

Mini-Review

Molecular Definition of Pseudohypoparathyroidism Variants

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Abbreviations: cAMP, 3',5'-cyclic adenosine monophosphate; AD-PHP1B, autosomal dominant PHP type Ib; AHO, Albright's hereditary osteodystrophy; AS, antisense transcript; DMR, differentially methylated region; Gs α , alpha-subunit of the stimulatory G protein; GPCR, G protein-coupled receptor; PHP, pseudohypoparathyroidism; PHP1A, PHP type Ia; PHP1B, pseudohypoparathyroidism type Ib; PHP2, pseudohypoparathyroidism type II; POH, progressive osseous heteroplasia; PPHP, pseudopseudohypoparathyroidism; PTH, parathyroid hormone.

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Abstract

Pseudohypoparathyroidism (PHP) and pseudopseudohypoparathyroidism (PPHP) are caused by mutations and/or epigenetic changes at the complex *GNAS* locus on chromosome 20q13.3 that undergoes parent-specific methylation changes at several differentially methylated regions (DMRs). *GNAS* encodes the alpha-subunit of the stimulatory G protein (Gs α) and several splice variants thereof. PHP type Ia (PHP1A) is caused by heterozygous inactivating mutations involving the maternal exons 1-13. Heterozygosity of these maternal *GNAS* mutations cause PTH-resistant hypocalcemia and hyperphosphatemia because paternal Gs α expression is suppressed in certain organs thus leading to little or no Gs α protein in the proximal renal tubules and other tissues. Besides biochemical abnormalities, PHP1A patients show developmental abnormalities, referred to as Albright's hereditary osteodystrophy (AHO). Some, but not all of these AHO features are encountered also in patients affected by PPHP, who carry paternal Gs α -specific mutations and typically show no laboratory abnormalities. Autosomal dominant PHP type Ib (AD-PHP1B) is caused by heterozygous maternal deletions within *GNAS* or *STX16*, which are associated with loss of methylation at the A/B DMR alone or at all maternally methylated *GNAS* exons. Loss of methylation of exon A/B and the resulting biallelic expression of A/B transcript reduces Gs α expression thus leading to hormonal resistance. Epigenetic changes at all differentially methylated *GNAS* regions are also observed in sporadic PHP1B, which is the most frequent PHP1B variant. However, this disease variant remains unresolved at the molecular level, except for rare cases with paternal uniparental isodisomy or heterodisomy of chromosome 20q (patUPD20q).

Key Words: pseudohypoparathyroidism, PTH, calcium, phosphate, cAMP, TSH, Gs-alpha, *GNAS*, *STX16*, epigenetics, parent-specific *GNAS* methylation.

This review will focus on pseudohypoparathyroidism type Ia (PHP1A) and the different variants of pseudohypoparathyroidism type Ib (PHP1B) with some discussion of pseudopseudohypoparathyroidism (PPHP) and its variant progressive osseous heteroplasia (POH), namely disorders that are all caused by impaired agonist-dependent 3',5'-cyclic adenosine monophosphate (cAMP) formation (1-5). The genetic defects that cause different forms of acrodysostosis will not be discussed, although some are caused by mutations in genes such as *PTHLH*, *PRKAR1A*, *PDE4D*, *PDE3A*, or *HDAC4*, which impair cAMP/protein kinase A signaling (6-11). Consequently, patients affected by acrodysostosis can have clinical, radiographic and/or laboratory features reminiscent of PHP1A, PHP1B, or PPHP. The first genetic defects responsible for PHP1A and PPHP, namely heterozygous mutations involving the *GNAS* exons encoding the alpha-subunit of the stimulatory G protein ($G\alpha$), were discovered more than 30 years ago. Much later the first genetic causes of PHP1B were identified, but most variants remain undefined at the molecular level. In fact, besides a rare form of sporadic PHP1B (sporPHP1B), only autosomal dominant PHP1B (AD-PHP1B) subtypes have been defined at the genetic and epigenetic level. SporPHP1B is the most frequent form of the disorder and although *GNAS* methylation changes are routinely assessed to establish the diagnosis of this PHP1B variant, the underlying genetic defects remain to be defined for most cases. In conjunction with the biochemical and epigenetic characterization of several genetically altered mouse strains, the molecular defects and *GNAS* methylation changes leading to the different PHP1B variants have enhanced the understanding of the underlying disease mechanisms that most likely involve abnormalities during oocyte development. These insights from mice with distinct mutations and from patients affected by PHP1B will also have implications for explaining the different abnormalities encountered in PHP1A and PPHP. This review will summarize the known genetic and epigenetic defects that cause the different forms of PHP and the insights gained into the intricate regulation at the *GNAS* locus that encodes $G\alpha$ and several additional transcripts.

First Description of a Novel Syndrome

The term pseudohypoparathyroidism (PHP) was first introduced in 1942 by Albright et al. to describe several patients who presented with hypocalcemia and hyperphosphatemia in association with obesity, short stature, short metacarpals and metatarsals, as well as neurocognitive impairment, namely clinical findings that are now referred to as Albright's hereditary osteodystrophy (AHO) (12). The

affected individuals showed no phosphaturic response to parathyroid extracts and the authors therefore concluded that the abnormal regulation of mineral ion homeostasis was due to renal resistance to parathyroid hormone (PTH) rather than PTH deficiency. These first descriptions of PHP patients laid the ground work for defining over the next several decades a more complete spectrum of the clinical and laboratory abnormalities associated with this rare and variable disorder.

First Insights: Impaired PTH-stimulated cAMP Generation in Pseudohypoparathyroidism

Shortly after the discovery that epinephrin and glucagon stimulate the generation of cAMP in liver homogenates (13), Aurbach and colleagues revealed that PTH increases the formation of this second messenger in plasma membranes from kidney and bone tissue (14-16). These authors had also shown that injection of PTH preparations into animals increases the urinary excretion of cAMP, and, with some delay, the urinary excretion of phosphate (17). They subsequently determined that PTH treatment rapidly increases urinary cAMP excretion in healthy humans and in patients with postsurgical hypoparathyroidism, but not in patients affected by PHP (18). These studies suggested a partial or total lack of PTH-sensitive adenylyl cyclase in the kidney of PHP patients and furthermore linked lack of the renal PTH-stimulated cAMP response to the blunted phosphaturic response noticed earlier by Albright et al. (12).

G-protein Deficiency Due to Heterozygous $G\alpha$ Mutations Causes PHP

In 1980, Farfel et al. (19) as well as Levine et al. (20) investigated extracts of erythrocyte membranes from patients who are now known to be affected by PHP1A, namely patients with characteristic AHO features, hypocalcemia, and impaired urinary excretion of cAMP and phosphate in response to exogenous PTH. Both groups of investigators revealed an approximately 50% reduction in the activity of a guanine nucleotide-binding protein (G-protein), later shown to be $G\alpha$ that couples various G protein-coupled receptors (GPCRs) to adenylyl cyclase and hence the formation of cAMP (1, 2). Because of the combination of PTH-resistant hypocalcemia and hyperphosphatemia, impaired PTH-stimulated urinary cAMP and phosphate excretion, AHO features, and reduced $G\alpha$ activity, this disorder is now referred to as PHP1A. Surprisingly, cell membranes from patients affected by PPHP (21) exhibited a similar reduction in G-protein activity as cells from patients affected by PHP1A, despite normal calcium and phosphate levels (see below).

After the molecular cloning of cDNAs encoding human $G\alpha$ (22) and defining the structure of its gene (*GNAS*) (23), nucleotide sequence analyses of genomic DNA from several PHP1A and PPHP patients revealed the first disease-causing mutations involving those *GNAS* exons that encode $G\alpha$ (24, 25). These *GNAS* mutations are heterozygous thus explaining, for both groups of patients, the approximately 50% reduction of $G\alpha$ protein and activity, which was observed in readily accessible cells of the patients but is expected to occur in all tissues (1, 2). *GNAS* mutations had provided essential first insights into the genetic defects underlying PHP1A. However, it remained uncertain as to why heterozygous $G\alpha$ mutations should lead to disease at all, until Davies and Hughes (26) documented that *GNAS* mutations involving exons 1-13 lead to AHO in combination with PTH-resistant hypocalcemia if inherited from a female, while inheritance of such mutations from a male leads only to AHO features, but not to hormonal resistance, that is PPHP. By now numerous $G\alpha$ mutations have been identified as causes of the different PHP variants (27); these are located on the maternal *GNAS* allele in patients affected by PHP1A and on the paternal *GNAS* allele in patients affected by PPHP (for databases listing the known mutations see www.hgmd.cf.ac.uk/ac/all.php or www.lovd.nl/GNAS).

A PHP Variant Without Apparent G-protein Deficiency

Besides reduced G-protein activity in PHP1A, Farfel et al. (19) reported normal activity levels for red blood cell membranes obtained from patients affected by postsurgical hypoparathyroidism, who showed cAMP and phosphate excretion in response to PTH, and from patients affected by PHP type II (PHP2), who showed, in response to PTH, an increase in urinary cAMP, but not in urinary phosphate excretion (28). Importantly, the authors also reported findings in a PHP variant in which patients present with hypocalcemia, elevated PTH levels, and impaired excretion of cAMP and phosphate in response to PTH, yet no AHO features. Samples from those patients exhibited normal G-protein activity, and therefore, this PHP variant has been referred to as PHP type Ib (PHP1B) (2).

The *GNAS* Complex Locus Gives Rise to Different Variants of $G\alpha$ and to Several Additional Transcripts

GNAS is a complex genetic locus that gives rise to several different transcripts (3) (Table 1). The best known and most widely studied *GNAS*-encoded protein is $G\alpha$, which comes in 4 different variants that are derived from

the same primary transcript initiated at exon 1. The long version of $G\alpha$ is encoded by exons 1-13, while the short version lacks the portion encoded by exon 3 and additional complexity is generated by the presence or absence of an extra CAG codon between the nucleotide sequences derived from exons E3 and E4 (22, 23). These transcripts were shown to be expressed at different levels in some tissues and although there is little evidence for functional differences between the 4 $G\alpha$ proteins (29, 30), a mutation in exon 3 that truncates the long, but not the short version of $G\alpha$, causes PHP1A (31).

Besides the 4 $G\alpha$ variants, 3 additional mRNAs are derived from distinct first exons and their promoters that splice onto *GNAS* exons 2-13. These alternative transcripts are A/B, extra-large form of $G\alpha$ (XLAs), and neuroendocrine secretory protein 55 (NESP55). The A/B transcript was initially thought to be nontranslated because exons A/B and 2 comprise no initiator methionine. However, through the use of an ATG in exon 3, the A/B transcript was subsequently shown to give rise to an amino-terminally truncated $G\alpha$ that reduces activity of the full-length G protein (32). The XLAs transcript encodes an extra-large $G\alpha$ variant comprising a unique protein sequence derived from exon XL followed by the portion of $G\alpha$ that is encoded by exons 2-13 (33). The NESP55 transcript encodes a neuroendocrine secretory protein with a molecular weight of 55 kDa; because of a termination codon within the first exon for this transcript, the nucleotide sequence derived from *GNAS* exons 2-13 are part of the 3'-noncoding region (34). In addition to these alternatively spliced sense mRNAs, an antisense transcript (AS) is derived from a fifth promoter on the opposite DNA strand (35). Furthermore, use of an alternative transcriptional start site within the XL exon can lead to a distinct mRNA and a protein termed XXLAs. Moreover, both XLAs and XXLAs transcripts contain a second open frame leading to gene products named ALEX or ALEXX. The ALEX protein has been shown to interact directly with XLAs (36).

Maternal $G\alpha$ Expression is Required for Normal Hormonal Responsiveness in Several Tissues

In 1969, as outlined above, Chase and colleagues showed that PPHP patients treated with PTH have a robust increase in urinary cAMP excretion that was indistinguishable from that in healthy controls and in patients affected by postsurgical hypoparathyroidism (18). Such patients were later shown to be carriers of heterozygous, loss-of-function $G\alpha$ mutations on the paternal *GNAS* allele, while patients affected by PHP1A carry such mutations on their maternal *GNAS* allele (Fig. 1). Heterozygosity for these mutations

Table 1. Alternative first *GNAS* exons giving rise to different RNA transcripts and proteins

Exons with promoters	RNA transcripts	Proteins	Normal methylation				Autosomal dominant PHP1B caused by maternal mutations				Sporadic PHP1B	
			Deletion of portions of <i>STX16</i> or of exon NESP		Deletion of exons AS3-4 with or without NESP		Deletion of portions of <i>STX16</i> or of exon NESP		Deletion of exons AS3-4 with or without NESP		Genetic defect unknown	Pat
			Mat	Pat	Mat	Pat	Mat	Pat	Mat	Pat		
NESP	NESP* → exons 2-13	NESP55	-	+	- or 0	+	+	- or 0	+	+	+	+
AS	AS exon 1-5	non-coding	+	-	+	-	-	-	-	-	-	-
XL	XL → exons 2-13	XL α s	+	-	+	-	-	-	-	-	-	-
A/B	A/B → exons 2-13	N-truncated G α	+	-	-	-	-	-	-	-	-	-
G α -exon 1	exons 1-13 (\pm exon 3; \pm CAG)	G α -long/short \pm Gln	Not methylated	-	Reduced or no G α expression from the paternal <i>GNAS</i> allele in some tissues	-	-	-	-	-	-	-

With exception of the unmethylated G α exon 1, the promoters of the alternative first exons undergo parent-specific methylation in healthy individuals. Maternal deletions involving *STX16* or NESP are the cause of some autosomal dominant PHP1B variants that are associated with loss of methylation at exon A/B, while maternal deletions that comprise exons AS3-4 with or without deletion of NESP lead to loss of all 3 maternal *GNAS* methylation imprints. In sporadic PHP1B there is loss of A/B methylation, often variable loss of methylation at the AS and XL, and gain of methylation at NESP. Some tissues, such as the proximal renal tubules, show reduced or no G α expression from the paternal *GNAS* allele, but the underlying silencing mechanism remains to be determined. *, termination codon in exon NESP. Abbreviations: AS, antisense transcript; G α , alpha-subunit of the stimulatory G protein; PHP1B, pseudohypoparathyroidism type 1b.

thus explained the equivalent reduction in G-protein activity observed for plasma membranes prepared from red blood cells or skin fibroblasts of patients affected by either PHP1A or PPHP. Importantly, these data indicated that G α expression from the maternal *GNAS* allele is essential for mediating PTH-induced urinary cAMP excretion, which is stimulated by the actions of PTH in the proximal renal tubules. These findings are consistent with the conclusion that this portion of the kidney relies on G α derived from the maternal *GNAS* allele. Subsequently, it was furthermore shown that G α is derived predominantly from the maternal allele in thyroid, gonads, pituitary, portions of the central nervous system, and brown adipose tissue. In these tissues, paternal G α expression is silenced through an as yet undefined mechanisms (37-43).

Silencing of paternal G α expression develops gradually, at least in the proximal renal tubules. Illustrating this, a PHP1A patient with a maternal *GNAS* mutation was shown to have a normal increase in PTH-stimulated urinary cAMP excretion at the age of 7 months, but then a markedly blunted response at the age of 3.9 years (44). Similarly, a patient with PHP1B revealed normal plasma PTH concentrations until the age of 2 years, when levels were shown to be elevated as evidence for acquired resistance to the hormone (45). Furthermore, studies in mice with maternal deletion of *Gnas* exon 1 revealed that paternal G α protein/mRNA expression in the proximal renal tubules declines over the first 2 months of life thus resulting gradually in PTH resistance and increased circulating PTH levels (46). Taken together, these findings indicate that G α expression in the renal proximal tubular cells is biallelic early in life, but the paternal contribution to total G α protein levels subsequently declines thereby explaining the delayed development of PTH resistance in the absence of maternally derived G α .

***GNAS* Undergoes Parent-specific Epigenetic Changes at Several Differentially Methylated Regions**

GNAS is one of only few genetic loci that undergo parent-specific changes in DNA methylation to limit protein expression to only one parental allele (33, 35, 47). The DMRs involving the promoters of exons AS, XL, and A/B are methylated only on the maternal *GNAS* allele; consequently, transcription from these promoters occurs only from the non-methylated paternal allele. The NESP promoter is methylated on the paternal allele and thus its mRNA is transcribed only maternally. In contrast, the G α promoter at exon 1 does not undergo parent-specific methylation. Nonetheless, its transcription is not always biallelic.

Human *GNAS* is located on the long arm of chromosome 20, while mouse *Gnas* is located on chromosome 2

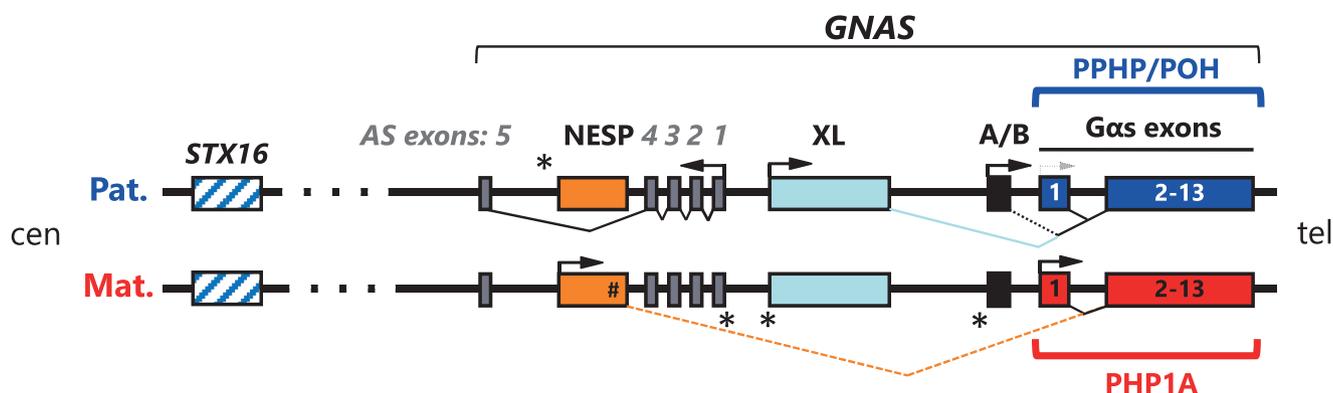


Figure 1. Organization of the *GNAS* locus. The *GNAS* complex gives rise to several imprinted sense and antisense transcripts. $G\alpha_s$, the most abundant product derived from this locus, is encoded by exons 1-13. This transcript is mostly biallelically expressed, except for few tissues like renal proximal tubule, thyroid, pituitary, brown adipose tissue, gonads, and various nuclei in the brain where paternal $G\alpha_s$ expression is partially or completely silenced through yet undefined mechanisms. The *GNAS* locus gives rise to 4 other transcripts, including the antisense transcript (AS), the NESP transcript encoding the neuroendocrine secretory protein 55 (NESP55), as well as the transcript encoding an extra-large form of $G\alpha_s$, named $XL\alpha_s$, and the A/B transcript (1A in mice) that may give rise to an amino-terminally truncated $G\alpha_s$. Promoters for XL, A/B and AS transcripts are methylated on the maternal allele and thus transcribed exclusively from the paternal allele. The NESP promoter is paternally methylated and active exclusively on the maternal allele. The NESP, $XL\alpha_s$, and A/B transcripts are derived from unique first exons that splice onto *GNAS* exon 2-13. Immediately centromeric of the XL promoter lies the promoter for the antisense transcript AS. Mutations involving any of the thirteen *GNAS* exons encoding $G\alpha_s$ are associated with AHO (Albright' hereditary osteodystrophy) with or without hormone resistance based on which parental allele is affected, namely pseudohypoparathyroidism 1A (PHP1A), if a mutation is present on the maternal allele (horizontal red bracket), or pseudopseudohypoparathyroidism (PPHP) and progressive osseous heteroplasia (POH), if a mutation is present on the paternal allele (horizontal blue bracket). Maternal and paternal *GNAS*-derived mRNAs are shown above and below the gene structure. Boxes indicate exons and splicing patterns are represented by broken/angled lines. Bent arrows indicate promoter start sites and direction of transcription. *Sites of differentially methylated regions (DMRs) on the maternal (XL, A/B, and AS) and the paternal (NESP) promoters.

(23, 48). In both species, the 4 DMRs undergo indistinguishable methylation changes and the corresponding promoters give rise to the same series of sense and antisense transcripts (33, 35, 49). Conclusions based on findings in mice with genetic alterations in *Gnas*, and in humans affected by PHP variants that are caused by different *GNAS* mutations have provided complementary insights into the underlying mechanisms by which $G\alpha_s$ expression is regulated in normal health and dysregulated in disease.

Pseudohypoparathyroidism Type Ia

PTH-resistant hypocalcemia and hyperphosphatemia are the most prominent features of PHP1A, but patients affected by this disorder can also develop resistance towards several additional hormones, including thyrotropin, calcitonin, and growth hormone-releasing hormone (2, 50, 51) and possibly melanocortin (52). Besides the AHO features, several additional clinical abnormalities are likely to be associated with a lack of maternal $G\alpha_s$, including hearing loss, decreased olfaction, sleep apnea, and asthma-like symptoms, thus implicating abnormal signaling at several additional GPCRs (4, 53). While several reports had provided no evidence for an obvious relationship between genotype and phenotype for PHP1A patients (27, 54-56), recent findings have indicated that PHP1A patients with deleterious truncating mutations show significantly more

subcutaneous calcifications than patients with missense mutations. In contrast, foreshortened metacarpals/tarsals were more frequently encountered with missense mutations, while obesity, short stature, and neurocognitive defects did not vary for patients with the different kinds of *GNAS* mutations (57).

Because of the parent-specificity of *GNAS* mutations and the involvement of different agonists acting through various distinct GPCRs, it is likely that a recently proposed new nomenclature for PHP and related diseases needs to be revised further because it focuses primarily on defects caused by impaired PTH- and PTHrP-dependent signaling, and does not take into account resistance towards other agonists and the parental allele carrying the *GNAS* mutation (58). However, the outlined guidelines have shown considerable promise by relying on few major clinical signs to suspect the diagnosis before proceeding with additional clinical and genetic assessment (59). Furthermore, the previously established nomenclature has equally severe limitations as the new one as it implies that endocrine abnormalities are restricted to PTH resistance and the term osteodystrophy refers to multiple abnormalities that affect not only the skeleton, but also neurocognition, weight regulation, and other developmental defects. Thus a further modified classification should be developed that focuses on impaired cAMP signaling rather than resistance to individual hormones.

Pseudopseudohypoparathyroidism

PPHP was also described first by Albright et al., when this group of investigators presented a patient with typical AHO features, yet without evidence for hormonal resistance and thus normal levels of serum calcium and phosphate (21). PPHP patients are now known to have some AHO features, such as short metacarpals and metatarsals, short stature, and round face, but usually without neurocognitive abnormalities and without obesity (4, 60-62), and only rare cases present with evidence for mild hormonal resistance (63). Furthermore, at birth individuals who are later diagnosed with PPHP are typically small for gestational age, particularly when paternal *GNAS* mutations affect exons 2-13, which suggests that paternal *XL α s* may have an important role during fetal development (64, 65). This conclusion is supported by findings in mice with targeted ablation of the XL exon, as these animals exhibit poor postnatal growth and survival (66). In addition, patients with maternal uniparental disomy of chromosome 20q (matUPD20q), who lack *XL α s* expression because the 2 maternal XL DMRs are both methylated, present with pre- and postnatal growth failure, hyperactivity, severe feeding difficulties, and short stature (67-69).

In addition to intrauterine and postnatal growth retardation and the subsequent development of several AHO features, some patients with paternal *GNAS* mutations develop POH. This more severe variant of PPHP is frequently caused by mutations involving the paternal *GNAS* exons 2-13, less often by exon 1 mutations. This also raises the question whether POH is related primarily to deficiency of paternal *XL α s* protein, rather than deficiency of paternal *G α* (5, 27, 70-72).

Different variants of pseudohypoparathyroidism type Ib

PHP1B occurs either as an autosomal dominant disorder or as a sporadic disease (AD-PHP1B and sporPHP1B, respectively). As in PHP1A, PTH resistance in renal proximal tubules and thus hypocalcemia and hyperphosphatemia despite elevated plasma PTH levels are the most obvious laboratory abnormalities in PHP1B. However, some resistance to thyrotropin is frequently noticed (4, 40, 73-76). PHP1B patients can also present with mild AHO features (4, 77-80) or even show bone abnormalities that are indistinguishable from those encountered in PHP1A or PPHP (81, 82). Analyses of erythrocyte membranes from PHP1B patients typically reveal normal *G α* bioactivity, which is different from the findings in PHP1A and PPHP; however, few PHP1B patients, particularly those with AHO features, show reduced *G α* activity, indicating that a threshold for *G α* activity could be

required for the development of some of these clinical aspects (83).

Patients affected by PHP1B display accelerated fetal growth resulting at birth in significantly higher weights and increased lengths, while patients affected by PHP1A are slightly smaller than average (62, 84). The increase in birth weight is particularly pronounced for patients with AD-PHP1B due to the 3-kb *STX16* deletion that results in loss of methylation at the exon A/B DMR, if these children are born to healthy female carriers of the genetic defect. Their birth weights differ from those of PPHP/POH patients with mutations involving *GNAS* exons E2-13 (65) by almost 4.5 standard deviation scores. This difference in birth parameters suggests a significant growth advantage due to loss of methylation at the exon A/B DMR (84).

Autosomal dominant forms of pseudohypoparathyroidism

A family in which several members had hypocalcemia and/or elevated PTH levels, but without obvious skeletal and other abnormalities, had been described by Winter and Hughes (85). Furthermore, in the report by Farfel et al. several members of a single family had presented with PTH-resistant hypocalcemia, but without AHO features and with normal G-protein activity in extracts from red blood cell membranes (19). In both kindreds, the disease followed an autosomal dominant mode of inheritance. Using genomic DNA from several subsequently identified families with autosomal dominant PHP1B (AD-PHP1B), the genetic defect was mapped to a region on chromosome 20q13.3 that is located centromeric of *GNAS* (86, 87). This led to the discovery of a recurrent 3-kb deletion in *STX16*, the gene encoding syntaxin 16, which is the most frequent cause of this PHP1B variant (3, 4, 88). Additional genetic mutations responsible for AD-PHP1B have since been identified, including several other deletions as well as different duplications and an inversion; these disease-causing mutations are all located within a region on the long arm of chromosome 20 that extends from *STX16* to *GNAS* (Fig. 2).

The 3-kb deletion as well as other *STX16* deletions, if located on the maternal allele, are associated with loss of methylation at the A/B DMR, but not at the AS and XL DMRs (45, 89, 90). Interestingly, deletion of the equivalent deletion in mice does not lead to loss of methylation at *Gnas* (91), which raises the possibility that the cis-acting elements regulating methylation at A/B and at the corresponding mouse 1A DMR are spatially distinct in humans and mice. Methylation changes restricted to the A/B DMR were furthermore documented for a maternal deletion extending from the NESP exon and its promoter to a region telomeric of AS exon 5 (92). This 18.9-kb deletion completely eliminates the NESP exon suggesting that the transcript/protein derived from this promoter is required for establishing

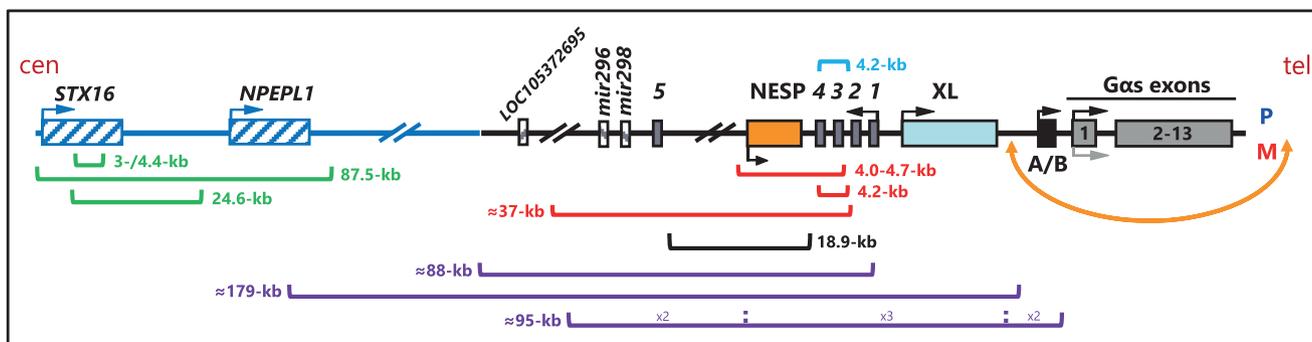


Figure 2. Genetic causes of autosomal dominant pseudohypoparathyroidism type 1b (AD-PHP1B). AD-PHP1B due to maternal deletions upstream of the $Gs\alpha$ -coding region that are associated with loss of methylation at one or more DMRs. Maternal deletions of *STX16* or *NESP* (green or black brackets) lead to loss of methylation specifically at the A/B DMR. The same epigenetic *GNAS* changes can also be due to a large genomic inversion (orange) or due to different duplications (purple brackets) involving the maternal allele. Deletion of AS exons 3-4 on the paternal allele (light blue bracket) leads to a partial loss of methylation at the paternal *NESP* DMR and a partial gain of methylation at the paternal A/B DMR. Maternal deletions that include AS exons 3-4 alone or in combination with exon *NESP* (red brackets) result in loss of methylation at all 3 maternal *GNAS* DMRs (A/B, XL, and AS). Boxes and connecting lines represent exons and introns, respectively. Arrows indicate direction of transcription. Brackets indicate deletions or duplications.

methylation at the exon A/B DMR (see below). Of note, the AS exons are not affected by this deletion, which is consistent with normal methylation at the AS and XL DMRs. Loss of methylation restricted to the A/B DMR can be caused also by different genetic duplications within *GNAS* (93-95). In addition, a large maternal inversion with the centromeric breakpoint located between exons XL and A/B leads to loss of methylation that is restricted to the A/B DMR (96).

In contrast to the *STX16* deletions that cause methylation changes restricted to the A/B DMR, maternal deletions involving either exons AS3-4 alone, or exons *NESP* and AS3-4 (97-99) lead to loss of methylation at all 3 maternal DMRs, namely AS, XL, and A/B. Identical epigenetic changes are observed when a similar deletion is introduced into the mouse (100). Interestingly, deletion of exons AS3-4 leads, when located on the paternal allele, to a partial loss of methylation at *NESP* and an incomplete gain of methylation at exon A/B (99), thereby providing insights into the underlying mechanisms responsible for establishing *GNAS* methylation imprints (see below).

Sporadic PHP1B

Sporadic (spor)PHP1B patients present with laboratory abnormalities that are largely indistinguishable from those observed in AD-PHP1B (73, 98, 101-105). These cases show a loss of methylation at the maternal *GNAS* exons AS, XL, and A/B, and, importantly, all sporPHP1B patients show a gain of methylation at the *NESP* DMR. In numerous sporadic PHP1B cases, the methylation changes within *GNAS* are incomplete, which is different from the uniform epigenetic changes encountered in the familial forms of the disease (73, 78, 98, 106, 107). Unlike autosomal dominant forms of PHP1B, the molecular causes of most sporPHP1B

patients have yet to be identified, except for those patients who have uniparental disomy involving the long arm of chromosome 20 (patUPD20q) (108-112).

Hypotheses Regarding the Mechanisms Leading to Abnormal *GNAS* Methylation

The *GNAS* methylation abnormalities that lead to the different familial and sporadic PHP1B variants occur most likely during oogenesis. Studies in mice show that the genome of oogonia undergoes complete demethylation during early oogenesis and subsequently 3 sites, namely AS, XL, and 1A, are remethylated before meiosis I (113). The epigenetic defects that underly the different inherited PHP1B variants characterized by loss of methylation at one or more of the maternal *GNAS* DMRs, namely AS, XL, and A/B, can thus be explained by disruption of the re-methylation events that occur during oocyte development.

The mouse *Nesp* DMR, which is completely unmethylated in sperm, does not get re-methylated until embryonic day 10.5 (114). It is therefore likely that methylation of the paternal and the maternal *NESP* DMRs, as encountered in most sporPHP1B patients (73, 98, 101-105), occurs postfertilization. One PHP1B family has been described in which 2 affected sisters show *GNAS* methylation changes that are similar to those encountered in sporPHP1B, including a gain of methylation at the maternal *NESP* DMR. Interestingly, however, linkage to the *STX16/GNAS* region was excluded in this family (103). This makes it likely that the primary genetic defect predicted for the affected members of this kindred, and possibly in at least some sporPHP1B cases, occurs outside of *GNAS* elsewhere in the genome.

The definition of several PHP1B cases that are associated with *STX16* or *GNAS* deletions, as well as findings in genetically modified mice provided important clues regarding the regulation of *GNAS* methylation. For example, a lack of paternal AS transcription caused by the introduction of a polyadenylation signal into AS exon 1 results in a lack of methylation at the paternal Nesp DMR and a concomitant gain of methylation at the paternal exon 1A (115, 116). Likewise, in one AD-PHP1B kindred, the deletion of exons AS3-4 on the paternal *GNAS* allele is associated with reduced methylation of the paternal NESP DMR and increased paternal A/B methylation. It therefore appears likely that a lack of full-length AS transcripts leads to active NESP transcription and hence methylation of the paternal A/B DMR (99). Taken together, findings in genetically altered mice and in one AD-PHP1B family show that active AS transcription is essential for enabling methylation at the NESP DMR.

In addition, a truncated maternal Nesp transcript in mice is associated with loss of methylation at the maternal 1A DMR (115, 116). Likewise, deletion of exon NESP on the maternal allele and thus a complete absence of NESP transcription, is associated in the affected members of an AD-PHP1B kindred with loss of methylation at exon A/B (92). These findings indicate that a maternal sense transcript derived from the NESP promoter is required for establishing methylation at the A/B DMR during oogenesis.

The importance of the *GNAS* locus for normal oocyte development is underlined by several recent findings. For example, *Gsa* was shown to play an essential role during oocyte maturation since Cre-mediated ablation of *Gnas* exon 1 under the control of the oocyte-specific *Zp3* promoter causes complete infertility (117). Furthermore, females affected by PHP1A or PPHP (118) preferentially transmit the mutant *GNAS* allele to the next generation. Similar transmission ratio distortion is observed for females who are carriers of *STX16* or *GNAS* mutations that cause AD-PHP1B when located on the maternal allele (119). In addition, females affected by AD-PHP1B due to a maternal mutation have significantly fewer offspring than unaffected females, who carry these mutations on their paternal allele. It is, however, unclear whether premature resumption of meiosis due to impaired *Gsa* expression is responsible for these latter findings.

In conclusion, the *GNAS* locus on chromosome 20q13.3, which encodes *Gsa* and several different splice variants thereof, is subject to complex regulatory mechanisms that include parent-specific methylation of several exons/promoters and a tissue-/cell-specific reduction in paternal *Gsa* expression through as-yet incompletely understood mechanisms. Three distinct, yet related diseases are caused by mutations in this genetic locus. PHP1A is caused by maternal mutations that directly impact the encoded *Gsa* protein, while

PPHP results when the same or similar mutations are located on the paternal allele. The autosomal dominant forms of AD-PHP1B are caused by maternal deletions, duplications, or an inversion involving *GNAS* or *STX16*, which lead to distinct methylation changes involving one or several different *GNAS* DMRs. With the exception of patients with patUPD20q, most sporadic PHP1B cases are unresolved.

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