

Gene Disruptions in Zebrafish

The zebrafish *Danio rerio* is a widely-used model organism that is not described in the book; a link to its genome database is found in Table 2.2. Zebrafish are easy to grow—they are a popular denizen of home aquariums—and provide a good model for analyzing many aspects of vertebrate development and evolution. Both fertilization and development occur externally, and the developing egg is transparent so that defects are easy to observe. Hundreds of mutants are known, most of them induced during random mutagenesis screens of the type described in Chapter 3, using either ENU or retroviral integration as the mutagen. This textbox describes recent advances which suggest that targeted gene disruptions may also be feasible.

Zinc finger nucleases provide sequence-specific double-stranded breaks. The strategy for targeted gene disruptions in zebrafish relies on the properties of a class of enzymes known as the zinc finger nucleases (ZFNs). These are fusion proteins made by attaching C2H2 zinc finger domains to the DNA cleavage domain of the endonuclease *FokI* (Panel A in the figure). *FokI* is a modular enzyme, with its endonuclease domain separable from its own DNA binding domain. In the ZFN fusion proteins, the endonuclease domain produces a double-stranded break, while the zinc fingers mediate the sequence-specific binding required to target the break to a particular site. Each zinc finger interacts with a different three-base target sequence, so different numbers and combinations of zinc fingers can be used to target different cleavage sites. Two different ZFNs are shown in Panel A of the figure at the end of this document, with slightly different zinc finger domains and thus different binding sites.

The targeted double-stranded breaks are then repaired in the cell by non-homologous end-joining, which often results in the addition or deletion of a few nucleotides at the site of the break. The addition or deletion of bases produces a frameshift mutation, knocking out the function of the targeted gene. Double-stranded breaks are also the necessary first step in homologous recombination, which opens the possibility that this method could be used in the future for targeted gene replacement, as described in Chapter 6 for yeast and mice; targeted gene replacement has yet to be carried out in zebrafish.

Targeting ZFNs to known genes. Two recent studies used different zinc finger domains and sequence targets to test the ability of ZFNs to make gene disruptions in zebrafish. The strategy is shown in Panel B of the figure, below. One study used ZFN proteins with three zinc fingers for targeting a nine-base sequence within the *kdr* gene; mutants in *kdr* have an embryonic vascular defect. The other study used ZFNs with four zinc fingers for targeting a twelve-base target site in the *golden* gene affecting pigmentation, and another ZFN with four different zinc fingers to target the *no tail* gene affecting tail morphology; the figure shows ZFNs with three zinc fingers for simplicity. All three of these genes have other mutant alleles and an easily observed mutant phenotype.

To make the gene disruption, a mixture of two different mRNAs with the coding region for the endonuclease domain in-frame and downstream of the zinc fingers was injected into the fertilized fish egg (Panel B of the figure). The egg translates the mRNA, producing fusion proteins that bind as dimers to their preferred sites within the target gene in the genome. Once bound, the dimeric protein cleaves the target DNA, producing a substrate for repair by non-homologous end-joining, and thereby generating a mutant; one of the small deletions of the *kdr* gene is shown in the figure.

Mutants were observed at a high rate, with as many as a third of the injected eggs producing a mutation in the targeted gene. Most but not all of the mutations were the small insertions or deletions arising from non-homologous end joining, indicating that the ZFN fusion protein is working as expected.

Because the mRNA is injected into the one-cell embryo as it is undergoing development, the gene disruption may not affect all of the cells of the adult fish. That is, the fish arising from the injected embryos could be mosaics, as shown at the bottom of Panel B in the figure. (Mosaicism is discussed in more detail in Chapter 9.) Breeding schemes are carried out to produce homozygotes for the newly induced mutations, which appear to be faithfully transmitted in the germline. As described in Chapter 6, a similar phenomenon is also seen in mice arising from altered embryonic stem cells, so it is not surprising that mosaicism was observed in fish. One intriguing modification was to inject the mRNA into eggs that are heterozygous mutant for the *golden* gene, so that homozygous *golden/golden* mutants could be recovered easily. This is a type of non-complementation screen, as described in Chapter 3, and works well for mutants like *golden* that are not lethal to the organism.

Current limitations and possible future directions. The key to the technique described here is the identification of an appropriate target site within the gene of interest. This involves not only finding a gene-specific sequence but also constructing zinc finger domains that will bind to that sequence. Three-base binding sequences are being determined for an increasing number of zinc finger domains, but correlating the available zinc finger domains with the sequence in the gene is the major constraint. Most of the

other laboratory manipulations involved in the protocol, such as injecting large number of fertilized eggs, screening them for mutant phenotypes, and breeding the mutant fish, are routine techniques in most zebrafish laboratories. Thus, the availability of ZFNs to bind the target sequences may be the primary limitation.

One potential concern with all targeting techniques involves off-target double-stranded breaks - that is, sequences in other genes that are the same or highly similar to the desired target sequence. This possibility was tested in these experiments by examining sequences that differ by one or a few nucleotides from the preferred target. Less than 1% of these potential off-target sites were mutated, indicating that the double-stranded break site is highly specific so long as the binding site for the zinc finger domains is well-established.

There is also a question of how many zinc fingers are needed for the specific targeting. There appear to be advantages and disadvantages to using more zinc fingers and a longer target sequence. The study using four zinc fingers and a 12-base recognition site was able to use a higher dosage of the ZFNs, possibly because no off-target sites were affected. In addition to producing a highly specific mutant, the higher dose also produced some fish that were homozygous mutant in the first generation, thus eliminating a breeding step. On the other hand, a gene construct with four defined zinc finger domains is harder to make than one with three domains, if only because the preferred binding site for most zinc fingers is not yet completely known.

In Chapter 6, we note that gene disruptions and gene replacements (that is, reverse genetic approaches) have two requirements: the ability to produce a directed mutation in the genes of a single cell; and the ability to grow an organism from that altered cell. The external fertilization and development of the fish allows for the second of these

criteria to occur routinely. There is no egg shell to penetrate as is found in flies and worms, and fish embryos are more easily accessible for manipulation than are mouse embryos. The ZFNs may provide the first of these criteria: a flexible method to direct mutations to one particular gene. The ability to make gene disruptions and potentially gene replacements in a vertebrate like the zebrafish is an exciting possibility for exploring vertebrate development, evolution, and disease.

References

Mutations in zebrafish--general

Amsterdam, A. and N. Hopkins, 2006. Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends in Genetics* 22: 473-478.

Targeted mutations using ZFNs

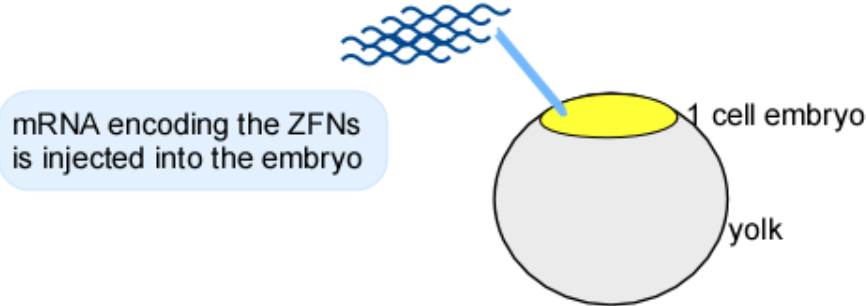
Doyon, Y. et al., 2008. Heritable targeted gene disruptions in zebrafish using designed zinc-finger nucleases. *Nature Biotechnology* 26: 702-708.

Meng, X, et al., 2008. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nature Biotechnology* 26: 695-701.

A. Zinc Finger Nucleases



B. Making Targeted Mutations



The ZFN proteins bind to their target sequences and produce a double-stranded DNA break



A deletion (or insertion) is made by non-homologous end-joining at the DS break



The mutation may affect all cells in the embryo or only some of the cells

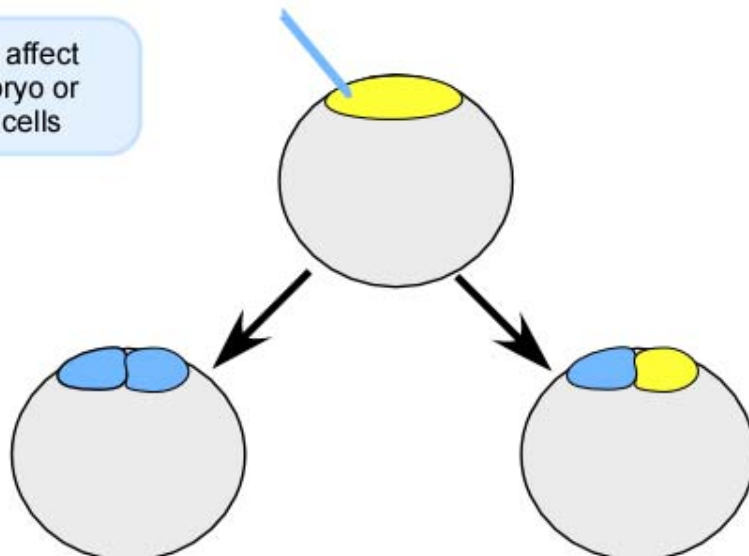


Figure Legend. Panel A depicts two different zinc finger nuclease (ZFN) fusion proteins. Each has three zinc finger domains that are not identical, as shown by the different shades of green. These zinc finger domains are fused to the coding region of the endonuclease domain from the FokI endonuclease in red.

Panel B illustrates the procedure. mRNA encoding the ZFNs is injected into the one-cell embryo. The embryo translates the mRNA to make ZFN proteins that bind to their target sequences. The nuclease acts as a dimer and produces a double-stranded DNA break. Note that one ZFN subunit binds to one 9-base sequence and the other subunit to a different 9-base sequence. The break and rejoining occurs between the two binding sequences. The rejoining can produce a deletion, as shown here for one of the mutations in the *kdr* gene, or an insertion. Since the mRNA is injected into a developing embryo, it is possible that only one of the cells in the two-cell embryo will have the mutation producing a genetic mosaic, similar to what happens with ES cells in the mouse. In this case, the mosaic fish need to be bred among themselves to produce a strain in which the gene is knocked out in all cells.