GENETIC ENGINEERING

Editing zebrafish becomes conditional

Burg, L. et al. *PLoS Genet* **14**, e1007754 (2018)

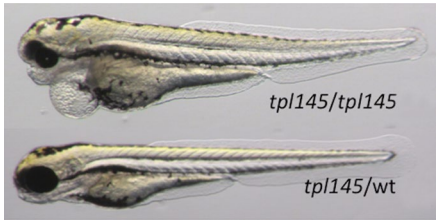
Mouse geneticists are lucky. Spoiled, you could say. For close to a quarter of a century now, they have been able to knock out genes not just comprehensively, throughout all the cells of a mouse, but also under specific, defined conditions—at a particular period in its development, say, or in selected tissues or cell types.

The same is not true for other vertebrates. Although the use of non-murine model species such as zebrafish has increased over the years, the tools to create conditional knockouts for these organisms have been absent. "Real conditional mutagenesis, to date, has only been possible in the mouse," says Darius Balciunas, a molecular geneticist at Temple University in Philadelphia who studies regeneration mechanisms.

That's a big problem, Balciunas explains. While knockouts have played a crucial role in identifying gene function, they can fall short. Some genes are so important to the developing organism that embryos lacking them are just not viable. Conditional knockouts allow researchers to delete a gene in a more limited manner, allowing the mutant to survive. They also provide a more nuanced and targeted way to study genes' roles in different biological processes.

The process for creating conditional knockouts in mice has traditionally involved delivering loxP sites—34-base-pair sequences derived from the bacteriophage P1—to both sides of an exon in a target gene. When a gene is 'floxed,' or flanked by LoxP sequences, it continues to be expressed unless a P1 enzyme called Cre recombinase inactivates it.

Floxing was first done in mice in 1994. Since then, molecular biologists have developed many mouse lines in which Cre recombinase is expressed in a tissue- or cell-specific manner, in such a way that it can be turned on pharmacologically. However, it's taken the more recent arrival of species-agnostic gene editing technologies like CRISPR/Cas9 to target specific points in the genome of animals like the zebrafish. In a recent report in *PLoS Genetics*, Balciunas's team describes how they've harnessed CRISPR/Cas9 to create a straightforward and accurate method for engineering selective mutations in zebrafish.



Heart defects observed in a conditional knock-out (top) compared to a wild-type zebrafish (bottom). Credit: Burg et al 2018. PLOS

The method was built on prior work from Balciunas' team, in which they developed an improved approach to genetically edit zebrafish to express epitopes for antibody binding experiments (*Sci Rep* **6**, 36986; 2016). In 1-cell zebrafish embryos, they deployed CRISPR/Cas9 to cut the DNA at a desired location, then used oligonucleotides—synthesized snippets of DNA—to deliver the epitope-coding sequences.

The approach worked so well that in the current work, the researchers set out to see whether it could be used to flox specific genes. They injected the CRISPR/Cas9 (along with guide RNAs that home it to the target gene) and the oligonucleotides for LoxP into embryos, then allowed the fish to grow up. Animals confirmed to carry a LoxP site were bred, and their offspring were edited with the method again to arrive at a second generation with the second LoxP site, ready for conditional testing.

Balciunas and his colleagues used the technique on four genes: tbx20, fleer, aldh1a2a, and tcf2, which are all required for development, meaning that homozygous mutants do not survive past the first few days. They generated fully conditional mutants for the first two genes and showed that floxed alleles were fully functional in their wild-type state—that is, in the absence of Cre recombinase. Injecting Cre recombinase mRNA into embryos led to complete loss of tbx20 and fleer function. They also tested floxed tbx20 mutants with a transgenic line expressing pharmacologically inducible Cre recombinase.

The technique's precision is not stellar: The engineered mutation integrates properly in only a small percentage of embryos. To find them, the researchers developed a screening protocol using a two-step form of PCR called nested PCR, which identifies DNA sequences with an extra degree of selectivity. "Often people start screening and they find incomplete integration, and then they give up—they say, 'Oh, this doesn't work,'" says Balciunas. "What we have found over the years is that this only means you have to work a little harder. If you screen enough fish, you will find one that has what you want."

Its magic, though, is its simplicity, says Mary Mullins, a cell and developmental biologist at the University of Pennsylvania who was not involved in the work. "There's nothing tricky—that's the lovely thing about it," she says. "There isn't a plasmid construct that has to be generated; you're just using an oligonucleotide."

There is some room for improvement, she notes. Currently, the two LoxP sequences must be integrated sequentially, which requires breeding two generations of animals. Balciunas agrees that the process takes a long time, much of it spent waiting for a generation of fish to grow up. "Roughly, a floxing experiment should take a year and a half of on-and-off work," he says. "So it is really important in selecting the target gene that you have really good prior evidence for thinking that this gene plays an important role."

For their part, Balciunas and his colleagues are ready to enjoy the fruits of their conditional knockout labor. They are completing the floxing of the remaining two genes described in the paper and are crossing conditional mutants with different Cre drivers to look at whether, when, in what cell types, and how the mutated genes are involved in regeneration.

"Now," he says, "the interesting biology is coming up."

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