Inheritable and Precise Large Genomic Deletions of Non-Coding RNA Genes in Zebrafish Using TALENs

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Abstract

Transcription activator-like effector nucleases (TALENs) have so far been applied to disrupt protein-coding genes which constitute only 2–3% of the genome in animals. The majority (70–90%) of the animal genome is actually transcribed as non-coding RNAs (ncRNAs), yet the lack of efficient tools to knockout ncRNA genes hinders studies on their *in vivo* functions. Here we have developed novel strategies using TALENs to achieve precise and inheritable large genomic deletions and knockout of ncRNA genes in zebrafish. We have demonstrated that individual miRNA genes could be disrupted using one pair of TALENs, whereas large microRNA (miRNA) gene clusters and long non-coding RNA (lncRNA) genes could be precisely deleted using two pairs of TALENs. We have generated large genomic deletions of two miRNA clusters (the 1.2 kb *miR-17-92* cluster and the 79.8 kb *miR-430* cluster) and one long non-coding RNA (lncRNA) gene (the 9.0 kb *malat1*), and the deletions are transmitted through the germline. Taken together, our results establish TALENs as a robust tool to engineer large genomic deletions and knockout of ncRNA genes, thus opening up new avenues in the application of TALENs to study the genome *in vivo*.

Introduction

TALENs are artificial nucleases that consist of the DNA binding domain from transcription activator like effectors (TALE) and a catalytic domain from FokI nuclease [1–2]. TALEs bind to DNA through the repeat domain, with one TALE repeat recognizing one DNA base. The base specificity is determined by the 12th and 13th amino acids called repeat-variable di-residues (RVDs) in each TALE repeat, with RVDs NI, NG, HD and NN recognizing adenine (A), thymine (T), cytosine (C) and guanine (G), respectively [3–4]. These RVDs-DNA pairings bring the FokI nuclease to a predetermined genomic locus to create DNA double-strand breaks (DSB). Repair of the DSB through the error-prone non-homologous end-joining pathway leads to small indels at the break site, thus enabling targeted gene disruption. So far, TALENs have been employed to disrupt specific genomic loci in yeast [5], worms [1,6–7], plants [8], zebrafish [9–13], medaka [14], rat [15], *Xenopus* [16], pig [17] as well as in cell lines [18–20] and human stem cells [21].

The indel-mutations generated by TALENs are often small (<30 bp) [22]. So far, these small indel-mutations have been used to disrupt the open reading frames (ORF) of the protein coding genes which constitute only 2–3% of the animal genome. The majority (70–90%) of the animal genome is actually transcribed, producing thousands of ncRNAs such as miRNAs, lncRNA, small interfering RNAs and PIWI-interacting RNAs [23–24]. Different

from the protein coding genes, the ncRNA genes contain no ORFs [25]. The functional regions of some ncRNAs such as lncRNAs are often unknown [25]. Other ncRNAs such as miRNAs often occur as gene clusters. Therefore, small sequence alterations may not be sufficient to disrupt the functions of these ncRNAs. Increasing evidence have demonstrated the functional roles of ncRNAs in a wide range of biological processes [26–28], yet the lack of efficient tools to knockout ncRNA genes in most animal species hinders studies on their *in vivo* functions.

Zebrafish is an important animal model to investigate gene functions. Besides the protein-coding genes, many ncRNA genes in the zebrafish genome are also transcribed [29–33]. In this study, we have developed strategies using TALENs to achieve precise and inheritable large genomic deletions and knockout of ncRNA genes in zebrafish.

Materials and Methods

Zebrafish Husbandry

AB zebrafish used in this study were maintained at 28°C in the zebrafish facility of the Chinese University of Hong Kong and the Institute of Hydrobiology. All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of the Chinese University of Hong Kong and the Institute of Hydrobiology.

Construction of Customized TALENs

The pCS2-TALEN-ELD/KKR plasmids were constructed as described [16]. Using the modified TALENs vectors, highly effective customized TALENs recognizing 12–31 bp half-sites could be assembled in five days. The whole procedure involves two digestion-ligation steps. The protocol to assemble TALENS was modified from a previous study [34]. The modular plasmids (60 ng each) were digested and ligated in a 10 ul volume containing 1 ul BsaI buffer (NEB buffer 4), 0.6 ul BsaI (6 U, NEB), 0.6 ul T4 ligase (1200 U, NEB) and 0.4 ul of 25 mM ATP. The reaction was performed on a PCR machine for 6 cycles of 20 min at 37°C and 10 min at 16°C, followed by heating to 50°C for 5 min and then to 80°C for 5 min. Thereafter, 1 ul Plasmid Safe DNase (10 U, Epicentre) was added and digested for 30 min. Five ul of the final products were used to transform competent cells. Five white clones were analyzed. The assembled array plasmids were isolated from the correct clones. The second digestion and ligation step was performed in 10 ul volumes containing 60 ng of each array plasmid, pCS2-TALEN-KKR or pCS2-TALEN-ELD, the last repeat plasmid, 1 ul of Esp3I buffer (NEB buffer 3), 0.6 ul of Esp3I (6 U, NEB), 0.4 ul of T4 ligase (800 U, NEB), and 0.4 ul of 25 mM ATP. The reaction was performed on a PCR machine for 6 cycles of 20 min at 37C and 10 min at 16°C, followed by heating to 50°C for 5 min and then to 80°C for 5 min. Five ul of the final products were used to transform competent cells. Plasmids were isolated from the correct clones for DNA sequencing. The zebrafish nanos-3′UTR was cloned into the 3′ end of the pCS2-TALEN-ELD/KKR coding sequence between XbaI and NotI. These vectors would be provided upon request.

TALEN mRNA Preparation, Microinjection and Mutation Detection

To prepare capped TALEN mRNA, the TALEN expression vectors were linearized by NotI and transcribed using the Sp6 mMESSAGE mMACHINE Kit (Ambion). TALEN mRNAs (100–500 pg) were microinjected into one-cell stage zebrafish embryos. The number of normal and deformed embryos were recorded at 24 hours post fertilization (day 1) and 48 hours post fertilization (day 2) (Table S1 in File S1). Two days after injection, genomic DNA was isolated from 8–10 pooled larvae with normal morphology. The target genomic region was amplified by limited cycles of PCR and subcloned to into pMD18-T (Takara) [35]. The mutation was analyzed by PCR or by sequencing. The primers used in this study are listed in Table S2 in File S1.

Mutation and Deletion Frequency Analysis

To determine the mutation frequency of each targeted locus and the fragment deletion frequency induced by the TALEN pair, genomic DNA was isolated from three replicates of 8–10 pooled zebrafish embryos at 48 hours post fertilization. For mutation frequency analysis, each target locus was amplified and subcloned into pMD18-T. Thirty-two single colonies were analyzed for each sample by PCR or subsequent sequencing. For genomic fragment deletion frequency analysis, real-time PCR was performed on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) using the SYBR Green I Kit. Standard curves were generated by serial dilution of the plasmid DNA. The genomic fragment deletion frequency was calculated as the copy number of genomic DNA with deletions divided by that of the total genomic DNA.

Suppression of Target GFP Expression by Wild-type or Mutated miR-1-2

The GFP sensor plasmid containing three imperfect complementary sites to miR-1-2 and the RFP indicator plasmid expressing the wild-type miR-1-2 precursor were from a previous study [35]. The miR-1-2 precursor mutants were generated by mutation PCR and cloned into the 3′ end of the pSP64T+dsRed coding sequence between EcoRI and XhoI. The 3′UTR sequence of cnn2 was cloned into pCS2-GFP-F vector between the XbaI and XhoI. GFP sensor (100 pg) was coinjected with the wild-type or mutated miR-1-2 (300 pg) into one-cell stage embryos. The embryos were photographed at 24 hours post fertilization.

Screening of Founders

The TALEN injected embryos were raised to adulthood and outcrossed with wild-type fish. For miR-1-1, genomic DNA from 32 pooled F1 embryos of each founder was amplified and subcloned. Thirty-two single clones were analyzed by PCR and sequencing. For genomic fragment deletions, 24 or 32 F1 embryos were collected from each founder and genomic PCR was performed to detect fragment deletion from each single embryo. The fragment deletions were subsequently confirmed by sequencing.

Results

Targeted Disruption of Individual miRNA Genes using a Single Talen Approach

MiRNAs are small non-coding RNAs regulating their targets by post-transcriptional mechanisms [26]. Loss-of-function of genes involved in the miRNA biogenesis pathway has revealed wide range biological functions of these tiny RNAs [26,37]. Here we investigate whether TALENs could be used to knockout miRNAs in zebrafish.

Using our optimized TALEN platform [16], we have assembled TALENs for two zebrafish miRNAs (miR-1-1 and miR-1-2). We placed the miRNA seed (a critical region for miRNA-mRNA pairing) at the spacer region where indels often occur. The assembled TALENs induced somatic mutations with high frequencies of up to 97% (Fig. 1A and Fig. S1). Nearly all these mutations altered the miRNA seed sequences, thus leading to loss-of-function of the miRNAs. Moreover, the indels also altered the hairpin structures of the pre-miRNAs (Fig. 1B), conceivably leading to aberrant miRNA biogenesis. Consistent with these predictions, functional studies indicated that the wild-type pre-miR-1-2 but not the pre-miR-1-2 mutants could effectively suppress the target GFP reporters containing either three imperfect complementary sites to miR-1-2 or the 3′UTR sequence of a reported miR-1-2 target gene [36] (Fig. 1C and Fig. 1D).

Precise Large Genomic Deletions of miRNA Clusters using a Dual Talen Approach

More than half of the miRNA genes occur as gene clusters in many vertebrates genome [38]. We thereafter asked whether TALENs could be used to knockout miRNA gene clusters. To knockout gene clusters of large genomic regions, we have devised a strategy that creates two DSBs simultaneously on each side of the targeted genomic fragment using two pairs of TALENs. Repair of the DSB by ligation of the broken ends would lead to disruption of each targeted locus and deletion of the flanked genomic fragment (Fig. 2A). To test this dual TALEN strategy we designed two pairs of TALENs to delete the zebrafish miR-17-92 cluster (1.2 kb) and the miR-430 cluster (79.8 kb) (Fig. 2B and Fig. 2F). While the miR-
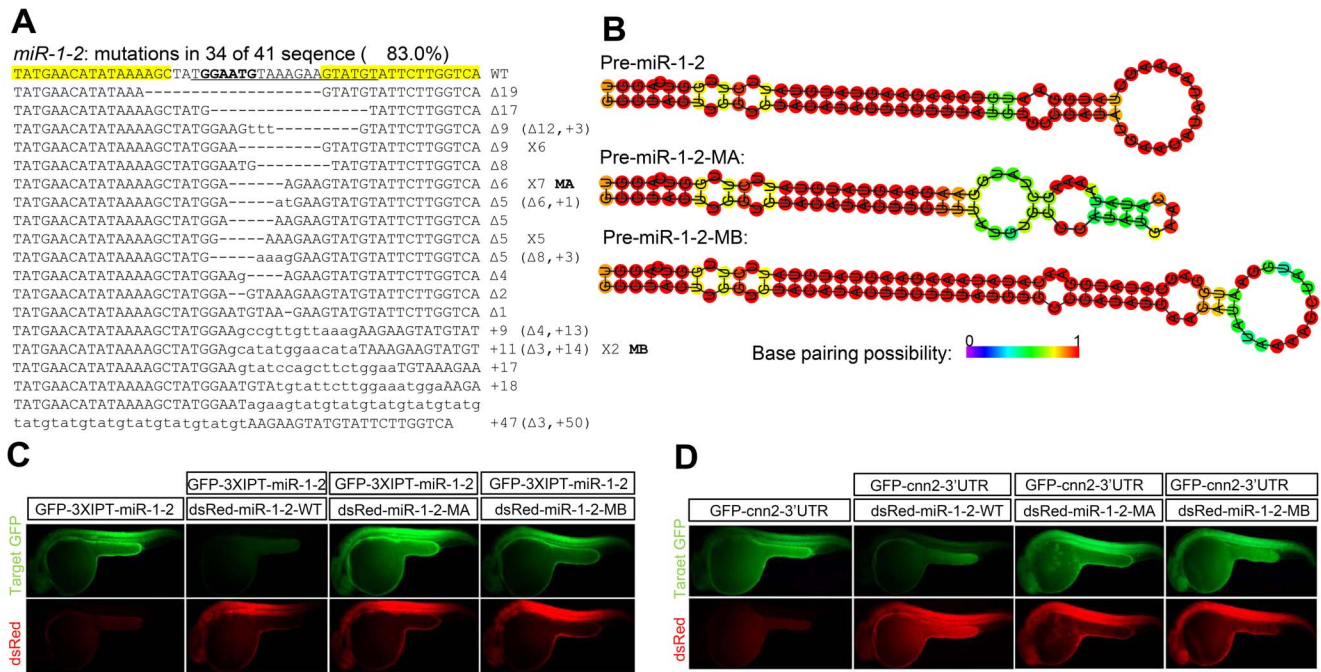
Figure 1. Targeted disruption of zebrafish *miR-1-2*. (A) Frequency and spectrum of TALEN induced *miR-1-2* mutations. The TALEN binding sites are shown in yellow background. DNA sequence encoding the mature miR-1-2 is underlined, with the seed sequence in bold. Deletions are indicated by dash lines and insertions are indicated by lowercase letters. The sizes of the insertions (+) or deletions (Δ) and the number of times each mutant allele appearing are shown on the right side of the mutant allele. **(B)** The hairpin structure of the wild-type pre-miR-1-2 and two pre-miR-1-2 mutants (MA and MB) in Panel A. **(C–D)** Functional suppression of target GFP expression by the wild-type or two mutated pre-miR-1-2. Messenger RNA of GFP sensor (GFP-3XIPT-miR-1-2 or GFP-cnn2-3'UTR) and RFP indicator expressing wild-type or mutant pre-miR-1-2 was co-injected into one-cell stage zebrafish embryos. Pictures were taken at 24 hours after injection. GFP-3XIPT-miR-1-2, GFP sensor containing three imperfect complementary sites to miR-1-2; GFP-cnn2–3'UTR, GFP sensor containing the 3'UTR sequence of *cnn2* (a miR-1-2 target gene); dsRed-miR-1-2, RFP indicator expressing mature miR-1-2.

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17-92 cluster encodes 6 miRNAs, the *miR-430* cluster is the largest miRNA cluster in vertebrates encoding 57 miRNAs. To create two concurrent DSBs on each targeted genomic region, mRNA of two pairs of TALENs were co-injected into one-cell stage zebrafish embryos. Two days after injection, genomic DNA was isolated from 8–10 pooled embryos. Primers were designed to detect deletion of the targeted genomic fragment (Table S2, Fig. S2 and Fig. S3 in File S1). PCR amplification of genomic DNA isolated from the pooled embryos indicated successful deletion of these miRNA clusters (Fig. 2C and Fig. S3 in File S1). Sequencing of the PCR products confirmed such successful deletions (Fig. 2D and Fig. 2G). All these deletions occurred accurately between the two targeted sites and there were none or few alien nucleotides retained after the large genomic deletions. Taken together, these results indicate that this dual TALEN strategy provides a powerful approach to generate precise large deletions in the genome.

The fragment deletion frequency of each targeted miRNA gene cluster and the indel-mutation frequency of each targeted locus produced by the dual TALEN approach were systematically analyzed. To evaluate the fragment deletion frequency, two pairs of primers were used to amplify genomic DNA with fragment deletions and a nearby undisrupted genomic region (Table S2 in File S1). Quantitative real-time PCR was performed to calculate the copy number of genomic DNA with fragment deletions with respect to that of the undisrupted genomic DNA. The mean deletion frequency was 4.2% for the *miR-17-92* cluster and 1.9% for the *miR-430* cluster in zebrafish embryonic cells respectively (Fig. 2E and Fig. 2H). The dual TALEN treatment produced indel mutations on each targeted locus with high frequency (Fig. 2E,

Fig. H and Table S3 in File S1). For both gene clusters, the fragment deletion frequency is lower than that of the indel mutations.

Precise Large Genomic Deletions of lncRNA Gene using the Dual talen Approach

Apart from small RNAs, the animal genome also produces large RNA transcripts called lncRNAs longer than 200 nucleotides that do not code for proteins [27,39]. In zebrafish, two recent studies annotated more than 1000 lncRNA genes that are expressed during early development [32–33]. To determine whether this dual TALEN strategy is also applicable to delete lncRNA genes, we have designed two pairs of TALENs to delete the 9.0 kb genomic region encoding the lncRNA *malat1* (Fig. 3A and Fig. 3B). Using this approach we obtained deletion of *malat1* in zebrafish somatic cells with a mean deletion frequency of 2.0% (Fig. 3D and Fig. S4 in File S1). The genomic sequence between the two TALEN targeting sites was accurately deleted (Fig. 3C). These results indicate that the dual TALEN strategy is a powerful approach to knockout lncRNA genes.

Germline Transmission of Knockout Genotypes

To test whether these knockout genotypes were inheritable, we have outcrossed the P0 fish raised from the TALEN injected embryos. For *miR-1-1*, 5 out of the 6 P0 fish transmitted the *miR-1-1* indel mutations with high frequencies (Fig. 4A). For the *miR-17-92* cluster, we have genotyped 24 individual F1 embryos from each of the 6 outcrossed P0 fish. Three out of the 6 P0 fish transmitted
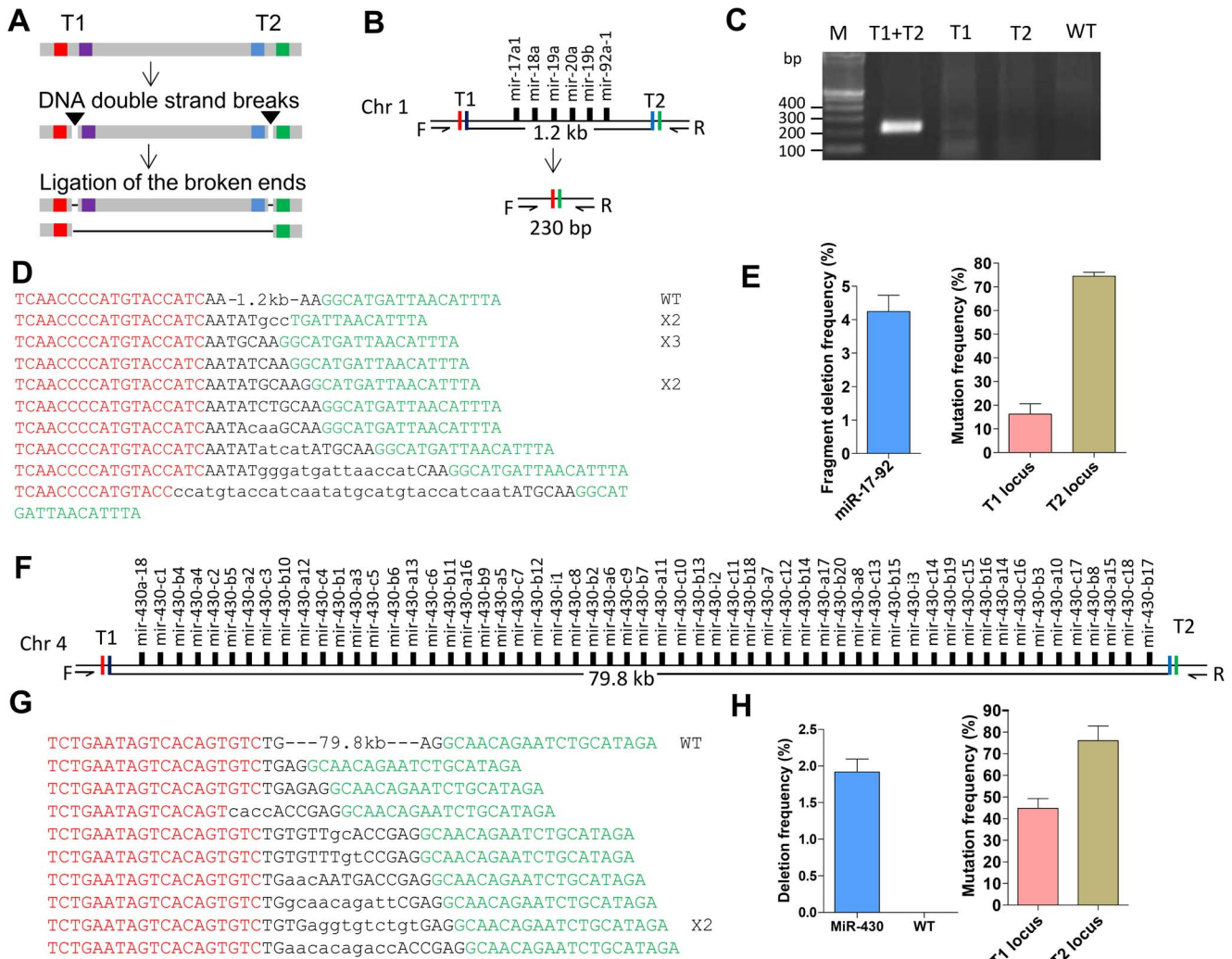
Figure 2. Precise large genomic deletions of miRNA gene clusters. (**A**) Schematic diagram of the large genomic deletion strategy using dual TALEN. The TALEN binding sites are shown in color. T1, TALEN pair 1; T2, TALEN pair 2. (**B**) Schematic representation of the zebrafish *miR-17-92* cluster and the designed TALENs for its deletion. After genomic deletions, a band of about 230 bp is expected to be amplified by PCR using the designed primers. (**C**) Genomic PCR showing deletion of the *miR-17-92* cluster. Gel picture showing PCR amplification of genomic DNA isolated from the pooled zebrafish embryos microinjected with two pairs of TALENs, one pair of TALENs or the wild-type control. (**D**) Sequencing results confirmed deletion of the *miR-17-92* cluster. Alien nucleotides inserted are indicated by lowercase letters. The number of times each mutant allele appearing are shown on the right side of the mutant allele. (**E**) Deletion frequency of the *miR-17-92* cluster and indel-mutation frequency of the T1 and T2 loci. Genomic DNA was isolated from pooled zebrafish embryos injected with the two pairs of TALENs. Data shown are mean values ± S.E.M from three replicates. (**F**) Schematic representation of the zebrafish *miR-430* cluster and the designed TALENs. (**G**) Sequencing results confirmed deletion of the *miR-430* cluster. Alien nucleotides inserted are indicated by lowercase letters. (**H**) Deletion frequency of the *miR-430* cluster and indel-mutation frequency of the T1 and T2 loci. Genomic DNA was isolated from pooled zebrafish embryos injected with the two pairs of TALENs. Data shown are mean values ± S.E.M from three replicates.
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the 1.2 kb *miR-17-92* deletions to 1/24, 3/24 and 6/24 F1 embryos respectively (Fig. 4B). For *malat1*, 4 out of the 32 P0 fish transmitted the 9.0 kb *malat1* deletions to 1/32, 2/32, 4/32 and 5/32 F1 embryos respectively (Fig. 4D).

For the larger *miR-430* cluster deletions, we have outcrossed 32 P0 fish but failed to obtain germline deletions, suggesting that large genomic deletions are not efficiently transmitted. To overcome this limitation, we have incorporated the zebrafish *nanos*-3′UTR into the TALEN construct (Fig. S5 in File S1). By screening 23 P0 fish raised from the TALEN-*nanos*-3′UTR injected embryos, we have obtained 1 founder transmitted the *miR-430* deletion to 3/32 embryos (Fig. 4C). These data suggested that the *nanos*-3′UTR

may increase the germline transmission frequency of large genomic deletions.

Discussion

Recently the ENCODE project estimated more than 70% of the human genome is transcribed into ncRNAs [40–41], indicating that ncRNAs represent a substantial portion of the transcriptome. However, because of the lack of an efficient ncRNA gene knockout platform in vertebrates, only a few of the ncRNA genes have been inactivated in mouse. The ability of engineered nucleases to target specific genomic loci across species makes them an attractive option to address this issue. However,
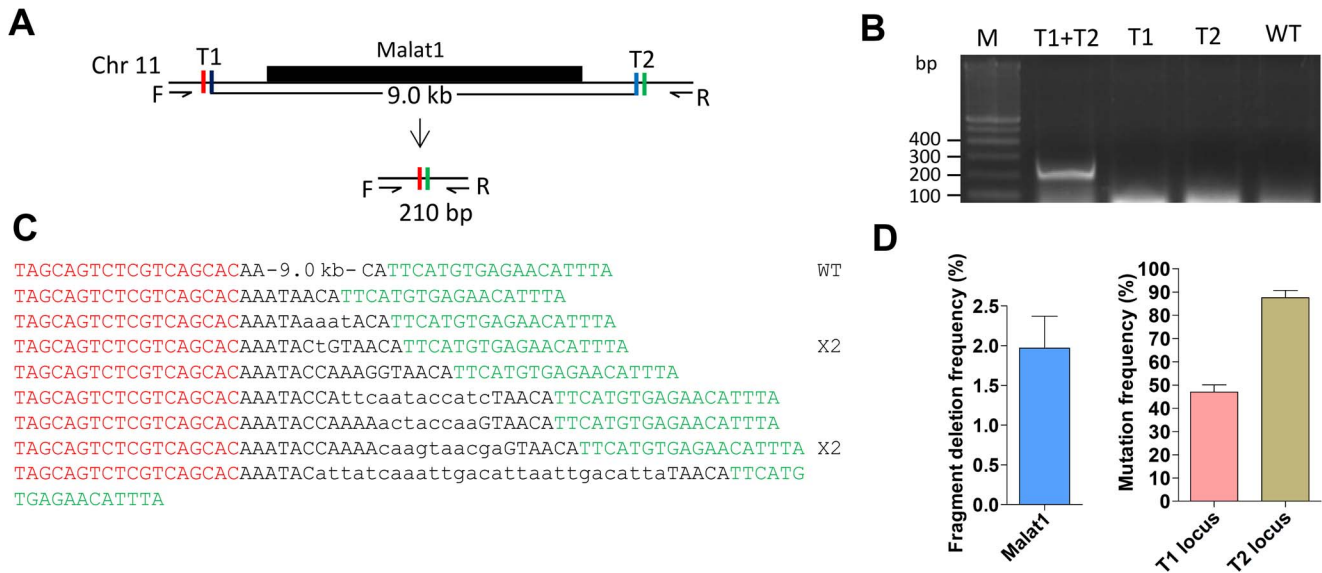
Figure 3. Precise large genomic deletion of a lncRNA gene. (**A**) Schematic representation of the zebrafish lncRNA *malat1* and the TALENs designed for its deletion. After the genomic deletion, a band of about 210 bp is expected to be amplified by PCR using the designed primers. (**B**) Genomic PCR showing the deletion of *malat1*. Gel picture showing PCR amplification of genomic DNA isolated from the pooled zebrafish embryos microinjected with two pairs of TALENs, one pair of TALENs or the wild-type control. (**C**) Sequencing results confirming *malat1* deletions. Alien nucleotides inserted are indicated by lowercase letters. The number of times each mutant allele appearing are shown on the right side of the mutant allele. (**D**) Deletion frequency of the *malat1* and mutation frequency of the two targeted loci. Genomic DNA was isolated from pooled zebrafish embryos injected with the two pairs of TALENs. Data shown are mean values ± S.E.M from three replicates.
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the engineered nucleases have been mainly used to induce small indel mutations. Because the functional regions of ncRNA gene are often unknown, knockout of an entire ncRNA gene is preferred to eliminate gene function. Here we test whether TALENs could be used to knockout of ncRNA genes in zebrafish.

Using zinc finger nucleases and TALENs, targeted fragment deletions have been recently reported in cell lines [17,42–44]. More recently, genomic deletions of an 800 bp fragment using TALENs have been reported in silkworm [7]. During the time this manuscript was reviewed, others have also reported that large

Figure 4. Germline transmission of the knockout genotypes. (**A**) Germline transmission of the *miR-1-1* mutations. The TALEN binding sites are shown in yellow background. DNA sequence encoding the mature miR-1-1 is underlined, with the seed sequence in bold. Deletions are indicated by dash lines and insertions are indicated by lowercase letters. The F0 germline deletion ratio is shown in square brackets. The ratios of mutated/total analyzed sequences from the pooled F1 embryos of each founder are shown in brackets. (**B**–**D**) Germline transmission of the *miR-17-92* deletions (**B**), *miR-430* deletions (**C**) and *malat1* deletions (**D**). The F0 germline deletion ratios are shown in square brackets and the F1 inheritance ratios are shown in brackets.
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genomic deletions could be efficiently engineered in zebrafish using a dual TALEN strategy [45–47]. In this study, we have demonstrated that the dual TALEN approach is a robust means to generate inheritable large genomic deletions in zebrafish. First, large genomic deletions (1–80 kb) could be efficiently generated in a few days and the deletions could be readily detected by genomic PCR. Second, all deletions have accurately occurred and none or few alien nucleotides are retained after the genomic deletions (Fig. S6 in File S1). Third, although the fragment deletion frequency is lower than that of the indel-mutation, these large deletions of about 9 kb could be efficiently transmitted through the germline.

Analysis of the transcriptome revealed that many small regulatory RNA are transcribed from the eukaryotic genome [40,48]. miRNA are small RNA molecules regulating the decay and translation of their target mRNAs. Mature miRNAs are generated from cleavage of the hairpin structure of pre-miRNAs by the RNAse III enzymes dicer [49]. In zebrafish, loss-of-function mutations of dicer in the maternal and zygotic embryos lead to multiple developmental defects followed by death on day 5 post-fertilization [50], indicating that miRNAs as a whole play essential roles in embryogenesis. But the exact functional roles of individual miRNAs or miRNAs clusters remain elusive. Moreover, most miRNAs expressed in zebrafish adult tissues may also regulate other physiological processes. In this study, we have demonstrated that large miRNA gene clusters could be precisely deleted using two pairs of TALENs, whereas individual miRNA genes could be inactivated by destruction of the miRNA hairpin structure and miRNA seed using one pair of TALENs, establishing TALEN as a robust method to knockout miRNA genes. Further studies will be performed to analyze the phenotypic changes resulting from deleting of individual miRNA and miRNA clusters.

Apart from small RNAs, the animal genome also produces many lncRNAs [27,39]. An increasing number of reports revealed that lncRNAs regulate many cellular processes ranging from maintaining embryonic stem cell pluripotency to epigenetic silencing of large genomic regions [28,39]. In zebrafish, more than 1000 lncRNA genes are expressed during early development [32–33]. Moreover, morpholino knockdown of lncRNAs lead to abnormal brain morphogenesis [33]. However, most lncRNAs are not well conserved and their functional regions are mostly unknown, thus limiting the applicability of the morpholino knockdown approach to study these lncRNAs. The dual TALEN strategy described in this study provides a powerful tool to knockout these lncRNA genes.

Germline transmission of the knockout genotypes is critical to obtain the homozygous gene knockout animals. In this study, we have demonstrated that deletions as long as 9.0 kb could be efficiently transmitted through the germline. To improve the germline integration efficiency of larger genomic deletions, we have incorporated the zebrafish nanos-3′UTR into the TALEN construct. The nanos 3′UTR was reported to protect mRNA from degradation in the primordial germ cells [51,52], thus may improve the germ cell target efficiency. Using this TALEN-nanos-3′UTR construct, we have demonstrated that large genomic deletions of the miR-430 cluster could be transmitted through the
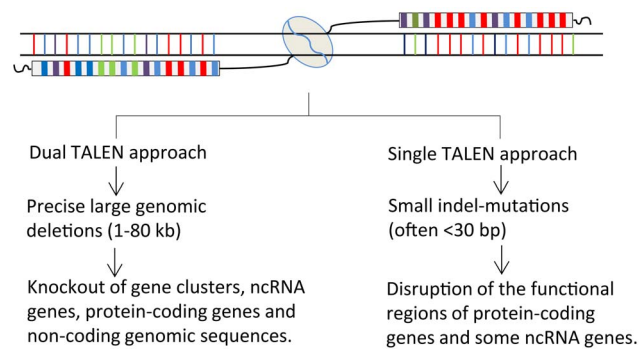

Figure 5. Schematic diagram illustrating knockout strategies using dual or single TALEN approaches. The dual TALEN approach could generate precise large genomic deletions (1–80 kb), allowing knockout of large gene clusters, ncRNA genes, protein-coding genes and non-coding regulatory sequences. The single TALEN approach could produce small indel-mutations of the targeted genomic loci (often <30 bp), allowing disruption of individual protein-coding genes or some individual ncRNA genes.
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germline. This strategy might also be applicable to engineer large genomic deletions in other species.

In summary, we have generated high frequency indel mutations of individual miRNA genes and precise large genomic deletions of non-coding RNA gene clusters in zebrafish using TALENs. Moreover, these deletions are inheritable. We propose that the same TALEN approach could be employed as a molecular tool to study the *in vivo* functions of ncRNA genes in other species. Moreover, the two TALEN strategies described in this study also allow one to knockout other ncRNA genes, entire protein coding genes, genomic regulatory sequences and to disrupt a small functional genomic region depending on the needs (Fig. 5).

Supporting Information

File S1 Supporting Figures and Tables.
(PDF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: YL WH CHKC. Performed the experiments: YL DL. Analyzed the data: YL DL HZ ZYZ WH CHKC. Contributed reagents/materials/analysis tools: YL CHKC. Wrote the paper: YL DL HZ ZYZ WH CHKC.

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