Genetics of Combined Pituitary Hormone Deficiency: Roadmap into the Genome Era

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The genetic basis for combined pituitary hormone deficiency (CPHD) is complex, involving 30 genes in a variety of syndromic and nonsyndromic presentations. Molecular diagnosis of this disorder is valuable for predicting disease progression, avoiding unnecessary surgery, and family planning. We expect that the application of high throughput sequencing will uncover additional contributing genes and eventually become a valuable tool for molecular diagnosis. For example, in the last 3 years, six new genes have been implicated in CPHD using whole-exome sequencing. In this review, we present a historical perspective on gene discovery for CPHD and predict approaches that may facilitate future gene identification projects conducted by clinicians and basic scientists. Guidelines for systematic reporting of genetic variants and assigning causality are emerging. We apply these guidelines retrospectively to reports of the genetic basis of CPHD and summarize modes of inheritance and penetrance for each of the known genes. In recent years, there have been great improvements in databases of genetic information for diverse populations. Some issues remain that make molecular diagnosis challenging in some cases. These include the inherent genetic complexity of this disorder, technical challenges like uneven coverage, differing results from variant calling and interpretation pipelines, the number of tolerated genetic alterations, and imperfect methods for predicting pathogenicity. We discuss approaches for future research in the genetics of CPHD. (*Endocrine Reviews* 37: 636–675, 2016)

I. Introduction

A. Definition, clinical features, and genetic complexity of CPHD

Combined pituitary hormone deficiency (CPHD) (also called panhypopituitarism) is a condition classically characterized by a shortage of GH and at least one other pituitary hormone. If GH is the only deficient hormone, a diagnosis of isolated GH deficiency (IGHD) is made. About 45% of IGHD cases evolve to CPHD after a median follow-up time of 5.4 years (1), and CPHD is more likely to develop in patients with more severe idiopathic IGHD (2). The prevalence of CPHD is estimated to be 1 in 8000 individuals worldwide (Genetics Home Reference at NIH, https://www.ghr.nlm.nih.gov). Clinically, CPHD is often discovered when children exhibit reduced growth velocity, although hormone deficiency at birth can cause hypoglycemia and sudden death, emphasizing the necessity for early detection and treatment (3, 4). The clinical workup for growth insufficiency includes measurement of sitting and standing height, circulating hormone levels, hormone secretion in response to stimulation, imaging of the brain and pituitary gland, and determination of bone age with hand x-rays (3, 5, 6). Imaging sometimes reveals a pituitary mass, and genetic testing can often predict whether the mass is likely benign, thus avoiding unnecessary surgery (7, 8). Family history is also important because mean parental height is used to calculate the child's target height. If there is no family history of growth insufficiency, the case is termed sporadic, and it could be genetic or environmental (see Genetic Terminology, Box 1). In familial cases involving multiple affected individuals, the likelihood is increased that the cause is genetic, although environmental factors could play a role in the expressivity or severity of the features.

CPHD is caused by both genetic and nongenetic factors including trauma, brain surgery, tumor, infection, chronic heavy metal poisoning, irradiation, and autoimmune diseases (9–15). Genetic defects causing CPHD typically result in insufficient anterior pituitary gland development and hormone secretion manifesting in early childhood. Mutations in genes expressed in the developing head, hypothalamus, and/or pituitary cause CPHD. The earliest acting genes in head development are often associated with craniofacial abnormalities in addition to pituitary dysfunction (syndromic CPHD), whereas genes expressed in the hypothalamus or intrinsic to the pituitary cause nonsyndromic CPHD. The genetic complexity of CPHD can be explained by the heterogeneity of underlying factors, which contributes in part to the broad phenotypic spectrum. To date, 30 genes have been reported to be involved in the pathogenesis of CPHD (Figure 1). *GLI2*, *HESX1*, *LHX3*, *LHX4*, *OTX2*, *POU1F1*, *PROP1*, and *SOX2* are the most studied ones (16–21).

Precise clinical phenotypic characterization of patients is a very important foundation for genetic studies. It is important to document the nature and progression of hormone deficiencies, take note of associated features, and eliminate cases that have a likely environmental etiology. Grouping of patients based on clinical phenotype can also help in identifying causal genes, which was done successfully with other endocrine disorders such as hypogonadotropic hypogonadism (HH), Kallmann syndrome (KS), and IGHD (22–25). As we learn more about the genetic etiology of CPHD, the data suggest that it is part of a spectrum disorder, with holoprosencephaly (HPE) and septo-optic dysplasia (SOD) at the severe end and HH and IGHD at the mild end of the spectrum (Table 1). The overlap derives from common developmental programs. For example, the eye, ear, nose, pituitary gland, and some cranial nerve ganglia are all derived from placodes in the developing head, and developmental regulation involves common genetic pathways (26). SOD features underdeveloped optic nerves, dysfunctional pituitary, and brain malformations. It results from altered craniofacial development in the midline and can be caused by mutations in *HESX1*, *OTX2*, *SOX2*, *SOX3*, and *PAX6* (26, 27). Other examples of CPHD with craniofacial involvement are patients with HPE, which occurs when the forebrain of the embryo fails to separate into two cerebral hemispheres with or without other craniofacial midline structural anomalies or eye defects. Genes in the sonic hedgehog (SHH) signaling pathway can cause both HPE and CPHD (28, 29).

HH and CPHD have overlapping genetic etiologies. HH is characterized by gonadotropin insufficiency, reduced sex steroid production, and delayed puberty or infertility (30). It can be caused by reduced production of GnRH due to failed migration of GnRH neurons during development, as well as impaired regulation of GnRH secretion. Mutations in many genes can cause HH, and some of these genes are also implicated in CPHD (*CHD7*, *PROKR2*, *WDR11*, *FGFR1*, and *FGF8*) (30–33) (Figure 1 and Table 1). In addition to X-linked or autosomal recessive Mendelian cases of HH, panel sequencing of known genes in large cohorts of patients provided evidence that individuals with HH sometimes carry deleterious alleles within multiple HH genes (30). CPHD may prove to be a multifactorial disease as well because identical mutations can produce a spectrum of phenotypic severity in different CPHD patients, and incomplete penetrance is not uncommon in family pedigrees. This suggests the involvement of multiple genes and/or environment in enhancing or suppressing the severity of the

Box 1. Genetic Terminology

Inheritance patterns

  Sporadic: No family history

  Familial: Multiple related individuals are affected.

  Autosomal: Chromosomes other than the sex chromosomes X and Y

  X-linked: The disorder is due to lesion on the X chromosome; typically boys are affected, receiving the mutant allele from an unaffected mother.

  Recessive: Affected individuals have a lesion in both alleles.

  Dominant: Affected individuals have a lesion in only one allele.

Effects of mutations

  LOF: Loss-of-function mutations result in lack of a gene product or an inactive one. Often recessive (see exception below).

  Dominant haploinsufficient: Loss of function of one allele is sufficient to cause the phenotype.

  Dominant negative: Mutant protein interferes with the function of the normal protein produced by the unaffected allele.

  GOF: Gain-of-function mutations cause over expression, mis-expression or excess function. Often dominant.

Mutations

  Missense: Nucleotide change results in an amino acid substitution.

  Nonsense: Nucleotide change causes a premature stop codon.

  Indel: Insertion or deletion of nucleotides.

  Frame shift: An indel that changes the reading frame of the protein.

  CNV: Copy number variants are gene duplications or deletions that result in overdosage or underdosage of genes, respectively.

  Splicing: Nucleotide changes that render the donor or acceptor sequences ineffective for splicing are commonly identified in exome sequencing, but changes within introns can create novel splice sites that disrupt gene function, and these are often missed.

  De novo mutation: The affected individual has a lesion that is not detected in either parent (see mosaicism below).

  Splice enhancer: DNA sequence motif that directs or improves splice site utilization. Mutations in exonic splice enhancers may not change the encoded amino acid but may still be deleterious because of the failure to utilize the appropriate splice site.

Phenotypic variability and penetrance

  Variable expressivity: Different individuals with the same genetic lesion can exhibit a range of symptoms from mild to severe.

  Incomplete penetrance: Not all individuals with the genetic lesion exhibit a clinical phenotype. Contrasts with fully penetrant, which means every individual with the lesion will be affected to some degree.

  Gonadal mosaicism: If a mutation occurs after fertilization, the individual may have germ cells that are normal as well as ones that carry the mutation. The mutation may or may not be detected in somatic tissue such as peripheral blood, which is often used to track mutations in a pedigree. If gonadal mosaicism is present but not detected, the mutation may be erroneously thought to be de novo, but there is a risk for additional affected children.

Miscellaneous

  SNP: Single nucleotide polymorphism.

  Digenic disorder: Two different mutated genes are required to cause the clinical phenotype.

  Double heterozygote: An individual who is heterozygous for variants in two different genes.

  Compound heterozygote: An individual with a different variant in each allele of the same gene.

  Hemizygote: An individual with only one copy of the gene either because the other copy is deleted or the gene is on the X-chromosome and the individual is male.

  Genetic heterogeneity: A disorder that can be caused by mutations in a variety of different genes.

Conventions are to indicate a mutation in the predicted protein (p.) with the normal or reference amino acid, the position of the change, and the alteration. Asterisk indicates a nonsense codon ie. p.W194*. For cDNA (c.), the nucleotide position is followed by the reference and change, ie, c.334C>Tindicates a C to T change. Ins and del indicate insertions and deletions, respectively.

features. Studies in mutant mice clearly document the effects of genetic and environmental factors, including maternal alcohol consumption on craniofacial development, and the fact that genetic lesions that are normally tolerated can sensitize the fetus to environmental challenges (34–36).

Height is a highly heritable trait, and it is estimated that genetic factors explain 80–90% of the variation (37). Numerous genome-wide association studies (GWAS) have been carried out on large population groups in an effort to identify the genetic factors that contribute to height (38–40). The loci identified to date explain about 30% of the heritability. Interestingly, a few genes implicated by GWAS are mutated in cases of GH insufficiency or resistance, including GLI2, GH1, GHR, and HHIP. In addition, association was found with HESX1 and POU1F1 in a pygmy population (41). Loci involved in skeletal dysplasia or intrauterine growth retardation are also implicated in height determination (42). Some of the missing heritability could be attributable to gene-gene interactions and epigenetic effects (43). For example, it is known that nutrition affects growth, and nutritional effects can be inherited trans generationally through incomplete erasure of epigenetic marks (44). It is notable that variation near the DNA methyltransferase gene, DNMT3A, is associated with height, and there

Figure 1.

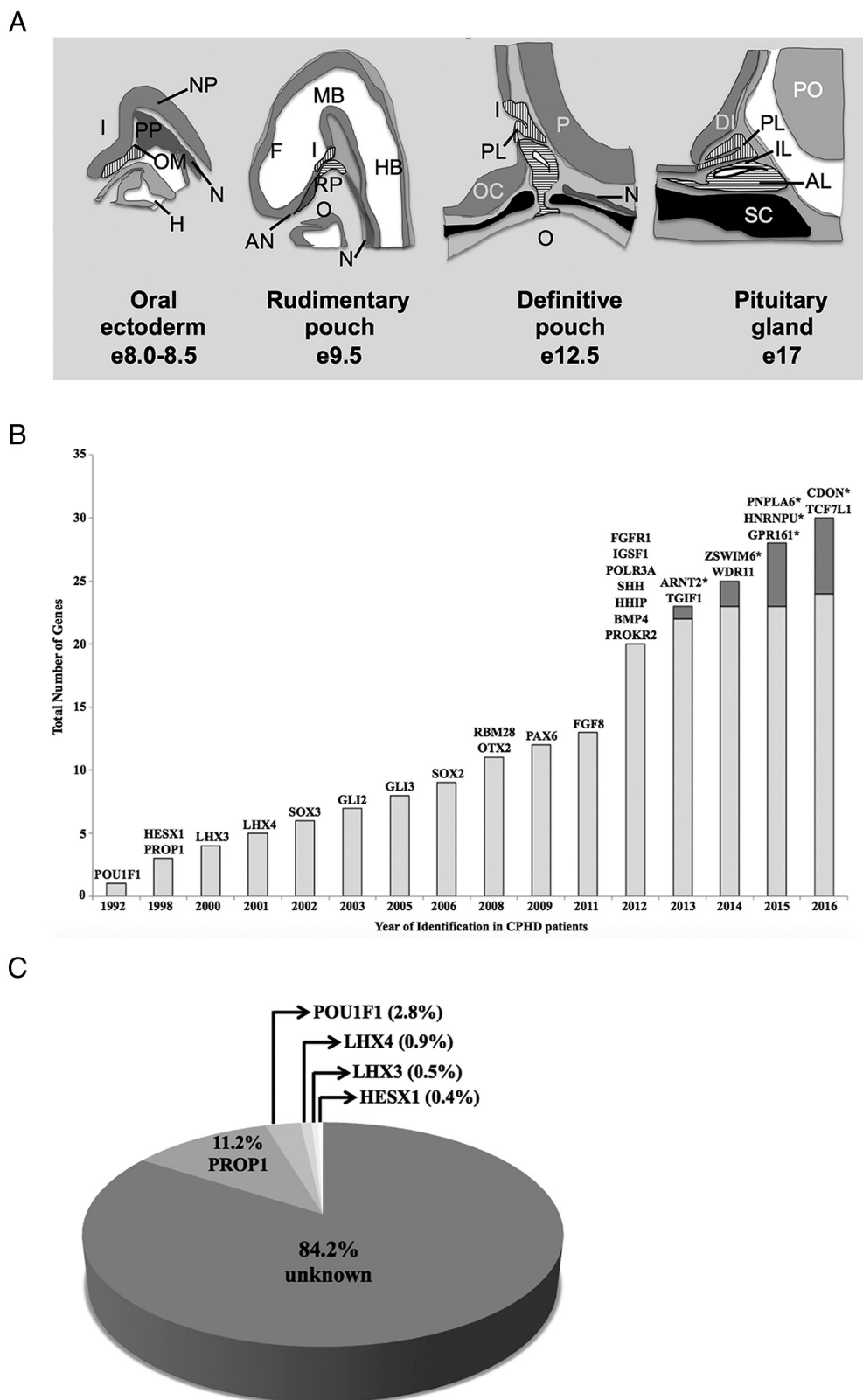Figure 1. Genetics of CPHD: timeline for discovery, cases explained, and developmental expression. A, Mouse pituitary development. Tissue fated to become Rathke's pouch is located at the anterior portion of the embryo. It is in constant contact with the neural ectoderm, which becomes the infundibulum. At e9.5, the oral ectoderm begins to invaginate to produce Rathke's pouch (horizontal hatching). At e12, the cartilage (black)

are several well-known syndromes that are imprinted and affect height (45). Epigenetic heredity is a determinant of adult height, but more research is needed to identify the specific contributions of epigenetic factors.

Most patients with CPHD (~84%) have no genetic diagnosis (Figure 1) (46–69). Gene identification methods for human diseases have evolved from positional cloning of single genes and Sanger sequencing to massively parallel sequencing. With reduced costs and increased application of next-generation sequencing (NGS) techniques, we expect that more novel genes and variants will be discovered. However, the assignment of pathogenicity and causality of genes and variants is not trivial, and there has not yet been a systematic review of this topic for CPHD. The purpose of this review is to document the inheritance mechanisms and pathogenicity of genes implicated in CPHD and its related diseases, to apply current criteria for classifying pathogenic variants, and to facilitate future novel gene discovery in CPHD. Other recent reviews have provided other perspectives on IGHD and CPHD (21, 25, 70–73).

B. Retrospective on gene discovery for CPHD in early years

There has been rapid progress in identifying genes that cause CPHD since 1992, and now a total of 30 genes are implicated, although the amount of evidence for individual genes is variable (Figure 1 and Table 2). We review the early discovery process here.

Figure 1. (Continued). begins to form, and Rathke's pouch pinches off. The posterior lobe (vertical hatching) is formed from evagination of neural ectoderm, and it produces FGF, which stimulates the growth of the pouch. The anterior and intermediate lobes are derived from Rathke's pouch and contain the major hormone-producing cell types by birth. The corresponding time in human development is weeks 4–9 (Carnegie stages 8–22) (418). Orientation: sagittal section with rostral to the left and dorsal at the top. I, infundibulum; H, heart; N, notochord; OM, oral membrane; PP, prechordal plate; F, forebrain; MB, midbrain; HB, hindbrain; AN, anterior neural pore; RP, Rathke's pouch; O, oral cavity; PL, posterior lobe; P, pontine flexure; OC, optic chiasma; IL, intermediate lobe; AL, anterior lobe; DI, diencephalon; PO, pons; SC, sphenoid cartilage. [Redrawn from H. Z. Sheng and H. Westphal: Early steps in pituitary organogenesis. *Trends Genet.* 1999; 15(6):236–240 (166), with permission. © Elsevier.] B, The timeline of gene discovery for CPHD. The light gray bars represent the total number of genes identified by the candidate screening methods; the dark gray bars represent the total number of genes identified by the WES. For each year, the identified gene names are at the top of the bar. *, Gene identified by WES. C, Pie chart indicating the familial and sporadic cases of CPHD that were explained by mutations in the genes *PROP1*, *POU1F1*, *LHX3*, *LHX4*, and *HESX1*. Data are summarized from an extensive literature survey, which illustrates the variation in frequency of *PROP1* mutations by ethnic groups (46). Because systematic screening has not yet been done for the other CPHD genes, it is not possible to estimate their frequency, but it is expected to be quite small.

1. Spontaneous mouse mutant strains reveal CPHD candidate genes

The first genes identified in CPHD patients were found after studies in mice implicated the genes in pituitary development and function. A spontaneous mutation causing dwarfism, infertility, and lethargy was reported in 1929. These mice, known as Snell dwarfs, were used to demonstrate that the pituitary gland regulates growth and the function of multiple target organs (74, 75). The molecular basis for the disorder was unknown until 1990 (76, 77). The pituitary transcription factor POU1F1 (previously known as PIT-1 and GHF-1) was discovered based on its ability to transactivate the GH and prolactin (PRL) genes (78, 79) and was tested as a candidate gene for the mutation. A recessive loss-of-function mutation in *Pou1f1* (p.W251C) was shown to be responsible for the GH, PRL, and TSH deficiency in these mutants. Two years later, a homozygous nonsense mutation in *POU1F1* gene was identified in a CPHD patient from consanguineous parents (80), which was the first report in humans of a defect in a transcription factor causing CPHD.

Similarly, the Ames dwarf mutation arose spontaneously in 1961, and in 1996 genetic mapping and positional cloning experiments demonstrated that these mice have panhypopituitarism caused by a p.S83P substitution in the paired-like homeodomain transcription factor, termed Prophet of *Pit-1* (*Prop1*) (81–83). Two years later, four CPHD families with homozygosity or compound heterozygosity for inactivating mutations of *PROP1* were identified (84).

The Ames and Snell dwarf mice are reasonable phenocopies of the human patients, although there are some differences. In humans, a differentiating diagnosis between *PROP1* and *POU1F1* patients can be the presence or absence of gonadotropin deficiency, respectively, in addition to low GH, TSH, and PRL (85). Both *Prop1*- and *Pou1f1*-deficient mice lack GH, TSH, and PRL, and both have low gonadotropins. However, supplementation with GH and T_4 is sufficient to restore fertility in both mutants (86, 87). Mice require thyroid hormone to develop the feedback loops that regulate gonadotropin function (88). Patients with *PROP1* mutations show progressive hormone deficiency that can eventually result in the life-threatening loss of ACTH, but adrenal function is preserved in *Prop1* mutant mice (89). However, it is important to note that mice homozygous for loss-of-function mutations in either *Prop1* or *Pou1f1* can manifest quite different symptoms on different genetic backgrounds, ranging from newborn lethality to a vigorous long life, even when environmental parameters like food intake and endemic diseases are invariant (90, 91). This demonstrates that the effects of both PROP1 and POU1F1 deficiency are enhanced or suppressed by ge-

Table 1.   Genes Implicated in Cases of CPHD Can Present With Other Clinical Phenotypes

	Phenotypes[a]				
Gene	**Craniofacial[b]**		**Pituitary[c]**		**Peripheral Tissues[d]**
ARNT2	Brain, eye			CPHD		Kidney, urinary tract
BMP4	Eye, craniofacial, cleft lip, palate, spina bifida aperta			CPHD		Renal hypodysplasia, hypospadias, polydactyly
CDON	HPE			(CPHD)		None
CHD7	Eye, craniofacial			CPHD		None
FGF8	SOD, KS: anosmia	HH[e]		CPHD	IGHD	Vacterl, hypospadia
FGFR1	Pfeiffer, Hartsfield, Jackson-Weiss syndromes			CPHD		None
GLI2	HPE, cleft lip, palate	HH		CPHD	IGHD	Polydactyly, cryptorchidism
GLI3	Pallister Hall syndrome, hypothalamic hamartoma, Greig cephalopolysyndactyly			CPHD	IGHD	Skeletal, polydactyly
GPR161	None			(CPHD)	(IGHD)	None
HESX1	SOD			CPHD	IGHD	None
HHIP	HPE			(CPHD)		None
HNRNPU	Lennox-Gastaut syndrome, CNS, epilepsy, intellectual disability			(CPHD)		None
IGSF1	None			CPHD, TSH only		Macro-orchidism
LHX3	Hearing			CPHD		Skeletal
LHX4	None			CPHD		None
OTX2	Eye, craniofacial			CPHD	IGHD	None
PAX6	Eye			CPHD		None
PNPLA6	Spastic paraplegia, Leber congenital amaurosis and other syndromes, eye, craniofacial	HH		CPHD		Muscle wasting
POLR3A	Hypomyelination, hypodontia	HH			(IGHD)	None
POU1F1	None			CPHD	IGHD	None
PROKR2	KS: anosmia	HH		(CPHD)	(IGHD)	Hirschsprung disease
PROP1	None			CPHD		None
RBM28	Alopecia, neurological defects, intellectual disability, tooth defects			(CPHD)		None
SHH	HPE, eye, dental anomalies			(CPHD)		None
SOX2	Eye, dental anomalies, hearing impairment	HH		CPHD		Micropenis
SOX3	Intellectual disability, abnormal facial features, speech difficulties, retrognathia, hearing impairment	HH		CPHD	IGHD	Sex reversal, digital anomalies, micropenis
TCF7L1	SOD			CPHD		None
TGIF1	HPE			CPHD		None
WDR11	Cleft palate, hearing, brain and dental abnormalities, KS: anosmia	HH		(CPHD)		None
ZSWIM6	Acromelic frontonasal dysostosis, brain			CPHD		Skeletal, cryptorchidism

[a] Phenotypes listed represent the range of symptoms reported in various individuals. The pituitary hormone deficiency and associated craniofacial and peripheral tissue phenotypes can vary and/or be absent.

[b] Craniofacial refers to all aspects of head development except the pituitary, which is listed separately.

[c] The parentheses indicate need for additional evidence or patient examples to add certainty to the role of the gene with the indicated pituitary phenotype.

[d] Peripheral tissue phenotypes are listed, except for those that are secondary to the pituitary hormone deficiencies.

[e] The HH can be of hypothalamic or pituitary origin. Cases were arbitrarily assigned to the pituitary category for simplicity.

netic variation inherent among inbred mouse strains. The mouse models provide excellent tools to identify such interacting genes (92, 93).

2. Genetically engineered mouse models identify CPHD candidate genes

The LIM homeodomain gene *Lhx3* (p-Lim or Lim3) is expressed in the developing pituitary gland. In 1996, the $Lhx3^{-/-}$ mutant mice were generated by homologous re-

combination in embryonic stem cells and were discovered to lack both the anterior and intermediate lobes of the pituitary gland (94). The pituitary primordium, Rathke's pouch (Figure 1), formed in the mutants but it failed to grow, and only *Pomc*-expressing cells were detected. Four years later, the human gene was cloned and characterized, and a homozygous *LHX3* defect was identified in patients with CPHD and rigid cervical spine with limited head rotation (95, 96).

Table 2. CPHD Genes Evaluated and Classified for the Causality

Gene Name	Evidence 1: Multiple Unrelated Patients	Evidence 2: Functional Studies	Evidence 3: Gene Expression	Evidence 4: Interactions and Pathways	Total Supporting Evidence
POU1F1	+	+	+	First gene discovered	4
PROP1	+	+	+	+	4
HESX1	+	+	+	+	4
LHX3	+	+	+	+	4
LHX4	+	+	+	+	4
OTX2	+	+	+	+	4
GLI2	+	+	+	+	4
SOX2	+	+	+	+	4
SOX3	+	+	+	+	4
FGF8	+	+	+	+	4
FGFR1	+	+	+	+	4
GLI3	+	+	+	+	4
PAX6	+	+	+	+	4
ARNT2		+	+	+	3
BMP4		+	+	+	3
IGSF1	+	+	+		3
PNPLA6	+	+	+		3
SHH	+		+	+	3
TCF7L1		+	+	+	3
ZSWIM6	+		+	+	3
CHD7			+	+	2
PROKR2			+	+	2
TGIF1			+	+	2
CDON				+	1
GPR161				+	1
HHIP				+	1
HNRNPU			+		1
POLR3A			+		1
RBM28			+		1
WDR11			+		1

Separation of groups of genes by line space indicate the groups of genes that are supported as CPHD candidates by different numbers of evidence.

Lhx4 is expressed in the pituitary primordium (Rathke's pouch) and persists in the anterior and intermediate lobes through adulthood (97). In 1997, genetically engineered *Lhx4*$^{-/-}$ mice were generated. They initially form Rathke's pouch normally, but very few cells undergo differentiation into the five anterior pituitary-specific cell lineages (97). The human *LHX4* was cloned, and a germline splice-site mutation in *LHX4* was found in a patient with CPHD and a small sella turcica in 2001 (98).

Hesx1 is expressed in embryonic stem cells and becomes restricted to the developing forebrain, hypothalamus, and Rathke's pouch (99). In 1998, mice homozygous for a *Hesx1* disruption were generated and found to exhibit variable anterior central nervous system (CNS) defects and pituitary dysmorphology (100). Afterward, patients with SOD and/or CPHD were screened for *HESX1* mutations, and siblings homozygous for a p.R160C substitution were identified (100).

3. Genes that cause severe craniofacial defects are associated with syndromic CPHD

The midline location of the pituitary gland and its close developmental association with the forebrain result in the

association of CPHD with HPE. HPE can be caused by mutations in *SHH*, *GLI2*, *PTCH1*, *TGIF*, *SIX3*, *ZIC2*, *NODAL*, *FOXH1*, *CDON*, *FGF8*, and *DISP1* (101–103). The SHH signaling pathway, which activates target genes under the control of the GLI family transcription factors, is the best studied cause of HPE. *GLI2* mutations were first identified in a genetic screen of 390 HPE patients (104). Three of the four index families carrying *GLI2* heterozygous mutations had hypopituitarism. Constitutive *Gli2* knockout mice die embryonically. Conditional deletion of *Gli2* in Rathke's pouch demonstrated that *Gli2* is required for normal GH, PRL, and ACTH production (105). *Shh* and *Gli2* signaling also control the diencephalic expression of *Bmp4* and *Fgf8*, which are necessary for stimulating anterior pituitary development (106). Mutations in these genes were later identified in patients with hypopituitarism (see section III.B).

4. CPHD genes revealed through detection of cytogenetic abnormalities

The involvement of *SOX3* gene with X-linked panhypopituitarism was discovered in 2002 through cytogenetic

testing and subsequent mutation analysis in a patient with mental retardation and IGHD (107). The patient had an in-frame duplication of 33 bp that expanded a polyalanine tract by 11 alanines. Later, in 2004, *Sox3* was studied in mice. *Sox3* is highly expressed in ventral diencephalon, and targeted disruption of *Sox3* leads to abnormal development of Rathke's pouch (108).

5. Genes identified through functional relationships with other CPHD genes

Heterozygous *OTX2* mutations were identified in patients with anophthalmia and microphthalmia in 2005 using a candidate gene screening approach (109, 110). Mouse studies showed that *Otx2* is required for activation of *Hesx1* expression in the developing forebrain (111), and *HESX1* mutations can cause eye and/or pituitary defects (100). Additionally, an OTX2 binding site was identified in the promoter of the *Hesx1* gene (112). The connection between OTX2 and HESX1 led to screening and identification of *OTX2* mutations in CPHD families with or without ocular abnormalities. A heterozygous frameshift mutation in *OTX2* was identified in a patient with anophthalmia, short stature, and GH deficiency, and a missense mutation was found in two unrelated children with CPHD who presented with neonatal hypoglycemia and deficiencies of GH, TSH, LH, FSH, and ACTH (53, 113). As early as 1995, however, mouse fetuses heterozygous for a genetically engineered *Otx2* knockout were discovered to have a highly variable phenotypic spectrum that is dependent upon the genetic background (35, 114). The phenotypes included pituitary aplasia, ocular malformations, facial clefting, and acephaly. Homozygotes had early embryonic lethality. The mechanism whereby *Otx2* haploinsufficiency causes hypopituitarism was revealed by specifically deleting *Otx2* in the neural ectoderm that gives rise to the ventral hypothalamus and posterior pituitary lobe. The hypoplasia of these regions led to reduced bone morphogenetic protein and fibroblast growth factor (FGF) signaling and, secondarily, reduced growth of the anterior lobe (115). Deletion in the anterior lobe had no consequences.

6. Summary

Early successes in identification of CPHD genes relied on phenotypic similarities between mouse models and human patients, the underlying knowledge of genetic pathways that regulate pituitary development, the occasional association of CPHD with genetic lesions that cause craniofacial anomalies, and cytogenetic findings. The emerging new paradigm is to conduct an unbiased search for new CPHD genes using NGS and utilize cell lines and animal models to demonstrate the functional relevance of

the genetic changes and understand the mechanism of action of the novel genes. Criteria are being established to ensure the authentic pathogenicity and causality of genetic variation, and we will apply the current guidelines to genes and variants implicated in CPHD (116, 117).

C. Criteria for identifying pathogenic genes and variants for CPHD

To date, most human CPHD genes were identified by Sanger sequencing of individual candidate genes, which is not an efficient approach for diseases with a great deal of genetic heterogeneity (118). Moreover, gene-by-gene screening is unlikely to identify digenic or multigenic disease unless all known genes are investigated in each patient. Whole exome sequencing (WES), currently the most commonly used massively parallel sequencing technique, examines the exons of all known protein-coding genes simultaneously and offers a powerful approach to rapidly screen for candidate disease-causing mutations, with a current diagnosis rate of 25% (119). The sequencing technique and data analysis pipeline of WES are not the focus of this review and have been comprehensively discussed elsewhere (120–122). The first case of a CPHD causative gene identified by WES was *ARNT2* (123). Since then, five other genes were implicated in CPHD by WES (Figure 1). We expect that application of WES and whole genome sequencing (WGS) will continue to increase our understanding of this disorder and provide valuable information about oligogenic disease.

The high rate of acquiring data by high throughput sequencing and the ambiguity in interpretation has prompted important dialog among genetic researchers and medical geneticists about assigning pathogenicity to genuine disease-causing genetic variants. In 2014, a group of 27 experts in genetic research, analysis, and clinical diagnostic sequencing published guidelines for investigating causality of sequence variants in human diseases (117). The challenges in assessing sequence variants in human disease are discussed in the guidelines, and a list of factors to consider at both the gene level and the variant level is provided for presumed monogenic diseases. One year later, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) updated their standards and guidelines for the interpretation of sequence variants (116). With awareness of the increased complexity in sequence interpretation during clinical genetic testing, the ACMG-AMP guidelines provided a stringent and semiquantitative set of criteria for variant classification. A recent pilot evaluation of the performance of ACMG-AMP guidelines on 99 variants among nine laboratories showed that consensus discussion and detailed review among the researchers greatly

improved the concordance of variant classifications (124). More effort is needed to increase application of a standardized approach for variant assessment and to achieve consistent concordance with variant interpretations. To date, there has not been a systematic overview in the literature of the pathogenicity of all the genes and variants that have already been reported to cause CPHD. It is timely and important to conduct such a survey before the field fully embraces the novel sequencing technologies for new gene identification in CPHD.

II. Classification and Review of CPHD Causative Genes and Variants

We present our methods for identifying genes implicated in CPHD and evaluating the pathogenicity using the currently accepted guidelines for genetic variation.

A. Gene-level assessment to confirm CPHD causative genes

Combining the search results from free (Online Mendelian Inheritance in Man [OMIM] and PubMed) and subscription (Human Gene Mutation Database [HGMD]) databases, we identified 30 genes that are associated with either nonsyndromic or syndromic CPHD in the literature by June 30, 2016. We applied the four categories of evidence suggested by MacArthur et al (117) to assess the causality of these genes for CPHD (Table 2). These lines of evidence include: 1) variants in the same gene and similar clinical phenotypes have been observed in multiple unrelated individuals; 2) functional studies, including biochemistry, cell culture, and/or animal models confirm the deleterious effects of detected variants in the genes; 3) the protein that the gene encodes is expressed in the appropriate developing tissue such as hypothalamus and/or pituitary, according to BioGPS (www.biogps.org), Gene Expression Database (GXD; http://www.informatics.jax.org/expression.shtml), and/or the internally maintained Embryonic Mouse Pituitary cDNA library (125) (https://sites.google.com/a/umich.edu/riken-database-2014/); and 4) the gene product interacts with other proteins or plays a role in the biological pathways that are implicated in the disease. Replication in unrelated individuals and functional studies are the most important types of evidence. We consider the genes that have at least two types of evidence, to date, that support their candidacy as causative genes for CPHD. These include the frequently screened and historically most relevant genes (*POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *SOX3*, *GLI2*, *SOX2*, and *OTX2*) and the genes that are recently identified or less commonly reported in CPHD (*BMP4*, *FGF8*, *FGFR1*, *GLI3*, *PAX6*, *IGSF1*, *SHH*, *ARNT2*,

TCF7L1, *CHD7*, *PROKR2*, *TGIF1*, *PNPLA6*, and *ZSWIM6*). Also, seven genes, *CDON*, *GPR161*, *RBM28*, *POLR3A*, *HHIP*, *WDR11*, and *HNRNPU*, are grouped together awaiting additional evidence to confirm causality. Among these, *CDON*, *GPR161*, and *HNRNPU* were discovered by WES, and the other four genes, *RBM28*, *POLR3A*, *HHIP*, *WDR11*, were from candidate gene screening.

B. Adapted workflow for variant-level classification of reported variants in CPHD patients

There are about 2,500 reported variants in the 30 CPHD-associated genes. For each variant, the classification criteria that we used are based on the ACMG-AMP guidelines, which is so far the most detailed and quantitative system for variant interpretation in genetic testing (116). Our retrospective review of variants implicated as causing CPHD focuses on specific criteria including minor allele frequency (MAF), which we expect to be rare in the case of a rare disease, computational prediction of a deleterious effect on function, and confirmation of reduced activity with functional assays. At the gene level, we weighed gene expression in the relevant tissues, gene function in pertinent biology pathways, variants in multiple families with similar phenotypes, and cosegregation of the clinical phenotype with the variant in a pedigree. We applied the weight and the rules for combining criteria in the ACMG-AMP guidelines to generate our workflow for the interpretation in loss-of-function variants and missense or in-frame indels (Figure 2). The MAF was determined from three public population databases: Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/), Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS/), and 1000 Genomes Project (1000G; http://browser.1000genomes.org/index.html). We used three in silico prediction programs for predicting the effects of missense variants: SIFT (http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and MutationTaster (http://www.mutationtaster.org/). SIFT predictions are classified as: tolerated and damaging. PolyPhen-2 predictions are classified as: benign, possibly or probably damaging. MutationTaster predictions are classified as: probably deleterious, known to be deleterious, probably harmless, known to be harmless. None of the programs that predict pathogenicity are perfect, which is an ongoing challenge for interpretation of genome sequence information. Functional studies are critical because prediction programs have both false negatives and false positives (117, 126, 127). Improved accuracy in prediction programs would be invaluable for the field because functional testing is particularly time consuming, but functional testing is likely to continue to be necessary and
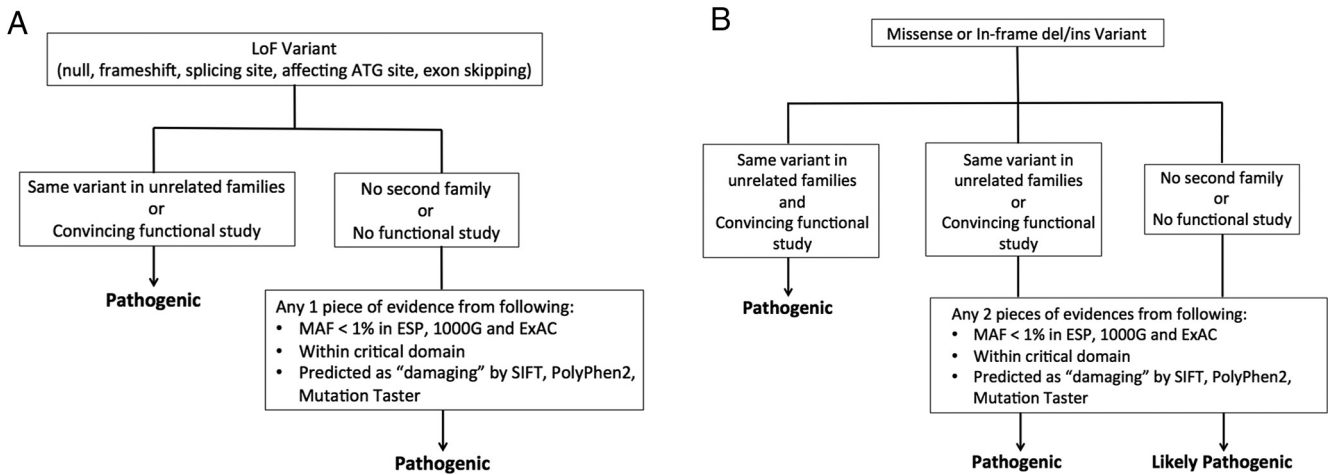
Figure 2.

Figure 2. Work flow for assessment on the reported genetic variants of CPHD. A, Loss-of-function (LoF) variants; B, missense or in-frame insertion and deletion (in/del) variants.

critically important for interpretation. Functional testing also has challenges, which we discuss in *Section IV.D*.

III. Re-evaluation on Pathogenicity and Penetrance of Reported Genes and Variants for CPHD

A. Frequently screened and currently most relevant genes with CPHD

1. PROP1

PROP1 is the most frequently mutated gene known to cause CPHD. Patients with mutations in *PROP1* are typically identified initially with short stature due to GH deficiency. Most patients also exhibit reduced TSH and PRL at the time of diagnosis. At the onset of puberty, many patients with *PROP1* mutations also exhibit LH and FSH deficiency and fail to develop secondary sexual characteristics. The loss of gonadotropins may also present as an evolving characteristic identified in adulthood. Many human patients also develop ACTH deficiency as they age (128). We hypothesize that the role of PROP1 in establishing pituitary stem cell pools and promoting pituitary stem cells to migrate and transition to differentiation is the basis for progressive hormone failure in humans (129). Magnetic resonance imaging (MRI) is invaluable for assessing whether the pituitary insufficiency is associated with other brain abnormalities, disrupted pituitary stalk, or ectopic posterior pituitary, which is among the varying range of physical features of the pituitary gland in patients with PROP1 mutations. Most cases display pituitary hypoplasia or aplasia; however, there are reports of pituitary hyperplasia, a waxing and waning of pituitary hyperplasia

to hypoplasia, as well as reports of pituitary masses (56, 128, 130–137).

The frequency of *PROP1* mutations varies among population groups and is highest in Eastern Europe and Russia (46–48, 64). Two of the most common variants identified are: a two base pair deletion, c.301_302delAG, which results in frameshift and early termination of the protein at amino acid 109; and c.150delA, which results in a frameshift at amino acid 53 and early termination at amino acid 164 (47). There are a couple of reports of heterozygous variants in *PROP1* with insufficient data to support the pathogenicity (138, 139). All of the other cases are either homozygous or compound heterozygous, recessive, mostly loss-of-function variants in *PROP1*. There are no convincing examples of incomplete penetrance, meaning individuals with two loss-of-function alleles but no endocrine disorder, yet there are examples of variable hormone profiles and age of onset within a pedigree, including one unusual case in which the patient reached a normal height but acquired CPHD as an adult (140). Interestingly, in one case with a homozygous p.W194* variant, the patient initially presented with HH and developed progressive GH and TSH deficiency during adulthood (141). In another familial case, with two affected brothers containing a compound heterozygous variant (c.150_150delA; c.334C>T), the patients were initially diagnosed with hypothyroidism due to only a slight deviation from normal linear growth. In subsequent follow-up exams, deficiencies in GH, LH, FSH, and PRL were also detected (136). Twenty-nine *PROP1* variants have been reported, including complete gene deletions (six variants), alterations in splicing (four variants), nonsense variants (eight variants),
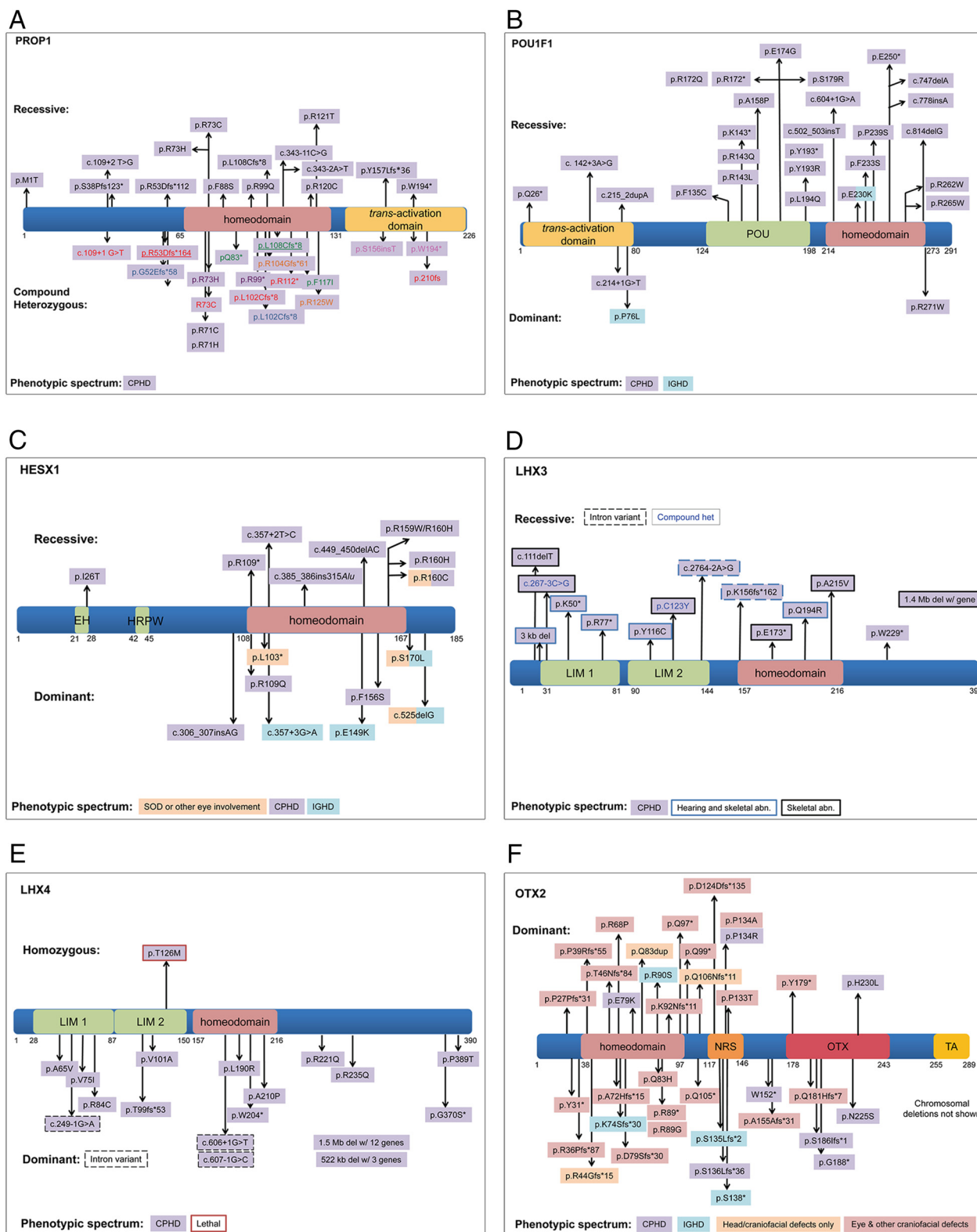
Figure 3.

Figure 3. Examples of pathogenic variants identified in CPHD genes.
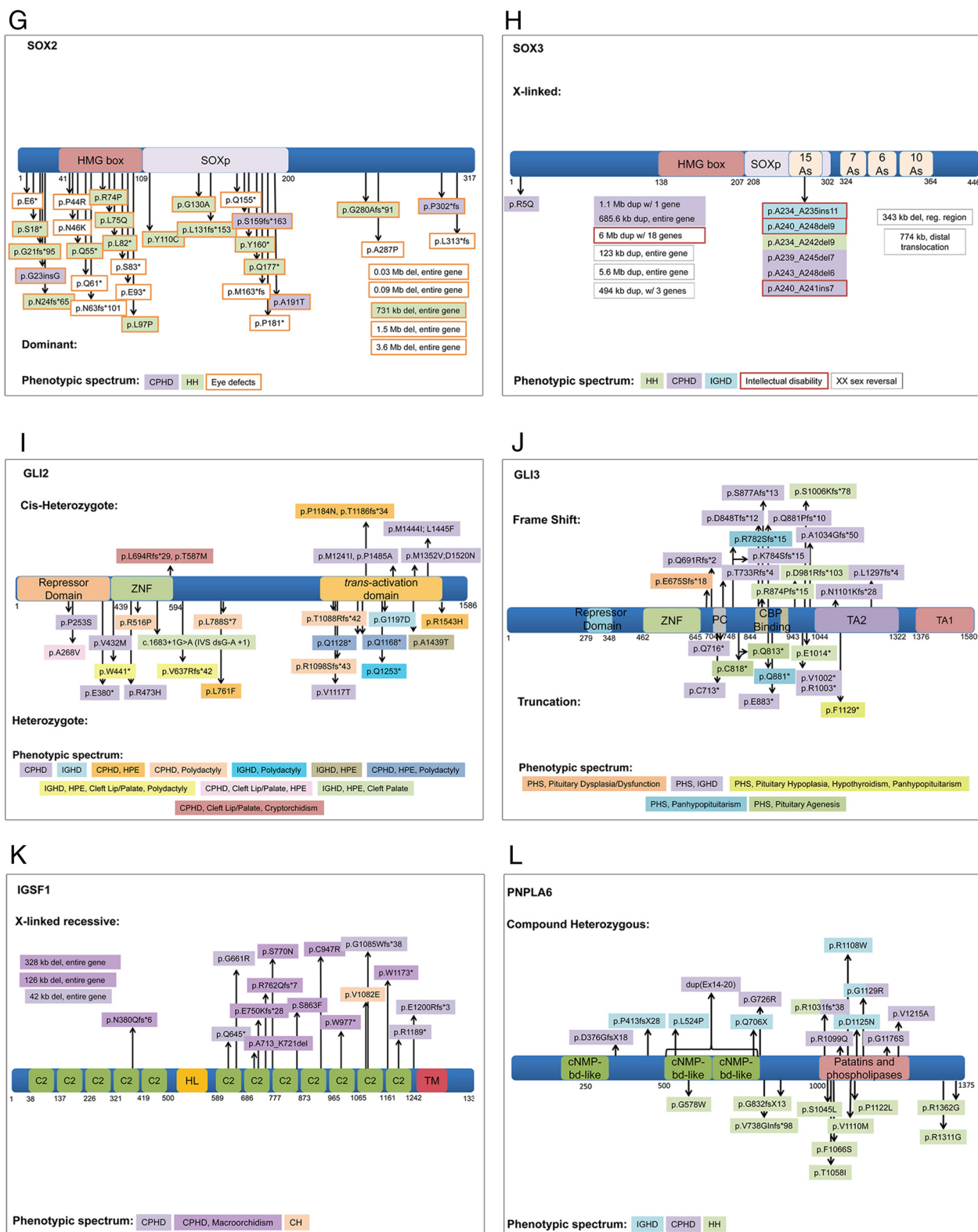
Figure 3.

Figure 3. (Continued).

and missense variants (11 variants). Fourteen of the 29 variants are proven pathogenic through functional studies, analysis of multiple carriers within a pedigree, analysis of multiple pedigrees, and allele frequency (Figure 3). Five variants are assumed pathogenic because they cause protein truncations.

2. POU1F1

POU1F1 is a pituitary-specific transcription factor necessary for expression of GH, TSH, and PRL. *POU1F1* contains a POU-specific and POU-homeodomain, which are both necessary for DNA binding. A total of 30 different variants in *POU1F1* have been described, and they are generally recessive with pituitary hypoplasia and CPHD. All patients with homozygous variants are familial, and all compound heterozygous cases but one are familial. About half of these variants have been proven to affect function with *in vitro* experiments. We determined that all of the homozygous and compound heterozygous variants are either likely pathogenic or pathogenic resulting in CPHD (Figure 3). There are three dominant-negative variants reported in *POU1F1* that are pathogenic. The *POU1F1* missense p.R271W acts as a dominant inhibitor of transcription resulting in CPHD (142). A few p.R271W cases exhibit cognitive impairment or hearing impairment, which may be due to hypothyroidism during pregnancy when the mother carries the same variant (143). Approximately half of the p.R271W variants are fully penetrant. Two cases are clearly incompletely penetrant, and the rest of the cases did not present enough family history to determine penetrance. A splicing variant at c.214+1G>T was also determined to be a dominant-negative variant, but it is incompletely penetrant (144). The third pathogenic dominant-negative variant identified is a missense p.P76L and results in IGHD. This variant is completely penetrant because all nine members of family carrying it exhibit the IGHD phenotype (145). Several other heterozygous variants in *POU1F1* have been reported but, due to a lack of patient data, cannot be labeled as pathogenic with certainty (58, 64, 146–149). A single case with a homozygous deletion of *POU1F1* together with the neighboring *CHMP2B* and *VGLL3* genes presented with an unusual syndromic phenotype. The child exhibited CPHD and hypoplastic pituitary typical for *POU1F1* mutations but had craniofacial defects and cognitive impairment and was initially unresponsive to GH therapy. The unusual phenotype may be due to the additive effects of deleting all three genes (150).

3. HESX1

HESX1 is a homeobox gene that plays an important role in early brain development. It is essential for the for-

mation of the pituitary and the development of the forebrain, including optic nerves (151, 152). Both recessive and dominant variants in *HESX1* can cause CPHD (Figure 3). All eight recessive mutations of *HESX1* gene can be classified as pathogenic and have been studied by functional assays or animal models (100, 153–158). Most of the recessive variants cause CPHD, except that homozygous p.R160C causes SOD and hypopituitarism. Most variants affect the DNA binding homeodomain or the engrailed homology domain that binds corepressors of the TLE family (p.I26T). Among the 14 heterozygous variants found in *HESX1*, four are loss-of-function variants (c.307_308insAG; c.525delG; c.308T>A, p.L103*; c.357+3G>A) and can be classified as pathogenic. Only one heterozygous missense variant, p.S170L, was identified in two unrelated families, and one family had two affected children with mild SOD. The rest of the heterozygous missense variants reported in *HESX1* are either incompletely penetrant or lack information on family segregation. More evidence is still needed to assign causality. Two heterozygous missense variants, p.H42Y and p.V75L, were identified in HH patients. The variant p.H42Y is rare in the population databases, but the prediction programs do not agree on whether it is likely damaging. The p.V75L variant is a novel one, not recorded in any population database, but MutationTaster predicts it would be tolerated. The functional effects of p.H42Y and p.V75L were not assessed. More evidence is needed to determine whether *HESX1* mutations cause HH.

4. LHX3

LHX3 is a member of the Lin11, Isl-1, and Mec-3 (LIM) homeodomain protein family of transcription factors. It is expressed in Rathke's pouch and the neural tube and is important for pituitary development and the organization of spinal cord neurons (159, 160). Variants in *LHX3* that cause CPHD are autosomal recessive and are mostly missense and nonsense mutations and include two complete gene deletions of *LHX3* (161–163) (Figure 3). GH and TSH are consistently reduced in patients with *LHX3* mutations, and in descending order PRL, LH/FSH, and ACTH production is also affected. Limited neck rotation is the most common accompanying symptom (164). In mice, *Lhx3* and *Lhx4* are expressed in the ventral motor neurons; therefore, the limited neck rotation may be due to abnormal innervation of the anterior neck muscles. Hyperplastic anterior pituitary gland, sensorineural hearing impairment, respiratory difficulties, and skeletal abnormalities were also seen in some cases (161–164). The variants found in *LHX3* do not cluster in any particular region or domain of the protein. Two heterozygous variants in *LHX3*, p.T63M and p.A322T, were found in patients

with CPHD (165). The p.A322T variant has a rare allele frequency of 0.003, but it is predicted to be benign using SIFT, which means it is unlikely to be causative. There was no allele frequency information for variant p.T63M, but it was also predicted to be benign.

5. LHX4

LHX4 also encodes a LIM homeodomain protein. Mutations in *LHX4* mostly result in CPHD cases with autosomal dominant inheritance and incomplete penetrance (50, 66, 98, 167) (Figure 3). There is only one documented homozygous *LHX4* variant (p.T126M) and it is associated with a lethal phenotype (168). Heterozygous *LHX4* variants are generally loss-of-function alleles, and patients carrying these heterozygous variants present with CPHD, short stature, small sella turcica and cerebellar defects, hypoplastic anterior pituitary, respiratory disease, genital malformations, some craniofacial features, and hypothyroidism (50, 66, 165–167, 169–173). In some cases, one parent carries the putative causative allele and has significant short stature, but without CPHD or any other phenotypic abnormality. There are at least five reports of de novo deletions encompassing *LHX4* (165, 170, 172). The clinical features of a patient with a de novo heterozygous 522-kb deletion including *LHX4*, *CEP350*, *QSOX1*, and *ACBD6* are highly similar to other heterozygous missense *LHX4* cases. Thus, the hemizygosity for the other genes is unlikely to contribute to this patient's phenotype (165). A 7.5-Mb duplication containing *LHX4* is associated with tall stature, cognitive deficits, and craniofacial dysmorphism, leaving open the possibility that *LHX4* overdosage could be problematic, but more studies are necessary to assess this definitively (174). Several *LHX4* variants of uncertain significance were reported in individuals with CPHD (66, 173). The variants p.242delK (MAF = 0.000364), p.N271S (MAF = not reported (n.r.)), and p.Q346R (MAF = $8.2e^{-6}$) are predicted to be benign and do not alter LHX4 transactivation properties in transfected cells (173). Although p.G370S (MAF = n.r.) has normal transactivation properties, it was predicted to be deleterious (66). Additional studies are warranted to determine whether the p.G370S variant affects LHX4-interacting proteins or in vivo function in zebrafish or mice (175, 176).

6. OTX2

OTX2 encodes orthodenticle homeobox 2, a member of the bicoid class of homeobox transcription factors. OTX2 is involved in the development of the brain and other head structures, most notably the eyes and pituitary gland (35, 114, 177, 178). Variants in *OTX2* account for approximately 2–3% of cases of anophthalmia or microphthalmia (179), and about one-third of these patients also have pituitary hormone deficiencies (180). Other symptoms, such as developmental delay, intellectual disabilities, and mouth and ear defects, can occur in variable combinations with eye and pituitary defects (180). All reported human *OTX2* variants are heterozygous, and 19 of 55 currently reported cases of *OTX2* mutations in humans present signs of pituitary disease (Figure 3). Of these, 37% are sporadic mutations, 42% are de novo, 16% are familial dominant with complete penetrance, and only 5% (one case) is familial dominant with incomplete penetrance, although incomplete penetrance is more common in *OTX2* variants overall at 22% (53, 109, 113, 179–204). Eye, pituitary, and craniofacial defects are also observed in *Otx2*-heterozygous mutant mice (35). *Otx2*-homozygous knockout mice die during midgestation, and there are at least two strain-specific modifiers that alter the penetrance of the heterozygous phenotype in mice (114). Thus, it is likely that genetic and/or environmental factors influence the penetrance of *OTX2* mutations in patients.

7. GLI2

Heterozygous, loss-of-function mutations in *GLI2* have been reported in 53 patients with a variety of clinical features with CPHD on the milder end of the spectrum (104, 205–218). There is no conclusive evidence of dominant-negative alleles. Ten of these variants can be classified as pathogenic based on functional studies and segregation in the pedigrees (Figure 3). In many cases, a pathogenic variant is associated with a variable phenotype and/or lack of penetrance in the family. For example, over 20% of the variants have one unaffected parent carrying the allele. *GLI2* variants are commonly associated with HPE, cleft palate, CPHD, and polydactyly. One-third of the patients with *GLI2* variants exhibit CPHD. Most of these CPHD patients (83%) are nonsyndromic in that they display no features characteristic of HPE, cleft palate, or polydactyly. IGHD (13%), hypopituitarism with unspecified hormone deficiencies (6%), and HH (2%) have also been associated with *GLI2* variants, but most of these cases (85%) have some features of HPE, cleft palate, or polydactyly. Combinations of HPE, cleft palate, and polydactyly without pituitary hormone insufficiency represent < 30% of recorded *GLI2* variants, with HPE presiding as the predominant phenotype within this group. Interestingly, two *GLI2* variants suspected to be causal are found in patients who also have variants in ZIC2 and SHH, two genes that can cause HPE. The combined effect of the two heterozygous genes could be contributing to the disease penetrance in these cases.

8. SOX2

SOX2 is a member of the high-mobility group (HMG) transcription factors related to SRY (sex-determining region Y). *Sox2* is important for embryonic development and is expressed in progenitor cells of many tissues including the developing brain, pituitary, and the otic and nasal placodes (219, 220). *SOX2* variants are autosomal dominant with incomplete penetrance (Figure 3). In some cases, the unaffected carrier mother has been shown to be a gonadal mosaic (221–223). The clinical features associated with *SOX2* mutations include eye disorders (anophthalmia and microphthalmia), brain malformations (usually hippocampal), intellectual disabilities, esophageal atresia, genital abnormalities, sensorineural hearing loss, and hypoplastic anterior pituitary. Pituitary masses have been observed in affected patients, but they do not progress (219). The hypothalamic-pituitary phenotypes caused by variants in *SOX2* include HH or CPHD (224–226), but endocrine testing was not performed in most of these individuals (222, 226–231). Most *SOX2* variants result in the absence or truncation of the gene affecting the HMG box domain (219, 225, 232–235). The eye and pituitary phenotype variability of *SOX2* mutations is demonstrated in a pedigree with a mother and two children who share a heterozygous deletion resulting in a frameshift truncating the SOX2 protein, p.G280Afs91* (236). The mother presented with HH and normal eyes, whereas the two children presented with eye defects, one with clinically normal pituitary function and the other with IGHD.

9. SOX3

SOX3 is another member of the SRY-related HMG transcription factor family. In familial cases of *SOX3*-related hypothalamic-pituitary phenotypes, CPHD is X-linked with incomplete penetrance. *SOX3* variants can cause CPHD (237–241), IGHD (107), or normosmic HH (32) with or without intellectual disability and anterior pituitary hypoplasia. Large duplications of Xq26 that included *SOX3* were found in individuals with hypopituitarism (238, 240). Some other cases of X-linked hypopituitarism suspected to be caused by *SOX3* dosage effects require further study to identify the precise lesions (242). Most mutations within the *SOX3* gene are insertions and deletions in the first polyalanine tract where poly-A expansions are associated with reduced transactivation and poly-A deletions are associated with increased transactivation (32, 107, 237, 239–241) (Figure 3). There is one documented amino acid substitution in *SOX3* in a male patient with CPHD (GH, LH, FSH deficiency) without intellectual disability (p.R5Q), which he inherited from his apparently unaffected mother (237). However, there was

no difference in transcriptional activity when compared with the wild-type SOX3, so the significance of this variant is not clear. An example of the incomplete penetrance of *SOX3* mutations is provided by a heterozygous female with CPHD and deletion of six alanines (237), with an unaffected carrier father. *SOX3* regulatory region rearrangements are shown to cause XX male sex reversal in mice and humans, which can be accompanied with low gonadotropin serum levels and intellectual disability (243–246).

B. Less frequently reported genes in CPHD cases

1. BMP4

The 37 documented *BMP4* mutations identified in patients are associated with a range of phenotypes that include cleft lip, cleft palate, eye and craniofacial defects, hypospadias, renal hypoplasia or dysplasia, spina bifida, and colon cancer. Most of the mutations are heterozygous (34 of 37), half are familial (17), and the other half are sporadic or de novo. Of the 17 familial mutations, only four are penetrant. The single reported case of a heterozygous *BMP4* mutation with a pituitary phenotype (p.R300P) is in a sporadic case with CPHD and a hypoplastic pituitary gland (247). The mutation prediction software suggested that it is pathogenic, and it is positioned in a highly conserved region of the *BMP4* gene, but no functional studies were carried out. Although we cannot conclude that this alteration is pathogenic, it is worth screening CPHD patients for lesions in *BMP4* because it contributes to the formation of Rathke's pouch in the mouse (248, 249).

2. FGF8

There are 23 mutations reported in *FGF8*, and most of the patients have KS (HH and anosmia). About half of the cases (11 of 23) are heterozygous and sporadic. The penetrance is unclear in many cases due to the lack of pedigree information. There are eight cases that are heterozygous and familial, but of those mutations, only one is penetrant. Interestingly, three different *FGF8* variants are reported to cause hypopituitarism (Supplemental Figure 1). A patient homozygous for a p.R189H variant had low ACTH, TSH, and PRL, along with HPE (103, 250). The parents of the patient are heterozygous and unaffected. The variant is predicted to be pathogenic according to mutation prediction software, and the MAF was 0.077. Based upon the above evidence, it is likely causative. A patient with CPHD and HH is heterozygous for an *FGF8* variant that compromised an exonic splicing enhancer site (c.216G>A p.T72T). No family history was reported, but the nucleotide change resulted in higher *FGF8* expression and

higher activity in cell culture (33). It is predicted to be disease causing and has a MAF of $4.13e^{-5}$. We concluded that this variant is pathogenic. Finally, a patient with low GH, thyroid hormone, LH, and FSH, hyposmia, and cleft palate is heterozygous for deletion of a thymine at position 574, which results in protein truncation and likely affects receptor interaction (c.574delT) (251). This variant is likely causative. A heterozygous p.Q216E variant was reported in a patient with low GH and SOD (103, 250). The p.Q216E variant has not been reported in the public population databases, but it is not predicted to be disease causing. Without more evidence, this variant cannot be labeled as causative. *FGF8* is expressed in the diencephalon and prospective hypothalamus during development in the human and mouse embryo (103, 250). Loss- and gain-of-function *Fgf8* experiments in the mouse highlight the importance of *Fgf8* in normal pituitary development. Ectopic expression of *Fgf8* in the mouse pituitary gland leads to a dysmorphic and hyperplastic pituitary gland that lacks GH and TSH expression (106), whereas reduced FGF8 expression in an *Fgf8* hypomorph causes pituitary hypoplasia (103, 250). The three patients with *FGF8* mutations and hypopituitarism provide convincing evidence that *FGF8* mutations are a cause of CPHD.

3. FGFR1

FGFR1 is one of four FGF receptors and plays an important role in the development of the nervous system. Human variants in *FGFR1* cause multiple diseases, such as Pfeiffer syndrome, Hartsfield syndrome, Jackson-Weiss syndrome, etc. Pathogenic variants in *FGFR1* are reported for HH with or without anosmia. There are several reports suggesting the oligogenic effects of *FGFR1* and other GnRH deficiency genes, such as *HS6ST1* (252) and *SEMA3A* (253). Heterozygous missense variants in the *FGFR1* gene were identified in unrelated SOD and CPHD patients (31, 33, 254). Three variants, p.S450F, p.P483S, and c.336C>T or p.T112T, were identified in SOD probands (33). The p.S450F and p.P483S variants were proved to be loss-of-function mutations by functional studies. The synonymous change c.336C>T was not observed in healthy controls or public datasets. This variant is predicted to generate a new exonic splicing enhancer binding site for the SRp40 splicing factor and/or to disrupt an overlapping putative exonic splicing enhancer octamer. Further investigation is needed to assess the functional effects of p.T112T. The heterozygous variant c.1342C>T, p.R448W was found in two unrelated CPHD patients. It is rare (MAF = 0.0008 in ExAC; not found in EVS and 1000G databases), located in the juxtamembrane domain of FGFR1 protein, and predicted to be damaging by SIFT, PolyPhen-2, and MutationTaster. It

has reduced transactivation activity in luciferase assays in transfected cells. Thus, *FGFR1* p.R448W is a pathogenic variant. The variant c.320C>T, p.S107L is located in the Ig-like C2-type 1 domain of FGFR1 protein and was found in two unrelated Japanese families. The MAF of p.S107L in the East Asian population is 4%, and both SIFT and PolyPhen-2 predict that p.S107L is a benign change. Therefore, p.S107L is unlikely to be a pathogenic variant. Two unrelated CPHD patients carry p.P772S, but there was no change in the activity of the p.P772S protein, and the variant p.P772S is homozygous in several cases from ExAC and 1000G databases. Thus, the p.P772S is likely to be a benign variant. The p.V102I change is a variant of uncertain significance because all the programs predict it to be benign, and the functional study showed only a mild reduction in protein activity. More information is needed to further evaluate p.V102I. Thus, of four *FGFR1* variants reported in CPHD patients, only p.R448W is clearly pathogenic.

4. GLI3

GLI3 is a zinc finger transcription factor and can act as a transcriptional activator or repressor of downstream targets of the SHH pathway. Heterozygous mutations in *GLI3* are responsible for most cases of Greig cephalopolysyndactyly syndrome and Pallister Hall syndrome. Greig cephalopolysyndactyly syndrome is associated with abnormal development of the limbs, head, and face and includes polydactyly, macrocephaly, and hypertelorism. Pallister Hall syndrome has some of the same features but also includes hypothalamic hamartoma and bifid epiglottis. Pallister Hall syndrome can also be associated with pituitary phenotypes including IGHD (255–258) and CPHD (256, 259, 260) with pituitary hypoplasia or agenesis (Figure 3). Many of the mutations are de novo, although there are a few examples of familial cases. There is a very strong correlation between the location of the mutation within the gene and the phenotype. Greig cephalopolysyndactyly syndrome is associated with gene deletions and loss-of-function mutations in the amino terminal third of the protein, which affect DNA binding in the zinc finger domain, and in the carboxy-terminal portion, which affects the transactivation domain. Pallister Hall syndrome is associated with mutations within the middle third of the protein that cause truncation before the transactivation domains, rendering the protein a constitutive repressor. In mice, *Gli2* and *Gli3* have overlapping functions in hypothalamic development (261). Thus, *GLI3* mutations may cause pituitary dysfunction that is secondary to hypothalamic defects.

5. IGSF1

The X-linked gene Ig superfamily member 1 (*IGSF1*) encodes a large, 150-kDa transmembrane protein. It contains 12 C2-type Ig-like loops in two groups of five and seven loops, respectively, separated by a hydrophobic linker (262, 263). It is thought that the nascent protein is cleaved in the hydrophobic linker to produce an N-terminal domain that is retained in the cytoplasm, whereas the larger C-terminal domain, containing a transmembrane protein, is translocated to the plasma membrane (264). *IGSF1* is expressed in many different tissues, in particular the testes and pituitary gland (265–268). Accordingly, 18 different human *IGSF1* variants have been reported, many with multiple affected family members across several generations, and some have also been found in unrelated families (265, 269–274) (Figure 3). Because the gene is X-linked, most reported patients have been males who are therefore hemizygous for *IGSF1*, presenting with central hypothyroidism (TSH deficiency) as the primary phenotype, with varying combinations of GH and/or PRL deficiencies and macroorchidism as a common symptom (Figure 3). Female carriers of *IGSF1* mutations were initially reported as unaffected, although more extensive studies have now shown that a subset (30–60%) of female carriers can be affected with central hypothyroidism, PRL deficiency, delayed age at menarche, and obesity (273, 275, 276). The gene is overall highly likely to be pathogenic for CPHD, with strong evidence from the human variants, and cell- and animal-based models have recapitulated some, but not all, of the phenotypes observed in the human variants. *Igsf1*-knockout mice also display central hypothyroidism, with defective pituitary TRH receptor (TRHR) signaling appearing to be the cause, although the mutant mice do not display the variety of other hormone deficiencies and macroorchidism observed in patients with *IGSF1* mutations (265).

6. PAX6

PAX6 is a highly conserved member of the paired homeodomain transcription factor family. Haploinsufficiency in *PAX6* has long been known to cause aniridia (277, 278). *PAX6* mutations provide a classic example of dosage-sensitive effects, in that homozygosity for loss-of-function mutations in mice cause anophthalmia, haploinsufficiency causes micro-ophthalmia, and overdosage also causes micro-ophthalmia (279, 280). During mouse pituitary development, *Pax6* is transiently expressed on the dorsal side of Rathke's pouch between embryonic day (e) 9.0 and e12.5, and it is thought to be involved in establishing a sharp boundary between dorsal and ventral cell types (281). But strain background appears to influence the features associated with *Pax6* loss of function (282,

283). A heterozygous deletion encompassing two genes and a *PAX6* enhancer was identified in a patient with IGHD, cleft palate, and optic disc cupping (200). The patient's clinically unaffected father carried the mutation, but he was mosaic for the deletion. The patient's brother had HPE, but no DNA was available for analysis. A heterozygous variant p.N116S (in the 422 amino acid isoform, or p.N130S in the 436 amino acid isoform) was identified in a Japanese male with CPHD and cryptorchidism, without ocular malformation (200). The mother is also heterozygous for this variant, but she is clinically unaffected. The p.N116S variant is not reported in the public population databases, and it is predicted as damaging by MutationTaster. The p.N116S variant is located within the paired homeodomain of the protein and affects the transactivation function of PAX6, but not the DNA binding ability (200). Over 350 heterozygous mutations in *PAX6* have been reported in individuals with ocular malformations, and pituitary hormone deficiency has only been reported for two patients. Therefore, *PAX6* mutations rarely cause pituitary abnormalities.

7. PROKR2

Heterozygous and homozygous mutations in the prokineticin receptor 2 (*PROKR2*) have been reported in patients with HH or KS, and less frequently in CPHD, IGHD, or Hirschsprung's disease. *PROKR2* encodes a G protein-coupled receptor (GPR73L1). Upon stimulation by the ligand PROK1, G proteins evoke an intracellular calcium release, and this feature is the basis for testing variant PROKR2 protein function in vitro. Functional links to causality in HH and KS are well established (284–290). *Prokr2* is highly expressed in the olfactory placode and GnRH neurons (291), and *Prokr2*$^{-/-}$ mice recapitulate the hypogonadal phenotype of humans with HH (292–294). Several individuals with HH or KS are heterozygous or compound heterozygous for *PROKR2* mutations and frequently carry one or more mutations in other genes mutated in HH or KS (digenic and multigenic cases) such as *KAL1*, *FGFR1*, *GNRHR*, *GPR54*, *KISS1*, and *NELF* (284, 295). Thus, HH and KS are excellent examples of oligogenic disease, and the involvement of *PROKR2* is strongly supported for this condition.

A frameshift and nine missense variants in *PROKR2* were found in patients with CPHD or IGHD (Supplemental Figure 1). It is not clear how mutations in *PROKR2* cause CPHD or IGHD. *Prokr2* is expressed in the developing brain and adult posterior pituitary gland, and variants in *PROKR2* were proposed to affect neuronal migration and/or development of the posterior lobe, which acts as a signaling center for stimulation of anterior pituitary growth (33, 287, 296). However, in late gestation,

Prokr2$^{-/-}$ mice have apparently normal expression of *Ghrh*, *Gh*, *Prl*, *Tshb*, and *Pomc* (288). Although *Prokr2*$^{-/-}$ mice were not examined postnatally, the existence of HH but not CPHD or IGHD suggests that mice do not model the human disorder perfectly. Alternatively, some individuals may have HH due to pathogenic variants in *PROKR2* and may be GH deficient due to variants in another gene or genes, resulting in a diagnosis of CPHD. In support of this idea, a patient with *PROKR2* p.R85H was carrying a heterozygous variant in *HESX1* (287). Additional studies are necessary to resolve this issue for other patients with *PROKR2* variants and a diagnosis of CPHD or IGHD.

8. SHH

In 2013, two heterozygous missense variants in the *SHH* gene were identified in CPHD patients (297, 298). Both variants are rare and exhibit incomplete penetrance. All three software programs predict that p.G427R is benign, and there was no evidence from functional studies to support the pathogenicity. The other variant, p.A226T, was originally reported in a patient with HPE (29). The software prediction programs list it as damaging/disease causing. Thus, "likely pathogenic" is a more accurate classification for p.A226T unless convincing functional studies are carried out.

9. TCF7L1

TCF7L1 gene encodes the transcription factor 7-like 1, which is a member of the T-cell factor (TCF)/lymphoid enhancer factor family. Within the nucleus, TCF/lymphoid enhancer factors interact with β-catenin to activate the expression of target genes (299, 300). The WNT signaling pathway and TCFs regulate proliferation of Rathke's pouch and differentiation of hormone-producing cells in the pituitary (301–306). In a recent study, *TCF7L1* expression was reported in the developing forebrain and pituitary gland. Mouse studies showed that it is required in the prospective hypothalamus to maintain normal expression of the hypothalamic signals involved in the induction and subsequent expansion of Rathke's pouch progenitors (307). Two heterozygous variants, p.R92P and p.R400Q, were identified in a screen of SOD patients and normal family members for mutations in *TCF7L1* by Sanger sequencing (307). The individual with the p.R92P variant has forebrain defects and normal pituitary function, and he inherited the variant from his unaffected father. The individual with the p.R400Q variant has SOD, absent posterior pituitary, and small anterior pituitary, and he is GH deficient. The variant was inherited from the unaffected mother, and two unaffected siblings also carried the variant. Although the p.R400Q is not completely penetrant, its pathogenicity was supported by: 1) reduced repressive function of TCF7L1 p.R400Q in vitro and in vivo; 2) the rare allele frequency (0.01% in ExAC); and 3) prediction that it is damaging by SIFT, PolyPhen-2, and MutationTaster. Thus, *TCF7L1* is a candidate gene for other cases of CPHD with the likely contribution of other genes or environmental factors.

10. TGIF1

TGIF1 encodes a homeodomain protein that represses TGFβ and retinoic acid signaling. TGFβ signaling has been shown in the paracrine communication of thyrotropes and folliculostellate cells in the regulation of TSH secretion (308). Many heterozygous loss-of-function mutations in *TGIF* are reported for HPE, and there is one report of *TGIF1* and CPHD with a central incisor, pituitary stalk disruption, and anterior lobe hypoplasia (p.Q267X), but no functional studies were carried out (297). *Tgif1* and *Tgif2* are expressed in the developing pituitary gland at e14.5 (125). The methods for testing *TGIF1* function are well established (297, 309), and the nonsense mutation seems likely to be a loss-of-function allele. Mice homozygous for a *Tgif* null mutation that deletes the entire gene are viable on mixed genetic backgrounds, but on a more pure C57BL/6J background, a proportion of the *Tgif1* null animals die perinatally (310). A different genetically engineered *Tgif1* mutation deleted exon 3 and is predicted to produce a truncated 80-amino acid protein (311). Mice heterozygous for this mutation do exhibit HPE, microcephaly, and exencephaly with high penetrance on the C57BL/6 background but are protected on the 129 strain background. Affected animals have altered expression of both *Pax6* and *Shh*. Mouse studies have demonstrated that *Tgif1* and *Tgif2* have overlapping function at gastrulation that is required for development of the anterior visceral endoderm and for normal *Otx2* expression (312). Although additional evidence is needed to support *TGIF1* as a cause of CPHD, the mouse studies strongly support the idea that it could have a role.

11. CHD7

Heterozygous loss of function in the chromodomain helicase DNA-binding protein 7 gene (*CHD7*) is the major causative factor for CHARGE syndrome (313, 314). Hormone defects include hypogonadism, growth failure with or without GH deficiency, and hypothyroidism (315). A CHARGE patient treated with GH exhibited improved growth rate and achieved target height (316). Two unrelated CHARGE syndrome children carrying rare variants in the *CHD7* gene were reported to have congenital hypopituitarism due to structural abnormalities of the pituitary gland (317). However, functional studies on these

two variants, p.P732A and c.IVS35+6T>C, have not been done. The missense variant p.P732A is rare, but both SIFT and PolyPhen-2 predict it as benign. *CHD7* is expressed in the pituitary and the hypothalamus (318). Mice heterozygous for a *Chd7* gene trap allele have HH due to decreased GnRH neurogenesis, and although they have growth insufficiency, GH and IGF-1 are normal (319). Additional studies are necessary to understand the growth insufficiency of individuals with *CHD7* mutations.

C. Genes implicated in CPHD that require additional evidence about causality

1. HHIP

Several members of the hedgehog signaling pathway have been implicated in HPE and abnormal pituitary function in humans and mice (218, 320). Because of this, a candidate gene mutation screening for *HHIP* (hedgehog-interacting protein) variants was carried out for 93 CPHD patients, and one patient was identified as heterozygous for a de novo c.-1G>C variant in the 5′-untranslated region (298). Because the −1 position of the Kozak consensus is 28% G and 45% C in humans, this change seems unlikely to affect translation (321). Consistent with this, cell culture studies did not show a significant functional effect. The patient was born preterm at 33 weeks in a breech delivery, with asphyxia requiring resuscitation and neonatal jaundice. She needed thyroid and glucocorticoid hormone replacement as a neonate and was still prepubertal at 13 years. It is difficult to rule out perinatal hypoxia as a contributor to her CPHD (322). Although *HHIP* is an excellent candidate gene for CPHD based on the known role of SHH signaling, the c.-1G>C variant appears benign. Mice heterozygous for a null allele of *Hhip* are unaffected, and homozygotes die at birth due to respiratory distress arising from developmental defects of the lung (323). They have patterning defects in multiple organs including the CNS and the pancreas, where endocrine cell proliferation is reduced (324, 325).

2. POLR3A

Polymerase RNA 3 DNA directed polypeptide A (*POLR3A*) encodes a subunit of RNA polymerase 3. It is implicated in 4H syndrome (hypomyelination, hypogonadotropic hypogonadism, and hypodontia), one of the POL3-related leukodystrophies (326). There is one documented patient with 4H and late onset GH deficiency, a compound heterozygous variant (p.R1005H, p.A1331T) with recessive inheritance (327). The p.R1005H variant (MAF = 0.00019) was predicted deleterious using SIFT but benign using PolyPhen-2. The p.A1331T variant (MAF = $8.24e^{-6}$) was predicted deleterious with both SIFT and PolyPhen-2. Approximately 50% of *POLR3A*

4H patients have short stature, but to date, there are no reports of endocrine evaluation to support GH deficiency or hypothyroidism as the cause of short stature in these patients (326).

3. RBM28

RNA binding motif protein 28 is involved with RNA processing, being a component of the spliceosomal small nuclear ribonucleoprotein complexes (328, 329). *RBM28* has been implicated in the development of alopecia, neurological defects, and endocrinopathy (ANE) syndrome. Individuals with mutations in this gene present with variable hair loss, intellectual disability, central adrenal insufficiency, and tooth abnormalities. There is one documented variant that is associated with recessive hypopituitarism: five siblings had a homozygous mutation, p.L351P (MAF = $6.59e^{-5}$), predicted to be deleterious using SIFT and PolyPhen-2. All five patients had CPHD and ANE syndrome (329, 330). The hormone deficiencies varied: all of the siblings had GH, LH, and FSH deficiencies, but only two had ACTH deficiencies, and one was PRL deficient. One of the five siblings also had anterior pituitary hypoplasia. *Rbm28* is expressed in the developing mouse pituitary gland at e12.5 and e14.5 (125). Although *RBM28* is an excellent candidate, the mechanism by which *RBM28* leads to CPHD remains to be elucidated.

4. WDR11

Mutations in the WD40 repeat containing protein WDR11 have been implicated in autosomal dominant HH and KS, and occasionally with craniofacial abnormalities including cleft palate, sensorineural hearing loss, and brain and dental abnormalities and WDR11 is expressed in the developing hypothalamus and pituitary (125, 331). Six unrelated patients with HH or KS contained sporadic heterozygous variations in the *WDR11* gene (331, 332). Four of the missense variants (p.A435T, p.H690Q, p.R448.5q, and p.R395W) were located in the EMX1 binding region, which has overlapping function with *OTX2*. Coimmunoprecipitation of EMX1 and WDR11 with these variants determined that p.A435T (MAF = 0.00047) and p.H690Q (MAF = $6.49e^{-5}$) completely disrupted EMX1 binding, p.R448.5q (MAF = 0.00045) reduced EMX1 binding, and p.R395W (MAF = 0.001) had no effect (331). These results do not align with SIFT and PolyPhen-2 predictions because p.R395W was predicted to be tolerated and possibly damaging by SIFT and PolyPhen-2, respectively. Variants p.A435T, p.R448.5q, and p.F1150L (MAF = 0.001) were predicted to be tolerated and benign, whereas p.H690Q was predicted to be deleterious and probably damaging by SIFT and PolyPhen-2.

However, the researchers found that the three variants that altered EMX1 binding were predicted by SPPIDER (http://sppider.cchmc.org/) (333) to alter protein-protein interactions. Given the expression pattern, association with *OTX2* and HH, *WDR11* may be a candidate gene for CPHD.

5. IFT172

The intraflagellar transport (IFT) 172 gene encodes a subunit of the B subcomplex of IFT. The IFT complex is important for ciliary assembly and maintenance and plays a role in hedgehog signaling (334). A patient with pituitary hypoplasia, ectopic posterior lobe, growth retardation, facial abnormalities, intellectual delay, skeletal abnormalities, and degeneration of kidney and eye was found to be a compound heterozygote for mutations in *IFT172*: p.Cys1727Arg and a novel splice site mutation in intron 4, c.337–2AC (335). He had a normal GH stimulation test but persistently low IGF-1 and a positive response to recombinant human GH therapy. His features are similar to patients with Mainzer-Saldino syndrome, which can be caused by mutations in *IFT140* or *IFT172*. These variants are predicted to be deleterious, but no functional studies were done. Although this patient did not have CPHD, his case suggests that *IFT172* could be another candidate gene for CPHD, and it would likely be associated with other developmental anomalies. Mice homozygous for null allele die during gestation with multiple anomalies including neural tube defects, VACTERL-like features, and homeotic transformations of the skeleton (336, 337). A conditional allele exists and could be used to study the effects of IFT172 on hypothalamic and pituitary development (338).

D. CPHD candidate genes discovered by WES

1. ARNT2

The *ARNT2* gene encodes a basic helix-loop-helix transcription factor: the aryl hydrocarbon receptor nuclear translocator. The only *ARNT2* mutation described to date is the c.1372_1373dupTC mutation, which occurred in a family of six generations with consanguineous marriages (123). Because of the consanguinity, autosomal recessive inheritance was predicted, and single nucleotide polymorphism (SNP) analysis was carried out on four affected individuals to identify regions of homozygosity. WES was carried out on one affected individual, and the segregation of the mutation was confirmed in the pedigree. The clinical presentation included CPHD, brain, eye, kidney, and urinary tract abnormalities in all individuals homozygous for the c.1372_1373dupTC mutation, and none of the heterozygotes. Two SNPs in the promoter region of

ARNT2 affect transcription, but their significance in human disease is not known (339). *Arnt2* has a broad expression pattern in the mouse embryo, including the CNS, spinal cord, hypothalamus, pituitary, eye, lung, and kidney, which correlates with the embryonic human expression (340). The patient phenotype is well connected to the developmental expression pattern. Mice homozygous for a null allele of *Arnt2* die shortly after birth of unknown cause, and they lack hypothalamic secretory neurons including vasopressin, oxytocin, CRH, TRH, and somatostatin (341, 342). This phenotype is similar to that of mice with a knockout of *Sim1*, which encodes a related protein that is known to dimerize with ARNT2, providing further support for the role of *ARNT2* in CPHD (343, 344).

2. ZSWIM6

ZSWIM6 is a transcription factor containing a zinc finger domain and a SWIM domain, which are important in DNA binding and protein-protein interactions, respectively. A heterozygous mutation in the sin-3 like domain of ZSWIM6 (p.R1163W) has been found in eight unrelated individuals with acromelic frontonasal dysostosis (345, 346). This disorder is characterized by severe frontonasal dysplasia, neurocognitive and motor delays, and preaxial polydactyly. Other associated variable features include hypopituitarism, absent olfactory bulbs, cryptorchidism, hypoplastic corpus callosum, and patellar hypoplasia. There was no detailed endocrine analysis of hypothalamic-pituitary function. Exome sequencing was carried out on two unrelated affected individuals and a trio with a mildly affected father, unaffected mother and affected child (345). A dominant mode of inheritance was initially considered, but no likely candidate variants were shared by the father and the affected child, and no variants were detected in unrelated affected individuals. An apparent de novo variant was found in the affected child from the trio, and the same de novo mutation was found in the two unrelated individuals that were sequenced. An unrelated adoptee carried the same mutation and may have been mosaic. Mosaicism could explain the mildly affected father as well, although the mutation was not detected in his DNA sample. Recently, four additional examples of unrelated individuals with acromelic frontonasal dysostosis were found to have the same p.R1163W variant (346). MutationTaster and in silico structural and atomic-resolution modeling predict that the p.R1163W variant is pathogenic, and primary osteoblast cell lines from patients, but not controls, had alteration in hedgehog signaling transcripts (345). This variant has not been identified in the normal population, but heterozygous deletions of *ZSWIM6* are found in unaffected individuals,

suggesting that the p.R1163W change is a gain-of-function mutation.

3. GPR161

G protein-coupled receptor 161 (*GPR161*) gene is located on 1q24.2. Activation of the receptor elevates cAMP, which leads to proteolytic conversion of Gli transcription factors (GLI2 and GLI3) into Gli repressor forms and inhibition of SHH signaling (347). There is a single report of CPHD caused by a homozygous missense *GPR161* mutation c.56T>A; p.L19Q in a consanguineous family. WES was carried out on two affected individuals and an unaffected sibling, and variants were filtered considering autosomal recessive inheritance. The parents were confirmed to be heterozygous for the p.L19Q variant, and segregation in the pedigree was consistent with autosomal recessive inheritance. The two affected individuals both have pituitary stalk interruption syndrome, other pituitary radiographic abnormalities, and GH deficiency. One sister has IGHD, but the other also has diabetes insipidus and central hypothyroidism. PolyPhen-2 predicts that the p.L19Q variant, in a highly conserved residue, is probably damaging. Structure prediction programs suggest that the variant is an extracellular loop that could affect ligand binding, but the ligand is not known, and SHH signaling is difficult to assess in fibroblasts. *Gpr161* is expressed in the developing mouse pituitary gland, but detailed expression studies have not been carried out (125). Without further information, it is difficult to be certain that *GPR161* p.L19Q causes hypopituitarism because: 1) this variant has a MAF of 2% among Asians and 3% overall, and there are seven homozygous individuals; 2) there is no functional study to prove that the p.L19Q variant is deleterious; and 3) we would expect a variant in *GPR161* to be gain of function in order to enhance the suppression of SHH signaling, which seems unlikely for a recessive inheritance pattern. However, *GPR161* also regulates WNT and retinoic acid signaling, which are important for normal pituitary development, and the effects of *GPR161* p.L19Q on these signaling pathways was not assessed (348–350). Identification of additional individuals with CPHD or IGHD and variants in *GPR161* could enhance the confidence that this gene has a role in pituitary development and function.

Studies in mice reveal that *Gpr161* is important for development of multiple organs, and genetic background can have a major effect on the penetrance of the phenotype (348). Homozygotes for a hypomorphic (reduced function) C-terminal truncation allele are generally not viable and have extensive craniofacial abnormalities, spina bifida, and limb defects. On certain genetic backgrounds the mutants are viable and have a belly spot and vacuolated

lens. SHH signaling is not affected in these mutants, but retinoic acid and WNT signaling are, and retinoic acid injection can rescue the neural tube defects. Homozygotes for a null allele have fully penetrant lethality, craniorachischisis, and craniofacial abnormalities (351). These data suggest that if *GPR161* p.L19Q is causal, the allele may be hypomorphic and influence retinoic acid signaling.

4. HNRNPU

HNRNPU (heterogeneous nuclear ribonucleoprotein U) is a member of the group of proteins that form complexes with nascent pre-mRNA transcripts in the nucleus of the cell. It is implicated in CNS abnormalities, with patients displaying learning disabilities and seizures. Some individuals are diagnosed with Lennox-Gastaut syndrome, which is characterized by epilepsy and occasionally impaired intellectual development (352). *HNRNPU* was first implicated as a causal gene for CPHD when found in a patient during WES of 119 trios with undiagnosed disorders who were referred for genetic testing (353). A splice site variant, c.1615−1 G>A, was found in a child with CPHD and epilepsy. It is predicted to be deleterious using SIFT and probably damaging by Poly-Phen-2. HNRNPU interacts with PITX2 and may be involved in the regulation of heterogeneous nuclear RNA stability (354). HNRNPU can also inhibit *GNRH* transcription (355, 356). Functional studies are needed to assess the effects of this variant on *HNRNPU* function. Mouse HNRNPU differs from human by only a single amino acid. Mice homozygous for a hypomorphic mutation in *Hnrnpu* exhibit early embryonic lethality at e9.5, growth retardation, and defects of the allantois (357). Mice homozygous for selective deletion of *Hnrnpu* have lethal cardiomyopathy and extensive defects in splicing mRNAs that are important for heart development (358).

5. PNPLA6

PNPLA6 encodes the patatin-like phospholipase domain containing protein 6, which is responsible for the de-esterification of membrane phosphatidylcholine into fatty acids and glycerophosphocholine. The first *PNPLA6* mutation implicated in a human disease was discovered in 2008 in a linkage study focused on a progressive spastic paraplegia and a distal muscle-wasting phenotype (359). Since then, autosomal recessive, compound heterozygous mutations have been found in individuals with a variety of neurodegenerative conditions including childhood blindness (360), Leber congenital amaurosis (360), Oliver MacFarlane syndrome, Laurence-Moon syndrome, Gordon Holmes syndrome, and Boucher-Neuhäuser syndrome (361). The distinction between the syndromes may be artificial, arising from variable clinical presentations. Indi-

viduals with Oliver MacFarlane or Laurence-Moon syndrome have congenital hypopituitarism including GH, TSH, and gonadotropin deficiency and chorioretinal atrophy. Craniofacial dysmorphology, ataxia, and paraplegia may also be evident. Boucher-Neuhäuser and Gordon Holmes syndromes have cerebellar ataxia and HH, but chorioretinal dystrophy is present only in the former (361, 362). Childhood blindness results from widespread photoreceptor neuron death (360). Homozygous and compound heterozygous mutations affecting the patatin-like phospholipase domain are fully penetrant. The mechanism underlying pituitary dysfunction may be impaired vesicular transport because *PNPLA6* mutant cells exhibit reduced secretion in response to stimulation (363). *PNPLA6* is expressed in the developing pituitary gland, and loss-of-function mutations have been identified in six unrelated individuals with pituitary dysfunction. Thus, *PNPLA6* should be considered in cases of CPHD or HH with features characteristic of *PNPLA6* associated syndromes. In mice, conditional inactivation of *Pnpla6* in the CNS causes neurodegeneration (364). However, fetuses homozygous for a null allele exhibit failed placental development, impaired blood vessel development, growth retardation by e7.5, and death by e9 (365). These results suggest that the human mutations in *PNPLA6* may be partial loss-of-function alleles.

6. CDON

CDON (cell adhesion associated, oncogene regulated) is a cell surface SHH-binding protein that promotes SHH signaling activity (366). Heterozygous loss-of-function mutations in *CDON* have been reported in HPE patients, including some with GH deficiency and absent pituitary (p.P689A and p.T790A, respectively) (102). $Cdon^{-/-}$ mice recapitulate the range of HPE phenotypes from unaffected or mildly affected to severe, depending on genetic background (367). Interestingly, maternal ethanol exposure synergizes dramatically with *Cdon* loss of function to produce highly penetrant HPE (36). On a 129S6 genetic background, $Cdon^{-/-}$ mice are unaffected, but if exposed to ethanol, 75% have HPE, providing strong evidence for gene-environment interaction. Cell-based assays using $Cdon^{-/-}$ mouse embryonic fibroblasts showed that the p.P689A variant is defective in conferring SHH-dependent induction of *Ptch1* and *Gli1*, but the p.T790A was not tested. By WES, a novel nonsense variant p.E922* was recently identified in a patient with pituitary stalk interruption syndrome and panhypopituitarism (368). The mother also carried this variant, and she had convergent strabismus that required surgical correction but normal height. This variant has never been reported in the public population database. The mutation of amino acid 922 to

a stop codon will result in a truncated CDON protein without the transmembrane and intracellular domain, which is expected to destroy function. Taken together, the GH-deficient HPE patient with p.689A, the p.E922* CPHD patient, and the studies in genetically engineered mice suggest that *CDON* is a candidate gene for other cases of CPHD.

E. Summary

We assessed the pathogenicity of reported variants in CPHD genes according to the current standards in the field. It is not surprising that most of the criteria were met by most of the earlier report and more established genes. More analysis is required to prove causality of some of the most recently reported candidates. Overall, WES has identified six new candidate genes for CPHD. To date, only *CDON*, *ZSWIM6*, and *PNPLA6* mutations have been found in multiple unrelated individuals. For disorders that are highly heterogeneous like CPHD, it can be helpful to take advantage of the online platform MatchMaker Exchange (http://www.matchmakerexchange.org/) to facilitate identifying cases with similar phenotypic and genotypic profiles among different research groups. In addition, functional studies are critical for testing the pathogenicity of the detected variants both in vitro and in vivo.

IV. Strategies to Improve Diagnostic Yield for CPHD and Future Directions

NGS tools have identified genetic variants responsible for many human diseases, and in some cases the molecular diagnosis has effectively guided physicians to effective therapeutic intervention (369). We expect that rigorous application of these methods will result in better molecular diagnosis of CPHD and provide new insight into pituitary organogenesis. However, the diagnostic yield for WES in a research setting is currently between 10 and 30% (370). The potential reasons for this and some strategies to improve the discovery of new genes and variants are discussed below.

A. Incomplete coverage of WES

WES is designed to identify coding variants and splice variants at intron-exon borders in all known genes, but in practice, the whole exome is not assayed because some areas of the genome are difficult to capture and/or sequence. For example, *SOX3* is 75% guanine and cytosine GC, and more than half of the *SOX3* coding region has < 20 fold coverage in the ExAC database (http://exac.broadinstitute.org/gene/ENSG00000134595). We

also experienced gaps in covering this gene in our WES analysis of CPHD patients (unpublished data). To avoid a false-negative result, such problematic areas should be screened by Sanger sequencing with modifications (371, 372), but this can be tedious. Improvements in high throughput capture and sequencing are emerging and are likely to continue to evolve, resulting in improved diagnostic yield (373). Some coverage gaps can be avoided using exome sequencing methods targeted to specific collections of candidate genes (Figure 4). This enriches the capture of a region of interest, reducing the likelihood of incomplete coverage. Targeted candidate gene sequencing methods can be applied to many samples at a time, providing an economical way to obtain deep coverage of genes in the panel (374). This can be a useful first step in determining causes of disease within a large sample set.

Although the targeted gene panels only provide information about the chosen genes or regions, they provide an economical, high throughput way to screen large sample sets for candidate genes at greater read depth than would be obtained with WES.

B. Genetic variants that are difficult to detect with WES (structural variants, splicing region, enhancers, and noncoding variants)

Rearrangements and copy number variations (CNVs), including insertions and deletions (indels) and duplications, are often missed by WES. Chromosomal microarray (SNP array) is a first-tier screen for genetic defects caused by CNVs and is recommended as a complement to exome sequencing (375). SNP arrays cover a wide area of the genome, including intergenic and nongenic regions. In-

Figure 4.
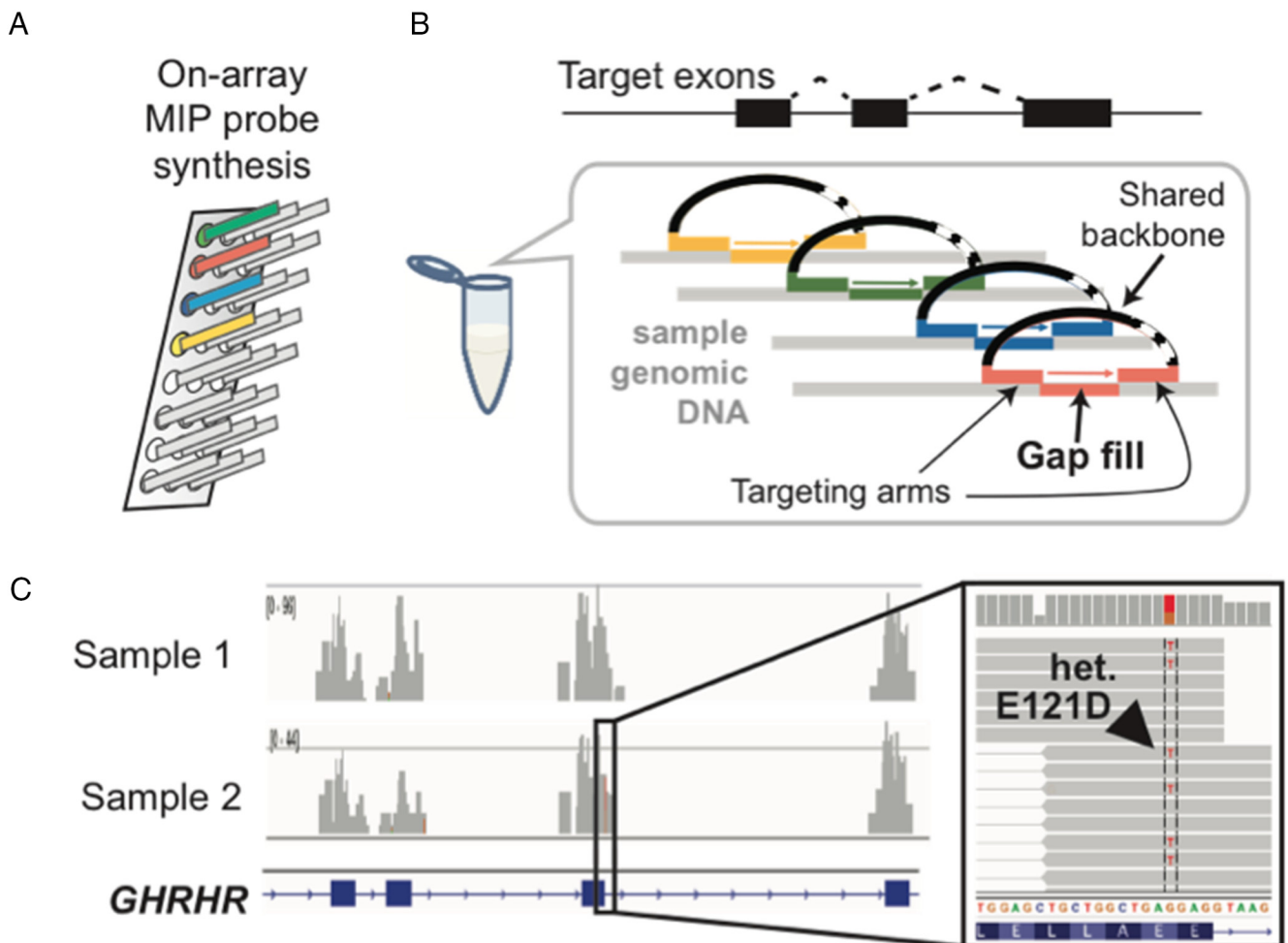
Figure 4. Molecular inversion probe (MIP) capture. A, MIP probe panels are synthesized in parallel on a microarray. B, In a single-well reaction, hundreds to thousands of exons are targeted in parallel by capture probes. As each probe anneals, the target is enzymatically copied and ligated to the probe backbone to yield a sequencing library. C, Example of MIPS sequencing data at *GHRHR* showing coverage for two samples, including a heterozygous missense SNV.

creasingly dense arrays, up to several million probes, offer high-resolution mapping of CNVs. SNP arrays typically detect a pathogenic CNV in 20% of the cases (376). WGS is another approach to identify lesions missed by WES, and as the cost of WGS drops, we expect it to replace WES. In many cases, the bioinformatics analysis must be specifically directed at examining mismatched paired-end reads to detect structural variation (377–379). If cytogenetic abnormalities or CNVs are known to be present from karyotype or chromosomal microarray analysis, jumping libraries can be carried out as an alternative to WGS to identify precise, gene-level information about the genetic lesion (380, 381).

Splice enhancers are sequences that enhance the proper usage of weak splice sites by serine-arginine splice regulators. Variations in exonic splice enhancers that do not change the encoded amino acid are an important cause of disease that is missed by most software prediction programs (382). Indeed, silent mutations in the exonic splice enhancers of the GH gene are an important contribution to autosomal dominant IGHD, which can progress to cause CPHD (383). Skipping exon 3 results in production of the 17.5-kDa GH isoform that blocks secretion of the 22-kDa biologically active form. Pharmaceuticals that improve the use of weaker splice sites are being explored as a treatment for this form of IGHD, and for prevention of the progression to CPHD (384). Improvements in software may make it easier to predict exonic splice enhancers and identify silent, pathogenic mutations.

Variation in regulatory elements is likely to be an important cause of disease (385), and 88% of the SNPs identified in GWAS are not in the exome (386). Taken together, these observations suggest that identifying regulatory regions of the genome (regulome) is an important area of future investigation. Many of the critical regulatory elements are species, developmental, and/or cell type specific. Analysis of the pituitary or hypothalamic regulome has not yet been included in the ENCODE project. Thus, more information is needed about the location of hypothalamic-pituitary regulatory elements for effective utilization of CNV and WGS information. We have recently identified the binding sites for PROP1 in rodent

Table 3. CPHD Gene Expression

| Gene | Fetal[a] | | | | Adult | | | |
| | Mouse | | Human | | Mouse | | Human | |
	Hypothalamus	Pituitary	Hypothalamus	Pituitary	Hypothalamus	Pituitary	Hypothalamus	Pituitary
POU1F1	−	x	?	x	−	x	−	x
PROP1	−	x	?	−	−	−	−	−
HESX1	−	x	x	x	−	−	−	−
LHX3	x	x	?	x	−	x	−	x
LHX4	x	x	?	−	−	−	−	−
OTX2	x	x	?	−	−	−	−	−
GLI2	x	x	x	x	−	x	−	−
SOX2	x	x	x	x	x	x	−	−
SOX3	x	−	?	−	−	−	−	−
FGF8	x	x	x	−	−	−	−	−
FGFR1	x	x	x	x	−	−	−	−
GLI3	x	−	?	−	−	−	−	−
PAX6	x	x	?	−	−	−	−	−
ARNT2	x	x	x	−	x	−	x	−
BMP4	x	x	x	−	−	−	−	−
IGSF1	x	x	?	x	x	x	x	x
PNPLA6	x	x	?	x	−	−	−	−
SHH	x	x	?	−	−	−	−	−
TCF7L1	x	x	x	x	−	−	−	−
ZSWIM6	x	x	?	−	−	−	−	−
CHD7	x	x	?	−	−	x	−	−
PROKR2	x	?	?	−	x	−	−	−
TGIF1	x	x	?	−	−	−	−	−
CDON	x	?	?	−	−	−	−	−
GPR161	?	x	?	−	x	−	−	−
HHIP	?	x	?	−	−	−	−	−
HNRNPU	?	?	?	x	−	−	−	−
POLR3A	−	−	?	−	−	−	x	x
RBM28	x	x	?	−	−	−	−	−
WDR11	?	x	?	−	−	−	−	−

[a] For many genes, numerous embryonic and fetal developmental ages have been examined in mouse. For human genes, information is available for 16-week gestation in Ref. 391. "x" menas enriched expression, "−" means the expression is not enriched, and "?" means unknown.

cell lines, which is an important step toward defining the pituitary regulome (129). A concerted effort to identify regulatory sites in the developing hypothalamus and pituitary gland would be valuable information as the field moves toward WGS analysis of CPHD.

C. Large number of tolerated variants: filtering by expression profiles and pathways

A challenge in interpreting WES data is the large number of tolerated, rare variants, which makes it difficult to filter the genes and nominate the appropriate candidate for functional studies. Often, WES is applied to trios including the unaffected parents and a single affected child. If the disorder is caused by a recessive mutation or de novo mutation, the list of candidate genes will usually be manageably short. If the disorder is caused by a dominant mutation with incomplete penetrance, then the list of candidate genes will be much longer, and it could be intractable. If there are multiple affected individuals in a pedigree, it is advisable to apply WES to the most distantly related affected individuals because they will have fewer rare variants in common that require filtering.

We reviewed the gene expression patterns of genes that are known to cause CPHD to determine whether gene expression profiles would be an appropriate way to prioritize candidate genes. Gene expression data from BioGPS (http://biogps.org) (387, 388) and MGI-GXD (Mouse Genome Informatics, http://www.informatics.jax.org; Gene Expression Database [GXD]) (389) were used to determine the enrichment of the 30 CPHD candidate genes in mouse and human hypothalamus and pituitary at different development stages. We considered gene expression enriched if its expression in the pituitary or hypothalamus was greater than three times the median in BioGPS or was present in the indicated tissue in MGI-GXD. For many genes, numerous embryonic and fetal developmental ages have been examined in the mouse hypothalamus and pituitary. Such extensive human gene expression analysis has yet to be performed. Human 16-week fetal pituitary expression and hypothalamic and pituitary expression for specific genes at various Carnegie stages were used to determine the developmental human gene expression for the 30 candidate genes (103, 123, 218, 232, 307, 362, 390, 391).

We found that the known genes have high brain and pituitary specificity for expression, and notably, expression is skewed to embryonic, but not adult stages (Table 3). Based on the expression patterns of these known genes, future candidate genes for CPHD causality should include genes that affect early head development, including anterior structures at gastrulation, craniofacial placode development, and the organogenesis of the hypothalamus and pituitary gland (21, 392–394). Expression profiling has been carried out in developing mouse pituitary gland at e12.5 and e14.5 (125), and in migrating neural crest and craniofacial placodes (e8.5 to e10.5) (395, 396). Numerous fetal-specific, novel expressed sequence tags were identified by expression analysis of fetal (male, 16 weeks) and adult human pituitary glands (391). A more comprehensive database on head, hypothalamic, and pituitary gene expression with spatiotemporal information would be valuable for advancing CPHD research.

Identifying genes in the CPHD interactome could be useful in filtering candidate genes for functional studies (397, 398). For example, several genes that encode members of the SHH signaling pathway are implicated in CPHD, and as more is learned about the mechanism of action of other known CPHD genes, pathway analysis could assist in prioritizing candidate genes. Knowledge of protein interactions with other proteins, DNA, RNA, and metabolites will also be valuable in understanding the mechanisms of disease.

D. Functional testing: genetic editing and animal models of human disease

As an increasing number of rare variants are docu-

Box 2. Main Points

Pituitary gland and hypothalamic development are dependent upon the expression of critical transcription factors and cell-cell signaling.

Mutations in these transcription factor and signaling pathway genes are responsible for some cases of pituitary hormone deficiency.

Thirty genes have been implicated in CPHD, but 84–90% of the cases still have unknown etiology.

The most commonly mutated gene in patients with combined hormone deficiency is the pituitary transcription factor *PROP1*, which accounts for 11% of cases.

The clinical presentation can vary among patients with the same mutation because of incomplete penetrance, variable expressivity, and/or the involvement of other genetic or environmental factors.

The genetics of CPHD place this phenotype in spectrum of disorders ranging from craniofacial defects such as holoprosencephaly to IGHD.

Advanced genome sequencing technology and bioinformatics hold promise for a better understanding of the genetic causes of this disorder.

Functional studies are necessary to assess the pathogenicity of novel genetic variants.

mented in both diseased and healthy populations, understanding which genetic changes are benign and which increase disease risk is the new frontier of translational research. Some of the most common functional characterizations involve cell transfection studies, in which the mutated gene of interest is transfected into a population of cells, and the function, expression, and stability of the mutated protein is characterized. Although imperfect, these assays can be scaled up to test many variants. Transfection studies can yield false negatives. For example, a transcription factor variant that does not influence DNA binding or transactivation of a consensus reporter gene in transient transfection may still have a negative effect on protein-protein interactions that are necessary for function and/or specificity in intact animals because these interactions and chromatin remodeling properties are not assayed in the cell culture system. For example, OTX2 interactions with LHX1, FOXA2, and LDB1 are important for its function (399–401). Protein interactions critical for LIM homeodomain proteins and POU1F1 are emerging, but many critical interactions are still unknown (142, 402).

Animal models are the "gold standard" for functional studies and can give invaluable insight into the mechanism of action. For example, mutations in *OTX2* fail to activate transcription of *POU1F1* and several other promoters in transfected cells, which clearly documented the deleterious effect of the variants on function. However, the mechanism of action does not involve regulation of *Pou1f1* because deletion of *Otx2* in the anterior pituitary has no effect, and the primary region of *Otx2* expression is in the ventral diencephalon (115). Disruption of *Otx2* in early head development and in the developing hypothalamus affects FGF signaling, which secondarily affects anterior pituitary growth. Another advantage of animal models is that they provide an opportunity to standardize the genetic background, which makes it easier to determine whether specific mutations are loss-of-function or hypomorphic alleles (390).

The time and expense associated with generating mouse models of human disease has led to the implementation of zebrafish as a high throughput, economical method of assessing function of human genetic variants. This approach is particularly relevant for CPHD studies. Pituitary development is conserved among vertebrates, and many of the genes that are critical for mammalian hypothalamic pituitary development have orthologs that function the same way in zebrafish (307, 403, 404). Zebrafish were used successfully to test *OTX2* mutations discovered in patients with the otocephaly-dysgnathia complex, and *prrx1*, *pgap1*, and *msx1* knockdown enhanced the severity of the phenotype (192). The use of

morpholinos to disrupt expression has been complicated by off-target effects and nonreproducible results, however (405). Genome-editing technology, using CRISPR/Cas9, is an alternative to morpholino studies in fish and has also facilitated generating mouse models of disease.

CRISPR is an endonuclease that can be targeted to a region of the genome in a highly specific manner. Briefly, CRISPR is transfected into a one-cell embryo with a single-guide RNA, targeting the endonuclease to the gene of interest, and the digestion and repair process disrupts it (406). In the presence of a donor DNA plasmid containing regions of homology to the cut region, knock-ins and floxed alleles are also possible (407–410). Technological improvements have increased specificity and efficiency and reduced off-target effects (411). This technology will allow us to understand how genes function in a physiological context in a variety of model organisms because it obviates the need for stem cells; all of the necessary materials can be injected into fertilized eggs or cells. Gene editing may have value for treating some genetic disorders (412), in addition to its role in generating animal models of disease.

E. Multifactorial disease

Systematic genetic screening for CPHD has only been reported for a few genes, and it is likely that there are additional genes to discover because approximately 84% of the cases are undiagnosed (46). The work summarized here makes it is clear that CPHD is a genetically complex disorder involving 30 or more genes. In addition, the responsible genes, range of syndromic features, and common occurrence of incomplete penetrance associated with CPHD suggest that it is an oligogenic disease and part of a spectrum disorder that includes craniofacial abnormalities. CPHD has genetic and phenotypic overlap with SOD, HPE, and HH, and there is evidence for multifactorial genetic contributions to these disorders (33, 413–415).

Our experience in screening a collection of sporadic and familial CPHD patients for mutations by WES is consistent with this hypothesis (unpublished results). In our screen of 69 exomes from 25 unrelated families, we were able to make a clear molecular diagnosis for only three of the probands. We found mutations in *HESX1* (416) and *GHRHR* (unpublished data). We did not identify another major, simple Mendelian gene like *PROP1*, which accounts for approximately 11% of cases worldwide (46). We did identify rare heterozygous variants that are predicted to be deleterious in six genes that have been implicated as PROP1 targets (129) or in HH, HPE, and SOD (unpublished data). Six probands have heterozygous, rare,

likely deleterious variants in two or more genes, suggesting digenic or oligogenic disease. These results suggest that CPHD, like HH, can arise from compounding effects of multiple genes and requires analysis of genetic load in cases and controls (353).

Given the large sample sizes that are necessary to identify modifier genes, it is possible that mice could be used to identify loci and genetic pathways that enhance and suppress incompletely penetrant CPHD phenotypes (92, 417). For example, two loci have been mapped that modify the craniofacial features of $Otx2^{+/-}$ mice, but the underlying genes have not been identified yet (114).

V. Conclusions

Over the past 24 years, 30 CPHD genes have been identified, which has resulted in a remarkable improvement in our basic understanding of the development of the hypothalamic-pituitary axis, the cellular and molecular regulation of hormone production and secretion, and the genetic basis for endocrine disorders (see Main Points, Box 2). The emergence of cheaper and efficient NGS technologies promises to advance both basic and clinical CPHD research. The benefits will extend beyond CPHD because many of the genes identified also affect craniofacial development, which is not uncommon and can have serious consequences for quality of life. As we enter the era of genomic medicine, we anticipate faster and more accessible genetic testing, enormous datasets, and a more complicated decision-making process to define the genetic causes for human diseases. We envision that the current NGS technologies will continue to improve in accuracy and diagnostic yield. The focus of this review has been to assess the candidates of CPHD at both the gene and variant levels to ensure that reporting of "causal" genetic defects has sufficient validation and supporting evidence, with the hope that this will be instructive for genetic analyses of other endocrine system disorders. We expect this review will provide a foundation for endocrinologists and other clinicians as they expand their work into precision medicine. Finally, we encourage collaborations and data sharing among the clinical genetic-testing laboratories and academic research groups in order to empower the identification of genes underlying rare genetic endocrine disorders.

Acknowledgments

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