

## MOLECULAR GENETICS OF HOLOPROSENCEPHALY

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### 1. ABSTRACT

Holoprosencephaly (HPE) is a common developmental defect of the human forebrain and midface. Pathological studies have identified different categories of severity of the brain and craniofacial malformations observed in HPE, although the variable clinical spectrum of HPE extends in unbroken sequence from alobar HPE and cyclopia to clinically unaffected carriers in familial HPE. The etiology of HPE is extremely heterogeneous including both environmental and genetic causes. Here we focus on molecular aspects of HPE in light of the recent identification of some of the genes causing human HPE and other candidate genes involved in forebrain development, through different approaches, such as *positional cloning* and *functional cloning*, based on animal models. These approaches will aid in the identification of additional genes involved in HPE and in a better understanding of the molecular genetics of brain development.

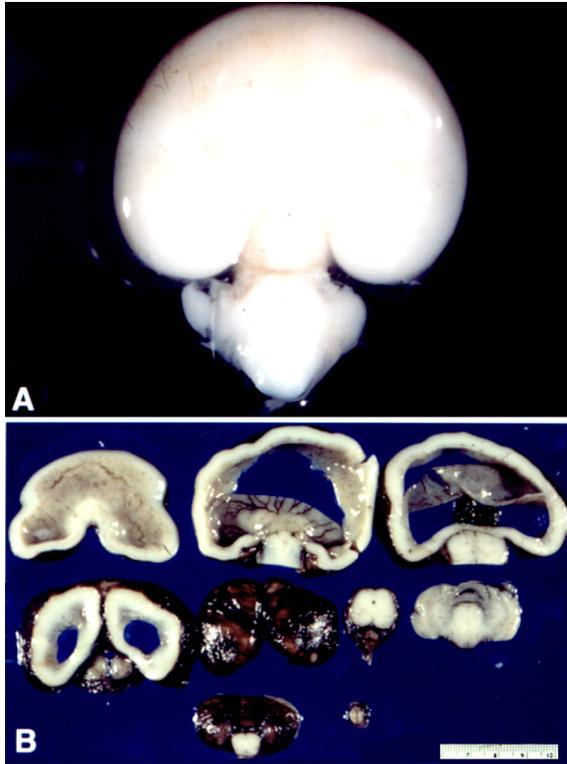
### 2. INTRODUCTION

Holoprosencephaly (HPE) is a common congenital malformation in which the forebrain (prosencephalon) fails to cleave along the mid-sagittal axis into distinct left and right hemispheres, into telencephalon and diencephalon, and into olfactory and optic bulbs tracts (1). During the neurulation process, the neural plate, derived from the ectoderm on the dorsal midline of the embryo, folds into the neural tube, whose most rostral end is destined to become the forebrain. Based on animal studies, the disruption of the normal dorsal-ventral patterning of the forebrain can result in HPE-like

malformations (2,3). The defect arises very early in the gastrulation stage of embryonic development. In the human, this corresponds to the third week of embryogenesis with the conversion of the two germ layers into a three-layered embryo (4).

Anatomical brain and craniofacial malformations are the principle diagnostic criteria for HPE (1,5). HPE is classified into three categories of severity (figure 1): alobar HPE, the most severe form, in which there is no interhemispheric fissure, a single brain ventricle is present, and there may be cyclopia and/or a proboscis-like nasal structure. In semilobar HPE, the interhemispheric fissure is present only posteriorly. In lobar HPE, the mildest form, most of the cerebral hemispheres and lateral ventricles are separated. Fusion of the most rostral and ventral portion of the telencephalon, and anomalies of the midline structures, such as thalami, corpus callosum, olfactory and optic bulbs may be present.

Phenotypic expression of HPE is quite variable and 70-80% of cases with alobar HPE have facies diagnostic for HPE (6-8). Cyclopia or synophthalmia, severe ocular hypotelorism with divided orbits, and a proboscis-like nasal structure are mostly associated with alobar HPE. Cebocephaly and median cleft lip are seen in alobar or semilobar HPE. Less severe facial dysmorphisms (figure 2) including ocular hypotelorism, iris coloboma, absence of nasal bones, single central incisor, unilateral or bilateral cleft lip/palate, and midface hypoplasia may be seen in any of the anatomic forms. Craniofacial anomalies



**Figure 1.** Brain anomalies in holoprosencephaly. (A) Alobar HPE of the forebrain without any identifiable midline structures in an 18-week gestation male fetus with triploidy. (B) Semilobar HPE with an incomplete interhemispheric fissure posteriorly (lower right) in a 30-week gestation male fetus with synophthalmia, a proboscis above the fused eye and a deletion for chromosome 18p. Same as Fig. 2C (Modified from 82).

not necessarily associated with abnormal brain imaging are considered HPE microforms, and include microcephaly, ocular hypotelorism, impaired sense of smell, or single central upper incisor. These microforms can be the only sign for a given individual of carrying an HPE gene and being at risk for having a child with HPE. Furthermore, up to 30% of obligate carriers of an HPE gene in autosomal dominant pedigrees are clinically unaffected (8-10). The wide variability in the clinical features is not only seen in sporadic HPE; even within a single pedigree, different members carrying a specific mutation in an HPE gene may present with the full range of HPE, HPE microforms, and even a completely normal phenotype (8,10,11). Thus, counseling for recurrence risk should be cautious in families with an HPE history.

The prevalence rate of HPE is 1 in 250 during embryogenesis (12), while it is estimated to be 1 in 10000 to 1 in 20000 among live births (13,14). The majority of HPE cases are apparently sporadic, although clear examples of autosomal dominant (AD) inheritance have been described (15). Pedigrees suggesting autosomal recessive and possibly X-linked inheritance have also been reported (8,9,15,16).

HPE is extremely heterogeneous with both teratogenic and genetic causes (7,9,16,17). The best

documented environmental factor in humans is maternal diabetes. Infants of diabetic mothers have a 1% risk (a 200-fold increase) for HPE (18). Maternal hypocholesterolemia during early gestation may be one of the environmental factors that contributes to the HPE outcome. HPE can be a manifestation of Smith-Lemli-Opitz (SLO) syndrome (19,20), caused by a defect in cholesterol biosynthesis (21-23). Other teratogens such as ethanol (24) and retinoic acid (25) have been associated with HPE in animal models. The effect of these factors in humans needs to be established.

### 3. Genetic basis of HPE

Different evidence support the genetic basis of HPE: approximately 18-25% of HPE cases have a recognizable monogenic syndrome (14, 26), and up to 45% of live births with HPE have non random chromosomal abnormalities (14). Furthermore, familial instances of HPE with several affected relatives have been reported in the literature; approximately 100 unrelated HPE families have been assembled by now in our laboratory.

Studying HPE on molecular level has led to the identification of the HPE genes: *Sonic Hedgehog (SHH)* (11,27), *ZIC2* (28), and *SIX3* (29), in addition to several candidate genes (30). The extensive search for mutations in these genes has provided an explanation of a portion of familial and sporadic HPE cases studied.

#### 3.1. Positional cloning approach

The initial approach to the identification of genes involved in HPE and forebrain development was *positional cloning*. This method came from the evidence of non-random cytogenetic rearrangements in patients with HPE. To date at least 12 loci located on 11 different chromosomes are thought to contain genes involved in HPE (31). For five of them, a minimal critical region has been identified: *HPE1* at 21q22.3, *HPE2* at 2p21, *HPE3* at 7q36, *HPE4* at 18p, and *HPE5* at 13q32 (31,32). In three of the loci (*HPE2*, *HPE3*, *HPE5*), an HPE gene has been identified and in a fourth one (*HPE4*), an excellent candidate gene is currently being studied.

*Sonic Hedgehog (SHH)* was the first HPE gene identified by this approach. *SHH* was found to map to 7q36 (27), a critical region where deletions and translocations had been reported in association with HPE (27,33,34). Moreover, a subset of AD HPE families were shown to be genetically linked to markers on 7q36 (15).

*Shh* is one of the three vertebrate Hedgehog genes (*Indian*, *Desert*, *Sonic*) that are homologous to the *Drosophila* segment polarity gene *hedgehog* (35). *Shh* encodes a secreted factor with morphogenic activity in a wide variety of tissues (for review see 31). In particular, its patterning activities in the entire extent of the nervous system including ventral forebrain (37,38) and eye (39) and in craniofacial structures (40) are consistent with the malformations present in HPE. *Shh* is expressed early in the development of the vertebrate notochord and floor plate (41,42), and plays a critical role in establishing ventral identity of the developing neural tube (43). Mice homozygous for a disrupted *Shh* gene show defects in the development of midline neural structures, lack ventral cells



**Figure 2.** Facial findings in holoprosencephaly and the HPE spectrum. (A) Cyclopia with cleft palate and absent nasal structure in a 16-week gestation fetus with alobar HPE and *del(2)(p21-p22.1)* who is deleted for *SIX3*. (B) Cyclopia with proboscis above the fused eye in a 28-week gestation fetus with alobar HPE and *del(2)(p21-p23)*. (C) Synophthalmia with proboscis in a 30-week gestation fetus with *del(18p)* who is deleted for *TGIF*. (D) Ethmocephaly, two closely spaced but separated eyes and a nose-like structure between the eyes in an infant of a diabetic mother. (E) and (F) Cebocephaly, ocular hypotelorism, downsloping palpebral fissures, and a blind-ended, single nostril in two newborn infants with alobar HPE. The etiology of HPE is unknown in (E). A *SHH* mutation is segregating with HPE in the family of (F). (G) – (K) Various clefts of the lips and/or palate in infants with HPE. (G) Microcephaly, midface hypoplasia with absence of the nasal bones and bilateral cleft lip and palate, and mental retardation, without any identifiable structural brain anomalies in a female with *SIX3* mutation. (H) Microcephaly, midface hypoplasia with absence of the nasal bones and midline cleft lip and palate in a female with semilobar HPE and *TGIF* mutation. (I) Microcephaly, midface hypoplasia with absence of the nasal bones and bilateral cleft lip and palate, in a female with semilobar HPE as part of Smith-Lemli-Opitz syndrome, characteristic biochemical defect in cholesterol metabolism and mutation in the gene coding for 7-dehydrocholesterol reductase. (J) Midface hypoplasia with absence of the nasal bones and bilateral cleft lip and palate, in a female with semilobar HPE, diabetes insipidus, *SHH* mutation and deletion for *TGIF* due to a cytogenetic deletion, *del(18p)*. (K) Microcephaly, ocular hypotelorism, bilateral inferior iris colobomata, repaired right-sided cleft lip and palate, developmental delay, and normal brain MRI in a male with *SHH* mutation. (L) Microcephaly, micro-ophthalmia, coloboma, and a small median philtrum pit in a female with semilobar HPE and *SIX3* mutation. (M) Microcephaly, flat nasal bridge in a female with semilobar HPE, diabetes insipidus, short stature and mutations in two genes: *SHH* and *ZIC2*. (N) Ocular hypotelorism and only mildly dysmorphic face in a male with a *ZIC2* mutation. (O) Microcephaly and a mildly dysmorphic face in a female with alobar HPE, profound developmental delay and *ZIC2* mutation. (P) Ocular hypotelorism and only mildly dysmorphic face in a female with alobar HPE and a *ZIC2* mutation. (Q) Microcephaly, and hypoplastic philtrum in a male with lobar HPE and *SHH* mutation (Son of individual in Fig. 2-7Y. One sib had alobar HPE). (R) Microcephaly, hypotelorism, and single central upper incisor, developmental delay and a normal brain scan in a male who is deleted for *TGIF* due to a ring r(18) chromosome and *del(18p)*. (S) Single central incisor and developmental delay in a male with *SIX3* mutation. Three sibs had alobar HPE and the same *SIX3* mutation. (T) Microcephaly, ocular hypotelorism, flat nose with no palpable cartilage, midface and philtrum hypoplasia, normal intelligence and normal brain MRI in a male with *SHH* mutation (Two sibs had alobar HPE). (U) Typical single maxillary incisor and absent superior labial frenulum in a male with HPE microsigns. (Same as in Fig. 2W). (V) Absent superior labial frenulum and repaired single central incisor in a female with HPE microsigns (Same as in Fig. 2Y). (W) HPE microsigns including single central incisor and ocular coloboma on the left eye in a male with above normal intelligence whose mother has anosmia. (X) Ocular hypotelorism, microcephaly, single incisor and normal intelligence in a male with a *SHH* mutation. Two of his children had alobar HPE and the same *SHH* mutation. (Y) Female with ocular hypotelorism, single central incisor who has two children with HPE. All three have a *SHH* mutation. (Modified from 83).

## Genetics of HPE

in the brain, and display craniofacial anomalies including cyclopia and a proboscis-like nasal structure (2). Thus, in addition to its chromosomal position, expression and functional data made *SHH* an excellent candidate gene for HPE.

The association of cytogenetic deletions including the *SHH* gene with HPE is consistent with a loss of *SHH* function (34). The complete coding region and exon-intron boundaries of *SHH* were screened for mutations in a large cohort of patients with HPE (10,11,44). Mutations were detected in 14 out of 78 (18%) clinical familial HPE cases and in 9 out of 266 (3.4%) clinical sporadic cases. Specifically, 37% of families showing AD transmission of the HPE spectrum, based on structural anomalies, carried a *SHH* mutation. The finding of *SHH* mutations in only a minority of the total cohort of HPE patients (6.7%) underscores the significant etiologic heterogeneity of this condition (10).

The second gene associated with HPE was identified as *ZIC2* which maps to the minimal critical region in 13q32 (*HPE5*) (28,45). Again, this chromosomal region containing *ZIC2* was found deleted in a series of patients with major congenital malformations, including brain anomalies such as HPE or exencephaly. The *Zic* gene family of transcription factors are expressed in early mouse development in a spatially restricted manner. *Zic2* is expressed in the dorsal neural tube, neural retina and distal limb bud (46). In humans, *ZIC2* showed expression only in fetal brain, in midline stripes adjacent to the expression of the *GLI* genes, mediators of the *SHH* pathway. In *Xenopus laevis*, *ZIC2* and *GLI* have been shown to function together in the regulation of patterned neural cell differentiation (47). *ZIC2* is homologous to the *Drosophila odd-paired* (*opa*) gene (48). *Opa* activates expression of genes such as *engrailed* and *wingless*, both of which are involved in the hh pathway (49).

Mutational analysis of *ZIC2* supports the hypothesis that it is indeed the HPE gene at locus *HPE5*. Heterozygous mutations were detected in HPE patients leading to either a truncated protein or to a slightly larger protein (through an alanine repeat expansion) with likely abnormal activities (28).

*SIX3* is the most recent HPE gene to be identified. The human *SIX3* maps to the *HPE2* locus on chromosomal region 2p21 (29). This region was defined by a set of six overlapping deletions and three clustered translocations in HPE patients (50). *SIX3* gene is homologous to the *Drosophila sine oculis* (*so*) / *optix* family of transcription factors, which plays a crucial role for the patterning of the visual system (51). It is involved in midline forebrain and eye formation in several organisms (52-54). Artificial expression of these genes in embryonic tissues has been shown to lead to ectopic eye formation (54). The *SIX/so* transcription factors are homeobox-containing genes also characterized by a contiguous *SIX* domain that participates as well in transcriptional activation (55). Although much has been elucidated about the *so* pathway in *Drosophila* and its complex interaction with

several different genes (reviewed in 30), its action in vertebrates and which genes it regulates need to be determined. Mutational analysis of *SIX3* in HPE patients has identified four different mutations in the homeodomain which are predicted to interfere with transcriptional activation (29).

The last gene implicated in the *positional cloning* approach for HPE is *TG-interacting factor* (*TGIF*), a candidate gene for the *HPE4* locus at 18p11.3 critical region (56). *TGIF* is expressed during early brain development in mice (57). It codes a transcription factor that acts as a repressor of retinoic acid (RA) regulated gene transcription, competitively inhibiting the binding of the retinoic X receptor (RXR) to a retinol-responsive promoter (58). Interestingly, prenatal RA exposure causes HPE-like malformations in humans and mice (25,59). More recently, a second role for *TGIF* as a repressor along the TGFbeta/Nodal-related signaling pathway has been identified. This aspect will be discussed in the “developmental pathways and functional approach” subsection (3.2.3.).

Four heterozygous missense mutations in *TGIF* have been recently identified in HPE patients and were not present in a normal control group (60). Given the role of *TGIF* in the RA pathway, these mutations may decrease *TGIF* repressor activity, causing increased signaling along the RA pathway and mimicking the effect of excessive retinoic acid exposure. To date, preliminary functional studies to determine the activity of the abnormal proteins showed a *TGIF* loss of function with at least one of the mutations located in *TGIF* homeodomain (Gripp et al., unpublished observations). This model is consistent with the observation that deletions of one copy of the *HPE4* gene result in HPE (56). It is of note that only 10% of patients carrying a *TGIF* deletion show HPE (8), in contrast to the high concordance of deletions at the *HPE2* (*SIX3*), *HPE3* (*SHH*), and *HPE5* (*ZIC2*) loci with HPE. It is possible that either maternal retinoic acid levels or altered activity in another protein could modify the effect of *TGIF*.

To date, three unrelated HPE cases have been reported in which individuals with a *SHH* mutation also had abnormalities in either *TGIF* or *ZIC2* genes (10). While several aspects of the interaction between HPE-associated signaling pathways are speculative, some links have been demonstrated in certain species (30). In chicken craniofacial primordia high doses of retinoic acid downregulate *Shh* expression (40). Thus, *TGIF* loss of function could result in decreased *SHH* expression, which could accentuate the effect of one allele of *SHH* with reduced or no activity.

In regard to the *SHH/ZIC2* combined mutation, it is possible that their biologic functions converge in a common pathway, and the decreased activity of one protein might negatively affect the other. In *Drosophila*, *opa* gene (*ZIC2* homologous) activates elements of the hh pathway (*engrailed* and *wingless*) (49). Functional studies of the mutations detected in *SHH*, *TGIF*, and *ZIC2* will be essential to confirm these hypotheses.

### 3.2. Developmental pathways and functional approach

A large number of candidate HPE genes are based on studies of genes involved in forebrain development in model organisms such as mouse, frog, chick and zebrafish, analyzing their loss of function or ectopic expression phenotypes. Models of signaling pathways associated with HPE and their interaction in vertebrates draw on data from lower organisms (for a review see 61).

#### 3.2.1. SHH signaling pathway

Both animal and human studies demonstrate that the Shh pathway is crucial for the normal progression of complex developmental processes. Several congenital disorders are caused by abnormalities in genes involved in the SHH pathway (36). This pathway is best defined in *Drosophila*, but many of the components are highly conserved in humans. Various genes within the SHH developmental pathway also become candidates for HPE.

Ptc, a multipass transmembrane protein, is the Hh receptor (62). In the absence of SHH, PTC represses Smoothed (SMO) signaling through direct binding. When SHH binds to PTC, SMO is released from inhibition, and activation of the SHH target genes proceeds. Given this function, we hypothesize that gain-of-function mutations in PTC or loss-of-function in SMO could lead to HPE, mimicking SHH haploinsufficiency. To date, mutational study of the *PTC* gene in HPE patients has resulted four different missense mutations that may prevent SHH signaling either by perturbing SHH binding or by inhibiting activation of SMO (63). Functional studies of these altered proteins are in progress.

There are three vertebrate homologues (*Gli1*, *Gli2*, *Gli3*) to the *Drosophila* zinc-finger transcription factor *cubitus interruptus* (*ci*), that regulate the transcription of SHH target genes through direct binding to their promoter elements (64). Two of them (*Gli1* and *Gli2*) are excellent candidate genes for HPE and are currently being screened in HPE patients. In *Xenopus*, *Gli1* mediates the floor plate cells and ventral neuron formation (65). *Gli1* is expressed in the ventral neural tube, and ectopic expression in transgenic mice can lead to activation of ventral markers (*Ptc*, *HNF-3b*, *Shh*) and suppression of dorsal markers (*Pax-3*, *AL-1*) (66). *Gli2* mutant mice lack floor plate and have abnormal development of the maxillary incisors (67).

Some of the SHH target genes are secreted morphogens that participate in a variety of developmental processes. The vertebrate *WNT* genes, homologues to the *Drosophila wingless* (*wg*), are expressed in the dorsal midline of the developing CNS and were shown to be required for notochord and dorsal neural tube development (68). *Bone morphogenetic proteins* (*BMPs*), homologues to *decapentaplegic* (*dpp*), are SHH target genes and also members of the TGFbeta gene family, required to activate the corresponding pathway. *BMPs* are expressed in the dorsal neural tube and act as regulators of the dorsal developing telencephalon (69). Ectopic expression studies of *BMP4* or 5 in chick prosencephalon result in HPE,

cyclopia and proboscis through a loss of ventral structures by cell death (3). The complexity of this system is consistent with the wide clinical variability seen in HPE and suggests possible interaction with other pathways.

#### 3.2.2. Cholesterol biosynthesis

Other genes in the SHH signaling pathway may also be implicated in the clinical expression of HPE. Shh protein is synthesized as a precursor that undergoes autocatalytic cleavage into a N-terminal (Shh-N) and a C-terminal (Shh-C) domain (70). During the autoprocessing reaction, a cholesterol moiety is covalently attached to the COOH-terminus of Shh-N (71,72). This modification is crucial for proper patterning activity.

Interestingly, HPE can be a severe manifestation of Smith-Lemli-Opitz (SLO) syndrome, which is caused by a defect in 7-dehydrocholesterol reductase, the final step in the cholesterol biosynthetic pathway (23,73). Thus, HPE in SLO may result from the inability of cholesterol to modify *SHH*, disrupting its proper patterning function. Furthermore, inhibitors of cholesterol synthesis in animal models in early gestation result in HPE-like malformations (74,75). Transgenic mice for a disrupted *megalyn*, a gene involved in cholesterol transport, show HPE (76). Based on these lines of evidence, maternal cholesterol levels in early gestation may be a crucial factor that contributes to the HPE outcome.

#### 3.2.3. TGFbeta signaling pathway

TGFbeta family of signaling molecules are involved in several developmental steps, including forebrain development, during gastrulation. It has been recently reported that several of these genes if mutated in animal models cause cyclopia. This pathway starts when a secreted extracellular TGFbeta member (*nodal*, *BMP4*, *activin*) binds to TGFbeta or TGFbeta-like receptors on the cell membrane that in turn activate the intracellular signal transducers SMADs. These various SMADs proteins interact with a common component, SMAD4 and with other repressor/activator elements within the nucleus, to regulate target gene expression (77).

Interestingly, the phenotype of the zebrafish mutants, *cyclops* (*cyc*) and *squint* (*sqt*), which are nodal-related genes, are consistent with human HPE. They both display cyclopia and defects in the prechordal plate and ventral nervous system (78). A third zebrafish mutant, *one-eyed pinhead* (*oep*), which is an essential nodal-extracellular cofactor, also shows lack of floor plate and cyclopia (79). Cyclopia has been reported at high frequency also in mice with heterozygous mutations in both *nodal* and *smad2* genes (80).

As mentioned in section 3.1., the HPE-candidate gene, *TGIF*, was shown to act *in vivo* as a corepressor of the SMAD2 protein (81), directing transcriptional silencing of target genes along the TGFbeta/Nodal-related signaling pathway. Thus, the detected mutations in the *TGIF* gene may lead to HPE through a “gain of repression” on Nodal-target-genes transcription along the TGFbeta pathway.

**Table 1.** Genetic factors for HPE: HPE genes and gene candidates for brain development (for references see 30)

SEARCH FOR HPE GENES	GENES	FUNCTIONS
<b>Cytogenetic loci</b>		
HPE1 21q22.3	?/exclusion of lanosterol synthase	
HPE2 2p21	SIX3	homeoprotein important for eye and forebrain development; SIX3 mutations cause HPE
HPE3 7q36	SHH	secreted signaling factor involved in embryonic patterning; mouse knock out has HPE phenotype; SHH mutations cause HPE
HPE4 18p11.3	TGIF	homeodomain protein that interacts with Smad2; competes with RXR for CRBII promoter site; TGIF mutations in human HPE
HPE5 13q32	ZIC2	zinc finger transcription factor; opa (Drosophila) activates en and wg; ZIC2 mutations cause HPE
HPE6 3p24-pter	Zinc finger gene, ZIC1/ZIC4?	
HPE7 13q12-q14	Forkhead homologue (FKHR)?	
HPE8 14q13	Thyroid Transcription Factor-1?	
HPE9 20p13	Homologue to mouse Coloboma?	
HPE10 1q42-qter	?	
HPE11 5p	?	
HPE12 6q26-qter	?	
<b>SHH signaling pathway</b>	PTC	transmembrane protein, SHH receptor; PTC mutations in human HPE
	SMO	transmembrane protein that interacts with PTC
	GLI family	zinc finger transcription factors, mediate SHH signaling; ectopic Gli-1 activates HNF-3beta, Ptc, Shh; Gli2 mouse mutants have HPE microsigns; GLI3 is associated with several human diseases
	WNT family	secreted segment polarity gene activated by SHH signaling
	BMP family	TGFbeta family secreted protein; target of SHH signaling; ectopic BMP4/5 in chick results in HPE phenotype
<b>Cholesterol metabolism</b>	7-deydrocholesterol reductase (SLO)	HPE in SLO patients; inhibitors of cholesterol synthesis inhibit Shh signaling and result in HPE phenotype in mice
	megalyn	mutations in mice result in HPE phenotype
<b>TGFbeta signaling pathway</b>	nodal	mutations cause cyclopia in animal models
	BMP4/5	(see above)
	oep	extracellular nodal cofactor; mutations cause cyclopia in zebrafish
	TGFbeta-like receptors	transduce TGFbeta signals activating Smads
	SMADs	mediators of signals to the nucleus; Smad2/nodal heterozygous mutant mouse has cyclopia
	TGIF	(see above)
<b>Forebrain development factors</b>	Dkk-1	Wnt agonist; if inhibited cause microcephaly and cyclopia
	HNF-3beta	expressed in head process and floor plate of neural tube; contains Gli binding site
	cerebrus	binds and represses nodal, BMP, Wnt
	floating head (flh)	mutants lack prechordal plate; synergic with cyclops in midline development
	bozozok	may be downstream Wnt and upstream TGFbeta signaling

### 3.3. Potential other HPE candidate genes

In addition to the better-defined genes reported above, other factors important in the forebrain patterning during early development could be considered HPE candidate genes. Some of them may be more relevant to HPE based on their expression patterns or their interaction with elements of the developmental pathways previously discussed (Table 1 and for a review see 30). *Dickkopf-1* (*dkk-1*) is a secreted protein required for head formation. It is a Wnt agonist and antibody inhibition of Dkk-1 results in microcephaly and cyclopia. *HNF-3b* is a transcription factor that contains a Gli binding site, required for the development of axial structures. It is expressed in the head process and floor plate of the neural tube. *Cerebrus* (*cer*) is a secreted factor that binds and represses nodal, BMP and Wnt. *Floatinghead* (*flh*) mutants lack prechordal plate. It is

synergistic with *cyclops* in midline development. *Masterblind* (*mbl*) is required for anterior structures and for *flh* expression. *Bozozok* (*boz*) is a homeoprotein that may act downstream of Wnt and upstream of TGFbeta signaling.

### 4. PERSPECTIVE

As the genetic factors involved in HPE are further identified, the complex relationships between these genes will be better appreciated. The significant etiologic heterogeneity of HPE underscores the importance of the identification of additional genes involved in HPE. It will be useful to continue the search of candidate genes considering both approaches: the “positional cloning” for the HPE loci cytogenetically defined and the “functional cloning” for those genes associated with multiple

developmental signaling pathways, analyzing their function and expression in animal models.

Mutations in the currently recognized HPE genes explain only a small portion of all HPE cases. As more candidate genes are identified and screened by mutational analysis in HPE patients, a more complete understanding of the numerous genetic factors which contribute to HPE will be elucidated, and a more accurate counseling for recurrence risk in families with a HPE history can be given.

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