

Functionally compromised *CHD7* alleles in patients with isolated GnRH deficiency

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Inactivating mutations in chromodomain helicase DNA binding protein 7 (*CHD7*) cause CHARGE syndrome, a severe multiorgan system disorder of which isolated gonadotropin-releasing hormone (GnRH) deficiency (IGD) is a minor feature. Recent reports have described predominantly missense *CHD7* alleles in IGD patients, but it is unclear if these alleles are relevant to causality or overall genetic burden of Kallmann syndrome (KS) and normosmic form of IGD. To address this question, we sequenced *CHD7* in 783 well-phenotyped IGD patients lacking full CHARGE features; we identified nonsynonymous rare sequence variants in 5.2% of the IGD cohort (73% missense and 27% splice variants). Functional analyses in zebrafish using a surrogate otolith assay of a representative set of these *CHD7* alleles showed that rare sequence variants observed in controls showed no altered function. In contrast, 75% of the IGD-associated alleles were deleterious and resulted in both KS and normosmic IGD. In two families, pathogenic mutations in *CHD7* coexisted with mutations in other known IGD genes. Taken together, our data suggest that rare deleterious *CHD7* alleles contribute to the mutational burden of patients with both KS and normosmic forms of IGD in the absence of full CHARGE syndrome. These findings (i) implicate a unique role or preferential sensitivity for *CHD7* in the ontogeny of GnRH neurons, (ii) reiterate the emerging genetic complexity of this family of IGD disorders, and (iii) demonstrate how the coordinated use of well-phenotyped cohorts, families, and functional studies can inform genetic architecture and provide insights into the developmental biology of cellular systems.

CHD7 | Kallmann syndrome | idiopathic hypogonadotropic hypogonadism | CHARGE syndrome | missense mutations

Inactivating mutations in the gene encoding chromodomain helicase DNA binding protein 7 (*CHD7*, MIM 608892) cause a severe developmental disorder that affects multiple organ systems and is referred to as CHARGE syndrome (Coloboma, Heart defects, choanal Atresia, Retardation of growth and development, Gonadal defects, and Ear/hearing abnormalities, MIM 214800) (1–3). More than 90% of the reported mutations causing this syndrome are de novo severe truncating lesions that are equally distributed across *CHD7*'s 37 exons and various functional domains (3–6). The “gonadal defect” in CHARGE patients is in fact hypogonadotropic hypogonadism, which is typically responsive to exogenous gonadotropin-releasing hormone (GnRH) administration: that is, they represent isolated GnRH deficiency (IGD) (7–11).

IGD is an inherited reproductive disorder caused by defects in the secretion of GnRH and its action on the anterior pituitary gonadotrope (12). Around 60% of IGD patients also manifest a variety of nonreproductive defects, principally anosmia, which defines Kallmann syndrome (KS), whereas the remainder exhibit a pure nonsyndromic neuroendocrine phenotype, referred to as normosmic IGD (nIGD) (12, 13). In KS, a shared migratory

defect of GnRH and olfactory axons produces IGD and anosmia, respectively, whereas other defects result from perturbation of other developmental pathways and processes (14). In contrast, the biological basis of nIGD cases resides in the failure of one or more hypothalamic neuroendocrine systems (12).

Several recent reports have suggested that rare sequence variants (RSVs) in *CHD7* occur in ~6% of IGD patients either with (9, 15) or without (8) any features of CHARGE syndrome. Although the initial report implicated *CHD7* in both KS and nIGD (8), two subsequent studies detected putative pathogenic *CHD7* RSVs in KS patients only (9, 16). However, the possible effects of these IGD-associated *CHD7* variants on protein function have not been investigated to date (8, 9, 15, 16). Therefore, we sought to: (i) determine the frequency spectrum, phenotypes, genotypes, and mode of inheritance of *CHD7* RSVs in a large, well-characterized cohort of IGD; (ii) characterize functionally a representative spectrum of these IGD- and CHARGE-associated RSVs; and (iii) consider whether differential activity of the protein, or loss thereof, contributes to KS/nIGD/CHARGE or other specific endophenotypes.

Significance

Inactivating mutations in the chromodomain helicase DNA binding protein 7 (*CHD7*) gene causes a severe developmental disorder known as CHARGE syndrome. Recently, several missense mutations in *CHD7* have been reported in isolated gonadotropin-releasing hormone (GnRH)-deficiency (IGD) patients who lack full CHARGE features. However, the precise functional consequence of these IGD-associated missense mutations on the activity of *CHD7* protein is not known. This study confirms the predominance of missense *CHD7* alleles in 5% of IGD patients and provides, to our knowledge, first experimental evidence that functionally compromised *CHD7* missense alleles contribute to the pathogenesis of both the anosmic and normosmic forms of IGD. These results imply a preferential sensitivity for *CHD7* dysfunction in the developmental ontogeny as well as neuroendocrine regulation of GnRH neurons in humans.

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Results

RSVs in *CHD7* Are Common in IGD Patients. All 37 exons and intron-exon junctions of *CHD7* were sequenced in 783 well-phenotyped patients with IGD. Following further detailed phenotypic review of patients with demonstrable mutations, four from the original cohort with an initial diagnosis of IGD were reclassified as CHARGE syndrome. Overall, we identified *CHD7* RSVs in 41 IGD patients (5.2%) lacking full CHARGE features (Fig. 1 and Table S1), a number consistent with previous reports (8, 16). Only 9 of 41 of these *CHD7*⁺ IGD patients showed evidence of minor CHARGE features (Table S2). In keeping with a recent report (16), a striking feature of this cohort was that, in contrast to the truncating *CHD7* mutations observed typically in patients with CHARGE, patients with IGD harbored predominantly missense variants ($n = 30$ of 41; 73%), with a smaller number of patients harboring predicted splice-variants ($n = 11$ of 41; 27%). All patients harboring *CHD7* RSVs were heterozygotes except for two individuals who each displayed two RSVs; however, no parental DNAs were available to determine their phase. All four patients who fulfilled the Verloes criteria for full CHARGE syndrome (17) harbored a single heterozygous *CHD7* RSV (one nonsense, one frameshift, and two missense variants) (Table S3). All RSVs identified in IGD patients and in full CHARGE patients are shown in Fig. 1.

IGD and CHARGE Patients Are Enriched for Functionally Deleterious RSVs. Computational analysis of the functional impact of the identified *CHD7* variants was performed using multiple software programs (*SI Materials and Methods*). Splice sequence analysis using three prediction programs predicted that only one putative splice variant would affect splice efficiency/utilization in two or more programs (Table S4). Modeling of the missense alleles produced significant discordance in the various predictions algorithms (Table S5). Given the known ambiguity of these prediction programs (18), we assessed the functionality of the IGD-associated RSVs using an in vivo zebrafish model. We selected a representative spectrum of 20 different nonsynonymous missense RSVs for extensive functional analyses based on their population frequency (Table S6) and associated human phenotypes (Tables S1–S3). The RSVs chosen for these analyses fell into three groups: (i) Group A (control alleles; $n = 3$): RSVs seen in IGD subjects but also seen with $\geq 0.5\%$ minor allele frequency (MAF) in the National Heart, Lung, and Blood Institute (NHLBI) exome database (p.S103T, p.M340V, p.L2984F) (18); (ii) Group B (alleles associated with IGD without full CHARGE; $n = 12$): RSVs (MAF < 0.5%) consisting of six alleles found in IGD subjects lacking any CHARGE features (p.Y1616C, p.G1982E, p.I2064V, p.I2232V, p.T2532M, p.Q2621E), three from IGD patients without any CHARGE features but who carried additional heterozygous mutations in other genes previously identified to be associated with IGD (p.G1845R, p.E1897K, p.A2789V), and three from patients with IGD who had minor CHARGE features (p.P940L, p.F1362L, p.R2065G); and (iii) Group C (alleles associated with full CHARGE; $n = 5$): RSVs (MAF < 0.5%) from CHARGE syndrome patients identified either in this study (p.V1021G, p.A1289V) or reported previously in

the *CHD7* database (p.I1028V, p.D1596G, p.D1812H) (www.chd7.org/molgenis.do) (6).

To test the consequences of these missense mutations on protein function, we injected a previously reported splice-blocking morpholino (MO) against *chd7* (*chd7*-MO) (19) into one- to four-cell-stage zebrafish embryos. Consistent with previous reports (19), injected embryos displayed abnormal otoliths at 3 days postfertilization (dpf), one of the many CHARGE phenotypes. We grouped the resulting embryos into four objective classes: normal, small (posterior otolith same size or smaller to anterior otolith), fused/three, or absent otoliths. Each of the 20 *CHD7* missense RSVs was tested for either its relative ability to rescue the morphant phenotype or its ability to phenocopy MO-induced otolith defects when overexpressed (under a dominant paradigm). For in vivo rescue, the *chd7*-MO (6 ng) and each mutant *CHD7* missense mRNA (5 pg) were injected and each experiment was scored blind to injection mixture and performed in triplicate. All morphant phenotypes could be rescued by coinjection of WT human *CHD7* mRNA (Fig. 2A, Inset F), whereas WT overexpression in the absence of MO produced no appreciable phenotypes (Fig. 2C). Because the mutant *CHD7* S103T control allele was able to rescue the morphant phenotype indistinguishable from WT human *CHD7* mRNA, all of the rescue experiments for mutant *CHD7* alleles were statistically compared against the S103T allele (Fig. 2B). Results for all mutant alleles on both assays are presented except for the Q2621E allele for which the rescue experiment failed and is not shown.

All three *CHD7* RSVs from group A (control alleles) were able to rescue morphant phenotypes in a manner indistinguishable from WT human *CHD7* and did not show any dominant activity upon overexpression (Fig. 2B and C). These data support the population frequency-derived prediction that group A alleles, with a MAF > 0.5%, are likely benign and bolstered confidence on the specificity of the assay. Among the 12 *CHD7* alleles found in IGD patients without full CHARGE (group B), five alleles (p.F1362L, p.G1845R, G1982E, p.I2064V, and p.I2232V) showed partial loss of function (LOF) in that the frequency of abnormal otolith phenotypes was significantly better than MO alone yet significantly worse than controls ($P < 0.05$ for each comparison, scored blind and in triplicate), indicating they were likely hypomorphic alleles (Fig. 2B). Four alleles (p.P940L, p.E1897K, p.T2532M, and p.Q2621E) showed a dominant effect in the overexpression assay (i.e., each induced the absence of otoliths in a small but significant number of embryos) ($P < 0.05$) (Fig. 2C). Three alleles (p.Y1616C, p.R2065G, and p.A2789V) tested benign in both assays (Fig. 2B and C). Thus, 75% (9 of 12) of IGD-associated (group B) missense RSVs tested in the functional assays were either hypomorphic or dominant, indicating an enrichment of functionally deleterious variants in this group compared with the controls in group A. Of the five alleles associated with the full CHARGE syndrome in group C, one was null (p.I1028V), two were hypomorphic (p.D1596G and p.D1812H), and two (p.A1289V and p.V1021G) were dominant (Fig. 2B and C).

Finally, integration of 3D structural modeling of *CHD7* using an automated homology model by SWISS-MODEL

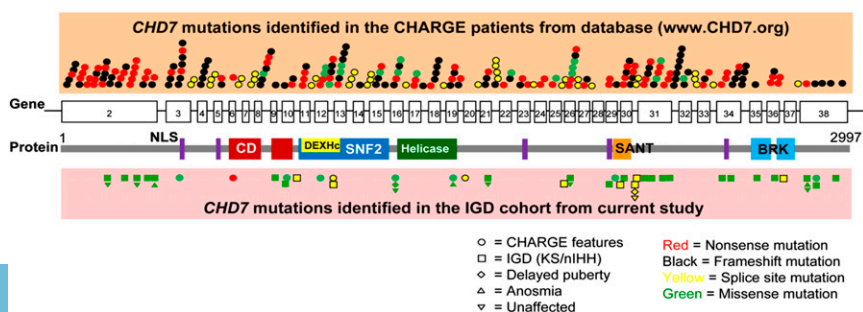


Fig. 1. *CHD7* protein domains and positions of RSVs identified in CHARGE syndrome and IGD. Both CHARGE- and IGD-associated variants in *CHD7* were equally dispersed across its 37 exons without mutational hot spots. Most mutations identified in CHARGE syndrome are nonsense or frameshift, whereas the vast majority of RSVs found in IGD are missense variants. RSVs identified in family members with delayed puberty, anosmia, and unaffected carriers were depicted along with RSVs seen in probands. BRK, Brahma and Kismet domain; CD, chromodomain; DEXHC, DEAD-like helicase superfamily including an ATP-binding domain; NLS, nuclear localization signal.

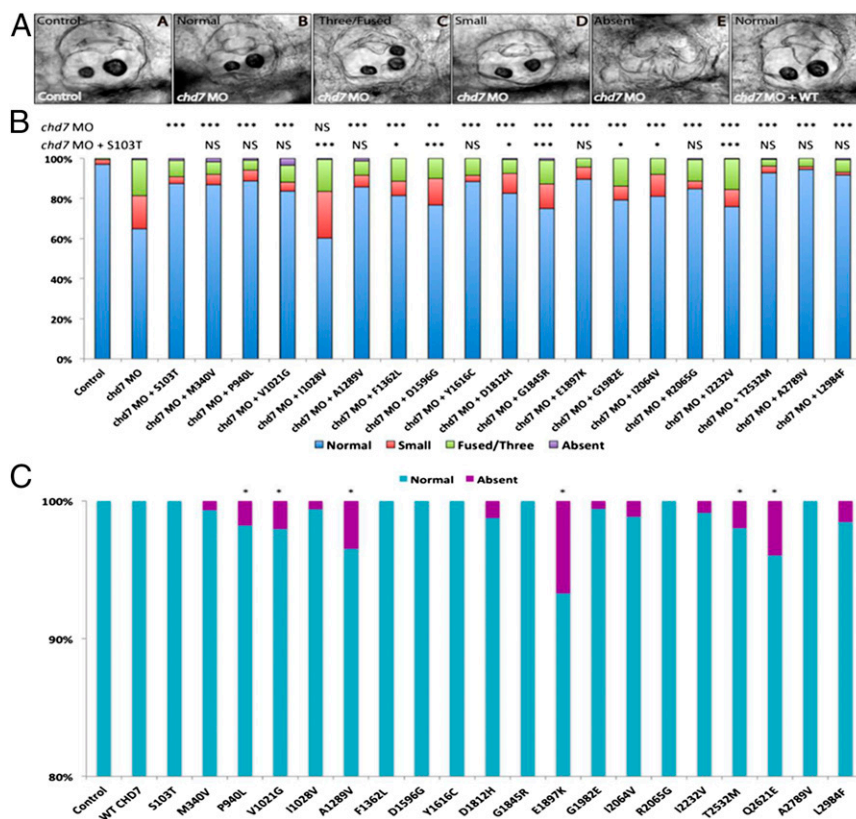


Fig. 2. Functional assessment of *CHD7* RSVs in zebrafish assay. (A) The otic vesicle of control-MO-injected zebrafish contains two otoliths (Inset A). Larva treated with an MO against *chd7* show normal (Inset B), three/fused (Inset C), or small (Inset D) or absent (Inset E) otoliths, whereas coinjection of WT human *CHD7* rescues the morphant phenotype showing normal two otoliths (Inset F). (B) In vivo rescue assay: Coinjection of human *CHD7* mRNA encoding mutant S103T allele along with *chd7* MO results in significant rescue of morphant phenotype indistinguishable from WT rescue. Coinjection of *chd7* MO with human *CHD7* mRNAs encoding the two other control alleles (p.M340V, p.L2984F) from group A (see main text) shows rescue similar to S103T allele. Five IGD-associated *CHD7* alleles (p.F1362L, p.G1845R, G1982E, p.L2064V, and p.L2232V) from group B (see main text) were partial LOF alleles (hypomorphs). Of the CHARGE-associated *CHD7* alleles (group C, see main text), one was complete LOF (null) (p.I1028V) and two were hypomorphic (p.D1596G and p.D1812H). The remaining *CHD7* alleles were benign on the rescue assay. (C) Overexpression of human mutant *CHD7* mRNAs in zebrafish: overexpression of the human WT *CHD7* mRNA did not induce any appreciable phenotypes. Four IGD-associated *CHD7* alleles (p.P940L, p.E1897K, p.T2532M, and p.Q2621E) from group B (see main text) and two CHARGE-associated *CHD7* alleles (p.A1289V and p.V1021G) from group C (see main text) showed a dominant effect in the overexpression assay. The remaining *CHD7* alleles were benign on the overexpression assay. *** $P < 0.0005$, ** $P < 0.005$, * $P < 0.05$; NS, not significant.

(swissmodel.expasy.org/) (20) or the FoldX protein design algorithm (21) predicted that four variants (p.P940L, p.V1021G, p.A1289V, p.G1982E) disrupted the 3D protein structure of *CHD7* (Fig. 3) and all of these four variants were also determined to be deleterious RSVs from the zebrafish experiments. These variants were located in the chromo, SNF2, or helicase domains, all known to play important roles in chromatin remodeling (22).

Deleterious *CHD7* Missense Variants Cause both KS and nIGD Without Full CHARGE. A total of 10 IGD subjects with both phenotypic forms ($n = 6$, KS; $n = 4$, nIGD) harbored pathogenic *CHD7* missense mutations (Table 1). Only two of these patients had additional features of CHARGE but none fulfilled the Verloes criteria for full typical/atypical CHARGE syndrome (Table 2). However, when taking into consideration the presence of any CHARGE feature in patients harboring these deleterious *CHD7* variants (Tables 1 and 2), patients with minor/full CHARGE were associated significantly with the KS phenotype ($P = 0.0165$), suggesting that the KS form of IGD is the primary allelic variant of CHARGE syndrome rather than its normosmic counterpart. However, the occurrence of deleterious *CHD7* RSVs in the normosmic patients suggests that *CHD7*'s effect may not be limited to GnRH migration alone. In addition, one nIGD subject (Pedigree #35) (Fig. 1 and Table 1) had an adult-onset presentation of IGD, further confirming a neuroendocrine role for

CHD7 in the pathogenesis of IGD. The clinical characteristics of patients with benign *CHD7* alleles are also shown in Table S7. The clinical characteristics of patients with *CHD7* alleles that were not tested in zebrafish assay are shown in Table S8.

Segregation Analysis Suggests Complex Inheritance Patterns. For functionally deleterious *CHD7* variants in the IGD pedigrees where inheritance information was available, none were de novo nor was there any apparent parent of origin bias (Fig. 4 and Table 1). These findings contrast previously reported CHARGE-causing variants that are typically de novo and show a preference for paternal inheritance (23). Segregation analysis also showed that the discovered *CHD7* alleles are not sufficient to explain the observed phenotypes (Fig. 4). In two families, the pathogenic *CHD7* variants were found in IGD patients who also carried a known pathogenic mutation in a second IGD gene (*FGFR1* in pedigree #24 and *GNRHR* in pedigree #25), raising the possibility that some *CHD7* alleles might interact genetically with other IGD mutations and thus contribute to the reported oligogenic nature of IGD (24) (Fig. 4 and Table 1). However, interpreting such genetic architecture based on a small number of candidate epistatic events must be done with caution because segregation analysis of the *CHD7* alleles was incomplete in these oligogenic pedigrees (Fig. 4). However, in both of these oligogenic pedigrees, the presence of another IGD-associated genetic variant did not

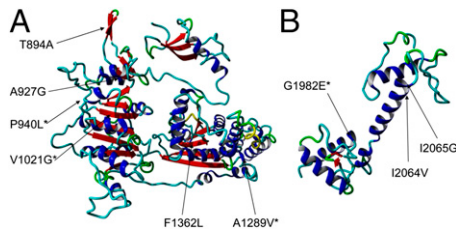


Fig. 3. Structural modeling of the CHD7 chromo- and helicase domains (amino acids 799–1511) (A) and SANT domain (amino acids 1964–2115) (B) showing four variants (p.P940L, p.V1021G, p.A1289V, and p.G1982E) that were predicted to be detrimental to protein stability by FoldX algorithm (asterisk).

appear to heighten the occurrence of any CHARGE-related phenotypes; that is, these patients exhibited IGD only. This observation suggests that these other IGD-causing genes probably lack direct functional synergy with *CHD7* in the developmental pathways involved in the non-GnRH organ systems involved in CHARGE syndrome.

Discussion

In this study, a large, well-phenotyped cohort of IGD patients, with both KS and nIGD that were assembled without preselection for or against known mutations in known IGD genes, were analyzed to assess the contribution of *CHD7* to IGD. In contrast to truncating *CHD7* mutations occurring in typical CHARGE syndrome, ~5.2% of IGD patients without full CHARGE features harbored heterozygous, nonsynonymous missense or splice-altering variants in *CHD7*. The observed prevalence and the predominantly missense nature of the *CHD7* variants is in accordance with recent reports (8, 16). However, to our knowledge for the first time, the functional impact of a representative subset of the missense variants in *CHD7* was assessed in a zebrafish assay and compared with functional effects of low-frequency missense RSVs represented in the public Exome Sequencing Project (ESP) database. Whereas all variants from the ESP database tested benign, ~75% of the tested IGD-associated *CHD7* RSVs were pathogenic, acting either as clear hypomorphs that were unable to rescue the *CHD7*-MO phenotype or as dominant alleles when overexpressed. On phenotypic analysis, most IGD-patients with

deleterious missense *CHD7* alleles did not fulfill full CHARGE criteria, thus providing functional molecular validation that the nonsyndromic form of IGD is indeed an allelic variant of CHARGE syndrome. Segregation analysis of deleterious *CHD7* variants shows incomplete penetrance and variable expressivity, suggesting that these deleterious variants contribute to the IGD phenotype but by themselves may be insufficient to cause it.

KS and nIGD represent the two distinct phenotypic forms of IGD. The KS phenotype results from the neurodevelopmental failure of GnRH neuronal migration during development and nIGD results from the neuroendocrine failure of GnRH secretion/action within the hypothalamus (12, 13). Whereas most genes exclusively cause either the KS or nIGD forms of the disease, a subset of genes (e.g., *FGF8/FGFR1*, *PROK2/PROKR2*) are associated with both KS and nIGD, indicating either (i) two potentially different pathophysiologic sites of actions on the ontogeny of GnRH neurons or (ii) the potential for dissociated effects on the ontogeny of GnRH neurons and olfactory axons, respectively (12, 13). Although the initial report by Kim et al. (8) implicated *CHD7* in both KS and nIGD, subsequent investigators have failed to identify any predicted pathogenic *CHD7* RSVs in nIGD (9, 16). However, some nIGD subjects did harbor rare *CHD7* alleles but these were deemed to be benign based on prediction programs, which are recognized to be prone to ambiguity (16). The present study was able to address these issues by performing functional studies and by examining the phenotypes of patients with pathogenic *CHD7* alleles, this study provides key insights into the role of *CHD7* in GnRH neuronal ontogeny. Functionally deleterious *CHD7* mutations were found in both KS and nIGD subjects, from which it can be inferred that *CHD7* likely has both distinct developmental as well as neuroendocrine roles. Moreover, four subjects with pathogenic *CHD7* alleles also had micropenis or cryptorchidism, implying an embryonic defect in GnRH development and secretion. These results also indicate that future genetic evaluations of all nonsyndromic KS and nIGD should be screened for *CHD7* for sequence variants.

To date, only a few cases of parent-to-child transmission have been reported in CHARGE pedigrees with *CHD7* mutation (23, 25). In addition, over 80% of CHARGE-causing *CHD7* mutations are null alleles (5, 6). In our IGD patients, however, not only were *CHD7* variants inherited (where possible to assess), but most IGD-associated *CHD7* variants were either hypomorphs

Table 1. Clinical characteristics of IGD patients without full CHARGE harboring pathogenic *CHD7* mutations by zebrafish model

Pedigree No.	Sex	Origin	De novo	Diagnosis	Olfaction	Nucleotide change	Amino acid change	Exon/Intron	Mutations in other genes
11*	M	Caucasian	NA	KS	Self-reported anosmia	2819C > T	P940L	10	None
12	M	Caucasian	NA	nIGD	Self-reported normal	2819C > T	P940L	10	None
18*	M	Caucasian	No	KS	UPSIT hyposmia	4084T > C	F1362L	19	None
24	F	Caucasian	No	KS	UPSIT anosmia	5533G > A	G1845R	26	<i>FGFR1</i> : 710G > A (G237D)
25	M	Asian	NA	nIGD	Self-reported normal	5689G > A	E1897K	29	<i>GNRHR</i> : 836G > A (C279Y) homozygote
28	M	Caucasian	NA	KS	Self-reported anosmia	5945G > A	G1982E	30	None
32	M	African American	NA	KS	UPSIT hyposmia	6190A > G	I2064V	31	None
35	M	Asian	NA	nIGD, adult onset	UPSIT normal	6694A > G	I2232V	31	None
38	F	African American	NA	nIGD	Self-reported normal	7595C > T	T2532M	34	None
44	M	Caucasian	NA	KS	Self-reported anosmia	8405G > A/7861C > G	G2802E/Q2621E	38/36	None

F, female; M, male; NA, not assessed; UPSIT, University of Pennsylvania Smell Identification Test.

*Proband with additional CHARGE features but not fulfilling Verloes CHARGE syndrome criteria.

Table 2. Additional CHARGE features present in IGD patients with CHD7 RSVs that were pathogenic in zebrafish model

Pedigree no.	Diagnosis	Mutation in											Verloes criteria for full CHARGE	
		CHD7	Second hits	C	H	A	RG	RD	G	HL	E	CLP		FA
11	KS	P940L	None	—	—	—	—	—	+	+	—	—	—	No
18	KS	F1362L	None	—	—	—	NA	+	+	+	—	—	—	No

A, atresia of choanae; C, coloboma; CLP, cleft lip/plate; E, external ear defect; FA, facial asymmetry; G, genital defects; H, heart defects; HL, hearing loss; RG, retarded growth; RD, retarded development.

*Probands with additional CHARGE features but not fulfilling Verloes CHARGE syndrome criteria.

or had a modest dominant effect. Thus, it appears that it is the nature and severity of these variants, rather than their location within the *CHD7* gene/protein that is critical for its selective perturbation on developing GnRH neurons producing IGD rather than their multiorgan involvement in CHARGE.

The present study has some limitations. The phenotypic details of additional CHARGE features in IGD patients, as well as phenotypes of parents, were partly ascertained by retrospective review of medical records, physician referral letters, and questionnaire data. Thus, it is possible that a prospective evaluation might uncover additional phenotypic features of CHARGE in these IGD patients. However, because the majority of our patients have had detailed physician reviews either via in-house or through referrals from multiple specialists, major CHARGE features are unlikely to have been missed. Typical of most sequencing studies, deep-intronic and regulatory-regions, deletions, and rearrangements were not evaluated. However, in previous studies of *CHD7* mutations, no such deletions have been found (6, 26). While IGD subjects underwent Sanger sequencing, control *CHD7* alleles were obtained from whole-exome sequencing data from the NHLBI ESP Exome Variant Server and, hence, potentially could be subject to platform-specific biases in variant ascertainment. However, reassuringly, limited validation of ESP singleton variants has been reported to be reliable (27) and hence biases, if any, are unlikely to be significant. Given the complexity of design and conduct of *CHD7* functional assays, only a subset of alleles were examined. However, multiple alleles tested consistently as hypomorphs in the rescue experiments, validating the observed findings. Similarly, some alleles displayed reproducible altered dominant phenotype upon overexpression whereas WT allele overexpression did not produce any altered phenotype. Moreover, all alleles predicted as destabilizing on automated homology modeling were found to be deleterious in zebrafish, further strengthening the observations. Two alternative splice products of the *CHD7* gene are known (28, 29) but the effect the RSVs identified in this study on these splice products were not examined in the present study. Finally,

functional assessment of *CHD7* alleles was ascertained using a surrogate otolith phenotype in zebrafish rather than GnRH or olfactory neuronal phenotypes. However, this assay is an out-standing proxy for *CHD7* function (19) and, hence, the functional deficits observed are unlikely to represent false-positive results. In contrast, there is a possibility that the alleles that tested benign in the otolith assays may actually be detrimental to protein function below the dynamic range of the assay, representing false-negative observations. In addition, four patients with documented hypomorphic/dominant alleles in this study also displayed hearing loss, thus confirming the biological relevance of the examined zebrafish phenotype (Tables 1 and 2 and Table S3).

The question of where *CHD7* fits into the developmental biology of the GnRH neurons remains a mystery. Mice with complete *Chd7* deficiency mirror the observed human phenotypes (delayed puberty, genital hypoplasia, hypogonadotropism) and display neurodevelopmental defects, including reduced GnRH neurons in the hypothalamus and reduced expression of *FGFR1* in the olfactory placode (30, 31). Recent work also suggests that *CHD7* regulates genes involved in neural crest guidance (32), and there is increasing evidence that KS may also result from impaired neural crest guidance (33). Taken together, these observations suggest that *CHD7* plays an important neurodevelopmental role in the formation of the hypothalamic-pituitary unit (31). That rare pathogenic *CHD7* variants are associated with nIGD suggests that in addition to its neurodevelopmental role, *CHD7* may well also influence subsequent neuroendocrine development pathways of GnRH neurons. The striking predominance of missense *CHD7* variants in IGD implies that this gene and its associated biological pathways are especially critical for the ontogeny of GnRH neurons. Hence, working out the function of *CHD7* via comparison of missense vs. truncating mutations might offer a route to unraveling its unique biological role in this complex system.

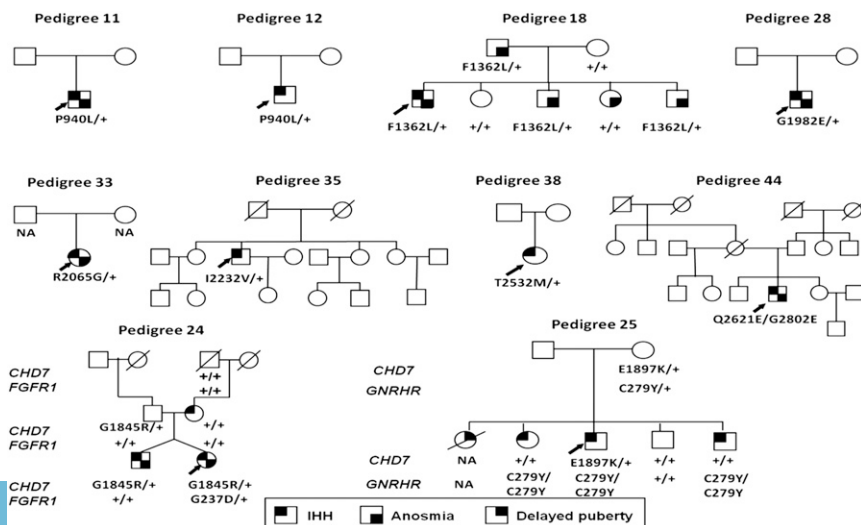


Fig. 4. Family pedigrees of probands with pathogenic *CHD7* mutations. Pedigrees #11, #12, #18, #28, #33, #35, #38, and #44 harbored *CHD7* mutations alone, and pedigrees #24 and #25 harbored *CHD7* mutations oligogenic with *FGFR1* and *GNRHR* genes, respectively. Among familial cases, pedigree #24 and pedigree #25 display incomplete penetrance for *CHD7* mutations and pedigree #18 displays variable expressivity of GnRH deficiency/anosmia phenotypes for the *CHD7* mutation. Probands are identified by arrows. “+” indicates WT allele.

Materials and Methods

Study Subjects and Phenotypic Information. Included in this study were 783 consecutive patients with IGD (367 KS, 411 nIGD) from the Reproductive Endocrine Unit of Massachusetts General Hospital, who underwent genetic screening for 14 genes associated with IGD (see below). Diagnostic criteria for IGD and CHARGE syndrome criteria are provided in *SI Materials and Methods*. Phenotypic records from individual medical charts, patient questionnaires, and referring provider's summary were reviewed to ascertain additional phenotypic information related to CHARGE syndrome, such as coloboma, choanal atresia, external ear defects, cardiovascular malformations, cleft lip/palate, and developmental delay. Study protocols were approved by the Massachusetts General Hospital/Partners Institutional Review Board and informed consent obtained from all participants.

Genetic Analyses. Genomic DNA was isolated from peripheral blood lymphocytes. In all IGD subjects, the 37 coding exons of *CHD7* (exons 2–38) and their intronic flanking regions were amplified by PCR and directly sequenced, as described previously (9). The GenBank accession number NM_017780.2 was used as reference sequence for *CHD7*. In addition, all coding exons and intronic flanking regions of the *GNRH1* (Gonadotropin-releasing hormone 1), *GNRHR* (Gonadotropin-releasing hormone receptor), *KISS1* (Kiss-1 metastasis-suppressor), *KISS1R* (Kiss1 receptor), *PROK2* (prokineticin 2), *PROKR2* (prokineticin receptor 2), *TAC3* (tachykinin 3), *TACR3* (tachykinin receptor 3), *FGF8* (fibroblast growth factor 8), *FGFR1* (fibroblast growth factor receptor 1), *KAL1* (kallmann syndrome 1 sequence), *HS6ST1* (heparan sulfate 6-O-sulfotransferase 1), and *NSMF* [NMDA receptor synaptotransmembrane signaling and neuronal migration factor; previously called *NELF* (nasal embryonic leutinizing hormone releasing hormone factor)] genes were amplified by PCR with specific primers and directly sequenced as described previously (24). Protein-altering variants in *CHD7* with a MAF < 0.5% in the NHLBI ESP Exome Variant Server (evs.gs.washington.edu/EVS/) (34) and not reported to be benign polymorphisms in previously published literature (5, 6), or in the *CHD7* database (www.CHD7.org) were considered as RSVs. In addition, splice-site altering variants in sequences within 8 bp of splice junctions (the consensus sequences are AG and GT for the donor and acceptor sites, respectively) with MAF < 0.5% in the NHLBI Exome Variant Server were

also considered as RSVs. For the other 13 IGD genes screened, previously published pathogenic RSVs were included in the oligogenicity analysis.

Generation of Zebrafish *chd7* Knockdown and Rescue Experiments. Zebrafish *chd7* knockdown was done using a splice-blocking MO (TTATTTCTG-GCACTAACCATGTCC), previously reported (19). One- to four-cell-stage zebrafish embryos were injected with 6 ng MO or 5 pg mRNA. Injected embryos were scored live at 3 dpf and classified according to the presence, amount, and size of otoliths. To confirm the specificity of the MO, rescue experiments were performed. Human full-length *CHD7* cDNA was kindly provided by Joanna Wysocka (Stanford School of Medicine, Stanford, CA) and Peter C. Scacheri (Case Western Reserve University School of Medicine, Cleveland, OH). Full-length WT *CHD7* was amplified and cloned into pCS₂. To generate *CHD7* mutations, primers were designed to introduce the specific change and the reaction was performed using QuikChange Site-Directed Mutagenesis kit (Stratagene). *CHD7* WT and mutations were confirmed through Sanger sequencing, cloned into pCS₂ vector, and transcribed in vitro with the SP6 Message Machine kit (Ambion). Next, 10 pg of mRNA was injected alone into zebrafish embryos. For rescue experiments, *CHD7* WT mRNA (5 pg) was coinjected with *chd7*-MO (6 ng).

Statistical Analysis. All zebrafish experiments were repeated at least three times and a Pearson's χ^2 test was used to determine significance between S103T allele rescue vs. mutant rescue and between WT vs. mutant *CHD7* allele overexpression. The χ^2 test was also used to compare the occurrence of KS vs. nIGD in the presence of pathogenic *CHD7* RSVs in IGD patients. A *P* value less than 0.05 were considered as statistically significant.

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