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## Genetics and Genomics of Congenital Heart Disease

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### Abstract

Congenital heart disease is the most common birth defect, and due to major advances in medical and surgical management, there are now more adults living with CHD than children. Until recently, the cause of the majority of CHD was unknown. Advances in genomic technologies have discovered the genetic etiology of a significant fraction of CHD, while at the same time pointing to remarkable complexity in CHD genetics. This review will focus on the evidence for genetic causes underlying CHD and discuss data supporting both monogenic and complex genetic mechanisms underlying CHD. The discoveries from CHD genetic studies draw attention to biological pathways that simultaneously open the door to a better understanding of cardiac development, and impact clinical care of CHD patients. Finally, we address clinical genetic evaluation of patients and families affected by CHD.

### Keywords

Congenital Heart Disease; Genetics, diagnostics; Genetics; genome

### CHD epidemiology: evidence for genetics underlying CHD

Congenital heart disease (CHD) is a structural abnormality of the heart and/or great vessels that is present at birth<sup>1</sup>. It is the most common birth defect, affecting approximately 1% of all liveborn infants<sup>2</sup>. CHD results from perturbation of the normal program of cardiac development (Fig. 1A). Historically, CHD has been categorized based on a combination of final anatomic and physiologic phenotypes (Fig. 1B), such as conotruncal defects that affect the ventricular septum and outflow tract (CTD), defects that lead to obstruction to left ventricular outflow (LVO), defects resulting from abnormal left-right relationships within the heart (heterotaxy, HTX), defects affecting the inflow such as the mitral and tricuspid valve abnormalities seen in atrioventricular canal defect, and a broad range of other defects

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including isolated atrial or ventricular septal defects<sup>3</sup>. Approximately one third of patients with CHD have disease that is categorized as severe (comprising univentricular hearts, HTX, CTD, atrioventricular canal defects, total anomalous pulmonary venous return, left ventricular outflow obstruction, and right ventricular outflow obstruction except isolated valvar pulmonary stenosis) and require intervention in the first year of life<sup>4</sup>. Despite progress in medical and surgical treatments, CHD remains the leading cause of mortality from birth defects in the developed world. Further, among the world's poorest populations, CHD has a greater contribution to cardiovascular disease associated disability-adjusted life-years than ischemic heart disease or stroke<sup>5</sup>.

The natural history of severe congenital heart disease was altered dramatically by the performance of the first systemic to pulmonary artery shunt procedure by Helen Taussig, Vivien Thomas and Alfred Blalock<sup>6</sup>. Since then, an almost universally lethal condition has become progressively more approachable through a combination of surgical, catheter-based and medical interventions. In the modern era, patients in developed countries undergoing CHD surgery, including those with complex CHD, have 10-year survival exceeding 80%<sup>1</sup>. This has resulted in an ever-increasing population of adults who are living with CHD, and there are now more people over the age of 18 with CHD than children with CHD<sup>7</sup>.

While hemodynamic management of CHD has improved dramatically, many patients with CHD have significant cardiac and extracardiac co-morbidities that impact their quality of life. Patients with repaired or palliated CHD are at risk for developing arrhythmias and myocardial dysfunction; in addition, 13.6% have associated extracardiac structural malformations, compared to 7% in the control population. Potentially the largest impact on quality of life in patients with CHD is from associated neurodevelopmental disabilities (NDD); the prevalence of NDD in the CHD population ranges from 10% in patients with mild CHD to over 50% in patients with severe CHD who require surgery during infancy<sup>8</sup>.

The underlying causes of CHD remains relatively poorly understood, and although it has long been thought to have both genetic and environmental contributions, the epidemiology of CHD points to genetics contributing to the majority of CHD. The overall incidence of CHD has been very stable at 0.8–1.1% of live births, with small changes in CHD incidence attributable to improved diagnostic methods such as increased detection of small septal defects via echocardiography<sup>9, 10</sup>. Although there is little evidence of temporal or geographic variation in overall incidence to suggest an environmental trigger, there are small differences in the types of CHD observed in different populations, such as the increased number of LVO lesions amongst white children compared to an increase in RV obstruction amongst Chinese children, which suggests population-specific genetic contributions<sup>2, 11</sup>.

Evidence supporting the genetic contribution to CHD can be gleaned from a number of sources. There is greater concordance of CHD in monozygotic than dizygotic twins<sup>12, 13</sup>, although there is evidence that twinning itself increases risk of CHD<sup>14, 15</sup>. The risk of recurrence of related forms of CHD among siblings is elevated, ranging from 3.4 for ASDs to 79.1 for heterotaxy in the Danish national cohort study<sup>16</sup>; there is a smaller, but still significantly increased risk recurrence for discordant CHD<sup>17</sup>. Additionally, rare Mendelian forms of CHD, comprising a small fraction of all cases, been described. These include forms

of ASD<sup>18</sup>, HTX<sup>19</sup>, severe mitral valve prolapse<sup>20, 21</sup>, and Bicuspid Aortic Valve<sup>22</sup>. The increased incidence of CHD in populations with high levels of consanguinity suggests a role for recessive genetic contributions<sup>23</sup>. However, it is striking that a large fraction of CHD, particularly of severely affected subjects, occurs in families with no other history of CHD. This suggests the possibility that a significant fraction of these cases is attributable to *de novo* genetic events including chromosomal abnormalities, smaller copy number variants and point mutations. The severity of CHD in these instances is likely to impair reproductive fitness, limiting transmission of these large-effect mutations and accounting for the absence of extended pedigrees supporting dominant modes of transmission.

Collectively, these findings point to a major genetic contribution to CHD (Fig. 1C). This observational data does not allow insight into whether CHD in individual subjects is attributable to single loci with large effect, a few loci with epistatic or additive interactions, polygenic effects of many loci, or various combinations of these models together. Additionally, the possibility of gene-environment interaction is an important consideration. The aggregate of genetic contributions to CHD are likely to not only underlie the structural CHD, but also be major contributors to CHD co-morbidities including heart failure, arrhythmia, neurocognitive outcomes, and even to the observation that cancer rates are higher in adults with CHD<sup>24</sup>. As CHD contributes to an ever-increasing amount of the overall burden of cardiovascular disease<sup>25</sup>, a thorough understanding of the underlying genetics will become ever more important to improved care of patients with CHD.

## Established genetic contributions to CHD

### Aneuploidy

Aneuploidies were the earliest identified genetic causes of CHD. Estimates of the proportion of CHD associated with cytogenetic abnormalities range from 9–18%<sup>26</sup>. The large number of genes that are dysregulated in the setting of aneuploidy results in effects on development that are often pleiotropic and severe, and 98% of fetuses with CHD and cytogenetic abnormalities have at least one extracardiac abnormality<sup>27</sup>. CHD is observed in 35–50% of liveborns with trisomy 21, 60–80% of liveborns with trisomy 13 and trisomy 18, and 33% with monosomy X. Further, there is a large effect on overall viability, as evidenced by the 33–42% incidence of aneuploidy amongst fetuses with prenatally diagnosed CHD, compared to 9–18% amongst neonates with CHD<sup>27</sup>. The types of CHD associated with specific aneuploidies covers a broad range of CHD phenotypes, although there are lesions that are more prominently associated with specific chromosomal abnormalities, such as atrioventricular septal defects in trisomy 21. The large numbers of genes with dosage disturbance in aneuploidy make it more challenging to pinpoint the underlying genetic and developmental mechanisms. However, insights have been gleaned from studies of patients with rare segmental trisomies affecting chromosome 21 suggesting that *DSCAM* and *COL6A* contribute to Down Syndrome-associated CHD<sup>28</sup>. Interestingly, overexpression of both *DSCAM* and *COL6A* in mice leads to heart abnormalities, while overexpression of either gene alone does not affect heart development<sup>29</sup>.

## Copy number variation

Copy number variation (CNV) refers to structural aberrations consisting of deletions or duplications ranging in size from 1 kb to several megabases and leading to altered dosage of genes encompassed by the CNV. Low-copy repeats and retrotransposons found throughout the genome form the substrate for CNV formation<sup>30</sup>. CNVs can occur *de-novo*, or be inherited. Millions of SNPs (Single Nucleotide Polymorphisms), each typically with population allele frequency >1%, can be simultaneously genotyped at very low cost through dense array-based platforms. This technology permits identification of regions of genome duplication and deletion in both coding and non-coding regions of the genome. More recently, CNVs have also been detected through whole exome and genome sequencing data. Comparison of dense array-based platforms with whole-exome sequencing showed that each strategy only identified ~70% of the CNVs that should have been detected,<sup>31</sup> and therefore together may provide substantial complementary information.

Several well-characterized large CNVs underlie recognized clinical syndromes that include CHD. Del22q11, a deletion of ~3mB resulting from flanking low-copy repeats, is the most common human microdeletion. It presents with a variable phenotype encompassing congenital heart disease, palate abnormalities, hypocalcemia, immunodeficiency, characteristic facial features, and neurodevelopmental abnormalities including learning disabilities and psychiatric disorders, also known as DiGeorge Syndrome and Velocardio-facial syndrome. Del22q11 includes the T-Box transcription factor *TBX1*, and haploinsufficiency for *TBX1* underlies the cardio-pharyngeal phenotype<sup>32-34</sup>. Recent work in mice haploinsufficient for *Tbx1* delineates regulation of H3K4me1 enrichment, providing an intriguing link between *TBX1* and chromatin remodeling in CHD<sup>35</sup>. Other CHD-associated CNVs that are well characterized include del8p23, which includes the cardiac transcription factor *GATA4* and manifests with a range of CHD along with developmental delay<sup>36</sup>; del7q11, the cause of William Syndrome, wherein the cardiac disease consists of supravalvar aortic and pulmonary stenosis and results from haploinsufficiency for Elastin<sup>37, 38</sup>; del 11q24-25 resulting in Jacobsen Syndrome<sup>39, 40</sup>. Recent analyses of larger cohorts of patients with CHD found several recurrent CNVs associated with CHD, including 1q21.1, 3p25.1, 16p13.11, 15q11.2 and 2p13.3<sup>31, 41</sup>.

Beyond specific syndromes associated with CNVs, their global contribution to CHD has been investigated in several large cohorts of patients with specific CHD: Tetralogy of Fallot<sup>41</sup>, Heterotaxy<sup>42</sup> and Hypoplastic Left Heart<sup>43-45</sup>, all of which show an overrepresentation of rare CNVs, and *de-novo* CNVs, in patients with CHD compared to controls<sup>46</sup>. An increased burden of CNVs was also detected in non-syndromic patients with mild-to-moderate severity CHD<sup>47</sup>. The availability of chromosomal microarray testing as a standard clinical test has increased the awareness of the contribution of CNVs to congenital heart disease, and clinical and research-based testing suggests that CNVs contribute to 10–15% of CHD<sup>48</sup>. As previously noted, given the inherent limitations of most commonly used platforms for CNV detection for optimal sensitivity, the contribution of CNVs to disease phenotypes may underestimate their contribution<sup>31</sup>.

## Inherited point mutations: Mendelian and inherited CHD

Remarkable insights into Mendelian and inherited forms of CHD have emerged from classic linkage analyses, positional cloning and targeted sequencing of CHD candidate genes. Many of the genes first implicated in inherited CHD are members of a core group of cardiac transcription factors that includes *NKX2.5*, the GATA family of zinc-finger proteins, T-box factors including *TBX5* and *TBX1* and MEF2 factors (reviewed in<sup>49-51</sup>). Mutations in *NKX2.5* were one of the first inherited point mutations clearly shown to cause human CHD. *NKX2.5* is a transcriptional regulator that interacts with *GATA4* to specify cardiac mesoderm, first identified in *Drosophila* mutants that had complete failure to form a heart tube<sup>52</sup>. Evaluation of large pedigrees that included individuals with isolated ASDs, and individuals with ASDs along with abnormalities of the conduction system subsequently identified *NKX2.5* mutation underlying both the ASD and conduction system defects. Notably, some affected individuals had the ASD alone, others had the conduction defect alone, and some had both the ASD and the conduction defect<sup>53</sup>. The phenotypic heterogeneity associated with *NKX2.5* mutations is remarkable, encompassing a wide range of CHD beyond ASDs, including HTX and Tetralogy of Fallot<sup>54</sup>. It is interesting to speculate whether the wide spectrum of CHD results from differences in genetic background, or interaction between an at-risk genotype and environmental influences that may include subtle variation in hemodynamics in-utero during critical times in cardiac development. *GATA4* is a zinc-finger transcription factor essential for cardiogenesis that directly associates with *NKX2.5*<sup>55</sup>. *GATA4* mutations were implicated in two families with CHD with cardiac septal defects<sup>56</sup>.

Mutations in *TBX5*, a T-box protein, were likewise implicated in two families with Holt-Oram Syndrome, a disease characterized by upper limb malformations and cardiac abnormalities (septation and conduction defects)<sup>57, 58</sup>. *TBX5* is notably expressed in both the developing forelimb buds and the heart, and similar to other T-box proteins, regulates cell fate and crucial developmental processes. Further evidence of causality has emerged from heterozygous *Tbx5* null mice displaying limb abnormalities, septal defects, deformed hearts, and other complex cardiac malformations<sup>59</sup>.

X-linked *ZIC3* mutations were identified in several multi-generational pedigrees, and were carried by family members with the mutation, some of whom by chance ended up with either complete *situs solitus* or complete *situs inversus* and functionally normal hearts<sup>19, 60, 61</sup>. *ZIC3* is a zinc-finger transcription factor that is required to form a functional left-right organizer<sup>62</sup> and is required to direct the directionality of heart looping. Since absence of left-right organizer function leads to random heart looping, these pedigrees show striking incomplete penetrance: some affected family members will have normal heart looping and appear phenotypically normal and transmit the disease allele, while others will have *situs inversus* or heterotaxy and complex CHD.

In addition to cardiac transcriptional regulators, genes coding for a variety of signaling molecules and cellular structural components have been identified in Mendelian inherited CHD. Dominantly inherited *NOTCH1* mutations were first described in 2 multigeneration pedigrees that included family members with bicuspid aortic valve with only hemodynamic impairment, while other family members have complex CHD including hypoplastic left

heart syndrome (HLHS)<sup>22</sup>. Since then, *NOTCH1* mutations have been found in additional CHD pedigrees<sup>63</sup>, and in ~5% of cases of one of the most common cardiac defects, bicuspid aortic valve, which is found in up to 2% of adults<sup>64</sup>. Similarly, mutations in *JAG1* were mapped to affected family members with Alagille syndrome, a multi-system disorder with diverse cardiac phenotypes. *JAG1* is one of the five ligands for receptors in the Notch signaling pathway, which results in localization of Notch to the nucleus and downstream activation of target genes. In addition, genes coding for the focal adhesion protein Tns1 (tensin 1) and the planar cell polarity protein Dchs1 (dachous1) were identified from several large pedigrees of severe mitral valve prolapse<sup>20, 21</sup>.

Less is known about the impact of recessive inheritance on CHD, although several lines of evidence support a recessive model contributing to some types of CHD. CHD is more prevalent in populations with a high degree of consanguinity<sup>23</sup>. Although there is an increase in all types of CHD in consanguineous populations, HTX and the associated complex cardiac malformations are observed at a higher frequency in consanguineous populations<sup>65</sup>. Pedigrees of consanguineous families with HTX identified recessively inherited mutations in genes including *SHROOM3*<sup>66</sup>(cytoskeletal protein), *WDR16*<sup>67</sup>(cilia-associated WD40 repeat-protein), and *MMP21*<sup>68</sup>(matrix metalloproteinase 21), and *NPHP4*<sup>69</sup> (nephronophthisis 4).

A different approach to understanding the recessive contribution to severe CHD was undertaken through an unbiased recessive mutagenesis screen in mouse<sup>70</sup>. Here, the offspring of ENU-mutagenized mice were intercrossed, and the pregnancies studied for any severe congenital heart disease by fetal ultrasound. This identified 61 genes contributing to recessively-inherited CHD. Several notable findings from this landmark study were that, of the 61 genes identified, 34 were cilia-related genes, several of which had previously been identified in human CHD. Further, cilia-related genes contributed to both HTX-type CHD, and CHD not associated with laterality defects. Together, the observations in a limited number of human pedigrees and in model organism suggest that recessive inheritance contributes to CHD, in particular to HTX-type (laterality) CHD.

## **Beyond large structural variation and Mendelian CHD: the impact of next generation sequencing on CHD genetics**

The explosion of technological approaches and analysis tools for next generation sequencing, which has occurred over almost a decade has opened the door for understanding the genetics of complex disease such as CHD. In particular, whole exome sequencing has allowed identification of mutations that were undefinable through traditional genomic methods, such as *de novo* variation, variants without clear Mendelian inheritance patterns, variants with marked reduced penetrance, and somatic alterations, among others.

### **Whole-exome sequencing**

The development of robust methods of whole-exome sequencing (WES) has created new opportunities for genomic discovery<sup>71, 72</sup>. The complete coding regions of the ~20,000 genes in the human genome plus their flanking splice sites comprises only ~33 Mb of DNA,

about 1% of the human genome sequence. Unbiased genetic discovery by positional cloning in humans, mice and fruit flies has demonstrated that the overwhelming majority of phenotypes caused by large-effect mutations are caused by coding sequence mutations. This has identified mutations in ~3,500 genes underlying known Mendelian phenotypes. The recognition that the 20,000 human genes are largely conserved across vertebrate phylogeny strongly suggests that mutation of most will lead to phenotypic consequences, although how many of these phenotypes will manifest as disease remains unknown. These observations motivated the development of methods for selective sequencing of this 1% of the genome, which now can be completed at about 20% of the cost of sequencing complete genomes, affording a very significant cost advantage and allowing large cohorts of patients with unexplained phenotypes to be sequenced. Sequential improvements have resulted in virtually complete detection of point mutations in the full coding region; challenges nonetheless remain in detecting certain CNVs and chromosomal translocations. WES can be used to identify genes that are mutated more often than expected by chance after accounting for sequencing 20,000 genes. This has allowed for the identification of novel disease genes for a range of disease phenotypes ranging from autism to congenital malformations to cancer through the analysis of transmitted, *de novo* and somatic mutations.

### Whole exome sequencing identifies *de novo* mutations in CHD

The first advance derived from applying next generation sequencing to the study of CHD was the discovery of the role of *de novo* mutation in CHD. Most CHD is sporadic: only 2.2% patients with CHD have affected first-degree relatives<sup>16</sup>. That sporadic disease such as CHD has stable incidence despite low reproductive potential suggests that new mutation occurs as existing mutations are lost due to impaired reproductive fitness, and suggests that *de novo* mutations underlie some CHD<sup>73</sup>. *De novo* mutations are on average more deleterious than inherited mutations, as there has been less evolutionary selection. These mutations occur at approximately  $1.8 \times 10^{-8}$ /nucleotide/generation, resulting in ~1 *de novo* mutations per coding-region (exome), and ~100–115 *de novo* mutations per genome<sup>74, 75</sup>. As spermatogenesis has many more germline cell divisions than oogenesis, this results in a 3.9:1 ratio of *de novo* mutations when comparing the paternal allele to the maternal allele<sup>76, 77</sup>. *De novo* mutations occur throughout the genome, but are not entirely randomly distributed. Factors that influence DNA mutation rate include high CpG density, segmental duplications, paternal age, and mutations conferring advantages during spermatogenesis.<sup>78</sup>

Since there is strong evidence for impaired reproductive fitness in a majority of CHD subjects, it is likely that *de novo* mutations confer a major contribution. This hypothesis has been tested in several studies that employed whole-exome sequencing of large cohorts of patient-parent trios affected by CHD. The first of these studies analyzed 362 trios with the patient affected by severe CHD. While the overall rate of *de novo* mutations was not significantly different between CHD cases and controls, there was a marked enrichment when stratifying for protein-altering *de novo* mutations in genes highly expressed in the developing heart (top quartile of gene expression in murine hearts at E9.5 and E14.5) in CHD cases. Further stratification by mutation type graded by stringency, from all protein-altering mutations to highly conserved missense and LOF mutations to LOF mutations alone, produced a significant rise in odds ratio. *De novo* mutations were found to

collectively contribute to 10% of severe CHD<sup>79</sup>. Moreover, an excess of *de novo* mutations was identified in chromatin remodeling genes that affected the reading, writing, and removal of two bivalent marks, H3K4 and H3K27 methylation, found at the promoters and enhancers of key developmental genes poised for activation<sup>79</sup>. Expanding the cohort size from 362 to 1,213 CHD trios comprising patients with the complete spectrum of CHD including less complicated CHD such as isolated atrial septal defects, in addition to moderate and severe CHD, reinforced the contribution of *de novo* mutations to ~10% of CHD. This cohort included patients with isolated CHD, CHD associated with known syndromes, and CHD with extracardiac malformations and/or neurodevelopmental abnormalities. More extensive phenotyping coupled with a larger patient cohort demonstrated that *de novo* mutations disproportionately contributed to CHD in patients with associated syndromes, extracardiac malformations and/or neurodevelopmental abnormalities. Notably, *de novo* mutations accounted for at least 20% of CHD with associated extracardiac and neurodevelopmental abnormalities<sup>75</sup>. While these mutations are more prominently associated with syndromic rather than non-syndromic CHD<sup>80</sup>, there is a small but measurable contribution to isolated CHD (CHD not associated with a known syndrome, and without any extracardiac malformations or neurodevelopmental abnormalities)<sup>81</sup>, which may become more clearly delineated when larger cohorts are analyzed<sup>8275</sup>.

## Biological pathways in CHD

The genetics underlying CHD have identified critical biological pathways involved in CHD including chromatin remodeling, Notch signaling, cilia function, sarcomere structure and function, and RAS signaling. These pathways are anticipated to provide direct insights into the mechanism of heart development, and provide insights into potential CHD co-morbidities such as ventricular dysfunction observed in the setting of sarcomere and RAS pathway mutations. Furthermore, identification of common developmental pathways shared between cardiac development and other systems, such as the nervous system in the setting of chromatin modifier mutations, and the respiratory system in the setting of cilia mutations, are anticipated to directly inform outcomes and prognosis for CHD patients. We will outline studies linking three of these pathways to CHD: Chromatin remodeling, Notch signaling and cilia genes.

## Chromatin modifiers and overlapping biology

As noted above, one of the most significant findings arising from the recent analysis of large cohorts of patients with CHD by whole-exome sequencing is the important role of mutations affecting chromatin regulating genes in CHD<sup>75, 79, 80</sup>. The first of these studies showed that *de-novo* mutations affecting chromatin regulating genes contribute to ~3% of CHD. This observation was reinforced by a larger analysis of 1,213 trios, noting LOF mutations in chromatin regulating genes in 25/1213 CHD cases, while only 3/900 controls ( $p=5.7 \times 10^{-11}$ ). All mutated genes with damaging mutations in cases and their affected chromatin marks are shown in Fig. 2a. Genes identified are involved in production, removal or reading of H3K4 methylation (H3K4me), H3K9 methylation (H3K9me), H3K27 methylation (H3K27me), H4K20 methylation (H4K20me), and ubiquitylation of H2BK120, which is required for H3K4 methylation<sup>79</sup>.



Chromatin regulating genes encompass ~ 600 genes that orchestrate dynamic gene expression during development by addition or removal of chemical marks on chromatin or by catalyzing changes in chromatin structure. The biological state of chromatin is controlled by ATP-dependent chromatin modifiers, including the Baf complex and Chd8, and by histone modifiers. Both have been linked to heart development; Baf60c regulates early heart development through cooperation with the *GATA4* transcription factor<sup>83</sup>. H3K4me and H3K27me constitute ‘bivalent’ marks that are found on the promoters and enhancers of key cardiac developmental genes poised for activation<sup>84</sup>. A significant burden of haploinsufficient (dominant) *de novo* mutations within these elements therefore indicate dosage sensitivity of the chromatin pathway in heart development. Although relatively rare, chromatin modifiers have been linked to isolated CHD such as the histone methyl transferase *PRDM6* which has been associated with non-syndromic Patent Ductus Arteriosus<sup>85</sup>.

The same control of gene expression required for normal cardiac development is also essential for brain development, and many chromatin regulating genes have been directly implicated in brain development, including members of the *BAF* complex, *CHD8*, *HDAC4* and polycomb group protein *EZH2*<sup>86</sup>. The overlap between specific chromatin regulators required for both heart and brain development remains unclear. Chromatin regulators are widely expressed, and mouse knockout frequently leads to very early lethality, precluding analysis of specific brain or heart phenotypes<sup>87</sup>. Heart-specific knockout of *Kmt2d*, a H3K4 methylase that is associated with Kabuki syndrome, results in very abnormal hearts with outflow tract septation defects in mice<sup>88</sup>. Mutations in chromatin modifying genes have been identified in patients with CHD have been associated with a range of syndromes, including Sotos Syndrome, Kabuki Syndrome, CHARGE and others (Fig. 2b). At the same time, genome sequencing studies in human neurodevelopmental and psychiatric disorders have identified mutations in chromatin modifying genes in Kleefstra, Schinzel-Giedion, Claes-Jensen, Weaver, Sotos, and Coffin-Siris syndromes among others. Although neurodevelopmental abnormalities are the most prominent feature, up to 50% of affected patients also have a CHD. These observations indicate that chromatin regulating mutations result in both cardiac and neurodevelopmental sequelae, and begin to shed light on potential developmental-genetic causes of the NDD associated with a subset of CHD patients.

### Notch pathway genes

Notch signaling is a highly conserved pathway mediating local intercellular communication that has important roles in a host of developmental processes that are very relevant to heart development, including formation of the left-right organizer<sup>89</sup>, blood vessel development<sup>90</sup> and ventricular chamber development<sup>91</sup>. Notch signaling provides a way for a Notch ligand from one cell to influence a directly neighboring cell via its Notch receptor (Fig. 3a) and determine cell fate. Upon binding of Notch ligand and receptor, signaling requires release of the Notch intracellular domain (NICD) via a coordinated series of tightly regulated steps including glycosylation of the receptor, ubiquitylation of the ligands by Mib1 and cleavage of the receptor triggered by Adam17/Tace. NICD can then function as a transcription factor regulating a large number of targets, including *SNAIL1*, *HES*, *HEY* and *NRARP*. Notch pathway genes implicated in CHD are outlined in Fig. 3b. This pathway was initially

implicated in CHD when *NOTCH1* mutations were identified in families with dominantly inherited bicuspid aortic valve and other left-ventricular outflow tract obstructive lesions<sup>22</sup>. Within a single family, *NOTCH* mutation associated with a range of CHD ranging from BAV to CoA to HLHS. Mutations affecting multiple components of Notch signaling (*NOTCH1*, *MAMLI*, *JAG1*) were significantly enriched in 51 families with multiple family members affected by a range of LVO-type CHD<sup>63</sup>. *NOTCH1* function does not appear to be restricted to left-sided heart development, as additional family studies identified high-impact *NOTCH1* mutations in families with both LVO-type CHD and TOF<sup>63</sup>.

Further, *NOTCH1* and Notch ligand *DLL4* mutations are the most common cause of Adams-Oliver Syndrome, a rare syndrome comprising CHD, aplasia cutis of the scalp and limb defects. Alagille syndrome (Arteriohepatic Dysplasia) is an autosomal dominant inherited syndrome characterized by cholestatic liver disease, variable degrees of kidney involvement and CHD that is most commonly TOF. Mutations in the Notch ligand *JAG1* are found in ~90% of patients with Alagille Syndrome<sup>92, 93</sup>, and mutations in the *NOTCH2* receptor are found in another 2% of individuals with Alagille Syndrome<sup>94</sup>. Similar to mutations in other Notch pathway members, *JAG1* mutations underlie both syndromic and isolated CHD, most notably TOF<sup>95</sup>. Finally, *GALNT11*, which is required for the S2 cleavage step of Notch receptor processing, has been linked to human HTX by affecting Notch-mediated specification of cilia function at the left-right organizer<sup>42, 89</sup>.

### Cilia genes

Mutations affecting cilia structure and function have been identified in patients with CHD, and notably, cilia mutations were the major class of mutations found in a recessive mouse screen for severe CHD<sup>70</sup>. Cilia are hair-like organelles found on the surface of most vertebrate cell types and serve a multitude of functions, including signaling, extracellular fluid propulsion and cell cycle control (Fig. 4a). Defects affecting cilia structure and/or function have been intimately linked to a group of diverse human disorders characterized by pleiotropic phenotypes including renal, neurological, sensory and laterality defects coined 'ciliopathies'. In heart development, the best understood role for cilia is establishing left-right (LR) asymmetry and determining the direction of heart looping. Here, a highly conserved ciliated left right organizer (LRO) utilizes cilia to generate and sense directional flow of extraembryonic fluid and transduce it in a polycystin-dependent manner to a calcium signal<sup>96-99</sup>. This triggers asymmetric gene expression in the lateral plate mesoderm, eventually leading to asymmetric heart looping. Due to the role of cilia in determining LR patterning, mutations affecting ciliary motility<sup>100</sup> and sensing<sup>101</sup> machinery result in HTX and CHD.

In mice, mutations in components of the dynein motor complex, such as left-right dynein (*Dnah11/Lrd*) and dynein heavy chain 5 (*Dnah5*), result in cardiac and visceral LR abnormalities<sup>102, 103</sup>. Not surprisingly, 6.5% of patients with PCD (primary ciliary dyskinesia), a disorder defined by abnormal ciliary motility in the airway epithelia, also display heterotaxy<sup>100</sup>. PCD is genetically highly heterogeneous, and there are currently 35 genes that have been linked to PCD. It remains unknown how many of the PCD genes cause CHD, since a diagnosis of PCD in patients with CHD is made more challenging, due to the

difficulty differentiating whether respiratory symptoms are primary, or secondary to the underlying cardiac pathology and the medical and surgical interventions required to manage the CHD. Other cilia genes that are not required for cilia motility and left-right development, but instead are involved in ciliogenesis or cilia-mediated sensation are also associated with CHD (Fig. 4b). It is interesting to speculate that cilia found in the developing heart and vasculature have a function in cardiac morphogenesis extending beyond their role in LR development<sup>104, 105</sup>, and that similar to mouse, cilia defects may underlie a broader range of human CHD than suspected to date.

## Future Efforts in CHD Genomics

### State of the art understanding of CHD genetics

The genetic basis of CHD has now been established in 1 out of 3 affected cases (Fig. 1 C). These comprise a broad array of genetic alterations in a large, heterogeneous group of genes. Earliest insights arose from aneuploidies in CHD. Likewise, there is a growing catalogue of CNVs in CHD, typified by well characterized deletions, such as del22q11. Given the large number of genes involved in aneuploidies and CNVs, identification of specific disease associated genes is challenging. In addition, inherited forms of CHD have been identified through traditional genetic tools, such as linkage. These linkage studies have ranged from mutations in cardiac transcription factors, such as *NKX2.5*, and *GATA4* to signaling molecules and cellular structural components, such as *NOTCH1* and *JAG1*. Through critical advances in next generation sequencing, our understanding of CHD biology has expanded rapidly over the past decade. Seminal studies have found that 10% of CHD is due to *de novo* mutations, which increases to greater than 20% when stratifying for CHD with associated EM or NDD. Indeed, certain biological pathways, including chromatin modification genes, cilia genes and the Notch signaling pathway, have been implicated in CHD, raising the possibility that environmental perturbations might phenocopy the effects of these mutations. Despite these significant advances, the genetic underpinnings of over 50% of CHD remain unknown. Barriers to a complete understanding of CHD genetics include the extreme genetic heterogeneity coupled with limited genotype-phenotype correlation. Some of the “unexplained CHD cases” could be due to mutations affecting the as of yet underexplored non-coding DNA, somatic mutations and gene-environment interactions as discussed below. In addition, CHD due to biallelic mutations has been underexplored, and thus far has largely focused on candidate gene analysis and familial CHD<sup>101</sup>. As new statistical approaches are coupled with larger CHD cohorts, the inherent challenges in an unbiased analysis of recessive mutations in sporadic CHD can be surmounted.

Most CHD is sporadic, with no affected family members. Beyond the 20% of sporadic CHD caused by *de-novo* CNVs and SNVs, it is likely that some CHD will be secondary to complex inheritance, wherein for example, a heterozygous mutation requires a modifier mutation, or the absence of a protective variant, in order to manifest as disease. This is supported by incomplete or non-penetrance in extended families carrying mutations in well-characterized CHD genes including *ZIC3* and *NOTCH1*<sup>19, 61, 106</sup>. Animal models provide an avenue for testing the complex trait hypothesis in a more controlled genetic environment, and for example the susceptibility to VSDs in mice heterozygous for mutation in the cardiac

transcription factor *Nkx2.5* was modified by loci on mouse chromosomes 6, 8 and 10<sup>107</sup>. Another example is provided by the observation that introduction of a null allele for the VEGF-A pathway gene *CRELD1* into a mouse model of Down Syndrome raises the incidence of AVSDs<sup>108</sup>, which supports prior observations on human Down Syndrome cohorts<sup>109</sup>. Correspondingly, several large GWAS studies in patients with CHD also identified possible loci influencing the susceptibility to CHD<sup>110, 111</sup>. In addition, it is also quite likely that since heart development appears to be highly dosage sensitive, some CHD may result from convergence of hypomorphic mutations in several components of a single pathway to exceed a threshold and manifest as disease. This model has previously been shown in a mouse model of congenital heart disease where the penetrance of the CHD phenotype is increased in mice compound heterozygous for *Zic3*<sup>(+/-)</sup>; *Nodal*<sup>(+/-)</sup><sup>112</sup>. As available gene- and protein-level interactome databases become more robust and comprehensive, it may very well become possible to link CHD genomics data with genetic interactome databases to better explore multigenic CHD.

### Exhausting the Coding Region: Number of Genes Contributing to CHD

Analysis of *de novo* mutations has illuminated the immense genetic heterogeneity underlying CHD pathogenesis. Recent work analyzing *de novo* mutations in 1,213 CHD subjects showed that ~392 genes, *albeit* with wide confidence intervals, collectively contribute to CHD. This estimation of the number of risk genes was performed using a maximum likelihood function, details of the simulation and derivation are noted in Homsy et al. and Iossifov et al.<sup>75, 113</sup>. Based on this function in 1,213 CHD cases, 392 genes were estimated to contribute to CHD<sup>75</sup>. An approach to identify a greater fraction of the CHD risk genes is to identify additional genes with more than one *de-novo* mutation in CHD patients. In a cohort double the size (2,426 trios), power simulations approximate 61 genes with more than one damaging mutation (~40 new genes in the additional 1,213 trios and 21 previously identified in the original 1,213 trios). To identify all 392 genes would require a significant increase in the number of CHD trios analyzed. Furthermore, recent work suggests that WES of 10,000 trios would permit ~80% saturation for detecting genes contributing to haploinsufficient syndromic CHD alone<sup>80</sup>; it is likely that a significantly larger number of trios will have to be analyzed to approach a complete gene set for *all* CHD. As sequencing continues to become faster and less expensive, it is anticipated that large-scale collaboration of CHD genetics programs such as the Pediatric Cardiac Genomics Consortium (PCGC)<sup>114</sup>, Pediatric Heart Network (PHN)<sup>115</sup>, and the UK10K consortium<sup>116</sup> could allow for capture of the estimated ~392 CHD risk genes, and make a previously daunting task achievable.

### Contribution of Somatic Mosaicism in CHD

The wealth of sequencing data at high coverage has prompted the search for genetic mosaicism. Genetic mosaicism is defined as the presence of having multiple populations of genetically distinct cells within an individual. Mosaic *de novo* variants have been shown to contribute up to 20% of sporadic cases in a number of developmental disorders, including Sturge Weber syndrome<sup>117</sup>, facioscapulohumeral muscular dystrophy<sup>118</sup>, and segmental neurofibromatosis<sup>119</sup>. There have also been clinical reports suggesting pathogenic mosaic CNVs in patients with CHD<sup>120</sup>. Small studies using array comparative genomic hybridization (aCGH) have not identified any CNVs with differential presence between

cardiac tissue and peripheral whole blood <sup>121</sup>. A recent study identified an excess of extreme allele-specific expression events in cardiac tissue from CHD patients compared to controls, and since only 15% of the ASE events were explained by genomic variants, it is possible that some of these were secondary to mosaicism <sup>122</sup>. However, such studies are limited due to sample size, lack of developmentally relevant cardiac tissues, and imperfect statistical tools to detect mosaic variation. Larger cohorts of sequencing data, continued developmental of analysis tools, and ascertainment of cardiac tissues could help in identification of mosaic mutations, such as (1) *de novo* mutations with mosaic tissue distributions with involvement of cardiac tissues or precursors that would directly influence heart development, and (2) parental mosaicism where the unaffected parents of an affected offspring with CHD harbors mutation in the germline and any somatic tissue not involved in cardiac developmental, such that the mutation is constitutively transmitted to the affected offspring.

### Environmental Phenocopies

Considering the overrepresentation of mutations in certain pathways, such as chromatin modifying genes, in which dosage sensitive mutations confer CHD, it is possible that environmental triggers phenocopy the effects of these mutations. Many environmental exposures have been studied through observational and epidemiological studies in CHD <sup>123</sup>. A large Canadian population study showed a modest association between folic acid supplementation and reduction in several subtypes of CHD <sup>124</sup>; of note is that a maternal *MTHFR* polymorphism contributes to the CHD risk pointing to gene-environment interaction in the development of CHD <sup>125</sup>. The best documented environmental exposure that contributes to CHD is maternal pre-pregnancy diabetes which leads to an adjusted relative risk for CHD of 4.0 <sup>126</sup>. The effect is independent of whether the mother is affected by type 1 or type 2 diabetes, and studies of the NOD (non-obese diabetic) mouse show that CHD in offspring correlates with elevated glucose during embryogenesis <sup>127</sup>. Especially in view of the increasing rates of type 2 diabetes in the younger population, this represents an important contributor to CHD; for example, maternal diabetes is thought to contribute to 6–8% of HLHS and TOF <sup>128</sup>. The potential for gene-environment interactions highlight the continued need to catalogue environmental exposures within a cohort which also has corresponding DNA sequencing data. This should further expand our understanding of the genetic and non-genetic mechanisms of CHD, and moreover tell us how these two etiologies may converge.

### Contribution of Non-Coding Mutations

The prominent role of transcriptional regulation in CHD predicts that mutations affecting regulatory elements will contribute to CHD. For example, homozygous variation in a *TBX5* enhancer was found in a patient with isolated septal defects <sup>129</sup>. An important obstacle of detecting non-coding mutations in CHD is to delineate cardiac-specific regulatory elements and promoters at appropriate developmental time-points. Projects, such as ENCODE and the Cardiovascular Genomic Consortium, continue to build these datasets, and thus may be helpful in identifying rare *de novo* events in these non-coding elements. Other sources of WGS discovery could focus on *cis*-acting regulatory sequences, allelic selective gene expression in regulatory elements, and identification of epistatic and modifying mutations in diseases with known coding mutations, but with poor penetrance. An example of the latter

has been shown in another developmental disorder, craniosynostosis, where rare *SMAD6* LOF mutations modified by a common variant in *BMP2* resulted in complete penetrance of this disease<sup>130</sup>. Exploration of the non-coding DNA will require whole genome sequencing (WGS), which provides the most comprehensive view of the genome. Beyond complete determination of mutations outside the coding region, WGS provides more complete coverage of the exome and leads to improved detection of exonic CNVs and translocations. The potential challenges of WGS include greater expense, larger amounts of acquired and stored data, and the greater challenge of interpreting sequence variation in non-coding DNA. At present, evidence that WGS comes close to WES in *efficiency* of discovery of rare mutations with large effect remains limited<sup>131</sup>. Moreover, the 10-fold lower conservation of enhancer sequences indicates a much lower power to find disease-related mutations and adds to the challenge. One study of patients with severe intellectual disability, however, identified a conclusive cause in 42% of patients by WGS, compared to 27% by WES; it is notable that many of the mutations identified by WGS in this study actually affected the exome<sup>132</sup>. This limitation of WES is progressively being overcome by improved capture technologies that generate progressively more complete coverage of all exonic sequences<sup>133</sup>. The genomic technologies applied to CHD gene discovery and CHD patient diagnosis are rapidly evolving. Currently, WGS is likely to contribute to understanding the genetic cause of CHD, but will be most effective when applied in patients without WES evidence of damaging *de novo* mutations or likely pathogenic dominant and recessive mutations.

## Clinical impact of CHD genetics

For CHD genetics to become part of standard care for CHD patients, there are a few essential considerations. First, testing should be broadly available, and specific testing (chromosomal microarray, karyotype, targeted sequencing, exome sequencing or genome sequencing) should be tailored to the specific patient's case with regard to type of CHD and presence or absence of extracardiac abnormalities. Second, the results of genetic testing for CHD should be actionable, i.e. impact management and hopefully contribute to improved outcome. Third, when a genetic cause for CHD can be identified, it becomes possible to provide much more specific information regarding recurrence risk in other family members; this will become increasingly important as many more patients with CHD reach reproductive age in the context of the growing population of adults with CHD. The extreme heterogeneity and variable expressivity of CHD make it difficult to directly link specific genes to specific outcomes. However, evidence is mounting that defects affecting specific gene ontologies and pathways predispose patients to defined groups of potential complications. These risks may be defined by the genetic defect, in addition to the specific anatomic-physiologic cardiac defect. We will focus on two outcomes associated with CHD for which there is mounting evidence that the genetic cause of the CHD is a major contributor: neurodevelopmental abnormalities, and surgical outcome focusing on respiratory complications. Other comorbidities such as renal and myocardial dysfunction may also be influenced by genetic findings contributing to the CHD<sup>134</sup>.

## Neurodevelopmental outcomes

One of the most impactful associations with CHD are neurodevelopmental abnormalities. They affect 10% of all patients with CHD, and 50% of patients with severe CHD<sup>135</sup>. The spectrum of associated neurodevelopment abnormalities includes intellectual disability, language deficits, autism spectrum, executive function deficits, deficits in non-verbal skills including motor skills and social cognition. Attention-deficit hyperactivity disorder is also observed at a prevalence of up to 3–4 times higher than the general population<sup>135, 136</sup>. Although the incidence of NDD is increased in the setting of complex CHD and CHD in the setting of known genetic syndromes, the underlying causes remain poorly defined. Investigation of risk factors for CHD-associated NDD has focused on the role of complications of cardiopulmonary bypass, effects of abnormal physiology preceding repair (including during fetal life), and complications of hospitalization including prolonged requirement for intensive care; it is likely that these factors interrelate with the genetic substrate<sup>137</sup>. Thus far, no dominant major contributor to the NDD associated with CHD has been identified, and it remains difficult to identify at-risk children prospectively. While some studies show a modest correlation with type of CHD and length of deep hypothermic arrest<sup>138</sup>, the most striking predictor of poor neurodevelopmental outcome at age two was preoperative microcephaly. Similarly, preoperative brain MRI has demonstrated white-matter abnormalities in 32% of newborns with D-Transposition of the Great Arteries or single ventricle, compared to none of the control infants<sup>139</sup>; both of these observations support the association of CHD and abnormal brain development independent of surgical management.

Although recent recommendations include developmental evaluation in a subset of high-risk patients with CHD, ND risk-stratification for CHD remains difficult. Poor neurodevelopmental outcomes are much more prevalent in CHD patients with a diagnosed genetic syndrome, and a link between genetic factors and weight growth and head circumference has been identified<sup>140</sup>. Genetic analysis of patients of 1,213 CHD patients revealed that *de novo* risk increases when stratifying for CHD cases with NDD and/or extracardiac manifestations (EM). Specifically, 10% of patients with CHD and NDD were found to be attributable to damaging *de novo* mutations, which increased to 20% when looking at patients with *both* NDD and EM. These mutations tended to occur in genes that were highly expressed in both the heart and the brain. Furthermore, within this set of cases, there were 66 genes, which were mutated in both CHD probands and seven published cohorts ascertained for neurodevelopmental phenotypes. These findings were suggestive of common genetic etiologies. Common pathways converged on chromatin modification, transcriptional regulations, Notch and Wnt Signaling, among other pathways in cardiac development.<sup>75</sup> These findings open an avenue to identification of CHD patients who are at risk for neurodevelopmental difficulties early during their clinical course. Several studies show that early interventions impact neurodevelopmental outcomes such as executive function in at-risk children<sup>141</sup>. Whether these or other interventions could also benefit children with CHD who are at high risk for neurodevelopmental sequelae still needs to be rigorously tested.

## Postoperative and Respiratory Outcomes

Surgical correction or palliation of even the most complex CHD has been one of the main drivers of the remarkable increase in survival for patients with CHD. One of the challenging aspects of caring for these patients is the variable outcome resulting from surgery for anatomically and physiologically identical CHD. Two of the most significant modulators of post-operative outcome that may be influenced by the genetic underpinnings of the CHD are respiratory complications and myocardial dysfunction, and the ability to identify at-risk patients preoperatively may allow improved surgical and post-operative care that is better tailored to the individual patient. Mutations in cilia genes are known to cause heterotaxy, and are also likely contributing to some types of non-heterotaxy CHD<sup>70, 100</sup>. In addition to CHD, cilia mutations cause PCD (Primary Ciliary Dyskinesia), a genetically heterogeneous disorder leading to pulmonary dysfunction, male infertility, and organ laterality defects<sup>142, 143</sup>. In the respiratory tract, PCD can result in immotile or dyskinetic cilia that fail to coordinate mucociliary clearance of pathogens and debris from the respiratory tract. Poor mucociliary clearance leads to infection and inflammation that damage the airway, and it is especially important to note that patients with ciliary dysfunction depend entirely on cough for mucociliary clearance, a function that is compromised in patients on mechanical ventilatory support such as post-operative CHD patients. With this in mind, it is not surprising that patients with airway ciliary dysfunction and heterotaxy with CHD have a higher rate of respiratory complication post-operatively compared with patients without airway ciliary dysfunction<sup>144</sup>; these findings suggest that prospective knowledge of which patients have airway ciliary dysfunction could improve postoperative outcome by tailored modifications to their respiratory care.

## Genetic testing in CHD

The utility of genetic information in tailoring care for the CHD patient, risk stratification, establishing prognosis, and counseling families affected by CHD is continually expanding as more is learned about the genetic contribution to CHD. Genetic testing is being offered to an increasing portion of CHD families, however, at this time there is little consensus on the type of testing, the specific clinical indication for testing, and the interpretation of testing results. Increasingly, specialty clinics in CHD genetics where testing can be ordered, and patient counseling provided by geneticists and genetic counselors working together with pediatric cardiologists are being offered to patients<sup>145</sup>. Below, we outline CHD genetic testing available on a clinical basis at the time of this writing; notably, the technology for genetic testing along with the interpretation of results is evolving at an extremely rapid pace, and it is distinctly possible that whole-exome or whole-genome sequencing will become more universally employed in the diagnosis and management of CHD in the very near future.

The most common clinical genetic tests utilized in CHD are karyotypes, chromosomal microarray, targeted FISH (Fluorescence in-situ hybridization), directed panel sequencing, and more recently whole-exome sequencing. The decision on which type of testing is appropriate depends on the clinical presentation. Karyotyping is commonly the first line of testing used. In studies of CHD patients admitted to a cardiac intensive care unit, or who had surgery in the first year of life, karyotyping yielded a diagnosis in 10.5%–23% of patients<sup>146, 147</sup>. The majority of the positive results from karyotyping were either Down



Syndrome or Turner Syndrome, both of which are often clinically recognized. Genome-wide microarray provides information on deletions and duplications including common CNVs associated with CHD such as del22q11 (DiGeorge Syndrome) and del7q11 (William Syndrome). Microarray has yielded a diagnostic result in 10–25% of patients tested, with an additional 8% of patients carrying a variant of unknown significance. Targeted FISH (fluorescent in-situ hybridization), most commonly focusing on the 22q11 deletion, identifies the presence or absence of the specific target at lower cost and slightly more rapid turnover than genome-wide microarray, and had a positive diagnostic rate of 12%. Whether targeted FISH is positive correlated most directly with the specific cardiac lesion being tested, with 25–50% of patients with interrupted aortic arch, pulmonary atresia with ventricular septal defect or truncus arteriosus having a positive 22q11 FISH<sup>147</sup>. It is important to note that the abnormalities detected by targeted FISH will also be detected by genome-wide microarray, and at least one study suggests that except in specific clinical scenarios, microarray testing is more cost effective<sup>146</sup>.

Finally, sequencing is increasingly being utilized to identify genetic causes of CHD at the clinical level. Targeted sequencing is being offered for panels of CHD candidate genes. Two studies using similar sets of 57 genes previously implicated in CHD to test a group of patients from non-syndromic CHD families with probable dominantly inherited CHD identified likely causative mutations in 25–46% of the families<sup>148, 149</sup>. This approach relies on variants segregating with disease within a family, and thus becomes more difficult in non-familial CHD, first because the composition of the currently utilized CHD gene sets in the targeted sequencing approach are biased towards inherited CHD, and second because variant interpretation in an isolated case is more challenging. When there is strong clinical suspicion for CHD associated with a syndrome that has known genetic cause, targeted sequencing of a gene or group of genes associated with that syndrome is indicated, and one study finds that targeted sequencing that is driven by clinical evaluation identified the etiology of CHD in 17% of the cases<sup>147</sup>. In specific syndromes that have been well characterized at the molecular level, such as Noonan Syndrome or Marfan Syndrome, the diagnostic yield can be as high as 80% (Noonan Syndrome) to 90% (Marfan Syndrome). Finally, whole-exome or even whole-genome sequencing, which have been extensively utilized in management of oncology patients, are increasingly being offered as a clinical tool in the care of CHD patients. Although these methods are more expensive and time-consuming than targeted sequencing, they are unbiased and allow re-analysis when additional clinical or genetic information come to light. Whole-exome sequencing has been successfully utilized clinically for almost a decade<sup>72</sup>, and is now becoming part of genetic testing for CHD<sup>150, 151</sup>. It provides information for the entire coding region of the genome at a cost of only 2–3 times the cost of targeted panels. The current clinical approach to WES in CHD is to obtain the data for the entire exome, and then interrogate gene sets that are driven by the clinical phenotype. If there are new pieces of clinical information obtained during the patient's course, it is possible to re-interrogate and look for mutations in a different set of genes. Further, if new genes are identified as possible etiologies for the patient's phenotype, it is possible to obtain patient data for those genes without reobtaining a sample and repeating sequencing. Because of the large amounts of data obtained by WES, the most challenging aspect of this methodology is identifying whether rare variants that are

biologically plausible, but have not previously been linked to disease (variants of unknown significance, VUS), are actually causal. Since *de-novo* mutations have been associated with CHD, interpretation of clinical WES results is greatly helped by obtaining parental samples; if a variant is not identified in either phenotypically normal parent, the likelihood that it is contributing to the patient's CHD is much more likely.

Genetic testing for CHD is increasingly becoming part of standard care, especially for severe CHD requiring intervention, and for CHD associated with extracardiac abnormalities. Specific testing should be strongly guided by the cardiac and extracardiac phenotype; a proposed strategy for clinical genetic testing in CHD is outlined in Figure 5. Phenotyping and family history are exceptionally important in this scenario, as they inform the type of testing, and will help guide the genetic testing laboratory. Equally important will be careful adjudication of those variants that are not known disease-causing variants, but variants of unknown significance (VUS). The first step here is to test whether the variant is *de-novo*, which greatly raises the likelihood that the variant is disease causing. Additional information on the likelihood that a given variant is disease-causing is provided by measures of evolutionary conservation of the position at which the variant is occurring, and by the biological impact of the resulting amino acid substitution. Computational algorithms that integrate sequence and functional parameters provide indices of whether a given missense variant is predicted to be damaging or benign are also able to add information on the likely pathogenicity of a sequence variant (Meta-SVM, Polyphen-2, SIFT, MutationTaster, among others) <sup>152</sup>. As more and increasingly complex technologies reveal an expanding array of genomic variation, criteria for interpretation of clinical genetic testing are being developed and standardized by workgroups including the American College of Medical Genetics and Genomics <sup>153</sup>. Finally, the implications of genetic testing for CHD patients and their families are significant, ranging from prognostic risk factors for neurodevelopmental outcome, to estimates of recurrence risk in siblings, and increasingly as CHD patients reach reproductive age, to recurrence risk in their own offspring. The increasing awareness of the major genetic contribution to CHD provides a strong argument for providing broad access for CHD patients and families to specialized cardiac genetics clinics that can provide high-quality genetic counseling, along with training of pediatric and adult cardiologists, and genetic counselors, in CHD genetics <sup>150</sup>.

## Summary

Genetics of CHD has made giant leaps forward in parallel with the evolution of genome analysis technologies. The suspicion that CHD is extremely heterogenic has been validated, and the anticipated complexity of CHD genetics further increased by relatively limited observed genotype-phenotype correlations. Even syndromes that were thought to be well-defined clinically, such as CHARGE and Kabuki, are showing tremendous variation in phenotype when they are defined on the basis of the molecular finding. It is distinctly possible that some of the outcome in CHD is substantially influenced by the underlying genetic cause, in addition to the morphology and hemodynamics that underpin the impressively successful medical and surgical management of CHD to date <sup>3</sup>. This observation drives the hope that early identification of genetic causes of CHD will allow more tailored management of CHD, and will hopefully improve the outcome especially with

respect to the many co-morbidities of CHD that have a profound impact in quality of life for patients living with CHD. For example, identification of neurodevelopmental risk genes can identify patients who can benefit from early intervention programs long before any clinical signs of NDD such as learning disabilities become apparent. In addition to the clinical implications of a more complete understanding of CHD genetics, the genes uncovered in human patients have already provided tremendous insights into the basic mechanisms underlying cardiac development.

Going forward, there is still much work to be done. Studies to date have at most defined the cause of 45–50% of CHD. Current analysis protocols are possibly underestimating the CNV and SNV contributions due to detection limitations and difficulty predicting whether identified variants are pathogenic or not. In addition, it is highly likely that some CHD is due to multi-locus inheritance, and that some is caused by mutations in non-coding DNA. As larger cohorts of CHD patients are being evaluated with progressively more comprehensive sequencing, it appears ever more likely that we will be able to identify the genetic underpinning of the majority of CHD, and to translate these findings into precision medicine for the care of CHD patients from infants to adults.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Non-standard abbreviations and acronyms

<b>CHD</b>	Congenital Heart Disease
<b>CTD</b>	Conotruncal Defects
<b>LVO</b>	Left-ventricular outflow defects
<b>HTX</b>	Heterotaxy-associated defects
<b>NDD</b>	Neurodevelopmental Disabilities
<b>RV</b>	Right Ventricle
<b>LV</b>	Left Ventricle
<b>ASD</b>	Atrial Septal Defect
<b>HLHS</b>	Hypoplastic Left Heart Syndrome
<b>CoA</b>	Coarctation of the Aorta
<b>BAV</b>	Bicuspid Aortic Valve

<b>TOF</b>	Tetralogy of Fallot
<b>AVSD</b>	Atrio-ventricular Septal Defect
<b>CNV</b>	Copy Number Variation
<b>WES</b>	Whole-Exome Sequencing
<b>WGS</b>	Whole Genome Sequencing
<b>LOF</b>	Loss of Function
<b>ASE</b>	Allele Specific Expression
<b>VUS</b>	Variant of Unknown Significance
<b>PCD</b>	Primary Ciliary Dyskinesia

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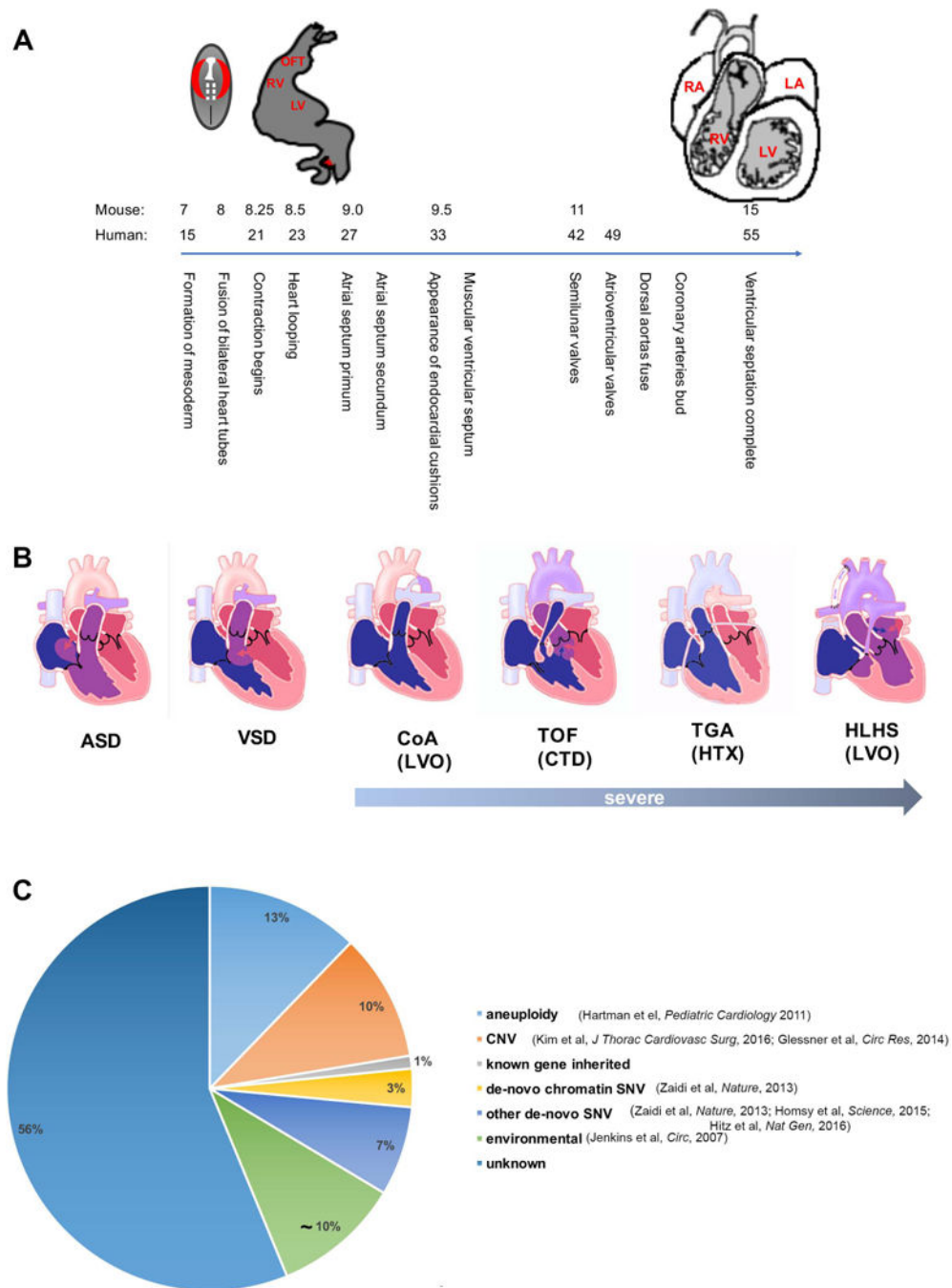
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**Figure 1.**

**A** Outline of human heart development. The X axis displays days of human and mouse gestation. **B** The spectrum of congenital heart disease from mild to severe. The lesions indicated as “severe” are expected to require intervention in the first year of life. ASD-Atrial Septal Defect, VSD-Ventricular Septal Defect, CoA- Coarctation of the Aorta, TOF-Tetralogy of Fallot, TGA- Transposition of the Great Arteries, HLHS- Hypoplastic Left Heart Syndrome. Classes of CHD based on proposed developmental-genetic mechanisms

are indicated in parentheses; LVO-Left Ventricular Outflow Obstruction, CTD- Conotruncal Defect, HTX- Heterotaxy. C Genetic causes of CHD identified to date.

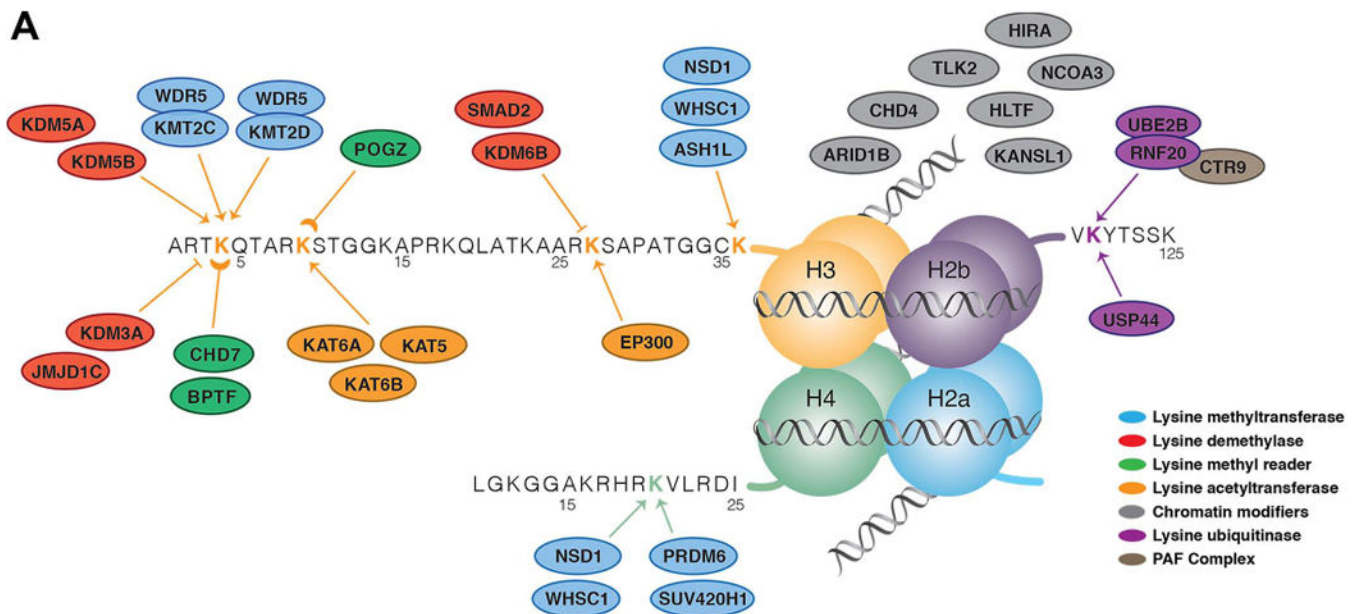
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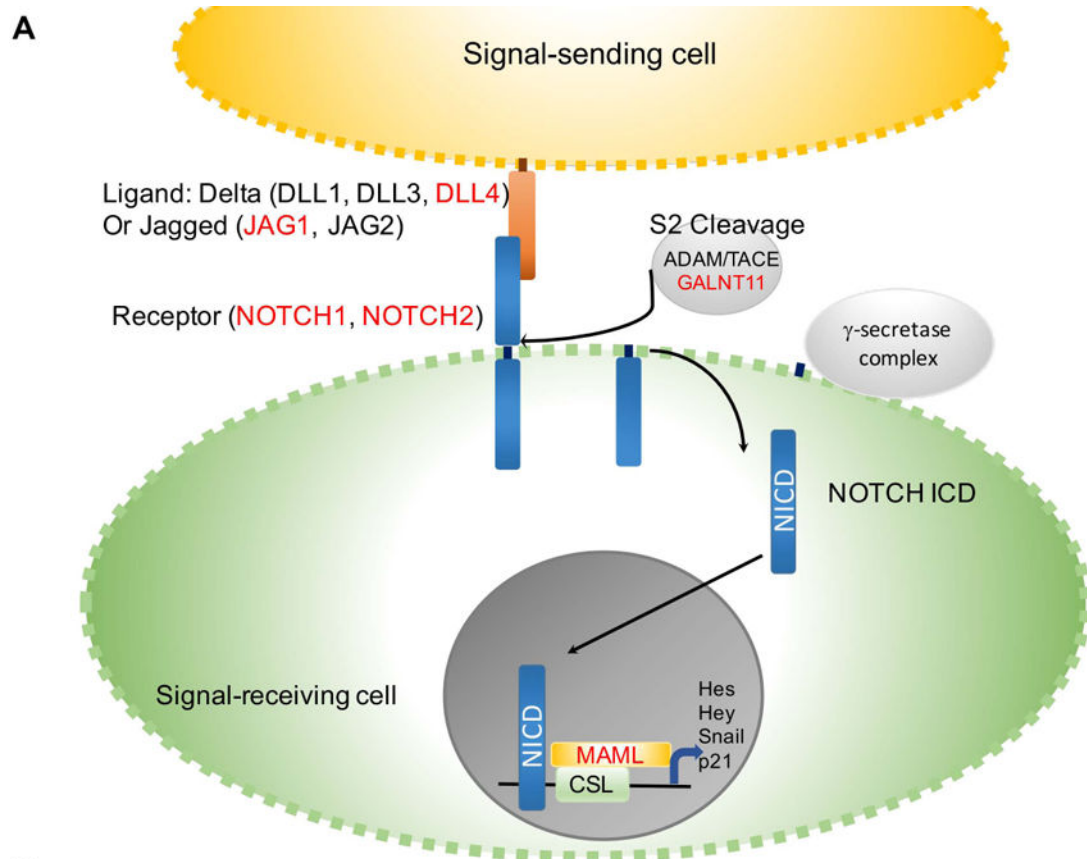
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**Figure 2.**

Chromatin remodeling genes in CHD. **A** Chromatin remodeling genes with mutations identified in CHD patients to date, highlighting the overlap between CHD and NDD genes. Nucleosomes with H3K4, H3K9, H3K27, H3K36 and H4K20 methylation and/or acetylation, and H2BK120 ubiquitylation are shown (adapted from Homsey et al, *Science* 2015). **B** Syndromes associated with chromatin remodeling gene mutations and their associated CHD and neurodevelopmental abnormalities.

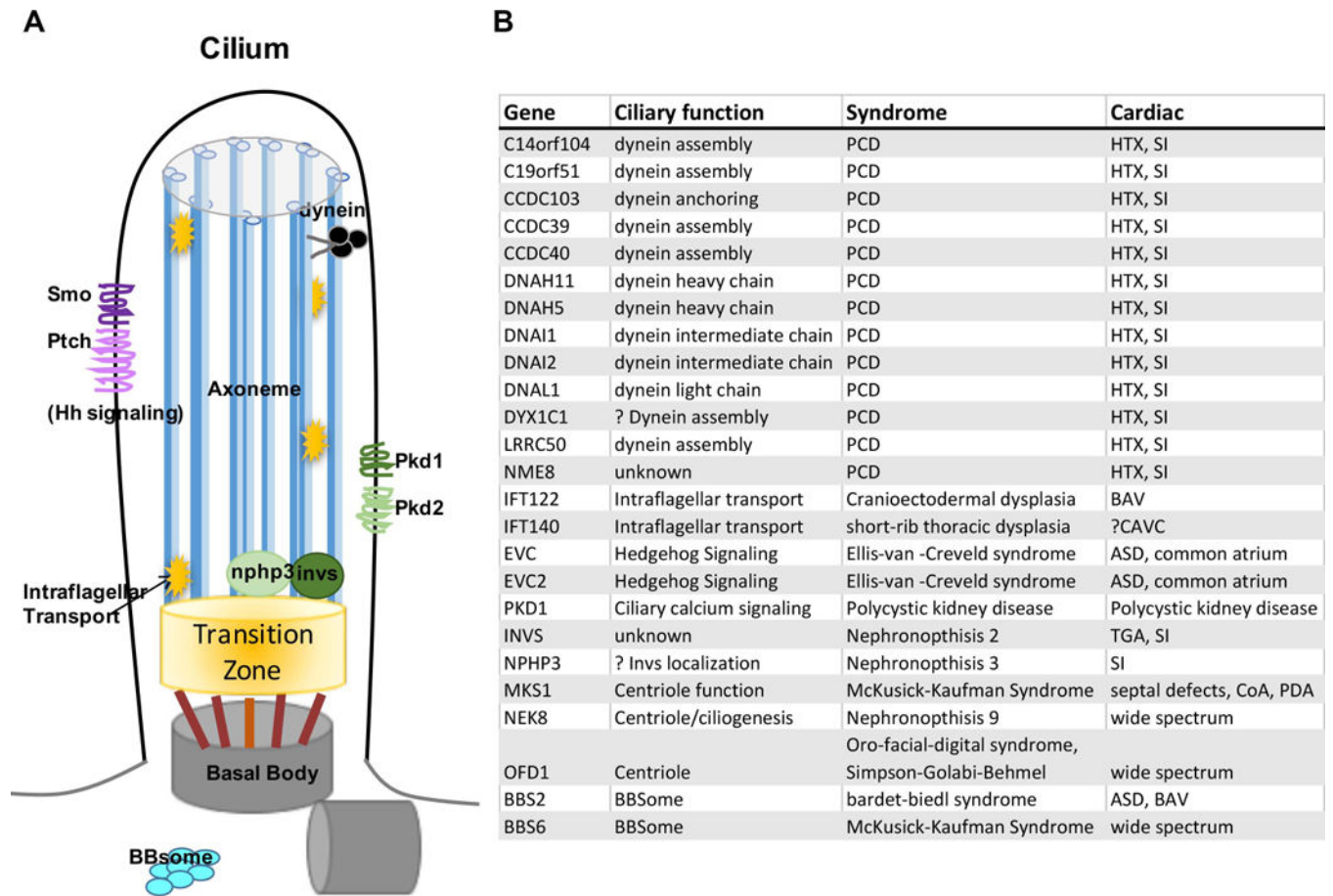


**B**

Gene	Cardiac Phenotype	OMIM Syndrome	Extracardiac
NOTCH1	BAV, CoA, HLHS, TOF, VSD	Adams-Oliver	Aplasia cutis congenita, brachydactyly, syndactyly, hemangioma
NOTCH2	TOF	Alagille	Dysmorphic facies, cholestatic liver disease, cystic kidneys
DLL4	VSD, TA	Adams Oliver	Aplasia cutis congenita, brachydactyly, syndactyly, hemangioma
JAG1	AS, TOF, peripheral pulmonary stenosis	Alagille	Dysmorphic facies, eye abnormalities, cholestatic liver disease, renal abnormalities, vertebral abnormalities, mental retardation (mild)
GALNT11	HTX	NA	
MAML2	Aortic Valve Disease	NA	

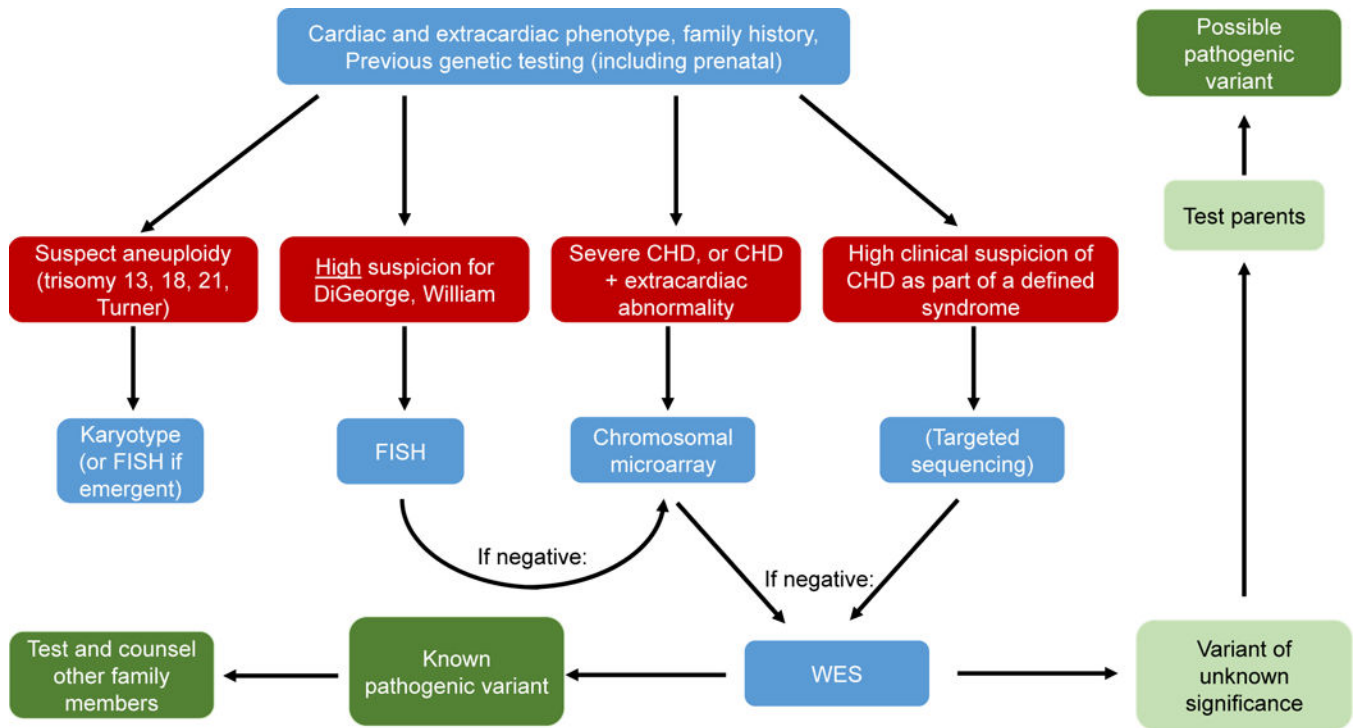
**Figure 3.**

NOTCH signaling in CHD **A** outline of NOTCH signaling pathway showing signal-sending cell in yellow, and signal receiving cell in green. **B** Syndromes and CHD associated with NOTCH pathway gene mutations.



**Figure 4.**

Cilia in CHD **A** Diagram of a cilium, showing the ciliary axoneme (blue) based on the mother centriole (gray) and linked via the transition zone (orange). **B** Syndromes and CHD linked to human cilia mutations.



**Figure 5.** Outline of proposed clinical genetic testing for patients with CHD