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Fishing for understanding: Unlocking the zebrafish gene editor's toolbox

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Abstract

The rapid growth of the field of gene editing can largely be attributed to the discovery and optimization of designer endonucleases. These include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regular interspersed short palindromic repeat (CRISPR) systems including Cas9, Cas12a, and structure-guided nucleases. Zebrafish (*Danio rerio*) have proven to be a powerful model system for genome engineering testing and applications due to their external development, high fecundity, and ease of housing. As the zebrafish gene editing toolkit continues to grow, it is becoming increasingly important to understand when and how to utilize which of these technologies for maximum efficacy in a particular project. While CRISPR–Cas9 has brought broad attention to the field of genome engineering in recent years, designer endonucleases have been utilized in genome engineering for more than two decades. This chapter provides a brief overview of designer endonuclease and other gene editing technologies in zebrafish as well as some of their known functional benefits and limitations depending on specific project goals. Finally, selected prospects for additional gene editing tools are presented, promising additional options for directed genomic programming of this versatile animal model system.

Keywords

Genome editing; zebrafish; designer nuclease; CRISPR; DNA repair; base editing

Introduction

One of the first applications of programmable designer endonucleases was published in 1996 in which a chimeric zinc finger was fused with the nuclease domain from FokI creating the zinc finger nuclease[2]. However, it was not until 2008 that the first targeted gene knockouts were reported in zebrafish using ZFNs[34]. Gene knockout and other mutant alleles are generated by the induction of double stranded breaks (DSBs) in the DNA double helix. These breaks are rapidly fixed by endogenous DNA repair pathways that can cause stochastic insertion or deletion (indel) mutations and can lead to disruption of the open

reading frame and subsequent protein structure. In the short period since then, the further optimization of ZFNs, TALENs[5], CRISPR systems[1] including the Cas9[67] as well as Cas12a (formerly known as Cpf1)[8,9] nucleases, and the structure guided nuclease[10] have facilitated unforeseen opportunities in the zebrafish engineering arena. Though knockdown approaches such as morpholino technology[11] provide robust reduction in gene expression, designer endonucleases can enable a gene knockout, which can lead to total ablation of gene products and the generation of mutant lines with relative ease. Even with these new technologies, the challenge to produce fully null mutants is still present. Total gene knockout is often confounded by genetic compensation[12,13], induction of unintentional hypomorphism[14], and other pathways that are not yet fully elucidated.

The DSBs induced by these technologies are often repaired by the high-efficiency, error-prone non-homologous end joining (NHEJ) pathway[15,16,17]. Less frequently, DNA breaks are repaired by the low efficiency, homology directed repair (HDR) group of pathways including homologous recombination that utilizes the homologous chromosome or exogenous DNA as a repair template[15,18]. In recent years, there has been further elucidation of another repair pathway, microhomology mediated end joining (MMEJ). MMEJ utilizes 5–25bp segments on the repair template that have homology around the break point and can assist in aligning the broken strands before joining[19,20,21]. The alignment conferred by the microhomologous regions can help MMEJ produce a desired and predictable outcome when used with a designer nuclease.

Technologies

Zinc Finger Nucleases – bringing designer nucleases to zebrafish

Cys2His2 zinc finger (ZF) sequence-specific DNA binding domains were first discovered in transcription factor IIIA from *Xenopus laevis*[22] and were subsequently engineered for customized nuclease application. ZF nucleases are made by the fusion of the programmable DNA binding domains with the fusion of the FokI endonuclease domain[23]. The native FokI type IIS restriction enzyme has separable DNA binding and nuclease domains and requires the formation of a dimer to be catalytically active[24]. ZFNs are currently engineered with three to six zinc finger domains comprised of ~30 amino acids each per domain with two beta strands and one alpha helix, with the alpha helix conferring recognition of 3 nucleotides[25]. For example, a single ZFN module containing 3 Cys2His2 domains is capable of recognizing 9nt of DNA sequence; a pair of three finger ZFNs are thus capable of recognizing 18nt, which can be sufficient for targeting a unique genomic locus in zebrafish[26]. FokI functions as a homodimer and requires the specific orientation and spacing of the ZFNs to induce a double stranded break (DSB) in the DNA at a desired gene region (Fig 1a). FokI can be further engineered to function as an obligate heterodimer to increase specificity of cleavage[27].

Microinjection of ZFN protein into zebrafish embryos has been shown to be well tolerated with as high as 5ng of ZFN mRNA being injected with minimal toxicity and appreciable efficacy[28,29]. However, the complex nature of ZFN synthesis and design such as unpredicted aberrant interactions between zinc finger subunits in addition to the high cost of construction has largely rendered ZFNs a less accessible technology to zebrafish researchers. However,

their pioneering work established gene editing in vertebrate embryos as a viable gene knockout approach that was subsequently deployed in other models such as mice[30] and rats[31].

TALENs

Transcription activator-like effectors (TALEs) are derived from plant pathogen *Xanthomonas* bacteria, a plant pathogen that hijacks host gene expression to favor its own survival[32,33,34]. The nuclease activity of transcription activator like effector nucleases (TALENs), like ZFNs, is conferred by the fusion of the FokI endonuclease to the TALE programmable DNA binding cassette[32]. The fused FokI functions as a homodimer that binds both sides of the target DNA and cleaves upon dimerization with the TALEN pairs[35]. TALE repeats are comprised of 33–35 amino acid repeats that are identical except for residues 12 and 13. These resides are referred to as the repeat variable diresidue (RVD) domain and are responsible for conferring one-to-one recognition of nucleotides[32]. TALENs are normally used as dimers with 15–20 TALE repeats binding on one strand separated by a spacer region of 15–20 nucleotides followed by another 15–20 TALE repeats binding the opposite strand; consequently, the DSB occurs at around the halfway point of the spacer where the fused FokI domains have the greatest probability of dimerization and subsequent DNA cleavage (Fig 1b).

Custom TALEN expression vectors are constructed using diverse approaches including Golden Gate[36], serial ligation[37], and ligation independent[38] methods. Conventional TALEN scaffolds function optimally at a locus with a thymidine at the 5’ end of the TALE binding region[39,32,40,36]. However, later scaffolds have since circumvented this preference without a substantive decrease in efficiency[41,42]. Due to their high efficacy when delivered *in vivo* by microinjection and new rapid synthesis protocols such as that developed by *Ma et al.*[43], TALENs remain a useful tool for genome engineering purposes, showing particular efficacy in zebrafish gene knock-in approaches [44] (see also below).

Though TALENs provide many solutions to the problems posed by ZFNs such as unpredicted interaction between the nuclease subunits, the detailed cloning methods involved in TALEN construction has rendered them useful in specific applications such as HR and HDR induction. The specialized employment of TALENs can be largely attributed to the highly accessible nature of CRISPR-Cas9 for NHEJ-based applications.

TALENs have had and continue to have tremendous impacts of the field of genome engineering. TALENs were one of the first technologies to achieve consistent targeted homologous recombination and paved the way for many later targeted integration technologies[45]. In addition to their pioneering work in integrating exogenous DNA, TALENs were one of the first technologies to remove large spans of DNA approaching nearly 20kb[46]. TALENs remain a useful tool and in fact have been recently shown to have powerful therapeutic application in chimeric antigen receptor T cell (CAR T) based modalities to treat various cancers[47]. In addition to therapeutic applications, TALENs continue to be streamlined technologies to investigate specific cell type function[48] as well as the expression of poorly understood regulatory pathways[49]

CRISPR-Cas9

Clustered regular interspersed short palindromic repeats (CRISPR) and CRISPR associated protein-9(Cas9)[1] have greatly expanded the genome engineering field. CRISPR systems have been elucidated to protect bacteria from exogenous DNA[50]. CRISPR RNA (crRNA) and a trans activating CRISPR RNA (tracrRNA) are transcribed from a CRISPR locus and are processed and incorporated into the mature Cas9 complex[51,52]. The CRISPR locus likewise encodes DNA of previously encountered pathogens in its spacer regions. The invading pathogenic DNA can later be transcribed and used as a guide and direct the binding of the mature Cas9 complex to the intruding virus. Once the complex is guided to the exogenous DNA, the DNA is cleaved and leads to subsequent degradation of the intruding DNA (Fig1c)[53,54,55]. The CRISPR–Cas9 system utilizes a protospacer adjacent motif (PAM), which is present in the exogenous DNA but absent in the encoded spacer region, to differentiate between the endogenous CRISPR spacer and the pathogenic DNA. In the commonly used CRISPR–Cas9 system derived from the human pathogen *Streptococcus pyogenes* (SpCas9), a native NGG 3' PAM sequence is required for efficient cleavage activity[56]. CRISPR–Cas9 has further been optimized for gene editing by adding a linker region between the tracrRNA and crRNA to form a single hybrid single guide RNA (sgRNA) to simplify molecular construction[57].

The SpCas9 system has since been outfitted for zebrafish by codon optimizing the Cas9 protein for zebrafish expression and adding an SV40 large T antigen nuclear localization signal to both the N and C termini[58]. This codon optimized Cas9 was subsequently cloned into vectors that facilitate the synthesis of capped and polyadenylated Cas9 mRNA via *in vitro* transcription from either SP6 or T3 promoters[58]. This method was further optimized by allowing the synthesis of gRNA generated by using two partially overlapping oligonucleotides in a clone-free manner[59]. One oligonucleotide is specific to the genomic loci of interest whereas the other is a generic oligonucleotide that can be used for all constructs and contains the necessary secondary structure. These oligonucleotides form a double stranded template by annealing at the designed 20nt overlap and are extended via polymerase chain reaction (PCR). The duplex DNA generated from this polymerization reaction can then be used for gRNA synthesis via *in vitro* transcription reaction[60] in a high throughput manner enabling one to synthesize hundreds of gRNA in just a few hours.

Injection of pre-formed gRNA/Cas9 ribonucleoprotein (RNP) complexes has likewise been optimized for zebrafish injections as early as the single cell stage[61,62]. Other Cas9 systems that have been shown to work well in zebrafish include forms fused to an NLS, and as fusions with several different fluorescent protein domains[62,63,64]. Other Cas9 mRNA constructs exist such as only C-terminal NLS or only N-terminal NLS tagged Cas9 as well as T7 promoter driven Cas9 for fine-tuned expression and mRNA. Many are now commercially available, reducing the requirement for gene editing to a single custom reagent. This simplification enables both the rapid deployment of this tool for many research studies and also opens the door to active learning classrooms such as an undergraduate development lab (Essner et al., personal communication).

Though efficient editing has been shown in zebrafish as well as other model systems, the CRISPR–Cas9 system is not without its drawbacks. Native SpCas9 has a requirement for an

NGG PAM sequence[56], a targeting limitation especially when targeting AT rich genomic regions such as introns. This obstacle is reduced as continued engineering of SpCas9 has led to variants with an NGA PAM[65,66] and is still in progress to utilize additional PAM targets. Whether these variants are as effective in zebrafish as they are in human DNA editing is yet to be described.

Off-targeting in zebrafish is an important consideration that can be addressed in several ways. First, outcrossing a founder animal results in the natural removal of 98% of DNA harboring any off-target edited (49 out of 50) non-linked mutant chromosomes[67]. Second, generating more than one allele can reduce the likelihood of a linked, second mutation found on the remaining selected chromosome.

Cas12a

Subsequent to the discovery and utilization of CRISPR–Cas9 as a genome editing tool, a bioinformatic expedition began to identify other bacterial immune systems with desirable qualities such as different PAM requirements and high efficiency nucleases with potential lower off target activity. This work led to the discovery of the CRISPR from *Prevotella* and *Francisella* 1 initially described as Cpf1 and now formally called Cas12a[688,69,70]. Cas12a contains a RuvC-like endonuclease domain that is similar to the Cas9 endonuclease domain, suggesting it has a similar role in adaptive immunity as a targeted endonuclease[71]. Additionally, Cas12a has fewer secondary structure requirements for gRNA, does not require a tracrRNA and utilizes only a sgRNA that can be self-processed[8]. Cas12a has a 5' TTTN PAM sequence preference[728], affording targeting into AT rich gene regions such as zebrafish introns that may be inaccessible with SpCas9. Additionally, Cas12a creates a staggered cut distal from the PAM by cleaving the DNA after the 18th base on the non-targeted strand and after the 23rd base on the targeted strand (Fig 1d). Unlike Cas9, this cleavage does not destroy the original target site and might facilitate greater efficiency of donation of exogenous DNA via single strand annealing[53] or homologous recombination approaches. All of these features could lead to simplified delivery in the zebrafish model.

However, initially described Cas12a systems do not work off-the-shelf in zebrafish as either RNP or mRNA[73]. Further optimization has led to functional systems for zebrafish[74], and Cas12a provides a unique opportunity for genome engineering in this model system due to its more compact size, AT rich PAM, and staggered DNA cutting. Cas12a can catalyze higher HDR-based repair in zebrafish relative to SpCas9, possibly due to its repeated cutting that could help drive HDR over nonspecific end joining[74].

Further, because Cas12a processes its own sgRNA, this allows for the possibility of Cas12a's sgRNAto be packaged into a smaller cassette being driven by an RNA Polymerase II promoter. Cas9 sgRNAs are large and require extensive processing, necessitating an RNA Polymerase III promoter such as U6 to drive their expression[75,76,77]. This added benefit provides a simplified opportunity for tissue-specific expression of Cas12a gRNA in zebrafish, which is challenging with current Cas9 gRNA systems. However, developing technologies are beginning to enable the use of RNA Polymerase II promoters for Cas9 sgRNA with the utilization of ribozymes and other molecular catalysts to process the sgRNA[78].

xCas9 – next generation of CRISPR targeting in zebrafish?

To circumvent the issue of the PAM requirement narrowing the target range of CRISPR–Cas9, researchers set out to develop a Cas9 with a broad PAM utilization. Though *Staphylococcus aureus* Cas9 (SaCas9) has been engineered previously to have an NNNRRT (R=A or G) PAM to increase its targeting range, it suffered a concomitant increase in off-target editing[79]. Using a variation of phage-assisted continuous evolution (PACE)[80], researchers evolved SpCas9 *in vitro* and selected for variants that bind a greater variety of PAM sequences. This led to the discovery of Cas9 variants, termed xCas9, that were capable of binding NG, NNG, GAA, GAT, and CAA PAMs[81]. These variants were assessed for genome engineering applications such as transcriptional activation by fusing xCas9 to the VP64 transcriptional activator[82], DNA cutting, and cytidine and adenine base editing by fusing xCas9 to third-generation (BE3) base editing architecture containing a nucleobase deaminase[83].

In all three parameters tested xCas9 was shown to have a significantly increased activity in each respective application relative to SpCas9. xCas9 provides a tool that has a broad range of PAM targets, greater specificity relative to SpCas9, and less off targets. This suggests that there is not an obligate trade-off between efficiency, PAM requirement, and specificity[84]. This xCas9 system currently has the largest potential genomic targeting range among known SpCas9 variants Though preliminary data for xCas9 appears promising, overall efficiency has not yet been established. Once cleavage kinetics and gene editing capabilities of xCas9 have been further assessed, this suggests the possibility of highly active and specific Cas9 variants that may be powerful tools in the zebrafish genome engineer's toolbox in the near future.

Structure guided nuclease – a DNA-guided gene editor

A DNA-guided endonuclease has been recently constructed known as the structure-guided nuclease (SGN)[10]. This gene editor is a fusion protein of flap-endonuclease-1 (FEN-1) and the cleavage domain of FokI. FEN–1 functions endogenously by recognizing single unpaired 3' nucleotide overhang "flaps" that occur during DNA replication and repair and removes the lesion for the DNA polymerase to fill in[85]. This structure-guided nuclease functions in concert with a pair of guide DNA (gDNA) molecules that have a single unpaired 3' nucleotide and produce a 3' overhang flap flanking the target site for the FEN-1 domain to recognize and cleave the target (Fig 2). Microinjection of SGN mRNA and gDNA results in the editing of endogenous zebrafish DNA, albeit with modest efficiency in this first generation tool[86,10]. Interestingly, the structure- guided nuclease preferentially creates large deletions instead of small indels that are observed with other engineered endonucleases[10]. This unique lack of PAM requirement and creation of large deletions suggests the possibility of exploiting the SGN's activity by insertion of large repair templates or producing efficient knockouts in zebrafish by removal of large regions of DNA in virtually any genomic locus. The founders of this technology report low overall editing efficacy and this technology has not yet been widely adopted at this time. With further validation and optimization, the SGN could be a powerful tool for the genome engineer in the near future.

Argonautes – the ups and downs of a genome tool

Argonaute proteins are enzymes involved in the processing and maturation of small RNA molecules involved in the RNA interference (RNAi) system that is implicated in eukaryotic gene regulation[87]. Promising Argonaute proteins are derived from *Rhodobacter sphaeroides* and *Thermus thermophiles* and are implicated in bacteria host defense[88]. These Argonautes have been shown to utilize DNA guides to induce RNA and DNA target cleavage[89]. Additionally, the Argonaute from the archaeon *Pyrococcus furiosus* (PfAgo) has been shown to use small 5' phosphorylated DNA guides to cleave both single and double stranded DNA at supraphysiological temperatures[90].

The search for DNA-guided Argonaute proteins that work at more physiological temperatures is ongoing. Though all claims for DNA editing activity has been retracted[91,92,93], the false-start from work with the *Natronobacterium gregoryi* Argonaute (NgAgo) suggests that NgAgo can be a potentially useful tool in zebrafish gene knockdown[94]. NgAgo and other Argonaute proteins therefore provide potential tools in the zebrafish engineering toolbox in addition to other gene knockdown technologies such as morpholinos, but lack the possibility of producing total gene knockouts at this time. As a result of the controversial past of NgAgo, researchers should proceed cautiously when investigating claims about potential editing capabilities and applications of this protein.

Oligo Mediated Repair

The previously mentioned gene editing tools can serve to produce gene knock outs or to knock in exogenous genomic sequences into the zebrafish genome. In addition to inserting novel genomic material into the zebrafish genome for analysis, many disease allele variants do not result in completely eliminated gene function, removing the possibility of using a simple knockout as the experimental paradigm[95]. Therefore it is important to be able to precisely program genetic information to express a foreign gene product or recapitulate a disease state. Designer nucleases introduce DSBs and subsequently activate repair and recombination pathways at the targeted zebrafish genomic locus[3,96]. Such repair pathways can be exploited using different approaches to produce a desired integration or mutant allele.

One such repair pathway is oligo mediated repair. Oligo mediated repair utilizes single stranded DNA (ssDNA) molecules with homology to the zebrafish genomic locus as a donor sequence to be used as a repair template following the induced DSB[97,98]. This approach replaces the host locus DNA with a delivered novel insert sequence. Oligo-mediated repair templates are easy to design and provide an efficient way to modify the host zebrafish locus. Using ssDNA that spans the region of a nuclease cut site, typically 1–10% of precise insertions can be detected via an oligo-mediated homology directed repair approach using TALENs and these integrations resulted in germline transmission[97,99,100]. Further, slightly larger segments such as loxP sites were also successfully inserted via ssDNA coinjection with TALENs to produce a potential conditional knockout allele that was also stably passed on to the germline[100]. However, donor templates are typically short (<100nt) focusing this approach typically on relatively smaller modifications.

One of the most promising newer technologies in oligo-mediated repair involves the utilization of asymmetric donor DNA in combination with SpCas9 to induce a DSB[101]. Dissociation of Cas9 from the substrate dsDNA locus is comparatively slow, resulting in Cas9 asymmetrically dissociating the 3' end of the cleaved DNA on the nontarget strand. By designing ssDNA similar in length to the cleaved DNA strand that is released first in the dissociation of the Cas9 complex, the rate of oligo-mediated repair in human cells when using SpCas9 can be increased by ~10x. With the further elucidation of Cas9 kinetics and ssDNA integration, this technology could be adapted to the zebrafish engineering sphere and produce high efficiency oligo-mediated repair by taking advantage of the asymmetric release of the dsDNA substrate.

Homologous Recombination

To achieve precise genome editing of larger DNA segments in zebrafish, investigators have deployed homologous recombination (HR). HR uses a double-stranded (dsDNA) donor with ~0.5–2kbp regions of homology on either side of the donor template to achieve precise replacement of up to several kb stretches of host sequence[102–104]. HR enables precise in-frame insertions of an entire protein coding sequence such as a reporter gene to track inheritance. Likewise, loxP sites can be introduced within the region of homology, but flanking the gene of interest, to produce conditional knockout zebrafish lines[102]. Using a designer nuclease with a dsDNA donor template has yielded up to 15% of injected zygotes with precisely edited genomes transmitted to progeny[102,103,45].

The general workflow to drive homologous recombination involves the injection of a designer nuclease and donor dsDNA flanked by ~1kb homology arms on either side of the inserted gene of interest. The 5' overhangs produced by TALENs as well as the blunt cuts induced by CRISPR–Cas9 both work in principle as substrates for homologous recombination. Donor dsDNA sequences are often designed such that integration destroys the nuclease recognition site and the integrated DNA is effectively protected from subsequent DNA cleavage once properly inserted. How injected DNA is recruited to the cleavage site in the zebrafish genome in not well understood. However, injection of circular donor DNA was shown to preferentially be used as a repair template relative to linear DNA to produce edited alleles using TALENs in zebrafish[102,103]. Utilizing a well-constructed designer nuclease in combination with a dsDNA donor plasmid are powerful tools to produce knock-in zebrafish that can be used to create stable transgenic lines for further investigation.

Though the above mentioned tools provide the opportunity for precise genetic knock-ins into the zebrafish genome there are several caveats to consider. The gene editing approaches in many published manuscripts seem to have a locus-specific effect on efficiency with variation between loci and different research groups. However, elucidation of other DNA repair pathways including MMEJ, single-stranded annealing (SSA), and alternative end joining (alt–EJ) pathways is beginning to indicate that there may be temporal and spatial preference for particular pathways. In fact, it has recently been shown that alt-EJ is favored in early development and this preference may be exploited to generate a desired mutant allele[105].

Further understanding and exploitation of these and other pathways may begin to yield precise knock-ins with more consistent results between trials and investigators.

Base Editing

Many genome editing technologies rely on exploiting various endogenous DNA repair pathways to facilitate the integration of exogenous DNA or produce a gene knockout by a frameshift mutation. An alternative editing system would function such to directly edit genomic DNA without relying on exogenous donor DNA. From this notion arose the base editor system (BE)[83]. The first generation base editor system (BE1) employed a catalytically inert Cas9 (dCas9) fused to a cytidine deaminase (APOBEC1) to induce C:G base pairs to be changed to T:A base pairs, as well as an XTEN linker region to facilitate structural mobility of the BE1 complex. To subvert possible mismatch repair (MMR) at the site of base editing and therefore restore the original C:G base pair, a uracil glycosylase inhibitor (UGI) was fused to the C terminus of BE1 creating the second generation base editor (BE2), which showed a 3-fold increase *in vitro* editing relative to BE1. Finally, *Komor et al.* hypothesized that nicking the DNA strand with the unedited G would stimulate newly synthesized DNA and induce MMR to preferentially resolve the U:G base pair into the desired U:A and subsequent T:A products. To facilitate this approach, the catalytic His residue at position 840 in the HNH domain of the dCas9 BE2[51] was restored to induce DNA nicking activity. This therefore produced the third generation base editor system (BE3) consisting of APOBEC-XTEN-dCas9(A840H)-UGI, which garners an efficient deamination window of ~5nt with preference to act from positions 4 to 8 within the protospacer, counting the end distal to the PAM as position 1 (Fig 3).

A BE3:sgRNA complex has been preliminarily tested in zebrafish with promising results[106,107]. BE3:sgRNA targeting the *tyrosinase* loci in the zebrafish genome was injected and assessed for base editing 4 days post injection via high throughput sequencing (HTS). BE3 showed minimum indel induction and modest mutations *in vivo* with as high as 7.7% efficiency of edited zebrafish alleles transmitted to the next generation. The base editing system therefore postulates a possible “DNA-free” editing system that does not rely on a donor DNA template, but can instead directly edit DNA *in vivo.*

Conclusion

Zebrafish are proving to be a powerful genetic and disease model due to their advantages such as conservation in biological function, high fecundity, rapid embryonic development, and external fertilization that facilitate embryo injection and analysis. The qualitative and quantitative data that can be obtained from the genotype and phenotype of zebrafish allow nearly unmatched high throughput screening relative to other vertebrate genetic models. These benefits also make zebrafish an outstanding system to investigate novel gene editing technologies.

Few current limits are found in the current genome engineering toolbox, but opportunities still remain. The development of a highly active DNA-guided gene editor could be optimized to leverage massively parallel DNA synthesis of oligonucleotides would be well-suited to the high throughput nature of the zebrafish model. Furthermore, perhaps the

genome engineer can consider a next generation genome editing tool that involves the direct editing of genomic DNA without having to employ exogenous DNA at all such as an optimized base editor system.

The zebrafish genome is net AT rich with open reading frames (ORFs) being largely GC rich[108]. Therefore, optimization of tools such as Cas12a that can take advantage of its unique PAM requirement or the structure guided nuclease that lacks a sequence requirement entirely will potentially facilitate improved targeting into introns and allow for insertion into these regions. Though CRISPR–Cas9 has brought the field of genome engineering into the mainstream view in recent years, novel genome editing technologies are improving at an unforeseen rate. With the assistance of the powerful zebrafish model, the genome engineer's toolbox continues to grow with increasingly specific and diverse activities for most any application the investigator may choose.

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Highlights

- The zebrafish is a powerful system for developing new genome engineering tools
- Designer nucleases enable precise editing of genomic DNA
- Gene editing tools from diverse origins from bacteria to artificially constructed tools provide unique advantages and limitations
- Exploiting endogenous DNA repair mechanisms can produce favored outcomes depending on gene editing tool application
- DNA can now be directly edited without the use of an exogenous repair template

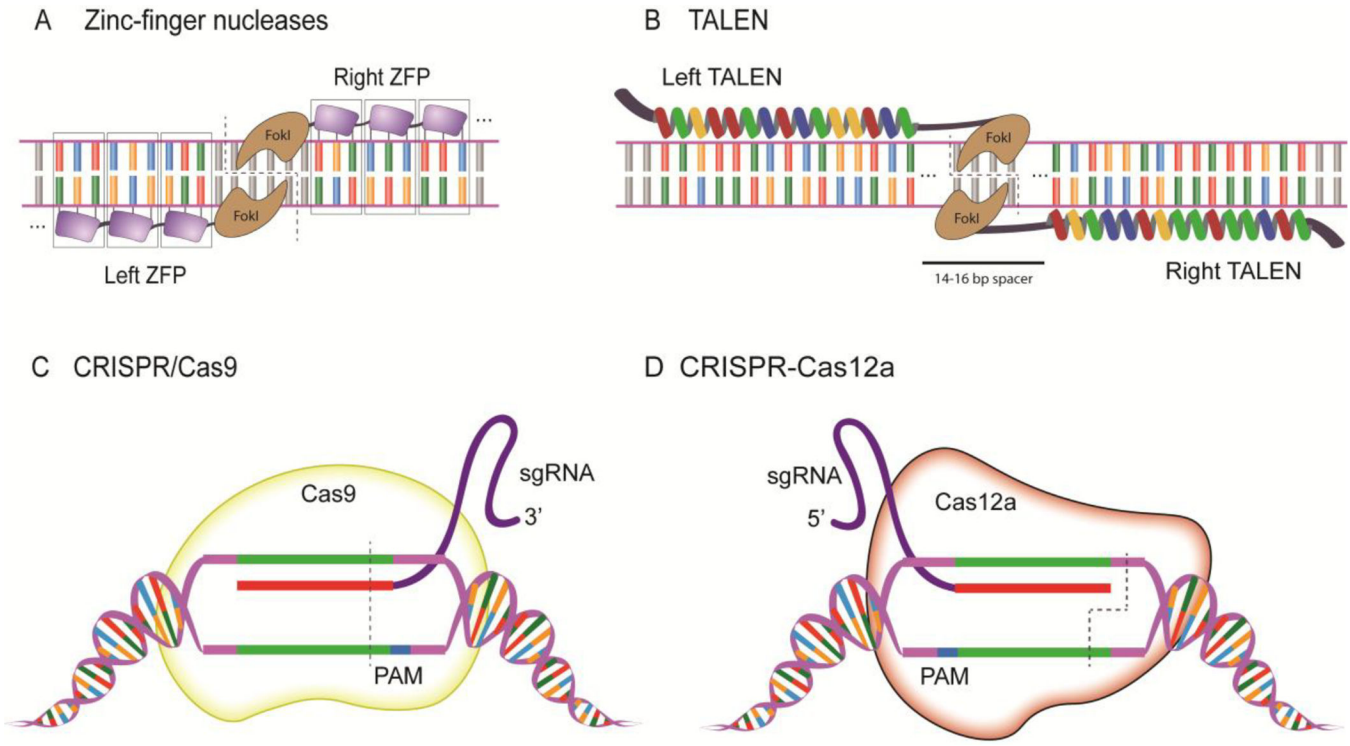

Fig 1. Established guided designer endonucleases used in zebrafish

(A) Zinc-finger nucleases recognize DNA by the fusion of 3 zinc finger recognition domains on either side of the DNA, specifically binding an 18nt region in this example, and the fused FokI dimerizes and catalyzes cleavage of the DNA. **(B)** An illustrative 15 repeat TALEN binds on either strand of the DNA separated by a spacer region, and the DSB occurs around the halfway point in the spacer region where the FokI domains dimerize.**(C)** SpCas9 recognizes the target sequence with the assistance of the sgRNA next to the 3' PAM sequence and induces a double stranded break 3 base pairs 5' from the PAM. **(D)** Cas12a recognizes the target sequence with the assistance of the sgRNA next to the 5' TTTN PAM sequence and cleaves the DNA at the 18th base on the non-targeted strand and after the 23rd base on the targeted strand to create sticky ends at the DSB. (see text)

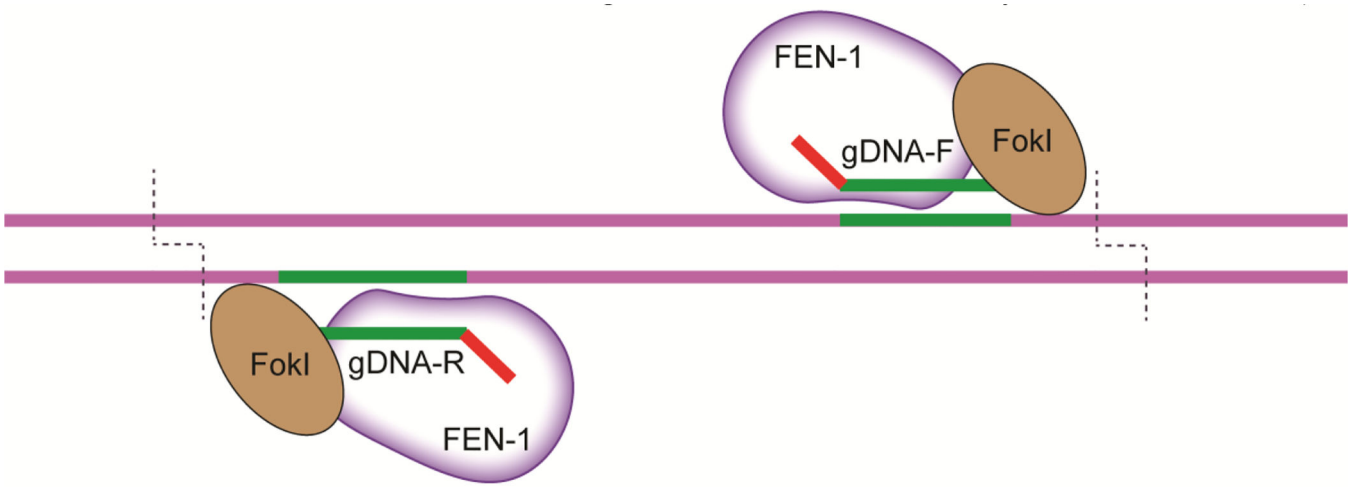

Fig 2. The structure-guided nuclease

Using a ssDNA guide of at least 20nt, the structure guided nuclease (SGN) dimer cleaves 9–10nt away from the 3' end of the gDNA on both strands of DNA and creates a large deletion of the intervening genomic locus[1086].

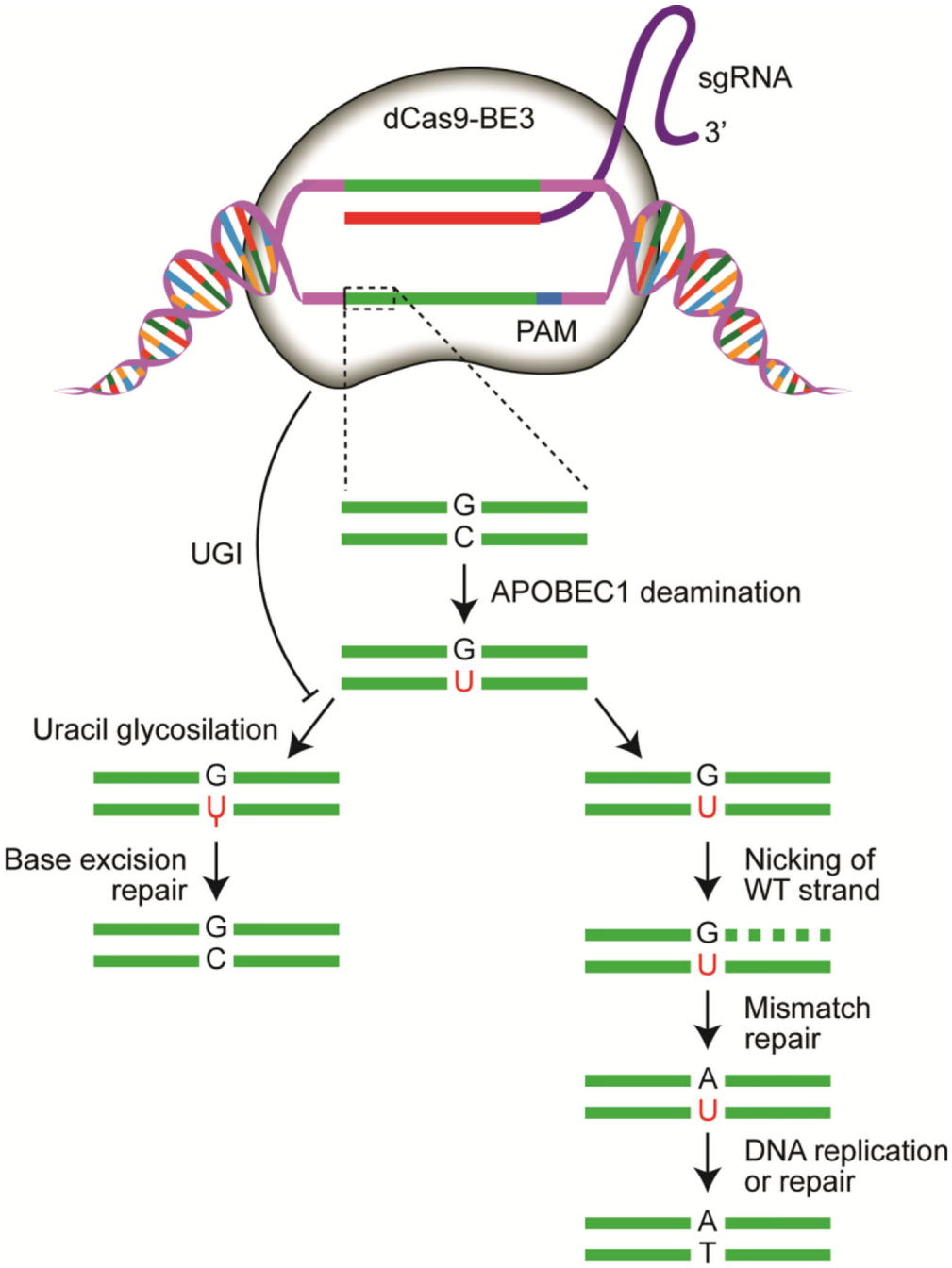

Fig 3. The base editor system for non-nuclease editing of the zebrafish genome

The third generation base editor (BE3) catalyzes G:C to an A:T deamination with the fused APOBEC1 nucleobase deaminase. Base excision repair is prevented by the fused uracil glycosylase inhibitor (UGI). The formation of the AT transition is further biased by introducing a nick on the non-edited strand to drive mismatch repair and subsequent resolution to the A:T basepair[106109].