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Genetic Architecture of Susceptibility to PCB126-Induced Developmental Cardiotoxicity in Zebrafish

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Variability in risk of developmental defects caused by dioxin-like compounds (DLCs) has been demonstrated within and among several vertebrate species. Beyond our knowledge of the aryl hydrocarbon receptor (AHR) and its role in mediating toxicity for this class of compounds, little else is known concerning precise downstream targets influencing this vulnerability. In the present study, zebrafish with divergent genetic backgrounds were screened for susceptibility to developmental cardiotoxicity caused by the prototypical DLC, 3,3′,4,4′,5-pentachlorobiphenyl (PCB126); a range up to ~40-fold differences was observed. Differentially sensitive zebrafish were chosen for a genetic cross, and the recombinant generation was used for genome-wide quantitative trait loci (QTL) mapping. Multiple QTLs were identified—several acting alone, one additively, and two others via epistatic interaction. Together, these QTLs account for 24% of the phenotypic variance observed in cardioteratogenicity resulting from PCB126 exposure (logarithm of the odds = 13.55, $p = 1.89 \times 10^{-10}$). Candidate genes in these QTL regions include the following: *ahr2*, *bcor*, and *capn1* (Chr 22); *e2f1* and *pdyn* (Chr 23); *ctnnt2*, *plcg1*, *eno3*, *tgm1*, and *tgm2* (interacting on Chr 23); and *vezf1* (Chr 15). These data demonstrate that DLC-induced cardiac teratogenicity is a multifactorial complex trait influenced by gene × gene and gene × environment interactions. The identified QTLs harbor many DLC-responsive genes critical to cardiovascular development and provide insight into the genetic basis of susceptibility to AHR-mediated developmental toxicity.

Key Words: **polychlorinated biphenyls; dioxin; heart; development; quantitative trait loci; genetic susceptibility; aryl hydrocarbon receptor.**

Polychlorinated dibenzo-*p*-dioxins and structurally related dioxin-like compounds (DLCs), including coplanar polychlorinated biphenyls (PCBs), are potent teratogens that persist in the environment and pose significant risk to human and ecological health. Amid the toxic effects associated with DLC exposure, a mounting body of evidence suggests that the cardiovascular system is one of the primary targets during development. Aberrant morphogenesis and dysfunction of the heart are observed following early-life stage exposure. Among vertebrates, this cardiotoxicity is evolutionarily conserved.

The cardioteratogenic effects of DLCs have been most extensively studied in zebrafish. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) arrests ventricular cardiomyocyte cell cycle (Antkiewicz *et al*., 2005), impairs atrioventricular and bulboventricular valve development (Grimes *et al*., 2008; Mehta *et al*., 2008), and prevents normal looping of the heart (Carney *et al*., 2006b). The hypoplastic heart is accompanied by a reduction in blood flow, pericardial edema, and a slowed heart beat with intermittent ventricular standstill (Antkiewicz *et al*., 2005). Morphological and functional deficits are also manifested in higher vertebrates following early-life stage DLC exposure.

Chicken embryos exposed to dioxin and dioxin-like PCBs exhibit compromised endocardial cushion, thin ventricular walls, a reduction in cardiomyocyte proliferation, reduced ventricular function, and edema (Ivnitski *et al*., 2001; Walker and Catron, 2000). Mice exposed *in utero* exhibit a dose-related decrease in heart-to-body weight ratio, interventricular septal defects, significant decrease in cardiomyocyte proliferation, and diminished heart rate (Lin *et al*., 2001; Thackaberry *et al*., 2005b).

Susceptibility to DLCs has been shown to vary within and among several piscine, avian, and mammalian species. Resistance to DLC-induced early-life stage effects has been observed in perch, tomcod, rainbow trout, and killifish populations residing in habitats with PCB-contaminated sediment (Carvalho *et al*., 2004; Forlin and Celander, 1995; Nacci *et al*., 1999; Wirgin *et al*., 2011; Yuan *et al*., 2006). Differences in susceptibility to DLC-induced cardiomyopathy between two breeds of chickens have been reported (Walker and Catron, 2000). Responsiveness to DLCs has also been

shown to differ among lines of inbred mice (reviewed in Nebert, 1989). Moreover, in humans, differences in the inducibility of DLC-responsive genes have been reported (Micka *et al*., 1997).

Susceptibility to environmental toxicants is determined by an individual's genetic background, environment, gene × environment interactions, and epistasis. In the postgenomic era, the interindividual variation that exists in DLC-induced cardiotoxicity can be leveraged to elucidate the genetic factors influencing vulnerability. Genome-wide comparisons of phenotypically discordant individuals can be made to link phenotypic variation to its underlying genetic basis, offering new insights into toxicological mechanisms and modes of action. In this study, a quantitative trait loci (QTL) analysis of PCB126-induced developmental cardiotoxicity was performed in zebrafish (*Danio rerio*). Genetically divergent strains of zebrafish were screened for differences in sensitivity to PCB126, and the most sensitive and tolerant strains were then chosen for a genetic cross. A bulk segregant analysis (BSA) was performed, comparing extreme discordant phenotypes from the recombinant generation. Candidate loci cosegregating with sensitivity differences were then chosen for QTL analysis. Herein, multiple QTLs are reported, identified from a genome-wide scan for single- and multiple-gene effects, including additive and epistatic interactions.

MATERIALS AND METHODS

Zebrafish maintenance, collection of embryos, and waterborne PCB126 exposure. Zebrafish strains AB, TL, TU, and WIK were obtained from the Zebrafish Information Network Repository (University of Oregon, Eugene, OR), strain EKW was obtained from Ekwill Fish Farms (Gibsonton, FL), and strain PKR originated from Parker Tropical Fish farm (Ruskin, FL). Zebrafish were maintained according to described procedures (Westerfield, 1995). Embryos were exposed to vehicle (0.1% dimethyl sulfoxide), graded concentrations of PCB126 (0–320 ppb), or [^{14}C]PCB169 (1μM; specific activity 12 μCi/μmol) for 1 h within 2 hours post-fertilization (hpf). At 144 hpf, larval zebrafish were evaluated for presence or absence of signs of DLC early-life stage toxicity—including abnormal looping of the heart, pericardial and yolk-sac edema, reduced heart rate, impaired swim bladder inflation, and craniofacial malformations. The proportion of individuals in each treatment group exhibiting overt toxic end points was determined, and the EC_{50} values for each strain were calculated using Probit Analysis software (U.S. Environmental Protection Agency, 2001).

DNA extraction and genotyping. Genomic DNA was extracted from larval fish using column-based kits (DNeasy; Qiagen). PCR was performed using a 3-primer probe system (2.0μM [FAM-CACGACGTTGTAAAAC-GAC] added to the 5′-end of the forward primer; Schuelke, 2000) to fluorescently label amplicons. PCR conditions also included: 20mM Tris-HCl, pH 8.4, 50mM KCl, 1.5mM $MgCl_2$, 1.0mM dNTPs, 0.2μM forward primer, 2.0μM reverse primer, 0.03 U of Taq, and 5 ng of template DNA per reaction. Thermal cycler (Tetrad; MJ Research/Bio-Rad) parameters were as follows: 3.0 min at 95°C (1 cycle); 30 s at 95°C, 30 s at 64°C, and 1.5 min at 72°C (repeat for 11 cycles, decreasing the annealing temperature by 0.8°C per cycle); 30 s at 95°C, 30 s at 54°C, and 1.5 min at 72°C (22 cycles); and then 15 min at 72°C (final extension). Amplified microsatellites were visualized using an ABI 3730 Genetic Analyzer (Applied Biosystems), and polymorphisms were scored using GeneMarker (v1.85; Softgenetics, LLC).

Genetic cross and BSA. Zebrafish from the most PCB-sensitive and PCB-tolerant strains were crossed following a classic F_2-intercross design. The recombinant generation (F_2) was exposed to PCB126 (80 ppb) and phenotyped to determine sensitivity. F_2 larvae were classified as "sensitive" if they exhibited significant edema, an incompletely looped (elongated) heart, and an obvious reduction in both heart rate and peripheral blood flow. Larvae were classified as "resistant" if they were indistinguishable from negative controls (no signs of toxicity). From these, two groups of 50—representing the most sensitive and most resistant fish—were pooled for BSA. The bulk samples were amplified with microsatellite markers ($N = 234$; Supplementary appendix 1) spanning the entire genome and were screened for skewed allelic frequency distributions dominated by the parental allele in its phenotypic class. Loci were identified as candidates if there was an overrepresentation of the sensitive parents' allele in the sensitive bulk or an overrepresentation of the resistant parents' allele in the resistant bulk.

QTL analysis. Genetic linkage maps were constructed using MAPMAKER/EXP (1994). QTL analysis was performed using R/qtl (Broman *et al*., 2003)—an interactive environment for mapping QTLs, implemented thru the statistical language/software R (Ihaka and Gentleman, 1996). Individual phenotypes were handled as binomial (sensitive vs. resistant) and quantitative (ranked by heart rate) traits. Heart rate was used as a noninvasive indicator of cardiotoxicity (Rubinstein, 2006) in order to preserve sample integrity and ensure that a sufficient quantity of DNA could be obtained from each individual for a genome-wide survey. One-dimensional (1D) genome scans were explored using marker regression and interval mapping (Feenstra *et al*., 2006; Haley and Knott, 1992; Sen and Churchill, 2001); two-dimensional (2D) scans were performed by multiple imputation (Sen and Churchill, 2001). Higher-order QTL models were also explored by multiple imputation. Logarithm of the odds (LOD) thresholds for genome-wide significance were estimated by permutation (Churchill and Doerge, 1994).

RESULTS

Interstrain Variation in Sensitivity to PCB126

Differences in susceptibility among genetically divergent strains of zebrafish were observed at 144 hpf (Fig. 1; Supplementary tables S1a and S1b) based on the presence or absence of cardinal signs of DLC early-life stage toxicity—including reduced peripheral blood flow, pericardial and yolk-sac edema, impaired swim bladder inflation, and craniofacial malformation (see Carney *et al*., 2006b for illustration of DLC developmental toxicity). EC_{50} values ranged from 9 to 336 ppb (95% confidence interval: 0–25 and 179–8561 ppb, respectively) (Fig. 1; Supplementary table S1a).

The most sensitive (TU) and tolerant (PKR) strains were also evaluated for a decrease in heart rate, a commonly used indicator of cardiac toxicity (reviewed in Rubinstein, 2006) sensitive to DLC exposure (Antkiewicz *et al*., 2005). Following exposure to PCB126 at 80 ppb—a concentration found to be highly toxic for TU but not for PKR (Supplementary table S1a)— significant differences in heart rate were observed between vehicle controls and PCB126-exposed TU fish at 72 and 144 hpf. No differences were observed in heart rate at either time point for strain PKR (Fig. 2A). Additionally, no differences were observed in uptake of radiolabeled PCB between the two respective strains (data not shown, $p = 0.20$).

Because 80 ppb PCB126 negatively impacts heart function and is teratogenic in sensitive TU fish but not the more

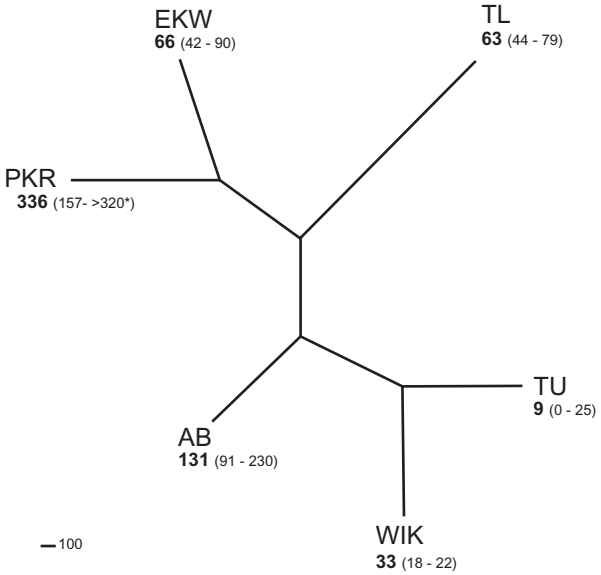

FIG. 1. The median effective concentration (EC_{50}) at which the teratogenic effects of PCB126 were observed among genetically divergent zebrafish strains. EC_{50} values were determined, as described in the ''Materials and Methods'' section. Branch lengths reflect genetic distance; numbers in parentheses represent 95% confidence intervals. Details regarding genetic differentiation and interstrain sensitivity differences can be found in Supplementary table S1.

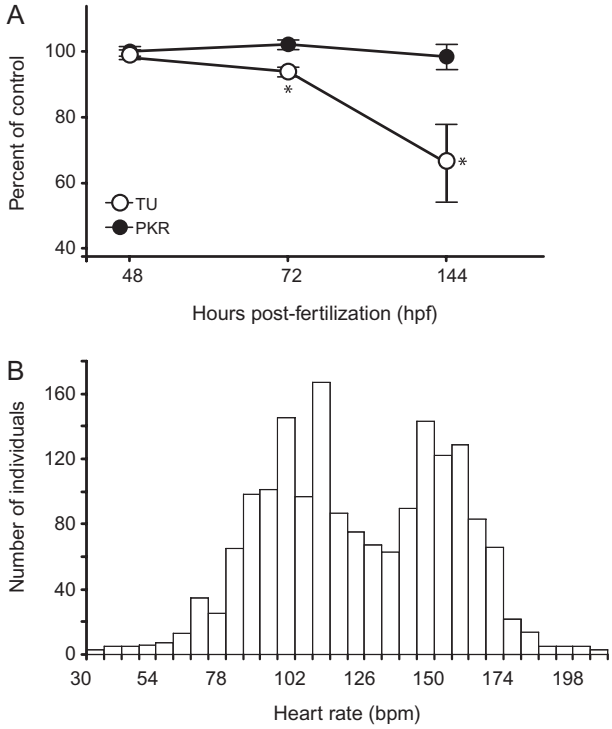

FIG. 2. Phenotypic differences in heart function (bpm). (A) At 48, 72, and 144 hpf, following PCB126 exposure (80 ppb), as observed in the parental generation represented by TU (○) and PKR (●). Error bars represent ± 1 SEM, *Indicates significantly different from respective control. (B) Phenotypic distribution of sensitivity in the recombinant F_2 generation. Heart rate (bpm), a noninvasive indicator of cardiotoxicity, is displayed.

tolerant PKR fish, this concentration was chosen to be used in subsequent exposures to maximize the genotype-to-phenotype signal for detecting loci associated with sensitivity differences.

Bulk Segregant Analysis

The genetic cross produced a recombinant population ($N = 1750$, F_2) segregating for sensitivity to PCB126. A broad bimodal distribution of cardiotoxicity (Fig. 2b) was observed at 144 hpf, confirming penetrance of the genetic information responsible for interstrain vulnerability differences to the experimental generation. The BSA of ''sensitive'' (unlooped heart, reduced or absent peripheral blood flow, pericardial edema, and a heart rate of $\leq$84 beats per minute [bpm]) versus ''resistant'' (no morphological signs of toxicity, heart rate of 132–156 bpm) individuals identified nine candidate microsatellite markers co-segregating with phenotypic differences (Fig. 3). 2To confirm the skewed allelic frequency distributions observed, each individual ($N = 100$) represented in the bulks was genotyped independently and strength-of-association with sensitivity was determined (Single-Marker analysis; QTL Cartographer v3.0). Following validation, highly significant ($p < 0.01$) genotype-phenotype associations were identified for Z21982/Chr15, Z10321/Chr22, and Z10949/Chr23. Subsequent fine-mapping efforts were concentrated on defining the chromosomal regions linked to these three loci.

Linkage Mapping

To define and narrow the chromosomal regions representing QTLs, F_2 larvae representing extreme discordant phenotypes ($N = 116$ sensitive; $N = 113$ tolerant) were genotyped with additional polymorphic microsatellite markers (Supplementary appendix 2) flanking the candidate loci identified by BSA. Due to the genomic location of the BSA candidate microsatellite markers, we also developed microsatellites located within the *ahr2* (GB#HQ116647), c*tnnt2a* (GB#HQ116648), *actc1b* (GB#HQ116648), and *myh7b* (GB#HQ116650) genes to test for genotype-phenotype associations.

Single-point analysis (Mapmaker/QTL) identified a total of nine linkage groups with a coverage ranging from 8.2 to 75.9 cM (Supplementary fig. S1a and appendix 2). A 2D scan of recombination fractions (Supplementary fig. S1b) using R/qtl (Broman *et al.*, 2003) verified the arrangement of these markers in each linkage group. The genetic maps representing the chromosomal regions genotyped are 100% concordant with the most current physical assembly of the zebrafish genome (Zv9, release 61, February 2011).

QTL Analysis

The 1D scan for main QTL controlling sensitivity to the teratogenic effects of PCB126 identified multiple QTL on

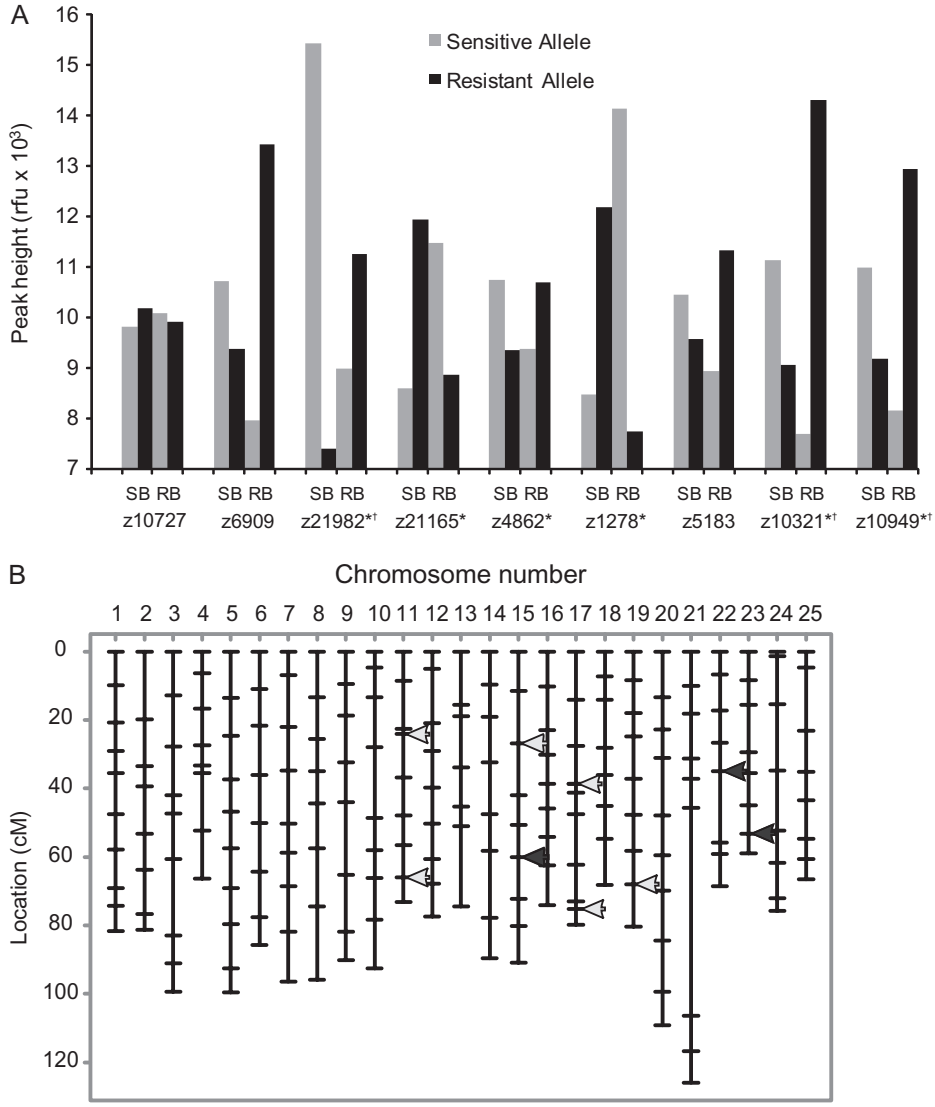

FIG. 3. Microsatellite markers identified by BSA which cosegregate with sensitivity differences observed in the F_2 bulks. (A) Relative peak intensities observed in sensitive and tolerant bulks for each candidate marker identified. Individuals from each bulk were genotyped independently to confirm skewed allele distributions (*$p = 0.05$, †$p < 0.01$). (B) Genomic location of candidates. Arrows indicate all candidate markers identified; solid arrows identify the loci found to be highly significant (qtl/Cartographer).

chromosomes (Chr) 22 and Chr 23 (Figs. 4 and 5). Disagreement exists between marker regression and interval mapping approaches regarding the predicted location of QTL on Chr 22. Marker regression identified zAHR2 as the most significant QTL (at 19.1 cM, LOD = 2.98, $p = 0.03$) on Chr 22 when treating susceptibility as a binomial trait (sensitive vs. resistant). The genetic architecture surrounding the zAHR2 QTL (Fig. 4) suggests the presence of two additional QTL on Chr 22: one 4.1 cM upstream (at 15 cM, LOD = 2.76, $p = 0.05$) and another 13.4 cM downstream (at 32.4 cM, LOD = 2.52, $p = 0.10$). Ranking discordant phenotypes by severity of cardiotoxicity increases the strength of the QTL at 32.4 cM to LOD 2.97 ($p = 0.03$). Contrary to marker regression, interval mapping predicts a single QTL downstream from the *ahr2* gene, just inside the adjacent interval (at 23 cM, LOD = 2.95, $p = 0.03$). On Chr 23, marker regression and interval mapping produced compatible results. The 1D scans identified two QTL—one at 62 cM (LOD = 3.81, $p = 0.003$) and the other at 75 cM (LOD = 3.43, $p = 0.007$).

To test for additive and epistatic interactions, a genome × genome comparison was performed. The 2D scan (Fig. 6) revealed additive relationships involving the QTLs identified by the 1D scan and an additional QTL on Chr 15 (at 46.4 cM, $LOD_{additive} = 5.77$, $p = 0.008$). The 2D scan also identified an epistatic interaction linked to markers at opposing ends of Chr 23 (cTNNT2::z59202). Alone, marker cTNNT2 cannot

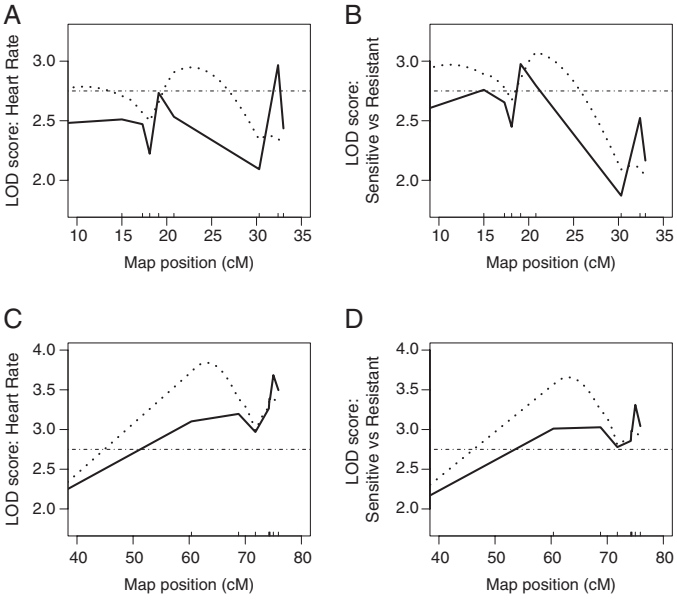

FIG. 4. LOD profiles of 1D genome scans for QTL associated with PCB126-induced cardioteratogenicity on Chr 22 (A and B) and Chr 23 (C and D). Sensitivity of each individual was considered as a binomial trait (sensitive vs. resistant; B and D) or quantitatively ranked by severity of cardiotoxicity (heart rate; A and C). Solid lines represent LOD profile determined by marker regression, dotted lines represent LOD profile determined by interval mapping, and the horizontal dashed line indicates significance threshold.

account for a significant portion of the phenotypic variance observed in the genetic cross (Table 1); however, when considered with z59202, the pair-wise interaction explains 14.01% of the phenotypic variance observed in sensitivity (LOD = 7.53, $p = 4.45 \times 10^{-05}$). In the full, multiple QTL model which allows for main, additive, and epistatic interactions, the total amount of variance in PCB126 sensitivity that can be explained by all QTL identified in this study is 24% (LOD = 13.55, $p = 1.89 \times 10^{-10}$).

DISCUSSION

The genetic architecture of interindividual variation in sensitivity to the cardioteratogenic effects of PCB126 reveals that susceptibility is a complex trait influenced by multiple loci. The identified QTL intervals harbor DLC-responsive genes known to have roles in cardiovascular development and function. Intriguing candidate susceptibility loci include genes involved in cell cycle control, endocardial and myocardial development, cardiac sarcomere assembly, and, notably, the aryl hydrocarbon receptor (AHR).

Main QTLs on Chr 22

The predicted position of the QTL on Chr 22 varies depending on the method used for linkage analysis (Fig. 4). Interval mapping generally offers an improvement over marker regression, utilizing information between markers to provide a refined estimate of QTL location (Broman and Sen, 2009). However, interval mapping can mistakenly estimate multiple, closely linked QTL as a single (ghost) QTL with a larger effect at the wrong position (reviewed in Kao and Zeng, 2010). In the present study, marker regression identified three closely linked QTLs on Chr 22. The central QTL corresponds to a marker located within the *ahr2* gene—the cytoplasmic receptor for dioxins and DLCs. The molecular mechanisms controlling DLC-induced teratogenesis are mediated by AHR, corroborating the results obtained by marker regression and lending support to the likelihood that the single QTL identified by interval mapping is indeed a ghost QTL.

The AHR has been shown to mediate sensitivity in multiple species. AHR-deficient mice are resistant to many effects of TCDD (Fernandez-Salguero *et al*., 1996), and morpholino gene knockdowns of the zebrafish *ahr2* (Prasch *et al*., 2003) afford protection against DLC-induced cardiotoxicity. In DLC-resistant Atlantic killifish (*Fundulus heteroclitus*), there is a genome-wide loss of responsiveness in AHR signaling (Whitehead *et al*., 2010), and suppression of the AHR pathway rescues DLC-sensitive killifish from cardiac teratogenesis (Clark *et al*., 2010). Moreover, a genetic mutation was recently identified in the *ahr2* gene of Atlantic tomcod which contributes to TCDD and PCB126 resistance (Wirgin *et al*., 2011).

Colocalizing with the QTL upstream from *ahr2* is an attractive candidate—the BCL6 corepressor gene (*bcor*). Carney *et al*. (2006a) identified *bcor* as a member of the heart-specific transcriptome following TCDD exposure in zebrafish. BCOR is a key transcriptional regulator during early embryogenesis and is associated with congenital cardiac abnormalities, including septal and mitral valve defects (Ng *et al*., 2004). BCOR regulates the NOTCH-signaling pathway (Sakano *et al*., 2010)—an evolutionarily conserved intercellular mechanism essential for proper cardiac development (High and Epstein, 2008). In zebrafish, the NOTCH-signaling pathway is disrupted following early-life stage exposure to TCDD. The timing and location of NOTCH dysregulation corresponds to a deficit in valve formation and ventricular myocardium in these fish (Mehta *et al*., 2008).

Colocalizing with the QTL downstream from *ahr2* is the *capn1* gene encoding for calpain-1, which is known to induce myocardial dysfunction (Papp *et al*., 2000). Interestingly, this marker becomes a highly significant QTL when differentially sensitive zebrafish are ranked by heart function (Fig. 4). CAPN1 also mediates the dioxin-induced activation and downregulation of the AHR (Dale and Eltom, 2006), further supporting a possible role in susceptibility to DLCs.

Main QTL on Chr 23

In vertebrates, DLC-associated cardioteratogenicity is characterized by a hypoplastic heart with fewer smaller cardiomyocytes. In all models, a decrease in cardiomyocyte

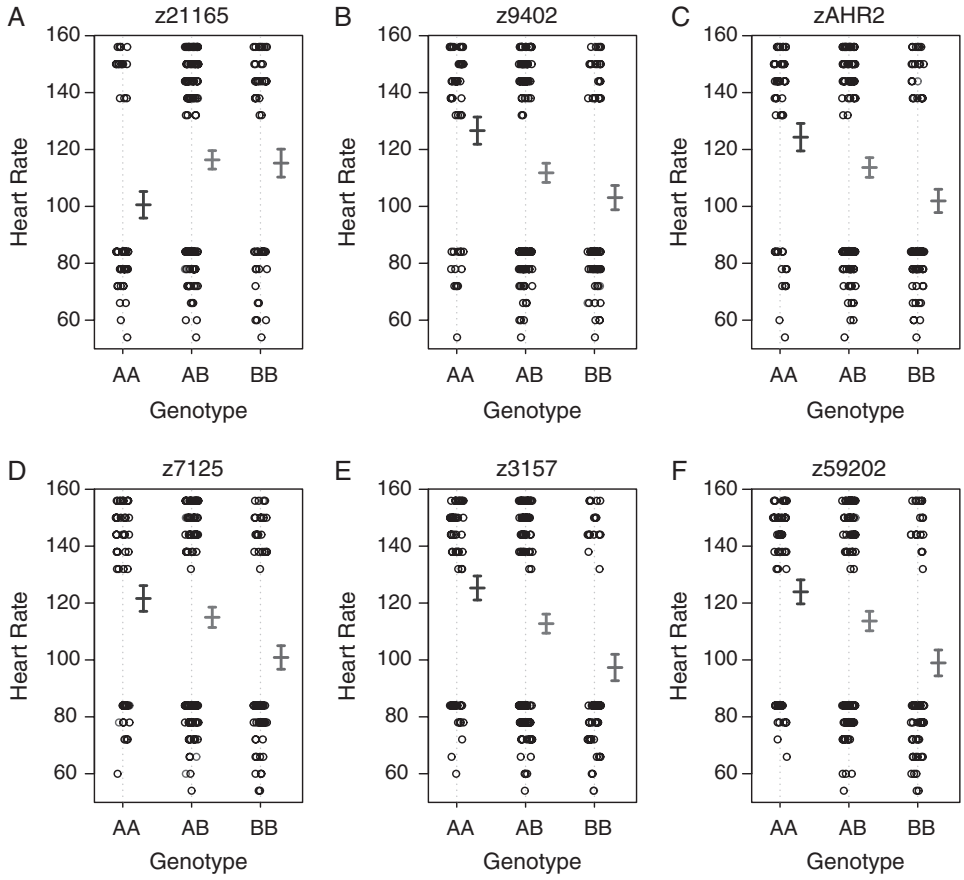

FIG. 5. Main effect plots displaying the phenotype-to-genotype relationship for each marker closest to the QTLs identified by the 1D scan (A–F). For each marker genotype, the individual phenotype is plotted, with the mean of genotype AA, AB, and BB. Error bars represent ± 1 SEM. Microsatellite allele sizes (bp) for ''AA'' and ''BB,'' respectively, are 139/139 and 157/157 (z21165), 260/260 and 258/258 (z9402), 221/221 and 229/229 (zAHR2), 220/220 and 184/184 (z7125), 104/104 and 128/128 (z3175), and 267/267 and 234/260 (z59202).

proliferation is observed (reviewed in Kopf and Walker, 2009). More than one gene colocalizing with the major Chr 23 QTL could contribute to the universal hypoplasia: transcription factor E2F1 (*e2f1*) and prodynorphin (*pdyn*). E2F1 is part of a transcriptional regulatory framework repressing the expression of genes involved in cell proliferation and organ morphogenesis following PCB exposure (Reymann and Borlak, 2006). In the heart-specific TCDD-responsive transcriptome, the expression of several G1/S-type cyclins under the control of E2F1 are repressed in both mice and zebrafish (Carney *et al.* 2006a; Thackaberry *et al.* 2005a). E2F1-dependent cell cycle progression is repressed following exposure to dioxins (Puga *et al.*, 2000), and combinatorial interactions of E2F1 and AHR have been documented (Marlowe *et al.*, 2008). PDYN orchestrates the differentiation of multipotent embryonic stem (ES) cells into beating cardiomyocytes by triggering the transcription of genes essential for cardiogenesis, including *Nkx*2.5 (Ventura and Maioli, 2000). Following exposure to TCDD, however, *Nkx*2.5 expression is repressed and fewer ES cells commit to becoming cardiomyocytes (Wang *et al.*, 2010).

A cluster of Cyp2 genes also map to the main Chr23 QTL. CYP2 enzymes are well known to be involved in eicosanoid synthesis and degradation; functions of eicosanoid second-messengers include organogenesis and other developmental functions (reviewed in Nebert and Karp, 2008).

Interacting QTLs on Chr 23

The systems that underlie cellular, developmental, and physiological processes are complex and contain many elements that interact and regulate one another. Epistatic interactions among loci (i.e., when the phenotypic effect of one locus depends on the genotype at a second locus) often make a substantial contribution to variation in complex traits—including cardiovascular disease (Tsai *et al.*, 2007). In the genetic cross described in this study, an interaction involving two QTLs on Chr 23, accounts for approximately half of the variance that can be explained by our model for sensitivity to PCB126-induced cardiotoxicity. Cross-referencing genes linked to the interacting QTL intervals with publicly available protein × protein interaction databases (Berger *et al.*, 2007) revealed an extensive

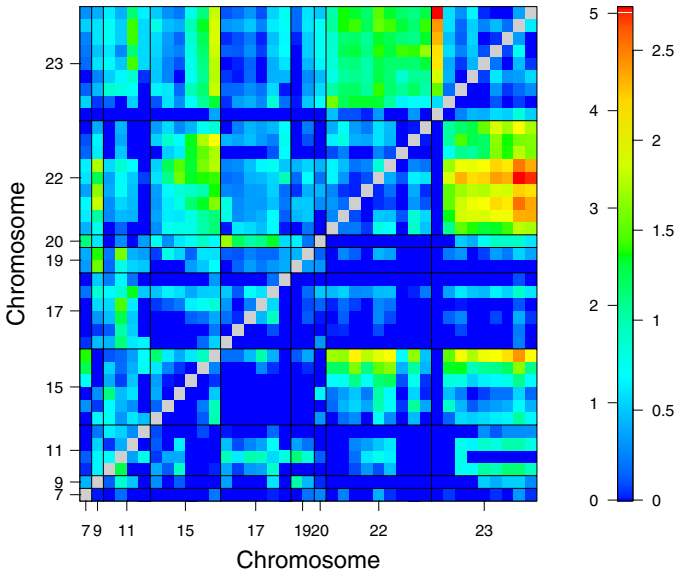

FIG. 6. Heat map of genome × genome scan for additive (lower diagonal) and epistatic (upper diagonal) interactions contributing to PCB126-induced cardiotoxicity. LOD legend for heat: Numbers on the left and right correspond to epistatic and additive LOD scores, respectively, for all pair-wise comparisons.

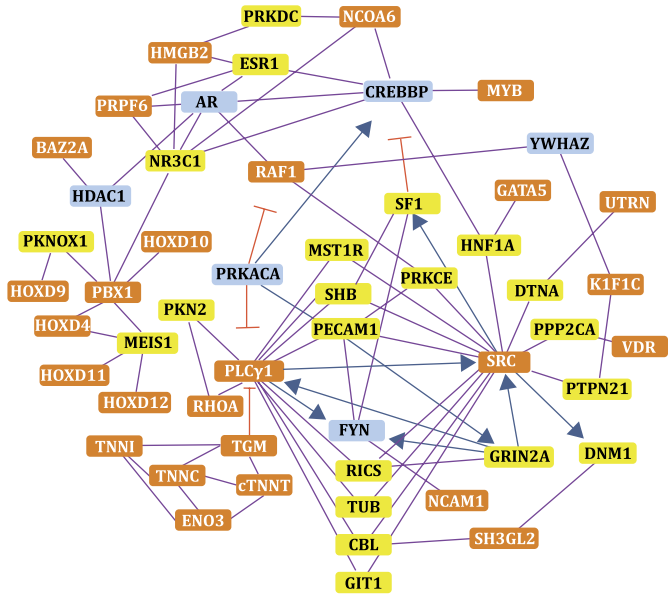

FIG. 7. Interactome of loci colocalizing with the two QTLs (orange boxes) representing the epistatic interaction (cTNNT2::z59202). Yellow boxes represent significant intermediates (z-score > 2.5). Blue boxes represent intermediates with a z-score < 2.5.

gene network (Fig. 7). This network contains loci responsive to DLC exposure that are known to have roles in cardiovascular development and function.

One of the interacting QTLs is anchored to a genetic marker located within the cardiac troponin T2 gene (*ctnnt2*), a component of the sarcomere responsible for heart contraction and found to be differentially expressed in DLC-sensitive versus tolerant Atlantic tomcod populations (Carlson *et al.*, 2009). Linked to *ctnnt2* is the plcγ1 gene encoding phospholipase Cγ1. PLCγ1-signaling provides the calcium transients required for ventricular contractility and affects key steps in vasculogenesis (Rottbauer *et al.*, 2005). The companion interacting QTL is represented by a genetic marker

TABLE 1
Percent Phenotypic Variance that Each QTL Represents

QTL	Chr	Position (cM)	Nearest marker	(%) Variance[a]	p value
Q1	15	46.4	z21165	4.43	0.005
Q2	22	15	z9402	5.64	0.001
Q3	22	19.1	AHR2	5.49	0.001
Q4	22	32.4	z7125	4.21	0.007
Q5	23	0	cTNNT2	1.47	0.187
Q6	23	62	z3157	7.05	0.000
Q7	23	75.9	z59202	5.94	0.000
Q5:Q7	23	0::75.9	cTNNT2::z59202	14.01	0.000

Note. Multiple QTL model: y ~ Q1 + Q2 + Q3 + Q4 + Q5 + Q6 + Q7 + Q5:Q7. Total % variance explained by fully interacting model = 23.86%, LOD = 13.55, $p = 2.6 \times 10^{-10}$.

[a]Single QTL model.

tightly linked to the muscle-specific gycolytic isoenzyme, β-enolase (*eno3*) and transglutaminase isoforms 1 and 2 (*tgm1* and *tgm2*). During development, ENO3 first appears in the cardiac heart tube and binds troponin with high affinity, where ATP is needed for contraction (Merkulova *et al.*, 1997). In the heart, transglutaminase is responsible for cross-linking troponins (Bergamini *et al.*, 1995; Gorza *et al.*, 1996), interacts directly with PLCγ1 as a negative effector (Murthy *et al.*, 1999), and has been implicated in multiple cardiac diseases (reviewed in Sane *et al.*, 2009). Moreover, cTNNT2, PLCγ1, TGM1, and TGM2 are all known to be perturbed following exposure to DLCs (Handley-Goldstone *et al.*, 2005; Rice and Cline, 1984; Rodriguez-Fragoso *et al.*, 2009; Rubin and Rice, 1988).

Other Candidate Loci

Localizing candidates for the QTL on Chr 15, which becomes significant when considering additive relationships with the other QTLs identified, will require additional mapping; the position of this QTL is unresolved (Supplementary fig. S2). However, under the assumption that a candidate is located nearby, the vascular endothelial zinc finger-1 gene (*vezf1*) colocalizes with this QTL (46.4 cM). VEZF1 regulates the expression of endothelin-1 (ET-1) (Aitsebaomo *et al.*, 2001), a gene found to be upregulated in the hearts of larval zebrafish (Carney *et al.*, 2006a) and fetal mice (Thackaberry *et al.* 2005a) following embryonic exposure to TCDD. In mice, elevated levels of ET-1 are associated with cardiac dysfunction (Lund *et al.*, 2003, 2006).

Genetic Susceptibility and DLCs

It is not surprising that the QTLs identified in this study contain a number of candidate loci that could contribute to the cardioteratogenic effects of DLCs. In eukaryotic genomes, genes with related functions tend to be spatially clustered and are often coexpressed as sequential components of a common pathway or biological process (reviewed in Hurst, 2004). Elucidating the precise gene in each QTL responsible for the observed differential sensitivity will require further mapping and demonstration of causality. Techniques—including positional cloning followed by targeted gene replacement, deletion mapping, morpholino knockdown experiments, and/or functional complementation—will be used to establish such a relationship. For many of the candidates that colocalize with the QTLs identified, morpholino knock downs in zebrafish and Xenopus have been described. Loss of function of cTNNT2 (Sehnert *et al*., 2002), PLCγ1 (Rottbauer *et al*., 2005), E2F1 (Waits, Bartman, and Nebert, unpublished data), BCOR (Hilton *et al*., 2007; Ng *et al*., 2004), and GATA5 (Peterkin *et al*., 2007) during development results in defects ranging from lack of heart beat to severe endocardial and myocardial hypoplasia—end points similar to those observed following embryonic exposure to DLCs. While a direct link between the candidate susceptibility loci and PCB126-induced cardioteratogenicity has not yet been firmly established, it is our hope that the information provided by this study, in conjunction with other related molecular and "omics" investigations, will contribute to the discovery of the precise molecular mechanisms involved.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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