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PERSPECTIVES

Pseudohypoparathyroidism, Gsα, and the *GNAS* Locus

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Described more than half a century ago, pseudohypoparathyroidism (PHP) is a disorder of hormone resistance in which the most prominent defect involves target organ resistance to the actions of parathyroid hormone (PTH), leading to hypocalcemia and hyperphosphatemia (1), combined with reduced serum concentrations of 1,25-dihydroxyvitamin D_3 (2;3). As an indication of PTH-resistance, rather than PTH-deficiency as in hypoparathyroidism, serum PTH levels are elevated and administration of exogenous biologically active PTH fails to result in an appropriate increase in urinary phosphate and cAMP excretion (1;4). PTH-resistance occurs in the proximal renal tubule, whereas no resistance appears to exist in other PTH target tissues, such as bone (5;6) and the thick ascending tubule (7), and it is probably because of these non-impaired PTH functions that patients can sometimes maintain normocalcemia without treatment. However, clinical manifestations of hypocalcemia, such as increased neuromuscular excitability or seizures, usually develop unless patients are appropriately treated with oral calcium supplements and 1,25- dihydroxyvitamin D. Treatment is also recommended for asymptomatic patients with normal calcium and phosphate levels if serum PTH levels are elevated, since long-term secondary hyperparathyroidism can result in severe hyperparathyroid bone disease (8). Since the first description of PHP by Albright and colleagues (1), different variants of this disorder have been defined (Table 1). Patients with PHP type-I exhibit both impaired nephrogenous cAMP generation

Table 1. Clinical and molecular features of patients with the different PHP-I forms.

	PTH-resistance	Additional hormone resistance	Typical AHO features	*GNAS* defects
PHP-Ia	Yes	Yes	Yes	Gsα mutations
PPHP	No	No	Yes	Gsα mutations
POH	No	No	No	Gsα mutations
PHP-Ib	Yes	Some cases	No	*STX16* or NESP55 deletions affecting *GNAS* imprinting

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and impaired phosphate excretion following exogenous PTH administration (1;4), while patients with PHP type-II exhibit a dissociation between these two responses, *i.e*., these patients have normal nephrogenous cAMP generation but impaired phosphate excretion (9). There are only a few cases of PHP-II in the literature, and the molecular defect responsible for PHP-II remains unknown. On the other hand, many more cases of PHP-I than PHP-II have been reported, and several different molecular defects have been associated with various subtypes of PHP-I. Therefore, we will provide our perspectives on the clinical and molecular definition of PHP-I and the subtypes thereof.

Phenotypic PHP-I Variants and Coding Gsα Mutations

Patients with PHP-Ia present with resistance to PTH and Albright's hereditary osteodystrophy (AHO), a constellation of physical features, which may include obesity, short stature, ectopic ossifications, brachydactyly and/or mental retardation. Most PHP-Ia patients also show resistance to other hormones, including thyroid stimulating hormone, gonadotropins, and growth hormone-releasing hormone (GHRH) (10-13). A common feature of these hormones, including PTH, is that their actions require cell surface receptors that couple to the stimulatory G protein. Accordingly, PHP-Ia is caused by heterozygous inactivating mutations located in *GNAS*, the gene encoding the α-subunit of the stimulatory G protein (Gsα) (Fig. 1).

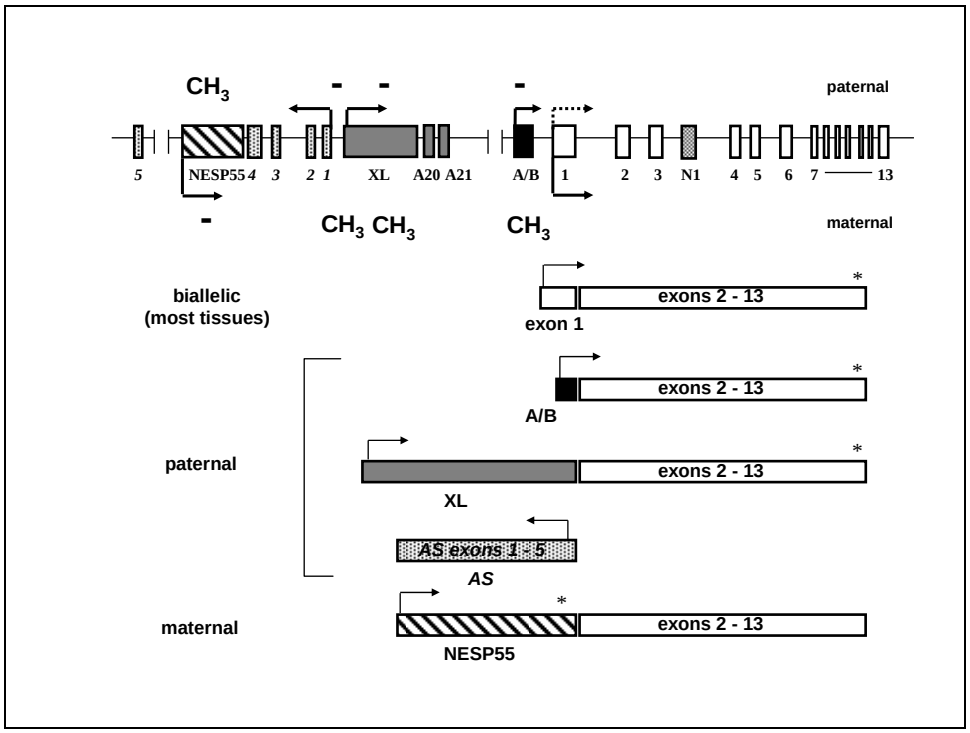

Figure 1: The complex *GNAS* locus gives rise to multiple transcripts. Boxes and connecting lines indicate exons and introns, respectively. Arrows indicate the direction of transcription. The five main transcriptional units derived from this locus and the utilized exons are indicated as rectangles below the gene depiction. Gsα transcripts are biallelically expressed except in a small number of tissues, including renal proximal tubules, thyroid, gonads, and pituitary, in which expression from the paternal allele is silenced (dashed arrow). XLαs, A/B and antisense (AS) transcripts are paternally expressed, and the NESP55 transcript is maternally expressed. Promoters of these latter four transcripts are either maternally or paternally methylated, as indicated by CH_3 (methylated CpGs) and - (unmethylated CpGs).

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Gsα is a ubiquitous signaling protein required for agonist activated stimulation of adenylyl cyclase, which in turn generates cyclic AMP (cAMP), an intracellular second messenger involved in numerous cellular responses throughout the body (14). Illustrating the importance of Gsα-mediated cellular responses, homozygous ablation of Gsα leads to embryonic lethality in mice (15-17). Numerous different *GNAS* mutations, including missense and nonsense amino acid changes, as well as insertions and deletions, have been identified in patients with PHP-Ia (see Online Mendelian Inheritance in Man #103580 at http://www.ncbi.nlm.nih.gov/ for a list of allelic variants). Consistent with their loss-of-function effects, these mutations are located in nearly all of the thirteen exons that encode Gsα and lead to an approximately 50% reduction in Gsα level/activity, which can be demonstrated in skin fibroblasts and erythrocyte membranes derived from PHP-Ia patients (11).

Inactivating Gsα mutations are also found in patients who display AHO features but not hormone resistance. This disorder has been named pseudopseudohypoparathyroidism (PPHP) (18). Patients with PHP-Ia and those with PPHP are typically found in the same kindreds, but never within the same sibship. While AHO occurs in the offspring irrespective of the gender of the parent transmitting the Gsα mutation, development of hormone resistance is subject to imprinting, such that it develops only in the offspring of female obligate carriers (19;20). Thus, paternal inheritance of a Gsα mutation results in PPHP (AHO only), whereas maternal inheritance of the same mutation results in PHP-Ia (AHO and hormone resistance). Consistent with the imprinted mode of inheritance observed for hormone resistance, recent investigations of the *GNAS* locus have revealed predominantly maternal expression of Gsα in some, but not all, tissues, including renal proximal tubules, thyroid, pituitary, and gonads (see below).

Heterozygous inactivating Gsα mutations are also found in patients with progressive osseous heteroplasia (POH), who have severe heterotopic ossifications that affect skeletal muscle and deep connective tissue (21). Some of the Gsα mutations found in POH patients are identical to those found in PHP-Ia or PPHP patients (21-23), and some patients with POH seem to present with hormone resistance and/or AHO features (22;24). It therefore appears likely that POH is an extreme manifestation of heterotopic ossifications associated with AHO. However, unlike AHO, which is thought to develop regardless of the parental origin of a Gsα mutation, POH appears to develop, in most cases, following paternal inheritance (23). The reasons for parental bias in the development of POH currently remain unknown, although it is possible that deficiency of other products of the *GNAS* gene, which show imprinted expression and are also disrupted by most of these mutations, contribute to the molecular pathology.

Gsα Shows Tissue-Specific Imprinting

Inactivating Gsα mutations can lead to various different phenotypes, and genomic imprinting appears to play an important role in the development of these phenotypes. Genomic imprinting refers to differential expression of genes specifically from either maternal or paternal alleles (25-27). This monoallelic, parental origin-specific expression correlates with allele-specific epigenetic marks within the imprinted gene (often at the promoter region), including methylation of the cytosine residues in CpG dinucleotides. Although the Gsα promoter itself lacks differential methylation (28-30), several studies have established that Gsα expression is predominantly maternal in a small number of tissues, *i.e.*, Gsα transcription from the paternal *GNAS* allele is silenced. Using genetically manipulated mice, Yu *et al*. (15) have shown that while *Gnas*(E2m+/p-) mice carrying a disrupted *Gnas* exon 2 on the paternal allele and wild-type litter mates have similar Gsα protein levels in the renal cortex, *Gnas*(E2m-/p+) mice carrying a disrupted *Gnas* exon 2 on the maternal allele almost completely lack Gsα in this tissue. Consistent with this finding, Gsα mRNA is nearly abolished in the renal proximal tubules of *Gnas*(E2m-/p+)

mice. Accordingly, *Gnas*(E2m-/p+) mice exhibit PTH-resistance, while *Gnas*(E2m+/p) mice appear to show normal PTH responsiveness (15). On the other hand, mice of these two genotypes have an equivalent, approximately 50% reduction of Gsα levels in the renal medulla compared to wild-type animals, indicating that Gsα is normally expressed from both parental alleles in this portion of the kidney. Recently, another mouse model carrying a point mutation in *Gnas* exon 6 (termed *oedematous-small* mutation) has also revealed PTH-resistance following maternal but not paternal transmission of the genetic defect (31;32). Thus, these findings from the *Gnas* knockout mouse models correlate well with the findings in kindreds with PHP-Ia and PPHP regarding PTH-resistance, and demonstrate the importance of Gsα imprinting in the pathogenesis of PHP-Ia. Unlike the patients, however, these two mouse models exhibit high early postnatal mortality (approximately 80% die before weaning) and various parental origin-specific defects, including those that involve energy and lipid metabolism (15;31;33;34). These phenotypes reflect disruption of the other imprinted transcripts that share exons with Gsα (see below), as verified by further investigations of the *Gnas*(E2m+/p-) and *Gnas*(E2m-/p+) mice and additional mouse models in which different *Gnas* exons are separately disrupted (16;35).

Analysis of various fetal and adult human tissues has also revealed imprinting of Gsα in some, but not all, tissues. Gsα expression appears to be predominantly maternal in the thyroid gland (36-38), gonads (36), and pituitary (39). Conversely, Gsα expression has been shown to be biallelic in a number of different fetal tissues (40), as well as in adult adrenal gland, bone, and adipose tissue (36;41). In addition, one study has demonstrated biallelic Gsα expression in human fetal renal cortices (42). Although apparently contradictory to the findings in mice (15), the latter finding may suggest that Gsα imprinting in this tissue is postnatal and/or occurs only in a small number of renal cortical cells. Overall, however, the tissue distribution of imprinted Gsα expression appears to correlate well with the tissue distribution of hormone resistance in patients with PHP-Ia. This is consistent with the prediction that an inactivating Gsα mutation leads to a dramatic decrease in Gsα level/activity and, thereby, hormone resistance only in tissues in which Gsα expression is predominantly maternal and only following maternal transmission. Conversely, the same mutation causes no detectable change in Gsα level/activity in the same tissues following paternal inheritance. Tissues in which Gsα is expressed biallelically are predicted to have approximately a 50% reduction of Gsα level/activity. While this reduction may be sufficient for maintaining normal cellular responses in some cells, it may lead to defective function in others, *i.e*., haploinsufficiency. In fact, because AHO features appear to develop independently of the gender of the parent transmitting the Gsα mutation, these features are thought to result from haploinsufficiency of Gsα signaling in various tissues. Providing evidence for this hypothesis, we have recently shown that in growth plates of chimeric mice, chondrocytes heterozygous for disruption of *Gnas* exon 2 undergo hypertrophic differentiation closer to the articular end of the developing bone, compared to wild-type chondrocytes (43). Nonetheless, given the patient-to-patient variability in the expression and severity of individual AHO features, it remains possible that imprinting of Gsα (or other imprinted *GNAS* transcripts) also contributes to the development of certain AHO features. This hypothesis is consistent with the predominantly paternal inheritance of POH (23).

The *GNAS* Locus Gives Rise to Multiple Imprinted Transcripts

Recent studies have revealed that in addition to Gsα, the *GNAS* locus gives rise to multiple coding and non-coding transcripts that show parental origin-specific expression (Fig. 1). Gsα is encoded by 13 exons that span about 20 kb of the genome (44). There are four different splice variants of Gsα formed through the alternative use of exon 3, as well as an additional codon inserted alternatively at the 5' end of exon 4

(45;46). In addition, the use of another alternative exon (N1) between exons 3 and 4 leads to a truncated Gsα mRNA and protein (47).

Recent studies of the genomic region comprising *GNAS* have led to the identification of several novel exons located upstream of exon 1, both in humans and mice. XLαs and neuroendocrine secretory protein-55 (NESP55) transcripts individually use separate upstream promoters and first exons that splice onto exons 2-13 of the Gsα transcript (29;30). However only XLαs and Gsα share protein sequence (48), since exons 2-13 in the NESP55 transcript are part of the 3' untranslated region (49;50). XLαs and NESP55 are oppositely imprinted; while XLαs is expressed from the paternal *GNAS* allele (29;30), NESP55, which is a chromogranin-like protein abundant in neuroendocrine tissues, is expressed from the maternal *GNAS* allele (30;50). XLαs also shows abundant expression in neuroendocrine tissues and the central nervous system, although its mRNA is detected in multiple other tissues, including adipose tissue, pancreas, and kidney (35;51;52). In addition to these protein products, the *GNAS* locus gives rise to a sense and an antisense non-coding transcript, each of which is expressed from the paternal allele. The transcript from the antisense strand (AS) has a promoter that overlaps with the promoter of the XLαs transcript (53;54). The non-coding transcript from the sense strand, termed A/B (also referred to as 1A or 1'), comprises exons 2-13, but uses, like the XLαs and NESP55 transcripts, a separate promoter and first exon (28;55;56). As in other imprinted genomic loci, and in contrast to the case with Gsα, the promoters of these additional *GNAS* transcripts are within differentially methylated regions (DMR), and the non-methylated promoter drives the expression in each case (28-30;50;53;54). Currently, the biological significance of the imprinted *GNAS* transcripts is poorly understood. As mentioned above, NESP55 belongs to the family of chromogranins and is associated with the constitutive secretory pathway in neurons and neuroendocrine cells (57). Targeted disruption of the NESP55 protein in mice leads to abnormal reactivity to novel environments (58). XLαs shares marked sequence identity with Gsα, including most of the domains shown to be functionally important for the latter (48). Accordingly, XLαs can mediate basal and agonist-induced adenylyl cyclase stimulation (59;60). Targeted disruption of the XLαs protein in mice, however, has thus far failed to reveal phenotypes that are unequivocally caused by a loss of the "Gsα-like" signaling activity of this protein *in vivo* (35). In fact, evidence from the XLαs knockout mice suggests that XLαs may oppose Gsα actions in certain tissues, such as brown fat (35). Nevertheless, mice lacking XLαs fail to adapt to feeding and show high early postnatal mortality. In addition, these mice show various defects in energy and glucose metabolism, including reduced adiposity in both brown and white fat and impaired metabolic responses to hypoglycemia (35).

Hormone-Resistance as a Result of Abnormal Gsα Imprinting

PHP-Ib defines those patients with PTH-resistance who lack AHO features (11). PHP-Ib patients usually also lack additional hormone resistance; however, mild TSH-resistance has been documented in some patients with this disorder (38;61;62). Although three brothers who had PTH-resistance with an apparent lack of AHO features (hence diagnosed with PHP-Ib) have been found to carry a Gsα point mutation predicted to cause selective uncoupling from the type-1 PTH receptor (63), findings in typical PHP-Ib patients rule out inactivating mutations within Gsα-coding *GNAS* exons (64;65). However, as in PHP-Ia, the genetic defect in an autosomal dominant form of PHP-Ib (AD-PHP-Ib) leads to hormone resistance only after maternal transmission, and has been mapped to a chromosomal region that comprises *GNAS* (66). Moreover, analysis of genomic DNA extracted from blood leukocytes shows imprinting abnormalities of *GNAS* in nearly all patients with PHP-Ib (61;67;68). Among the various imprinting defects, the most consistent one is a loss of methylation at exon A/B, accompanied by biallelic expression of this transcript; these

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epigenetic changes are proposed to be the cause of hormone resistance observed in patients with PHP-Ib (67). Demonstrating that defective *GNAS* imprinting alone can lead to PTH-resistance, a sporadic case of PHP-Ib has been shown to have paternal uniparental isodisomy of chromosome 20q and, as a consequence, a "paternal-only" imprinting profile throughout the *GNAS* locus (62).

In multiple kindreds with AD-PHP-Ib, we detected a heterozygous identical 3-kb deletion located approximately 200 kb upstream of *GNAS* (69) (Fig. 2). Interestingly, this deletion, flanked by direct repeat elements, removes exons 4-6 of the neighboring *STX16* locus, which encodes syntaxin-16, a member of the SNARE family of proteins involved in intracellular trafficking (70;71). Affected individuals inherit the 3-kb deletion from female obligate carriers, while unaffected carriers inherit the same deletion from male obligate carriers, which is consistent with the imprinted mode of inheritance documented for AD-PHP-Ib. In one AD-PHP-Ib kindred, we discovered another heterozygous, maternally inherited *STX16* deletion that is 4.4-kb in size (72) (Fig. 2). This novel deletion overlaps with the frequent 3-kb deletion and removes exons 2-4. Thus, both the 3-kb and 4.4-kb deletions are predicted to lead to expression of an inactive syntaxin-16 protein from one allele. Nevertheless, given that PHP-Ib develops after maternal inheritance of the genetic defect only, which is consistent with the inheritance mode of the identified mutations in affected individuals, loss of one copy of *STX16* could cause PHP-Ib only if this gene were also imprinted. However, based on methylation and allelic expression analyses using lymphoblastoid cells derived from PHP-Ib patients and normal controls, *STX16* does not appear to be imprinted (72), and therefore it appears unlikely that syntaxin-16 is involved in the molecular pathogenesis of PHP-Ib.

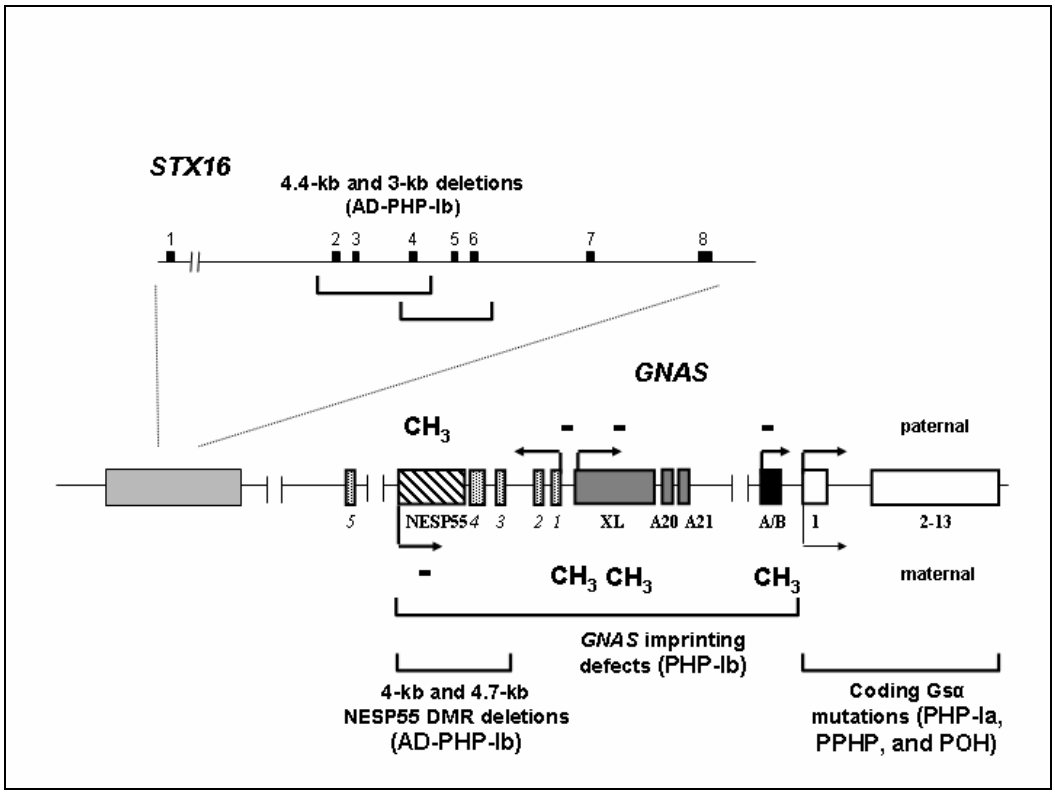

Figure 2: Location of the genetic and epigenetic defects identified in patients with various forms of PHP-I. Maternally inherited, heterozygous loss-of-function mutations are found in patients with PHP-Ia. The same mutations are inherited paternally in patients with PPHP and in most patients with POH. Identical heterozygous 3-kb deletions and a single 4.4-kb deletion within the *STX16*

locus have been identified in unrelated AD-PHP-Ib kindreds in whom affected individuals show isolated loss of exon A/B imprinting. These deletions remove several *STX16* exons and are predicted, upon maternal inheritance, to disrupt a putative *cis*-acting regulatory element required for exon A/B imprinting. *STX16* is located approximately 200 kb upstream of *GNAS*. A 4-kb and a 4.7-kb deletion of the NESP55 DMR have been identified in two unrelated AD-PHP-Ib kindreds in which affected individuals show loss of all maternal *GNAS* imprints. These deletions are predicted, upon maternal inheritance, to disrupt a putative *cis*-acting regulatory element required for the imprinting of the entire *GNAS* maternal allele.

Thus far, all patients with deletions at the *STX16* locus that have been examined show a loss of methylation at exon A/B but lack any other *GNAS* imprinting abnormalities (69;73-75). The strong correlation between these genetic and epigenetic defects suggests that the identified deletions disrupt a *cis*-acting long-range regulatory element required for the imprinting of exon A/B. It is possible that this putative element is located in the region removed by both deletions, *i.e*., an approximately 1-kb sequence comprising *STX16* exon 4 and flanking intronic sequences. Similar imprinting regulatory elements with such long distance effects have been identified in other imprinted loci (27); however, there appears to be no significant sequence homology between the region deleted in AD-PHP-Ib patients and the previously characterized imprinting control elements. Future studies are required to discover the nature of this putative long-range regulatory element controlling *GNAS* imprinting.

Most sporadic and some familial PHP-Ib cases exhibit imprinting defects not only at exon A/B, but also at other *GNAS* DMRs (61;67;76). In two such kindreds, in which affected individuals show a “paternal-only” imprinting pattern throughout the *GNAS* locus, we have recently identified deletions that remove the DMR at exon NESP55, which also included exons 3 and 4 of the AS transcript (76) (Fig. 2). In affected individuals, these deletions are on the maternal allele and, therefore, explain the apparent gain of methylation observed at the NESP55 DMR. Of note, when examined in terms of *GNAS* methylation status, unaffected carriers in these two kindreds exhibit an apparent loss of NESP55 methylation. Since maternal inheritance of each deletion results in loss of imprinting of all the maternally imprinted *GNAS* regions, it appears likely that the NESP55 DMR comprises yet another *cis*-acting regulatory element required for the establishment and/or maintenance of imprinting on the maternal *GNAS* allele. The region carries two putative sites for the CCCTC binding factor (CTCF), a *trans*-acting factor that shows methylation-sensitive enhancer-blocking activity in other imprinted loci (77;78). The significance, if any, of these putative CTCF binding sites in maintaining *GNAS* imprinting, and whether they normally serve as boundary elements, remains to be determined.

NESP55 deletions similar in size to those found in the two AD-PHP-Ib kindreds have not been detected in a number of sporadic PHP-Ib cases that also exhibit broad *GNAS* imprinting defects (unpublished data, Bastepe and Jüppner). Considering that the parents bear no clinical or epigenetic defects and that some sporadic cases share maternal haplotypes with their unaffected siblings (unpublished data, Bastepe and Jüppner), it is possible that *de novo* smaller deletions within the NESP55 DMR cause the observed imprinting defects and PTH-resistance in some sporadic cases. However, it is also conceivable that the sporadic PHP-Ib cases carry defects in a gene that is distinct from *GNAS*. Therefore, identification of the genetic defects in sporadic PHP-Ib cases may help delineate the boundaries of the putative imprinting regulatory element in this region or reveal additional players that contribute to the mechanisms underlying the imprinting of Gsα in different tissues.

Regulation of Tissue-Specific Gsα Imprinting

The tissues in which Gsα expression is predominantly maternal include those that exhibit resistance to their hormones in PHP-Ib, *i.e*., renal proximal tubule and thyroid.

Thus, it appears likely that the loss of exon A/B imprinting on the maternal allele, seen in nearly all PHP-Ib cases, leads to a silencing in *cis* of Gsα expression in renal proximal tubules and thyroid. Given that this epigenetic defect makes the maternal allele behave like the paternal allele with respect to imprinting, it is predicted that the normal silencing of Gsα on the paternal allele involves the exon A/B DMR. Recently, Williamson *et al*. (32) and Liu *et al*. (79) have independently generated mice with targeted deletion of exon 1A (the mouse ortholog of exon A/B). Paternal, but not maternal, deletion of exon 1A results in Gsα overexpression in brown adipose tissue and renal proximal tubules without any effects on Gsα expression in other tissues in which Gsα is normally biallelic, thus demonstrating the importance of this region for tissue-specific Gsα imprinting. However, since both of these targeted deletions remove exon A/B promoter activity, it remains unclear whether silencing of Gsα is mediated by an intact A/B transcript, A/B promoter activity, or a non-methylated exon A/B region. Considering that the A/B transcript and the Gsα transcript are expressed together in many tissues (53;54;80), a promoter competition model appears unlikely (unless a model that involves promoter competition in specific tissues is invoked). A more likely model could involve the binding of a tissue-specific *trans*-acting factor to the exon A/B region in a methylation-sensitive manner. For example, this *trans*-acting factor could limit the effect of a putative enhancer element on the Gsα promoter, or it might act as a repressor to directly suppress the activity of that promoter. Additional studies are required to elucidate the tissue-specific mechanisms underlying the silencing of Gsα.

Summary and Conclusions

The different variants of PHP-I appear to be caused by mutations that affect Gsα activity and/or expression, and the tissue-specific imprinting of Gsα expression plays a major role in phenotypic presentation. The *GNAS* locus gives rise, in addition to Gsα, to several other transcripts that show parental origin-specific expression in nearly all tissues. While some of these additional *GNAS* transcripts, such as XLαs, are also disrupted by mutations found in different PHP forms (PHP-Ia, PPHP, and POH), their cellular functions currently remain unclear. Future studies are required to address whether deficiency of *GNAS*-derived proteins other than Gsα also contributes to the complex phenotypes associated with PHP. In order to improve our knowledge of this group of disorders and the role of *GNAS*, it is also important to conduct careful clinical comparisons among different PHP patients carrying *GNAS* mutations of different nature and parental origin.

Conflict of Interest: The authors have declared that no conflicts of interest exist.

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