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Insights into the genetic architecture of congenital heart disease from animal modeling

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ABSTRACT

Congenital heart disease (CHD) is observed in up to 1% of live births and is one of the leading causes of mortality from birth defects. While hundreds of genes have been implicated in the genetic etiology of CHD, their role in CHD pathogenesis is still poorly understood. This is largely a reflection of the sporadic nature of CHD, as well as its variable expressivity and incomplete penetrance. We reviewed the monogenic causes and evidence for oligogenic etiology of CHD, as well as the role of *de novo* mutations, common variants, and genetic modifiers. For further mechanistic insight, we leveraged single-cell data across species to investigate the cellular expression characteristics of genes implicated in CHD in developing human and mouse embryonic hearts. Understanding the genetic etiology of CHD may enable the application of precision medicine and prenatal diagnosis, thereby facilitating early intervention to improve outcomes for patients with CHD.

Keywords: Congenital heart disease; Genetic modifier; Single cell sequencing; *De novo* mutation; Protective variant; Common copy number variant

OVERVIEW OF CHD

Congenital heart disease (CHD) is characterized by structural abnormalities in the heart and/or great vessels at birth (Triedman & Newburger, 2016). CHD is among the most common birth defects, accounting for approximately 8–11 out of 1 000 live births (Botto et al., 2001; Ferencz et al., 1985; Hoffman & Kaplan, 2002; Marelli et al., 2007), and is a leading cause of childhood morbidity and mortality worldwide. CHD encompasses many subtypes ranging from relatively mild lesions, such as ventricular septal defect (VSD), atrial septal defect (ASD), and bicuspid aortic valve (BAV), to severe

lesions, such as hypoplastic left heart syndrome (HLHS) and transposition of the great arteries (TGA). Additionally, 13.6% of live births with CHD also exhibit extracardiac congenital anomalies (Egbe et al., 2014).

With advances in surgical and medical interventions, one-year mortality for infants with critical CHD has decreased from 32.6% in 1979–1993 to 17.5% in 1994–2005 (Oster et al., 2013). More than 90% of children with CHD who survive their first year of life now survive to adulthood, resulting in a growing population of adults with CHD that far exceeds the number of babies born annually with CHD. Causes of CHD include genetic factors, which account for the majority of CHD (Zaidi & Brueckner, 2017), and nongenetic factors, which account for about 10% of CHD (Jenkins et al., 2007), such as maternal exposure to teratogens, infectious agents (Fahed et al., 2013), and various environmental factors.

ETIOLOGY OF CHD

Familial studies show high recurrence risk, suggesting that 90% of CHD is likely to have a genetic etiology (Nora et al., 1969; Øyen et al., 2009), but this is complicated by incomplete penetrance and variable expressivity. Epidemiological studies have indicated that a genetic or environmental cause can be identified in 20% to 30% of CHD (Cowan & Ware, 2015), with gross chromosomal abnormalities or aneuploidy found in 9%–18% of all CHD and pathogenic copy number variants (CNVs) (rare or *de novo* CNVs) found in 3%–25% of syndromic CHD and 3%–10% among sporadic nonsyndromic CHD. *De novo* mutations account for approximately 10% of severe CHD and 28% of CHD with extracardiac and/or neurodevelopmental defects. At present, only 1.8% of CHD cases occur due to rare inherited mutations (Homsy et al., 2015; Jin et al., 2017; Zaidi & Brueckner, 2017) (Figure 1).

Identification of genetic variants linked to CHD have been made possible by several large-scale human sequencing projects, including the Pediatric Cardiac Genomics Consortium (PCGC) (Pediatric Cardiac Genomics Consortium Writing Committee et al., 2013), Deciphering Developmental Disorders Project (DDD) (Firth et al., 2011), Framingham Heart Study 100K Project (Larson et al., 2007), and
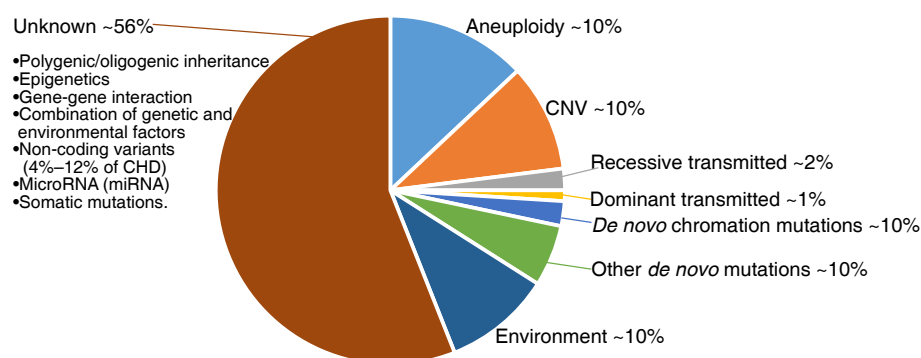
Figure 1 Distribution of different genetic causes of CHD

Competence Network for Congenital Heart Defects (Germany) (Pickardt et al., 2016). Data from these studies have significantly advanced our understanding of the genetic landscape of CHD, with more than 400 genes implicated to play a role in CHD. Different genetic variations contribute to CHD, including chromosomal abnormalities, CNVs, single-nucleotide variants, and small deletions/insertions (InDels) (Figure 1). These genetic variations can be inherited or occur *de novo* (present in probands but absent in parents), and depending on the specific type of genetic change, can impact gene dosage, involve missense variants that cause dominant gain-of-function effects, or more commonly, result in recessive loss-of-function (LOF) due to nonsense, canonical splice-site, frameshift indel, or start loss mutations. Genetic variations can also occur in noncoding sequences, causing dysregulated gene expression. Genomic analysis of CHD patients has revealed differences in the genetic architecture of nonsyndromic and syndromic CHD (Sifrim et al., 2016), with *de novo* variants specifically enriched in syndromic CHD and inherited coding variants linked to nonsyndromic CHD.

The genetics underpinning most CHD cases remain unexplained, a reflection of the considerable gap in our current knowledge regarding the genetic etiology of CHD. It is likely that a large proportion of unexplained cases may be oligogenic or exhibit multifactorial etiology involving interactions between genetic and environmental factors. Recent evidence suggests that noncoding variants may impact the expression of CHD-associated genes contributing to CHD, especially *de novo* noncoding variants within enhancers and RNA-binding protein sites, potentially underlying 4%–12% of CHD cases (Morton et al., 2022). Other proposed genetic mechanisms include microRNA (miRNA), somatic mutations, and epigenetic contributions (Hsieh et al., 2020; Manheimer et al., 2018; Morton et al., 2022; Smith et al., 2015; Xu et al., 2009; Zaidi et al., 2013) (Figure 1).

The genetic mechanisms contributing to CHD pathogenesis are complex and many informative reviews have discussed the causes and genetic architecture of CHD as well as the biological pathways involved in its pathogenesis (Diab et al., 2021; Fahed et al., 2013; Morton et al., 2022; Nees & Chung, 2020; Pierpont et al., 2018; Triedman & Newburger, 2016; Williams et al., 2019; Zaidi & Brueckner, 2017). In this review, we discuss recent genetic discoveries in CHD, focusing on examples of CHD-associated genes validated by animal models and insights gained from scRNA-seq data obtained from developing embryonic hearts in humans (Asp et al., 2019; Miao et al., 2020), mice (Hill et al., 2019), and chickens (Mantri et al., 2021). This recent research has shed new light on the cardiac-specific expression of genes linked to CHD and

revealed the complex cellular and molecular mechanisms involved in cardiovascular development that may contribute to the pathogenesis of CHD.

Clinical genetic testing and diagnosis of CHD

While many genes have been implicated in CHD, only a small proportion of CHD cases (3%–5%) have been found to have definitive diagnostic causal mutations in well-established CHD genes (van der Bom et al., 2011).The overall diagnostic yield from genetic testing of infants with CHD using karyotyping, fluorescence *in situ* hybridization (FISH), and chromosome microarray analysis is 15%–25%, up to 30% for syndromic cases with extracardiac defects (Nees & Chung, 2020). Whole-exome sequencing (WES) has provided clinical diagnosis in 12.7% of all CHD cases, accounting for 11.5% of isolated CHD and 14.7% of CHD associated with extracardiac anomalies (Mone et al., 2021), while whole-genome sequencing (WGS) has identified 12.6%–31% of familial cases of CHD (Alankarage et al., 2019; Reuter et al., 2020). Thus, genetic testing may benefit pregnancy management, clinical monitoring, and genetic counseling.

Genetic etiology of syndromic CHD

Many syndromic CHD cases arise from either chromosome dosage abnormalities, such as trisomy 13, 18, and 21 and Turner Syndrome, or structural variants including CNVs (Jenkins et al., 2007; Morton et al., 2022; Nees & Chung, 2020). For such syndromic disorders, the specific genes responsible for CHD in most cases remain poorly understood. A list of syndromes associated with CHD and the genes thought to underlie pathogenesis (Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018) are summarized in Table 1. Analysis of scRNA-seq data from developing human, mouse, and chicken hearts show that most genes causing syndromic CHD are broadly expressed in all cardiac cell types (Figure 2). This is consistent with the observation of severe cardiac abnormalities in CHD associated with these genes and suggests the developmental etiology of syndromic CHD may involve perturbation of multiple cardiac cell types. The *FBN1* gene, responsible for causing Marfan syndrome, is predominantly expressed in endocardial cells and cardiac fibroblasts, and is mainly located at the base of the outflow tract (OFT) and in the valve apparatus (Figure 2), consistent with the fact that *FBN1* mutations are often associated with mitral valve prolapse, ascending aortic dilatation, and aortic dissection (Damrauer et al., 2019). Similar cell type-specific expression across species indicates that mice and chicken are suitable animal models to study the functional roles and pathogenic mechanisms of genes related to human CHD.

Table 1 Monogenic conditions causing syndromes with CHD phenotype

Syndrome	Causal gene(s)	Associated CHD	% CHD	References
Adams-Oliver	*DLL4, DOCK6, EOGT, NOTCH1, ARHGAP31, RBPJ*	BAV, PDA, PS, VSD, ASD, TOF	20	Nees & Chung, 2020; Pierpont et al., 2018
Alagille	*JAG1, NOTCH2*	Branch pulmonary artery stenosis, TOF, PA	90–95	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
Axenfeld-Rieger	*FOXC1*	ASD, AS, PS, TOF, BAV, TA	Unknown	Nees & Chung, 2020
Baller-Gerold and Rothmund-Thomson	*RECQL4*	VSD, TOF, subaortic stenosis	25	Nees & Chung, 2020; Pierpont et al., 2018
Bardet-Biedl	*BBS2, BBS6*	AS, PS, PDA, cardiomyopathies	7–50	Nees & Chung, 2020
Beckwith Wiedemann	*CDKNIC*	VSD, HLHS, PS	6.5	Pierpont et al., 2018
Cantu	*ABCC9*	Cardiomegaly, ventricular hypertrophy, PDA, BAV	60–75	Nees & Chung, 2020; Pierpont et al., 2018
Carpenter	*RAB23*	ASD, VSD, TOF, PDA, PS	18–50	Nees & Chung, 2020; Pierpont et al., 2018
Cardiofaciocutaneous	*BRAF, KRAS, MAP2K1, MAP2K2*	PS, ASD, VSD, HCM	71–75	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
Coffin-Lowry	*RSK2*	LVNC, MVP, AVA	5–14	Fahed et al., 2013
Congenital heart defects, dysmorphic facial features, and intellectual developmental disorder	*CDK13*	ASD, VSD, PS	56	Nees & Chung, 2020
Char	*TFAP2B*	PDA, VSD	26–75	Fahed et al., 2013; Nees & Chung, 2020
CHARGE	*CHD7*	TOF, PDA, DORV, AVSD, VSD	75–85	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
Coffin-Siris	*ARID1B, SMARCA4, SMARCB1, ARID1A, SMARCB1, SMARCE1*	ASD, VSD, PS, AS, dextrocardia, CoA, PDA, TOF	20-44	Nees & Chung, 2020; Pierpont et al., 2018
Cornelia deLange	*NIPBL, SMC3, SMC1L1*	VSD, ASD, PS, PDA	13–70	Nees & Chung, 2020; Pierpont et al., 2018
Costello	*HRAS*	PS, ASD, VSD, HCM, arrhythmias	50–60	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
Duane-radial ray syndrome DDRS (Okihiro syndrome)	SALL4	VSD, PFO, TOF	Unknown	Fahed et al., 2013
Ellis-van Creveld	*EVC, EVC2*	Common atrium	60–75	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
Fragile X	*FMR1*	MVP, aortic dilation	10–20	Nees & Chung, 2020; Pierpont et al., 2018
Genitopatellar or Ohdo/SBBYS	*KAT6B*	ASD, VSD, PFO	50	Nees & Chung, 2020
Heterotaxy	*GDF1, NODAL, ZIC3*	Pulmonary venous anomalies, atrial anomalies, AVSD, PS, AS, conotruncal anomalies	>90	Nees & Chung, 2020
Holt-Oram	*TBX5*	ASD, VSD, AVSD, conduction defects	75–85	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
Johanson-Blizzard	*UBR1*	Dysplastic mitral valve, PDA, VSD, ASD, dextrocardia	10	Nees & Chung, 2020
Kabuki	*KDM6A, KMT2D*	CoA, BAV, VSD	30–50	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
Kleefstra	*EHMT1*	ASD, VSD, TOF, PDA, CoA, BAV	40–45	Nees & Chung, 2020
Koolen-De Vries	*KANSL1*	ASD, VSD, PDA, BAV, PS	39	Nees & Chung, 2020
Loeys-Dietz	*TGFBR1, TGFBR2, SMAD3*	BAV, PDA, ASD, MVP	30–50	Nees & Chung, 2020
Mandibulofacial dysostosis, Guion-Almeida Type	*EFTUD2*	ASD, VSD, PDA	30–60	Nees & Chung, 2020
Marfan	*FBN1*	AR, MVP	80	Nees & Chung, 2020
Mental retardation, autosomal dominant	*KAT6A*	PDA, ASD, VSD	Unknown	Nees & Chung, 2020
Mowat-Wilson	*ZEB2*	VSD, CoA, ASD, PDA, PS	50	Nees & Chung, 2020; Pierpont et al., 2018
Myhre	*SMAD4*	ASD, VSD, PDA, PS, AS, CoA	60	Nees & Chung, 2020
Nephronophthisis and Meckel-Gruber-like syndrome	*NPHP3*	AS, ASD, PDA	20	Nees & Chung, 2020
Neurofibromatosis	*NF1*	PS, CoA, MR, PDA, VSD, AS, AR, ASD	2–15	Nees & Chung, 2020

Syndrome	Causal gene(s)	Associated CHD	% CHD	References
Nance-Horan	*NHS*	TOF, VSD, PDA	<10	Pierpont et al., 2018
Noonan	*PTPN11, KRAS, SOS1, RAF1, BRAF, MEK1, HRAS, NRAS, SHOC2, CBL, NF1, RIT1, SOS2, LZTR1*	PS, HCM, ASD, TOF, AVSD, VSD, PDA	75–90	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
Noonan syndrome with multiple lentigines	*PTPN11*	HCM, conduction abnormalities	80	Nees & Chung, 2020
Oculofaciocardiodental (OFCD)	*BCOR*	ASD, VSD, PS, AS, PDA, dextrocardia, DORV	66–74	Nees & Chung, 2020
Orofaciodigital	*OFD1*	ASD, AVSD, HLHS	33–100	Nees & Chung, 2020
Peter's plus	*B3GLCT/B3GALTL*	ASD, VSD, PS, subvalvular AS	25–30	Nees & Chung, 2020; Pierpont et al., 2018
Polycystic kidney disease, autosomal dominant	*PKD1*	MVP, ASD, PDA	10–20	Nees & Chung, 2020
Renal-hepatic-pancreatic dysplasia/Nephronophthisis	*NEK8*	Cardiomegaly, HCM, septal defects, PDA	Unknown	Nees & Chung, 2020
Roberts	*ESCO2*	ASD, AS	20–50	Nees & Chung, 2020; Pierpont et al., 2018
Robinow	*ROR2, WNT5A*	PS, VSD, ASD, DORV, TOF, CoA, TA	15–30	Nees & Chung, 2020; Pierpont et al., 2018
Rubinstein-Taybi	*CBP,EP300*	PDA, VSD, ASD	30–33	Nees & Chung, 2020; Pierpont et al., 2018
Saethre-Chotzen	*TWIST*	VSD	<10	Pierpont et al., 2018
Short rib polydactyly type I	*DYNC2H1*	TGA, DORV, DOLV, AVSD, HRH	<25	
Sifrim-Hitz-Weiss	*CHD4*	PDA, ASD, VSD, BAV, TOF, CoA	Unknown	Nees & Chung, 2020
Simpson-Golabi-Behmel	*GPC3*	TGA, VSD, PVS, CoA, AS, PDA, BAV, CM	26	Pierpont et al., 2018
Smith-Lemli-Opitz	*DHCR7*	AVSD, ASD, VSD	50	Nees & Chung, 2020; Pierpont et al., 2018
Sotos	*NSD1*	ASD, PDA, VSD	8–50	Nees & Chung, 2020; Pierpont et al., 2018
Syndromic microphthalmia/Pulmonary hypoplasia-diaphragmatic hernia-anophthalmia-cardiac defect (PDAC)	*STRA6*	ASD, VSD, PS, PDA, PA, TOF, CoA, TA	50	Nees & Chung, 2020
Townes-Brocks	*SALL1*	VSD, ASD, PA, TA	20–30	Nees & Chung, 2020; Pierpont et al., 2018
Weill-Marchesani	*ADAMTS10*	MVP, AS, PS	50	Nees & Chung, 2020
Williams-Beuren	*ELN*	SVAS, PAS, VSD, ASD	80	Pierpont et al., 2018

AR, aortic regurgitation; AS, aortic stenosis; AA, aortic atresia; ASD, atrial septal defect; AVA, aortic valve anomaly; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; BPV, bicuspid pulmonary valve; CFC, cardiofaciocutaneous; CHARGE, coloboma, heart defects, choanal atresia, retarded growth and development, genital anomalies, and ear anomalies; CM, cardiomyopathy; CoA, coarctation of the aorta; DEX, dextrocardia; DOLV, double-outlet left ventricle; DORV, double-outlet right ventricle; HCM, hypertrophic cardiomyopathy; HD, heart disease; HLHS, hypoplastic left heart; HRH, hypoplastic right heart; IAA, interruption of aortic arch; LVNC, left ventricular noncompaction; MR, mitral regurgitation; MVP, mitral valve prolapse; OFD, oral-facial-digital; PA, pulmonary atresia; PAS, pulmonary artery stenosis; PDA, patent ductus arteriosus; PE, pericardial effusion; PPS, peripheral pulmonary stenosis; PS, pulmonary stenosis; PVS, pulmonary stenosis; PFO, patent foramen ovale; RVOTO, right ventricular outflow tract obstruction; SVAS, supravalvular aortic stenosis; TR, tricuspid regurgitation; TA, truncus arteriosus; TAPVR, total anomalous pulmonary venous return; TGA, transposition of great arteries; TOF, tetralogy of Fallot; VACTERL, association of vertebral defects, anal atresia, cardiac defects, tracheoesophageal fistula, renal and limb anomalies; VR, vascular ring; and VSD, ventricular septal defect.

Genetic etiology of nonsyndromic CHD

The majority of nonsyndromic CHD occurs sporadically, without other organ involvement (Garg, 2006). Genetic heterogeneity, incomplete penetrance, and variable expressivity characterize isolated nonsyndromic CHD cases, making genetic analysis challenging. While more than 400 genes have been implicated in CHD (Morton et al., 2022; Zaidi et al., 2013), the genetic cause of most isolated CHD cases remains unknown. While the list of genes involved in isolated CHD is rapidly expanding with advances in omics technologies, verifying gene-disease association and the pathogenicity of rare variants in CHD candidate genes remains difficult. Mutations in genes associated with nonsyndromic CHD cases are usually identified through familial studies, with further verification using DNA sequencing data from other CHD patient cohorts.

Genes associated with nonsyndromic CHD cases can be divided into three functional categories, including transcription factors, signaling molecules, and structural proteins, essential for normal cardiac development (Fahed et al., 2013). Genes in each functional category known to cause human CHD, as collected from review papers (Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018), are shown in Table 2. Many genes are associated with several CHD phenotypes, most of which have been identified in only a few families comprising a relatively small number of CHD subjects. Hence, in many instances, gene-disease association remains tentative and awaits further confirmation. In many cases, no data are available from animal modeling to accelerate the validation process.
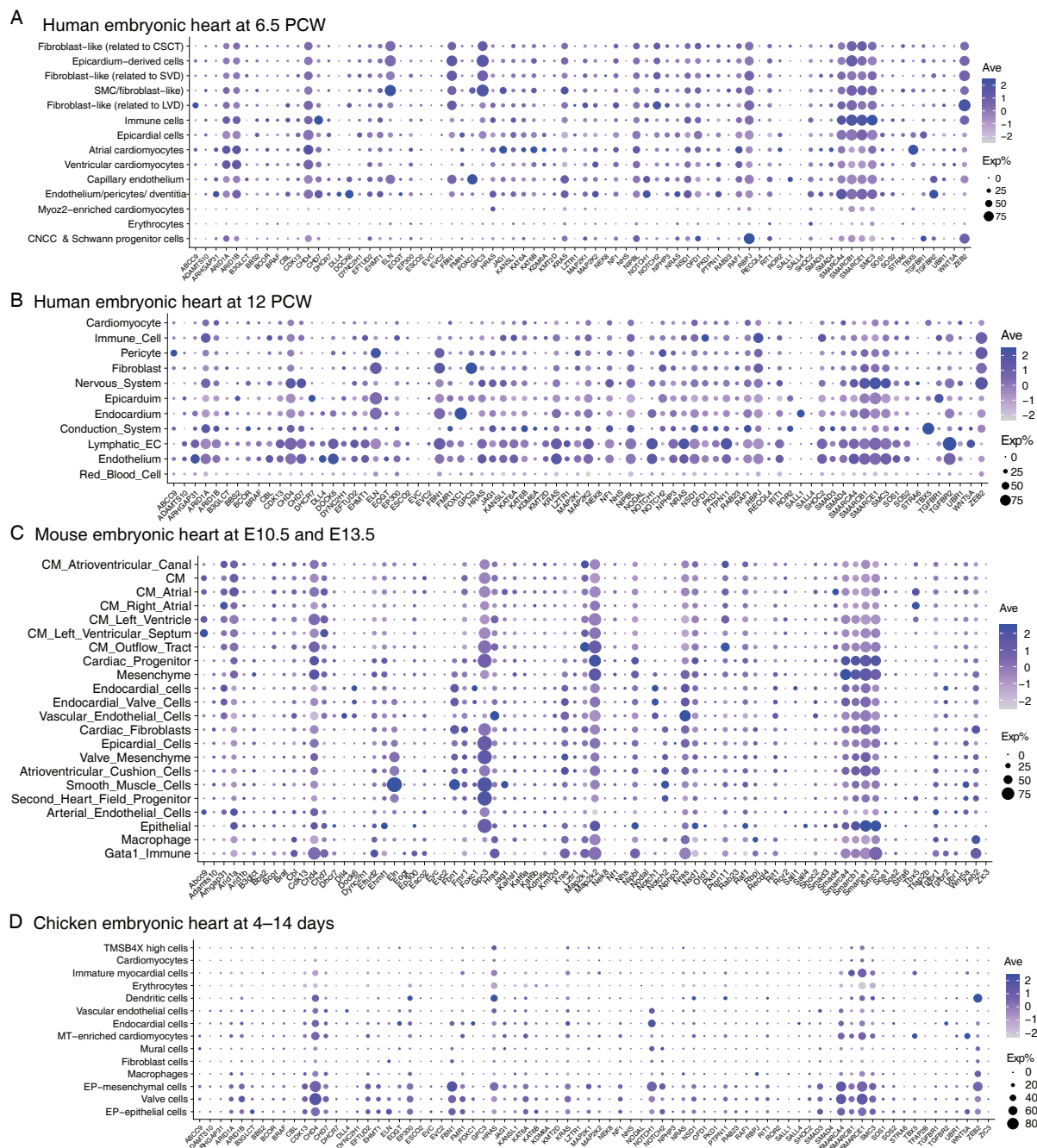
Figure 2 Cardiac cellular expression of genes causing syndromes with CHD phenotype in developing chicken, mouse, and human heart

A–D: Data adapted from scRNA-seq of human embryonic heart at 6.5 (A) (Asp et al., 2019) and 12 (B) (Miao et al., 2020) postconceptional weeks (PCW) and mouse (Hill et al., 2019) (C) and chicken (Mantri et al., 2021) (D) embryonic heart. Size of dot indicates percentage of cells expressing that gene within a cluster (Exp%). Gene list for syndromic CHD was summarized from previous reviews (Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018). CNCC: Cardiac neural crest cells. CSCT: Cardiac skeleton connective tissue. EP: Epicardial cells. EC: Endocardial cells. LVD: Larger vascular development. SVD: Smaller vascular development. CM: Cardiomyocytes.

Interestingly, certain genes causing syndromic CHD have also been found to cause isolated CHD. For example, missense and null mutations in *JAG1* encoding a ligand for Notch1 are responsible for Alagille syndrome, an autosomal-dominant multisystem disorder (Li et al., 1997; Oda et al., 1997), while frameshift and missense mutations in *JAG1* are associated with tetralogy of Fallot (TOF) or pulmonary stenosis without Alagille syndrome (Bauer et al., 2010). Disruption of *ELN*, a critical extracellular matrix component of vascular tissue, can cause Williams-Beuren Syndrome with cardiovascular and connective tissue abnormalities (Francke,

1999), while point mutations or small intragenic deletions in *ELN* have been identified in familial supravalvular aortic stenosis and other large artery stenoses without Williams-Beuren Syndrome (Hayano et al., 2019; Micale et al., 2010). *TFAP2B* is a transcription factor that leads to Char syndrome characterized by patent ductus arteriosus, dysmorphic facial features, and hand anomalies (Satoda et al., 2000). Splicing and small deletion mutations in the *TFAP2B* gene are also identified in patent ductus arteriosus without Char syndrome features (Chen et al., 2011; Khetyar et al., 2008). Mutations in the *TBX5* transcription factor can cause Holt-Oram syndrome

Table 2 Genes that cause isolated CHD

Gene	Cardiovascular phenotype	OMIM	Mis_z	pLI	References
Transcription factors and co-factors					
ANKRD1	TAPVR	609599	0.19	0.00	Fahed et al., 2013
CITED2	ASD; VSD	602937	−0.47	0.76	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
ZFPM2	TOF, DORV	603693	1.00	0.21	Fahed et al., 2013
GATA4	ASD, PS, VSD, TOF, AVSD, PAPVR	600576	0.67	0.49	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
GATA5	ASD, BAV, TOF, VSD, DORV	611496	1.07	0.28	Nees & Chung, 2020
GATA6	ASD, TOF, PS, AVSD, PDA, OFT defects, VSD	601656	1.28	1.00	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
HAND1	SV, VSD	602406	−0.28	0.11	Nees & Chung, 2020
HAND2	TOF	602407	1.18	0.40	Fahed et al., 2013; Nees & Chung, 2020
IRX4	VSD	606199	−0.20	0.01	Fahed et al., 2013
MED13L	TGA	608771	3.69	1.00	Nees & Chung, 2020; Pierpont et al., 2018
MEIS2	ASD, VSD, CoA	601740	2.46	1.00	Nees & Chung, 2020
MESP1	ASD, VSD, TOF, CoA, DORV, AA	608689	0.35	0.00	Lahm et al., 2013; Werner et al., 2016; Zhang et al., 2017
NR2F2	AVSD, AS, CoA, VSD, HLHS, TOF, DORV	107773	3.60	0.99	Nees & Chung, 2020; Pierpont et al., 2018
NKX2-5	ASD, VSD, TOF, HLH, CoA, TGA, DORV, IAA, OFT defects	600584	0.20	0.95	Nees & Chung, 2020; Pierpont et al., 2018
NKX2-6	PTA	611770	0.26	0.02	Nees & Chung, 2020; Pierpont et al., 2018
TBX1	TOF, (22q11 deletion syndromes)	602054	0.74	0.84	Fahed et al., 2013; Nees & Chung, 2020
TBX5	AVSD, ASD, VSD, (Holt Oram syndrome)	601620	1.19	1.00	Fahed et al., 2013; Nees & Chung, 2020
TBX20	ASD, MS, VSD	606061	1.68	0.97	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
TFAP2B	PDA, (Char syndrome)	601601	1.29	0.99	Fahed et al., 2013
ZIC3	TGA, PS, DORV, TAPVR, ASD, HLH, VSD, Dextrocardia, L-R axis defects	300265	2.52	0.92	Fahed et al., 2013
Receptors, ligands, and signaling					
ACVR1	AVSD	102576	0.33	2.34	Nees & Chung, 2020; Pierpont et al., 2018
ACVR2B	PS, DORV, TGA, Dextrocardia,	602730	2.04	0.83	Fahed et al., 2013
ALDH1A2	TOF	603687	1.44	0.36	Fahed et al., 2013
CFC1	TOF; TGA; AVSD; ASD; VSD; IAA; DORV	605194	0.11	−0.22	Fahed et al., 2013
CRELD1	ASD; AVSD	607170	0.17	0.00	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
FOXH1	TOF, TGA	603621	−2.55	0.04	Fahed et al., 2013
GDF1	Heterotaxy, TOF, TGA, DORV	602880	1.69	0.41	Fahed et al., 2013
GJA1	ASD, HLH, TAPVR, (Oculodentodigital dysplasia)	121014	1.28	0.16	Nees & Chung, 2020; Pierpont et al., 2018
JAG1	PAS, TOF, (Alagille syndrome)	601920	3.25	1.00	Nees & Chung, 2020; Pierpont et al., 2018
LEFTY2	TGA, AVSD, IAA, CoA, L-R axis defects, IVC defects	601877	0.58	0.00	Fahed et al., 2013
NODAL	TGA, PA, TOF, DORV, Dextrocardia, IVC defect, TAPVR, AVSD	601265	0.97	0.97	Fahed et al., 2013; Nees & Chung, 2020
NOTCH1	BAV, AS, CoA, HLH	190198	3.45	1.00	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
PDGFRA	TAPVR	173490	1.94	1.00	Nees & Chung, 2020; Pierpont et al., 2018
SMAD2	HTX, DORV, ASD, VSD, PDA	601366	3.66	1.00	Nees & Chung, 2020
SMAD6	BAV, CoA, AS	602931	−0.59	0.00	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
TAB2	OFT defects	605101	1.61	1.00	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
TDGF1	TOF, VSD	187395	−0.06	0.00	Fahed et al., 2013
VEGFA	CoA, OFT defects	192240	0.00	0.47	Fahed et al., 2013
Structural proteins					
ACTC1	ASD	102540	4.52	0.74	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
DCHS1	MVP	603057	2.40	1.00	Pierpont et al., 2018
ELN	SVAS, PAS, PS, AS, (Williams-Beuren syndrome)	130160	0.05	0.00	Nees & Chung, 2020; Pierpont et al., 2018
MYH11	PDA, Aortic Aneurysm	160745	1.44	0.77	Nees & Chung, 2020; Pierpont et al., 2018
MYH6	ASD, TA, AS, PFO, TGA	160710	0.86	0.00	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
MYH7	Ebstein Anomaly, ASD, NVM	160760	3.93	0.00	Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018
MYBPC3	ASD, PDA, VSD, MR	600958	1.45	0.00	Nees & Chung, 2020

Phenotypes in parentheses denote syndromes or extracardiac manifestations associated with gene mutations. Mis_z: Z score indicating gene intolerance to missense variation. pLI: Score indicating gene intolerance to a loss-of-function variation. OMIM: Online Mendelian Inheritance in Man. Miz_z and pLI score were downloaded from the Genome Aggregation Database.

with cardiac and limb anomalies but are also observed in isolated CHD (Smemo et al., 2012).

Unlike the broad expression profiles of genes linked to syndromic CHD, genes causing isolated CHD are often more restricted and are associated with specific cardiac cell types (Figure 3). Four (*ACTC1*, *MYH6*, *MYH7*, and *MYBPC3*) out of

seven genes associated with isolated CHD encode structural proteins expressed in cardiomyocytes, while *DCHS1*, which causes mitral prolapse, is enriched in the endocardium and *ELN*, which causes aortic defects, is enriched in endocardial cells and cardiac fibroblasts. In addition, seven (36.8%) out of 19 genes encoding transcription factors (*CITED2*, *GATA4*,
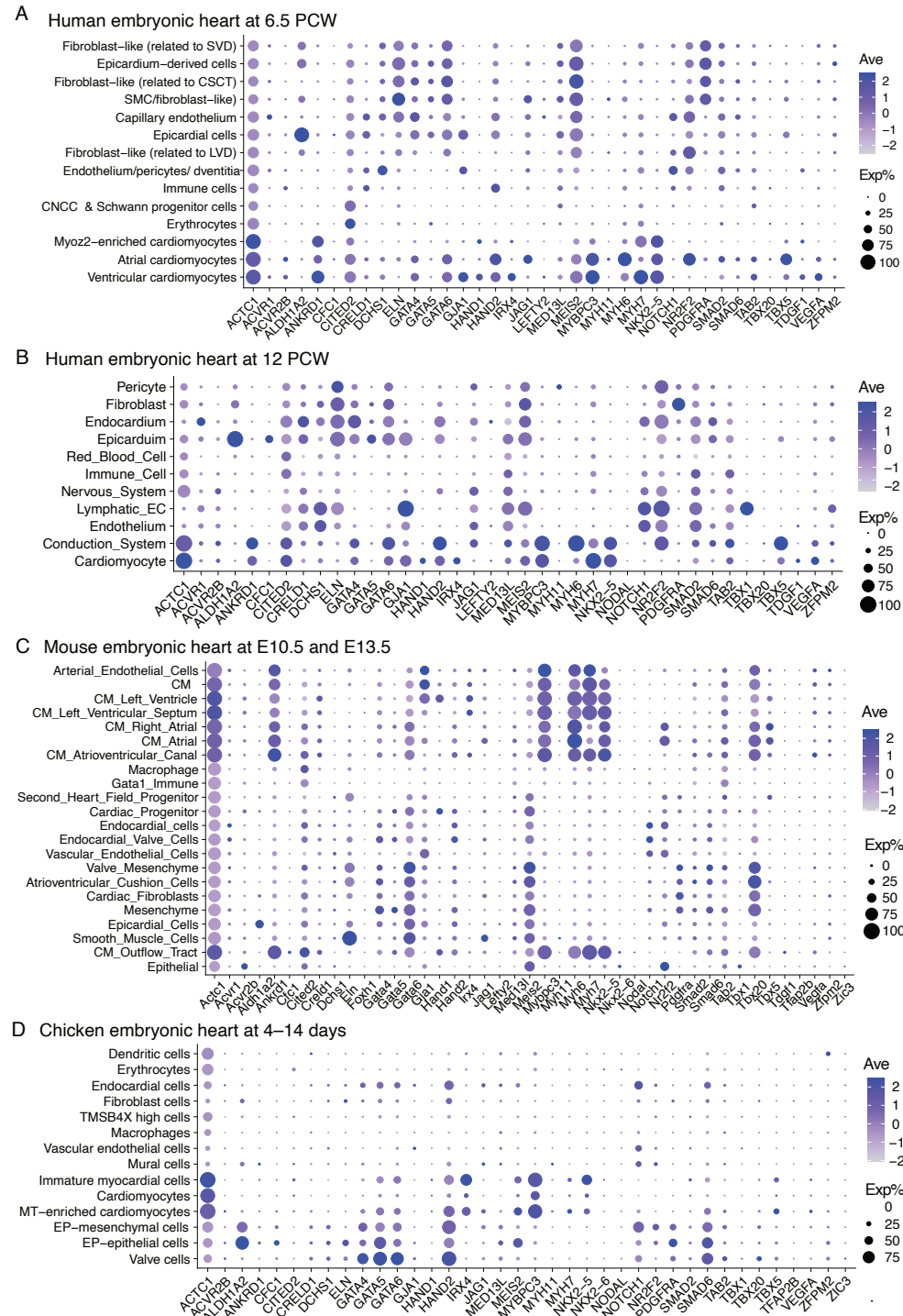
Figure 3 Cardiac cellular expression of genes causing isolated CHD in developing chicken, mouse, and human heart

A–D: Data adapted from scRNA-seq of human embryonic heart at 6.5 (A) (Asp et al., 2019) and 12 (B) (Miao et al., 2020) PCW and mouse (Hill et al., 2019) (C) and chicken (Mantri et al., 2021) (D) embryonic heart. Size of dot indicates percentage of cells expressing that gene within a cluster (Exp%). Gene list for isolated CHD was summarized from previous reviews (Fahed et al., 2013; Nees & Chung, 2020; Pierpont et al., 2018). CNCC: Cardiac neural crest cells. CSCT: Cardiac skeleton connective tissue. EP: Epicardial cells. EC: Endocardial cells. LVD: Larger vascular development. SVD: Smaller vascular development. CM: Cardiomyocytes.

GATA6, *HAND2*, *NR2F2*, *NKX2-5*, and *TBX5*), and three (16.7%) out of 18 genes related to receptor signaling (*ALDH1A2*, *TAB2*, and *VEGFA*) are expressed in cardiomyocytes in the human embryonic heart. *PDGFRA* is expressed in fibroblasts from the heart OFT, while *PDGFRA* mutations in humans and mice can also lead to inflow tract anomalies (Bleyl et al., 2010), as well as outflow tract CHD in knockout mouse models (Aghajanian et al., 2017).

De novo mutations causing CHD

Due to the complexity and heterogeneity of CHD, nearly 60% of cases remain of unknown etiology. Human genetic studies using large-size CHD probands from PCGC-obtained WES data revealed that *de novo* coding variants (DNVs) in several hundred genes contribute to approximately 8% of CHD cases, including 3% of isolated CHD subjects, 28% with both neurodevelopmental and extracardiac congenital anomalies, and 10% of severe CHD cases (Homsy et al., 2015; Jin et al., 2017; Zaidi et al., 2013). In addition, predicted functional noncoding DNVs may account for a similar fraction of CHD cases via disruption of transcriptional and post-transcriptional regulation of cardiac development (Richter et al., 2020), although the genetic mechanism needs further investigation (Richter et al., 2020). Coding DNVs linked to CHD are enriched in syndromic CHD patients, while inherited protein-altering variants are enriched in nonsyndromic CHD patients. *De novo* CNVs may account for only a small fraction of CHD cases (Soemedi et al., 2012; Zaidi et al., 2013).

Genes associated with histone modifications and chromatin remodeling make up 30% of *de novo* mutations related to CHD (Diab et al., 2021; Ohtani & Dimmeler, 2011; Wang et al., 2022; Zaidi et al., 2013), associated with a wide spectrum of CHD phenotypes, including left ventricular outflow obstruction (LVOTO), conotruncal defects, and heterotaxy (Homsy et al., 2015; Jin et al., 2017; Zaidi et al., 2013). Previous analysis of mice with mutations in *Sap130*, a constituent of the histone deacetylase complex, revealed that mutation in this chromatin modifier caused left ventricular hypoplasia (Liu et al., 2017; Yagi et al., 2018) and hypoplastic left heart syndrome (HLHS). Thus, mutant mice with double homozygous *Pcdha9* and *Sap130* mutations exhibit HLHS. Mutations in *Pcdha9*, encoding a protocadherin cell adhesion protein, can cause isolated BAV when present alone, but in combination with *Sap130* mutation can cause aortic hypoplasia/atresia associated with HLHS, as seen in double *Sap130/Pcdha9* mutant mice with HLHS (Liu et al., 2017).

Mice lacking *HDAC1* or *HDAC2* encoding histone deacetylases result in cardiac defects (Montgomery et al., 2007), while mouse embryos lacking *HDAC3* show an increase in transforming growth factor-β1 (TGF-β1) bioavailability with outflow tract abnormalities, including double-outlet right ventricle (DORV), BAV, VSD, and embryonic lethality (Lewandowski et al., 2015). Furthermore, defects in *Smarcd3* encoding Baf60c, a subunit of the chromatin remodeling complex, can also cause outflow tract remodeling defects reminiscent of human CHD (Lickert et al., 2004). These findings in humans and mice suggest that DNVs, especially DNVs in chromatin-modifying proteins, may play an important role in human CHD. Further functional validation using modeling of predicted CHD pathogenic DNVs using CRISPR gene-edited mice should provide the necessary experimental validation of their role in human CHD pathogenesis. Such animal models can also help elucidate the cellular and molecular mechanisms contributing to the developmental etiology of CHD.

Cilia-related CHD genes

Cilia are hair-like, microtubule-based cellular organelles that play an essential role in many cellular processes, as well as cardiovascular development and CHD pathogenesis. Cilia are broadly involved in CHD pathogenesis, including mediation of second heart field cell migration into the outflow tract, outflow tract alignment and septation through regulation of the planar cell polarity (PCP) pathway (Gibbs et al., 2016), and endocardial-mesenchymal transition (EMT) in valvular morphogenesis (Toomer et al., 2019). Moreover, many cilia-transduced cell signaling pathways play important roles in cardiovascular development, such as Shh, Tgfb, Wnt, and Pdgf signaling. Cilia function in mechanosensation, cardiac fibrosis, and heart regeneration (Djenoune et al., 2022; Gabriel et al., 2021), and play an important role in the regulation of left-right patterning required for establishing left-right asymmetry of the cardiovascular system (Caspary et al., 2007; McGrath et al., 2003) (Nakhleh et al., 2012) (Figure 4). Unsurprisingly, certain genes identified in CHD are required for normal left-right patterning. As this asymmetry is essential for establishing normal systemic-pulmonary circulation for oxygenation of blood, complex and lethal CHD cases are often those associated with mutations causing disturbance in laterality (Gabriel & Lo, 2020; Gabriel et al., 2021; Li et al., 2015).

The first evidence for the importance of cilia in CHD pathogenesis came from large-scale forward genetic screening of chemically mutagenized mice (Li et al., 2015). As mice with CHD invariably die before birth or neonatally, the use of fetal ultrasound imaging for screening has made it possible to recover mutations causing CHD. A previous study involving echocardiography and ultrasound scanning of 87 355 chemically mutagenized C57BL/6J fetal mice, identified 218 CHD mouse models displaying a wide spectrum of phenotypes, similar to those observed clinically in human patients (Li et al., 2015). Further WES study of 113 mutant lines with severe CHD identified 91 recessive CHD mutations in 61 genes, including 34 cilia-related genes and 16 cilia signaling-related genes, indicating the central importance of cilia-related genes in CHD pathogenesis (Figure 4). Further investigations utilizing WES data have provided additional evidence supporting the involvement of cilia-related genes in CHD pathogenesis. For example, a WES study of 249 TGA patients showed enrichment in ciliary genes harboring rare, potentially damaging variants (Liu et al., 2020). WES studies conducted on the PCGC cohort also demonstrated that cilia and cilia-related genes are significantly enriched in rare and damaging recessive mutations identified in CHD, including in patients with laterality defects, such as heterotaxy and dextro-transposition of the great arteries (d-TGA) (Jin et al., 2017; Watkins et al., 2019). Of note, many cilia-related genes causing CHD are also associated with various ciliopathies, a broad spectrum of recessive disorders involving mutations in cilia-related genes. With increasing access to human sequencing data, many cilia-related genes have been implicated in human CHD, but the function of cilia in heart development and CHD pathogenesis still require further elucidation based on cellular and molecular studies.

Most cilia-related genes causing CHD are broadly expressed at low levels in all cardiac cells, except for
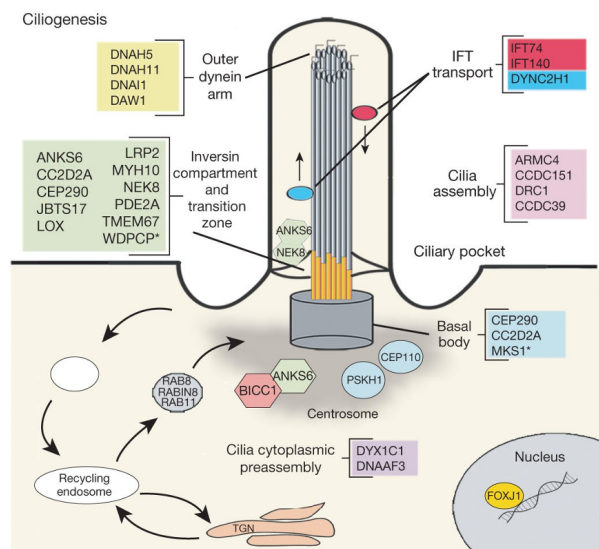
Figure 4 CHD genes in cilia recovered from mouse mutagenesis screening

Diagram of biological context of ciliary genes in vesicular and endocytic trafficking. AP, adaptor protein complex; MVB, multivesicular body; Ub, ubiquitination. Adopted with permission from Li et al. (2015).

C21orf59, which is enriched in endocardial cells and fibroblasts, and *DCHS1*, *NPHP3*, and *RNF20*, which are specifically expressed in the endocardium (Figure 5). Certain genes, such as *GALNT11*, *MKKS*, and *PKD2*, also exhibit higher expression broadly. This broad expression of cilia-related genes in different cell types in the heart is consistent with the expectation that most cells have a primary cilium, except at the time of mitosis. Interestingly, compared to other cardiac cell types, cilia-related genes have much higher expression in the endocardium, consistent with the role of cilia in regulating EMT, a process essential for endocardial cushion mesenchyme formation by endocardial EMT. Consistent with this, previous studies have shown that numerous ciliary proteins are highly expressed in the endocardial cushion, with about 60% of patients with ciliopathies and mutations in cilia-related genes displaying defects in the endocardial cushion (Koefoed et al., 2014; Sund et al., 2009).

Role of genetic modifiers in cardiac disease and CHD pathogenesis

The incomplete penetrance and variable expressivity of CHD suggest that genetic modifiers may play an important role in CHD pathogenesis. Large-scale next-generation sequencing data from humans (Boycott et al., 2013) have provided evidence for incomplete penetrance, such that not all individuals carrying a known pathogenic mutation will manifest the disease. For example, a study of 13 adults with pathogenic mutations known to cause severe Mendelian disorders reported no clinical manifestations of disease (Chen et al., 2016). These 13 individuals, identified from genomic analysis of 589 306 individuals, possessed homozygous (autosomal recessive disease) or heterozygous (autosomal dominant disease) mutations known to cause severe Mendelian diseases before the age of 18, and yet showed no sign of disease, suggesting a protective genetic mechanism involving genetic modifiers that may allow these subjects to survive disease free. Obtaining experimental evidence to support the existence of such genetic suppressors impacting human disease penetrance is challenging and requires animal models

to verify the impact of genetic suppressors on disease phenotypes. Two notable studies have provided recent evidence from animal modeling, confirming the existence of variants exerting genetic modifier effects on the heritability and penetrance of human cardiac disease. One study showed that a genetic modifier may act as a suppressor to rescue otherwise dominant lethal mutations (Teekakirikul et al., 2022), while the other study showed that a genetic modifier may exacerbate mutations that only exert borderline deleterious effects to cause disease (Gifford et al., 2019).

Protective variant suppresses dominant lethal mutation causing ASD

Recently, a *TLN2* variant was identified to mediate heritable transmission of an embryonic lethal mutation in *TPM1*, a sarcomeric actin-binding protein required for cardiac muscle contractility (Teekakirikul et al., 2022). This *TPM1* mutation showed dominant inheritance linked to a large ASD in eight individuals of a five-generation pedigree. It is extremely rare, observed in only three other individuals worldwide, based on the ClinVar database (Teekakirikul et al., 2022). Mouse and frog modeling showed that this *TPM1* mutation disrupts cardiac contractile function, causing heartbeat initiation failure and early lethality in mouse embryos at embryonic day 8.5 (E8.5). This explains the extreme rarity of this mutation in the human population. Nevertheless, the propagation of this mutation in eight individuals over five generations would suggest the existence of a closely linked protective variant that may suppress the deleterious effects of the dominant lethal *TPM1* mutation. This was confirmed with the discovery of a second mutation in *TLN2*, another myofilament actin-binding protein in the same chromosome interval as the *TPM1* mutation. Double CRISPR knockin of the *Tpm1/Tln2* mutations allowed mice to survive with a normal heartbeat and a large ASD similar to that observed in the patients (Teekakirikul et al., 2022) (Figure 6). Collectively, these findings provide a paradigm in which protective variants can increase genetic resilience by allowing the propagation of otherwise embryonically lethal mutations. These findings also highlight a previously unknown role of *TPM1* mutation in the large ASDs.

Genetic modifier promoting left ventricular noncompaction (LVNC) cardiomyopathy

Another recent study using animal modeling showed that genetic modifiers can also increase disease penetrance by promoting the deleterious effects of variants that otherwise may not cause disease on their own. A *NKX2.5* mutation was identified as a genetic modifier that can promote childhood-onset LVNC (Gifford et al., 2019) from missense mutations in *MKL2* and *MYH7*. Notably, a child with LVNC inherited *MYH7* and *MKL2* variants from their affected asymptomatic father as well as a rare *NKX2-5* variant from their unaffected mother (Gifford et al., 2019). Functional experiments using CRISPR gene-edited mouse models found that the triple-heterozygous *Mkl2/Myh7/Nkx2-5* mutant mice exhibited LVNC, with deep trabeculations in the left ventricular wall. In contrast, mice with single or double mutations in the three genes exhibited no or only subtle cardiac defects. Based on analysis of different mouse models, the *NKX2-5* variant acted as a genetic modifier to promote LVNC from the *MKL2/MYH7* missense mutations (Gifford et al., 2019).

Role of common variants in pathogenesis of LVOTO-CHD

As the cause of most CHD cases remains unexplained, common variants have been suggested to contribute to the

Figure 5 Cardiac cellular expression of genes causing syndromes with CHD phenotype

A–C:Data adapted from scRNA-seq of human embryonic heart at 6.5 (A) (Asp et al., 2019) and 12 (B) (Miao et al., 2020) PCW and chicken (Mantri et al., 2021) (C) embryonic heart. Size of dot indicates percentage of cells expressing that gene within a cluster (Exp%). List of reported cilia-related genes linked to human CHD was obtained from a previous review (Djenoune et al., 2022). CNCC: Cardiac neural crest cells. CSCT: Cardiac skeleton connective tissue. EP: Epicardial cells. EC: Endocardial cells. LVD: Larger vascular development. SVD: Smaller vascular development.

genetic etiology of CHD. While genome-wide association studies (GWAS) have identified common variants associated with specific types of CHD, these only account for a small proportion of cases (Cordell et al., 2013a, 2013b; Jiang et al., 2018; Nees & Chung, 2020; Teekakirikul et al., 2021). Whether these are susceptibility loci or truly causative of disease needs to be further investigated. Exploring the potential role of common variants in human disease is inherently challenging, as such variants are unlikely to be under strong selection and may have more subtle effects on phenotypes. Nevertheless, a recent study revealed a common deletion copy number variant (delCNV) in the protocadherin *PCDHA* gene cluster that may contribute to human CHD, comprising left ventricle and left ventricular outflow tract defects, collectively referred to as LVOTO lesions.

HLHS mutant mice exhibit a digenic etiology for HLHS, comprising mutations in *Pcdha9* and *Sap130* (Liu et al., 2017). *Pcdha9* alone can cause BAV, one of the most common CHD types seen in the white adult population (1%–3%) (Siu & Silversides, 2010). A common delCNV that spans *PCDHA9*

has been reported in the human genome, suggesting a possible link between *PCDHA* delCNV and BAV (Teekakirikul et al., 2021). The prevalence of the 16.8 kb *PCDHA* delCNV is most common in Europeans (6%–11%), less common in Africans/African Americans (5%–7%), and least common in Southeast Asians (0%–3%) (Noonan et al., 2003; Teekakirikul et al., 2021), similar to the racial prevalence of BAV (Chandra et al., 2012; Li et al., 2017). A case-control association study identified two common delCNVs (16.8 kb and 13.6 kb delCNV) in the *PCDHA* gene cluster spanning *PCDHA8* and *PCDHA9* and significantly associated with BAV, HLHS, and other LVOTO-CHD (Teekakirikul et al., 2021) (Figure 7) in white, Chinese, and black LVOTO patients. Overall, these findings are consistent with those obtained in the HLHS mouse model (Liu et al., 2017). Immunostaining showed PCDHA protein expression in the developing aorta, both in aortic media and cushions, which was markedly reduced in *Pcdha9* mutant mouse embryos (Teekakirikul et al., 2021). Unlike common variants identified in GWAS, mouse model findings suggest that the *PCDHA* delCNVs are genetic causes of BAV and

Figure 6 Rescue of *Tpm1* embryonic lethality and recapitulation of ASD in *Tpm1/Tln2* double-KI mice

Adopted with permission from Teekakirikul et al. (2022). A: Significant rescue of heartbeat was observed in *Tpm1/Tln2* double-KI versus *Tpm1* KI mice. *n*: Number of embryos analyzed. Data were analyzed using Fisher's exact test. B: Representative histopathology and immunofluorescent staining of E16.5 *Tpm1/Tln2* double-KI verse wild-type (WT) mouse hearts. Upper left panel shows normal atrial septum in WT mouse embryo, while upper right shows a large ASD in a CRISPR double-*Tpm1/Tln2*-KI embryo. Arrowhead points to atrial septum in WT embryo, which is not observed in mutant double-KI embryo. Immunofluorescent staining of sections shows TPM1 and α-actinin expression in sarcomeric structures in hearts of WT (left) and *Tpm1/Tln2*-double-KI (right) embryos. LV, left ventricle; RV, right ventricle. C: Quantification shows decreased α-actinin/TPM1 ratio in heart tissue of *Tpm1/Tln2*-double-KI (*n*=3) vs. WT embryos (*n*=3). Data shown as mean±standard error of the mean (*SEM*) were analyzed using unpaired Student's *t*-test. D: Efficiency of double *Tpm1/Tln2* KI in E16.5–17.5 embryos with or without ASD. Note, ASD was only observed in embryos with effective *Tpm1/Tln2* double KI. *n*: Number of embryos analyzed with or without ASD phenotype. E: Diagram showing *TPM1* and *TLN2* in cardiac muscle and potential impact of deleterious K5del *TPM1* mutation and its buffering by *TLN2* variant.

LVOTO phenotypes, not merely susceptibility loci. These results further demonstrate the value of leveraging animal models for functional modeling and investigations to elucidate the genetic mechanisms of CHD.

CONCLUSIONS AND FUTURE PERSPECTIVES

As one of the most common birth defects, CHD remains a leading cause of newborn morbidity and mortality. It is characterized by complex genetics, further complicated by incomplete penetrance and variable expressivity. While studies investigating the genetic etiology of CHD have focused on rare pathogenic mutations, the roles of common variants and variants that may exert protective effects warrant further investigation to provide a more complete picture of the complex genetics of CHD. Despite the challenges posed by CHD genetics research, the integration of multiomics data and CRISPR gene-editing techniques for rapid animal model production should help verify the role of patient-recovered
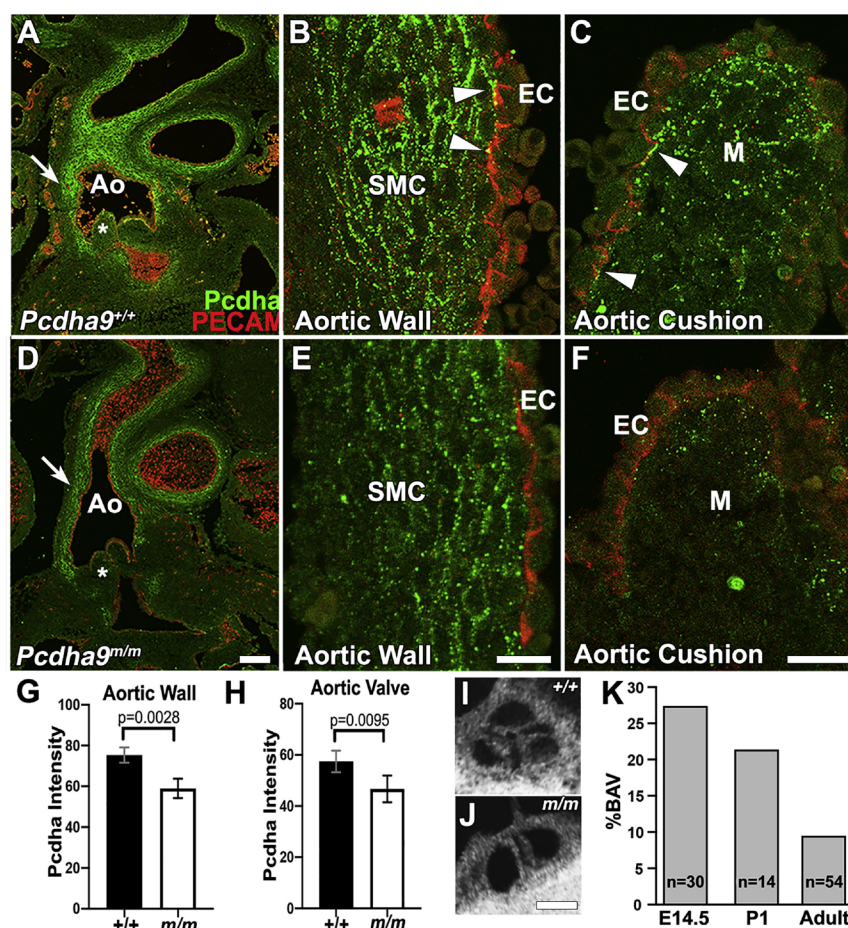
Figure 7 PCDHA protein expression in *Pcdha9* mutant mice and knockdown of *Pcdha9* in mice

Adopted with permission from Teekakirikul et al. (2021). A–C: Immunostaining and confocal imaging show PCDHA protein expression in WT E14.5 mouse embryo aorta (A), with magnified views showing expression in smooth muscle cells (SMCs) on intimal side (B), and in mesenchyme (M) of aortic cushion. C: Occasional staining was also observed at the interface between endothelial (PECAM positive) and underlying SMCs. Scale bar, 25 mm. D–F: Immunostaining and confocal imaging of PCDHA protein expression in E14.5 CRISPR-targeted *Pcdha9$^{m/m}$* mutant embryo showed reduced immunostaining, indicating reduced PCDHA protein expression in aorta (D). Magnified views show reduced immunostaining in both aorta wall (E) and aortic cushion (F). Scale bar, 25 mm. G, H: Quantification of staining intensity showed reduced PCDHA protein expression in both aorta wall (G) and aortic cushion (H) of *Pcdha9$^{m/m}$* embryos. Values are mean of 5 SD with statistical analysis conducted using *t*-test. I, J: Episcopic confocal microscopy images of tricuspid aortic valve at P1 (I) and BAV at P1 (J). K: Percentage of *Pcdha9* mutant animals found to have BAV phenotype at E14.5, P1, and adult.

sequence variants in CHD. In addition, the use of patient-derived induced pluripotent stem cells for differentiation into cardiac cell lineages and 3D organoid culture modeling should provide new avenues for rapid disease modeling, especially for selected CHD phenotypes, such as those involving defects in valvular morphogenesis. The integration of multiomics data and animal modeling should provide valuable insights into the genetic basis of CHD, enabling accurate and early diagnosis. Such research should also facilitate the identification of new drug targets for the development of therapies that may improve the long-term outcomes for patients with CHD.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.Z. and C.W.L. conceived the project and jointly wrote the manuscript. All authors read and approved the final version of the manuscript.

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