Heritable Targeted Gene Disruption in Zebrafish Using Designed Zinc Finger Nucleases

Yannick Doyon1,2, Jasmine M McCammon1,4, Jeffrey C Miller2, Farhoud Faraji2, Catherine Ngo2, George E Katibah2, Rainier Amora2, Toby D Hocking2, Lei Zhang2, Edward J Rebar2, Philip D Gregory2, Fyodor D Urnov1,2, and Sharon L Amacher1,3

1 Department of Molecular and Cell Biology and Center for Integrative Genomics, University of California, Berkeley, CA 94720-3200, USA
2 Sangamo BioSciences, Inc., Pt. Richmond Tech Center, 501 Canal Blvd., Suite A100, Richmond, CA

Abstract

We describe here the use of zinc finger nucleases (ZFNs) for somatic and germline disruption of genes in zebrafish (Danio rerio), where targeted mutagenesis was previously intractable. ZFNs induce a targeted double-strand break in the genome that is repaired to generate small insertions and deletions. We designed ZFNs targeting the zebrafish golden and no tail/Brachyury genes. In both cases, injection of ZFN-encoding mRNA into 1-cell embryos yielded a high percentage of animals carrying distinct mutations at the ZFN-specified position and exhibiting expected loss-of-function phenotypes. Disrupted ntl alleles were transmitted from ZFN mRNA-injected founder animals in over half the adults tested at frequencies averaging 20%. The frequency and precision of gene disruption events observed, in combination with the ability to design ZFNs against any locus, open fundamentally novel avenues of experimentation, and suggest that ZFN technology may be widely applied to many organisms that allow mRNA delivery into the fertilized egg.

The zebrafish has proven to be an excellent model for vertebrate development and disease due to its rapid development, transparent embryos, and its relatively facile forward genetics. Techniques for reverse genetic approaches in zebrafish, however, are limited to mRNA knockdown strategies using modified antisense oligomers (morpholinos) and TILLING for point mutations in a locus of interest. Both strategies have shortcomings and are limited in scope: morpholinos are active for only the first few days of development, hence their transient effect fails to impact on juvenile or adult phenotypes, while TILLING is both time-consuming and less effective for intron-rich genes with small exons because the chances of obtaining null or hypomorphic alleles are reduced. Conventional “gene targeting,” a
powerful technique for gene disruption in mouse embryonic stem cells often requires positive-negative selection with cytotoxic drugs, which is inapplicable in the context of a vertebrate embryo.

The resistance of the genome in most metazoa to targeted sequence alteration has recently been overcome through the use of zinc finger nucleases (ZFNs). Initially developed to cleave DNA in vitro, ZFNs are a fusion between the cleavage domain of the type IIS restriction enzyme, FokI, and a DNA recognition domain containing 3 or more C2H2 zinc finger motifs. The heterodimerization at a particular position in the DNA of two individual ZFNs in precise orientation and spacing leads to a double-strand break (DSB) in the DNA. DSB repair pathways operate in all eukarya, and include non-homologous end-joining (NHEJ, the direct ligation of the two DNA ends, frequently with loss or gain of small amounts of DNA sequence), as well as homology-directed repair. Importantly, Carroll and colleagues exploited NHEJ-based repair of a ZFN-induced DSB to disrupt a gene in Drosophila and an intergenic region in C. elegans, thus establishing the potential of this technology in reverse genetic applications, recently expanded to mammalian tissue culture cells. Here, using golden/slc24a5 (gol) and no tail/Brachyury (ntl) as test loci, we investigated the feasibility of using designed ZFNs for reverse-genetic applications in zebrafish.

The gol pigment locus encodes a transmembrane protein, Slc24a5, and was chosen for an initial test of ZFN-driven editing because the lack of embryonic pigmentation caused by homozygous viable gol mutations make scoring for ZFN activity by loss-of-function straightforward. The C2H2 zinc finger is a versatile DNA binding motif capable of recognizing a broad range of sequences, and efforts over the past 14 years have yielded a large number of zinc fingers aimed at recognizing investigator-specified DNA triplets (see Supplementary Information online). As a consequence, simple in silico analysis can illuminate a high number of potential zinc finger proteins (ZFPs) for any given target gene. We describe here an experimentally straightforward two-step approach to identify those particular ZFPs that will yield robust ZFNs for in vivo gene disruption, and its application to the zebrafish gol and ntl genes.

We analyzed in silico the cDNA sequence of the gol gene and assembled a panel of 4-finger ZFPs (see Materials and Methods, Supplementary Information, and Supplementary Fig. 1 online) directed against a range of distinct positions in the gol locus (Supplementary Table 1 online). To limit the number of ZFN pairs to be tested in vivo, the affinity of the ZFPs for their target sequence was measured using an ELISA assay (Supplementary Fig. 2 online). Next, to determine which of these ZFPs will form pairs most likely to be active in zebrafish gene disruption, we developed a budding yeast-based system to rapidly measure ZFN activity (Fig. 1A–B; Supplementary Fig. 3 online; a detailed description is provided in Supplementary Information online).

In building this system, we made use of the well-established fact that while NHEJ operates relatively inefficiently in budding yeast, a DSB induced between two short direct repeats spaced by a heterologous sequence of up to 25 kb leads to resection of the entire intervening DNA, and the restoration of chromosome integrity via the single-strand annealing of the two
repeats 15. We disabled the open reading frame for a secreted form of alpha-galactosidase (encoded by the MEL1 gene) with the gol sequence and placed this construct into a specific position of the budding yeast genome. Experiments using a control construct, in which the DSB between the direct repeats was induced by the well-studied yeast endonuclease, HO, validated the dynamic range and low background of the system in reporting the efficiency of DSB induction by the nuclease (Fig. 1C–D). We then screened the panel of gol-targeting ZFNs for DNA cleavage activity in a budding yeast reporter strain carrying their target sequence (Fig. 1A). This analysis identified active ZFN pairs directed against exons 4, 8, and 9 of the gol gene (Fig. 2A) – we focused on the exon 4 and 9-targeting ZFNs in subsequent experiments (Fig. 2B, Supplementary Fig. 4 online).

Following screening in budding yeast, the ZFNs identified as active in that system were tested for native gene disruption activity at their intended target locus in zebrafish: the ZFN expression constructs were transcribed and capped in vitro, and the resulting mRNAs were injected into 1-cell zebrafish embryos heterozygous for the golb1 mutation 12. For the majority of our experiments we used ZFNs carrying obligate-heterodimer forms of the FokI endonuclease domain 16; these inhibit ZFN homodimerization and thus improve specificity by limiting cleavage at off-target sites recognized by ZFN homodimers 16. At 2 days post fertilization (dpf), golb1 heterozygotes normally have dark eye pigmentation (Fig. 2C, upper left); however, we detected clones of unpigmented cells in the eye, potentially representing loss of gol function on the non-golb1 chromosome, in up to 32% of ZFN-injected, but not in control, embryos (Table 1 and Fig. 2C). A clear positive correlation was observed between extent of mosaicism and amount of ZFN mRNA injected (Table 1). At the highest doses of ZFNs, over 30% of injected embryos exhibited pigmentation mosaicism in the eye (Table 1). Also at the highest ZFN doses, a small fraction of ZFN-injected embryos exhibited mild to moderate developmental defects, such as a bent body axis or mild head necrosis. However, even at the highest concentrations tested, the majority of embryos showing pigment mosaicism were indistinguishable from wild-type embryos (69–90% depending on the ZFN pair tested) (Table 1).

To investigate whether these phenotypes were the consequence of somatic loss-of-function mutations resulting from repair of ZFN-induced DSBs by NHEJ, we genotyped the gol locus in injected embryos. We used PCR to amplify a 312 bp region including the ZFN target site, with total genomic DNA from individual fish with gol clones serving as the input for each PCR reaction. Consistent with the phenotypes observed above, we found a broad range of loss-of-function alleles in the genomes of ZFN-injected animals, but not in controls (data not shown). These included deletions ranging in size from 7 to 65 bp and insertions of 4 to 12 bp (Fig. 2D). Taken together, these experiments demonstrated that a single injection of ZFN-encoding mRNA into an early zebrafish embryo can result in the efficient mutation of the locus targeted by the ZFNs to yield alleles that confer loss-of-function phenotypes.

To confirm the generality of the observations above, we next targeted the no tail/Brachyury (ntl) gene, a major regulator of early embryogenesis. The choice of target gene was in large part inspired by the fact that the study of early development is a major direction of biological investigation in zebrafish as a model system. The ntl gene encodes an essential T-box transcription factor required for proper mesoderm formation; zebrafish embryos

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homozygous for a null ntl mutation lack a notochord and a tail. To test whether ZFNs can induce somatic mutations in the ntl gene, we followed the same scheme described for the gol-targeting ZFNs (ZF design, ELISA in vitro binding assay, and in vivo yeast-based activity assay) (Fig. 3A; Supplementary Table 2 and Fig. 5 online). For these experiments, we focused our design efforts on exon 2, the location of a well-studied null allele of the gene (Fig. 3B).

Using lead proteins (Supplementary Fig. 6 online) identified in the yeast-based assay (Fig. 3A) for our initial effort in ntl disruption, we injected mRNA encoding high-fidelity, obligate-heterodimer 16 ZFNs into 1-cell embryos from a cross between wild-type and ntl<sup>b195</sup> heterozygous fish. Since half the injected embryos carry the ntl<sup>b195</sup> null allele, we hypothesized that these embryos would reveal ZFN-driven mutations of the wild-type allele on the other chromosome. Strikingly, we observed that 16–27% of injected embryos displayed a ntl-like phenotype (Fig. 3D), either mimicking the null phenotype (Fig. 3C) or a less severe phenotype typical of a hypomorphic allele, ntl<sup>b487</sup> (Supplementary Fig. 7 online). We noted that even high ZFN mRNA doses were well-tolerated by the embryos (Fig. 3D).

We genotyped individual ntl-like embryos at the ZFN target stretch using a rapid assay based on a mismatch-sensitive endonuclease 16 that allows one to measure the fraction of chromatids carrying mutations relative to the wild-type sequence. This analysis showed that the fraction of mutated chromatids ranged from 3 to 17% in the majority of embryos (Supplementary Fig. 8 online). To further analyze the types of mutations induced, we sequenced a 226 bp region surrounding the DSB site from ZFN-injected embryos, and observed a broad range of deletions and insertions precisely at the targeted locus (Fig. 3E). The mutant sequences shown in Fig. 3E appear to represent alleles generated by NHEJ-induced repair of the ZFN-cleaved chromatid, because chromatids bearing the ~1.5 Kb ntl<sup>b195</sup> insertion (see Fig. 3B) are too large to be amplified and analyzed.

Given our published data on the feasibility of generating single-step biallelic genome editing events using ZFNs in human cells 18, we analyzed whether wild-type embryos injected with high-fidelity ntl-targeting ZFNs displayed ntl mutant phenotypes. In these experiments, we found that juvenile and adult fish had posterior tail truncations (20% [3/15] and 21% [12/58] among stocks injected with 1 ng and 5 ng ZFN Pair 2, respectively, and 7.7% [4/52] among stocks injected with 5 ng ZFN Pair 3) (Fig. 4A). We sequenced the ntl locus surrounding the ZFN-cleavage site from a small posterior tissue sample from each of three tailless embryos and found that ntl mutant-bearing amplicons represent a substantial fraction of the total (Sample 1, 5/25 (20%) ntl-bearing chromatids, 2 different alleles; Sample 2, 3/30 (10%) ntl-bearing chromatids, 1 allele; Sample 3, 8/29 (28%) ntl-bearing chromatids, 4 different alleles) (Supplementary Fig. 9 online).

Interestingly, when ntl-targeting ZFNs carrying wild-type FokI cleavage domains were injected into wild-type embryos, a high frequency of embryos exhibiting a ntl phenotype were observed; sequencing a 226 bp region surrounding the DSB site revealed that each of 3 representative embryos carried between 64–81% disrupted ntl alleles (Table 2; Supplementary Fig. 10 online). Given that a single wild-type ntl allele is sufficient for a
normal phenotype, these data suggest that ZFNs can induce biallelic disruption of a target gene locus after mRNA injection into the 1-cell embryo.

To demonstrate that ZFNs can effectively induce mutations in the germline, wild-type embryos injected with ntl-targeting high-fidelity 16, obligate-heterodimer ZFNs were raised to sexual maturity and screened. We found that ZFN-injected fish breed normally and give clutches of similar size as when we intercross our non-ZFN injected wild-type stocks. Eggs from ZFN-injected females were fertilized in vitro with sperm from a male heterozygous for the ntlb195 allele. Of 7 females analyzed, 4 generated ntl progeny (Table 3; Fig. 4B) at frequencies ranging between 1–13% as gauged by this complementation cross (Table 3). To measure the frequency of gametes carrying gene disruptions, we directly genotyped the chromatid provided to the progeny (both wild-type and ntl) by four of the founder mothers, and found that the germline carried mutations at frequencies ranging from 5–32% (Table 3). Direct sequencing confirmed these estimates and revealed that three founders carried at least two new alleles, and one founder carried at least one (Fig. 4C). In addition to founders identified using complementation crosses and chromatid genotyping (Table 3), we have identified an additional 2 carriers (germline transmission rates of 33% and 53%) using complementation crosses and an additional 5 carriers (germline transmission rates of 6–25%) by analyzing haploids generated from founder females. Taken together, the data show that over 60% of ntl ZFN-injected founders (11/18) carry mutations at the ntl locus, with an average germline frequency of 20%.

To determine the level of specificity of ZFN function, an experimentally determined DNA binding consensus was derived for each ZFN (see Materials and Methods, Supplementary Fig. 11 online for experimental details). This information was then used to search the zebrafish genome for sites showing the highest similarity to their intended target site (Supplementary Fig. 11 online). To experimentally determine whether ZFNs cleave at these alternative “off-target” sites, we directly analyzed the top 5 potential off-target loci for each ntl-targeting ZFN pair. We used PCR to amplify the cognate chromosomal loci from phenotypically ntl progeny embryos (Table 3) carrying ZFN-induced mutations at the ntl gene, and analyzed them by direct sequencing, loss-of-RFLP, and mismatch endonuclease assays. All the chromatids we analyzed were wild-type (Supplementary Fig. 11 online). These results confirm that ZFNs can be used to specifically and efficiently create heritable mutant alleles at loci of interest.

Finally, to show that ZFN-induced alleles can be propagated in subsequent generations, we outcrossed two ntl ZFN-injected females (Females C and D, Table 3) and recovered heterozygous carrier individuals at frequencies consistent with the germline frequency of the mutation in each founder female (Table 3), indicating that heterozygosity for the new induced alleles was completely viable. As stocks reached sexual maturity, fish were intercrossed to siblings and backcrossed to founders. We recovered ntl mutant phenotypes in the expected ratios and observed no non-ntl phenotypes among F2 progeny. While our analysis has failed to reveal ZFN-induced mutations at loci other than ntl, in the hypothetical possibility that an undesired secondary mutation was occurring at a rate as high as 10%, it would rapidly segregate from the desired ntl mutation even after one generation.
Our data show ZFNs to be highly effective tools for inducing mutations at a specified locus in the zebrafish, the rapid production of embryos and adult animals with corresponding loss-of-function phenotypes (Tables 1 and 2) and adults carrying new alleles (Table 3). To serve this purpose, ZFNs can be injected as mRNA, thus allowing only transient nuclease expression, which we have shown to be sufficient to introduce a stable genetic mark on the targeted locus. The experiments in the present work describe the successful disruption of 3 distinct positions at 2 separate genetic loci and the use of ZFN injection into wild-type embryos to yield phenotypes expected from a loss of targeted gene function (Table 2). In addition, we show that ZFNs can be used to generate novel germline mutations with high specificity of action (Table 3; Supplementary Fig. 11 online).

The C2H2 zinc finger motif 19 has been engineered to recognize many distinct DNA sequences 14,20,21 (see also Supplementary Information online), and this has paved the way for the design of ZFNs used in efficient genome editing at a broad range of investigator-specified endogenous loci, including a mutation hotspot in a human monogenic disease locus 18. As our data show, the combination of this versatile platform with a rapid yeast-based assay for the identification of maximally active engineered ZFNs, open fundamentally novel avenues of experimentation in the zebrafish, and suggest that ZFN technology may be widely applied to many organisms for which reverse genetics strategies are currently unavailable.

MATERIALS AND METHODS

ZFN constructs

Detailed information, including full ZFN sequences, expression vector nomenclature, and a description of the design, assembly, and validation process, is provided in Supplementary information. Zinc finger proteins against zebrafish gol and ntl were designed and validated biochemically in vitro as described previously 18, 22, and cloned into expression vectors to generate zinc finger nucleases. In all experiments, the highfidelity, obligate-heterodimer 16 ZFN forms were used, except the experiments in Table 2 (also described in Supplementary Fig. 10 online), for which ZFNs carried wild-type FokI. In all other aspects, the nucleases had the same architecture as previously described 18. Note that requests for ZFN reagents other than those described here should be directed to zfn@sial.com, or via http://www.sigma.com/zfn.

Yeast assay

Detailed methods are provided in Supplementary Information. All strains were derived from the S288C parental strain 69-1B (MATa, his3Δ200, lys2-128Δ, leu2Δ1). The SSA reporter constructs were assembled into the HO targeting vector HO-poly-KanMX4-HO 23 by cloning two overlapping and non-functional fragments of the MEL1 gene under the control of the PGK1 promoter (Supplementary Fig. 3 online). ZFN were expressed using a galactose-inducible promoter 24, and inductions were performed as described 25 following the scheme presented in Fig. 1B. The quantitative α-galactosidase assay was performed as described with minor modifications 26.
Zebrfish mutant alleles, stocks, and husbandry

Zebrfish embryos were obtained by in vitro fertilization and natural spawnings of adults maintained at 28.5°C on a 14 h light, 10 h dark light cycle and staged as described. The golden (golb1) and no tail (ntlb195) mutant alleles used have been previously described 12,28. The ntlb487 allele was identified in a forward genetic screen performed in Eugene, Oregon, and failed to complement two other ntl alleles (S.L. Amacher and C.B. Kimmel, unpublished data). Transheterozygotes were obtained in expected Mendelian ratios in at least two independent pairwise crosses (71/326 [22%] for ntlb160/ntlb487 and 135/542 [25%] for ntlb459/ntlb487). Homozygous ntlb487 embryos have a less severe phenotype than that of the null allele, often having a more extensive, forked tail and patches of notochord tissue (Supplementary Fig. 7 online), in contrast to the complete lack of tail and notochord in null mutants 17.

Preparation and microinjection of ZFN mRNA

Matched golden ZFN pairs were subcloned into a pVAX vector with coding sequences separated by a 2A ribosome stuttering sequence for all experiments except those shown in Supplementary Fig. 10 online, which were done with separate expression constructs (see Supplementary Information online for vector maps, construct sequences, and recombinant DNA protocols and Supplementary Table 4 for plasmid names). In all cases, plasmids were linearized with Eag I and transcribed in vitro using the T7 mMessage mMachine transcription kit (Ambion, Austin, TX) to generate a single capped transcript containing both open reading frames. Approximately 5 nl of capped mRNA was injected into 1-cell zebrafish embryos at concentrations ranging from 0.04 ng/nl to 1 ng/nl.

Analysis of genome editing at ZFN target sites

A 312 bp region surrounding the gol/slc24a5 Pair 14/15 cleavage site was amplified by PCR with primers 5′-ATCTGATATGGCCATGTCCAACATCG-3′ and 5′-GGAACAATCCCATACGCTCCTGCAG-3′ and a 226 bp region surrounding the ntl/Brachyury Pair 2/3 cleavage site with primers 5′-ACGAATGTTTCCCGTGCTCAGAGCC-3′ and 5′-GCTGAAAGATACGGGTGCTTTCATCCAGTGCG-3′. To analyze ZFN-induced mutations eliminating the BsrDI restriction site that lies between the left and right ZFN binding sites, PCR products were digested with BsrDI and resolved on a 2% agarose gel. BsrDI resistant sequences were 226 bp, while wild-type sequences were digested to 176 and 50 bp fragments. Heterozygous individuals carried all 3 band sizes. PCR products were ligated into the pCR® 4-TOPO® vector for sequencing (Invitrogen, Carlsbad CA).

For analysis of potential off-target action by the ZFNs, the position weight matrix (i.e., an experimentally determined consensus DNA binding site) for each ZFN was determined using an in vitro site selection method (Supplementary Information online). The off-target sites, and all the genotyping data, are provided in Supplementary Fig. 11 online.
Scoring of mosaic pigmentation in larval eyes

Two days after injection of gol ZFN mRNA, embryos were immobilized by immersion in 0.004% Tricaine and each eye was carefully evaluated for the appearance of small to large patches of unpigmented cells using an Olympus SZ60 dissecting microscope. Uninjected embryos had uniformly darkly pigmented eyes. Embryos were also scored for developmental defects.

In situ hybridization of ntl ZFN injected embryos

In order to analyze subtle notochord defects in ntl ZFN mRNA-injected embryos that may not have been detected by morphological analysis, embryos were sorted by phenotype and processed for ntl expression by whole mount in situ hybridization as previously described 29.

Imaging

Live and fixed embryos were mounted between bridged coverslips or in 3% methylcellulose and imaged on a Zeiss Axioplan 2 upright microscope. Digital images were taken with a Zeiss Axiocam camera.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Yeast-based system of identification of maximally active ZFNs

(A) Components of yeast-based chromosomal reporter system: expression vectors for the ZFNs (top), target gene integrated into the chromosome at the HO locus (middle). After a double-strand break (DSB) is induced at the target by a pair of ZFNs, it is processed via single-strand annealing (SSA) to repair the MEL1 reporter gene, the activity of which can be rapidly assayed in liquid culture. (B) Outline of procedure for ZFN activity measurement in yeast. (C) Reporter gene restoration is dependent on a precisely targeted DSB. A reporter gene was engineered as in Fig. 1A, except it carried a recognition site of the HO
endonuclease. MeI1 activity is only observed following induction of expression of the endonuclease (“+gal”), and of its target in the reporter locus (bottom row). (D) Exceedingly low frequency of spontaneous MEL1 gene repair. Essentially no MeI1 activity is observed when HO expression is not induced (left panel), with the exception of very low-frequency spontaneous MEL1 restoration events (right panel), visualized as small sectors of blue cells within otherwise white colonies. As shown in the middle, induction of HO endonuclease expression converts the overwhelming majority of the cells in the sample to a MEL1 state.
Figure 2. Injection of golden ZFN-encoding mRNA into zebrafish embryos induces targeted loss-of-function mutations in somatic cells

(A) Twenty one pairs of high-fidelity, obligate-heterodimer 16 ZFNs (Supplementary Table 1) targeting the gol locus were screened for activity in yeast using the assay shown in Fig. 1, with the exception that Me11 activity was assayed in the yeast growth medium, rather than relying on the number of MEL1-positive colonies. For each ZFN pair, activity in the medium of the Me11 enzyme is listed in mU, as detected after induction by galactose. (B) Schematic of the gol locus indicating recognition sites of three ZFN pairs chosen for further testing. ZFN pair 1 recognizes a site within Exon 4 upstream of the loss-of-function golb1 allele, whereas ZFN pairs 14 and 15 both recognize a site within the last exon upstream of coding sequence for two putative C-terminal transmembrane helices. (C) Injection of ZFN pair 14 mRNA (5 ng) into golb1 heterozygous 1-cell embryos induces somatic loss-of-function mutations in the wild-type gol allele. The 2 dpf embryo on the top left shows no evidence of loss of heterozygosity; the eyes are uniformly and darkly pigmented like wild-type embryos. Single or multiple patches of unpigmented cells can be observed in gol ZFN-injected embryos (remaining three panels), and sometimes mosaic patches were seen in both eyes. Except for these pigment clones, the embryos shown were phenotypically normal. (D) When PCR products from pooled individuals were sequenced, deletions and insertions typical of NHEJ were observed. In some cases, these are in frame and in other cases are frameshifts.
Figure 3. Injection of no tail ZFN-encoding mRNA into zebrafish embryos induces targeted loss-of-function mutations in somatic cells

(A) Five high-fidelity, obligate-heterodimer 16 ZFN pairs (Supplementary Table 2) targeting the ntl locus were screened for activity using the yeast-based assay shown in Fig. 1. See Fig. 2A legend for details. (B) Schematic of the ntl locus indicating the recognition site of the ZFN pairs chosen for further testing. ZFN pairs 2 and 3 recognize the same site within Exon 2 just upstream of the site of the loss-of-function ntlb195 insertion allele. (C) Injection of 5 ng ntl ZFN pair 2 mRNA into 1-cell ntlb195 heterozygous embryos induces mutations in the
wild-type ntl allele. Compared to uninjected embryos at 1 dpf (top panel), ZFN-injected embryos lack a notochord and tail like null mutants, or have patchy notochords and forked tails (inset), like hypomorphic ntl mutants. (D) Graph showing the percentage of ntl<sup>b195</sup> heterozygous embryos displaying wild-type or ntl mutant phenotypes after injection of 5 ng mRNA encoding either ZFN pair 2 or ZFN pair 3. A small percentage of injected embryos were defective (“unscored”). Approximately one-quarter of the ntl-like embryos were slightly more necrotic than the typical ntl mutant (4/17 for ZFN pair 2 and 5/18 for ZFN pair 3). (E) When PCR products from pooled ntl-like individuals were sequenced, deletions and insertions typical of NHEJ were observed. In some cases, these are in frame and in other cases cause frameshifts.
Figure 4. Injection of *no tail* ZFN-encoding mRNA in wild-type embryos creates novel *ntl* mutations that are transmitted through the germline

(A) Some juvenile fish derived from wild-type embryos injected with mRNA encoding *ntl*-targeting ZFNs (both conventional and high-fidelity FokI domains, the latter shown here) show posterior truncations. Right two panels show posteriorly truncated juveniles; left panels are normal-appearing siblings. (B) *ntl* phenotypes observed in progeny of ZFN-injected founder animals in complementation crosses at 2 dpf. Wild-type embryos injected with mRNA encoding high-fidelity, *ntl*-targeting ZFNs were grown to adulthood and eggs from founder females were fertilized in vitro with sperm from a *ntl*^b195^ heterozygous male. Representative progeny from this complementation test with Founder female A are shown in the right panel. (C) Novel *ntl* alleles (7 total) carried by founders that gave phenotypically *ntl* progeny in complementation cross (see also Table 3). Founder A was derived from a wild-type embryo injected with ZFN pair 2 (5 ng mRNA), and founders B through D from wild-type embryos injected with ZFN pair 3 (5 ng mRNA).
TABLE 1

ZFNs directed to the zebrafish golden gene induce somatic loss-of-function phenotypes.

| ZFN pair | Dose (ng) | Normal embryos | Abnormal embryos |
|----------|-----------|----------------|------------------|
|          |           | WT eye pigment | gol clones       | WT eye pigment | gol clones | Not scored | Total WT eye pigm | Total gol clones |
| 14       | 0.2       | 54/58 (93%) | 1/58 (2%)       | 2/58 (3%)      | 0/58 (0%) | 1/58 (2%)  | 56/58 96%        | 1/58 2%         |
|          | 1.0       | 39/42 (93%) | 2/42 (5%)       | 0/42 (0%)      | 1/42 (2%) | 0/42 (0%)  | 39/42 93%        | 3/42 7%         |
|          | 5.0       | 26/49 (53%) | 11/49 (22%)     | 0/49 (0%)      | 5/40 (10%)| 7/49 (14%) | 26/49 53%        | 16/49 32%       |
| 15       | 0.2       | 42/45 (93%) | 1/45 (2%)       | 0/45 (0%)      | 0/45 (0%) | 2/45 (4%)  | 42/45 93%        | 1/45 2%         |
|          | 1.0       | 26/29 (90%) | 3/29 (10%)      | 0/29 (0%)      | 0/29 (0%) | 0/29 (0%)  | 26/29 90%        | 3/29 10%        |
|          | 5.0       | 24/37 (65%) | 9/37 (24%)      | 0/37 (0%)      | 3/37 (8%) | 1/37 (3%)  | 24/37 65%        | 12/37 32%       |
|          | 0.2       | 11/11 (100%)| 0/11 (0%)       | 0/11 (0%)      | 0/11 (0%) | 0/11 (0%)  | 11/11 100%       | 0/11 0%         |
| 1        | 1.0       | 48/50 (96%) | 0/50 (0%)       | 0/50 (0%)      | 2/50 (4%) | 48/50 96%  | 0/50 0%          |                |
|          | 5.0       | 45/70 (64%) | 7/70 (10%)      | 9/70 (13%)     | 2/70 (3%) | 7/70 (10%) | 45/70 77%        | 9/70 13%        |
|          | 7.0       | 78/123 (63%)| 26/123 (21%)    | 12/123 (10%)   | 3/123 (2%)| 4/123 (3%) | 90/123 73%       | 29/123 24%      |

\(^1\) High-fidelity, gol-targeting ZFN mRNA was injected into 1-cell embryos heterozygous for the gol^{b1} allele at the indicated dose. Eye pigmentation mosaicism was scored at 48 hpf and embryos having at least one clone of unpigmented cells in an otherwise dark eye were scored as having gol eye clones. Representative examples are shown in Fig. 2C.

\(^2\) Most embryos in this category had slight to moderate developmental defects. Common syndromes were a bent body axis or slight head necrosis. The embryos in the “Not scored” column had severe developmental defects that precluded scoring of eye pigmentation mosaicism.
TABLE 2

ZFNs directed to the zebrafish no tail gene induce loss-of-function mutations on both chromatids early in development

| ntl ZFN pair | Dose (ng) | Embryos injected | Live embryos at 24h | Scoreable embryos at 24h | WT phenotype | ntl phenotype | WT ISH phenotype | ntl ISH phenotype |
|-------------|-----------|------------------|---------------------|--------------------------|--------------|---------------|------------------|------------------|
| 2           | 0.2 each  | 100              | 94/100 (94%)        | 88/100 (88%)             | 68/88 (77%)  | 20/88² (23%)  | N.D.             | N.D.             |
|             | 1.0 each  | 101              | 87/101 (86%)        | 60/101 (59%)             | 17/60 (28%)  | 43/60³ (72%)  | N.D.             | N.D.             |
|             | 6.0 each  | 120              | 39/120 (33%)        | 24/120 (20%)             | 4/24 (17%)   | 20/24⁴ (83%)  | 3/17 (17%)       | 14/17 (83%)      |

¹The ZFN pairs contained wild-type, rather than high-fidelity 16 FokI endonuclease domains, and were injected as mRNA into 1-cell wild-type zebrafish embryos.

²All of the 20 ntl-like embryos had a hypomorphic ntl b487 phenotype.

³13/43 (30%) had a null ntl phenotype, the remaining 70% had hypomorphic phenotypes.

⁴Virtually all embryos had a null ntl phenotype; approximately half were more necrotic than usual (see also Supplementary Fig. 10 online).
ZFNs directed to the zebrafish *no tail* gene induce loss-of-function germline mutations

| Founder | Complementation testing data | Chromatid genotyping data | Total |
|---------|------------------------------|--------------------------|-------|
|         | WT progeny | ntl progeny | Unscored progeny | % germline | WT progeny | ntl progeny | Unscored progeny |       |
| A       | 109/118 92.4% | 9/118 7.6% | 0/118 0% | 15.3% | 11/96 11.5% | 9/9 100% | N.A. | 20/105 19% |
| B       | 77/82 93.9% | 1/82 1.2% | 4/82 4.9% | 2.4% | 1/77 1.3% | 1/1 100% | 2/4 50% | 4/82 4.9% |
| C       | 37/50 74% | 3/50 6% | 10/50 20% | 12% | 9/37 24.3% | 3/3 100% | 3/7 42.9% | 15/47 31.9% |
| D       | 12/15 80% | 2/15 13.3% | 1/15 6.4% | 26.7% | 2/12 16.7% | 2/2 100% | 0/1 0% | 4/15 26.7% |

1 These progeny could not be conclusively phenotyped.

2 The ZFN target site overlaps a BsrDI restriction site. The chromatids were genotyped by amplifying the ZFN targeted stretch by PCR using primers that do not amplify the *ntl* allele, and measuring the frequency of disrupted alleles by determining the fraction of BsrDI-resistant PCR products.