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Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs

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ABSTRACT

Transcription activator-like effector nucleases (TALENs) are powerful new research tools that enable targeted gene disruption in a wide variety of model organisms. Recent work has shown that TALENs can induce mutations in endogenous zebrafish genes, but to date only four genes have been altered, and larger-scale tests of the success rate, mutation efficiencies and germline transmission rates have not been described. Here, we constructed homodimeric TALENs to 10 different targets in various endogenous zebrafish genes and found that 7 nuclease pairs induced targeted indel mutations with high efficiencies ranging from 2 to 76%. We also tested obligate heterodimeric TALENs and found that these nucleases induce mutations with comparable or higher frequencies and have better toxicity profiles than their homodimeric counterparts. Importantly, mutations induced by both homodimeric and heterodimeric TALENs are passed efficiently through the germline, in some cases reaching 100% transmission. For one target gene sequence, we observed substantially reduced mutagenesis efficiency for a variant site bearing two mismatched nucleotides, raising the possibility that TALENs might be used to perform allele-specific gene disruption. Our results suggest that construction of one to two heterodimeric TALEN pairs for any given gene will, in most cases, enable researchers to rapidly generate knockout zebrafish.

INTRODUCTION

Customized endonucleases have become indispensable tools for genome editing in human somatic and pluripotent stem cells, rats, *Drosophila*, *Caenorhabditis elegans*, plants and zebrafish (1–5). These enzymes are engineered to create site-specific double-strand deoxyribonucleic acid (DNA) breaks (DSBs) that can be repaired by non-homologous end joining, resulting in insertions and deletions (indels) that disrupt the reading frame and function of the targeted gene (6–13). Alternatively, DSBs can also be repaired via homologous recombination (HR), thereby incorporating specific sequence modifications provided on a donor template (14–17).

One class of endonucleases that has drawn much interest is the transcription activator-like effector nucleases (TALENs) (18–23). These enzymes consist of a fusion between the non-specific cleavage domain from the naturally occurring Type IIS FokI endonuclease and a transcription activator-like (TAL) effector DNA recognition domain. The TAL effector recognition domain comprises an N-terminal domain that recognizes a 5′ T in the binding site, a variable number of highly conserved 33–35 amino acid repeats each capable of specifying a single DNA nucleotide, and C-terminal sequence from naturally occurring TAL effector proteins. The identities

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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of two amino acids within each repeat (known as repeat variable di-residues or RVDs) are associated with different DNA-binding specificities. Repeats bearing different RVDs have been identified that recognize each of the four DNA nucleotides, and these domains can be assembled into arrays capable of binding virtually any DNA sequence that begins with a 5′ T (18). The simplicity of design, robust activity and nearly limitless targeting range of the TALENs make this platform very attractive for genome editing.

Previously, we and another group showed that TALENs could effectively induce targeted disruption of four different endogenous genes in zebrafish but germline transmission was only demonstrated for two of these genes (19,20). In this study, we sought to perform tests with a larger number of genes to determine the success rate for obtaining active TALENs capable of robustly cleaving zebrafish genes, to assess the efficiency of transmitting TALEN-induced mutations through the germline and to compare the activities of TALENs that harbor homodimeric or obligate heterodimeric FokI cleavage domains. We addressed these questions by examining the activities of homodimeric and heterodimeric TALEN pairs designed for target sites present in six different zebrafish genes. Our results demonstrate that most TALENs are highly active in zebrafish, that TALEN-induced mutations can be efficiently transmitted through the zebrafish germline and that heterodimeric TALENs show similar or greater activities than their homodimeric counterparts. Our findings provide strong support for the idea that TALENs will enable facile generation of zebrafish knockout lines for nearly all endogenous zebrafish genes.

MATERIALS AND METHODS

Zebrafish care

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

Construction of TALEN expression vectors

Homodimeric TALENs harboring a wild-type FokI nuclease domain were assembled using the fast ligation-based automatable solid-phase high-throughput (FLASH) method as described previously (18). Heterodimeric TALENs were generated by digesting sequence-verified homodimeric TALEN clones with NheI and BamHI and cloning them into expression vectors MLM290/292 that contain EL and KK heterodimeric FokI domains (available through the nonprofit plasmid repository, Addgene), or equivalent vectors that contain additional N496D or H537R mutations (yielding ELD and KKR heterodimeric FokI domains) described by Doyon *et al.* (24). The nucleotide sequences of the ELD and KKR nuclease expression vectors can be found in Supplementary Figure S1. The nucleotide sequences of the TALE repeat arrays and the full amino acid sequences of the TALENs used in this study can be found in Supplementary Figures S2 and S3.

Production of TALEN mRNAs

TALEN expression vectors were linearized with *Pme*I, which were then used for *in vitro* transcription using the mMESSAGE mMACHINE® T7 ULTRA kit (Ambion). The transcribed RNAs were polyadenylated using the reagents in the same kit.

Microinjection and analysis of somatic mutations

Approximately 2 nl of the TALEN messenger ribonucleic acid (mRNAs, 300 pg/nl) was injected into one-cell stage zebrafish embryos. On the next day, the surviving injected embryos were grouped into either 'normal' or 'deformed' phenotypes. Genomic DNA was extracted from pools of 12 embryos from each 'normal' group as previously described (25). Targeted genomic loci were amplified using primers designed to anneal approximately 150–200 base pairs upstream and downstream from the expected cut site. A list of the primers used in this study is provided in Supplementary Figure S4. The resulting polymerase chain reaction (PCR) product was cloned into pGEM-5Zf(+) using the pGEM-T Easy kit (Promega). After transformation of the ligated vectors, single colonies were reinoculated for plasmid DNA isolation and sequencing. The sequence of each clone represents one amplified allele. In this study, we sequenced approximately 48–96 clones for each target site and determined somatic mutation rates based on the percentages of the mutated alleles. According to statistical analysis, we will have a greater than 95% probability of detecting at least one mutant allele by screening 48 clones for a TALEN pair that has a mutation efficiency of 6.25% or higher. Similarly, we will have greater than 95% confidence for detecting at least one mutant allele by screening 96 clones for a TALEN pair that has mutation efficiency of 3.125% or higher. Therefore, given the number of clones we sequenced in our experiments, we may not be able to reliably detect indel mutations for TALEN pairs that have targeting efficiencies below 3–6%.

Founder screen

Potential founders were crossed with wild-type zebrafish. Three to four days post-fertilization, progeny were lysed individually in 25 µl of the alkaline lysis buffer (25 mM NaOH, 0.2 mM ethylenediaminetetraacetic acid) and heated at 95°C for 30 min. Subsequently, the DNA solution was neutralized using 25 µl of the neutralization buffer (40 mM Tris-HCl). Samples were spun at 3000 rpm for 5 min, and the supernatant contained extracted genomic DNA. In general, 12 embryos from each potential founder were screened for the presence of TALEN-induced mutations by PCR amplifying the region surrounding the relevant TALEN cleavage site using a 6-FAM-labeled fluorescent forward primer and a regular reverse primer (Supplementary Figure S4). The fluorescent PCR products were analyzed on ABI 3730xl DNA analyzer to evaluate their sizes (25). For sequence confirmation, genomic DNAs from single embryos were amplified using targeted loci-specific primers, and the PCR products were cloned into pGEM-5Zf(+)

vector as described above. The resulting clones were sequenced and the mutations were determined.

RESULTS

Activities of obligate heterodimeric TALENs in zebrafish

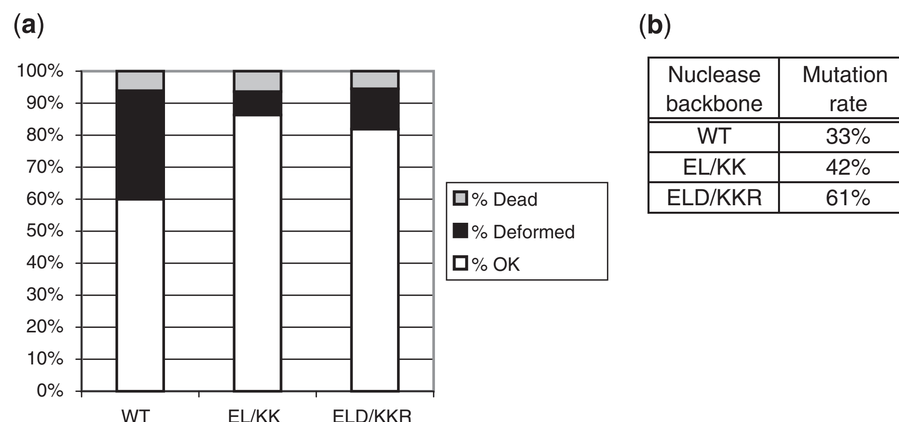
In previous work, we showed that TALENs harboring wild-type homodimeric FokI nuclease domains could efficiently induce targeted mutations in zebrafish, whereas Huang *et al*. successfully targeted two zebrafish genes using TALENs harboring modified heterodimeric FokI nuclease domains (19,20). We wished to compare the activities of homodimeric TALENs to corresponding heterodimeric TALENs. Obligate heterodimeric nuclease domains should reduce unwanted homodimeric pairing between two identical TALEN molecules, thereby increasing the formation of the desired heterodimer and reducing potential 'off-target' effects caused by homodimers. Heterodimeric FokI domains used in engineered ZFNs have shown reduced off-target cleavage events in human cells as well as reduced cytotoxicity (24,26–28). However, obligate heterodimeric ZFNs have also been reported to possess less activity than their homodimeric counterparts, and therefore, we were interested in investigating the activities of TALENs made with heterodimeric FokI domains (24,26).

For an initial experiment, we chose two pairs of heterodimeric FokI domains referred to as EL/KK and ELD/KKR that have been previously described and used for engineering heterodimeric ZFNs (24,26). [Note that these FokI domains differ from the ones used by Huang *et al*. (20).] Specifically, we made heterodimeric versions of a homodimeric TALEN pair, which we had previously shown sounded redundant can induce somatic indel mutations in 33% of *gria3a* gene alleles in zebrafish embryos (19). We found that both heterodimeric *gria3a* TALEN pairs induced smaller numbers of deformed embryos (~20% of deformed and dead embryos for both EL/KK and ELD/KKR versus ~40% of those for the homodimeric pair) and were also moderately more active in their abilities to induce somatic gene mutations in the *gria3a* gene (42% for EL/KK and 61% for ELD/KKR) than the homodimeric pair from which they were derived (Figure 1 and Supplementary Figure S5). These results suggest that TALENs constructed with either EL/KK or ELD/KKR heterodimeric FokI domains are not only well tolerated in zebrafish embryos but also induce high rates of target site cleavage. Because the ELD/KKR heterodimers were more active than the EL/KK heterodimers in both our TALEN experiment and previously published studies performed with ZFNs in human cells (24), we chose to utilize ELD/KKR TALENs for our subsequent experiments.

Success rates and mutagenic efficiencies of homodimeric and heterodimeric TALENs

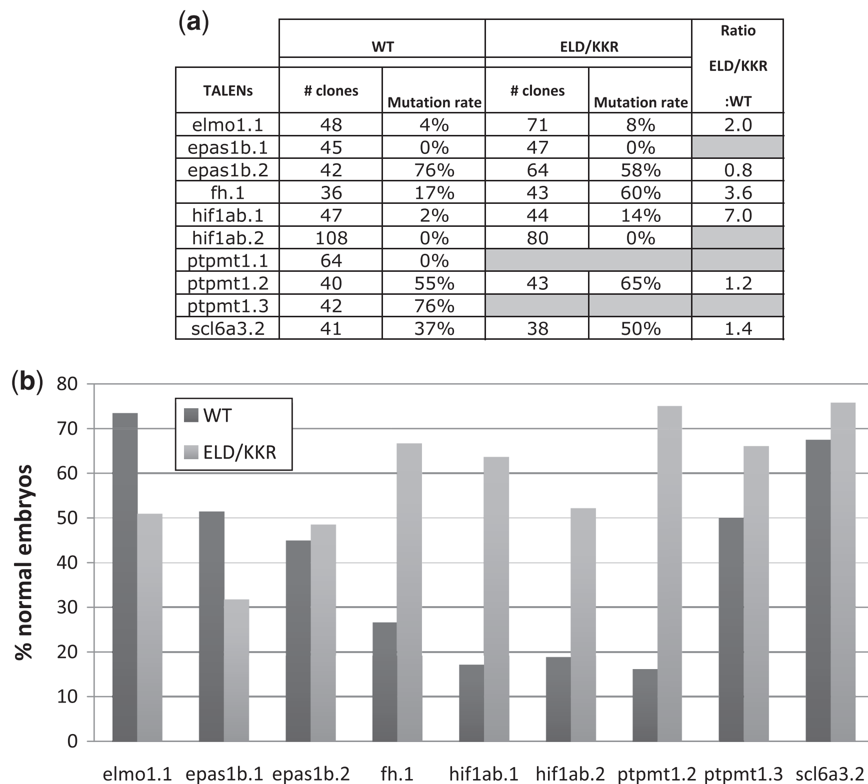
To better define the success rates and the effectiveness of the TALEN platform for mutating endogenous zebrafish genes, we performed a larger-scale test of homodimeric and heterodimeric TALENs. On the basis of the results of a recent systematic and large-scale test of various length TALENs we recently performed in human cells (18), we chose to construct TALENs composed of 16.5 TALE repeats in all of our studies.

We initially designed and constructed homodimeric TALENs for 10 target sites found in the first 3 exons of 6 endogenous zebrafish genes (Supplementary Figure S6). Among these genes, we had previously targeted *hif1ab* and *slc6a3* (also known as *dopamine transporter*, *dat*) with customized ZFNs but at sites toward the end of their coding regions (10). We found that 7 of these 10 TALEN pairs efficiently induced indels at their targeted loci in the TALEN mRNA-injected embryos with somatic mutation rates ranging from 2 to 76% (Figure 2a and Supplementary Figure S7). In addition, for all six genes we sought to mutate, we successfully identified at least one pair of TALENs that cleave efficiently within the first three exons (the reliable detection limit of our assay is



Nuclease backbone	Mutation rate
WT	33%
EL/KK	42%
ELD/KKR	61%

Figure 1. Targeting efficiencies and toxicities of homodimeric and heterodimeric TALENs in zebrafish. (**a**) Percentages of dead, deformed and normal (OK) embryos at 1 day after injection with TALEN-coding mRNAs. The total number of embryos scored (n) is 165, 110 and 127 for WT, EL/KK and ELD/KKR, respectively. (**b**) Mutation rates in somatic zebrafish cells are determined as described in 'Materials and Methods' section and are shown here. Mutated sequences can be found in Supplementary Figure S5. The TALENs constructed in two heterodimeric frameworks showed increased targeting efficiencies and reduced toxicities compared to their homodimeric counterparts.



(a)

TALENs	WT # clones	WT Mutation rate	ELD/KKR # clones	ELD/KKR Mutation rate	Ratio ELD/KKR :WT
elmo1.1	48	4%	71	8%	2.0
epas1b.1	45	0%	47	0%	
epas1b.2	42	76%	64	58%	0.8
fh.1	36	17%	43	60%	3.6
hif1ab.1	47	2%	44	14%	7.0
hif1ab.2	108	0%	80	0%	
ptpmt1.1	64	0%			
ptpmt1.2	40	55%	43	65%	1.2
ptpmt1.3	42	76%			
scl6a3.2	41	37%	38	50%	1.4

Figure 2. Engineered TALENs are highly efficient in targeting endogenous zebrafish genes. (**a**) Frequencies of mutations induced by homodimeric and heterodimeric TALENs targeting 10 endogenous loci in zebrafish. The number of clones depicts the total number of alleles including wild-type and mutant alleles that have been sequenced. The ratios of mutation rates between heterodimeric and homodimeric TALENs are also shown. (**b**) Comparison of the toxicity induced by homodimeric and heterodimeric TALENs. The percentages of normal embryos at 1 day after injection with TALEN-coding mRNAs are shown. A total of 30–189 embryos were scored for each TALEN pair. The number of embryos scored for each TALEN pair is shown in Supplementary Figure S8.

approximately 3–6% as discussed in the 'Materials and Methods' section). Our large-scale test suggests that TALENs have a high success rate in zebrafish and that active nucleases can robustly mutagenize their targeted loci.

To investigate the activities of heterodimeric TALENs, we constructed ELD/KKR versions of 8 of the 10 homodimeric TALEN pairs described above and then tested their abilities to induce somatic mutations in zebrafish. In general, heterodimeric TALENs showed comparable or higher activities (from 0.8- to 7-fold) than their homodimeric counterparts (Figure 2a and Supplementary Figure S7). Interestingly, TALENs that failed to show activities as homodimers were also inactive as heterodimers. Taken together, these results suggest that, in contrast to the results of others in human cells (24), the ELD/KKR heterodimeric TALENs have equivalent or higher activities than homodimeric TALENs in zebrafish.

Given the reported improved specificity of heterodimeric nucleases relative to homodimeric nucleases (24,28), we compared the toxicities of matched homodimeric and heterodimeric TALENs. To do this, we compared the percentages of normal embryos one day after microinjection of mRNAs encoding homodimeric and heterodimeric TALENs. Our results show that homodimeric TALENs exhibit variable levels of toxicities (16–73% of normal embryos), while heterodimeric TALENs are more consistently well tolerated (32–75% of normal embryos) (Figure 2b). Therefore, these results suggest that the ELD/KKR heterodimeric TALENs may provide a less toxic alternative to homodimeric TALENs for mutagenizing zebrafish.

Preferential allelic disruption of a zebrafish gene using TALENs

While performing sequence analysis of a clutch of epas1b.2-targeted embryos, we unexpectedly identified a two base pair polymorphism (AA→GC) in one allele that falls within the left target half-site of the TALENs targeted to that gene. In the embryos injected with homodimeric TALENs targeted to the epas1b.2 site, we found that the allele with the correct target sequence was mutated with a frequency of 76%, whereas the allele with the target sequence harboring the two mismatches were mutated with a frequency of only 2% (Figure 3). Similarly, heterodimeric TALENs targeted to the epas1b.2 site-induced mutations in 58% of the alleles with the correct target sequences but in only 3% of the alleles bearing the 2-bp mismatch (Figure 3). These data indicate that both homodimeric and heterodimeric TALENs can exhibit strong selectivity toward their intended target sequences, with the ability to discriminate

```
epas1b.2:
SQT418/419  Mutations in 32 of 42 sequences: ~76.2%
GCTACAATACTCCCACTGAAATGACAGATGCAGACAGACTCATGGACAGTTGGTATC WT

<-----------------------------------GACTCATGGACAGTTGGTATC -103
GCTACAATACTCCCACTGAAAT--------------GACTCATGGACAGTTGGTATC -14
GCTACAATAagacagacaa------------AGACAGACTCATGGACAGTTGGTATC -12 (-22 and +10)
GCTACAATACTCCCACTGAAAT----------GACAGACTCATGGACAGTTGGTATC -10 [x21]
GCTACAATACTCCCACTGAAA----------AGACAGACTCATGGACAGTTGGTATC -10 [x3]
GCTACAATACTCCCACTGAAATGA------CAGACAGACTCATGGACAGTTGGTATC -6  [x2]
GCTACAATACTCCCACTGAAATGACA---GCAGACAGACTCATGGACAGTTGGTATC -3  [x2]
CTCCCACTGAAATGACAGATGCAGACAGactcccactgaaatgacagatgcagacag +29

epas1b.2:
WH7/8       Mutations in 37 of 64 sequences: ~57.8%
GCTACAATACTCCCACTGAAATGACAGATGCAGACAGACTCATGGACAGTTGGTATC WT

<-------------------------------GACAGACTCATGGACAGTTGGTATC -90
<------------------------------AGACAGACTCATGGACAGTTGGTATC -53
<------------------------------AGACAGACTCATGGACAGTTGGTATC -41
GCTACA---------------------------ACAGACTCATGGACAGTTGGTATC -27
GCTACAATACTCCCACTGAAAT----------GACAGACTCATGGACAGTTGGTATC -10 [X27]
GCTACAATACTCCCACTGAAATGA------CAGACAGACTCATGGACAGTTGGTATC -6  [X4]
GCTACAATACTCCCACTGAAATGACAGcaatc-ACAGACTCATGGACAGTTGGTATC -1  (-6 and +5)
GCTACAATACTCCCACTGAAATactcccactgaaaAGACAGACTCATGGACAGTTGG +4  (-9 and +13)

epas1b.2:
SQT418/419  Mutations in 2 of 92 sequences: ~2.2%
GCTACAATACTCCCACTGAAATGACAGATGCAGACAGACTCATGGACAGTTGGTATC WT

GCTACGCTACTCCCACTG-------tcccaCAGACAGACTCATGGACAGTTGGTATC -7  (-12 and +5)
GCTACGCTACTCCCACTGAAAT----------GACAGACTCATGGACAGTTGGTATC -10

epas1b.2:
WH7/8       Mutations in 2 of 76 sequences: ~2.6%
GCTACAATACTCCCACTGAAATGACAGATGCAGACAGACTCATGGACAGTTGGTATC WT

GCTACGCTACTCCCACTGAAAT----------GACAGACTCATGGACAGTTGGTATC -10
GCTACGCTACTCCCACTGAAATGACAGACTCAtccatGACAGACTCATGGACAGTTG +4
```

Figure 3. Frequencies and sequences of somatic mutations induced by TALENs at their intended or variant target loci. Both homodimeric and heterodimeric TALENs targeting epas1b.2 induced high rates of indel mutations at their intended target loci. However, their targeting efficiencies at a variant target locus with a 2 base pair mismatch were reduced significantly. The TALEN constructs used and their mutation rates are as indicated (SQT418/419, homodimeric TALEN constructs; WH7/8, ELD/KKR heterodimeric TALEN constructs). The wild-type sequence is shown at the top with TALEN binding sites marked in yellow. Deletions are indicated by gray highlighted red dashes and insertions by blue highlighted lower case letters. The sizes of the indels are labeled to the right of each sequence (+, insertion; –, deletion). The number of times each mutant allele was isolated is shown in brackets.

between alleles and sites that differ by as few as two nucleotides.

High-transmission rates of heritable mutations induced by TALENs

The published literature to date describes only two examples for which TALEN-induced mutations have been transmitted through the germline in zebrafish (20). By using two TALEN pairs described in our previous report (for *gria 3a* and *hey2*) (19) and six pairs from this study (for *elmo1*, *epas1b*, *fh*, *hif1ab*, *ptpmt1* and *slc6a3*), we performed founder screens for a total of eight different zebrafish genes. We successfully identified founders for all of these eight genes and found that both homodimeric and heterodimeric TALENs induce heritable mutations that can be transmitted through the germline (Figure 4). For the *epas1b*, *fh* and *gria3a* gene targets, we found that all the potential fish we screened (from two to eight fish for each gene target) could transmit heritable mutations with percentages of mutant embryos from each founder ranging from 25 to 75% (Figure 4). Of note, the somatic mutation rates for those three TALEN pairs were 76% for *epas1b*, 60% for *fh* and 33% for *gria3a* (Figures 1b and 2a). For *hey2*, *hif1ab*, *ptpmt1* and *slc6a3* [the somatic mutation rates for these TALEN pairs ranged from 11 to 65%, Figure 2a (19)], we were able to identify one or more founders for each of these gene targets by screening 4–12 potential founder fish (12 embryos from each fish). Even for the *elmo1* TALEN pair that induced a somatic mutation rate of only 8% (Figure 2a), we identified one founder after screening 22 fish (12 embryos from each fish) (Figure 4). As has been observed previously with ZFNs, for several TALEN pairs, we observed more than one type of heritable mutation from a single founder, suggesting that those mutations occurred independently in different germ cells within the same animal (Figures 4 and 5). Overall, our results demonstrate that TALENs are highly efficient and sufficiently non-toxic to zebrafish embryonic cells so

elmo1: elmo1.1 (WH 1/2) 1 out of 22 fish screened is a founder.

#	transmission rate	# of embryos	# of mutant	Mutations[a]
1	8.3%	12	1	1(-11 bp)

epas1b: epas1b.2 (SQT418/419) 2 out of 2 fish screened are founders.

#	transmission rate	# of embryos	# of mutant	Mutations[a]
1	62.5%	24	15	15(-10 bp)
2	41.7%	12	5	2(-10 bp), 3(+1 bp)

fh: fh.1 (WH 9/10) 3 out of 3 fish screened are founders.

#	transmission rate	# of embryos	# of mutant	Mutations[a]
1	33.3%	12	4	3(-7 bp), 1(-17 bp)
2	33.3%	12	4	2(-7 bp), 2(-12 bp)
3	41.7%	12	5	2(-7 bp), 3(-8 bp)

gria3a: #1295/#1260 8 out of 8 fish screened are founders.

#	transmission rate	# of embryos	# of mutant	Mutations[a]
1	75.0%	12	9	5(-9 bp),2(-7 bp),2(-11 bp)
2	58.3%	12	7	5(-9 bp), 2(-4 bp)
3	58.3%	12	7	1(-11 bp), 2(-6 bp), 2(-22 bp), 2(-4 bp)
4	58.3%	12	7	4(-5 bp),3(-6 bp)
5	50.0%	10	5	2(-4 bp), 2(-3 bp), 1(-2 bp)
6	33.3%	12	4	2(+11 bp), 2(-6 bp)
7	33.3%	12	4	3(-3 bp), 1(-4 bp)
8	25.0%	12	3	3(-4 bp)

hey2: #1297/#1257 3 out of 12 fish screened are founders.

#	transmission rate	# of embryos	# of mutant	Mutations[a]
1	25.0%	12	3	2(-2 bp), 1(-8 bp)
2	41.7%	12	5	5(-7 bp)
3	8.3%	12	1	1(-3 bp)

hif1ab: hif1ab.1 (WH 11/12) 1 out of 4 fish screened is a founder.

#	transmission rate	# of embryos	# of mutant	Mutations[a]
1	8.3%	12	1	1(-4 bp)

ptpmt1: ptpmt1.2 (WH 13/14) 4 out of 9 fish screened are founders.

#	transmission rate	# of embryos	# of mutant	Mutations[a]
1	8.3%	12	1	1(+7 bp)
2	8.3%	12	1	1(+7 bp)
3	8.3%	12	1	1(-13 bp)
4	25.0%	12	3	2(-9 bp), 1(+4 bp)

slc6a3: slc6a3.2 (SQT 426/427) 1 out of 8 fish screened is a founder.

#	transmission rate	# of embryos	# of mutant	Mutations[a]
1	8.3%	12	1	1(-17 bp)

Figure 4. TALENs induce high rates of heritable mutations. Fish that had been previously injected with either homodimeric or heterodimeric TALENs were screened for founders carrying heritable mutations. The targeted gene and the TALEN pairs injected are indicated at the top of each panel. Individual embryos from each potential founder were lysed for PCR analysis. Indel mutations were identified by the presence of the PCR size variants. Except for hey2 and gria3a, the sequences of the mutant alleles have been determined by sequencing (Figure 5). TALEN 1295/1260, 1297/1257 and the SQT constructs encode homodimeric TALEN pairs, whereas the WH constructs encode ELD/KKR heterodimeric TALENs. [a]The number of embryos that possessed indel mutations are shown outside of the parentheses, and the sizes of the indels are shown inside the parentheses. Some of the mutation sequences are shown in Figure 5.

```
elmo1:        elmo1.1       (WH1/2)
WT   TCCGGCAGACATCGTGAAGGTGGCCATCGAGTGGCCTGGGGCCTTCCCCAAA

MT1  TCCGGCAGACATCGTGAAGGTGG-----------CCTGGGGCCTTCCCCAAA    -11


epas1b:       epas1b.2      (SQT418/419)
WT   TACCAACTGTCCATGAGTCTGTCTGCATCTGTCATTTCAGTGGGAGTATTGTA

MT1  TACCAACTGTCCATGAGTCTGT----------CATTTCAGTGGGAGTATTGTA   -10
MT2  TACCAACTGTCCATGAGTCTGT----------CATTTCAGTGGGAGTATTGTA   -10
MT2  TACCAACTGTCCATGAGTCTGTCTGCATtCTGTCATTTCAGTGGGAGTATTGT   +1


fh:           fh.1          (WH9/10)
WT   TTTGATGGATCTCTGAGCGGCCCGCAGATCTGACAAACTCGCGCTGAAGCGA

MT1  TTTGATGGATCTCT-------ttcacacgCTGACAAACTCGCGCTGAAGCGA    -7 (-15 and +8)
MT1  TTTGATGGATCTCTGA-----------------CAAACTCGCGCTGAAGCGA    -17
MT2  TTTGATGGATCTCTGAGCGGCC-------CTGACAAACTCGCGCTGAAGCGA    -7
MT2  TTTGATGGATCTCTGAGCGGCC------------AAACTCGCGCTGAAGCGA    -12
MT3  TTTGATGGATCTCTGAGCGGCCC-------TGACAAACTCGCGCTGAAGCGA    -7
MT3  TTTGATGGATCTCTGAGCGGCCC--------GACAAACTCGCGCTGAAGCGA    -8


hif1ab:       hif1ab.1      (WH11/12)
WT   TACTGGAGTTGTCACTGAAAAGAAAAGGTAGGGATTCCCGTTTTTGCTTGGA

MT1  TACTGGAGTTGTCACTGAAAAGAAAAGG----GATTCCCGTTTTTGCTTGGA    -4


ptpmt1:       ptpmt1.2      (WH13/14)
WT   TCATATTCTTCATTCATGGTAATTACTCCTCTTACTTTTTCATTTTGGACCA

MT1  TCATATTCTTCATTCATGGTAATTACTCCTCTTAcctcttaCTTTTTCATTT    +7
MT2  TCATATTCTTCATTCATGGTAATgaatttcattcttttcaaCTTTTTCATTT    +7(-11 and +18)
MT3  TCATATTCTTCATTCATGGTA-------------CTTTTTCATTTTGGACCA    -13
MT4  TCATATTCTTCATTCATGG---------CTCTTACTTTTTCATTTTGGACCA    -9
MT4  TCATATTCTTCATTCATGGTAATTACTtactCCTCTTACTTTTTCATTTTGG    +4


slc6a3:       slc6a3.2      (SQT426/427)
WT   TAGAAGAGCGGCATCCCGGCGATCACCATGAAGAAGAGATACGGCACCAGGA

MT1  TAGAAGAGCGGCATCCCGGCG-----------------ATACGGCACCAGGA    -17
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Figure 5. Sequences of heritable mutations induced by TALENs. For six of the eight genes that are shown in Figure 4, we have determined the sequences of all the mutant alleles identified in the progeny of the founders by sequencing. Potential founders were generated by injection of either homodimeric (SQT constructs) or heterodimeric (WH constructs) TALEN mRNAs as indicated. The MT numbers shown at the left of the sequences represent the founder numbers, which correspond to the founder numbers shown in Figure 4. Sometimes different mutant alleles were found in different embryos from a single founder. For the sequences, the wild-type sequence is shown at the top with TALEN binding sites marked in yellow. Deletions are indicated by gray highlighted red dashes and insertions by blue highlighted lower case letters. The sizes of the indels are labeled to the right of each sequence (+, insertion; −, deletion).

as to enable efficient transmission of mutations through the germline.

DISCUSSION

TALENs have been shown previously to be capable of generating mutations in endogenous zebrafish genes (19,20), but success rates, somatic mutation rates, toxicities and germline transmission efficiencies determined from large-scale studies have not yet been reported. In addition, the most recent generation of obligate heterodimeric FokI nuclease domains have not yet been used with TALENs in zebrafish (24). In this study, we sought to use the FLASH method recently described by our group to perform a large-scale assessment of the TALEN platform for mutating zebrafish genes (18).

Our results show that both homodimeric and heterodimeric TALENs can efficiently modify their targeted loci in zebrafish. Seven of the 10 homodimeric TALEN pairs constructed induced mutations with efficiencies as high as 76%. For eight of the 10 homodimeric TALEN pairs, we constructed obligate heterodimeric versions using the ELD/KKR FokI nuclease domains. We found that these heterodimeric TALEN pairs exhibited either comparable or, in most

cases, increased somatic mutation activities (ranging from a two- to seven-fold increase). This finding was somewhat surprising because it differs from what has been observed with the activities of heterodimeric ELD/KKR ZFNs in human cells where these nucleases show somewhat lower activities than their homodimeric counterparts (24). The heterodimeric TALENs also appear to exhibit less cytotoxicity than their matched homodimeric counterparts as judged by the percentage of normal embryos observed after injection of TALEN-encoded mRNAs. This finding is consistent with the observation that heterodimeric ZFNs possess improved specificities relative to matched homodimeric ZFNs (24, 28). Our results show that TALENs assembled on the FLASH platform have a high success rate (as measured by their abilities to induce mutations in somatic zebrafish cells) and that active nucleases can efficiently mutagenize their intended targets, a finding consistent with our recent demonstration that FLASH-assembled TALENs have a >88% success rate for targeting endogenous genes in human cells and that active nucleases induce mutations with high efficiencies (18). In addition, our findings suggest that heterodimeric ELD/KKR TALENs may be more active and less toxic than their homodimeric counterparts. Taken together, we conclude that heterodimeric ELD/KKR TALENs are preferable to homodimeric TALENs for use in zebrafish.

Our data provide a larger scale demonstration that TALEN-induced mutations can be successfully transmitted through the germline. Previous reports with TALENs in zebrafish have shown germline transmission for only two zebrafish genes (20). In this report, we show that TALEN-induced mutations in eight different endogenous gene targets can be transmitted through the germline using either homodimeric or heterodimeric TALENs. In some cases, we found that all the fish we screened were founders. Our results suggest that founders can typically be identified after screening as few as 5–10 fish injected with TALEN mRNAs if the somatic mutation rate of the TALENs is above 10%. Nevertheless, transmissible mutations may still occur even if the somatic mutation rate falls below 10%, as in the case of *elmo1* and as has been shown in a previous report (20). In this study, more than 70% of the effective homodimeric or heterodimeric TALEN pairs we tested resulted in a somatic mutation rate above 10%.

Interestingly, we found that one of our TALEN pairs exhibits a high degree of specificity for its intended target site. This pair showed greatly reduced activity at a polymorphic target allele that contains two mismatches from the intended sequence. Targeting efficiency for this polymorphic sequence was decreased significantly for both homodimeric and heterodimeric TALENs. This result indicates that TALENs can exhibit strong specificity for their intended binding sequence even when an alternative target bears as few as two differences. This finding parallels that observed for a TALEN pair targeted to the human *CCR5* gene, which showed selectivity against an off-target site that differed by only two mismatches from the intended site (29). Our result also strongly suggests the exciting possibility that it may be possible to design allele-specific TALENs that can selectively mutagenize one allele when two different alleles are present in a single cell. Additional experiments will be needed to test whether this finding can be generalized to other target sites.

The results of our study demonstrate that the TALEN technology is a simple, robust and efficient platform for performing targeted gene disruption in zebrafish. Our findings lead to some practical suggestions for investigators interested in using this technology: (i) Most genes can be successfully targeted by generating TALENs for just one or two sites, (ii) heterodimeric ELD/KKR TALENs are superior to homodimeric TALENs due to their higher activities and lower toxicities and (iii) founders can be identified by screening as few as a handful of fish. The results of this report, together with another recent study showing that TALENs have an essentially limitless targeting range and can be rapidly assembled in high-throughput using the FLASH assembly method (18), strongly suggest that it may now be feasible to rapidly create very large collections of mutant zebrafish. We also note that all TALENs used in this and our previous study (19) are built on a particular architecture that has been shown to yield nucleases that can efficiently modify genes in *C. elegans* (22), rats (30) and human somatic (18, 23) and pluripotent stem cells (21). A number of other architectures for building TALENs have been described (20, 29, 31–42), and it will be of interest to see whether TALENs built on these other platforms also show similarly high success rates and efficiencies in zebrafish.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–8.

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