PERSPECTIVES

INNOVATION

TILLING — a high-throughput harvest for functional genomics

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The availability of the whole genome sequence of many model organisms, combined with well-established chemical mutagenesis methods and cost-effective high-throughput DNA genotyping, allows mutations to be identified for virtually any gene. Recently dubbed TILLING (for targeting induced local lesions in genomes), this general method is gaining popularity. In this article, I discuss some of the TILLING methods that are available, the successes that have been reported for several organisms and the future outlook for such methods.

Without doubt, one of the most important tools in biological research is mutational analysis. Our understanding of the basic mechanisms of disease, development, cell biology and metabolism has been transformed by the systematic application of mutational analysis.

So, you want mutants?

Traditionally, forward genetics, driven by the identification of mutant phenotypes, has been the most widely used approach. Most of what we understand about the genetic control of animal and plant development derives from systematic screens for mutations that produce visible phenotypes that result from defective developmental processes. For example, the hierarchy of gene activation that underlies the establishment of the segmented body plan of *Drosophila melanogaster* was deduced by Nüsslein-Volhard and Wieschaus from a large collection of mutations that affect patterns of the larval cuticle[1]. The forward-genetic approach, driven by phenotypic identification, is so powerful that mouse geneticists, who have the tools to generate individual mutations at will, sometimes find large-scale chemical mutagenesis very informative[2,3].

There are, however, limitations imposed by forward-genetic screens that necessitate the development of generic reverse-genetic methods. For example, now that we know the whole genome sequence of many organisms, we are aware that genetic complexity will make many mutations essentially undetectable in typical screens for phenotype. In addition, many genes present such small targets that it is unfeasible to screen numbers that are large enough to identify a mutation. Finally, with traditional forward genetics, rare phenotypes or rare mutations that produce a given phenotype can be missed simply because of the vast number of genomes that need to be screened to obtain a hit.

Reverse genetics generally refers to the generation or targeted discovery of a mutation in a gene that is known by its sequence. In mice, reverse genetics is usually carried out using homologous recombination in embryonic stem (ES) cells, which allows a precise mutation to be constructed in nearly any gene[4].

A less precise reverse-genetic approach in *Drosophila* and *Caenorhabditis elegans* involves using libraries of individuals who each carry a transposable element insertion[5–7]. Such libraries are useful as many of the insertions have been mapped and some will disrupt the expression of nearby genes. In the case of *Drosophila* P-ELEMENTS, imprecise excision can be driven to generate a mutation in the nearest gene. Recently, another reverse-genetic system has been developed for *Drosophila* that uses homologous recombination. In this system, the yeast Flp recombinase is used to excise a transgene targeting-construct and a site-specific endonuclease (I-SceI) is used to cleave the excised product, which stimulates homologous recombination[8]. The system has its obvious attractions; however, it is a lengthy procedure that requires the generation of specific transgenic flies.

“Without doubt, one of the most important tools in biological research is mutational analysis.”

For *Xenopus* and zebrafish researchers, reverse genetics of a sort is achieved by the disruption of translation or splicing of specific mRNAs using antisense MORPHOLINO OLIGONUCLEOTIDES (MOs), which have a modified backbone that is resistant to degradation by endogenous nucleases. MOs are now widely used and can, in most cases, produce PHENOCOPIES of known mutations[9]. Although MOs are extremely useful, they are not a substitute for mutations. MOs only produce loss of function rather than altered function and as they are diluted by cell division and growth, they limit studies to the early stages of development.

Although the existing reverse-genetic approaches are effective, the main disadvantage is that they tend to be organism specific. A general method that is applicable to many organisms would allow genetic studies in organisms that were not accessible before. The good news is that there is such a method.

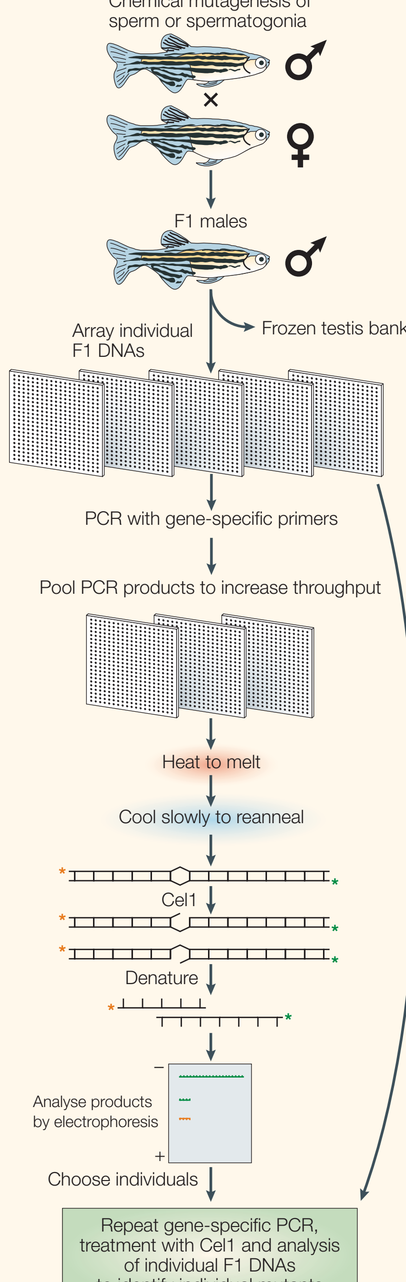


Figure 1 | **The TILLING process.** In this case, F1 zebrafish carriers of mutations are generated after either spermatogonial or sperm mutagenesis. Testes are frozen for future recovery of mutations and genomic DNAs are collected from individual F1 fish. DNA samples can be pooled for genotyping, which involves gene-specific PCR with labelled primers, melting and re-annealing, followed by treatment with Cel1 endonuclease and analysis on automated sequencing machines. The identification of mutants is rapid because differential double-end labelling allows mutations to be detected on complimentary strands. Once a mutation has been identified in a pool, genotyping is repeated for individual DNA samples from that pool to identify the individual mutants. Adapted with permission from REF. 11 © (2001) American Society of Plant Biologists.

Box 1 | Chemical mutagens and TILLING

The chemical mutagens that will be useful for TILLING are those that generate point mutations. Alkylating agents, such as ethyl methanesulfonate (EMS) or *N*-ethyl- *N*-nitrosourea (ENU), directly modify bases of the DNA and, on replication, these bases pair inappropriately, which leads to base changes. For example, EMS reacts with guanine to make a base that will pair with T instead of C, leading in most cases to G:C → A:T transitions[41]. By contrast, whereas ENU can also generate O^6-ethylguanine modifications, several studies indicate that numerous A:T → T:A TRANSVERSIONS occur, which are probably the result of O^4-ethylthymidine modifications[42–44]. So, different alkylating mutagens can produce different constellations of mutations that lead to different projected codon changes.

TILLING will give you mutants

S. Henikoff and colleagues have assembled a process that is a good general solution to the problem of identifying mutations in genes that are known only by their sequence[10–12]. They call the method TILLING (for targeting induced local lesions in genomes), partly to reflect their first application of the method to the identification of mutations in the plant *Arabidopsis thaliana*.

So, how does TILLING work? FIGURE 1 shows the TILLING process as applied to zebrafish instead of *Arabidopsis*. First, chemically induced mutation-carrying gametes (or living individuals) are collected (FIG. 1; BOX 1). The absolute number of gametes or individuals that carry mutations depends on several factors, such as the size of the gene and the frequency of induced changes, but it will be in the order of thousands. For each individual, a sample of DNA is taken for high-throughput analysis of heterozygosity. PCR is used to generate amplified fragments of exons from each individual or from pools of carriers. The PCR products are melted and re-annealed and then digested in the PCR mix using Cel1 endonuclease (FIG. 2) — a recently identified endonuclease from celery, which reliably cleaves at mismatched bases in heteroduplex DNA[13–15]. The digested PCR products are then analysed by gel electrophoresis. Using differentially labelled fluorescent forward- and reverse PCR-primers, automated analysis is carried out on DNA sequencing machines. Once mutations are identified, they can be verified by sequencing. The stored gametes from carriers or live carriers can then be used to generate families of carrier individuals and crosses can be done to test the function of the gene (FIG. 1).

In addition to being a general method for the identification of mutations in an arbitrary gene, TILLING also provides an ALLELIC SERIES of mutations. In a relatively unbiased way, many amino-acid substitutions, splicing mutations and stop mutations can be identified by TILLING. This has several advantages over other methods. For example, with insertional mutagenesis, complete loss-of-function alleles are typically generated. With an allelic series, however, which is not biased by phenotypic selection, partial loss-of-function and novel-function alleles can often provide a more informative insight into the true function of a gene product. In addition, by using different chemical mutagens, it is possible to identify different constellations of mutations. This has the added advantage of increasing the diversity of an allelic series, raising the possibility of identifying specific amino-acid changes that are made interesting by results from other biochemical, structural or genetic studies.

TILLING in any organism

TILLING has been embraced by the *Arabidopsis* research community and has been made widely accessible through the *Arabidopsis* TILLING Project (see online links box). The success with *Arabidopsis* has inspired researchers who work with other model organisms to use the method, which has the potential to be applied to many other genetic systems, especially animal species for which there are no other, or limited, reverse-genetic methods. Although the *Arabidopsis* research community has the distinct advantage that seeds are relatively easy to store and distribute, there are good reasons to believe that community-wide efforts to support TILLING for other organisms should be undertaken. For example, animal stock centres would be ideally equipped to handle the establishment, storage and distribution of mutagenized stocks, and DNA sequencing facilities would be well-suited for the high-throughput genotyping that TILLING requires. In addition to the logistics of organizing a community effort, there are several factors that should be given consideration for a TILLING project in any organism.

Mutagenesis method. Of primary importance is the method that is used to generate chemically induced mutations. To ensure that useful mutations are identified, a sensible balance must be struck between the amounts of mutagenesis and the number of individuals

analysed. There are well-established chemical mutagenesis methods for many organisms and many of these are likely to be directly applicable to a TILLING project.

One consideration, especially for vertebrate species, is whether to mutagenize sperm or SPERMATOGONIA (mitotic sperm stem cells). Higher rates of mutagenesis mean that fewer F1 individuals need to be screened to uncover desired mutations. Mutagenesis of mature sperm, either *in vivo* or *in vitro*, yields high rates of mutation, but it also leads to a mosaic distribution of mutations in F1 animals[16–18]. This happens because each strand of DNA in the mature sperm is independently mutagenized and the strands segregate to distinct BLASTOMERES during the first cell division after fertilization. In addition, sperm mutagenesis can produce mutations, such as transpositions and chromosomal deficiencies, which would not be detected using a single-nucleotide genotyping method[19]. Such mutations could indeed present problems in the analysis of identified point mutations that cause phenotypes that are not associated with the gene under study. Spermatogonial mutagenesis, by contrast, produces non-mosaic F1 individuals, as pre-meiotic germ cells are the target of this mutagenesis scheme and it largely produces point mutations[16,20]. Spermatogonial mutagenesis, however, can produce mutation rates that are at least an order of magnitude lower than sperm mutagenesis, meaning that many more F1 individuals need to be screened[16,21,22]. However, if the genotyping method used is efficient and cost effective, then processing more individuals might be desirable.

Gamete storage versus maintenance of carrier stocks. A second important factor to consider is how induced mutations will be maintained while carrier DNA is analysed. For plants, gamete storage and mutation recovery is a relatively simple matter of maintaining seeds from collections of M2 individuals[23,24]. For many animal species, however, the storage of gametes can be problematic. With zebrafish, for example, although there are published methods for sperm freezing and recovery, the reliability of the technique for a large number of individuals is difficult to assess and would need to be carefully monitored to ensure that mutations are efficiently recovered[25]. It makes sense to maintain live populations of F1 individuals for several reasons. The number of usable individuals is effectively doubled, as both males and females can be used. With zebrafish, which are reproductively active for more than two years, DNA can be extracted from caudal fins, which regenerate, and live fish can be maintained for breeding when a useful mutation has been identified. In addition, maintaining live populations eliminates the cumbersome task of collecting gametes and ensuring the recovery of mutations. One important concern for live storage, however, is that loss of individuals is unavoidable and if a particular individual is relied on then there is no recourse for recovering the mutation. In addition, there are notable space issues for the maintenance of live stocks, especially if the mutagenesis rates mean that thousands of individuals need to be maintained. For example, with zebrafish, individual fish could be housed in single tanks or individuals could be pooled, leaving the job of identifying a specific carrier until after TILLING. For other species, such as *Drosophila*, there is no choice: live stocks need to be maintained, as there are no other effective methods for long-term maintenance of a genetic trait.

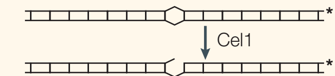


Figure 2 | **The Cel1 endonuclease.** Cel1 endonuclease will cleave heteroduplex DNA at any single base-pair mismatch. The top panel depicts a single base mismatched heteroduplex DNA end-labelled on one strand. Cel1 cuts single strands leaving both full-length substrate (F) and cut fragment (I). All possible mismatch combinations are effectively cleaved by Cel1. Reproduced with permission from REF. 13 © (1998) Oxford University Press.

Glossary

ALLELIC SERIES
A series of different genotypes, or alleles, of a specific gene, that are often associated with different phenotypes.

BLASTOMERES
Cells of the early cleavage-stage embryo.

DENATURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY
(dHPLC). A rapid chromatography method that can be used to distinguish heteroduplex from homoduplex DNA. It can detect single base differences between alleles.

HEDGEHOG
The Hedgehog proteins are a class of secreted cell–cell signalling molecule. The name derives from the appearance of embryonic *Drosophila melanogaster* mutants that lack *hedgehog* gene function. There are many vertebrate genes that encode Hedgehog homologues.

M2
Second generation mating of a specific mutagenized individual.

MATERNAL CONTRIBUTION
In many organisms, mRNA and proteins are stored in the egg before fertilization.

microRNAs
Short (22 bp), non-coding RNAs that are probably involved in gene regulation.

MORPHOLINO OLIGONUCLEOTIDE
A DNA analogue in which the bases are linked to a six-membered morpholine ring.

P-ELEMENTS
Transposable elements that are widely used for mutating and manipulating the *Drosophila* genome.

PHENOCOPY
The production of a phenotype, which closely resembles a phenotype that normally results from a specific gene mutation.

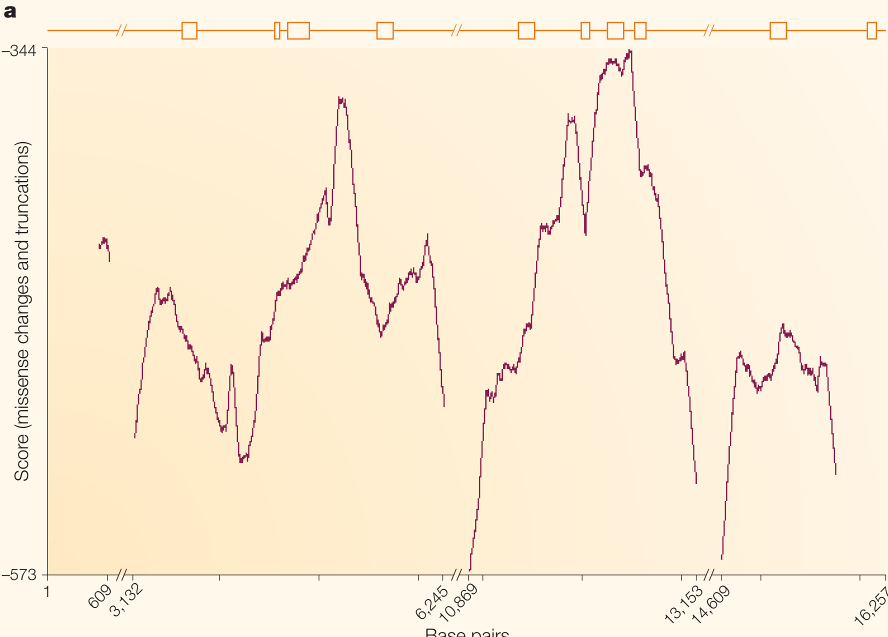
PHOSPHONOPEPTIDE NUCLEIC ACID
As with morpholino oligonucleotides, these have a modified backbone that is resistant to nuclease digestion, yet allows hybridization with complementary DNA or RNA molecules and can be used to interfere with protein synthesis of specific target mRNAs.

PRIMORDIAL GERM CELLS
Germline cells at all stages of development from the time when this lineage is formed until they arrive at the gonad and start differentiating into gametes.

SPERMATOGONIA
The mitotically dividing stem cells of the male germline, the descendants of which ultimately become mature sperm.

TRANSVERSION
A point mutation in which a purine base is substituted for a pyrimidine base and vice versa; for example, an A:T → C:G transversion.

WNT
The Wnt proteins are a class of secreted cell–cell signalling molecule. The name derives from a fusion of two original names. In *Drosophila melanogaster,* Wingless (like Hedgehog) is involved in patterning the early embryo. In vertebrates, Int-1 was the first member of this class of protein to be discovered, and was identified in a screen for viral cancer-causing insertions in mice.



b

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MQNSHSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQTHADAKVQVLDNENVS
NGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVSKIAQYKRECPSIFAWEIRDRLLSEGVC
TNDNIPSVSSINRVLRNLASEKQQMGADGMYDKLRMLNGQTGSWGTRPGWYPGTSVPGQPTQD
GCQQQEGGGENTNSISSNGEDSDEAQMRLQLKRKLQRNRTSFTQEQIEALEKEFERTHYPDVF
ARERLAAKIDLPEARIQVWFSNRRAKWRREEKLRNQRRQASNTPSHIPISSSFSTSVYQPIPQ
PTTPVSSFTSGSMLGRTDTALTNTYSALPPMPSFTMANNLPMQPPVPSQTSSYSCMLPTSPSV
NGRSYDTYTPPHMQTHMNSQPMGTSGTTSTGLISPGVSVPVQVPGSEPDMSQYWPRLQ
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Figure 3 | **CODDLE analysis of the mouse *Pax6* gene.** The CODDLE (for codons optimized to discover deleterious lesions) program uses genome sequence data as input and determines which exons are most likely to yield nonsense and missense mutations, taking into account the mutagen that was used and the genome being studied. In this example, CODDLE selects a region that spans exons 7, 8 and 9 with a good probability of generating missense and nonsense mutations under *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis. **a** | The graphical output of the CODDLE program. The orange line and boxes across the top indicate the *Pax6* gene organization, and the graph indicates the changes and truncations score. **b** | The amino acids of exon 7 are depicted in blue, exon 8 in magenta and exon 9 in orange. Overall, 12 codons could be mutated to a stop codon after ENU mutagenesis (bold red). After selecting the exons that are most likely to be mutagenized, CODDLE runs another program, Primer3, to generate PCR primers for the amplification of the selected exons.

Sequence analysis. In the overall design of sets of primers for TILLING, it is helpful to identify coding regions with the highest probability of producing nonsense or useful missense mutations. The proWeb project at the Fred Hutchinson Cancer Research Center has developed web-based sequence analysis tools to assist in this process (see online links box). The program CODDLE (for codons optimized to discover deleterious lesions) takes genome sequence data as input, determines which exons are most likely to yield nonsense and missense mutations given a particular type of mutagen, then runs another program, Primer3, to generate PCR primers to amplify selected exons[26] (FIG. 3) (see online links box).

After mutations have been identified and sequenced, the program PARSESNP (for project aligned related sequences and evaluate SNPs) shows the locations of induced polymorphisms, including restriction enzyme polymorphisms (see online links box). With a homology model of related sequences for the target protein, PARSESNP produces predictions of the severity of missense changes[27].

For the recovery of mutations after identification, it is useful to have a routine method to genotype individual carriers. Neff and colleagues have developed a useful method that is known as dCAPS (for derived cleaved amplified polymorphic sequence), which uses a mismatched PCR primer to generate restriction enzyme polymorphisms in the amplified product, allowing mutant and wild-type alleles to be distinguished[28]. A web-based program is used to design primers and identify restriction sites (see online links box)[29].

Genotyping methods. Another factor to consider is the choice of genotyping method. Although Cel1 is clearly an effective way to detect heterozygous mutations, even in pools of individuals, other methods might be more applicable for an existing set-up or might provide more information. Perhaps the most complete information is given by resequencing the target exons of each individual in the array. This was done on an array of F1 progeny of *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized zebrafish for the *rag1* gene — from 2,679 F1 carriers, 15 mutations were identified, including 9 amino-acid substitutions and 1 premature stop codon mutation[30].

Initially, DENATURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (dHPLC) was used to detect mutations in TILLING in *Arabidopsis*, but the Henikoff group now use Cel1 instead, mainly because samples can be pooled to increase throughput and reduce costs[10,11]. As another alternative, a gene-disruption method that involves a yeast-based translational screening assay has been used to identify mutations for rats[31]. This method is designed to detect premature stop codons in the target gene. In essence, an open reading frame of target-gene cDNA or genomic DNA that is taken from F1 progeny of mutagenized animals is PCR amplified and inserted in frame into a yeast vector that is driven by the constitutive ADH1 promoter to generate a functional ADE2 fusion protein. Any premature stop codons in the target-gene open reading frame will lead to the formation of red-coloured yeast colonies instead of the white colonies that are formed when the functional ADE2 fusion protein is present. This method has the advantage of allowing the efficient detection of nonsense mutations and the detection of mutations that are manifest in expressed mRNA, but it cannot detect the more subtle alleles that can be identified by the Cel1 or re-sequencing strategies. Overall, the method might prove to be too cumbersome for a general application.

As the technologies for mutation detection improve, both in terms of throughput and expense, the arrayed gamete resources will remain useful and can be analysed using many distinct methods.

> "[TILLING] has the potential to be applied to many other genetic systems, especially animal species for which there are no other, or limited, reverse-genetic methods."

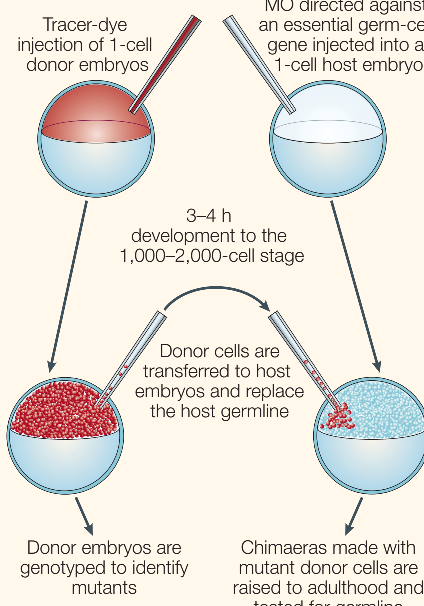

Figure 4 | **Generation of zebrafish germline chimaeras.** Wild-type host embryos are injected with an antisense morpholino oligonucleotide (MO) that specifically kills germ cells. Labelled donor cells are transplanted from potentially mutant donor embryos and some of these cells replace the germline of the host embryos. The donor embryos are genotyped to test whether they are homozygous carriers of the mutations. Host chimaeras that are known to contain mutant donor tissue are raised to adulthood and are tested for transmission of mutations.

More considerations and an application

TILLING offers a reverse-genetic approach for organisms for which there are few other possibilities. For zebrafish and *Xenopus*, however, oligonucleotides with modified backbone structures, such as MOs or PHOSPHONOPEPTIDE NUCLEIC ACIDS, are now routinely used to disrupt gene expression[32–34]. With these approaches, it is possible to know the loss-of-function phenotype during early development for a given gene. For TILLING, this knowledge can function as a useful pre-screen to motivate a concerted effort to uncover mutations in the gene.

TILLING also allows the identification of mutations that would be silent in the context of forward mutagenesis screens. Some of these mutations will be silent because of the overlapping function of genes and lack of an overt phenotype. In addition, in species such as fish and amphibia, MATERNAL CONTRIBUTIONS can often mask the function of genes if only the zygotic function has been disrupted. To address the overlapping functions of genes, it would be useful to have a means to examine the loss of several genes simultaneously. Moreover, to address maternal versus zygotic function of genes, it would be useful to have a systematic means of disrupting maternal contributions. Antisense MOs can be used to disrupt the expression of several genes simultaneously, but this approach is limited[35]. A new method, which has been developed for zebrafish, allows the generation of germline chimaeras. The method, which entails the targeted destruction of endogenous PRIMORDIAL GERM CELLS in host embryos followed by the seeding of new donor germ cells, will allow the routine generation of single, double or triple maternal–zygotic mutant embryos[36] (FIG. 4). When combined with mutations that have been identified by TILLING, this method has the potential to address some difficult problems in vertebrate genetics. For example, suppose that you want to examine the simultaneous loss of function of all of the known Gli proteins in a vertebrate. The Gli proteins are a class of zinc-finger transcription factor that are involved in, among other things, the transduction of HEDGEHOG signals[37]. For two zebrafish loci, *detour* and *you-too*, which encode Gli1 and Gli2 respectively, there are mutant alleles, but for the gene encoding the third Gli protein, Gli3, no mutant alleles have been identified[38]. With TILLING, mutant *gli3* alleles can be isolated and by using the new germline chimaera method, triple-mutant *detour;you-too;gli3* embryos could be generated.

Two TILLING successes in zebrafish

Very recently, TILLING has been used to identify mutations in two zebrafish genes. In one study, mutant alleles of the gene that encodes the enzyme Dicer1, which is necessary for the production of microRNAs, were isolated. The central finding of this study is that Dicer1, and therefore microRNAs by implication, are essential for normal development in vertebrates[39]. In the second study, TILLING identified a nonsense mutant allele of the gene that encodes zebrafish adenomatous polyposis, a tumour suppressor that is normally involved in WNT signal transduction, and identified a previously unknown role for Wnt signalling in cardiac-valve formation[40]. These successes highlight the general applicability of TILLING for the detection of mutations and will undoubtedly stimulate more diverse research communities, not previously able to carry out mutational analysis, to add this powerful research tool to their bag.

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Competing interests statement

The author declares that he has no competing financial interests.

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Autobiography

As an undergraduate at the University of Colorado, Boulder, USA, Derek Stemple worked with Dick McIntosh on microtubule dynamics in living mammalian cells. His Ph.D. was completed under the supervision of David Anderson at the Caltech, where he identified a stem cell for mammalian neural crest. With Wolfgang Driever at the Massachusetts General Hospital, Boston, USA, he participated in a systematic screen for mutations that affect zebrafish embryogenesis. He has lived and worked in England since 1997. He began independent work at the National Institute for Medical Research, London, and is at present at the Wellcome Trust Sanger Institute, Cambridge. His research interests include the development of the notochord, muscular dystrophy and early vertebrate development.

URLs

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Further Information

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