Development of Thyroid Deiodinase Knockout *Xenopus tropicalis* Using CRISPR/Cas12a Gene Editing Towards Establishment of an Amphibian Adverse Outcome Pathway

Sally Mayasich[1,2], Philip DeGoey[2], Jonathan Haselman[2], Sigmund Degitz[2]

[1]ORISE/ORAU Research Participation Program, [2]U.S. EPA, ORD, CCTE, Great Lakes Toxicology & Ecology Division (GLTED), Duluth, MN

Sally Mayasich | mayasich.sally@epa.gov | 218-529-5122

www.epa.gov

Introduction and Objectives

Three iodothyronine deiodinase enzymes, identified in both mammals and amphibians, are selenoproteins anchored in the cell membranes. DIO1 and DIO2 catalyze the removal of an iodine from thyroid hormones to activate the hormone in specific tissues at appropriate times, and the DIO3 inactivates the hormone when it is no longer needed. We used the clustered regularly interspaced short palindromic repeat (CRISPR) /Cas12a (Cpf1) system to disrupt type 2 and type 3 iodothyronine deiodinase enzyme genes (***Xtdio2*** and ***Xtdio3***) in *Xenopus tropicalis* zygotes. The objectives of gene disruption are to:

- Significantly decrease or knock out the expression of a specific protein target in the thyroid system to
- Identify molecular initiating events, key events and adverse outcomes for Adverse Outcome Pathway (AOP) development to
- Predict the effects in amphibians of chemicals that inhibit deiodinases. For example, our ToxCast chemical screening identified several strong inhibitors of *Xenopus* dio3 activity.*

CRISPR/Cas12a (LbCpf1) crRNA design and microinjection methods

- Guide RNA design was aided using CRISPRscan and CRISPR RGEN tools Cas-designer. The CRISPR guide RNA oligos (crRNA) were synthesized by Integrated DNA Technologies. Effective crRNAs were selected through *in vitro* digestion of a PCR-generated genomic DNA segment containing the target site, incubated with a ribonucleoprotein (RNP) complex formed between *X. tropicalis dio2* or *dio3* crRNA and LbCpf1 enzyme (New England Biolabs; NEB). The NEB protocol was used, with modifications.
- *X. tropicalis* adults were induced to spawn by hCG injection; eggs were collected and treated with L-cysteine rinse; single-cell zygotes were sorted into 1/9x Modified Ringers with 3% Ficoll.
- Preparation of the microinjection cocktail followed Moreno-Mateos et al. (2017), with modifications. Single-cell-stage fertilized embryos (zygotes) were injected with ~2 nl (20 fmol) of either the *dio2* or *dio3* RNP complex in Cpf1 working buffer (20mM Hepes pH 7.5, +KCl+MgCl2+TCEP+glycerol) with 0.01% phenol red.
- Following 24-30 hours of incubation at 25°C in 1/9x MR, embryos were transferred to 4.5 L flow-through tanks delivering Lake Superior Water @ ~25 mL/min.

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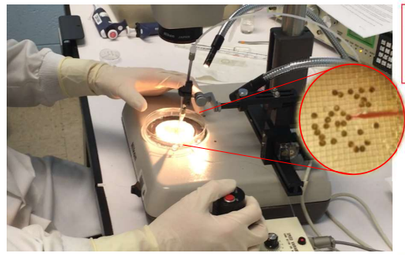
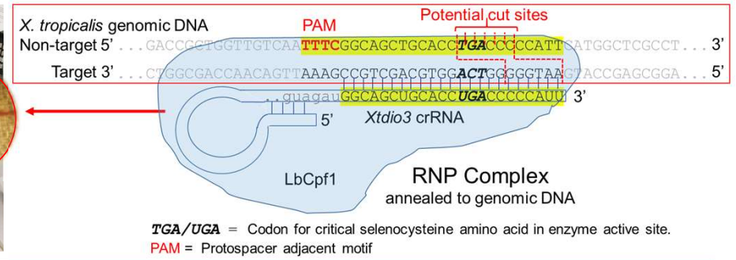
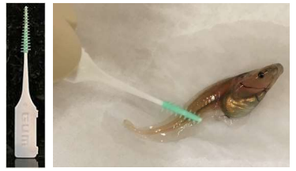
Microinjection of RNP complexes into *X. tropicalis* zygotes

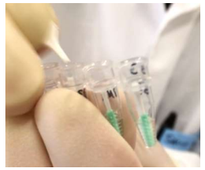




Rapid, non-invasive swab DNA extraction and polymerase chain reaction (PCR) genotype screening

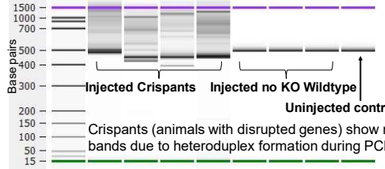


GUM® softpick swab; no anesthesia needed.



Pre-score and snap handle.

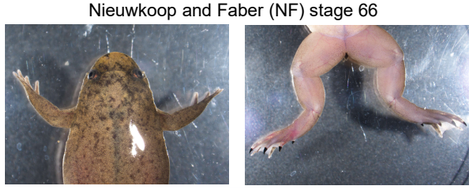
Tadpoles in individual beakers until DNA extraction, PCR and Bioanalyzer ("electrophoresis") results are complete (total 3-4 hrs).



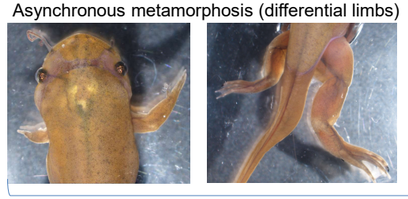
Crispants (animals with disrupted genes) show multiple bands due to heteroduplex formation during PCR.

Results and Adverse Outcome Pathway (AOP) Development

Mock injected control at 45 days
Nieuwkoop and Faber (NF) stage 66



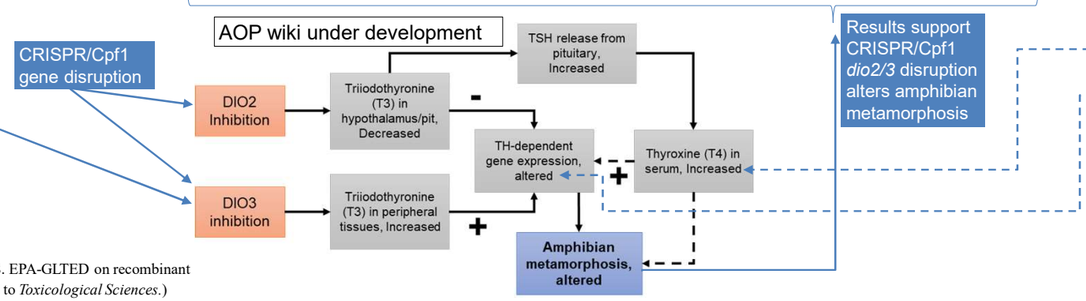
dio2 Crispant at 44 days
Asynchronous metamorphosis (differential limbs)



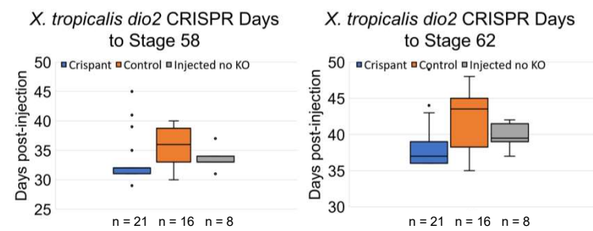
dio3 Crispant at 26 days
Precocious metamorphosis/early tail and gill resorption

Chemicals that could alter amphibian metamorphosis through inhibition of type 3 deiodinase enzyme.*	Xenopus dio3 IC50 (µM)
Chlorothalonil	0.67
Fipronil	0.88
Fluazinam	2.27
Triflumizole	2.77
Pentachloropyridine	6.17
SSR69071	7.83
Quinoxyfen	7.88
Bisphenol A diglycidyl ether	8.91
Pirimicarb	11.53
Octhilinone	13.05
Dichlone	13.8

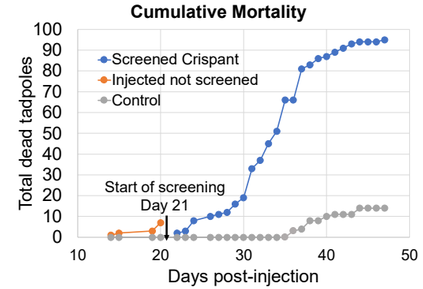
*Results of *in vitro* screening assays performed at U.S. EPA-GLTED on recombinant *X. laevis* dio3 enzyme (Mayasich et al. to be submitted to *Toxicological Sciences*.)



Accelerated metamorphosis in *dio2* crispants

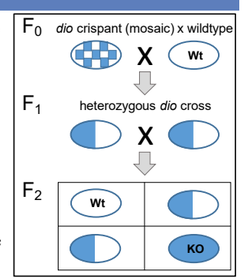


Precocious metamorphosis leads to high mortality in *dio3* crispants



Future Directions

- Surviving crispants will be cross-bred to attempt complete knockout of the *dio2* and *dio3* genes in *X. tropicalis* F2 generation.
- Sequencing;
- serum analyses for T3 and T4; and
- TH-dependent gene expression analyses will be conducted.



Acknowledgements

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