Rapid Mutation of Endogenous Zebrafish Genes Using Zinc Finger Nucleases Made by Oligomerized Pool ENgineering (OPEN)

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(Article begins on next page)
Rapid Mutation of Endogenous Zebrafish Genes Using Zinc Finger Nucleases Made by Oligomerized Pool ENGineering (OPEN)

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Abstract

Background: Customized zinc finger nucleases (ZFNs) form the basis of a broadly applicable tool for highly efficient genome modification. ZFNs are artificial restriction endonucleases consisting of a non-specific nuclease domain fused to a zinc finger array which can be engineered to recognize specific DNA sequences of interest. Recent proof-of-principle experiments have shown that targeted knockout mutations can be efficiently generated in endogenous zebrafish genes via non-homologous end-joining-mediated repair of ZFN-induced DNA double-stranded breaks. The Zinc Finger Consortium, a group of academic laboratories committed to the development of engineered zinc finger technology, recently described the first rapid, highly effective, and publicly available method for engineering zinc finger arrays. The Consortium has previously used this new method (known as OPEN for Oligomerized Pool ENGineering) to generate high quality ZFN pairs that function in human and plant cells.

Methodology/Principal Findings: Here we show that OPEN can also be used to generate ZFNs that function efficiently in zebrafish. Using OPEN, we successfully engineered ZFN pairs for five endogenous zebrafish genes: ttr2, dopamine transporter, telomerase, hif1aa, and gridlock. Each of these ZFN pairs induces targeted insertions and deletions with high efficiency at its endogenous gene target in somatic zebrafish cells. In addition, these mutations are transmitted through the germline with sufficiently high frequency such that only a small number of fish need to be screened to identify founders. Finally, in silico analysis demonstrates that one or more potential OPEN ZFN sites can be found within the first three coding exons of more than 25,000 different endogenous zebrafish gene transcripts.

Conclusions and Significance: In summary, our study nearly triples the total number of endogenous zebrafish genes successfully modified using ZFNs (from three to eight) and suggests that OPEN provides a reliable method for introducing targeted mutations in nearly any zebrafish gene of interest.

Introduction

Engineered zinc finger nucleases (ZFNs) form the basis of a broadly applicable technology for highly efficient genome modification [1–6]. ZFNs function as dimers [7] with each monomer consisting of an engineered zinc finger array (typically composed of three or four fingers) fused to a non-specific cleavage domain from the FokI endonuclease [8,9]. Zinc finger arrays in ZFNs can be engineered to bind target DNA sequences of interest [10–17], thereby enabling the introduction of double-strand DNA breaks (DSBs) into specific genomic sequences.

ZFNs can be used to alter endogenous genes in Drosophila and mammalian cells with absolute efficiencies ranging from 1%–50% [18–26]. ZFN-induced DSBs can be repaired by non-homologous end-joining (NHEJ), an imperfect process which frequently results in the creation of insertions and deletions (indels) at the site of the break. Alternatively, repair of a ZFN-induced DSB by homologous recombination (HR) with an appropriately designed exogenous
“donor template” (an approach known as “gene targeting”) can be used to introduce a specific mutation near the break or to insert a DNA sequence at the the break.

Recent proof-of-principle studies have shown that ZFNs can also be used to create targeted NHEJ-mediated knockout mutations in endogenous zebrafish genes. Wolfe and Lawson created ZFN-induced knockouts in the kdr gene [27] while Amacher and colleagues mutated the golden and tfl genes [20]. These results demonstrate that ZFNs can provide an important genetic capability previously unavailable to researchers in the zebrafish field and have created much excitement in the community.

An important question raised by these groundbreaking studies is how can the typical zebrafish researcher generate the customized ZFNs required to practice this targeted knockout technology [29]. The Wolfe and Lawson kdr ZFNs [27] were made using a modified version of a previously described two-stage optimization strategy [30]. This approach is very difficult for the non-specialist scientist to practice because it requires the construction and interrogation of three partially randomized zinc finger libraries and of a secondary recombinant library derived from the outputs of the initial three libraries. The tfl and golden ZFNs used by Amacher and colleagues were constructed using a proprietary engineering platform developed by Sangamo BioSciences, Inc. [28]. ZFNs made by this proprietary method can be purchased from Sigma-Aldrich but the high fee charged per ZFN pair [31] may make it difficult for most labs to purchase ZFNs for more than one or two genes of interest. A third method previously used to make ZFNs (for use in other cell types) is the “modular assembly” approach in which zinc fingers with pre-selected specificities are joined together [32–35]. However, a recent large-scale assessment of the modular assembly method demonstrated that it is highly inefficient with a success rate for making functional ZFN pairs that is at best ~6% [36].

The Zinc Finger Consortium recently described the development and validation of a rapid, highly effective, and publicly available method for engineering zinc finger arrays termed OPEN (for Oligomerized Pool ENgineering) [26]. OPEN requires the construction of only a single recombinant zinc finger library (smaller than 106 in size) and yields ZFNs that function with high efficiencies in human and plant cells [26]. The method accounts for the context-dependent DNA-binding activities of zinc fingers, a parameter that previous studies have suggested is important for creating arrays with high DNA-binding affinities and specificities [30,37–42]. In direct comparisons, OPEN exhibited a much higher success rate for yielding functional ZFNs than the modular assembly method [26]. In indirect comparisons performed with different target sites, ZFNs made by OPEN also exhibited activities and toxicities comparable to ZFNs made by the proprietary Sangamo BioSciences approach [26].

In this study, we use a modified and more rapid version of OPEN to generate ZFNs for five endogenous zebrafish gene targets. We show that these OPEN ZFNs efficiently induce indel mutations in their respective endogenous gene targets in somatic zebrafish cells. In addition, we demonstrate germline transmission of ZFN-induced mutations for four of the five gene targets. Finally, we use in silico analysis to show that one or more potential OPEN ZFN target sites can be found within the first three coding exons of more than 25,000 transcripts derived from endogenous zebrafish genes. Our results demonstrate that OPEN can rapidly generate ZFNs for efficient mutation of endogenous genes in zebrafish and provide strong additional support for its use with this important model organism.

Results

Using OPEN to engineer zinc finger arrays for endogenous zebrafish gene targets

We used the OPEN method to engineer zinc finger arrays for potential ZFN target sites in five different endogenous zebrafish genes: dopamine transporter (dit), hypoxia-inducible factor 1α (hif1α), tolubarine, transferrin receptor 2 (tfr2), and gridlock. The targeted genes differ widely in size, genomic location, and functional class (channel, receptor, enzyme, transcription factor) and were selected for their relevance to ongoing zebrafish research projects or for their general utility for the zebrafish community. We used the web-based ZiFiT v3.0 software program (http://bindr.gdb.iastate.edu/ZiFiT/) [26] to identify potential target sites in the coding sequences of these genes. Ten selections (one for each half-site in the five full ZFN target sites) were performed using an improved, more rapid version of our recently described OPEN method (Figure 1). Alterations made to the original method included miniaturization of the selections so that they can be performed using multi-channel pipets, multi-well (24-well) blocks, and smaller amounts of solid and liquid media (see Materials and Methods). These alterations have led to a substantial increase in the speed of the procedure: as many as 48 selections can now be completed by two individuals in less than 8 weeks time.

The OPEN selections we performed successfully yielded multiple active zinc finger arrays for all 10 target half-sites (Table 1). As before, we defined successful arrays as those that can activate transcription of a lacZ reporter gene by three-fold or more in the B2H system [26]. Previous studies have shown that zinc finger arrays which activate transcription above this threshold in the B2H system possess high affinity and high specificity for their cognate DNA binding site [30]. For comparison, we also tested the zinc finger arrays from the kdr ZFNs previously made by Wolfe and Lawson [27] on their respective target half-sites. Interestingly, we found that although one of the arrays (ZFP1) activated transcription more than three-fold in the B2H system, the other (ZFP2) failed to show any activation (Table 1). The lower activity of the kdr ZFP2 zinc finger array in the B2H system may be due to low DNA-binding specificity as well as to the lower stringency of the bacterial one-hybrid (B1H) system used to select the kdr-targeted zinc finger arrays (see Discussion below).

Efficient somatic cell mutation of endogenous zebrafish genes using OPEN ZFNs

We next tested the abilities of zinc finger arrays obtained by OPEN to induce mutations when expressed as ZFNs in somatic zebrafish cells. To do this, we chose one zinc finger array for each ZFN target half-site and tested pairs as ZFNs (highlighted in bold italics in Table 1). To test the robustness of our OPEN selections, we chose zinc finger arrays with high (but not always the highest) B2H fold-activation for testing as ZFN pairs; however, all arrays tested met the minimum three-fold B2H activation threshold described above. DNA fragments encoding these zinc finger arrays were cloned into ZFN expression vectors previously constructed by the Joung lab (see Materials and Methods for details) [26]. The ten resulting vectors encode ZFNs consisting of a FLAG epitope tag, an SV40 nuclear localization signal, and a zinc finger array fused to an obligate heterodimeric FokI nuclease domain [43]. These vectors also harbor a bacteriophage T7 promoter positioned upstream of the ZFN coding sequence. As a positive control, we also constructed two additional ZFN expression plasmids which encoded obligate heterodimeric ZFNs harboring the Wolfe/Lawson kdr zinc finger arrays (ZFP1 and ZFP2) [27]. We note that these control kdr ZFN plasmids are identical to our OPEN ZFN expression vectors except for the sequences encoding the zinc

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finger arrays. We transcribed RNA from each of these 12 ZFN expression plasmids and performed poly A-tailing of the RNA as described in Materials and Methods.

In an initial control experiment to test whether our ZFN vectors and experimental conditions would work efficiently in zebrafish, we injected 100 embryos each with 100 pg of purified RNA made from the pair of vectors encoding the Wolfe/Lawson kdr ZFNs (50 pg of RNA encoding each ZFN). As shown in Figure 2, we observed that approximately 79% of the embryos were dead or exhibited a highly deformed “monster” phenotype, consistent with previously published experiments performed with these ZFNs [27]. Furthermore, we observed that we could not inject more than 100 pg of RNA/embryo without causing death in almost all embryos [data not shown]. To assess whether targeted mutagenesis of kdr occurred at the somatic cell level, we harvested genomic DNA from a pool of 10 embryos two days post-injection and sequenced the region of the kdr gene targeted by the Wolfe/Lawson ZFNs using a limited cycle PCR/DNA-sequencing method previously described and validated by the Joung lab for quantitation of mutations in a population of alleles [26]. As shown in Figure 3A, 10% of the kdr alleles we sequenced harbored insertions or deletions at the site of the ZFN-induced DSB, a
Table 1. Recognition helix (RH) amino acid sequences and B2H activities of zinc finger arrays for endogenous zebrafish gene targets.

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<th>Site Name</th>
<th>F1 subsite/RH sequence</th>
<th>F2 subsite/RH sequence</th>
<th>F3 subsite/RH sequence</th>
<th>Mean B2H fold-activation</th>
<th>S.D. of B2H fold-activation</th>
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</table>
endogenous genes in somatic zebrafish cells.

We conclude that OPEN ZFNs can efficiently induce mutations at indel mutations are predicted to create frameshift mutations with mutagenesis rates ranging from 3%–20%. Nearly all of these deletions at the ZFN cleavage site for all five endogenous genes targets. As shown in Figures 3B–3F, we observed insertion or deletions at the ZFN cleavage site for all five endogenous genes targets. We tested whether ZFN-induced mutations observed in somatic cell experiments described above were allowed to mature to adulthood and crossed with wild-type fish (fish in which gridlock had been targeted have not yet reached maturity and therefore have not yet been tested). To identify founders, we analyzed individual embryos from these crosses using either direct DNA sequencing or a restriction digest assay that checks for the loss of a restriction site located at the ZFN-induced DSB (see Materials and Methods). As shown in Table 2, founders were identified at frequencies of ~6%, 33%, 25%, and 50% for mutations in the dat, tf2, telomerase and hif1aa genes, respectively. The percentages of embryos harboring ZFN-induced mutations from founders ranged from 9% to 60% (Table 2). We sequenced the mutations from a subset of these embryos to determine the molecular nature of the indels and found both frame-shifted and frame-preserved mutations (Figure 4).

Efficient germline transmission of mutations induced by OPEN ZFNs

We tested whether ZFN-induced mutations observed in somatic zebrafish cells could be transmitted efficiently through the germline. Injected embryos remaining from four of the five somatic cell experiments described above were allowed to mature to adulthood and crossed with wild-type fish (fish in which gridlock had been targeted have not yet reached maturity and therefore have not yet been tested). To identify founders, we analyzed individual embryos from these crosses using either direct DNA sequencing or a restriction digest assay that checks for the loss of a restriction site located at the ZFN-induced DSB (see Materials and Methods). As shown in Table 2, founders were identified at frequencies of ~6%, 33%, 25%, and 50% for mutations in the dat, tf2, telomerase and hif1aa genes, respectively. The percentages of embryos harboring ZFN-induced mutations from founders ranged from 9% to 60% (Table 2). We sequenced the mutations from a subset of these embryos to determine the molecular nature of the indels and found both frame-shifted and frame-preserved mutations (Figure 4). We conclude that mutations generated by OPEN ZFNs undergo efficient germline transmission in zebrafish.

In silico identification of OPEN ZFN targets within endogenous zebrafish genes

Using a program similar to the web-based software program ZiFiT v3.0 [26,44], we searched for sites in endogenous zebrafish genes that could potentially be targeted by OPEN (see Materials and Methods). To do this, we searched the first three coding exons of 29,291 protein-coding gene transcripts that have known mapped chromosomal locations and that are present in the Ensembl Danio rerio database (release 51; http://www.ensembl.

Table 1. cont.

<table>
<thead>
<tr>
<th>ZFN Name</th>
<th>Site Name</th>
<th>F1 subsite/RH sequence</th>
<th>F2 subsite/RH sequence</th>
<th>F3 subsite/RH sequence</th>
<th>Mean B2H fold-activation</th>
<th>S.D. of B2H fold-activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>OZ489</td>
<td>TRR2_2R</td>
<td>QROALDR</td>
<td>QDQNLTR</td>
<td>VQGNLAR</td>
<td>5.52</td>
<td>0.66</td>
</tr>
<tr>
<td>OZ490</td>
<td>TRR2_2R</td>
<td>QROALDR</td>
<td>QATNLOR</td>
<td>VGSNLR</td>
<td>5.23</td>
<td>0.26</td>
</tr>
<tr>
<td>OZ491</td>
<td>TRR2_2R</td>
<td>SAQALAR</td>
<td>QDQNLAR</td>
<td>VGSNLR</td>
<td>6.03</td>
<td>1.78</td>
</tr>
<tr>
<td>OZ492</td>
<td>TRR2_2R</td>
<td>QROALDR</td>
<td>QDQNLSR</td>
<td>VGSNLR</td>
<td>5.63</td>
<td>0.42</td>
</tr>
<tr>
<td>OZ493</td>
<td>TRR2_2R</td>
<td>QROALDR</td>
<td>QGQNLR</td>
<td>VGSNLSR</td>
<td>6.57</td>
<td>0.12</td>
</tr>
<tr>
<td>OZ494</td>
<td>TRR2_2R</td>
<td>QROALDR</td>
<td>QDQNLTR</td>
<td>VGSNLR</td>
<td>6.76</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Each OPEN zinc finger array was assigned an OZ designation which permits their unique identification in the web-based Zinc Finger Database (ZiFDB) program [47]. Previously published zinc finger arrays targeted to the kdr gene (isolated by B1H selection) [27] are also shown. Each nine bp target site was named as follows: “gene name or abbreviation”, “exon number”, and “L” or “R” indicating left or right half-site. The amino acids selected in the three zinc finger recognition helices of each array are shown (residues are shown left to right in the order 1, 2, 3, 4, 5, 6 numbered relative to the helix start). B2H values that fall below the cut-off of three-fold activation in the B2H system are italicized. The names of zinc finger arrays tested as ZFNs in zebrafish are shown in bold italics. Abbreviations key: DT = dopamine transporter, HIF = hif1aa, Telo = telomerase, TR2 = transferrin receptor 2, and Grck = gridlock.

doi:10.1371/journal.pone.0004348.t001
In this report, we used the recently described OPEN zinc finger engineering method to rapidly generate ZFNs that can modify endogenous zebrafish gene targets with high efficiency. In less than two months, we generated ZFNs for target sites in five different biologically important genes. The five pairs of ZFNs we tested can efficiently generate mutations at their intended endogenous gene target in somatic zebrafish cells. In addition, we demonstrated that mutations induced by OPEN ZFNs can be efficiently transmitted through the germline. These results suggest that only a small number of fish need to be screened to identify founders, consistent with previously published results [27,28]. Our results nearly triple the total published number of endogenous zebrafish genes successfully modified using ZFNs from three (kdr, golden, and ntl) to eight and demonstrate that OPEN is a highly effective ZFN engineering method for creating targeted mutations in zebrafish.

In silico analysis indicates that as many as 25,174 zebrafish gene transcripts have one or more potential OPEN ZFN target sites in their first three coding exons and as many as 20,418 transcripts have two or more sites in the first three coding exons. Our
Rapid Mutation by OPEN ZFNs

A. $kdr$ (10 pooled embryos, 40 sequences total):

<table>
<thead>
<tr>
<th>ZFN-L</th>
<th>ZFN-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGATGGAGATACACACTTCAGCATATGAGGGACAGCTCACTTCATTGACAGGACTG</td>
<td>WT [36x]</td>
</tr>
</tbody>
</table>

CTGATGGAGATACACACTTCAGCATATGAGGGACAGCTCACTTCATTGACAGGACTG | Δ3 [1] |
CTGATGGAGATACACACTTCAGCATATGAGGGACAGCTCACTTCATTGACAGGACTG | Δ5 [1] |
CTGATGGAGATACACACTTCAGCATATGAGGGACAGCTCACTTCATTGACAGGACTG | +4 [1] |
CTGATGGAGATACACACTTCAGCATATGAGGGACAGCTCACTTCATTGACAGGACTG | +3 [1] |

Mutations in 4 of 40 sequences: 10%

B. $tfr2$ (10 pooled embryos, 45 sequences total):

<table>
<thead>
<tr>
<th>ZFN-L</th>
<th>ZFN-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAGTCAGGCCGAGAAGCCCTCTATTTGAGTAACAGATTGGTCTGACACTGG</td>
<td>wild-type [36x]</td>
</tr>
</tbody>
</table>

GAGTCAGGCCGAGAAGCCCTCTATTTGAGTAACAGATTGGTCTGACACTGG | Δ20 [3x] |
GAGTCAGGCCGAGAAGCCCTCTATTTGAGTAACAGATTGGTCTGACACTGG | +4 [1x] |
GAGTCAGGCCGAGAAGCCCTCTATTTGAGTAACAGATTGGTCTGACACTGG | +5 (Δ11 and +16) [1x] |

Mutations in 9 of 45 sequences: 20%

C. dopamine transporter (12 pooled embryos, 47 sequences total):

<table>
<thead>
<tr>
<th>ZFN-L</th>
<th>ZFN-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTGTTTACAGCTGTTGACACCCTCTTCAGCTGCCCAGGTCAATTCTCTTTT</td>
<td>wild-type [42x]</td>
</tr>
</tbody>
</table>

GTGTTTACAGCTGTTGACACCCTCTTCAGCTGCCCAGGTCAATTCTCTTT | Δ7 (Δ13 and +6) [1x] |
GTGTTTACAGCTGTTGACACCCTCTTCAGCTGCCCAGGTCAATTCTCTTT | +3 [1x] |
GTGTTTACAGCTGTTGACACCCTCTTCAGCTGCCCAGGTCAATTCTCTTT | +4 [1x] |
GTGTTTACAGCTGTTGACACCCTCTTCAGCTGCCCAGGTCAATTCTCTTT | +4 [2x] |

Mutations in 5 of 47 sequences: 10.6%

D. telomerase (10 pooled embryos, 40 sequences total):

<table>
<thead>
<tr>
<th>ZFN-L</th>
<th>ZFN-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGGCTCGCCTCCTGCTAGTGCAAGAAGCCTGCTGCGTTG</td>
<td>wild-type [35x]</td>
</tr>
</tbody>
</table>

GGGCTCGCCTCCTGCTAGTGCAAGAAGCCTGCTGCGTTG | Δ13 [1x] |
GGGCTCGCCTCCTGCTAGTGCAAGAAGCCTGCTGCGTTG | +0 (Δ6 and +6) [1x] |
GGGCTCGCCTCCTGCTAGTGCAAGAAGCCTGCTGCGTTG | +4 [1x] |
GGGCTCGCCTCCTGCTAGTGCAAGAAGCCTGCTGCGTTG | +7 (Δ4 and +11) [1x] |
GGGCTCGCCTCCTGCTAGTGCAAGAAGCCTGCTGCGTTG | +14 (Δ2 and +16) [1x] |

Mutations in 5 of 47 sequences: 12.5%

E. hif1aa (4 pooled embryos, 32 sequences total):

<table>
<thead>
<tr>
<th>ZFN-L</th>
<th>ZFN-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCCGGGCCCCTCTGATCGCTGCACTGCACTGCATGAGGCTGCGCTGCG</td>
<td>wild-type [30x]</td>
</tr>
</tbody>
</table>

TCCGGGCCCCTCTGATCGCTGCACTGCACTGCATGAGGCTGCGCTGCG | Δ25 [1x] |
TCCGGGCCCCTCTGATCGCTGCACTGCACTGCATGAGGCTGCGCTGCG | +2 [1x] |

Mutations in 2 of 32 sequences = 6.25%

F. gridlock (5 pooled embryos, 33 sequences total):

<table>
<thead>
<tr>
<th>ZFN-L</th>
<th>ZFN-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGCACTCCATCTCTGGCTGTTCTGCAGCAGGCTGAGGCTCCC</td>
<td>wild-type [32x]</td>
</tr>
</tbody>
</table>

GCATCCATCTCTGGCTGTTCTGCAGCAGGCTGAGGCTCCC | Δ40 [1x] |

Mutation in 1 of 33 sequences = 3.03%
previous experience using OPEN to make ZFNs for endogenous human gene targets suggests that the success rate of this method is high but not 100% [26]. Assuming that a similar experience holds true in zebrafish, a prudent strategy might be to target more than one sequence in a gene of interest to improve the chances of successfully mutating that gene. Our analysis shows that most zebrafish gene transcripts possess multiple potential OPEN ZFN target sites and therefore suggests that a large number of genes will be targetable by such a strategy. As the academic community collectively gains experience using OPEN ZFNs in zebrafish (and other organisms), we anticipate that algorithms for identifying potential OPEN ZFN target sites will also continue to improve and evolve, thereby allowing for targeting of fewer sites per gene. Nonetheless, our results strongly suggest that OPEN in its current

### Table 2. Frequencies of mutations from founder analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th># of fish screened</th>
<th># of mutant founder fish</th>
<th>transmission rate for each mutant founder</th>
</tr>
</thead>
<tbody>
<tr>
<td>tfr2</td>
<td>12</td>
<td>4</td>
<td>1/10 (10%) 1/10 (10%) 2/11 (18%) 6/10 (60%)</td>
</tr>
<tr>
<td>dat</td>
<td>17</td>
<td>1</td>
<td>6/30 (20%)</td>
</tr>
<tr>
<td>telo</td>
<td>8</td>
<td>2</td>
<td>1/11 (9%) 1/11 (9%)</td>
</tr>
<tr>
<td>hif1 aa</td>
<td>2</td>
<td>1</td>
<td>1/11 (9%)</td>
</tr>
</tbody>
</table>

Numbers of fish screened for mutant founders are shown for each gene. For each mutant founder identified, the transmission rate is shown as the # of mutant embryos/# of embryos screened and as a percentage of mutant embryos identified (in parentheses). tfr2 = transferrin receptor 2; dat = dopamine transporter; telo = telomerase; hif1 aa = hypoxia-inducible factor.

doi:10.1371/journal.pone.0004348.t002

Figure 3. Frequencies and sequences of ZFN-induced mutations in somatic zebrafish cells. For each gene targeted by ZFNs, the wild-type sequence is shown at the top with ZFN binding sites marked. Deletions are indicated by grey highlighted red dashes and insertions by blue highlighted lower case blue letters. The number of times each wild-type mutant allele was isolated is shown in brackets.

doi:10.1371/journal.pone.0004348.g003

Figure 4. Sequences of ZFN-induced mutations transmitted through the germline. For each target gene, the wild-type sequence is shown at the top with ZFN binding sites marked and the mutated alleles found in founder progeny are shown below the wild-type sequence. Each mutant sequence shown was isolated from progeny of different founders. Deletions are indicated by grey highlighted red dashes and insertions by blue highlighted lower case blue letters.

doi:10.1371/journal.pone.0004348.g004

A. **tfr2:**

- **ZFN-L:**
  - GAGTCAGGCGGAGAGCCCCCTCATTGAGTGAAGCTGTGCCGCTGG
  - wild-type

- **ZFN-R:**
  - GAGTCAGGCGGAGAGCCCCCTCATTGAGTGAAGCTGTGCCGCTGG
  - mutant founder 1 (+4)

  - GAGTCAGGCGGAGAGCCCCCTCATTGAGTGAAGCTGTGCCGCTGG
  - mutant founder 2 (+7 [+9 and Δ2])

  - GAGTCAGGCGGAGAGCCCCCTCATTGAGTGAAGCTGTGCCGCTGG
  - mutant founder 3 (Δ2)

  - GAGTCAGGCGGAGAGCCCCCTCATTGAGTGAAGCTGTGCCGCTGG
  - mutant founder 4 (Δ2)

B. **dopamine transporter:**

- **ZFN-L:**
  - GTGTTTACGCTGTGGACGACCTTCGGCGGAGGCTCAATTCTCTTT
  - wild-type

- **ZFN-R:**
  - GTGTTTACGCTGTGGACGACCTTCGGCGGAGGCTCAATTCTCTTT
  - mutant founder 1 (Δ6)

C. **telomerase:**

- **ZFN-L:**
  - GGCTCCGGCCACCTCCTACATCGCTCAAGAGCTGGCTGGG
  - wild-type

- **ZFN-R:**
  - GGCTCCGGCCACCTCCTACATCGCTCAAGAGCTGGCTGGG
  - mutant founder 1 (Δ4)

  - GGCTCCGGCCACCTCCTACATCGCTCAAGAGCTGGCTGGG
  - mutant founder 2 (Δ5)

D. **hif1aa:**

- **ZFN-L:**
  - CCACCACCACCCCATACCTGCTGGGAGGCTGGCAAGACAT
  - wild-type [30x]

- **ZFN-R:**
  - CCACCACCACCCCATACCTGCTGGGAGGCTGGCAAGACAT
  - mutant founder 1 (+5)

Rapid Mutation by OPEN ZFNs
form can be used to engineer ZFNs for a high percentage of zebrafish genes.

The reagents we used to construct and express ZFNs in our experiments are part of the OPEN Zinc Finger Consortium platform for engineering zinc finger arrays. Zinc finger arrays constructed by OPEN can be excised as XbaI/BamHI fragments and cloned directly into expression plasmids which then encode FLAG-tagged, NLS-tagged, obligate heterodimeric ZFNs. ZFN-encoding RNA can be directly transcribed from these plasmids using T7 RNA polymerase. OPEN Zinc Finger Consortium reagents are publicly available to academic laboratories through either Addgene (a non-profit plasmid distribution service; see http://www.addgene.org/zfc) or the Joung lab. All engineering and validation steps in the OPEN method are carried out in simple and inexpensive E. coli-based systems. Collectively, these reagents provide academics with an important platform which enables rapid engineering of ZFNs for zebrafish genes.

When compared with ZFNs previously made to the kdr gene using a bacterial one-hybrid-based method, OPEN ZFNs were equally efficient at inducing mutations at their respective target sites but showed less toxicity and teratogenesis, even when larger amounts of ZFN-encoding RNA were injected. (Others have also noted the relatively greater toxicity of the kdr ZFNs compared with the golden and ntl ZFNs made using the Sangamo BioSciences zinc finger engineering technology [29,45] although we note that those comparisons were made between experiments which used different ZFN expression vectors.) The difference in toxicity we observed between the kdr ZFNs and our OPEN ZFNs is most likely due to the quality of the zinc finger arrays because all other sequences in the expression vectors are otherwise identical. One possible explanation for the greater toxicity of the kdr ZFNs is that the B1H system used to create them might permit identification of zinc finger arrays with lower specificities than those identified by the OPEN B2H system. Consistent with this, we note that one of the zinc finger arrays (ZFP2) used to make the kdr ZFNs failed to activate transcription in the B2H system (Table 1) and therefore would not have been identified as a positive clone if the kdr site had been targeted using the OPEN method. This result suggests that the B1H method used to engineer the kdr zinc finger arrays may be less stringent than the B2H-based OPEN approach, perhaps due to the use of a multi-copy reporter in the B1H system as opposed to the single-copy reporter used in the B2H system. This difference might reduce the selective pressure for DNA-binding specificity since the target DNA site in the B1H system will be present at a higher concentration in the bacterial cell relative to the “non-specific” DNA of the E. coli chromosome. An important priority for future work will be to examine the spectrum and range of “off-target” sites altered by OPEN ZFNs, perhaps using methods previously described by other groups [27,28].

The ability of OPEN to rapidly yield high quality ZFNs for a large number of different target sites should also improve prospects for using ZFNs to induce precise homologous recombination (HR) events at endogenous zebrafish genes. Repair of a ZFN-induced DSB by HR with an appropriately designed exogenous “donor template” (a process known as ZFN-induced gene targeting) has been used to introduce specific alterations or insertions with high efficiencies at endogenous genes in Drosophila [18,19], plant [Townernd et al., manuscript submitted], and human cells [23–26,43]. However, our experience using ZFNs in human cells suggests that ZFN-induced homologous recombination can be much more challenging to implement than ZFN-induced mutagenic NHEJ-mediated repair. For example, not all ZFNs that can induce NHEJ at their target sites will necessarily promote efficient HR (M. Maeder, S. Begannya, and J.K. Joung, unpublished results). Thus, the ability to use the OPEN method to rapidly engineer ZFNs with both high activities and low toxicities for many different target sites should greatly enhance prospects for successfully using ZFNs to induce specific HR events in zebrafish.

Materials and Methods

OPEN selections of zinc finger arrays

Zinc finger arrays were selected using the OPEN method essentially as previously described [26] but with a small number of alterations that improve the speed and throughput of the protocol. We briefly summarize the overall protocol here with greater detail provided for steps of the protocol we altered for this report. A more detailed step-by-step protocol for performing OPEN selections is forthcoming (Maeder et al., manuscript in preparation) and is currently available upon request from the Joung lab. To create recombinant libraries of zinc finger arrays for use in OPEN selections, zinc finger pools for target triplet subsites [26] were amplified by PCR using primers and conditions as previously described [26]. Amplified finger pool products were purified on 10% polyacrylamide gels and then fused together by PCR to create random combinations of three-finger arrays. These fusion PCR reactions were performed with equal concentrations of the three purified finger pool fragments and using primers and cycling conditions as previously described [26]. The resulting PCR product encoding a collection of three-finger arrays was purified on a 5% polyacrylamide gel and treated with Pfu polymerase and T4 polynucleotide kinase to create ligation-ready overhangs [26]. This fragment was then ligated to pBR-UV3-GP-FD2 vector that had been digested with the restriction enzyme BsdI. The resulting plasmids express the collection of zinc finger arrays as FLAG-tagged Gal4P fusions in the B2H system. Electroporation was then used to introduce these ligation products into XL-1 Blue E. coli cells and into a B2H selection strain harboring the full target DNA sequence of interest. For the selections described in this report, we grew selection strains as 1 ml cultures in 24-well, 10 ml-capacity

| Table 3. Summary of potential OPEN ZFN target sites identified in zebrafish transcripts |
|---------------------------------------------|----------------|----------------|----------------|
| Number of transcripts with:                | ...in the first coding exon | ...in the first two coding exons | ...in the first three coding exons |
| One or more potential ZFN sites...         | 14,623          | 21,781          | 25,174         |
| Two or more potential ZFN sites...         | 9,292           | 15,961          | 20,418         |

A total of 29,291 gene transcripts derived from the Ensembl release 51 Danio rerio database were analyzed for potential OPEN ZFN target sites (see Materials and Methods for additional details). doi:10.1371/journal.pone.0004348.t003
pyramidal well blocks in a Microtittertron shaker (Appropriate Technical Resources, Inc.) at 350 rpm, 37°C, 80% humidity. These cultures were grown in NM medium supplemented with 30 µg/ml chloramphenicol, 30 µg/ml kanamycin, and 50 µM IPTG. Following overnight growth, selection strain cultures were infected with a matched combinatorial zinc finger array phagemid phage library constructed as described above. Following phage infection, 4 ml of NM medium [46] supplemented with 30 µg/ml chloramphenicol, 30 µg/ml kanamycin, and 50 µM IPTG was added to the cells which were then shaken on the Microtittertron shaker for 1.5 hrs. The infected cells were then spun down and 4ml of the supernatant removed. The cell pellet was then resuspended in the remaining 1 ml of liquid media and 250 µl of this resuspension was plated on two different NM/CCK medium plates containing 50 µM IPTG, 10 mM 3AT, and 20 µg/mL streptomycin or 50 µM IPTG, 25 mM 3AT, and 40 µg/mL streptomycin. After 36–48 hours of incubation, colonies were harvested from the highest stringency plate yielding at least 1000 colonies as previously described [26]. The resulting cell suspension was then diluted with 4.5 ml 2XYT media at least 1000 infected/transformed cells) was then plated on a square 100mm NM medium agar plate supplemented with 50 µg/ml chloramphenicol and 30 µg/ml kanamycin to an OD600<0.1 in the 10 ml-capacity well of a 24-well block and allowed to grow for 1 hour in the Microtittertron shaker as described above. This subculture was infected with 10^10 kanamycin transducing units of M13K07 helper phage and then grown for six hours in the Microtittertron shaker. Phage-containing culture supernatants were harvested by filtering the cell cultures through a 0.22 µm polyethersulfone syringe filter.

In the second stage of OPEN selection, selection strain cells were again grown in 24-well blocks but in 1 ml of NM medium supplemented with 30 µg/ml chloramphenicol, 30 µg/ml kanamycin, and no IPTG. This overnight culture was infected with ~6x10^7 ampicillin-transducing units (ATU) of zinc finger-encoding phagemid phage rescued from the initial stage of selection. Following infection, 400 µl of NM medium supplemented with 30 µg/ml chloramphenicol and 30 µg/ml kanamycin was added to the cells which were then shaken on the Microtittertron shaker for 1.5 hrs. 375 µl of this infected culture (corresponding to ~5x10^3 infected/transformed cells) was then plated on a square 100mm x100mm NM medium agar plate supplemented with 100 µg/ml carbenicillin, 30 µg/ml chloramphenicol, 30 µg/ml kanamycin and containing gradients of 3-aminoxizole (from 0 to 80 mM) and streptomycin (from 0 to 100 µg/ml). Gradient plates were poured as previously described [26].

**Construction of ZFN expression vectors**

DNA sequences encoding zinc finger arrays identified by OPEN were transferred to ZFN expression vectors by using the phagemids encoding these arrays as templates for PCR reactions using primers OK.1677 and OK.1678 ([Supplemental Table 26](#)). The resulting DNA fragments (encoding the zinc finger arrays) were digested with Xbal and BamH1 and cloned into Xbal/BamH1-digested ZFN expression vectors pMLM335 or pMLM336 [26]. The pMLM335 and pMLM336 vectors encode previously described obligate heterodimeric ZFNs [43]. Final sequence-verified plasmids were prepared using a QiAgen HiSpeed Midiprep kit using RNase free reagents and stored in RNase-free Eppendorf Safe Lock Tubes.

**Preparation of ZFN-encoding RNA**

ZFN expression vectors were linearized with PseI (an enzyme which cleaves just 3’ to the end of the ZFN coding sequence) and transcribed in vitro using the T7 mMessage mMACHINE kit (Ambion). The transcribed ZFN RNAs were then polyadenylated using the Poly(A) Tailing kit (Ambion).

**Injection of zebrafish and analysis of somatic mutations**

Approximately 2 nl of the ZFN RNA (at concentrations of 50–400 pg/µl) was injected into one-cell stage zebrafish embryos. Two days following fertilization, the surviving injected embryos were grouped into either “normal” or “deformed” phenotypes. Genomic DNA was extracted from pools of 4–12 embryos from each “normal” group using DNA extraction buffer (10 mM Tris, pH 0.9, 200 mM NaCl, 10 mM EDTA, 0.5% SDS, 100 µg/ml Proteinase K), followed by phenol/chloroform extraction and ethanol precipitation. The DNA was resuspended in 40 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA).

2.5 µl of the resulting genomic DNA was then used as template for a PCR reaction using Platinum Taq DNA Polymerase High Fidelity enzyme (Invitrogen) with primers designed to anneal approximately 150 to 200 bp upstream and downstream from the expected mutation. The resulting PCR product was cleaned up using a QIAGEN MinElute PCR purification kit and then ligated using a ZeroBlunt TOPO kit (Invitrogen) into linearized pCR4 Blunt-TOPO vector. The ligation was transformed into Mach1 T1-bacteriophage resistant E.coli (Invitrogen) and plated on LB plates containing 50 µg/ml kanamycin. Following incubation overnight at 37°C, colonies were picked from these plates and inoculated into 700 µl TB medium containing 50 µg/ml kanamycin in 96-well blocks with 1ml pyramidal-bottom wells. These blocks were shaken at 900 rpm, 37°C, and 80% humidity in a Microtittertron shaker. Plasmid DNA was isolated from these cultures and sent for sequencing using the “T3 sequencing” primer ([Supplemental Table 26](#)).

**Identification and sequencing of germline transmitted mutations**

Potential founders were crossed with wild-type zebrafish. One to three dpf (days post fertilization), progeny were lysed individually in lysis buffer (10 mM Tris, pH 8.0, 2 mM EDTA, 0.1% Triton X-100, 100 µg/ml Proteinase K) and incubated at 50°C overnight. For each target gene, 10–12 embryos from each potential founder were screened for the presence of ZFN-induced mutations by amplifying the region surrounding the relevant ZFN cleavage site by PCR and then using either restriction digest- and/or DNA sequencing-based assays: For the tf2 gene, we used primers OK.1922 and OK.1923 to amplify the region surrounding the ZFN target site by PCR and the resulting ~405 bp product from each embryo was directly sequenced with primer Tfr2-seq ([Supplemental Table 26](#)). For the dopamine transporter gene, we used primers OK.1916 and OK.1917 ([Supplemental Table 26](#)) to amplify the region surrounding the ZFN target site by PCR and the resulting ~418 bp product was digested with the restriction enzyme ApeI. The PCR product from a wild-type allele will yield 5 fragments of 251, 68, 52, 25 and 7-bp sizes. Introduction of indel mutations at the ZFN target site will cause disruption of the ApeI site and result in the appearance of an additional 120-bp fragment which is detectable on a 3% agarose gel. PCR fragments from selected progeny that showed evidence for loss of the ApeI site were blunt-end cloned into the pCR4 Blunt-TOPO vector as described above and sequenced with the “T3 sequencing” primer. For the telomerase gene, we used primers OK.1928 and OK.1930 ([Supplemental Table 26](#)) to amplify the surrounding region by PCR and the resulting ~306 bp product was digested with the enzyme BspHI. The PCR product from a wild-type allele will contain only one BspHI site. Introduction of indel mutations at the ZFN target site will disrupt the BspHI site thereby resulting in the generation of PCR products resistant to digestion by BspHI. PCR
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fragments from progeny that were resistant to BspHI were blunt-end cloned into the pCR4 Blunt-TOPO vector as described above and sequenced with the “T3 sequencing” primer. For the hifaa gene, we used primers OK.1934 and OK.1935 (Supplemental Table 26) to amplify the surrounding region by PCR and the resulting ~401 bp product was digested with the enzyme BfdI. The PCR product from a wild-type allele will contain only one BfdI site. Introduction of indel mutations at the ZFN target site will disrupt the BfdI site thereby resulting in the generation of PCR products resistant to digestion by BfdI. PCR fragments from progeny that were resistant to BfdI were blunt-end cloned into the pCR4 Blunt-TOPO vector as described above and sequenced with the “T3 sequencing” primer.

Identification of potential OPEN ZFN target sites in zebrafish transcripts

ZFN target sites were generated from Danio rerio chromosomal contigs (Zv7) and gene table files (updated July 2008) from Ensembl (http://www.ensembl.org) for all mapped chromosomal protein coding transcripts. ZFN target sites were identified that can be targeted using currently available OPEN reagents [26] and that possess a spacer of 5, 6, or 7 nucleotides between the target half-sites. Only ZFN sites whose spacer falls entirely within an exon were identified as potential targets. In addition, because all previous 9 bp sites successfully targeted by OPEN to date have possessed at least one GNN triplet [26], we eliminated ZFN sites harboring one or more half-sites that are devoid of GNN triplets. Finally, because OPEN selections are performed in E. coli, ZFN sites containing either a dam or a denA methylation site in either half-site were also eliminated from the target list.

Supporting Information

Table S1 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 1. Potential OPEN ZFN target sites within transcripts were identified as described in Materials and Methods. Gene ID and Transcript ID are from the Ensembl Danio rerio release 51 database. “Strand” indicates whether the “Target Site” occurs on the forward (+) or reverse (−) strand. “ZFN Spacer Length” indicates the length of the spacer sequence located between the ZFN half-sites (5, 6, or 7 bps). “Coding Sequence Length” indicates the total nucleotide length of the coding sequence within the transcript and “ZFN Cleavage Site” indicates the nucleotide position of the cleavage site (i.e.-the first base of the “Target Site”) within the coding sequence.

Table S2 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 2. Data presented as described in the legend to Table S1.

Table S3 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 3. Data presented as described in the legend to Table S1.

Table S4 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 4. Data presented as described in the legend to Table S1.

Table S5 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 5. Data presented as described in the legend to Table S1.

Table S6 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 6. Data presented as described in the legend to Table S1.

Table S7 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 7. Data presented as described in the legend to Table S1.

Table S8 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 8. Data presented as described in the legend to Table S1.

Table S9 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 9. Data presented as described in the legend to Table S1.

Table S10 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 10. Data presented as described in the legend to Table S1.

Table S11 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 11. Data presented as described in the legend to Table S1.

Table S12 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 12. Data presented as described in the legend to Table S1.

Table S13 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 13. Data presented as described in the legend to Table S1.

Table S14 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 14. Data presented as described in the legend to Table S1.

Table S15 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 15. Data presented as described in the legend to Table S1.

Table S16 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 16. Data presented as described in the legend to Table S1.
Table S17 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 17. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s017 (2.02 MB XLS)

Table S18 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 18. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s018 (2.13 MB XLS)

Table S19 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 19. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s019 (2.06 MB XLS)

Table S20 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 20. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s020 (2.79 MB XLS)

Table S21 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 21. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s021 (1.72 MB XLS)

Table S22 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 22. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s022 (2.11 MB XLS)

Table S23 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 23. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s023 (1.98 MB XLS)

Table S24 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 24. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s024 (1.46 MB XLS)

Table S25 Potential OPEN ZFN target sites in gene transcripts encoded on zebrafish chromosome 25. Data presented as described in the legend to Table S1. Found at: doi:10.1371/journal.pone.0004348.s025 (1.58 MB XLS)

Table S26 Sequences of primers used in this study Found at: doi:10.1371/journal.pone.0004348.s026 (0.02 MB XLS)

Author Contributions
Conceived and designed the experiments: JEF JRJY MLM DR JDS RTP JKJ. Performed the experiments: JEF JRJY MLM DR JDS. Analyzed the data: JEF JRJY MLM DR JDS. Contributed reagents/materials/ analysis tools: MLM DR JDS RTP JKJ. Wrote the paper: JEF JRJY JDS RTP JKJ.

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