2006).

Standard MOs injected into the early embryo have global effects on target protein expression; however, more restricted application of MOs can be obtained through different applications of the technology. By delaying the timing of the MO injection, their effect can be limited to dorsal forerunner cells within the early embryo (Amack and Yost, 2004). Alternately, the utilization of caged MOs allows restricted knockdown in a defined region of the embryo (Shestopalov et al., 2007). Caged MOs contain an additional complementary sequence joined through a photocleavable linker that inhibits function; subsequent irradiation of the desired tissue with UV light relieves inhibition within a specific region of the embryo, allowing defined spatial and temporal control of gene knockdown (Shestopalov et al., 2007). Notably, regionalized activation can be achieved through two-photon excitation, enabling gene knockdown in single cells (Ouyang et al., 2009). MOs have also been applied in adults to study the effect of targeted gene (Thummel et al., 2006; Thummel et al., 2007) or miRNA (Yin et al., 2008) knockdown on fin regeneration by employing a combination of direct injection followed by electroporation to achieve broad efficacy in the local tissue.

MOs, like any other knockdown-based approach, have a number of limitations that constrain their application. Since target inhibition is not achieved via a defined genetic lesion, the degree of knockdown can be variable, and the duration of efficacy in the embryo is limited to ~3 days (Bill et al., 2009; Nasevicius and Ekker, 2000; Smart et al., 2004). An additional potential problem is spurious phenotypes due to off-target effects. The most frequent complications arise from MO-induced neuronal apoptosis that results from upregulation of the p53 pathway, although this effect can be partially ameliorated by coinjecting a p53-MO (Robu et al., 2007). Because the off-target effects of MOs can result in confounding influences on development, associated phenotypes are typically verified by employing at least two different MOs that target independent regions of the RNA transcript and, if feasible, performing a rescue experiment (Eisen and Smith, 2008). With the advent of improved reverse genetic approaches, it is now also possible to generate a null allele to validate the observed MO-dependent phenotype.

Targeting Induced Local Lesions in Genomes

Targeting Induced Local Lesions in Genomes (TILLING) was the first reverse genetic approach in zebrafish to successfully yield germline mutations in a desired target gene. This method, which
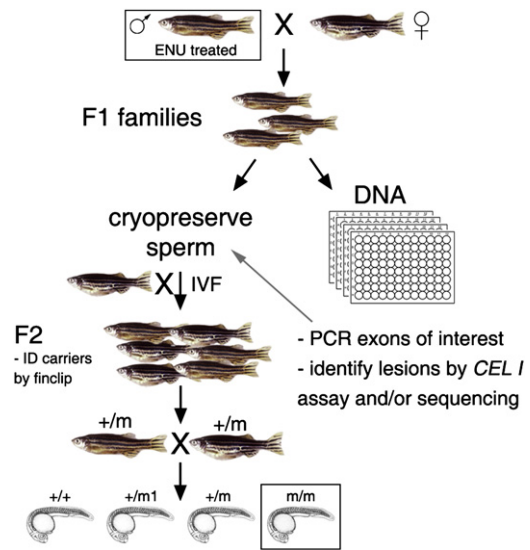

Figure 4. Overview of TILLING Mutant Identification
F1 males from the ENU-mutagenesis protocol are sacrificed, where samples of cryopreserved sperm and genomic DNA from each individual are archived. Genomic DNA from these animals is screened in a high-throughput method for individuals bearing lesions in the desired gene. Sperm from each identified carrier is used to generate F2 animals through in-vitro fertilization (IVF), and heterozygous carriers are then identified by genotyping.

combines extensive mutagenesis of an organism's genome with a comprehensive analysis of loci in a mutagenized population, allows rare mutagenic events to be recovered for subsequent phenotypic analysis. TILLING was originally developed by the Henikoff laboratory to screen libraries of EMS-treated *Arabidopsis* for desired mutant alleles (Colbert et al., 2001; McCallum et al., 2000) and was subsequently adapted by Wienholds and colleagues to the zebrafish (Wienholds et al., 2002; Wienholds et al., 2003b). In this implementation, adult zebrafish males are mutagenized with ENU, as in phenotype-based screens, and are used to generate F1 families (Figure 4). Subsequently, multiple sperm samples from each individual F1 male are cryopreserved while corresponding genomic DNA is isolated and archived for lesion analysis. If space permits, DNA can be isolated from finclips and the F1 population can be maintained as a living library (Wienholds et al., 2003b).

Lesions within the library are identified through the analysis of PCR amplicons spanning genomic regions of interest, where the analyzed regions are typically early exons or those containing critical functional domains in which a nonsense mutation would likely be inactivating. It is also possible to identify inactivating splice-site mutations as well as missense mutations for the purpose of creating an allelic series of hypomorphic mutants. In the most common implementation, mutations are detected by denaturation and reannealing of pooled PCR products from multiple library members, followed by digestion with the mismatch-specific endonuclease *CEL I* (Oleykowski et al., 1998), which cleaves heteroduplex DNA (Colbert et al., 2001). Potential lesions identified as *CEL I* cleavage events are subsequently characterized by sequencing the genomic region of the putative carrier. Following validation, the cryopreserved sperm is used for in vitro fertilization to generate an F2 family, in which

one-half of the animals will be carriers of the desired allele (Figure 4). Heterozygous carriers, identified through finclip and genotyping, are subsequently in-crossed to generate embryos in which one-quarter carry homozygous mutations within the desired gene.

Given the large number of F1 fish that need to be screened to find a lesion in a gene of interest, the initial identification step using *CEL I* can prove to be a bottleneck. As an alternative, direct sequencing of genomic regions of interest can be employed (Wienholds et al., 2002). Direct sequencing has the additional advantage that SNPs within the genome, which can give confounding signals in the *CEL I* assay, are readily classified. The development of massively parallel sequencing platforms provides new avenues for rapidly screening TILLING libraries, just as it can dramatically increase the rate of discovery of ENU mutations identified through forward genetic screens (Gupta et al., 2010b; Zhang et al., 2009). In a single lane of Illumina sequencing, due to its inherent capacity, samples from a pool of fish can be multiplexed enabling thousands of exons to be simultaneous screened (D. Stemple, personal communication). This approach holds great promise for the rapid identification of large numbers of mutant alleles from mutagenized libraries, with the potential for even greater throughput as the number and length of sequencing reads continues its rapid increase with each new generation of instruments (Mardis, 2011).

TILLING has generated zebrafish lines bearing mutations in a number of notable genes. *rag1*, which is required for V(D)J-recombination and proper development of T cells and B cells in mouse (Mombaerts et al., 1992), was the first mutant allele identified. Interestingly, *rag1* mutant zebrafish are viable and not obviously immunocompromised (Wienholds et al., 2002), but do have significantly lower lymphocyte populations (Petrie-Hanson et al., 2009), suggesting that this line may serve as a platform for generating adult zebrafish that can be used for transplantation and xenograft experiments. Numerous disease-related alleles have been identified through TILLING, including mutations in *tp53* (Berghmans et al., 2005), *ptena/b* (Faucherre et al., 2008), and *vhl* (van Rooijen et al., 2009), which have been subsequently used to investigate cancer biology to great effect (Berghmans et al., 2005; Ceol et al., 2011; Dovey et al., 2009; Faucherre et al., 2008; Patton et al., 2005). TILLING alleles have also provided novel insights into vertebrate development. A notable example is a truncation allele identified in the *dicer1* gene (Wienholds et al., 2003a), which is required for proper cleavage of microRNA precursors (Hutvágner et al., 2001; Ketting et al., 2001). Zygotic *dicer1* mutants have suppressed mature miRNA levels and late larval lethality (Wienholds et al., 2003a). Giraldez et al. subsequently utilized this line to generate embryos lacking both maternal and zygotic *dicer1* function to reveal an essential requirement for miRNAs in repressing maternal mRNAs at the maternal-zygotic transition (Giraldez et al., 2005; Giraldez et al., 2006).

As with large-scale forward genetic screens, TILLING projects entail a significant degree of effort and investment. Since ENU generates germline mutational loads of $\sim$1 in every 10^5 bp (de Bruijn et al., 2009), exhaustive mutagenic coverage of the genome ($\sim$1 nonsense mutation per kb) requires many thousands of F1 animals (Moens et al., 2008). To enable high-throughput screening of libraries for multiple targets, initial studies utilized large-scale DNA analysis equipment (Wienholds et al., 2003b), which can be cost prohibitive for smaller groups. While deep sequencing strategies can streamline the screening process, these approaches also require access to specialized equipment and a significant investment in computational resources and personnel. Fortunately, consortiums of laboratories (Moens, Solnica-Krezel, and Postlethwait) or centers (Sanger Institute) have been established where the community can request genes to be analyzed via their respective pipelines (Moens et al., 2008). In both cases, web-accessible interfaces are available to track the status of requested alleles (http://www.zfishtilling.org/zfish/ and http://www.sanger.ac.uk/Projects/D_rerio/zmp/). To date, these groups have generated over 1000 mutant alleles in genes of interest with a focus on nonsense or splice-site disrupting mutations to ensure abrogation of gene function.

As with mutants that are identified from forward genetic screens, F1 carriers from TILLING libraries also harbor a large number of other genetic lesions due to the high mutagenic load imposed by the mutagenesis strategy. Since current ENU regimens for TILLING induce $\sim$1 lesion per 10^5 bp, it is likely that more than 100 of these will be tightly linked ($\leq$10 centimorgans) to the desired mutation, although only a fraction will occur in coding sequence or other functional elements. As a consequence, care must be taken to ensure that an observed phenotype segregates with the modification at the genomic locus of interest. To rule out confounding effects from linked mutations, more than one mutant allele should be characterized or the phenotype should be rescued through the expression of a wild-type version of the allele (provided either by transgene or mRNA injection). Alternately, as the cost of sequencing continues to decrease, it should be possible in the near future to directly sequence the genome of carriers of interest to identify all of the lesions that are present that may contribute to an observed phenotype.

Retroviral and Transposon-Mediated Mutagenesis
As with ENU, insertional mutagens, such as a retrovirus or transposon, can also be utilized for reverse genetic strategies to identify modified alleles of a target gene. These systems have an important advantage over ENU in that the insertion site provides a readily identifiable tag that simplifies screening for carriers of a particular disrupted allele within the library (Jao et al., 2008). Moreover, the inserted DNA sequence can be tailored to interfere with gene expression, while also providing a readout of the expression pattern of the "captured" gene through the incorporation of an expressed tag, such as GFP (Jao et al., 2008; Sivasubbu et al., 2007). By including recombination sites (e.g., loxP elements) flanking these gene-breaking elements, it is also possible to generate conditional alleles in zebrafish that are dictated by the spatial and temporal expression of the complementary recombinase (Petzold et al., 2009).

Building on technical advances for forward genetic screens in zebrafish (Chen et al., 2002; Golling et al., 2002), Burgess and colleagues have generated a pilot-scale insertional mutagenesis library with VSV pseudotyped murine moloney leukemia virus to examine the feasibility of using this approach for a reverse genetic screen (Wang et al., 2007). In this case, founder fish

and F1 males bearing a high insertional load are generated as in a forward genetic screen. Subsequently, sperm are cryopreserved and DNA archives are constructed in analogy to TILLING libraries. Wang et al. were able to achieve high levels of proviral insertions in founder animals that allowed the analysis of F1 animals harboring on average 10 retroviral insertions. Approximately 40% of the mappable hits in the genome fell in genes. Although the vast majority of these (92%) were intronic, 132 integrations (59%) were in the first intron, which often reduced gene expression to < 10%–30% of normal levels (Wang et al., 2007). While some genes will not be readily accessible by this approach (e.g., those with a single exon), this work suggests that it will be possible to identify retroviral insertions in the majority of zebrafish genes.

Insertional mutagenesis utilizing the *Tol2* transposon has also been employed for gene inactivation. A number of different gene-breaking constructs have been employed in this context to interfere with the transcription or splicing of genes neighboring the insertion, including gene (Clark et al., 2004; Clark et al., 2011; Kawakami et al., 2004) and enhancer trap constructs (Balciunas et al., 2004; Parinov et al., 2004; Sivasubbu et al., 2007). A small-scale screen has been performed in zebrafish to determine the frequency of gene inactivation by enhancer trap *Tol2* insertions, where the choice of carriers was guided by the insertions that generated spatially restricted GFP expression patterns (Sivasubbu et al., 2007). This analysis revealed that only a modest number of *Tol2* insertions (4%) disrupted gene function sufficiently to produce an observed early embryonic phenotype. One notable advantage of the transposase-mediated mutagenesis is the ability to excise the mutagenic insertions through reintroduction of the transposase to test for causality (Urasaki et al., 2008). This is feasible, even though transposon excision is imperfect, because most of these insertions are intergenic or intronic, where small postexcision mutations often will not impact gene function.

Both the retroviral and transposon-based insertional mutagenesis systems have the potential to provide collections of disrupted alleles for the majority of genes within the zebrafish genome. Like TILLING, this potential is further enhanced by advances in sequencing technology, which lend themselves to the rapid, parallel identification of insertions throughout the genome in a large number of carriers (Jao et al., 2008). The analysis of retroviral insertions has been successfully adapted to the Illumina paired-end sequencing platform, allowing proviral insertions to be mapped in many hundreds of F1 animals in a single lane of sequencing (S. Burgess, personal communication). Excitingly, this portends the rapid identification of a large number of insertional mutants. A caveat of most previous insertional approaches was the inability to generate full null alleles in most instances; instead, most alleles were hypomorphs that reduce, sometimes dramatically, gene expression. This deficiency has recently been addressed by the construction of more efficient gene-breaking cassettes for Tol2-based mutagenesis (Clark et al., 2011). These new constructs can function as conditional alleles, as they encode loxP sites flanking the mutagenic cassette for excision when Cre recombinase is supplied through mRNA injection or tissue specific Cre expression. Like ENU mutants, F1 founders from insertional mutagenesis typically carry high insertional loads, requiring appropriate out-crossing

and careful identification of carriers in subsequent generations prior to phenotypic analysis.

Targeted Gene Inactivation via Zinc Finger Nucleases

An exciting recent advance that should enable more widespread reverse genetic approaches in zebrafish is the development of Zinc Finger Nucleases (ZFNs), which allow the introduction of targeted lesions in the zebrafish genome. The Chandrasegaran laboratory described the first ZFNs (Kim et al., 1996), which are a chimeric fusion between a Cys_2His_2 Zinc Finger Array (ZFA) that provides the sequence specificity and the cleavage domain of *FokI* endonuclease (Figure 5A) (Urnov et al., 2010). Seminal biochemical (Miller et al., 1985), structural (Fairall et al., 1993; Lee et al., 1989; Pavletich and Pabo, 1991), and specificity modification (Greisman and Pabo, 1997; Isalan et al., 2001; Rebar and Pabo, 1994; Segal et al., 1999) studies laid a foundation whereby ZFAs can be engineered to recognize a wide variety of DNA sequences, facilitating the construction of ZFNs that can be targeted to a desired genomic locus. DNA cleavage by the attached nuclease domain requires dimerization (Bitinaite et al., 1998), and consequently a pair of ZFNs must bind with the proper orientation and spacing to generate a double-stranded break (DSB) (Bibikova et al., 2001; Händel et al., 2009). Subsequent repair of the DSB in vivo by nonhomologous end joining is occasionally imperfect, resulting in the introduction of insertions or deletions at the site of ZFN cleavage.

The potential utility of ZFNs for the modification of animal genomes was first demonstrated by the Carroll laboratory in the fruit fly (Beumer et al., 2008; Bibikova et al., 2002; Bozas et al., 2009) and was subsequently adapted for germline gene inactivation in zebrafish (Doyon et al., 2008; Meng et al., 2008). For this purpose, a pair of ZFNs with specificity for an early coding exon of the target gene is created, and then mRNAs encoding these ZFNs are injected into one-cell embryos (Figure 5B). Once translated, the ZFNs, if sufficiently active, generate lesions at the desired genomic locus. Typically the presence of ZFN-induced somatic lesions is verified in a subset of the injected embryos prior to raising potential founders to adulthood. Carriers of potentially deleterious lesions (insertions or deletions leading to frameshifts) are subsequently identified by genotyping embryos generated through an outcross. These founders are then out-crossed to generate F1 families, and heterozygous carriers, identified through finclip and genotyping, are subsequently in-crossed to generate embryos in which one-quarter carry homozygous mutations within the desired gene.

Generating ZFNs with high in vivo activity at the desired target site has been the primary bottleneck limiting the broad implementation of this technology. ZFN activity can be influenced by the specificity of each incorporated ZFA (Cornu et al., 2008; Urnov et al., 2005), the dimerization potential of the nuclease domain (where obligate heterodimers have been engineered to reduce off-target cleavage events [Doyon et al., 2011; Miller et al., 2007; Söllü et al., 2010; Szczepek et al., 2007]), the composition and length of the linker joining ZFA and nuclease domain (Bibikova et al., 2001; Händel et al., 2009; Shimizu et al., 2009), and, in zebrafish embryos, the injected dose of the ZFN pair (Doyon et al., 2008; Gupta et al., 2011a). The ZFA is typically composed of three to six fingers, with each finger recognizing
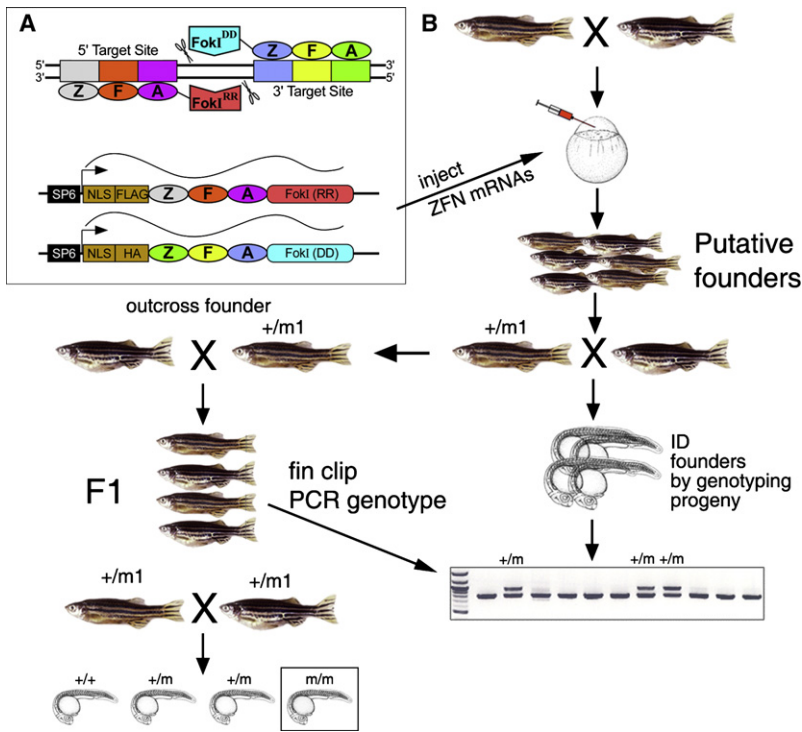
Figure 5. Overview of ZFN-Based Gene Inactivation
(A) A pair of ZFNs are designed to bind neighboring sequences within the target gene of interest. DNA recognition is mediated by the ZFA, while the attached *FokI* nuclease domain generates a double-stranded break (DSB) upon dimerization.
(B) mRNAs encoding each ZFN are prepared and then injected into one-cell embryos. Putative founders from these injections are raised to adulthood and out-crossed to identify carriers and the mutant alleles they transmit. Founders harboring interesting alleles are out-crossed to generate an F1 population, and heterozygous F1 carriers are identified and then in-crossed to provide homozygous mutant embryos for phenotyping.

roughly 3 bp of DNA (Urnov et al., 2010). Consequently, a pair of ZFNs will combine to recognize 18–36 bp, depending on the number of incorporated fingers.

Two general approaches exist for creating ZFNs with novel specificity (outside their direct purchase from Sigma-Aldrich): ZFAs can be assembled from an archive of single-finger (Carroll et al., 2006; Kim et al., 2009; Wright et al., 2006) or two-finger modules (Sander et al., 2011) engineered to recognize specific DNA subsites; alternately, ZFAs can be selected from a library of fingers where the specificity determinants have been randomized (Greisman and Pabo, 1997; Hurt et al., 2003; Isalan et al., 2001; Maeder et al., 2008; Meng et al., 2008). Bacterial one-hybrid (Meng et al., 2008) and two-hybrid (Hurt et al., 2003) methods have been described that provide a facile method for performing ZFA selections, and the two-hybrid method has been further refined with the creation of preselected libraries that simplify the selection process (Maeder et al., 2008). Although selections are more labor intensive, ZFNs utilizing selected ZFAs reportedly have higher success rates than those utilizing ZFAs assembled from single finger archives (Ramirez et al., 2008), as selection methods inherently identify finger sets that are complementary for DNA recognition. Nonetheless, single-finger assembly methods have proven successful in generating targeted null alleles with specific phenotypes in zebrafish (Bussmann et al., 2011; Cifuentes et al., 2010; Siekmann et al., 2009). Moreover, a context-dependent ZFA assembly method (CoDA) employing two-finger modules has recently achieved success rates of ~50% when prescreening activity of these ZFAs in a bacterial two-hybrid system (Sander et al., 2011).

ZFN-mediated gene inactivation in zebrafish is sufficiently robust that, in some cases, ZFN-treated animals can transmit

mutant alleles as if they are heterozygous carriers (Doyon et al., 2008; Meng et al., 2008). ZFNs appear to cause minimal collateral damage to the genomes of treated zebrafish (Doyon et al., 2008; Meng et al., 2008), although it is clear that injected nucleases are toxic to the embryo (Doyon et al., 2008; Foley et al., 2009b; Meng et al., 2008). Massively parallel sequencing has been used to probe the extent of off-target lesions at 141 sites within the genome for one set of nucleases (Gupta et al., 2011a). This analysis revealed that only a handful of off-target sites were appreciably cleaved and that incorporating ZFAs with higher specificity reduces the frequency of these events (Gupta et al., 2011a). To support this technology, zebrafish-centric protocols (Foley et al., 2009a; McCammon and Amacher, 2010) and web-based tools for ZFN design (Mandell and Barbas, 2006; Meng et al., 2008; Reyon et al., 2011; Sander et al., 2010) have been constructed, and convenient yeast reporter assays (Doyon et al., 2008; Zhang et al., 2010) have been developed to identify active ZFNs prior to their in vivo utilization. Members of the Zinc Finger Consortium are also selecting a modest number (~80) of ZFNs for community-derived target genes.

To date, phenotypes have been described for ZFN-induced germline lesions in five genes (Bussmann et al., 2011; Cifuentes et al., 2010; Doyon et al., 2008; Meng et al., 2008; Siekmann et al., 2009). In several of these cases, ZFN-targeted alleles have provided new insights into developmental processes. For example, ZFN-generated lesions in *cxcr4a* (Bussmann et al., 2011; Siekmann et al., 2009) and *cxcl12b* (Bussmann et al., 2011) have revealed a role for chemokine signaling in vasculature patterning in both the head and trunk of the early embryo, where both genes play complementary roles in the angiogenic properties of vessels in response to hemodynamic flow. Likewise, analysis of MZ*ago2* mutants acquired through ZFN mutagenesis revealed an unexpected role for Ago2 in dicer-independent processing of a subset of miRNAs (Cifuentes et al., 2010). Given the demonstrated efficacy of this approach, ZFN-generated mutant alleles will become an increasingly important tool for deciphering gene function.

Despite the tremendous advances in the past few years, ZFNs still have their limitations. Gaps in our understanding of sequence-specific DNA recognition by zinc fingers still restrict

our ability to construct ZFNs targeting *any* desired site in a genome, although this should continue to improve as the knowledge base grows. More troublesome have been efforts to utilize ZFNs to initiate homology-directed repair from an exogenously supplied template to generate tailor-made genomic alterations (Urnov et al., 2010), such as the creation of conditional alleles. Although this approach has proven successful in human cells (Porteus and Baltimore, 2003; Urnov et al., 2005), fruit fly (Bozas et al., 2009), mouse (Cui et al., 2011; Meyer et al., 2010), and rat (Cui et al., 2011), it has proven elusive in zebrafish.

Transgenesis

In other model systems, conditional transgenic strategies have been a valuable reverse genetic approach to elucidate gene function by allowing overexpression of wild-type, dominant-active, or dominant-negative versions of a desired gene. Transgenic systems that provide precise temporal or spatial control of gene activation or inhibition allow more detailed analysis of gene function. Several of these approaches have been successfully validated in zebrafish, leading to increased application in recent years. We provide an overview of two of these approaches (GAL4/UAS and Cre/loxP), which have been widely applied in other models to great effect and can now be readily employed in the zebrafish.

Among the most powerful conditional transgenic expression strategies in *Drosophila* is the bipartite Gal4/UAS system (Brand and Perrimon, 1993). In this case, two separate transgenic lines are generated: one in which a tissue-specific or inducible promoter drives expression of the yeast transcription factor GAL4 and a second line in which the GAL4-responsive Upstream Activating Sequence (UAS) coupled to a minimal promoter drives expression of the desired transgene. In most cases, the transgene encodes a dominant gain or loss of function allele. Upon crossing GAL4- and UAS-bearing transgenic lines, the resulting progeny will display UAS-driven transgene expression only in GAL4-expressing cells (Brand and Perrimon, 1993). The GAL4/UAS system was initially employed in zebrafish using the *heat shock protein 70* (*hsp70*) promoter to drive GAL4 in a temperature-inducible manner (Scheer and Campos-Ortega, 1999). Subsequent application of this line has been instrumental in demonstrating the importance of Notch signaling in multiple cell types later in development, including differentiation of glia, arterial endothelial cells, and hematopoietic stem cells (Burns et al., 2005; Lawson et al., 2001; Scheer et al., 2001). Despite the success of these initial examples of the GAL4/UAS system, its application has seen limited use in the zebrafish community. This is likely due to the lack of applicable driver lines, as well as problems with somatic silencing of the multicopy UAS sequence in responder lines (Goll et al., 2009). In the former case, recent use of the Tol2 transposable element has enabled generation of several hundred enhancer trap GAL4 lines (Asakawa et al., 2008; Scott et al., 2007), which are available to the research community (Kawakami et al., 2010) and will likely increase the use of this approach for dissecting developmental processes in the zebrafish.

An alternative conditional transgenesis strategy is the Cre/loxP system, which has been widely applied in mouse (Gu et al., 1994; Hamilton and Abremski, 1984; Nagy, 2000). Like GAL4/UAS, Cre/loxP is a bipartite transgenic approach in which

a transgenic driver line expresses the bacteriophage Cre recombinase in a tissue-specific manner (Nagy, 2000). The responder line typically bears a transgene in which a strong ubiquitous promoter drives expression of a "stop" cassette flanked by direct repeat loxP sites upstream of a sequence encoding a dominant gain- or loss-of-function allele. The stop cassette normally prevents expression of the active transgene, but in the presence of Cre, recombination between the flanking loxP sites removes these inhibitory sequences. It is further possible to control the temporal activity of the Cre recombinase by fusing it to a form of the estrogen receptor ligand-binding domain that has been modified to bind to tamoxifen (Metzger et al., 1995). For functional analysis, Cre/loxP was initially utilized in zebrafish to enable conditional expression of oncogenic forms of human KRAS and MYC, allowing the induction of tumor formation (Le et al., 2007) or leukemia (Feng et al., 2007) in adults. In this initial work, Cre expression was made inducible by means of the *hsp70* promoter. While functional manipulations using Cre/loxP approaches have been limited, their application has been increasing for lineage tracing, because unlike GAL4/UAS, Cre-mediated recombination permanently marks the target cell and its progeny (Zinyk et al., 1998). In particular, tamoxifen-inducible forms of Cre, as well as inducible responder lines, have allowed precise lineage tracing of progenitors populations in both the developing pancreas and regenerating heart (Hesselson et al., 2009; Kikuchi et al., 2010; Wang et al., 2011). Like GAL4/UAS, the widespread application of Cre/loxP technology is somewhat hindered by the lack of appropriate driver lines, although initial efforts using the Tol2 backbone to establish enhancer trap Cre drivers are encouraging (Boniface et al., 2009). Based on these early efforts and the ability to perform lineage tracing and genetic manipulations, as well as the possibility for mosaic analysis, it is likely that Cre/loxP approaches will continue to gain in popularity within the zebrafish community.

Reverse Genetics—Perspectives and Future Directions

Zebrafish should continue to expand in popularity as a vertebrate developmental model due to ease of in vivo visualization of developmental processes coupled with the advent of new and improved reverse genetic approaches that will facilitate the rapid creation of interesting null and hypomorphic alleles. In particular, massively parallel sequencing has the potential to revolutionize the recovery of mutant alleles from insertional and chemical mutagenic libraries, which could supercharge developmental studies in this model and pave the way for large-scale phenotypic studies of mutant populations. Likewise, the continued technological improvements in artificial nucleases (ZFNs, meganucleases [Silva et al., 2011], and TALENs [Miller et al., 2011]) have the potential to facilitate the rapid creation of tailor-made genomic changes through the development of knockin methodologies. This advance would allow the facile creation of a range of human disease models in zebrafish. One caveat to utilization of the zebrafish model over other vertebrate systems is the potential greater functional complexity that is inherent due to the additional genome duplication of teleost fish (Amores et al., 1998; Ekker et al., 1992; Prince et al., 1998), where these gene duplications could complement null alleles that are generated at a particular locus. The new reverse genetic tools should help to address this question, and the ease of transgenesis and

transplantation in zebrafish will nicely complement the existing array of powerful forward genetic tools for the implementation of genetic modifier screens to more broadly investigate gene function at a systems level. The recent demonstration of rapid and effective chemical genetic screens in the embryo, which have demonstrated the ability to probe both developmental (North et al., 2009; North et al., 2007) and behavioral functions (Peal et al., 2011; Rihel et al., 2010), also holds fantastic potential to facilitate the discovery of new therapeutics based on observed effects in vivo, as opposed to classical target-based cell culture assays.

Conclusion

Powerful forward and reverse genetic approaches have been established for the zebrafish that facilitate the detailed study of gene function in development and disease. Many of these methods have also found broad application for the study of gene function in other prominent vertebrate models, where, in some cases, even more sophisticated tools are available (e.g., the generation of conditional alleles in the mouse). The recent development of reverse genetic techniques that are species agnostic (ZFNs) should allow both established (zebrafish and rat) and new vertebrate models to play more prominent roles in analyzing gene function. Given the dual nature of conservation in developmental pathways—that they are used similarly in related organisms and that they are often used reiteratively in disease processes—the ability to apply both forward and reverse genetic approaches in the zebrafish and other model organisms will continue to provide important new insights into both vertebrate development and human disease.

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