

Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases

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We describe the use of zinc-finger nucleases (ZFNs) for somatic and germline disruption of genes in zebrafish (*Danio rerio*), in which 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 (ntl)* genes and developed a budding yeast-based assay to identify the most active ZFNs for use *in vivo*. Injection of ZFN-encoding mRNA into one-cell embryos yielded a high percentage of animals carrying distinct mutations at the ZFN-specified position and exhibiting expected loss-of-function phenotypes. Over half the ZFN mRNA-injected founder animals transmitted disrupted *ntl* alleles at frequencies averaging 20%. The frequency and precision of gene-disruption events observed suggest that this approach should be applicable to any loci in zebrafish or in other organisms that allow mRNA delivery into the fertilized egg.

The zebrafish has proven to be an excellent model for vertebrate development and disease¹ 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)² and TILLING for point mutations by detection of heterozygosity in a locus of interest, and subsequent sequencing, among a library of chemically mutagenized gametes. Both strategies have shortcomings and are limited in scope: morpholinos are active for only the first few days of development and do not affect juvenile or adult phenotypes, whereas 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 reduced³. Conventional gene targeting, a powerful technique for gene disruption in mouse embryonic stem cells⁴, often requires positive-negative selection with cytotoxic drugs⁵, which is inapplicable in the context of a vertebrate embryo.

The resistance of the genome in most metazoa to targeted sequence alteration⁵ has recently been overcome through the use of zinc-finger nucleases (ZFNs). Initially developed to cleave DNA *in vitro*⁶, ZFNs

are a fusion between the cleavage domain of the type IIS restriction enzyme *FokI* and a DNA-recognition domain containing three or more C₂H₂ zinc-finger motifs. 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 DNA⁷. DSB repair pathways operate in all eukarya, and include nonhomologous 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 repair⁸. NHEJ-based repair of a ZFN-induced DSB has been exploited to disrupt a gene in *Drosophila*⁹ and an intergenic region in *Caenorhabditis elegans*¹⁰, thus establishing the potential of this technology in reverse genetic applications, recently expanded to mammalian tissue culture cells¹¹. Here, using *golden/slc24a5 (gol)* and *ntl* as test loci, we investigated the feasibility of applying designed ZFNs to reverse genetics in zebrafish.

The *gol* pigment locus encodes a transmembrane protein, Slc24a5 (ref. 12), and was chosen for an initial test of ZFN-driven editing because the lack of embryonic pigmentation caused by homozygous viable *gol* mutations makes scoring for ZFN activity by loss of function straightforward¹³. The C₂H₂ zinc finger is a versatile DNA binding motif capable of recognizing a broad range of sequences¹⁴, and efforts over the past 14 years have yielded a large number of zinc fingers aimed at recognizing investigator-specified DNA triplets (see **Supplementary Methods** 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 four-finger ZFPs (see **Supplementary Methods** 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*, we measured the affinity of the ZFPs for their target sequence using an enzyme-linked immunosorbent (ELISA) assay (**Supplementary Fig. 2** online). Next, to determine which of these ZFPs will form pairs most likely to be active

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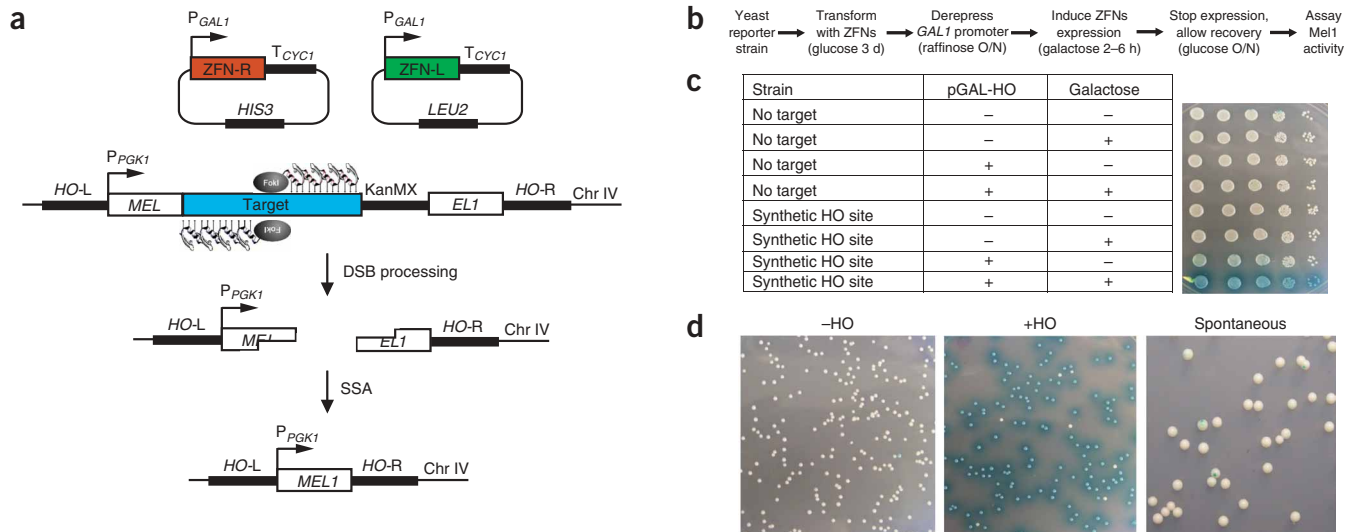


Figure 1 Yeast-based system for identification of maximally active ZFNs. **(a)** Components of yeast-based chromosomal reporter system: expression vectors for the ZFNs (top), target gene integrated into the chromosome at the HO locus (middle). After a double-strand break (DSB) is induced at the target 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 **a**, except it carried a recognition site of the HO endonuclease. Mel1 activity is only observed after induction of expression of the endonuclease (+gal) in the presence of its target in the reporter locus (bottom row). **(d)** Exceedingly low frequency of spontaneous *MEL1* gene repair. Essentially no Mel1 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.

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 Methods online).

In building this system, we made use of the well-established fact that whereas 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 by the single-strand annealing of the two repeats¹⁵. We disabled the open reading frame for a secreted form of alpha-galactosidase (encoded by the yeast *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 ZFNs targeting exons 4 and 9 in subsequent experiments (Fig. 2b, Supplementary Fig. 4 online).

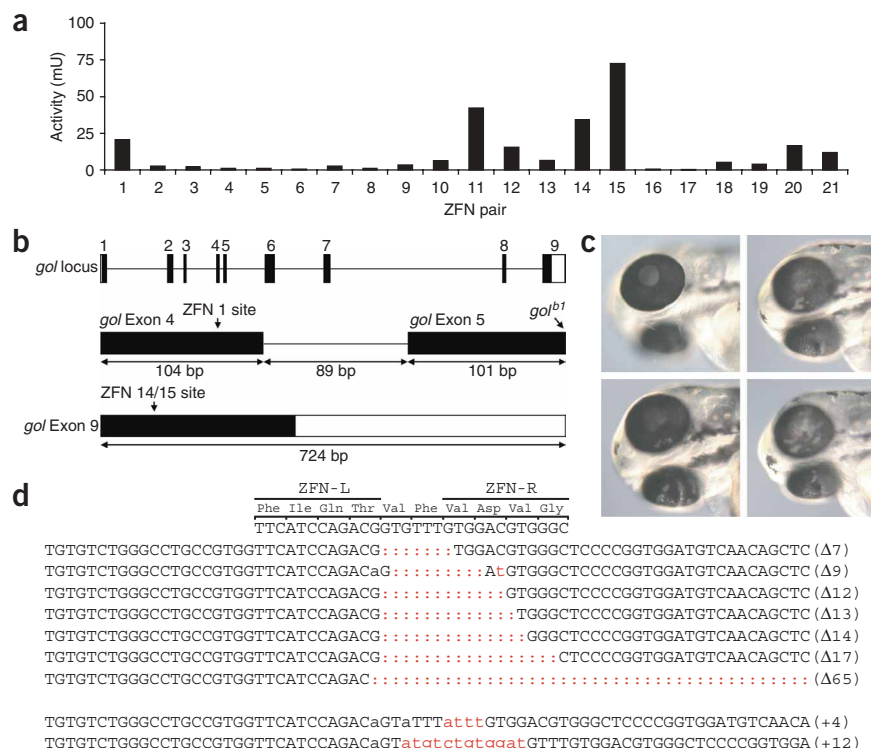
After screening in budding yeast, the ZFNs identified as active in that system were tested for native gene disruption activity at their intended target locus in zebrafish: the ZFN expression constructs were transcribed and capped *in vitro*, and the resulting mRNAs were injected into one-cell zebrafish embryos heterozygous for the *gol*^{b1} mutation¹². For the majority of our experiments, we used ZFNs carrying obligate-heterodimer forms of the *FokI* endonuclease domain¹⁶; these inhibit ZFN homodimerization and thus improve specificity by limiting cleavage at off-target sites recognized by ZFN

homodimers¹⁶. At 2 d post-fertilization (d.p.f.), *gol*^{b1} 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-*gol*^{b1} chromosome, in up to 32% of ZFN-injected, but not 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, >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 pigmentation mosaicism were otherwise 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 7–65 bp and insertions of 4–12 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 *ntl* gene, a major regulator of early embryogenesis. The choice of this target gene was largely inspired by the fact that the study

Figure 2 Injection of *gol* ZFN-encoding mRNA into zebrafish embryos induces targeted loss-of-function mutations in somatic cells. (a) Twenty-one pairs of high-fidelity, obligate-heterodimer¹⁶ ZFNs (Supplementary Table 1) targeting the *gol* locus were screened for activity in yeast using the assay shown in Figure 1, with the exception that Mel1 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 Mel1 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 *gol*^{bl} 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 *gol*^{bl} heterozygous one-cell embryos induces somatic loss-of-function mutations in the wild-type *gol* allele. The 2 d.p.f. 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.



of early development is a major focus of biological investigation in the zebrafish model system. The *ntl* gene encodes an essential T-box transcription factor required for proper mesoderm formation; zebrafish embryos homozygous for a null *ntl* mutation lack a notochord and a tail¹⁷. To test whether ZFNs can induce somatic mutations in the *ntl* gene, we followed the same scheme described for the *gol*-targeting ZFNs (ZFN design, ELISA *in vitro* binding assay, and *in vivo* yeast-based activity assay) (Fig. 3a; Supplementary Table 2 and

Supplementary 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-heterodimer¹⁶ ZFNs into one-cell embryos from a cross between wild-type and *ntl*^{bl195} heterozygous fish. Because half the injected embryos carry the

Table 1 ZFNs directed to the zebrafish *gol* gene induce somatic loss-of-function phenotypes

ZFN pair ^b	Dose (ng)	Normal embryos		Abnormal embryos ^a			Total WT eye pigment	Total <i>gol</i> clones
		WT eye pigment	<i>gol</i> eye clones	WT eye pigment	<i>gol</i> eye clones	Not scored		
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%)	5/49 (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%
1	0.2	11/11 (100%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	11/11 100%	0/11 0%
	1.0	48/50 (96%)	0/50 (0%)	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%)	54/70 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%

^aMost 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. ^bHigh-fidelity, *gol*-targeting ZFN mRNA was injected into one-cell embryos heterozygous for the *gol*^{bl} allele at the indicated dose. Eye pigmentation mosaicism was scored at 2 d 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 Figure 2c.

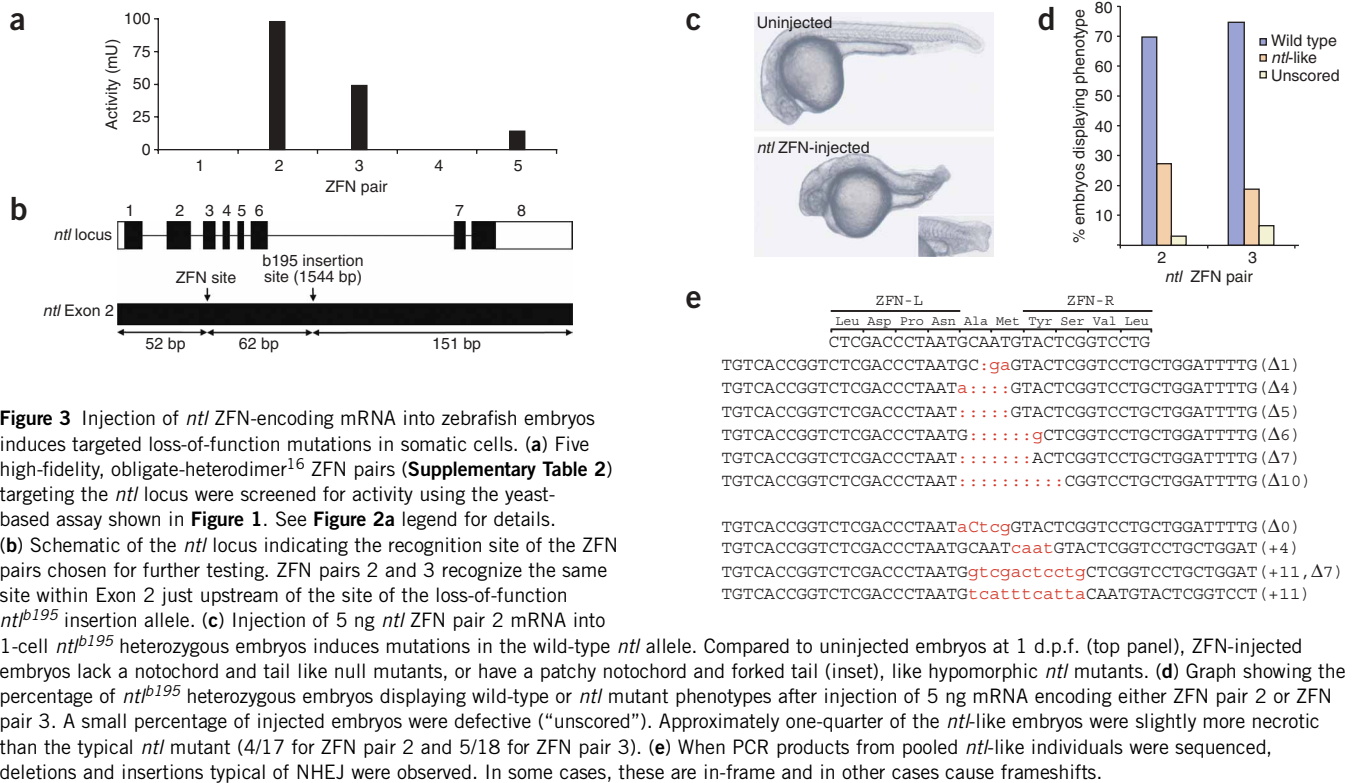


Figure 3 Injection of *ntl* ZFN-encoding mRNA into zebrafish embryos induces targeted loss-of-function mutations in somatic cells. **(a)** Five high-fidelity, obligate-heterodimer¹⁶ ZFN pairs (**Supplementary Table 2**) targeting the *ntl* locus were screened for activity using the yeast-based assay shown in **Figure 1**. See **Figure 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 *ntl*^{b195} insertion allele. **(c)** Injection of 5 ng *ntl* ZFN pair 2 mRNA into 1-cell *ntl*^{b195} heterozygous embryos induces mutations in the wild-type *ntl* allele. Compared to uninjected embryos at 1 d.p.f. (top panel), ZFN-injected embryos lack a notochord and tail like null mutants, or have a patchy notochord and forked tail (inset), like hypomorphic *ntl* mutants. **(d)** Graph showing the percentage of *ntl*^{b195} 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 (“unscorable”). 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.

ntl^{b195} null allele, we hypothesized that these embryos would reveal ZFN-driven mutations of the wild-type allele on the other chromosome. Notably, we observed that 16–27% of injected embryos displayed a *ntl*-like phenotype, either mimicking the null phenotype¹⁷ or a less severe phenotype typical of a hypomorphic allele, *ntl*^{b487} (**Fig. 3c–d** and **Supplementary Fig. 7** online). 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 endonuclease¹⁶ that allows one to measure the fraction of chromatids carrying mutations

relative to the wild-type sequence. The fraction of mutated chromatids ranged from 3–17% 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 **Figure 3e** appear to represent alleles generated by NHEJ-induced repair of the ZFN-cleaved chromatid, because chromatids bearing the ~1.5-kb *ntl*^{b195} insertion (**Fig. 3b**) are too large to be amplified and analyzed.

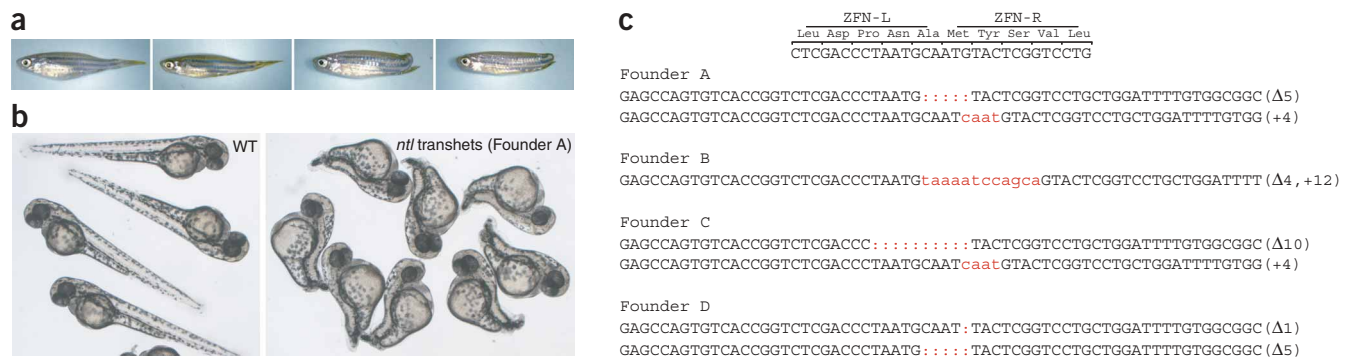


Figure 4 Injection of *ntl* ZFN-encoding mRNA in wild-type embryos creates novel *ntl* mutations that are transmitted through the germ line. **(a)** Some juvenile fish derived from wild-type embryos injected with mRNA encoding *ntl*-targeting ZFNs (both conventional and high-fidelity *FokI* domains, 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 d.p.f. 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 (seven 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 2 ZFNs directed to the zebrafish *ntl* gene induce loss-of-function mutations on both chromatids early in development

<i>ntl</i> ZFN pair ^a	Dose (ng)	Embryos injected	Live embryos at 24 h	Scoreable embryos at 24 h	WT phenotype	<i>ntl</i> phenotype	WT ISH phenotype	<i>ntl</i> ISH phenotype
2	0.2 each	100	94/100 (94%)	88/100 (88%)	68/88 (77%)	20/88 ^b (23%)	N.D.	N.D.
	1.0 each	101	87/101 (86%)	60/101 (59%)	17/60 (28%)	43/60 ^c (72%)	N.D.	N.D.
	6.0 each	120	39/120 (33%)	24/120 (20%)	4/24 (17%)	20/24 ^d (83%)	3/17 (17%)	14/17 (83%)

^aThe ZFN pairs contained wild-type, rather than high-fidelity¹⁶ *FokI* endonuclease domains and were injected as mRNA into 1-cell wild-type zebrafish embryos. ^bAll of the 20 *ntl*-like embryos had a hypomorphic *ntl*^{b487} phenotype. ^c13/43 (30%) had a null *ntl* phenotype; the remaining 70% had hypomorphic phenotypes. ^dVirtually all embryos had a null *ntl* phenotype; approximately half were more necrotic than usual (see also **Supplementary Fig. 10** online).

Given our published data on the feasibility of generating single-step biallelic genome editing events using ZFNs in human cells¹⁸, 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 fish and found that *ntl* mutant-bearing amplicons represent a substantial fraction of the total (sample 1, 5/25 (20%) *ntl*-bearing chromatids, two different alleles; sample 2, 3/30 (10%) *ntl*-bearing chromatids, one allele; sample 3, 8/29 (28%) *ntl*-bearing chromatids, four different alleles) (**Supplementary Fig. 9** online).

When *ntl*-targeting ZFNs carrying wild-type *FokI* cleavage domains were injected into wild-type embryos, a high frequency of embryos exhibiting an *ntl* phenotype was observed; sequencing of a 226-bp region surrounding the DSB site revealed that each of three representative embryos carried between 64 and 81% disrupted *ntl* alleles (**Table 2** and **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 one-cell embryo.

To demonstrate that ZFNs can effectively induce mutations in the germ line, we raised wild-type embryos injected with *ntl*-targeting high-fidelity¹⁶, obligate-heterodimer ZFNs to sexual maturity and screened them. 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 *ntl*^{b195} allele. Of seven females analyzed, four generated *ntl* progeny (**Table 3**; **Fig. 4b**) at frequencies ranging from 1–13% as gauged by a 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 germ line 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 identified an additional two carriers (germline transmission rates of 33% and 53%) using complementation crosses and an additional five carriers (germline transmission rates of 6–25%) by analyzing haploids generated from founder females. Taken together, the data show that >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, we derived an experimentally determined DNA-binding consensus site for each ZFN (**Supplementary Fig. 11** online). This information was then used to search the zebrafish genome for sites showing the highest similarity to each ZFN's intended target site (**Supplementary Fig. 11** online). To experimentally determine whether ZFNs cleave at these alternative 'off-target' sites, we directly analyzed the top five potential off-target loci for each *ntl*-targeting ZFN pair. We used PCR to amplify the cognate chromosomal loci from progeny embryos with the *ntl* phenotype (**Table 3**) carrying ZFN-induced mutations at the *ntl* gene, and analyzed them by direct sequencing, loss of restriction-fragment-length polymorphism and mismatch endonuclease assays (primers used are listed in **Supplementary Table 3**). All the chromatids we analyzed were wild type (**Supplementary Fig. 11** online). These results confirmed 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. Our analysis did not reveal ZFN-induced mutations at loci other than *ntl*; however, were an undesired secondary mutation to occur at a rate as high as 10%, it would rapidly segregate from the desired *ntl* mutation even after one generation.

Table 3 ZFNs directed to the zebrafish *ntl* gene induce loss-of-function germline mutations

Founder	Complementation testing data				Chromatid genotyping data ^a (frequency of <i>ntl</i> allele disruption)			
	WT progeny	<i>ntl</i> progeny	Unscored progeny ^b	% germline	WT progeny	<i>ntl</i> progeny	Unscored progeny	Total
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%)

^aThe 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*^{b195} allele and measuring the frequency of disrupted alleles by determining the fraction of BsrDI-resistant PCR products. ^bThese progeny could not be conclusively phenotyped.

The data presented here show the utility of ZFNs for inducing mutations at a specified locus in the zebrafish and for rapidly producing embryos and adult animals with corresponding loss-of-function phenotypes (Tables 1 and 2) and adults carrying new alleles (Table 3). Although ZFNs injected as mRNA are expressed transiently, this is sufficient to introduce a stable genetic mark. We describe the successful disruption of three distinct positions at two 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 for the intended locus (Table 3 and Supplementary Fig. 11 online).

The C₂H₂ zinc-finger motif^{19,20} has been engineered to recognize many distinct DNA sequences^{14,21} (Supplementary Methods), 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 locus¹⁸. Our data show that the combination of this versatile platform with a rapid yeast-based assay for the identification of maximally active engineered ZFNs allow targeted genetic modification in the zebrafish, and suggest that ZFN technology may be applicable to many organisms for which reverse-genetics strategies are currently unavailable.

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 Methods, Supplementary Figures 1,4,6 and Supplementary Tables 1,2,4. 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 high-fidelity, obligate-heterodimer¹⁶ ZFN forms were used, except the experiments in Table 2 (also described in Supplementary Fig. 10), for which ZFNs carried wild-type *FokI*. In all other aspects, the nucleases had the same architecture as previously described¹⁸.

Yeast assay. Detailed methods are provided in Supplementary Methods. All strains were derived from the S288C parental strain 69-1B (*MATa*, *his3A200*, *lys2-128δ*, *leu2A1*). The SSA reporter constructs were assembled into the *HO* targeting vector *HO*-poly-KanMX4-*HO*²³ by cloning two overlapping and nonfunctional fragments of the *MEL1* gene under the control of the *PGK1* promoter (Supplementary Fig. 3). ZFN were expressed using a galactose-inducible promoter²⁴, and inductions were performed as described²⁵ following the scheme presented in Figure 1b. The quantitative α -galactosidase assay was performed as described with minor modifications²⁶.

Zebrafish mutant alleles, stocks and husbandry. Zebrafish 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 described²⁷. The *gol*^{b1} and *ntl*^{b195} mutant alleles used have been previously described^{12,28}. The *ntl*^{b487} allele was identified in a forward genetic screen performed in Eugene, Oregon, and failed to complement two other *ntl* alleles (S.L.A. and C.B. Kimmel, unpublished data). Transheterozygotes were obtained in expected mendelian ratios in at least two independent pairwise crosses (71/326 [22%] for *ntl*^{b160}/*ntl*^{b487} and 135/542 [25%] for *ntl*^{b459}/*ntl*^{b487}). Homozygous *ntl*^{b487} 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), in contrast to the complete lack of tail and notochord in null mutants¹⁷.

Preparation and microinjection of ZFN mRNA. Matched *gol* or *ntl* 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, which were done with separate expression constructs (see Supplementary Figs. 4,6 and Supplementary Tables 1,2 for vector maps, construct sequences, and recombinant DNA protocols and Supplementary

Table 4 for plasmid names). In all cases, plasmids were linearized with *EagI* and transcribed *in vitro* using the T7 mMessage mMachine transcription kit (Ambion) to generate a single capped transcript containing both open reading frames. Approximately 5 nl of capped mRNA was injected into one-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'-GGAACAATCCCATACGCTCTGCAG-3' and a 226-bp region surrounding the *ntl* 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, whereas wild-type sequences were digested to 176- and 50-bp fragments. Heterozygous individuals carried all three band sizes. PCR products were ligated into the pCR 4-TOPO vector for sequencing (Invitrogen).

For analysis of potential off-target action by the ZFNs, the position weight matrix (that is, an experimentally determined consensus DNA binding site) for each ZFN was determined using an *in vitro* site selection method (Supplementary Methods). The off-target sites, and all the genotyping data, are provided in Supplementary Fig. 11.

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.** To analyze subtle notochord defects in *ntl* ZFN mRNA-injected embryos that may not have been detected by morphological analysis, we sorted embryos by phenotype and processed for *ntl* expression by whole mount *in situ* hybridization as previously described²⁹.

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.

Requests for materials. For zinc-finger nuclease reagents described in the paper Supplementary Table 4, furnov@sangamo.com; for ZFN reagents other than those described here, zfn@sial.com or through <http://www.sigma.com/zfn>.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

F.D.U. and S.L.A. conceived the project; Y.D., J.M.M., J.C.M., L.Z., E.J.R., F.D.U. and S.L.A. designed experiments; Y.D., J.M.M., F.F., C.N., G.E.K., R.A., T.D.H. and S.L.A. performed experiments; Y.D., J.M.M., P.D.G., F.D.U. and S.L.A. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturebiotechnology/>.

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