

Crosstalk between AHR and Wnt signaling through R-Spondin1 impairs tissue regeneration in zebrafish

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ABSTRACT Exposure to dioxins, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), causes a wide array of toxicities in vertebrates, which are mostly considered to be mediated through the inappropriate activation of the aryl hydrocarbon receptor (AHR) signaling pathway. Although transcriptional regulation by AHR is widely studied, the molecular mechanisms responsible for the adverse outcomes after AHR activation are largely unknown. To identify the important downstream events of AHR activation, we employed the zebrafish caudal fin regeneration model, where AHR activation blocks the regenerative process. Comparative toxicogenomic analysis revealed that both adult and larval fins respond to TCDD during regeneration with misexpression of Wnt signaling pathway members and Wnt target genes. R-Spondin1, a novel ligand for the Wnt coreceptor, was highly induced, and we hypothesized that misexpression of R-Spondin1 is necessary for AHR activation to block regeneration. Partial antisense repression of R-Spondin1 reversed the inhibitory effect of TCDD, and tissue regeneration was restored. This finding demonstrates that inhibition of regeneration by TCDD is mediated by misinduction of R-Spondin1. Because R-Spondin1 signals through the Wnt coreceptor LRP6, we further demonstrated that the TCDD-mediated block in regeneration is also LRP6 dependent. Collectively, these results indicate that inappropriate regulation of R-Spondin1/LRP6 is absolutely required for TCDD to inhibit fin regeneration.—Mathew, L. K., Sengupta, S. S., LaDu, J., Andreasen, E. A., Tanguay, R. L. Crosstalk between AHR and Wnt signaling through R-Spondin1 impairs tissue regeneration in zebrafish. *FASEB J.* 22, 3087–3096 (2008)

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THE ARYL HYDROCARBON RECEPTOR (AHR) is a basic-helix-loop-helix (bHLH) transcription factor, well known for its ability to bind to environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1, 2). The well-accepted mechanism of action of TCDD is that the ligand-bound AHR translocates to the nucleus, where it binds with its dimerization partner, Ah receptor nuclear translocator (ARNT). The AHR-ARNT complex binds to specific enhancer elements, which results in the transactivation of a variety of genes that presumably play a role in producing diverse toxicities, including reproductive and

developmental toxicity, immunotoxicity, cardiotoxicity, teratogenicity, and neurotoxicity at low exposure levels (reviewed in ref. 3). Even though the transcriptional regulation of AHR has been widely studied, the molecular mechanisms of AHR-dependent toxicities are poorly understood. To understand the complex molecular mechanisms after AHR activation, an *in vivo* research model easily amenable to molecular and genetic manipulation would be very useful.

Zebrafish caudal fin regeneration is a well-established research model that has been utilized to identify the basic principles of tissue regeneration (4, 5). Zebrafish regenerate their caudal fins by a process referred to as epimorphic regeneration. Immediately after surgical amputation, epithelial cells begin to migrate over the injured site, forming a wound cap, which is followed by the dedifferentiation of cells proximal to the amputation plane into a cluster of pluripotent cells referred to as the blastema (4–6). The blastema cells further proliferate and differentiate into the cell types required to complete the regenerative outgrowth. This complex process is tightly regulated by multiple signaling pathways, and interference by external stimuli could modulate the regeneration process. We have previously demonstrated that TCDD, an AHR ligand, inhibits zebrafish fin regeneration at both adult and larval stages (7, 8). We also reported that the inhibition of fin regeneration by TCDD is absolutely AHR2 and ARNT1 dependent (7). Therefore, we developed this inhibition of regeneration phenomenon as an *in vivo* platform to identify the molecular signaling pathways affected downstream of AHR activation.

Through microarray and morphological analysis, we have previously demonstrated that TCDD affects several components involved in cellular differentiation and extracellular matrix (ECM) composition in adult tissue regenerates (9, 10). To determine whether the gene expression changes are conserved between the adult and larval fin regeneration models, we performed microarray analysis using the larval regeneration model. Comparative genomic analysis revealed that AHR activation results in the misexpression of a number of Wnt signaling genes in both regeneration systems, and we conclusively demon-

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strate that crosstalk between AHR and Wnt signaling is responsible for the impairment of fin regeneration.

MATERIALS AND METHODS

Zebrafish embryos

Fertilized embryos from AB strain zebrafish (University of Oregon, Eugene, OR, USA) were used for all the experiments. All embryos were reared in our laboratory according to standard procedures, and each experimental group consisted of 12 larvae (36). Sox9b mutant line was a gift from Dr. John H. Postlethwait (University of Oregon, Eugene, OR, USA).

Chemicals

TCDD (>99% pure) was obtained from Chemsyn (Lenexa, KS, USA), and the larval exposures were done at a concentration of 1 ng/mL. 6-Bromoindirubin-3'-oxime (BIO) was bought from EMD Biosciences (San Diego, CA, USA), and the larvae were exposed at a final concentration of 10 μ M.

Fin RNA isolation

Caudal fins from 2-day-old embryos were amputated, and the animals were exposed to either dimethyl sulfoxide (DMSO; vehicle control) or TCDD (>99% pure; Chemsyn) in the water for 1 h (Supplemental Fig. S1). After several rinses in TCDD-free water, the larvae were reared until 2 and 3 days postamputation (dpa) when their regenerating fin tissue was amputated and collected for RNA analysis. RNA was extracted from the fin tissue using the RNAqueous Micro kit (Ambion, Austin, TX, USA). Three groups at each time point and treatment, each comprising 150 larval fins, were pooled to make an individual replicate. The quality and quantity of RNA was analyzed by UV absorbance. The abundance of ribosomal RNA and degree of degradation were determined in electropherogram patterns using the 2100 Bioanalyzer and RNA 6000 Nano chips (Agilent Technologies, Palo Alto, CA, USA).

Affymetrix microarray processing

The microarray processing using the Affymetrix platform was performed by the Center for Genome Research and Biocomputing at Oregon State University (Corvallis OR, USA). A total of 100 ng of RNA from the larval fin tissue (+/-TCDD) at 2 and 3 dpa was used to generate biotinylated complementary RNA (cRNA) using the Two-Cycle Target Labeling kit (Affymetrix, Santa Clara, CA, USA). In short, the different RNA samples were reverse-transcribed using a T7-(dT)₂₄ primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and double-stranded cDNA was synthesized. Another round of double-stranded cDNA synthesis was conducted using the previously synthesized cDNA as the template. Biotinylated cRNA was synthesized from the double-stranded cDNA using T7 RNA polymerase and a biotin-conjugated pseudouridine containing nucleotide mixture provided in the IVT Labeling Kit (Affymetrix). The biotinylated cRNA was quantified, and 10 μ g of purified and fragmented cRNA from each experimental sample was hybridized to zebrafish genome arrays (Zebrafish430_2) according to the Affymetrix GeneChip Expression Analysis Technical Manual (701021 Rev. 5). Arrays were scanned with an Affymetrix scanner 3000. Each array image was visualized to discount artifactual signals, scratches, or debris. Experiments were certified under Minimum Information About a Microarray Experiment (MIAME) standards.

Data analysis was performed by importing the Affymetrix .CEL files into GeneSpring 7.1 software (Agilent Technologies). The files were gene chip-robust multiarray (GC-RMA) processed to discount for background signal, and each transcript was normalized to the median signal to allow comparison between arrays on a relative scale for each gene. The differential gene expression changes by TCDD during regeneration was performed by comparing the experimental samples from the vehicle *vs.* TCDD-exposed larvae at 2 and 3 dpa by 1-way ANOVA assuming equal variance ($P < 0.05$). Only genes that were at least 1.7-fold differentially expressed from the vehicle gene levels were considered for analysis. The annotation of genes was conducted by taking into account the sequence similarity to known mammalian proteins that was determined by conducting a basic local alignment search tool (BLAST) search of each Affymetrix probe set against the Sanger database (http://www.sanger.ac.uk/Projects/D_rerio/). Moreover, other databases, such as Genbank (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the Zebrafish Affy Chip Annotation Project at Children's Hospital Boston (<http://134.174.23.160/zfaca/hash/master020106public.aspx>), were used simultaneously. Experiments were MIAME certified, and the raw data are listed at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/projects/geo/>; series record GSE10184).

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

From the larval fin tissue (+/-TCDD), total RNA was isolated in triplicate at 2 and 3 dpa ($n=150$ /group). cDNA was prepared from 100 ng of total RNA per group using Superscript II (Life Technologies, Gaithersburg, MD, USA) and oligo(dT) primers in a 20 μ l volume. qRT-PCR was performed using gene-specific primers (Supplemental Table S5) with the Opticon 2 real-time PCR detection system (MJ Research, Waltham, MA, USA). According to the manufacturer's instructions (Finnzymes, Espoo, Finland), 1 μ l of cDNA was used for each PCR using DyNAmo SYBR green qPCR kit. All experimental samples were normalized to their β -actin abundance, and quantitative differences between biological samples were determined by normalizing all samples to a common reference sample. Agarose gel electrophoresis and thermal denaturation (melt curve analysis) were performed to analyze the formation of specific PCR products. Statistical significant differences of mRNA abundance were assessed by 1-way ANOVA on \log_{10} transformed data using Tukey's method ($P < 0.05$) (SigmaStat Software, Chicago, IL, USA).

Oligonucleotides

Oligonucleotide primers were synthesized by MWG-Biotech (High Point, NC, USA). Forward primers are prefixed with an F corresponding to sense strands, and antisense reverse primers are designated with an R. Primers were designed to amplify sequences within the Affymetrix probe set sequence. Sequences for each primer can be found in Supplemental Table S6.

Cloning of zebrafish LRP6 (zFLRP6) gene

The human LRP6 peptide sequence was blasted against the Sanger database, and a putative gene (Ensemble gene ID ENSDARG00000063702) with 66% peptide similarity was identified. Three predicted transcripts were enlisted for this gene; based on the number of exons and splicing similarity, we pursued with the transcript ID ENSDART00000093327. Forward and reverse primers were designed from either ends of the predicted coding sequence, and PCR was performed

using KOD hot start DNA polymerase (Novagen, San Diego, CA, USA) to clone the full-length gene (predicted sequence is 4767 bp). The gel-purified PCR product was incubated with *Taq* polymerase at 72°C for 1 min to add T at the blunt end and then inserted to the TOPO-XL vector (Invitrogen, Carlsbad, CA, USA). The full-length sequence of zfLRP6 gene was performed by designing primers within the fragment after each DNA sequencing step. The full-length coding sequence of the zfLRP6 gene from the start to the stop codon is 4863 bp (Genbank No. EU395842).

In situ hybridization

In situ localization of mRNA was performed on the regenerating fin at respective time points, as described previously (37, 38). The *msxe* and *dlx5a* probes were obtained from Atsushi Kawakami (Tokyo Institute of Technology, Yokohama, Japan) (19). The *Raldh2* probe was prepared by cloning the cDNA by RT-PCR from the RNA isolated from the whole adult zebrafish. *~din1* probe was generated by cloning the cDNA by RT-PCR from the adult zebrafish RNA.

Morpholinos (MOs)

The R-Spondin1 gene was specifically targeted by designing a splice junction MO at the intron 1-exon 2 boundary (Gene Tools, Philomath, OR, USA). The sequence of the fluorescein-tagged R-Spondin1 MO is 5'-GTGCTTACTGATG-GAGAAAAGACAG-3'. A splice junction MO at the exon 3-intron 4 boundary of the LRP6 gene was designed to transiently knock down the transcript. The sequence of LRP6 MO is 5'-AGGTGTTCTGACCTGCTGGAGCCGT-3'. MOs were diluted to 3 mM in 1× Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6) as described (39). A standard control MO (Gene Tools, Philomath, OR, USA) (5'-CTCTTACCTCAGT-TACAATTTATA 3') was used as the control MO. Approximately, 2 nl of 0.3 mM R-Spondin1 MO and 0.5 mM LRP6 MO solution was microinjected into the embryos at the 1–2 cell stage. The control, R-Spondin1, and LRP6 morphants were amputated at 2 days postfertilization (dpf) and exposed to vehicle or TCDD for 1 h, then the larvae were raised for 3 days at 28°C.

RESULTS

Gene expression changes in larval regenerating tissue after AHR activation

Previously, we reported that TCDD inhibits both adult and larval fin regeneration (7, 8). To elucidate the molecular signaling events downstream of AHR activation, microarray analysis was conducted using regenerating fin tissue isolated from larval fish in the presence and absence of TCDD (Supplemental Fig. S1). Alterations in gene expression by TCDD were evaluated by first filtering for genes that were at least 1.7-fold differentially abundant at 2 and 3 dpa from their time-matched control. One-way ANOVA analysis was performed between the time-matched vehicle and TCDD-exposed gene list for statistical significance. A total of 1097 genes was differentially expressed by TCDD exposure (Supplemental Fig. S2A). The genes were categorized into functional groups based on sequence homology and the identified function (Supplemental Fig. S2B). Components related to signal transduction, ECM remodeling, cytoskeleton dynamics, and Wnt signaling pathway were the predominant functional

groups significantly affected by TCDD (Supplemental Tables S1 and S2). Transcripts from different functional groups that were differentially expressed by AHR activation were validated by qRT-PCR (Supplemental Fig. S3A, B), illustrating the validity of the microarray results. R-Spondin1, a recently identified Wnt ligand, was one of the most highly induced transcripts at 2 and 3 dpa. In contrast, Sox9b, a transcription factor that plays a major role in chondrogenesis regulation, was the gene mostly repressed by TCDD. Promoter analysis of 5000 bp upstream sequence of R-Spondin1 revealed a single AHR response element (AHRE) and a number of cis-elements for other transcription factors. However, no AHREs are present on the Sox9b promoter. The role of these cis-regulatory elements in mediating the AHR-dependent induction of R-Spondin1 is an important future goal. Collectively, these larval regeneration responses to TCDD are consistent with our previous adult fin regeneration microarray studies (10) and prompted to us to perform comparative toxicogenomic analysis between these two distinct platforms.

Comparative toxicogenomic approach reveals misregulation of Wnt signaling by AHR activation

Because fin regeneration is inhibited by AHR activation in both life stages, the key molecular events controlling this phenomenon may be conserved. The transcriptional response to TCDD in larval fin regeneration system was compared with our previously published gene expression analysis data on adult fin regeneration after TCDD exposure (9) (Supplemental Fig. S4). We generated a list of genes that were differentially regulated at least 1.7-fold in response to TCDD in both adult and larvae regenerating fins at any time point. Approximately 50 genes were similarly enhanced and 150 transcripts were repressed due to AHR activation in the two regenerative models (Supplemental Tables S3 and S4). Notably, R-Spondin1 and Sox9b showed the greatest induction and repression, respectively, during larval and adult fin regeneration after exposure to TCDD. The similar expression pattern of 200 genes in response to AHR activation in the regenerating fin tissue in two different life stages with different structural architecture suggests common mechanisms of action for the inhibitory phenotype. It is noteworthy that transcripts related to ECM metabolism, cell adhesion, and migration dominated the list of differentially expressed genes after TCDD exposure in both the models.

Misregulation of Wnt signaling by AHR activation is mediated through R-Spondin1

In addition to the overexpression of R-Spondin1, numerous Wnt signaling targets were also altered by AHR activation, in both regeneration models (Table 1). The identification of Wnt target genes was performed with reference to the Wnt home page (<http://www.stanford.edu/~rnusse/wntwindow.html>) and recognized more than 20 Wnt target genes that were modulated by AHR activation in these two regenerative models (Table 1). The pattern of the expression of these Wnt target

TABLE 1. *Wnt signaling pathway members and target genes*

Member or target	Fold difference (<i>P</i> value)				
	Larvae		Adult		
	2 dpa	3 dpa	1 dpa	3 dpa	5 dpa
span class=SpellE Wnt pathway members					
R-spondin-1	9.02 (0.04)	15.41 (0.01)	9.40 (0.01)	20.46 (0.01)	15.29 (0.00)
TCI (C8orf4)		7.82 (0.05)			
Dickkopf-1		3.35 (0.02)			
Frizzled 7a	3.45 (0.02)	3.16 (0.00)	5.44 (0.01)	3.84 (0.00)	3.10 (0.01)
WNT inhibitory factor 1		2.93 (0.01)			
Casein kinase 1, epsilon		2.51 (0.01)			
SOX3	2.79 (0.04)	2.56 (0.02)			1.95 (0.03)
Frizzled 10	0.58 (0.05)	0.33 (0.01)			
Frizzled 8a		0.47 (0.05)			
wnt11r					0.32 (0.03)
Wnt target genes					
Fgf20a		11.20 (0.02)			2.98 (0.02)
jagged1a		6.23 (0.04)			2.20 (0.03)
Claudin-1		3.94 (0.01)			
VEGF		3.82 (0.01)			
Cyclooxygenase 2	2.03 (0.03)	3.29 (0.02)			
Glucagon		3.12 (0.02)			
Cholecystokinin		2.86 (0.01)	5.62 (0.01)	6.35 (0.01)	4.18 (0.02)
Follistatin		2.83 (0.01)			
CYR 61		2.64 (0.01)			0.22 (0.03)
CTGF		2.26 (0.01)			
	2.26 (0.04)	2.17 (0.01)			
TIMP2	1.96 (0.04)	2.01 (0.01)			2.77 (0.05)
				0.07	0.09
Sox9b	0.04 (0.02)	0.17 (0.02)	0.11 (0.02)	(<0.01)	(<0.01)
cyp26a1					0.26 (0.00)
Runx2a					0.40 (0.00)
twist1					0.35 (0.01)
c-jun					0.49 (0.03)
Lysyl oxidase		0.10 (0.01)	0.42 (0.01)		0.46 (0.05)
				0.22	0.31
Connexin 43		0.55 (0.02)	0.54 (0.01)	(<0.01)	(<0.01)
Coll1a1		0.19 (0.02)	0.37 (0.02)	0.40 (0.02)	
Periostin	0.45 (0.04)	0.36 (0.04)			
dapper 2				0.35 (0.00)	
IGF-2				0.47 (0.02)	
cmyc				0.50 (0.00)	
				0.52	
Snail1a		0.48 (0.01)		(<0.01)	

genes suggested that TCDD improperly activates Wnt signaling pathway in tissue regenerates. *In situ* localization of *r-spondin1* revealed high induction of the transcript in the fin tissue of TCDD-exposed larvae, consistent with the microarray and qRT-PCR results (Fig. 1). The localization pattern of *r-spondin1* in the regenerating fin tissue was unique, as it was expressed in a few rows of cells surrounding the notochord, in proximity to the regenerating fin tissue. Because R-Spondin1 is an upstream modulator of Wnt signaling, we hypothesized that AHR-dependent misexpression of R-Spondin1 may dictate the downstream gene expression changes that collectively result in impaired cellular differentiation, ECM remodeling, and cell adhesion and migration. If the induction of R-Spondin1 is required for TCDD to block the regenerative growth, antisense repression of R-Spondin1 should permit regeneration in the presence of TCDD. A splice junction MO

was designed to target R-Spondin1 gene in the IIE2 boundary (Fig. 2A). It is noteworthy that complete knock-down of R-Spondin1 resulted in the lethality of all the microinjected embryos (morphants) at 24 hpf, suggesting an important functional role for this gene during development (data not shown). To avoid a lethal response, we titrated the level of the fluorescent tagged R-Spondin1 MO to obtain partial suppression of the gene to a level that did not completely impair embryonic development. The morphants were screened for fluorescence, and the animals with similar intensity were selected for the study. The amputated control and R-Spondin1 morphants at 48 hpf were then exposed to vehicle or TCDD and raised for 3 days. The control and R-Spondin1 morphants exposed to vehicle regenerated the caudal fins, whereas TCDD blocked the regeneration of control morphants (Fig. 2B). However, partial suppression of R-Spondin1 using splice

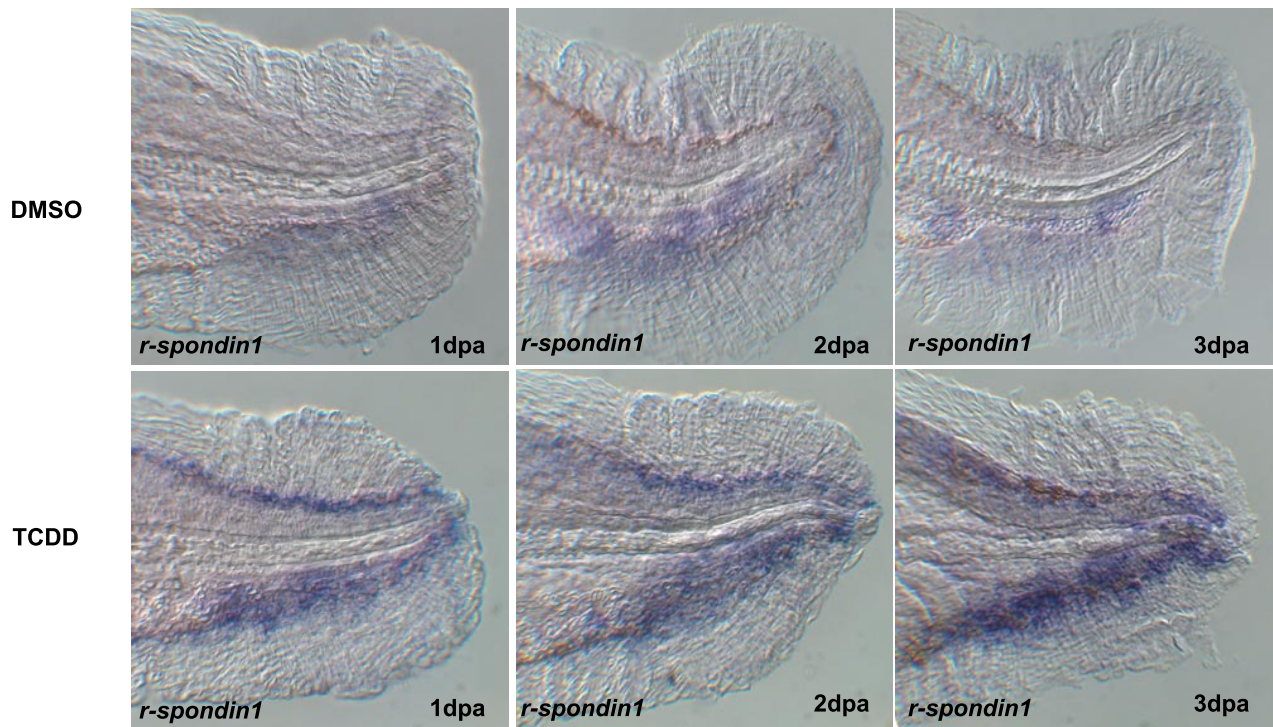


Figure 1. Localization of R-Spondin1 in the larval fin tissue after exposure to TCDD. *In situ* hybridization of *r-spondin1* in larval regenerating fin tissue exposed to vehicle or TCDD. A thick row of cells surrounding the notochord in proximity to the regenerating fin tissue expresses *r-spondin1* in TCDD exposed larvae.

blocking MOs abrogated the TCDD-dependent block of regeneration, suggesting that inappropriate induction of R-Spondin1 is required for TCDD to impair regeneration

(Fig. 2B). The partial repression of R-Spondin1 transcript was analyzed by RT-PCR using specific primers on control and R-Spondin1 morphants at 3 dpf (Fig. 2A, C). These

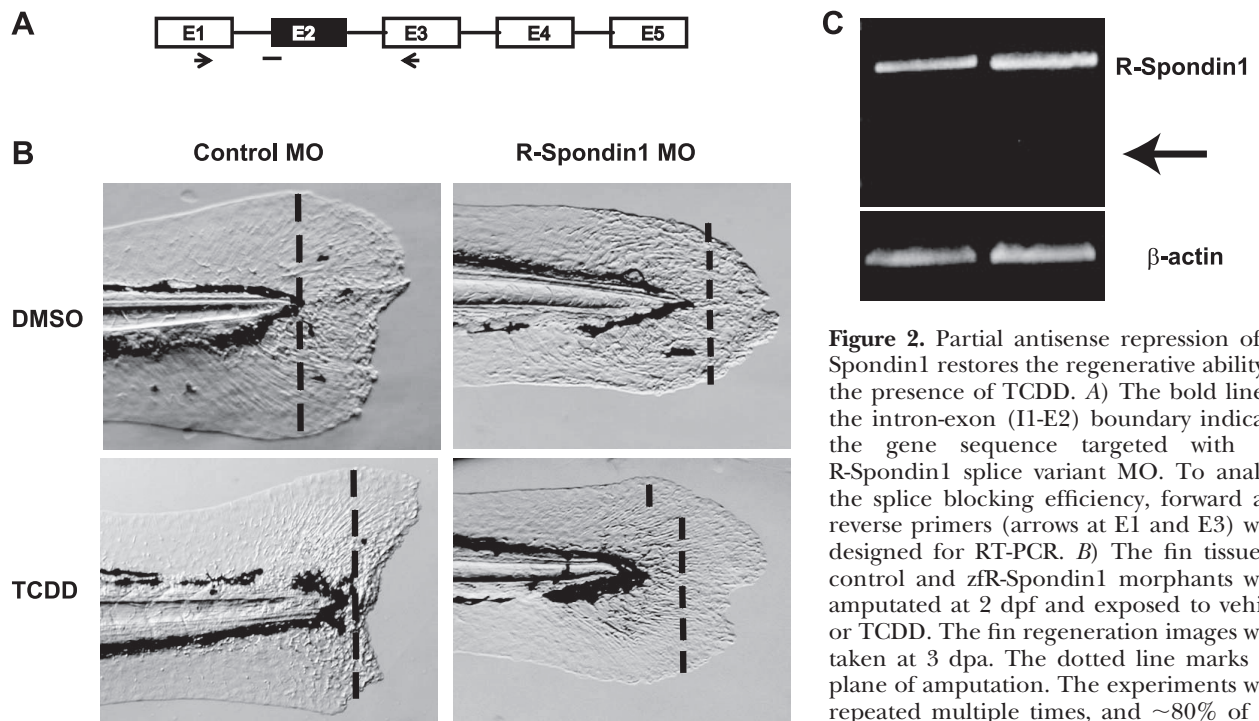


Figure 2. Partial antisense repression of R-Spondin1 restores the regenerative ability in the presence of TCDD. *A*) The bold line at the intron-exon (I1-E2) boundary indicates the gene sequence targeted with the R-Spondin1 splice variant MO. To analyze the splice blocking efficiency, forward and reverse primers (arrows at E1 and E3) were designed for RT-PCR. *B*) The fin tissue of control and z*R-Spondin1* morphants were amputated at 2 dpf and exposed to vehicle or TCDD. The fin regeneration images were taken at 3 dpa. The dotted line marks the plane of amputation. The experiments were repeated multiple times, and ~80% of the R-Spondin1 morphants did not elicit inhibition of regeneration after TCDD exposure ($n=70/85$). *C*) Analysis of R-Spondin1 transcript in control and R-Spondin1 morphants (3 dpf) after qRT-PCR, followed by agarose gel electrophoresis (2%). The arrow points to the misspliced R-Spondin1 variant after the loss of the targeted exon2. β -Actin expression was used as the loading control.

of regeneration after TCDD exposure ($n=70/85$). *C*) Analysis of R-Spondin1 transcript in control and R-Spondin1 morphants (3 dpf) after qRT-PCR, followed by agarose gel electrophoresis (2%). The arrow points to the misspliced R-Spondin1 variant after the loss of the targeted exon2. β -Actin expression was used as the loading control.

results are significant and demonstrate for the first time that an adverse *in vivo* response to AHR activation can be circumvented by modulating a single downstream gene. Moreover, these studies identify a functional crosstalk between the AHR and Wnt signal transduction pathways.

LRP6 is required for TCDD-dependent inhibition of regeneration

Recent studies suggest that R-Spondin1 signaling is mediated directly or indirectly through the Wnt coreceptor LRP6 (11, 12). To underscore the indispensable role of R-Spondin1 signaling to this inhibitory response, we hypothesized that if R-Spondin1 signaling mediates through LRP6, antisense repression of LRP6 should permit regeneration even in the presence of TCDD. We designed a splice junction MO against the LRP6 gene in the E3I4 boundary (Fig. 3A). Similar to the R-Spondin1 MO experiments, the complete knockdown of LRP6 elicited lethality, implicating its functional importance during development. Further titration experiments, along with the screening for fluorescence of the control and LRP6 morphants, enabled the selection of the animals for the studies. The caudal fins of the selected control and LRP6 morphants were amputated at 48 hpf and exposed to vehicle or TCDD for 1 h and allowed to regenerate for the 3 days. The control and LRP6 morphants exposed to vehicle completely regenerated the fin tissue, suggesting that partial reduction of LRP6 gene did not affect the normal regeneration process. The control morphants exposed to TCDD elicited inhibition of regeneration, whereas LRP6 morphants regenerated their lost fin tissue in the presence of TCDD (Fig. 3B). This finding suggests that the misexpression of R-Spondin1 requires LRP6 in order to block tissue regeneration. We confirmed the efficiency of the splice

junction LRP6MO by PCR (Fig. 3C). This result underscores our findings that AHR activation affects tissue regeneration by interfering with the normal functional role of Wnt signaling.

Improper activation of Wnt signaling impairs regeneration

Our microarray data and functional studies with R-Spondin1 support the hypothesis that AHR activation is inappropriately activating the Wnt signaling pathway through R-Spondin1, which causes the inhibition of regeneration. Inhibition of Wnt signaling also blocks fin regeneration, indicating a complex functional role for Wnts in tissue regeneration (6). To directly test the consequence of improper activation of Wnt signaling during regeneration, we used BIO, a specific pharmacological inhibitor of glycogen synthase kinase-3 (GSK-3), which is a negative regulator of canonical Wnt signaling pathway (13). Two-day-old larvae were amputated and exposed to vehicle or BIO (10 μ M) for 3 days continuously. BIO exposure inhibited regeneration phenotypically similar to TCDD exposure (Fig. 4A), suggesting that inappropriate overactivation of Wnt signaling inhibits the regenerative capacity.

Because our results suggest that TCDD overactivates the Wnt signaling pathway, we performed comparative *in situ* hybridization analysis between TCDD- and BIO-exposed larvae using molecular markers that specifically define the wound epithelium (*dlx5a*) and the blastema (*msxe* and *raldh2*). When compared with the vehicle control, the expression of *dlx5a*, *msxe*, and *raldh2* were lost in the regenerates of TCDD-exposed animals at 1 dpa (Fig. 4B), indicating improper formation of the wound epithelium and the blastema. We further analyzed the expression of the above-described markers on BIO-exposed larvae. Similar to the TCDD

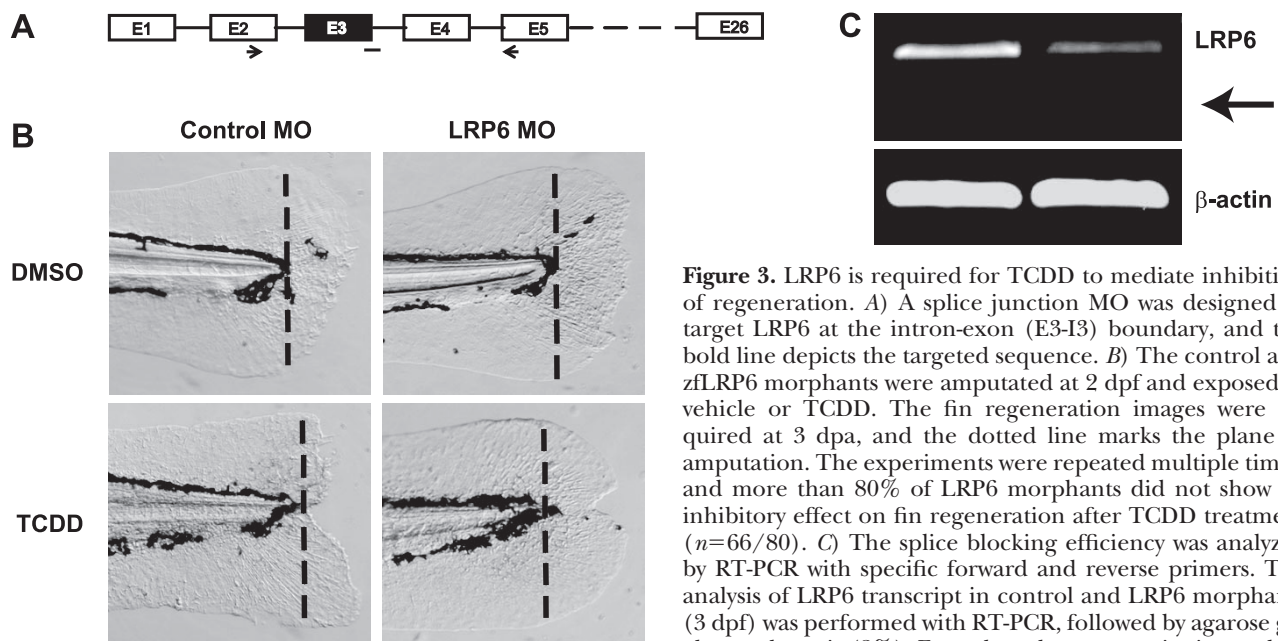


Figure 3. LRP6 is required for TCDD to mediate inhibition of regeneration. A) A splice junction MO was designed to target LRP6 at the intron-exon (E3-I3) boundary, and the bold line depicts the targeted sequence. B) The control and zfLRP6 morphants were amputated at 2 dpf and exposed to vehicle or TCDD. The fin regeneration images were acquired at 3 dpa, and the dotted line marks the plane of amputation. The experiments were repeated multiple times, and more than 80% of LRP6 morphants did not show an inhibitory effect on fin regeneration after TCDD treatment ($n=66/80$). C) The splice blocking efficiency was analyzed by RT-PCR with specific forward and reverse primers. The analysis of LRP6 transcript in control and LRP6 morphants (3 dpf) was performed with RT-PCR, followed by agarose gel electrophoresis (2%). Even though nonquantitative, a clear

reduction of the LRP6 primary transcript was found in the LRP6 morphants. The arrow points to the misspliced LRP6 variant after the loss of the targeted exon. β -Actin expression was used as the loading control.

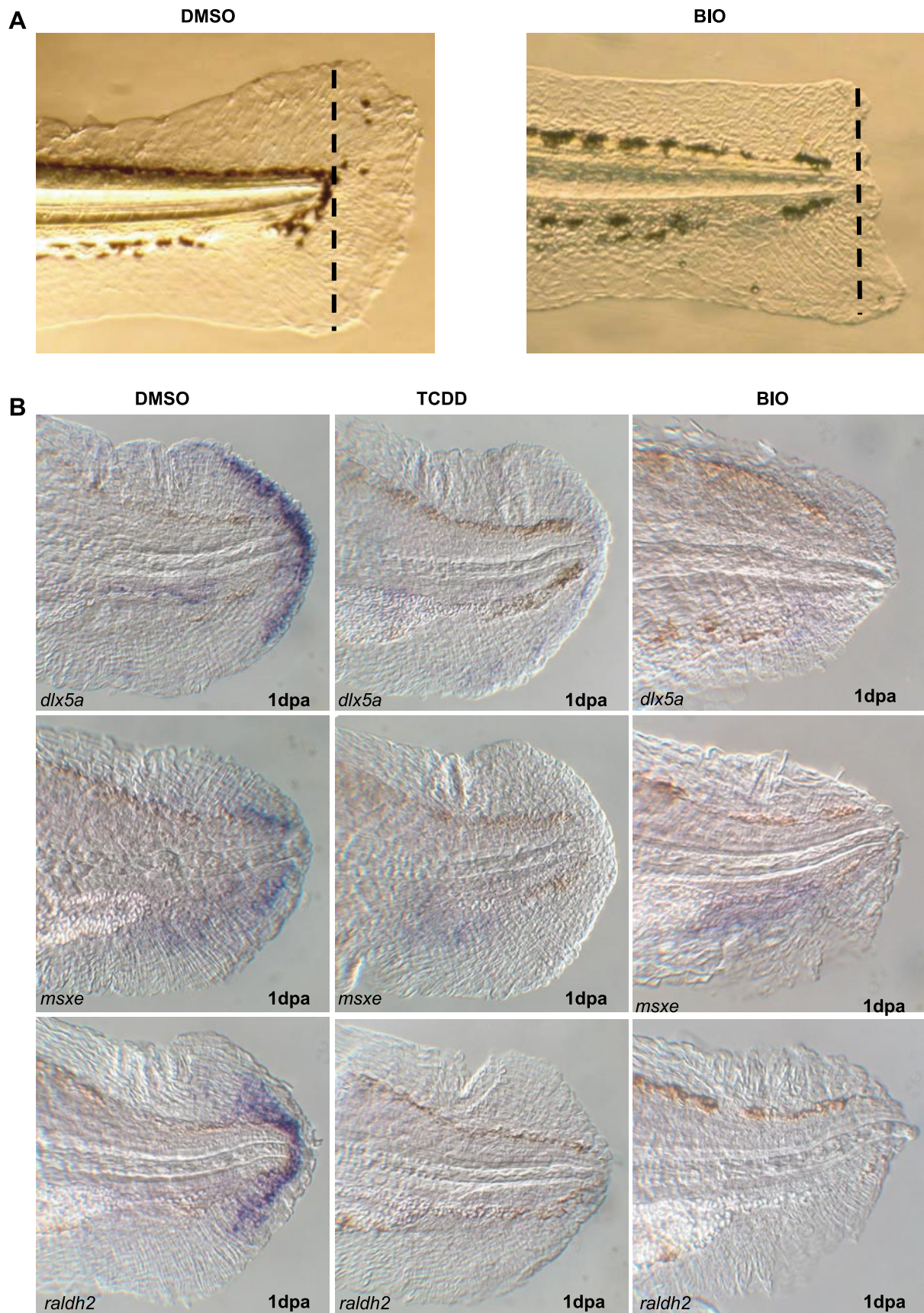


Figure 4. GSK3 inhibitor BIO impairs regeneration. *A*) Two-day-old larvae were amputated and exposed to vehicle or BIO (GSK3 inhibitor) continuously. Images are representative, acquired at 3 dpa. *B*) *In situ* hybridization with *dlx5a*, *msxe*, and *raldh2* was performed to analyze the integrity of wound healing and blastema formation after exposure to TCDD and BIO at 1 dpa. The images are representative of multiple experiments and more than 25 images per treatment.

response, the expression of *dlx5a*, *msxe*, and *raldh2* was lost in the regenerating fin tissue at 1 dpa in the BIO exposed larvae (Fig. 4B), suggesting that inappropriate activation of Wnt signaling inhibits fin regeneration by impairing wound epithelium and blastema formation. These results illustrate the importance of appropriate Wnt signaling regulation for tissue regeneration and support our hypothesis that TCDD exposure elevates the expression of the Wnt ligand R-Spondin1, leading to overactivation of the Wnt signaling pathway.

Impaired regeneration by AHR activation is not completely Sox9b dependent

Because R-Spondin1 is a Wnt ligand, we further analyzed the relation between R-Spondin1 and a Wnt target gene that was misexpressed following AHR activation. *Sox9b* was identified as a Wnt target gene in mesenchymal cells during the process of chondrogenic differentiation, and this gene was strongly repressed following the activation of the Wnt signaling pathway (14–16). Notably, in both adult and larval fin regeneration models, *sox9b* was the most repressed gene by TCDD, and we proposed that the activation of Wnt signaling by AHR activation through R-Spondin1 was responsible for the repression. We performed functional regeneration studies using *sox9b* homozygous mutants to determine whether the repression of *sox9b* was sufficient to impair regeneration. The amputated *sox9b* mutants regenerated their fin tissue by 3 dpa, but with defective cartilaginous-like support structures and actinotrichia, resulting in a delicate unsupported new tissue (Supplemental Fig. S5). This finding is important because our previous immunohistochemical and histological studies in the adult fin regeneration system clearly demonstrated that TCDD affects the ECM components in the regenerating fin tissue (10). Because SOX9 is a master regulator of chondrogenesis (17, 18), these results indicate that the reduced abundance of *sox9b* by AHR activation could explain the impairment of cartilage-like structures. Moreover, these results indicate the involvement of additional targets downstream of R-Spondin1, and parallel to *sox9b*, which contributes to the inhibition phenotype.

DISCUSSION

Zebrafish caudal fin regeneration is an established model to investigate the basic biological mechanisms of tissue regeneration. Adult- and larval-stage zebrafish share the same remarkable ability to regenerate the caudal fins following amputation (7, 19). We have reported that TCDD inhibits both life stages of caudal fin regeneration. The toxicity studies associated with exposure to halogenated aromatic hydrocarbons such as TCDD have resulted in the identification of AHR as an essential ligand activated transcription factor that mediates most if not all of the toxic effects of TCDD. What remain mostly unknown are the molecular events downstream of AHR activation that elicit adverse tissue specific responses. To address this information gap, we

have exploited a TCDD-dependent block in fin regeneration in order to identify the molecular signaling pathways affected after AHR activation (7, 8). In addition to our regeneration studies, a previous report demonstrated that treatment of mice with TCDD after partial hepatectomy resulted in the suppression of liver regeneration (20). Therefore, because TCDD has a global effect on tissue regeneration, identification of the signaling pathways that interact with AHR will also provide a better understanding of the basic regeneration pathways. Global gene expression analysis in the adult fin regeneration after TCDD exposure revealed that AHR activation modulates a cluster of genes involved in cellular differentiation and ECM metabolism (9). Similar to the adult model, TCDD most profoundly altered the abundance of transcripts involved in signal transduction, metabolism, and composition of the ECM during larval fin regeneration.

Comparative analysis performed between the two fin regeneration models revealed a concordance in the pattern of gene expression between these two models in response to TCDD. Antisense repression of R-Spondin1 resulted in the inability of TCDD to impair regeneration, indicating that misinduction of this gene was absolutely required for AHR activation to inhibit regeneration. The R-Spondin family belongs to a newly identified class of secreted proteins and includes four members (R-Spondin1–4). Each R-spondin contains a leading N-terminal signal peptide, two furin-type cysteine-rich domains, one thrombospondin-type domain, and a C-terminal basic amino acid-rich domain (21, 22). R-Spondins have been identified as novel ligands for the Fdz/LRP receptor complex and induce the β -catenin/TCF-dependent gene activation (23). Recent studies suggest that human R-Spondin1 is a high-affinity ligand for LRP6, and binding of this ligand to the receptor results in the phosphorylation of LRP6 and activation of β -catenin signaling (12). However, another recent study suggests that R-Spondin1 does not directly interact with LRP6 but instead prevents the DKK1/Kremen-mediated internalization of LRP6, facilitating increased LRP6 levels on the cell surface (11). However, both studies acknowledge that LRP6 is required for R-Spondin1 signaling. Even though R-Spondins act through β -catenin stabilization and may synergize with Wnt proteins, multiple *in vitro* and *in vivo* studies in different species suggest that the effect may not be completely dependent on the canonical Wnt signaling pathway (21, 24).

Because LRP6 is required for R-Spondin1 signaling, we further tested the functional requirement of LRP6 in the AHR activation-mediated inhibition of fin regeneration phenomenon. We observed that partial suppression of LRP6 is sufficient to prevent the inhibitory effect of TCDD on fin regeneration. This demonstrates that R-Spondin1 requires LRP6 to mediate its effect and also underscores our hypothesis that AHR activation improperly activates the canonical Wnt signaling pathway. These results are important as a previous study reported that increased Wnt/ β -catenin signaling augments adult zebrafish fin regeneration (6). However, our TCDD and BIO results suggest that overactivation of Wnt/ β -catenin signaling can also impair regenera-

tion. This finding leads to a question about the threshold level of the Wnt signaling pathway that is required for the completion of the regenerative process. Analysis of molecular markers suggests that both TCDD and BIO exposure affect blastema formation by inhibiting the cellular differentiation process. Previous studies have reported that even though Wnt signaling is required for hematopoietic stem cell self-renewal, constitutive activation of β -catenin signaling blocked multilineage differentiation, affecting the stem cell maintenance (25, 26). Another recent study demonstrated that muscle stem cells from aged mice convert from a myogenic to fibrogenic lineage, resulting in tissue fibrosis, and the lineage conversion was associated with activation of canonical Wnt signaling pathway (27). Altogether, these studies indicate that inappropriate activation of Wnt/ β -catenin signaling affects the differentiation process during fin regeneration. Also, these results demonstrate the absolute requirement for a fine balance of Wnt signaling for the process of epimorphic regeneration.

The functional importance of R-Spondin genes was revealed from a few recent genetic studies and identified the association of R-Spondin mutations with human disease conditions (28, 29). A rare autosomal recessive disease condition known as anonychia is linked to the mutation of R-Spo4. Another recessive syndrome characterized by XX sex reversal, palmoplantar hyperkeratosis, and predisposition to squamous cell carcinoma is due to the mutation of R-Spondin1 (28, 29). Mutation of R-Spondin1 causes the XX sex reversal to male, and this effect may be due to the induction of SOX9 promoting testis development. More clearly, it has been proposed that R-Spondin1 regulates the expression of SOX9 and that the mutation of R-Spondin1 resulted in the inappropriate induction of SOX9 levels, which indeed resulted in the XX sex reversal (28). Therefore, normal expression of R-Spondin1 will result in the repression of SOX9, which will lead to the proper development of the female gonads. These human studies are consistent with our earlier supposition that AHR-dependent increased expression of R-Spondin1 directly leads to decreased expression of Sox9b. Moreover, SOX9 is also reported to be a Wnt target gene (15, 16, 30); hence, we propose that the reduced level of Sox9b is caused by the inappropriate induction of R-Spondin1. In addition, SOX9 is a transcription factor as well as a master regulator that controls chondrogenesis and ECM composition (17, 30, 31), and *sox9b* expression in the basal epidermal cell layer of the distal part of the adult regenerating fin tissue has been previously reported (32). However, *sox9b* mutants regenerated their fin tissue with defective chondrogenesis, suggesting that repression of *sox9b* could explain the TCDD-dependent changes in ECM transcript abundance and the observed pathology.

In addition to our report of improper activation of Wnt signaling by AHR activation, other studies have demonstrated the interference of AHR with other developmental signaling pathways. AHR influences TGF- β signaling members in a ligand-dependent or -independent manner (33–35). A recent report has illustrated the ligand-independent role of AHR in

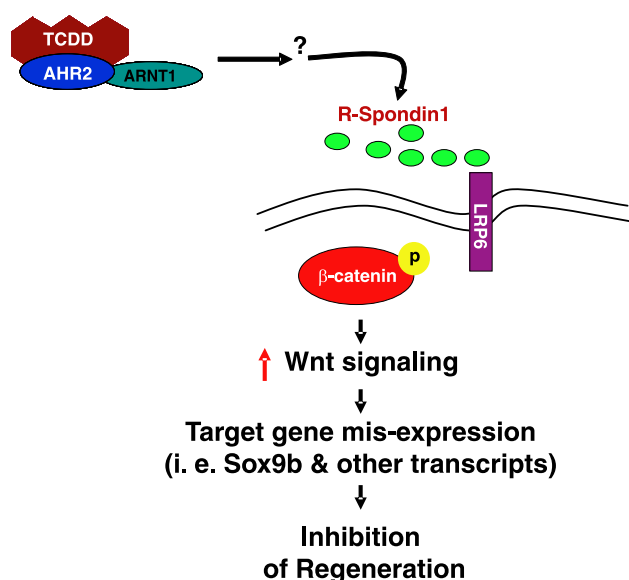


Figure 5. Proposed model of mechanism. AHR activation during fin regeneration improperly expresses R-Spondin1, which mediates through LRP6 to activate the Wnt/ β -catenin signaling. Activation of Wnt/ β -catenin signaling results in the stabilization of β -catenin, which in turn causes the misexpression of various Wnt target genes including *sox9b*. The functional consequences of these events collectively results in inhibition of fin regeneration.

reversing the proliferative and gene expression phenotype of AHR $^{-/-}$ fibroblasts by inhibiting TGF- β signaling (33). Another study has demonstrated that addition of TGF- β 3 into the palate culture model completely prevented the TCDD-mediated block of palatal fusion (34). Our report is the first to demonstrate the complete reversal of an adverse outcome of AHR activation by modulating the expression of a single gene in a whole animal and establishes functional crosstalk between AHR and Wnt signaling pathways *in vivo* (Fig. 5). It is noteworthy that misregulation of Wnt signaling causes a wide variety of adverse effects, such as developmental defects and cancer, and these new findings put forward the potential role of environmental AHR ligands in the etiology of different disease conditions. It is also important to understand precisely how AHR activation regulates the expression of R-Spondin1 and future studies could reveal the mechanisms that mediate the misexpression of R-Spondin1. FJ

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