HUMAN GENETICS

De novo mutations in congenital heart disease with neurodevelopmental and other congenital anomalies

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Congenital heart disease (CHD) patients have an increased prevalence of extracardiac congenital anomalies (CAs) and risk of neurodevelopmental disabilities (NDDs). Exome sequencing of 1213 CHD parent-offspring trios identified an excess of protein-damaging de novo mutations, especially in genes highly expressed in the developing heart and brain. These mutations accounted for 20% of patients with CHD, NDD, and CA but only 2% of patients with isolated CHD. Mutations altered genes involved in morphogenesis, chromatin modification, and transcriptional regulation, including multiple mutations in *RBFOX2*, a regulator of mRNA splicing. Genes mutated in other cohorts examined for NDD were enriched in CHD cases, particularly those with coexisting NDD. These findings reveal shared genetic contributions to CHD, NDD, and CA and provide opportunities for improved prognostic assessment and early therapeutic intervention in CHD patients.

Extracardiac congenital anomalies (CAs, structural or functional anomalies that arise in utero) occur in approximately 13% of newborns with congenital heart disease (CHD), including 2% with a genetic syndrome, which is almost twice the prevalence observed in infants without CHD (*1*). Newborns with CHD are also at risk for the emergence of neurodevelopmental disorders (NDDs), including cognitive, motor, social, and language impairments. NDDs occur in 10% of all children with CHD and in 50% with severe CHD (*2*). Explanations to account for the high frequency of CA and NDD in CHD patients include embryonic circulatory deficits and stresses associated with postnatal therapeutic interventions (*3*), but these hypotheses remain unproven.

We sequenced exomes in 1213 CHD trios (probands and their unaffected parents) enrolled in the Pediatric Cardiac Genetics Consortium (PCGC) (*4*) or the Pediatric Heart Network (*5*), after excluding CHD cases with clinically recognized genetic syndromes. Analyses included 353 previously reported CHD trios (*6*). We compared de novo mutations identified in CHD that occurred in isolation, or accompanied by CA, NDD, or both (phenotypes in table S1 and database S1). Previously sequenced trios ($n = 900$) from the Simons Foundation Autism Research Initiative Simplex Collection, each consisting of the unaffected parents and sibling of a child with autism spectrum disorder, served as control trios (*7*–*9*).

CHD and control probands were analyzed for de novo mutations (databases S2 and S3). To evaluate the significance of mutation frequencies, we adapted a recently reported de novo expectation model (*10*) to assess mutation rates by variant class [synonymous, loss of function (LoF; such as nonsense, frameshift, or canonical splice disruptions), or missense]. We derived gene-based rates of de novo mutation from local sequence context and adjusted by per-base coverage separately in case and control cohorts (databases S4 and S5). We extended the model by merging all possible transcripts to obtain transcript-independent probabilities and by adding rates for deleterious missense variants predicted by the Meta-SVM score (D-Mis) (*11*). This yielded an overall mean expected mutation rate of 1.1 de novo variant per proband.

The expected and observed numbers of de novo mutations in each variant class in all CHD and control study participants (Table 1) were compared using a Poisson distribution. De novo mutation rates per variant class were accurately predicted in controls, replicating previous model validations (*10*). However, among all CHD trios, we detected significant enrichment (i.e., observed divided by expected frequencies) of LoF and D-Mis variants of 1.3 ($P = 0.0016$) and 1.6 ($P = 1.8 \times 10^{-10}$), respectively, across all genes. The combination of LoF+D-Mis variants (hereafter denoted as "damaging") was 1.4-fold enriched in CHD cases as compared to expectation, similar to the observed case versus control comparison (table S2). This burden persisted after excluding 353 previously studied CHD trios (table S3) and was found in each CHD category (conotruncal defects, left ventricular outflow tract obstruction, and "other"), except for heterotaxy, which showed no excess (table S4).

Damaging de novo mutations were markedly increased in CHD cases (enrichment = 2.4, $P = 5.1 \times 10^{-24}$) among 4420 genes in the top quartile of expression during heart development [high heart expression, HHE (*6*)] (Table 1). Conversely, controls had no significant enrichment in de novo mutations in HHE genes. Neither cases nor controls were enriched in de novo mutations among genes within the lower three quartiles of developing heart expression (LHE) (Table 1). From the observed and expected values, we estimated that 58% of these damaging de novo mutations contributed to CHD.

Twenty-one genes had multiple damaging de novo mutations only in cases, an unlikely chance

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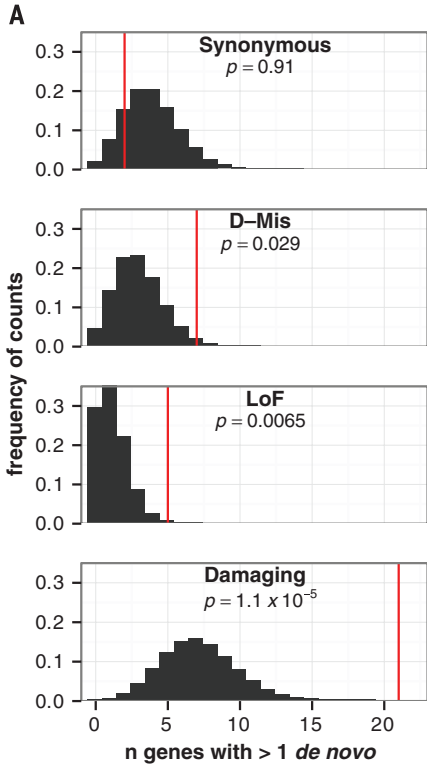

B

Gene	LoF	D-Mis	p
PTPN11	0	4	2.90 x 10^{-11}
KMT2D	4	2	4.10 x 10^{-09}
RBFOX2	3	0	3.40 x 10^{-08}
KDM5B	3	0	2.88 x 10^{-06}
KRT13	0	2	1.01 x 10^{-05}
MYH6	0	3	2.41 x 10^{-05}
CAD	0	3	3.73 x 10^{-05}
NAA15	2	0	4.67 x 10^{-05}
SMAD2	1	1	1.08 x 10^{-04}
RABGAP1L	1	1	4.01 x 10^{-04}
POGZ	1	1	4.33 x 10^{-04}
JAG1	1	1	4.47 x 10^{-04}
GANAB	1	1	4.52 x 10^{-04}
DTNA	1	1	4.68 x 10^{-04}
PPL	1	1	5.98 x 10^{-04}
CHD7	2	0	6.16 x 10^{-04}
ZEB2	1	1	6.18 x 10^{-04}
FBN1	0	2	6.79 x 10^{-04}
CHD4	0	2	1.16 x 10^{-03}
AHNAK	1	1	2.88 x 10^{-03}
NOTCH1	1	1	4.35 x 10^{-03}

Fig. 1. Genes with multiple de novo mutations are candidate CHD risk genes. (**A**) Histograms show the expected distribution of the number of genes containing multiple de novo mutations (empirically derived using 1M permutations, black) and the observed number of genes with multiple mutations in cases (red line) for each class. *P* values were calculated by permutation. (**B**) Twenty-one genes with multiple damaging de novo mutations in cases. *P* values are from Poisson test against expectation, with significance threshold <9 × 10^{-7}. Further details are shown in table S6.

Table 1. De novo enrichment in cases versus controls by expectation analysis. *n*, number of de novo mutations; rate, number of de novo mutations divided by the number of individuals in the cohort (*N*); enrichment, ratio of observed to expected numbers of mutations; HHE, high heart-expressed genes (top quartile of expression); LHE, lower heart-expressed genes (bottom three quartiles of expression); D-Mis, damaging missense predicted by Meta-SVM; Damaging, D-Mis+LoF. Bold numbers indicate enrichment >2 or *P* < 0.005.

	Cases, *N* = 1213						Controls, *N* = 900					
	Observed		Expected		Enrichment	*P*	Observed		Expected		Enrichment	*P*
	n	Rate	*n*	Rate			*n*	Rate	*n*	Rate		
All genes												
Total	1273	1.05	1312.7	1.08	1.0	0.87	925	1.03	979.7	1.09	0.9	0.96
Synonymous	277	0.23	371.4	0.31	0.7	1	229	0.25	277.4	0.31	0.8	1
Missense	846	0.70	824.9	0.68	1.0	0.24	614	0.68	615.6	0.68	1.0	0.53
D-Mis	212	0.17	133.1	0.11	1.6	**1.8 × 10^{-10}**	119	0.13	99.3	0.11	1.2	0.03
LoF	150	0.12	116.5	0.10	1.3	**0.0016**	82	0.09	86.7	0.10	0.9	0.71
Damaging	362	0.30	249.5	0.21	1.4	**1.5 × 10^{-11}**	201	0.22	186.0	0.21	1.1	0.14
HHE genes												
Total	448	0.37	372.4	0.31	1.2	**7.8 × 10^{-05}**	271	0.30	277.7	0.31	1.0	0.66
Synonymous	81	0.07	103.5	0.09	0.8	0.99	80	0.09	77.3	0.09	1.0	0.39
Missense	288	0.24	234.3	0.19	1.2	**0.00038**	163	0.18	174.7	0.19	0.9	0.82
D-Mis	99	0.08	40.6	0.03	**2.4**	**7.7 × 10^{-15}**	37	0.04	30.3	0.03	1.2	0.13
LoF	79	0.07	34.5	0.03	**2.3**	**6.2 × 10^{-11}**	28	0.03	25.7	0.03	1.1	0.35
Damaging	178	0.15	75.1	0.06	**2.4**	**5.1 × 10^{-24}**	65	0.07	55.9	0.06	1.2	0.13
LHE genes												
Total	825	0.68	940.3	0.78	0.9	1	654	0.73	702.1	0.78	0.9	0.97
Synonymous	196	0.16	267.8	0.22	0.7	1	149	0.17	200.1	0.22	0.7	1
Missense	558	0.46	590.5	0.49	0.9	0.91	451	0.50	440.9	0.49	1.0	0.32
D-Mis	113	0.09	92.4	0.08	1.2	0.021	82	0.09	69.0	0.08	1.2	0.069
LoF	71	0.06	82.0	0.07	0.9	0.9	54	0.06	61.1	0.07	0.9	0.83
Damaging	184	0.15	174.4	0.14	1.1	0.24	136	0.15	130.1	0.14	1.1	0.31

occurrence (Fig. 1A; median expected = 7; $P = 1.1 \times 10^{-5}$ by permutation), suggesting that these genes are likely to be pathogenic for CHD (Fig. 1B). Indeed, this list includes seven genes previously implicated in CHD (*PTPN11, KMT2D, CHD7, MYH6, JAG1, NOTCH1,* and *ZEB2*). Enrichments were not observed among genes with multiple de novo synonymous variants in CHD cases or across any variant class among controls (Fig. 1A and table S5). A variety of cardiac malformations were associated with mutations in each of these 21 genes (Fig. 1B and table S6). From simulations based on these data (*12*), we estimate that de novo mutations in ~392 HHE genes contribute to CHD pathogenesis (fig. S1).

Within this 21-gene set, *PTPN11, KMT2D* (*MLL2*), and *RBFOX2* each had significantly more damaging de novo mutations than expected (Bonferroni corrected threshold for genome-wide significance = $P < 9 \times 10^{-7}$; Fig. 1B). *RBFOX2*, an RNA-binding protein that regulates alternative splicing, has not been previously implicated in CHD. *RBFOX2* harbored three distinct de novo LoF mutations, a highly significant finding (Fig. 1B; $P = 3.4 \times 10^{-8}$). Additionally, we previously identified a de novo copy number loss that encompasses *RBFOX2* in another CHD proband (*13*). These four probands all had hypoplastic left heart syndrome (HLHS). *RBFOX2* is critical for zebrafish heart development (*14*) and regulates

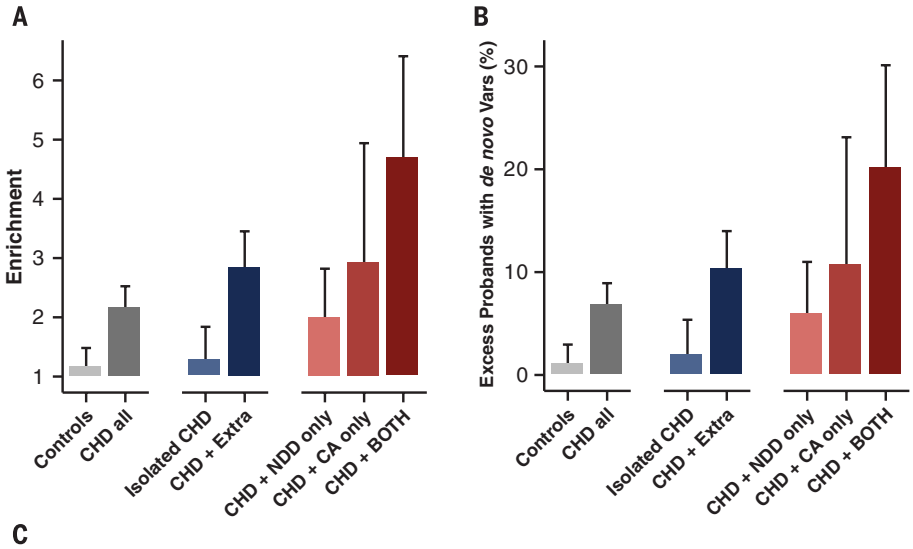

Fig. 2. Burden of damaging de novo mutations in HHE genes among CHD cases with extracardiac phenotypes. (**A**) The enrichment (ratio of observed to expected) of damaging de novo mutations in HHE genes is shown for each phenotype (±95% confidence interval). Case probands were excluded if they carried de novo mutations in known CHD syndrome genes (n = 19), had unknown extracardiac phenotype for both NDD and CA (n = 6), or had one unknown phenotype and were negative for the other (n = 273). Cases with either CA or NDD and unknown status for the other phenotype (n = 97) were included in the "Extra" category but excluded from the "only" categories. (**B**) Percent excess of individuals carrying damaging de novo mutations in HHE genes by indicated phenotype (±95% confidence interval). An explanation of the calculation is provided in (*12*). (**C**) Table of observed and expected de novo rates for the indicated variant classes by phenotype.

C

	Observed		Expected		Enrichment	p
	n	Rate	n	Rate		
Isolated CHD (356)						
Synonymous	17	0.05	30	0.09	0.6	1
Missense	83	0.23	69	0.19	1.2	0.052
D-Mis	17	0.05	12	0.03	1.4	0.097
LoF	11	0.03	10	0.03	1.1	0.43
Damaging	28	0.08	22	0.06	1.3	0.12
CHD + Extra (559)						
Synonymous	38	0.07	48	0.09	0.8	0.94
Missense	130	0.23	108	0.19	1.2	0.022
D-Mis	49	0.09	19	0.03	**2.6**	**4.3×10^{-09}**
LoF	49	0.09	16	0.03	**3.1**	**2.2×10^{-11}**
Damaging	98	0.18	35	0.06	**2.8**	**1.1×10^{-18}**
CHD + NDD only (252)						
Synonymous	22	0.09	22	0.09	1.0	0.49
Missense	46	0.18	49	0.19	0.9	0.67
D-Mis	15	0.06	8	0.03	1.8	0.026
LoF	16	0.06	7	0.03	**2.2**	**0.003**
Damaging	31	0.12	16	0.06	2.0	**0.00038**
CHD + CA only (72)						
Synonymous	4	0.06	6	0.08	0.7	0.86
Missense	19	0.26	14	0.19	1.4	0.11
D-Mis	9	0.12	2	0.03	**3.7**	**0.00089**
LoF	4	0.06	2	0.03	2.0	0.15
Damaging	13	0.18	4	0.06	**2.9**	**0.00074**
CHD + Both (138)						
Synonymous	6	0.04	12	0.09	0.5	0.98
Missense	43	0.31	27	0.19	1.6	**0.0022**
D-Mis	17	0.12	5	0.03	**3.7**	**7.4×10^{-06}**
LoF	23	0.17	4	0.03	**5.9**	**4.1×10^{-11}**
Damaging	40	0.29	8	0.06	**4.7**	**5.6×10^{-15}**

epithelial-mesenchymal transitions (EMTs) (*15*). Disruption of EMTs is felt to underlie HLHS pathogenesis (*16*). We observed significant enrichment of damaging mutations in *RBFOX2* target genes (*17*) in CHD cases (1.9-fold, $P = 6.6 \times 10^{-8}$) but not controls (table S7).

Analyses of gene (GO) and human phenotype (HP) ontologies revealed enrichment of damaging de novo mutations in genes involved in anatomic structure morphogenesis (GO:0009653; 2.4-fold; Bonferroni $P = 3.4 \times 10^{-14}$), cardiovascular system development (GO:0072358; 3.2-fold; Bonferroni $P = 7.5 \times 10^{-9}$), neurodevelopmental abnormality (HP:0012759; 2.6-fold; Bonferroni $P = 1.8 \times 10^{-6}$), and others (database S6). We replicated the reported excess of de novo LoF mutations affecting genes involved in chromatin modification (*6*), even after including only newly studied cases (GO:0016568, 5.1-fold enrichment, P value = 7.2×10^{-5}; database S7). In the full CHD cohort, there were 25 de novo LoF mutations in chromatin-modifying genes, a 5.3-fold enrichment over expectation ($P = 5.7 \times 10^{-11}$, table S8; fig. S2), strongly supporting the conclusion that these damaging de novo mutations have large effects on CHD risk.

We examined the prevalence of damaging de novo mutations in CHD with or without NDD and/or CA (Fig. 2) after excluding 19 participants found to have de novo mutations in known syndromic CHD genes and 279 participants with uncertain NDD/CA status. Damaging de novo mutations in HHE genes were not significantly enriched in 356 participants with isolated CHD or in controls but were ~3-fold enriched in 559 CHD cases with CA and/or NDD (CHD + Extra, $P = 1.1 \times 10^{-18}$), including 97 probands diagnosed with either NDD or CA but unknown for the other phenotype. Excluding these 97 probands, we observed a 4.7-fold enrichment of damaging de novo mutations in HHE genes among 138 CHD cases with both NDD and CA ($P = 5.6 \times 10^{-15}$), a 2-fold enrichment in CHD cases with only NDD (252 probands, $P = 3.8 \times 10^{-4}$), and a 2.9-fold enrichment in CHD cases with only CA (72 probands, $P = 7.4 \times 10^{-4}$). By comparing de novo rates in cases against expectation, we estimate that damaging de novo mutations in HHE genes contributed to 20% of CHD with both NDD and CA (95% confidence interval 12 to 30%), 10% (7 to 14%) of CHD with CA and/or NDD, 10% (2.5 to 23%) and 6% (2 to 11%) of CHD with CA only or NDD only, respectively, and 2% (0.5 to 5%) of isolated CHD (Fig. 2B). These results implied frequent pleiotropic effects of de novo mutations in CHD and raise the possibility that mutations in these same genes might also contribute to nonsyndromic NDD and/or other CA. We find that genes mutated in CHD are not only enriched for high expression in the developing heart, they are also enriched for high expression in the developing brain (table S9).

To further explore these pleiotropic effects, we considered whether genes with damaging de novo mutations in CHD with NDD overlapped with 1161 genes (database S8) found to contain damaging de novo mutations in seven cohorts ascertained for NDD phenotypes excluding CHD (published NDD, P-NDD gene set) (*7*, *18*–*23*). Sixty-nine genes (table S10) with damaging de novo mutations ($n = 85$ mutations) were shared in CHD and P-NDD cohorts, far more than expected by chance (expected = 32 mutations; 2.6-fold enrichment; $P = 8.9 \times 10^{-15}$, Fig. 3A and table S11). HHE genes were particularly enriched among P-NDD genes that were mutated in CHD (4.4-fold for all CHD cases, $P = 1.2 \times 10^{-23}$; Fig. 3A and table S11). Moreover, genes mutated both in P-NDD and CHD cohorts are in the top quartile of both developmental heart and brain expression far more than expected by chance (observed = 38, expected = 11, $P = 6.1 \times 10^{-11}$, binomial test, Fig. 3B). The input of these 69 overlapping genes into GO ontology analysis revealed significant terms that were broadly involved in the regulation of developmental transcription programs. These included 19 chromatin modifiers (GO:0016568 9.3-fold, $P = 8.5 \times 10^{-10}$; database S9 and fig. S2), including genes responsible for altering the methylation, acetylation, or ubiquitination status of numerous regulatory lysine residues on the nucleosome. Additionally, there were 32 transcriptional regulators (GO:0006355 2.8-fold $P = 1.5 \times 10^{-4}$; database S9), including genes involved in Wnt (*CTNNB1*, *DVL3*, and *LRP5*) and Notch (*NOTCH1* and *EP300*) signaling, important pathways in cardiac development. These findings demonstrate shared genetic etiologies for CHD and NDD patients and confirm the pleiotropic effects of mutations in these genes. Because it is unlikely that many NDD-ascertained patients with these

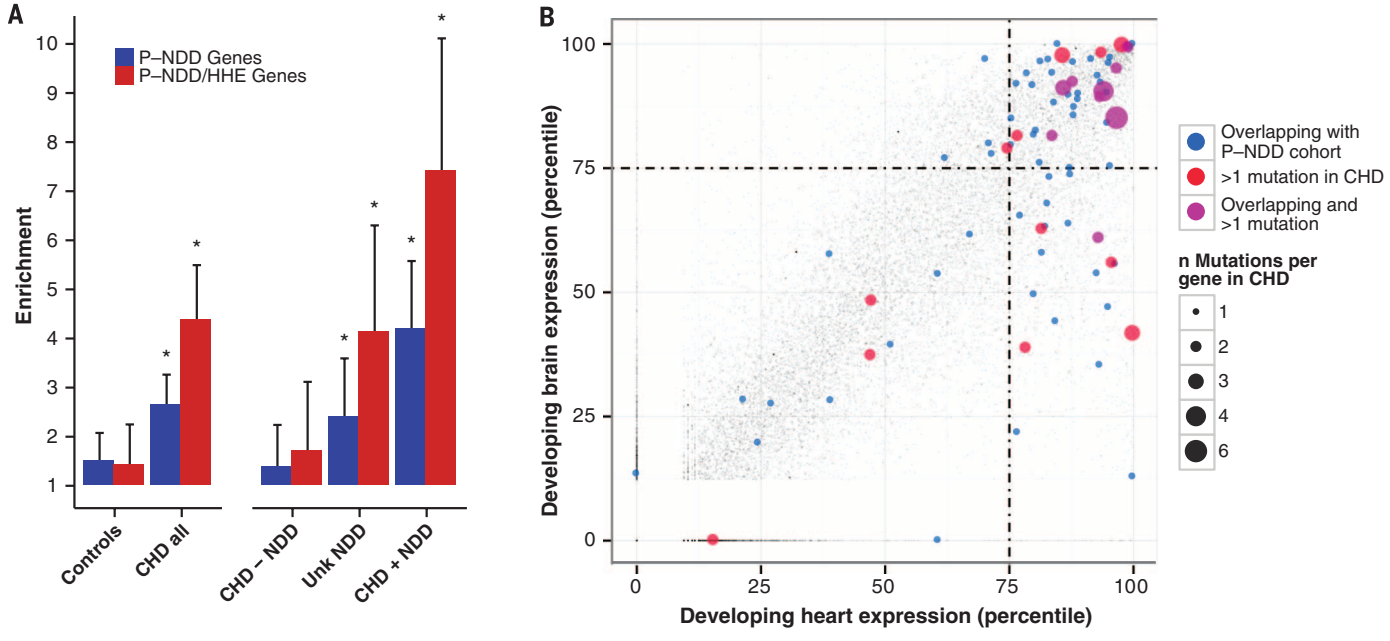

Fig. 3. Genes containing de novo mutations in CHD cases show pleiotropic developmental effects. (**A**) Individuals with CHD carry an excess of damaging de novo mutations among 1161 genes identified by containing damaging de novo mutations in seven published studies of NDD (P-NDD cohort) (*7*,*18*–*23*). All CHD cases were subdivided by NDD status (CHD + NDD: $n = 417$ patients; CHD – NDD: $n = 440$; unknown NDD: $n = 363$). P values ≤ 0.005 as indicated (stars) were calculated from a Poisson test against a model-derived distribution (values in table S11). The P-NDD gene set (blue) was further filtered for HHE genes (P-NDD/HHE, red, 564 genes). Enrichments are shown ±95% confidence intervals. (**B**) Percentile gene expression ranks (100 = high) are shown for all genes (gray) in the developing brain and heart, highlighting 69 genes with damaging de novo mutations in both CHD cases and the P-NDD cohort (blue or purple). Genes with multiple de novo mutations in CHD (red or purple) are shown. The point size represents the numbers of de novo events.

mutations had clinically important CHD, and not all CHD patients with these mutations have NDD (table S10), our findings also indicate that these mutations have variable expressivity, including isolated CHD, isolated NDD, or both.

Consistent with these observations, CHD participants with damaging de novo mutations in these 69 overlapping genes (Fig. 3A) had a significantly increased risk of NDD (absolute risk of 73%, odds ratio 3.1, $P = 7.9 \times 10^{-5}$, Fisher exact test). Damaging mutations (28 mutations in 27 participants) in chromatin modifiers showed the highest risk of NDD (19 participants with NDD, 8 with unknown NDD status due to age <1 year at evaluation). Moreover, the marked enrichments in damaging de novo mutations among P-NDD genes with HHE (Fig. 3A and table S11), 7.4-fold in 413 CHD cases with NDD ($P = 3.9 \times 10^{-22}$), 4.1-fold in 362 CHD infants with unknown NDD status ($P = 2.2 \times 10^{-7}$), and no significant enrichment in 438 CHD cases without NDD ($P = 0.075$), strongly imply a future risk of NDD among CHD infants with these variants. These observations suggest that genotype is a strong predictor for future development of NDD in CHD infants. Despite these highly significant findings, our estimates are based purely on statistical grounds and limited to in silico predictions of damaging variants, a caveat that should be considered when extrapolating these results to identify causative or predictive mutations in individual patients.

Contemporary therapeutic interventions have substantially improved survival among newborns with serious CHD. Despite these advances, many lifelong medical issues remain. The demonstration that damaging de novo gene mutations cause CHD, particularly when associated with NDD and other congenital anomalies, has both clinical and research implications. First, clinical genotyping may help stratify CHD patients and identify those at high risk for NDD, enabling surveillance and early interventions to improve school performance, employability, and quality of life. Second, the pleiotropic consequence of these mutations implies that further study of these genes may uncover critical regulation of broad developmental programs. Finally, the high frequency of mutation in transcriptional regulators suggests that mutations in regulatory elements (promoters and enhancers) may be additional causes of CHD, particularly isolated CHD.

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SUPPLEMENTARY MATERIALS

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De novo mutations in congenital heart disease with neurodevelopmental and other congenital anomalies

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