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NOTCH, a new signaling pathway implicated in Holoprosencephaly

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ABSTRACT

Genetics of Holoprosencephaly (HPE), a congenital malformation of the developing human forebrain, is due to multiple genetic defects. Most genes that have been implicated in HPE belong to the Sonic Hedgehog (SHH) signaling pathway. Here we describe a new candidate gene isolated from array CGH redundant 6qter deletions, *DELTA Like 1* (*DLL1*), which is a ligand of NOTCH. We show that *DLL1* is co-expressed in the developing chick forebrain with *Fgf8*. By treating chick embryos with a pharmacological inhibitor, we demonstrate that DLL1 interacts with FGF signaling pathway. Moreover, a mutation analysis of *DLL1* in HPE patients, revealed a three-nucleotide deletion. These various findings implicate DLL1 in early patterning of the forebrain and identify NOTCH as a new signaling pathway involved in HPE.

INTRODUCTION

Holoprosencephaly (HPE) is the most common congenital malformation of the developing human brain. It is characterized by defective division of the forebrain into distinct left and right hemispheres (1). The clinical spectrum of HPE includes a broad range of malformations of the brain and face. In the most severe form (alobar HPE), the forebrain consists of a single ventricle, and the midline structure is missing; in semi lobar forms, the frontoparietal lobes are fused and the interhemispheric fissure is present posteriorly; in lobar HPE, a distinct hemispheric fissure is present; in microform HPE, the least severe form, midline structures are present and only mildly affected.

The etiology of HPE is highly heterogeneous and complex, presumably involving numerous genes and environmental factors. Chromosome aberrations have provided an important clue for the initial location of genes involved in HPE (2). From karyotype analyses, various genomic regions have been described as containing HPE candidate genes. Candidate genes namely *SHH*, *SIX3 TGIF* and *ZIC2* (3-7) have been identified in these loci. Mutations or deletions in these main genes account for about 28% of cases, and all cases are heterozygous (8-10). Other genes, belonging to brain development pathways, like Hedgehog or Nodal, were then tested for mutations and subsequently validated for HPE: *NODAL, TDGF1, FOXH1, PTCH1, GLI2, DISP1* (11-14). However, mutations or deletions in these genes occur infrequently and are generally associated with HPE microforms (4).

The phenotypic variability of HPE patients with particular gene mutations cannot currently be explained by single-gene haploinsufficiency. However, a multihit hypothesis would be consistent with the variable penetrance of familial mutations associated with this disease. HPE might result from two or more independent genetic lesions impacting forebrain formation. Indeed, the detectable mutations in HPE genes observed in patients may represent only one of several discrete steps needed to produce this abnormality.

The forebrain consists of several functionally and morphologically distinct structures. It acquires its regional specialization under the influence of several patterning centers that are recruited as early as the gastrulation stage (2). In the developing prosencephalon, opposed ventralizing and dorsalizing activities of Hedgehog (HH) and Bone Morphogenetic Protein (BMP) signaling pathways have key roles in the specification of dorso-ventral polarity (15). In addition to SHH signaling, FGF is required to elicit full specification of the ventral telencephalon. The involvement of FGF during midline development has been demonstrated by detailed observations of telencephalic conditional FGF receptor mice (16). Therefore, partition of the forebrain and formation of the midline require delicate balance between SHH and FGF signaling.

We previously performed genome-wide screening for microscopic anomalies in our HPE cohort using comparative genome hybridization arrays (17). Seventeen % of the HPE patients tested displayed *de novo* microrearrangements, demonstrating the high prevalence of genomic imbalances in cases of HPE and strongly supporting the notion of a multigenic origin of this developmental disorder. Here, we report four redundant chromosomal deletions overlapping the 6qter region in unrelated patients suffering severe HPE or microforms. Bioinformatics investigations of on this region allowed us to define a minimal critical deleted region of 2.2 Mb, containing a pertinent candidate gene, *DLL1*. DLL1 is a ligand of NOTCH and is consequently a major actor in many developmental processes (18). Although NOTCH signaling has been described to be important during the neurogenic phase of forebrain development (19), no link to HPE has previously been described. Here, we provide the first evidences that *DLL1* haploinsufficiency contributes to HPE.

RESULTS

Microarray analysis and determination of the break point

We used array-CGH to identify microrearrangements. The Agilent Human genome CGH array (Agilent Technologies, Santa Clara, CA, USA) used for patients 1, 2 and 3 has been described previously (17). Patient 4 was tested with the same Agilent CGH array but with the 105K microarray.

Patient 1 displayed a large hemizygous deletion of 15.7 Mb from 6q24 to the terminal end (table 1 and Fig. 1). This deletion was initially detected by MLPA and confirmed with the 44K oligonucleotide Agilent Array. According to the NCBI map viewer, this region encompasses 82 genes. No notable findings were observed in any other region except for copy number variations (CNV). FISH analysis of the parents showed that they did not have this deletion and thus proved *de novo* occurrence.

Patient 2 displayed a hemizygous deletion of 12.3 Mb (Table 1 and Fig. 1) from 6q25.3 to the telomere. No other chromosomal abnormality was observed.

Patient 3 displayed a hemizygous deletion of 5.2 Mb from 6q26 to the terminal end (Table 1 and Fig. 1) encompassing 37 genes. This deletion was associated with an 8 Mb duplication of 20p-ter.

Patient 4 displayed a hemizygous deletion of a maximum size of 2.2 MB from 6q27 to the terminal end involving 14 genes (Tables 1,2 and Fig. 1). Parental FISH analysis showed no evidence of a similar loss in either of the parents.

DLL1 is ranking first by Gene prioritization

The region deleted from patient 4 (168,690,655-170,899,992) was 2.2 Mb long and includes 14 RefSeq as listed in the University of California Santa Cruz (UCSC) Genome browser (Table 2). None of the 14 genes that map in this region has previously been clearly associated with a human brain disorder. Ten have no pathological findings associated with them, five have no annotation in OMIM, and four are open reading frames that are not annotated at all.

We used the prioritization software Endeavour (20) to identify candidate genes. We listed the 10 known HPE genes as a reference set: *SHH, SIX3, ZIC2, TGIF, GLI2, TDGF1, FOXH1, NODAL, PTCH1* and *DISP1* (4). The information available for each of the 10 annotated genes within the 2.2 Mb deletion was then compared with these reference set. The *DELTA-LIKE 1* (*DLL1*) gene was identified as the highest priority gene, which is not surprising as it is the only gene associated with developmental processes amongst the 10 genes.

Consequently, we have extended the application of Endeavour software to the 37 genes mapping in a larger region of 7.2 Mb encompassing the 2.2 Mb deleted region. Indeed, genomic deletions can contribute to phenotypic differences by modifying not only the expression levels of genes within the aneuploid segments but also of neighboring, normal copy-number genes (21). This position effect has been reported as far as 5 Mb away from the breakpoints. The *DLL1* gene was again identified as the highest priority gene in this extended region.

Molecular screening of the *DLL1* gene in HPE patients: identification of a 3 bp-deletion

We systematically sequenced the eleven exons and flanking intronic regions of the DLL1 gene from each of 100 HPE probands of our cohort (17). A heterozygous 3-bp deletion affecting two codons (NM_005618.3:c.1802_1804del) was identified in exon 9 from a patient with semilobar HPE (Fig. 2). The mutation was inherited from the father who underwent septoplasty of the nasal septum but did not display obvious typical form of HPE. It leads to

the substitution of two amino-acids (aspartic acid and isoleucine) by one valine (p.Asp601_Ile602delinsVal). Both substituted aminoacids are conserved through seven species from Xenopus to mammals (Fig. 2), and map within an ubiquitin motif in the intracellular domain of DLL1 (22). This mutation was not found in 103 ethnically matched controls. Furthermore, it was not detected in the pilot data from the 1000 Genomes Project (http://www.1000genomes.org) and not reported as a common variant in the Alamut software (Interactive Biosoftware).

Expression of *Dll1* during early development of the chick forebrain is coincident with sites of FGF signaling

We studied the expression pattern of *Dll1* and in particular whether it is expressed in areas of the forebrain that are abnormal in cases of HPE. The dynamic expression of *Dll1* during somitogenesis has been well described (23, 24), but less is known about its expression in the forebrain at the stage of HPE development. We used whole-mount *in-situ* hybridization to study the expression patterns of *Dll1* from gastrulation to stage HH14 (anlagen of the optic vesicle) in chick embryos (Fig. 3). *Dll1* was first detected at the onset of gastrulation (HH5), in the extending head fold, and then the level of expression rapidly increased as the cranial neuropore closes (Fig. 3a, c-e). At HH10, the anterior neuropore is closed and the prosencephalon has been formed. Subsequently, as the optic vesicles begin to invaginate, *Dll1* was clearly detected in the cranial neuropore. *In situ* hybridization for *Dll1* revealed a strong, well-defined domain that extended from the most anterior facial region to the anterior boundary of the eye and covered the rostro-ventral portion of the prosencephalon (Fig. 3e). In older embryos, transcripts persisted in the ventral developing head (Fig. 3i). Interestingly, a similar pattern of expression has been reported for *Fgf8* in the head process (25). However, unlike *Dll1*, *Fgf8* expression is not detected at stage HH5 in the extending head fold but

appears in the anterior neuropore at HH8 (Fig. 3b). At stage 10, *Fgf8* and *Dll1* are similarly expressed in the rostro-ventral portion of the prosencephalon. To determine whether *Dll1* and *Fgf8* are expressed in the same tissue, we performed *in situ* hybridization for both *Fgf8* and *Dll1* on consecutive histological sections at HH11 (Fig. 3e-h). Transcripts of both genes were found in the surface ectoderm and neurectoderm in histological sections.

The observed expression pattern of *Dll1* closely coincides with known sites of *Fgf8* expression in the anterior region of chick embryo (25) in which FGF signaling plays a key regulator function during anterior forebrain development (26).

FGF signaling is required to maintain *Dll1* expression in the telencephalon

FGF signaling, like SHH signaling, is essential for the generation of ventral cell types in the telencephalon. We therefore investigated whether FGF signaling had a role in regulating the expression of *Dll1*. We developed a drug-based strategy that allowed us to block endogenous FGF signals from the onset of gastrulation. SU5402, is an efficient *in vitro* pharmacological inhibitor of FGFR (27) and as proved to be a useful reagent for reducing FGF signaling activity *in vivo* (26). Therefore, we developed a protocol to use SU5402 to block FGF signals in chick whole embryos from the onset of gastrulation (HH4) to the primary regionalization of the forebrain (HH12).

We first tested a variety of concentrations to determine the maximal dose that allows head development. We treated embryos with SU5402 for periods of 24 hours from pregastrulation stages (HH4) to the 16 somites stage (HH12). We observed that 100 % (n = 23/23) of the embryos treated with a concentration of 10 µM SU5402 showed severe morphological defects all along the antero-posterior axis with anterior truncation as previously described in Zebrafish (26, 28). These defects were in all cases associated with substantial down-regulation of *Dll1* expression in the anterior neuropore (Fig. 4b) and a posteriorization of the

Dll1 signal in the presomitic mesoderm. Since this down-regulation of *Dll1* may be linked to the severe phenotype of the treated-embryos, we have used a lower concentration of SU5402. In contrast, this treatment (5 µM of inhibitor) gave milder defects: no anterior truncation was observed and the embryonic axis was normal, as shown by the normal expression of *Dll1* in the presomitic mesoderm (Fig. 4c). Interestingly, *Dll1* expression was still very substantially reduced in 50 % (11/22) of the embryos, and lower than normal in the other 50% (Fig. 4c). This suggests that FGF signaling is important to maintain *Dll1* expression in the anterior neuropore.

Inhibiting SHH signaling does not disrupt *Dll1* expression in the anterior neuropore

We next tested whether perturbations of SHH signaling disrupted *Dll1* expression in the head level. To block SHH signaling, chick embryos were cultured in the presence of cyclopamine, a plant-derived steroidal alkaloid cyclopamine. Cyclopamine directly antagonizes the SHH signal activation component Smoothened (29). The drug was applied at HH4 (before the onset of gastrulation) at a concentration of 5µM (30). At this dose, cyclopia was observed in 100% of the treated embryos (n=27) and there was no noticeable effect on *Dll1* expression in the prosencephalon (Fig. 4d). Thus, we conclude that *Dll1* expression in the early prosencephalon is not regulated by SHH signaling.

DISCUSSION

The inheritance of HPE is complex and results from interplay between multiple genetic factors (2, 31). Discovery of new causative genes for such defects in human is challenging because of genetic heterogeneity and variable penetrance. In view of these complexities, we used an integrated human genetic approach and an appropriate animal model to study genetics

of HPE. This strategy allowed us to describe a new risk locus for HPE in 6qter that contains an excellent candidate gene, *Dll1*. We also provide experimental data linking this gene with HPE syndrome.

In a previous array-CGH study we demonstrated the high prevalence of rearrangements in HPE patients (4, 17). Here, we describe rearrangements in overlapping regions in four patients leading us to focus on a hemizygous deletion in the qter extent of the long arm of chromosome 6 (6qter), common to these four cases. In a context of a rare disease, this rate of 6qter deletion is very significant.

Rare cases of abnormalities of the terminal long arm of chromosome 6 have been reported in the literature, most having been revealed by standard cytogenetic analysis and most in live born children. The 6qter deletion syndrome has various characteristic features including mental retardation, facial dysmorphism, seizure and brain abnormalities, including dysgenesis of corpus callosum (32, 33). These cases may represent microforms of HPE (34). Some cardiac and ophthalmic abnormalities have also been reported but were mostly associated with very large 6q deletions (35).

The deletions in our four patients were of between 15.7 Mb and 2.2 Mb. There is no evident correlation between the size of the deletions and the HPE severity; indeed, both the 12.3 Mb deletion and the smallest deletion of 2.2 Mb were associated with alobar HPE, the most severe form. Such heterogeneity is a common feature in HPE; it has been extensively described for patients with alterations in the *SHH*, the main gene in HPE (36, 37). This contributed to our suspicion that only one gene within the region deleted in our four patients was responsible for HPE. We therefore assumed that the minimal deleted region of 2.2 Mb, extending from nucleotide 168,690,655 to 170,899,992 of 6qter, contained a novel HPE gene. This deleted region contains 14 genes and Endeavour was used to prioritize them (20). This

analysis identified Delta-like 1 (*DLL1*) as the best candidate gene. We also tested for a long-range effect of the minimal deletion, by including the 5 Mb flanking the minimal region (21) and nevertheless, *DLL1* was persistently the best candidate. DELTA proteins function as cell-surface ligands for NOTCH receptors in a highly conserved signal transduction mechanism. Most importantly, the NOTCH plays a role in patterning various tissues during embryonic development.

Of the other genes present in this region, *TBP* is a potential candidate gene. *TBP* encodes the TATA-binding protein, which is a general binding transcription factor and it has been reported to be a good candidate for mental retardation (38). However, TBP was not scored highly by Endeavour in our analysis and no relation with brain development has been attributed to this gene by explorations with mutant mice (39).

Although we cannot formally exclude the possibility that haploinsufficiency of other 6qter genes contributes to the phenotype, the identification of a 3-bp deletion in a highly conserved region within the *DLL1* gene in an independent case of HPE is strong support for haploinsufficiency of this gene participating in the HPE phenotype. This 3-bp deletion, leading to the substitution of two aminoacids (aspartic acid and isoleucine) by a valine, is not reported as a common variant in the Alamut software. All Notch ligands share a similar architecture: an N-terminal region required for receptor binding, a Delta/Serrate/lag-2 domain, a variable number of EGF like repeats, a transmembrane segment and a relatively short cytolasmic tail (40). The 3-bp deletion maps within a putative ubiquitin motif in the intracellular domain of DLL1 (22). Notably, it has been shown that ubiquitination of Notch ligands is essential for effective Notch activation (41-43). Additionally, this deletion implicates aminoacid residues that are conserved through species from Zebrafish to chimpanzee, consistent with this variant having functional significance (Fig. 2).

That the father of the patient carrying the 3-bp deletion transmitted the mutation but did not display patent HPE is not surprising in view of the incomplete penetrance of the disease. Indeed, the majority of HPE mutations are inherited and variable expressivity among HPE family members is characteristic of this pathology (10). It indicates that additional factors contribute to the severity of the phenotype (2). One significant mutation of *Dll1* was found in a series of 100 HPE patients. Similar findings have been described for several HPE genes, in fact, mutations of the *TDGF1*, *DISP1*, *GLI2*, NODAL, *FOXH1* or *PTCH1,* collectively explain 1% of the studied cases and are often associated to microforms (44).

We report here that *Dll1* is expressed during the first stages of brain development consistent with it having a role in forebrain development. We provide the first detailed description of *Dll1* expression in the developing forebrain and show that it corresponds to known sites of FGF signaling. *Dll1* is transiently expressed during forebrain formation in a spatially and tissue-restricted manner at a critical period of forebrain development. Expression of *Dll1* in the head region was first detected in head fold-stage embryos in the presumptive forebrain and high levels of *Dll1* transcripts did accumulated in the telencephalon at the time of anterior neuropore closure. From this stage, the pattern of *Dll1* expression in the forebrain is remarkably similar to that of *Fgf8* in the anterior neuropore.

FGF signaling, like SHH signaling, is essential for the generation of ventral cell types in the telencephalon. Telencephalon phenotypes in various animal models attributed to reduced FGF activity include midline defects (28, 45). Moreover, there is evidence that FGFs act in a dose-dependent manner to pattern the ventral telencephalon (46). Most significantly, heterozygous microdeletions and mutations affecting FGF8 have been described in HPE cases, implicating the FGF signaling pathway in human HPE (47, 48).

The overlapping expression patterns of the *Dll1* and *Fgf8* genes suggest an interaction between these two signaling pathways. To test this possibility, we used a pharmacological approach to inactivate FGF signaling and examine the consequences for *Dll1* expression. We exploited *ex ovo* chick embryo cultures (so called roller cultures) to test the effects of inhibition of the FGF signaling during brain formation. Reducing FGF signaling by SU5402 treatment during early brain development totally inhibited *Dll1* expression specifically in the severely disorganized telencephalon. At lower concentration of inhibitor, while the telencephalon is not truncated expression was still down regulated in the anterior neuropore. These observations suggest that FGF signaling is required to maintain (49) the expression of *Dll1* in the telencephalon. However, the onset of *Fgf8* expression is around the 4-somite stage (HH8), whereas *Dll1* expression is detectable before the head-fold stage (HH5). Thus, it is unlikely that *Fgf8* initiates *Dll1* expression in the head process as *Dll1* is expressed first in this tissue.

As the most frequent cause of holoprosencephaly is lack of SHH signaling, we also examined the expression of *Dll1* in a hedgehog-deficient chick model. The findings that *Dll1* expression was normal suggest that this gene is not regulated by SHH signaling pathway.

The evolutionarily conserved NOTCH signaling in vertebrates is thought to act predominantly in a ligand/receptor–like manner and mediate various cell-fate decisions important for the morphogenesis and development of numerous organs (18). As a result, the molecular mechanism of this pathway has been the subject of intensive research (19). Interestingly, DLL1-NOTCH signaling interacts with FGF signaling during the clock mechanism process to regulate *Hes7* leading to the formation of somites (49). Also, FGF signaling is required for expression of *Dll1* in the spinal cord stem zone (50). These various findings support the existence of an interaction between *Fgf8* and of *Dll1* to cooperatively regulate ventral telencephalon patterning.

However, the function of DLL1 during forebrain regionalization is still unclear. *Dll1* mouse mutants die around day 11; although some midline defects have been observed, no typical HPE phenotype has been described (51, 52). Moreover, Dll1 haploinsufficiency in adult mice leads to a reduce body weight (53) Nevertheless no investigations of possible fine structural abnormalities of the forebrain have been done. Targeted mutations of the *Notch1* or *Notch2* gene give rise to widespread cell death and result in embryonic death around E10.5 (54-56). Interestingly, telencephalic-specific deletion of *Notch1* results in a reduction in the size of the ganglionic eminence, a ventral structure of the forebrain (57). While these animal models strongly implicate a perturbation of the NOTCH signaling pathway in the onset of HPE, it also demonstrate that mutation in Dll1 has a modest deleterious functional effect on the brain. Therefore, it is reasonable to conclude that additional genetic or environmental factors, presently unknown, would be required to produce severe HPE. In fact, although the number of instances is small, some cases of HPE are the result of two or more independent genetic lesions impacting common or interacting developmental pathways during forebrain development (58). It is therefore likely that our patients carry other deleterious mutations. However no co-morbid alterations have been identified to date. In a forthcoming study, a whole exome sequencing of these cases would provide evidence for this hypothesis.

In conclusion, our data demonstrate that dysregulation of *DLL1* confers susceptibility to the occurrence of HPE. Therefore, *DLL1* is another of the large set of genes that, when structurally altered, can lead to HPE disorders. Most importantly, this study provides evidence for a role of the NOTCH signaling pathway in early forebrain development that is linked to the FGF signaling pathway. Future directions should be centered on screening genetics elements of the NOTCH pathway in HPE patients.

MATERIALS AND METHODS

Patient reports

Patient 1: The proband was a female foetus diagnosed with semi-lobar HPE. No familial history has been noted, although there were two previous first-trimester miscarriages. Termination of pregnancy occurred at week 14 of gestation in a context of intrauterine growth restriction. Anatomopathological examination confirmed semi-lobar HPE with complete corpus callosum agenesis, thalami fusion, facial dysmorphy including hypotelorism, median cleft lip and palate, and associated malformations including abnormal lung lobulation.

Patient 2: The first pregnancy of a 35 year-old woman was terminated at week 21 of gestation for major hydrocephalus and spina bifida observed in the female foetus. Anatomopathological examination revealed that the foetus had multiple congenital anomalies including alobar HPE, facial dysmorphism (bilateral microphthalmia, severe hypotelorism, single nostril, maxillary hypoplasia) and lumbar rachischisis associated with intrauterine growth restriction.

Patient 3: The first pregnancy in this family was terminated for HPE, but unfortunately no sample for this foetus was available. The two other brothers were diagnosed with microforms. They both share some common features: developmental delay, speech delay, behaviour disorder and clinical examination revealed eyes-squint and dimples. The elder brother underwent surgery for ventricular septal defect and patent ductus arteriosus. In both cases, brain MRI showed enlarged ventricles and callosal dysgenesis. The younger brother had a posterior fossa arachnoids cyst or a small cerebellum, and a primitive gyral pattern in frontal and left parietal lobe. These cases will be referred to hereafter as patient 3.

Patient 4: The index case is a newborn girl first diagnosed with hydrocephalus. Complementary exams confirmed that she had in fact an alobar form of HPE.

Patient 5: This boy was born with non characteristic facial features and absence of corpus callosum. He presented mental retardation and feeding intolerance. Semi lobar HPE was confirmed by MRI. The *DLL1* gene in this patient was sequenced.

Array-CGH:

Oligonucleotide array-CGH was performed using the Agilent Human Genome CGH microarray (Agilent Technologies, Santa Clara, CA, USA). Depending on the patient 44K, 105K or 244K oligonucleotides arrays were used, as previously described (17). Microarrays were scanned using the Agilent scanner G2565BA. Images were extracted using Agilent Feature Extraction software and data were analysed with Agilent DNA Analytics and Nexus Copy Number softwares to identify chromosome aberrations. Reference genomic DNA was from single male or female individuals.

Gene prioritization

We attempted to identify candidate genes using a prioritization software, Endeavour (20). Endeavour uses a variety of different types of data to prioritize a set of genes based on their similarities with known candidate genes considered as training genes. These data include those from the literature, functional annotation (GeneOntology), and those concerning microarray expression, protein domains (InterPro and Motif) and Disease Probabilities (59). The software provides an overall ranking by combining the rankings from each category. Here, we used the known HPE genes as a training set (4).

Roller-tube culture and chemical inhibition in chick

Fertile hens eggs were incubated in a humidified room at 38°C. The Embryos were staged according to Hamburger and Hamilton (60) (Hamburger and Hamilton, 1992) and were

collected at stage 4 (HH4) and cultured as described (61). Folded and sealed embryos along the longitudinal axis were transferred to 5ml plastic bottles containing 500µl of Liebovitz medium. The bottles were placed on a roller apparatus rotating at 30revs/minute, inclined at an angle of about 10 degrees in a 38°C incubator. Development was then allowed to proceed for 24 hours.

Loss of function experiments were performed with the following compounds: cyclopamine (Sigma) dissolved at 1mg/ml stock in 2-hydroxypropyl-ß-cyclodextrin (HBC, Sigma); SU5402 (Calbiochem), dissolved in 25 mM stock in DMSO. Embryos were treated with cyclopamine or SU5402 added to the culture medium continuously from the gastrula stage (HH4). Control embryos were treated with DMSO.

Section and whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed with chicken embryos using anti-sense digoxigenin-labeled riboprobes as previously described (62). For *in situ* hybridization with paraffin sections, embryos were fixed by 4% paraformaldehyde in PBS (1h, 4°C) and embedded in paraffin wax. Section 7 µm thick were cut, placed on slides and rehydrated in water: 120µl of the RNA probes (diluted 1/100 in the hybridization buffer) was applied to each slide. Plasmid carrying chick *Fgf8* (*EcorI*-T7; gift of Sophie Creuzet) and chick *Dll1* (*NotI*-T3; gift of Frank Schubert) were used as templates to generate antisense riboprobes.

Sequencing

All patient and control samples were collected after obtaining informed consent according to the guidelines of our institutional review boards. The exons of the *DLL1* gene were amplified by PCR and analysed by direct sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and the ABI Prism 3130 Genetic Analyzer.

The gene mutation nomenclature used in this article follows the recommendations of den Dunnen and Antonarakis (2001) (63).

The patients 1, 2, 3 and 5 described in this paper were tested for mutations and deletions in *SHH*, *ZIC2*, *SIX3, TGIF* and *DLL1* but none of them had anomalies in these genes.

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FIGURE LEGENDS

Figure 1: Fine mapping of chromosome aberrations in 6q25.3-6q27.

Four deletions (red bars) were identified in HPE cases.

Figure 2: *Dll1* mutation in a HPE case

(A) Partial DLL1 reference-sequence-read traces and corresponding traces of deletion as identified in patient 5. A heterozygous 3 bp-deletion implicating two codons (yellow box, NM_005618.3:c.1802_1804del) was identified in exon 9. This mutation leads to the

substitution of two amino-acids (aspartic acid and isoleucine) by one valine (grey box; p.Asp601_Ile602delinsVal). (B) Conservation of deleted DLL1 amino acids residues in several tetrapod and fish lineages (red bracket). The deletion occurs within an ubiquitination sites (ub); prediction of ubiquination sites is based on the preference for acidic residues adjacent to the target lysine (64).

Figure 3: Expression of chick *Dll1* and comparison with *Fgf8* expression between stage HH5+ and HH13 in the brain region. (a-f) ventral (i) lateral views. *In situ* hybridization analysis of *Dll1* (a,c-e, g,i) and *Fgf8* (b,f,h) in whole chick embryos. (a) Early neural fold stage chick embryo, expression is in the posterior mesoderm and primitive streak. Note the absence of *Dll1* transcripts from the Hensen's Node (HN). Expression is first detected in the Head Fold (HF). (b) Note the absence of *Fgf8* transcripts from the head fold at this stage. The asterix marks the anterior region at Head Fold stage (HH5+) from which Fg8 transcripts are absent. (c,d) *Dll1* is expressed in the anterior neuropore of the head process. (e,i) At stage HH11+ and HH13, *Dll1* is expressed in the presumptive telencephalon (T). (g,h) *In situ* hybridization with digoxigenin-labelled *Dll1* or *Fgf8* antisense probes on frontal histological sections at HH11+. The histological sections presented are consecutive sections. Note that *Dll1* and *Fgf8* are both expressed in the surface ectoderm and neurectoderm. O, Optic vesicle; PM, Presomitic Mesoderm; I, Isthmus; SE, Surface Ectoderm.

Figure 4: Down-regulation of *Dll1* in embryos treated with the FGF inhibitor SU5402 but not those treated with cyclopamine. Whole-mount *in situ* hybridization of HH12 cultured embryos. Embryos are viewed laterally. (a) *Dll1* expression in control embryos, DMSO (control) treatment has no detectable effect on the embryo. The *Dll1* signal is detected in ventral telencephalon (T) and presomitic mesoderm (PM). (b) High concentration of

SU5402 (10μM) caused severe telencephalic abnormality and downregulation of *Dll1*. (c) A lower concentration of SU5402 affected telencephalon morphogenesis less severely, but Dll1 is still abolished or significantly reduced in the telencephalon. (d) Treatment of embryos with cyclopamine did not inhibited *Dll1* expression.

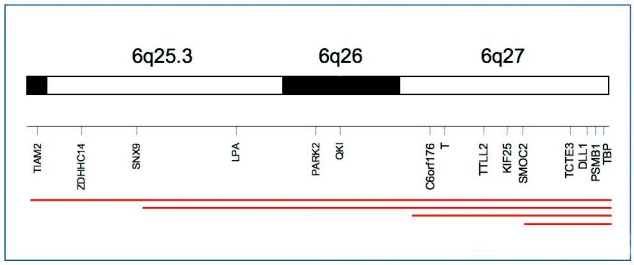
Table 1: Major clinical features of individuals with deletions of 6qter and summary of the breakpoints.

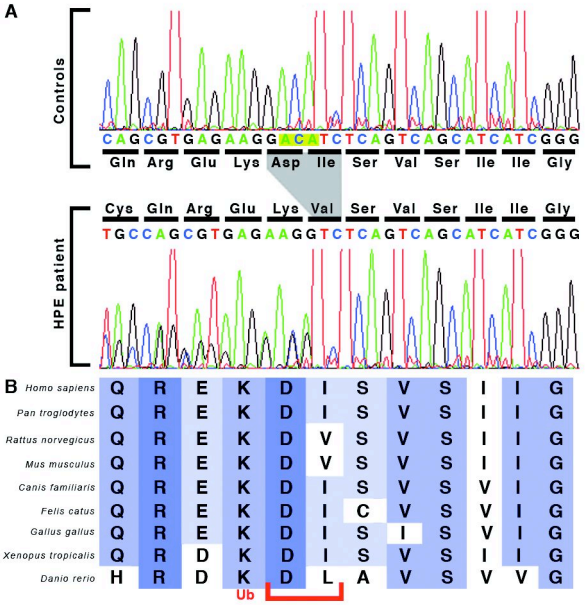
Patient	HPE type	Gender	Array	Cytogenetic bands	Minimal deletion (hg18)	Size (Mb)
1	Semilobar	F (foetus)	44k	6q24-6qter	155,166,802-170,899,992	15,7
2	Alobar	F (Foetus)	44k	del6q25-6qter	158,361,721-170,899,992	12,3
3	Microform	M (newborn)	244k	Del6q26-6qter	165,7767853-170,899,992	5,2
4	Alobar	F (newborn)	105k	Del6q27-6qter	168,690,655-170,899,992	2,2

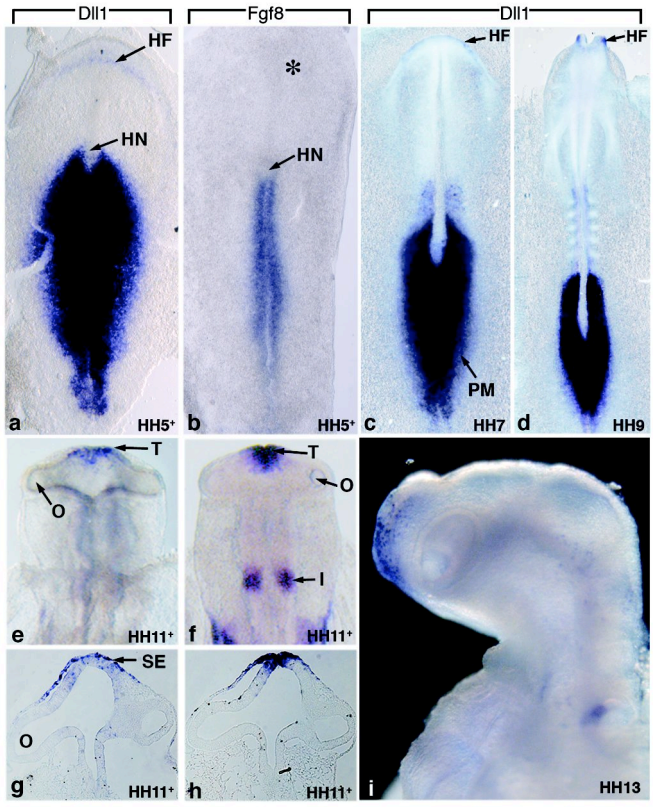
Table 2: List of the 14 genes located in the 2.2 Mb deleted region

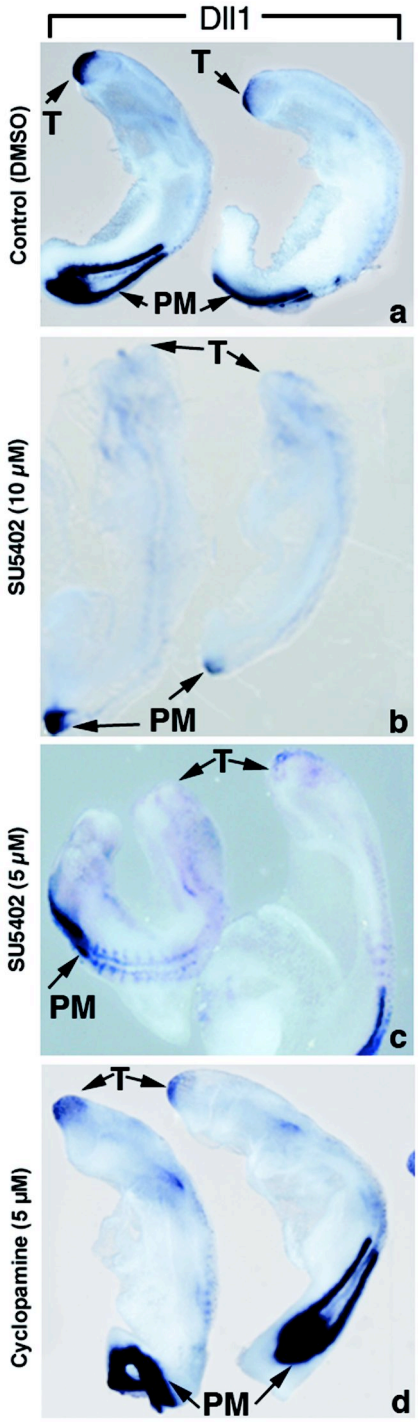
Genes	Name	Position	OMIM	Pathology
SMOC2	SPARC related molecular Calcium binding 2	168584880-168810596	607223	COPD susceptibility
THBS2	Thrombospondin 2	169357800-169396062	188061	Lumber disc herniation
WDR27	WD repeat domain 27	169811555-169844084	None	None
C6orf120	Chromosome 6 open reading frame 120	169844182-169848327	None	None
PHF10	PHD finger protein 10	169845926-169865909	613069	None
TCTE3	T-complex-associated-testis-expressed 3	169882140-169893563	186977	None
C6orf70	Chromosome 6 open reading frame 70	169895885-169923542	None	None
C6orf122	Chromosome 6 open reading frame 122	169930811-169940846	None	None
C6orf208	Chromosome 6 open reading frame 208	169932342-169943796	None	None
DLL1	Delta-like protein 1 precursor (Drosophila Delta homolog 1)	170591294-170599697	606582	None
FAM120B	Family with sequence similarity 120B	170457769-170556162	612266	None
PSMB1	Proteasome (prosome, macropain) subunit, beta type, 1	170686134-170704312	602017	None
TBP	TATA box binding protein	170705396-170723872	600075	Spinocerebelar ataxia, Parkinson disease, Huntington disease like 4
PDCD2	Programmed cell death 2	170732762-170735673	600866	Lymphomas

None: no link to human disease identify by screening various genetic databases and literature.


6q25.3
6q26
6q27
TiAM2
ZDHHC14
SNX9
LPA
PARK2
QKI
C6orf176
T
TTLL2
KIF25
SMOC2
TCTE3
DLL1
PSMB1
TBP


A
Controls
CAGCGTGAGAAGGACATCTCAGTCAGCATCATCGGG
Gln Arg Glu Lys Asp Ile Ser Val Ser Ile Ile Gly
HPE patient
Cys Gln Arg Glu Lys Val Ser Val Ser Ile Ile Gly
TGCCAGCGTGAGAAGGTCTCAGTCAGCATCATCGGG
B
Homo sapiens Q R E K D I S V S I I G
Pan troglodytes Q R E K D I S V S I I G
Rattus norvegicus Q R E K D V S V S I I G
Mus musculus Q R E K D V S V S I I G
Canis familiaris Q R E K D I S V S V I G
Felis catus Q R E K D I C V S V I G
Gallus gallus Q R E K D I S I S V I G
Xenopus tropicalis Q R D K D I S V S I I G
Danio rerio H R D K D L A V S V V G
Ub


Dll1
Fgf8
Dll1
HF
HN
HF
HF
PM
a HH5+
b HH5+
c HH7
d HH9
T
O
T
O
I
e HH11+
f HH11+
SE
O
g HH11+
h HH11+
i HH13


Dll1
Control (DMSO)
T
T
PM
a
SU5402 (10 μM)
T
PM
b
SU5402 (5 μM)
T
PM
c
Cyclopamine (5 μM)
T
PM
d