Genetic analysis of CHARGE syndrome identifies overlapping molecular biology

Amanda Moccia, MS1, Anshika Srivastava, PhD1, Jennifer M. Skidmore, BS2, John A. Bernat, MD, PhD3, Marsha Wheeler, PhD4, University of Washington Center for Mendelian Genomics, Jessica X. Chong, PhD4, Deborah Nickerson, PhD4, Michael Bamshad, MD4, Margaret A. Hefner, MS5, Donna M. Martin, MD, PhD1,2 and Stephanie L. Bielas, PhD1

INTRODUCTION

Epigenomic regulation is essential for human development. De novo pathogenic variants in genes that encode chromatin effectors are important for developmental transcriptional plasticity and make important genetic contributions to developmental disorders. CHARGE syndrome (MIM 214800) is an autosomal-dominant multiple congenital anomaly condition. Individuals with CHARGE syndrome present with a combination of distinct findings such as coloboma of the eye, choanal atresia, cleft palate, vestibular abnormalities, cranial nerve and other brain anomalies, and characteristic dysmorphology particularly of the ear and face.1 It occurs in about 1 in 10,000 births worldwide.2 CHARGE syndrome can be caused by heterozygous pathogenic variants in CHD7, encoding chromodomain helicase DNA binding protein 7, an adenosine triphosphate–dependent chromatin remodeler.3 However, a pathogenic variant in CHD7 is present in the majority of affected individuals. However, no causal variant can be found in 5–30% (depending on the cohort) of individuals with a clinical diagnosis of CHARGE syndrome.

Methods: We performed whole-exome sequencing (WES) on 28 families from which at least one individual presented with features highly suggestive of CHARGE syndrome.

Results: Pathogenic variants in CHD7 were present in 15 of 28 individuals (53.6%), whereas 4 (14.3%) individuals had pathogenic variants in other genes (RERE, KMT2D, EP300, or PUF60). A variant of uncertain clinical significance in KDM6A was identified in one (3.5%) individual. The remaining eight (28.6%) individuals were not found to have pathogenic variants by WES.

Conclusion: These results demonstrate that the phenotypic features of CHARGE syndrome overlap with multiple other rare single-gene syndromes. Additionally, they implicate a shared molecular pathology that disrupts epigenetic regulation of multiple-organ development.

Key Words: CHARGE; chromatin; exome; genetics; oligogenicity

1Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan, USA; 2Department of Pediatrics, University of Michigan Medical School, Ann Arbor, Michigan, USA; 3Department of Pediatrics, University of Iowa Carver College of Medicine, Iowa City, Iowa, USA; 4University of Washington Center for Mendelian Genomics, University of Washington, Seattle, Washington, USA; 5Department of Pediatrics, Saint Louis University School of Medicine, St. Louis, Missouri, USA.

Correspondence: Stephanie L. Bielas (sbielas@umich.edu) or Donna M. Martin (donnamm@umich.edu)

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genes other than CHD7 might explain the genetic etiology in individuals not previously diagnosed with CHD7 pathogenic variants. We performed WES for a cohort of 28 individuals with clinical features of CHARGE who had no previous history of genetic testing or tested negative for pathogenic variants, deletions, or duplications in CHD7. WES revealed pathogenic variants in genes previously shown to cause other developmental disorders, including Rubinstein–Taybi syndrome (EP300), Kabuki syndrome (KMT2D), Verheij syndrome (PUF60, MIM 615583), and RERE-associated developmental disorder (RERE, MIM 616975). These results illustrate a higher degree of oligogenicity than previously recognized for individuals with clinical CHARGE features, suggesting common underlying molecular biological mechanisms that contribute to development of affected organs and tissues.

**MATERIALS AND METHODS**

**Subject recruitment and sample collection**

The study protocol was approved by the Institutional Review Board for the Protection of Human Subjects at the University of Michigan Medical School. Individuals with clinical features of CHARGE and their family members were recruited using standard practices at the 12th International CHARGE Syndrome Foundation conference (Chicago, Illinois, 2015), the Pediatrics Genetics Clinic at the University of Michigan C. S. Mott Children’s Hospital, and external health-care facilities. We included individuals with features of CHARGE syndrome for which a genetic etiology had not been identified and excluded individuals with features of CHARGE syndrome with a previously identified CHD7 pathogenic variant. Study participation involved informed written consent, a clinical history questionnaire, and either a saliva or peripheral blood sample. DNA was extracted per the manufacturer’s instructions from saliva samples using DNA Genotek Prep-IT L2P (catalog no. PT-L2P, Ottawa, ON, Canada) or blood samples using Qiagen DNeasy Blood & Tissue Kit (catalog no. 69504, Hilden, Germany). DNA quantification was employed using Life Technologies Quant-iT PicoGreen dsDNA Assay Kit (catalog no. P7589, Eugene, OR, USA) with a microplate reader to obtain 3 μg of DNA for whole-exome sequencing (WES).

**Exome sequencing**

DNA samples were submitted to the University of Washington Center for Mendelian Genomics (UW-CMG) where library construction, WES, and analysis were performed. Briefly, sequencing libraries were generated for each DNA sample in an automated, 96-well plate format (PerkinElmer Janus II). Sample libraries were constructed from 1 μg of genomic DNA, which underwent a series of shotgun library construction steps including acoustic sonication (Covaris), end-repair, A-tailing ligation of unique sequencing adaptors, and polymerase chain reaction amplification. Sample libraries were hybridized to the Nimblegen SeqCap EZ v2.0 target (~36.5 Mb) in multiplex for a period of 72 h. Captured DNA was then purified, polymerase chain reaction amplified, and normalized for sequencing.

Captured DNA was sequenced on Illumina HiSeq machines using paired-end sequencing. Raw sequence data in FASTQ format was aligned to the human genome reference hg19 using the Burrows–Wheeler Aligner algorithm for the generation of BAM files.9 The quality of each sample was assessed for coverage (80% of sequenced target with ≥20× coverage and 90% of target with ≥8× coverage) and transition/transversion ratios. Additionally, samples were quality controlled to confirm sex using PLINK (v1.90b2m) software, and estimations of kinship were corroborated with pedigrees using KING v.1.4.0 software.

**Variant detection, interpretation, and validation**

Variants and indels were detected and genotyped using HaploTypeCaller from Genome Analysis Toolkit with hard filtering parameters. A multisample VCF was generated for all samples following Genome Analysis Toolkit best practices.10 The multisample VCF was annotated using the Variant Effect Predictor (VEP) tool v.83 within GEMINI (http://uswest.ensembl.org/info/docs/tools/vep/index.html). Copy-number variants (CNVs) were detected from exome sequence data using the program CoNIFER.11 GEMINI v.0.19.1 was used to filter variants and indels detected in affected individuals.12 Variants were excluded if allele frequency in reference populations (ExAC, National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP) 6500, or 1000 Genomes) exceeded 0.005 or exceeded 0.05 in the UW-CMG internal dataset. Variant impact determined by Variant Effect Predictor was also utilized to exclude variants and indels with an impact severity of “low” (e.g., intergenic, intronic, synonymous, 5’UTR, and 3’UTR variants). Variant prioritization was based on the following parameters: variant impact, presence in a disease-associated gene, conservation of impacted nucleotide by genomic evolutionary rate profiling (GERP), or predicted deleteriousness (combined annotation dependent depletion score (CADD) > 15).13,14 Interpretation of detected CNVs was based on size, frequency in the UW-CMG internal database, and overlap with known disease-associated regions. In addition, CNVs were filtered based on phenotypic overlap according to the American College of Medical Genetics and Genomics guidelines for postnatal CNV calling.15 Sanger sequencing was used to validate rare, protein-altering variants. Variant segregation was also used to confirm inheritance patterns when family member samples were available.

**RESULTS**

**Cohort**

We assembled a cohort of 28 affected individuals and their parents (Table 1). Proband ranged in age from 18 months to 40 years, with 14 probands being <18 years of age. The majority of probands (26/28) presented with two or more major clinical CHARGE criteria, achieving a diagnosis of...
CHARGE as defined by Hale et al. Proband 517 also exhibited minor clinical features according to the diagnostic criteria (Table 1 and Supplementary Table 1 online). Nineteen of the families in the cohort were trios, one a duo, and the remaining eight were proband only. A previous history of negative CHD7 genetic testing was reported for 12 probands with the remaining 16 probands reporting no prior history of clinical genetic testing at the level of CHD7 or WES.

CHD7
WES and analysis were conducted at the UW-CMG. Fifteen probands, one of whom had a previous history of negative clinical CHD7 genetic testing, were found to have pathogenic variants in CHD7 (GenBank NM_001042681) by WES (Table 2). Of these 15 CHD7 variants, 11 were designated as de novo because the variant was absent in parental samples and one variant was inherited from a clinically affected mother. For three probands, parental DNA was unavailable for segregation analysis. The majority of CHD7 variants (13) were classified as either a stop-gained or frameshift, consistent with the previously described allelic spectrum of CHD7 variants that cause CHARGE syndrome. One CHD7 splicing variant and one missense variant were also detected. Seven of the 15 CHD7 variants were described in 1000 Genomes with an association with CHARGE syndrome by the Human Gene Mutation Database or ClinVar. Otherwise, all CHD7 variants were absent in ExAC, 1000 Genomes, and the NHLBI ESP Exome Variant Server. CNV analysis of WES data did not reveal pathogenic changes (Supplementary Table 3).

RERE
Proband 517, a female at the age of 8 years at the time of study recruitment, presented with a medical history consistent with CHARGE syndrome. Notably, she exhibited bilateral choanal atresia, right inferior iris coloboma, abnormal external ears, progressive sensorineural hearing loss, and bilateral cochlear dysplasia identified by temporal bone computed tomography. Additionally, proband 517 was also noted to exhibit growth retardation, short stature, microcephaly, developmental delay, and truncus arteriosus repaired surgically at 1 month of age. Consistent with the results of her previous chromosomal microarray and CHD7 genetic testing, proband 517 was not found to harbor a pathogenic variant in CHD7 or CNV by WES (Supplementary Table 2). Rather, a de novo in-frame duplication (c.4313_4318dupTCCACC (p. Leu1438_His1439dup) (GenBank NM_001042681); hg19 chr1: 8,418,276) in the RE (arginine–glutamic acid) dipeptide repeats gene RERE was identified. This in-frame duplication occurs in a histidine-rich region of RERE (UniProt QP2R6), and is absent in publicly available databases ExAC, 1000 Genomes, and the NHLBI ESP Exome Variant Server. The RERE variant detected for proband 517 is the same duplication identified for male subject S2 by Fregeau et al. who exhibits similar features to proband 517 including choanal atresia, coloboma, growth retardation, short stature, developmental delay, and microcephaly. Such phenotypic overlap suggests that this variant contributes to the clinical findings of proband 517.

KMT2D and KDM6A
Variants in KMT2D and KDM6A, genes that underlie Kabuki syndrome, were identified in our cohort. Proband 518, a 16-year-old female, had clinical features of CHARGE syndrome (Table 2). Specifically, her medical history was notable for external ear abnormalities, mild hearing loss in the right ear, moderate to severe hearing loss in the left ear, long palpebral fissures, everted lashes, and persistent fingertip pads. Multiple ophthalmologic abnormalities were also noted, including bilateral chiorioretinal colobomas, strabismus, and nystagmus. In addition, proband 518 exhibits cardiac and renal abnormalities, small stature, precocious puberty, developmental delay, and skeletal-related problems including kyphosis and scoliosis. However, previous genetic testing did not identify a pathogenic variant in CHD7. WES reconfirmed previous negative CHD7 genetic testing results and identified a single-nucleotide duplication in KMT2D (c.9602dupT (p. Ser3202Glufs*13) (GenBank NM_003482); hg19 chr12: 49,431,536) (Supplementary Table 2). This duplication is predicted to result in a frameshift, generation of a premature stop codon in the central portion of KMT2D (UniProt O14686), and nonsense-mediated decay. The c.9602dupT
Table 2  Proband, CHARGE major diagnostic features, gene variants, and pathogenicity evidence

| Proband | CHARGE major criteria | Gene | Genomic position | cDNA variant | Protein alteration | CADD score | GERP score | Inheritance | Status |
|---------|-----------------------|------|------------------|--------------|-------------------|------------|------------|-------------|--------|
| Coloboma | Choanal atresia or cleft palate | Abnormal external, middle, or inner ears | Pathogenic CHD7 | |
| 502     | +                     | +    | +                | +            | CHD7             | chr8: 61,761,664 | c.5355G>A | p.Trp1785* | 48      | 5.87   | De novo | Known |
| 503     | +                     | +    | -                | +            | CHD7             | chr8: 61,655,332 | c.1342_1343delAG | p.Arg448Glufs*126 | N/A | 5.59   | De novo | Novel |
| 504     | +                     | +    | +                | +            | CHD7             | chr8: 61,754,595 | c.4835delA | p.Asn1612Ilefs*28 | N/A | 3.85   | Unknown | Novel |
| 505     | +                     | +    | -                | +            | CHD7             | chr8: 61,773,655 | c.7802dupA | p.Tyr2601* | N/A | 5.73   | De novo | Novel |
| 506     | +                     | -    | +                | +            | CHD7             | chr8: 61,736,406 | c.3209delT | p.Val1070Glyfs*2 | N/A | 5.69   | De novo | Novel |
| 507     | +                     | +    | -                | +            | CHD7             | chr8: 61,742,962 | c.3606_3616dupAGAAACTATTA | p.Le1206lysfs*9 | N/A | 5.47   | De novo | Novel |
| 508     | +                     | +    | -                | +            | CHD7             | chr8: 61,757,970 | c.5210+2T>C | 22.6 | 5.48   | De novo | Novel |
| 509     | +                     | -    | -                | +            | CHD7             | chr8: 61,714,150 | c.2440C>T | p.Gln814* | 44      | 6.17   | Inherited | Known |
| 510     | +                     | +    | +                | +            | CHD7             | chr8: 61,750,761 | c.4480C>T | p.Arg1494* | 48      | 4.42   | Unknown | Known |
| 511     | +                     | -    | +                | +            | CHD7             | chr8: 61,763,056 | c.5409T>G | p.Tyr1803* | 49      | 1.18   | De novo | Novel |
| 512     | +                     | -    | +                | +            | CHD7             | chr8: 61,654,916 | c.925C>T | p.Gln309* | 40      | 5.56   | Unknown | Known |
| 513     | +                     | +    | +                | +            | CHD7             | chr8: 61,765,241 | c.6079C>T | p.Arg2027* | 49      | 3.64   | De novo | Known |
| 514     | +                     | -    | -                | +            | CHD7             | chr8: 61,734,662 | c.2915A>G | p.Gln972Arg | 24.7 | 5.53   | De novo | Novel |
| 515     | +                     | +    | +                | +            | CHD7             | chr8: 61,736,402 | c.3205C>T | p.Arg1069* | 45      | 5.69   | De novo | Known |
| 516     | +                     | +    | +                | +            | CHD7             | chr8: 61,735,210 | c.3106C>T | p.Arg1036* | 45      | 5.53   | De novo | Known |
| 517     | +                     | +    | -                | +            | RBE              | chr1: 8,418,276 | c.4313_4318dupTCCACC | p.Leu1438_His1439dup | N/A | 5.61   | De novo | Known |
| 518     | +                     | -    | +                | -            | KMT2D            | chr12: 49,431,536 | c.9602dupT | p.Ser3202Glufs*13 | N/A | –0.809 | Unknown | Novel |
| 519     | +                     | +    | +                | -            | KDM6A            | chrX: 44,820,553 | c.250A>G | p.Lle84Val | 17.16 | 5.66   | X-linked recessive | Novel |
| 520     | –                     | +    | +                | -            | E3P00            | chr22: 41,553,171 | c.3262-2A>G | 24.4 | 5.7    | De novo | Novel |
| 521     | +                     | -    | -                | -            | PURF60           | chr8: 144,900,664 | c.389G>A | p.Arg130His | 25.9 | 5.18   | De novo | Novel |

CADD, combined annotation dependent depletion; cDNA, complementary DNA; GERP, genomic evolutionary rate profiling; known, identified in CHD7 database and defined as pathogenic; N/A, not applicable; novel<sup>a</sup>, a splice-site variant is observed one nucleotide away at c.5210+3A>G and defined as pathogenic.

Known or novel status of CHD7 variants were defined according to presence in the CHD7 database at http://molgenis51.gcc.rug.nl on 29 June 2017. Known CHD7 variants were also observed in 1000 Genomes with an association to CHARGE syndrome by the Human Gene Mutation Database or ClinVar.
variant in KMT2D is absent from the ExAC, NHLBI ESP Exome Variant Server, and 1000 Genomes databases, and resembles previously described KMT2D variants ascribed to Kabuki syndrome.19

Proband 519, a male just under 18 months of age at the time of recruitment, had clinical findings consistent with CHARGE syndrome including bilateral choanal atresia, bilateral iris coloboma and asymmetric microphthalmia, and low external ear placement on the left side with normal hearing, but no CHD7 pathogenic variant. A family history of unilateral choanal atresia in a maternal great-grandfather was also reported. WES confirmed absence of a pathogenic CHD7 variant (Supplementary Table 2). A hemizygous missense variant in KDM6A (c.250A>G (p.Ile84Val) (GenBank NM_021140); hg19 chrX: 44,820,553) was identified just N-terminal to the first tetratricopeptide motif (UniProt O15550). A GERP conservation score of 5.660 and a CADD score of 17.16, along with the variant’s absence from publicly available databases ExAC, 1000 Genomes, and the NHLBI ESP Exome Variant Server, suggest deleteriousness. The inheritance of the KDM6A variant from a self-reported unaffected mother is unexpected as carrier mothers are reported to exhibit mild features of Kabuki syndrome, but this may reflect favorable skewing of X-inactivation.19 Consequently, we classify the p.Ile84Val variant observed in proband 519 as a variant of uncertain clinical significance. Further experimental studies including X-inactivation profiling, and a clinical evaluation of the mother of proband 519 would be required to change the variant classification.

EP300

Proband 520 was an 11-year-old male with a clinical diagnosis of CHARGE syndrome with a normal chromosomal microarray and CHD7 sequencing. Significant findings included right-sided choanal atresia, sensorineural hearing loss, external ear abnormalities, dysphagia, developmental delay, growth deficiency, a ventricular septal defect, and patent ductus arteriosus with spontaneous closure. He was born prematurely with polyhydramnios. Facial features included level palpebral fissures, a short nose with anteverted nares, an underbite with a symmetric facial grimace, and left-sided esotropia. He was found to have a de novo splice acceptor variant in EP300 3’ to exon 18 (c.3262-2A>G (GenBank NM_001429); hg19 chr2: 41,553,171). Pathogenic variants in EP300 are a minor cause of Rubinstein–Taybi syndrome (RTS) (OMIM 180849).20 The c.3262-2A nucleotide encodes a protein involved in pre-messenger RNA splicing. PUF60 encodes a protein involved in pre-messenger RNA splicing. Similarly, PUF60 missense variants ascribed to Kabuki syndrome, but this may reflect favorable skewing of X-inactivation. Consequently, we classify the p.Ile84Val variant observed in proband 519 as a variant of uncertain clinical significance. Further experimental studies including X-inactivation profiling, and a clinical evaluation of the mother of proband 519 would be required to change the variant classification.

FLNA

Proband 522 was a 10-year-old male with clinical features suggestive of CHARGE syndrome including bilateral retinal colobomas, bilateral cleft lip and palate, bilateral hypoplastic vestibular apparatus and semicircular canals, and abnormal external ears. He also has a history of hearing loss, resolved patent ductus arteriosus, developmental delay, dysphagia, left hydrolephrosis, hiatal hernia, genital anomalies, and normal brain imaging. Consistent with his previous genetic workup, proband 522 was not found to have variants impacting CHD7 or CNVs by WES. Instead, he was found to have a missense variant (c.2309A>G (p.Asn770Ser) (GenBank NM_001456); hg19 chrX: 153,591,124) in the rod-domain repeat 6 (UniProt P21333-2) of filamin A (FLNA) that was inherited from his unaffected mother. Variants in FLNA often cluster in the calponin homology domain 2 and the rod-domain repeat 10, and are associated with several X-linked disorders together termed otopalatodigital spectrum disorders including otopalatodigital syndrome types I and II (MIM 311300 and 304120), Melnick–Needles syndrome (MIM 309350), and frontometaphyseal dysplasia type I (MIM 305620). Some clinical features of proband 522 overlap with features of FLNA-associated conditions, specifically otopalatodigital syndrome type 1, including hearing loss and bilateral cleft lip/palate. However, he had only mild syndactyly of the left second and third toes, which is less severe than the skeletal or limb abnormalities typically observed in FLNA-related disorders. Important in our analysis was additional genetic testing of an unaffected male sibling, who was found to be...
positive for the c.2309A > G FLNA variant. This information provided support for a variant classification of likely benign according to American College of Medical Genetics and Genomics guidelines.

**DISCUSSION**

We report results of WES of a cohort of 28 individuals with clinical features of CHARGE syndrome. Pathogenic variants in CHD7 were discovered in 15/28 (53.6%) individuals and 4/28 (14.3%) individuals had pathogenic variants in four genes other than CHD7, including RERE, KMT2D, EP300, and PUF60. Our results suggest that careful phenotype–genotype analysis in the setting of comprehensive sequencing studies can uncover new relationships between genes and phenotypes, and sheds light on the underlying molecular pathology contributing to CHARGE syndrome and related disorders.

Our findings broaden the spectrum of clinical features associated with pathogenic variants in genes such as EP300. RTS is characterized by intellectual disability, growth delay, and distinctive craniofacial and digital dysmorphisms.24 CHARGE can typically be differentiated from RTS by the presence of semicircular canal dysgenesis, choanal atresia, facial asymmetry, gondal hypoplasia, and syndrome-specific dysmorphology. While hearing loss has been reported in individuals with EP300-associated RTS, the choanal atresia remains unique to proband 520.25 It is possible that choanal atresia is a low-penetrance phenotype of EP300-associated RTS, but remains seldom detected due to the limited number of individuals with EP300 variants to date. This illustrates how the unbiased use of WES can broaden the phenotypic spectrum associated with known disease genes to uncover novel phenotypic overlap between developmental disorders. Alternatively, dual molecular diagnoses may account for his unique RTS presentation. Although WES did not uncover any additional variants suggestive of this alternative, the possibility remains that proband 520 possesses an undiscovered genetic or nongenetic etiology.

The substantial clinical overlap between RERE-associated developmental disorder and CHARGE syndrome raises the possibility of classifying RERE as a genetic cause of CHARGE syndrome. Heterozygous pathogenic variants in RERE have been reported in 10 individuals with complex and variable phenotypes that overlap with those observed in CHARGE syndrome, including structural brain malformations, intellectual disability, seizures, coloboma, sensorineural hearing loss, choanal atresia, cardiac malformations, and gastroesophageal reflux disease.18 Clinical evaluation of individuals with RERE pathogenic variants shows that 2 of the 10 individuals meet clinical criteria for CHARGE syndrome.17,18 Inner ear dysplasia and hypogonadotropic hypogonadism are not reported in RERE-associated developmental disorder and the craniofacial features differ from those commonly observed in CHARGE syndrome (square shaped facies, auricular anomalies, facial asymmetry, and seventh nerve palsy). These differences suggest that pathogenic variants in RERE and CHD7 are the genetic etiologies of distinct disorders.

Our results show that complex phenotypes presenting as CHARGE syndrome may be caused by pathogenic variants in CHD7 or genes associated with other syndromes. The high degree of phenotypic overlap among these disorders raises the question of whether CHARGE syndrome should be considered as a single-gene disorder associated only with pathogenic variants in CHD7 or an oligogenic disorder.26 Isolated features of CHARGE, such as congenital heart disease and congenital hypogonadotropic hypogonadism, can occur due to pathogenic variants in multiple individual genes, and are therefore considered to be oligogenic.27 Careful attention to both clinical diagnosis and molecular results is sure to continue shedding light on this important question.

Phenotypic overlap between CHARGE and Kabuki syndromes is well described, and may reflect direct interactions between CHD7, KMT2D, and KDM6A or shared effects on DNA methylation.28,29 Furthermore, overlapping features with Rubinstein–Taybi, Verheij, and RERE-associated syndromes may also indicate shared molecular pathology between proteins encoded by CHD7, KMT2D, KDM6A, EP300, PUF60, and RERE, the genetic findings in this study.21,30–34 Such functional synergy is supported by experimental observations from primary cells derived from individuals with CHARGE syndrome and animal models. RERE is a coregulator of retinoic acid (RA) signaling and forms a complex with NR2F2 (nuclear receptor subfamily 2, group F, member 2), P300, and retinoic acid receptors (RARs/RXRs), which is recruited to the retinoic acid response element (RARE) of retinoic acid transcriptional targets, such as FGF8 (Figure 1).32 Activation of RAREs by RA promotes an exchange of repressive histone modification (H3K27Me3) in favor of activating ones (H3K27Ac) and translocation of nucleosomes to expose DNA to initiate transcription, functions attributed to KDM6A, P300, CHD7, and PUF60 respectively. A shared molecular biology is also supported by evidence of direct interaction between CHD7 and P300-incorporating complexes.34

This functional synergy may be pertinent to understand the epistatic relationship between CHD7 and FGF8 discovered in the Chd7 knockout mouse.35 While mice lacking one working copy of Chd7 or Fgf8 exhibit normal cerebellar vermis development, those lacking one functional copy each of Chd7 and Fgf8 exhibit cerebellar vermis hypoplasia. Importantly, CHD7 and FGF8 do not directly interact and the molecular underpinning of this epistatic effect has not been described. Fgf8 is an RA-responsive gene. In the absence of RA, RAR/RXR heterodimers bind to RARE sequences, accompanied by histone deacetylases, H3K27 methyltransferase polycomb repressive complex 2 (PRC2), and histone H3K4 mono- and dimethyltransferase KMT2D to repress transcription of RA-responsive genes.31 In response to RA the RERE/NR2F2/P300 complex binds retinoic acid receptors at RAREs.32 Activation of the RAREs promotes nucleosome translocation, an important function of CHD7 that allows for KDM6A occupancy concomitant with H3K27 demethylation and activating H3K27 acetylation by P300, setting the stage for
Figure 1 Model of molecular synergy implicated by genetic determinants (asterisks) that share clinical features of CHARGE syndrome. Co-regulatory retinoic acid response elements (red DNA; RAREs) control cell-specific transcription of retinoic acid (RA) responsive genes, like FGF8 (orange DNA). (a) In the absence of RA, polycomb repressive complex 2 (PRC2) and KMT2D are recruited to RAREs, where they catalyze H3K27Me3 and H3K4Me1 respectively, promoting FGF8 transcriptional repression. (b) In response to RA, the RERE/NR2F2/P300 complex forms, binding nuclear heterodimers of retinoic acid receptors (RAR/RXR) at RAREs. Histone acetyltransferase P300 and KMT2D cooperate to activate enhancer and enhancer-promoter looping that requires nucleosome translocation along the DNA by CHD7. KDM6A demethylates the repressive H3K27Me3 histone modifications. Enhancer-promoter looping is stabilized by the mediator complex (gray oval). CHD7 translocates nucleosomes to permit transcription of FGF8 by RNA pol II. PUF60 helps form the transcription bubble required for RNA Pol II transcription and pre-messenger RNA splicing.

Classifying variants is an inherent challenge of WES interpretation, as demonstrated by the FLNA variant identified in proband 522. Such an example illustrates the value of testing for segregation of the allele with the disorder among family members, especially for genes like FLNA that are associated with diverse malformations and exhibit complicated patterns of variation and X-inactivation. In total, 8/28 (28.6%) probands in our cohort had no pathogenic variant detected by WES. Further genome-wide analysis may be instrumental in identifying an underlying genetic etiology for these individuals. It is possible that pathogenic variants in noncoding regions may be identified to cause CHARGE syndrome. This potential etiology is particularly relevant for CHARGE, since human CHD7 is a large gene (188 kb) and CHD7 is known to bind preferentially to tens of thousands of H3K4me-enriched regions of the genome, including promoters, enhancers, and superenhancers. Other potential causes of CHARGE syndrome that could escape detection by standard chromosomal microarray and single-gene, gene panel, or whole-exome sequencing include (i) differences in DNA methylation, (ii) cryptic chromosomal rearrangements, and (iii) somatic mosaicism. A recent study showed, for example, that DNA methylation signatures differ greatly between individuals with CHARGE and Kabuki syndromes, despite extensive overlap in clinical presentation. Potential environmental causes of features of CHARGE syndrome may include RA exposure or vitamin D deficiency, which also present with craniofacial malformations, ocular and auricular anomalies, and heart defects. Further studies using larger cohorts of individuals with CHARGE and those with related clinical features, accompanied by methylation and genome-sequencing studies, will help explore and distinguish among these possibilities.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE
The authors declare no conflict of interest.
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