A Review of the Functional Roles of the Zebrafish Aryl Hydrocarbon Receptors

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RUNNING HEAD: Functional roles of zebrafish AHRs

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Abstract

Over the last two decades, the zebrafish (*Danio rerio*) has emerged as a stellar model for unraveling molecular signaling events mediated by the Aryl Hydrocarbon Receptor (AHR), an important ligand-activated receptor found in all eumetazoan animals. Zebrafish have three AHRs – AHR1a, AHR1b, and AHR2, and studies have demonstrated the diversity of both the endogenous and toxicological functions of the zebrafish AHRs. In this contemporary review, we first highlight the evolution of the zebrafish *ahr* genes, and the characteristics of the receptors including developmental and adult expression, their endogenous and inducible roles, and the predicted ligands from homology modeling studies. We then review the toxicity of a broad spectrum of AHR ligands across multiple life stages (early stage, and adult), discuss their transcriptomic and epigenetic mechanisms of action, and report on any known interactions between the AHRs and other signaling pathways. Through this article, we summarize the promising research that furthers our understanding of the complex AHR pathway through the extensive use of zebrafish as a model, coupled with a large array of molecular techniques. Since much of the research has focused on the functions of AHR2 during development and the mechanism of TCDD toxicity, we illustrate the need to address the considerable knowledge gap in our understanding of both the mechanistic roles of AHR1a and AHR1b, and the diverse modes of toxicity of the various AHR ligands.

Introduction

Aryl Hydrocarbon Receptor (AHR)

The Aryl Hydrocarbon Receptors (AHRs) are ligand-dependent transcription factors that mediate a wide range of biological and toxicological effects in animals (Abel and Haarmann-Stemmann 2010; Barouki et al. 2007; Esser et al. 2009; Hankinson 1995; Nguyen et al. 2018; Safe et al. 2013). Although several endogenous ligands have been identified since the discovery of the AHR in 1976 (Poland et al. 1976), the focus has been on characterizing the toxicity of numerous environmental chemicals including the halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs), many of which cause toxicity via the AHR signaling pathway (Denison and Nagy 2003; Nguyen and Bradfield 2008). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an HAH, is the most potent and thoroughly investigated of the known AHR ligands and it elicits many species- and tissue-specific toxicological effects (Couture et al. 1990; Denison and Nagy 2003; Mandal 2005). By virtue of its limited metabolism (Vinopal and Casida 1973), TCDD is typically utilized as the prototypical molecular probe to study the signaling events downstream of AHR activation (Poland and Kende 1976), and forms the basis of investigation of many of the AHR-dependent mechanisms reviewed in this paper.

Canonical signaling for the AHRs, which are part of the basic Helix-Loop-Helix Per-Arnt-Sim (bHLH/PAS) family of proteins, involves the conversion into an active form that can dimerize with another bHLH/PAS protein, the AHR nuclear translocator (ARNT) (Hoffman et al. 1991; Kewley et al. 2004). In their latent and unbound state, the AHRs are found in the cytoplasm and are stably associated with two molecules of the 90kDa molecular chaperone heat shock protein 90 (Hsp90), p23, and AHR-interacting protein (AIP/XAP2/Ara9) (Carver and Bradfield 1997; Kazlauskas et al. 1999; Ma and Whitlock 1997; Perdew 1988). Upon ligand binding, the AHR is activated and the AHR/Hsp90 complex translocates to the nucleus where Hsp90 is exchanged for the partner protein, ARNT (Hoffman et al. 1991; Reyes et al. 1992; Swanson 2002). The AHR/ARNT heterodimer recognizes and regulates transcription of downstream genes such as the cytochrome P450 family of genes (CYPs) and the aryl hydrocarbon receptor repressor (AHRR) via aryl hydrocarbon response elements (AHREs; also known as DREs or XREs) in their promoter regions (Mimura et al. 1999; Watson and Hankinson 1992). The CYP1s are amongst the most well-studied AHR gene targets and are involved in both the metabolic activation and detoxification of the various AHR ligands (Nebert et al. 2004). In addition to the CYPs and AHRR, the AHR can also directly or indirectly regulate expression of a large battery of genes, the identities and functions of which are still being discovered (Abel and Haarmann-Stemmann 2010; Beischlag et al. 2008). While our review predominantly focuses on what we know about canonical AHR signaling in zebrafish, we acknowledge that the AHRs have several non-canonical functions as well (Jackson et al. 2015). Some examples include AHR as an E3 ubiquitin ligase in cytosol (Ohtake et al. 2007), its interaction with p300, pRb, and E2F (Marlowe et al. 2004; Puga et al. 2000), and as a partner for KLF6 (Wright et al. 2017) and RelB (Vogel et al. 2007).

Evolution of the AHR in different species

The AHR is an ancient protein found in all eumetazoan animals, indicating that it originated more than 600 million years ago (Hahn et al. 2017). A fundamental difference between AHRs in invertebrates and vertebrates is that most of the vertebrate AHR proteins exhibit high-affinity binding of halogenated and non-halogenated aromatic hydrocarbons,

whereas all invertebrate AHRs examined to date lack that ability and appear to have roles primarily in developmental processes (Butler et al. 2001; Hahn 2002). During animal evolution, AHR genes have been duplicated, including in early vertebrate evolution (a tandem duplication and an expansion associated with two early vertebrate, whole-genome duplication events) and in specific vertebrate lineages, especially fish (Hahn et al. 2017). These duplications, coupled with some lineage-specific gene losses, result in the presence of between one and five AHR genes per species.

An important difference between AHR signaling in mammals and fishes is that most mammals—including most of those used as models in toxicology research—possess a single AHR gene, whereas most fishes have multiple AHRs. Fish typically possess four AHR genes—two pairs of tandem AHR1-AHR2—although additional gene duplications and losses have led to some variation, including in zebrafish (Hahn et al. 2006). It is not clear why fish have retained multiple AHR genes, including the AHR2 paralogs that have been lost from most mammals, as well as the additional duplicates of AHR1 and AHR2 resulting from a fish-specific whole genome duplication event that occurred ~350 million years ago (Amores et al. 1998; Glasauer and Neuhauss 2014). The maintenance of multiple AHRs in modern fish is notable considering that more than 80% of the gene duplicates formed during the fish-specific whole genome duplication were subsequently lost. The prevailing hypothesis for retention of gene duplicates is that they have become more specialized by partitioning the multiple functions of their common ancestor (subfunctionalization; (Amores et al. 1998; Force et al. 1999; Lynch and Force 2000)). However, they may also evolve new functions (neofunctionalization). Therefore, fish models can serve as an ideal platform to study the role of AHR in both physiology and toxicology.

Zebrafish as a toxicological model organism

Zebrafish is a well-established vertebrate model for studying embryonic development and developmental toxicology, and has been used extensively to unravel AHR pathway complexity (Garcia et al. 2016; Sipes et al. 2011; Teraoka et al. 2003a). Zebrafish embryos are transparent, and they develop externally and rapidly, with primary organogenesis complete around 48 hours post fertilization (hpf), and the heart, liver, and brain well developed by 120 hpf (Kimmel et al. 1995). To this end, most early stage toxicity studies are conducted with morphological, behavioral, and molecular evaluations occurring within the first 120 hours of development (Nishimura et al. 2016). Zebrafish also possess high genetic relatedness to humans; 76% of human genes have a zebrafish ortholog, and 82% of human genes that cause disease are present in zebrafish, increasing the translational value of the zebrafish model (Howe et al. 2013). Furthermore, zebrafish share similar morphology, physiology, and xenobiotic metabolic pathways with mammals (Diekmann and Hill 2013), possessing direct orthologs of the human CYP1 enzymes like *cyp1a* and *cyp1b1*, in addition to *cyp1c1* and *cyp1c2* that lack human orthologs (Goldstone et al. 2010).

The zebrafish AHRs

Zebrafish possess three AHR genes (*ahr1a*, *ahr1b*, and *ahr2*) that were named at the time of discovery according to their hypothesized evolutionary relationships to the AHR genes in other fish. Thus, the *ahr1* genes were thought to be most closely related to the *ahr1* genes of other fish and to the mammalian AHR (Andreasen et al. 2002a; Karchner et al. 2005); the designation “a” and “b” reflected the initial conclusion that *ahr1a* and *ahr1b* were paralogs formed during the fish-specific whole genome duplication, and was consistent with the standard zebrafish nomenclature for such paralogs (Karchner et al. 2005). More recent analysis taking into account new AHR sequences from a variety of species, combined

with analysis of shared synteny between zebrafish and human chromosomes, suggested that zebrafish *ahr1a* is orthologous[1] to the mammalian AHR, rather than a paralog of *ahr1b* resulting from the fish-specific whole genome duplication (Hahn et al. 2017). Zebrafish *ahr2* is orthologous to *ahr2b* genes of other fishes (Karchner et al. 2005; Tanguay et al. 1999).

Further insight into the relationships of zebrafish *ahr* genes to AHR genes in other vertebrates can be obtained by additional analyses of shared synteny, which can complement gene phylogenies to help reveal evolutionary relationships (Postlethwait 2007). A comparison of the shared synteny among AHR-containing chromosomes in zebrafish, human, mouse, and chicken using *Genomicus* (Muffato et al. 2010; Nguyen et al. 2018) is illustrative (**Fig. 1**). The zebrafish ***ahr1a*** gene is located on chromosome 16 (Andreasen et al. 2002a; Barbazuk et al. 2000; Hahn et al. 2017; Le Beau et al. 1994). Zebrafish chromosome 16 exhibits extensive shared synteny with human chromosome 7, mouse chromosome 12, and chicken chromosome 2 – the locations of the canonical *AHR* genes in each of these species (**Fig. 1A**). This supports the earlier suggestion that *ahr1a* is the ortholog of human *AHR* (Hahn et al. 2017). A reciprocal analysis of shared synteny using the AHR on human chromosome 7 as the reference gene (**Fig. 1C**) confirms the relationship between AHR-containing human chromosome 7, mouse chromosome 12, chicken chromosome 2, and zebrafish chromosome 16, and also reveals the loss of the predicted paralog of *ahr1a* that would have been expected from the fish-specific whole genome duplication (see chromosome 19). Zebrafish ***ahr2*** and ***ahr1b*** are located on chromosome 22 (Karchner et al. 2005; Tanguay et al. 1999; Wang et al. 1998a). This chromosome exhibits extensive shared synteny with chicken chromosome 7, the location of two additional chicken *AHR* genes, designated *AHR2* and *AHR1B* (Lee et al. 2013; Yasui et al. 2007) (**Fig. 1B**). Both zebrafish chromosome 22 and chicken chromosome 7 exhibit shared synteny with human chromosome 2 and mouse chromosome 1, which lack the AHR2-AHR1 pair, confirming the loss of these genes in the mammalian lineages leading to human and mouse (**Fig. 1B**).

See **Table 1** for more details on the three zebrafish *ahr* genes and their respective translation products.

The presence of three AHRs in zebrafish is intriguing. Despite the plethora of research that has been conducted, we are only beginning to understand their functional roles in development and in adult tissues, and we do not yet have a clear picture of the extent to which each paralog is involved in endogenous vs. toxicological roles. In this paper, we survey current AHR zebrafish toxicology research, and identify specific knowledge gaps and opportunities for future research. We begin by reviewing the receptor characteristics (Section 1), followed by early stage toxicity (Section 2) and interaction of AHRs with other signaling pathways (Section 3), and conclude with adult toxicity and potential AHR-associated epigenetic effects (Section 4).

Section 1. Receptor characteristics

General characteristics of the zebrafish AHRs, beginning with their baseline and chemically induced expression (Section 1.1) and endogenous roles in both developing and adult zebrafish (Section 1.2) are summarized in **Table 2.** We later

[1] Orthology refers only to evolutionary relationships, and does not necessarily imply identical functions. Two genes are orthologous if they have descended from the same gene in the most recent common ancestor of the species in which they are found (Fitch WM. 1970. Distinguishing homologous from analogous proteins. Syst Zool. 19(2):99-113).

elucidate the inducible roles of the three AHRs along with their known binding partners (Section 1.3), and conclude this section by reviewing homology modeling of the three receptors (Section 1.4).

Section 1.1. Expression of AHRs in zebrafish

Transcriptomic, *in situ* hybridization, and immunohistochemical techniques have been used to understand the developmental, tissue-specific, and chemically induced expression of the AHRs. ***Ahr2*** mRNA is expressed during normal zebrafish development in several regions including the head and the trunk (Andreasen et al. 2002b; Sugden et al. 2017); expression is detected as early as 5 hpf and does not change through 120 hpf (Andreasen et al. 2002b; Tanguay et al. 1999). Upon zebrafish embryonic exposure to TCDD, *ahr2* expression increases and is detected in several locations across zebrafish development from 24 to 120 hpf (Andreasen et al. 2002b; Garcia et al. 2018a; Karchner et al. 2005; Tanguay et al. 1999). Other chemicals such as beta-naphthoflavone (BNF), a synthetic flavonoid commonly used as a surrogate model PAH (Poland and Kende 1976; Sugden et al. 2017), cardiosulfa, a sulfonamide drug (Ko et al. 2009), and the polychlorinated biphenyl, PCB-126 (Kubota et al. 2014) induce *ahr2* expression in developing zebrafish. On the other hand, exposure to benzo[a]pyrene (BaP) and some other oxy-PAHs significantly reduce *ahr2* expression suggesting the complexity of AHR regulation by different PAHs (Cunha et al. 2020). In adults, *ahr2* mRNA is detected in the brain, heart, muscle, swim bladder, liver, gill, skin, eye, kidney, fin both in unexposed and TCDD-exposed animals (Andreasen et al. 2002a). An antibody to AHR2 has been used to investigate AHR2 function in zebrafish cell culture (Wentworth et al. 2004) and in a heterologous cell system (Evans et al. 2008), but has not been used successfully *in vivo*.

Ahr1a mRNA is expressed during normal zebrafish development; expression is detected from 24 hpf, increases by 72 hpf, and stays relatively constant through 120 hpf (Andreasen et al. 2002a; Karchner et al. 2005). *Ahr1a* has more restricted expression patterns compared to *ahr2* and is detected weakly in the liver at 52 hpf (Sugden et al. 2017), in a regenerating fin 3 days post amputation (Mathew et al. 2006), and in the adult brain (Webb et al. 2009), liver, heart, swim bladder, and kidney (Andreasen et al. 2002a). Upon embryonic exposure to TCDD, *ahr1a* expression significantly increases at 72 and 120 hpf (Andreasen et al. 2002a; Karchner et al. 2005), while BNF slightly induces expression of *ahr1a* in 48 hpf zebrafish (Sugden et al. 2017). There are no published antibodies experimentally shown to detect AHR1a protein expression in zebrafish.

Like *ahr2* and *ahr1a*, ***ahr1b*** mRNA is expressed during normal development; expression is detected from 24 hpf and is increased at 48 and 72 hpf (Karchner et al. 2005). *Ahr1b* mRNA is highly expressed in the developing eye (Karchner et al. 2017; Sugden et al. 2017). Unlike *ahr2* and *ahr1a*, *ahr1b* expression does not change after exposure to TCDD or BNF (Karchner et al. 2005; Sugden et al. 2017; Ulin et al. 2019). However, BaP exposure increased expression of *ahr1b* in 72 hpf zebrafish (Huang et al. 2012), while low level pyrene exposure did not induce expression of any of the three *ahr* genes (Zhang et al. 2012). A rabbit polyclonal antibody targeting the AHR1b protein (Ulin et al. 2019) detected protein expression by western blot in 24-hpf zebrafish. Using this same antibody, Karchner et al. performed immunohistochemical staining of 96-hpf larvae and showed that, like its mRNA, the AHR1b protein is also expressed in the developing eye, including the retinal inner and outer plexiform layers (Karchner et al. 2017). Overall, these studies show that the spatiotemporal expression of the AHRs is dependent on the specific AHR receptor.

Section 1.2. Endogenous AHR roles in zebrafish

To effectively study the endogenous and toxicological roles of the zebrafish AHRs, reverse genetics tools including transient knockdown of translation using morpholino oligonucleotides (Heasman 2002; Timme-Laragy et al. 2012b), and stable and heritable genetic knockout lines have been generated. While both of these tools can greatly enable the understanding of the functions of the zebrafish AHRs, we acknowledge their limitations here. In addition to their specific targets, morpholinos can nonspecifically affect expression of other targets so without rigorous controls it can be challenging to conclude whether an observed morpholino phenotype is due to its specific or off-target effects (Kok et al., 2015; Stainier et al., 2017). On the other hand, heritable mutations—especially those generating premature termination codons and nonsense-mediated decay of the resulting mRNA—can be subject to genetic compensation, where in response to the mutation, cells upregulate related genes that rescue the mutant phenotype (El-Brolosy et al. 2019; Ma et al. 2019; Rossi et al. 2015). Additionally, a mutation presumed to be loss-of-function might be rescued by altered mRNA processing, such as exon-skipping or alternative splicing, that produces a functional or partly functional protein (Anderson et al. 2017). This means that a heritable mutation that produces no phenotype could be a false negative result. It is important to take these drawbacks of both morpholinos and knockout lines into consideration while interpreting the results of the studies presented below.

Both splice-blocking and translation-blocking morpholinos have been designed for *ahr1a* (Incardona et al. 2005; Seifinejad et al. 2019), *ahr1b* (Goodale et al. 2012; Ulin et al. 2019), and *ahr2* (Bugel et al. 2013; Prasch et al. 2003; Teraoka et al. 2003b). Morpholino knockdown of *ahr2* does not produce visible phenotypes (Dong et al. 2004; Prasch et al. 2003) likely due to the incomplete and transient receptor knockdown, prompting several groups to generate stable AHR mutant lines. The first functional AHR2 knockout line was established using Targeted Induced Local Lesions in Genomes (TILLING) (Goodale et al. 2012). Later, Transcription Activator-Like Effector Nucleotide (TALEN)-Mediated Mutagenesis was used to generate AHR1a, AHR1b, and AHR2 mutants (Sugden et al. 2017). More recently, two CRISPR-Cas9 AHR2 homozygous mutant lines (Garcia et al. 2018a; Souder and Gorelick 2019) and CRISPR-Cas9 AHR1a and AHR1b homozygous mutant lines (Karchner et al. 2017; Souder and Gorelick 2019) have been established. These mutant lines are being intensively used to study both the endogenous and toxicological roles of the AHRs; the endogenous roles are reviewed here while the toxicological roles are examined in later sections.

Some studies have suggested that all three AHRs are dispensable specifically for embryonic vascular patterning, and normal larval fin development and jaw growth (Souder and Gorelick 2019; Sugden et al. 2017). However, AHR2-null background zebrafish have fin and craniofacial malformation as adults (Garcia et al. 2018a; Goodale et al. 2012; Souder and Gorelick 2019), and both abnormal larval and adult behavior (Garcia et al. 2018a; Knecht et al. 2017b; Wu et al. 2019). AHR2-null zebrafish are also largely infertile and show decreased survival and diminished reproductive health (Garcia et al. 2018a). Loss of AHR2 does not affect basal developmental mRNA expression of *cyp1a, ahr1b, ahrra, ahrrb, cyp1b1, cyp1c1, cyp3a65, slincR,* and *sox9B*, all known AHR-regulated genes (Garcia et al. 2018a; Goodale et al. 2012; Prasch et al. 2003). On the other hand, AHR2 is important for endogenous *cyp1a* expression specifically in the developing zebrafish eye but not in the trunk or brain (Sugden et al. 2017). Only the lack of all three AHRs caused a complete loss of *cyp1a* mRNA expression (but not *cyp1b1* expression) throughout the developing zebrafish (Sugden et al.

2017). These studies demonstrate the various possible roles of AHR2 in maintaining normal morphology and development. AHR2 also binds endogenous AHR ligands identified in other systems. Formylindolo[3,2-b]carbazole (FICZ), a tryptophan oxidation product formed upon exposure to UV or visible radiation, binds both AHR2 and AHR1b *in vitro* and induces expression of *cyp1a* and *cyp1b1* in an AHR2-dependent manner (Jonsson et al. 2009). Morpholino knockdown experiments illustrate that FICZ has increased and decreased toxicity in the absence of *cyp1a* and *ahr2* respectively, suggesting that the biological effects of FICZ are AHR2-dependent and regulated by its Cyp1a-mediated metabolism (Wincent et al. 2016). Zebrafish have also been used to define the endogenous roles of 3α,5α-tetrahydrocorticosterone and 3α,5β-tetrahydrocorticosterone (5α- and 5β-THB). These neuroactive steroids induce AHR2-dependent *cyp1a*, *mbp*, and *sox10*, the latter two of which are markers for myelinating cells (Wu et al. 2019). 5α-THB exposure also alters zebrafish larval behavior in an AHR2-dependent manner suggesting the importance of THB-AHR2 signaling in normal nervous system development (Wu et al. 2019).

There are no identified endogenous roles for **AHR1a** in zebrafish. AHR1a does not seem to play a role in normal development, larval feeding, or endogenous Cyp1a protein expression evidenced from AHR1a mutant fish that appear normal (Sugden et al. 2017). Further, neither AHR1a nor **AHR1b** morphants or mutants display overt phenotypes (Garner et al. 2013; Goodale et al. 2012; Sugden et al. 2017). However, a recent study showed that morpholino knockdown of *ahr1a* led to loss of hypocretin/orexin expression and developmental deformities (Seifinejad et al. 2019). Additionally, another study identified crosstalk between AHR1b and Nrf signaling during zebrafish development (Ulin et al. 2019). Many have suggested that partial overlapping functional redundancy of AHR2 and AHR1b allows for compensatory activity when AHR2 is lost (Prasch et al. 2003; Sugden et al. 2017); however, additional research is needed to clarify this. It is possible that the level of investigation to date has been insufficient to identify and confirm subtle development roles for these orthologs.

Section 1.3. Inducible roles of the zebrafish AHRs

To understand the inducible roles of the zebrafish AHRs, a combination of *in vitro* binding studies, transactivation assays in COS-7 mammalian cells, and *in vivo* zebrafish developmental studies has been utilized.

AHR2 is a functional receptor whose signaling is modulated not only by its various ligands, but also by its binding partners and downstream genes. Zebrafish have two ARNT genes, *arnt1* and *arnt2*, each present as three splice forms (ARNT1a, 1b, 1c, and ARNT2a, 2b, 2c) (Prasch et al. 2006; Tanguay et al. 2000; Wang et al. 1998b; Wang et al. 2000). AHR2 is capable of binding ARNT1b, ARNT1c, ARNT2b, and ARNT2c *in vitro* but only the complexes of AHR2 with ARNT1b, ARNT1c, or ARNT2b are able to promote transactivation by inducing AHRE-driven transcription with TCDD (Prasch et al. 2006; Tanguay et al. 2000). It was later shown using morpholino studies that both AHR2 and some form of the ARNT1 protein, but not the ARNT2 protein, are required for generating toxic responses to TCDD in developing zebrafish (Antkiewicz et al. 2006; Prasch et al. 2004; Prasch et al. 2006). It is not yet known how the AHR2-ARNT complexes mediate responses induced by other ligands. Furthermore, the functions of the various splice variants of the ARNTs are yet to be elucidated. AHR signaling can also be subjected to down-regulation by proteasomal degradation of AHR2 (Wentworth et al. 2004) as well as by transcriptional repression of its target genes by the aryl hydrocarbon receptor repressor (AHRR). Zebrafish have two distinct AHRRs, AHRRa (originally designated AHRR1) and AHRRb (AHRR2),

which are co-orthologs of the mammalian AHRR (Evans et al. 2005). Both AHRRa and AHRRb are induced in an AHR2-dependent manner similar to *cyp1a*, only by compounds that activate the AHR signaling pathway (Evans et al. 2005; Garcia et al. 2018a; Jenny et al. 2009; Timme-Laragy et al. 2007). AHRRa blocks AHR2 function by competing for binding to AHREs as well as by a transrepression mechanism that is independent of DNA binding (Evans et al. 2008). Knockdown of AHRRa, but not AHRRb, using a morpholino in the absence of TCDD exposure, phenocopied TCDD developmental toxicity and caused a large number of gene expression changes compared to wild type fish, while knockdown of either AHRRa or AHRRb enhanced TCDD-induced pericardial edema (Aluru et al. 2014; Jenny et al. 2009). These results suggest that while AHRRa is involved in regulating constitutive AHR signaling, both AHRRa and AHRRb play a role in modulating TCDD developmental toxicity. The ability of AHRRa knockdown to both phenocopy TCDD toxicity (in the absence of TCDD exposure) and enhance TCDD toxicity is consistent with a role for AHRRa in controlling constitutive AHR activity (in unexposed embryos), and a role for the AHR2-dependent induction of AHRRa after TCDD exposure to limit the AHR-dependent TCDD effects in a negative feedback loop. Further, zebrafish embryos in which AHRRb or both AHRRs (but not AHRRa alone) were knocked down had increased TCDD-induced expression of *cyp1a, cyp1b1,* and *cyp1c1* at 72 hpf, suggesting that AHRRb may have a role in controlling TCDD-activated AHR signaling (Jenny et al. 2009). To date, we do not know how AHRRa or AHRRb interact with AHR1a or AHR1b. Future work in single and double mutant lines for the AHRRs and AHRs will enhance our understanding of their interactions and functions in zebrafish.

The zebrafish **AHR1a** is a functional receptor *in vivo* (Goodale et al. 2012), but does not bind the canonical exogenous ligand, TCDD, in an *in vitro* heterologous cell system (Andreasen et al. 2002a; Karchner et al. 2005); the receptor is able to bind ARNT2b, can recognize AHREs more weakly compared to AHR2, and lacks transactivation activity with all ARNT2 proteins *in vitro* (Andreasen et al. 2002a). **AHR1b** was identified as a fully functional zebrafish receptor when assessed *in vitro* and in a heterologous cell system (Karchner et al. 2005). TCDD can bind AHR1b which interacts with ARNT2b, and promotes transactivation with efficacy comparable with that of AHR2 but with an 8-fold lower sensitivity (Karchner et al. 2005). It remains unknown to what extent AHR1a and AHR1b are able to interact with the ARNT1 proteins.

Section 1.4. Homology modeling of zebrafish AHRs

Several *in silico*-based modeling studies have investigated the structure and ligand binding properties of the three zebrafish AHRs (Bisson et al. 2009; Fraccalvieri et al. 2013; Zhang et al. 2018a; Zhang et al. 2018b). Using molecular dynamics simulations, it was determined that TCDD and many dioxin-like compounds interact with six amino acid residues in the AHR2 ligand-binding domain (Zhang et al. 2018b). The results supported, for the first time, the finding that polychlorinated diphenylsulfides can bind and activate AHR2 (Zhang et al. 2018b). Similarly, 2,2,',4,4'5-penta-BDE (BDE-99) is able to bind to both the zebrafish AHR2 as well as the Pregnane X Receptor (PXR) (Zhang et al. 2018a). While the ligand-binding pocket was more compact in the Bisson model (Bisson et al. 2009) compared to Fraccalvieri (Fraccalvieri et al. 2013), both models predicted that TCDD binds to AHR2 and AHR1b, but not AHR1a. Using site-directed mutagenesis coupled with functional analyses, it was determined that AHR1a was not able to bind TCDD because of differences in three amino acid residues in the ligand binding domain of AHR1a compared to that of AHR2

(Fraccalvieri et al. 2013). The differences make the AHR1a binding cavity much shorter than that of AHR2 with too limited space for TCDD binding. The Bisson model (Bisson et al. 2009) has also been utilized to predict binding with molecular docking of structurally different AHR ligands to the three zebrafish AHRs, summarized in **Table 3.** The table reveals that in general, xenobiotic ligands bind to more than one zebrafish AHR, making it likely that their overall toxicity is mediated by a combination of the receptors.

Overall, the studies reviewed in this section demonstrate that the three zebrafish AHRs are diverse in their local expression patterns, with only partial overlap in developmental and adult expression indicating cell-type-specific regulation. AHR2 and AHR1a are more widely expressed compared to AHR1b, and while AHR2 has been associated with normal developmental and physiological functions, such roles are not yet apparent for AHR1a and AHR1b. Importantly, all three receptors bind a variety of ligands evidenced by empirical and homology modeling studies. Clearly, the changing levels of expression of all three receptors across development testify to their dynamic nature and allude to the complexity of accurately understanding the AHRs' functional roles at different life stages.

Section 2. Early stage toxicity

In this section, we discuss ligands that produce adverse developmental effects dependent on the presence of each of the zebrafish AHRs. The majority of the research has focused on AHR2 (Section 2.1) and environmental contaminants including PAHs, TCDD, polychlorinated biphenyls (PCBs), and pharmaceuticals. It is noteworthy that several PAHs also activate AHR1a (Section 2.2) and AHR1b (Section 2.3), and below we specifically review the Cyp1a expression patterns dependent on the three AHRs.

Section 2.1. AHR2

Despite much research focused on TCDD, several studies have explored a diversity of xenobiotics and suggest that *in vivo* toxicity may be mediated by more than one zebrafish AHR (Goodale et al. 2012). Majority of the research conducted so far utilizes morpholino knockdown (with regulation of *cyp1a* induction as confirmation for knockdown) to reveal receptor-dependent toxicity effects; however, as groups are beginning to generate stable genetic knockout lines, more AHR2 mutant studies are being conducted. While morpholino knockdown can inform on which of the three receptors are important for mediating toxicity, only mutant studies with complete knockouts can definitively demonstrate toxicologically functional roles for the AHR paralogs. In this section, we focus on the xenobiotics whose toxicity is mediated primarily by AHR2 to collate what we know about AHR2's functionality. We will begin by reviewing the early stage toxicity of PAHs (Section 2.1.1) and other xenobiotics (Section 2.1.2), then we will summarize what we know about TCDD (Section 2.1.3) early stage toxicity in zebrafish. The functional role of AHR2 upon exposure to diverse ligands is summarized in **Table 4.**

Section 2.1.1. Polycyclic Aromatic Hydrocarbons (PAHs)

Many PAHs cause dioxin-like AHR2-dependent phenotypic endpoints including pericardial and yolk sac edemas, bent axes, cardiotoxicity, and eye and jaw malformations. Morpholino knockdown studies suggest that the following PAHs cause developmental toxicity primarily via AHR2: BaP (Cunha et al. 2020; Incardona et al. 2011), retene (Scott et al.

2011), benz[a]anthracene (BAA) (Incardona et al. 2006), pyrene (Incardona et al. 2005), and 1,9-benz-10-anthrone (BEZO) and Benz(a)anthracene-7,12-dione (7,12-B[a]AQ) (Goodale et al. 2015). Dozens of different PAH exposures have been associated with altered embryonic and larval behavior (Geier et al. 2018a); however only little is known about AHR2's role in behavioral outcomes. Specifically, BaP-exposed wild type zebrafish exhibit a hyperactive swimming response in the 120-hpf larval photomotor response (LPR) assay while BaP-exposed AHR2 mutants do not display a significantly altered LPR (Knecht et al. 2017b). This suggests that disruption of the AHR2 signaling pathway can lead to detrimental consequences to nervous system development and functioning. Some AHR2 knockdown studies revealed that many PAHs, including phenanthrene, dibenzothiophene, and benzo[k]fluoranthene (BkF), produce adverse developmental outcomes independent of AHR2 (Incardona et al. 2005; Incardona et al. 2011), despite inducing AHR2-dependent Cyp1a protein expression (Incardona et al. 2005; Incardona et al. 2011; Shankar et al. 2019). It is possible that the developmental toxicity produced by these PAHs is mediated by other zebrafish AHRs, or that incomplete morpholino knockdown confounded these studies. One recent morpholino study however, showed that exposure to BkF and three other fluoranthenes produced caudal fin duplication that is AHR2-dependent (Garland et al. 2020). This suggests that AHR2 may mediate specific malformations such as the fin duplication, while chemical interaction with other receptors such as AHR1b may mediate other developmental toxicity endpoints. Future studies testing the toxicity of these PAHs in both AHR1b and AHR2-null backgrounds are crucial to verify these results.

Both *cyp1a* mRNA and protein expression are frequently used as indicators of AHR activation by PAHs. In general, PAHs that elicit AHR2-dependent toxicity also induce *cyp1a* mRNA expression (Goodale et al. 2015; Knecht et al. 2013). Studies have also demonstrated that the obligate AHR isoforms for PAH toxicity can be inferred by determining the larval Cyp1a protein expression pattern. For example, developmental exposure to oxy-PAHs 7,12-B[a]AQ, BEZO, and BaP produce Cyp1a protein expression in the vasculature that is partially or fully dependent on AHR2 (Goodale et al. 2015; Incardona et al. 2011; Knecht et al. 2013). PAHs such as chrysene, retene, BAA, BkF, 1,6-dinitropyrene, benzo[j]fluoranthene, dibenzo[a.h]pyrene, dibenzo[a,i]pyrene, and benzo[b]fluoranthene (**Table 4**) induce Cyp1a protein in both the skin and the vasculature, among other regions. Vascular Cyp1a expression in response to these latter PAHs is only partially reduced upon AHR2 knockdown; however, Cyp1a expression in the skin is lost, demonstrating its complete AHR2-dependence. Similarly, while the surrogate model PAH, BNF, induces *cyp1a* mRNA expression in the skin and vasculature, only the expression in the skin is completely lost in AHR2 mutants (Sugden et al. 2017). Because of this differential, we recently reported that induction of Cyp1a in the skin is a more robust and reliable biomarker for AHR2 activation in developing zebrafish (Shankar et al. 2019). Studies have also identified nitro-PAHs such as 7-nitrobenz[a]anthracene and 3,7-dinitrobenzo[k]fluoranthene that do not cause visible developmental malformations at the tested concentrations but produce AHR2-dependent Cyp1a expression in a variety of organs (Chlebowski et al. 2017). Thus, these chemicals activate AHR2 without causing visible developmental toxicity, indicative of a potential adaptive response by Cyp1a. It is also possible that the absence of developmental toxicity is due to the lack of sustained activation of AHR2 by these chemicals, which has been hypothesized for other AHR agonists such as retene (Billiard et al. 1999). Future work assessing toxicity with Cyp1a inhibition and with daily renewal of the chemical exposure solution will help clarify this hypothesis.

Several studies have investigated the specific functional role of Cyp1a induction in PAH toxicity, and the apparent direct role for Cyp1a in PAH toxicity is chemical substrate-dependent. Typically, studies utilize *cyp1a* morphants, or known Cyp1a competitive inhibitors such as alpha-naphthoflavone (ANF) or fluoranthene. Retene (Scott et al. 2011) and BAA (Incardona et al. 2006) cause AHR2-dependent, but Cyp1a-independent cardiovascular developmental toxicity in zebrafish. On the other hand, *cyp1a* knockdown delays the toxic effects of pyrene, but fails to entirely protect the developing zebrafish from toxicity (Incardona et al. 2005). *Cyp1a* morphants also display enhanced toxic responses to the strong AHR ligand BkF, suggesting a protective role for Cyp1a in BkF toxicity (Incardona et al. 2011). Strong AHR agonists BkF (Garner et al. 2013; Van Tiem and Di Giulio 2011) and BaP (Garner et al. 2013; Jayasundara et al. 2015), and the weak AHR ligand phenanthrene (Brown et al. 2015) were more developmentally toxic when combined with fluoranthene, suggesting that inhibition of Cyp1a-mediated metabolism can enhance toxicity of these PAHs. While the phenanthrene + fluoranthene toxicity was not AHR2-dependent (Brown et al. 2015), AHR2 knockdown offered a protective role against the cardiotoxicity induced by the BkF+ fluoranthene and BaP+ fluoranthene mixtures (Garner et al. 2013). Similarly, the AHR2-dependent toxicity of BNF synergistically increased in combination with either ANF or a *cyp1a* morpholino, further demonstrating that Cyp1a can play an important protective role against PAH toxicity (Billiard et al. 2006). It was later shown that ANF did not act as an AHR antagonist, but rather a Cyp1a enzyme inhibitor, potentially prolonging the time the AHR was being activated, enhancing developmental toxicity (Timme-Laragy et al. 2007). Other than one study demonstrating that *cyp1b1* did not seem to play a role in PAH toxicity (Timme-Laragy et al. 2008), the roles of the other zebrafish *cyp* genes in PAH toxicity are not well understood.

For PAHs whose toxicity is AHR2-dependent based on morpholino studies, there have been a number of corresponding whole-embryonic, genome-wide transcriptomic studies (Fang et al. 2015; Goodale et al. 2015; Hawliczek et al. 2012; Shankar et al. 2019) albeit without comparing gene expression profiles in the presence and absence of AHR2. One study however identified several novel genes and potential mechanisms specifically in the developing zebrafish heart that could mediate cardiotoxicity via AHR2 upon exposure to BaP, fluoranthene, and BaP+ fluoranthene (Jayasundara et al. 2015). It was concluded that AHR2-dependent cardiotoxicity of BaP+ fluoranthene was mediated, at least in part, by perturbations to Ca^{2+} homoeostasis. Genome-wide transcriptomic studies for PAHs known to be primarily AHR2 agonists show that despite activating the same receptor, the transcriptomic changes downstream of AHR2 are ligand-dependent (Goodale et al. 2015; Shankar et al. 2019). We hypothesize that either slight differences in how chemicals bind to the receptor, or the formation of metabolites that can activate their own receptors, or a combination of the two, are contributing to the ligand-dependent gene expression profiles. Future work investigating these hypotheses is needed to illuminate more specific interactions between different ligands and the zebrafish AHRs. Some studies have utilized quantitative PCR to measure expression of specific genes including the *cyps* (mentioned above), and have identified genes that are regulated via AHR2 upon exposures to BAAQ (*cyp1b1*, *wfikkn1*, *gstp2*, *igfbp1a*) (Goodale et al. 2015), BEZO (*gstp2*, *igfbp1a*, *arg2*), and BkF (*cyp1a*, *cyp1b*, *cyp1c*, *gstp2*, *gpx1*, *gclc*) (Van Tiem and Di Giulio 2011). While several of these genes have been identified and well-studied (eg: *gpx1, gstp2, gclc* are involved in antioxidant responses), elucidation of functions of some genes such as *wfikkn1* is ongoing.

Section 2.1.2. Other xenobiotics: mixtures, pharmaceuticals, and halogenated hydrocarbons

AHR2 can at least partially mediate toxicity of **cigarette smoke** (Massarsky et al. 2016) and **PAH-containing soil extracts** from a gasworks, a former wood preservation site, and a coke oven site (Wincent et al. 2015). A recent study found that the developmental cardiotoxicity effects of both the individual chemicals and the **mixture of BaP and the oxy-PAH, 6H-benzo[cd]pyren-6-one** were significantly reduced upon *ahr2* knockdown (Cunha et al. 2020). Unlike these mixtures, **weathered crude oil** consisting of lower molecular weight PAHs, elicits morphological deficits and cardiotoxicity in an AHR2-independent manner, highlighting the potential influence of the structure and size of PAHs (Incardona et al. 2005). One study investigated Cyp1a protein expression induced in 120-hpf zebrafish after exposure to an **environmentally relevant PAH mixture**. Upon *ahr2* knockdown, Cyp1a vascular expression was eliminated, but there was production of Cyp1a protein in the liver attributed to the loss of AHR2 leading to the production of metabolites that had a higher affinity for AHR1a. Independently knocking down AHR1a or AHR1b did not alter Cyp1a protein expression; however, a triple morpholino knockdown of all three AHRs reduced Cyp1a protein expression (Geier et al. 2018b). These results reiterate the need for considering the functional roles of all three zebrafish AHRs, especially when studying the mechanisms of toxicity of complex mixtures.

The zebrafish AHR2 mediates developmental toxicity of **other small molecules and pharmaceuticals**. Although all three AHRs can bind leflunomide, an anti-inflammatory drug (Bisson et al. 2009; Goodale et al. 2012), AHR2 mediates the bulk of its Cyp1a vascular expression at 120 hpf (Goodale et al. 2012; O'Donnell et al. 2010). Its metabolite A771726 is not an AHR2 agonist (O'Donnell et al. 2010). The small molecule sulfonamide, cardiosulfa, also produces AHR2-dependent cardiotoxicity in developing zebrafish as seen in *ahr2* morpholino knockdown studies (Ko et al. 2009; Ko and Shin 2012). Similar to TCDD (reviewed below), Cyp1a neither reduces nor exacerbates cardiosulfa toxicity (Ko and Shin 2012). As noted above, the indole FICZ can cause developmental toxicity in an AHR2-dependent manner, especially when Cyp1a activity is inhibited or reduced by genetic knock-down (Jonsson et al. 2009; Wincent et al. 2016).

Other organic compounds such as phenanthroline (Ellis and Crawford 2016) and two halogenated carbazoles (Fang et al. 2016) are associated with PAH and TCDD-like developmental toxicity respectively, and the fungicide, paclobutrazol (Wang et al. 2015) causes digestive tract toxicity, all of which are AHR2-mediated. Some flame-retardant chemicals appear to buck the trend. For instance, *ahr2* knockdown does not reduce the cardiotoxicity associated with exposure to mono-substituted isopropyl triaryl phosphate (mITP), a major component of Firemaster 550 commercial mixture (Gerlach et al. 2014; McGee et al. 2013). However, *ahr2* knockdown prevents vascular Cyp1a protein expression in response to mITP, suggesting that the mixture does activate AHR2 (Gerlach et al. 2014). An AHR antagonist (CH223191) was able to block heart malformations induced by mITP but it was suggested that CH223191 antagonizes another target in addition to AHR (Gerlach et al. 2014; McGee et al. 2013).

PCB-126 (3,3',4,4',5-pentachlorobiphenyl) is one of the most potent AHR agonists (Kafafi et al. 1993), and is associated with developmental toxicity in zebrafish (Grimes et al. 2008). *Ahr2* knockdown greatly reduces PCB-126-induced cardiac effects and mortality, but only provides minimal protection against the abnormal inflation of the swim bladder (Garner et al. 2013; Jonsson et al. 2007). A follow-up study showed that, at a lower PCB-126 exposure concentration of 5 nM, *ahr2* gene knockdown prevented the swim bladder phenotype. This suggests that, again, incomplete *ahr2* morpholino

knockdown was probably operant and thus insufficient to block toxicity at the higher concentration (Jonsson et al. 2012). This pattern was similar to *ahr2* knockdown that partly mitigated cardiotoxicity caused by a lower TCDD exposure concentration of 0.3 ppb, but not at higher concentrations of 0.5 and 1ppb (Dong et al. 2004). *Ahr2* knockdown also significantly reduces *cyp1a*, *cyp1b1*, *cyp1c1*, and *cyp1c2* mRNA expression, and Cyp1a protein activity produced by PCB-126 exposure (Garner et al. 2013; Jonsson et al. 2007; Jonsson et al. 2012). One recent study determined that PCB-126 exposure not only caused increased expression of few AHR target genes (*ahrra*, *tiparp*, and *nfe2l2b*), but also led to their mRNA being hypermethylated (Aluru and Karchner 2020); future work to understand the specific role of this post-transcriptional modification is needed. Similar to TCDD, PCB-126 is not metabolized and accumulates in zebrafish, which leads to persistent expression of target genes (Garner and Di Giulio 2012; Meyer-Alert et al. 2018). Waits & Nebert used a quantitative trait locus (QTL) approach to investigate the genetic basis for zebrafish embryo susceptibility to PCB-126-induced developmental cardiotoxicity. Among the top-ranked QTLs was a region on chromosome 22 that includes *ahr2* and *ahr1b*, implicating one or both of these receptors in having a role in PCB-126 toxicity (Waits and Nebert 2011).

Section 2.1.3. TCDD

Developmental toxicity

TCDD is the most studied AHR2 ligand. In experiments using *in vitro*-translated AHR proteins or expression in heterologous cells, TCDD does not bind or activate AHR1a, but it binds AHR2 and AHR1b and activates them with comparable efficacies (Andreasen et al. 2002a; Karchner et al. 2005). When zebrafish are developmentally exposed to TCDD, they display reduced survival, and several phenotypes such as (but not limited to) cardiotoxicity, pericardial and yolk sac edemas, and craniofacial malformations (Henry et al. 1997). The various adverse developmental outcomes are reviewed in (Carney et al. 2006b). Knockdown and knockout studies have demonstrated the role of AHR2 in mediating TCDD-induced pericardial and yolk sac edemas, cardiovascular and craniofacial malformations, decrease in body length, and increased apoptosis, in addition to a significant increase in the mRNA levels of *cyp1a*, *cyp1b1*, *cyp1c1*, and *cyp1c2* in zebrafish (Carney et al. 2004; Dong et al. 2004; Garcia et al. 2018a; Goodale et al. 2012; Jonsson et al. 2007; Prasch et al. 2003; Souder and Gorelick 2019; Teraoka et al. 2003b; Yin et al. 2008). One study also demonstrated that the constitutive activation of the AHR2 in zebrafish cardiac myocytes not only led to TCDD-like cardiotoxicity, but also other defects in craniofacial development and failure to form swim bladders, suggesting the importance of the heart as a target organ (Lanham et al. 2014). We note that *ahr2* knockdown, however, was unable to protect against TCDD-induced inhibition of swim bladder inflation and mortality; this was attributed to the short half life of morpholinos after injection or a potential role of the other zebrafish AHRs (Prasch et al. 2003). Future work clarifying these results in an AHR2-null background zebrafish is necessary.

Mechanisms of TCDD developmental toxicity

The mechanisms of TCDD toxicity in humans and several vertebrate model organisms, including zebrafish, have been reviewed in (Carney et al. 2006b; King-Heiden et al. 2012; Yoshioka et al. 2011; Yoshioka and Tohyama 2019). TCDD-induced toxicity in zebrafish is associated with an array of transcriptomic changes, including modest changes to microRNA expression, in both developing zebrafish and in specific adult organs such as the heart (Alexeyenko et al. 2010; Carney et al. 2006a; Chen et al. 2008; Garcia et al. 2018b; Handley-Goldstone et al. 2005; Jenny et al. 2012). The

gene expression changes may be a function of a) large-scale toxicological phenotypes associated with TCDD, b) downstream effects of the AHR2/ARNT1 complex binding AHREs of various genes, or c) the interaction of the AHR with other pathways or transcription factors (Carney et al. 2006b). Here, we review what is known about the role of AHR2-regulated genes in TCDD-induced developmental malformations in zebrafish.

Binding of TCDD to AHR2 induces expression of the *cyp1* gene family. Upon exposure to TCDD, *cyp1a* mRNA and protein are expressed early in development in a variety of organs at the different development stages (Andreasen et al. 2002b; Kim et al. 2013; Yamazaki et al. 2002; Zodrow et al. 2004). One study noted that the Cyp1a protein is first localized to the skin and the vasculature after which it transitions to the vasculature, kidney, and liver by 120 hpf (Andreasen et al. 2002b). When AHR2-null zebrafish are exposed to TCDD, Cyp1a protein expression at 120 hpf is almost completely prevented (Goodale et al. 2012). It was initially thought that the induction of *cyp1a* is required for TCDD developmental toxicity (Teraoka et al. 2003b); however, a later study demonstrated that, consistent with mammalian literature, TCDD produces developmental toxicity endpoints independent of *cyp1a* (Carney et al. 2004). TCDD also induces expression of *cyp1b1*; however, this does not appear to have a direct role in TCDD-induced pericardial edema and craniofacial malformations (Yin et al. 2008). *Cyp1c1* and *cyp1c2* likely play roles in TCDD-induced circulation failure in the mid-brain but the exact mechanism is unknown (Kubota et al. 2011). Unlike the other zebrafish *cyp1s*, *cyp1d1* does not seem to be transcriptionally activated by TCDD or PCB126 (Goldstone et al. 2009).

Cyclooxygenase-2 (COX-2) enzymes, a family of heme-containing enzymes thought to be involved in acute inflammatory responses, have been studied in the context of the AHR signaling pathway in zebrafish and other model organisms. Zebrafish have two *cox-2* genes, *cox2a* and *cox2b* (Ishikawa et al. 2007); both are induced in an AHR2-dependent manner upon exposure to PCB-126, but future work is needed to identify whether these genes are direct targets of AHR2 (Jonsson et al. 2012). To our knowledge, induction of these genes has not been demonstrated upon TCDD exposure. However, it is suggested that *cox2a*, in combination with the thromboxane receptor and thomboxane A synthase 1 (also known as *cyp5a*), is involved in local circulation failure in the dorsal midbrain of developing zebrafish (Teraoka et al. 2009). Another study discovered the role of the *cox2b*-thromboxane pathway in TCDD-induced AHR2-dependent "precadiac" edema, the increased area of the small cavity between the heart and the body wall (Teraoka et al. 2014). The study demonstrated that knockdown of *cox2b*, but not *cox2a,* prevented formation of the precardiac edema in the TCDD-exposed zebrafish, and also showed the involvement of the thromboxane pathway, concluding that thromboxane release by TCDD probably led to the edema in the developing zebrafish. Additionally, other factors such as oxidative stress are involved, as an antioxidant was able to inhibit both precardiac edema and circulation failure caused by TCDD exposure (Dong et al. 2004).

The sry box containing 9b (*sox9b*) gene, a critical chondrogenic transcription factor, has been linked to cardiotoxicity caused by TCDD (Hofsteen et al. 2013b). Upon exposure to TCDD, *sox9b* expression is significantly reduced in an AHR2-dependent manner (Garcia et al. 2018a; Xiong et al. 2008), and one study found that *sox9b* knockdown resulted in TCDD-like heart malformations (Hofsteen et al. 2013b). Morpholino knockdown of *sox9b* also caused a phenotype similar to TCDD-induced jaw malformation, and restoration of *sox9b* with mRNA injection prevented craniofacial malformations suggesting *sox9b*'s role in TCDD-induced craniofacial defects (Xiong et al. 2008). A *sox9b* promoter - eGFP transgenic reporter fish (uw101Tg) (Plavicki et al. 2014) was produced and used to identify the TCDD-induced

repression of *sox9b* in the developing zebrafish heart and brain (Garcia et al. 2017; Hofsteen et al. 2013b). A mechanism for linking activation of AHR2 and *sox9b* repression was recently suggested (Garcia et al. 2017; Garcia et al. 2018b). The *sox9b* long intergenic noncoding RNA (*slincR*) is significantly induced by TCDD in an AHR2-dependent manner and appears to interact with the 5' untranslated region of the *sox9b* gene (Garcia et al. 2017; Garcia et al. 2018b). Exposure of *slincR* morphants to TCDD resulted in altered jaw cartilage structure and reduced incidence of hemorrhaging, suggesting a possible functional role of *slincR* in both TCDD-induced craniofacial malformations and cardiotoxicity (Garcia et al. 2018b). The study also highlighted several PAHs that induce *slincR* expression at high levels without causing *sox9b* repression, indicating that *slincR* could not only have tissue-specific effects but could also regulate other genes beyond *sox9b* (Garcia et al. 2018b).

A member of the forkhead box family of transcription factors, originally designated *foxq1b* but now known as *foxq1a*, is highly induced by TCDD exposure at a rate faster than *cyp1a* in developing zebrafish, in an AHR2-dependent manner (Planchart and Mattingly 2010). *In situ* hybridization experiments showed that the transcript is expressed in the jaw primordium and is hypothesized to play a role in craniofacial abnormalities (Planchart and Mattingly 2010). TCDD also induces the paralogous gene *foxq1b* in zebrafish (Hahn et al. 2014). More work is needed to identify the functional role of the *foxq1* paralogs in the TCDD toxicity pathway.

Section 2.2. AHR1a

Initial *in vitro* studies concluded that AHR1a was non-functional because it did not bind TCDD and was transcriptionally inactive when expressed in cells together with ARNT2b (Andreasen et al. 2002a). These results are supported by *in vivo* studies in AHR1a mutant fish, from which it was concluded that AHR1a was not required for TCDD-induced toxicity and Cyp1a activity in zebrafish (Souder and Gorelick 2019). BNF also does not activate AHR1a, and it was suggested that the zebrafish *ahr1a* is a possible pseudogene (Karchner et al. 2005). However, more recent *in vivo* studies demonstrate that AHR1a is functional and can be activated by chemicals including leflunomide (Goodale et al. 2012), the oxy-PAH xanthone (Knecht et al. 2013), several nitro-PAHs like 5-nitroacenaphthalene, 9-nitrophenanthrene, and 7-nitrobenzo[k]fluoranthene (Chlebowski et al. 2017), and the parent PAHs pyrene (Incardona et al. 2006) and chrysene (Incardona et al. 2005). Upon *ahr1a* knockdown and developmental exposure to each of these chemicals, either a reduction of toxicity (Chlebowski et al. 2017; Incardona et al. 2006) or a reduction of induced Cyp1a protein expression (Chlebowski et al. 2017; Goodale et al. 2012; Incardona et al. 2005; Knecht et al. 2013) was confirmed. Furthermore, AHR1a is the dominant receptor involved in regulating induction of larval hepatic Cyp1a; *ahr1a* knockdown reduces Cyp1a liver expression induced by pyrene (Incardona et al. 2006); leflunomide (Goodale et al. 2012), xanthone (Knecht et al. 2013), and the nitro-PAHs, 5-nitroacenaphthalene, 9-nitrophenanthrene, and 7-nitrobenzo[k]fluoranthene (Chlebowski et al. 2017).

In contrast to the above-mentioned studies, Garner et al. found that morpholino knockdown of *ahr1a* exacerbated the developmental toxicity caused by both PCB-126 and a mixture of PAHs, BkF and fluoranthene (Garner et al. 2013). Although *ahr1a* knockdown did not affect *cyp1a*, *cyp1b1*, and *cyp1c1* gene expression, Cyp1a protein activity, measured using the ethoxyresorufin-O-deethylase (EROD) assay, increased. From this study, the authors hypothesized that AHR1a likely mimics AHRR and consequently, the absence of AHR1a results in excessive AHR2 signaling and enhances the

cardiotoxicity measured by pericardial edema (Garner et al. 2013). The study also highlights that AHR1a seems to inhibit Cyp1a protein activity, as *ahr1a* knockdown led to increased activity. This was in contrast to AHR2, which mediated an increase in Cyp1a activity (Garner et al. 2013). Another study found that the prevalence of mITP-induced cardiotoxicity, but not its severity, increased when all three *ahr*s were knocked down compared to just *ahr1b*/*ahr2* knockdown, suggesting that AHR1a may play a role in mITP-induced cardiotoxicity (Gerlach et al. 2014). However, it is noteworthy that *cyp1a* transcript expression was not altered by *ahr1a* knockdown like it was by *ahr1b*/*ahr2* knockdown, indicating that mITP likely does not activate AHR1a (Gerlach et al. 2014). Overall, AHR1a appears to have relevance and ligand-specific functions that are currently enigmatic. **Table 5** summarizes the effects mediated by AHR1a in developing zebrafish.

Section 2.3. AHR1b

Zebrafish morpholino studies reveal that AHR1b does not play a role in early life toxicity caused by PCB-126, a PAH mixture of BkF and fluoranthene (Garner et al. 2013), or TCDD (Souder and Gorelick 2019). Although *ahr1b* knockdown did not prevent mITP-induced cardiotoxicity in zebrafish, there was a significant decrease in the prevalence of mITP-induced pericardial edema in an AHR2 mutant line injected with the *ahr1b* morpholino compared to control morpholino-injected AHR2 mutants. This suggests AHR1b's possible role in mediating mITP-induced cardiotoxicity (Gerlach et al. 2014). Additionally, studies suggest that AHR1b may be involved in not only developmental toxicity, but also adult toxicity effects of chemicals like TCDD, some PAHs, and PCB-126 (Garner et al. 2013; Goodale et al. 2012); however, a closer look with histopathology or immunohistochemistry may be necessary to reveal possible subtle effects missed in gross morphology studies. It was also suggested that AHR1b could be functionally redundant with AHR2, but so far, it seems evident that AHR2 has a greater role in regulating the expression of xenobiotic-metabolizing enzymes and mediating toxicity compared to AHR1b (Garner et al. 2013).

While knockdown studies have not definitively shown a role for AHR1b in the developmental toxicity of xenobiotics, AHR1b appears to be important for leflunomide-induced Cyp1a protein expression in the vasculature, but not in the liver (Goodale et al. 2012). AHR1b may also play a role in Cyp1a protein expression induced by mITP in the vasculature, heart, and liver (Gerlach et al. 2014), and by nitro-PAHs like 7-nitrobenzo[k]fluoranthene in the vasculature, skin, and the neuromasts (Chlebowski et al. 2017). *Ahr1b* knockdown in mITP-exposed zebrafish also reduced *cyp1a* mRNA levels (Gerlach et al. 2014). These studies demonstrate that AHR1b can be activated by various chemicals and they concur with earlier studies that showed AHR1b is a fully functional receptor (Karchner et al. 2005) with partially overlapping functions with AHR2. Unlike AHR2 and AHR1a, AHR1b does not appear to mediate Cyp1a expression in any specific tissue. **Table 6** summarizes the evidence for AHR1b's role in developmental toxicity in zebrafish.

Overall, AHR2 is predominant in mediating the early stage toxicity of a large variety of ligands. AHR1a and AHR1b can also mediate developmental toxicity albeit to a lesser extent, and this supports the idea that the three AHRs have partitioned multiple AHR roles. The three AHRs have distinct ligand profiles, and even when different chemicals activate the same receptor, the downstream gene expression and developmental toxicity endpoints can be considerably different, suggesting ligand-specific activation of the AHRs.

Section 3. Interaction between AHR and other pathways

In addition to the direct AHR-mediated toxicity, the AHRs and AHR-responsive genes can directly or indirectly interact with genes from several different signaling pathways while modulating toxicological responses to a ligand. The developmental zebrafish model provides an ideal platform to study these interactions, since most signaling mechanisms are concurrently and dynamically at play during development. In this section, we focus on studies that have explored the crosstalk between AHR signaling and other pathways using embryonic zebrafish.

Section 3.1. AHR and oxidative stress

Oxidative stress - the disruption of redox signaling and control (Jones 2006) - is a well-studied toxicological phenomenon that occurs in response to several classes of chemicals that produce reactive oxygen species (ROS) or disrupt thiol homeostasis (Di Giulio and Hinton 2008; Sies et al. 2017). AHR-mediated oxidative stress (Di Giulio and Hinton 2008) occurs through a variety of mechanisms, including stimulation of inflammatory responses and induction of pro-oxidant enzymes such as xanthine oxidase and CYP-dependent monooxygenases, which can release ROS or generate redox-cycling metabolites (Dalton et al. 2002; Reichard et al. 2006). Developmental exposures to AHR ligands such as PAHs (Van Tiem and Di Giulio 2011), oxy-PAHs (Knecht et al. 2013), heterocyclic and nitro-PAHs (Chlebowski et al. 2017), or PCB-126 (Liu et al. 2016) result in induction of redox-responsive antioxidant genes such as glutathione peroxidase (*gpx1*), glutamate cysteine ligase (*gclc1*), and superoxidase dismutase (*sod1*). In addition, PCB-126 also induces a significant increase in lipid peroxidation, a result of ROS-induced cellular damage (Liu et al. 2016). In fact, AHR activation and antioxidant responses act synchronously in response to toxicant exposures - this was evidenced by the mirroring of the activities of total SOD and Cyp enzymes in whole homogenates of fish exposed to the PAHs, phenanthrene and anthracene (Wang et al. 2018). Taken together, these studies suggest a robust antioxidant response as well as some levels of oxidative damage associated with AHR activation. A number of knockdown studies have also supported these outcomes. For example, *ahr2* knockdown blocks the increased expression of *gpx1*, *gclc1* and *sod1* by the AHR agonist BkF (Van Tiem and Di Giulio 2011). *Ahr2* knockdown also prevents the induction of ROS and 8-OHdG (8-hydroxy-2′ -deoxyguanosine, a marker of oxidative DNA damage) by the chlorinated solvent trichloroethylene (TCE) (Jin et al. 2020), although wild type TCE-exposed embryos do not show an induction of *cyp1a1* or *ahr2* transcripts. Nevertheless, these studies confirm the specific role of AHR2 in mediating both oxidative damage and antioxidant responses.

A major driver for AHR-induced antioxidant responses is the crosstalk between AHR and their prime regulator, Nrf2 (Baird and Yamamoto 2020). This mechanism is particularly important for AHR2 ligands such as TCDD, which do not undergo substantial metabolism and hence, redox cycling (Dietrich 2016). Nrf2 (also called Nfe2l2) is a transcription factor that regulates the expression of a number of antioxidant enzymes such as NAD(P)H:quinone oxidoreductase (NQO1) as well as xenobiotic-metabolizing enzymes such as glutathione-S-transferases (GSTs) (Dietrich 2016). In mammals, AHR regulates Nrf2 expression and Nrf2 mediates the AHR-dependent induction of several xenobiotic-metabolizing enzymes by TCDD (Miao et al. 2005; Yeager et al. 2009). Zebrafish have two Nrf2 genes, *nrf2a* and *nrf2b,* both of which contain AHREs within their promoter regions (Timme-Laragy et al. 2012a). A number of chemicals such as TCDD (Hahn et al. 2014; Timme-Laragy et al. 2012a), PAHs (Knecht et al. 2013), and PCBs (Timme-Laragy et al.

2012a; Timme-Laragy et al. 2015) induce *nrf2a* or *nrf2b* mRNA expression at different life stages, in an AHR2-dependent manner (Timme-Laragy et al. 2012a). For example, embryonic exposures to the oxy-PAH 7,12-B[a]AQ results in increased expression of *nrf2* and *nqo1* in addition to genes associated with the glutathione redox cycle (*gst*, *gpx,* and *sod* families) (Knecht et al. 2013). One study showed that although exposures to PCB-126 in wild type embryos did not elicit any antioxidant responses, an *nrf2a* mutant displayed altered both basal expression and PCB-inducibility of certain *ahr*, *nrf2* and *gst* family genes (Rousseau et al. 2015). Other *nrf2*-family genes and AHR forms may also be involved in this cross-talk; for example, AHR1b regulates the constitutive and TCDD-inducible expression of *nrf2a* as well as other members of the *nrf* gene family, *nrf1a* and *nrf1b* (Ulin et al. 2019). These studies suggest that antioxidant responses to TCDD, PCBs, and PAHs may be driven by a combination of oxidative stress and AHR-Nrf2 crosstalk. Indeed, it is well known that the glutathione and Nrf2 pathways are interdependent, and *nrf2* knockdown can perturb the glutathione redox state (Sant et al. 2017). In contrast, TCDD, a strong inducer of *nrf2* in both zebrafish (Hahn et al. 2014; Ulin et al. 2019), and in mammals (Miao et al. 2005; Yeager et al. 2009), does not induce expression of antioxidant genes such as *sod*, *gst* or *nqo1* (Alexeyenko et al. 2010; Hahn et al. 2014). Overall, these studies provide evidence of the complexity of the role of AHR, the glutathione redox state, and the Nrf2 pathway in trying to maintain oxidative homeostasis in response to xenobiotics.

Section 3.2. AHR-Wnt crosstalk and tissue regeneration

While mammals, including humans, have a limited regenerative capacity restricted to some organs such as liver and skin, other vertebrates possess high regenerative capacity of the heart, liver, limbs, etc (Marques et al. 2019). The process of regeneration involves cellular migration, blastema formation, differentiation, and proliferation that are all regulated by multiple signaling pathways (Akimenko et al. 2003; Santamaria and Becerra 1991), and external stressors can potentially inhibit regeneration (Mathew et al. 2009). Zebrafish, in particular, has been widely used as a model for studying tissue regeneration following surgical amputation of organs (Akimenko et al. 2003). Exposure to AHR ligands such as TCDD and leflunomide (an anti-inflammatory drug) following fin amputation results in a failure of adult and larval fin regeneration (Andreasen et al. 2007; Mathew et al. 2006; O'Donnell et al. 2010; Zodrow and Tanguay 2003). Morpholino knockdown of *ahr2* and *arnt1* results in both unexposed and TCDD-exposed morphants showing normal fin regeneration, indicating that the inhibition of regeneration by TCDD is AHR2 and ARNT1-dependent (Mathew et al. 2006). Following TCDD exposures, adult regenerative tissues also show widespread changes in the transcripts that regulate cellular differentiation, cartilage, collagen, cell growth, tissue regeneration, and extracellular matrix - all important factors involved in tissue regeneration (Andreasen et al. 2007). Specifically, TCDD exposure is associated with both transcriptional activation of R-spondin 1, and a repression of *sox9b* (a transcription factor regulated by AHR2, as discussed previously) in embryos (Mathew et al. 2007). R-spondin 1 is a Wnt/β catenin signaling gene that contains an AHRE in its promoter region. Morphants resulting from partial suppression of both R-spondin 1 and LRP6, a Wnt co-receptor, show normal caudal fin regeneration following TCDD exposure, demonstrating that activation of these Wnt signaling genes is required for TCDD to inhibit regeneration (Mathew et al. 2007). This result is also supported by the induction of a number of other Wnt/β-catenin signaling genes by TCDD in regenerating tissues. In conjunction with transcriptional activation of R-spondin 1, the expression of *sox9b* is repressed within regenerating fin tissues after TCDD exposure (Mathew et al. 2007). Interestingly, although *sox9b* morphants show some levels of regeneration of caudal fins,

the regenerative tissue still possesses defective structures, indicating that this process is not completely dependent on *sox9b* (Mathew et al. 2007). In humans, SOX9 is also a Wnt-target gene and is directly regulated by R-spondin 1 (Yano et al. 2005). Furthermore, SOX9 also inhibits expression of β catenin-associated genes and promotes degradation of β catenin (Yano et al. 2005). Therefore, it is likely that the inverse expression patterns between R-spondin1 and *sox9b* observed within regenerative fin tissues results from a crosstalk between the AHR and Wnt signaling mechanisms to regulate tissue regeneration. In addition to the proposed AHR-Wnt crosstalk, other AHR-mediated mechanisms can govern tissue regeneration, depending on the tissue type. For example, one study showed that TCDD exposures of adult zebrafish with partially amputated hearts led to an inhibition of regeneration of myocardial tissues, but there was no impact on *sox9b*, R-spondin 1, or other Wnt signaling genes although, as seen with caudal fin amputation, expression of genes associated with tissue regeneration and extracellular matrix was altered (Hofsteen et al. 2013a). The lack of change in transcript levels of *sox9b* and fin tissue regeneration, while largely governed by similar molecular factors, have some differences; for example: while fin regeneration is co-regulated by Wnt signaling, myocardial regeneration is co-regulated by TGFβ and NFκβ pathways (Sehring et al. 2016). Despite these differences, the studies show that only the chemical activation of AHR inhibits tissue regeneration.

Section 3.3. Estrogen receptor (ER)

Both mammalian and zebrafish studies show clear evidence of crosstalk between AHR and ER. In mammals, AHR interactions with estrogen signaling pathways have long been known to occur through a variety of mechanisms (Safe and Wormke 2003; Swedenborg and Pongratz 2010), including the role of AHR as an E3 ubiquitin ligase controlling proteasomal degradation of ER (Ohtake et al. 2007; Ohtake et al. 2003; Wormke et al. 2003). It is not known whether fish AHRs can act in this way, but there is other evidence for AHR-ER crosstalk. In zebrafish, C*yp3c* can be induced by both AHR and ER ligands, suggesting that there may be a crosstalk between these two receptor mechanisms in regulation of CYP3 (Shaya et al. 2019). The direct interaction between AHR and ER pathways has been shown in other studies where the transcriptional induction of ER-target *cyp19b* or vitellogenin by ER ligands 17α-ethynylestradiol and 17ß-estradiol, were reversed by TCDD (Bugel et al. 2013; Cheshenko et al. 2007). In addition, this effect was partially blocked by ANF, an AHR antagonist (Cheshenko et al. 2007). Interestingly, a chemically induced pan-ER inhibition does not block TCDD-induced, AHR2-mediated cardiotoxicity, suggesting that AHR2 is not a constitutive partner of ER (Souder and Gorelick 2019). However, these authors also conclude that an AHR-ER crosstalk may be tissue-dependent. This was supported by another study, where 17β-estradiol increased *ahr1a* mRNA expression only in the 4-day old zebrafish brain, but not in other organs (Hao et al. 2013). Likewise, an adult zebrafish study showed that TCDD inhibited levels of the genes regulating the estrogen receptor as well as estrogen synthesis and follicular development in the zebrafish ovary (King-Heiden et al. 2008), highlighting the role of the AHR-ER crosstalk in the reproductive system (discussed later). Therefore, interactions between AHR and ER in zebrafish is likely complicated and highly dependent on the specific target tissues.

Section 3.4. Pregnane X receptor (PXR)

AHR displays some levels of crosstalk with the transcription factor PXR, which regulates a number of CYP2 and CYP3 enzymes and is involved in detoxification of an array of xenobiotics, primarily steroids. Embryonic zebrafish exposures to either pregnenolone (a PXR agonist) or PCB-126 (an AHR agonist) result in the increased transcript levels of *pxr, ahr2,*

cyp1a as well as a number of *cyp2* and *cyp3* genes (Kubota et al. 2015). Furthermore, knockdown of *ahr2* reverses the PCB 126-induced transcriptional activation of *cyp1a* and *pxr,* as well as *cyp2* and *cyp3* genes. Taken together, these studies suggest an inevitable crosstalk between AHR and PXR in regulation of *cyp2* and *cyp3* genes.

Section 3.5. Fibroblast growth factor (FGF)

Studies have explored the interaction between AHR and the FGF pathway in developmental processes. In mammals, the FGF pathway gene, *fgf21,* is a known AHR target gene (Cheng et al. 2014). In zebrafish, similar to AHR, the FGF pathway is known to independently regulate tissue regeneration. However, a study comparing fin regeneration after exposure to TCDD as well as an FGF pathway inhibitor, SU5042, showed that, although both AHR activation and FGF inhibition lead to inhibition of fin regeneration, the phenotypes were morphometrically different and there was no evidence of interaction between the two pathways in the regenerative process (Mathew et al. 2006).

In summary, we note that much of the work concerning crosstalk with other signaling pathways has focused on AHR2. Although it is evident that the AHR2 signaling pathway interacts with several other transcription factors and signaling pathways, the interactions are highly complex, and we have only begun to understand them in zebrafish.

Section 4. Adult toxicity, epigenetics and multigenerational effects

Compared to the many studies assessing the role of AHRs in during development, only a limited number have investigated the role of the zebrafish AHRs in both post-developmental physiology in juveniles and adults, and across generations. To our knowledge, AHR1a- or AHR1b-specific adult and multigenerational toxicity after exposure to xenobiotic chemicals has not been investigated. In this section, we first review the functional roles of AHR2 in TCDD-induced adult toxicity (Section 4.1) of the reproductive (Section 4.1.1) and musculoskeletal (Section 4.1.2) systems, and then describe what is known about the epigenetic effects (Section 4.2) of the ligands TCDD, PCB-126, and BaP, whose toxicity endpoints are mediated primarily by AHR2.

Section 4.1. Adult toxicity

Section 4.1.1. Reproductive System

AHR plays a modest constitutive role in the reproductive system; a study with AHR mutants showed altered follicular development in ovaries of the AHR2-null zebrafish compared to wild type zebrafish (Garcia et al. 2018a). However, more profound effects on reproductive organs have been shown to be triggered by xenobiotic activation of AHR2 by TCDD (King-Heiden et al. 2012) and these impacts are expected due to the crosstalk between AHR and ER as described in the previous section. Indeed, dietary TCDD exposure reduced mRNA levels of genes regulating the estrogen receptor and follicular development in adult zebrafish ovaries, while inducing expression of *cyp1a* (King-Heiden et al. 2008). Additionally, zebrafish exposed to TCDD at 3- and 7- weeks post fertilization displayed reduced fecundity and reduced percentage of fertilized eggs (Baker et al. 2013). Paired spawning also showed that these impacts were independent of the sex of the fish. While female fish displayed abnormalities in ovarian structures, the testes of TCDD-exposed male fish displayed decreased spermatozoa with increase in spermatogonia, decreased germinal epithelial

thickness, and altered responses in genes regulating testis development and steroidogenesis (Baker et al. 2016). Interestingly, TCDD-exposed fish also experienced a prevalent shift towards feminization, but a significant percentage of female fish possessed male gonads (Baker et al. 2013). From these studies, it is evident that both AHR knockout (Garcia et al. 2018a) and its activation (other studies) result in reproductive deficiencies, suggesting that any disruption to the normal AHR signaling mechanism can have deleterious effects on reproductive physiology. These effects on the reproductive system may also be mediated through AHR-associated epigenetic mechanisms, discussed in Section 4.2. Taken together, these studies unequivocally highlight the significant role of AHR in reproductive development, sex determination, and reproductive functions.

Section 4.1.2. Musculoskeletal System

TCDD exposures have also been shown to affect musculoskeletal development in adults, with zebrafish exposed to TCDD at 3- and 7- weeks post fertilization displaying skeletal deficits during adulthood, including skeletal kinks, shortened jaw structures, and abnormal operculum and bone structures (Baker et al. 2013). Interestingly, AHR2-null fish also showed similar deficits in skeleton and fins, including defective fins, abnormal dentary, operculum and frontal structures, smaller orbital and supraorbital bones (Baker et al. 2014b; Garcia et al. 2018a; Goodale et al. 2012; Souder and Gorelick 2019). These identical responses both to AHR2 deficiency and AHR2 activation mimic the equivocality of responses on reproductive development and highlight the need for more detailed studies on the role of AHR2 in musculoskeletal development. They also reiterate the sensitive nature of the AHR2 signaling pathway, where any alteration – either its deficiency or activation – can have profound impacts on the musculoskeletal system.

Section 4.2. Epigenetics and multigenerational effects

TCDD exposure results in several transgenerational effects (Baker et al. 2014a; Baker et al. 2014c; King-Heiden et al. 2005; Meyer et al. 2018) which have been reviewed recently (Viluksela and Pohjanvirta 2019). It has been hypothesized that epigenetic mechanisms mediate these effects of TCDD, although specific epigenetic modifications have not been identified as having a causal role in the responses observed in F1 and F2 generations (Baker et al. 2014a). There is increasing evidence in other organisms showing how epigenetic modifications are related to AHR signaling and TCDD toxicity (Patrizi and Siciliani de Cumis 2018; Viluksela and Pohjanvirta 2019). The expression of *cyp1a1* and *cyp1b1* in the F1 generation of a TCDD-exposed zebrafish lineage was significantly higher than in control-lineage F1 animals, suggesting an AHR role (Olsvik et al. 2014). Within the genome region queried, there was no effect of TCDD on the methylation pattern of the *ahr2* promoter in developing zebrafish (Aluru et al. 2015). However, the study found altered promoter methylation of AHR target genes, *ahrra* and *c-fos*. TCDD exposure also altered *dnmt* expression in the F0 generation, and *in vitro* transactivation studies identified that three of the five tested *dnmt* promoters caused transactivation of luciferase reporter by AHR2/ARNT2 in the presence of TCDD (Aluru et al. 2015). A recent study that examined genome-wide changes in DNA methylation in adult testes of zebrafish exposed to TCDD found differential methylation of genes involved in reproductive and epigenetic processes (Akemann et al. 2018) and some of these histological and transcriptomic effects persisted in subsequent F1 and F2 generations (Meyer et al. 2018), suggesting a methylation-dependent transfer of biomarkers across generations. **PCB-126** exposure of adult zebrafish caused extensive alteration in genome-wide DNA methylation patterns in liver and brain, which were not strongly correlated with altered

gene expression, suggesting a complex relationship between DNA methylation and gene regulation (Aluru et al. 2018). Taken together, these studies suggest the potential role of methylation-dependent epigenetics in driving TCDD- and PCB-126-induced reproductive outcomes.

BaP exposure leads to transgenerational effects including alterations to locomotor activity, decreased heartbeat and mitochondrial function, reduced hatch rate, reduced egg production and offspring survival, increased mortality and incidence of malformations up to the F2 generation (Corrales et al. 2014a; Corrales et al. 2014b; Fang et al. 2013; Knecht et al. 2017a). These effects were likely a result of global hypomethylation in conjunction with alterations of expression of developmental and cancer-related genes in BaP-exposed F0 zebrafish (Corrales et al. 2014a; Fang et al. 2013; Knecht et al. 2017a). *Dnmt* expression was generally reduced with BaP exposure, an effect that was further strengthened by AHR2 knockdown (Knecht et al. 2017a). This was unexpected and may have been due to a mechanism different from AHR2/ARNT acting via AHREs in the *dnmt* promoters. Another study found that, following BaP exposure, *dnmt1* and *dnmt3a* had increased mRNA expression in 96 hpf larvae and in adult brains; however, the role of the AHRs in mediating the altered expression was not investigated (Gao et al. 2017).

In summary, the functional role of AHR2 in mediating adult toxicity and epigenetic perturbation is in the early stages of investigation and more studies are being conducted across several labs to better understand these mechanisms.

Conclusions and future directions

The studies conducted so far have demonstrated the vast diversity of both the endogenous and toxicological functional roles of the zebrafish AHRs. Research in zebrafish (and other fish models) has enhanced our understanding of AHR biology in all its richness, including endogenous and toxicological AHR roles. This research has complemented studies in other animal models, and in particular has contributed to knowledge about AHR functions during vertebrate development. Additionally, research on the zebrafish AHRs has explored and revealed the heterogeneity of ligands that are able to bind to each of the three receptors. The majority of the research so far on all aspects of functionality has focused on AHR2; future work concentrating on how AHR1a and AHR1b contribute to normal physiology and toxicity of xenobiotics will not only inform us of their roles, but could also reveal unknown functions of the AHR, many of which are conserved across vertebrates. Further, understanding the downstream signaling events upon AHR activation has centered on TCDD as a ligand. Exploration of the functions of AHR-regulated genes upon exposure to other chemicals will facilitate a gene biomarker approach to further characterize and classify xenobiotics that act via AHR. With the remarkable diversity of AHR ligands, the wide-ranging downstream AHR-regulated genes, and the crosstalk interactions with other signaling pathways, it is clear we must discern how the activation of the AHRs by its various ligands can differentially modulate signaling pathways that dictate biological outcomes.

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Conflict of Interest

The authors declare no potential conflicts of interest with respect to this article.

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Figures

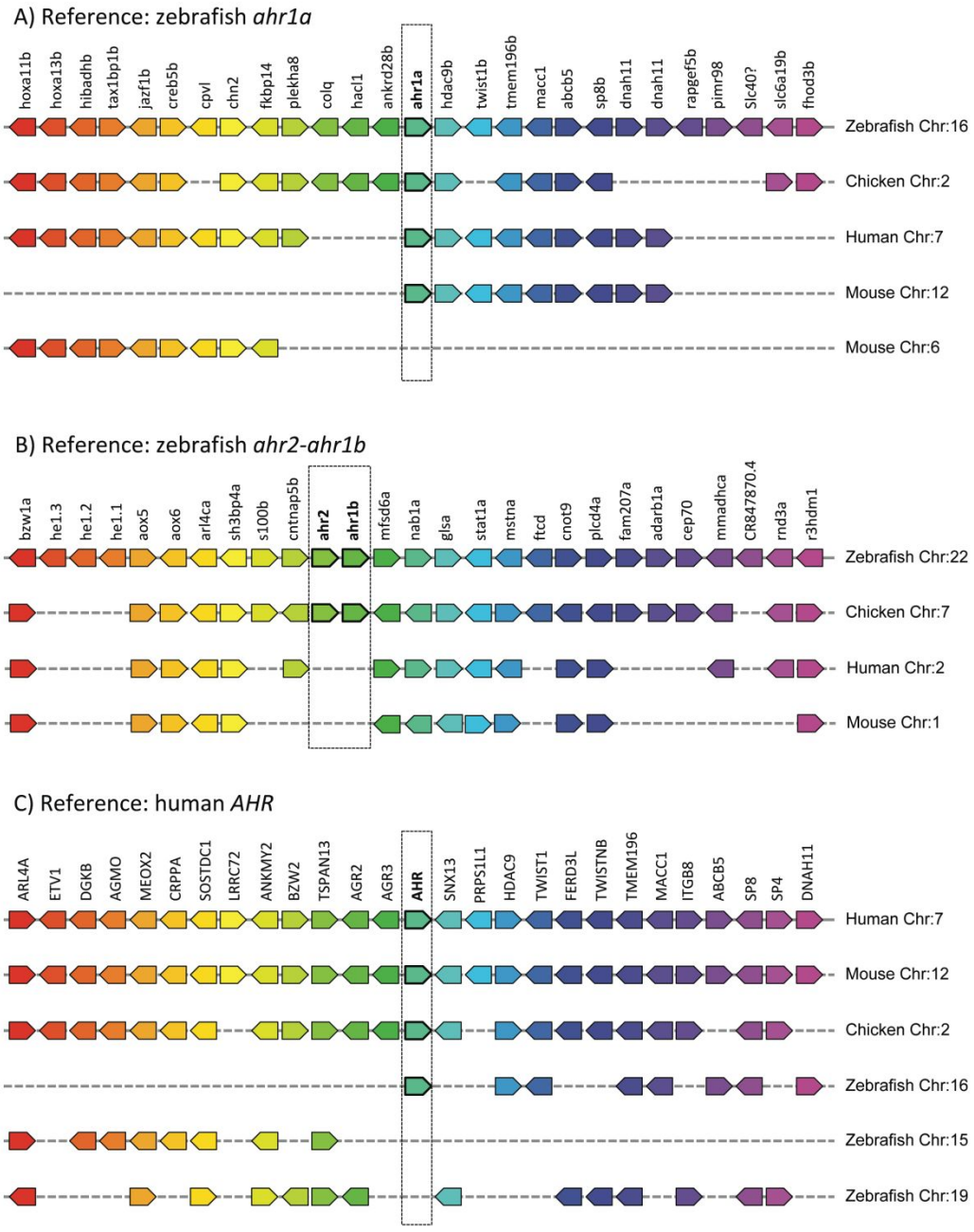

Figure 1. Shared synteny between zebrafish and other vertebrate *AHR* genes. Shared synteny was analyzed using *Genomicus* (versions 93.0 and 100.01) (Muffato et al. 2010; Nguyen et al. 2018) with manual curation using *Ensembl*. The AlignView tool in *Genomicus* was used to visualize the syntenic relationships. Genomes for human (*Homo sapiens*), mouse (*Mus musculus*), and chicken (*Gallus gallus*) were used to illustrate syntenic relationships with the chromosomes containing three zebrafish *ahr* genes. The panels show the shared synteny obtained when using **A)** zebrafish *ahr1a* (chromosome 16), **B)** zebrafish *ahr2-ahr1b* (chromosome 22), and **C)** human *AHR* (chromosome 7) as reference genes. The genes on either side of the reference gene are shown in the correct order and orientation. Orthologs (or in some cases paralogs) of the reference gene and its flanking genes are shown in the same color, below the reference chromosome, organized by species and chromosome. The position and order of genes below the reference chromosome do not necessarily reflect their position and order on the indicated chromosome.

Tables

Table 1. Zebrafish AHR genes and their respective translation products.

Characteristic	AHR2	AHR1a	AHR1b
Zebrafish chromosome/linkage group	22	16	22
Mammalian orthologs	--	AHR	--
Amino acid length	1027 aa	805 aa	954 aa
Predicted molecular mass of protein	113 kDa	90.4 kDa	104.8 kDa
Overall amino acid identity comparison with human AHR	51 %	52 %	67 %
Amino acid identity comparison with human AHR ligand binding domain	71 %	68 %	71 %
Overall amino acid identity comparison with AHR1b	45 %	44 %	100 %
Conserved N-terminal halves identity comparison with AHR1b protein	66 %	63 %	100 %

Table 2. Receptor characteristics (developmental baseline and TCDD-induced mRNA expression, endogenous ligands and roles, and binding partners) of AHR2, AHR1a, and AHR1b. See text for citations.

Characteristic	AHR2	AHR1a	AHR1b
Earliest detected expression	**5 hpf** (Andreasen et al. 2002b; Tanguay et al. 1999)	**24 hpf** (Andreasen et al. 2002a; Karchner et al. 2005)	**24 hpf** (Karchner et al. 2005)
mRNA localization during development	**Several regions including the head and trunk** (Andreasen et al. 2002b; Sugden et al. 2017)	**Liver at 52 hpf** (Sugden et al. 2017)**, regenerating fin** (Sugden et al. 2017)	**Developing eye** (Karchner et al. 2017; Sugden et al. 2017)
mRNA localization in adults	**Brain, heart, muscle, swim bladder, liver, gill, skin, eye, kidney, fin** (Andreasen et al. 2002a)	**Brain** (Webb et al. 2009)**, liver, heart, swim bladder, and kidney** (Andreasen et al. 2002a)	**Unknown**
Effect of TCDD exposure on mRNA expression	**Increase in expression** (Andreasen et al. 2002b; Garcia et al. 2018a; Karchner et al. 2005; Tanguay et al. 1999)	**Increase in expression** (Andreasen et al. 2002a; Karchner et al. 2005)	**No change in expression** (Karchner et al. 2005; Ulin et al. 2019)
Endogenous roles	**Several at both embryonic/larval and adult life stages** (See Section 1.2.)	**Possible roles in hypocretin/orexin signaling** (Seifinejad et al. 2019)	**Crosstalk detected with NRF signaling** (Ulin et al. 2019)
Known endogenous ligands	**FICZ** (Jonsson et al. 2009; Wincent et al. 2016), **3α,5α-tetrahydrocorticosterone and 3α,5β-tetrahydrocorticosterone (5α- and 5β-THB)** (Wu et al. 2019)	**None identified**	**FICZ** (Jonsson et al. 2009)
Endogenous Cyp1a expression regulation	**Expression in the developing zebrafish eye but not in the trunk or brain** (Sugden et al. 2017)	**None**	**None**
***In vitro* binding with ARNTs**	**ARNT1b, 1c, 2b, 2c** (Prasch et al. 2006; Tanguay et al. 2000)	**ARNT2b** (Andreasen et al. 2002a)	**ARNT2b** (Karchner et al. 2005)
***In vitro* binding with TCDD**	**Yes** (Prasch et al. 2006; Tanguay et al. 2000)	**No** (Andreasen et al. 2002a; Karchner et al. 2005)	**Yes** (Karchner et al. 2005)
***In vitro* transactivation activity with TCDD**	**Yes** (Prasch et al. 2006; Tanguay et al. 2000)	**Not applicable**	**Yes but less sensitive than AHR2** (Karchner et al. 2005)
ARNT required for *in vivo* activation	**ARNT1** (Antkiewicz et al. 2006; Prasch et al. 2004; Prasch et al. 2006)	**Not applicable**	**Unknown**

Table 3. Predicted binding of different ligands to the zebrafish AHRs.

Ligand	AHR2	AHR1a	AHR1b	References
Anthracene	Yes	Not tested	Not tested	(Goodale et al. 2015)
Anthrone derivative SP600125	Yes	Not tested	Not tested	(Goodale et al. 2015)
BAA	Yes	Not tested	Not tested	(Goodale et al. 2015)
BaP	Yes	Not tested	Not tested	(Goodale et al. 2015)
BEZO	Yes	Not tested	Not tested	(Goodale et al. 2015)
CH223191	Yes	Yes	Weak	(Gerlach et al. 2014)
Leflunomide	Yes	Yes	Yes	(Bisson et al. 2009; Goodale et al. 2012; O'Donnell et al. 2010)
***ortho*-mITP**	Yes	No	No	(Gerlach et al. 2014)
***meta*-mITP**	Yes	Yes	Weak	(Gerlach et al. 2014)
***para*-mITP**	Weak	Yes	No	(Gerlach et al. 2014)
TCDD	Yes	No	Yes	(Bisson et al. 2009)
NPAHs, HAHs, amino PAHs	Yes	Yes	Yes	(Chlebowski et al. 2017)

Table 4. Developmental toxicity endpoints and CYP1A expression patterns mediated by AHR2 from morpholino knockdown studies.

Xenobiotic ligand	Endpoints mediated by ligand *x* AHR2	Results of AHR2 knockdown	References
	Polycyclic Aromatic Hydrocarbons (PAHs)		
Pyrene	- Pericardial edema, cell death in the neural tube, anemia - CYP1A protein expression	- Prevention of morphological effects - Reduced vasculature expression	(Incardona et al. 2005)
Chrysene	- CYP1A protein expression	- Prevention of epidermal expression - *No change to vasculature expression*	(Incardona et al. 2005)
Dibenzothiophene	- CYP1A protein expression	- Reduction of weak vascular expression - *No change to cardiotoxicity*	(Incardona et al. 2005)
Phenanthrene	- CYP1A protein expression	- Reduction of weak vascular expression - *No change to cardiotoxicity*	(Incardona et al. 2005)
Benz[a]anthracene (BAA)	- Pericardial edema - Intracranial hemorrhage - CYP1A protein expression	- Reduction in prevalence of pericardial edema - Prevention - Reduction in ventricular myocardium and epidermis - *No reduction in endocardial and other vascular endothelial* CYP1A *protein induction*	(Incardona et al. 2006; Incardona et al. 2011)
Benzo[a]pyrene (BaP)	- Pericardial edema - CYP1A protein expression Larval hyperactive swimming response	- Partial protection - Loss of myocardial expression - *No reduction in endocardial expression* - Decreased larval swimming response	(Cunha et al. 2020; Incardona et al. 2011; Knecht et al. 2017b)
Benzo[k]fluoranthene (BkF)	- CYP1A protein expression	- Markedly reduced epidermal CYP1A expression - *No reduction in endocardial expression or pericardial edema*	(Incardona et al. 2011)

Retene	- Pericardial edema - CYP1A protein expression	- Prevalence and severity of pericardial edema lower - Reduced epidermal expression - *No change to vascular expression*	(Scott et al. 2011)
Benz(a)anthracene-7,12-dione (7,12-B[a]AQ)	- Morphology malformations: axis, trunk, brain, yolk and pericardial edemas, circulation, eye and jaw malformations - CYP1A protein expression	- Prevention - Prevention of vasculature expression	(Goodale et al. 2015; Knecht et al. 2013)
1,9-benz-10-anthrone (BEZO)	- Morphology malformations: axis, trunk, brain, yolk and pericardial edemas, circulation, eye and jaw malformations	- Prevention	(Goodale et al. 2015)
1,6-dinitropyrene,	- CYP1A protein expression	- Reduction in skin and vasculature	(Chlebowski et al. 2017)
benzo[j]fluoranthene, dibenzo[a.h]pyrene, dibenzo[a,i]pyrene, and benzo[b]fluoranthene	- CYP1A protein expression	- Reduction in vasculature and prevention of skin expression	(Shankar et al. 2019)
6H-benzo[cd]pyren-6-one	- Cardiotoxicity	- Prevention	(Cunha et al. 2020)
Other chemicals: mixtures, pharmaceuticals, indoles, and halogenated aromatic hydrocarbons			
PAH-containing soil extracts from a gasworks, a former wood preservation site, and a coke oven	- Edema and viability	- Decrease in edema occurrence, and increase in viability	(Wincent et al. 2015)
Total particulate matter (cigarette smoke)	- Several morphological endpoints, viability, hatching success	- Reduction in morphological endpoints, increased viability and hatching success	(Massarsky et al. 2016)

Environmentally relevant PAH mixtures ("Supermix 3" and "Supermix 10")	- CYP1A protein expression	- Supermix 3: decrease invasculature expression and increase in liver expression Supermix 10: persistence of both vasculature and liver expression	(Geier et al. 2018b)
BaP and 6H-benzo[cd]pyren-6-one	- Cardiotoxicity (pericardial edema and string heart formation)	- Prevention	(Cunha et al. 2020)
Cardiosulfa (sulfanomide drug)	- Cardiotoxicity	- Reduction	(Ko et al. 2009; Ko and Shin 2012)
Leflunomide	- CYP1A protein expression	Reduction	(Goodale et al. 2012; O'Donnell et al. 2010)
3,3',4,4',5-pentachlorobiphenyl (PCB-126)	- Cardiotoxicity and mortality	- Reduction	(Garner et al. 2013; Jonsson et al. 2007)
Mono-substituted isopropyl triaryl phosphate (mITP) (a major component of Firemaster 550, a flame retardant mixture)	- CYP1A protein expression	- Reduction	(Gerlach et al. 2014)
Paclobutrazol (fungicide)	- Digestive tract toxicity	- Reduction	(Wang et al. 2015)
Formylindolo[3,2-b]carbazole (FICZ)	- Mortality, pericardial edema, circulatory failure	- Prevention	(Wincent et al. 2016)
Phenanthroline	- PAH-like toxicity	- Reduction	(Ellis and Crawford 2016)
2,7-dibromocarbazole and 2,3,6,7-tetrachlorocarbazole	- TCDD-like developmental toxicity	- Reduction	(Fang et al. 2016)
3α,5α-tetrahydrocorticosterone (5α-THB)	- Enhanced larval locomotor activity	- Blocked enhancement - *AHR2 knockdown did not block 5β-THB-induced enhanced larval locomotor activity*	(Wu et al. 2019)
2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)	- Developmental toxicity (multiple endpoints)	- Prevention - *No change to inhibition of swim bladder inflation and mortality*	See text

Table 5. Developmental toxicity endpoints and CYP1A **expression patterns mediated by AHR1a from morpholino knockdown studies.**

Xenobiotic ligand	Endpoints mediated by ligand *x* AHR1a	Results of AHR1a knockdown	References
Chrysene	- Vasculature CYP1A expression	- Reduction - *No change to epidermal* CYP1A *expression*	(Incardona et al. 2005)
Pyrene	- Liver abnormalities, pericardial edema, neural tube cell death	- Reduction - *No change to dorsal curvature caused by pyrene exposure*	(Incardona et al. 2006)
Pyrene	- Liver CYP1A expression	- Reduction - *No change to vascular* CYP1A *expression*	(Incardona et al. 2006)
Leflunomide	- Liver CYP1A expression	- Reduction	(Goodale et al. 2012)
BkF + FL, PCB-126	- Pericardial effusion - CYP1A activity	- Greater pericardial effusion - Increased activity	(Garner et al. 2013)
Xanthone	- Several developmental endpoints - Liver CYP1A expression	- Attenuated malformations - Reduction	(Knecht et al. 2013)
mITP	- Pericardial edema	- Increased prevalence	(Gerlach et al. 2014)
5-nitroacenaphthalene	- Pericardial and yolk sac edemas	- Reduction	(Chlebowski et al. 2017)
5-nitroacenaphthalene, 9-nitrophenanthrene, and 7-nitrobenzo[k]fluoranthene	- Liver CYP1A expression	- Reduction	(Chlebowski et al. 2017)

Table 6. Developmental toxicity endpoints and CYP1A expression patterns mediated by AHR1b from morpholino knockdown studies.

Xenobiotic ligand	Endpoints mediated by ligand *x* AHR1b	Results of AHR1b knockdown	References
Leflunomide	- Vasculature CYP1A expression	- Prevention	(Goodale et al. 2012)
mITP	- Pericardial edema	- Reduction in prevalence of pericardial edema	(Gerlach et al. 2014)
7-nitrobenzo[k]fluoranthene	- Vasculature, skin, neuromast CYP1A protein expression	- Slight reduction	(Chlebowski et al. 2017)

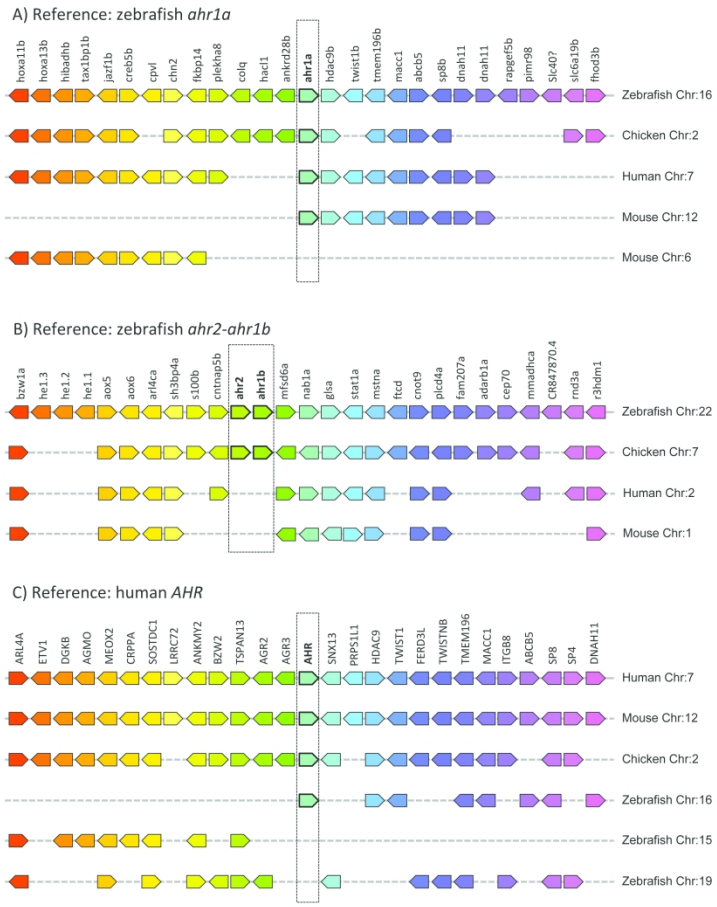

Figure 1. Shared synteny between zebrafish and other vertebrate AHR genes. Shared synteny was analyzed using Genomicus (versions 93.0 and 100.01) (Muffato et al. 2010; Nguyen et al. 2018) with manual curation using Ensembl. The AlignView tool in Genomicus was used to visualize the syntenic relationships. Genomes for human (Homo sapiens), mouse (Mus musculus), and chicken (Gallus gallus) were used to illustrate syntenic relationships with the chromosomes containing three zebrafish ahr genes. The panels show the shared synteny obtained when using A) zebrafish ahr1a (chromosome 16), B) zebrafish ahr2-ahr1b (chromosome 22), and C) human AHR (chromosome 7) as reference genes. The genes on either side of the reference gene are shown in the correct order and orientation. Orthologs (or in some cases paralogs) of the reference gene and its flanking genes are shown in the same color, below the reference chromosome, organized by species and chromosome. The position and order of genes below the reference chromosome do not necessarily reflect their position and order on the indicated chromosome.