

Genetic and environmental risk factors in congenital heart disease functionally converge in protein networks driving heart development

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Congenital heart disease (CHD) occurs in ~1% of newborns. CHD arises from many distinct etiologies, ranging from genetic or genomic variation to exposure to teratogens, which elicit diverse cell and molecular responses during cardiac development. To systematically explore the relationships between CHD risk factors and responses, we compiled and integrated comprehensive datasets from studies of CHD in humans and model organisms. We examined two alternative models of potential functional relationships between genes in these datasets: direct convergence, in which CHD risk factors significantly and directly impact the same genes and molecules and functional convergence, in which risk factors significantly impact different molecules that participate in a discrete heart development network. We observed no evidence for direct convergence. In contrast, we show that CHD risk factors functionally converge in protein networks driving the development of specific anatomical structures (e.g., outflow tract, ventricular septum, and atrial septum) that are malformed by CHD. This integrative analysis of CHD risk factors and responses suggests a complex pattern of functional interactions between genomic variation and environmental exposures that modulate critical biological systems during heart development.

genetics | transcriptional profiles | systems biology | developmental biology

Congenital heart disease (CHD) is a prevalent birth defect that can occur from genetic variations, environmental exposures, and other factors. Genetic risk factors for CHD include Mendelian mutations, copy number variants (CNVs), translocations, and single nucleotide polymorphisms (SNPs) (1–3). The cardiac responses to some genetic risks have been identified through transcriptional analyses of model organisms engineered to carry human CHD mutations (4). There are many environmental risk factors for CHD, including pesticides and therapeutic agents (5), and some of these are known to target particular genes. Although the cardiac molecular responses to environmental teratogens remain largely unexplored, transcriptional profiles of zebrafish exposed to retinoic acid have been characterized (6). As CHD risk factors and responses rarely incriminate the same genes nor directly impact a limited set of biological pathways, whether these converge on discrete molecular programs involved in heart development is unknown.

We used systems biology analyses to integrate the heterogeneous risk factors and response involved in CHD. Given the extraordinary conservation of genes and processes involved in heart development across species (7–10), it is possible to integrate data from humans and model organisms in a straight

forward manner. Moreover, our analyses capitalize on a series of previously published and validated functional molecular networks, which direct the development of distinct anatomical structures of the human heart (11). These networks provided a developmental scaffold onto which risk and responder datasets of CHD were assessed. The hypothesis underlying our work is that these integrative analyses might elucidate unsuspected functional relationships and provide unique insights into the molecular basis of heart defects.

To address this hypothesis, we assembled gene sets of CHD risk factors (risk genes hereafter), and transcriptional responses to CHD risk factors (responder genes hereafter). Risk genes included previously reported Mendelian mutations, SNPs, CNVs, translocations, and genes that are the direct targets of teratogens, such as retinoic acid receptors (environmental target genes hereafter). Responder genes included those with altered cardiac expression in model organisms with human monogenic CHD mutations (Mendelian responders hereafter) or following exposure to teratogens (environmental responders hereafter). We interrogated the risk and responder datasets for “direct convergence” in which the same genes were directly impacted and for “functional convergence” in which genes encoding different proteins within a discrete network of heart development (11) were impacted (Fig. 1). Unlike functional convergence, direct convergence can be analyzed by overlapping gene sets without prior knowledge of developmental programs.

Whereas we observe little evidence for direct convergence, our analyses revealed significant functional convergence of the risk and responder datasets in the context of well-defined, but highly polygenic gene sets that participate in biological networks at specific stages of heart development. Significant functional convergence of the risk and responder datasets occurred in gene sets that form networks driving the development of the ventricular

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Table 1. Human risk datasets

Type of risk factor	Description	Source for dataset
CNVs and translocations: Cohort A	Four hundred three rare structural variants (spanning 709 genes) identified in 136 trios with isolated CHD.	Ref. 3 and this study. Details, chromosomal coordinates, and gene identifiers are provided in <i>SI Appendix</i> and <i>Dataset S1</i> .
CNVs and translocations: Cohort B	One thousand five hundred forty-four rare structural variants (spanning 657 genes) identified in 526 probands with isolated or syndromic CHD.	This study. Details, chromosomal coordinates, and gene identifiers are provided in <i>SI Appendix</i> and <i>Dataset S2</i> .
Mendelian CHD genes	Forty-six genes with mutations that cause Mendelian forms of CHD.	Curated from the literature. Details and gene list are provided in <i>SI Appendix</i> and <i>Dataset S3</i> .
SNP-associated genes	Six genes containing SNPs that are associated with CHD.	Curated from the literature. Details and gene list are provided in <i>SI Appendix</i> and <i>Dataset S3</i> .
Environmental target genes	Seven genes (e.g., retinoic acid receptor) that are direct targets of environmental CHD teratogens (e.g., retinoic acid).	Curated from the literature. Details and gene list are provided in <i>SI Appendix</i> and <i>Dataset S3</i> .

convergence between the risk and responder datasets in 17 of 19 networks. Moreover, these results did not reflect shared genes among different networks being tested (details in *SI Appendix*).

Lack of Direct Convergence of the Risk and Responder Datasets.

Despite observing that some genes are represented in more than one of the risk and responder datasets, these genetic and environmental risk factors did not converge on a limited gene set or pathway. Fig. 3 illustrates the very few genes (boxed in red) present within three networks (Fig. 2) that were identified in more than one risk or responder dataset. Analyses of the larger set of networks (*SI Appendix*, Figs. S1–S16) confirmed this observation.

To statistically test for direct convergence between the risk and responder datasets, we made pairwise comparisons without mapping the datasets to developmental networks (Fig. 4). Among responder datasets, we observed a trend toward direct convergence, but this generally did not extend across all of the risk datasets and sharply contrasted to the functional convergence observed when the risk and responder datasets were integrated into the developmental networks. Notably, the genetic and environmental risk factors rarely converged on the same genes. Moreover, whereas some CHD risks impacted well-characterized developmental signaling pathways, these pathways were individually associated with only a few of the risk factors examined here. Thus, directly comparing the risk and responder datasets would suggest that these genes were not functionally related or involved in a common molecular program for heart development.

Enrichment of Heart Development Genes in Risk and Responder Datasets.

We considered whether the lack of direct convergence of the risk and responder datasets reflected “noise,” i.e., the inclusion of genes in the datasets that are not directly involved in heart development. To allow a normalized comparison between the risk and responder datasets despite considerable differences in gene counts (and experimental platforms underlying these data), we examined dataset-specific enrichments of heart development genes in each of the individual risk and responder datasets compared with null distributions. We observed that each of the individual datasets were significantly enriched for genes present in the 19

developmental networks underlying cardiac anatomical structures (Fig. 5A; $P < 5.0e-4$ to $P = 0.019$ for the individual datasets using a permutation test), indicating noise in each of these datasets cannot explain the observed lack of direct convergence.

That significant enrichment of heart development genes was observed in the analyses of structural variants was particularly noteworthy, given the inclusion of many genes within each deleted or duplicated genomic segment. As some of these genes are unlikely to be involved in CHD, including them should limit the statistical power of these analyses. To validate the positive signal associated with the analysis of cohort A (*Dataset S1*), we studied a second independent dataset of rare CNVs derived from cohort B (*Dataset S2*). Despite the considerable diversity of heart malformations, and the likely inclusion of many genes not involved in CHD in these CNVs, data from cohort B replicated the findings for cohort A (Fig. 5A, $P = 3.0e-3$, using a permutation test).

To confirm that the differences in sizes between the datasets were not confounding this analysis, we applied another test based on probability distributions of the observed overlaps between networks and individual datasets (which normalizes for size differences between the datasets; *SI Appendix*). This test corroborated and strengthened the results in Fig. 5A: All risk and responder datasets were enriched for heart developmental genes ($P < 5.0e-4$ to $P = 0.002$ for the individual datasets using a permutation test; *SI Appendix*, Fig. S17).

As a control for both tests, we also performed comparable analyses for six control datasets unrelated to CHD. Analogous to the risk and responder datasets assembled for CHD, we gathered risk and responder datasets for Crohn disease (*SI Appendix* and ref. 16) and additional datasets of genes altered by rare structural variants (CNVs $<1\%$ population frequency) in three independent cohorts of healthy individuals (the National Institute of Neurological Disorders and Stroke, the Human Genome Diversity Project, and cohort C, detailed in *SI Appendix*). Analyses of each of these six control datasets showed no enrichment for genes involved in the heart developmental networks (*SI Appendix*, Fig. S18 and Fig. 5B) and confirmed that the observed enrichment (Fig. 5A) is specific to the CHD-related datasets of risk and responder genes.

Table 2. Genetic and environmental responder datasets

Type of responder	Description	Source for dataset
Mendelian responder genes	Six hundred seventy-three differentially expressed genes in the hearts of mice with CHD mutations in <i>Gata4</i> and <i>Nkx2-5</i> .	This study. Details, and gene list are provided in <i>SI Appendix</i> and <i>Dataset S6</i> .
Environmental responder gene	Two hundred forty differentially expressed genes in the hearts of zebrafish after embryonic exposure to teratogenic doses of retinoic acid.	Curated from the literature. Gene list is provided in ref 5.

A Outflow Tract Network

	ACVR2B	AMH	BMPER	CITED2	CITED4	EGLN3	FGF6	FGF7	FOG2	GATA4	GJA1	HIF1A	JUN	MAKP2K1	NF1	NKX2-5	NROB2	NR1H2	NRBF2	NTF3	PLXND1	POR	PTPN11	RARA	RARB	RARG	RXRA	RXRG	TBX2	TBX5
SNPs																														
MM																														
MR																														
SVA																														
SV ^B																														
ET																														
ER																														

C Atrial Septal Network

	DLL1	FOXH1	FOXM1	GATA4	JAG1	NKX2-5	NOTCH2	PRKCH	PTPN11	TBX5
SNPs										
MM										
MR										
SVA										
SV ^B										
ET										
ER										

B Ventricular Septal Network

	ACVR2B	ADRBK2	BMPER	CAV1	CCND2	CITED1	CITED2	CRKL	CXCR4	DBP	DLL1	DTX3	FGF7	FKBP1A	FOG2	FOXH1	GATA4	HXB2	HXB3	JAG1	JUN	LAMB3	MEF2A	MYBL2	NF1	NKX2-5	NOTCH2	NTF3	PCSK6	PDK1	PTPN11	RXRA	TBX5	TWIST1
SNPs																																		
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Fig. 3. Few genes are shared between CHD risk and responder datasets. Each panel shows all genes from any risk or responder dataset that overlaps with the gene set represented in the outflow tract (A), ventricular septum (B), and atrial septum (C) developmental network. Datasets are color coded as detailed in Fig. 2 and abbreviated as: SNPs, single nucleotide polymorphisms; MM, Mendelian mutations; MR, Mendelian responders; SVA and SVB, structural variants in cohorts A and B respectively; ET, environmental targets; ER, environmental responders. Despite the significant functional convergence between the risk and responder datasets in each of the networks (Fig. 2), only a few genes (highlighted in red) are identified in multiple risk datasets.

the application of contemporary sequencing technologies. We suggest that this strategy may inform other complex and congenital disorders.

Our analyses incorporated risk and responder datasets that span more than 2,100 unique genes. Whereas each dataset was enriched for genes encoding proteins in the heart developmental networks, only 15 genes (*ACTC1*, *CCND2*, *FOXM1*, *IGFBP5*, *JAG1*, *MYH6*, *NOTCH1*, *PCSK6*, *PLCB1*, *RAFI*, *RARG*, *RARA*, *TBX5*, *TMOD1*, and *TWIST1*) were found in multiple risk or response datasets, as well as in one of the 19 heart developmental networks. Seven of these genes (*CCND2*, *FOXM1*, *IGFBP5*, *PCSK6*, *PLCB1*, *TWIST1*, and *TMOD1*) are not recognized as Mendelian causes of CHD; nor are these genes the direct targets of teratogens. Further study of these genes in cardiac development and CHD may be warranted (discussion in *SI Appendix*).

Another unexpected result from our analyses was that sequence and structural variants, both rare and common, converged on large polygenic gene sets required for heart development, but

did not point to a shared subset of genes. Although more tests will be necessary to fully interpret these observations, it is tempting to speculate that functional convergence might help to explain the “missing heritability” paradox in complex disorders (22) including CHD. As the extent of individual genomic variation is more widely appreciated, we expect that both deleterious and mitigating influences on the highly polygenic molecular networks driving heart development will be discovered and that individual patients could have deleterious sequence or structural variation in several genes across a network.

Whereas we recognize limitations in these analyses (details in *SI Appendix*), recent studies support functional convergence of risk factors in heart and other developmental networks. For example, pairs of CNVs within a genome convey greater risk for severe developmental delay than individual CNVs (23), and de novo exon mutations in autism patients physically interact in protein interaction networks (19, 20). Moreover, emerging studies of the combined effect of environment and genetics in animal models also support functional convergence (discussion in *SI Appendix*).

Given these analyses and the complexity of CHD, we speculate that malformations arise from a multidimensional combinatorial space of perhaps tens or hundreds of sequence and structural variants that may perturb several different members of large developmental networks in a single patient. The individual genetic risk factors might be very rare, and the specific combination of risk factors in many patients unique, which could account for the considerable phenotypic heterogeneity observed in CHD patients with identical mutations in established CHD genes. Although speculative at this point, these hypotheses should be amenable to testing using systems-based approaches to integrate personal sequence variations in the context of heart developmental networks.

Improved understanding of the functional convergence of risk factors in highly polygenic disorders such as CHD may suggest new prevention strategies and diagnostic methods based on genetics. Despite critical advances in surgical and catheterization-based CHD therapies that have reduced childhood mortality to less than 5% in developed countries, life-long cardiovascular

	Risk Genes				Responder Genes	
	SNPs	Mendelian Mutations	CNVs	Environment	Mendelian Mutations	Environment
Risk Genes	SNPs	NA	$P = 1.0$	NA	$P = 1.0$	$P = 1.0$
	Mendelian Mutations		$P = 0.96$	$P = 1.0$	$P = 0.03$	$P = 1.0$
	CNVs			$P = 1.0$	$P = 1.0$	$P = 1.0$
	Environment				$P = 1.0$	$P = 1.0$
Responder Genes	Mendelian Mutations					$P = 7.0e-3$
	Environment					

Fig. 4. Pairwise comparisons of direct convergences of risk and responder datasets. Genes (Table 1) were compared using hypergeometric statistics to assess significance of overlap between datasets of varying sizes and corrected for multiple testing. Shading denotes significant (red) or insignificant (grey) direct convergence. SNPs within Mendelian CHD genes and environmental target genes are not independent and were excluded (NA).

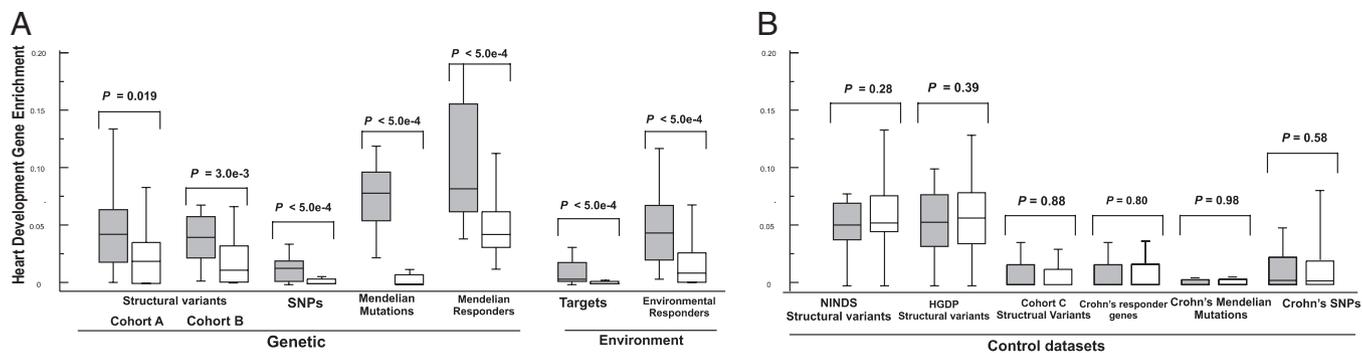


Fig. 5. Risk and responder datasets are enriched for heart developmental genes. (A) Box-and whisker plots showing the fraction of genes shared between the individual risk and responder datasets (Tables 1 and 2) and each of the 19 cardiac developmental networks, compared with dataset-specific null distributions. Each dataset shows significant enrichment for heart developmental genes represented in one or more of the networks ($P < 5.0 \times 10^{-4}$ to $P = 0.016$ for the individual datasets of risk or responder genes, respectively) after taking into account the size and composition of each dataset. Note that the enrichment of genes within structural variants identified in cohort A are replicated in an independent dataset from cohort B. (B) Analyses of control datasets of comparable complexity to the CHD risk and responder datasets showed no enrichment for genes within 19 heart developmental networks. Distributions are plotted as their median, first, and third quartile (box) and minimum and maximum (whiskers). Gray boxes, dataset-specific enrichment; white boxes, dataset-specific null distribution.

problems often remain after corrective procedures (24, 25). Moreover, the mortality of CHD is still very high in developing countries (26). Systems-based insights into cardiac developmental networks and CHD risk factors may lead to improved genetic diagnoses, treatment, and eventually prevention strategies and promote deeper understanding of how an individual's genome and environmental interactions result in disease.

Methods

All studies conformed to the Helsinki Declaration of the World Medical Association. Human datasets were acquired using protocols approved by institutional human research committees. Animal studies were performed using protocols approved by the Animal Care and Use Committee of Harvard Medical School. All risk, responder, and control gene sets, the cardiac developmental networks, and the random gene sets used in the permutation analyses are available at www.cbs.dtu.dk/suppl/dgfi. Details for the risk and responder datasets are provided in *SI Appendix*.

Statistical Tests. Functional convergence was assessed using permutation tests to measure the likelihood of observing similar or higher degrees of convergence on protein sets matched to the proteins in the specific cardiac development network being tested using data from the protein-protein interaction database InWeb (18, 27, 28). Direct convergence was assessed from the significance of pairwise overlaps between risk and responder datasets, calculated using a hypergeometric distribution. Enrichment of heart developmental genes in datasets was assessed by measuring the fraction of genes affected in each of the corresponding 19 protein networks and comparing the number of affected genes with a dataset-specific null distribution using a two-sample Kolmogorov-Smirnov test. (For more details, see *SI Appendix*.)

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