Efficient In Vivo Genome Editing Using RNA-Guided Nucleases

Published in final edited form as:
Nat Biotechnol. 2013 March ; 31(3): 227–229. doi:10.1038/nbt.2501.

Efficient *In Vivo* Genome Editing Using RNA-Guided Nucleases

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems have evolved in bacteria and archaea as a defense mechanism to silence foreign nucleic acids of viruses and plasmids. Recent work has shown that bacterial type II CRISPR systems can be adapted to create guide RNAs (**gRNAs**) capable of directing site-specific DNA cleavage by the Cas9 nuclease *in vitro*. Here we show that this system can function *in vivo* to induce targeted genetic modifications in zebrafish embryos with efficiencies comparable to those obtained using ZFNs and TALENs for the same genes. RNA-guided nucleases robustly enabled genome editing at 9 of 11 different sites tested, including two for which TALENs previously failed to induce alterations. These results demonstrate that programmable CRISPR/Cas systems provide a simple, rapid, and highly scalable method for altering genes *in vivo*, opening the door to using RNA-guided nucleases for genome editing in a wide range of organisms.

Bacteria and archaea have evolved an elegant adaptive defense mechanism which uses clustered regularly interspaced short palindromic repeats (CRISPR), together with CRISPR-associated (Cas) proteins, to provide acquired resistance to invading viruses and plasmids[1–3]. The type II CRISPR/Cas system relies on uptake of foreign DNA fragments into CRISPR loci[4] and subsequent transcription and processing of these CRISPR repeat-spacer arrays into short CRISPR RNAs (**crRNAs**)[5], which in turn anneal to a trans-activating crRNA (**tracrRNA**) and direct sequence-specific silencing of foreign nucleic acid by Cas proteins[5–7] (Figure 1A). Recent *in vitro* studies have shown that a single synthetic guide RNA (**gRNA**), consisting of a fusion of crRNA and tracrRNA, can direct Cas9-mediated cleavage of target DNA[6] (Figure 1B). However, an important question that currently remains unanswered is whether CRISPR/Cas-based systems can have broad utility

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Conflict of Interest Statement

J.K.J. has a financial interest in Transposagen Biopharmaceuticals. J.K.J.'s interests were reviewed and are managed by Massachusetts General Hospital and Partners HealthCare in accordance with their conflict of interest policies.

for performing genome editing in a wide variety of whole organisms as has been shown with other technologies such as zinc finger nucleases (**ZFN**s)[8] or transcription activator-like effector nucleases (**TALEN**s)[9]. This capability of ZFNs and TALENs to mediate targeted *in vivo* modification of genomes has enabled both genetic studies and the development of disease models in a broad range of organisms that were previously difficult to alter. Here we explore the abilities of customizable gRNAs and Cas9 nuclease to efficiently modify endogenous genes *in vivo* in zebrafish embryos and show that this system provides a rapid and robust alternative to ZFNs and TALENs for performing genome editing in whole organisms.

To establish whether gRNAs can direct Cas9 nuclease-mediated alteration of endogenous genes *in vivo* in zebrafish, we constructed expression vectors that enable T7 RNA polymerase-mediated production of a capped, poly-adenylated mRNA encoding the monomeric Cas9 nuclease and of a customizable gRNA bearing 20 nucleotides (**nts**) of sequence complementary to a target site (Figure 1C). The sequence of our gRNA differs from another used previously *in vitro*[6] in that it contains additional tracrRNA-derived sequences at its 3' end (compare Figure 1B and 1C; also see Supplementary Table 1). For initial experiments, we designed and constructed a gRNA that harbors a targeting region complementary to a sequence in the *fh* gene (site #1) (Supplementary Table 2 and **Methods**).

To optimize the quantity of each RNA species to use for genome editing, we microinjected varying amounts of *fh*-targeted gRNA and Cas9-encoding mRNA into one-cell stage zebrafish embryos and then assessed the frequency of altered alleles in single embryos using a T7 Endonuclease I (**T7EI**) assay (**Methods**). We observed robust induction of targeted insertion/deletion mutations (**indels**) at all concentrations of RNAs tested (mean frequencies ranging from 10.0 to 52.7%) and in nearly all individual embryos tested (Supplementary Table 3). We note that the highest mean frequency of mutations was obtained when injecting a solution containing 12.5 ng/μl RNA and 300 ng/μl Cas9-encoding mRNA (Supplementary Table 3) and we therefore used these concentrations for all subsequent experiments. Sequencing of mutated *fh* alleles revealed indels that begin within or encompass the 5' end of the DNA sequence complementary to the gRNA (Supplementary Figure 1). This pattern of mutations is consistent with the expected induction of a Cas9-induced double-stranded break (**DSB**) at this position[6] within the genomic *fh* target site followed by error-prone NHEJ-mediated repair.

To test the robustness of the gRNA/Cas9 system in zebrafish, we constructed ten additional gRNAs targeting another sequence in the *fh* gene (site #2) and sites in nine additional endogenous genes (Supplementary Table 2). Strikingly, we found that for eight of the ten sites we targeted, co-injection of gRNA with Cas9-encoding mRNA induced high frequencies of targeted indels at these sites in all individual embryos tested (Table 1). Mean frequencies of mutagenesis for these eight successfully targeted sites ranged from 24.1% to 59.4% as judged by T7EI assay (Table 1) and did not appear to depend upon which DNA strand (sense or anti-sense) was targeted by the gRNA. Of note, we obtained high efficiencies of mutagenesis at two sites in the *gsk3b* and *drd3* genes which we had been unable previously to alter using TALENs (Supplementary Table 4). For the remaining six successful targets, the mutation rates we observed were comparable to those we previously observed at targets in these same genes using ZFNs and/or TALENs (Table 1 and Supplementary Table 4). DNA sequencing of mutated alleles for all eight of these target sites confirmed the efficient introduction of targeted indels at the expected genomic locations (Figure 2 and Supplementary Figure 2). The lengths of indel mutations induced by RNA-guided Cas9 are similar to those of mutations induced by ZFNs and TALENs previously made by our groups (Supplementary Figure 3a). Furthermore, the nature of the

mutations (i.e.—the relative abundance of insertions and deletions) also appears to be similar among all three platforms (Supplementary Figure 3b). Our results strongly suggest that the gRNA/Cas9 platform has a high success rate in zebrafish with a total of 9/11 (or >80%) of the sites we targeted showing robust alterations.

RNA-guided nucleases provide an important complementary technology to TALENs and ZFNs for genome editing in whole organisms. Only one customized gRNA is required to target a specific sequence in contrast to the need to design and assemble two TALENs or ZFNs for each site. gRNAs are encoded on short ~100 bp sequences and are therefore much simpler and easier to construct than TALENs or ZFNs. The short length of gRNA sequences also avoids undesirable complications associated with longer (typically 3 kb or more) and highly repetitive TALEN-encoding vectors (e.g. —delivery using viral vectors, challenges with DNA sequencing, potential for recombination). Furthermore, we successfully used our gRNA/Cas9 reagents to efficiently mutagenize sites in endogenous zebrafish genes that we were previously unable to alter previously using TALENs. It will be of interest to determine going forward why these TALENs fail to mutagenize their targets with high efficiency and also how gRNA-guided Cas9 nucleases are able to successfully alter such sites.

In its current implementation, our gRNA/Cas9 system described above can in principle target any sequence of the form 5'-GG-N_{18}-NGG-3'. Such sites occur once in every 128 bps of random DNA sequence. Constraints on the range of targetable sequences are due to sequence requirements imposed by the T7 promoter used to make gRNAs (GG at the 5' end of the transcript) and by the requirement for a PAM sequence (NGG) in genomic DNA just 3' to the target site[6]. Previous studies suggest that the T7 promoter requirement for a pair of guanines at the 5' end of the transcript could be relaxed to allow for an adenine at either position[10]. Loosening this constraint would enable targeting of sequences of the form 5'-(G/A)(G/A)-N_{18}-NGG-3', which occur once in every 32 bps of random DNA sequence. To simplify the identification of targetable sites, we have updated our web-based ZiFiT Targeter program[11, 12] with this new functionality (http://zifit.partners.org/ZiFiT_Cas9). Future studies should be directed at performing larger-scale tests of the targeting range of the gRNA/Cas9 system, as has been done recently with TALENs in human cells[13], and at understanding why some gRNAs fail to mediate efficient sequence alterations (e.g.—the two failed gRNAs among the 11 we tested) and whether such failures can be predicted in advance.

It is important to emphasize that the modified CRISPR/Cas platform described here can be rapidly adopted by any researcher seeking to modify the genome of any organism into which RNA can be introduced. Our plasmids expressing short ~100 nt gRNAs with customized targeting regions can be easily and rapidly assembled simply by ligating pairs of short annealed oligonucleotides into our T7 promoter-based gRNA vector (**Methods**). This process is considerably simpler than other publicly available methods for assembling TALEN- or ZFN-encoding plasmids[9] and therefore should be readily amenable to automation and high-throughput use. The cost of making gRNA expression plasmids will be relatively lower than even that of making TALENs since oligonucleotides can be commercially ordered in large-scale at low cost and only a simple ligation reaction is required. In addition to facilitating target site identification, the updated ZiFiT Targeter program also provides the sequences of oligonucleotides required to construct customized gRNAs. All plasmids described in this report will be available through the non-profit reagent distribution service Addgene (http://www.addgene.org/crispr-cas).

Previous studies from our groups and others have shown efficient germline transmission occurs for all engineered ZFNs and TALENs that exhibit somatic mutation rates of 2% or greater in the embryos that develop normally after microinjection[14–17]. We note that all of

the active gRNA/Cas9 nuclease combinations described in this report exhibit somatic mutation rates well above 10% and that these alterations were detected in normally developing embryos. Therefore, we expect that germline transmission of Cas9-induced mutations will be as efficient as those induced by ZFNs or TALENs.

Another important question to address in future studies will be the genome-wide specificity of RNA-guided Cas9 nucleases. A previous *in vitro* study has suggested that the 3' end of the gRNA target recognition sequence may be the most critical for specificity[6] but whether this will also be true in cells or *in vivo* remains to be determined. We note that the toxicity induced by gRNA/Cas9-encoding mRNA in zebrafish (as judged by the numbers of deformed and dead embryos; Supplementary Figure 4) appeared to be variable among the different gRNAs tested with no direct correlation to their abilities to induce indels at the intended target sites, a phenomenon we have also observed with ZFNs and TALENs in previous studies[14, 16, 18, 19]. The frequencies of deformed or dead embryos are comparable to what we have observed in previous experiments using ZFNs[14, 19] and TALENs[16, 18].

Our results provide the largest set of endogenous genes modified by RNA-guided Cas9 nucleases to date and demonstrate the robustness of this platform *in vivo* for facile and efficient genetic modification of zebrafish. In addition, the small size of gRNAs and the need for only a single monomeric Cas9 nuclease (rather than pairs of dimeric ZFNs or TALENs) are characteristics that make this system potentially ideal for performing multiplex genome editing. The demonstration that customized RNA-guided nucleases can be used to efficiently induce site-specific modifications *in vivo* in zebrafish will encourage wider use of this robust and easy-to-use technology in a broad range of other whole organisms.

Methods

Cas9 nuclease expression plasmid

DNA encoding the Cas9 nuclease was amplified from the pMJ806 vector (Addgene Plasmid #39312) by PCR using the following primers, which add a T7 promoter site 5' to the translational start codon and a nuclear localization signal at the carboxy-terminal end of the Cas9 coding sequence: OMM704: 5'-ataagaatgcggccgctaatacgactcactatagggagagccgccaccATGGATAAGAAATACTCAATAGGCTTAG -3' OMM705: 5'-gtacataccggtcatcctgcagctccaccgctcgagactttcctcttcttcttgggagaaccGTCACCTCCTAGCTGAC -3' The resulting PCR product was digested with the NotI and AgeI restriction enzymes and inserted into plasmid pMLM651. The resulting vector has a unique PmeI restriction site positioned 3' to the end of the Cas9 coding sequence that can be used to linearize the plasmids prior to run-off *in vitro* transcription.

gRNA expression vector

Vector pDR274 harboring a T7 promoter positioned upstream of a partial gRNA sequence (full DNA sequence provided in Supplementary Figure 5) was designed and constructed by commercial DNA synthesis (Integrated DNA Technologies). To construct plasmids encoding gRNAs bearing customized 20 nt targeting sequences, we digested pDR274 with BsaI restriction enzyme and then cloned a pair of appropriately designed and annealed oligonucleotides into this vector backbone. The annealed oligonucleotides have overhangs that are compatible with directional cloning into the BsaI-digested pDR274 vector. The sequences of the annealed oligonucleotides are listed in Supplementary Table 2.

Web-based ZiFiT Targeter Software

The ZiFiT Targeter website (http://zifit.partners.org/ZiFiT_Cas9) was updated to include an option to identify potential target sites for our RNA-guided Cas9 system. Users can query up to 96 sequences at once and indicate the specific nucleotide that they are interested in altering. ZiFiT Targeter will analyze these query sequences and return sites that either flank the nucleotide of interest, or, are as close to it as possible. If no nucleotide of interest is indicated, the program will identify target sites closest to the center of the query sequence. By default, ZiFiT Targeter will identify sites that meet the following criteria: 5'-GG-$(N)_{18}$-NGG-3'. The 5' GG dinucleotide is part of the T7 promoter and users can remove this constraint if they wish. ZiFiT Targeter also returns a downloadable list of the sequences of oligonucleotides that need to be synthesized and cloned into the pDR274 vector to create a gRNA expression vector for each target site of interest.

Zebrafish care

All zebrafish care and uses were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Production of gRNA and Cas9 mRNA

gRNAs were transcribed using the DraI-digested gRNA expression vectors as templates and the MAXIscript T7 kit (Life Technologies). The Cas9 mRNA was transcribed using PmeI-digested Cas9 expression vector and the mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). Following completion of transcription, the poly (A) tailing reaction and DNase I treatment were performed according to the manufacturer's instructions. Both the gRNA and the Cas9-encoding mRNA were then purified by LiCl precipitation and re-dissolved in RNase-free water.

Microinjection of zebrafish embryos and evaluation of nuclease-associated toxicity

gRNA and Cas9-encoding mRNA were co-injected into one-cell stage zebrafish embryos. Unless otherwise indicated, each embryo was injected with 2 nl of solution containing ~12.5ng/μl of gRNA and ~300ng/μl of Cas9 mRNA. On the next day, injected embryos were inspected under stereoscope and were classified as dead, deformed or normal phenotypes. Only embryos that developed normally were assayed for target site mutations using T7 Endonuclease I assay or DNA sequencing (see below). Genomic DNA was extracted from either single embryos or a pool of ten embryos as previously described[21].

T7 Endonuclease I (T7EI) mutation detection assays

Targeted genomic loci were amplified from genomic zebrafish DNA using primers designed to anneal approximately 150 to 200 base pairs upstream and downstream from the expected cut site and Phusion Hot Start II high-fidelity DNA polymerase (New England Biolabs) according to the manufacturer's instructions. A list of the primers used in this study is provided in Supplementary Table 5. PCR products were purified with Ampure XP (Agencourt) according to the manufacturer's instructions. T7 Endonuclease I assays were performed and estimated NHEJ frequencies were calculated as previously described[13].

DNA Sequencing of Mutated Endogenous Gene Target Sites

Each target locus was amplified by PCR from the genomic DNA of ten injected embryos. The resulting PCR products were cloned into a plasmid using the pGEM-T kit (Promega) or Zero Blunt TOPO PCR cloning kit (Life Technologies). Following transformation of these reactions, plasmid DNAs isolated from overnight cultures of single colonies were sequenced (Massachusetts General Hospital DNA Sequencing Core). Mutated alleles were identified by comparison to the wild-type unmodified sequence. Single base substitutions, deletions, or

insertions were not designated as mutant alleles because we could not exclude the possibility that these alterations might also be generated by the PCR amplification process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a National Institutes of Health (NIH) Director's Pioneer Award DP1 GM105378 (J.K.J.), NIH R01 GM088040 (J.K.J. & R.T.P.), NIH P50 HG005550 (J.K.J.), NIH K01 AG031300 (J.-R.J.Y.), and a Massachusetts General Hospital Claflin award (J.-R.J.Y.). We thank George Church, John Aach, and Prashant Mali for sharing unpublished results and helpful discussions.

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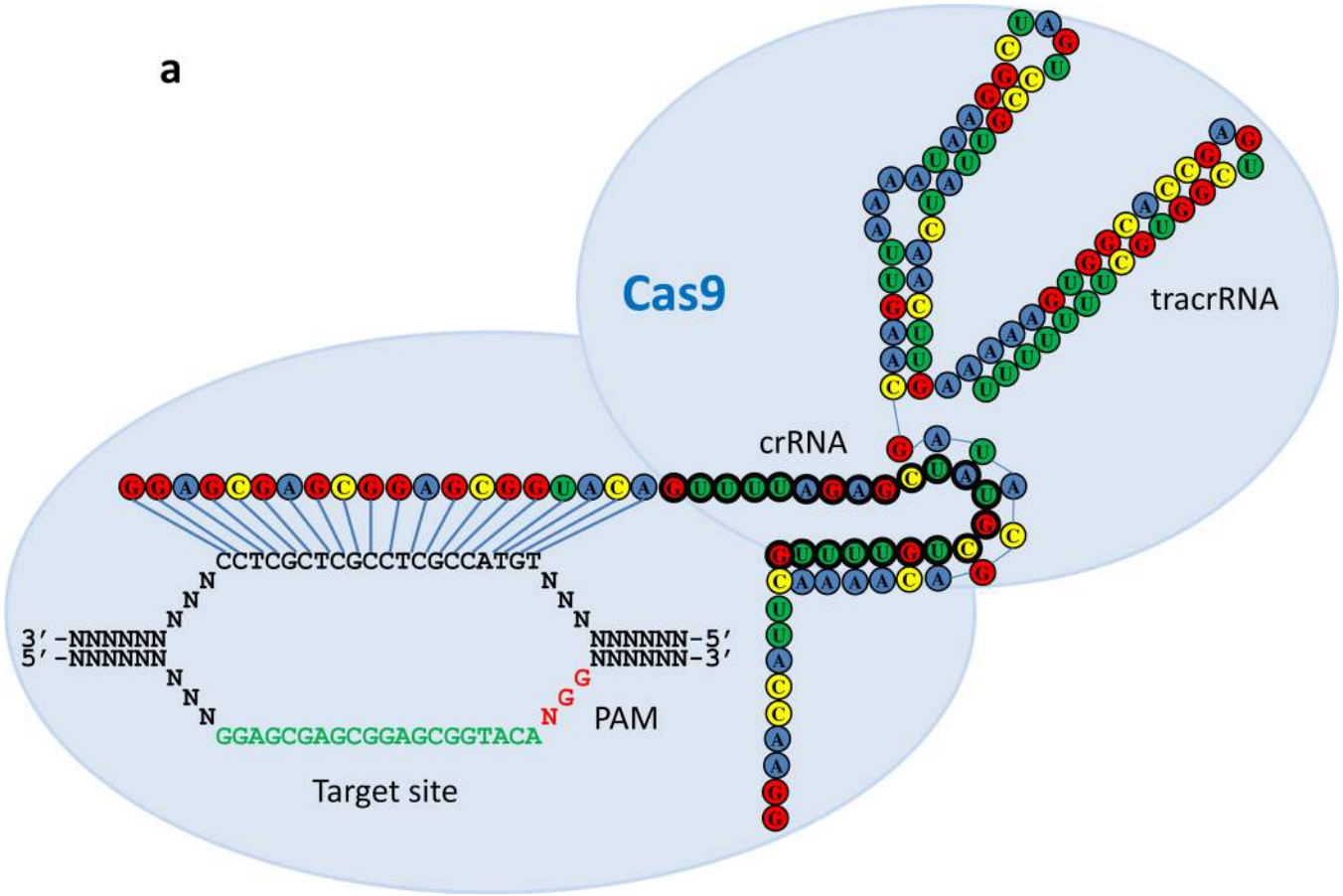

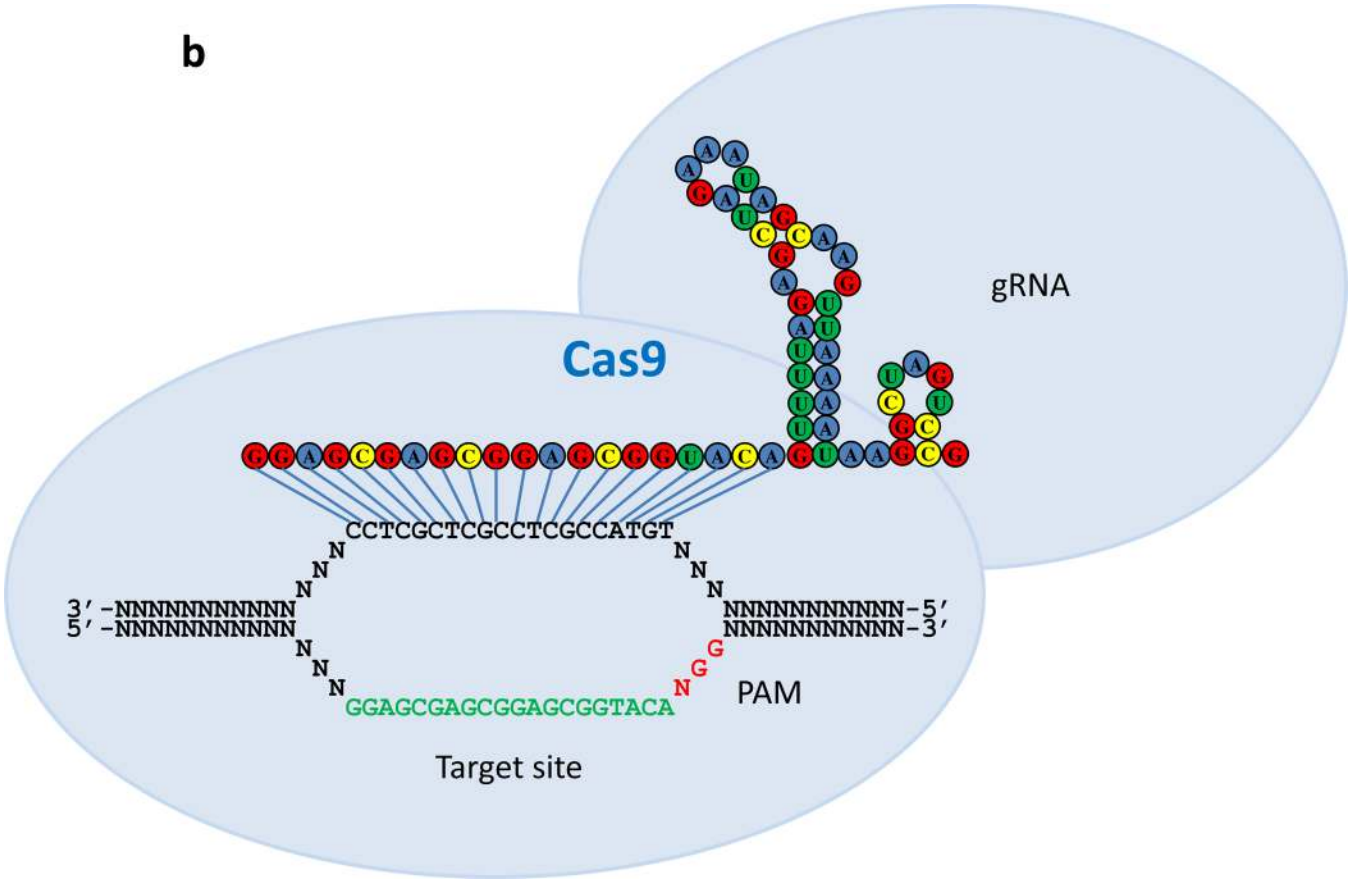

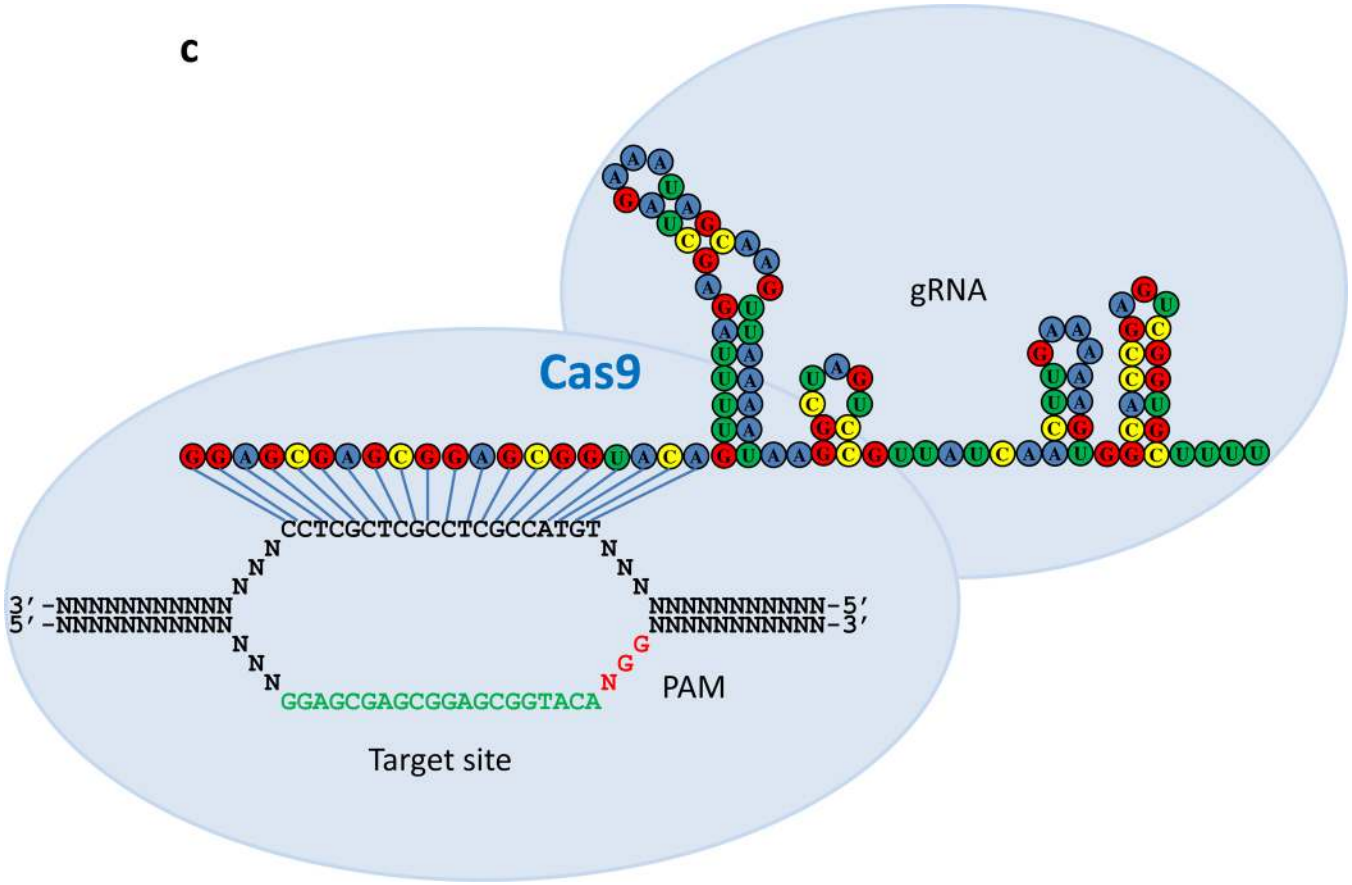

Figure 1.

Schematic illustrating naturally occurring and engineered RNA-guided nuclease systems. (**A**) Naturally occurring dual RNA-guided Cas9 nuclease. crRNA interacts with the complementary strand of the DNA target site harboring an adjacent PAM sequence (green and red text, respectively), tracrRNA base pairs with the crRNA, and the overall complex is recognized and cleaved by Cas9 nuclease (light blue shape). Folding of the crRNA and tracrRNA molecules depicted as predicted by Mfold[20] and the association of the crRNA to the tracrRNA depicted is partially based on the model previously proposed by Jinek et al[6]. (**B**) Engineered gRNA/Cas9 system previously used *in vitro*. gRNA composed of portions of the crRNA and tracrRNA from (A) is illustrated interacting with the DNA target site. Folding of gRNA is as predicted by Mfold[20]. (**C**) Modified engineered gRNA/Cas9 system used *in vivo* in this study. Components are illustrated the same way as in (B) except the gRNA contains additional sequence from the 3' end of the tracrRNA. Folding of gRNA is as predicted by Mfold[20].

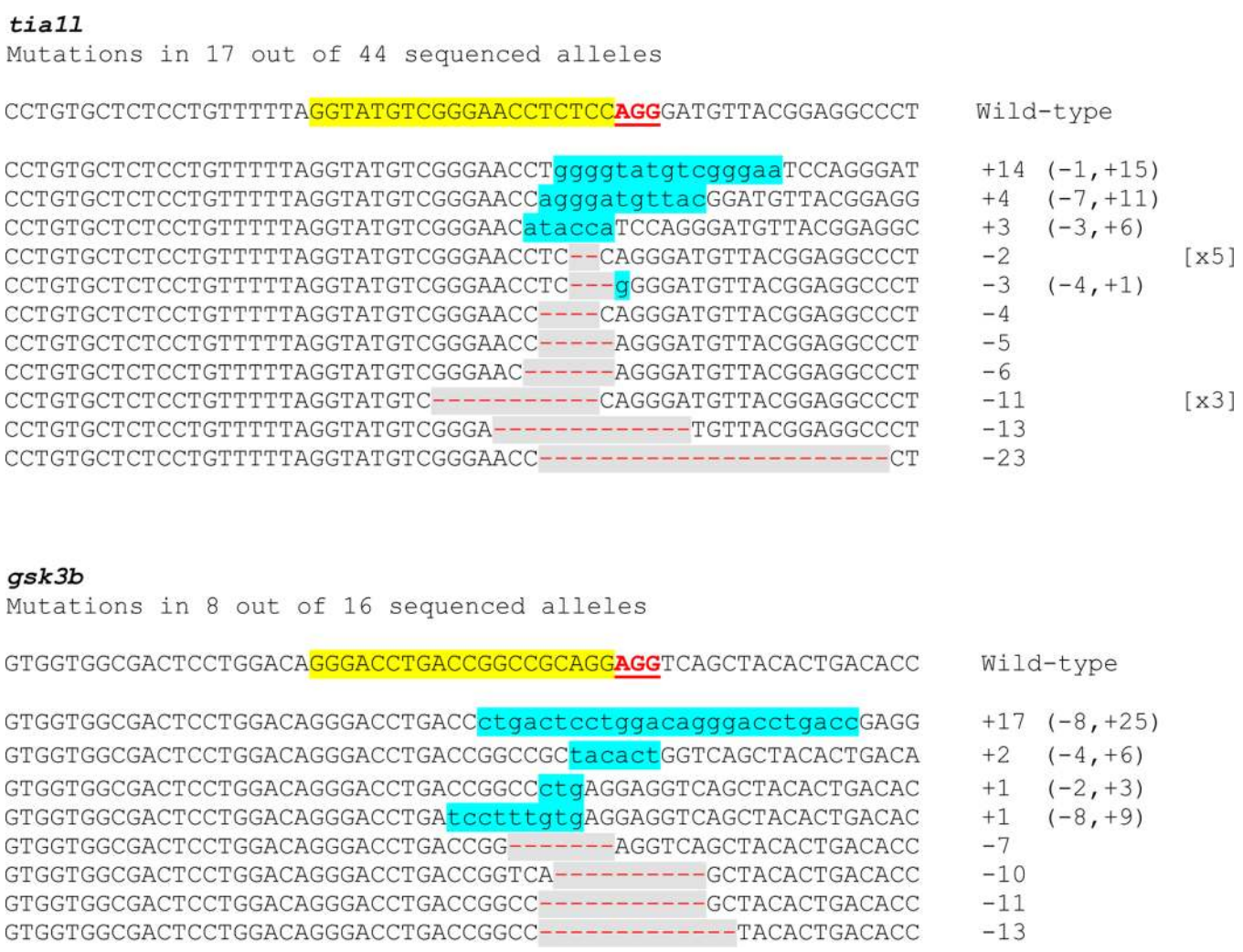

Figure 2.

Targeted indel mutations induced by engineered gRNA/Cas9 at the *tia1l* and *gsk3b* genes. For each gene, the wild-type sequence is shown at the top with the target sites highlighted in yellow and the PAM sequence highlighted as red underlined text. Deletions are shown as red dashes highlighted in grey and insertions as lower case letters highlighted in blue. The net change in length caused by each indel mutation is to the right of each sequence (+, insertion; −, deletion). Note that some alterations have both insertions and deletions of sequence and in these instances the alterations are enumerated in the parentheses. The number of times each mutant allele was isolated is shown in brackets.

Table 1

Mutation frequencies induced by customized gRNA/Cas9 nucleases at 10 endogenous gene target sites in the zebrafish genome

Mutagenesis frequencies in the *fh* gene (site #1) induced by various concentration of gRNA and Cas9 mRNA. For each set of RNA concentrations used, up to five individual embryos were assessed for indel mutation frequency using the T7EI assay (Online Methods). Mean frequencies for each set of concentrations are also shown with standard errors of the mean.

Gene	Indel Mutation Frequency										
	Embryo # 1	Embryo # 2	Embryo # 3	Embryo # 4	Embryo # 5	Embryo # 6	Embryo # 7	Embryo # 8	Embryo # 9	Embryo # 10	Mean ± SEM
***fh* (site #2)**	62.05%	52.16%	50.82%	60.18%	64.73%	66.13%	61.18%	55.45%	64.96%	55.95%	59.4 ± 1.7 %
apoea	6.28%	15.91%	2.70%	3.72%	40.78%	55.62%	6.56%	59.61%	11.67%	37.87%	24.1 ± 7.0 %
gria3a	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
th1	15.08%	29.26%	51.11%	41.21%	46.37%	45.85%	53.43%	19.99%	15.92%	37.53%	35.6 ± 4.6 %
rgs4	53.45%	42.14%	50.85%	44.44%	48.67%	26.43%	27.35%	15.12%	19.13%	30.13%	35.8 ± 4.4 %
tia1l	59.64%	59.88%	63.12%	57.33%	67.91%	58.02%	61.54%	56.39%	14.36%	72.18%	57.0 ± 5.0 %
tph1a	7.32%	37.81%	28.59%	49.83%	40.92%	41.24%	37.49%	44.56%	41.84%	30.75%	36.0 ± 3.7 %
slc6a3.2	0.00%	N/A	N/A	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
gsk3b	39.02%	4.94%	55.62%	4.31%	44.09%	19.12%	24.31%	3.07%	43.11%	33.31%	27.1 ± 6.0 %
drd3	32.46%	20.34%	13.80%	34.20%	44.24%	33.13%	20.14%	23.17%	30.75%	31.35%	28.4 ± 2.8 %