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ARTICLE

A cAMP-specific phosphodiesterase (*PDE8B*) that is mutated in adrenal hyperplasia is expressed widely in human and mouse tissues: a novel *PDE8B* isoform in human adrenal cortex

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Bilateral adrenocortical hyperplasia (BAH) is the second most common cause of corticotropin-independent Cushing syndrome (CS). Genetic forms of BAH have been associated with complex syndromes such as Carney Complex and McCune–Albright syndrome or may present as isolated micronodular adrenocortical disease (iMAD) usually in children and young adults with CS. A genome-wide association study identified inactivating phosphodiesterase (PDE) 11A (*PDE11A*)-sequencing defects as low-penetrance predisposing factors for iMAD and related abnormalities; we also described a mutation (c.914A>C/H305P) in cyclic AMP (cAMP)-specific *PDE8B*, in a patient with iMAD. In this study we further characterize this mutation; we also found a novel *PDE8B* isoform that is highly expressed in the adrenal gland. This mutation is shown to significantly affect the ability of the protein to degrade cAMP *in vitro*. Tumor tissues from patients with iMAD and no mutations in the coding *PDE8B* sequence or any other related genes (*PRKAR1A*, *PDE11A*) showed downregulated PDE8B expression (compared to normal adrenal cortex). Pde8b is detectable in the adrenal gland of newborn mice and is widely expressed in other mouse tissues. We conclude that *PDE8B* is another PDE gene linked to iMAD; it is a candidate causative gene for other adrenocortical lesions linked to the cAMP signaling pathway and possibly for tumors in other tissues.
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Introduction

Micronodular adrenocortical disease (MAD) is a distinct type of corticotropin-independent bilateral adrenocortical hyperplasia (BAH) and is associated with Cushing syndrome (CS) in children and young adults.[1] MAD is characterized by multiple nodules, capsular deficits that often result in extra-adrenal cortical excrescences

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and hyperplasia of the main cortical tissue; the adrenal medulla is normal in this condition.[1–3] A rare variant of MAD is heavily pigmented due to the accumulation of lipofuscin in the cortical cells; this form is known as primary pigmented nodular adrenocortical disease (PPNAD).[1,3] All forms of BAH have been linked to abnormalities of the cyclic AMP (cAMP) signaling pathway:[4–6] *GNAS* is mutated in BAH in the context of McCune–Albright syndrome,[4] and *PRKAR1A* mutations cause sporadic and familial PPNAD, the latter as part of Carney complex.[5]

Recently, a single-nucleotide polymorphism-based genome-wide association (GWA) study identified inactivating *PDE11A*-sequencing defects in 7 out of 17 patients with iMAD.[7] *PDE11A* encodes a dual-specificity PDE that binds both cAMP and cGMP, and is expressed in a number of endocrine tissues, including the adrenal cortex.[7–11] Adrenocortical hyperplasia in the presence of a partially inactivated PDE is likely to be caused by high cellular cAMP levels in a manner analogous to endocrine tumor formation in McCune–Albright syndrome.[7,12]

The GWA study identified a number of other chromosomal loci as potentially linked to the development of iMAD. Among them, the chromosomal locus 5q13 containing the gene for a cAMP-specific PDE, *PDE8B*, was the second most favored by all analyses, including loss of heterozygosity and transmission disequilibrium testing (Horvath *et al* (2006)[7], supplementary data available at http://www.nature.com/ng/archive/suppinfo/index.html). *PDE8B* was also shown to have significantly higher expression in the adrenal gland compared to all other cAMP-specific PDEs, including *PDE1A*, *PDE4A*, *PDE4B*, *PDE4C*, *PDE4D*, *PDE7A* and *PDE9A* (Horvath *et al* (2006)[7], supplementary data). We then identified a single germline *PDE8B* missense substitution (c.914A>C/H305P) in a patient with iMAD, which we reported as a brief case report.[13]

In the present report, we expand this observation on the identified *PDE8B* mutation and the involvement of this gene in adrenal hyperplasias; we characterized further the mutation and identified a novel *PDE8B* isoform that is expressed in adrenocortical and other tissues, and, finally, examined *PDE8B* expression in the developing and adult mouse tissues.

Materials and methods

Subjects and their samples

The institutional review boards of NICHD, NIH, approved the genetic investigation of patients with adrenocortical tumors under NICHD protocols 95-CH-0059 and 00-CH-0160 after the patients had given informed consent; 22 unrelated pediatric patients with isolated BAH were included in this study, including the 17 patients described previously.[7] The affected individuals ranged in age from 1 to 14, with an average age of 6.2 years. Patients who were diagnosed with CS by standard clinical criteria and testing underwent adrenalectomy. The controls included in this study have been described elsewhere.[7,14]

DNA, RNA, protein extraction and sequence analysis

Blood and tissue samples were collected, as previously described.[14] DNA, RNA and proteins were extracted from whole blood and cell cultures using standard procedures.

All the primers used for the PCR amplification of the PDE8B coding regions and exon–intron junctions are listed in Table 1. After the amplification, the PCR products were agarose gel purified (Minelute; Qiagen) and bi-directionally sequenced on 3130 × l Genetic Analyzer (Applied Biosystems). Sequences were analyzed using Vector NTI 10 software (Invitrogen).

5′-RACE

Human adrenal total RNA (Ambion) was subjected to RNA ligase-mediated 5′-RACE (RLMRACE) using the GeneRacer (Invitrogen) kit according to the manufacturer's instructions. Briefly, 1 μg total RNA was reverse transcribed with Superscript III RT (Invitrogen), and cDNA was subjected to two successive rounds of PCR with Accuprime Taq polymerase (Invitrogen) using primer R3: CACTGGGGTGAT CTTCACG-TGCTGTTGG and nested primer R2: CCTGCCA CTCCTTTCCCTTCTTGATGCAT. The resulting amplimer was TA-subcloned and fully sequenced.

Generation of PDE8B c.914C>A substitution-bearing constructs

Wild-type *PDE8B1* (Origene) was used to first generate the isoform that was identified in the adrenal gland. The endonucleases *Eco*RI and *Apa*I were used according to the manufacturer's instructions (New England Biolabs). The first exon was excited from *PDE8B1* and replaced with the newly identified exonic sequence from our RACE products. Further, c.914C>A substitution was introduced using overlapping PCR with the following primers:

F1: CAGTGTTTACAGCATTAGATCACTGTC;
R1: CAGACAGAGCCTCCTTCATTCAGATAT;
F2: ATATCTGAATGAAGGAGGCTC-TGTCTG;
R2: ATCTCTAGAACTCTGTCCAAGGCTTC.

Transfection experiments were performed as previously described.[14] cAMP levels were measured by cAMP-Glo Assay (Promega) as recommended by the manufacturer.

Mouse expression studies

Pde8b mice expression studies were performed in collaboration with Phylogeny (www.phylogenyinc.com). Tissues were obtained from e9.5, e15.5, p1, p10 and adult mice. Frozen tissues were cut into 8- to 10-μm sections, fixed for 60 min in cold acetone, air-dried and then stored at room temperature. The antibody used in this study (rabbit polyclonal antibody to human PDE8B

Table 1 Primers used for PDE8B sequencing

Exon no.	*Forward primer sequence (5′–3′)*	*Reverse primer sequence (5′–3′)*
1	gaggaagatggcccaaaag	gctcccatcatctccacaaa
2	agtgcacacggtggcataat	gagccgagactgagcctcta
3	gctgggattacaggcatgag	ggagagagaaatctacaggaagga
4	tcctaatccacaagggcatc	aacaaacaaaaccccccaaag
5	ctgctggagctttctctgct	gtcctggggcttaatttcct
6	caagcactttgaacaccttga	tgccattcattgcctgttta
7	ttgggagacatcagcattca	tcagtattctttgcacagcttga
8	cttcctacggggcacaca	ccagattcacttgagttccaaa
9	aggcattgggaaatgtaacg	gctcatactggcatttcaagc
10	atgtgtgggctctgtgtgaa	aaagatttgtcagaggaaccaaa
11	tggtgtatgtctttcatctgttca	tgccataaaggaagattcaagg
12	gcccggcctaatgtttattt	ccctccaaagagacgacaaa
13	ctcctgcctccaaagttcc	ctctcacagaacccgcttg
14	tcttttggattctgggcata	cagcctcatggaaagacaaa
15	atcccagttctccacgttct	tgcaattcttactctaactgtgctc
16	cttccagctagtcccatttga	caggggccatagtctctctg
17	cccctgtgtgtgtgaagcta	tggcagaaaggtccatgtc
18	cgcctctccacatctaggtc	gaactcactgaagaccaaatgagat
19	ctggggaaaatggaatagca	ttcatccccaaggaaaacaa
20	acaggtggtgacagggactt	aaggaaccagaagcctaccc
21	aggcttgagagtgggaaggt	ctgctggggatgcaaagaat
22A	gatcccaaacttgtcccaga	CAGGATGACAGCAGAGCAAA[a]
22B	GTTTGAGGCTTCCATCTGACA	AGCCATAGAGGCTGTGAAGC
22C	CATCTCCCAGGATGGTGACT	ACTTGGTGTAGTGCCTCGTG
22D	CTTCTGGCCGATGGTATGAC	ccctatcccctcccaaaat

[a]Capital letters indicate exonic primers.

synthetic peptide) was purchased from Abcam (catalog no. ab14621). This antibody has no cross-reactivity with PDE8A or any other PDE family members. Slides were washed in PBS and then incubated with antibody and revealed according to peroxidase technique. To detect nonspecific immunoreactivity, normal rabbit serum was used as a control.

To perform IHC, the sections were hydrated in PBS (from Invitrogen 10×, pH 7.2), blocked in TBST (Tris-Buffered Saline with Tween 20, Dako catalog no. S3006) for 5 min, peroxidase-blocked (0.03% H_2O_2 in diH_2O) for 5 min and rinsed (20 dips) in PBS. PDE8B antibody diluted 1:50 with antibody diluent (Dako catalog no. S0809) was applied to the sections for 60 min at room temperature, then rinsed with PBS for 5 min. Avidin/biotin blocking solution (Dako catalog no. X0590) was applied for 20 min followed by a PBS rinse for 5 min. Goat anti-rabbit antibody (Vector BA-1000) was applied at a dilution of 1:200 for 20 min at room temperature followed by a rinse with PBS for 5 min. The detection reagent Vectastain Elite (Vector PK-6100) was applied for 30 min and DAB (Dako catalog no. K3468) for 5 min at room temperature. This procedure produced brown immunostaining within cell cytoplasm or around cells. The sections were rinsed in diH_2O, counterstained with hematoxylin, rinsed again in diH_2O, dehydrated in a series of alcohols and xylene and then coverslipped. All sections were counterstained with hematoxylin and photographed under brightfield illumination.

Results

PDE8B mutation analysis in patients with iMAD and CS

A total of 22 subjects with iMAD and CS were studied, of whom 18 were Caucasians, two African-Americans, one Hispanic and one South Asian (from Pakistan). All samples were negative for *PRKAR1A*, *MYH8* and *GNAS* mutations (data not shown). This cohort included the 17 patients screened for the presence of *PDE11A*-inactivating mutations and has been described previously.[7] The single base substitution (c.914A>T/H305P) that was reported elsewhere[13] was found in the linker region between the PAS (period, aryl-hydrocarbon receptor nuclear translocator (ARNT) and single-minded) and the catalytic domain of PDE8B in a female patient with CS (CAR 559.03). As we reported elsewhere, the patient had presented in early childhood with obesity, hypercholesterolemia and growth abnormalities due to iMAD;[13] she had inherited the mutation from her father (CAR 559.01), who was not known to have CS, but whose subsequent investigation revealed a phenotype consistent with mild iMAD: he had abnormal midnight cortisol levels,[13] obesity, hypertension and, on computed tomography, mild BAH, more prominent on the left adrenal gland (Figure 1). The c.914A>T *PDE8B* mutation has not been found in 1030 unrelated control individuals from a variety of populations, including 285 controls from a mostly Latin-American-based population sample (Coriell Institute; http://www.coriell.org/) and 745 individuals enrolled in the New York Cancer Project (NYCP) study.[14]

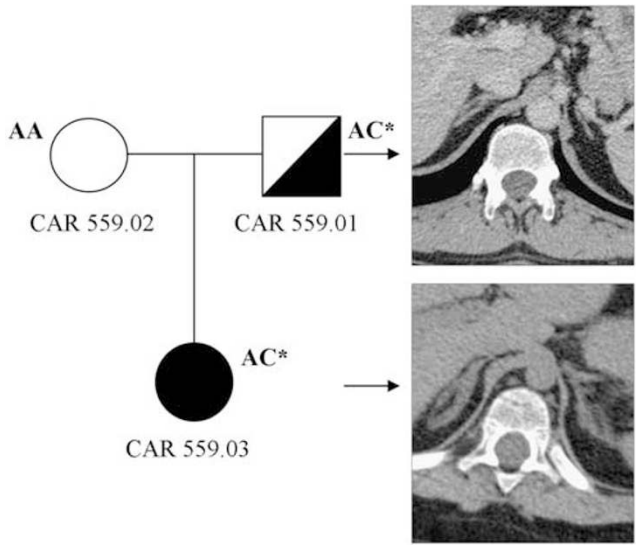

Figure 1 Computed tomography (CT) of the adrenal gland of our patient (CAR 559.03) and her father (CAR 559.01), who is also carrier of the H305P mutation in PDE8B.

In vitro studies of the effect of H305P mutation; novel PDE8B isoform

The H305P mutation affects an evolutionarily conserved residue of the PDE8B protein (Figure 2a). To estimate the effect on the protein function, we performed *in vitro* studies on HEK293 cells: the c.914A>T substitution led to significantly higher cAMP level in cells transfected with the *PDE8B* bearing the substitution.[13]

During our attempts to amplify the whole open reading frame (ORF) of *PDE8B* from adrenal tissue, we identified the presence of an isoform shorter than the previously published one with a different 5′-end. We performed 5′-RACE and isolated a distinct, novel isoform (Figure 2b). The novel isoform includes one additional 5′-located exon of 53 bp that contains an in-frame initiation codon, accompanied by classical *Kozak* motif; the deduced protein sequence of 12 aa is presented in Figure 2c. This exon was located 30 473 bp 5′ of the known transcription start site, and its upstream sequences displayed clear promoter and regulatory features. Similar to the already known transcription initiation site, a CpG island was located between positions −245 and 454 of the newly identified initiator codon. In contrast to all the known *PDE8B* isoforms, the novel isoform skips the currently recognized exon 1, known as the longest exon of the gene (Figure 2d). Sequencing of the newly identified exon in our 22 patients did not reveal any alterations from the published genomic sequence (data not shown). Quantitative PCR showed highest expression of the novel isoform in the pancreas, followed by prostate, testis, heart, kidney, ovary and adrenal (Figure 2e). The ability of the novel isoform to degrade cAMP was similar to *PDE8B1*, as shown by our transfection experiments on HEK293 cells; this was compared with the wild-type, known ORF and the mutant c.914A>T form (Figure 3).

PDE8B expression in normal and a series of adrenocortical tumors

We assessed *PDE8B* expression in a panel of three normal and six adrenocortical tumor samples using western blot analysis. These samples have been screened for *CTNNB1*-, *GNAS*-, *PDE11A*- and *PDE8B*-sequence defects and none was found (data not shown). All six samples contained lower PDE8B protein levels despite the absence of mutations in the coding sequence of *PDE8B* (Figure 4). Interestingly, quantitative PCR on the same adrenocortical samples did not show differential *PDE8B* expression between normal and tumor tissues (data not shown).

Pde8b protein expression in mice

We studied *Pde8b* protein expression in embryonic and adult mice; a summary of Pde8b distribution at stages e15.5, p1, p10 and p77 is presented in Table 2. PDE8b was undetectable at stage e9.5 in the embryo, but occurs in e15.5 mouse tissues (Figure 5a). At this stage, Pde8b was found primarily in the embryonic adipose tissue primordium forming the future hibernation gland that is located in the space between the scapulae (Figure 5b). Little Pde8b was found in the e15.5 liver and heart ventricle (Figure 5c and d respectively). Pde8b distribution formed islets around groups of liver cells and between the heart ventricle muscle fibers; this type of extracellular labeling was not seen in other regions.

Relative to the embryonic stage, a much greater amount of Pde8b was detected in the p1 mouse. Pde8b was labeled in the adipose tissue, especially in the hibernating gland situated between the scapulae (Figure 5e). Also evident was the presence of Pde8b in the heart and liver, stomach mucosa, and in the convoluted tubules of the kidney (Figure 5f–i). The way the labeling is distributed in these organs (around, rather than within the cells) suggests the presence of Pde8b on cell surfaces. Pde8b was detected on the surface of cardiomyocytes, the epithelial-cell apical portion of the stomach and the brush border surface of the presumptive kidney distal tubules. Alternatively, this pattern of labeling may be attributable to the presence of Pde8b in the interstitial space between cells.

By postnatal day 10, a decrease of Pde8b was observed in many tissues, including the adipose tissue (Figure 5j). Taking into account mass and distribution, however, the adipose tissue still can be considered to contain more Pde8b than any other tissue. Lower Pde8b levels were detected in the heart ventricle tissue.

In the liver, the pattern of Pde8b changed from extracellular in the embryo to intracellular in the adult (Figure 5k). Barely detectable Pde8b was seen in the epithelium of the apical portion of the lung bronchioles

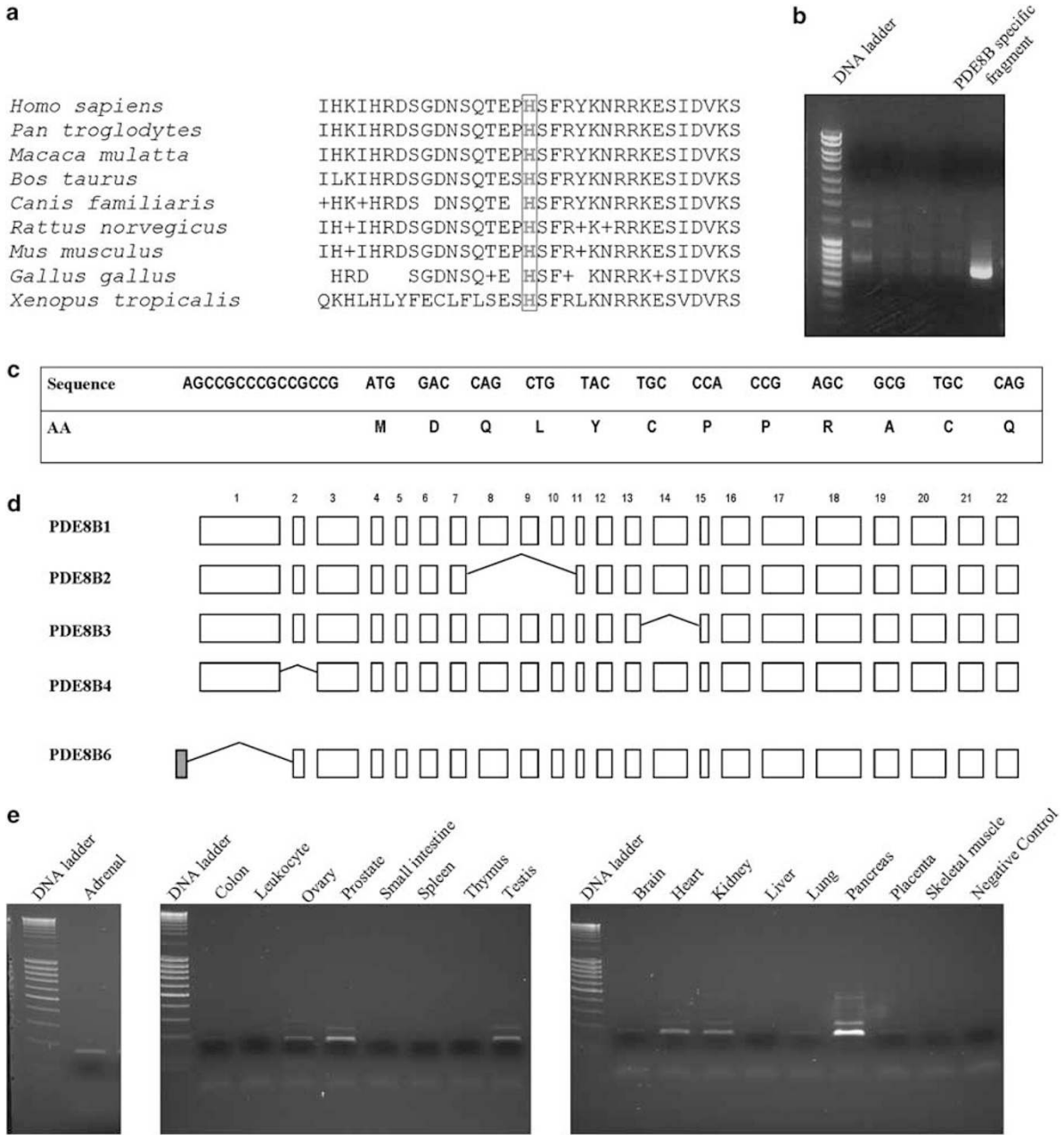

Figure 2 (**a**) Conservation of His at position 305 of PDE across different species; (**b**) gel electrophoresis on the products generated by 5′-RACE; the adrenal-specific fragment that was further used to identify the 5′-end of the novel isoform is shown; (**c**) nucleotide and amino-acid sequence of the novel exon; (**d**) *PDE8B* isoforms generated by alternative splicing; the newly identified exon is shown in red; (**e**) expression of the novel isoform across panel of 17 normal human tissues.

(Figure 5l). The pancreas exhibited faint labeling in the endocrine pancreatic islets and even less in the exocrine pancreatic acini (Figure 5m). There was detectable Pde8b in the vas deferens (data not shown). In the male reproductive organs, detectable Pde8b occurred in the seminiferous tubules of the testis, consistent with the distribution of the spermatogonia, but not spermatozoa (Figure 5n).

Discussion

The reported P305H substitution in PDE8B was present in family members with very mild to a clinically significant phenotype in terms of the severity of CS.[13] We have reported before similar differences in the phenotype of iMAD;[7,14] it appears that PDE-sequence defects are acting as predisposing factors to the development of nodularity

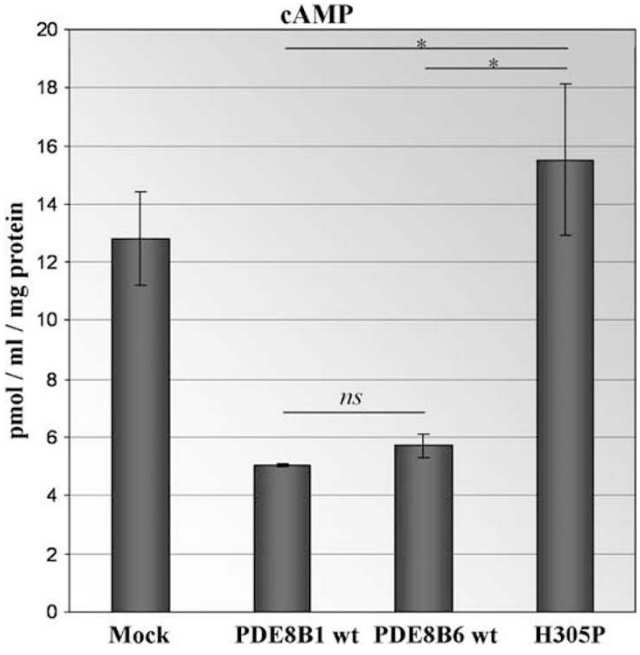

Figure 3 *In vitro* experiments with *PDE8B* ORF construct; asterisk indicates significance of the observed difference between the wild-type and mutant constructs ($P<0.05$).

and they are not causative *per se*. This is consistent with the relatively high (for a rare disease) frequency of *PDE11A*-sequencing defects in the general population,[14] although the P305H *PDE8B* mutation was not found in more than 2000 control chromosomes.

Although the cause of all forms of BAH studied to date seems to be linked to increased cAMP signaling, the histopathological changes in the adrenal glands of patients with the various mutations or functional abnormalities of this pathway differ significantly and overlap only partially.[1] *PRKAR1A* mutations are associated with PPNAD,[5]

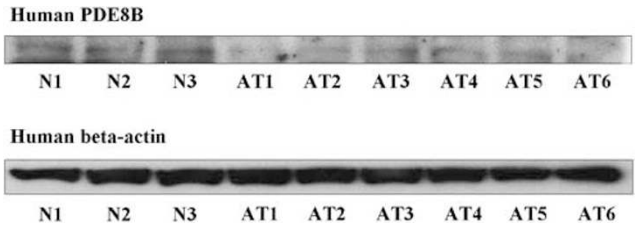

Figure 4 Western blot analysis of PDE8B in three normal (N) and six tumor adrenocortical (AT) tissue samples from patients with no mutations in the coding sequence of *PDE8B*. Lower protein levels in the tumor samples compared to the normal tissues were observed. The protein expression is controlled by β-actin.

Table 2 IHC distribution of PDE8B

Tissue	*e15.5 (sex not determined)*	*p1*	*p10*	*p77*
Adipose tissue axillae (M)	+++++/−	+++++/−	+++/−	ne
Adipose tissue axillae (F)		+++++/−	+++/−	++/−
Adipose tissue scapulae (M)	++/−	+++++/−	+++/−	++/−
Adipose tissue scapulae (F)		+++++/−	++++/−	+++/−
Adrenal gland (M)	ne	+/−	ne	ne
Adrenal gland (F)		+/−	ne	ne
Adipose around blood vessels (M)	−/−	++/−	+++/−	++/−
Adipose around blood vessels (F)		+++/−	+++/−	++/−
Brain (M)	+/−	+/−	+/+	ne
Brain (F)		+/−	+/+	ne
Stomach (M)	−	++/−	+/+	ne
Stomach (F)		+++/−	+/+	ne
Small intestine (M)	−/−	+++/−	++/−	ne
Small intestine (F)		+++/−	+++/−	+++
Heart, ventricle (M)	+/−	++/−	+/−	−/−
Heart, ventricle (F)		++/−	+/−	+/−
Kidney (M)	++/−	++/−	ne	ne
Kidney (F)		+++/−	ne	ne
Liver (M)	+++/−	++/−[a]	++/−	+++/−
Liver (F)		++/−[a]	++/−	+++/−
Lung, bronchus (M)	+/−	+/−	−/−	−/−
Lung, bronchus (F)		+/−	+/−	−/−
Skeletal muscles (M)	+/−	−/−	+/−	−/−
Skeletal muscles (F)		+/−	+/−	−/−
Pancreatic islets (M)	ne	−/−	−/−	ne
Pancreatic islets (F)		−/−	−/−	++/−
Spleen (M)	ne	+/−	ne	ne
Spleen (F)		++/−	ne	+/−
Testis	+/−	±	+/−	−/−

[a]Relates to PDE8B localization in embryonic and newborn hepatic tissue in the form of islets.
Score: ne, not examined; −, undetectable; +, weak; +++, medium; +++++, strong immunoreaction presented as PDE8B/IgG.

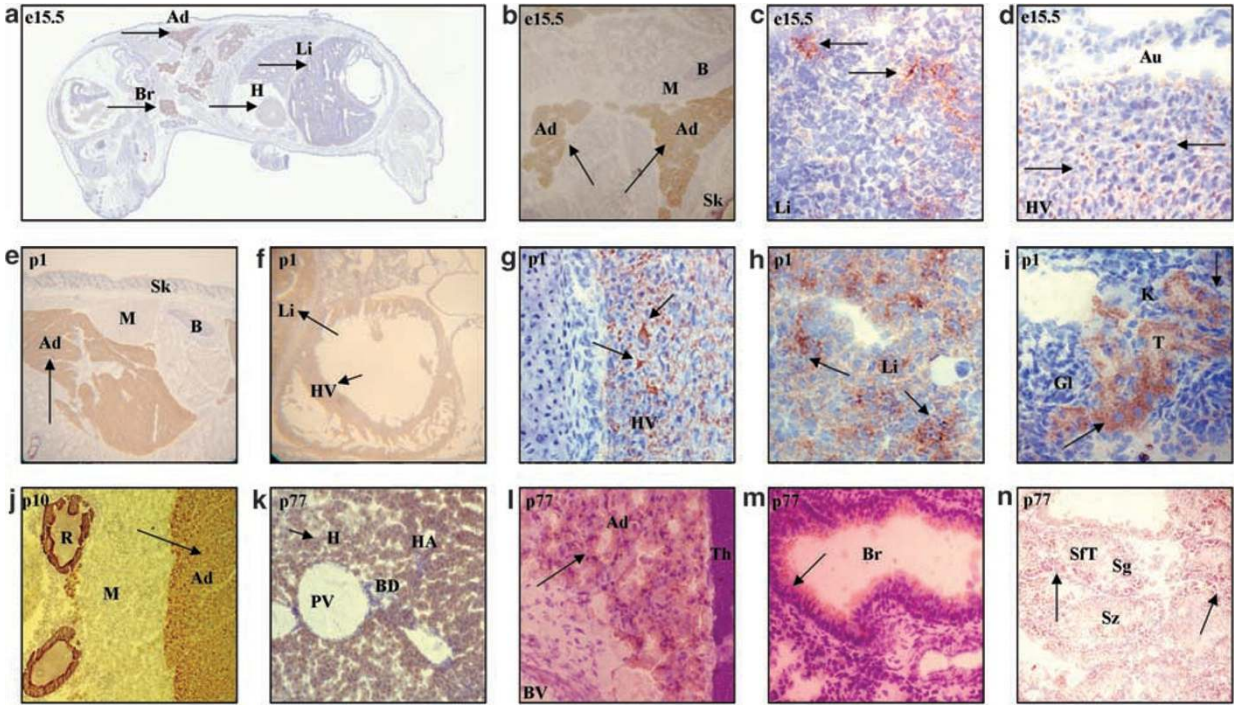

Figure 5 (**a**) Brown PDE8B immunostaining (arrows) in the mouse embryo on day 15.5, seen in a whole-body section (Br – brain; H – heart; Li – liver; Ad – adipose tissue, upper arrow indicates the hibernating gland); (**b**) brown PDE8B immunostaining (arrows) in the adipose tissue; upper arrow indicates the hibernating gland (B – bone; M – skeletal muscle; Sk – skin); (**c**) immunostaining in embryonic liver showing brown labeling forming the islets in between the liver cells (arrows); (**d**) PDE8B in the heart ventricle (HV) forming small spots between muscle fibers (arrows). The atria (Au) do not show any labeling; (**e**) brown PDE8B immunostaining in the adipose tissue of the hibernating gland of a newborn mouse, arrow indicates the hibernating gland; (**f**) PDE8B immunostaining (arrows) in the heart ventricle and liver of a newborn mouse; (**g**) the wall of the heart ventricle; PDE8B labeling (arrow) forms a network around the muscle fibers; (**h**) liver; PDE8B labeling (arrow) seen as a heterogeneous distribution throughout the liver parenchyma; (**i**) kidney tubules, most likely distal tubules, but not glomeruli, showing PDE8B labeling (arrow); Gl – glomerulus; K – kidney; T – tubules; (**j**) intense PDE8B immunostaining (arrow) in the adipose tissue; R – rib; (**k**) PDE8B immunostaining (arrow) in the liver parenchyma; blood vessels, including branches of the portal vein and hepatic artery, and bile duct remain unlabeled (BD – bile duct; H – hepatocyte; HA – hepatic artery; PV – portal vein branch); (**l**) PDE8B immunostaining (arrow) in the brown adipose tissue surrounding the thymus; thymus and blood vessels remain unstained (BV – blood vessel; Th – thymus); (**m**) light PDE8B immunostaining (arrow) in the epithelial cells of the bronchioles (Br – bronchiole); (**n**) PDE8B immunostaining (arrow) in the seminiferous tubules of the adult mouse testis showing spermatogonia to be slightly labeled, spermatozoa to be unstained (SfT – seminiferous tubule; Sg – spermatogonia; Sz – spermatozoa).

whereas *PDE11A* and possibly *PDE8B* mutations seem to predispose to a variety of lesions from isolated (without any other associated tumors) PPNAD to nonpigmented iMAD and other forms of BAH;[7,13,14] *GNAS* mutations are associated with the mostly macronodular and clearly nonpigmented form of BAH that one sees in McCune–Albright syndrome.[4] The identification of more mutations in these and other PDEs will shed more light on this apparent lack of tight genotype–phenotype correlation, which is somewhat unexpected as tissues affected by PPNAD are almost identical histologically.

PDE8B encodes cAMP-specific PDE with the highest affinity to cAMP among all the known PDEs.[15,16] Although involvement in some environment signal transduction has been suggested by the presence of REC (receiver) and PAS domains, no ligand has yet been identified, and the function of PDE8B remains largely unknown. As in other PDEs, the *PDE8B* locus is quite complex and encodes multiple isoforms, arising mainly from alternative splicing and displaying tissue-specific expression.[9,15–18] We describe here a novel isoform that is highly expressed in endocrine tissues, including the adrenal gland, where it shows the highest expression level compared to other cAMP-degrading PDEs. The high adrenal-specific expression levels, its significant affinity to cAMP and the overall importance of cAMP signaling for adrenocortical tumorigenesis suggest that PDE8B could be a candidate for further investigations of its role in adrenal (and possibly other endocrine) tumors.

We detected decreased PDE8B protein levels in adrenocortical tumor samples that are negative for germline mutations in the gene. The real-time PCR experiments did not show significant alteration of the *PDE8B* mRNA levels in the same tumors, thus suggesting posttranscriptional events involved in the regulation of expression. The human microRNA databases list four miRNAs that are

predicted to target *PDE8B*: hsa-miR-412, hsa-miRNA-770-5p, hsa-miRNA-520d-5p and hsa-miRNA-493 (www.microRNA.org). All four of them recognize 5′-located regions of the gene that do not overlap with the c.914A>C substitution. Ongoing experiments assess the ability of those miRNAs to silence PDE8B expression *in vitro*.

Mouse studies showed that predominant expression of Pde8b in the adipose tissue was detected in both embryonic and adult mouse. At embryonic stage e15.5, Pde8b was labeled primarily in the adipose tissue primordium forming the future hibernation gland. Late in gestation, the hibernation gland accumulates fat to serve as an immediate energy reserve at birth. Although the hibernation gland does not yet contain fat at stage e15.5, the presence of Pde8b may indicate that it plays a role in processes related to fat intake and storage. The adipose remained to be the tissue with the highest Pde8b expression in newborn (p1) and adult mice (p10 and p77), although slight decrease in the protein levels has been observed after birth.

Besides its role in the energy intake and storage, the adipose tissue has been recently recognized to have an important endocrine function, and leptin has been the first characterized hormone that is secreted by the adipocytes.[19] Several studies from the last decade have contributed to the elucidation of the endocrinology of adipose tissue and its secretion of adipokines.[20,21] The early and stable Pde8b expression in mouse adipocytes is in need of further investigation. A recent study explores the stimulatory effect of adipose tissue-derived factors on adrenal steroidogenesis and secretory function.[22] An important cross talk between adipose tissue and adrenocortical function is supported by the study of Lamounier-Zepter *et al*[23] on A-ZIP/F1 transgenic mice. This and previous studies have suggested that leptin deficiency may be one of the causes for increased adrenal glucocorticoid production.[24] As leptin signaling has been suggested to involve another cAMP-specific PDE, PDE3B, a similar role could be speculated for Pde8b.[25]

Apart from the adipose tissue, an early Pde8b labeling was detected in the liver and the heart ventricle. The type of labeling of Pde8b in these two tissues was suggestive of extracellular or membranous localization of the protein. The same localization of Pde8b was observed later at p1 stage in the newborn mouse liver and heart where, however, significantly higher levels of the protein were detected. In addition, at p1 stage, Pde8b was detected on the surface of the epithelial-cell apical portion of the stomach and the brush border surface of the presumptive kidney distal tubules. At p10 stage, a slight decrease in the Pde8B expression was detected in almost all of the tissues. Considering ontogeny, differences in the subcellular Pde8b localization were noted, especially in the liver. In the embryonic and newborn mouse liver, Pde8b seemed to exhibit a rather membranous (or extracellular) localization pattern, whereas in the adult mouse liver, Pde8B was localized in the cytoplasm. This observation requires further specifically designed subcellular localization studies including *in situ* hybridization to define the cells that are expressing the gene during the time when extracellular localization is observed.

In conclusion, we describe here the expression studies of a mutation of *PDE8B* causing iMAD and the identification of a novel expression isoform of this gene that is expressed in the adrenal gland. We also present expression studies for this gene in normal mouse tissues; these studies suggest a wider role of PDE8B in endocrine function.

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Conflict of interest

The authors declare that they have no competing financial interests.

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