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## Reproductive Endocrine Phenotypes relating to *CHD7* mutations in humans

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### Abstract

Mutations in the gene *CHD7* cause CHARGE syndrome, a rare multi-organ syndromic disorder. Gonadal defects are common in individuals with CHARGE syndrome (seen in ~60–80% of cases) and represent the letter “G” in the CHARGE syndrome acronym. The gonadal defect in CHARGE syndrome results from congenital deficiency of the hypothalamic hormone Gonadotropin-releasing hormone (GnRH), which manifests clinically as pubertal failure and infertility, and biochemically as hypogonadotropic hypogonadism (low sex steroid hormone levels with inappropriately normal or low gonadotropin levels). In addition to the gonadal endocrine abnormalities, in a small minority of individuals with CHARGE, additional endocrine defects including growth hormone deficiency, multiple pituitary hormone deficits and primary hypothyroidism may also be seen. *CHD7* mutations disrupt the targeting of olfactory axons and the migration of GnRH-synthesizing neurons during embryonic development, resulting in congenital idiopathic hypogonadotropic hypogonadism (IHH) and anosmia (or hyposmia), two features that define human Kallmann syndrome. Since Kallmann syndrome is as one of its constituent phenotypes in individuals with CHARGE, recent studies have investigated the role of *CHD7* mutations in individuals with IHH and established that deleterious missense mutations in *CHD7* are associated with Kallmann syndrome as well as normosmic form of IHH. These missense mutations affect the ATPase and nucleosome remodeling activities of the *CHD7* protein. These observations suggest that *CHD7* protein function is critical for the ontogeny of GnRH neurons and neuroendocrine regulation of GnRH secretion.

### Keywords

CHARGE syndrome; Kallmann syndrome; Isolated GnRH deficiency; idiopathic hypogonadotropic hypogonadism; *CHD7*; GnRH

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**Conflicts of Interest: None**

## Introduction

CHARGE syndrome (**C**oloboma, **H**ear defects, choanal **A**tresia, **R**etardation of growth and development, **G**onadal defects, and **E**ar/hearing abnormalities) is a rare multi-organ syndromic disorder in which haploinsufficiency in the gene *CHD7* accounts for nearly 70% of cases (Vissers et al. 2004). It is now well established that individuals with CHARGE display a wide spectrum of pleiotropic phenotypes, which have resulted in the definition of ‘major’ and ‘minor’ clinical criteria for clinical diagnosis of this developmental disorder (Hale et al. 2016; Jongmans et al. 2006). The letter “**G**” in the CHARGE acronym refers to gonadal defects which represents one of the four minor diagnostic criteria that clinically defines CHARGE syndrome (van Ravenswaaij-Arts et al. 2015). The gonadal defects in CHARGE were initially recognized in male infants who displayed genital hypoplasia characterized by micropenis and/or cryptorchidism (Pagon et al. 1981). It is now well established that these gonadal defects result from gonadotropin-releasing hormone (GnRH) deficiency leading to idiopathic hypogonadotropic hypogonadism (IHH) that can manifest in both sexes (Balasubramanian et al. 2014; Kim et al. 2008; Marcos et al. 2014). While gonadal defects are seen in a substantial proportion of individuals with CHARGE, additional endocrine abnormalities such as short stature, growth hormone deficiency, and multiple pituitary hormone deficits have also been infrequently reported in CHARGE (Dorr et al. 2015; Esposito et al. 2014; Gregory et al. 2013; Aramaki et al. 2006; Asakura et al. 2008; Pinto et al. 2005). In addition, several patients with just IHH without the pleiotropic spectrum of CHARGE features are also known to harbor presumed pathogenic *CHD7* variants (Kim et al. 2008; Balasubramanian et al. 2014). This review will focus on the endocrine phenotypes associated with CHARGE syndrome, the *CHD7* mutational spectrum in humans with IHH without full CHARGE features, and the molecular basis for reproductive endocrine phenotypes seen in individuals with *CHD7* mutations, and discuss the specific role of *CHD7* in GnRH neuronal ontogeny.

## Endocrine abnormalities in CHARGE syndrome

### Congenital Idiopathic Hypogonadotropic Hypogonadism (IHH), due to GnRH deficiency

The most common endocrine phenotype seen ~60–80 in % of individuals with CHARGE is hypogonadotropic hypogonadism (HH) (Bergman, Janssen, et al. 2011). Since penile growth and descent of the testes are dependent on an intact hypothalamic-pituitary-gonadal (HPG) axis *in utero* and during the neonatal period (Grumbach 2005), congenital hypogonadism due to hypothalamic/pituitary dysfunction manifests itself readily in boys who display micropenis and/or cryptorchidism. Hence, in male patients, HPG axis defects (micropenis and/or cryptorchidism) were recognized very early in the initial reports of individuals with CHARGE, thus constituting the “**G**” (genital hypoplasia) in the CHARGE association (Pagon et al. 1981). In contrast to boys with CHARGE in whom these HPG defects are readily evident, girls do not manifest any evident external genitalia phenotypes. Thus, the gonadal defects in females with CHARGE were not recognized initially and only became evident when these patients with features of CHARGE failed to enter normal puberty (Wheeler et al. 2000). In both sexes, the cause of the HPG axis dysfunction and resultant

genital/gonadal defects was initially unclear. Subsequently, a series of case reports firmly established that the genital defects in boys with CHARGE as well as the lack of puberty in females with CHARGE were secondary to hypogonadotropic hypogonadism [HH] (Oley, Baraitser, and Grant 1988; Harvey, Leaper, and Bankier 1991; Pinto et al. 2005; Wheeler et al. 2000). HH can result from either GnRH-related defects in the hypothalamus and/or defects in anterior pituitary function and further insights into the precise etiology of HH in CHARGE came from the study of the olfactory phenotypes in CHARGE children. Fetal studies in CHARGE children showed that olfactory bulb agenesis was one of their most consistent morphological features (Teixeira et al. 2010; Sanlaville et al. 2006). Olfactory testing and/or MRI imaging in individuals with CHARGE with gonadal defects revealed defective olfaction/olfactory bulb development in a significant number of patients (Pinto et al. 2005; Chalouhi et al. 2005). This phenotypic combination of HH and anosmia is well recognized as Kallmann syndrome (KS) (also referred as olfactogenital syndrome), a phenotypic form of congenital idiopathic hypogonadotropic hypogonadism (IHH) that results from a shared migratory defect of the GnRH and olfactory axons (Balasubramanian et al. 2010; Schwanzel-Fukuda and Pfaff 1989). Kallmann syndrome results from a pathological sequence during embryonic development wherein olfactory sensory neurons are prematurely interrupted in their migratory journey from the nose to the brain, resulting in concomitant arrest of migration of GnRH-synthesizing neurons that migrate alongside the olfactory fibers (Schwanzel-Fukuda and Pfaff 1989; Teixeira et al. 2010). The KS form of IHH is distinct to the other major phenotypic form of IHH in which individuals exhibit GnRH deficiency but without olfactory deficits, i.e. normosmic IHH [nIHH], which represents a neuroendocrine defect in GnRH secretion once the GnRH neurons reach the hypothalamus (Balasubramanian et al. 2010). Thus, the presence of KS in individuals with CHARGE confirms that HH in CHARGE is due to embryonic GnRH deficiency, indicating a primary defect in GnRH neuronal migration/integrity rather than a primary pituitary defect. In addition, the presence of anosmia has been shown to be highly predictive of HH in CHARGE (Bergman, Bocca, et al. 2011). In keeping with these observations, majority of individuals with CHARGE have otherwise normal anterior pituitary hormone function and anatomy of the pituitary/sella, confirming hypothalamic GnRH deficiency (Aramaki et al. 2006; Asakura et al. 2008; Pinto et al. 2005). In terms of therapy, boys with micropenis require testosterone or gonadotropin therapy during infancy to restore penile growth. Cryptorchidism may require orchidopexy for repositioning the testes in the scrotum, a critical element for subsequent normal gonadal function as well as future fertility. In adolescent individuals with CHARGE who display absence of puberty and HH, induction of puberty in the form of testosterone/gonadotropins in boys and estradiol/progestin therapy in females is indicated. For adult CHARGE patients (both men and women) who seek fertility, induction of fertility requires the use of either exogenous gonadotropin therapy or pulsatile GnRH therapy, monitored and overseen by a specialist reproductive endocrinologist. Patients undergoing such fertility therapy should also be referred to the Clinical Genetics team for appropriate genetic counseling and determination of recurrence risk for CHARGE in the offspring. The precise therapeutic regimens for induction of puberty and fertility are beyond the scope of this review, but are described in detail elsewhere, both by the author's group (Balasubramanian and Crowley 1993) and others (Boehm et al. 2015; Palmert and Dunkel 2012). In addition to hormone replacement, additional monitoring required for individuals

with CHARGE includes bone age estimation and follow-up with serial bone density measurements to diagnose and treat their potential osteopenia/osteoporosis. Although some patients with genetic forms of IHH are known to “reverse” their hypogonadotropism in adulthood (Raivio et al. 2007), to-date, reversal of HH in patients with the full CHARGE spectrum has not been reported (see below for reversal of HH in patients with *CHD7* mutations associated with IHH alone but without full CHARGE spectrum).

### Growth defects and other anterior pituitary hormone defects

The “**R**” in the CHARGE acronym denotes retardation of growth and development. These growth defects in individuals with CHARGE often become evident in the postnatal period and their etiology is often multifactorial, including feeding difficulties, poor nutrition, gastro-esophageal reflux, renal dysfunction, cardiac dysfunction and rarely, Growth Hormone (GH) deficiency (Zentner, Layman, et al. 2010). In reports where GH axis was specifically examined in individuals with CHARGE, ~9% exhibited suboptimal GH levels requiring treatment and these subjects respond favorably to exogenous GH replacement (Shoji et al. 2014; Asakura et al. 2008; Pinto et al. 2005; Dorr et al. 2015; Esposito et al. 2014). Rarely, individuals with CHARGE show evidence of combined pituitary hormone deficiency (combination of GH, LH/FSH, TSH, ACTH) and such cases can demonstrate radiological evidence of anterior pituitary hypoplasia (Pinto et al. 2005) or ectopic posterior pituitary (Gregory et al. 2013) suggesting that rare subsets of patients with CHARGE may manifest structural pituitary abnormalities that result in combined pituitary hormone dysfunction. In terms of therapy, appropriate hormone replacement (thyroxine, growth hormone, steroid replacement) is indicated for these anterior pituitary hormone dysfunctions. Although the majority of individuals with CHARGE display endocrine phenotypes relating to hypothalamic/pituitary defects, primary hypothyroidism has also been reported rarely in CHARGE (Aramaki et al. 2006; Asakura et al. 2008).

## Molecular and biologic basis of endocrine defects in CHARGE

### *CHD7* mutations in patients with full CHARGE syndrome

Nearly 70–90% of patients with suspected CHARGE syndrome harbor pathogenic heterozygous mutations in the *CHD7* gene (Zentner, Layman, et al. 2010; Bergman, Janssen, et al. 2012; Janssen et al. 2012), located on chromosome 8q12.1, that encodes the evolutionarily conserved Chromodomain Helicase DNA binding [CHD] protein 7, a member of the larger CHD family that functions as an ATP-dependent chromatin remodeling protein (Manning and Yusufzai 2017; Bouazoune and Kingston 2012). The CHD family of proteins have pleotropic functions: chromatin remodeling, regulation of embryonic stem cell pluripotency, methylated histone binding, DNA binding, transcriptional regulation, cell cycle regulation, and regulation of apoptosis (He et al. 2016; Manning and Yusufzai 2017; Zentner, Hurd, et al. 2010). The vast majority of *CHD7* mutations demonstrable in individuals with full CHARGE spectrum are truncating mutations (frameshift; non-sense) that are distributed across all 37 coding exons, suggesting haplo-insufficiency of *CHD7* as the major molecular mechanism for this syndrome (Zentner, Layman, et al. 2010). In addition to these truncating mutations, splice variants, missense mutations and structural variants (exonic deletions, whole gene or chromosomal deletions and chromosomal

rearrangements) leading to loss of function can also cause CHARGE (Harvey, Leaper, and Bankier 1991). The vast majority of all *CHD7* mutations are *de novo* and hence most patients present as sporadic cases with a small minority of *CHD7* mutations showing familial inheritance in an autosomal dominant manner (Zentner, Layman, et al. 2010; Delahaye et al. 2007). Rarely, germline mosaic *CHD7* variants have also been reported (Jongmans et al. 2008).

## Missense allelic variants in *CHD7* can cause IHH in the absence of full CHARGE features

### Enrichment of *CHD7* missense mutations in IHH patients

Although patients with typical CHARGE features are easily recognized clinically, more recent reports have begun to recognize a growing complexity of this diagnosis with the milder end of the CHARGE spectrum occurring in individuals with *CHD7* mutations who exhibit clinical features that do not fulfill the clinical criteria for CHARGE. Since CHARGE syndrome may include Kallmann syndrome as one of its constituent phenotypes, two initial studies used a candidate gene approach to test the hypothesis that IHH may represent the milder end of *CHD7*-related CHARGE syndrome. By sequencing the *CHD7* gene in IHH (either KS or normosmic form of IHH [nIHH]), Jongmans *et al* identified 3/38 KS patients harboring *de novo* *CHD7* mutations (2 stop-mutations and 1 missense) whereas the normosmic IHH patients were negative for *CHD7* variants (Jongmans et al. 2009). However, all 3 KS patients with *CHD7* variants, upon additional phenotypic review, universally exhibited major CHARGE features. In contrast, Kim et al, identified 7 IHH patients (3KS, 4 nIHH) harboring *CHD7* mutations (2 intronic mutations leading to exon skipping and 5 missense mutations), all of whom lacked major CHARGE phenotypes, thus implicating *CHD7* allelic variants as a cause of both KS AND nIHH forms of IHH without CHARGE features (Kim et al. 2008). In view of the conflicting data as to whether *CHD7* mutations are capable of causing isolated KS without full CHARGE, Bergman et al, examined 36 KS patients in whom they identified 3 with *CHD7* mutations (2 nonsense, and 1 *de novo* missense) (Bergman, de Ronde, et al. 2012). However, all 3 subjects displayed additional CHARGE features, leading to their conclusion that *CHD7* mutations do not cause isolated IHH.

### Pathogenic missense *CHD7* alleles are enriched in IHH

Given the uncertainty of the role of *CHD7* in IHH in the absence of full CHARGE and to address the functional consequences of *CHD7* variants associated with IHH, we examined 783 well-characterized IHH patients for *CHD7* variants (Balasubramanian et al. 2014). Functional validation of a representative subset of IHH- and CHARGE- associated *CHD7* variants was performed using zebrafish injected with a splice-blocking morpholino against *Chd7*, in which otolith morphology was used as a surrogate phenotype to assess *CHD7* pathogenicity. Overall, 5.2% (n=41 patients) harbored heterozygous *CHD7* variants of whom only 9 showed evidence of minor CHARGE features while the rest had no CHARGE features. Strikingly, in contrast to the truncating *CHD7* mutations observed typically in patients with CHARGE, patients with IHH harbored predominantly missense variants. Next,

a representative spectrum of 20 different non-synonymous missense *CHD7* variants were subjected to functional analyses in zebrafish based on their population frequency and associated human phenotypes: (i) Control alleles: n=3; all with population minor allele frequency (MAF) < 0.5%; (ii) 12 IHH alleles with MAF < 0.5% associated with IHH without full CHARGE and (iii) 5 CHARGE alleles with MAF < 0.5% that were known to cause full CHARGE. Functional studies in zebrafish tested the relative ability of each disease-associated missense allele to rescue the morphant phenotype compared to control alleles or their ability to phenocopy MO-induced otolith defects when over-expressed. While all control missense alleles tested benign on the zebrafish assay, 9/12 IHH-associated missense alleles tested deleterious, displaying either hypomorphic or dominant phenotypes. All CHARGE-associated alleles were also functionally deleterious. In addition, three-dimensional structural modeling of CHD7 using an automated homology modeling [SWISS-MODEL(<http://swissmodel.expasy.org/>)] or the FoldX protein design algorithm (Balasubramanian et al. 2014) identified 4 *CHD7* missense variants that disrupted the three-dimensional protein structure of CHD7, all of which were also determined to be deleterious rare sequence variants from the zebrafish experiments. Thus, this large study confirmed that *CHD7* missense mutations can cause IHH without CHARGE.

The above functional studies provided molecular validation that allelic missense *CHD7* variants cause IHH and select *CHD7* mutations that have been functionally determined to be deleterious and linked to IHH are shown in Figure 1. This significant excess of deleterious *CHD7* missense mutations in IHH compared to the CHARGE syndrome was also confirmed by another independent study (Marcos et al. 2014). These observations suggest an exquisite dose-function-responsiveness for CHD7 protein function in relation to the varied developmental processes and organ systems that are critically regulated by CHD7. Hypomorphic/dominant *CHD7* alleles may preferentially disrupt only the GnRH/HPG axis when compared to the multi-organ systems affected by null mutations. Taken together, these genetic and phenotypic studies suggest that the ontogeny of GnRH neurogenesis is particularly susceptible to the mildest dysregulation of CHD7 function. In this regard, some of the IHH patients with *CHD7* missense alleles also display hearing loss and hence it can be hypothesized that semi-circular canal development/function may also be equally susceptible to these allelic defects, but this requires additional targeted auditory phenotyping studies in *CHD7*-related IHH subjects.

While these data implicating the missense *CHD7* alleles are compelling, given the large size of the *CHD7* gene, it is important to recognize that not all rare *CHD7* missense variants are necessarily pathogenic. Indeed, the recent expanded population level sequencing data available through the Exome Aggregation Consortium (ExAC) show that *CHD7* missense mutations are not constrained in the human genome (Karczewski et al. 2017). Indeed, in our in-house sequencing data, a burden test of *CHD7* missense mutations in IHH compared to ExAC shows no statistical enrichment for *CHD7* missense mutations with MAF < 1%. However, the IHH cohort was statistically enriched for *CHD7* missense variants with MAF < 0.1% (ultra-rare variants) (R.B unpublished observations) (Table 1). This excess of *CHD7* ultra-rare variants suggests that the rarity of the *CHD7* variants may be a reliable proxy for likely pathogenicity. Moreover, while the ExAC data does not show constraint for missense mutations across the whole *CHD7* gene, exon-specific constraint analysis for *CHD7* in the

ExAC data reveals significant constraint for missense mutations in exons 10–29 (Samocha et al from ExAC consortium, personal communication). These constrained exons correspond to amino acid positions 952–2065 which encompass the critical CHD7 domains (SNF2, helicase, and SANT domains), all known to impact chromatin remodeling (Bergman, Janssen, et al. 2011). These exon-specific constraint data are largely in keeping with the most recent zebrafish missense mutation modeling data (Balasubramanian et al. 2014), confirming that the IHH-associated pathogenic *CHD7* missense alleles disrupt key evolutionarily constrained amino acid residues that are critical for normal function of the CHD7 protein.

### **Pathogenic CHD7 alleles cause both KS and nIHH, including reversible forms of GnRH deficiency and contribute to oligogenic inheritance in IHH**

In contrast to the highly penetrant olfactory dysfunction in full CHARGE syndrome, missense alleles in *CHD7* are associated with both KS and nIHH (Kim et al. 2008; Balasubramanian et al. 2014). These observations suggest distinct neurodevelopmental and neuroendocrine roles for CHD7 function. In addition, whereas IHH has been previously thought to be a life-long disorder, it is now clear that ~10% of IHH patients may experience of spontaneous recovery of HPG axis function later in adulthood (Raivio et al. 2007). While this phenotypic subset has been association with patients harboring specific milder mutational forms of IHH [e.g. mutations in the *TAC3/TACR3* genes (Sidhoum et al. 2014)], a recent report identified a patient with IHH and *CHD7* truncating mutation who experienced spontaneous reversal of his IHH (Laitinen et al. 2012). Hence, serial clinical examination and intermittent cessation of hormonal therapy and biochemical re-evaluation for hypogonadism may be relevant for patients with *CHD7* related IHH who display clinical features that may signal reversal. Features that should prompt consideration of such reversal of GnRH deficiency include: spontaneous growth of testes while off testosterone therapy, eugonadal testosterone levels off therapy, or the appearance of spontaneous menses/fertility in females. Although IHH subjects harboring pathogenic *CHD7* missense mutations typically do not fulfill the clinical criteria for CHARGE diagnosis, known CHARGE features such as hearing loss and cleft lip/palate have been shown to be enriched in IHH patients harboring *CHD7* variants upon more careful phenotyping (Marcos et al. 2014; Costa-Barbosa et al. 2013). Therefore, the presence of hearing loss and/or cleft lip/palate in IHH patients should alert clinicians to search for *CHD7* mutations as this information will be critical for diagnosis as well as genetic counseling given the autosomal dominant mode of transmission associated with these variants.

In contrast to the *de novo* and paternal origin of mutations associated with severe CHARGE (Pauli et al. 2012), the vast majority of IHH-associated *CHD7* variants is inherited, often from unaffected parents, and exhibit no apparent parent of origin bias (Balasubramanian et al. 2014). In addition, in reports where segregation analysis of *CHD7* missense alleles is available, there is considerable incomplete penetrance for the IHH phenotype in those harboring *CHD7* missense mutations (Marcos et al. 2014; Balasubramanian et al. 2014). Moreover, in two families reported in our study, pathogenic *CHD7* variants were found in IHH patients who also carried a known pathogenic mutation in a 2nd gene known to be associated with IHH (e.g. *FGFR1* and *GNRHR*), suggesting that some *CHD7* alleles

contribute to the known oligogenic architecture of IHH (Balasubramanian et al. 2014). Although the remaining individuals with *CHD7* mutations did not harbor any additional known IHH gene variants, it is noteworthy that only 40% of IHH patients have mutations in the genes identified so far and hence some of these individuals with *CHD7* mutations with IHH may harbor variants in novel IHH genes that are yet to be identified.

### **Pathogenic *CHD7* missense alleles disrupt nucleosome remodeling activity of *CHD7***

The precise molecular mechanisms underlying missense mutations causing IHH has been recently examined. Using site-directed mutagenesis, Bouazoune et al engineered *CHD7* missense mutations identified in IHH and examined ATPase and nucleosome remodeling activity of mutant and WT *CHD7* (Bouazoune and Kingston 2012). Three chromodomain mutations previously linked to IHH were examined: one mutation affecting a highly conserved sequence motif in the first chromodomain (S834F) and two mutations affecting the second chromodomain of *CHD7* (K907T; T917M). The mutant S834F protein completely abolished both ATPase and nucleosome remodeling activities by *CHD7*. In contrast, the mutant K907T protein, and to a lesser extent, T917M protein, showed only reductions in ATPase activity and nucleosome-remodeling capabilities compared with WT. These results suggest the milder missense *CHD7* alleles linked to IHH impair *CHD7* related nucleosome remodeling function and this defect may underlie the pathologic human phenotypes such as IHH.

### **Insights into the role of *CHD7* gene in the ontogeny of GnRH neurons: Lessons from humans with IHH, CHARGE and from mouse models of CHARGE syndrome**

The precise role of *CHD7* in the ontogeny of the GnRH neurons is under active investigation. Comprehensive human and mouse embryonic expression analysis shows ubiquitous *CHD7* expression during early development (Layman et al. 2009; Sanlaville et al. 2006; Bergman et al. 2010; Layman, Hurd, and Martin 2011). Specifically, in relation to the recognized endocrine phenotypes that primarily affect the neuroendocrine hypothalamus and pituitary, *CHD7* is widely expressed in the early in the undifferentiated neuroepithelium, neural crest derived mesenchyme, and later in the nasal epithelia, olfactory bulbs and nerves, and also in the anterior and median lobes of pituitary (Layman et al. 2009; Sanlaville et al. 2006; Bergman et al. 2010; Layman, Hurd, and Martin 2011). This pattern of expression is consistent with the endocrine phenotypes of HH and other rare anterior pituitary hormone defects in CHARGE.

### **Embryonic GnRH neuronal migration and pubertal timing is disrupted in human CHARGE syndrome and murine CHARGE syndrome models**

Given the intricate relationship and joint origin of the olfactory axons and the GnRH neurons, studies in a human fetus with CHARGE syndrome has confirmed that the CHARGE fetus showed arhinencephaly (olfactory bulb agenesis) with absence of GnRH neurons in the forebrain (Teixeira et al. 2010). The migrating olfactory and GnRH neurons accumulated in the fronto-nasal region with the formation of bilateral spherical structures



consistent with neuromas near the cribriform plate, implicating a significant migratory defect (Teixeira et al. 2010). In keeping with these observations, *Chd7* deficient mice display smaller olfactory bulbs, reduced number of olfactory sensory neurons, defective senses of smell with loss of odor-evoked electro-olfactogram responses, reduced hypothalamic GnRH neuronal numbers, hypoplastic genitalia, hypogonadotropism and impaired pubertal timing (Bergman et al. 2010; Layman, Hurd, and Martin 2011; Layman et al. 2009). In addition, they show significant defects in neural stem proliferation with reductions in production, proliferation and regeneration of olfactory sensory neurons (Layman et al. 2009). These complementary observations in humans and mice provide compelling evidence that the reproductive deficits in CHARGE are secondary to impaired GnRH migration and consequent GnRH deficiency. The GnRH deficiency in CHARGE syndrome, thus results from the olfactogenital pathological sequence, implicating the disruption of olfactory axon migration to the forebrain as the primary cause of the defective migration of GnRH synthesizing cells, which normally migrate along these nerve fibers.

### **Pathogenic CHD7 mutations in humans cause both KS and nIHH suggesting additional role(s) for CHD7 in GnRH neuronal integrity beyond its migratory defects**

KS and nIHH represent the two distinct phenotypic forms of IHH, the former pointing to early neurodevelopmental failure of GnRH neuronal migration during development while nIHH represents neuroendocrine failure of GnRH secretion/action within the hypothalamus, i.e. much later in their developmental history (Balasubramanian et al. 2010). The association of pathogenic *CHD7* mutations with both KS and nIHH is thus intriguing and suggests that *CHD7* may have temporal and spatially distinct actions on GnRH neurons, i.e. initially during embryonic development and then also later in the regulation of GnRH neuronal function in the hypothalamus. Alternatively, deleterious missense *CHD7* alleles associated with IHH may cause subtle olfactory phenotypes that may require detailed olfactory imaging studies to fully assess olfactory function. Another possibility is that olfactory phenotype may be weakly penetrant in the context of *CHD7* missense alleles and in this regard, it is notable that in *Chd7* mutant mice, the penetrance of olfactory agenesis is significantly lower than that seen in human individuals with CHARGE (Bergman et al. 2010), suggesting that *CHD7* disruption can result in variable olfactory defects. However, the precise role of *CHD7* mutations in nIHH is still unclear and requires further evaluation.

### ***CHD7* regulates neural crest related genes and *CHD7* related CHARGE/IHH represents a likely ‘neurocristopathy’**

Cranial neural crest cells are a transient, multi-potent, migratory cellular population (Bronner 2015) arising adjacent to the olfactory placode from where GnRH neurons also emerge. It has been hypothesized that the myriad organ systems affected by CHARGE relate to abnormalities in neural crest development and in their various crest-derived cell types (Siebert, Graham, and MacDonald 1985). Recent studies in humans (Bajpai et al. 2010; Schulz et al. 2014) and *Xenopus* (Schulz et al. 2014) have shown an evolutionarily conserved role for *CHD7* in the formation of multipotent migratory neural crest cells and for activation of their transcriptional circuitry. Genome-wide microarray expression analysis on wild-type and *Chd7*<sup>-/-</sup> mouse embryos has identified 98 differentially expressed genes

downstream of CHD7, several of which are known to be involved in neural crest cell and axon guidance (Schulz et al. 2014). Accumulating data from mouse and zebrafish suggest that a small (~30%) but significant subset of GnRH neurons may have a neural crest origin (Whitlock et al. 2006; Forni et al. 2011). In addition, biallelic expression of *Chd7* has been shown to be essential for maintaining appropriate levels of *Fgf8* expression during cerebellar development (Basson et al. 2008; Yu et al. 2013). *FGF8* is involved in the maintenance of progenitor status and in fate determination of cranial neural crest cells (Shao et al. 2015). Given that *FGF8* mutations are also known to cause Kallmann Syndrome (Falardeau et al. 2008), the emerging evidence of epistatic interactions between *Chd7* and *Fgf8* provides validation to the relevance of neural crest cells to GnRH neurogenesis. Recently, mutations in *SOX10*, a key neural crest cell specific transcription factor has also now been shown to be an important genetic cause for the KS form of IHH (Pingault et al. 2013). *Chd7* is known to interact with *Sox10* (Hebert et al. 2003) and recent zebrafish *Chd7* morphant model of CHARGE has also shown *Sox10* deregulation is an important driver of the neural crest-derived aspects of CHARGE syndrome. In addition to its interaction with *Sox10*, *Chd7* has also been identified as a *Sox2* transcriptional cofactor with direct physical interaction between *Sox2* and *Chd7* with overlapping genome-wide binding sites (Engelen et al. 2011; Puc and Rosenfeld 2011), again attesting to a critical role for CHD7 during embryonic neurogenesis. Taken together, several of the CHARGE features, including IHH, may represent direct phenotypic consequences of neural crest dysregulation, supporting the hypothesis that CHD7-associated CHARGE and IHH may represent novel neurocristopathies.

## Conclusions and future directions

Endocrine abnormalities constitute a significant morbidity to patients with CHARGE syndrome that should be screened for and treated. Disruption of *CHD7* in humans causes a spectrum of human phenotypes ranging from severe CHARGE phenotypes at one of the spectrum while allelic phenotypes affecting selected organ systems, such as those leading to IHH lie at the other end of the spectrum. Also, this spectrum of disorders may represent novel neural crest cell related human phenotypes. Although, it is clear that CHD7 protein's chromatin remodeling activity is critical for the ontogeny of olfactory and GnRH neurons during development, the precise cellular mechanisms that underlie the disruption of this dynamic migratory journey remain to be fully deciphered. In addition, humans with *CHD7* mutations, especially missense alleles, display incomplete penetrance and are likely to harbor additional epistatic mutations in other genes that are yet to be fully identified. With increasing availability of next generation sequencing, additional putatively causal *CHD7* alleles and mutations in other novel genes governing GnRH ontogeny are likely to be uncovered. However, to fully understand the molecular and biologic basis of CHD7-associated CHARGE and IHH, there is an urgent need for developing high throughput and rapidly accessible assay platforms for ascertaining the functional consequence of these mutations. Such functional studies are imperative for accurate clinical interpretation of identified *CHD7* mutations and genetic counseling of patients and their families. This pressing need for understand the systems biology of CHD7-related human disorders can only be met by developing multidisciplinary collaborative teams involving clinicians,

physician-scientists, geneticists and basic investigators. Detailed phenotyping of patients harboring *CHD7* mutations will uncover the true spectrum of phenotypes associated with these mutations and accurate functional interpretation of these mutations will require development of patient-derived induced pluripotent cell models.

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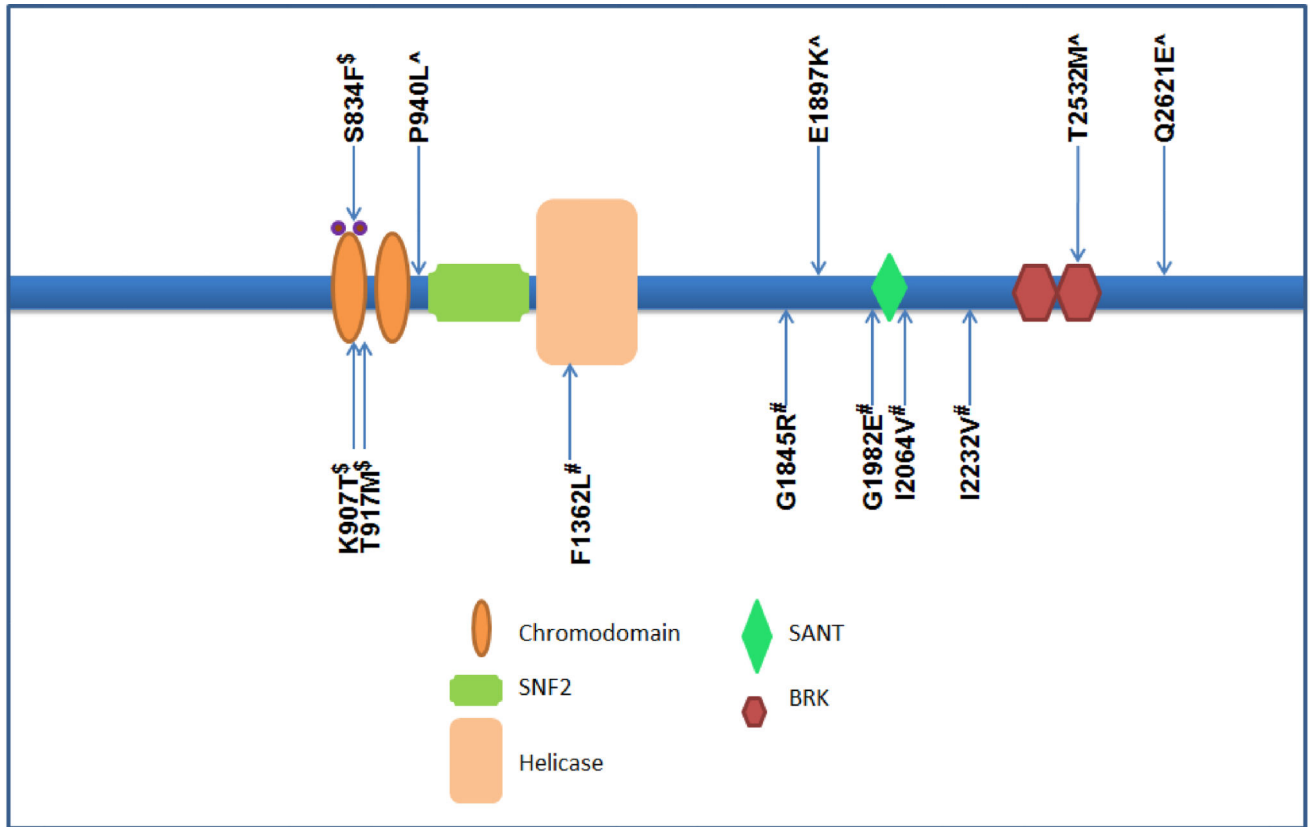
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**Figure 1.**

Schematic of CHD7 protein, its domains and published IHH-associated functionally validated *CHD7* mutations, missense and two deleterious splice variants are shown. <sup>^</sup>Variants pathogenic and shown to be dominant on zebrafish assays (Balasubramanian and others 2014); <sup>#</sup>Variants pathogenic and shown to be hypomorphic in zebrafish assays (Balasubramanian and others 2014); <sup>\$</sup>Variants pathogenic and shown to be loss of function in ATPase and nucleosome remodelling *in vitro* assays (Bouazoune and Kingston 2012).



**Table I**

Rare heterozygous variant burden for CHD7 missense mutations in IHH vs. ExAC database

Minor Allele Frequency of <i>CHD7</i> missense variant	Cases (n=703)	Controls (n=33,370)	Odds Ratio (95% CI)	P-value (Fischer's Exact)
< 1%	40/703 (5.7%)	2108/33370 (6.3%)	0.9 (0.6–1.2)	0.58
< 0.5%	32/703 (4.5%)	1316/33370 (3.9%)	1.2 (0.8 –2.7)	0.38
< 0.1%	32/703 (4.5%)	1005/33370 (3%)	<b>1.5 (1.1 –2.2)</b>	<b>0.03</b>

Cases represent 703 IHH patients (Non-Finnish European ancestry) from the MGH Reproductive Endocrine Unit. Controls represent 33,370 subjects of Non-Finnish European ancestry in the ExAC database (Karczewski et al. 2017).

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