

Using fish models to understand the role of aryl hydrocarbon receptor (AHR)-interacting protein (AIP) in controlling sensitivity and resistance to dioxin-like compounds *in vivo*

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Abstract

Humans are exposed to chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) that cause toxicity through activation of the aryl hydrocarbon receptor (AHR). There is inter-individual variation in sensitivity to the effects of AHR ligands, but it is not fully explained by variation in the AHR. A clue to the genetic mechanisms underlying differential sensitivity to AHR agonists has emerged from studies of Atlantic killifish (*Fundulus heteroclitus*) populations with evolved tolerance to PCBs, TCDD, and PAHs. Genomic studies of these populations identified AHR-interacting protein (AIP/Ara9/XAP2) as the strongest candidate resistance gene. However, the precise role of AIP in the mechanism of resistance is unknown. To understand the role of AIP in the toxicity of dioxin-like compounds *in vivo*, we used CRISPR-Cas9 to generate AIP loss-of-function alleles in killifish and zebrafish (*Danio rerio*). Homozygous mutant killifish and zebrafish die during larval development—by 30 and 12 d postfertilization, respectively—whereas heterozygous mutants develop, survive, and reproduce normally. During embryonic and early larval stages, homozygous mutant zebrafish exhibit reduced sensitivity to embryotoxic effects of exposure to 3,3',4,4',5-pentachlorobiphenyl (PCB126) and TCDD. Gene expression profiling of *aip*-deficient larvae revealed hundreds of differentially expressed genes. PCB126 induced similar sets of well-known AHR-regulated genes in mutant and wild-type larvae, although with reduced magnitude overall in AIP mutants. This study highlights the important role of AIP in fish larval development and demonstrates that AIP status can influence the response of vertebrate embryos to dioxin-like compounds *in vivo*.

Keywords: dioxins; developmental toxicity; molecular mechanisms; polychlorinated biphenyls; receptors; zebrafish

Humans are exposed to a variety of halogenated and nonhalogenated aromatic hydrocarbons that cause toxicity through activation of the aryl hydrocarbon receptor (AHR) signaling pathway. AHR agonists include chlorinated dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), non-*ortho*-substituted polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and a variety of natural products, including microbial metabolites (Poland and Knutson 1982; Denison et al. 2011; Avilla et al. 2020; Dong and Perdew 2020). There is inter-individual variation in sensitivity to effects of AHR ligands in human cells and tissues (Harper et al. 2002; Nebert et al. 2004; Okey et al. 2005a, 2005b; Lu et al. 2010; Dornbos et al. 2016), but the mechanisms are poorly understood. Although differences in the AHR itself

explain differences in sensitivity among some vertebrate species and strains (Poland et al. 1994; Pohjanvirta et al. 1998; Lavine et al. 2005; Karchner et al. 2006; Farmahin et al. 2013), studies of human AHR variants have yielded contradictory results that do not fully explain individual differences in sensitivity (Kawajiri et al. 1995; Wong et al. 2001a, 2001b; Harper et al. 2002; Nebert et al. 2004; Okey et al. 2005a, 2005b; Kovalova et al. 2016). This suggests that variation in other components of the AHR pathway may contribute to differences in sensitivity among individuals.

The investigation of genes and allelic variants underlying natural disease resistance or evolved adaptation to environmental exposures can reveal genetic modifiers of toxicity (Harper et al. 2015; MacArthur 2016; Vittecoq et al. 2018). A clue to the genetic

mechanisms underlying differential sensitivity to AHR agonists has emerged from the study of fish populations experiencing long-term exposure to PCBs, TCDD, and PAHs. Several exposed populations of the Atlantic killifish (also known as mummichogs; *Fundulus heteroclitus*) have independently evolved resistance to these toxicants, resulting in a >100-fold reduction in sensitivity (Nacci et al. 2010). AHR signaling is impaired in these killifish, as demonstrated by resistance to early life stage toxicity of a variety of AHR ligands (Nacci et al. 1999, 2010) and by the widespread loss of inducibility of AHR-regulated CYP1A (reviewed in Nacci et al. 2010) and many other AHR target genes (Whitehead et al. 2010, 2012; Oleksiak et al. 2011; Reid et al. 2016). Genome-level studies of multiple sensitive and resistant killifish populations using different genomic approaches have identified the AHR-interacting protein (AIP; also known as Ara9 and XAP2; Kuzhandaivelu et al. 1996; Carver and Bradfield 1997; Ma and Whitlock 1997; Meyer et al. 1998) as the strongest candidate for a major resistance gene in these populations (Nacci et al. 2016; Reid et al. 2016; Osterberg et al. 2018; Miller et al. 2024).

AIP is an evolutionarily conserved (Aforei et al. 2018) immunophilin homolog that acts as a chaperone for AHR and a variety of other proteins (Trivellin and Korbonits 2011; Kazzaz et al. 2024), but its functions are not well defined. In mammalian cells *in vitro*, AIP enhances the stability of AHR (Meyer and Perdew 1999; LaPres et al. 2000; Meyer et al. 2000; Lees et al. 2003) and regulates its subcellular localization (LaPres et al. 2000; Petruilis et al. 2003; Ramadoss et al. 2004; Pollenz and Dougherty 2005). AIP also plays an essential role in AHR signaling during mouse embryonic development (Lin et al. 2008). Although AIP's participation in AHR-dependent signaling is complex and not fully understood, the evidence supports the idea that AIP has a key role in regulating AHR function. Consistent with this, AIP can enhance the response of AHR to β -naphthoflavone in a yeast expression system (Carver et al. 1998) and in mammalian cells (LaPres et al. 2000), and mammalian cells deficient in AIP or with impaired AHR–AIP interactions have altered sensitivity to TCDD (Ma and Whitlock 1997; LaPres et al. 2000; Kazlauskas et al. 2002; Hollingshead et al. 2004).

AIP is also a human disease gene. Single-copy germline mutations of AIP predispose humans to familial isolated pituitary adenomas (FIPA) (Vierimaa et al. 2006; Georgitsi et al. 2007; Beckers et al. 2013; Daly and Beckers 2015). Over 100 different AIP variants have been found in FIPA patients (Loughrey and Korbonits 2019); most involve missense or nonsense mutations that affect AIP function (Chahal et al. 2010; Beckers et al. 2013; Gadelha et al. 2013). The underlying mechanism by which AIP variants lead to tumor formation is not known, but the frequency of pathogenic AIP variants supports the idea that variation in AIP can have important functional consequences. Understanding how AIP contributes to altered chemical sensitivity may help to illuminate the overall role of AIP in vertebrate systems, including in FIPA.

Most research on AIP function in relation to the AHR signaling pathway has been conducted *in vitro* and in cell culture; there are few *in vivo* studies, in part because loss-of-function alleles of AIP are embryo-lethal (Lin et al. 2007, 2008; Aforei et al. 2018). Only one *in vivo* study has addressed AIP's role in sensitivity to AHR ligands (Nukaya et al. 2010). In that study, hepatocyte-specific AIP deficiency in mice caused reduced sensitivity to TCDD-induced hepatotoxicity and reduced induction of some AHR target genes (*Cyp1b1*, *Ahr*) but not others (*Cyp1a1*, *Cyp1a2*) (Nukaya et al. 2010). The authors suggested that AIP may contribute to

differential sensitivity to the effects of AHR agonists among individuals and species.

To better understand the role of AIP in evolved resistance to TCDD, PCBs, and PAHs in killifish and its role more generally in controlling sensitivity to AHR agonists during embryonic development *in vivo*, we used CRISPR-Cas9-mediated genome editing to generate germ-line loss-of-function mutations in Atlantic killifish and zebrafish (*Danio rerio*). Here, we report the initial findings from these lines, including larval lethality, altered gene expression, and altered sensitivity to embryotoxic effects of TCDD and a dioxin-like PCB.

Materials and methods

Choice of models

The original associations between AIP and sensitivity to dioxin-like compounds were made using populations of Atlantic killifish (Nacci et al. 2016; Reid et al. 2016; Osterberg et al. 2018; Miller et al. 2024), a well-known environmental model species (Burnett et al. 2007; Reid et al. 2017). We have successfully used CRISPR-Cas9 to perform genome-editing in killifish (Aluru et al. 2015), so our initial AIP loss-of-function experiments were done in this species. Subsequently, we generated AIP loss-of-function mutants in zebrafish, an established laboratory model for investigating the developmental toxicology of dioxin-like compounds (Shankar et al. 2020). More generally, the zebrafish embryo is a powerful *in vivo* model or tool (Sive 2011) for investigating developmental processes, screening chemicals, and studying mechanisms of developmental toxicity (Truong et al. 2014; Garcia et al. 2016; Horzmann and Freeman 2018). Fundamental features of vertebrate developmental signaling pathways and mechanisms are conserved in zebrafish (Garcia et al. 2016), supporting the use of this model species to address both basic research questions in vertebrate developmental biology and translational research relevant to human disease (Tal et al. 2020). Key technical advantages of zebrafish include external development of nearly transparent embryos, small size, rapid development (<72 hr to hatch), and short generation time (Kimmel et al. 1995). A whole genome sequence has revealed extensive orthology with human genes (Howe et al. 2013). The much shorter generation time of zebrafish (3 mo) versus killifish (12 to 24 mo) means that gene targeting and subsequent experimental manipulations can be performed much more efficiently in zebrafish. Because of this, we focused on the zebrafish mutants for most of the experimental work reported in this paper.

Fish husbandry

All animal husbandry practices at the Woods Hole Oceanographic Institution (WHOI) were approved by WHOI's Animal Care and Use Committee (Assurance number D16-00381 from the Office of Laboratory Animal Welfare of the U.S. National Institutes of Health). The work conducted at EPA falls under approved Animal Care and Use Protocols Eco23-03-002, Eco23-07-001, Eco26-04-002, and Eco26-08-001.

Mature male and female Atlantic killifish were collected from Scorton Creek (Sandwich, MA) using minnow traps, as described previously (Aluru et al. 2015). This reference population is known to be sensitive to dioxin-like compounds (Bello et al. 2001; Nacci et al. 2010). Fish were maintained at WHOI and at EPA in tanks with continuous flow-through seawater (SW) and fed TetraMin Tropical Flakes (Tetra, Blacksburg, VA, United States) or Cool Mix Flakes (Brine Shrimp Direct, Ogden, UT). Fertilized embryos were generated as described previously (Aluru et al. 2015).

Zebrafish (*Danio rerio*) from the Tupfel/Long fin mutation (TL) wild-type strain were used in all experiments. Adult zebrafish were reared at WHOI in a recirculating system (Iwaki Aquatic Systems, Holliston, MA, United States) at $28.0^{\circ} \pm 0.5^{\circ}\text{C}$, 14:10 h light:dark cycle (photoperiod) and pH of 7.2 to 7.8. The fish were fed twice daily with brine shrimp (*Artemia salina*) and microencapsulated feed (GEMMA Micro 300 micro-pellets; Skretting USA, Tooele, UT). Embryos were collected from multiple tanks of group matings of approximately 15 females and 7 males per tank. Successfully fertilized eggs were maintained in 0.3x Danieau's solution [17.4 mM NaCl, 0.2 mM KCl, 0.12 mM MgSO_4 , 0.18 mM $\text{Ca}(\text{NO}_3)_2$, and 1.5 mM HEPES, pH 7.3] and monitored daily. Some AIP mutant zebrafish (described below) were transferred to the Sinnhuber Aquatic Research Laboratory (Corvallis, Oregon), where they were reared under conditions described previously (Truong et al. 2014) and in accordance with Institutional Animal Care and Use Committee protocols at Oregon State University.

Genome editing using CRISPR-Cas9 in killifish and zebrafish

Genome editing followed procedures that we described previously (Aluru et al. 2015), with modifications as described below. Single guide RNAs (sgRNAs) were designed using the ZiFiT Targeter website (http://zifit.partners.org/ZiFiT_Cas9). The sgRNA sequences are provided in Fig. S1. The zebrafish and killifish genomes were screened for off-target sites of candidate gRNAs using fuzznuc (<http://emboss.open-bio.org/rel/rel6/apps/fuzznuc.html>). For the killifish studies, one sgRNA targeting exon 2 was prepared as described (Aluru et al. 2015). To generate sgRNAs for the zebrafish studies, 54 nucleotide oligomers targeting exons 2 and 5 were designed following the EnGen sgRNA synthesis kit instructions (New England BioLabs, Ipswich, MA) and obtained from Eurofins Genomics (Louisville, KY). The sgRNAs were purified with the RNA Clean & Concentrator kit (Zymo Research, Irvine, CA). The sgRNA/Cas9 protein mixtures consisted of 80 ng/ μL sgRNA and 25 ng/ μL Cas9 protein. Two nanoliters of the mixture was microinjected into one-cell stage embryos as described previously (Aluru et al. 2015).

Killifish

During the summer 2015 and 2016 breeding seasons, killifish embryos were injected at WHOI with one sgRNA targeting AIP exon 2. Larvae were transferred to the Atlantic Coastal Environmental Sciences Division, U.S. Environmental Protection Agency, Narragansett, RI, where they were raised until sexually mature (1 to 2 yr). Potential founders (F0) were bred and the F1 larvae were raised until they could be fin-clipped and genotyped at the AIP locus. F1 larvae carrying a mutant allele were raised to adulthood, crossed with wild-type killifish, and the offspring carrying the mutant allele (heterozygous F2) were raised and used for subsequent breeding experiments. The AIP mutant killifish line was designated *aip*^{wh71}; additional details are provided in Results.

Zebrafish

SgRNAs targeting exons 2 and 5 of zebrafish AIP were injected separately into TL zebrafish embryos between July and December 2017. Potential founders (F0) were identified by breeding and genotyping the embryos. Identified founders were subsequently bred and the F1 larvae were raised until they could be fin-clipped and genotyped at the AIP locus. F1 larvae carrying mutant alleles were raised to adulthood, crossed with wild-type

(TL) zebrafish, and the offspring carrying mutant alleles (heterozygous F2) were raised and used for subsequent breeding experiments. The 2 mutant lines were designated *aip*^{wh86} (generated using an sgRNA targeting exon 2) and *aip*^{wh239} (generated using an sgRNA targeting exon 5); additional details are provided in Results.

Mutation detection and genotyping

Somatic and germline mutations were detected using the Surveyor mutation detection kit (Transgenomic Inc., Omaha, NE, United States) following the manufacturer's instructions (Qiu et al. 2004). For genotyping, genomic DNA from single larvae or adult tail fin clips was isolated by incubating the tissue at 95°C in 100 μL (adult) or 50 μL (larvae) of 50 mM NaOH for 5 min. The DNA solution was neutralized by adding 10% (v/v) of 1 M Tris (tris (hydroxymethyl)aminomethane) (MS-222), pH 8 followed by centrifugation for 1 min at 14,000 rpm. PCR was performed with 1 μL of the supernatant. Genotyping was carried out by amplifying a small region surrounding the mutation site using GoTaq polymerase mix (Promega, Madison, WI), followed by direct sequencing (Eurofins, Louisville, KY or Sequegen, Worcester, MA). TaqMan genotyping was also utilized for screening zebrafish AIP mutants. In that case, genomic DNA from single larvae was isolated by incubating the tissue at 95°C in 40 μL of 50 mM NaOH for 5 min. The DNA solution was neutralized by adding 4 μL of 1 M Tris, pH 8 and then 200 μL of water, followed by centrifugation for 1 min at 14,000 rpm. Primer and probe sequences and PCR conditions are provided in Fig. S2.

Cloning and [³⁵S] expression analysis of mutant alleles

Gonads were dissected from adult heterozygous *aip*^{wh86} and *aip*^{wh239} zebrafish (2 individuals each). Total RNA was isolated using the Aurum total RNA mini kit (BioRad) and cDNA was synthesized using the iScript kit (BioRad). Full-length AIP coding region was amplified with primers AIP-Fwd (5'-CTACACTCCA CAGGGCAACACTGC-3') and AIP-Rev (5'-GTGCATGTGCTCC AACTTCTCTCC-3') and using Advantage Polymerase (TakaraBio). Amplified DNA fragments were purified from agarose gels using the QIAEXII kit (QIAGEN) and ligated into the pGEM-T Easy plasmid (Promega). Ligation reactions were transformed into DH-5- α competent cells (New England Biolabs). Plasmid DNAs were isolated (Promega) and inserts were confirmed by restriction digestion. Four clones for each AIP mutant construct were sequenced fully using primers AIP-Fwd, AIP-Rev, Seq1 (5'-CC CTCTGGTTTCTCTGTCCTACTACG-3'), and Seq2 (5'-TCATAGTAC TGACCCAGAAGAAGC-3'). Plasmid DNA for one clone each for *aip*^{wh86} and *aip*^{wh239} was cut with *Sall*/*Apal* restriction enzymes and ligated into the pcDNA3.1 vector (Invitrogen) cut with *XhoI*/*Apal* using the Quick Ligase kit (New England Biolabs). The resulting expression constructs were sequenced from the ends using the T7 and BGHRev universal primers to confirm accurate cloning. *In vitro* transcription and translation in the presence of [³⁵S] methionine was performed with the T7-TnT kit (Promega) according to manufacturer's instructions, followed by SDS-PAGE on 15% acrylamide gel and autoradiography to confirm protein expression.

Assessment of mutant survival and phenotypes

To evaluate the survival of mutant fish, we crossed confirmed heterozygous mutants. Embryos were collected and observed once or twice each day throughout development. Embryos or larvae that died or were moribund were sampled. Surviving

larvae were sampled at 60 d postfertilization (dpf; killifish) or 14 dpf (zebrafish) for genotyping. The numbers of clutches and embryos per clutch are provided in figure legends and in [Tables S1](#) and [S2](#). Survival data were analyzed using Kaplan–Meier survival analysis in Prism version 10 (GraphPad Software, LLC).

Chemical exposures

Rationale for exposure experiments

Several exposure experiments were performed with AIP mutant zebrafish embryos (summarized in [Table 1](#) and [Fig. 1](#), with additional details in the [Supplementary Figures](#)). All experiments were performed using offspring generated from a cross of heterozygous mutant parents, which allowed us to compare the responses of the 3 genotypes (homozygous wild-type, heterozygous mutant, and homozygous mutant) from the same breeding. With this design, each embryo or larva must be genotyped after phenotyping and prior to pooling for subsequent gene expression analyses. For each experiment, we generated the embryos through group breeding of approximately 15 to 20 heterozygous adult pairs. Thus, although the embryos for each experiment were generated from a single breeding, they represent genetic contributions from multiple parents.

The goal of the exposures was to assess the sensitivity of mutant embryos and larvae to 2 potent dioxin-like compounds, TCDD and PCB126, which have been shown to cause developmental toxicity in zebrafish, including cardiovascular and craniofacial abnormalities ([Henry et al. 1997](#); [Jönsson et al. 2007](#); [Grimes et al. 2008](#); [Goodale et al. 2012](#)). Because these initial experiments were somewhat exploratory, the concentrations, timing and duration of exposure, and sampling times varied among experiments ([Fig. 1](#)). Exposures were conducted by exposing the embryos to toxicant (TCDD or PCB126) or vehicle (DMSO) in single petri dishes (experiments 1 to 5) or triplicate 96-well plates (experiment 6). The concentrations of TCDD and PCB126 used had been shown previously to cause developmental toxicity in most of the exposed embryos or larvae ([Henry et al. 1997](#); [Jönsson et al. 2007](#); [Grimes et al. 2008](#); [Goodale et al. 2012](#)). The DMSO concentration varied among experiments (0.1% or 0.02%) but in all cases was the same in toxicant and vehicle dishes within an experiment and was well below concentrations that

cause toxicity in zebrafish embryos (>1%; ([Hoyberghs et al. 2021](#))).

Phenotype scoring was carried out by individual observation of each embryo under the microscope prior to sampling, noting the presence of defects such as yolk sac edema, pericardial edema, and in a subset of experiments, jaw malformation and tubular heart. Representative photos of edema phenotypes are shown in [Fig. S3](#).

- **Experiment 1** (*aip^{wh239}*; TCDD): Tanks containing confirmed heterozygous mutant zebrafish were bred. At 31 h postfertilization (hpf), embryos were exposed to TCDD (2 nM) or DMSO (0.02%) for 1 h in glass petri dishes (50 ml of exposure medium and 50 embryos per dish), rinsed, and placed in individual wells of 48-well plates for observation. The concentration of TCDD (2 nM) was selected based on previous observations that it induces AHR-mediated phenotypes in zebrafish ([Jenny et al. 2009](#)). Embryos and larvae were scored for cardiovascular phenotypes (pericardial edema) each day. At 6 d postfertilization (dpf), larvae were anesthetized with MS222, transferred to a microscope slide, and a small section of the tail removed with a scalpel and used for genotyping as described above.
- **Experiment 2** (*aip^{wh239}*; PCB126): Embryos were exposed to 3,3',4,4',5-pentachlorobiphenyl (PCB126; 10 nM ([Jönsson et al. 2007](#))) or DMSO (0.02%) from 6 to 24 hpf in glass petri dishes (50 ml of exposure medium and 44 embryos per dish), rinsed, and placed in individual wells of 48-well plates for observation. Embryos and larvae were scored for cardiovascular and craniofacial phenotypes each day. At 72 hpf, larvae were anesthetized with MS222 and processed as described in Experiment 1.
- **Experiment 3a** (*aip^{wh239}*; PCB126): 100 embryos each were exposed to PCB126 (10 nM) or DMSO (0.02%) starting at 6 hpf. At 24 hpf (18 h dosing), embryos were rinsed and placed in clean 0.3x Danieau's solution. At 72 hpf, individual larvae were transferred to a small petri dish of MS-222 for a few minutes and then onto a microscope slide. Tails were cut with a scalpel for genotyping and the remaining body was frozen in liquid nitrogen for RNA. For RNA isolation, 5 genotyped embryos were randomly pooled for each replicate sample, with 4 replicate pools for each genotype and treatment.

Table 1. Exposure experiments conducted in AIP-deficient zebrafish.

Expt.	Mutant line	Chemical (concentrations)	Exposure timing	Observation/sampling	Endpoints ^a	Embryos (#)
1	<i>aip^{wh239}</i>	TCDD (2 nM)	31–32 hpf	6 dpf	E	50
		DMSO (0.02%)				49
2	<i>aip^{wh239}</i>	PCB126 (10 nM)	6–24 hpf	3 dpf	E, J, H	42
		DMSO (0.02%)				40
3a	<i>aip^{wh239}</i>	PCB126 (10 nM)	6–24 hpf	3 dpf	E (3 dpf), RNA-seq	96
		DMSO (0.02%)			E (6 dpf)	99
		PCB126 (10 nM)		6 dpf		85
		DMSO (0.02%)				48
3b	<i>aip^{wh86}</i>	DMSO (0.1%)	120–126 hpf	126 hpf	RNA-seq	114
4	<i>aip^{wh86}</i>	PCB126 (10 nM)	6–24 hpf	3 dpf	E	84
		DMSO (0.02%)				84
5	<i>aip^{wh86}</i>	PCB126 (10 nM)	6–24 hpf	4 dpf	E	81
		DMSO (0.02%)				73
6	<i>aip^{wh86}</i>	TCDD (1 nM)	6–120 hpf	5 dpf	deformities	91
		TCDD (10 nM)				96
		DMSO (0.1%)				85

^a All larvae were genotyped at the end of the experiments. TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PCB126, 3,3',4,4',5-pentachlorobiphenyl; hpf, hours postfertilization; dpf, days postfertilization; E, pericardial and yolk-sac edema; J, jaw malformation; H, heart malformation (tube heart); RNA-seq, RNA-sequencing. For additional details, see text, [Fig. 1](#), and [Figs S5–S11](#).

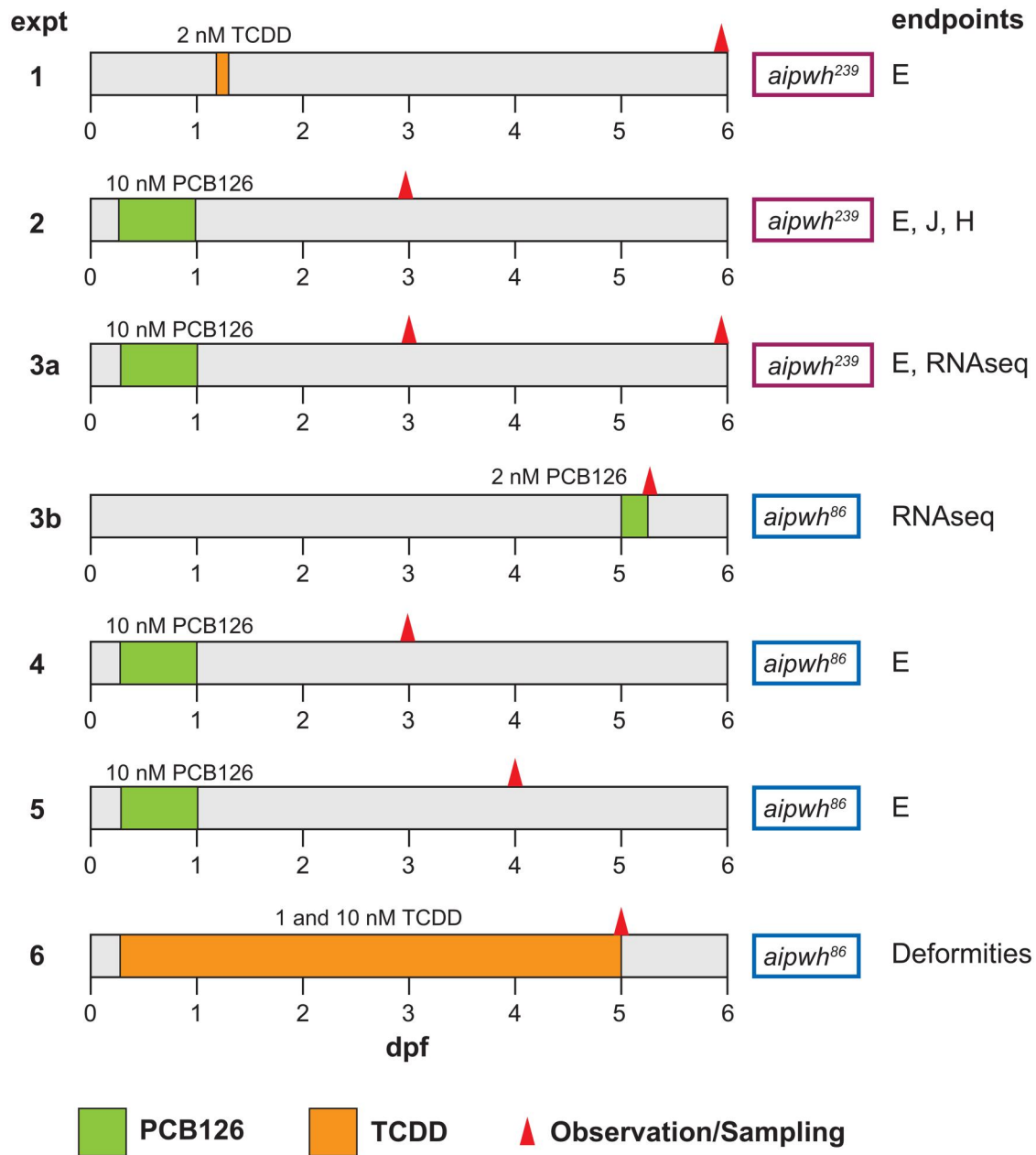


Fig. 1. Exposure experiments conducted in AIP-deficient zebrafish. The figure illustrates the various exposure designs, sampling times, mutant lines, and endpoints used in the different experiments. For additional details, see the text, [Table 1](#), and [Figs S5–S11](#). Abbreviations: TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCB126: 3,3',4,4',5-pentachlorobiphenyl; dpf: Days postfertilization; E: Pericardial and yolk-sac edema; J: Jaw malformation; H: Heart malformation (tube heart); RNAseq: RNA-sequencing.

Another set of exposed larvae was observed until 6 dpf, when they were phenotyped and sampled for genotyping.

- **Experiment 3b** (*aip^{wh86}*; DMSO): Embryos were exposed to PCB126 (2 nM) or DMSO (0.1%) in glass petri dishes (75 ml exposure medium and 60 larvae per dish) for 6 h at 120 hpf. Immediately after exposure, larvae were placed in Eppendorf tubes with 117 μ L of RNA lysis buffer (see RNA-sequencing) and frozen at -80°C for RNA isolation. The next day, larvae were homogenized in lysis buffer and 3 μ L samples of each lysate were added to 147 μ L nuclease-free water in wells of a 96-well plate and frozen at -20°C for later genotyping. The rest of the lysate was frozen at -80°C for later RNA isolation. After genotyping, thawed lysates were pooled by genotype and

treatment (4 pools, 5 lysates per pool) and used for RNA isolation. For technical reasons, the PCB data from this experiment were not usable, so we focused only on the data from DMSO-exposed larvae of the 3 genotypes.

- **Experiment 4** (*aip^{wh86}*; PCB126): Embryos were exposed to PCB126 (10 nM) or DMSO (0.02%) from 6 to 24 hpf in glass petri dishes. At 24 hpf (18 h dosing), embryos were rinsed and placed in clean 0.3x Danieau's solution. At 3 dpf, phenotypes were scored and individual embryos were processed for DNA isolation and genotyping.
- **Experiment 5** (*aip^{wh86}*; PCB126): Embryos were exposed to PCB126 (10 nM) or DMSO (0.02%) from 6 to 24 hpf in glass petri dishes. At 24 hpf (18 h dosing), embryos were rinsed and

placed in clean 0.3x Danieau's solution. At 4 dpf, phenotypes were scored and individual embryos were processed for DNA isolation and genotyping.

- **Experiment 6 (*aip^{wh86}*; TCDD):** This experiment was conducted on embryos obtained from *aip^{wh86}* adult fish shipped to Oregon State University, following their established protocols (Truong et al. 2011, 2014). Embryos in triplicate 96-well plates filled with 100 μ L of embryo medium per well were exposed to TCDD (1 or 10 nM) or DMSO (0.1%) (32 embryos at each concentration per plate) from 6 to 120 hpf. At 24 and 120 hpf, larvae were assessed for 15 morphological endpoints (Truong et al. 2011, 2014) and then individual embryos were processed for DNA isolation and TaqMan genotyping.

RNA-sequencing

Total RNA from experiments 3a and 3b was isolated using the Aurum total RNA mini kit (BioRad) following manufacturer's instructions and was quantified using a NanoDrop spectrophotometer. Total RNA was checked for quality using an Agilent Bioanalyzer or Fragment Analyzer. All samples had RNA integrity numbers or RNA quality numbers between 9 and 10. Illumina Stranded mRNA libraries from 4 biological replicates from each treatment (DMSO and PCB126) were sequenced on 2 lanes of the Illumina NovaSeq or X-plus platforms with single-end 100bp reads. Library construction and sequencing were performed at the Tufts University Core Facility (Boston, MA). Approximately 30 to 40 million reads were obtained for each sample. Raw reads were preprocessed and mapped to the zebrafish genome (Ensembl version 110) using STAR 2.7.10b (Dobin and Gingeras 2015). Approximately 93% of the reads from each sample were mapped uniquely to the genome. Using genomic coordinates, mapped reads were counted using HTseq-count (Andrews 2010). Raw data files are deposited in the GEO database (GEO accession numbers GSE292310 and GSE295203). Integrative Genomics Viewer (IGV version 2.3) was used to visualize aligned reads (Thorvaldsdóttir et al. 2013).

Data analysis

Statistical analysis was performed using the edgeR package (Robinson et al. 2010). The EdgeR FilterByExpr command was used to filter out genes with low expression. Sources of variation in the RNAseq data were determined using normalized reads by generating a multiple-dimensional scaling (MDS) plot using EdgeR. Differential gene expression analysis between different genotypes and exposure conditions was done using quasi-likelihood F-test (Chen et al. 2016). Genes with statistically significant (FDR < 0.05) differential expression in comparisons of genotypes or PCB126 exposure were annotated using the Ensembl BioMart tool and by BLAST search of NCBI protein sequence database. Gene set enrichment analysis was performed with iDEP (Ge et al. 2018) to detect statistically significantly enriched pathways and Gene Ontology (GO) terms. Venn diagrams of gene IDs were generated for determining overlaps between data sets (<https://bioinformatics.psb.ugent.be/webtools/Venn/>).

Results

Generation of AIP-mutant killifish and zebrafish Killifish

From a tank of potential founders (F0), F1 offspring with 1-, 19-, and 21-bp deletions were identified. The fish with a 19-bp deletion (a male) was raised to adulthood, crossed with a wild-type

female killifish, and the offspring carrying the mutant allele (heterozygous F2) were raised and used for subsequent breeding experiments.

The 19-bp deletion in the killifish AIP gene (designated *aip^{wh71}*) leads to a frameshift and premature termination codon (Fig. 2). The mutant allele is predicted to encode a truncated protein of 71 amino acids (aa) (7.9 kDa), of which 55 are from the wild-type protein and 16 are missense amino acids.

Zebrafish

Potential founders that had been injected with sgRNA targeting exon 5 produced offspring (F1) containing 2-bp, 5-bp, 7-bp, 11-bp, and 57-bp deletions. Offspring heterozygous for the 5-bp deletion were raised to adulthood and they (or their heterozygous offspring) were used for subsequent breeding experiments. This exon 5 mutant allele (designated *aip^{wh239}*) encodes a truncated 26.5 kDa protein (239 aa, including 17 missense aa, vs 342 aa (38.3 kDa) for wild-type AIP) that lacks 2 of the 3 TPR (tetratricopeptide) domains and the C-terminal α -helix, domains that interact with AHR (Carver et al. 1998; Meyer and Perdew 1999; Bell and Poland 2000; Kazlauskas et al. 2000) (Fig. 2).

Male and female founders injected with sgRNA targeting exon 2 were crossed and the offspring (F1) were genotyped. Fish with a 2-bp deletion was identified and raised to adulthood. This exon 2 mutant allele (designated *aip^{wh86}*) encodes a truncated 9.6 kDa protein of 86 aa (including 4 missense aa) that lacks all of the TPR domains and the C-terminal α -helix (Fig. 2).

Survival of AIP mutant embryos and larvae Killifish

We crossed heterozygous (HET; +/-) AIP mutant killifish and followed their offspring for 60 d to determine whether complete loss of AIP function affects embryo or larval survival. In 3 independent clutches of killifish embryos, we found that no homozygous mutant (HMZ; -/-) larvae survived past 30 dpf (Fig. 3A). Thus, loss of AIP function appears to be lethal in killifish larvae.

All subsequent experiments were performed using the 2 zebrafish AIP mutant lines, because of their more consistent breeding, shorter development time, and ease of manipulation as compared with killifish.

Zebrafish

F1 *aip^{wh239}* AIP heterozygotes (+/-) were crossed. Embryos and early larvae genotyped between 3 and 6 dpf displayed the expected Mendelian ratios (+/+ : +/- : -/-, 1:2:1), demonstrating that the AIP mutation is not embryonic lethal (Table S1). Embryos and early larvae from *aip^{wh86}* HET intercrosses also survived through 6 dpf, demonstrating that this AIP mutation also is not embryonic lethal.

To assess the effects of AIP loss-of-function on later larval development, we crossed HET mutant parents and followed the offspring through larval development, followed by genotyping of dead larvae and then genotyping of all survivors at 14 dpf. For both *aip^{wh239}* and *aip^{wh86}* mutants, HMZ larvae began to die at 7 to 8 dpf, with 100% mortality by day 12 (Fig. 3B and C). The mean time to death did not differ significantly between mutant lines (*aip^{wh239}*: 9.4 ± 1.3 d, N=4 clutches; *aip^{wh86}*: 9.5 ± 1.2 d, N=5 clutches) (Table S2).

Larval phenotypes

HMZ mutant larvae of both zebrafish lines (*aip^{wh86}* and *aip^{wh239}*) exhibited progressive changes in morphology prior to death. Many HMZ larvae did not inflate their swim bladders or formed

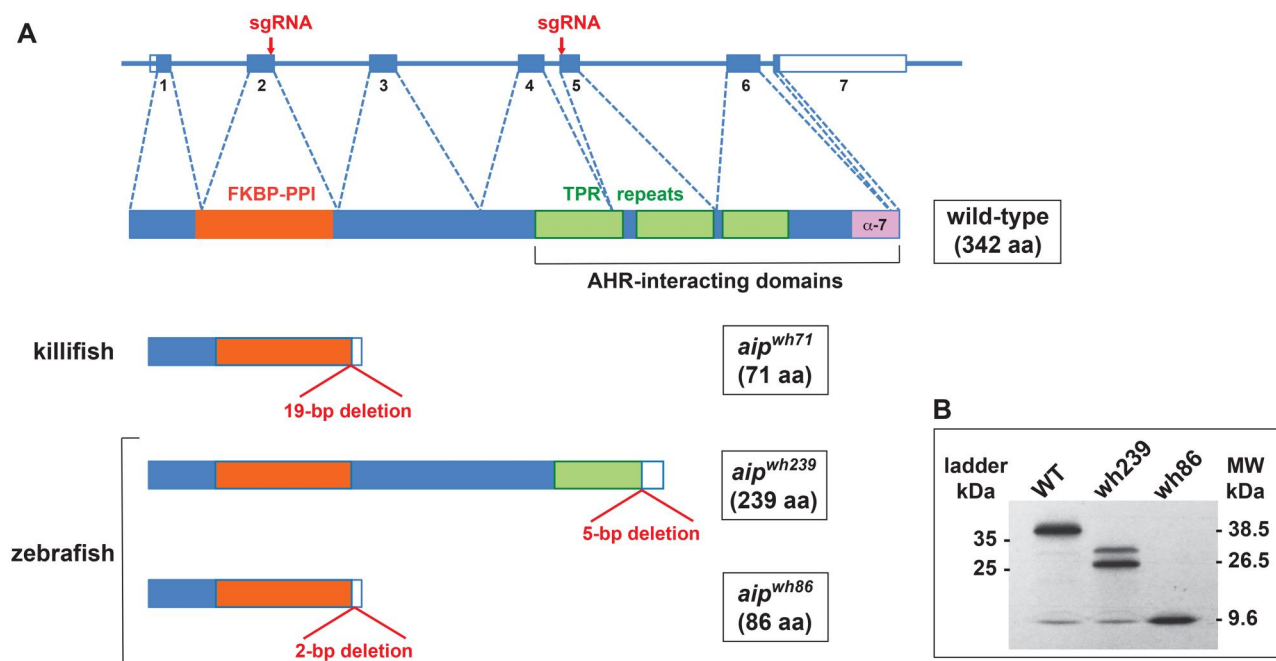


Fig. 2. Killifish and Zebrafish AIP mutants generated by CRISPR-Cas9. **A) Top:** The gene and mRNA structures of AIP are illustrated. **Bottom:** All alleles encode frameshift mutations that cause the addition of missense amino acids (red) and premature termination codons, leading to predicted truncated proteins. **B)** The zebrafish mutant cDNAs were cloned and expressed by in vitro transcription and translation in the presence of [³⁵S]methionine, confirming that they encode truncated proteins. The double band in the middle lane may reflect a partial read-through of the premature termination codon introduced by the frameshift mutation; however, any additional amino acids would be mis-sense residues. Abbreviations: SgRNA: Single guide RNA; FKBP-PPI: FK506-binding protein—peptidyl-prolyl isomerase domain; TPR: Tetratricopeptide; AHR: Aryl hydrocarbon receptor.

small ones that collapsed by 9 to 10 dpf (Fig. S4). Jaw malformations of variable severity were seen in HMZ larvae. Morphological changes in the digestive tract, liver, and brain suggestive of necrosis was evident starting at 9 dpf, shortly before death (Fig. S4). A more detailed histological analysis of these changes will be reported in a subsequent paper. The HET larvae of both zebrafish lines were morphologically and behaviorally similar to wild-type (WT) larvae.

Loss of AIP affects sensitivity to dioxin-like compounds

In light of the identification of AIP as a candidate resistance gene in PCB- or PAH-exposed killifish populations (Nacci et al. 2016; Reid et al. 2016; Osterberg et al. 2018; Miller et al. 2024), we hypothesized that zebrafish with partial or complete loss of AIP function would be less sensitive to dioxin-like compounds. We tested this hypothesis in a series of experiments using each of the zebrafish AIP mutant lines (*aip^{wh86}* and *aip^{wh239}*) exposed to the AHR ligands PCB126 or TCDD (Table 1). Although the complete loss of zygotic AIP is lethal by 12 dpf, the embryos and larvae appear unaffected during the embryonic and early larval stages, allowing us to test their sensitivity to PCB126 and TCDD. In these experiments, which were done in offspring of heterozygote incrosses, treatment-related phenotypes were scored, after which individual larvae were genotyped and the phenotypic data were sorted by genotype.

We performed 3 experiments using exon 5 (*aip^{wh239}*) zebrafish to assess the effect of this AIP mutation on embryo/larval sensitivity to TCDD or PCB126. In experiment 1, all larvae exposed to TCDD (2 nM) from 31 to 32 hpf developed the classical signs of TCDD toxicity, including pericardial and yolk-sac edema, by 6 dpf. However, in the HMZ mutant larvae, the severity of the effects was less than in WT or HET larvae; no HMZ mutant larvae

exhibited the severe edema seen in other genotypes (Fig. S5). Similar results were obtained in experiment 2 when embryos were exposed to PCB126 (3,3',4,4',5-pentachlorobiphenyl; 10 nM) from 6 to 24 hpf. In this experiment, we assessed pericardial and yolk-sac edema, jaw malformations, and defective looping during heart development (tube heart)—all of which are characteristic of fish embryos exposed to dioxin-like compounds—at 3 dpf after embryo exposure to PCB126. The *aip^{-/-}* larvae tended to have lower deformity scores, which reflected a lower incidence and severity of tube heart (Fig. S6). In experiment 3a, in which PCB126-exposed embryos were scored for edema at 3 dpf, *aip^{-/-}* larvae had a lower overall incidence of edema as compared with *aip^{+/+}* or *aip^{+/-}* larvae and none of the *aip^{-/-}* larvae had severe edema (Fig. S7).

Additional exposure experiments were performed using heterozygote intercrosses of the exon 2 (*aip^{wh86}*) zebrafish. In experiment 4, offspring of *aip^{wh86/+}* fish were exposed to 10 nM PCB126 from 4 to 24 hpf. At 3 dpf, the *aip^{-/-}* larvae had a lower overall incidence of edema and reduced severity of edema as compared with *aip^{+/+}* or *aip^{+/-}* larvae (Fig. 4A; Fig. S8). In a similar experiment (experiment 5) in which larvae were assessed at 4 dpf, there was a reduction in the incidence of edema in PCB126-exposed *aip^{-/-}* and *aip^{+/-}* larvae as compared with *aip^{+/+}* larvae (Fig. S9).

To obtain a more detailed assessment of genotype-specific differences in sensitivity to TCDD, in experiment 6 we performed an exposure experiment using the OSU phenotyping platform, which includes assessment of 15 morphological phenotypes (Truong et al. 2011, 2014). Embryos were exposed to TCDD (1 or 10 nM) from 6 to 120 hpf and then phenotypes were assessed at 24 and 120 hpf (Truong et al. 2011, 2014). Although *aip^{+/+}* or *aip^{+/-}* larvae exposed to either concentration of TCDD exhibited a significant increase in treatment-related deformities, the *aip^{-/-}* larvae were less sensitive to 1 nM TCDD (Fig. 4B; Fig. S10).

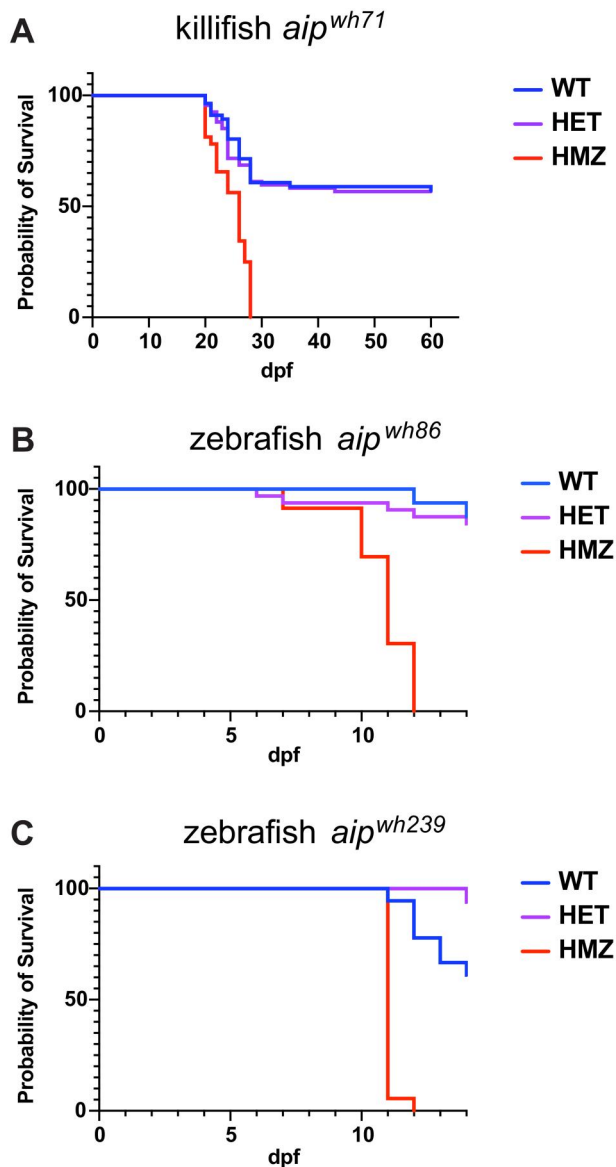


Fig. 3. Survival of AIP mutant fish. A) Killifish. Heterozygous *aip*^{wh71} killifish were crossed and dead or moribund embryos and larvae were sampled and genotyped as described in *Materials and Methods*. At the end of 2 mo, all surviving larvae were fin-clipped and genotyped. The graph shows combined results from 2 independent clutches, totaling 162 larvae (56 wild-type, 67 heterozygous, 32 homozygous). B, C) Zebrafish. Heterozygous *aip*^{wh86} (B) and *aip*^{wh239} (C) zebrafish were crossed and dead or moribund embryos and larvae were sampled and genotyped as described in *Materials and Methods*. After 14 dpf, all survival larvae were genotyped. The graph shows representative results from 4 or 5 clutches (Table S2). Abbreviations: WT: Wild-type, HET: Heterozygous, HMZ: Homozygous, dpf: Days postfertilization.

Collectively, these results from fish from 2 independent *aip*-mutant lines suggest that the loss of AIP function does not completely protect zebrafish against the embryo/larval toxicity of dioxin-like compounds but may reduce their sensitivity to these effects.

Altered gene expression in AIP mutant zebrafish (3 and 5 dpf)

To determine the effect of *aip* loss-of-function on constitutive and PCB-inducible gene expression, RNA sequencing was performed on *aip*^{wh239} larvae from experiment 3a (exposed to

PCB126 from 6 to 24 hpf and sampled at 3 dpf) and *aip*^{wh86} larvae from experiment 3b (sampled at 5 dpf). Analysis of the read data by multi-dimensional scaling (MDS) revealed clustering of samples by genotype and treatment (Fig. 5A; Fig. S11A). The complete sets of differentially expressed genes can be found in [Supplementary Data File 1](#) (experiment 3a) and [Supplementary Data File 2](#) (experiment 3b).

Constitutive gene expression (DMSO vehicle control)

There were no differentially expressed genes (DEGs) between the HET *aip*^{wh239} mutants and WT larvae from the same parents, consistent with the overlap of these 2 clusters in the MDS plot. Gene expression in the DMSO-exposed HMZ *aip*^{wh239} mutants differed substantially from that in the DMSO-exposed WT and HET, with 1433 (849 up, 584 down) and 1111 (478 down, 633 up) DEGs, respectively. Genes down-regulated in the HMZ *aip*^{wh239} mutants as compared with WT larvae included *aip* itself (11-fold), as well as another AHR pathway gene, *ahrrb* (10-fold), and an AHR-regulated lncRNA (*slincR*; 1.7-fold) ([Supplementary Data File 1](#)). Pathway analysis revealed that the down-regulated gene set was enriched in Biological Process (BP), Molecular Function (MF), and KEGG pathway GO terms related to ribosome biogenesis, DNA replication, and DNA repair. The upregulated gene set showed enrichment in GO terms small molecule metabolic process, cellular catabolic process, monooxygenase activity, and transporter activity (Fig. S12; [Supplementary Data File 3](#)).

Similar results were obtained from the *aip*^{wh86} larvae sampled at 5 dpf. Gene expression in the DMSO-exposed HMZ *aip*^{wh86} mutants differed substantially from that in the DMSO-exposed WT and HET larvae, with 1028 (430 down, 598 up) and 791 (277 down, 514 up) DEGs, respectively (Fig. S11B). In addition to *aip* (12-fold), genes down-regulated in the HMZ *aip*^{wh86} mutants as compared with WT larvae included *ahrra* (6-fold), *slincR* (3.7-fold), and several odorant and olfactory receptor genes ([Supplementary Data File 2](#)). GO term and KEGG pathway enrichment analysis of downregulated genes indicated that cell cycle process, chromosome segregation, sensory perception, G protein-coupled receptor activity, and metabolism of xenobiotics by cytochrome P450 were enriched. The up-regulated genes were enriched in GO terms such as transmembrane transport and muscle contraction (Fig. S13; [Supplementary Data File 3](#)).

In data sets from both AIP mutant lines, a few of the most highly differentially expressed genes are located on chromosome 1, near *aip* ([Supplementary Data File 4](#)); some of these may represent allele-specific expression of genes in linkage disequilibrium with *aip* (White et al. 2022) rather than genes whose expression was affected by the loss of AIP. Since the *aip*^{wh86} and *aip*^{wh239} mutant lines originated from independent founders, the chromosome 1 DEGs that are shared by the 2 mutant lines are less likely to represent allele-specific expression.

PCB126 effects on gene expression in AIP mutant zebrafish (3 dpf)

In the *aip*^{wh239} larvae from experiment 3a exposed to PCB126 and sampled at 3 dpf, PCB126-induced changes in gene expression were found in all genotypes, with patterns of DEGs that included both shared and genotype-specific changes and both up- and down-regulated genes (Fig. 5B, left panel; [Supplementary Data File 1](#)). A PCA plot of the average normalized reads from DMSO- and PCB126-exposed WT, HET, and HMZ mutant larvae shows the effect of PCB126 exposure (shift on the horizontal axis) superimposed on an effect of the loss of AIP (shift on the vertical axis) (Fig. 5B, right panel). The set of 147 DEGs shared by all genotypes

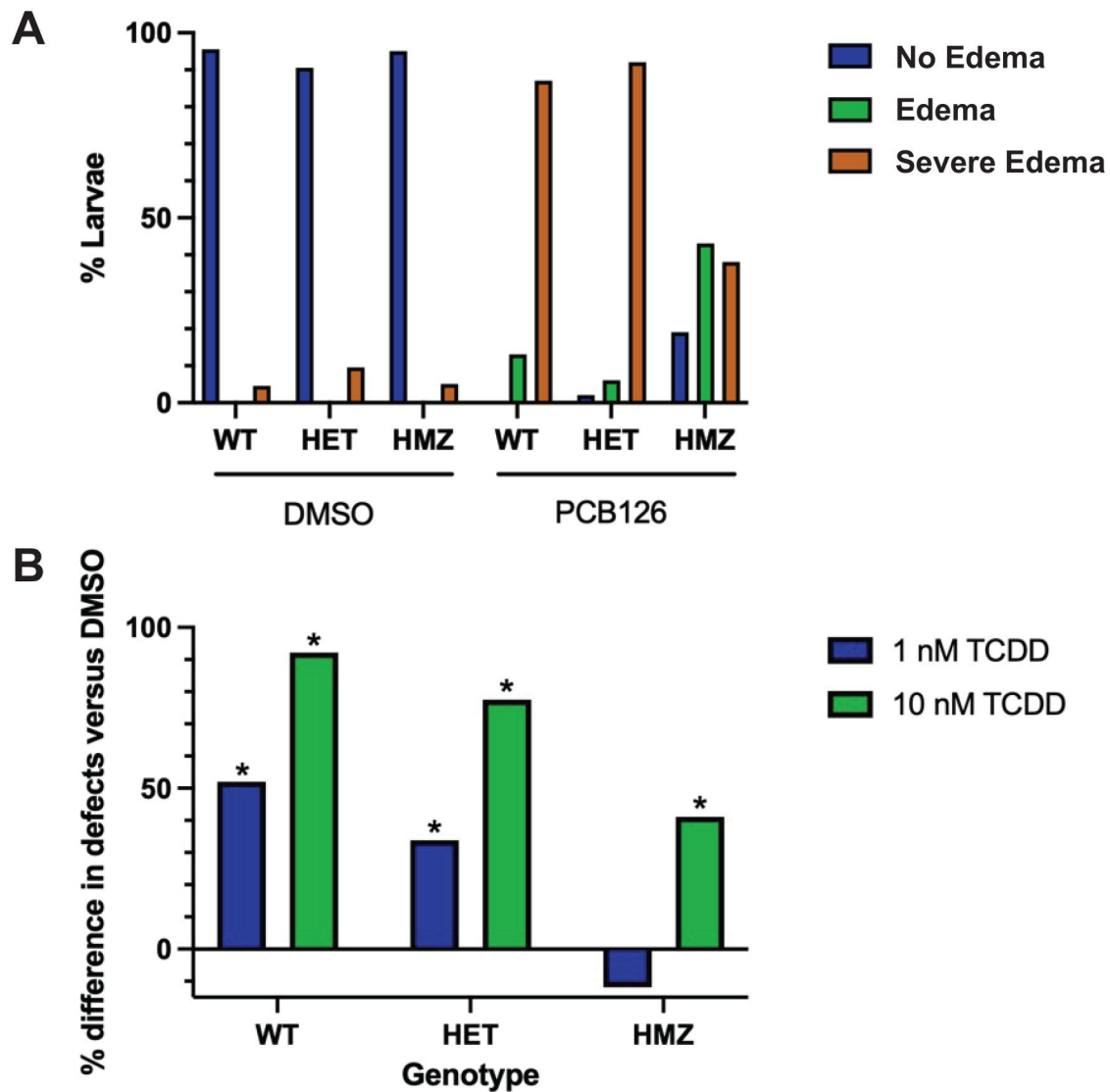


Fig. 4. Sensitivity of AIP mutant zebrafish larvae to the developmental toxicity of dioxin-like compounds. A) PCB126 (experiment 4). Heterozygous *aip^{wh86}* zebrafish were crossed and their embryos were exposed to DMSO (0.02%) or PCB126 (10 nM) from 6 to 24 hpf. At 72 hpf, the degree of edema was scored and the larvae were genotyped. Additional information about this experiment can be found in Fig. S8. B) TCDD (experiment 6). Heterozygous *aip^{wh86}* zebrafish were crossed and their embryos were exposed in 96-well plates to DMSO (0.1%) or TCDD (1 or 10 nM) from 6 to 120 hpf. At 24 and 120 hpf, larvae were assessed for 15 morphological endpoints and then genotyped. To account for some background defects in the AIP mutants, a background correction was applied and the percent difference in defects relative to DMSO controls was determined. *P < 0.05 vs. DMSO (Fisher's exact test). Additional information about this experiment can be found in Fig. S10. Abbreviations: TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCB126: 3,3',4,4',5-pentachlorobiphenyl; WT: Wild-type; HET: Heterozygous; HMZ: Homozygous.

included well-known AHR-regulated genes such as *cyp1a*, *cyp1b1*, *cyp1c1*, *cyp1c2*, *ahrra*, *ahrrb*, *foxq1a*, *foxq1b*, *fgf7*, *ugt1b1*, *ugt1b5*, *sult6b1*, *nfel2b*, *tiparp*, *slincR*, *cyb5a*, *ahr2* (all up-regulated), *sox9b* (down-regulated), and others (Supplementary Data File 1). Although similar sets of genes were induced in both WT and HMZ mutant larvae, the magnitude of induction (PCB126 fold change vs DMSO) was generally less in the HMZ mutants, as indicated by a slope of 0.6712 in the correlation analysis of up-regulated genes (Fig. 5C). Similarly, the fold down-regulation by PCB126 exposure in the HMZ mutants was muted (slope of 0.498) as compared with that in the WT larvae (Fig. 5D). In addition, there were a few genes for which more substantial differences in the degree of induction between the WT and HMZ mutant fish were observed. For example, the fold-induction of *cyp1a*, *cyp1c1*, *cyp1c2*, and *fgf7* was less in HMZ mutants than in the WT fish (Fig. 5C). Notably, both *ahrr* paralogs and the *slincR* long

noncoding RNA were more highly induced in the *aip^{wh239}* HMZ mutants than in the WT larvae (Fig. 5C). This may be related to their down-regulation in the DMSO-exposed HMZ larvae compared with DMSO-exposed WT larvae (Fig. S14A); thus, although the fold-induction of these genes by PCB is greater in HMZ as compared with WT larvae, on an absolute basis the induced transcript levels in PCB-exposed HMZ larvae were equal to or less than those of PCB-exposed WT larvae (Fig. S14B).

There were 116 genes that were differentially expressed after PCB126 treatment in WT and HET but not HMZ (Fig. 5B, left panel). Most of these (91%) had fold-change values less than 2. Although pathway analysis of this gene set was not informative, it is notable that 6 genes in hypoxia response pathways [*egln3*, *vegfd*, *egln2*, *p4ha1b*, *hif1an* (*hif-1*), and *hif1al* (*hif3*)] were induced by PCB126 in WT and HET but not HMZ mutant fish. At the opposite end of the spectrum were 398 genes that were differentially

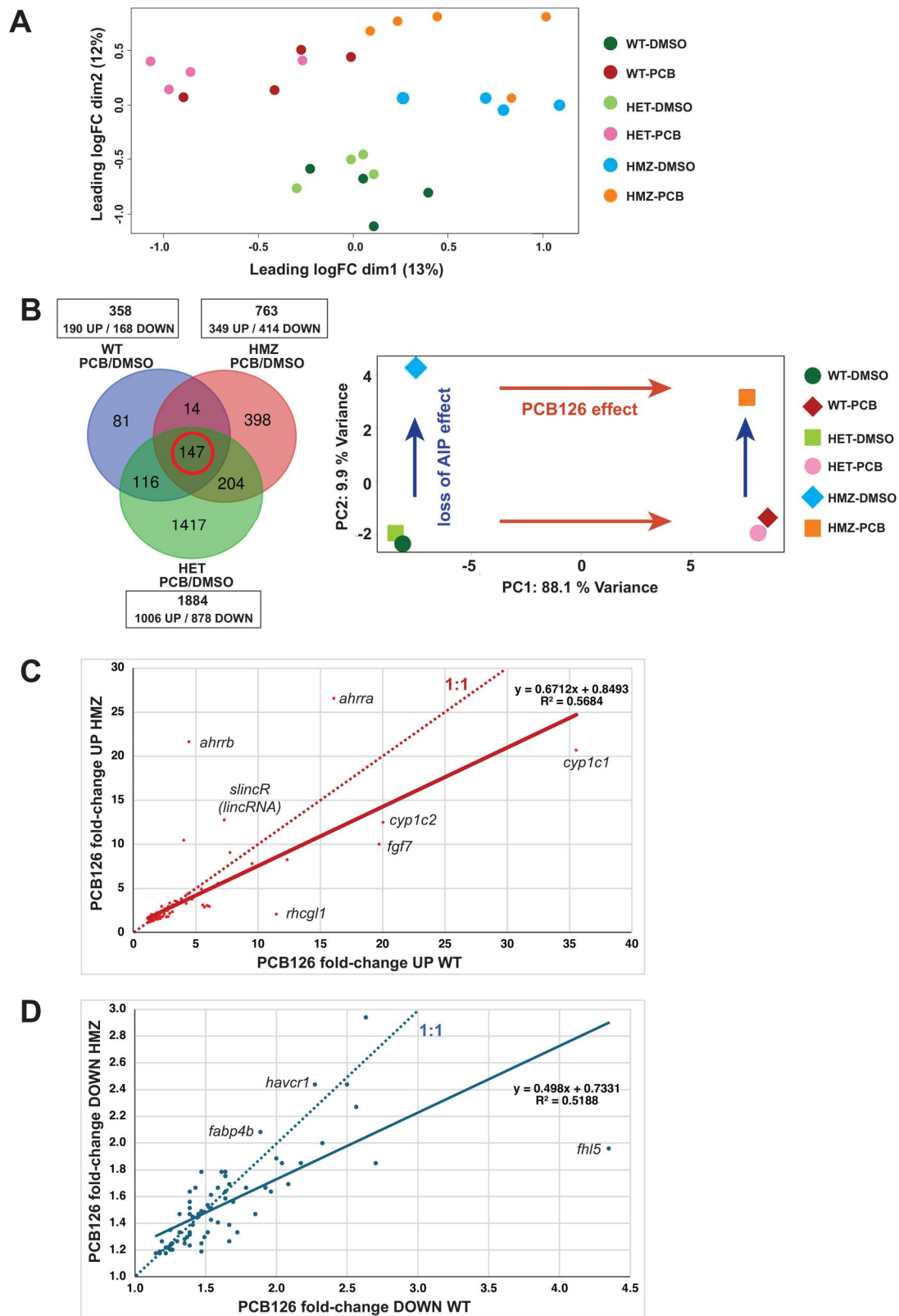


Fig. 5. Gene expression in *aip*^{wh239} mutants exposed to PCB126 (experiment 3a). Heterozygous *aip*^{wh239} zebrafish were crossed and their embryos were exposed in 96-well plates to DMSO (0.1%) or PCB126 (10 nM) from 6 to 24 hpf. At 72 hpf, larvae were genotyped and sampled for RNAseq. A) Multi-dimensional scaling (MDS) plot. B, left panel. Venn diagram showing the number of differentially expressed genes (DEGs) as a result of exposure to PCB126 in the 3 genotypes. B, right panel. Principle components analysis (PCA) plot of average normalized reads from each treatment group. C) Comparison of the fold-induction (up-regulation) of gene expression by PCB126 in HMZ *aip* mutant vs WT larvae. (CYP1A has been omitted from the correlation analysis). The linear regression (solid red line) is shown, along with the line of equal induction magnitude (1:1; dashed red line). D) Comparison of the fold down-regulation of gene expression by PCB126 in HMZ *aip* mutant vs WT larvae. The linear regression (solid blue line) is shown, along with the line of equal magnitude (1:1; dashed blue line). Additional information about this experiment can be found in Fig. S7. Abbreviations: PCB126: 3,3',4,4',5-pentachlorobiphenyl; WT: Wild-type; HET: Heterozygous; HMZ: Homozygous.

expressed in HMZ but not WT or HET fish after PCB126 treatment (Fig. 5B, left panel). Again, most of these (88%) had fold-change values less than 2-fold and pathway analysis was not informative. Interestingly, about a quarter of the genes in both of these gene sets (28/116 and 125/398) were also dysregulated in DMSO-treated HMZ mutants as compared with WT fish (Supplementary Data File 1).

GO term enrichment analysis of the genes differentially expressed after PCB126 exposure revealed a variety of Biological Process (BP), Molecular Function (MF), and KEGG pathway GO terms (Supplementary Data File 3). One interesting difference was the downregulation of visual perception process (9 genes) and photoreceptor activity (13 genes) in response to PCB exposure in WT and HET larvae, but not in HMZ larvae (Supplementary Data File 3).

Presence of maternal wild-type AIP mRNA in mutant embryos

The gene expression results stimulated us to take a closer look at the possible persistence of maternally supplied, wild-type *aip* transcripts in mutant embryos and larvae. The WT, HET, and HMZ embryos used for all exposure experiments were produced by heterozygous parents, so these embryos may have carried both mutant and wild-type *aip* mRNA supplied by the mother. We therefore examined our RNA-seq datasets for the presence of wild-type and mutant transcripts. We found that wild-type transcripts are present in HMZ larvae at 3 and 5 dpf, although at levels well below those found in WT or HET larvae (~3% of WT levels; Fig. 6A and B). Similar results were also found in 5 dpf larvae of both mutant lines after crossing onto an AB genetic background (Perone et al. 2024). In addition, we found that in both HET and HMZ larvae, mutant *aip* transcripts were present at levels much lower than expected (Fig. 6A and B), again suggesting that the mutant transcripts undergo nonsense-mediated decay. The persistent presence of wild-type maternal transcripts even at 3 and 5 dpf—when most other maternal transcripts have been degraded (Giraldez 2010)—suggests that the homozygous mutant

larvae may not be completely lacking functional AIP protein at these early larval stages.

Discussion

AIP is a chaperone protein with important but poorly understood roles in regulating AHR-dependent signaling. Most of what we know about the function of AIP in AHR signaling has been obtained from *in vitro* and cell culture experiments in mammalian systems. Here, inspired by studies in fish populations with evolved resistance to dioxin-like compounds that revealed AIP as a major resistance gene (Nacci et al. 2016; Reid et al. 2016; Osterberg et al. 2018; Miller et al. 2024), we sought to explore the role of AIP in fish development and in controlling the sensitivity to the developmental toxicity of dioxin-like compounds *in vivo*. Our initial results demonstrate that loss of AIP leads to complete larval lethality in 2 species of fish and that, before they begin to decline, early zebrafish larvae have reduced sensitivity to 2 potent dioxin-like compounds. These results set the stage for future studies to elucidate the mechanisms by which AIP acts to control the sensitivity to dioxin-like compounds *in vivo*.

AIP loss-of-function is lethal in killifish and zebrafish

Our initial experiments with fish expressing putative loss-of-function alleles of AIP showed that both killifish (several clutches from one line) and zebrafish (multiple clutches from 2 independent lines) exhibited 100% lethality during mid- to late-larval stages (by 30 dpf in killifish and 12 dpf in zebrafish). This finding was not a complete surprise, because AIP loss-of-function is embryo-lethal in mice (Lin et al. 2007) and flies (Aflorei et al. 2018) and causes a severe phenotype in worms (Chen et al. 2017). Mice lacking AIP die between embryonic days 10 and 18 and exhibit multiple cardiovascular defects (Lin et al. 2007). Similarly, loss-of-function mutations in *Drosophila* cause death at an early larval stage (Aflorei et al. 2018). Thus, AIP-null killifish and zebrafish resemble other models, but delayed lethality allows experiments to be done using early life stages—through at least 5 dpf in

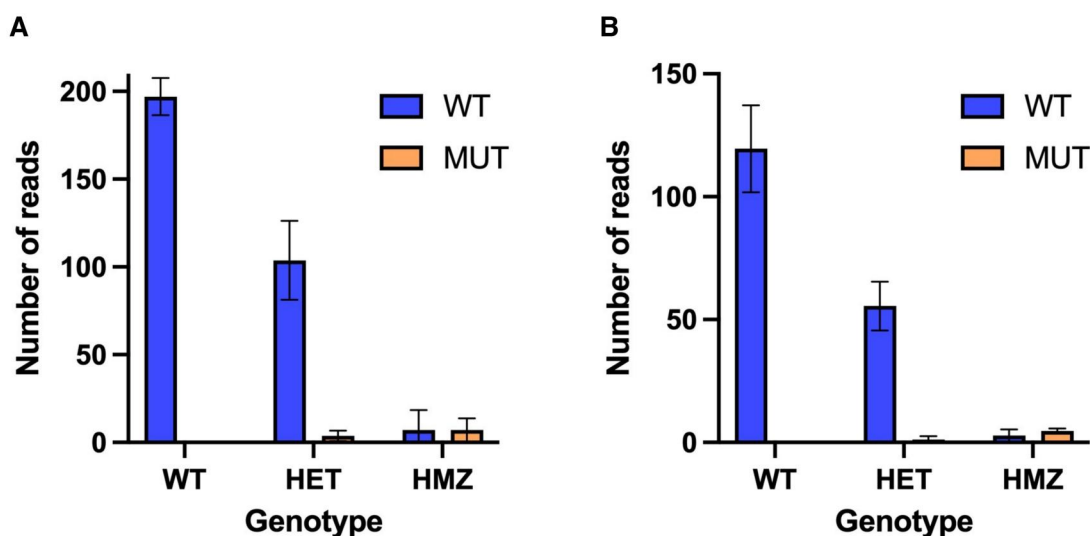


Fig. 6. Wild-type (WT) and mutant (MUT) AIP transcripts in WT, HET, and HMZ mutant *aip^{wh239}* larvae at 3 dpf (A, experiment 3a) and *aip^{wh86}* larvae at 5 dpf (B, experiment 3b). Transcript numbers were assessed using RNA-seq data. Data represent the number of reads that overlap the deletion site in the *aip* gene, averaged over 4 replicate samples. The numbers include both maternal and zygotic transcripts. Results show that HMZ mutant embryos have some WT maternal transcripts at 3 and 5 dpf. Mutant transcripts appear to be rapidly degraded, suggesting nonsense-mediated decay. Abbreviations used: WT: Wild-type; HET: Heterozygous; HMZ: Homozygous.

zebrafish—when the larvae appear completely normal. Thus, we were able to use zebrafish embryos and early larvae to assess whether loss of AIP might affect the sensitivity to dioxin-like compounds.

Zebrafish AIP mutants have reduced sensitivity to the developmental toxicity of TCDD and PCB126

Based on their results from mice with hepatocyte-specific loss of AIP, Nukaya et al. (2010) hypothesized that variation in AIP status may contribute to differential sensitivity to the toxicity of AHR agonists. In a series of experiments using 2 independent zebrafish *aip*-mutant lines (*aip*^{wh86} and *aip*^{wh239}), we sought to determine whether loss of AIP in embryos and early larvae would affect the sensitivity to 2 highly toxic AHR ligands. We found that the homozygous mutant offspring of heterozygous fish exhibited reduced sensitivity to the developmental cardiovascular toxicity of AHR ligands PCB126 and TCDD. Notably, the AIP mutants were not completely insensitive to these compounds, which differs from the dramatic protection provided by loss of AHR2 (Goodale et al. 2012; Garcia et al. 2018; Souder and Gorelick 2019) or ARNT1 (Edwards et al. 2023) in zebrafish.

The reduced sensitivity of AIP-mutant zebrafish also was more subtle than the substantial degree of PCB resistance that has evolved in some populations of Atlantic killifish (Nacci et al. 2010). Although we cannot quantify the reduced sensitivity of the HMZ AIP-mutant zebrafish because we did not perform dose-response experiments with TCDD or PCB126, the differences among WT, HET, and HMZ mutants clearly did not approach the >100-fold reduction in sensitivity observed in PCB-resistant killifish populations (Nacci et al. 2010). This could reflect a species difference between zebrafish and killifish in their response to loss of AIP function. However, it is perhaps more likely that the PCB resistance in killifish is not a simple AIP loss-of-function defect, but rather something more complex. Our ongoing experiments exploring the functional consequences of single-nucleotide variants of AIP (including some found in PCB-resistant killifish) may shed light on such a mechanism.

Zebrafish AIP mutants exhibit altered gene expression and inducibility

Because zebrafish larvae with complete loss of AIP survive and appear normal through 5 dpf, we performed an unbiased assessment of gene expression using RNAseq in *aip*^{wh239} larvae at 3 dpf and *aip*^{wh86} larvae at 5 dpf to identify changes caused by the loss of AIP, before the larvae begin to exhibit pathological changes and reduced survival. The large decrease in *aip* mRNA in the homozygous mutant larvae suggests that, because of the premature termination codons in the 2 mutant alleles, the mutant mRNAs undergo degradation by nonsense-mediated decay (NMD). RNA degradation can lead to compensation by transcriptional adaptation (Rossi et al. 2015; El-Brolosy et al. 2019; Elizalde and Gorelick 2023), but the larval lethal phenotype of our mutants indicated that transcriptional adaptation did not occur or was incomplete. Consistent with that notion, we found no potential compensatory increases in expression of *aip*-related genes, including *aip*-like 1 (*aip1l*) (Iribarne et al. 2017) or other immunophilin-related genes, with the possible exception of a small increase in expression of *fkbp8* (1.23-fold; Supplementary Data File 1).

An unexpected result was the strong down-regulation of both paralogs of the aryl hydrocarbon receptor repressor (*ahrra* and *ahrrb*) and the lncRNA *slincR* in the homozygous mutant larvae.

These genes are regulated by the AHR (Evans et al. 2005; Garcia et al. 2017), but other AHR-regulated genes did not have reduced expression in the mutants. One possibility is that the constitutive expression of *ahrra*, *ahrrb*, and *slincR* is especially sensitive to changes in AHR activity that may result from the loss of AIP. Because AHR proteins are negative regulators of AHR function (Mimura et al. 1999; Evans et al. 2008), the down-regulation of *ahrra* and *ahrrb* also could help to compensate for any reduction in AHR-dependent signaling in unexposed AIP mutant larvae.

Pathway enrichment analysis of the genes differentially expressed between homozygous *aip* mutant larvae and wild-type larvae revealed a variety of pathways that defy a simple interpretation. The complexity of these gene expression data may reflect the variety of proteins that are known to interact with AIP (Trivellin and Korbonits 2011; Kazzaz et al. 2024). Among the pathways enriched in our data set was G-protein coupled receptor (GPCR) activity, which is reminiscent of the cAMP and GPCR-related pathways dysregulated in Aip-knockout mouse embryo fibroblasts (Tuominen et al. 2015) and the similar pathways disrupted in tumors from humans with FIPA (Tuominen et al. 2015; Bizzi et al. 2019).

In the response of mutant embryos to PCB126, there were many apparent differences in DEGs among the 3 genotypes. Despite these differences, an examination of known AHR2-regulated genes (e.g. those identified earlier; Garcia et al. 2018; Shankar et al. 2019, 2021) did not reveal dramatic differences in the set of genes altered by PCB126 between homozygous mutants and wild-type or heterozygous mutants, but there was an overall decrease in the average magnitude of induction or down-regulation (Fig. 5C and D). The degree of induction of *cyp1a*, *cyp1c1*, *cyp1c2*, and *fgf7*—all known AHR target genes—was lower in the HMZ mutants as compared with WT larvae. There were some exceptions, however. *ahrra*, *ahrrb*, and *slincR* were more strongly induced in the HMZ mutants as compared with WT, but this appeared to be secondary to their constitutive down-regulation in DMSO-exposed homozygous mutants. Thus, we found differential induction of some but not all AHR-regulated genes. Our results differ in detail but are qualitatively similar to those of Nukaya et al. (2010), who found that although the induction of *Cyp1a1* and *Cyp1a2* by TCDD was not affected in liver of mice with hepatocyte-specific loss of Aip, the induction of 2 other AHR-regulated genes (*Ahrr* and *Cyp1b1*) was reduced. Similarly, the induction of CYP1B1 by AHR ligand kynurenine in rat GH3 cells was reduced when AIP expression was partially silenced by siRNA (Lecoq et al. 2016). In our studies, there may have been cell-specific differences in response that were not captured by our whole-embryo RNA-seq but might be revealed by single-cell RNA-sequencing.

A complicating factor in the present study is the presence of some maternal, wild-type AIP in the homozygous *aip* mutant offspring, an unavoidable consequence of the necessity of using heterozygous intercrosses to generate homozygous mutant embryos. AIP mRNA is a maternal-zygotic transcript (White et al. 2017; Bhat et al. 2023; Sur et al. 2023). Thus, there was a low level of maternal wild-type transcripts that persisted in the mutant embryos and larvae. The amounts were small (~3% of WT); thus, our experiments could be considered as a very strong (97%) knock-down rather than an absolute knock-out. Whether the small amounts of wild-type AIP present in mutant embryos influenced our results is unclear; sensitivity to AHR agonists has not previously been examined in any *in vivo* system with partially reduced AIP expression. Because maternal transcripts typically are degraded as development progresses, our initial exposure

design (exposure to PCB126 from 6 to 24 hpf; sampling at 3 dpf) may not have been optimal for detecting an effect of the loss of zygotic *aip* expression. Additional studies using later exposures may be needed to better understand the effect of *aip* loss of function on AHR-mediated effects. Additional insight into the effect of AIP loss-of-function and its effects on AHR signaling would be facilitated by the generation of maternal-zygotic AIP mutants (Shi 2022) in which wild-type AIP mRNA is completely absent in embryos.

Future directions and implications for PCB-resistant killifish

The results reported here highlight the need for additional research to understand the roles of AIP in embryonic and larval development and in controlling AHR signaling during these early life stages. We are currently performing a detailed histopathological investigation and analysis of gene expression in the AIP mutant zebrafish to identify the earliest changes and the progression of events leading to lethality in larvae between 5 and 12 dpf (Perone et al. 2024; Stinson et al. 2025). Because variation in AIP is strongly associated with the development of pituitary adenomas in humans and other mammals (Loughrey and Korbonits 2019; Kazzaz et al. 2024), we also are investigating pituitary phenotypes in these fish (Stinson et al. 2025).

To further understand the mechanism by which altered AIP expression or function leads to reduced sensitivity to DLCs, it will be necessary to obtain a more detailed view of AHR function in AIP mutant fish. AHR levels and function are influenced by the presence of AIP (LaPres et al. 2000). Of particular relevance for our study, loss of AIP in mouse hepatocytes causes a reduction in AHR protein levels, which may contribute to the protection from TCDD hepatotoxicity and reduced sensitivity to induction of some AHR-regulated genes (Nukaya et al. 2010). We currently lack the tools to assess the levels of the 3 zebrafish AHRs (e.g. specific antibodies or fish with epitope-tagged AHRs), but this is a goal of future research.

Another goal is to examine whether the effect of AIP loss-of-function on the sensitivity to AHR agonists is ligand-specific. Evolved resistance of killifish to AHR ligands has been demonstrated in fish populations in which the primary AHR-active contaminants include PCBs, TCDD, or PAHs (Nacci et al. 2010; Whitehead et al. 2017). If AIP deficiency leads to reduced levels of AHR protein, as shown in some studies (LaPres et al. 2000; Pollenz and Dougherty 2005; Nukaya et al. 2010), this could result in ligand-specific effects because of *receptor reserve* ("spare receptors"), a ligand-specific property of receptor-mediated responses (Kenakin 1999). Thus, highly efficacious ligands such as TCDD, which can cause a maximal response by binding to a small fraction of the total AHR pool, may be less affected by reductions in AHR levels than are low-efficacy AHR ligands, which must occupy more of the AHR protein pool to achieve the same degree of response (Poland 1991). There is evidence for spare AHRs in some systems (Karenlampi et al. 1988; Poland 1991; Hestermann et al. 2000) and one previous study has suggested the possibility of ligand-specific AIP function (Petrulis et al. 2003). The concept of spare receptors could also explain gene-specific effects of loss of AIP that have been reported previously (Nukaya et al. 2010; Lecoq et al. 2016) and in the present manuscript.

Our research on AIP in killifish and zebrafish was motivated by the finding, from genomic studies of multiple sensitive and resistant killifish populations, that this gene was the strongest candidate for a major PCB and PAH resistance gene (Nacci et al.

2016; Reid et al. 2016; Osterberg et al. 2018; Miller et al. 2024). Evolved PCB resistance in another species of fish (Atlantic tomcod) was also hypothesized to involve AIP (Wirgin et al. 2011). Because the function of AIP in fish has not been examined previously, in the current studies, we sought to use a loss-of-function approach to provide some initial information about how AIP might be involved in fish development and the developmental toxicity of dioxin-like compounds. In light of the incomplete, species-specific, and sometimes contradictory results from research on the role of AIP in regulating AHR signaling in mammalian systems (Hollingshead et al. 2004; Trivellin and Korbonits 2011; Kazzaz et al. 2024), we can imagine multiple potential mechanisms by which AIP could modulate AHR signaling in fish with evolved PCB resistance. Such mechanisms might include, for example, altered levels of AIP protein (either up or down, from changes in mRNA expression or altered protein stability) or sequence variation that affects AIP–AHR interactions. These and other possible mechanisms are being explored in ongoing research from our laboratories.

Although AHR status does not affect the incidence of pituitary adenomas in FIPA patients (Shen et al. 2024), epidemiological studies have suggested an association between exposure to dioxin-like compounds and the severity of this condition (Pesatori et al. 2008; Cannavò et al. 2010; Cannavo et al. 2016, 2017). Thus, understanding the role of AIP in controlling sensitivity to AHR agonists in fish models may shed light on the factors that determine differential susceptibility of humans to dioxin-like compounds and on the role of AIP in human disease.

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Supplementary material

Supplementary material is available at *Toxicological Sciences* online.

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Conflicts of interest

None declared.

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