Unraveling the role of AHR gene duplication in PAH toxicity in zebrafish

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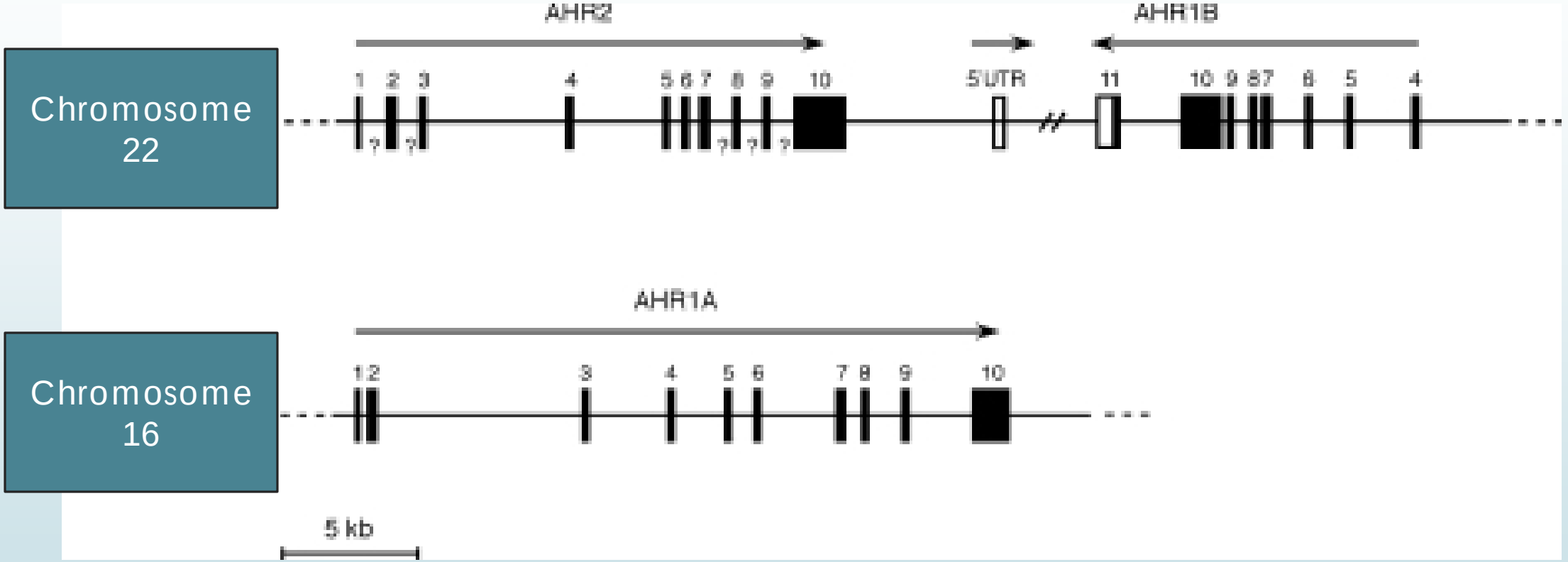
Outline

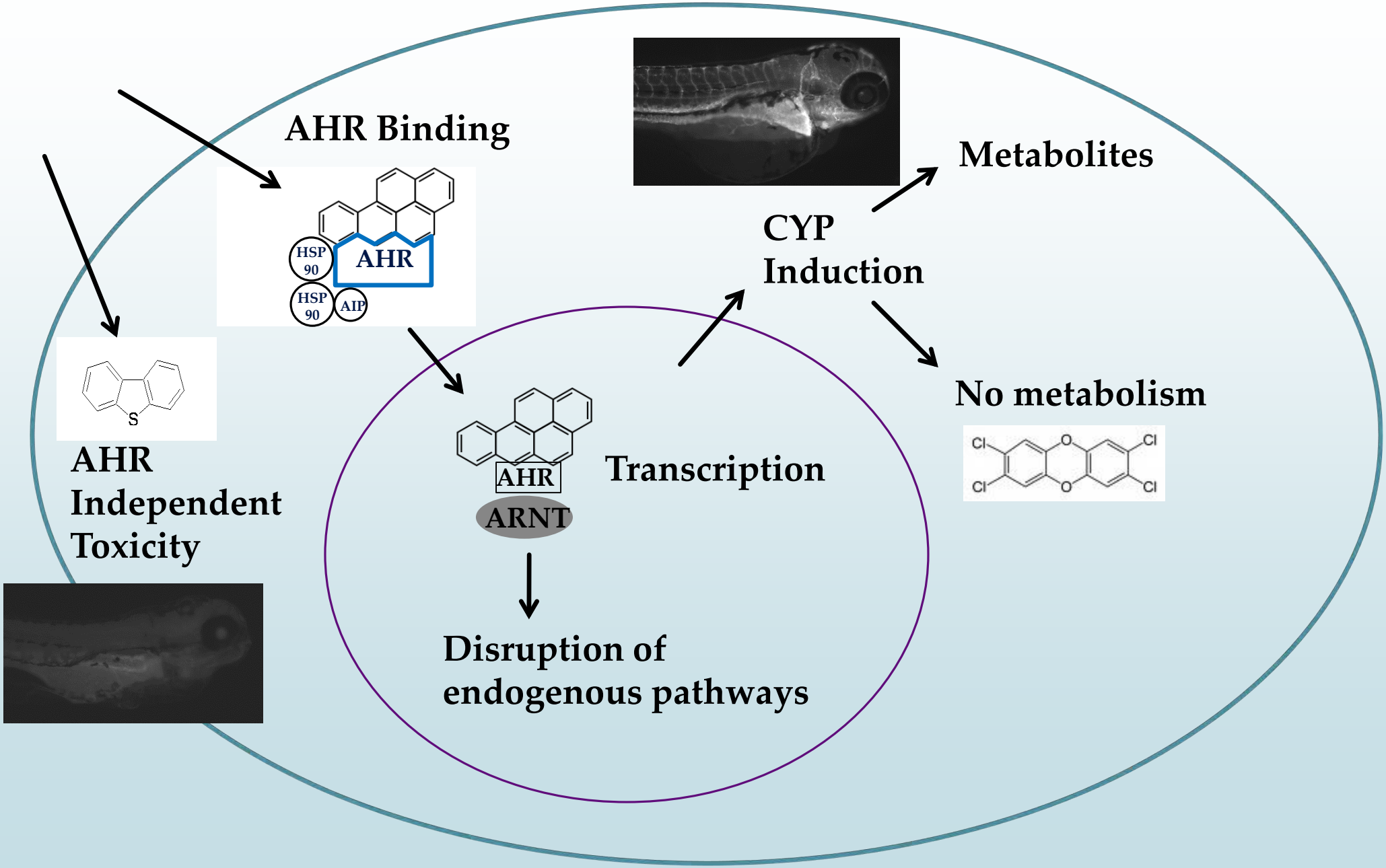
- A little history on zebrafish AHR genes
- PAH toxicity
- Classifying PAHs
- AHR Downstream events
- AHR regulated non coding RNAS

AHR Gene Duplication



AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes
Sibel I. Karchner, Diana G. Franks, Mark E. Hahn, Biochem J. 2005 Nov 15; 392(Pt 1): 153–161.

The AHR and PAH pathways of toxicity

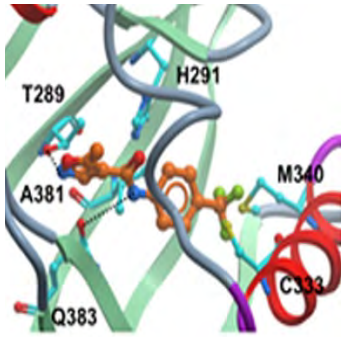


Predicting AHR Ligands

Modeling a “Target” Zebrafish AHRs

Zebrafish three AHRs

- AHR2 primary mediator of toxicity
- AHR1A deficient in TCDD binding and transactivation activity
- AHR1B functional but no known toxicological roles



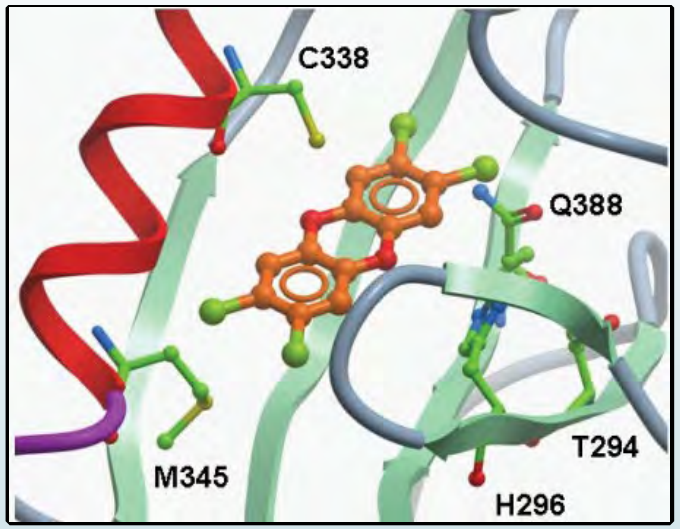
AHR Homology Model

- AHR ligand binding domain models built using NMR structure of HIF2α (PAS domain)
- Mouse, rat, human, zebrafish
- Performed molecular docking of putative AHR ligands

Bisson, W.H. et al. 2009, J Med Chem. O’Donnell, E.F. et al. 2010, PLOS One

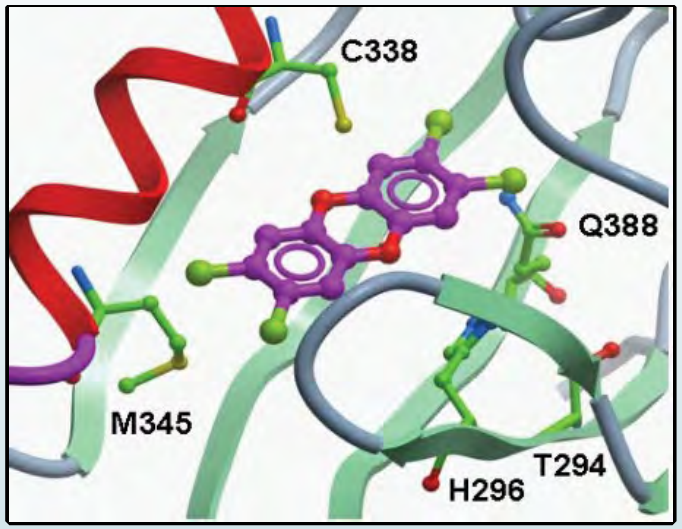
TCDD Molecular Docking with the Zebrafish AHRs

AHR2



-3.97

AHR1B



-4.86

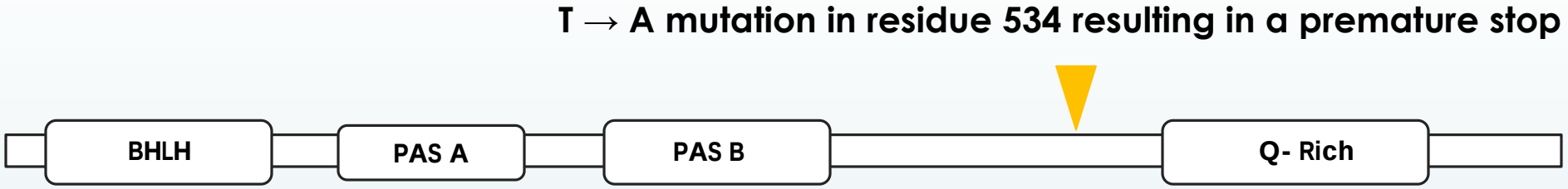
AHR1A

Unable to dock

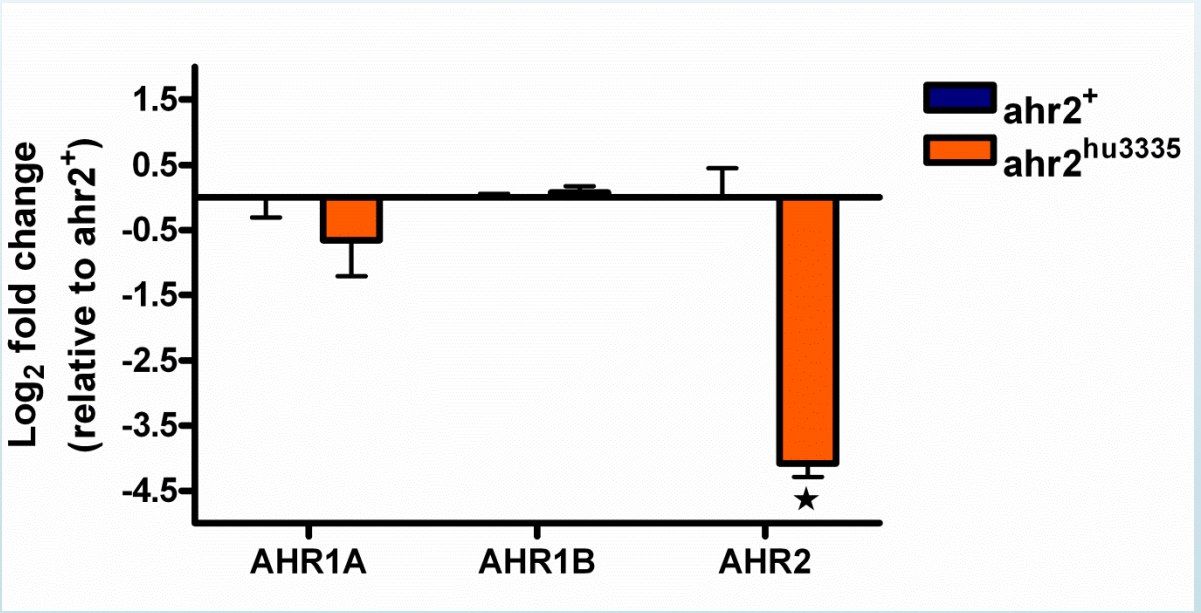
Predicted binding energy (kcal/mole)

Bisson, W.H. et al. 2009, J Med Chem.

The *ahr2*^{hu3335} Zebrafish Line

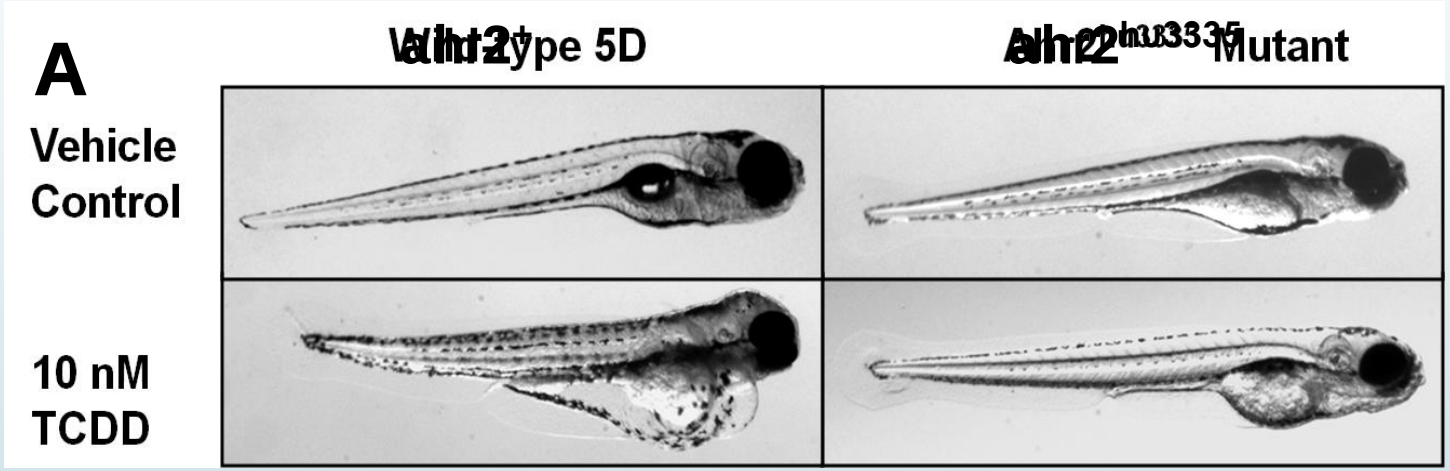


- Truncated protein is predicted to be non-functional
- Basal mRNA expression suggests mutant ahr2^{hu3335} transcript is degraded



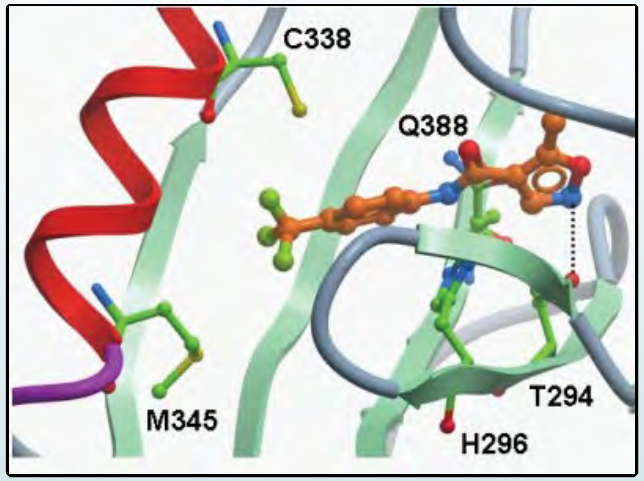
Goodale et al. PloS one 2012

Ahr2^{hu3335} Mutants Are Resistant to TCDD-Induced Developmental Toxicity



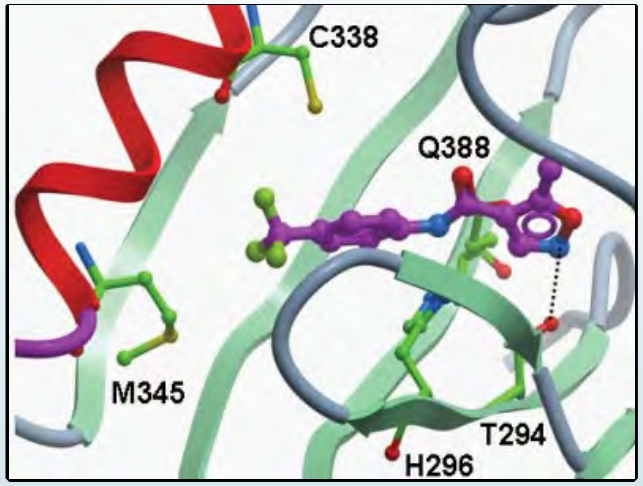
Leflunomide Molecular Docking

AHR2



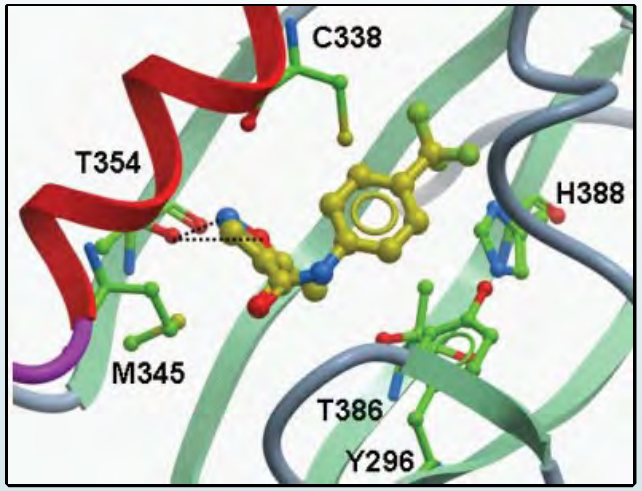
-2.13

AHR1B



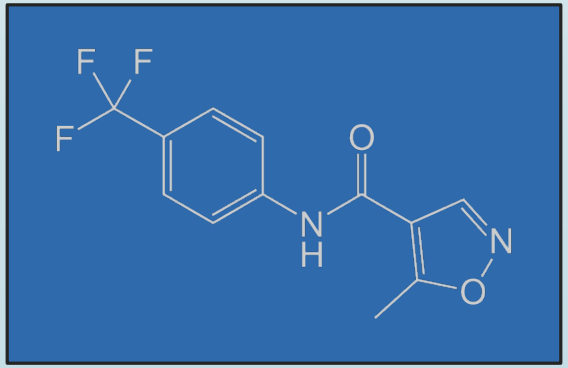
-1.97

AHR1A



-2.19

Predicted binding energy
(kcal/mole)



O'Donnell, E.F. et al. 2010, PLOS One

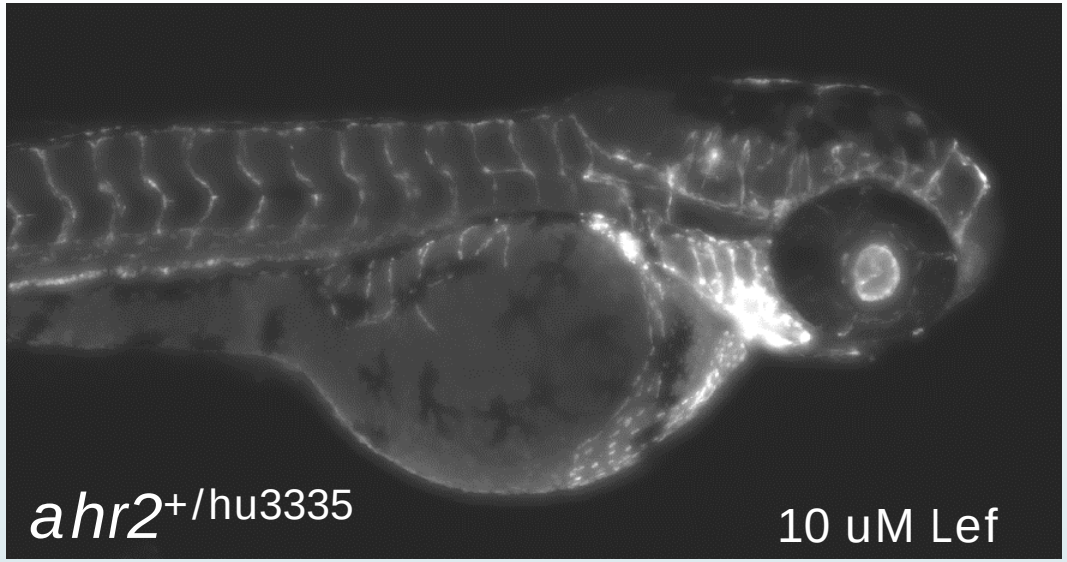
Leflunomide-induced CYP1A expression is partially AHR2 dependent

AHR Status

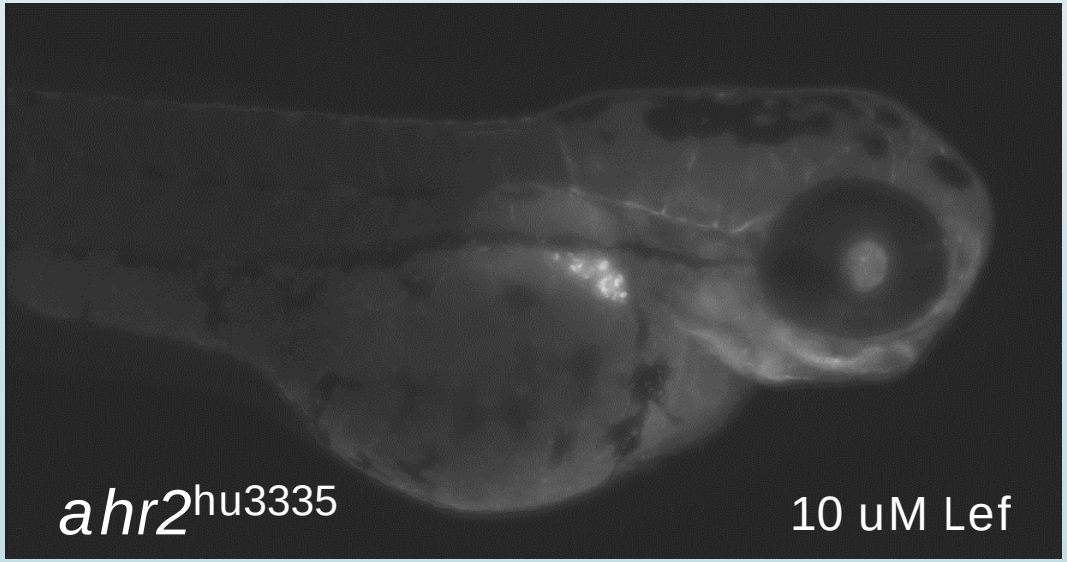
Expressed

Not Expressed

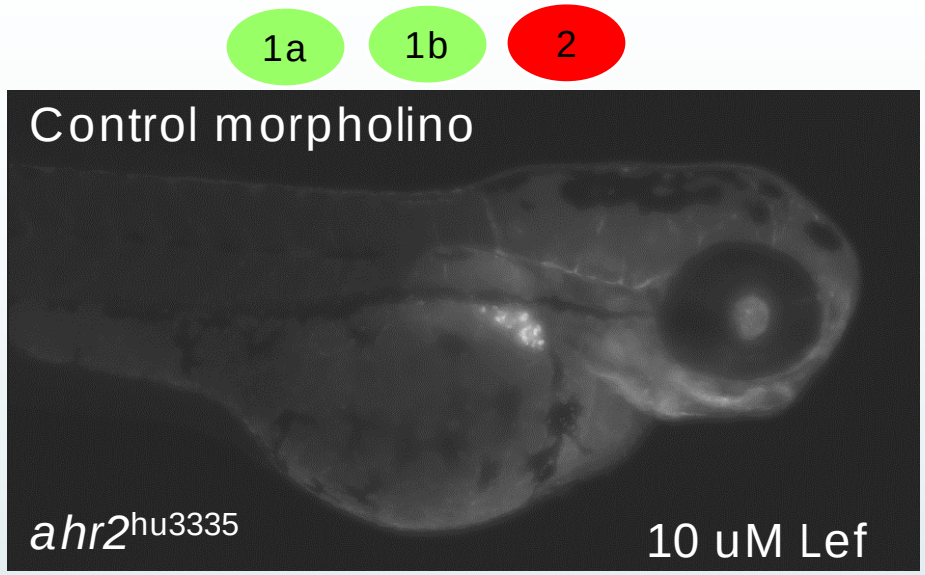
1a 1b 2

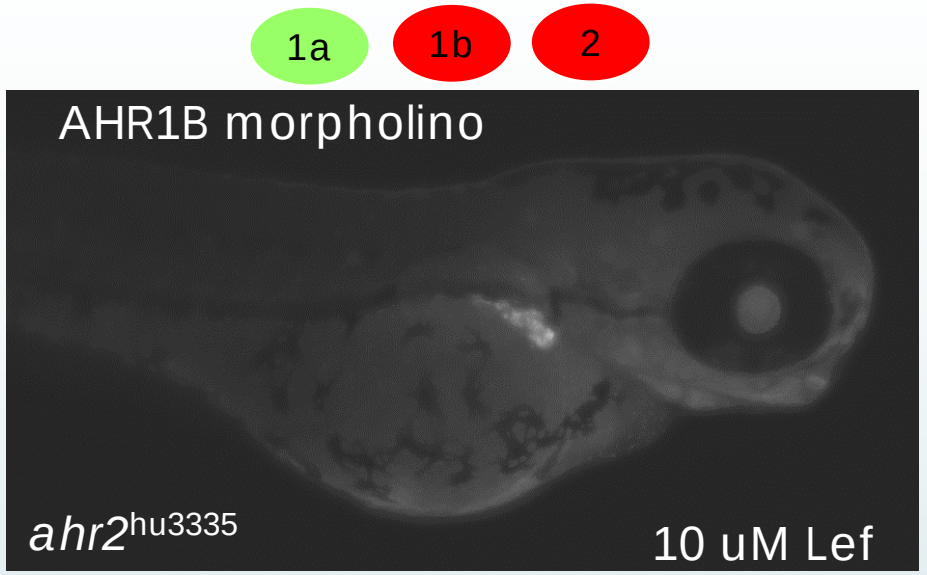


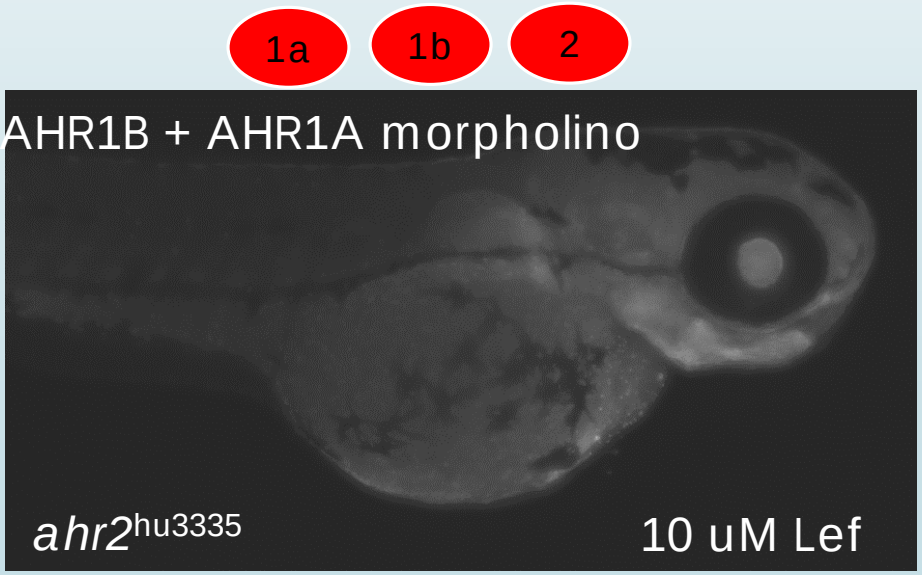
1a 1b 2

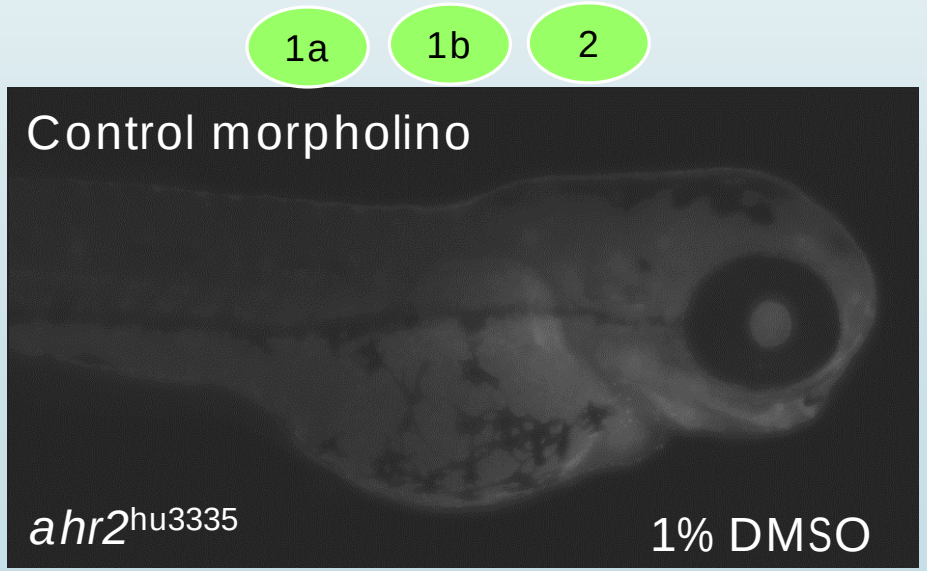


AHR1A Dependent CYP1A Expression

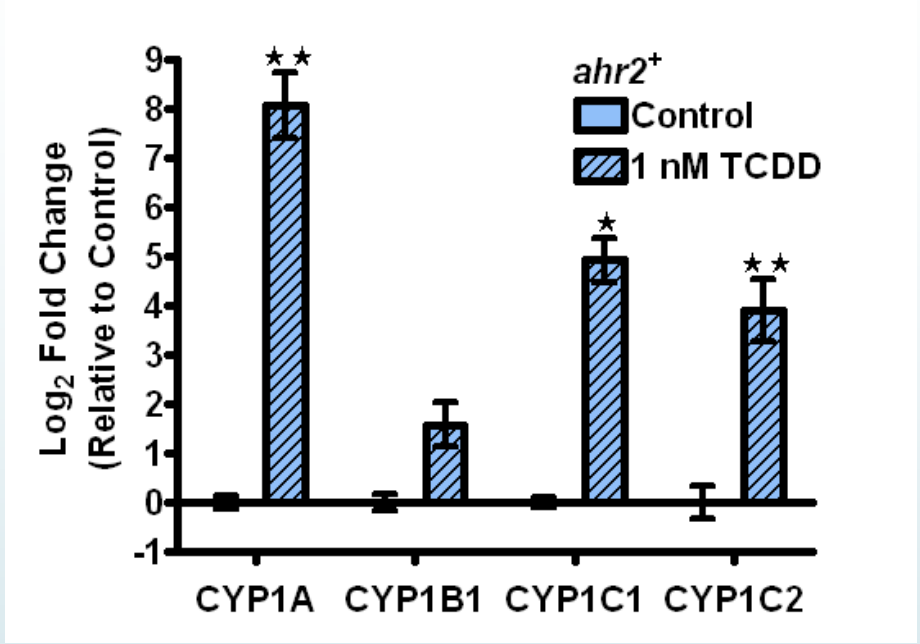


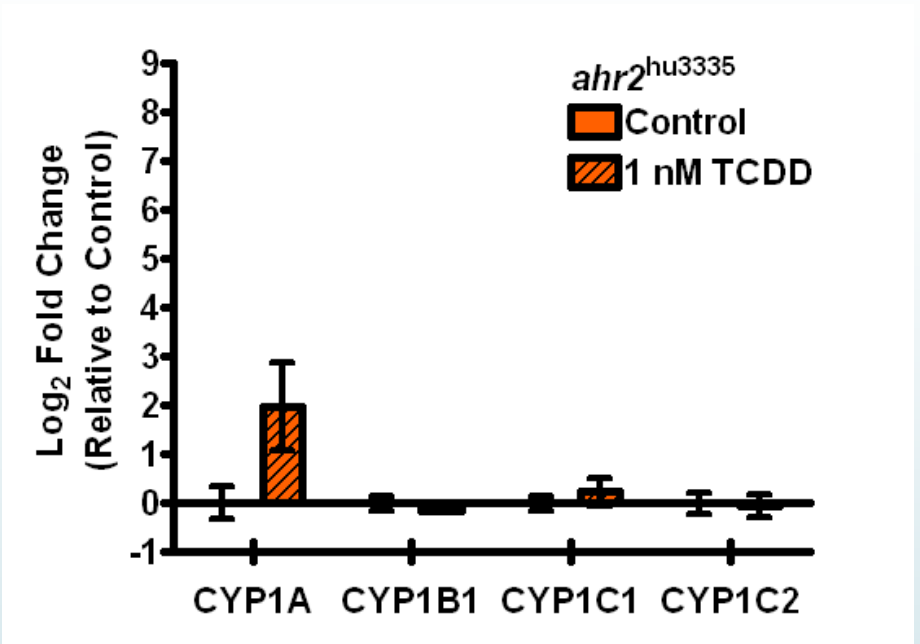


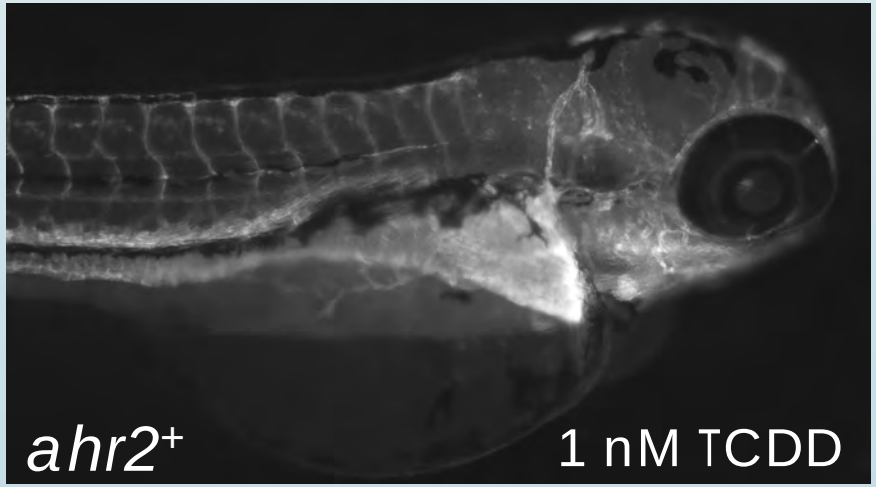


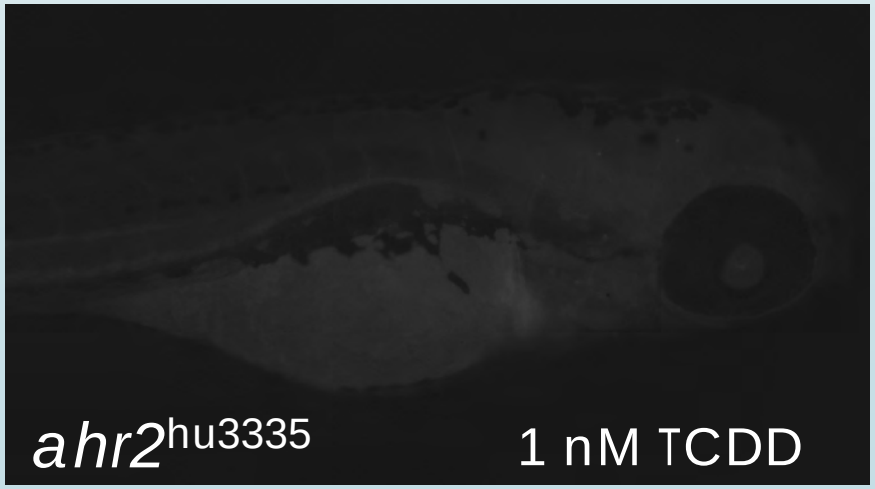


ahr2 Mutants Are Resistant to TCDD-induced CYP Expression Changes

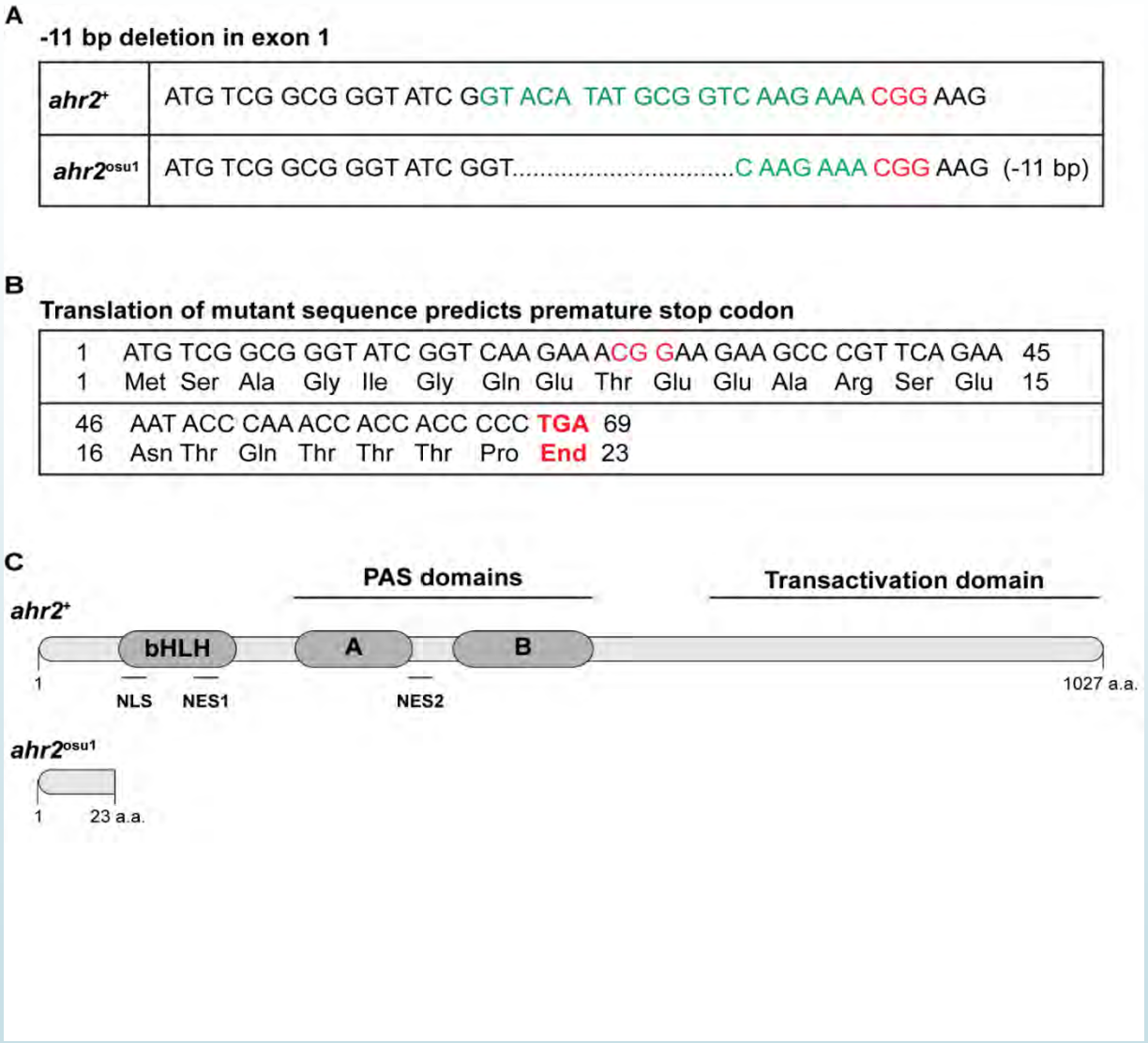


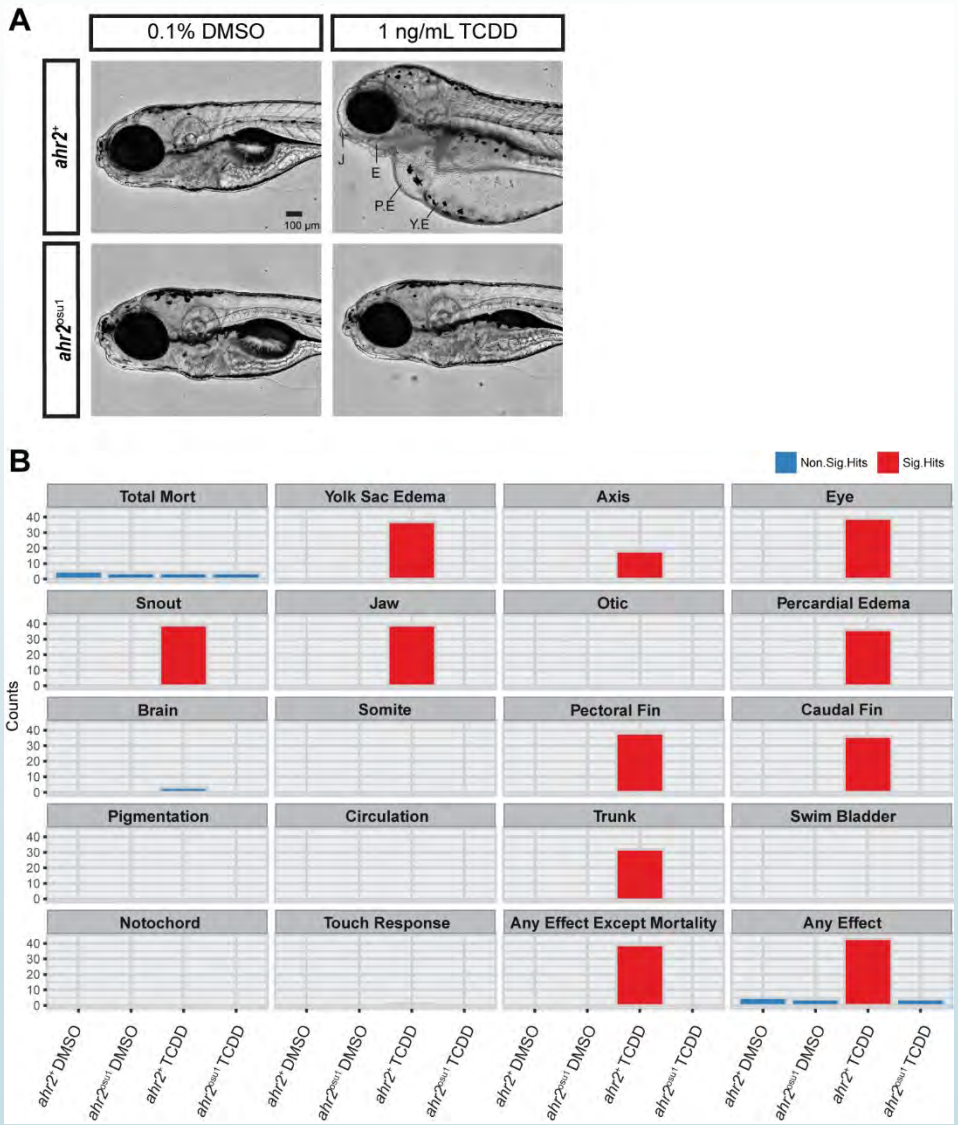






AHR2 importance confirmed in CRISPR/Cas9 line

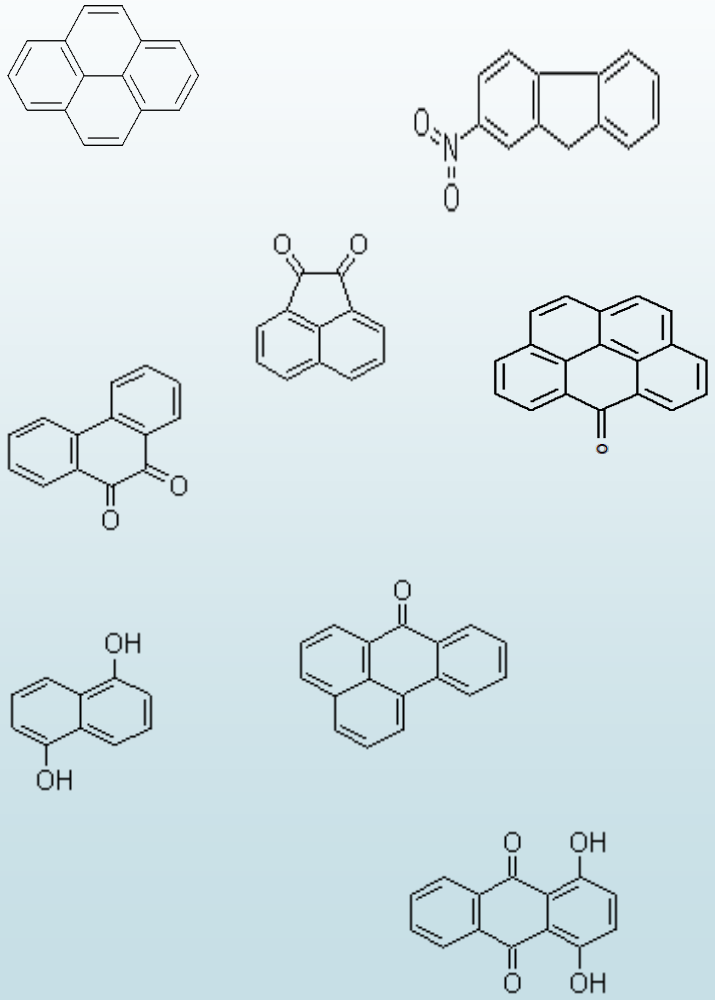




Garcia GR, Bugel SM, Truong L, Spagnoli S, Tanguay RL. AHR2 required for normal behavioral responses and proper development of the skeletal and reproductive systems in zebrafish. PloS one. 2018;13(3)

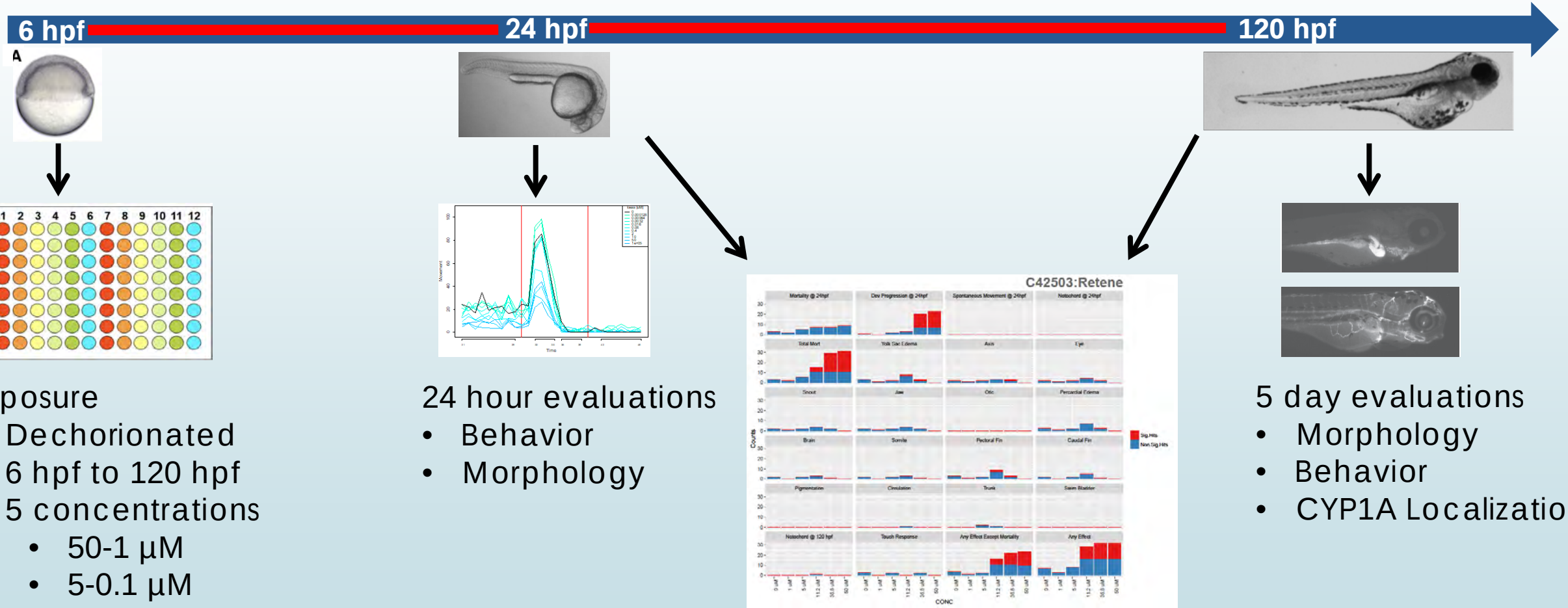
OSU/PNNL SuperFund Research Program
Polycyclic Aromatic Hydrocarbon Mixtures: New Technologies and Emerging Health Risks

Toxicity Mechanisms for Most PAHs are Unknown



- Environmental samples can contain 100’s PAHs
- Environmentally Dynamic
- Parent, substituted compounds
- Toxicity data is scarce for substituted PAHs
- PAHs induce AHR-dependent and AHR-independent developmental toxicity, dependent on structure
- We lack the structural basis for developmental and neurotoxicity

High-Throughput Screening of PAHs

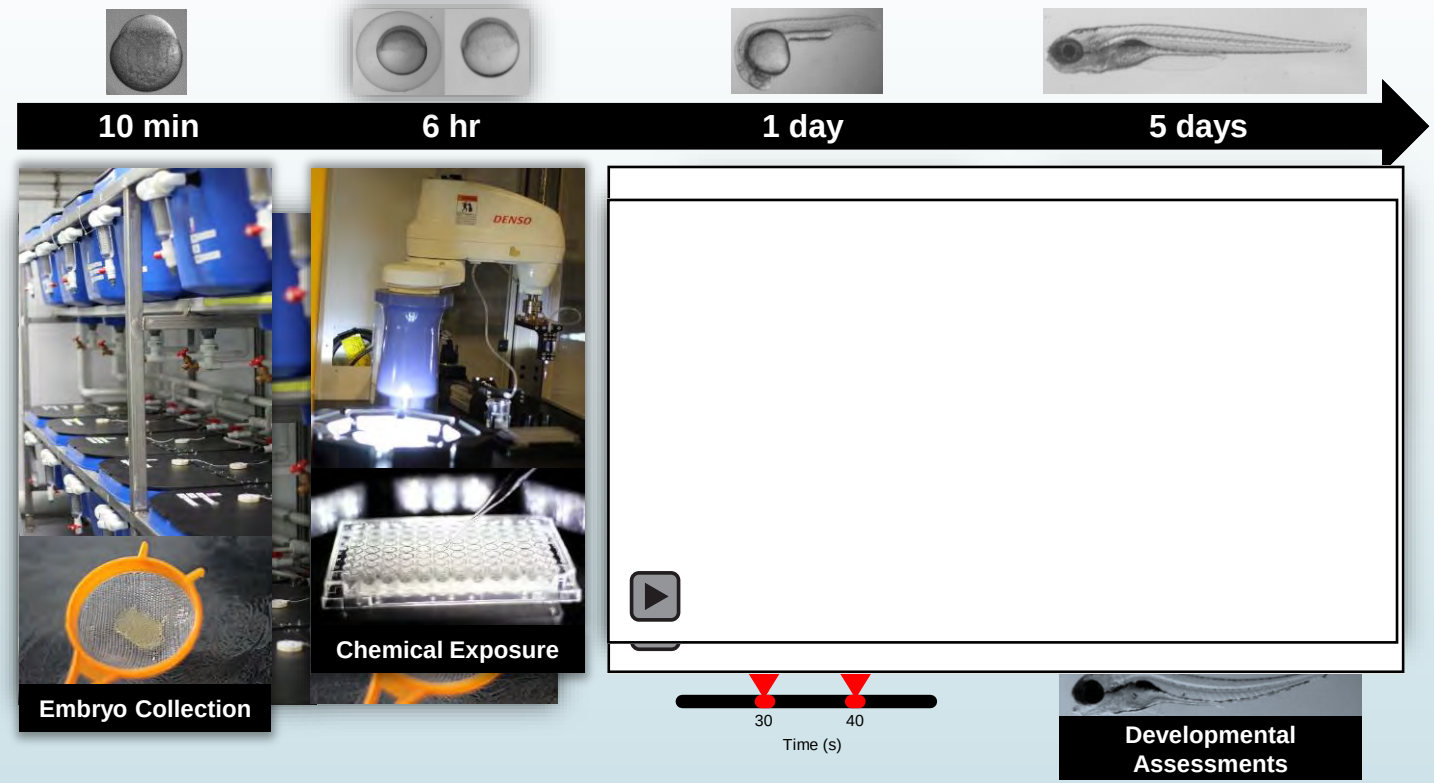


Exposure
- Dechorionated
- 6 hpf to 120 hpf
- 5 concentrations
  - 50-1 µM
  - 5-0.1 µM
- N=32

24 hour evaluations
- Behavior
- Morphology

5 day evaluations
- Morphology
- Behavior
- CYP1A Localization

HTS Platform for Chemical Screening



Truong et al. (2014) Toxicol Sci 137: 212-233.

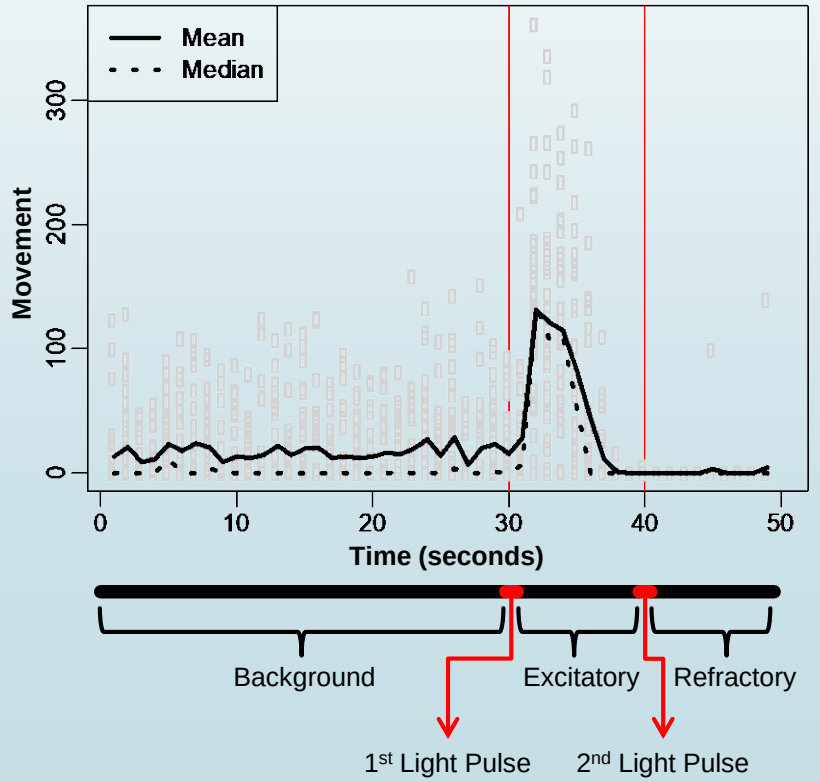
Mandrell, D., **Truong, L.**, et al . 2012. Automated zebrafish chorion removal and single embryo placement: Optimizing throughput of zebrafish developmental toxicity screens. *Journal of Laboratory Automation 17 (1) 66-74.*

All dependent on a custom made LIM system “ZAAP”

What About The 24 hpf Photomotor Response (EPR)?

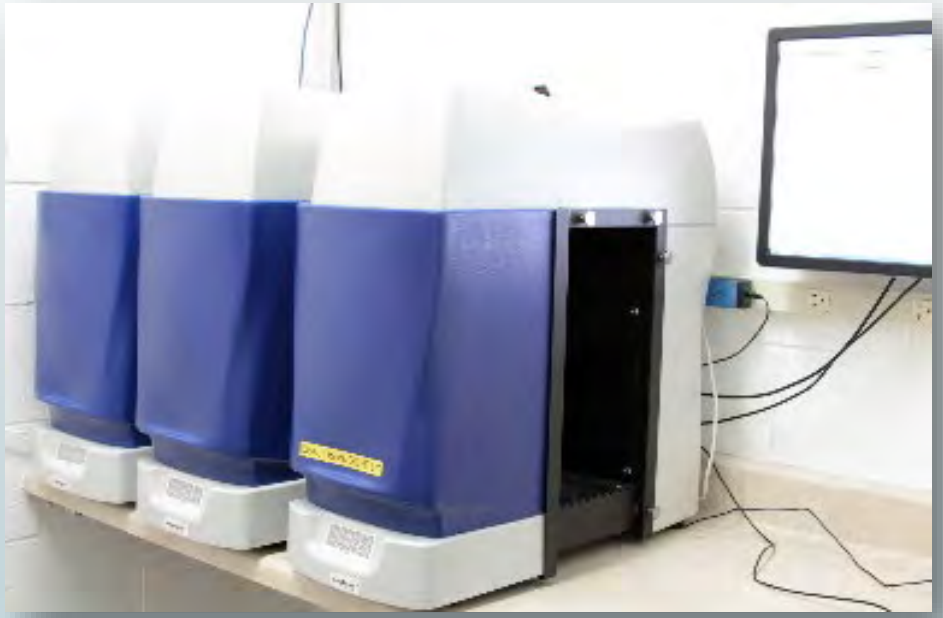
- Control fish (in the absence of chemical) will respond after the 1st light pulse (**Excitatory** interval) but not after the 2nd light pulse (**Refractory** interval).

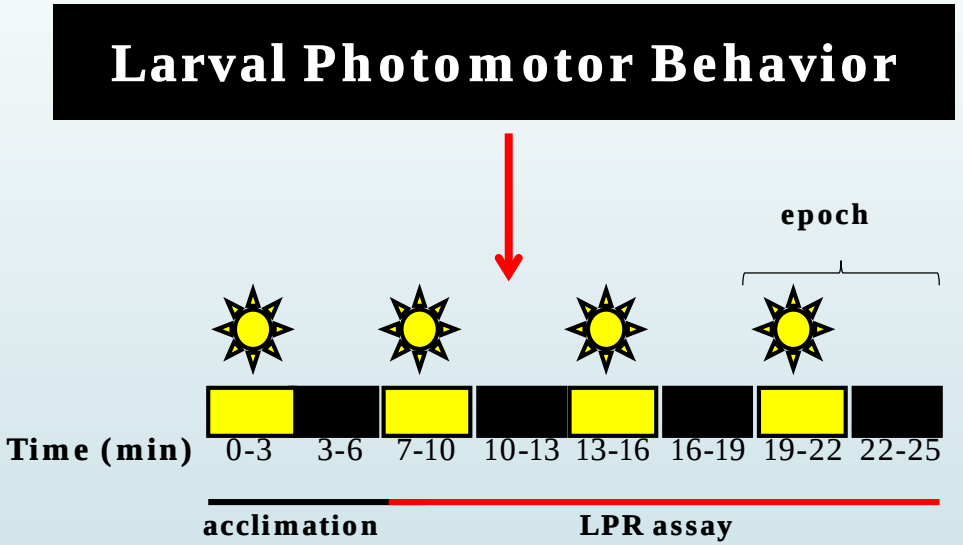
EPR recording (50 seconds)



Larval Photomotor Response (LPR) Behavioral Testing (5 day Larvae)

Distance Moved During Alternating Periods of Light and Dark

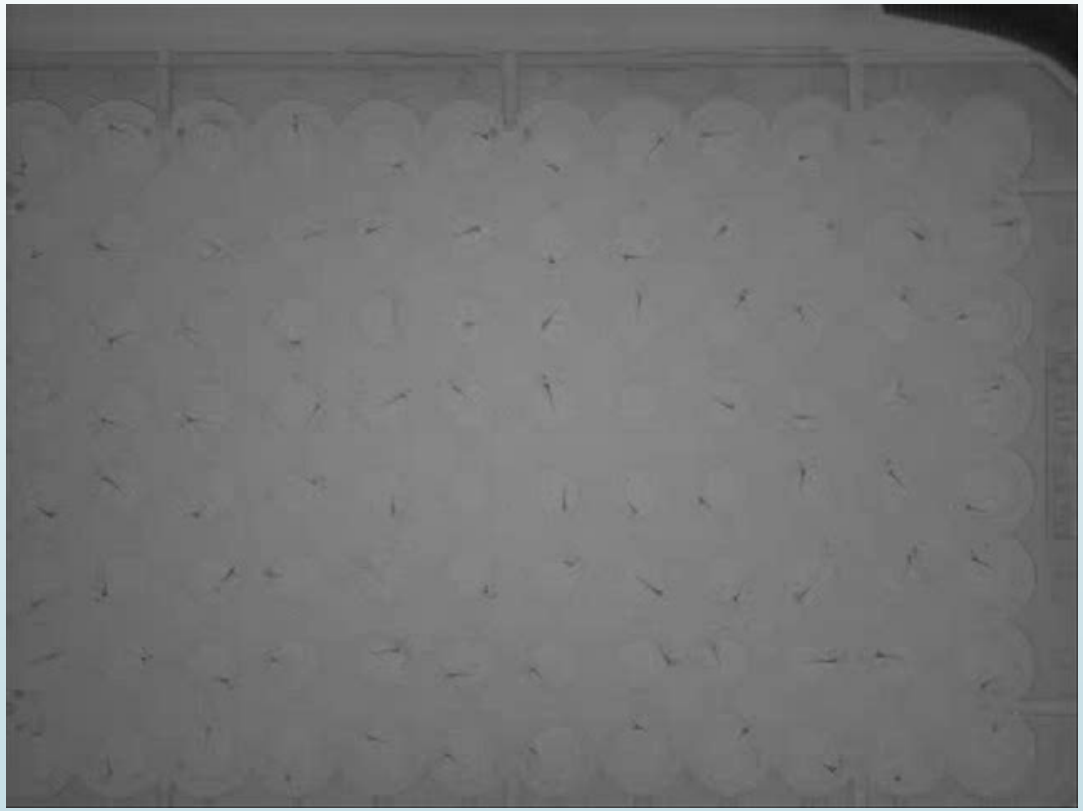


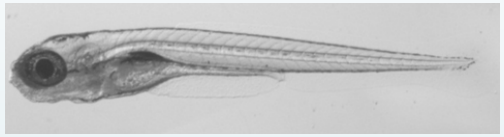


The LPR assay is 18 mins, with a 6 min acclimation period. Statistical significant was determined using a K-S test with a $p<0.01$, and a minimum of 30% AUC change (relative to control).

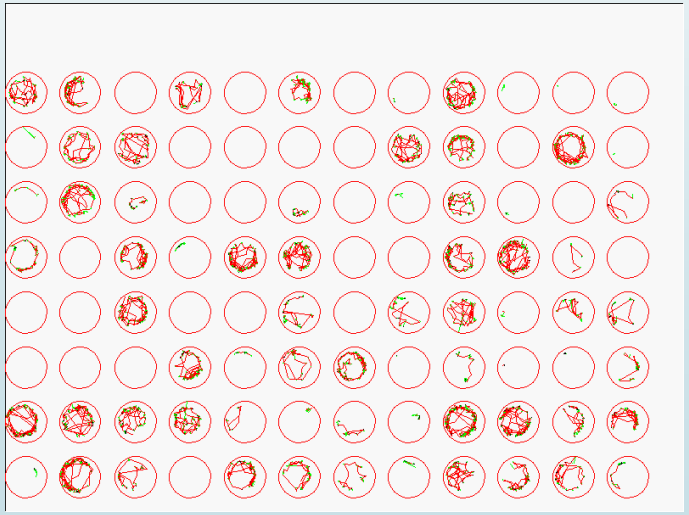
Larval Photomotor Response (LPR) Behavioral Testing (5 day Larvae)

96-well plate

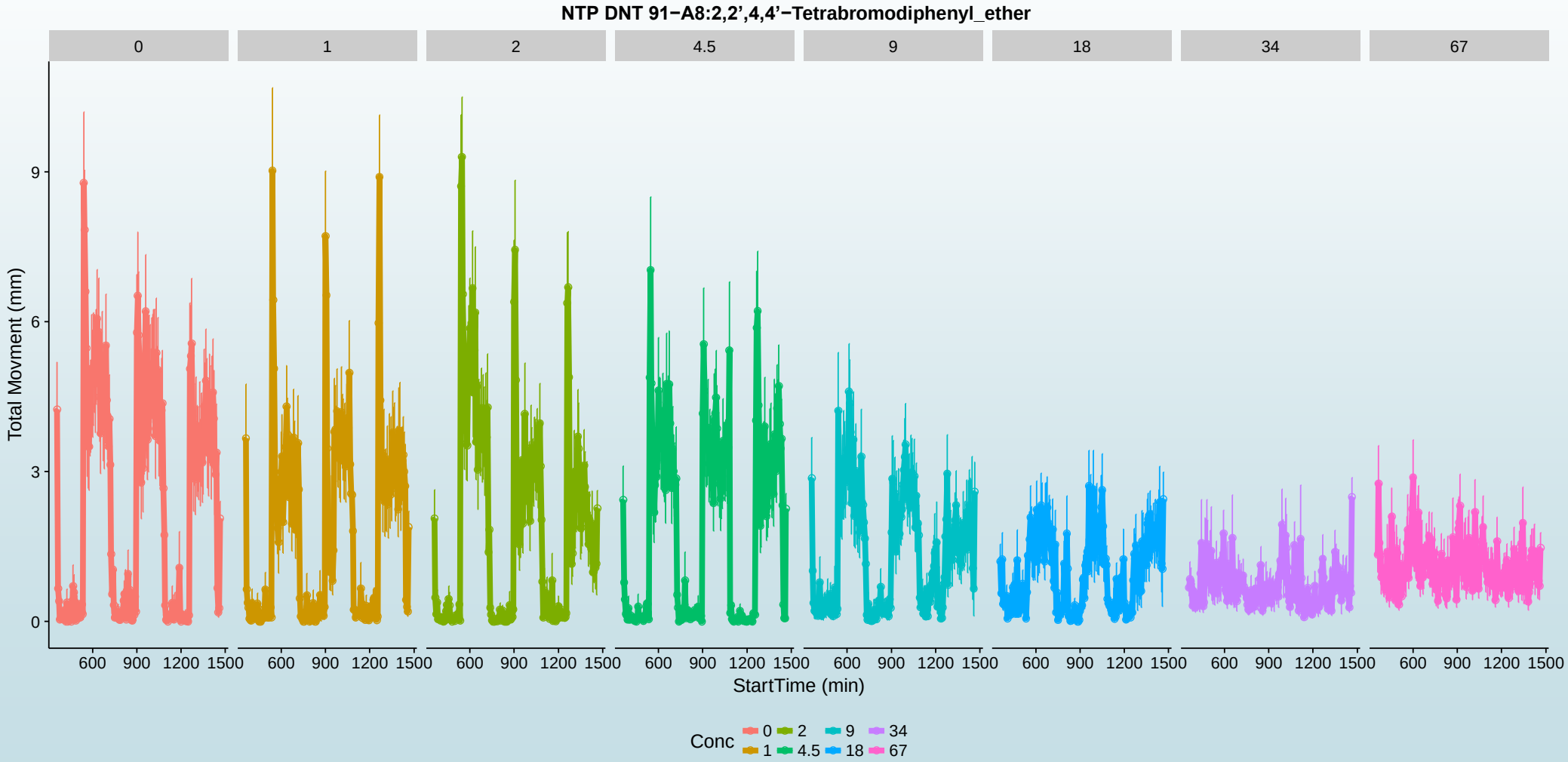




Distance moved over time

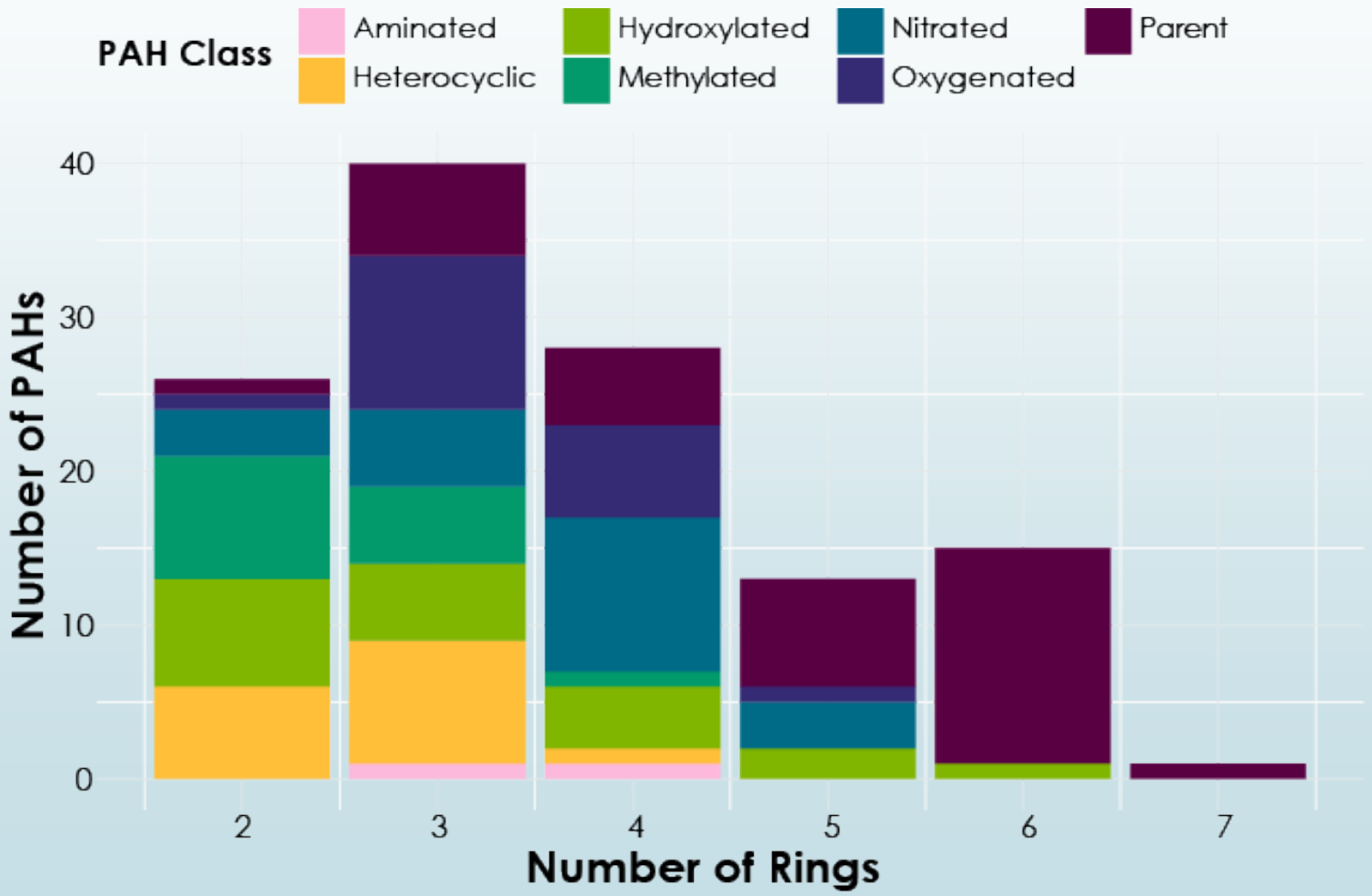


Larval Photomotor Response (LPR) Example Output (5 day Larvae)

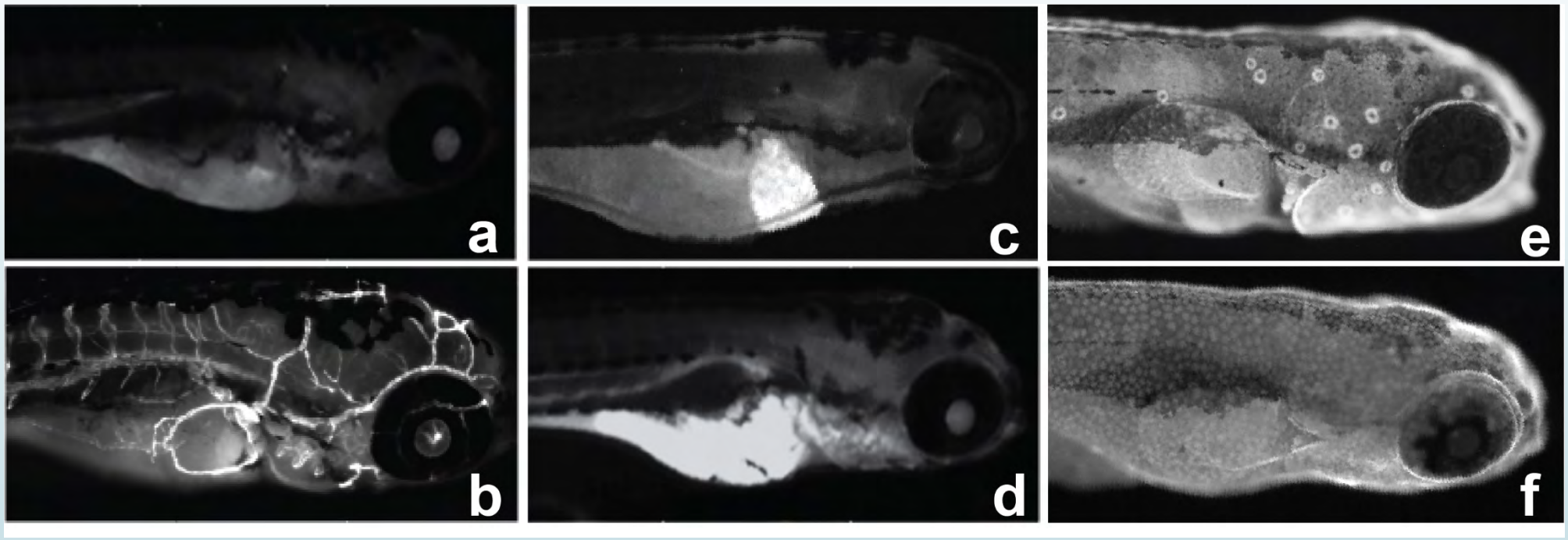


Comparative PAH Screening Effort

Developed a Library of PAHs for Comparative Analysis

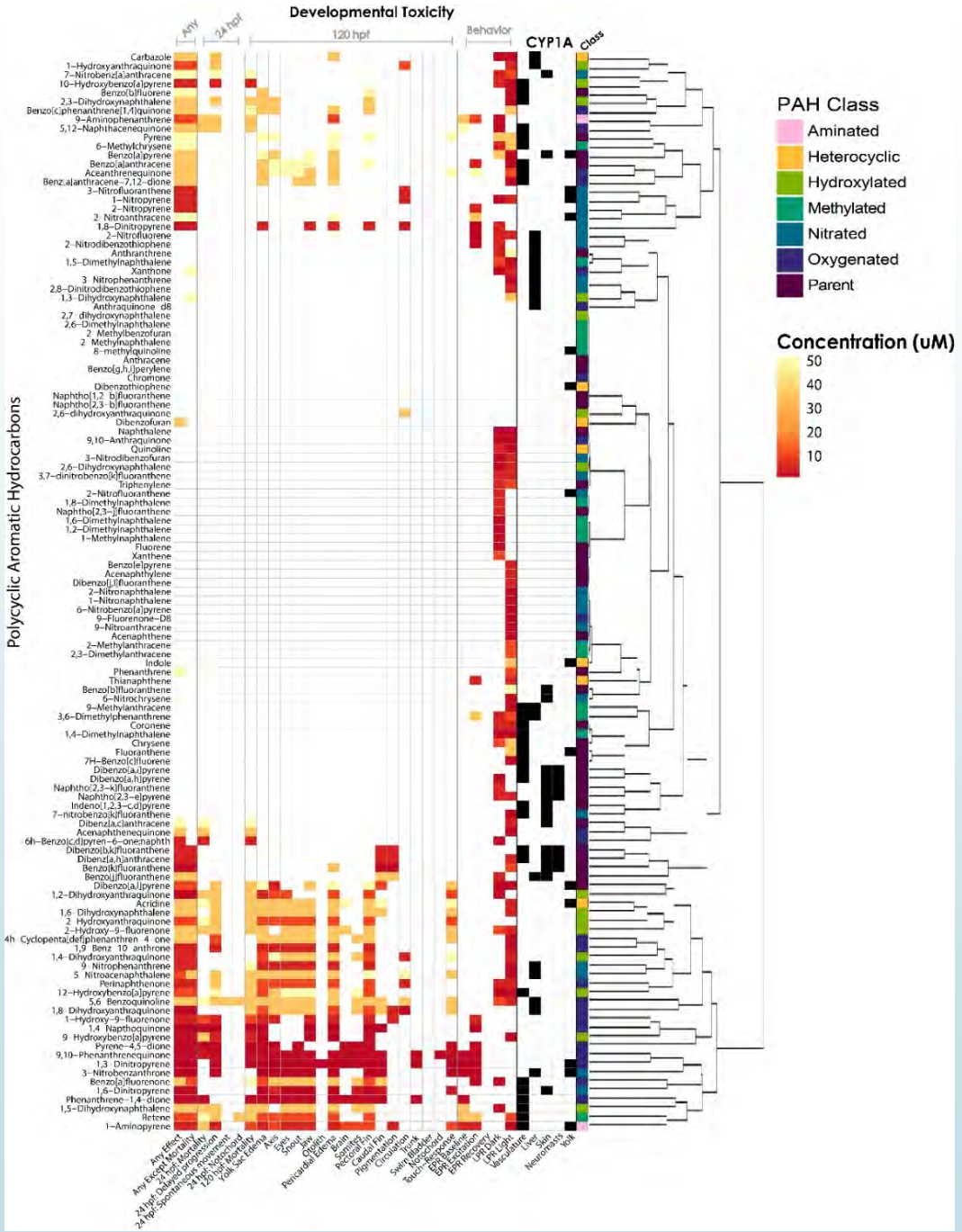


CYP1A Expression Patterns as a Biomarker of AHR Activation

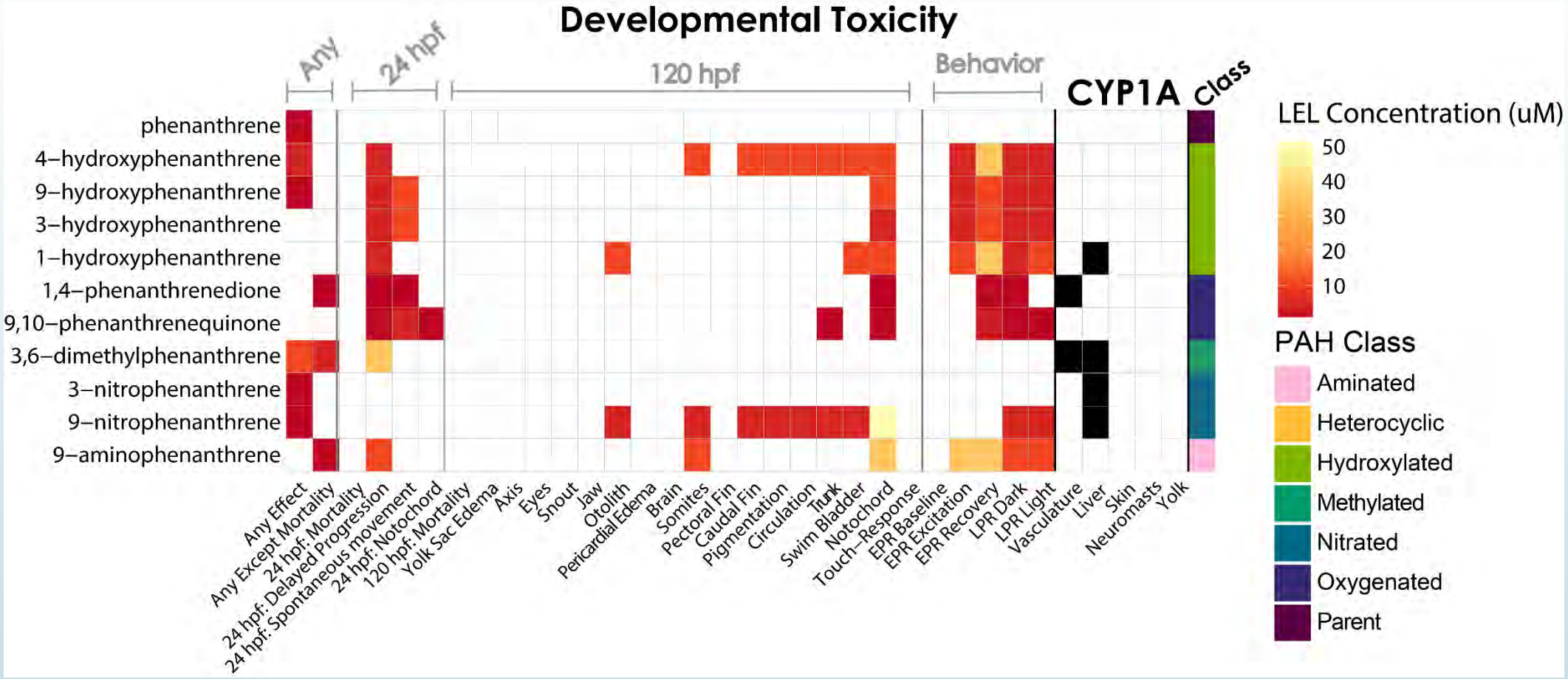


120 hpf

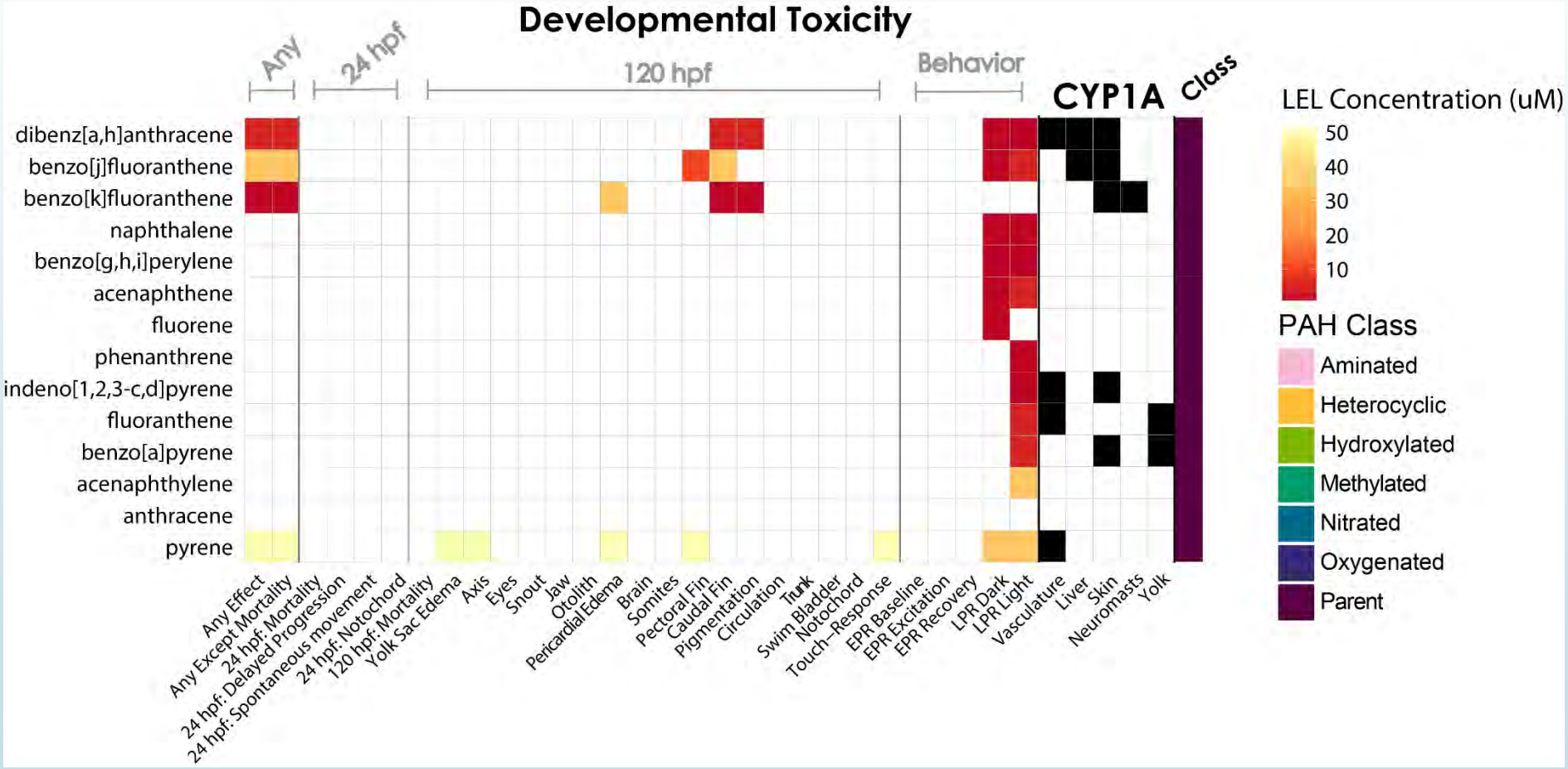
Summary of Results



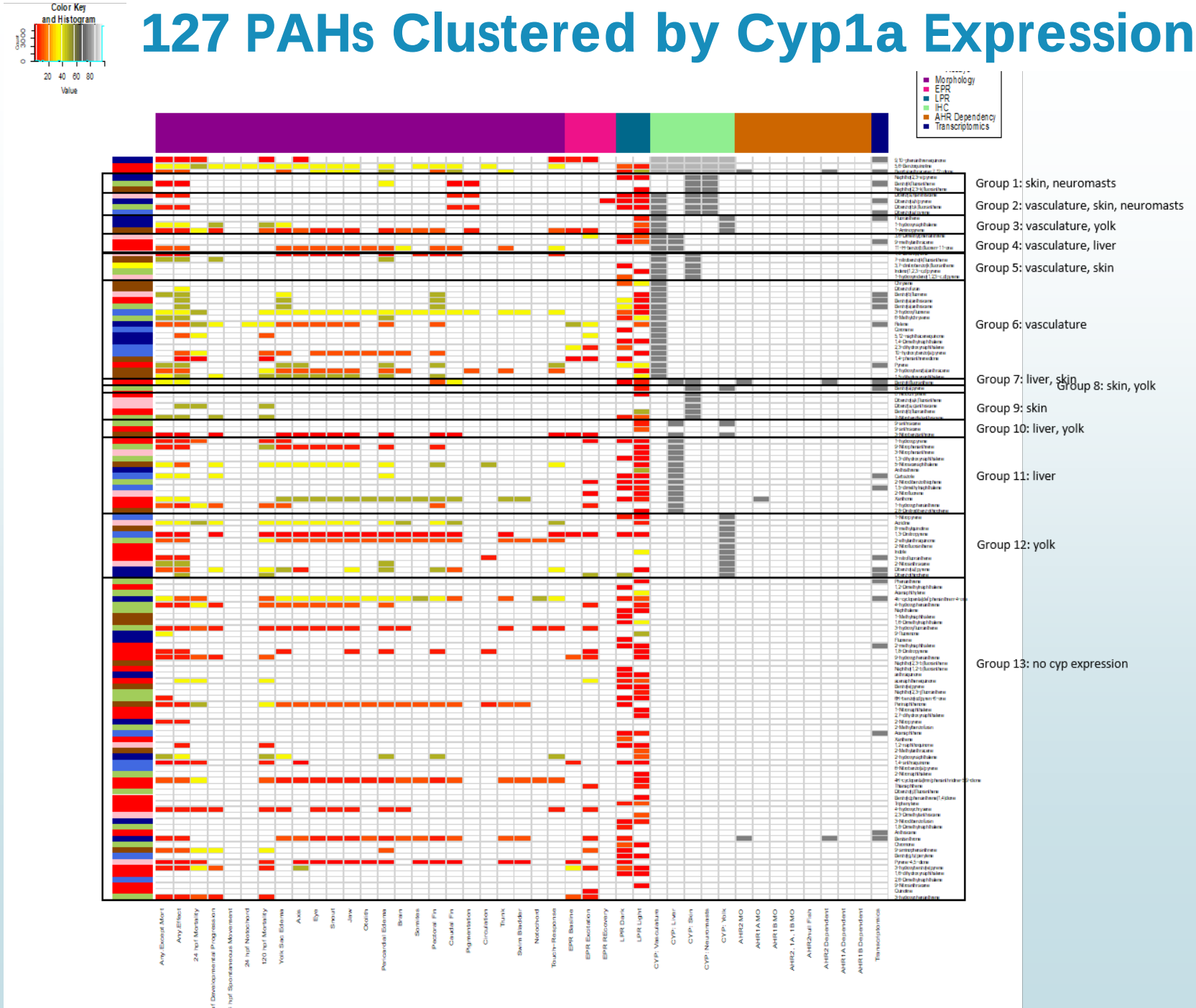
Example Parent and Derivatives



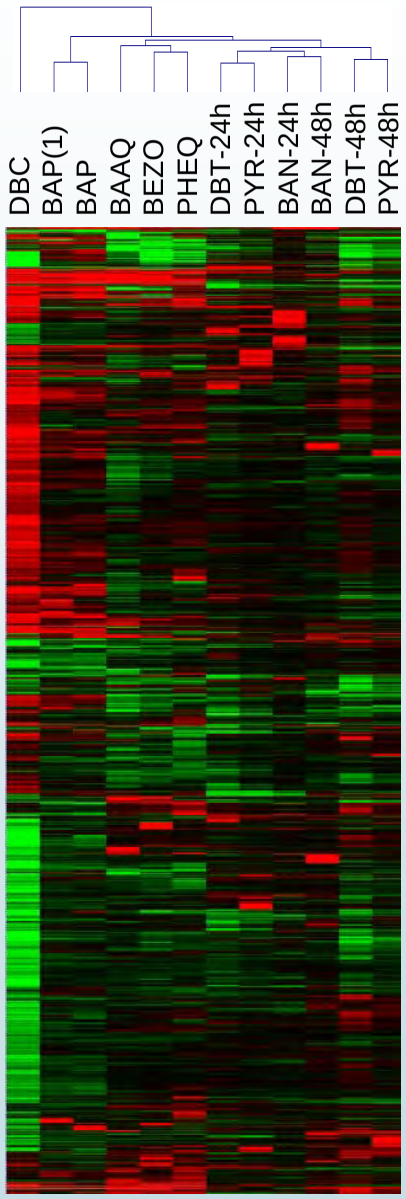
16 EPA Priority PAHs Do Not Reflect Full Range of Effects

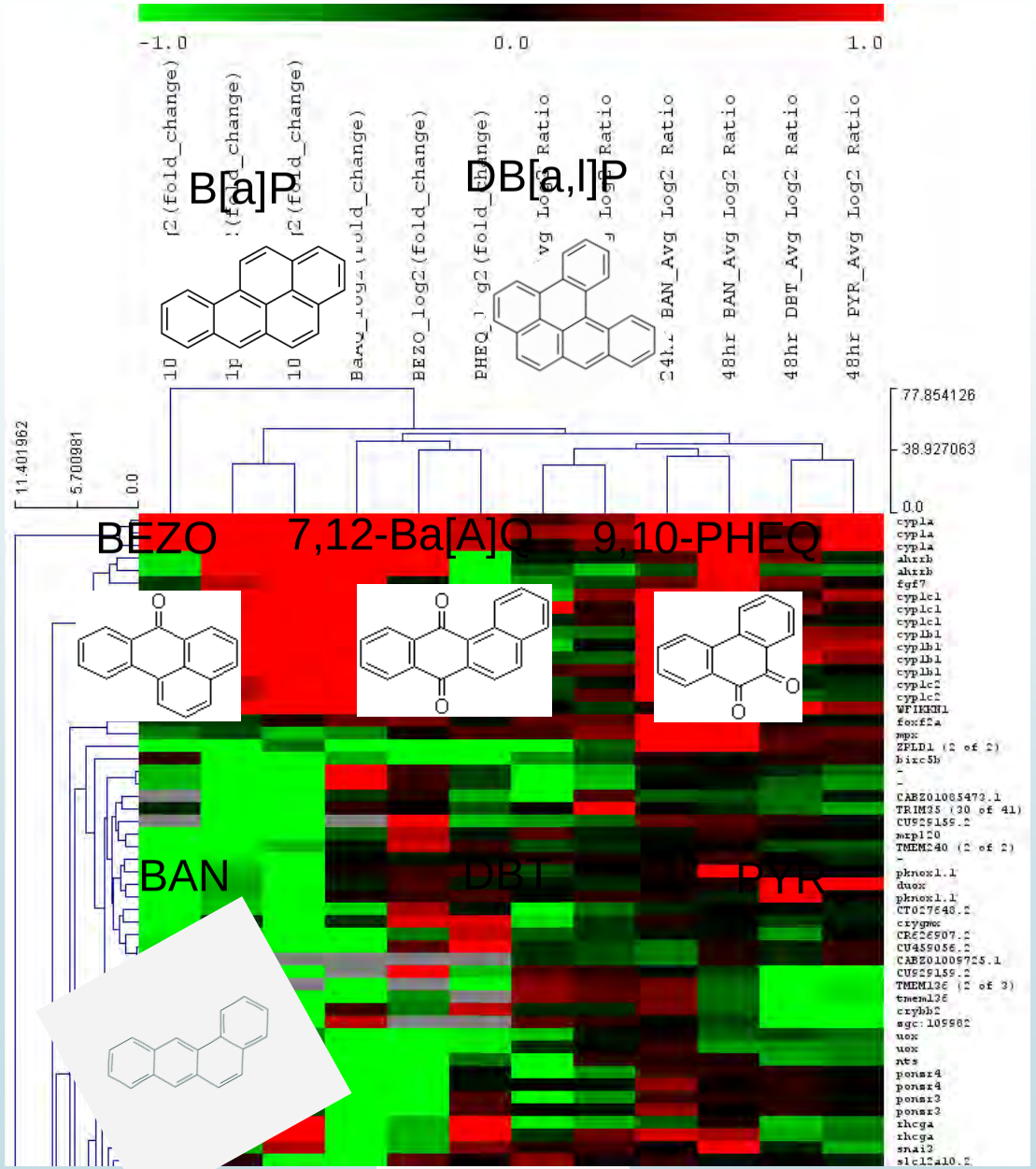


127 PAHs Clustered by Cyp1a Expression Patterns



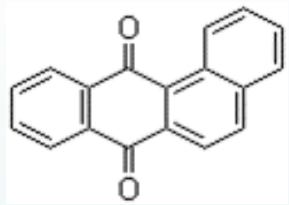
RNA-SEQ Analysis (Embryonic Expression)



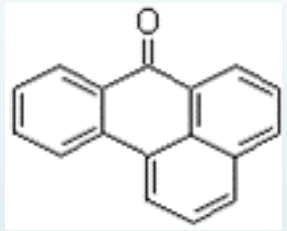


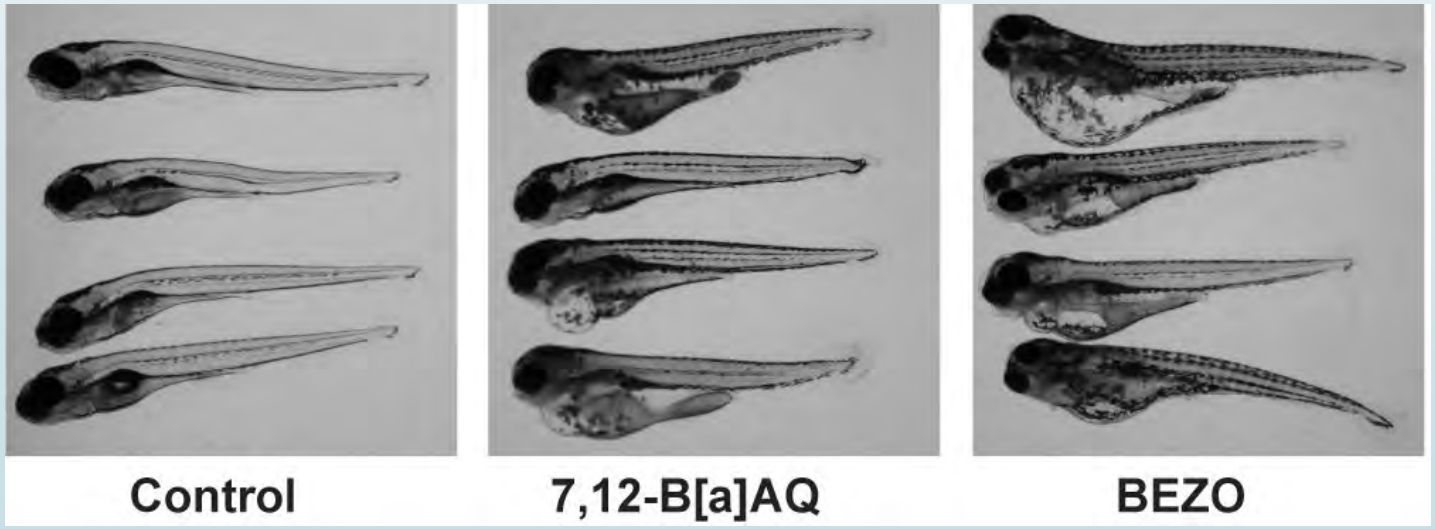
Case Study – two OPAHs

Benz[a]anthracene-7,12-dione
(7,12-Ba[A]Q)

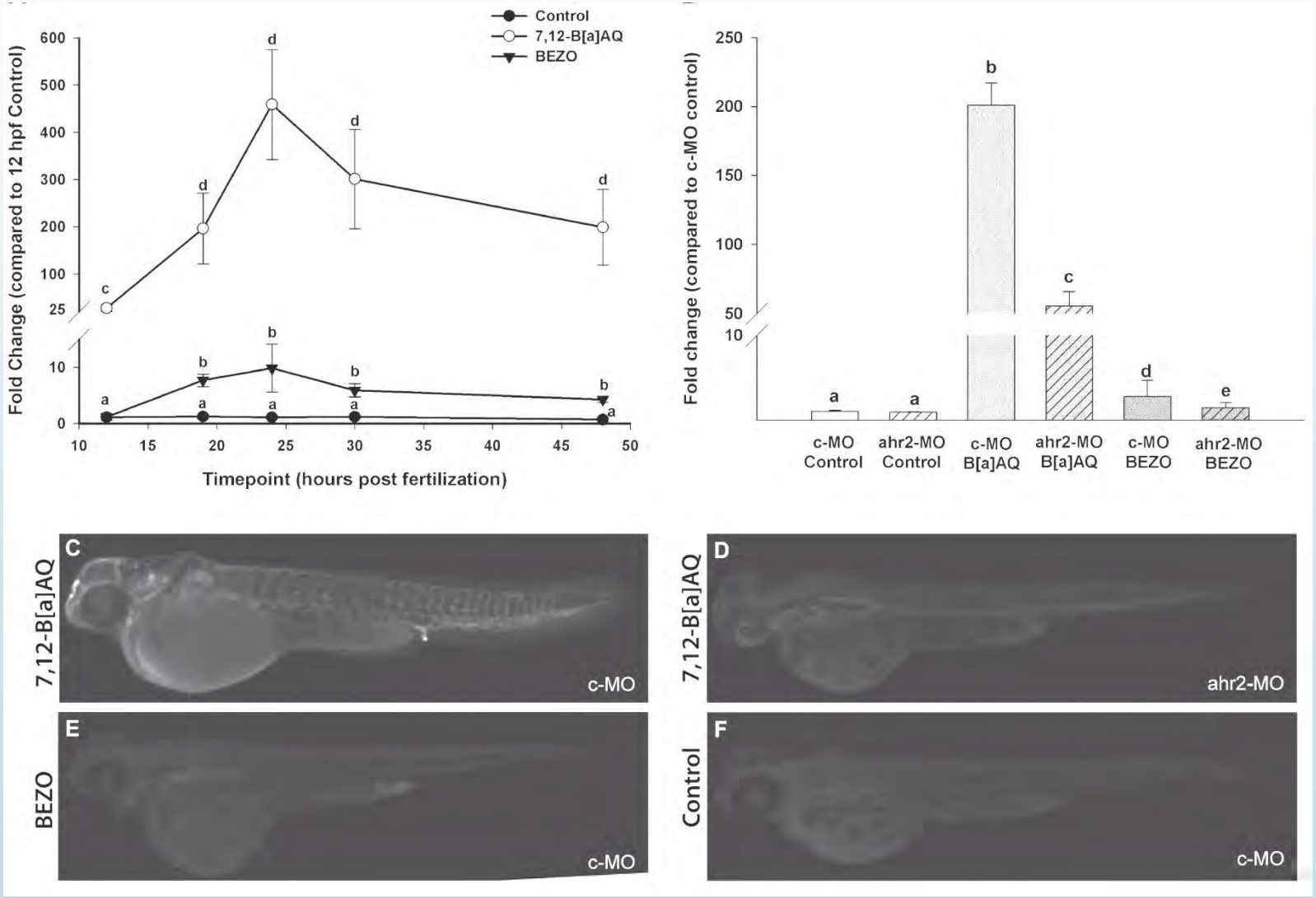


1,9-Benz-10-anthrone (BEZO)

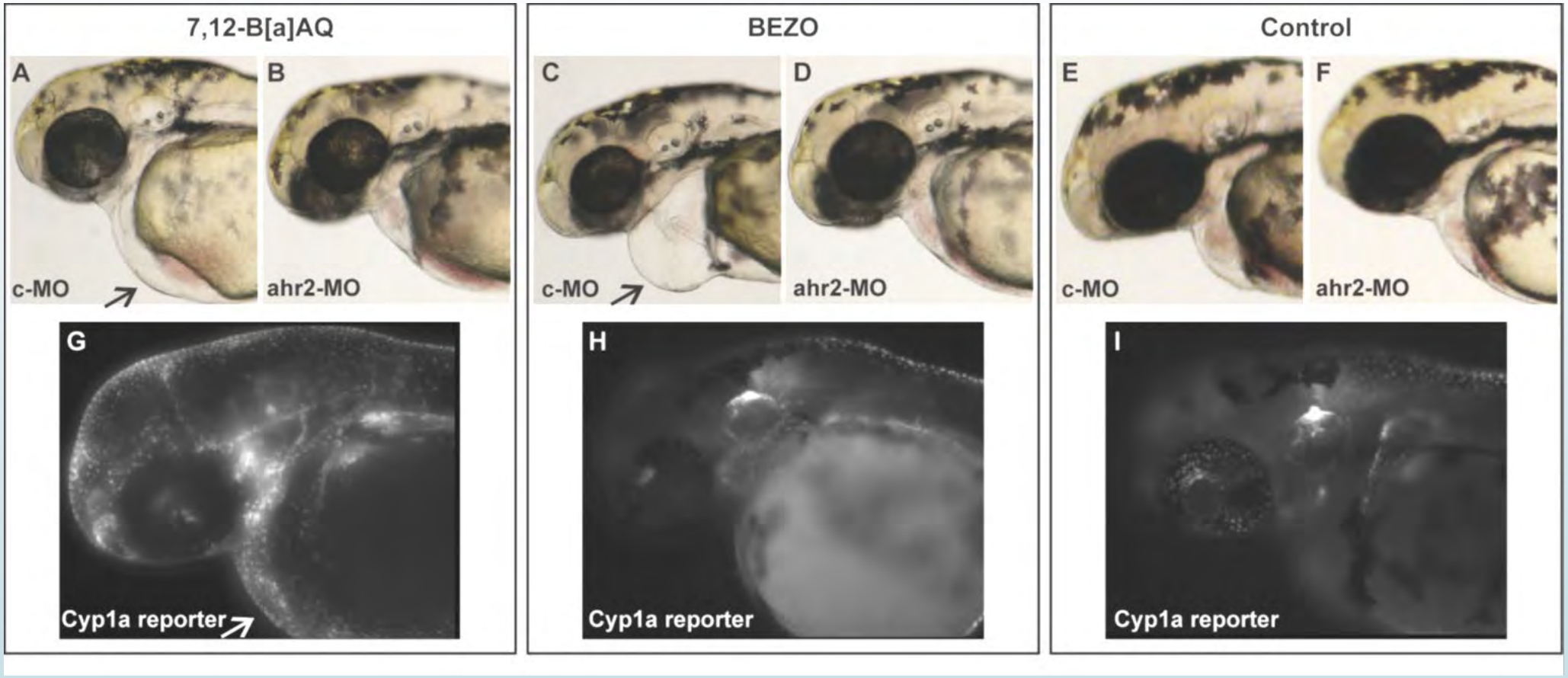




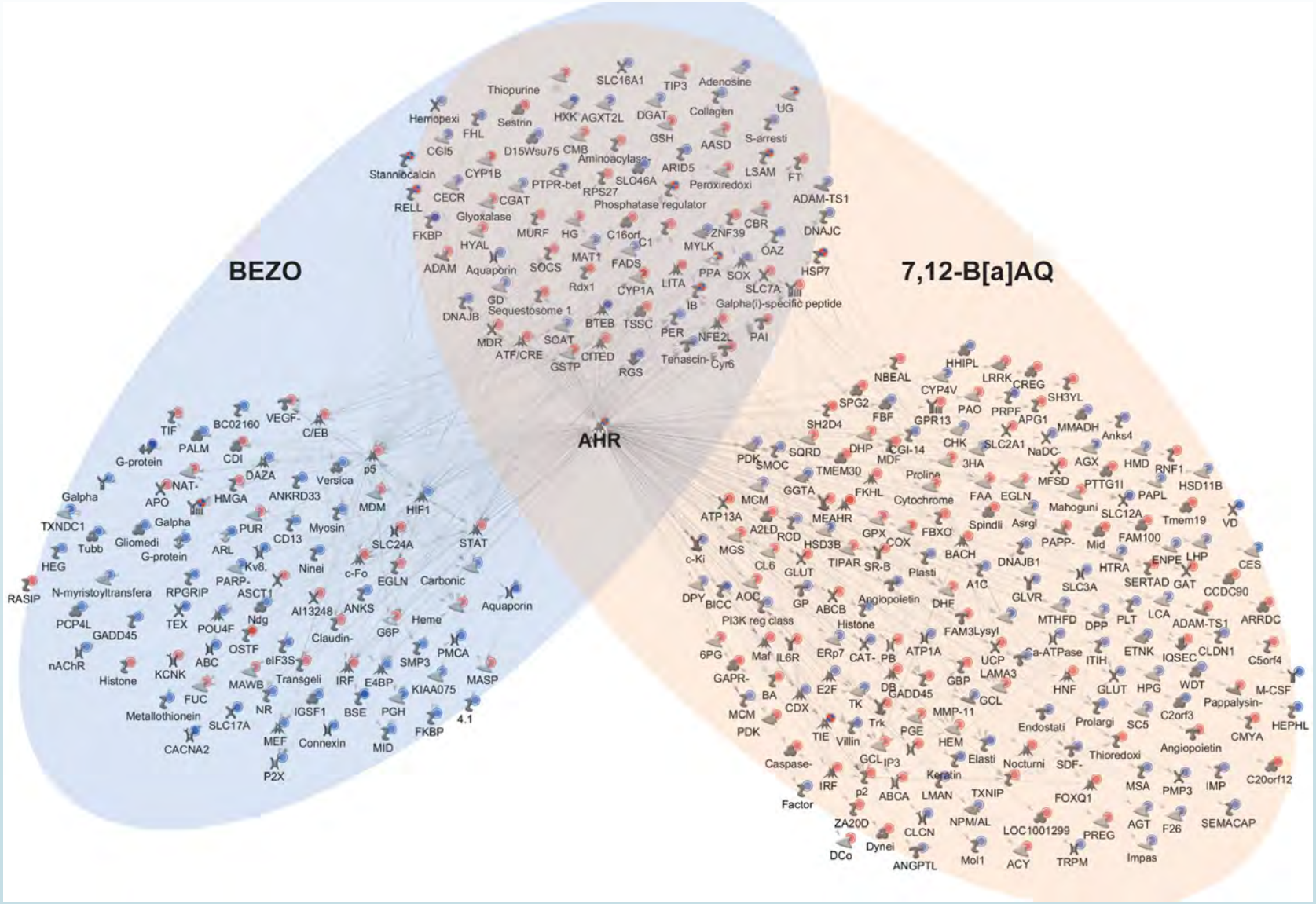
Differential Downstream AHR Activation (Measured CYP1A Expression)



Overt Toxicity for both – AHR2 Dependent



Complexity of Defining Molecular Responses – Even if MIE is known



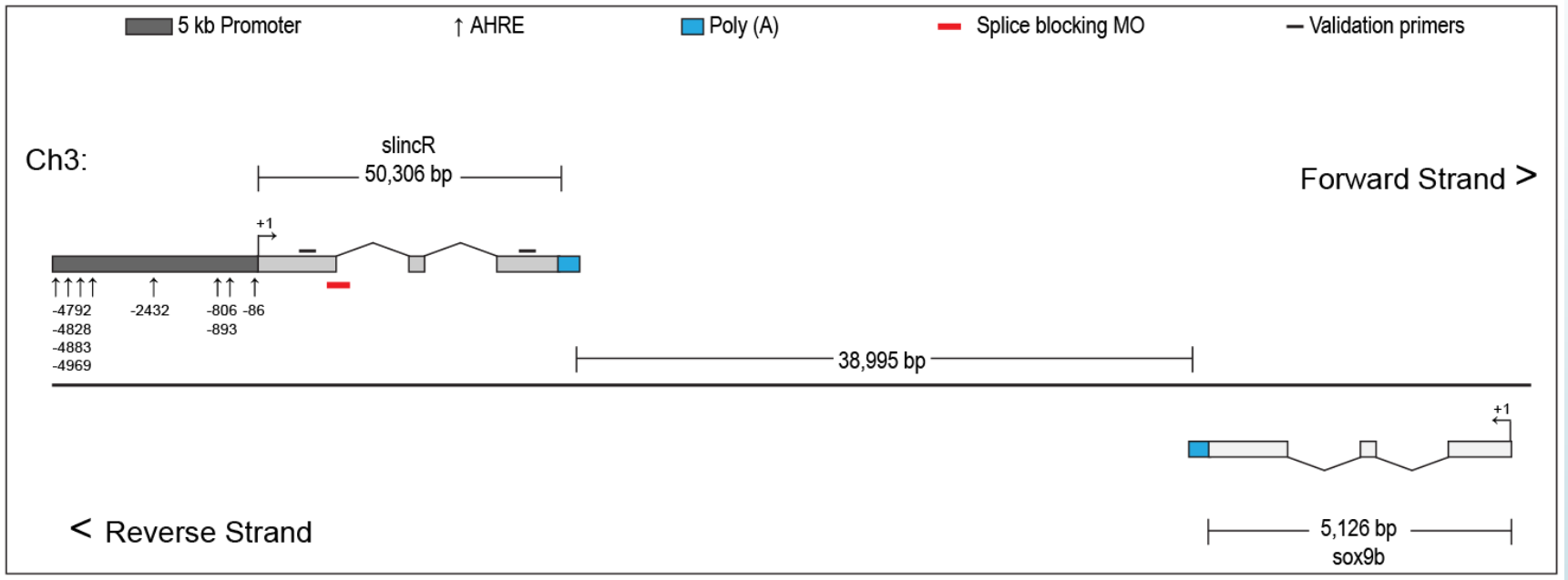
Looking Downstream of AHR2 activation

Novel Transcript identified as long non-coding RNA (lncRNA) and mapped adjacent to Sox9b gene

Top significantly elevated transcripts identified from RNA-Seq in whole 48 hpf embryo exposure to 10 µM B[a]AQ

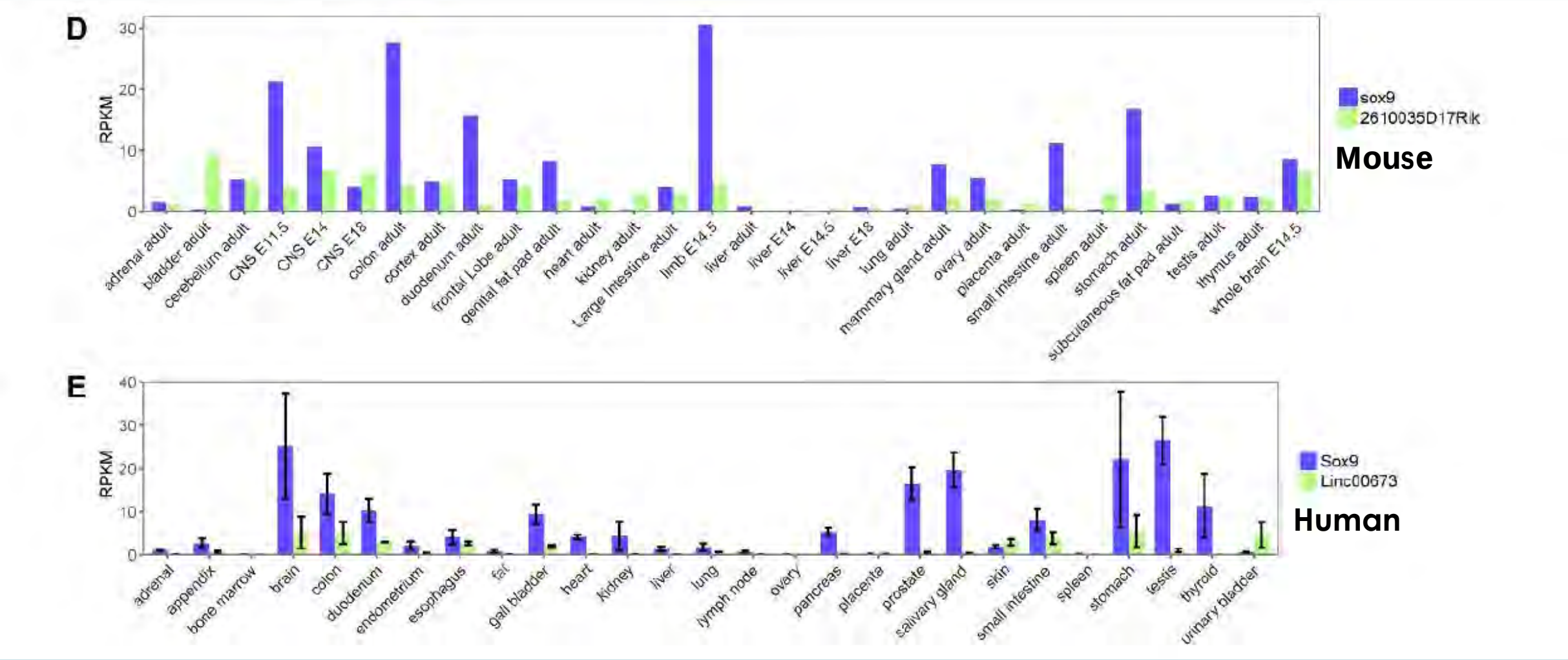
Gene	log2 (fold change)
cyp1a	7.9
cyp1c1	4.8
cyp1c2	4.6
cyp1b1	3.6
novel transcript	**3.2**

sox9b **l**ong **i**ntergenic **n**on-**c**oding **R**NA (slincR)



- Spatial arrangement conserved
- Adjacency of AHREs in promoter region of lncRNA conserved
- Expect lncRNA-target regulation to be similar between fish and mammals

Sox9b and “SlincR” Expression in Mammalian Tissues



RNA-seq expression data from NCBI BioProjects PRJNA66167 (Mouse) and PRJEB4337 (Human)

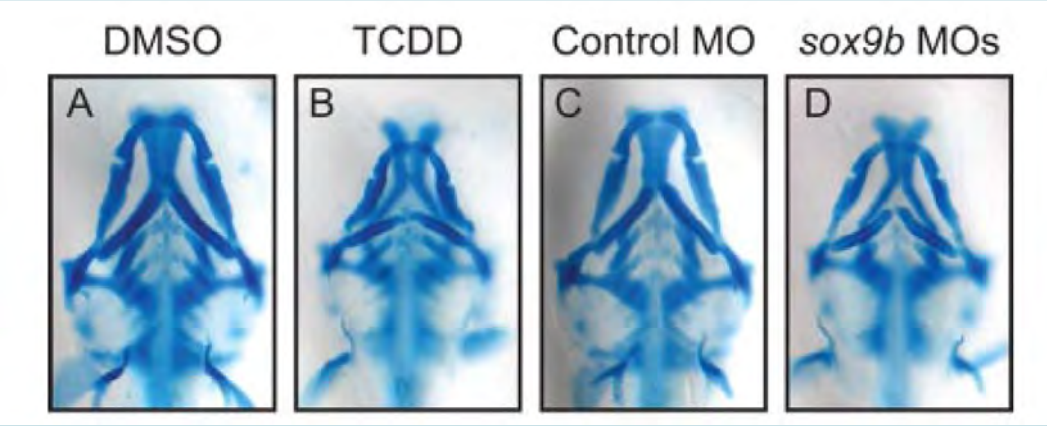
Sox9 Importance for Toxicology and Human Health

Sox9 in humans:

- Dysregulation of Sox9 has been implicated in skeletal deformities, fibrosis, and cancer (Pritchett 2011)

Upon developmental exposure to TCDD, zebrafish Sox9b:

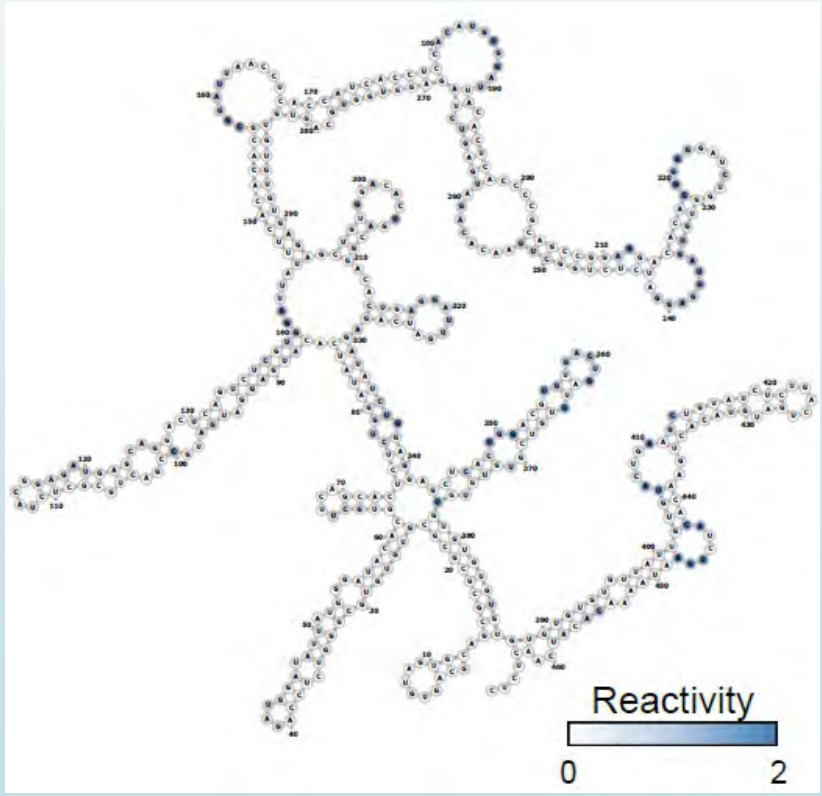
- Is one of the most reduced transcripts in several target organ toxicities
- Is hypothesized to have a **causal** role in TCDD toxicity



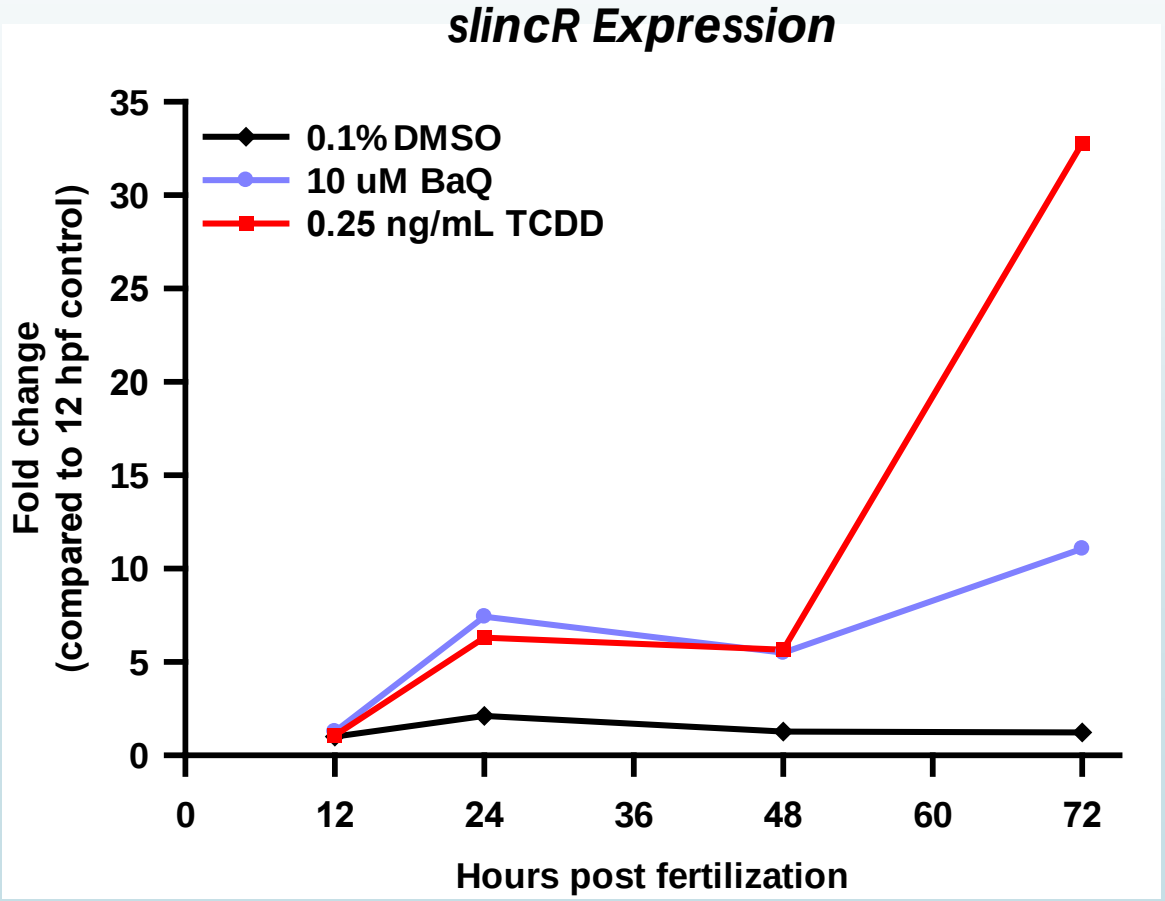
- The mechanism of Sox9b repression was unknown

Predicted slincR Structure

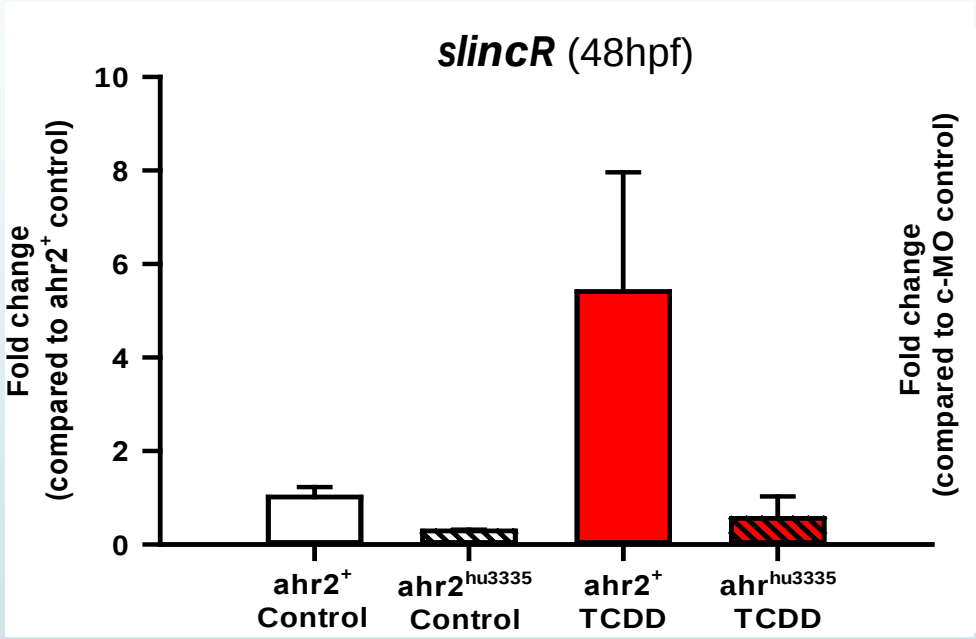
Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE)

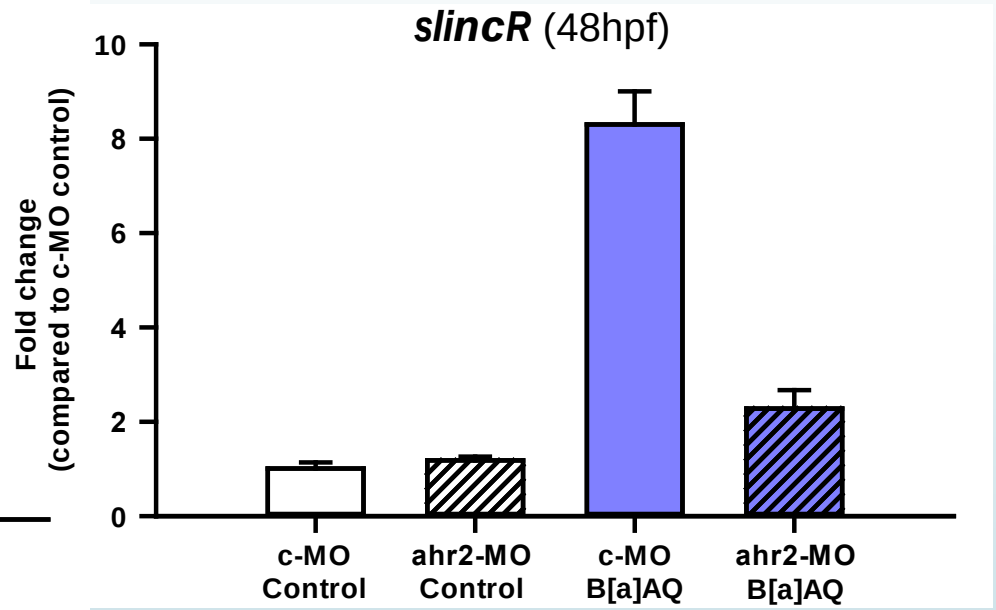


slincR Expression is Elevated by Other AHR Ligands

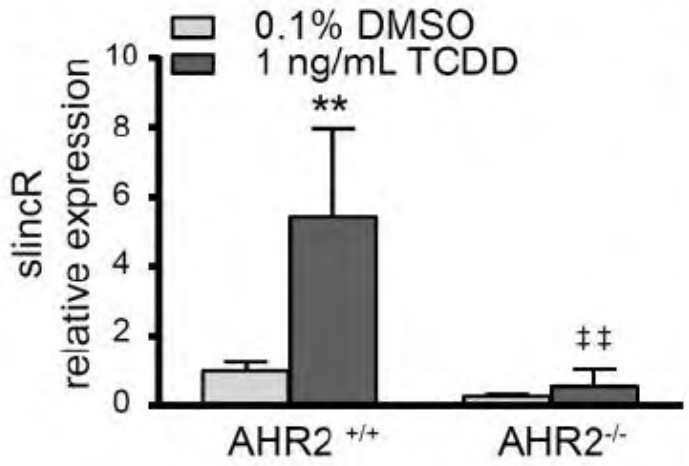


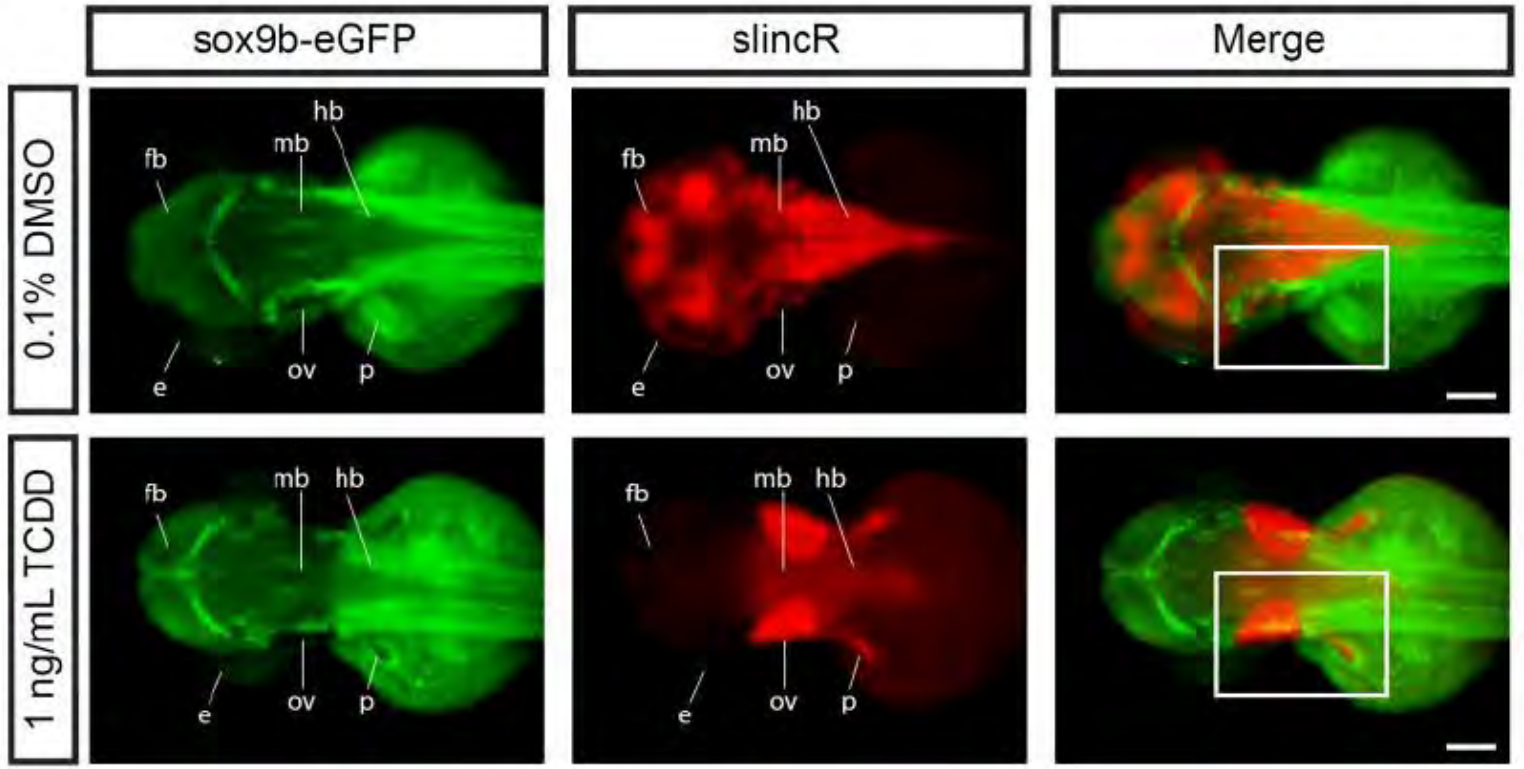
Induction of *slincR* Expression Requires AHR2





Expression of Sox9b and SlicnR Expression

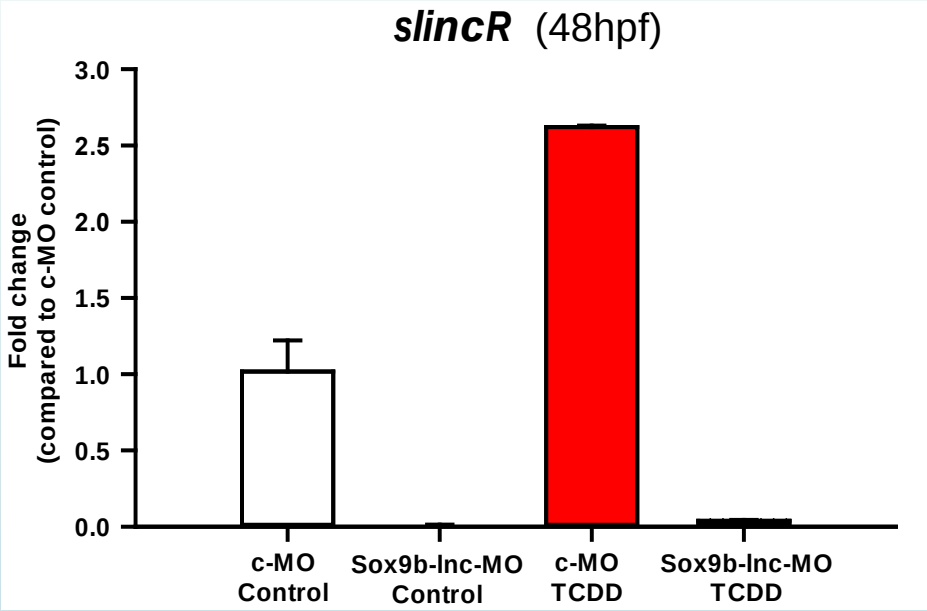


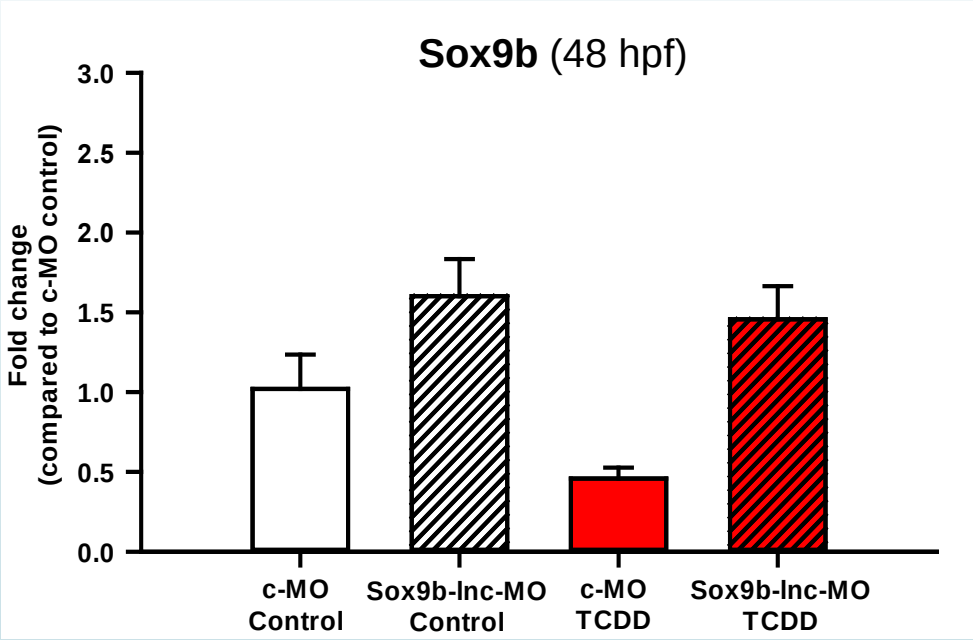


48 hpf

Knockdown of *slincR* Results In a Relief in Repression of Sox9b

Confirmation of Knockdown:



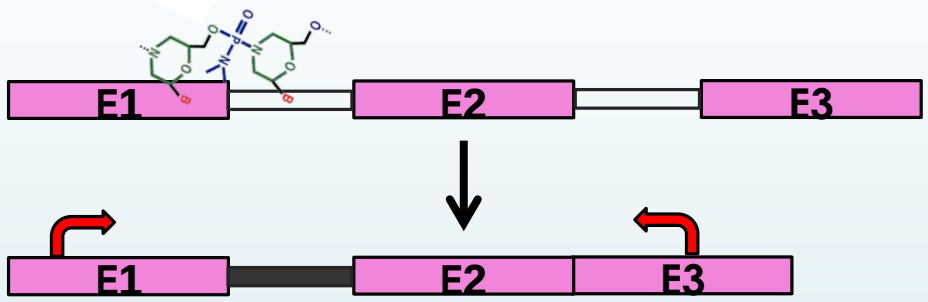


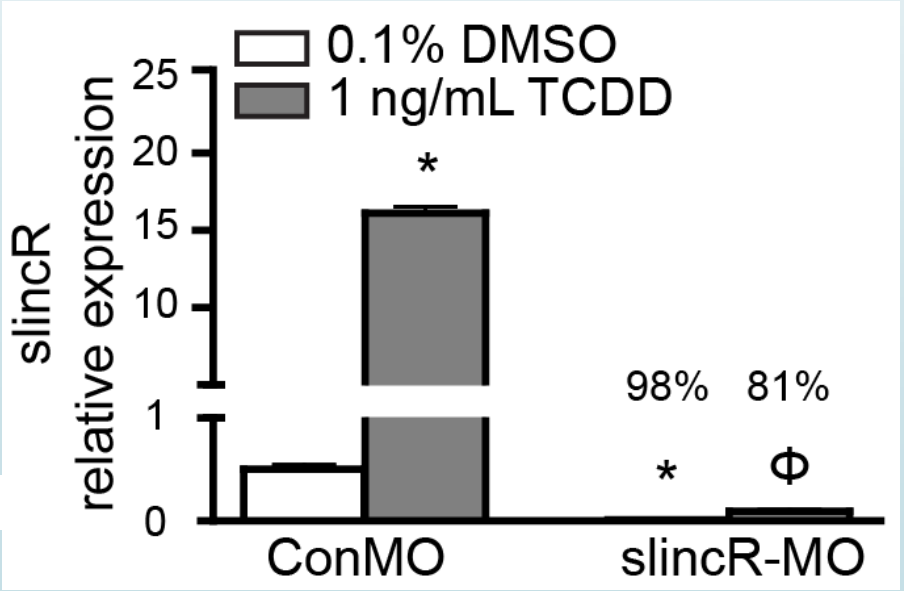
What is the transcriptional impact of knocking slincR down?

RNA-Seq Experimental Objectives:

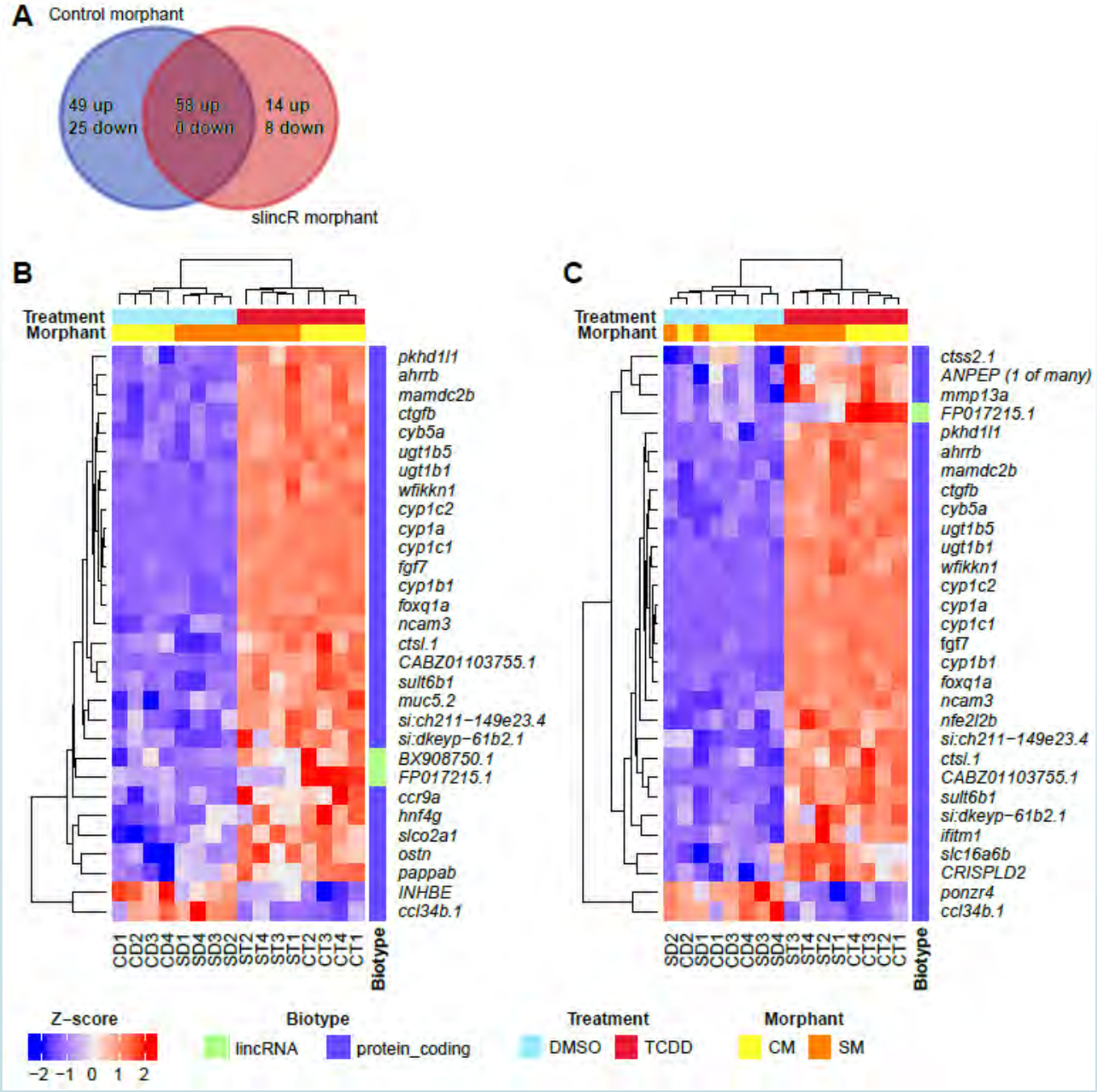
Identify the transcripts, pathways, and biological processes altered or regulated by slincR expression during normal development and during AHR2 activation

slincR splice blocking morpholino design:



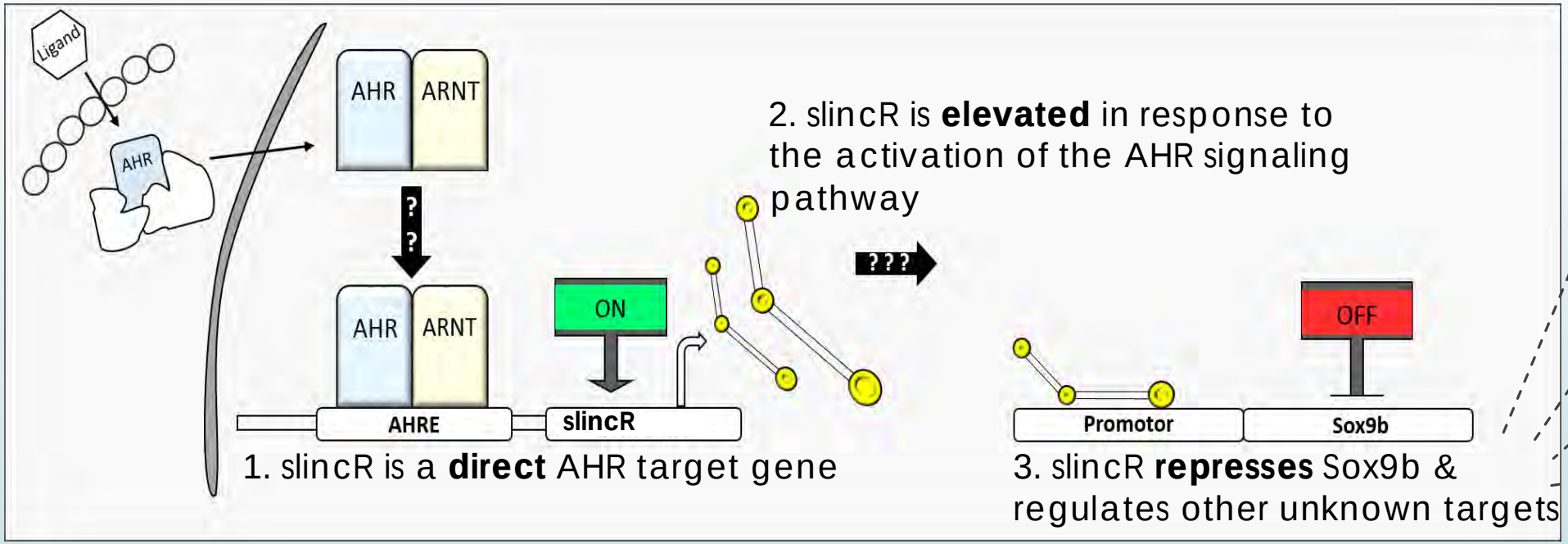


SlincR Transcriptional regulation



Garcia et al. Signaling events downstream of AHR activation that contribute to toxic responses: The functional role of an AHR-dependent long non-coding RNA (slincR) using the zebrafish Model In press EHP

Proposed mechanism of AHR-dependent control of gene expression during normal development



4. Altered biological processes

- **Translation (↓)**
- **Inflammation (↓)**
- **Cell adhesion (↑)**
- **Notch signaling (↑)**

To Summarize

- AHR gene duplication – zebrafish have three functional AHRs
- Increasingly possible to predict ligand/AHR binding
- Platform for structure-based PAH screening
- Complex downstream AHR targets
- AHR/SlincR/Sox9b relationship
- Highly amenable for mixture assessments

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