Supplementary Materials

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Supplementary Discussion

Calculated frequencies of cells bearing bi-allelic alterations in mutagenized embryos

We observed mean mutagenesis frequencies of endogenous zebrafish gene loci in the pooled genomic DNA of ten injected embryos that ranged from ~24 to 60% as judged by the T7EI assay (**Table 1** and **Supplementary Table 3**). Assuming that the frequency of mutations is independent, the percentage of cells bearing bi-allelic alterations in an embryo would be expected to be approximately the square of the observed mutagenesis frequency. For example, if the allele mutagenesis frequency was 50% then the expectation is that approximately 25% of cells in that population would bear bi-allelic alterations (0.5*0.5 = 0.25). Using this equation, we calculate that ~6 to 36% of cells should bear bi-allelic mutations for the various loci we successfully targeted. However, the actual percentages of cells with bi-allelic alterations may be even higher because: (1) the T7EI assay can underestimate frequencies of mutations on the high end of the range and (2) our experience using engineered nucleases in other cell types suggests that the probabilities of mutagenic events are not always completely independent.

***fh* (site #1)**

Mutations in 47 out of 88 sequenced alleles

```
GAGAGCAGTAGTTCCGCCCCGGTCGCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    Wild-type

GAGAGCAGTAGTTCCGCCCCCGGagatgtaccgctccgctcgccatgtacCGCCATGTAC    +26 (-1,+27)
GAGAGCAGTAGTTCCGCCCCCGGaatgttccgtcTCGCCATGTACCGCTCCGCTCGCTCC    +11
GAGAGCAGTAGTTCCGCCCCCGGTaccgccccccggtacCGCCATGTACCGCTCCGCTCGC    +14
GAGAGCAGTAGTTCCGCCCCCGGTaccatgtacCGCCATGTACCGCTCCGCTCGCTCCCT    +9
GAGAGCAGTAGTTCCGCCCCCGGcagtagtTCGCCATGTACCGCTCCGCTCGCTCCCTGC    +7
GAGAGCAGTAGTTCCGCCCCCGGccatgtacCGCCATGTACCGCTCCGCTCGCTCCCTGC    +7  (-1,+8)
GAGAGCAGTAGTTCCACCCCagttgaaaaTCGCCATGTACCGCTCCGCTCGCTCCCTGCA    +6  (-3,+9)
GAGAGCAGTAGTTCCGCtctgacttgacCGCCATGTACCGCTCCGCTCGCTCCCTGCATC    +4  (-7,+11) [x2]
GAGAGCAGTAGTTCCGCCCCCatgtTCGCCATGTACCGCTCCGCTCGCTCCCTGCATCGC    +2  (-2+4)
GAGAGCAGTAGTTCCGCCCCCGGagTCGCCATGTACCGCTCCGCTCGCTCCCTGCATCGC    +2
GAGAGCAGTAGTTCCGCCCCCGcccCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -1  (-4,+3)
GAGAGCAGTAGTTCCGCCCC----CGCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -4
GAGAGCAGTAGTTCCGCCCCC---CGCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -3
GAGAGCAGTAGTTCCGCCCCCG---GCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -3
GAGAGCAGTAGTTCCGCCCC----CGCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -4          [x5]
GAGAGCAGTAGTTCCGCCC----TCGCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -4
GAGAGCAGTAGTTCCGCCCCCGG------aatgtacagCCGCTCGCTCCCTGCATCGCTT    -6  (-15,+9)
GAGAGCAGTAGTTCCGCCC------GCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -6
GAGAGCAGTAGTTCCG-------TCGCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -7
GAGAGCAGTAGTTCCGCCCC--------ATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -8          [x2]
GAGAGCAGTAGTTCCGCat---------ATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -9  (-11,+2)
GAGAGCAGTAGTTC----------TCGCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -9
GAGAGCAGTAGTTCCGCCCCCagcagtagtc----------CTCGCTCCCTGCATCGCTT    -10 (-20,+10)
GAGAGCAGTAGTTCC----------GCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -10         [x7]
GAGAGCAGTAGTTCCGCC----------ATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -10
GAGAGCAGTAGTTC-----------GCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -11
GAGAGCAGTAGTTCCGCCCCCGGgggaa-----------GCTCGCTCCCTGCATCGCTT    -12 (-17,+5)
GAGAGCAGTAGTTCCGCCCCCG--------------CTCCGCTCGCTCCCTGCATCGCTT    -14
GAGAGCAGTAGT----------------TGTACCGCTCCGCTCGCTCCCTGCATCGCTT    -17
GAGAGCAGTAGTTCCGCCCC-------------------GCTCGCTCCCTGCATCGCTT    -20
GAGAGCAGTAGTTCCGC-----------------------------TCCCTGCATCGCTT    -29
GAGAGCAGTAGTTCCGCCtccgcc-----------------------------------    -50 (-56,+6)
GAGAGCAGTAGTTCCGCCCCC--------------------------------------    -53
GAGAGCAGTAGTTC-----------------------------------------------    -66
GAGAGCAGTAGTTCCGCCCCCGG------------------------------------    -92
```

Supplementary Figure 1 Targeted indel mutations induced by engineered gRNA/Cas9 at the *fh* gene (site #1). Alleles shown were amplified from pooled genomic DNA isolated from ten embryos that had been injected with 36.2 ng/ul of gRNA and 100 ng/ul of Cas9 mRNA (embryos injected at these concentrations of RNA were used because these conditions yielded one of the highest mean mutation frequencies in the optimization experiments shown in **Supplementary Table 1**). The wild-type sequence is shown at the top with the reverse complement of the target site highlighted in green and the reverse complement of 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.

***fh* (site #2)**
Mutations in 20 out of 20 sequenced alleles

```
AGTTCCGCCCCCGGTCGCCATGTACCGCTCCGCTCGCTCCCTGCATCGCTTCAGCGCGAG    Wild-type

AGTTCCGCCCCCGGTCGCCATtccgcccccggtcgCCGCTCCGCTCGCTCCCTGCATCGC    +11 (-3,+14)
AGTTCCGCCCCCGGTCGCCA----CCGCTCCGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -4           [x4]
AGTTCCGCCCCCGGTCGC--------CGCTCCGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -7
AGTTCCGCCCCCGGTC--------------CGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -14
AGTTCCGCCCCCG--------------CTCCGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -14
AGTTCCGCCC------------------TCCGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -18
AGTTCCGCC-------------------CCCGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -19
AGTTCCGCCCCCGCTCGC--------------------TCCCTGCATCGCTTCAGCGCGAG    -19          [x3]
AGTTCCGC--------------------TCCGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -20
AGTTCCGCTC--------------------CGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -20          [x3]
AGT---------------------------CGCTCGCTCCCTGCATCGCTTCAGCGCGAG    -27
AGTTCCGCCCCCGGTCGCCA----------------------TGCATCGCTTCAGCGCGAG    -21
AGTTCCGCCCCCGGTCGCCATGTcgctc--------------------------------    -45 (-50,+5)
```

th1
Mutations in 20 out of 64 sequenced alleles

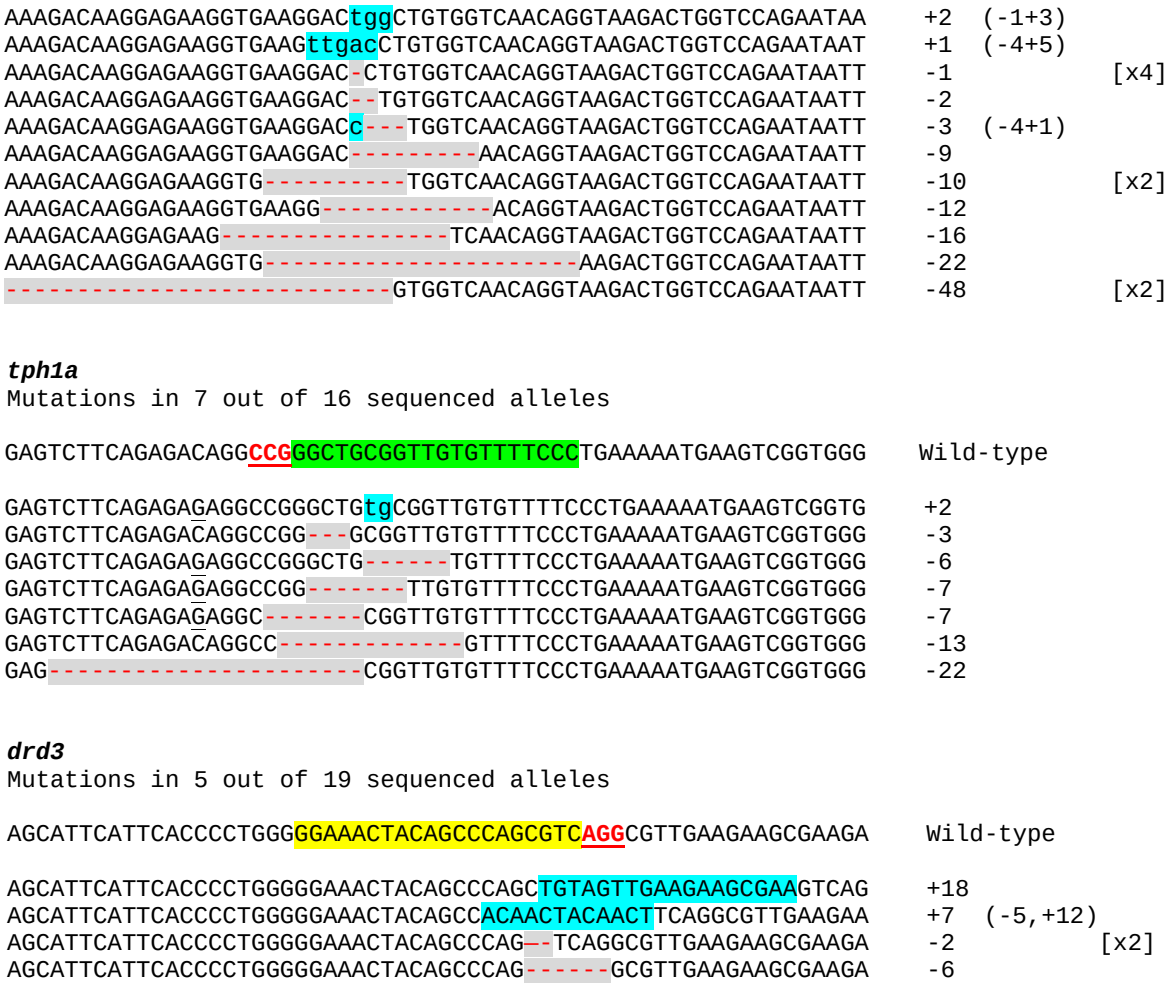
```
AGGCGGCAGAGTTTGATCGAGGATGCGCGTAAGGAGCGCGAGGCGGCGCCGCGGCGGCG    Wild-type

AGGCGGCAGAGTTTGATCGAGGATGCGCGTAAGGcggcgcgtaaGGCGGCGGCCGCGGCG    +3  (-7,+10)
AGGCGGCAGAGTTTGATCGAGGATGCGCGTAAGGAGCGCGcgAGGCGGCGGCCGCGGCGG    +2
AGGCGGCAGAGTTTGATCGAGGATGCGCGTAAGGAGGcgGCGAGGCGGCGGCCGCGGCGG    +2  (-1,+3)
AGGCGGCAGAGTTTGATCGAGGGTGCGCGTAAGGAG----AGGCGGCGGCCGCGGCGGCG    -4
AGGCGGCAGAGTTTGATCGAGGATGCGCGTA-----CGCGAGGCGGCGGCCGCGGCGGCG    -5
AGGCGGCAGAGTTTGATCGAGGATGCGCGTAAG------GAGGCGGCGGCCGCGGCGGCG    -6           [x7]
AGGCGGCAGAGTTTGATCGAGGATGCGC----------GAGGCGGCGGCCGCGGCGGCG    -11          [x2]
AGGCGGCAGAGTTTGATCGAGGATGCGCGTAAGG-----------CGGCCGCGGCGGCG    -12
AGGCGGCAGAGTTTGA----------------------CGAGGCGGCGGCCGCGGCGGCG    -22
AGGCGGCAGAGTTTGATCGAGGATGCG-----------------------CGCGGCGGCG    -23
AGGCGGCAGAGTTTGATCGAGGATGCGCGTAAGGAG-------------------------    -28
AGGCGGCAGAGTTTGATCGAGGATGC----------------------------GCGGCG    -28
AGGCGGCAGAGTTTGATCGAGGATGCGCGTAA---------------------------G    -27
```

apoea
Mutations in 11 out of 38 sequenced alleles

```
CAGGGGCGATTCCTGTTTCAGGATGAGCCAAGAAGCCGCTGGGAAGAGGCCGTGGATCAG    Wild-type

CAGGGGCGATTCCTGTTTCAGGATGAGCCAAGAAGCC----tGAAGAGGCCGTGGATCAG    -4  (-5,+1)
CAGGGGCGATTCCTGTTTCAGGATGAGCCAAGAAGa----GGGAAGAGGCCGTGGATCAG    -4  (-5,+1)  [x2]
CAGGGGCGATTCCTGTTTCAGGATGAGCCAAGAAG-----GGGAAGAGGCCGTGGATCAG    -5           [x2]
CAGGGGCGATTCCTGTTTCAGGATGAtccacg-----GCTGGGAAGAGGCCGTGGATCAG    -5  (-11,+6)
CAGGGGCGATTCCTGTTTCAGGATGAGCCAAGAAG------GGAAGAGGCCGTGGATCAG    -6
CAGGGGCGATTCCTGTTTCAGGATGAGCCAAG-----------AAGAGGCCGTGGATCAG    -11          [x3]
CAGGGGCGATTCCTGTTTCAGGAaa-----------------GAAGAGGCCGTGGATCAG    -17 (-19,+2)
```

rgs4
Mutations in 20 out of 43 sequenced alleles

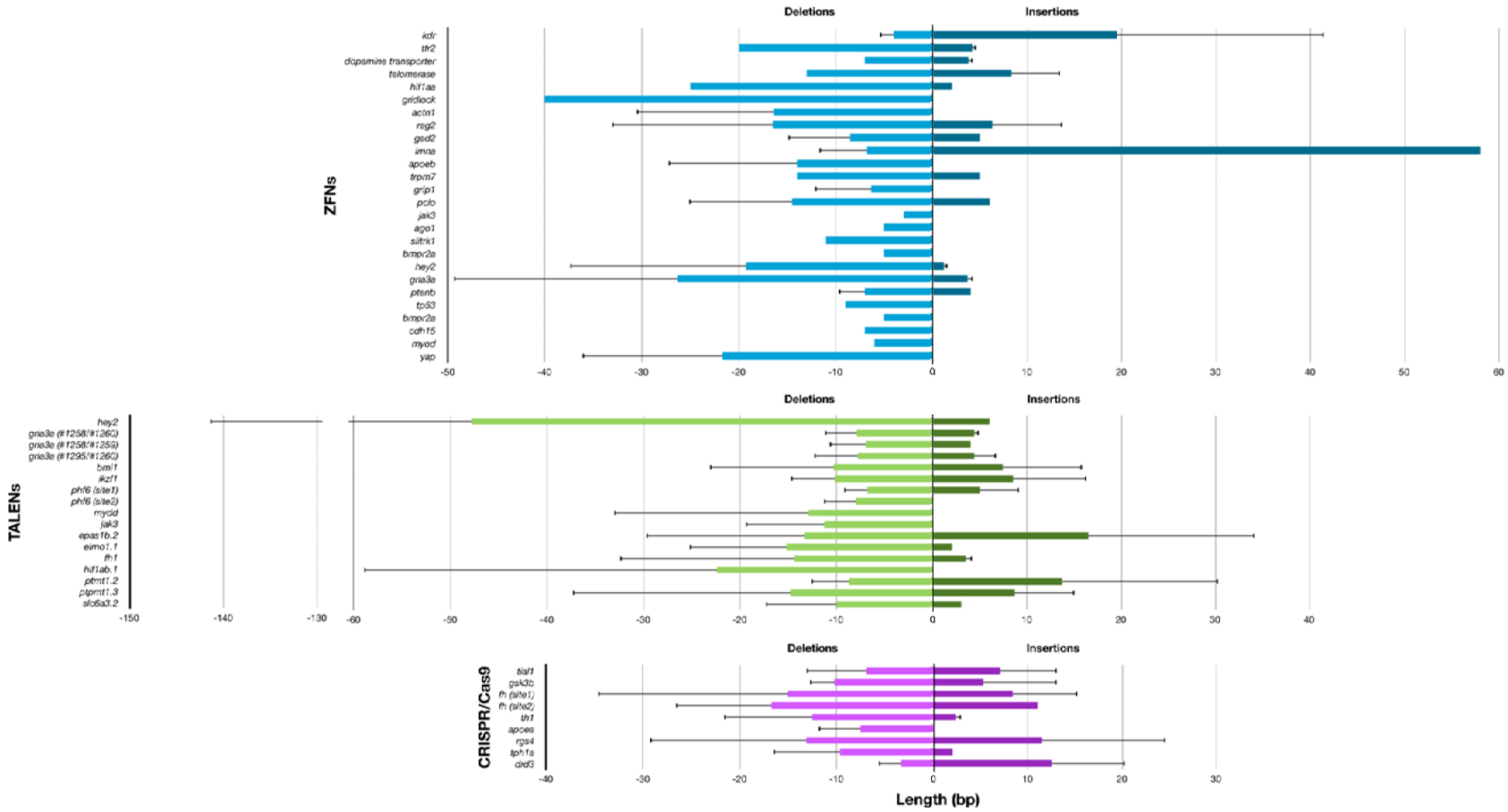
```
AAAGACAAGGAGAAGGTGAAGGACACTGTGGTCAACAGGTAAGACTGGTCCAGAATAATT    Wild-type

AAAGACAAGGAGAAGGTGAAGGACAacaggtaaaagctttaatttttttcacagttgaag    +35
AAAGACAAGGAGAAGGTGAAGGACAacaggacaaaggacaaCTGTGGTCAACAGGTAAGA    +16
AAAGACAAGGAGAAGGTGAAGGACAacaggtaagaaggTGTGGTCAACAGGTAAGACTGG    +12 (-1+13)
AAAGACAAGGAGAAGGTGAAGGACAacCTGTGGTCAACAGGTAAGACTGGTCCAGAATAA    +2
```



Supplementary Figure 2 Targeted indel mutations induced by engineered gRNA/Cas9 at the *fh* (site #2), *th1, apoea, rgs4*, *tph1a,* and *drd3* genes. 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. For some genes, the target site is on the reverse complement strand and in these cases the reverse complement of the target site is highlighted in green and the reverse complement of the PAM site is 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. A minor sequence polymorphism observed in the *tph1a* gene is underlined.

For each gene, the sequences of alleles shown were amplified from pooled genomic DNA isolated from ten embryos. The mutation efficiencies at these genes have also been assessed in single embryos by T7EI assay as shown in **Table 1**. See also **Figure 1d**.

a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Insertions
ZFNs
kdr
tlr2
dopamine transporter
telomerase
hif1aa
gridlock
actn1
rag2
gad2
imna
apoeb
trpm7
grip1
pcfo
jak3
ago1
siltrk1
bmpr2a
hey2
gria3a
ptenb
tp53
bmpr2a
cdh15
myod
yap
-50
-40
-30
-20
-10
0
10
20
30
40
50
60
Deletions
Insertions
TALENs
hey2
gria3a (#1258/#1260)
gria3a (#1258/#1259)
gria3a (#1295/#1260)
bmi1
ikzf1
phf6 (site1)
phf6 (site2)
mycid
jak3
epas1b.2
eimo1.1
fh1
hif1ab.1
ptmt1.2
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slc6a3.2
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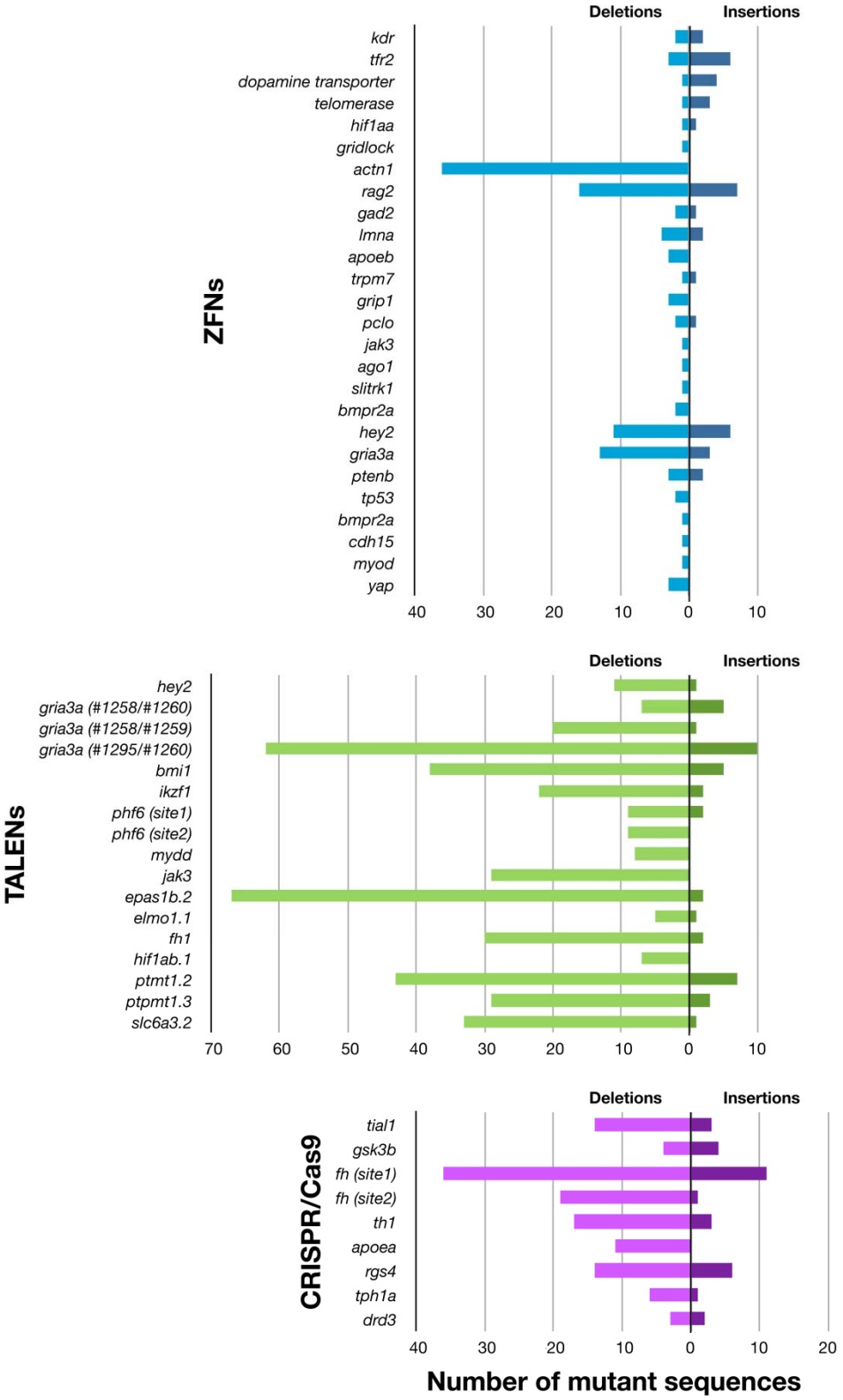
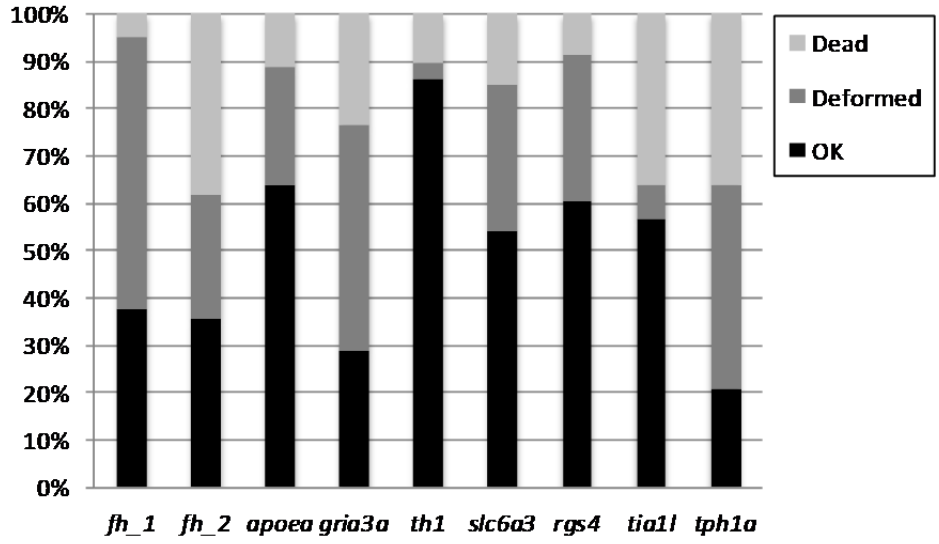
-140
-130
-60
-50
-40
-30
-20
-10
0
10
20
30
40
Deletions
Insertions
CRISPR/Cas9
tiaf1
gsk3b
fh (site1)
fh (site2)
th1
apoes
rgs4
tph1a
drd3
-40
-30
-20
-10
0
10
20
30
Length (bp)

b


Deletions
Insertions
ZFNs
kdr
tfr2
dopamine transporter
telomerase
hif1aa
gridlock
actn1
rag2
gad2
lmna
apoeb
trpm7
grip1
pclo
jak3
ago1
slitrk1
bmpr2a
hey2
gria3a
ptenb
tp53
bmpr2a
cdh15
myod
yap
40 30 20 10 0 10
Deletions
Insertions
TALENs
hey2
gria3a (#1258/#1260)
gria3a (#1258/#1259)
gria3a (#1295/#1260)
bmi1
ikzf1
phf6 (site1)
phf6 (site2)
mydd
jak3
epas1b.2
elmo1.1
fh1
hif1ab.1
ptmt1.2
ptpmt1.3
slc6a3.2
70 60 50 40 30 20 10 0 10
Deletions
Insertions
CRISPR/Cas9
tial1
gsk3b
fh (site1)
fh (site2)
th1
apoea
rgs4
tph1a
drd3
40 30 20 10 0 10 20
Number of mutant sequences

Supplementary Figure 3 Comparison of the length and types of indel mutations induced by ZFNs, TALENs, and gRNA-targeted Cas9 nuclease. (**a**) Mean lengths of deletion and insertion mutations are shown for various endogenous zebrafish gene targets altered by ZFNs (blue colored bars), TALENs (green colored bars), and gRNA-targeted Cas9 nuclease (purple colored bars). Error bars represent standard deviations. (**b**) Numbers of deletion and insertion mutant sequences for various endogenous zebrafish gene targets altered by ZFNs, TALENs, and gRNA-targeted Cas9 nuclease are shown (color-coded as in (a)). For both (a) and (b), mutation data for ZFNs and TALENs were derived from previously published studies that used ZFNs and TALENs made by the Joung lab[1-5] and mutation data for gRNA/Cas9 were from the experiments of this report.



Supplementary Figure 4 Toxicities of engineered gRNA/Cas9 nucleases in zebrafish embryos. 2 nl containing ~12.5 ng/µl of gRNA and ~300 ng/µl of Cas9-encoding mRNA were injected into 1-cell stage zebrafish embryos. Names of the target genes are shown on the x-axis. One day following injection, numbers of normal (OK), deformed and dead embryos were scored. Bars indicate the percentages of the embryos in each phenotypic category. Between 77 to 198 embryos were scored for each target site.

```
                  +
TAATACGACTCACTATAGGAGAGACCGAGAGAGGGTCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTAT
    T7 promoter      BsaI         BsaI                  Guide RNA
CAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTAAAAAGCTTGGATCGACGAGAGCAGCGCGACTGGATCTGTCGCCCGTCTCAA
                                  DraI
ACGCAACCCTCCGGCGGTCGCATATCATTCAGGACGAGCCTCAGACTCCAGCGTAACTGGACTGCAATCAACTCACTGGCTCACCT
TCCGGTCCACGATCAGCTAGAATCAAGCTGACTAGATAAACTGGCCGTCGTTTTACACGGG
                                          M13 primer
TGGGCCTTTCTTCGGTAGAAAATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACC
ACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAGGTAACTGGCTTCAGCAGAGCGCAGATACC
AAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCC
TGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGG
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AGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGC
TTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCATCGATTTTTGTGATGCTCGTCA
GGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGGTGATTACATTTAGGTCCTCATTA
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GAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCACCAACCAAACCGTTAT
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AGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCTGGGATCGC
AGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGCC
TGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAAT
CGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCG
CGGCTTCAAGCAAGACGTTTCCCGTTGAATATGGCTCATTTTAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGC
CCGGTAGTGATCTTATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAAGTCAAAAGCCTCCGGTCGGAGGCTTTTGA
CTTTCTGCTATGGAGGTCAGGTATGATTTAAATGGTCAGTATTGAGCCTCAGGAAACAGCTATGACATCAAGCTGACTAGATAATC
TAGCTGATCGTGGACCGATCATACGTATAATGCCGTAAGATCACGGGTCGCAGCACAGCTCGCGGTCCAGTAGTGATCGACACTGC
TCGATCCGCTCGCACCGCTAGC
```

Supplementary Figure 5 Full DNA sequence of gRNA expression vector pDR274. T7 promoter is underlined and the start site of transcription (+1) is marked with a + and highlighted in green. The pair of BsaI restriction sites used for cloning are indicated in bold text and the DraI restriction site used to linearize the plasmid for run-off transcription is shown in bold, italicized text. The "stuffer" sequence that is replaced with the annealed oligonucleotides to create customized gRNAs is highlighted in yellow and the full length gRNA is shown as italicized underlined text. The sequence of an M13 primer binding site is shown as underlined text.

Reference	gRNA sequence (5’ to 3’)	Length
Jinek et al., *Science* 2012	NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCG	62 nts
This work	GGNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	100 nts

Supplementary Table 1 Sequences of gRNAs used in previously published *in vitro* work (Jinek et al., 2012[6]) and in the current *in vivo* study.

Target gene	Target site (5' - 3') (PAM is underlined)	Oligonucleotide 1 (5' - 3')	Oligonucleotide 2 (5' - 3')
apoea	GGATGAGCCAAGAAGCCGCTGGG	TAGGATGAGCCAAGAAGCCGCT	AAACAGCGGCTTCTTGGCTCAT
gria3a	GGTGGTATTTTTTTGAGTGTGGG	TAGGTGGTATTTTTTTGAGTGT	AAACACACTCAAAAAAATACCA
th1	GGATGCGCGTAAGGAGCGCGAGG	TAGGATGCGCGTAAGGAGCGCG	AAACCGCGCTCCTTACGCGCAT
fh (Site #1)	GGAGCGGTACATGGCGACCGGGG	TAGGAGCGGTACATGGCGACCG	AAACCGGTCGCCATGTACCGCT
fh (Site #2)	GGAGCGAGCGGAGCGGTACATGG	TAGGAGCGAGCGGAGCGGTACA	AAACTGTACCGCTCCGCTCGCT
slc6a3	GGTGCCGTATCTCTTCTTCATGG	TAGGTGCCGTATCTCTTCTTCA	AAACTGAAGAAGAGATACGGCA
rgs4	GGAGAAGGTGAAGGACACTGTGG	TAGGAGAAGGTGAAGGACACTG	AAACCAGTGTCCTTCACCTTCT
tia1l	GGTATGTCGGGAACCTCTCCAGG	TAGGTATGTCGGGAACCTCTCC	AAACGGAGAGGTTCCCGACATA
tph1a	GGGAAAACACAACCGCAGCCCGG	TAGGGAAAACACAACCGCAGCC	AAACGGCTGCGGTTGTGTTTTC
gsk3b	GGGACCTGACCGGCCGCAGGAGG	TAGGGACCTGACCGGCCGCAGG	AAACCCTGCGGCCGGTCAGGTC
drd3	GGAAACTACAGCCCAGCGTCAGG	TAGGAAACTACAGCCCAGCGTC	AAACGACGCTGGGCTGTAGTTT

Supplementary Table 2 Eleven zebrafish gene sites targeted in this study and oligonucleotides used to make the associated customized gRNA expression vectors.

gRNA (ng/ul)	Cas9 mRNA (ng/ul)	Indel Mutation Frequency					
		Embryo #1	Embryo #2	Embryo #3	Embryo #4	Embryo #5	Mean ± SEM
5	100	15.5%	15.9%	0.0%	29.5%	47.1%	21.6% ± 7.9%
12.5	100	39.5%	40.4%	25.5%	51.6%	26.9%	36.8% ± 4.8%
25	100	3.9%	12.5%	10.8%	12.6%	10.1%	10.0% ± 1.6%
36.7	100	14.3%	44.3%	29.9%	31.7%	46.8%	33.4% ± 5.8%
12.5	300	57.8%	57.3%	61.7%	35.5%	51.3%	52.7% ± 4.6%

Supplementary Table 3 Mutation frequencies in the fh gene (site #1) induced by various concentrations 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 mutation frequencies of the five individual embryos for each set of concentrations are also shown with standard errors of the mean.

Gene	Nuclease Platform	Target sequence (5' to 3')	Indel Mutation Frequency (%)
fh	TALENs	TCGCTTCAGCGCGAGTTTGTCAGATCTGCGGGCCGCTCAGAGATCCATCAAA	60.0
th1	TALENs	TCTCAGAAGTTTGTTGGGAGGCGGCAGAGTTTGATCGAGGATGCGCGTAAGGA	51.4
tia1l	TALENs	TGTTACGGAGGCCCTCATCCTGCAAGTGTTCTCTCAGATCGGCCCCTGCAAGA	76.3
apoea	TALENs	TTTCAGGATGAGCCAAGAAGCCGCTGGGAAGAGGCCGTGGATCAGTTCTGGA	20.6
rgs4	TALENs	TGCCAAAGATATAAAACATAAGATTGGCTTCCTGCTTCAAAAGCCAGATCCA	24.1
tph1a	TALENs	TGAACAAATCTGCTTTCACGAAGATCGAGGAGAATAAAGACAACAAAACAGA	21.9
drd3	TALENs	TCATTCACCCCTGGGGGAAACTACAGCCCAGCGTCAGGCGTTGAAGAAGCGA	0
gsk3b	TALENs	TGGCGACTCCTGGACAGGGACCTGACCGGCCGCAGGAGGTCAGCTACACTGA	0
slc6a3	TALENs	TCCTGGTGCCGTATCTCTTCTTCATGGTGATCGCCGGGATGCCGCTCTTCTA	50.0
gria3a	TALENs	TCGTCCAATAGCTTCTCAGTCACGCACGCCTGTGAGTTTCTGCTCTTTA	61.0
gria3a	ZFNs	AGCTTCTCAGTCACGCACGCCTGTGAGTTT	25.8

Supplementary Table 4 Previously determined TALEN- and ZFN-induced mutation frequencies for the ten genes targeted in this study. Indel mutation frequency was determined as previously described[3]. Data for the *gria3a*, *fh*, and *slc6a3.2* genes were previously published[1, 3] and the remaining data are unpublished results from our groups.

Target site	Primer Name	Primer sequence (5'-3')	Experiment
fh	JY165	CAGGCTGTTGAACCGTAGATTTAGT	T7E1 and sequencing
	JY166	TCCACATGTTTTGAGTTTGAGAGTC	
th1	JY190	GGAGATGTAAATCACCTCCATCTGA	T7E1 and sequencing
	JY191	ATGTTAGCCTACCTCGAAAACCTTC	
tia1l	JY198	CCTGTGCTCTCCTGTTTTTAGGTAT	Sequencing
	JY199	AACATGGTAAGAAGCGTGAGTGTTT	
	oFYF414	TGAAAACGTGGCAGAAATGA	T7E1
	oFYF415	GGATTTATGCAGCCCAGAGA	
apoea	JY184	CATGCCAATTAAATTTGTCAAAACA	T7E1 and sequencing
	JY185	TTGAGATGTTTCAAAGCGTTTACTC	
rgs4	JY236	TATGCTTGCATAAATTGAGCGTCTA	T7E1 and sequencing
	JY237	TGAAATAAGCCATGGTAAATCACAC	
tph1a	JY192	TTGGCAAGAGAACTATGAGTGAATG	T7E1 and sequencing
	JY193	AAATAAAACCTCACGTTACCTGGAA	
drd3	JY220	ACACTGCATGTTGTCAAGCATTTAT	T7E1 and sequencing
	JY221	CTTACTTCCAAATAAACTGCCCAAG	
gsk3b	JY186	AGTATGATTGGTGGAACACAGGAAT	T7E1 and sequencing
	JY187	CTTACCTTAAATCGCTTGTCCTGAA	
slc6a3	JY155	GTTCCCATACCTGTGCTACAAGAAC	T7E1 and sequencing
	JY156	ATTTGTGTGTGTCTTCCATCTGAGT	
gria3a	JY027	TCGCCGTTCAGCTCTACAACAC	T7E1 and sequencing
	JY028	TCAAACCCACGTCTTTGGTGAG	

Supplementary Table 5 List of PCR primers used in this study

Supplementary References

1. Cade, L. et al. Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs. *Nucleic Acids Res* **40**, 8001-8010 (2012).
2. Moore, F.E. et al. Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). *PLoS One* **7**, e37877 (2012).
3. Sander, J.D. et al. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol* **29**, 697-698 (2011).
4. Sander, J.D. et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods* **8**, 67-69 (2011).
5. Foley, J.E. et al. Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN). *PLoS One* **4**, e4348 (2009).
6. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).