Title: Clinical and molecular effects of CHD7 in the heart

Running title: CHD7 and the heart

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ABSTRACT

Heart defects are a frequent cause of morbidity and mortality in CHARGE syndrome, caused by loss-of-function mutations in *CHD7*. We reviewed the clinical and molecular aspects of CHD7 related to the cardiovascular manifestations of the syndrome.

The type of heart defects in patients with *CHD7* mutations is variable, with an overrepresentation of AVSD and outflow tract defect including aortic arch anomalies. *Chd7* haploinsufficiency in mouse is a good model for studying the heart effects seen in CHARGE syndrome. Mouse models reveal a role for *Chd7* in multiple linages during heart development. Formation of the great vessels requires *Chd7* expression in the pharyngeal surface ectodermal via cell non-autonomous effects, likely on the neural crest cells. In cardiogenic mesoderm *Chd7* is required for atrioventricular cushion development and septation of the outflow tract. Knowledge concerning the function of CHD7 in the heart is emerging and indicates CHD7 may act in concert with transcription factors such as TBX1 and SMADs to regulate genes such as p53 and the cardiac transcription factor *NKX2.5*.

KEYWORDS

CHD7, CHARGE syndrome, congenital heart defects

INTRODUCTION

Loss-of-function mutations in *CHD7* (MIM 608892) are the major cause of CHARGE syndrome (MIM 214800) [Vissers et al., 2004]. CHARGE syndrome is a variable multiple congenital malformation syndrome including heart defects. Haploinsufficiency of *CHD7* alters the transcription of tissue-specific target genes that are normally regulated by CHD7 or complexes containing CHD7 [Schnetz et al., 2009; Schnetz et al., 2010; Zentner et al., 2010]. In this paper, we will focus on the effect of CHD7 in the heart from a clinical point of view and relate it to knowledge on the molecular development of the heart.

HEART DEFECTS IN PATIENTS WITH PATHOGENIC *CHD7* MUTATIONS

Since cardiac malformations are a well-recognized feature of CHARGE syndrome, studies of heart defects were performed decades prior to the identification of *CHD7* as the major cause [Cyran et al., 1987; Lin et al., 1987; Wyse et al., 1993]. While studies differed, outflow tract defects, persistent ductus arteriosus (PDA), atrioventriculoseptal defect (AVSD) and ventriculoseptal defects were most frequent.

After the identification of the *CHD7* gene, several studies focused on the phenotype of patient with a *CHD7* mutation, including heart defects. Heart defects were identified in 66% and 92% of patients [Jongmans et al., 2006; Jyonouchi et al., 2009]. One large study focused on the type of cardiac defect in 299 patients with pathogenic *CHD7* mutations [Corsten-Janssen et al., 2013]. 74% of the patients with pathogenic *CHD7* mutations had a heart defect. Heart defects occurred significantly more often in patients with truncating mutations than in patients with a missense or splice-site mutation (80% vs 58%). The type of cardiovascular defect diagnosed in patients with pathogenic *CHD7* mutation is

variable, with outflow tract and septal defect being the most prevalent. As shown in Figure 1 compared to a group of non-syndromic heart malformations, an overrepresentation of AVSD, PDA and outflow tract defects was seen [Corsten-Janssen et al., 2013]. Also, aortic arch anomalies, including abnormal branching such as aberrant subclavian artery, were frequently present in the cohort [Corsten-Janssen et al., 2016].

CHD7 MUTATIONS IN COHORTS OF PATIENTS WITH HEART DEFECTS

Pathogenic *CHD7* mutations may cause a small subset of CHARGE manifestations. For example a pathogenic *CHD7* mutation was identified in a girl who only expressed a typical external ear anomaly and a heart defect [Bergman et al., 2011]. ~~Indeed pathogenic *CHD7* mutations have been identified in studies that analyzed *CHD7* in patients expressing only a few features of CHARGE syndrome~~, Similarly, CHD7 mutations have been described in patients with just hypogonadotropic hypogonadism and anosmia. [Bergman et al., 2011]. The identification of a *CHD7* mutation is important for counseling on recurrence risk, prenatal options and clinical follow up [Bergman et al., 2011]. Since *CHD7* causes congenital heart defects in most people harboring a pathogenic *CHD7* mutations [Corsten-Janssen et al., 2013], several studies analyzed *CHD7* in cohorts of patients with cardiovascular malformations. An overview of the different studies is shown in Table 1.

Screening of *CHD7* only in a cohort of 67 patients with isolated heart defects [Qi et al., 2008] and in a cohort of 46 patients selected for their type of heart defect and one other feature of CHARGE syndrome [Corsten-Janssen et al., 2014] identified no pathogenic mutations. These studies indicate *CHD7* mutations are not a major cause of congenital heart defects, even when patients are selected for extra-cardiac features. However, in two larger retrospective clinical studies in 310 patients with bicarotid trunk (the most common variation in aortic arch branching)

and in 257 patients with tetralogy of Fallot with pulmonary stenosis, CHARGE syndrome was diagnosed in around 1% of the patients [Oswal et al., 2014; Reinshagen et al., 2014]. Although the main intention of these studies was not to search for syndromes, the results suggest *CHD7* mutations could be identified by screening *CHD7* in larger cohorts.

Next generation sequencing techniques have made it possible to screen multiple genes involved cardiac development, including *CHD7*.. An exome sequencing analysis of 362 parent-child trios, with the child having a severe congenital heart defect, revealed a *de novo* truncating *CHD7* mutation in a proband with syndromic tetralogy of Fallot [Zaidi et al., 2013]. Retrospectively based on the guidelines for *CHD7* analysis, this patient might have been eligible for *CHD7* analysis based on the extra-cardiac features (cleft lip and palate, inguinal hernia, micropenis, sensorineural hearing loss, abnormal neurological development) [Bergman et al., 2011]. In a cohort of 81 patients with AVSD, an enrichment of rare and rare potentially damaging *CHD7* mutations was identified while screening 112 AVSD-related genes. The patients had syndromic as well as non-syndromic heart defects. A contribution of *CHD7* variants to AVSD was suspected by the authors, although the pathogenic effect of the rare *CHD7* variants identified in this study has not been proven [D'Alessandro et al., 2015].

In conclusion, currently direct analysis of *CHD7* only seems warranted when congenital heart defect is present with at least one other major CHARGE feature. *CHD7* should be added to gene panels for heart defect, especially syndromic heart defects.

GENERAL INFORMATION ON HEART DEVELOPMENT

Before considering how CHD7 orchestrates the complex events of cardiac morphogenesis it is useful to reprise the relevant developmental biology (for further review see reference [Epstein, 2010]). The heart is the first organ required for embryonic survival. The main cellular contribution to cardiac structures is provided by mesodermal derivatives, although the other germ layers (ectoderm and endoderm) also have a vital role to play. The cardiac mesoderm cells are located in the cardiac crescent and the two arms of the crescent migrate medially to form the primitive heart tube. The heart chambers from via a process of "ballooning" out from this tube. The cardiac mesoderm itself contains two groups of cells generally thought to comprise distinct linages that give rise to specific regions of the heart. The left ventricle and parts of the atria are derived from the so called first heart field. The right ventricle, much of the outflow tract, parts of the atria and atrial septum are derived from second heart field cells that migrate to the poles of the early heart tube. Thus, second heart field cells are added after the first heart field contribution. Complex morphogenetic events are required to establish the four-chambered heart. The heart undergoes looping such that the left and right atria become aligned with their respective ventricles. In addition the single artery emanating from the heart, the outflow tract, septates into the pulmonary artery and aorta. The region between the atria and ventricles forms swellings called cushions composed of a jelly like substance. The endothelial lining over the cushions undergoes an endothelial to mesenchymal transition with the resulting cells migrating into the cushions. The cushions are remodeled to form the atrioventricular valves. An analogous process is required in the outflow tract to create the aortic and pulmonary valves and to form an effective septum between these two vessels. Notably, the septation of the ventricles and outflow tract is dependent upon a normal contribution from an additional, neuroectodermally derived, group of cells - the cardiac neural crest linage (cNCC). *CHD7*, being expressed in all germ layers and the neural crest during heart development, has the potential to influence many relevant events.

The analysis of mouse (and other animal) models has shed light upon some of these processes, as described below. The relationship between these various cell linages, subsequent heart structures and pathways impacted by loss of CHD7 activity is presented schematically in Figure 2.

CHD7 MOUSE HAPLOINSUFFICIENCY MODELS AND THE HEART

Chemical mutagenesis yielded a number of mutations in *Chd7*, several of which were studied prior to knowledge of the underlying genetic etiology of CHARGE syndrome [Alavizadeh et al., 2001; Bosman et al., 2005]. These mice were identified through circling or other behavioural phenotypes linked to inner ear abnormalities. More recently, "gene trap" mutants have been studied, and in these animals the coding region of *Chd7* is replaced by a reporter gene that provides a read out of *Chd7* expression [Hurd et al., 2007; Randall et al., 2009]. These mouse models recapitulate the cardiac phenotype seen in the human syndrome well, with ventriculoseptal defects, atrial septal defects and interruption of the aortic arch type B (IAA-B) reported. However, *Chd7* null mice die at mid gestation and have severe growth delay so work on these mice has been limited.

CHD7 AND THE P53 PATHWAY

During an analysis of mice containing one wild type and one mutant, transcriptionally dead variant of the tumour-suppressor protein p53, it was noted that the embryonic phenotypes obtained were characteristic of CHARGE syndrome [Van Nostrand et al., 2014]. In this model the mutant p53 protein stabilized and hyperactivated wild-type p53, resulting in ectopic activation of p53 target genes which triggered cell-cycle arrest or

apoptosis. CHD7 was found to bind the p53 promoter and down regulate p53 expression, and CHD7 loss in mouse NCCs or samples from patients with CHARGE syndrome resulted in p53 activation [Van Nostrand et al., 2014]. Moreover, when *Chd7* mutants were bred to give homozygous null *Chd7* embryos in the context of a single p53 loss of function allele, the *Chd7* null phenotype was partially rescued, suggesting a role for p53 downstream of CHD7. However, the rescue did not permit normal heart development, but appeared to allow a further 24h of development compared to *Chd7* nulls. The effect might be relatively non-specific via an inhibition of apoptosis in null embryos by heterozygosity for p53. It is interesting to note that CHD7 regulates rRNA biosynthesis via the Treacle protein mutated in Treacher Collins syndrome [Zentner et al., 2010] and that *Tcof1* mutants are similarly rescued by crossing to heterozygous p53 loss of function mice [Jones et al., 2008]. In general it is worth noting that the growth retardation and developmental delay in constitutive *Chd7* null mutants makes it difficult to disentangle cause versus effect when considering the poor heart morphogenesis seen in such mutants.

CHD7 AND THE BMP PATHWAY

While CHD7 binds to linker DNA between nucleosomes, it does not contain motifs that allow sequence-specific DNA binding and therefore recruitment to chromatin sites must be via protein interactions either with other transcriptional regulators, histone modifications, or both. CHD7 was identified as a protein partner of SMAD1, a receptor SMAD that interacts with SMAD4 to regulate transcription upon activation of the BMP pathway [Liu et al., 2013]. Of interest, the complex was found to regulate *NKX2.5*, an important transcription factor expressed in both heart fields and required for heart development. *NKX2.5* demonstrates haploinsufficiency in humans, with mutations being associated with arrhythmia

and atrial septal defect.

TISSUE SPECIFIC REQUIREMENTS FOR *CHD7*

It is clear from work conducted in human embryonic stem cells (hESCs) and mouse embryos that CHD7 has a major role in neural crest cell development. In hESCs CHD7 is complexed with another chromatin remodeling protein called PBAF (polybromo- and Brg1/Brahma-associated factor (BAF)). Together they act at the enhancer of the *SOX9* transcription factor gene, itself a key component of the NCC gene regulator network [Bajpai et al., 2010]. CHD7 also partners with BRG1 to regulate the SEMA3-family receptor *PlexinA2* which is required for outflow tract formation [Li et al., 2013]. Indeed, *Sema3c*, the specific Sema3 involved in this process is down-regulated in *Chd7* null embryos [Schulz et al., 2014]. However, NCCs secrete *Sema3c* and SEMA3 signalling to NCCs is not required for cardiac outflow tract septation [Plein et al., 2015], so additional mechanisms or linages must be involved (see mesoderm, below).

As mentioned above, there is substantial evidence from *in vitro* work that CHD7 is involved in NCC development. In fish NCC migration, fate choice and differentiation are all affected in *chd7* morphants [Balow et al., 2013; Patten et al., 2012] (although with the caveat that NCC seems particularly susceptible to off-target morpholino effects [Boer et al., 2016]). It is therefore surprising that while NCC specific knockout of *Chd7* results in craniofacial abnormalities, cardiac defects were not reported [Sperry et al., 2014]. This agrees with genetic rescue experiments of Randall et al. [2009]. In this paradigm, embryos heterozygous for *Chd7* following a gene trap can be induced, via Cre recombinase expression,

to re-express *Chd7* from the trapped allele in specific tissues. Thus, Cre-containing embryos will be dizygous in the targeted tissue and heterozygous in all others. Rescue of *Chd7* expression in NCC, using *Wnt1Cre*, failed to correct abnormalities of the pharyngeal arch arteries (PAAs). On the other hand, the *Ap2αCre* driver, which rescues expression in the pharyngeal surface ectodermal (PSE) layer of the embryo (as well as NCC), does rescue these phenotypes. This implies that there is a non-autonomous role of CHD7 in the PSE whereby it controls signalling required for the NCC to make its required contribution to cardiovascular development. This is reminiscent of tissue specific requirements for the TBX1 transcription factor [Calmont et al., 2009], an issue revisited below.

The role of CHD7 in cardiac progenitors was analyzed using a *Mesp1Cre* driver. *Mesp1* is expressed in the common progenitor cells of both the first and second heart field, together with cells of the craniofacial mesoderm that will form skeletal muscle of the head and neck [Diogo et al., 2015]. As well as cardiomyocytes, *Mesp1* linage cells can give rise to endothelial cells, smooth muscle and mesenchymal cells such as fibroblasts. Embryos null for *Chd7* in the *Mesp1Cre* linage bypassed the early lethality noted in the constitutive nulls but died from E14.5 to birth [Payne et al., 2015]. In embryos surviving to E15.5 surface hemorrhage was observed, a sign of possible heart failure or defective vessel development. A range of cardiovascular defects was present in these embryos. IAA-B and aberrant subclavian artery reflected abnormal growth and remodeling of the PAA system. Severe intracardiac defects were observed, which recapitulated to varying extents a rare human heart malformation called the Holmes heart. Late gestation hearts had both a double inlet left ventricle (with small right ventricle) and a double outlet right ventricle with a single atrioventricular valve. There was occasional instance of common arterial trunk. These phenotypes likely reflected an earlier function of CHD7 since the protein itself was not detectable in hearts of E13.5 embryos. Analysis of the transcriptome of *Mesp1Cre*-

Chd7 conditionally mutant hearts at E11.5 and E12.5 identified several dysregulated pathways; including down regulation of *Sema3c* in both the second heart field and ingressing NCCs. Knockout of *Chd7* specifically in the endothelial cell linage did not yield any evidence of vessel defect so the surface hemorrhage and edema may be due to heart failure. This is supported by the observation that the expression of several genes encoding proteins involved in excitation contraction coupling was altered. Indeed, cultured cardiomyocytes from *Mesp1Cre-Chd7* conditional hearts displayed aberrant calcium currents, suggesting that cardiomyocyte function is disturbed in these mutants [Payne et al., 2015].

CLINICAL AND MOLECULAR OVERLAP OF CHARGE SYNDROME WITH OTHER HEART DEFECT SYNDROMES

The overlap between the cardiac defects seen in CHARGE syndrome and 22q11.2 deletion syndrome (MIM192430 and 188400) had already been noticed by Lin et al in 1987 [Lin et al., 1987]. Since then many publications have focused on the overlap that includes, in addition to heart defects, cleft palate, developmental delay, renal abnormalities and hearing loss [Corsten-Janssen et al., 2012; Jyonouchi et al., 2009]. Focusing on heart defects, the outflow tract abnormalities, and especially the aortic arch anomalies occur in both syndromes. Indeed, *CHD7* mutations have been identified in patients with a 22q11.2 deletion phenotype and, vice versa, 22q11.2 deletions have been identified in patients suspected of CHARGE syndrome [Corsten-Janssen et al., 2012; Randall et al., 2009; Sanka et al., 2007]. The aplasia or hypoplasia of the 4th PAA mentioned above in the context of *Chd7* haploinsufficiency is a frequent finding in *Tbx1* heterozygous embryos [Randall et al., 2009]. Like *Chd7*, expression of *Tbx1* in the PSE is required for PAA formation. Moreover, embryos doubly heterozygous for both *Chd7* and *Tbx1* have more frequent and more severe great vessel defects, suggesting that these two transcriptional regulators operate in the same developmental

pathway [Randall et al., 2009]. This may relate to their effects on chromatin. TBX1 is known to recruit histone methylases to chromatin, TBX1 chromatin binding is associated with the H3K4me1 modification [Fulcoli et al., 2016], and CHD7 chromatin binding peaks coincide with peaks of H3K4me1 [Schnetz et al., 2009; Schnetz et al., 2010]. Thus, one scenario is that TBX1 establishes a chromatin modification that is interpreted by CHD7 which remodels the nucleosomes at these sites.

The clinical and molecular overlap of CHARGE syndrome with other syndromes is more extensive than 22q11.2 deletion syndrome alone as is shown in Table 2. It is interesting to note that Kabuki syndrome (MIM147920), which also has clinical overlap with CHARGE syndrome, can be caused by mutations affecting lysine-specific methyltransferase 2D (KMT2D, MIM602113) [Butcher et al., 2017; Schulz et al., 2014; Verhagen et al., 2014]. KMT2D and CHD7 have a molecular link through interactions with the same chromatin machinery (members of the WAR complex) [Schulz et al., 2014], and also share gene targets [Butcher et al., 2017]. Exome sequencing of patients with sporadic congenital heart defects revealed a relative excess of mutations in genes encoding similar chromatin modifiers (that in particular regulate methylation at lysine 4 of histone H3) pointing to the importance of these proteins in normal cardiovascular development [Zaidi et al., 2013]. As chromatin methylation (and other modifications) may be influenced by environmental factors, for instance via homocysteine and the one-carbon cycle, they may be nodes for gene-environment interactions [Selhub, 2002].

In conclusion, the cardiac phenotype in patients with *CHD7* mutation is variable, but a relative over representation of AVSD and outflow tract defects including aortic arch anomalies is seen. *Chd7* deficient mice have an overlapping cardiac phenotype with human CHARGE patients.

Molecular studies have elucidated some underlying mechanisms and genetic interactions during cardiac development by which congenital heart defect arises in patients with *CHD7* mutations and overlapping heart syndromes. *Chd7* has a role in several cell linages, for example in the PSE for remodeling of the pharyngeal arch arteries and in mesoderm for atrioventricular cushion development and septation of the outflow tract. CHD7 protein likely interacts with other gene-regulators such as TBX1 and SMADs to regulate genes as the cardiac transcription factor *NKX2.5*. Rare variants in these genes may contribute to the genetic architecture of sporadic congenital heart defect.

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CONFLICTS OF INTEREST

None

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FIGURE LEGENDS

Figure 1: Type of heart defects caused by CHD7 mutations

The distribution of congenital heart defects in a group of 202 patients with a *CHD7* mutation (A), and in 1007 patients with non-syndromic congenital heart defects (B), adapted from reference [Corsten-Janssen et al., 2013]. The combination of AVSD, outflow tract defects and PDA cover about 52% of the heart defects in the *CHD7* mutation group, while these types of heart defects cover only 12% in the non syndromic group.

AVSD, atrioventricular septal defects; LVOTO, left ventricular outflow tract obstruction; OFT, outflow tract defects; PDA, patent ductus arteriosus; RVOTO, right ventricular outflow tract obstruction.

Pathways And Linages in which CHD7 is Active During Heart Development

The diagram presents a schematic of the heart and proximal vessels at around E10.5 of mouse gestation and 4-5 weeks in humans. The different linages described in the review are color coded as indicated. Structures may comprise more than one lineage e.g. mesoderm and neural crest in the PAAs, OFT and semilunar valves. Groups of cells or linages are underlined: NCC, neural crest cells; FHF, first heart field; SHF, second heart field. The approximate anatomic location of their contribution is indicated by the curved arrows. Thus, NCC contributes to the smooth muscle of the PAAs, and SHF mesoderm to the endothelial lining with both linages contributing to the outflow tract. The official name of genes/protein/pathways implicated in the process is given. Non italicized names represent protein interactors of CHD7, in italics are gene or gene families that are directly and/or indirectly regulated by CHD7. Autonomous/non-autonomous indicates where the effect is within a specific cell linage or where cell-cell signalling processes are involved.

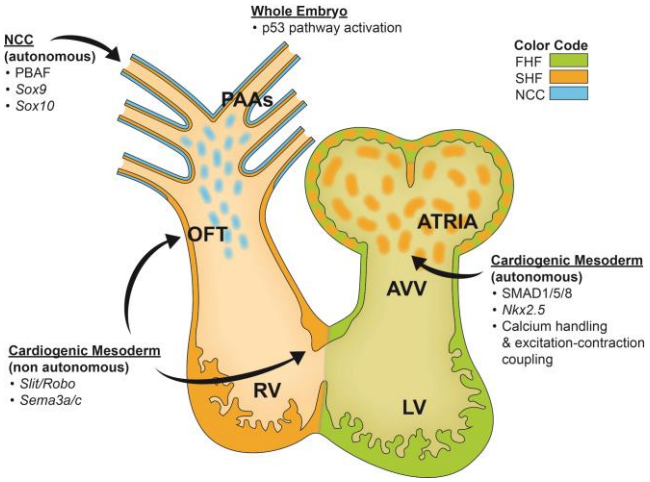

Table: clinical and molecular overlap between heart syndromes

	CHARGE syndrome	***Mandibulofacial dysostosis***	***Pallister Hall syndrome***	***Alagille syndrome***	***Kabuki syndrome***	***Syndromic microphtalmia type 3***	***22q11.2 deletion syndrome***	***Mowat Wilson syndrome***
	CHD7	***EFTUD2***	***GLI3***	***JAG1***	***KMT2D***	***SOX2***	***TBX1***	***ZEB2***
Cardiac phenotype	Variable, ASD, AVSD, OFT, PDA, VSD	ASD, VSD	Coa, PDA, VSD	PPS, ASD, VSD, TOF, Coa	Variable, ASD, Coa, VSD	PDA, VSD	Variable, OFT	ASD, PDA, PS, VSD
Coloboma/microphtalmia	+		+			+	rare	+
Cranial nerve defects	+	-	-	-	-	-	-	-
Choanal atresia	+	rare	-	-	-	-	rare	-
Cleft lip and/or palate	+	+	+		+		+	+
Short stature	+	+	+	-	+	+	+	+
Intellectual disability/ developmental delay	+	+	-	rare	+	+	+	+
Genital hypoplasia	+	-	+	-	+	+	-	-
External ear anomaly	+	+	+	-	-	-	-	+
Hearing loss	+	+	-	-	+	+	-	-
SCC dysplasia	+	-	-	-	-	-	-	-
Esophageal atresia	+	rare	-	-	-	+	-	-
Renal anomalies	+	-	+	+	+	-	+	-
Anal atresia	rare	-	+	-	+	-	-	-
Vertebral anomalies	rare	-	+	+	+	+	+	-
Limb anomalies	rare	+	+	+	-	-	-	-
Imunnodeficiency	rare	-	-	-	-	-	+	-
Other		trigonocephaly, malar and mandibular hypoplasia	multiple buccal frenula, bifid epiglottis, laryngeal cleft, pre-auricular skintag	cholestasis, posterior embryotoxon, cerebrovascular accidents	persistent fetal finger pads, facial dysmorphism e.g. eversion of the lateral third of the lower eyelid	anophtalmia, anterior pituitary hypoplasia	velopharyngeal incompetence, psychiatric disorder, hypocalciemia	facial dysmorphism e.g. uplifted earlobe, epilepsy

In this table clinical information is given on genetic heart syndromes that have a clinical overlap with CHARGE syndrome. Most of the genes mentioned in this table have a molecular link with CHD7 as well. SOX2 and CHD7 have been shown to physically interact with JAG1 and GLI3 as target genes [Engelen et al., 2011]. CHD7 and KMTD2 interact with members of the same chromatin remodelling machine and have common target genes [Butcher et al., 2017; Schulz et al., 2014; Verhagen et al., 2014]. TBX1 and CHD7 are both needed for normal PAA development, and CHD7 alters the expression of TBX1 in inner ear neurogenesis [Hurd et al., 2010; Randall et al., 2009]. For EFTUD2 and ZEB2 only clinical overlap has been described so far [Luquetti et al., 2013; Wenger et al., 2014].

ASD, atrial septal defects; AVSD, atrioventriculoseptal defect; Coa, coarctation of aorta; OFT, outflow tract anomalies; PDA, patent ductus arteriosus; PPS, Peripheral pulmonary artery stenosis; PS, Pulmonary artery stenosis; SCC, semicircular canals; TOF, tetralogy of Fallot; VSD, ventricular septal defect.

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