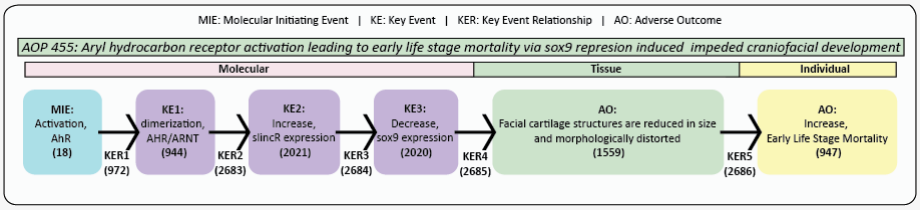
AOP ID and Title:

AOP 455: Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development
Short Title: Ahr mediated early stage mortality via craniofacial malformations

Graphical Representation



Authors

Prarthana Shankar, Ph.D., US EPA Mid-Continent Ecology Division, Duluth, MN, USA (pshankar@usgs.gov)

Dan Villeneueve, Ph.D., US EPA Mid-Continent Ecology Division, Duluth, MN, USA (villeneuve.dan@epa.gov)

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Abstract

The Aryl Hydrocarbon Receptors (Ahrs) are evolutionarily conserved ligand-dependent transcription factors that are activated by structurally diverse endogenous compounds as well as environmental chemicals such as polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons. Ahr activation leads to several transcriptional changes that can cause developmental toxicity resulting in mortality. Evidence was assembled and evaluated for a novel adverse outcome pathway (AOP) which describes how Ahr activation (molecular initiating event; MIE) can lead to early-stage mortality (adverse outcome; AO), via *SOX9*-mediated craniofacial malformations. Using a key event relationship (KER)-by-KER approach, we collected evidence using both a narrative search, and through systematic review based on detailed search terms. Weight of evidence for each KER was assessed to inform overall confidence of the AOP. The AOP links to previous descriptions of Ahr activation (ex: AOPs 21 and 150), and connect them to two novel key events (KEs), increase in *slincR* expression, a newly characterized long non-coding RNA with regulatory functions, and suppression of *SOX9*, a critical transcription factor implicated in chondrogenesis and cardiac development. In general, confidence levels for KERs ranged between medium and strong, with few inconsistencies, as well as several opportunities for future research identified. While majority of the KEs have only been demonstrated in zebrafish with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as an Ahr activator, evidence suggests that the two AOPs likely apply to most vertebrates, and many Ahr activating chemicals. Addition of the AOP into the AOP-Wiki helps expand the growing Ahr-related AOP network to nineteen individual AOPs, of which six are endorsed or in progress, and the remaining 13 relatively underdeveloped.

Background

<<<<<The key events (KEs) associated with AOPs 455 and 456 are predominantly similar, with the exception of KE4 in each AOP. KE4 in AOP 455 is designated an AO and is Event 1559: “Facial cartilage structures are reduced in size and morphologically distorted”, and KE4 in AOP 456 is Event 317: “Altered, Cardiovascular development/function.” While AOP 456 may be of higher biologically relevance, both AOPs are ecologically important and contribute significantly to the growing network of AOPs beginning with the activation of the Aryl hydrocarbon receptor (Ahr). Since both AOPs have several overlapping KEs, some redundant text is to be expected in the individual AOP-Wiki pages.>>>>>

The Aryl Hydrocarbon Receptor (Ahrs) are evolutionarily conserved ligand-dependent transcription factors that can be activated by a wide range of structurally diverse compounds (Denison and Nagy 2003; Hahn et al. 2017). The Ahrs have critical physiological roles in normal development of both vertebrates and invertebrates, and several endogenous Ahr ligands, such as retinoic acid and metabolites of tryptophan, have been identified (Esteban et al. 2021; Nguyen and Bradfield 2008). In addition, Ahr activation by environmental pollutants including halogenated aromatic hydrocarbons (HAHs), polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) can lead to a variety of adverse health effects, such as dysfunction to the immune, reproductive, and cardiovascular systems (Hansen et al. 2014; Hernandez-Ochoa et al. 2009; Stevens et al. 2009; Zhang 2011), as well as improper development and neurobehavior (Garcia et al. 2018a). Ahr activation is also associated with tumor promotion and carcinogenesis (Safe et al. 2013). Several studies in model organisms such as zebrafish and rodents have shown that Ahr-deficient animals in gene knock-out studies have either diminished or no harmful effects from exposure to Ahr activating environmental pollutants (Fernandez-Salguero et al. 1996; Garcia et al. 2018a; Goodale et al. 2015; Harrill et al. 2016), highlighting the significance of the receptors in mediating toxicity of Ahr-active chemicals.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a bioaccumulative and highly toxic HAH, is typically used as the prototypical molecular probe to investigate Ahr-related outcomes and is thus one of the most thoroughly investigated of the known Ahr agonists. One notable difference between dioxins such as TCDD, and labile PAHs for example, is that TCDD exposure leads to prolonged and continuous receptor activation, which is different from PAH-induced transient receptor activation that is generally considered an adaptive response. However, significant dioxin-like toxicity, including generation of oxidative stress, has been demonstrated in several organisms exposed to PAHs. Toxicity is generally attributed to the generation of harmful reactive metabolites, or from environmentally relevant chronic PAH exposures that can induce sustained Ahr activation (Billiard et al. 1999). Further, any differences in Ahr-dependent toxicity among species is likely because of the presence of multiple Ahr

isoforms, in combination with their differential binding affinities to a specific chemical (Doering et al. 2013; Doering et al. 2018; Karchner et al. 2006). Regardless, it is widely accepted that upon activation of the Ahrs, a cascade of complex molecular events ensues, leading to crosstalk signaling and pathophysiological effects. While several possible lesser-understood signaling pathways exist (Sondermann et al. 2023; Wright et al. 2017), the most widely described and major signaling route is the canonical Ahr signaling pathway.

Canonical Ahr signaling involves the conversion of the inactive Ahr, which is present in the cytoplasm, to its active form that can translocate to the nucleus and dimerize with the Ahr nuclear translocator (ARNT) (Wright et al. 2017). The Ahr-ARNT heterodimer can consequently regulate transcription of several downstream genes either indirectly, or directly, which is the case for the cytochrome P450s (*CYPs*) that are induced via the direct binding of the heterodimer to the aryl hydrocarbon response elements (Ahres, or XREs or DREs) (Lo and Matthews 2012). To help organize the complexity of the concurrent regulation of 1000s of genes by the Ahr signaling pathway, as well as consequent toxicity effects, scientists have begun to organize existing evidence in the form of Adverse Outcome Pathways (AOPs) (Ankley et al. 2010) and AOP networks (Knapen et al. 2018). There are currently nineteen Ahr-related AOPs in the AOP-Wiki (as of April 10th, 2023; aopwiki.org), with six AOPs included in The Organization for Economic Co-operation and Development's (OECD) Work Plan that are open for comments, and the remaining 13 relatively under developed. With the rapid rate at which new research on Ahr-mediated toxicity is being conducted, there is still extensive scope for assembly of existing and novel biological data into actionable knowledge that can support decision-making around Ahr-related environmental effects and disease outcomes.

Besides being highly relevant and important toxicity phenotypes in both humans and other vertebrates, both craniofacial malformations and cardiovascular toxicity are easily observable and measurable in zebrafish, and have been identified upon exposure to various Ahr activating environmental chemicals (Antkiewicz et al. 2005; Henry et al. 1997; Li et al. 2014). Importantly, developing zebrafish exposed to TCDD have severe heart and vasculature malformations, in addition to jaw structure impairments that occur secondarily to inhibited chondrogenesis (Carney et al. 2006). One of the genes whose expression is most reduced in the jaw upon TCDD exposure in zebrafish is ***sox9b, sry-box containing gene 9b*** (Xiong et al. 2008). This gene, one of two zebrafish paralogs of the *SOX9* gene, is a critical transcription factor that has been implicated in several processes including chondrogenesis and cardiac development, in addition to skeletal development, male gonad genesis, and cancer progression (Lefebvre and Dvir-Ginzberg 2017; Panda et al. 2021). Based on current knowledge, primarily from developmental zebrafish studies, it is apparent that there are strong relationships between Ahr, SOX9, and craniofacial (AOP 455) or cardiovascular (AOP 456) malformations that can be causally linked in an AOP network.

The two AOPs also provide weight of evidence for the inclusion of a nove**long non-coding RNA (lncRNA)** as a key event. LncRNAs are transcripts longer than 200 nucleotides that do not encode functional proteins, but have their own promoters and the ability to be processed (spliced and polyadenylated) similar to mRNAs (Mattick et al. 2023). The nature of lncRNAs is such that they have diverse functions and can regulate gene expression at multiple levels, including by interacting with DNA, RNA, proteins, and altering transcription of both neighboring and distant genes (Statello et al. 2021). Importantly, there is growing recognition for the link between exposure to chemicals, differential expression profiles of lncRNAs, and consequent toxicity (Dempsey and Cui 2017). Specific to the proposed AOPs, evidence suggests an important role for the recently discovered lncRNA, "sox9b long intergenic non-coding RNA" (*slincR*) in the Ahr signaling toxicity pathway via its interaction with the transcription factor, SOX9 (Garcia et al. 2017). Thus, the ability of SOX9 to interact with Ahr signaling, paired with its functional versatility, implicates it as a critical player in the Ahr toxicity pathway, by mediating disruptions to both craniofacial and cardiovascular development.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	18	Activation, AhR	Activation, AhR
2	KE	944	dimerization, AHR/ARNT	dimerization, AHR/ARNT
3	KE	2021	Increase, slincR expression	Increase, slincR expression
4	KE	2020	Decrease, sox9 expression	Decrease, sox9 expression
6	AO	947	Increase, Early Life Stage Mortality	Increase, Early Life Stage Mortality
5	AO	1559	Facial cartilage structures are reduced in size and morphologically distorted	Smaller and morphologically distorted facial cartilage structures

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation, AhR	adjacent	dimerization, AHR/ARNT	High	Moderate
dimerization, AHR/ARNT	adjacent	Increase, slincR expression	Moderate	Moderate
Increase, slincR expression	adjacent	Decrease, sox9 expression	Moderate	Moderate
Decrease, sox9 expression	adjacent	Facial cartilage structures are reduced in size and morphologically distorted	High	Low

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Facial cartilage structures are reduced in size and morphologically distorted	adjacent	Increase, Early Life Stage Mortality	Low	Low
Activation, AhR	non-adjacent	Decrease, sox9 expression	Moderate	Low
Increase, slincR expression	non-adjacent	Facial cartilage structures are reduced in size and morphologically distorted	Moderate	Moderate
Activation, AhR	non-adjacent	Increase, Early Life Stage Mortality	High	Moderate
Activation, AhR	non-adjacent	Facial cartilage structures are reduced in size and morphologically distorted	High	High

Stressors

Name	Evidence
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	

Overall Assessment of the AOP

See details below.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
mouse	Mus musculus	Low	NCBI
human	Homo sapiens	Low	NCBI
Sebastiscus marmoratus	Sebastiscus marmoratus	Low	NCBI
Salmo salar	Salmo salar	Low	NCBI
chicken	Gallus gallus	Low	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage and Sex

The relationships between Ahr, Arnt, *slincR* and *sox9b*, and cardiac and craniofacial malformations have been well established in developing zebrafish, specifically as embryos, and thus sex is not a relevant parameter.

Taxonomic

Evidence gathered suggests that the domain of applicability covers most vertebrates, from fish to humans and other wildlife. It is important to highlight that while all the relationships within the AOP, except the link between craniofacial malformations and early life stage mortality, have been observed definitively in one species (*Danio rerio*), there is strong evidence for specific KERs in species other than zebrafish. For example, Ahr's evolutionarily conserved role as a master regulator of toxicity of several environmental pollutants has been shown in animals including fish, birds, rodents, and humans (Hahn et al. 2017). Another example is SOX9's highly conserved role as a critical transcriptional factor in craniofacial and cardiac development in animals such as fish, rodents, and amphibians (Garside et al. 2015; Lee and Saint-Jeannet 2011). While there is strong evidence for Ahr activation leading to *sox9b* repression in developing zebrafish, this relationship has been identified in other fish species such as white sturgeon and Atlantic salmon (Doering et al. 2016; Olufsen and Arukwe 2011). Additionally, while *slincR* has only been described in zebrafish so far, it is worth noting that putative mouse and human orthologs have been identified (Garcia et al. 2018b), increasing the possibility that the zebrafish-specific results can be translated to other organisms. The formation of craniofacial structures is a predominantly evolutionarily conserved dynamic and complex process that begins early in embryonic development (Helms et al. 2005; Kuratani 2005). Consequently, craniofacial development can be thought of as a potential target of disruption in early embryos, and an AOP network around this key event would be highly relevant to both humans and general wildlife. Thus, the different lines of evidence suggest that the taxonomic domain of applicability for the two proposed AOPs can likely cover most vertebrates.

Essentiality of the Key Events

Direct evidence for the essentiality of several of the key events in the AOP has been provided by gene modification and knockout studies of the Ahr, *slincR*, and *sox9b* (one of two orthologs of *SOX9*) genes primarily in zebrafish. Highlights of the most important studies are provided here:

Event ID	Key Event	Evidence	Essentiality/Assessment
18	Activation, AhR	Strong	1. Several studies in model organisms such as zebrafish and rodents have shown that Ahr-deficient animals in gene knock-out studies have either diminished or completely nonexistent harmful effects of both TCDD and several PAHs, including craniofacial defects (Fernandez-Salguero et al. 1996; Garcia et al. 2018a; Goodale et al. 2015; Harrill et al. 2016). 2. Ahr2 knock-out in zebrafish with 1ng/mL TCDD exposure had significantly diminished slincR expression at 48 hpf (Garcia et al. 2017). 3. Ahr2 knockout zebrafish with 1ng/mL TCDD exposure did not have significantly reduced sox9b expression at 48 hpf (Garcia et al. 2018a).
944	Dimerization, AHR/ARNT	Strong	Canonical Ahr signaling involves the conversion of the inactive Ahr, which is present in the cytoplasm, to its active form that can translocate to the nucleus and dimerize with the Ahr nuclear translocator (ARNT) (Wright et al. 2017). Evidence suggests that the Ahr/ARNT heterodimer can consequently regulate gene expression within the Ahr signaling cascade.
2021	Increase, slincR expression	Strong	1. When slincR expression is knocked down using a morpholino, normal sox9b expression levels and spatial pattern are altered during zebrafish development (Garcia et al. 2017). Specifically, in slincR morphants exposed to DMSO or TCDD, sox9b expression was significantly higher than in control morphant zebrafish. 2. When slincR expression is knocked down using a morpholino, several downstream target genes of sox9b, such as, notch3, adamts3, fabp2, sfrp2, and fgfr3 were altered in their gene expression compared to control morphants (Garcia et al. 2017). 3. While both control and slincR morphant zebrafish exposed to TCDD displayed cartilage structure defects, the slincR morphants had an abnormal junction between hyosymplectic and ceratohyal cartilages in comparison to the control morphants (Garcia et al. 2018b), suggesting slincR's role in the craniofacial malformation caused due to TCDD exposure.
2020	Decrease, sox9 expression	Strong	1. Sox9b morpholino knockdown of zebrafish led to severe jaw malformations by 72hpf – the meckel's, palatoquadrate, and the ceratohyal cartilage structures in sox9b morphants had the same defects as when zebrafish are exposed to the Ahr activating chemical, TCDD (Xiong et al. 2008). 2. Sox9a morpholino knockdown as well as CRISPR-Cas9 knockout in zebrafish has implicated sox9a as necessary for normal cartilage development (Koskinen et al. 2009; Lin et al. 2021). 3. Investigations into the mutations in the human sox9 coding sequence have identified two novel deletions in the upstream region associated with pierre robin sequence (PRS), characterized by severe jaw malformations and clefting (Gordon et al. 2014).

1559	Facial cartilage structures are reduced in size and morphologically distorted	Low	1. Studies in developing zebrafish and mummichog (*Fundulus heteroclitus*) have found that low concentrations of TCDD or PCB126 exposure can lead to subtle malformations in the lower jaw, in addition to reduced feeding capabilities of the fish (Couillard et al. 2011; King Heiden et al. 2009). However, both studies observed a reduction in feeding even in the fish that did not display jaw malformations, consequently, reduced feeding was not directly linked to an inability to capture prey due to the craniofacial deformity. Overall, it is likely that a combination of different malformations (ex: effects on both the heart and jaw) contribute to Ahr activation-induced mortality 2. These results are corroborated by an evaluation of TCDD toxicity in seven fish species, where despite the observation of craniofacial malformations in all species, TCDD toxicity, including mortality, decreased once exogenous feeding began suggesting the lack of a strong causal link between craniofacial malformations and poor survival (Elonen et al. 1998).
947	Increase, Early Life Stage Mortality	N/A	This is the terminal key event in the AOP and hence its essentiality for downstream events cannot be evaluated.

Weight of Evidence Summary

Biological Plausibility

- Ahr – strong : Strongest Biological Plausibility evidence for AOPs 455 and 456 comes from our extensive understanding of the Ahr signaling pathway in multiple different organisms. The functional roles of Ahr and its binding partners, including ARNT, have been well-studied (Fujii-Kuriyama and Kawajiri 2010), and it is well known that the Ahr signaling pathway mediates a variety of physiological and toxicological functions (Larigot et al. 2018).
- slincR and sox9 – strong: Strong evidence for the Biological Plausibility of*slincR* having a role in AOPs 455 and 456 comes from the nature of lncRNAs which is such that they have diverse functions and can regulate gene expression at multiple levels, including by interacting with DNA, RNA, proteins, and altering transcription of both neighboring and distant genes (Statello et al. 2021). Additionally, slincR (in situ hybridization) and sox9b (immunohistochemistry for sox9b-eGFP) are expressed in adjacent and overlapping tissues through multiple stages of zebrafish development, such as in the eye, otic vesicle, and in the lower jaw (Garcia et al. 2017) providing one line of evidence for slincR being able to regulate sox9b gene expression. Further, a capture hybridization analysis of RNA targets (CHART) experiment in both DMSO- and TCDD-exposed 48 hpf zebrafish identified enrichment of slincR in the 5'UTR of the sox9b locus (Garcia et al. 2018b) pointing to possible interaction between slincR and sox9b.
- slincR and craniofacial development - strong: Across multiple stages of zebrafish development, slincR is expressed in the jaw/snout region, as well as in the eye and otic vesicle (Garcia et al. 2017). In addition, upon exposure to TCDD (a strong Ahr activating chemical), slincR expression increases in both the otic vesicle, as well as the lower jaw/snout region (Garcia et al. 2017). Knockdown of slincR expression in developing zebrafish also alters expression of sox9b, as well as certain downstream targets of sox9, such as notch3, adamts3, fabp2, sfrp2, and fgfr3 (Garcia et al. 2017). These are different lines of Biological Plausibility evidence for slincR being a mediator between Ahr activation and craniofacial/cartilage malformations.
- Sox9 and craniofacial development - strong: Strongest Biological Plausibility evidence comes from studies in multiple species showing the spatiotemporal expression of sox9 in the developing cartilage structures of the jaw suggesting possible role of sox9 in both craniofacial development and dysfunction. For example, in mice, sox9 mRNA is widely expressed in the condylar anlage and Meckel's cartilage (Shibata et al. 2006), and the sox9 protein in the tissue layer of secondary cartilage (Hirouchi et al. 2018; Zhang et al. 2013). Additionally, sox9 is expressed widely during palatogenesis (Nie 2006; Watanabe et al. 2016) and is also found in the temporomandibular joint of developing mice (TMJ) (Wang et al. 2011). There is some evidence for sox9 being expressed in the condyle cartilage, as well as the proliferative layer and in the chondrocytes of developing rats (Al-Dujaili et al. 2018; Rabie and Hägg 2002). Similarly, sox9 expression has been found in developing cartilage structures of rabbits, duck, quail, zebrafish and salmon, and opposum to name a few animals, increasing the strength of the biological plausibility of sox9 being involved in craniofacial development and consequently, the signaling mechanisms preceding craniofacial malformations.
- Craniofacial malformations and early-stage mortality - moderate: It is reasonable to infer that malformed jaw structure of animals in the wild could impact their feeding success, leading to reduced growth and possible early mortality. However, few studies have demonstrated the relationships between jaw malformations, reduced feeding, and mortality, especially in fish (Noble et al. 2012). Impacts on animals to capture prey can also lead to population-wide changes to both the predators and prey (Weis et al. 2001), constraining foraging patterns and thus recruitment success.

Dose Concordance

- Ahr activation leading to early life stage mortality has been well-studied. The KER page (https://aopwiki.org/relationships/984) has examples in difference species for empirical evidence for this relationship.
- Strongest evidence for dose concordance between Ahr activation, slincR induction, and sox9 repression comes from a developing zebrafish study that utilized TCDD as the Ahr activating chemical. The concentration-response experiment showed that cyp1a (biomarker for Ahr activation) and slincR expression increased in parallel as TCDD exposure concentration increased, and that cyp1a and slincR are induced at TCDD exposure concentrations lower than concentrations at which

sox9b is repressed (Garcia et al. 2018b).
    - Both cyp1a and slincR were significantly induced starting at 0.0625 ng/mL TCDD exposure.
    - Significant cyp1a (~log2FC = 6) and slincR (~log2FC = 2) inductions were detected at 0.0625 ng/mL TCDD, while significant sox9b repression (~log2FC = -1) was detected only at 0.5ng/mL TCDD.

- (Garcia et al. 2018b) also showed that with increasing concentrations of TCDD, the severity of overall developmental malformations, including pericardial edema (indicator of potential cardiotoxicity) and jaw malformations increased.

- Strong dose concordance has been determined between cardiovascular malformations and early life stage mortality (please see KER page: https://aopwiki.org/relationships/1567), however, to the best of out knowledge, no systematic effort has been performed to identify "dose concordance" evidence for the KER between craniofacial malformations and early life stage mortality.

Uncertainties, inconsistencies, data gaps

While we have listed out various possible uncertainties, inconsistencies, and data gaps in the respective KER pages, here we highlight the most important ones:

1. One possible inconsistency in the literature is that not all ARNT isoforms in a particular species (for example, zebrafish) are important for mediating *in vivo* toxicity (Prasch et al. 2004), and future research could help clarify the relative influence of the different Ahr binding partners. The most well-studied Ahr binding partner is ARNT and it does appear to be important for TCDD toxicity – hence it is included as a KE in AOPs 455 and 456.
2. While the relationships in AOPs 455 and 456 have been definitively shown with TCDD as the activating chemical, future research must investigate the KERs with other Ahr activators, such as PAHs and other HAHs. Similarly, future research in organisms other than zebrafish, will add significantly to the weight of evidence for AOPs 455 and 456.
3. One inconsistency comes from a study exposing 16 individual PAHs to developing zebrafish where none were associated with a significant decrease in *sox9b* expression, despite six inducing both *cyp1a* and *slincR* expression (Garcia et al. 2018b). It is possible that the PAHs that are rapidly metabolized (unlike TCDD) induce different gene expression changes upon Ahr activation, or that the *slincR*/*sox9b* gene expression alterations are tissue-specific and are thus unable to be resolved consistently in whole animal transcriptomic studies.
4. Morpholino knockdown of sox9b in zebrafish led to a significant increase in slincR expression suggesting that slincR and sox9b may share overlapping regulatory networks that is not fully understood (Garcia et al., 2018).
5. We note that slincR is not the only mechanism of regulation of sox9. Other studies have found evidence for different regulatory mechanisms of sox9, but the circumstances under which different pathways are turned on is still unknown (Dash et al., 2021; Fu et al., 2018). Thus we highlight that in the contact of AOPs 455 and 456, induction of slincR expression is likely a better biomarker of the AOP compared to reduction of sox9 expression.
6. Impact of absence of slincR has only been studied with morpholino knockdown experiments (Garcia et al., 2017; Garcia et al., 2018), which have two relevant drawbacks: 1. Inability to maintain slincR repression by 72 hpf since morpholinos are transient in nature, and 2. Incomplete functional knockout which prevents us from understanding the true impact of the absence of slincR. Future studies using CRISPR-Cas-generated knockout lines, for example, will help overcome both limitations.
7. Few studies have showed an opposite relationship between sox9 expression and the size of cartilage structures.
    a. Conditional knockout of setdb1 (histone methyltransferase) specifically in the murine Meckel's cartilage led to and enlargement of the cartilage structure as well as the proliferation of chondrocytes, however, sox9 expression was significantly repressed (Yahiro et al., 2017).
    b. Experimental unilateral anterior crossbite created in rats led to decreased ratio of the hypertrophic cartilage layer in the experiment group, which was evidence for obvious cartilage degradation. This was accompanied by induction of sox9 expression (Zhang et al., 2013b).
8. One recent zebrafish study using the CRISPR-Cas9 tool, demonstrated that sox9a but not sox9b was required for normal cartilage development (Lin et al., 2021). This is inconsistent with all previous research showing the importance of both sox9a and sox9b for cartilage development in zebrafish.

Quantitative Consideration

Strongest quantitative understanding for the AOPs 455 and 456 is between the MIE (Activation, Ahr) and the AO (Increase, Early Life Stage Mortality) and is described in detail in the KER page (Event 984; https://aopwiki.org/relationships/984). Additionally, for the halogenated aromatic hydrocarbons (HAHs), we have a moderate quantitative understanding of the binding affinity of the different chemicals to the Ahr which partially led to the widespread use of the toxic equivalency factor (TEF) concept for humans, fish, and other wildlife risk assessment (Van den Berg et al. 1998). On the other hand, models that currently exist for chemicals such as the polycyclic aromatic hydrocarbons (PAHs) are often considered oversimplified due to the possible differences in receptor binding affinity and consequent differential metabolism and toxicity (Billiard et al. 2008). Nevertheless, we highlight that TCDD has been identified as the prototypical stressor for both AOPs 455 and 456, and the TEF concept could be leveraged to determine total toxic equivalencies (TEQs) for dioxin-like chemicals based on the known concentrations at which TCDD can induce different key events of the AOPs.

The presence of two measurable gene expression events (*SOX9* and *slincR*) as well as easily observable zebrafish toxicity phenotypes in AOPs 455 and 456 has given opportunity for the beginning of our quantitative understanding of the pathways. Garcia et al (Garcia et al. 2018b) conducted a TCDD concentration-response experiment (0 – 1.0 ng/mL) in developing zebrafish and determined that after just 1 h of exposure at 6 hpf, the number of zebrafish with malformations in the developing jaw and pericardial edema was statistically significant at 0.25 ng/mL TCDD. The study also measured *cyp1a*, *slincR*, and *sox9b* expression, and showed significant *cyp1a* (a measure of Ahr activation) and *slincR* induction from 0.0625 ng/mL, and a trend for *sox9b* repression from 0.125 ng/mL which was significant from 0.5 ng/mL TCDD exposure compared to the DMSO vehicle control. Additionally, *slincR* morpholino knockdown which reduced *slincR* expression by 98% in control animals, and by 81% in TCDD-exposed zebrafish compared to their respective control morphants (Garcia et al. 2017) significantly altered *sox9b* spatial and quantitative expression (Garcia et al. 2017), as well as had impacts on both craniofacial development and the cardiovascular system of developing zebrafish (Garcia et al. 2018b). While this preliminary quantitative understanding between several of the relationships in the two AOPs is not available for other chemicals, taxonomic groups, or species, the TEF concept is still the most plausible and feasible method of risk assessment for dioxin-like chemicals even if they have broad species-specific responsiveness (Van den Berg et al. 1998).

Considerations for Potential Applications of the AOP (optional)

With the diversity of ligands that bind and activate the Ahrs, and the variety of biological and toxicological functions these receptors are involved in, AOPs describing different aspects of the Ahr signaling pathway could provide immense potential for cross-chemical and cross-taxa extrapolations. Additionally, the AOP networks can help prioritize the most relevant mechanistic data for regulatory decision making, while also identifying critical knowledge gaps for future research. Several *in vitro* and *in silico* assays are being leveraged to identify chemical structures that activate the Ahr (Larsson et al. 2018). A deeper understanding of the mechanisms of toxicity endpoints can not only help illuminate the specific conditions under which malformations might occur, but it can also provide phenotypic-specific genetic biomarkers, such as *slincR* and *SOX9 as well as VEGF and COX2.*These can be easily measured in short-term *in vivo* exposures as evidence for progression along an Ahr-mediated adverse outcome pathway. As such, both the current AOPs and the broader AOP network can support tiered and hypothesis directed testing strategies based on *in vitro* or *in silico* screening results. From an environmental monitoring standpoint, the novel AOPs provide one or more reliable effects-based indicators (ex: *slincR* or *SOX9*) that could serve as early warning signs before the onset of deformities or mortality. Assuming the biomarkers are conserved across species, which is likely the case for *slincR* and *SOX9*, gene expression measurements could also be used for predicting toxicant responses across a broad diversity of phylogenetic groups. Overall, the two proposed AOPs have the potential to: 1. Expand on the Ahr-related AOP network to gain a more comprehensive view of Ahr-related processes to support regulatory decisions, and 2. Integrate *in vivo* measures of gene expression response into the risk assessment paradigm for Ahr activating pollutants to enable extrapolations across both chemicals and taxa, while also identifying key differences between them.

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Appendix 1

List of MIEs in this AOP

Event: 18: Activation, AhR

Short Name: Activation, AhR

Key Event Component

Process	Object	Action
aryl hydrocarbon receptor activity	aryl hydrocarbon receptor	increased
aryl hydrocarbon receptor binding	aryl hydrocarbon receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:21 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2	MolecularInitiatingEvent
Aop:57 - AhR activation leading to hepatic steatosis	MolecularInitiatingEvent
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyria	MolecularInitiatingEvent
Aop:150 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF	MolecularInitiatingEvent
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	MolecularInitiatingEvent
Aop:151 - AhR activation leading to preeclampsia	MolecularInitiatingEvent
Aop:414 - Aryl hydrocarbon receptor activation leading to lung fibrosis through TGF-β dependent fibrosis toxicity pathway	MolecularInitiatingEvent
Aop:415 - Aryl hydrocarbon receptor activation leading to lung fibrosis through IL-6 toxicity pathway	MolecularInitiatingEvent
Aop:416 - Aryl hydrocarbon receptor activation leading to lung cancer through IL-6 toxicity pathway	MolecularInitiatingEvent
Aop:417 - Aryl hydrocarbon receptor activation leading to lung cancer through AHR-ARNT toxicity pathway	MolecularInitiatingEvent
Aop:418 - Aryl hydrocarbon receptor activation leading to impaired lung function through AHR-ARNT toxicity pathway	KeyEvent
Aop:419 - Aryl hydrocarbon receptor activation leading to impaired lung function through P53 toxicity pathway	KeyEvent
Aop:420 - Aryl hydrocarbon receptor activation leading to lung cancer through sustained NRF2 toxicity pathway	MolecularInitiatingEvent
Aop:439 - Activation of the AhR leading to metastatic breast cancer	MolecularInitiatingEvent
Aop:455 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	MolecularInitiatingEvent
Aop:456 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	MolecularInitiatingEvent
Aop:458 - AhR activation in the liver leading to Subsequent Adverse Neurodevelopmental Outcomes in Mammals	MolecularInitiatingEvent
Aop:494 - AhR activation leading to liver fibrosis	MolecularInitiatingEvent
Aop:459 - AhR activation in the thyroid leading to Subsequent Adverse Neurodevelopmental Outcomes in Mammals	MolecularInitiatingEvent
Aop:563 - Aryl hydrocarbon Receptor (AHR) activation causes Premature Ovarian Insufficiency via Bax mediated apoptosis	MolecularInitiatingEvent

Stressors

Name
Benzidine
Dibenzo-p-dioxin
Polychlorinated biphenyl
Polychlorinated dibenzofurans
Hexachlorobenzene
Polycyclic aromatic hydrocarbons (PAHs)

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebra danio	Danio rerio	High	NCBI

Term	Scientific Term	Evidence	Links
Gallus gallus	Gallus gallus	High	NCBI
Pagrus major	Pagrus major	High	NCBI
Acipenser transmontanus	Acipenser transmontanus	High	NCBI
Acipenser fulvescens	Acipenser fulvescens	High	NCBI
rainbow trout	Oncorhynchus mykiss	High	NCBI
Salmo salar	Salmo salar	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
Ambystoma mexicanum	Ambystoma mexicanum	High	NCBI
Phasianus colchicus	Phasianus colchicus	High	NCBI
Coturnix japonica	Coturnix japonica	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
Microgadus tomcod	Microgadus tomcod	High	NCBI
Homo sapiens	Homo sapiens		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The AHR structure has been shown to contribute to differences in species sensitivity to DLCs in several animal models. In 1976, a 10-fold difference was reported between two strains of mice (non-responsive DBA/2 mouse, and responsive C57BL/6 14 mouse) in CYP1A induction, lethality and teratogenicity following TCDD exposure[3]. This difference in dioxin sensitivity was later attributed to a single nucleotide polymorphism at position 375 (the equivalent position of amino acid residue 380 in chicken) in the AHR LBD[30][19][31]. Several other studies reported the importance of this amino acid in birds and mammals[32][30][22][33][34][35][31][36]. It has also been shown that the amino acid at position 319 (equivalent to 324 in chicken) plays an important role in ligand-binding affinity to the AHR and transactivation ability of the AHR, due to its involvement in LBD cavity volume and its steric effect[35]. Mutation at position 319 in the mouse eliminated AHR DNA binding[35].

The first study that attempted to elucidate the role of avian AHR1 domains and key amino acids within avian AHR1 in avian differential sensitivity was performed by Karchner *et al.*[22]. Using chimeric AHR1 constructs combining three AHR1 domains (DBD, LBD and TAD) from the chicken (highly sensitive to DLC toxicity) and common tern (resistant to DLC toxicity), Karchner and colleagues[22], showed that amino acid differences within the LBD were responsible for differences in TCDD sensitivity between the chicken and common tern. More specifically, the amino acid residues found at positions 324 and 380 in the AHR1 LBD were associated with differences in TCDD binding affinity and transactivation between the chicken (Ile324_Ser380) and common tern (Val324_Ala380) receptors[22]. Since the Karchner et al. (2006) study was conducted, the predicted AHR1 LBD amino acid sequences were been obtained for over 85 species of birds and 6 amino acid residues differed among species[14][37] . However, only the amino acids at positions 324 and 380 in the AHR1 LBD were associated with differences in DLC toxicity in ovo and AHR1-mediated gene expression in vitro[14][37][16]. These results indicate that avian species can be divided into one of three AHR1 types based on the amino acids found at positions 324 and 380 of the AHR1 LBD: type 1 (Ile324_Ser380), type 2 (Ile324_Ala380) and type 3 (Val324_Ala380)[14][37][16].

- Little is known about differences in binding affinity of AhRs and how this relates to sensitivity in non-avian taxa.
- Low binding affinity for DLCs of AhR1s of African clawed frog (*Xenopus laevis*) and axolotl (*Ambystoma mexicanum*) has been suggested as a mechanism for tolerance of these amphibians to DLCs (Lavine et al 2005; Shoots et al 2015).
- Among reptiles, only AhRs of American alligator (*Alligator mississippiensis*) have been investigated and little is known about the sensitivity of American alligator or other reptiles to DLCs (Oka et al 2016).
- Among fishes, great differences in sensitivity to DLCs are known both for AhRs and for embryos among species that have been tested (Doering et al 2013; 2014).
- Differences in binding affinity of the AhR2 have been demonstrated to explain differences in sensitivity to DLCs between sensitive and tolerant populations of Atlantic Tomcod (*Microgadus tomcod*) (Wirgin et al 2011).
    - This was attributed to the rapid evolution of populations in highly contaminated areas of the Hudson River, resulting in a 6-base pair deletion in the AHR sequence (outside the LBD) and reduced ligand binding affinity, due to reduces AHR protein stability.
- Information is not yet available regarding whether differences in binding affinity of AhRs of fishes are predictive of differences in sensitivity of embryos, juveniles, or adults (Doering et al 2013).

The AhR is a very conserved and ancient protein (95) and the AhR is present in human and mice (96–98)The AhR is present in human physiology and pathology. The AhR is highly expressed at several important physiological barriers such as the placenta, lung, gastrointestinal system, and liver in human (Wakx, Marinelli, Watanabe). In these tissues, the AhR is involved in both detoxication processes involving xenobiotic metabolizing enzymes such as cytochromes P450, and in immune functions translating chemical signals into immune defence pathways (Marinelli, Stobbe). Moreover, it has a regulatory role in human dendritic cells and myelination (Kado, Shackleford). The lung constitutes another barrier exposed to components of air pollution such as particles and hydrocarbons (air pollution, cigarette smoke). The AhR detects such hydrocarbons and protects the pulmonary cells from their deleterious effects through metabolization. The regulatory effect on blood cells of the AhR, balancing different related cell types, can be extended to the megakaryocytes and their precursors; indeed, StemRegenin 1 (SR1), an antagonist of the AhR increases the human population of CD34+CD41low cells, a fraction of very efficient precursors of proplatelets (Bock). The occurrence of a nystagmus has been subsequently diagnosed in humans bearing a AhR mutation (Borovok).

In human cancer, the AhR has either a pro or con tumor effect depending on the tissue, the ligand, and the duration of the activation (Zudaire, Chang, Litzenburg, Gramatzki, Lin, Wang). In human breast cancer, the AhR is thoughts to be responsible of its progression (Goode, Kanno, Optiz, Novikov, Hall, Subramaniam, Barhoover). In human mammary benign cells, Brooks et al. noted that a high level of AhR was associated with a modified cell cycle (with a 50% increase in population doubling time in cells expressing the AhR by more than 3-fold) and EMT including increased cell migration. Narasimnhan et al. found that suppression of the AhR pathway had a pro-tumorigenic effect in vitro (EMT, tumor migration) in triple negative breast cancer.

Many endogenous and exogenous ligands are present for the AhR in human (Optiz, Adachi, Schroeder, Rothhammer). Indoles, such as indole-3-carbinol or one of its secondary metabolites, 3-3'- Diindolylmethane, are degradation products found in cruciferous vegetables and characterized as AhR ligands (Ema, Kall, Miller) they are also inducers of the human and rat CYP1A1 (Optiz). FICZ is the most potent AhR ligand known to date: it has a stronger affinity than TCDD for the human AhR (TCDD Kd=0.48 nM/FICZ Kd=0.07 nM) (Coumoul).

Key Event Description

The AHR Receptor

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) superfamily and consists of three domains: the DNA-binding domain (DBD), ligand binding domain (LBD) and transactivation domain (TAD)[1]. Other members of this superfamily include the AHR nuclear translocator (ARNT), which acts as a dimerization partner of the AHR [2][3]; Per, a circadian transcription factor; and Sim, the “single-minded” protein involved in neuronal development [4][5]. This group of proteins shares a highly conserved PAS domain and is involved in the detection of and adaptation to environmental change[4].

Investigations of invertebrates possessing early homologs of the AhR suggest that the AhR evolutionarily functioned in regulation of the cell cycle, cellular proliferation and differentiation, and cell-to-cell communications (Hahn et al 2002). However, critical functions in angiogenesis, regulation of the immune system, neuronal processes, metabolism, development of the heart and other organ systems, and detoxification have emerged sometime in early vertebrate evolution (Duncan et al., 1998; Emmons et al., 1999; Lahvis and Bradfield, 1998).

The molecular Initiating Event

Figure 1: The molecular mechanism of activation of gene expression by AHR.

The molecular mechanism for AHR-mediated activation of gene expression is presented in Figure 1. In its unliganded form, the AHR is part of a cytosolic complex containing heat shock protein 90 (HSP90), the HSP90 co-chaperone p23 and AHR-interacting protein (AIP)[6]. Upon ligand binding, the AHR migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with ARNT[7]. The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene expression[6]. Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-beta, interleukin-1 beta), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are regulated through this mechanism [6][8][7][9].

AHR Isoforms

- Over time the AhR has undergone gene duplication and diversification in vertebrates, which has resulted in multiple clades of AhR, namely AhR1, AhR2, and AhR3 (Hahn 2002).
- Fishes and birds express AhR1s and AhR2s, while mammals express a single AhR that is homologous to the AhR1 (Hahn 2002; Hahn et al 2006).
- The AhR3 is poorly understood and known only from some cartilaginous fishes (Hahn 2002).
- Little is known about diversity of AhRs in reptiles and amphibians (Hahn et al 2002).
- In some taxa, subsequent genome duplication events have further led to multiple isoforms of AhRs in some species, with up to four isoforms of the AhR (α, β, δ, γ) having been identified in Atlantic salmon (*Salmo salar*) (Hansson et al 2004).
- Although homologs of the AhR have been identified in some invertebrates, compared to vertebrates these AhRs have differences in binding of ligands in the species investigated to date (Hahn 2002; Hahn et al 1994).

Roles of isoforms in birds:

Two AHR isoforms (AHR1 and AHR2) have been identified in the black-footed albatross (*Phoebastria nigripes*), great cormorant (*Phalacrocorax carbo*) and domestic chicken (*Gallus gallus domesticus*)[10]. AHR1 mRNA levels were similar in the kidney, heart, lung, spleen, brain, gonad and intestine from the great cormorant but were lower in muscle and pancreas. AHR2 expression was

mainly observed in the liver, but was also detected in gonad, brain and intestine. AHR1 levels represented a greater proportion (80%) of total AHR levels than AHR2 in the cormorant liver[10], and while both AHR isoforms bound to TCDD, AHR2 was less effective at inducing TCDD-dependent transactivation compared to AHR1 in black-footed albatross, great cormorant and domestic chicken[11][10].

- AhR1 and AhR2 both bind and are activated by TCDD*in vitro* (Yasui et al 2007).
- AhR1 has greater binding affinity and sensitivity to activation by TCDD relative to AhR2 (Yasui et al 2007).
- AhR1 is believed to mediate toxicities of DLCs, while AhR2 has no known role in toxicities (Farmahin et al 2012; Farmahin et al 2013; Manning et al 2012).

Roles of isoforms in fishes:

- AhR1 and AhR2 both bind and are activated by TCDD*in vitro* (Bak et al 2013; Doering et al 2014; 2015; Karchner et al 1999; 2005).
- AhR1 has greater sensitivity to activation by TCDD than AhR2 in red seabream (*Pagrus major*), white sturgeon (*Acipenser transmontanus*), and lake sturgeon (*Acipenser fulvescens*) (Bak et al 2013; Doering et al 2014; 2015)
- AhR2 has greater binding affinity or activation by TCDD than AhR1 in zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*) (Karchner et al 1999; 2005).
- AhR2 is believed to mediate toxicities in fishes, while AhR1 has no known role in toxicities. Specifically, knockdown of AhR2 protects against toxicities of dioxin-like compounds (DLCs) and polycyclic aromatic hydrocarbons (PAHs) in zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*), while knockdown of AhR1 offers no protection (Clark et al 2010; Prasch et al 2003; Van Tiem & Di Giulio 2011).

Roles of isoforms in amphibians and reptiles:

- Less is known about AhRs of amphibians or reptiles.
- AhR1 is believed to mediate toxicities in amphibians (Hahn 2002; Lavine et al 2005; Oka et al 2016; Shoots et al 2015). However, all AhRs of amphibians that have been investigated have very low affinity for TCDD (Hahn 2002; Lavine et al 2005; Oka et al 2016; Shoots et al 2015).
- Both AhR1s and AhR2 of American alligator (*Alligator mississippiensis*) are activated by agonists with comparable sensitivities (Oka et al 2016). AhRs of no other reptiles have been investigated.

Role in mammals

AhR expression is essentially ubiquitous in mammals consistent with a broad-spectrum homeostatic role, however expression levels varying widely across tissues with the liver, thymus, lung, kidney, spleen, and placenta exhibiting greatest expression (Harper PA). Additionally, AhR expression is developmentally regulated, and more recent evidence indicates a role for the AhR in developmental process affecting hematopoiesis, immune system biology, neural differentiation, and liver architecture (Wright E J) . AHR is involved in regulating the rate of apoptosis of oocytes in germ cell nests during embryonic life and in regulating survival of oocytes in the fetal and neonatal ovary. Specifically, studies have shown that ovaries obtained from AHRKO mice on ED13.5 and cultured for 72 h in the absence of hormonal support with the aim of inducing apoptosis, contained higher numbers of non-apoptotic germ cells compared to wild-type (WT) ovaries cultured in the same conditions (Hernández-Ochoa)

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Transactivation Reporter Gene Assays (recommended approach)

Transient transfection transactivation

Transient transfection transactivation is the most common method for evaluating nuclear receptor activation[12]. Full-length AHR cDNAs are cloned into an expression vector along with a reporter gene construct (chimeric luciferase, P-lactamase or CAT reporter vectors containing the appropriate response elements for the gene of interest). There are a number of commercially available cell lines that can serve as recipients for these vectors (CV-1, HuH7, FLC-7, LS174T, LS180 MCF-7, HEC1, LLC-PK1, HEK293, HepG2, and Caco-2 cells)[12]. The greatest advantage of using transfected cells, rather than primary cell cultures, is the assurance that the nuclear receptor of interest is responsible for the observed induction. This would not be possible in a primary cell culture due to the co-regulation of different receptors for the same target genes. This model makes it easy to compare the responsiveness of the AHR across multiple species under the same conditions simply by switching out the AHR clone. One disadvantage to the transient transfection assay is the inherent variability associated with transfection efficiency, leading to a movement towards the use of stable cell lines containing the nuclear receptor and reporter gene linked to the appropriate response elements[12].

Luciferase reporter gene (LRG) assay

The described luciferase reporter gene (LRG) assays have been used to investigate activation of AhRs of:

- Humans (*Homo sapiens*) (Abnet et al 1999)
- Species of birds, namely chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*), Japanese quail (*Coturnix japonica*), and common tern (*Sterna hirundo*) (Farmahin et al 2012; Manning et al 2013), Mutant AhR1s with ligand binding domains resembling those of at least 86 avian species have also been investigated (Farmahin et al 2013). AhR2s of birds have only been investigated in black-footed albatross (*Phoebastria nigripes*) and common cormorant (*Phalacrocorax carbo*)

(Yasio et al 2007).
- American alligator (*Alligator mississippiensis*) is the only reptile for which AhR activation has been investigated (Oka et al 2016), AhR1A, AhR1B, and AhR2 of American alligator were assayed (Oka et al 2016).
- AhR1 of two amphibians have been investigated, namely African clawed frog (*Xenopus laevis*) and salamander (*Ambystoma mexicanum*) (Lavine et al 2005; Shoots et al 2015; Ohi et al 2003),
- AhR1s and AhR2s of several species of fish have been investigated, namely Atlantic salmon (*Salmo salar*), Atlantic tomcod (*Microgadus tomcod*), white sturgeon (*Acipenser transmontanus*), rainbow trout (*Onchorhynchys mykiss*), red seabream (*Pagrus major*), lake sturgeon (*Acipenser fulvescens*), and zebrafish (*Danio rerio*) (Andreasen et al 2002; Abnet et al 1999; Bak et al 2013; Doering et al 2014; 2015; Evans et al 2005; Hansson & Hahn 2008; Karchner et al 1999; Tanguay et al 1999; Wirgin et al 2011).

For demonstrative purposes, a luciferase reporter gene assay used to measure AHR1-mediated transactivation for avian species is described here. However, comparable assays are utilized for investigating AHR1s and AHR2s of all taxa. A monkey kidney cell line (Cos-7) that has low endogenous AHR1 expression was transfected with the appropriate avian AHR1 clone, cormorant ARNT1, a CYP1A5 firefly luciferase reporter construct and a *Renilla* luciferase vector to control for transfection efficiency. After seeding, the cells were exposed to DLC and luciferase activity was measured using a luminometer. Luminescence, which is proportional to the extent of AHR activation, is expressed as the ratio of firefly luciferase units to *Renilla* luciferase units [13]. This particular assay was modified from its original version to increase throughput efficiency; (a) cells were seeded in 96-well plates rather than Petri dishes or 48- well plates, (b) DLCs were added directly to the wells without changing the cell culture medium, and (c) the same 96-well plates were used to measure luminescence without lysing the cells and transferring to another plate. Similar reporter gene assays have been used to measure AHR1 activation in domestic and wild species of birds, including the chicken, ring-necked pheasant (Phasianus colchicus), Japanese quail (Coturnix japonica), great cormorant, black-footed albatross and peregrine falcon (Falco peregrinus).[14][13][15][11][16][17]

Transactivation in stable cell lines

Stable cell lines have been developed and purified to the extent that each cell contains both the nuclear receptor and appropriate reporter vector, eliminating the variability associated with transfection [12]. A stable human cell line containing a luciferase reporter driven by multiple dioxin response elements has been developed that is useful in identifying AhR agonists and antagonists[18]. An added benefit of this model is the potential to multiplex 3 assays in a single well: receptor activation, cell viability and enzyme activity[12]. Such assays are used extensively in drug discovery due to their high throughput efficiency, and may serve just as useful for risk assessment purposes.

Ligand-Binding Assays

Ligand binding assays measure the ability of a test compound to compete with a labeled, high-affinity reference ligand for the LBD of a nuclear receptor. It is important to note that ligand binding does not necessitate receptor activation and therefore cannot distinguish between agonists and antagonists; however, binding affinities of AHR ligands are highly correlated with chemical potencies[19] and can explain differences in species sensitivities to DLCs[20][21][22]; they are therefore worth mentioning. Binding affinity and efficacy have been used to develop structure-activity relationships for AHR disruption[20][23] that are potentially useful in risk-assessment. There has been tremendous progress in the development of ligand-binding assays for nuclear receptors that use homogenous assay formats (no wash steps) allowing for the detection of low-affinity ligands, many of which do not require a radiolabel and are amenable to high throughput screening[24][12]. This author however was unable to find specific examples of such assays in the context of AHR binding and therefore some classic radioligand assays are described instead.

Hydroxyapatite (HAP) binding assay

The HAP binding assay makes use of an *in vitro* transcription/translation method to synthesize the AHR protein, which is then incubated with radiolabeled TDCPP and a HAP pellet. The occupied protein adsorbs to the HAP and the radioactivity is measured to determine saturation binding. An additional ligand can also be included in the mixture in order to determine its binding affinity relative to TCDD (competitive binding)[25][22]. This assay is simple, repeatable and reproducible; however, it is insensitive to weak ligand-receptor interactions[22][21][26].

Whole cell filtration binding assay

Dold and Greenlee[27] developed a method to detect specific binding of TCDD to whole mammalian cells in culture and was later modified by Farmahin et al.[21] for avian species. The cultured cells are incubated with radiolabeled TCDD with or without the presence of a competing ligand and filtered. The occupied protein adsorbs onto the filter and the radioactivity is measured to determine saturation binging and/or competitive binding. This assay is able to detect weak ligand-receptor interactions that are below the detection limit of the HAP assay[21].

Protein-DNA Interaction Assays

The active AHR complexed with ARNT can be measured using protein-DNA interaction assays. Two methods are described in detail by Perez-Romero and Imperiale[28]. Chromatin immunoprecipitation measures the interaction of proteins with specific genomic regions *in vivo*. It involves the treatment of cells with formaldehyde to crosslink neighboring protein-protein and protein-DNA molecules. Nuclear fractions are isolated, the genomic DNA is sheared, and nuclear lysates are used in immunoprecipitations with an antibody against the protein of interest. After reversal of the crosslinking, the associated DNA fragments are sequenced. Enrichment of specific DNA sequences represents regions on the genome that the protein of interest is associated with *in vivo*. Electrophoretic mobility shift assay (EMSA) provides a rapid method to study DNA-binding protein interactions in vitro. This relies on the fact that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments. The protein-DNA complex components are then identified with appropriate antibodies. The EMSA assay was found to be consistent with the LRG assay in chicken hepatoma cells dosed with dioxin-like compounds[29].

In silico Approaches

In silico homology modeling of the ligand binding domain of the AHR in combination with molecular docking simulations can provide valuable insight into the transactivation-potential of a diverse array of AHR ligands. Such models have been developed for multiple AHR isoforms and ligands (high/low affinity, endogenous and synthetic, agonists and antagonists), and can accurately predict ligand potency based on their structure and physicochemical properties (Bonati et al 2017; Hirano et al 2015; Sovadinova et al 2006).

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List of Key Events in the AOP

Event: 944: dimerization, AHR/ARNT

Short Name: dimerization, AHR/ARNT

Key Event Component

Process	Object	Action
protein dimerization activity	aryl hydrocarbon receptor	increased
protein dimerization activity	aryl hydrocarbon receptor nuclear translocator	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:150 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF	KeyEvent
Aop:21 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2	KeyEvent
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	KeyEvent
Aop:151 - AhR activation leading to preeclampsia	KeyEvent
Aop:455 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	KeyEvent
Aop:456 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	KeyEvent
Aop:563 - Aryl hydrocarbon Receptor (AHR) activation causes Premature Ovarian Insufficiency via Bax mediated apoptosis	KeyEvent

Stressors

Name
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)
Stressor:147 Dibenzo-p-dioxin
Polychlorinated biphenyl
Polychlorinated dibenzofurans
Polycyclic aromatic hydrocarbons

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	High	NCBI

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
mouse	Mus musculus	High	NCBI
Coturnix japonica	Coturnix japonica	High	NCBI
Phasianus colchicus	Phasianus colchicus	High	NCBI
rainbow trout	Oncorhynchus mykiss	High	NCBI
Pagrus major	Pagrus major	High	NCBI
Acipenser fulvescens	Acipenser fulvescens	High	NCBI
Acipenser transmontanus	Acipenser transmontanus	High	NCBI
Salmo salar	Salmo salar	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
human	Homo sapiens	High	NCBI
Ambystoma mexicanum	Ambystoma mexicanum	High	NCBI
Microgadus tomcod	Microgadus tomcod	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic Presence of ARNT genes:

- ARNTs have been identified in all tetrapods investigated to date (Drutel et al 1996; Hirose et al 1996; Hoffman et al 1991; Lee et al 2007; Lee et al 2011).
- ARNTs have been identified in a great phylogenetic diversity of fishes, including early fishes (Doering et al 2014; 2016).
- ARNT has been identified in investigated invertebrates (Powell-Coffman et al 1998).

Taxonomic Applicability of Heterodimerization of ARNT isoforms with AhR isoforms:

- In mouse (*Mus mus*) and chicken (*Gallus gallus*) both the ARNT1 and ARNT2 were capable of heterdimerizing with AHR and interacting with dioxin-responsive elements on the DNA *in vitro* (Hirose et al 1996; Lee et al 2007; Lee et al 2011; Prasch et al 2004). However, no studies have yet confirmed involvement of both ARNT1 and ARNT2 *in vivo*.
- In zebrafish, all adverse effects of DLCs so far examined *in vivo* are mediated solely by ARNT1 based on knockdown studies, although ARNT2 is capable of heterodimerizing with AHR2 and interacting with dioxin-responsive elements on the DNA *in vitro* (Prasch et al 2004; Prasch et al 2006). In addition to AHRs of zebrafish, AHRs of Atlantic salmon (*Salmo salar*), Atlantic tomcod (*Microgadus tomcod*), mummichog, rainbow trout, and red seabream (*Pagrus major*) have been demonstrated to heterodimerize with ARNT1 *in vitro* (Abnet et al 1999; Bak et al 2013; Hansson & Hahn 2008; Karchner et al 1999; Wirgin et al 2011), while AHRs of white sturgeon (*Acipenser transmontanus*), and lake sturgeon (*Acipenser fulvescens*) have been demonstrated to heterodimerize with ARNT2 *in vitro* (Doering et al 2014b; 2015b; Prasch et al 2004; 2006).

This mechanism is conserved across species. Mammals possess a single AHR, whereas birds and fish express multiple isoforms, and all three express multiple ARNT isoforms. Not all of the isoforms identified are functionally active. For example, killifish AHR1 and AHR2 are active and display different transcription profiles, whereas zebrafish AHR2 and ARNT2 are active in mediating xenobiotic-mediated toxicity and AHR1 is inactive (Hahn et al. 2006; Prasch et al. 2006).

Key Event Description

Structure and Function of ARNT

- The aryl hydrocarbon receptor nuclear translocator (ARNT) is a member of the Per-Arnt-Sim (PAS) family of proteins (Gu et al 2000).
- PAS proteins share highly conserved PAS domains (Gu et al 2000).
- PAS proteins act as transcriptional regulators in response to environmental and physiological cues (Gu et al 2000).
- ARNTs have numerous key roles in vertebrates related to responses to developmental and environmental cues.

Isoforms of ARNT:

- Over time ARNT has undergone gene duplication and diversification in vertebrates, which has resulted in three clades of ARNT, namely ARNT1, ARNT2, and ARNT3.
- Each clade can include multiple isoforms and splice variants (Hill et al 2009; Lee et al 2007; Lee et al 2011; Powel & Hahn 2000; Tanguay et al 2000).
- ARNT1s have been demonstrated to function predominantly through heterodimerization with the aryl hydrocarbon receptor (AhR) and hypoxia inducible factor 1 α (HIF1α) (Prasch et al 2004; 2006; Wang et al 1995).

- ARNT2s are believed to function predominantly through heterodimerization with Single Minded (SIM) (Hirose et al 1996).
- ARNT3s, which are also known as ARNT-like (ARNTL), Brain and Muscle ARNT-like-1 (BMAL1), or Morphine Preference 3 (MOP3), are believed to function predominantly through heterodimerization with Circadian Locomotor Output Cycles Kaput (CLOCK) (Gekakis et al 1998).

Roles of ARNTs in mammals:

- ARNT1 functions in normal vascular and hematopoietic development (Kozak et al 1997; Maltepe et al 1997; Abbott & Buckalew 2000).
- ARNT2 functions in development of the hypothalamus and nervous system (Hosoya et al 2001; Keith et al 2001).
- ARNT3 functions in biological rhythms (Gekakis et al 1998).

- Several isoforms of ARNT have recently been identified in mammalian and aquatic species based on their sequence identity to ARNT. They are grouped into four ARNT types that include: ARNT (HIF-1), ARNT2, BMAL1 (ARNT3, MOP3, JAP3, ARNTL1, TIC), and BMAL2 (ARNT4, ARNTL2, MOP9) (Dougherty et al 2010).

- Coexpression of ARNT with AhR is crucial for steroid synthesis, secretion, and cellular functions during non-pregnancy, pregnancy, and pseudopregnancy. In ovarian follicles, AhR and ARNT are expressed in the follicular epithelia of primordial and growing follicles, contributing to oocyte maturation and follicular development starting from the primary follicle stage. ARNT also regulates ovarian steroid hormones, such as estradiol and progesterone, impacting ovarian physiology. Knockout studies in mice show that ARNT is essential for embryonic development, with embryos failing to survive beyond day 9.5 due to growth retardation, such findings highlight ARNT's vital role in reproduction and development (Hasan et al 2003, Khorram et al 2002).

Roles of ARNTs in other taxa:

- ARNTs have been demonstrated to have roles in development of the heart, brain, liver, and possibly the peripheral nervous system in zebrafish (*Danio rerio*) (Hill et al 2009).
- Roles of ARNTs in other taxa have not been sufficiently investigated to date.

Interaction with AHR

- Both ARNT1s and ARNT2s are able to heterodimerize with AhR and interact with dioxin-responsive elements on the DNA i*n vitro* systems (Hirose et al 1996; Lee et al 2007; Lee et al 2011; Prasch et al 2004).
- Selective knockdown of ARNTs in zebrafish (*Danio rerio*) demonstrates that ARNT1s, but not ARNT2s, are required for activation of the AhR *in vivo* (Prasch et al 2004; 2006).
- In limited investigations ARNT3 has not been demonstrated to interact with the AHR either*in vivo* or *in vitro* (Jain et al 1998).

Upon ligand binding, the aryl hydrocarbon receptor (AHR) migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with AHR nuclear translocator (ARNT) (Mimura and Fujii-Kuriyama 2003). The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene expression (Fujii-Kuriyama and Kawajiri 2010). Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-beta, interleukin-1 beta), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are regulated through this mechanism (Fujii-Kuriyama and Kawajiri 2010; Giesy et al. 2006; Mimura and Fujii-Kuriyama 2003; Safe 1994).

How it is Measured or Detected

AhR/ARNT heterodimerization can be measured in several ways:

1) The active AHR complexed with ARNT can be measured using protein-DNA interaction assays. Two methods are described in detail by Perez-Romero and Imperiale (Perez-Romero and Imperiale 2007). Chromatin immunoprecipitation measures the interaction of proteins with specific genomic regions in vivo. It involves the treatment of cells with formaldehyde to crosslink neighboring protein-protein and protein-DNA molecules. Nuclear fractions are isolated, the genomic DNA is sheared, and nuclear lysates are used in immunoprecipitations with an antibody against the protein of interest. After reversal of the crosslinking, the associated DNA fragments are sequenced. Enrichment of specific DNA sequences represents regions on the genome that the protein of interest is associated with in vivo. Electrophoretic mobility shift assay (EMSA) provides a rapid method to study DNA-binding protein interactions in vitro. This relies on the fact that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments. The protein-DNA complex components are then identified with appropriate antibodies. The EMSA assay was found to be consistent with the luciferase reporter gene assay (in chicken hepatoma cells dosed with dioxin-like compounds (Heid et al. 2001).

2) Species-specific differences in dimerization and differences in dimerization between ARNT isoform and AhR isoform combinations have been assessed through luciferase reporter gene (LRG) assays utilizing COS-7 cells transfected with expression constructs of AhR and ARNT isoforms of mammals, birds, and fishes (Abnet et al 1999; Bak et al 2013; Doering et al 2014; 2015; Hansson & Hahn 2008; Hirose et al 1996; Karchner et al 1999; Lee et al 2007; Lee et al 2011; Prasch et al 2004; Wirgin et al 2011). However, this method is indirect as it also includes binding of a ligand to the AhR, and interaction of the AhR/ARNT heterodimer with dioxin-responsive elements on the DNA.

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Event: 2021: Increase, slincR expression

Short Name: Increase, slincR expression

Key Event Component

Process	Object	Action
gene expression		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:455 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	KeyEvent
Aop:456 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Danio rerio	Danio rerio	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

- slincR was discovered and characterized in developing zebrafish (Garcia et al., 2017; Garcia et al., 2018).
- Additionally, putative mammalian orthologs have also been identified using the slncky Evolution Browser (Garcia et al., 2018). The mouse ortholog was identified from an unpublished RNA sequencing dataset from male and female mouse urogenital epithelial tissue exposed to TCDD using a combination of proximity to the sox9 locus and TCDD-induced gene expression. Only one lncRNA (2610035D17Rik) matched the criteria. The human ortholog (LINC00673) of the mouse lncRNA was identified using slncky. Expression of both the mouse and human lncRNA orthologs from NCBI were consistent with zebrafish slincR expression.

Key Event Description

Descriptions of the KE comes from two studies that discovered and described slincR in zebrafish (Garcia et al., 2017; Garcia et al., 2018).

- The **s**ox9b **l**ong **i**ntergenic **n**on-**c**oding **R**NA or slincR is a novel long non-coding RNA (lncRNA) that was recently discovered in developing zebrafish
- slincR gene expression is dependent on Aryl hydrocarbon receptor (Ahr) activation, with slincR induced up to ~$\log_2$FC=5 in whole-animal zebrafish exposed to the potent Ahr ligand, TCDD. This induction takes place only in the presence of a functional Ahr protein. SlincR is also induced by multiple other Ahr ligands.

- slincR is located approximately 40,000 bp upstream and antisense of the sox9b gene locus in zebrafish. sox9b is one of the most reduced transcripts in the jaw when zebrafish are exposed to TCDD (Xiong et al., 2008), and is one of two zebrafish paralogs of sox9, a critical transcription factor that has been implicated in several processes including chondrogenesis and cardiac development, in addition to skeletal development, male gonad genesis, and cancer progression (Panda et al., 2021; Lefebvre et al., 2017).
- slincR was found to be enriched in the 5'UTR of the sox9b gene, suggesting possible interactions between slincR and sox9b. A slincR morpholino experiment demonstrated that slincR is required for sox9b repression.
- Morpholino knockdown of slincR showed slincR's ability to regulate cartilage development, and play a role in TCDD-induced hemorrhaging, both via whole-animal transcriptomics and phenotypic analyses.

How it is Measured or Detected

slincR gene expression can be measured by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) and has been measured in embryonic zebrafish at 48 and 96 hours post fertilization (hpf) (Garcia et al., 2017).

slincR tissue localization of expression can be measured by in situ hybridization and has been measured in embryonic zebrafish at 24, 36, 48, 60, and 72 hpf (Garcia et al., 2017).

slincR molecular localization can be measured by capture hybridization analysis of RNA targets (CHART) and was measured in 48 hpf zebrafish embryos (Garcia et al., 2018).

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Event: 2020: Decrease, sox9 expression

Short Name: Decrease, sox9 expression

Key Event Component

Process	Object	Action
gene expression		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:455 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	KeyEvent
Aop:456 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Description

- The sox family of proteins are a group of highly conserved transcriptional regulators that are present in most groups of animals from invertebrates and unicellular organisms (Phochanukul and Russell 2010) to the more complex vertebrates.
- Sox proteins are characterized by containing the highly conserved high mobility group (HMG) domain, and around 20 different sox proteins have been discovered in mice and humans to date (Jo et al., 2014).
- Sox9, which is part of the soxE subgroup, was initially discovered as the gene underlying campomelic dysplasia (CD), a haplosufficiency disorder characterized by abnormal chondrogenesis, as well as autosomal XY sex reversal from males to females (Wagner et al., 1994).
- Since then, sox9 has been implicated in several functions such as in chondrogenesis, skeletal development, male gonad genesis, development of mesodermal tissues such as cardiac valves and septa, and pyloric sphincter, in ectoderm development (neural stem cells, gliogenesis, and neural stem cells), in hair follicle stem cells, retinal progenitor cells, and the otic placode, and during endoderm development impacting the pancreas, liver, intestine, and lungs. The developmental functions of sox9 have been comprehensively reviewed (Jo et al., 2014; Kawaguchi 2013; Lee and Saint-Jeannet 2011; Lefebvre and Dvir-Ginzberg 2017).
- Several of sox9's functions are hypothesized to take place as a result of its role as a repressor of the Wnt/B-catenin signaling pathway. Of note, the canonical Wnt signaling pathway promotes chondrocyte differentiation in a sox9-dependent manner (Yano et al., 2005).
- Sox9b (one of two paralogs of the sox9 gene in zebrafish) is one of the most reduced transcripts in the jaw upon TCDD exposure in zebrafish which causes severe lower jaw defects (Xiong et al., 2008), supporting role of sox9's repression in craniofacial defects.

How it is Measured or Detected

The studies cited in the KE Description used common methods such as RT-qPCR, to measure sox9 expression. It should be noted that the starting level of sox9 expression can be important to determine the magnitude of its repression that is needed to have a significant effect on craniofacial effects (for example), but there still exists a knowledge gap regarding this aspect.

List of Adverse Outcomes in this AOP

Event: 947: Increase, Early Life Stage Mortality

Short Name: Increase, Early Life Stage Mortality

Key Event Component

Process	Object	Action
embryonic lethality		increased
mortality		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:150 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF	AdverseOutcome
Aop:21 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2	KeyEvent

AOP ID and Name	Event Type
Aop:455 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	AdverseOutcome
Aop:456 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	AdverseOutcome

Stressors

Name
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Foetal	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

All members of the subphylum vertebrata are susceptible to early life stage death (Weinstein 1999).

Key Event Description

Increased early life stage mortality refers to an increase in the number of individuals dying in an experimental replicate group or in a population over a specific period of time.

In Birds:

Early life stage mortality occurs at any stage in development prior to birth/hatch and is considered embryolethal.

In Fishes:

Early Life Stage Mortality refers to death prior to yolk sac adsorption and swim-up.

How it is Measured or Detected

In birds it may be identified as failure to hatch or lack of movement within the egg when candled; heartbeat monitors are available for identifying viable avian and reptillian eggs (ex. Avitronic's Buddy monitor). In mammals, stillborn or mummified offspring, or an increased rate of resorptions early in pregnancy are all considered embryolethal, and can be detected using ultra-high frequency ultrasound (30-70 MHz; a.k.a. ultrasound biomicroscopy) (Flores *et al.* 2014). In fishes, mortality is typically measured by observation. Lack of any heart beat, gill movement, and body movement are typical signs of death used in the evaluation of mortality.

Regulatory Significance of the AO

Poor early life stage survival is an endpoint of major relevance to environmental regulators, as it is likely to lead to population decline. Early-life stage, acute and chronic test guidelines have been established by the Organisation for Economic Co-operation and Development (OECD), U.S. Environmental Protection Agency (EPA) and Environment and Climate Change Canada (ECCC), and are currently used in risk assessments to set limits for safe exposures. Aquatic test guidlines are most prevalent and include OECD210, OECD229, EPA850.1400 and ECCC EPS 1/RM/28 for fish and OECD241 for frogs.

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Event: 1559: Facial cartilage structures are reduced in size and morphologically distorted

Short Name: Smaller and morphologically distorted facial cartilage structures

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:274 - Histone deacetylase inhibition leads to impeded craniofacial development	AdverseOutcome
Aop:455 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	AdverseOutcome

Biological Context

Level of Biological Organization

Tissue

Key Event Description

In order for cartilage structures to form, chondrocytes need to secrete large amounts of collagen. One outcome of the disturbances of NCC migration and differentiation is the distortion and reduced size of facial cartilage structures.

How it is Measured or Detected

The appearance of facial cartilage structures is readily visible in ventral views of zebrafish embryos at 5 days post fertilization. Such structures as the ceratohyal, Meckel's cartilage, and the palatoquadrate are visible using fluorescent collagen reporter lines. Measurements of the angle formed by the ceratohyal provide a quantitative readout.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 972: Activation, AhR leads to dimerization, AHR/ARNT

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF	adjacent	High	Moderate
Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2	adjacent	High	Moderate
Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	adjacent	High	Moderate
AhR activation leading to preeclampsia	adjacent		
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	adjacent	High	Moderate
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	adjacent	High	Moderate
Aryl hydrocarbon Receptor (AHR) activation causes Premature Ovarian Insufficiency via Bax mediated apoptosis	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI

Term	Scientific Term	Evidence	Links
Danio rerio	Danio rerio	High	NCBI
rainbow trout	Oncorhynchus mykiss	High	NCBI
Pagrus major	Pagrus major	High	NCBI
Acipenser fulvescens	Acipenser fulvescens	High	NCBI
Salmo salar	Salmo salar	High	NCBI
Acipenser transmontanus	Acipenser transmontanus	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
Ambystoma mexicanum	Ambystoma mexicanum	High	NCBI
Microgadus tomcod	Microgadus tomcod	High	NCBI
human	Homo sapiens	High	NCBI
Gallus gallus	Gallus gallus	High	NCBI
Phasianus colchicus	Phasianus colchicus	High	NCBI
Coturnix japonica	Coturnix japonica	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

- The aryl hydrocarbon receptor (AhR) and aryl hydrocarbon receptor nuclear translocator (ARNT) are highly conserved and ancient proteins with homologs having been identified in most major animal groups, apart from the most ancient lineages, such as sponges (Porifera) (Hahn et al 2002).
- *In vitro* dimerization of AhRs and ARNTs have been demonstrated in mammals, birds, reptiles, amphibians, teleost and non-teleost fishes, and some invertebrates (Butler et al 2001; Emmons et al 1999; Hahn et al 2002; Powell-Coffman et al 1998).

Key Event Relationship Description

In its unliganded form, the AHR is part of a cytosolic complex containing heat shock protein 90 (HSP90), the HSP90 co-chaperone p23 and AHR-interacting protein (AIP) (Fujii-Kuriyama *et al.* 2010). Upon ligand binding, the aryl hydrocarbon receptor (AHR) migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with AHR nuclear translocator (ARNT) (Mimura and Fujii-Kuriyama 2003).

AhRs can heterodimerize with ARNT1 and ARNT2 isoforms in order to activate reporter constructs in transfected cells and recognize response elements in gel shift assays in all investigated vertebrates, including birds, fishes, and reptiles (Abnet et al 1999; Andreasen et al 2002a; 2002b; Bak et al 2013; Doering et al 2014; Doering et al 2015; Farmahin et al 2012; 2013; Hansson & Hahn 2008; Karchner et al 1999; 2006; Lavine et al 2005; Shoots et al 2015; Tanguay et al 1999; 2000; Wirgin et al 2011).

Evidence Supporting this KER

Biological Plausibility

The mechanism of AHR-mediated transcriptional regulation is well understood (Fujii-Kuriyama and Kawajiri 2010).

Numerous PAS proteins are known to interact with each other in response to environmental and developmental cues through dimerization at their PAS domains (Pohjanvirta 2012).

Empirical Evidence

ARNT is a necessary dimerization partner for the transcriptional activation of AHR regulated genes (Hoffman et al. 1991; Poland et al. 1976). The AHR/ARNT complex was confirmed following in vitro exposure to halogenated aromatic hydrocarbons using an electrophoretic mobility shift assay; a dose-dependent supershift in DNA-binding was observed using specific antibodies in chicken and human cell lines (Heid et al. 2001).

- Unliganded AhR exists as a cytosolic 9S form, while in the presence of a ligand the AhR exists as a nuclear 6S form. ARNT exists as a nuclear 6S form (Okey 2007).
- The 6S form of AhR is approximately 210 kDa. Ligated AhR is approximately 100 kDa and ARNT is approximately 110 kDa (Elferink et al 1990; Swanson et al 1993).
- Dimerization of AhRs with ARNTs has been demonstrated in all invertebrate and vertebrate species so far investigated (Butler et al 2001; Emmons et al 1999; Hahn et al 2002; Powell-Coffman et al 1998).
- Heterodimers are not formed on response elements in gel shift assays in the absence of AhR and/or ARNT (Tanguay et al 2000).

Uncertainties and Inconsistencies

- There are uncertainties in the precise physiological and toxicological roles of different AhR clades (AhR1, AhR2, AhR3) and isoforms (α, β, δ, γ).
- There are uncertainties in the precise physiological and toxicological roles of different ARNT clades (ARNT1, ARNT2, ARNT3) and isoforms (a, b, c).
- Nothing is known about differences in binding affinity of AhR for ARNT and of the AhR/ARNT heterodimer for DNA among species and taxa.
- There is uncertainty in whether anthropogenic contaminants that act as ligands of the AhR and lead to dimerization of AhR with ARNT in vertebrates also act as ligands in invertebrates.

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Relationship: 2683: dimerization, AHR/ARNT leads to Increase, slincR expression

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	adjacent	Moderate	Moderate
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
mouse	Mus musculus	High	NCBI
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

- Ahr activation (and thus, the dimerization of Ahr/ARNT) resulting in significant slincR induction of expression has only been investigated in zebrafish and mice (Garcia et al., 2017; Garcia et al., 2018).

Key Event Relationship Description

- Dimerization of Ahr/ARNT take place when the ligand-activated Ahr translocates to the nucleus from the cytoplasm.
- The Ahr/ARNT heterodimer can recognize aryl hydrocarbon response elements (AHREs), also known as xenobiotic response elements (XREs) or dioxin response elements (DREs), in the promoter region of downstream genes to regulate gene expression (Schmidt and Bradfield 1996). The target genes can either increase or decrease in their expression.
- slincR expression significantly increases when zebrafish are exposed to TCDD, and the slincR promoter includes the core AHRE (5’-T/GCGTG-3’) in multiple locations (Garcia et al., 2017), suggesting that slincR is a direct downstream target of the Ahr/ARNT heterodimer.

Evidence Supporting this KER

Biological Plausibility

- Eight putative AHREs have been identified in the slincR promoter of the zebrafish gene (Garcia et al., 2017).
- The potential orthologs of slincR in the mouse and human genomes also have conserved AHREs (Garcia et al., 2018).

Empirical Evidence

Empirical evidence and essentiality of KE_{up} for KE_{down} to occur

- Expression of slincR is significantly increased when zebrafish are exposed to TCDD (Garcia et al., 2017). TCDD is a strong Ahr activating ligand that causes the dimerization of Ahr and ARNT, and ARNT1 in zebrafish has been shown to be required for TCDD-induced toxicity (Prasch et al., 2006).
- When AHR2-null zebrafish generated using CRISPR-Cas9 are exposed to TCDD, slincR expression at 48 hours post fertilization (hpf) is significantly lower than wildtype zebrafish exposed to TCDD (Garcia et al., 2017).
- slincR expression is significantly induced upon exposure to several polycyclic aromatic hydrocarbons (PAHs), many of whom are Ahr activating chemicals (Garcia et al., 2018). The PAHs that induce slincR expression are retene, benzo[j]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, and benzo[b]fluoranthene.
- TCDD-exposed embryonic mouse urogenital tissue samples showed significant increase in expression of the mouse slincR ortholog (2610035D17Rik) compared to the vehicle control, DMSO (Garcia et al., 2018).

Uncertainties and Inconsistencies

- Certain PAHs, such as fluoranthene, phenanthrene, and 9-methylanthracene that significantly induce cyp1a greater than $\log_2 FC = 2$, indicating that Ahr has been activated, do not induce slincR expression in zebrafish (Garcia et al., 2018).

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Relationship: 2684: Increase, slincR expression leads to Decrease, sox9 expression

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	adjacent	Moderate	Moderate
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
mouse	Mus musculus	Moderate	NCBI
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

- The interaction between SlincR and sox9 (sox9b, specifically) has only been investigated in zebrafish.

Key Event Relationship Description

- Sox9b (one of two paralogs of the sox9 gene in zebrafish) is one of the most reduced transcripts in the jaw upon TCDD exposure in zebrafish (Xiong et al., 2008).
- One question that remained unanswered was the possible mechanisms by which Ahr was regulating sox9b expression, given that even though there are eight putative AHREs near the sox9b promoter, its repression in zebrafish does not occur immediately after TCDD exposure (Xiong et al. 2008).
- slincR is a long non-coding RNA (lncRNA) that was recently discovered in zebrafish. Multiple lines of evidence from zebrafish experiments point to slincR being the intermediate between Ahr activation and sox9b repression (Garcia et al., 2017; Garcia et al., 2018).

Evidence Supporting this KER

Biological Plausibility

- The nature of lncRNAs is such that they have diverse functions and can regulate gene expression at multiple levels, including by interacting with DNA, RNA, proteins, and altering transcription of both neighboring and distant genes (Statello et al., 2021).
- slincR (in situ hybridization) and sox9b (immunohistochemistry for sox9b-eGFP) are expressed in adjacent and overlapping tissues through multiple stages of zebrafish development, such as in the eye, otic vesicle, and in the lower jaw (Garcia et al., 2017).

- A capture hybridization analysis of RNA targets (CHART) experiment in both DMSO- and TCDD-exposed 48 hpf zebrafish identified enrichment of slincR in the 5'UTR of the sox9b locus (Garcia et al., 2018) suggesting possible interaction between slincR and sox9b.

Empirical Evidence

Empirical evidence and essentiality of KE_{up} for KE_{down} to occur

- Upon Ahr activation with TCDD, slincR expression significantly increases at concentrations lower (0.0625 ng/mL) than when sox9b expression is significantly repressed (0.5 ng/mL) demonstrating that slincR induction precedes sox9b repression.
- When slincR expression is knocked down using a morpholino, normal sox9b expression levels and spatial pattern are altered during zebrafish development (Garcia et al., 2017). Specifically, in slincR morphants exposed to DMSO or TCDD, sox9b expression was significantly higher than in control morphant zebrafish.
- When slincR expression is knocked down using a morpholino, several downstream target genes of sox9b, such as, notch3, adamts3, fabp2, sfrp2, and fgfr3 were altered in their gene expression compared to control morphants (Garcia et al., 2017).

Uncertainties and Inconsistencies

- Six individual PAHs, retene, benzo[j]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, and benzo[b]fluoranthene, significantly induced slincR expression in whole-animal zebrafish, however no repression of sox9b was detected in any of the PAHs (Garcia et al., 2018).
- Morpholino knockdown of sox9b in zebrafish led to a significant increase in slincR expression suggesting that slincR and sox9b may share overlapping regulatory networks that is not fully understood (Garcia et al., 2018).
- We note that slincR is not the only mechanism of regulation of sox9. Other studies have found evidence for different regulatory mechanisms of sox9, but the circumstances under which different pathways are turned on is still unknown (Dash et al., 2021; Fu et al., 2018).

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Garcia GR, Shankar P, Dunham CL, Garcia A, La Du JK, Truong L, Tilton SC, Tanguay RL. 2018. Signaling events downstream of ahr activation that contribute to toxic responses: The functional role of an ahr-dependent long noncoding rna (slincr) using the zebrafish model. Environ Health Perspect. 126(11):117002.

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Relationship: 2685: Decrease, sox9 expression leads to Smaller and morphologically distorted facial cartilage structures

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
rat	Rattus norvegicus	High	NCBI
chicken	Gallus gallus	High	NCBI
mouse	Mus musculus	High	NCBI
Sebastiscus marmoratus	Sebastiscus marmoratus	High	NCBI

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

- Evidence suggests that sox9 and its function in craniofacial development (and thus its repression leading to craniofacial malformations), is an evolutionarily conserved phenomenon. This relationship appears to exist in almost all vertebrates and invertebrates that encode one or more versions of the sox9 gene.

Key Event Relationship Description

- Sox9 is an important transcriptional regulator that has been implicated in several functions including craniofacial development, specifically via chondrogenesis or the formation of cartilage structure (Lefebvre and Dvir-Ginzberg 2017).
- Additionally, exposure of different animals to relevant environmental pollutants leads to a significant decrease of sox9 expression (Garcia et al., 2017; Shi et al., 2017; Tussellino et al., 2016).
- This KER provides lines of evidence linking the sox9 repression to alterations in craniofacial development and function.

Evidence Supporting this KER

KER 2685 concordance
table: https://aopwiki.org/system/dragonfly/production/2022/10/20/71wqxtrj9g_Concordance_Table_sox9_to_craniofacial_clean.pdf

Biological Plausibility

- Compelling biological plausibility evidence comes from studies in multiple species showing the spatiotemporal expression of sox9 in the developing cartilage structures of the jaw.
  - In mice, sox9 mRNA is widely expressed in the condylar anlage and Meckel's cartilage (Shibata et al. 2006), and the sox9 protein in the tissue layer of secondary cartilage (Hirouchi et al., 2018; Zhang et al., 2013) suggesting roles of sox9 in chondrogenesis. Additionally, sox9 is expressed widely during palatogenesis (Nie 2006; Watanabe et al., 2016), and is also found in the temporomandibular joint of developing mice (TMJ) (Wang et al., 2011). There is a some evidence for sox9 being expressed in the condyle cartilage, as well as the proliferative layer and in the chondrocytes of developing rats (Al-Dujaili et al., 2018; Rabie and Hägg 2002).
  - Sox9 is expressed within the developing chondrocytes of rabbits (Huang et al., 2015).
  - Sox9 is expressed in the different regions of the jaw cartilage structure of developing chickens (Hu et al., 2008), duck, and quail (Eames and Schneider 2008).
  - In zebrafish, sox9b has spatiotemporal expression patterns in and around perichondrial cells (Burns et al., 2015), and generally in the lower jaw region of developing sox9b reporter zebrafish (Garcia et al., 2017) (Burns et al., 2016). Sox9 expression has been detected in the dentition of atlantic salmon as well (Huysseune et al., 2008).
  - Compared to mice, sox9 expression was identified earlier in the cranial analgen of opposum embryos demonstrating species-specific sox9 expression spatiotemporal patterns (Wakamatsu et al., 2014).
- Few studies have found evidence for relationships between sox9 and molecular signaling pathways that are important for normal development of the craniofacial region. The lines of evidence provide some mechanisms by which sox9 can be involved when disruption of craniofacial cartilage takes place.
  - Fertilized chicken eggs infected with retroviruses coding **BMP** had sox9 expression induced in different regions of the cartilage structure (Hu et al., 2008).
  - Exogenous **BMP4** added to mouse mandibular explants leads to induction of sox9 expression within 24 hours (Semba et al., 2000). Similarly rat organ cultures exposed to **BMP7** for eight days had significantly increased sox9 expression as well as more bone and cartilage, as well as an induction of chondrocyte proliferation and differentiation (Cowan et al., 2006).
  - In mouse and chicken in vitro culture systems, addition of **BMP** induced chondrogenesis, while epidermal growth factor (EGF) repressed both chondrogenesis and also led to sox9 repression (Nonaka et al., 1999).
  - **Noggin**-soaked beads inserted into stage 15 or stage 20 chicken embryos had increased sox9 expression in the maxillary mesenchyme which was associated with ectopic cartilage growth in the stage 15 embryos, and loss of bones in the stage 20 animals (Celá et al., 2016).
  - **Fibroblast growth factors (FGFs)** which play a fundamental role in cartilage formation were added to chicken pluripotent mesenchymal cells which led to both sox repression as well as depression of cartilage matrix production (Bobick et al., 2007). In the case of one rat study, FGF10 electroporated via an expression vector increased both sox9 expression as well as the size of the Meckel's cartilage (Terao et al., 2011).
  - Condylar cartilage explants cultured with a **notch signal inhibitor** had sox9 expression increased as well as a decrease of proliferation as measured by cyclic B1 expression (Serrano et al., 2014).
  - **Wnt** signaling inhibited by dickkopf-1 in chicken embryos had sox9 expression downregulated, in addition to defects of the maxilla and hypoplasia of the premaxilla and palatine bones (Shimomura et al., 2019).

Empirical Evidence

Empirical evidence and essentiality of KE_{up} for KE_{down} to occur

- Developing zebrafish exposed to 1ng/mL TCDD had several deformations in the jaw region including inthe Meckel's cartilage which exhibited decreased calcein staining of calcium, and in the craniofacial skeleton which had reduced ossification of the dermal bones. These deformities were associated with a significant reduction of sox9b expression (Burns et al., 2015; Garcia et al., 2017).

- Developing rockfish exposed to 0.5, 5 and 50nM pyrene had decrease of sox9a expression in the craniofacial skeleton region (such as the Meckel's cartilage, ceratobranchial and pectoral fin blastemas) in a concentration-dependent manner, as well as severe craniofacial deformities starting at 0.5 nM pyrene exposure (Shi et al., 2012).
- In one study, zebrafish injected with sox9b morpholinos had several jaw malformations by 72 hours post fertilization (hpf). Additionally cartilage staining of the jaw showed that the Meckel's, palatoquadrate, and ceratohyal cartilages were smaller and malformed compared to control morphants (Xiong et al., 2008). A rescue experiment with sox9b mRNA led to 14% of mRNA-injected animals showing normal jaw phenotype.
- Sox9a morpholino knockdown in zebrafish also led to a blockage of cartilage formation (Koskinen et al., 2009).
- In one study, zebrafish exposed to procymidone (0, 10, 100, and 1000 ng/L) significantly repressed sox9 expression (paralog not mentioned) at the 100 and 1000 ng/L concentrations. Significant lower jaw malformations such as, shortened mandibular arch and lower jaw length, were observed at the same concentrations (Wu et al., 2018).
- Investigations into the mutations in the human sox9 coding sequence have identified two novel deletions in the upstream region associated with pierre robin sequence (PRS) (Gordon et al., 2014).
- Sox9 expression purposely ablated in mouse cranial neural crest cells led to an inactivation of sox9 as well as the absence of the condylar cartilage and TMJ malformations (Wang et al., 2011).
- Parathyroid hormone exposure in mice as well as primary chondrocyte cultures from the mandibular condylar cartilage of mice exposed to the hormone had significantly increased sox9 expression as well as a significant increase in cell proliferation in the cartilage and cartilage thickness (Dutra et al., 2021).
- Intermittent hypoxia exposure-induced underdeveloped mandibular ramus and condyles is associated with downregulation of sox9 expression in rats (Lekvijittada et al., 2021). Similarly intermittent nasal obstruction (mouth breathing) in four-week-old rats let to a decrease of sox9 expression in the mesenchymal stem cells of the condylar cartilage, as well as all mandibular parameters being significantly smaller (Wang et al., 2019). However no differences in proliferative ability was seen.
- Mice with altered functional loading had a loss of condylar cartilage, loss of density of mandibular condylar subchondral bone, as well as a decrease in early chondrocyte differentiation, all of which were associated with a decrease in sox9 expression in the jaw region (Chen et al., 2011; Chen et al., 2009). On the other hand, repeated mechanical loading applied to rats had increased sox9 expression as well as a promotion of condylar cartilage growth (Ng et al. 2006).

Uncertainties and Inconsistencies

- Few studies have showed an opposite relationship between sox9 expression and the size of cartilage structures.
  - Conditional knockout of setdb1 (histone methyltransferase) specifically in the murine Meckel's cartilage led to and enlargement of the cartilage structure as well as the proliferation of chondrocytes, however, sox9 expression was significantly repressed (Yahiro et al., 2017).
  - Experimental unilateral anterior crossbite created in rats led to decreased ratio of the hypertrophic cartilage layer in the experiment group, which was evidence for obvious cartilage degradation. This was accompanied by induction of sox9 expression (Zhang et al., 2013b).
- One recent zebrafish study using the CRISPR-Cas9 tool, demonstrated that sox9a but not sox9b was required for normal cartilage development (Lin et al., 2021). This is inconsistent with all previous research showing the importance of both sox9a and sox9b for cartilage development in zebrafish.

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Relationship: 2686: Smaller and morphologically distorted facial cartilage structures leads to Increase, Early Life Stage Mortality

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fish	fish	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Development	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Key Event Relationship Description

- This KER describes evidence for craniofacial malformations (including smaller and morphologically distorted facial cartilage structure) leading to early life stage mortality.
- Most of the evidence comes from biological plausibility, with some supporting evidence from the mammalian literature.
- The occurrence of craniofacial malformations in fish and wildlife is still regarded as an adverse outcome in several regulatory contexts. For example, animal craniofacial deformities, specifically in birds and fish, are a commonly cited Beneficial Use Impairment (BUI) at Great Lakes Areas of Concern (AOCs) (IJC 1991). Significant reductions in the occurrence and prevalence of deformities at these sites is a criterion for delisting. Consequently, craniofacial malformation is an important phenotype for risk assessment of Ahr activating chemicals, even if it does not directly lead to reduced survival.

Evidence Supporting this KER

Biological Plausibility

- It is reasonable to infer that malformed jaw structure of animals in the wild could impact their feeding success, leading to reduced growth and possible early mortality. Impacts on animals to capture prey can also lead to population-wide changes to both the predators and prey (Weis et al. 2001), constraining foraging patterns and thus recruitment success.

Uncertainties and Inconsistencies

- Few studies have demonstrated the relationships between jaw malformations, reduced feeding, and mortality in fish (Noble et al. 2012).
- Specifically, studies in developing zebrafish and mummichog (*Fundulus heteroclitus*) have found that low concentrations of TCDD or PCB126 exposure can lead to subtle malformations in the lower jaw, in addition to reduced feeding capabilities of the fish (Couillard et al. 2011; King Heiden et al. 2009). However, both studies observed a reduction in feeding even in the fish that did not display jaw malformations, consequently, reduced feeding was not directly linked to an inability to capture prey due to the craniofacial deformity. Overall, it is likely that a combination of different malformations (ex: effects on both the heart and jaw) contribute to Ahr activation-induced mortality.
- The above results are corroborated by an evaluation of TCDD toxicity in seven fish species, where despite the observation of craniofacial malformations in all species, TCDD toxicity, including mortality, decreased once exogenous feeding began suggesting the lack of a strong causal link between craniofacial malformations and poor survival (Elonen et al. 1998).
- An important caveat to the conclusions based on laboratory studies is that the relative abundance of food in an experimental test system relative to the natural environment may hide minor impacts on feeding efficiency that could be more impactful in nature. However, field-based investigations of the effect of craniofacial malformations on prey capture, feeding, and overall ecological fitness are difficult to address due to inherent complexities of the ecosystem.

Quantitative Understanding of the Linkage

Known modulating factors

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List of Non Adjacent Key Event Relationships

Relationship: 2688: Activation, AhR leads to Decrease, sox9 expression

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	non-adjacent	Moderate	Low
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
human	Homo sapiens	Moderate	NCBI
Salmo salar	Salmo salar	Moderate	NCBI
Sebastiscus marmoratus	Sebastiscus marmoratus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

- The relationship between Ahr activation and sox9 repression is best studied in developing zebrafish. Some supporting evidence comes from salmon larvae, as well as human lung cells, suggesting that this relationship is highly evolutionarily conserved among vertebrates (at least), but also likely tissue-specific.

Key Event Relationship Description

- The Ahr is a ligand activated transcription factor that is capable of regulating gene expression of several genes, all belonging to the Ahr signaling cascade (Larigot et al., 2018).
- Canonical Ahr signaling involves receptor translocation from the cytoplasm to the nucleus, followed by Ahr-ARNT heterodimerization. The heterodimer then recognizes Aryl hydrocarbon response elements (AHREs) in the promoter regions of

different genes to regulate their expression (Swanson 2002). Indirect gene regulation is also possible, with the downstream target genes interacting with other signaling pathways (Mathew et al., 2008).

- Sox9 is one proposed indirect gene within the Ahr signaling cascade.Sox9b, one of two paralogs of the sox9 gene in zebrafish, is one of the most reduced transcripts in the jaw upon TCDD exposure in zebrafish (Xiong et al., 2008). Thus, there exists a non-adjacent relationship between Ahr activation and the repression of sox9.

Evidence Supporting this KER

KER 2688 concordance
table: https://aopwiki.org/system/dragonfly/production/2022/10/20/7inngdvxht_Concordance_Table_AHR_to_sox9_clean.pdf

Biological Plausibility

- Evidence for biological plausibility comes from Ahr's ability to interact with several molecular signaling pathways, including the Wnt-beta catenin pathway (Mathew et al. 2008). Sox9 is one important member of the Wnt-beta catenin signaling pathway, specifically as it relates to chondrogenesis (Sinha et al., 2021; Topol et al., 2009.

Empirical Evidence

Empirical evidence and essentiality of KE_{up} for KE_{down} to occur

- Developing zebrafish exposed to 1ng/mL TCDD significantly repress sox9b (one of two paralogs of sox9 in zebrafish) by approximately 2-fold in the heart tissue at 72 hours post fertilization (hpf) (Hofsteen et al., 2013).
- sox9b is significantly repressed in 72-hpf whole animal zebrafish exposed to 2nM TCDD(Jenny et al., 2009). In the same study, knockdown of AHRRb caused a significant decrease in sox9b mRNA expression in the absence of TCDD exposure, suggesting some level of endogenous Ahr control of sox9.
- Whole animal zebrafish exposed to a concentration range of TCDD significantly repress sox9b from 0.5 ng/mL exposure concentration, with cyp1a, a biomarker of Ahr activation, significantly induced from 0.0625 ng/mL (Garcia et al., 2018b).
- A sox9b reporter zebrafish line exposed to 1ng/mL TCDD showed a trend for sox9b repression measured using qRT-PCR (Garcia et al., 2017).
- 96-hpf zebrafish exposed to 1ng/mL TCDD induces cyp1a and represses sox9b in parallel in isolated jaw tissue over multiple time points after exposure (Xiong et al., 2008).
- In regenerating fin tissue after 2 or 3 days post caudal fin amputation of 48-hpf zebrafish exposed to 1ng/mL TCDD, sox9b was one of the most repressed transcripts (Mathew et al., 2008).
- Additionally, one of the most repressed genes in caudal fins from adult zebrafish IP injected with TCDD was also sox9b (Andreasen et al., 2006).
- In whole atlantic salmon larvae exposed to 1 or 10 ng/L PCB-77, sox9 mRNA was significantly reduced (by 50% compared to controls) only till 500 dd, after which, non-significant or significant increases were detected at both concentrations (Olufsen and Arukwe 2011).
- In A549 pulmonary epithelial cells, individual exposures to TCDD, the PAHS Benzo[a]pyrene and benzo[k]fluoranthene, as well as a non-cytotoxic concentration of ambient aerosol particle fraction PM0.5, significantly repressed sox9 expression while also inducing cyp1a expression (Prochazkova et al., 2018; Simeckova et al., 2019.
- Developing zebrafish exposed to 0.5, 5, and 50 nM pyrene (a known Ahr activating chemical), had concentration-dependent significant sox9 repression in the craniofacial skeleton (seen using in situ hybridization), as well as concentration-dependent craniofacial deformations (Shi et al., 2012).
- Ahr2 knockout zebrafish with 1ng/mL TCDD exposure did not have significantly reduced sox9b expression at 48 hpf (Garcia et al., 2018a).
- In one study, adult white sturgeon were exposed to equipotent concentrations of TCDD, PCB-77, and BaP. Repression of sox9 transcript was identified in the livers of fish exposed to all three chemicals (Doering et al., 2016).

Uncertainties and Inconsistencies

- Whole animal zebrafish exposed to several individual PAHs, many of whom significantly induce cyp1a by 48 hpf, do not cause significant repression of sox9b (Garcia et al., 2018b). The PAHs are retene, benzo[j]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]pyrene, benzo[b]fluoranthene, fluoranthene, phenanthrene, and 9-methylanthracene. Dibenzo[a,i]pyrene was the only PAH from the list that showed a trend for sox9b reduction. One explanation is that the possible tissue-specific sox9b repression was not enough to capture expression changes in this whole-animal study where zebrafish were exposed to Ahr activators not as strong as TCDD.
- A microarray study investigating gene expression changes in the jaw primordium of zebrafish exposed to TCDD from 1 to 24 hpf did not include either paralog of sox9 in the top downregulated gene list (Planchart and Mattingly 2010). It is possible that sox9 was not present in the microarray.
- In a human glioblastoma cell culture study, sox9 was repressed when ARNT2 was knocked down, in addition to the study identifying potential binding regions of ARNT2 in the regulatory region of sox9 (Bogeas et al., 2018). While no functional studies were conducted, it is possible that there may be cell-specific direct regulation of sox9 by Ahr/ARNT.
- In frozen human lung tumor samples, expression of sox9 was significantly higher in smokers compared to in samples from non-smokers. Additionally, in adenocarcinomas in smoking women, sox9 expression was relatively high. Of note, these results were accompanied by the lack of induction of Ahr expression (Szymanowska-Narloch et al., 2013).

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Relationship: 2690: Increase, slincR expression leads to Smaller and morphologically distorted facial cartilage structures

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

Evidence for this KER comes from zebrafish studies.

Key Event Relationship Description

- Craniofacial malformations, including due to small and distorted facial cartilage structures, are a common phenotypic endpoint detected upon exposure to a variety of environmental chemicals (Huang et al., 2021).
- Craniofacial development is a highly complex and coordinated process involving both environmental and genetic factors, and thus the mechanisms leading up to its disruption are expected to be complicated (Raterman et al., 2020).
- This KER describes one molecular player (slincR) that is involved in both normal craniofacial development as well as chemical exposure-induced facial cartilage structures.

Evidence Supporting this KER

Biological Plausibility

- Across multiple stages of zebrafish development, slincR is expressed in the jaw/snout region, as well as in the eye and otic vesicle (Garcia et al., 2017).
- Upon exposure to TCDD (a strong Ahr activating chemical), slincR expression increases in both the otic vesicle, as well as the lower jaw/snout region (Garcia et al., 2017).
- Knockdown of slincR expression in developing zebrafish, alters expression of sox9b (a critical transcription factor that regulates cartilage development (Lefebvre et al., 2017)), as well as certain downstream targets of sox9, such as notch3, adamts3, fabp2, sfrp2, and fgfr3 (Garcia et al., 2017).

Empirical Evidence

Empirical evidence and essentiality of KE_{up} for KE_{down} to occur

- While both control and slincR morphant zebrafish exposed to TCDD displayed cartilage structure defects, the slincR morphants had an abnormal junction between hyosymplectic and ceratohyal cartilages in comparison to the control morphants (Garcia et al., 2018), suggesting slincR's role in the craniofacial malformation caused due to TCDD exposure.

Uncertainties and Inconsistencies

- DMSO-treated slincR morphants did not show any changes to craniofacial cartilage structure (Garcia et al., 2018).
- Impact of absence of slincR has only been studied with morpholino knockdown experiments (Garcia et al., 2017; Garcia et al., 2018), which have two relevant drawbacks: 1. Inability to maintain slincR repression by 72 hpf since morpholinos are transient in nature, and 2. Incomplete functional knockout which prevents us from understanding the true impact of the absence of slincR. Future studies using CRISPR-Cas-generated knockout lines, for example, will help overcome both limitations.

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Relationship: 984: Activation, AhR leads to Increase, Early Life Stage Mortality

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF	non-adjacent	High	Moderate
Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2	non-adjacent	High	Moderate
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	non-adjacent	High	Moderate
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	High	NCBI
Japanese quail	Coturnix japonica	High	NCBI
Ring-necked pheasant	Phasianus colchicus	High	NCBI
turkey	Meleagris gallopavo	High	NCBI
bobwhite quail	Colinus virginianus	High	NCBI
American kestrel	Falco sparverius	High	NCBI
Double-crested cormorant	Double-crested cormorant	High	NCBI
Eastern bluebird	Eastern bluebird	High	NCBI
zebrafish	Danio rerio	High	NCBI
Fundulus heteroclitus	Fundulus heteroclitus	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Oncorhynchus mykiss	Oncorhynchus mykiss	Moderate	NCBI
Xenopus laevis	Xenopus laevis	Low	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

- Overall, this KER is believed to be applicable to all vertebrates based on mortality as a result of exposure to known agonists of the AhR (Buckler et al 2015; Cohen-Barnhouse et al 2011; Elonen et al 1998; Johnson et al 1998; Jung et al 1997; Kopf & Walker 2009; Park et al 2014; Tillitt et al 2016; Toomey et al 2001; Walker et al 1991; Wang et al 2013; Yamauchi et al 2006; Zabel et al 1995).

- The correlation between AHR-mediated reporter gene activity and embryo death has been demonstrated in species of birds and fishes (Doernig et al 2018).
- Less is known about differences in binding affinity of AhRs and how this relates to sensitivity in reptiles or amphibians.
- Low binding affinity for DLCs of AhR1s of African clawed frog (*Xenopus laevis*) and axolotl (*Ambystoma mexicanum*) has been suggested as a mechanism for tolerance of these amphibians to DLCs (Lavine et al 2005; Shoots et al 2015).
- Among reptiles, only AhRs of American alligator (*Alligator mississippiensis*) have been investigated and little is known about the sensitivity of American alligator or other reptiles to DLCs (Oka et al 2016).
- Among fishes, great differences in sensitivity to DLCs are known both for AhRs and for embryos among species that have been tested (Doering et al 2013; 2014; 2018).
- Differences in binding affinity of the AhR2 have been demonstrated to explain differences in sensitivity to DLCs between sensitive and tolerant populations of Atlantic Tomcod (*Microgadus tomcod*) (Wirgin et al 2011).

Key Event Relationship Description

The aryl hydrocarbon receptor is commonly known for its involvement in xenobiotic metabolism and clearance, but it also regulates a number of endogenous processes including angiogenesis, immune responses, neuronal processes, metabolism, and

development of numerous organ systems (Duncan et al., 1998; Emmons et al., 1999; Hahn et al 2002; Lahvis and Bradfield, 1998). Strong AHR agonists that cause sustained AHR activation interfere with the receptor's endogenous role in embryogenesis, which causes numerous developmental abnormalities and ultimately leads to embryonic death (Kopf and Walker 2009; Carreira et al 2015).

It's important to note that his relationship only applies to AHR agonists that cause sustained AHR activation. Strong AHR agonists that are rapidly metabolized, such as polycyclic aromatic hydrocarbons, only cause transient AHR activation leading to an alternate mode of toxicity.

This Key Event Relationship describes the indirect link between the Molecular Initiating Event (activation of the AhR) and the Adverse Outcome (increased early life stage mortality).

Evidence Supporting this KER

Biological Plausibility

AHR Ligand Binding Domain

- Mammalian and avian sensitivity to DLCs ultimately comes down to the identity of two particular amino acids in the ligand binding domain (LBD) of the AHR: positions 375 and 319 in mice and 380 and 324 in birds.
  - A 10-fold difference between two strains of mice (non-responsive DBA/2 mouse, and responsive C57BL/6 14 mouse) in CYP1A induction, lethality and teratogenicity following TCDD exposure (Poland et al. 1976), was attributed to a single nucleotide polymorphism at position 375 (Ema et al. 1994; Poland et al. 1994; Poland and Knutson 1982).
  - Several other studies reported the importance of this amino acid in birds and mammals (Backlund and Ingelman-Sundberg 2004; Ema et al. 1994; Karchner et al. 2006; Murray et al. 2005; Pandini et al. 2007; Pandini et al. 2009; Poland et al. 1994; Ramadoss and Perdew 2004).
- The amino acid at position 319 plays an important role in ligand-binding affinity to the AHR and transactivation ability of the AHR, due to its involvement in LBD cavity volume and its steric effect (Pandini et al. 2009).
  - Mutation at position 319 in the mouse eliminated AHR DNA binding (Pandini et al. 2009).

Using AHR LBD Constructs to Determine Avian Sensitivity

- Using chimeric AHR1 constructs combining three AHR1 domains (DBD, LBD and TAD) from the chicken (highly sensitive to DLC toxicity) and common tern (resistant to DLC toxicity), Karchner and colleagues (2006), showed that amino acid differences within the LBD were responsible for differences in TCDD sensitivity between the chicken and common tern.
  - They specifically attributed positions 324 and 380 with differences in TCDD binding affinity and transactivation between the chicken (Ile324_Ser380) and common tern (Val324_Ala380) receptors.
- The LBD of over 85 bird species have since been analyzed to find that 6 amino acid residues differed among species (Farmahin et al. 2013; Head et al. 2008), but only positions 324 and 380 in the AHR1 LBD were associated with differences in DLC toxicity in ovo and AHR1-mediated gene expression in vitro (Farmahin et al. 2013; Head et al. 2008; Manning et al. 2012).
  - Based on these results, avian species can be divided into one of three AHR1 types based on the amino acids found at positions 324 and 380 of the AHR1 LBD: type 1 (Ile324_Ser380; most sensitive), type 2 (Ile324_Ala380; moderately sensitive) and type 3 (Val324_Ala380; least sensitive) (Farmahin et al. 2013; Head et al. 2008; Manning et al. 2012).
  - A sampling of bird species and their AHR LBD category is described in table 1. A list of 86 species and their subtype can be found in Farmahin et al. (2013).

AHR1 LBD Types.png

Empirical Evidence

Mammals:

- AhR deficient strains of mice (*Mus musculus*) are unaffected by exposure to agonists of the AhR (Fernandez-Salguero et al 1996).
- Strains of mice that express AhRs with lesser affinity for agonists are more tolerant to adverse effects of exposure relative to strains of mice that express AhRs with greater affinity for agonists (Bisson et al 2009; Ema et al 1993).

Birds:

Binding of dioxin-like compounds (DLCs) to avian AHR1 (Farmahin et al. 2014; Karchner et al. 2006) and AHR1-mediated transactivation measured using luciferase reporter gene (LRG) assays have been demonstrated in domestic and wild species of birds (Farmahin et al. 2012; Farmahin et al. 2013b; Fujisawa et al. 2012; Lee et al. 2009; Manning et al. 2012; Mol et al. 2012), and binding affinity was found to be strongly correlated with embryotoxicity (Manning et al. 2012) .

Fish:

- Knockdown of the AhR2 prevents mortality following exposure to agonist of the AhR in fishes (Clark et al 2010; Hanno et al 2010; Prasch et al 2003; Van Tiem & Di Giulio 2011). Relative potencies of dioxin-like compounds for activation of AHR2 alpha of rainbow trout (*Oncorhynchus mykiss*) is predictive of relative potencies for early life stage mortality (Abnet et al 1999).
- AhR2-mediated transactivation measured using luciferase reproter gene (LRG) assays have been demonstrated in 8 species of freshwater and marine fishes to strongly correlate with early life stage mortality (Doering et al 2018). However, AhR1-mediated transactivation does not (Doering et al 2018). Further, the slope and y-intercept for the relationship between AhR2-mediated transactivation and early life stage mortality in fishes are not statistically different from the slope and y-intercept for the relatoinship between AhR1-mediated transactivatoin and embryotoxicity (Doering et al 2018).

Amphibians:

- AhR1s of amphibians studied to date are insensitive to activation by dioxin-like compounds*in vitro*, while amphibians studies to date are extremely tolerant to adverse effects of exposure to dioxin-like compounds *in vivo* (Jung et al 1997; Lavine et al 2005; Shoots et al 2015).

Invertebrates:

- Chemicals that activate the AhR of vertebrates are not known to bind AhRs of invertebrates and increased mortality in invertebrates has never been observed as a result of exposure to these agonists (Hahn 2002; Hahn et al 1994).

Uncertainties and Inconsistencies

Interestingly, interference with endogenous AHR functions, either by knock-out or by agonist exposure during early development, causes similar cardiac abnormalities (Carreira et al 2015). Although this is counterintuitive, it demonstrates that the AHR has an optimal window of activity, and deviation either above or below this range results in toxicity.

Uncertainites:

- Only limited AhR activation information and mortality information is currently available for reptiles and amphibians.
- Despite decades of research into the molecular initiating event (i.e., binding of chemicals to the AhR) and resulting adverse outcomes (i.e. mortality), less is known about the precise cascade of key events that link activation of the AhR to the adverse outcome (Doering et al 2016).
- However, hundreds to thousands of different genes are regulated, either directly or indirectly, by activation of the AhR, which presents major uncertainties in the precise pathway of key events or whether perturbation to multiple pathways is the cause of mortality (Brinkmann et al 2016; Doering et al 2016; Huang et al 2014; Li et al 2013; Whitehead et al 2010).
- Despite these uncertainties in the AOP, considerable research has investigated the indirect relationship between activation of the AhR and increased mortality among different chemicals, species, and taxa (Doering et al 2013).

Inconsistencies:

- There are no currently known inconsistencies between AhR activation and increased mortality among vertebrates.

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Relationship: 2689: Activation, AhR leads to Smaller and morphologically distorted facial cartilage structures

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
mouse	Mus musculus	High	NCBI
American mink	Neovison vison	High	NCBI
human	Homo sapiens	Moderate	NCBI
chicken	Gallus gallus	High	NCBI
Japanese quail	Coturnix japonica	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

- This KER provides some highlights for the relationship between Ahr signaling activation and craniofacial formation disruptions, including those directly associated with cartilage structure malformation.
- Several Ahr activating chemicals have been associated with the disruption of jaw formation in animals such as fish and mink (Hornung et al. 1999; Render et al. 2000), providing evidence for the KER.

Evidence Supporting this KER

KER 2689 concordance table: https://aopwiki.org/system/dragonfly/production/2022/10/20/14h2wanxmd_Concordance_Table_AHR_to_craniofacial_clean.pdf

Biological Plausibility

- Primary biological plausibility evidence comes from studies using techniques such as in situ hybridization and immunohistochemistry to identify Ahr and Ahr-related gene and protein expression in lower jaw structures of a variety of animals. For example, Ahr mRNA and protein are present in mouse craniofacial tissue (Abbott et al. 1994a; Abbott et al. 1998), Ahr and Arnt protein are expressed in human embryonic palatal cells (Abbott et al. 1994b), and Ahr2 and cyp1a are expressed in the craniofacial region (including the Meckel's cartilage) of zebrafish (Mattingly et al. 2001; Teraoka et al. 2002).

Empirical Evidence

Empirical evidence and essentiality of KE_{up} for KE_{down} to occur

Some examples for empirical evidence from exposures to known Ahr activators:

- TCDD exposure to developing mice causes cleft plate and negative effects on cell proliferation in the jaw region (Tao et al. 2020).
- Beta-naphthoflavone (BNF) exposure to mink decreased squamous epithelial proliferation and caused lesions in mink jaws (Matz et al. 2019).
- In zebrafish, PCB-126 exposure caused impaired lower jaw growth, and TCDD exposure decreased craniofacial cartilage size, chondrocyte size and number, and tended to decrease chondrocyte proliferation, while also causing the Meckel's and palatoquadrate cartilages to be malformed (Burns et al. 2015; Grimes et al. 2008; Liu et al. 2016; Xiong et al. 2008).
- Chick embryos exposed to mono-ortho-chlorinated chlorobiphenyls at high concentrations led to mortality of the embryos, with the surviving embryos displaying shortened beaks (as well as microphthalmia, degenerative hepatic lesions and edema) (Brunstrom 1990).
- Japanese Quail and Common Pheasant embryos exposed to TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), and 2,3,7,8-tetrachlorodibenzofuran (TCDF), and White Leghorn Chicken embryos exposed to TCDD and PeCDF all displayed bill deformities including incomplete or lack of upper/lower beak or crossbill (Cohen-Barnhouse et al., 2011). Japanese Quail in particular had % deformities that increased in a concentration-depedent manner (0.42 - 23 g/egg) for each chemical.

Some examples for essentiality evidence:

- Ahr2 morpholino knockdown in zebrafish exposed to 0.4 ng/mL TCDD provided partial protection from TCDD-induced jaw length shortening (Prasch et al. 2003).
- Ahr-null mice were protected from TCDD-induced cleft palate (Mimura et al. 1997).

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