OXFORD

Briefings in Functional Genomics, 15(4), 2016, 322–330

doi: 10.1093/bfgp/elv051
Advance Access Publication Date: 9 December 2015
Review paper

Genome editing in zebrafish: a practical overview

Robert Sertori, Monique Trengove, Faiza Basheer, Alister C. Ward and Clifford Liongue

Correspondence author: Alister C. Ward, School of Medicine, Deakin University, Pigdons Road, Geelong Victoria 3217, Australia. Tel.: +61-3 5227 2041; Fax: +61-3 5227 2945; E-mail: award@deakin.edu.au

Abstract

Zebrafish is a powerful model for the study of vertebrate development, being amenable to a wide range of genetic and other manipulations to probe the molecular basis of development and its perturbation in disease. Over recent years, genome editing approaches have become increasingly used as an efficient and sophisticated approach to precisely engineer the zebrafish genome, which has further enhanced the utility of this organism. This review provides a practical overview of genome editing and its application in zebrafish research, including alternate strategies for introducing and screening for specific genetic changes.

Key words: zebrafish; genome editing; zinc finger nucleases; TALENs; CRISPR; HDR

Introduction

Zebrafish represents a powerful vertebrate model for the investigation of development and its disruption, with broad conservation of key genes and developmental pathways between zebrafish and humans [1]. The transparent externally developing zebrafish embryos are extremely accessible, providing exquisite opportunities for manipulation and analysis. Forward genetic screens are possible in zebrafish using chemical- or transposon-mediated random mutagenesis to identify genes affecting specific biological processes in either larvae or adults [2–4]. Reverse genetic methodologies can also be used to study the effects of gene ablation, either transiently using morpholino-mediated gene knockdown [5] or permanently using targeting-induced local lesions in genome (TILLING) [6]. In addition, genes are able to be overexpressed transiently by injection of mRNA or DNA [7], or stably via transgenesis, including conditional/inducible approaches [8].

A variety of genome-editing approaches, based on zinc finger nucleases (ZFNs) [9, 10], transcription activator-like effector nucleases (TALENs) [11–13] or clustered regularly interspaced short palindromic repeats (CRISPR) [14–16], have more recently provided additional capabilities to efficiently achieve specific gene manipulation. These different strategies use alternate mechanisms to induce a double-stranded break (DSB) at a specific site(s) in the zebrafish genome; both ZFNs and TALENs rely on protein–DNA interactions to provide sequence specificity, while CRISPR involves RNA/DNA targeting. However, all three methodologies subsequently invoke common native repair processes to achieve a range of permanent mutagenic outcomes in a reliable and efficient manner. This review will outline the alternative genome editing methodologies for specific cleavage, the strategies used to introduce a variety of genetic changes taking advantage of native repair processes and the approaches used for screening and propagation of successfully manipulated genes.

Sequence-specific targeting

Zinc finger nucleases

ZFNs were the first genome-editing strategy to be applied to zebrafish. ZFNs act as artificial restriction enzymes, being a chimeric fusion between an array of sequence-specific, DNA-binding Cys_2His_2 zinc-finger domains (ZFDs) and the non-sequence-specific FokI endonuclease [17, 18]. Each ZFD is composed of 30 amino acids that recognize a specific nucleotide triplet and are used in an array of three to six domains, giving each ZFN a 9–18 bp target site [19]. Moreover, ZFNs are designed in pairs, with each member recognizing a different chromosomal

Robert Sertori is a PhD student using TALENs to investigate cytokine receptors in zebrafish immunity.
Monique Trengove has recently complete a PhD examining zebrafish SOCS proteins utilizing ZFN and TILLING approaches.
Faiza Basheer is a PhD student applying TALEN and CRISPR to the study of aberrant cytokine receptor signalling in disease.
Alister C. Ward has a long-standing interest using zebrafish as a genetic model for understanding cytokine signaling and haematopoiesis.
Clifford Liongue is an Alfred Deakin Postdoctoral Research Fellow who has pioneered the use of genome editing in zebrafish.

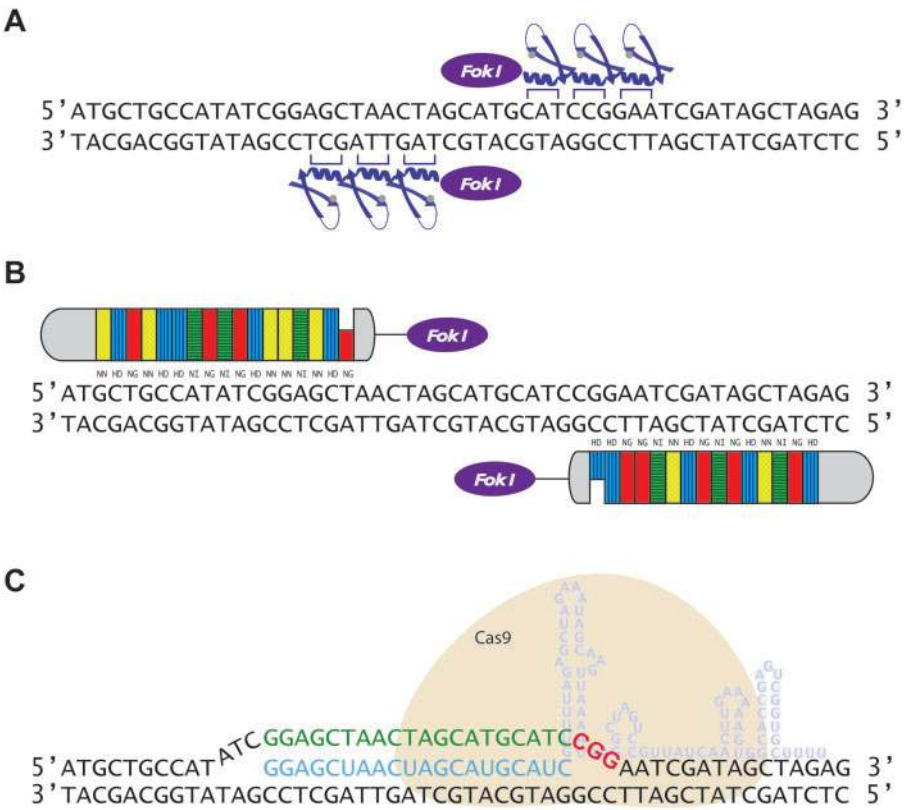

Figure 1. Genome targeting by ZFNs, TALENs and CRISPR. (**A**) ZFN-mediated targeting. Zinc finger domains are shown schematically, with the 3 bp sequence targeted by each indicated, and the fused FokI domains shown. Binding of a pair of ZFNs with the correct geometry at the target site allows FokI nuclease dimerization, facilitating the introduction of a DSB into the DNA. (**B**) TALEN-mediated targeting. TALEN RVDs are coded (NG: solid; NN: checked pattern; NI: horizontal stripes; HD: vertical stripes) with the nucleotides they target indicated, and the fused FokI domains shown. Appropriate FokI nuclease dimerization again facilitates DSBs at the target site. (**C**) CRISPR-mediated targeting. The guide RNA is shown alongside the nucleotides it binds to. This targets the Cas9 (shaded) adjacent to the PAM motif (NGG), where it generates a DSB. (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)

strand with a small spacer that enables FokI assembly with a geometry that allows the nuclease domains to dimerize (Figure 1A). Therefore, DNA cleavage only occurs when the two ZFNs bind adjacently, such that a DSB is introduced [20, 21].

The make-up of ZFNs is flexible and can be customized to recognize any sequence of interest [22, 23]. ZFNs can be designed to specific targets using online tools, including Zinc Finger Tools [24] and ZiFiT Targeter [25, 26], with the encoding sequences able to be synthesized by commercial suppliers or generated in-house by context-dependent assembly method [27], oligomerized pool engineering [28] or modular assembly [29].

ZFN pairs are delivered by co-injection of mRNAs encoding the individual ZFNs into one-cell zebrafish embryos. This approach has a reported mutagenic frequency of 1.6–33% [25, 30] and good germ line transmission, and is able to generate both heterozygous and homozygous knockout organisms with a wide variety of mutations at the target site [20, 21]. New ZFN construction methods have been developed including the Sigma CompoZr Custom Zinc Finger Nuclease Kit that has shown up to 98% mutagenic efficiency [25].

The major limitations of this approach are the high failure rates to generate ZFNs by modular assembly [31] and cutting at off-target sites (those varying 1–4 bp from target site) [20, 32]. One study reported off-targeting in ~1% of morphologically normal embryos but at a greater frequency in phenotypically abnormal embryos [20]. Delivery of ZFNs at an appropriate concentration can minimize such off-target events [33].

TALENs

TALENs also represent engineered restriction enzymes consisting of fusions between a DNA binding region and FokI that work in pairs [34]. In this case the DNA binding domain is based on an array of Tal effectors, originally discovered in the plant pathogen *Xanthomonas* [35]. These consist of a 33–35 amino acid repeat containing two hypervariable amino acids at positions 12 and 13, termed repeat variable diresidues (RVDs), which each bind to a specific target base [34, 36, 37]. From the wide variety available, the commonly used RVDs are NN to bind guanine, NI to bind adenine, HD to bind cytosine and NG to bind thymine [34]. Between 12 and 31 repeats are fused in an array, followed by a 20-amino acid 'half repeat' before a 63-amino acid C terminal region fused to FokI sequences [38, 39] (Figure 1B). Like ZFNs, the FokI endonuclease of each TALEN pair must dimerize to introduce a DSB.

TALENs targeting specific sites can be designed with the help of online tools, such as TAL Effector Nucleotide Targeter 2.0 at Cornell University [40], and the encoding sequences either synthesized by commercial suppliers or constructed using low-cost kits, such as the 'Golden Gate' assembly system [41]. The latter method relies on the conjugation of half TALEN pairs consisting of 10 repeats and adding the remaining repeats through sequential cutting and ligation, eventually splicing with sequences encoding FokI. An updated system, the Golden Gate Platinum assembly kit, involves conjugation of four RVDs together in the first step, which greatly increases the ease of TALEN generation [42].

Table 1. Comparison of genome editing technologies

Property	ZFNs	TALENs	CRISPR
Recognition motif	Zinc finger	Tal effector	Guide RNA
Specificity	18–36 bp (3 bases per finger)	40 bp (1 base per effector)	20 bp (1 base per base)
Targetability	Broad	Broad	Limited by motifs
Reported efficiency	1.6–33%	10–98.5%	Up to 100%
Off target effects	High	Low	High

TALEN pairs are also delivered by injection of encoding mRNAs, and have been used to generate a wide variety of mutations in zebrafish [39, 43–46], with up to 98.5% efficiency [47]. When compared with ZFNs, TALENs are more reliable, with higher efficiency, but also greater target specificity leading to reduced off-target effects [47, 48].

CRISPR

The Clustered, Regularly Interspaced, Short Palindromic Repeat (CRISPR)/CRISPR-associated 9 (Cas9) system has proven to be a simple, efficient and reliable alternative approach for genome editing in zebrafish [49, 50]. This system is derived from the adaptive immune mechanisms of bacteria and archaea that is used to defend against viruses and plasmids [14, 15, 51]. It consists of a CRISPR RNA (crRNA) array made of short foreign DNA fragments derived from viruses and/or plasmids that are retained as a memory of previous exposures, a trans-activating crRNA (tracrRNA) that mediates the processing of the crRNA array into short fragments and a Cas nuclease [51, 52]. A chimera of the crRNA and tracrRNA, known as the single-guide RNA (sgRNA), has been generated for ease of gene manipulation [53]. The 'guide' sequence within the sgRNA recognizes and binds to a complementary 20 bp DNA target adjacent to a protospacer adjacent motif (PAM) sequence, NGG, and the DNA is cleaved by Cas at the RuvC and HNH sites lying upstream [53].

sgRNA specific for a target site can be designed using ZiFiT Targeter [16, 26] and ordered as an oligonucleotide for subsequent cloning and *in vitro* transcription. This is co-injected into zebrafish embryos along with capped and poly A-tailed mRNA encoding Cas9. CRISPR-Cas9 gene editing has proven to be highly specific, being more efficient and easy to customize compared with other approaches. This approach can also be used to generate tissue-specific knockouts, transcribing the gRNA ubiquitously using a U6 promoter, while limiting expression of Cas9 with a tissue-specific promoter [54]. CRISPR can also be multiplexed by injection of multiple sgRNAs, allowing simultaneous targeting of multiple genes or locations within a gene [55]. The biggest limitation of this methodology is its inability to target all sequences, relying on specific sequence motifs to be present (Table 1).

Repair mechanisms

The DSBs generated by the various genome editing approaches are subsequently repaired through native repair processes [22].

Non-homologous end joining

Non-homologous end joining (NHEJ) is an error-prone process that occurs in the absence of any repair template, resulting in the efficient introduction of random insertion/deletion (indel) mutations, some of which will result in ablation of gene function [19] (Figure 2). NHEJ is primarily aimed at the generation of loss-of-function alleles, with indels introduced in coding exons, causing frame shift mutations and premature stop codons, leading to truncation of the encoded protein or other changes that serve to disrupt an essential domain [56, 57].

Microhomology end joining

Another process for repairing DSBs is microhomology end joining (MHEJ), which relies on small regions of homology adjacent to the target site for repair [58]. The occurrence of frameshift versus in-frame mutations is greatly affected by these regions of microhomology. However, careful analysis of the target site using prediction tools can maximize the chance of generating frameshift mutations [59].

Homology-dependent repair

In contrast to NHEJ/MHEJ, homology-dependent repair (HDR) uses a donor template for repair that displays homology to the cleavage site, thereby facilitating the introduction of specific genetic alterations in an efficient manner [44]. The repair template is exogenously introduced either as a double-stranded DNA construct with flanking insertion sequences in the homology arms either linear or circular, or as a single-stranded DNA oligonucleotide. The HDR approach can introduce a full range of genetic alterations through recombination of the repair template with the target locus [56, 57].

Homology-independent repair

Homology-independent repair (HIR) relies on ligation of cleaved donor DNA into a similarly cleaved target site using genome editing technologies to facilitate the repair in injected embryos. This technique has shown up to 33% efficiency in achieving targeted insertion [60].

Suggested workflow

A suitable genome editing strategy is designed using online resources in concert with sequencing of the target location to avoid polymorphisms at the target site and the necessary reagents ordered or generated in-house. Injected embryos are initially screened by appropriate method to determine cleavage efficiency. Efficiencies of 10% or more are acceptable for the generation of indels but optimization is suggested, as it will decrease the amount of subsequent screening. To identify founder (F0) fish, their progeny are screened, with in-crosses used to decrease the amount of screening.

Positive founders are then out-crossed with wild-types, with the aim of producing at least three independent lines that are propagated for further analysis. F1 embryos are raised to adulthood with a fin clip used to genotype and identify carriers, which are subsequently out-crossed with wild-types. F2 carriers are identified and can be in-crossed to produce homozygotes at Mendelian ratios. If viable, homozygote mutant zebrafish can

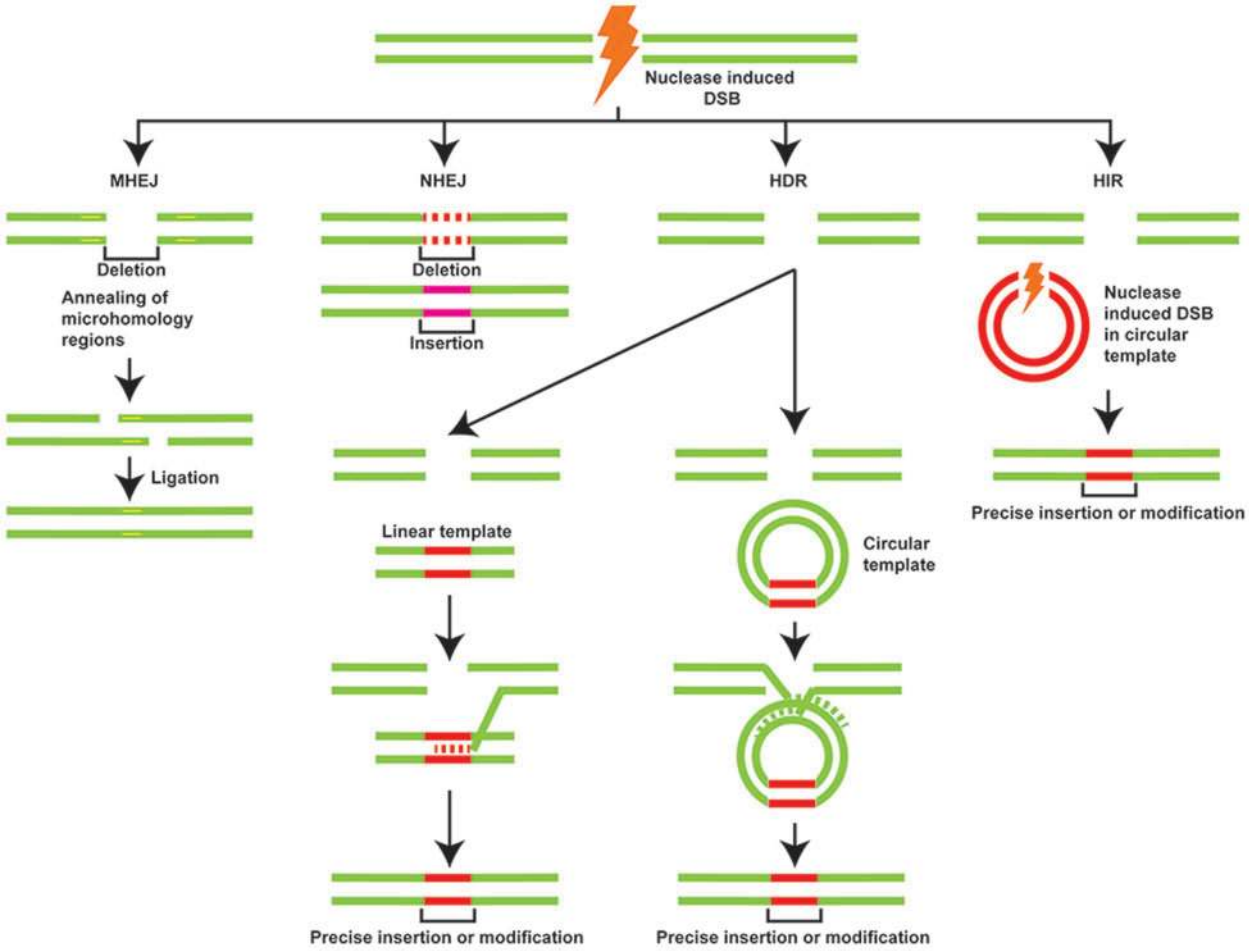

Figure 2. Utilization of endogenous repair process for a variety of genome editing outcomes. DSBs introduced by alternative genome targeting approaches are repaired by MHEJ, NHEJ, HDR or HIR. MHEJ and NHEJ occur in the absence of donor template and result in insertions or deletions (indels), which are predictable in the case of MHEJ but essentially random for NHEJ. In HDR, a transgene donor template can be provided either in linear or circular form, facilitating targeted insertion or modification, while in HIR a circular donor template is the target of the DSB. (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)

be used to create stable lines, or alternatively maintained as a heterozygote for phenotypic analysis (Figure 3).

Screening

Overview

There are a range of techniques available to screen the injected founders and their progeny for correct genome targeting. Each involves DNA extraction and polymerase chain reaction (PCR) amplification of the region around the target site for subsequent analysis. The approach selected is based on the type of mutation, likely efficiency and other practical considerations. As the efficiency of genome editing improves, screening is increasingly becoming the bottle-neck, and so throughput and cost are important considerations.

Specific methodologies

The simplest method for screening is based on differential amplicon size. Agarose gel electrophoresis can resolve differences in amplicon length down to ~10% of the amplicon size. This approach is simple, quick and applicable to high throughput, but is practically effective only for large deletions, such as whole exon deletion using genome editing technologies to target two sites simultaneously.

Several approaches are based on the generation of heteroduplexes formed by the presence of induced mutations. Using TILLING, these heteroduplexes are partially digested by a nuclease such as CelI [61] or T7 endonuclease I [16, 62] and separated by acrylamide gel electrophoresis. This is a sensitive technique allowing simultaneous screening of up to eight pooled samples [63], providing reasonable throughput. However, this approach can give a large number of false positives that increase the cost of screening by requiring larger numbers of samples to be sequenced.

One of the most promising screening technologies is High Resolution Melt (HRM) analysis, which involves the quantitative analysis of DNA melt curves. Genomic samples are saturated with DNA binding dye and fluorescence measured as the temperature is increased. HRM can detect single-nucleotide changes, even when present in relatively low frequency [64, 65], while different length amplicons typically produce different curves, allowing allelic series to be identified before sequencing. This represents an efficient and effective approach, although the presence of polymorphisms can complicate the interpretation/usefulness significantly.

Quantitative PCR (qPCR) can be used to detect indels by designing a primer set that flanks the target region and a second set where the 3′ end is targeted to the mutational hot spot region, usually the middle four to five bases of the spacer region in ZFNs and TALENs and the four bases upstream of the PAM

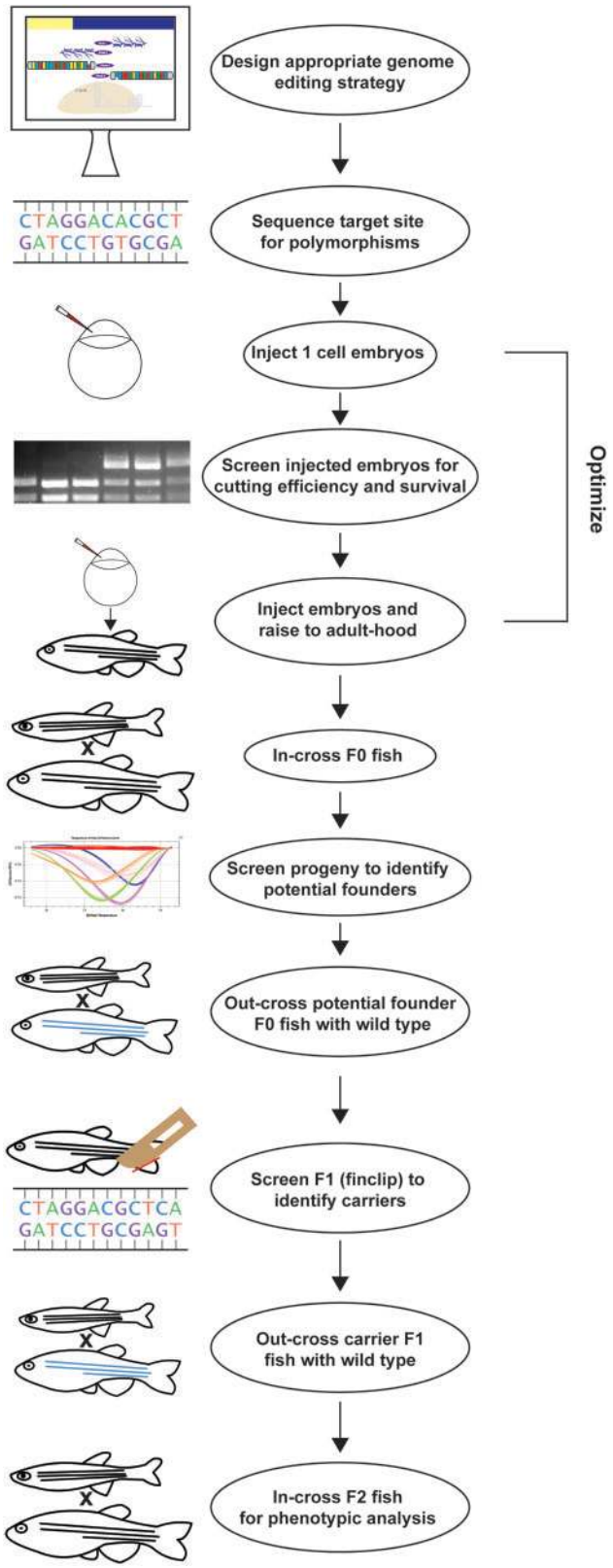

Figure 3. Typical workflow for genome editing. Schematic depiction of steps in genome editing including genome editing design, target site pre-screening, injection of embryos and subsequent strategies for the identification and propagation of specific mutants. (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)

when targeting with CRISPR. The presence of mutant RNA will decrease the amplification efficiency, resulting in decreased relative expression. Using this methodology, mutation efficiencies as low as 10% can be detected [66].

Other approaches screen for the specific sequence change. For example, restriction fragment length polymorphism (RFLP) analysis involves designing an editing site coincident with a restriction enzyme site—or introducing one through HDR—allowing identification of potential mutants by the loss/gain of a restriction site in an amplified product, resulting in altered fragment sizes following digestion. This strategy works most effectively if there is an additional site for the enzyme away from the targeted sequence, which can be used to ensure efficient digestion, thereby avoiding false positives/negatives. This approach is straightforward and has similar throughput to endonuclease assay digestion, but requires a suitable site for screening and can be expensive when more obscure enzymes are needed.

For any genome editing project, sequencing is always required. The screening techniques mentioned above can only indicate that a change is present, but sequencing alone can fully characterize the molecular details of each targeting event. Sanger sequencing is reliable and easy to interpret when compared with a wild-type sequence analysed in parallel. Potential mutants can also be screened by NextGen sequencing approaches, which offer high throughput for screening of multiple loci [67] (Figure 4) (Table 2).

Potential problems and some solutions

Genome editing technology is still in its relative infancy, with a number of problems commonly experienced. However, the methodology is rapidly evolving to address these issues. For example, off-targeting remains a key problem. To reduce off-targeting of both ZFNs and TALENs, FokI variants have been generated carrying mutations in the dimerization helices such that only heterodimerization is possible, thereby reducing off-targeting. These include Q486E:I499L and E490K:I538K (EL+KK) [9] or R487D and D483R (D+R) [68] with complementary FokI variants attached to each DNA binding segment. To increase cleavage efficiency, mutants have also been generated in the cleavage domain of FokI, such as S418P and K441E, which show a 3- to 6-fold increase in mutagenesis rates compared with wild-type FokI [69]. Combining cleavage and dimerization mutants further increases effectiveness particular for NHEJ. To increase the efficiency of HDR, an inactivating mutation, D450A [70, 71], has been incorporated into one of the FokI cleavage domains so that a single strand cut or 'nick' will be introduced [72], which inhibits formation of indels associated with NHEJ [73]. Variants of Cas9 have also been generated to reduce off-target mutations, including nickase mutants and FokI fusions that require two adjacent sgRNAs to introduce a DSB, thereby doubling the size of the target recognition sequence [74]. Combined with the available bioinformatics tools for design [75], off-targeting can be greatly minimized. In addition, several methods exist to detect off-target events, most underpinned by Next-Gen sequencing, such as unbiased identification of DSBs enabled by sequencing (GUIDE-seq), *in vitro* nuclease-digested genome sequencing (Digenome-seq) and high-throughput genomic translocation sequencing [75]. Analysis of multiple independent alleles and extensive back-crossing provide additional strategies to circumvent potential off-target issues.

For CRISPR, the sites available for targeting are limited by sequence requirements of the promoter chosen to initiate the transcription of the sgRNA and also the (PAM) motif. For

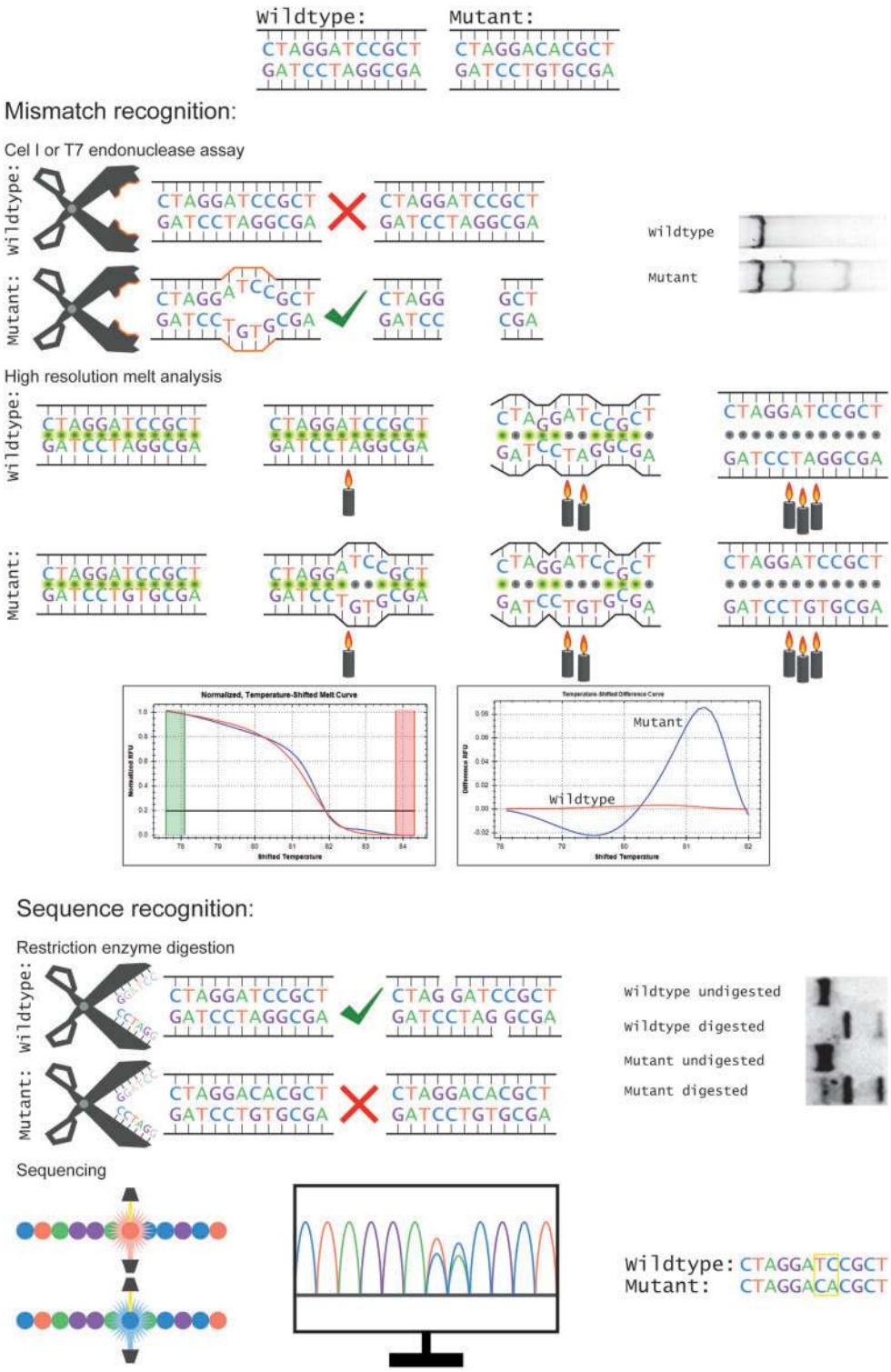

Figure 4. Key molecular methodologies for mutant screening. Screening for introduced mutations relies on two underlying approaches, mismatch recognition or sequence recognition of an amplicon including the target site. Cel I or T7 endonucleases will cleave the DNA at a mismatch generating cleavage products of the wild-type amplicon. High-resolution melt (HRM) analysis uses fluorescence to detect altered melt temperatures between wild-type and mutant. Restriction enzymes identify the loss of a specific sequence in a mutant with an uncleaved amplicon in the mutant digest. Sanger sequencing directly distinguishes mutant and wild-type sequences by termination fluorescence, which shows a characteristic double-peak pattern on the chromatogram. (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)

instance, the use of T7-based RNA has limited targeting potential owing to the first transcribed base using T7 polymerase incorporating a GG to start the sgRNA, which in concert with PAM requirements limits the targeting potential to sites with the sequence context of $GGN_{19}GG$ [16], although this can also be overcome by designing shorter guides that have a 5′ mismatch $gGN_{19}GG$ [76]. Use of other promoters to initiate transcription such as U6, which only incorporates a single G, can also

Table 2. Comparison of screening methodologies to identify mutations

	Size	Mismatch recognition			Sequence recognition		
Property	Electrophoresis	Endonuclease digestion	HRM	qPCR	RFLP	Sanger sequencing	Next-Gen sequencing
Cost	$	$$	$	$	$-$$	$$	$$-$$$
Accuracy	Low	Low	Medium	Medium	Medium	Definitive	Definitive
Speed	4 h	8–24 h	3 h	3 h	8–24 h	4–55 h	2 days

increase potential targets to $GN_{20}GG$ sites [77]. Recently, a PAM mutant D1135V/R1335Q/T1337R was generated allowing modest targeting of NGAG sites [78], which greatly increases the targeting ability of CRISPR.

The effectiveness and efficiency of genome editing approaches can vary widely, depending on the target sequence. Apparent 'non-functionality' can be owing to several factors, including single nucleotide polymorphisms and other sequence variants, methylation and alternate modifications, as well as inaccessible chromatin. A variety of approaches can circumvent these issues, including prior sequencing of relevant genomic regions of in-house fish and targeting of multiple target sites for each gene. The efficiency of CRISPR can be further increased by directly injecting Cas9 protein or protein/sgRNA complex to bypass endogenous transcription of Cas9 RNA [67, 79], while codon-optimized and green fluorescent protein (GFP)-tagged versions of Fok1 and Cas9 are available [54, 80, 81]. For HDR, the use of longer homology arms has been shown to increase targeting efficiency [82], and the purification of donor DNA with a nucleotide removal kit can increase the survival of injected embryos [83].

Finally, as a result of the widespread utilization of genome editing and morpholino-mediated gene knockdown in parallel, it has become apparent that different phenotypes result, with so-called 'morphants' typically having more pronounced/extensive phenotypic alterations than 'knockouts'. Two reasons have been proposed — firstly, that morpholinos result in off-target/non-specific effects [84] and, secondly, that mutations induce genetic compensation that serves to mask phenotypic changes [85] — which are not mutually exclusive. Practical approaches to provide further confidence about observed phenotypes include the analysis of multiple alleles, genetic rescue, combining morpholinos and mutants, as well as global transcriptome analysis.

Conclusions

Zebrafish has proven to be a robust model for studying the genetic basis of development and its disruption in disease. Genome editing provides an expanded repertoire of possibilities to efficiently create virtually any genetic manipulation. This will continue to enhance the use of zebrafish, particularly for modelling human disease.

Key Points

- Zebrafish can be manipulated by ZFNs, TALENs and CRISPR.
- Both random (MHEJ, NHEJ) and targeted (HDR, HIR) mutants are possible.
- New approaches are being rapidly developed to increase efficiency and sophistication.

Acknowledgements

The authors acknowledge support of Australian Postgraduate Award (M.T. and R.S.) or Deakin University International Postgraduate Scholarship (F.B.), Alfred Deakin Postdoctoral Research Fellowship (C.L.) as well as support from the Centre for Molecular and Medical Research (A.C.W.).

References

1. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 2007;**8**:353–67.
2. Mullins MC, Hammerschmidt M, Haffter P, *et al*. Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr Biol* 1994;**4**:189–202.
3. Patton EE, Zon LI. The art and design of genetic screens: zebrafish. *Nat Rev Genet* 2001;**2**:956–66.
4. Gibert Y, Trengove MC, Ward AC. Zebrafish as a genetic model in pre-clinical drug testing and screening. *Curr Med Chem* 2013;**20**:2458–66.
5. Bill BR, Petzold AM, Clark KJ, *et al*. A primer for morpholino use in zebrafish. *Zebrafish* 2009;**6**:69–77.
6. Kettleborough RN, Bruijn E, Eeden F, *et al*. High-throughput target-selected gene inactivation in zebrafish. *Methods Cell Biol* 2011;**104**:121–7.
7. Hogan BM, Verkade H, Lieschke GJ, *et al*. Manipulation of gene expression during zebrafish embryonic development using transient approaches. *Methods Mol Biol* 2008;**469**:273–300.
8. Mosimann C, Zon LI. Advanced zebrafish transgenesis with Tol2 and application for Cre/lox recombination experiments. *Methods Cell Biol* 2011;**104**:173–94.
9. Miller JC, Holmes MC, Wang J, *et al*. An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol* 2007;**25**:778–85.
10. Porteus MH, Baltimore D. Chimeric nucleases stimulate gene targeting in human cells. *Science* 2003;**300**:763.
11. Christian M, Cermak T, Doyle EL, *et al*. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 2010;**186**:757–61.
12. Wood AJ, Lo TW, Zeitler B, *et al*. Targeted genome editing across species using ZFNs and TALENs. *Science* 2011;**333**:307.
13. Cade L, Reyon D, Hwang WY, *et al*. Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs. *Nucleic Acids Res* 2012;**40**:8001–10.
14. Deveau H, Garneau JE, Moineau S. CRISPR/Cas system and its role in phage-bacteria interactions. *Ann Rev Microbio* 2010;**64**:475–93.
15. Horvath P, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. *Science* 2010;**327**:167–70.

16. Hwang WY, Fu Y, Reyon D, *et al*. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 2013;**31**:227–9.
17. Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci USA* 1996;**93**:1156–60.
18. Wolfe SA, Nekludova L, Pabo CO. DNA recognition by Cys2His2 zinc finger proteins. *Ann Rev Biophys Biomol Structure* 2000;**29**:183–212.
19. Doyon Y, McCammon JM, Miller JC, *et al*. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* 2008;**26**:702–8.
20. Meng X, Noyes MB, Zhu LJ, *et al*. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol* 2008;**26**:695–701.
21. Urnov FD, Rebar EJ, Holmes MC, *et al*. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 2010;**11**:636–46.
22. Gaj T, Gersbach CA, Barbas CF, III. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotech* 2013;**31**:397–405.
23. McCammon JM, Doyon Y, Amacher SL. Inducing high rates of targeted mutagenesis in zebrafish using Zinc Finger Nucleases (ZFNs). *Meth Mol Biol* 2011;**770**:505–27.
24. Carroll D, Morton JJ, Beumer KJ, *et al*. Design, construction and in vitro testing of zinc finger nucleases. *Nat Protoc* 2006;**1**:1329–41.
25. Sood R, Carrington B, Bishop K, *et al*. Efficient methods for targeted mutagenesis in zebrafish using zinc-finger nucleases: data from targeting of nine genes using CompoZr or CoDA ZFNs. *PLoS One* 2013;**8**:e57239.
26. Sander JD, Maeder ML, Reyon D, *et al*. ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool. *Nucleic Acids Res* 2010;**38**:W462–8.
27. Sander JD, Dahlborg EJ, Goodwin MJ, *et al*. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods* 2011;**8**:67–9.
28. Maeder ML, Thibodeau-Beganny S, Sander JD, *et al*. Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. *Nat Protoc* 2009;**4**:1471–501.
29. Zhu C, Smith T, McNulty J, *et al*. Evaluation and application of modularly assembled zinc-finger nucleases in zebrafish. *Development* 2011;**138**:4555–64.
30. Liu Y, Kretz CA, Maeder ML, *et al*. Targeted mutagenesis of zebrafish antithrombin III triggers disseminated intravascular coagulation and thrombosis, revealing insight into function. *Blood* 2014;**124**:142–50.
31. Ramirez CL, Foley JE, Wright DA, *et al*. Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat Methods* 2008;**5**:374–5.
32. Pingoud A, Silva GH. Precision genome surgery. *Nat Biotechnol* 2007;**25**:743–4.
33. Foley JE, Maeder ML, Pearlberg J, *et al*. Targeted mutagenesis in zebrafish using customized zinc-finger nucleases. *Nat Protoc* 2009;**4**:1855–67.
34. Joung JK, Sander JD. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol* 2012;**14**:49–55.
35. Boch J, Scholze H, Schornack S, *et al*. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 2009;**326**:1509–12.
36. Bogdanove AJ, Voytas DF. TAL effectors: customizable proteins for DNA targeting. *Science* 2011;**333**:1843–6.
37. Deng D, Yan C, Pan X, *et al*. Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 2012;**335**:720–3.
38. Miller JC, Tan S, Qiao G, *et al*. A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol* 2011;**29**:143–8.
39. Bedell VM, Wang Y, Campbell JM, *et al*. In vivo genome editing using a high-efficiency TALEN system. *Nature* 2012;**491**:114–18.
40. Doyle EL, Booher NJ, Standage DS, *et al*. TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. *Nucleic Acids Res* 2012;**40**:W117–22.
41. Cermak T, Doyle EL, Christian M, *et al*. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res* 2011;**39**:e82.
42. Sakuma T, Ochiai H, Kaneko T, *et al*. Repeating pattern of non-RVD variations in DNA-binding modules enhances TALEN activity. *Sci Rep* 2013;**3**:3379.
43. Sander JD, Cade L, Khayter C, *et al*. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol* 2011;**29**:697–8.
44. Huang P, Xiao A, Zhou M, *et al*. Heritable gene targeting in zebrafish using customized TALENs. *Nat Biotechnol* 2011;**29**:699–700.
45. Zu Y, Tong X, Wang Z, *et al*. TALEN-mediated precise genome modification by homologous recombination in zebrafish. *Nat Methods* 2013;**10**:329–31.
46. Tatsumi Y, Takeda M, Matsuda M, *et al*. TALEN-mediated mutagenesis in zebrafish reveals a role for r-spondin 2 in fin ray and vertebral development. *FEBS Lett* 2014;**588**:4543–50.
47. Dahlem TJ, Hoshijima K, Jurynec MJ, *et al*. Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. *PLoS Genet* 2012;**8**:e1002861.
48. Mussolino C, Morbitzer R, Lütge F, *et al*. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucl Acids Res* 2011;**39**:9283–93.
49. Talbot JC, Amacher SL. A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles. *Zebrafish* 2014;**11**:583–5.
50. Blackburn PR, Campbell JM, Clark KJ, *et al*. The CRISPR system–keeping zebrafish gene targeting fresh. *Zebrafish* 2013;**10**:116–18.
51. Garneau JE, Dupuis MÈ, Villion M, *et al*. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 2010;**468**:67–71.
52. Gasiunas G, Barrangou R, Horvath P, *et al*. Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci USA* 2012;**109**:E2579–E86.
53. Jinek M, Chylinski K, Fonfara I, *et al*. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012;**337**:816–21.
54. Ablain J, Durand EM, Yang S, *et al*. A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Dev Cell* 2015;**32**:756–64.
55. Jao LE, Wente SR, Chen W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci USA* 2013;**110**:13904–9.
56. Ran FA, Hsu PD, Wright J, *et al*. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 2013;**8**:2281–308.
57. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* 2014;**32**:347–55.

58. McVey M, Lee SE. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet* 2008;**24**:529–38.
59. Bae S, Kweon J, Kim HS, *et al*. Microhomology-based choice of Cas9 nuclease target sites. *Nat Methods* 2014;**11**:705–6.
60. Auer TO, Duroure K, De Cian A, *et al*. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res* 2014;**24**:142–53.
61. Oleykowski CA, Bronson Mullins CR, Godwin AK, *et al*. Mutation detection using a novel plant endonuclease. *Nucleic Acids Res* 1998;**26**:4597–602.
62. Auer TO, Duroure K, Concordet JP, *et al*. CRISPR/Cas9-mediated conversion of eGFP- into Gal4-transgenic lines in zebrafish. *Nat Protoc* 2014;**9**:2823–40.
63. Moens CB, Donn TM, Wolf-Saxon ER, *et al*. Reverse genetics in zebrafish by TILLING. *Brief Funct Genomic Proteomic* 2008;**7**:454–9.
64. Martino A, Mancuso T, Rossi AM. Application of high-resolution melting to large-scale, high-throughput SNP genotyping: a comparison with the TaqMan method. *J Biomol Screen* 2010;**15**:623–9.
65. Parant JM, George SA, Pryor R, *et al*. A rapid and efficient method of genotyping zebrafish mutants. *Dev Dyn* 2009;**238**:3168–74.
66. Yu C, Zhang Y, Yao S, *et al*. A PCR based protocol for detecting indel mutations induced by TALENs and CRISPR/Cas9 in zebrafish. *PLoS One* 2014;**9**:e98282.
67. Gagnon JA, Valen E, Thyme SB, *et al*. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLoS One* 2014;**9**:e98186.
68. Szczepek M, Brondani V, Buchel J, *et al*. Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat Biotechnol* 2007;**25**:786–93.
69. Guo J, Gaj T, Barbas CF, III Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases. *J Mol Biol* 2010;**400**:96–107.
70. Waugh DS, Sauer RT. Single amino acid substitutions uncouple the DNA binding and strand scission activities of Fok I endonuclease. *Proc Natl Acad Sci USA* 1993;**90**:9596–600.
71. Bitinaite J, Wah DA, Aggarwal AK, *et al*. FokI dimerization is required for DNA cleavage. *Proc Natl Acad Sci USA* 1998;**95**:10570–5.
72. Ramirez CL, Certo MT, Mussolino C, *et al*. Engineered zinc finger nickases induce homology-directed repair with reduced mutagenic effects. *Nucleic Acids Res* 2012;**40**:5560–8.
73. Kim E, Kim S, Kim DH, *et al*. Precision genome engineering with programmable DNA-nicking enzymes. *Genome Res* 2012;**22**:1327–33.
74. Jamal M, Khan FA, Da L, *et al*. Keeping CRISPR/Cas on-Target. *Curr Issues Mol Biol* 2015;**20**:1–20.
75. Koo T, Lee J, Kim JS. Measuring and reducing off-target activities of programmable nucleases including CRISPR-Cas9. *Mol Cells* 2015;**38**:475–81.
76. Moreno-Mateos MA, Vejnar CE, Beaudoin JD, *et al*. CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nat Methods* 2015;**12**:982–8.
77. Yin L, Maddison LA, Li M, *et al*. Multiplex conditional mutagenesis using transgenic expression of Cas9 and sgRNAs. *Genetics* 2015;**200**:431–41.
78. Kleinstiver BP, Prew MS, Tsai SQ, *et al*. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 2015;**523**:481–5.
79. Sung YH, Kim JM, Kim HT, *et al*. Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. *Genome Res* 2014;**24**:125–31.
80. Liu D, Wang Z, Xiao A, *et al*. Efficient gene targeting in zebrafish mediated by a zebrafish-codon-optimized cas9 and evaluation of off-targeting effect. *J Genet Genomics* 2014;**41**:43–6.
81. Huang P, Xiao A, Tong X, *et al*. Targeted mutagenesis in zebrafish by TALENs. *Methods Mol Biol* 2016;**1338**:191–206.
82. Shin J, Chen J, Solnica-Krezel L. Efficient homologous recombination-mediated genome engineering in zebrafish using TALE nucleases. *Development* 2014;**141**:3807–18.
83. Bedell VM, Ekker SC. Using engineered endonucleases to create knockout and knockin zebrafish models. *Methods Mol Biol* 2015;**1239**:291–305.
84. Kok FO, Shin M, Ni CW, *et al*. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev Cell* 2015;**32**:97–108.
85. Rossi A, Kontarakis Z, Gerri C, *et al*. Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* 2015;**524**:230–3.