

**PTH1R MUTATIONS ASSOCIATED WITH OLLIER DISEASE RESULT IN RECEPTOR LOSS OF FUNCTION**

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**PTHR1 MUTATIONS ASSOCIATED WITH OLLIER DISEASE RESULT IN  
RECEPTOR LOSS OF FUNCTION**

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### Abstract

PTHR1 signalling pathway is critical for regulation of endochondral ossification. Thus, abnormalities in genes belonging to this pathway could potentially participate in the pathogenesis of Ollier disease / Maffucci's syndrome, two developmental disorder defined by the presence of multiple enchondromas. In agreement, a functionally deleterious mutation in PTHR1 (p.R150C) was identified in enchondromas from two of six unrelated patients with enchondromatosis. However, neither the p.R150C mutation (26 tumors) nor any other mutation in the *PTHR1* gene (11 patients) could be identified in another study. To further define the role of PTHR1 signalling pathway in Ollier disease and Maffucci syndrome, we analysed the coding sequences of four genes (*PTHR1*, *IHH*, *PTHrP* and *GNAS1*) in leucocyte and/or tumor DNA from 57 and 23 patients affected with Ollier disease or Maffucci syndrome respectively. We identified three previously undescribed missense mutations in PTHR1 in patients with Ollier disease at the heterozygous state. Two mutations (p.G121E, p.A122T) were present only in enchondromas, and one (p.R255H) in both enchondroma and leukocyte DNA. Assessment of receptor function demonstrated that these three mutations impair PTHR1 function, either by reducing the affinity of the receptor for PTH or reducing receptor expression at the cell surface. These mutations were not found in DNA from 222 controls. Including our data, PTHR1 functionally deleterious mutations have now been identified in 5/31 enchondromas from Ollier patients. These findings provide further support for the idea that heterozygous mutations in PTHR1 that impair receptor function participate in the pathogenesis of Ollier disease in some patients.

## INTRODUCTION

Enchondromatosis (OMIM 166000) or Ollier disease (World Health Organization terminology) (1) is a developmental disorder defined by the presence of multiple enchondromas (2-5). Typically, these cartilaginous lesions have an asymmetric distribution, but important variability is seen in the age of onset of the disease and the size, number, location, and evolution of the enchondromas. Most patients have bilateral enchondromatosis, but there is a tendency for one side of the body to be more severely affected. The condition in which multiple enchondromatosis is associated with vascular anomalies characterized by the presence of fusiform cells and high frequency of mesenchymal tumors is known as Maffucci syndrome (2, 6). Enchondromas in Ollier disease present a significant risk of malignant transformation into chondrosarcoma, which usually occurs in young adults, and thus occurs at an earlier age than is observed in patients with isolated chondrosarcoma (2, 7-9). The reported incidence of malignant transformation is even higher in Maffucci's syndrome, the prognosis of which is more severe than that of Ollier disease (2, 3). The association of Ollier disease with other tumors, particularly ovarian juvenile granulosa cell tumors has been reported (2, 10-12).

Enchondromas are almost exclusively localized in the metaphysis of long bones and in the small bones of the hands and feet (2, 3, 13). The tumors initially develop close to the growth plate cartilage where endochondral bone ossification occurs and then migrate progressively towards the diaphysis. Endochondral bone ossification is a highly regulated process that requires the differentiation of mesenchymal cells into hypertrophic chondrocytes and the subsequent replacement of a cartilaginous matrix by mineralized bone. It has been postulated that enchondromas result from abnormalities in signaling pathways controlling the proliferation and differentiation of chondrocytes, leading to the development of intraosseous cartilaginous foci. There are few reports of cytogenetic evaluation of benign enchondromas in Ollier disease, and no tumor-specific chromosomal abnormalities have been associated with enchondromas, or chondrosarcomas in these diseases (14-16). Studies evaluating

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3 isolated chondrosarcomas have usually revealed heterogeneous and complex changes (16),  
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5 although loss of heterozygosity at chromosomal band 9p21 has been observed in several  
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7 cases, and is associated with a loss of expression of the INK4A/p16 protein, a tumor  
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9 suppressor gene involved in control of the cell cycle (17). Expression of parathyroid related  
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11 peptide (PTHrP), its receptor PTHR1, and BCL2 may be correlated with the grade of  
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13 malignancy in chondrosarcoma (18-21).  
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16 PTHrP and Indian Hedgehog (IHH) acting on their respective receptors PTHR1 and  
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18 PTCH1 participate in a tightly coupled signalling relay that is critical for the regulation of  
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20 chondrocyte differentiation and endochondral ossification (22). Thus, abnormalities in these  
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22 genes could potentially participate in the pathogenesis of Ollier disease / Maffucci's  
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24 syndrome. In agreement with this hypothesis, a functionally deleterious mutation in PTHR1  
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26 (p.R150C) was identified in enchondromas from two of six unrelated patients with  
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28 enchondromatosis (23). However, neither the p.R150C mutation (26 tumors) nor any other  
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30 mutation in the *PTHR1* gene (11 patients) could be identified in another study (24). These  
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32 findings suggest that the molecular defects associated with enchondromatosis may be  
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34 heterogeneous, but no other candidate genes, including those participating in the PTHrP /  
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36 IHH pathway, have been evaluated.  
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40 To further define the role of PTHR1 signalling pathway in Ollier disease / Maffucci  
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42 syndrome, we analysed the coding sequences of PTHR1 and three other genes (PTHrP,  
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44 IHH,  $Gs\alpha$ ) implicated in this pathway in two large cohorts of patients. In this study, we have  
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46 i) identified three new mutations in PTHR1 in patients with Ollier disease, ii) characterized  
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48 the abnormalities in receptor function resulting from these mutations, and iii) through  
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50 modelling of the N-terminal ectodomain, evaluated the impact of the PTHR1 mutations on the  
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52 receptor function.  
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## MATERIALS AND METHODS

### Patients

Informed consent was obtained for all patients. The study was performed in two series of patients. The first series comprised 46 patients (29 females, 17 males) from a French cohort. The clinical characteristics of the patients are presented supplementary table 1. Data were obtained by review of clinical charts, contact with physicians in charge of the patients, and through a questionnaire sent to the patients in collaboration with the French association for Ollier disease / Maffucci syndrome. Patient age at diagnosis ranged from 1 to 36 years (median 6.4 years). All patients were affected by at least three enchondromas. Unilateral body involvement was present in 23 patients (52%), and bilateral involvement with a marked asymmetry was noted in 9 patients. No patients had spinal involvement. Two patients (HP17 and HP55) were affected by chondrosarcomas. One patient (HP17) had Maffucci syndrome. For all patients, the diagnosis of exostosis was excluded. Sixteen females (59%) and four (26%) had a height  $\geq$  50 percentile ( $p=0.06$  by Fisher's exact test). In all patients, serum calcium and phosphorus levels were within the normal range for the age (data not shown). Leukocyte DNA was obtained from all patients; tumor DNA was available for 12 patients, including the patient with Maffucci syndrome (10 enchondromas and 2 chondrosarcomas).

The second series comprised 34 patients from a Belgium cohort, 12 patients affected by Ollier disease (5 females, 7 males; age at the time of the study 6-38 years), and 22 patients affected by Maffucci syndrome (11 females, 11 males; age at the time of the study 15-52 years) (supplementary table 2). Leukocyte DNA was obtained from all patients except one; tumor DNA was available for 3 patients affected with Ollier disease and 13 patients with Maffucci syndrome (10 enchondromas, 2 chondrosarcomas, one spindle cell hemangioendothelioma).

Genomic DNA from 222 Caucasians was used as a control group.

## Sequence analysis

Genomic DNA was extracted from peripheral blood and/or tumors obtained at the time of surgery using a QIAamp DNA purification Kit (Qiagen SA, Courtaboeuf, France). Intronic, and when required, exonic primers were used to amplify all coding exons and intron-exon junctions for the *PTHR1*, *IHH*, *PTHrP* and *GNAS1* ( $Gs\alpha$  exons 1-13) genes. *PTHR1* gene was analysed in all leukocyte and tumor DNA samples from the French and Belgium cohorts. *IHH* and *PTHrP* were analysed in all leukocyte and tumor DNA from the French cohort. The *GNAS1* gene (exons 1-13 of *Gsa*) was analysed in tumor (9 samples) and leukocyte DNA from 4 patients from the French cohort. The sequences of primers are available upon request. PCR products were analysed by direct sequencing (french patients and 112 controls) or by prescreening using SSCP/heteroduplex analysis combined with sequencing of abnormally migrating fragments (belgium patients and 110 controls).

PCR products were sequenced in both directions. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied biosystems, Courtaboeuf, France), and analysed using an ABI PRISM 3100 sequencer (Applied Biosystems) and Beckman CEQ2000 fluorescent capillary sequencer for the french and belgium cohorts respectively.

Nucleotide positions are numbered from the ATG start codon in the cDNA (sequence accession numbers: *PTHR1*, [NM\\_000316](#); *IHH*, [NM\\_002181](#); *PTHrP* transcript variant 1, [NM\\_198965](#); *PTHrP* transcript variant 2, [NM\\_002820](#); *PTHrP* transcript variant 3, [NM\\_198964](#), *GNAS1*, [NM\\_000516](#)). All nucleotide changes (except for previously reported polymorphisms) were verified by resequencing of a different PCR product. Only silent and missense nucleotide changes in the coding region are reported. However, for all polymorphisms identified in introns, it was verified that they were not predicted to affect splicing ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)).

## Site directed mutagenesis



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3 Full length wild-type (WT) complementary DNA (cDNA) encoding the human PTHR1  
4 (PTHR1) was inserted into the pEGFP-N2 N-terminal fusion protein vector (Clontech  
5 Laboratories, Mountain View, CA) upstream of the enhanced green fluorescent protein gene  
6 (25). Missense mutations were introduced in the WT PTHR1 cDNA construct using the  
7 Quick Change Site directed Mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by  
8 complete sequencing of cDNA inserts (oligonucleotide sequences available on request).  
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### 16 **Cell culture and transient transfection**

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18 The function of all mutated PTHR1 receptors were studied following transient  
19 expression in CHO cells. Mutant p.R150C, p.G121E, p.A122T PTHR1 were also studied  
20 following expression in COS-7. CHO and COS-7 cells were cultured in 24 well plates as  
21 previously described (26-28). Transfection of CHO cells were performed using 1.5  $\mu$ l of  
22 FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) with 1  $\mu$ g pEGFPN2  
23 plasmid encoding wild-type or mutant PTHR1. Transfection of COS-7 cells were performed  
24 using lipofectamine 2000 (Invitrogen SARL, Cergy Pontoise, France) and 500 ng DNA / well  
25 as previously described (26, 28).  
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### 38 **[<sup>125</sup>I] PTH 1-34 binding, immunological assessment of receptor expression, and cAMP** 39 **measurement**

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42 Techniques used to assess PTH 1-34 binding, PTHR1 cell membrane expression,  
43 and cAMP production and have been described (26-28). Briefly, the binding properties of  
44 wild-type and mutated hPTHR1 receptors were determined by competitive inhibition of [<sup>125</sup>I]  
45 (Nle<sup>8,18</sup>Tyr<sup>34</sup>)human PTH (1-34) amide ([<sup>125</sup>I]-PTH1-34) (Amersham Biosciences UK Ltd,  
46 Buckinghamshire, England) binding to transfected cells by increasing concentrations of  
47 unlabeled (Nle<sup>8,18</sup>Tyr<sup>34</sup>) human PTH (1-34) (Bachem UK Ltd, Merseyside, England) (PTH1-  
48 34) as in (25, 28). The specific binding was calculated as the difference between [<sup>125</sup>I]-  
49 peptide bound and the non-specific binding (i.e., that measured in the presence of 8x10<sup>-7</sup>M  
50 unlabeled PTH 1-34). To determine the IC<sub>50</sub>, binding data was fitted to a sigmoid curve with  
51 variable slope (Graphpad Prism 4, GraphPad Software Inc., San Diego, CA). Immunological  
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3 assessment of cell surface receptor expression was performed using a rabbit polyclonal  
4 antibody raised against a 17 amino acid epitope present in exon E2 of the human PTHR1  
5 (90E-S-E-E-D-K-E-A-P-T-G-S-R-Y-R-G-106R) (PTHR1 E2 antibody) and a secondary [<sup>125</sup>I]-  
6 labelled donkey anti-rabbit immunoglobulin antibody (Amersham biosciences UK Ltd,  
7 Buckinghamshire, England) as previously described (29). For the determination of  
8 intracellular cAMP production, cells were incubated without or with hPTH1-34 under  
9 continuous agitation in 0.5 ml of culture medium containing 0.1% (w/v) bovine serum albumin  
10 and 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, Saint-Quentin Fallavier, France).  
11 After a 20 min incubation at 37 °C, the medium was removed and cells were lysed with 1 M  
12 perchloric acid. The cAMP present in the lysate was measured by radioimmunoassay as  
13 described (26). Results are expressed as pmol of cAMP/well. Experiments were performed  
14 at least three times in duplicate with two different plasmid preparations.  
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### 32 **Confocal laser scanning microscopy**

33 CHO cells were transiently transfected with the WT or mutant PTHR1 cDNA inserted  
34 into the pEGFP plasmid. Forty-eight h after transfection, unpermeabilized cells were  
35 incubated for 30 min at 4°C with the PTHR1 antibody used for the assessment of PTHR1  
36 expression (PTHR1 E2 antibody), washed extensively with phosphate-buffered saline, and  
37 incubated for 30 min at 4°C with a secondary rhodamine-labelled donkey anti-rabbit  
38 immunoglobulin. Cells were fixed in 1% paraformaldehyde. Glass coverslips were mounted  
39 and examined by confocal laser scanning microscopy (CLSM-510-META, Zeiss, Germany)  
40 equipped with epifluorescent optics (x63 NA 1.3 oil-immersion objective). Simultaneous two-  
41 channel recording was performed with a pinhole size of 90 µm using excitation wavelengths  
42 of 488/588 nm, a 510/580 double dichroic mirror, and a 515-545 bandpass fluorescein  
43 isothiocyanate filter together with a 590-nm long pass filter. Double-labelled cells were  
44 analyzed separately to avoid spillover between channels. In all experiments, omission of the  
45 primary antibody was confirmed to result in no detectable staining.  
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3 **Location of mutations in the structure of the hPTHr1 N-terminal ectodomain (N-ter)**  
4 **complexed to PTH.**  
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8 In order to locate the spatial position of the PTHR1 mutations, we plotted each residue in the  
9 recently published structure of the N-ter of PTHR1 (PDB ID code 3C4M) bound to PTH (15-  
10 34) obtained by crystallization and X-ray diffraction (30).  
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## RESULTS

### Sequencing of candidate genes

*PTHrP*, *IHH* and *Gsα* were only tested in the French cohort. No variant alleles were identified in the coding sequence of the *PTHrP* gene in either leukocyte or tumor DNA in the 50 patients studied whereas three previously described silent polymorphisms (rs3731878, p.T200T; rs3731881, p.P251P; rs394452, p.T376T), and one previously undescribed heterozygous variant (p.Y180Y / c.540T>C; HP 65) were identified in *IHH*. The allele frequency of the known polymorphisms did not differ from that reported in the NCBI SNP database (not shown). Three previously described silent polymorphisms (rs7121, p.I131I; rs3730171, p.I186I; rs8386, p.N371N) were identified in *Gsα*. Again, their allele frequency did not differ from that reported in the NCBI SNP database (not shown).

Seven heterozygous previously unreported PTHR1 SNPs, 4 silent (p.D30D, p.A72A, p.T163T, p.V455V) and 3 non-synonymous (p.G121E, p.A122T, p.R225H), were identified only in samples from the 80 patients with Ollier disease / Maffucci syndrome (supplementary tables 1 and 2). The three previously undescribed non-synonymous mutations were all found in patients with Ollier disease. Two of these mutations (p.G121E, p.A122T) were observed in DNA from enchondromas, but not in leukocyte DNA; the R255H mutation was detected both in enchondroma and leukocyte DNA. In addition, two known non-synonymous polymorphisms (p.G100D, p.E546K) were identified in patients and controls (p.G100D, 1 patient and 1 control; p.E546K, 3 patients and 6 controls) (23, 31). The allele frequency of the common synonymous PTHR1 polymorphism p.N463N (rs186987) was similar in the patient and control populations, and to that described in the NCBI SNP database. Two unreported heterozygous polymorphisms, one synonymous (p.S492S) and one non-synonymous (p.P581R), were each identified in one control subject.

### Functional characterization of mutant and wild-type PTHR1

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Functional studies evaluating the impact of all nonsynonymous SNPs identified in patients with Ollier disease, including the three new alterations identified in this study (p.G121E, p.A122T, p.R225H), the mutation described by Hopyan et al. (p.R150C), and the two known polymorphisms (p.G100D, p.E546K) were performed (functional characterization of p.E546K, but not p.G100D, has been previously reported (23, 31)). In addition, The p.H223R mutation identified in patients with Jansen's metaphyseal chondrodysplasia was evaluated (32).

*Ligand Binding.* WT and mutant PTHR1 cell surface expression and affinity was assessed by the inhibition of [<sup>125</sup>I] hPTH1-34 binding by increasing concentrations of unlabelled hPTH1-34. For cells expressing WT PTHR1, half maximal displacement of bound radiolabelled PTH was obtained with approximately  $3 \times 10^{-10}$  M unlabelled hPTH1-34 (figure 1A and table 1). Similar results were obtained for cells transfected with PTHR1 receptors carrying the nonsynonymous mutations p.G100D, p.A122T, p.R150C and p.E546K (figure 1A and table 1). In contrast, a marked reduction in [<sup>125</sup>I]hPTH(1-34) maximal radioligand binding and binding affinity was observed for cells expressing PTHR1 carrying the p.G121E and p.R255H mutations (figure 1A and table 1). The IC<sub>50</sub> for these mutants was at least 50-fold higher than that observed for WT PTHR1 (>15 nM), but a precise value could not be determined because binding affinities > 15 nM cannot be determined using our approach. The binding properties of PTHR1 carrying the p.G121E, p.A122T and p.R150C mutations were also studied in COS-7 cells; results similar to those observed in CHO cells were obtained, except for an approximate one log right shift in binding affinity observed for WT and mutant PTHR1. Thus, p.A122T and p.R150C PTHR1, maximal binding and binding affinity were similar to that of WT PTHR1; for p.G121E PTHR1, maximal binding and binding affinity were decreased. As previously described, maximal radioligand binding by the constitutively active p.H223R mutant was approximately 50% lower than that of WT-PTH1, with a similar half maximal displacement of bound PTH (figure 1A and table 1).

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3 *Immunological assessment of PTHR1 cell-surface expression.* In order to quantify receptor  
4 expression, we assessed cell surface expression of wild-type and mutant receptors using a  
5 polyclonal primary antibody directed against an extracellular epitope encoded by E2 of  
6 PTHR1, and a secondary iodinated antibody (29). Expression of p.A122T, p.R150C and  
7 p.E546K PTHR1 were similar to that of WT PTHR1 (figure 2 A and table 1), while expression  
8 of p.G121E and p.R255H PTHR1 were approximately 70% lower than that of WT PTHR1  
9 (figure 2A and table 1). These results are consistent with the results obtained in the  
10 radioreceptor studies described above. Antibody binding for the p.G100D mutation was less  
11 than 5% of that observed for the WT receptor (figure 2A and table 1). The p.G100D mutation  
12 is located within the epitope against which the antibody was raised. As these cells show  
13 normal binding of radiolabelled PTH, this result indicates that the p.G100D mutation disrupts  
14 the epitope preventing recognition by this antibody. Expression of the p.H223R mutant was  
15 approximately 25 % lower than that of WT-PTHR1, results consistent with previous studies  
16 (32).  
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### 36 **Analysis of PTHR1 expression using confocal microscopy**

37 To evaluate the distribution of PTHR1 expression in CHO cells, cells were transfected with  
38 an expression vector producing GFP-tagged PTHR1, and 48 hours after transfection,  
39 expression of PTHR1 on the surface of non-permeabilized cells was evaluated using the  
40 PTHR1 E2 antibody and a rhodamine-labelled secondary antibody. Cell surface expression  
41 was clearly observed for WT PTHR1 and p.A122T, p.R150C and p.E546K PTHR1 (Figure 3).  
42 In contrast, although some cell surface expression was detected, a diffuse green labelling  
43 was observed in cells transfected with p.G121E and p.R255H PTHR1. This labeling pattern  
44 indicates that the receptors were expressed but not appropriately addressed to the plasma  
45 membrane. Similar results were observed in cells expressing p.H223R PTHR1, as  
46 previously reported. The thin green labelling observed at the cell periphery without  
47 cytoplasmic staining in cells transfected by p.G100D PTHR1 indicates that this mutant is  
48 correctly addressed to the cell surface.  
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## cAMP production

The functional consequences of the PTHR1 mutations was assessed by comparing basal and agonist-stimulated cAMP production in cells expressing WT and mutant receptors (Table 1). Except for the constitutionally active p.H223R PTHR1, basal cAMP production in cells transfected with mutants PTHR1 was not significantly different from that measured for WT receptors (Figure 2B).

In cells expressing the WT PTHR1, cAMP production increased 15-fold over basal, and half maximal stimulation of cAMP production was obtained with approximately  $6 \times 10^{-10}$  M hPTH(1-34) (Figure 1B and Table 1). When challenged with increasing amounts of hPTH(1-34), three patterns of cAMP accumulation was observed in cells expressing PTHR1 carrying nonsynonymous polymorphisms: (i) For cells expressing p.A122T and p.R150C PTHR1, an approximately 50% decrease in maximal ligand-induced cAMP production was observed, but the EC50 was similar to that of the WTPTHR1. As previously described, a similar decrease in the maximal stimulation fold of cAMP production without change in EC50 was also observed in cells expressing the H223R mutation. (ii) For cells expressing p.G121E and p.R255H PTHR1, a marked shift to the right in the dose response curve was observed (EC50  $29.9 \times 10^{-9}$  M and  $15 \times 10^{-9}$  M respectively). cAMP stimulation fold over basal by  $10^{-7}$  M hPTH(1-34) was 77 and 95 %, respectively for cells expressing p.G121E and p.R255H PTHR1 compared to that of cells expressing the WT PTHR1. (iii) For cells expressing the p.G100D and p.E546K polymorphisms, results were similar to those obtained for WT PTHR1, indicating that these nonsynonymous mutations do not affect receptor function.

A summary of the impact of the nonsynonymous mutations evaluated in this study on the expression and function of PTHR1 is shown in Table 2.

**Location of mutations in the structure of the hPTH1 N-terminal ectodomain (N-ter) complexed to PTH.**

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3 In the structure described by Pioszak and Xu (30), the PTHR1 N-ted (PDB ID code 3C4M)  
4 contains one major  $\alpha$  helix (residues 33–57) and two antiparallel  $\beta$  sheets ( $\beta$  -strands 1 and  
5 2: residues 110–120),  $\beta$  -strands 3 and 4 (residues 130–140) stabilized by 3 disulfide bonds  
6 linking Cys<sup>48</sup>-Cys<sup>117</sup>, Cys<sup>108</sup>-Cys<sup>148</sup>, and Cys<sup>131</sup>-Cys<sup>170</sup> (Figure 4). This structured core  
7 represents a typical sushi domain present in several structures of class B GPCR N-ted (30,  
8 35). The ligand binding ridge is localized along the structured core of the N-ted (30) (Figure  
9 4). The exon E2 sequence present in the PTHR1 N-ted is not included within the structured  
10 core, and lies in front of the first  $\beta$  sheet (dotted line, figure 4).  
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21 The localization of amino acid changes present in the PTHR1 N-ted identified in this  
22 and previous studies, including the p.P132L mutation (Blomstrand chondrodysplasia) (25, 33,  
23 34), and inducing changes in PTHR1 function is shown in Figure 4. These mutations are all  
24 included within the structured core of the N-ted of the PTH receptor, but outside the putative  
25 binding ridge. The p.G121E and p.A122T mutations, which respectively reduced ligand  
26 affinity and maximal cAMP production, are located in a loop between the  $\beta$ 2 and  $\beta$ 3 sheets.  
27 The p.R150C mutation, which reduced maximal cAMP production, is located in a loop  
28 between  $\beta$ 3 sheet and the disulfide bridge Cys<sup>131</sup>-Cys<sup>170</sup> (Figure 4). The p.P132L mutation is  
29 localized close to a stretch of four residues (aa 135-138) involved in direct contacts between  
30 PTH and PTHR1 N-ted. In contrast, the p.G100D mutation, which does not modify PTHR1  
31 function, is located in exon E2. Because the p.R255H mutation is not included in the  
32 PTHR1-N-ted, it could not be positioned in the structure.  
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## DISCUSSION

To further define the role of PTHR1 signalling pathway in Ollier disease and Maffucci syndrome, we have analysed the coding sequences and the intron-exon boundaries of four genes participating in this pathway in a large cohort of patients. In this study, we identified three previously undescribed missense mutations in PTHR1 in patients with Ollier disease. Two of these mutations were present only in enchondromas, and one in both enchondroma and leukocyte DNA. The assessment of receptor function demonstrated that all these mutations impair PTHR1 function, either by reducing the affinity of the receptor for PTH or reducing receptor expression at the cell surface. Structural modelling of PTHR1 indicated that the deleterious mutations associated with Ollier disease and located within the N-ted all lie within the structured core of the N-ted. Functionally deleterious PTHR1 mutations have now been identified in 5 of 31 enchondromas from patients with Ollier disease (this study + (23, 24), whereas such mutations have not be found in DNA from 222 control patients (this study) evaluated by the same techniques. These findings provide further support for the idea that heterozygous mutations in PTHR1 that impair receptor function can participate in the pathogenesis of Ollier disease in some patients.

### *PTHR1 mutations and the pathogenesis of Ollier disease*

It has been suggested that enchondromas result from abnormalities in signalling pathways controlling proliferation and differentiation of chondrocytes, leading to the development of the intraosseous cartilaginous foci. In particular, PTHR1 regulates the switch from proliferating to hypertrophic chondrocytes, and thereby influences the number of cells expressing Indian HedgeHog (IHH). IHH is a member of a family of morphogen proteins that are important for embryonic patterning, and is highly expressed in the transition zone between proliferating and hypertrophic chondrocytes. This multifunctional protein stimulates chondrocyte proliferation, predominantly through a PTHrP-independent mechanism, and also delays their hypertrophy by increasing PTHrP synthesis by periarticular chondrocytes. Thus

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3 PTHR1 and IHH signalling pathways are tightly coupled, although both exert functions  
4 independently of each other. Reduced PTHR1 signalling would be expected to impair this  
5 coupling process necessary for harmonious chondrocyte proliferation and differentiation,  
6 thereby contributing to the development of enchondromas.  
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11 Our results and those from the literature indicate that missense PTHR1 mutations may  
12 be involved in the development of enchondromas in a minority of patients. Three amino-acid  
13 substitutions in PTHR1 were identified in the present study that impaired the ability of the  
14 mutant receptors to stimulate cAMP production, due to 1) either a decrease in receptor  
15 expression and ligand affinity (p.G121E and p.R255H), or 2) to suboptimal agonist-induced  
16 cAMP production despite normal PTHR1 expression (p.A122T). Two of these mutations  
17 were somatic and the third one likely germline. None of these receptors displayed  
18 constitutional activity, whereas, as expected, increased constitutional activity was observed  
19 for the PTHR1 expressing the p.H223R mutation identified in patients with Jansen's  
20 metaphyseal chondrodysplasia. We also evaluated the functional properties of the p.R150C  
21 mutation, previously identified in two patients with Ollier disease by Hopyan et al. (23).  
22 Consistent with the results of Hopyan et al., we found that PTH-induced cAMP production  
23 was reduced in cells transiently expressing this receptor, but in contrast to the previous  
24 report, receptor expression was normal. Thus, the apparent increase in constitutional activity  
25 reported by these authors following correction for receptor expression (23) was not observed  
26 in our study. The reason why the p.R150C receptor expression was normal in our  
27 experiments and decreased in Hopyan's studies are not clear, but are probably due to  
28 differences in experimental conditions. Nevertheless, all mutant receptors identified  
29 exclusively in patients with Ollier disease are characterized by impaired ligand-induced  
30 cAMP production.  
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55 It is noteworthy that for three of the five patients with Ollier disease in whom PTHR1  
56 mutations were identified in enchondromas, similar mutations were not detected in peripheral  
57 blood leukocytes (this study and reference) (31). Because multiple enchondromas were  
58 present in these patients, the findings are consistent with the hypothesis that the mutations  
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3 occurred during development, resulting in genetic mosaicism in these individuals. Further  
4 studies evaluating multiple tumors and other tissues from such patients will be required to  
5 further support this idea. Nevertheless, sensitive screening for PTHR1 mutations in this  
6 disease appears to require the evaluation of tumoral tissue, because mutations have now  
7 been identified in 5/31 enchondromas, but only 2/58 samples of leukocytes DNA evaluated  
8 by the same approach ( $p < 0.05$  by Fischer's exact test).  
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12 In the course of these studies we also evaluated samples obtained from patients with  
13 Maffucci syndrome (enchondromas.chondrosarcomas,  $n=12$ , blood leukocytes,  $n=13$ ), but  
14 deleterious mutations in PTHR1 were not identified. Because of the small number of patients  
15 studied, however, the frequency of finding PTHR1 mutations was not significantly different  
16 comparing patients with Ollier's disease and Maffucci syndrome (5/31 and 0/12 positive  
17 tumors, respectively,  $p=0.3$  by Fischer's exact test). Further work is required to assess the  
18 role, if any, of PTHR1 mutations in Maffucci syndrome.  
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23 Other findings are consistent with the conclusion that abnormalities in the PTHR1 /  
24 IHH pathway can be linked to the development of endochondromas. Transgenic mice  
25 expressing the mutant p.R150C PTHR1 under the control of the collagen type II promoter  
26 develop tumors that are similar to those observed in human enchondromatosis (23). A  
27 contribution of a dysregulation of IHH signalling pathway to the development of  
28 enchondromas is also supported by the development of enchondromas in mice  
29 overexpressing the Hedgehog (Hh) transcriptional regulator, Gli2, and the activation of a  
30 Hedgehog-responsive Gli2-luciferase reporter construct by the p.R150C *PTHR1* mutant (23).  
31 Finally, downregulation of IHH/PTHrP signalling as a result of EXT mutation has been shown  
32 to play a role in osteochondroma formation (35).  
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55 Even when present, it is not certain that the PTHR1 mutations identified are sufficient  
56 alone to induce enchondromas. The p.R150C mutation has been identified both in a patient  
57 with Ollier disease and in a parent who had other skeletal abnormalities without  
58 enchondromas. In this regard, it will be interesting to evaluate family members of patients  
59 expressing the p.R255H PTHR1 mutation identified in this study, but such clinical samples  
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3 are not currently available. Indeed, the PTHR1 loss of function mutations identified in Ollier  
4 disease are expressed in the heterozygous state. PTHR1 dimerization has not been  
5 documented for receptors of this family, rendering a dominant negative effect of the mutant  
6 receptor unlikely. These findings support the hypothesis that, even when a PTHR1 mutation  
7 is present, a combination of genetic events, germline and/or somatic, are required for the  
8 development of enchondromas.  
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11 The nature of such putative genetic abnormalities remains to be defined.  
12 Abnormalities involving other participants in the PTHR1 pathway are potential candidates.  
13 For three of these genes, PTHrP, IHH and GNAS1 (G $\alpha$ ), no missense mutation were  
14 identified in these studies. Although these negative results do not exclude the possibility that  
15 genetic abnormalities at these loci can contribute to the development of multiple  
16 enchondromas, our findings suggest that such abnormalities are not a frequent cause.  
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#### 18 Other PTHR1 polymorphisms

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20 Currently, only two missense polymorphisms in PTHR1 have been described in  
21 population studies, p.G100D and p.E546K. In the course of these studies, both of these  
22 polymorphisms were identified with apparently similar frequency in controls and patients with  
23 Ollier disease / Maffucci syndrome. We evaluated the functional properties of receptors  
24 carrying both of these polymorphisms, but no abnormalities were identified. Amino-acid  
25 p.G100 is located in exon E2 of the PTHR1; this exon is not present in other family II GPCR,  
26 and its deletion has been previously shown not to affect PTHR1 function (36). Therefore the  
27 lack of deleterious effect of the p.G100D mutation was not unexpected. The p.E546K  
28 polymorphism has previously been extensively characterized by Schipani et al., and no  
29 abnormalities were found (31).  
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#### 31 *3D spatial location of mutations*

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33 In order to better analyse the mechanism through which mutations may affect  
34 receptor function, we represented the recently resolved crystal structure of the PTHR1 N-ter-  
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3 PTH complex (PDB ID code 3C4M) (30), and positioned the PTHR1 mutations identified in  
4 this and previous studies.  
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7 The observation that the functionally deleterious PTHR1 mutations (p.G121E, p.A122T,  
8 p.R150C and p.P132L) are all located within the structured core, but outside the putative  
9 ligand binding ridge indicates that the mutations may not directly impact receptor-ligand  
10 interactions, but rather induce changes in the three dimensional organization of the N-ter  
11 structured core necessary for PTH recognition and receptor activation. In support of this, the  
12 p.P132L substitution (Blomstrand chondrodysplasia) (25, 33, 34) appears to disrupt an  
13 interaction required to hold the structure of the N-ter and PTH-PTH1R signaling (30). An  
14 analysis performed using a model of the VPAC1 receptor (also a class II GPCR) (37)  
15 supports this conclusion. In this model, proline-87 in the VPAC1 receptor, which  
16 corresponds to proline-132 in PTHR1, is also included within the structured core of the N-ter  
17 of the VPAC1 receptor, but outside the binding ridge. The substitution of P87 by alanine  
18 induced an important decrease in the affinity of VIP for its receptor (unpublished data). In  
19 contrast, substitution of proline-87 by glycine, which introduces a flexible point in the  
20 structured core, does not affect VPAC1 receptor function (38). The p.G100D mutation, which  
21 does not modify PTHR1 function, is not located within exon E2 because the p.R255H  
22 mutation is not included in the PTHR1-N-ter, no structure-function correlates could be  
23 obtained for this mutation.  
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In conclusion, this study provides further evidence that functionally deleterious mutations in PTHR1 are present in a subset of patients with Ollier disease, and therefore offers support to the idea that abnormalities in signalling pathways controlling chondrocyte proliferation and differentiation can contribute to the pathogenesis of this disorder. Our results emphasize that the genetic abnormalities responsible for this disease are likely to be both heterogeneous and multi-factorial. The evaluation of additional candidate genes in large cohorts of patients may prove rewarding.

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## LEGEND FIGURES

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Figure 1. Functional evaluation of wild-type and mutant PTHR1 expressed in CHO cells. (A). Binding of [<sup>125</sup>I] PTH 1-34 by cells incubated with radio ligand only (maximal binding) and in the presence of increasing concentrations of unlabeled hPTH 1-34. The maximal binding measured in cells transfected with the WT-PTHR1 was set as 100%. Results were corrected for non-specific binding measured in the presence of 8x10<sup>-7</sup> M PTH1-34. (B) cAMP accumulation in response to increasing concentrations of hPTH 1-34. Results are expressed as stimulation fold over basal. CHO cells were transfected with 1 µg plasmid DNA coding for the WT or mutant PTHR1 and functional studies were performed 48 h later as described in the methods. Results are the mean of at least three experiments performed in duplicate with two plasmid preparations. SB: specific binding. WT: wild-type.

Figure 2. (A) Immunological assessment of cell-surface expression of wild-type and mutant PTHR1 expressed in CHO cells. Transfected cells were incubated sequentially with polyclonal rabbit anti-human PTHR1 antibody and [<sup>125</sup>I]-labeled anti-rabbit immunoglobulin antibody. Results are expressed as % [<sup>125</sup>I]-labeled anti-rabbit immunoglobulin antibody bound in cells transfected with the WT-PTHR1. p.G100D substitution disrupts the epitope recognition by the PTHR1 antibody (see results) (B). Basal cAMP production in cells transfected with WT or mutant PTHR1. Basal cAMP accumulation is expressed as picomoles cAMP / well normalized to cell-surface expression as determined in (2A). Expression of the mutant p.G100D PTHR1 was considered to be that of WT PTHR1, based on results from radioligand binding and confocal immunofluorescence experiments (see results). CHO cells were transfected with 1 µg plasmid DNA coding for the WT or mutant PTHR1 and experiments performed 48 h later. Results are the mean ± SEM of at least three experiments performed in duplicate with two plasmid preparations.

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3 Figure 3. Confocal immunofluorescent microscopy analysis of wild-type and mutant PTHR1  
4 tagged with GFP and using sequentially polyclonal rabbit anti-human PTHR1 antibody and  
5 rhodamine-labeled anti-rabbit immunoglobulin antibody, without cellular permeabilization.  
6 Colocalisation (originally yellow, and falsy colored in white for clarity) of green GFP tagged  
7 PTHR1 and the rhodamine labelled secondary antibody against the PTHR1 primary antibody  
8 (directed against the E2 extracellular epitope) indicates cell surface expression. No  
9 colocalization was observed in cells transfected with the p.G100D PTHR1, indicating that the  
10 p.G100D mutation prevents PTHR1 recognition by the PTHR1 antibody (see results). CHO  
11 cells were transfected with 1 µg plasmid DNA coding for the WT or mutant PTHR1, and  
12 confocal immunofluorescent microscopy analysis was performed as described in the material  
13 and methods.  
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29 Figure 4. Representation of the recently resolved crystal structure of the PTH–PTHr1 N-ter  
30 complex (PDB ID code 3C4M) (30), and position the PTHR1 mutations identified in this and  
31 previous studies. The figures shows ribbon representation of receptor N-ter. Grey: PTHR1  
32 N-ter main chain; grey, dashed line: exon E2 (residues 57-105); yellow: β sheets; black:  
33 disulfide bonds between residues Cys<sup>48</sup>-Cys<sup>117</sup>, Cys<sup>108</sup>-Cys<sup>148</sup>, and Cys<sup>131</sup>-Cys<sup>170</sup>); red: PTH.  
34 The mutated amino-acids are indicated in blue. All mutated amino-acids are located within  
35 the structured core.  
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Fig 1

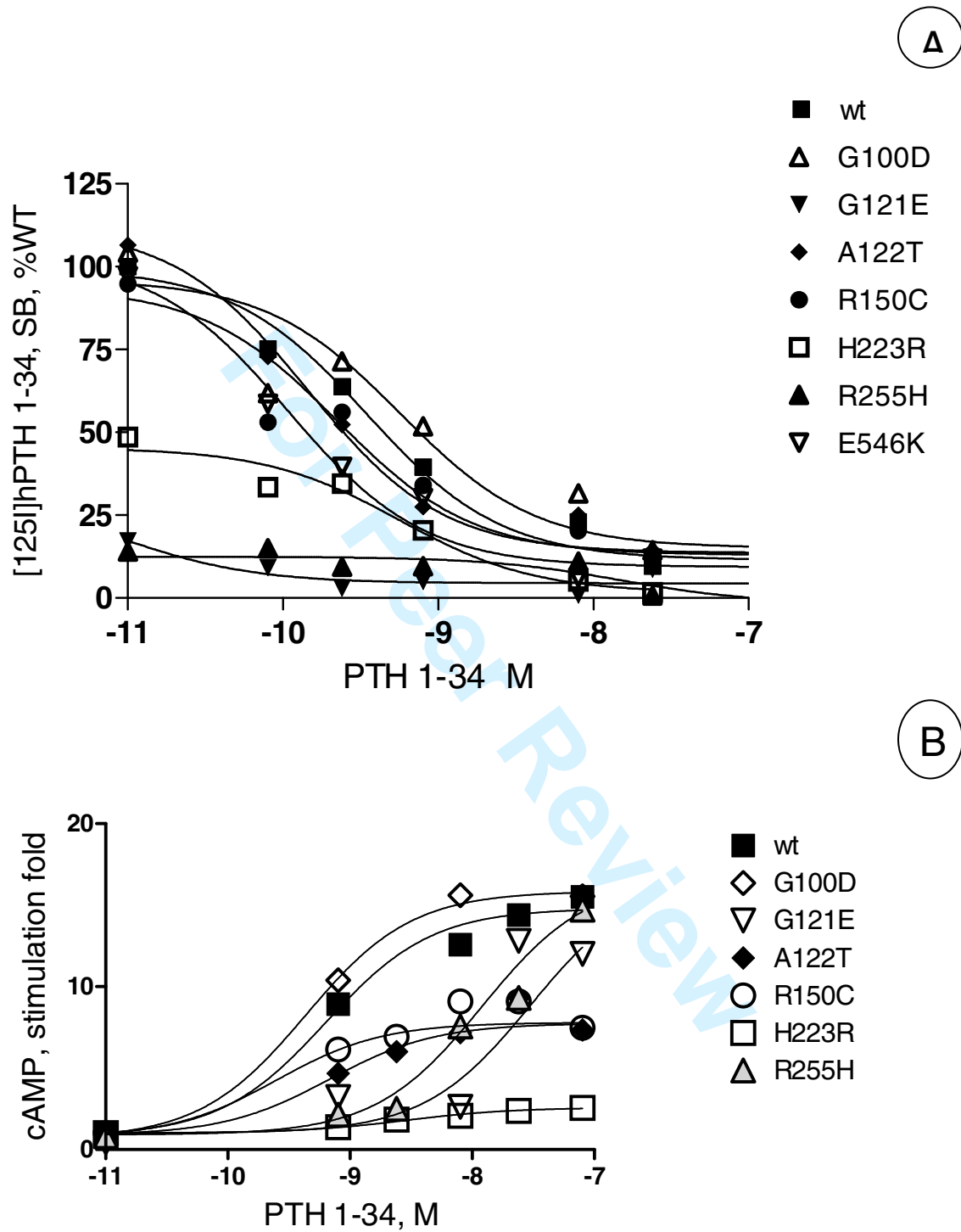
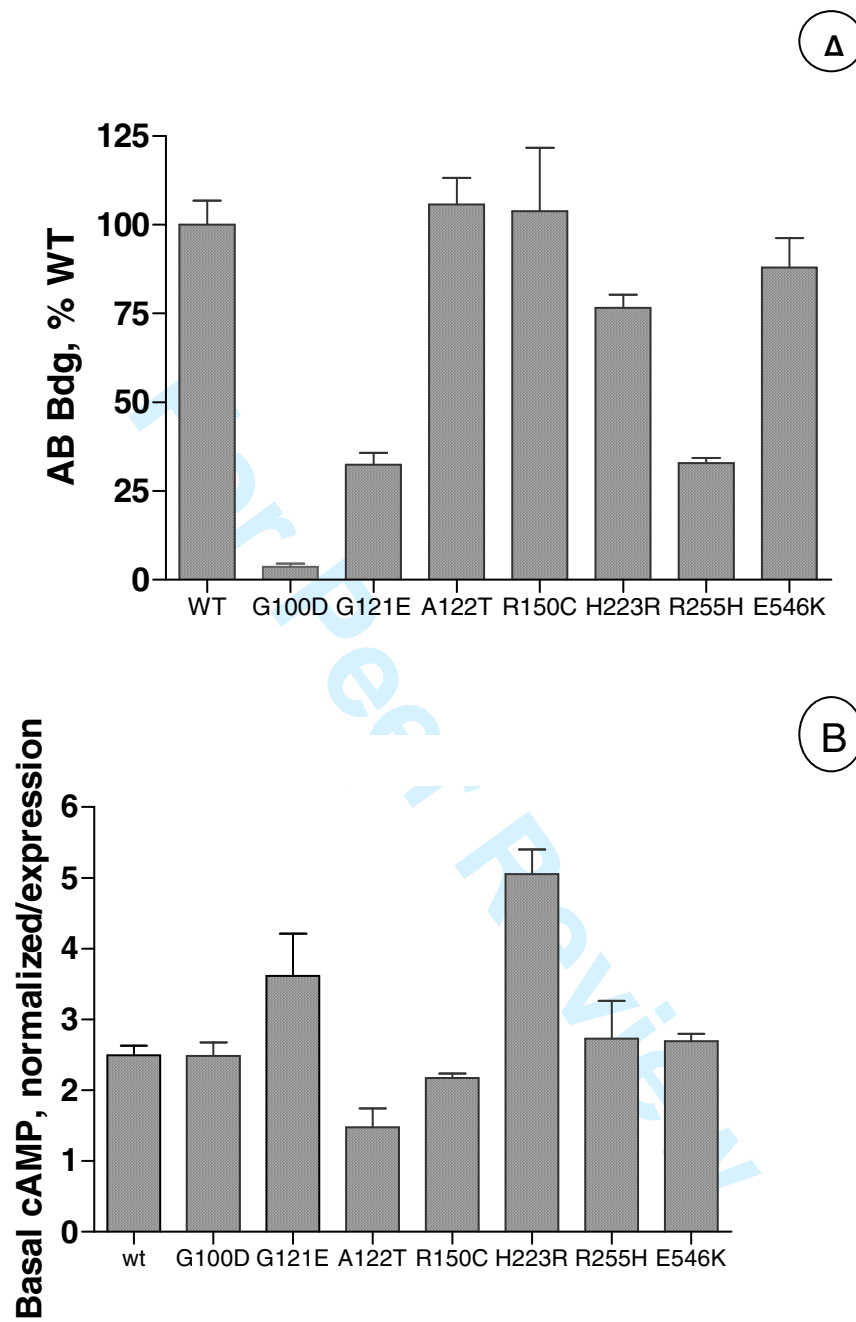


Fig 2





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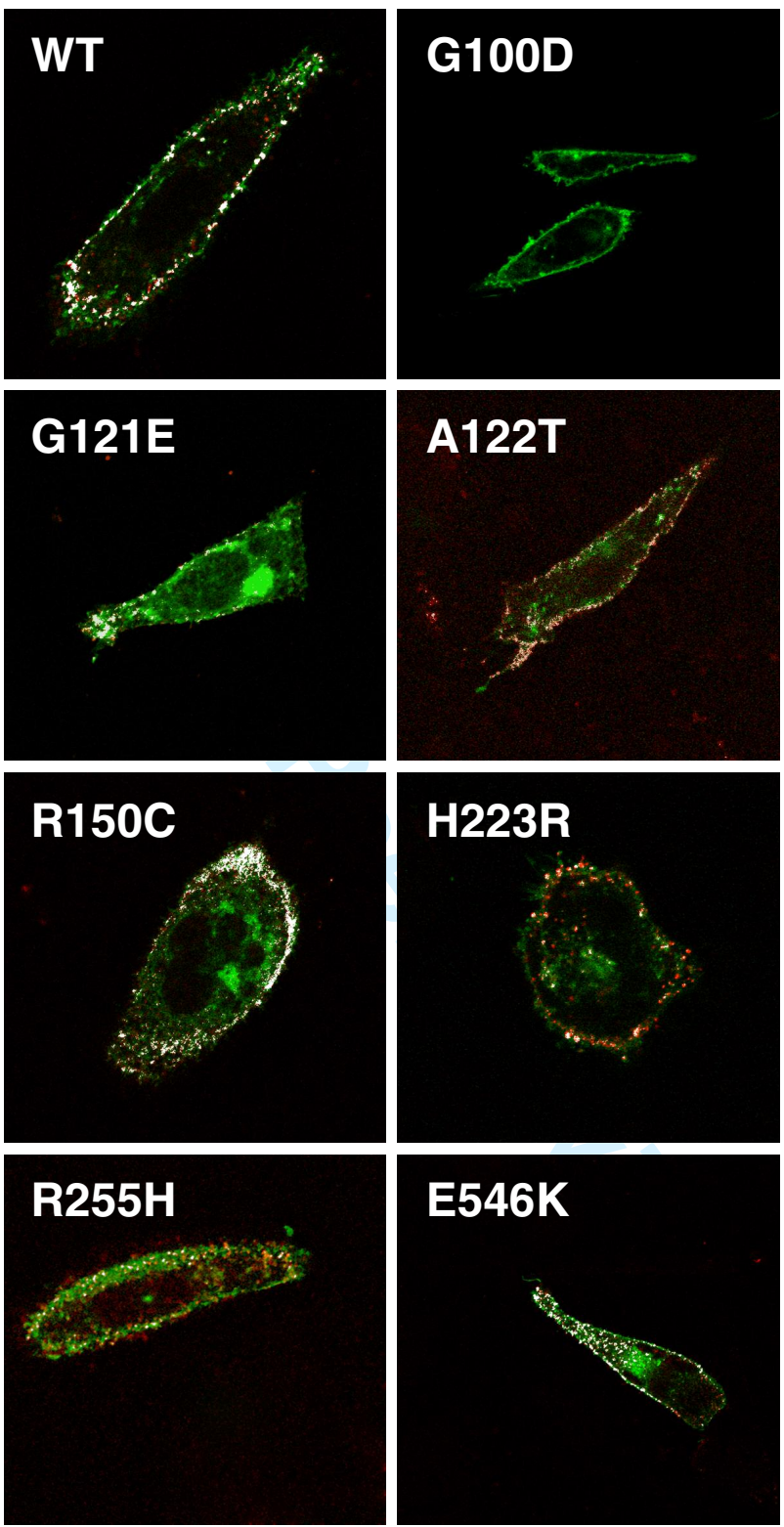


Figure 3



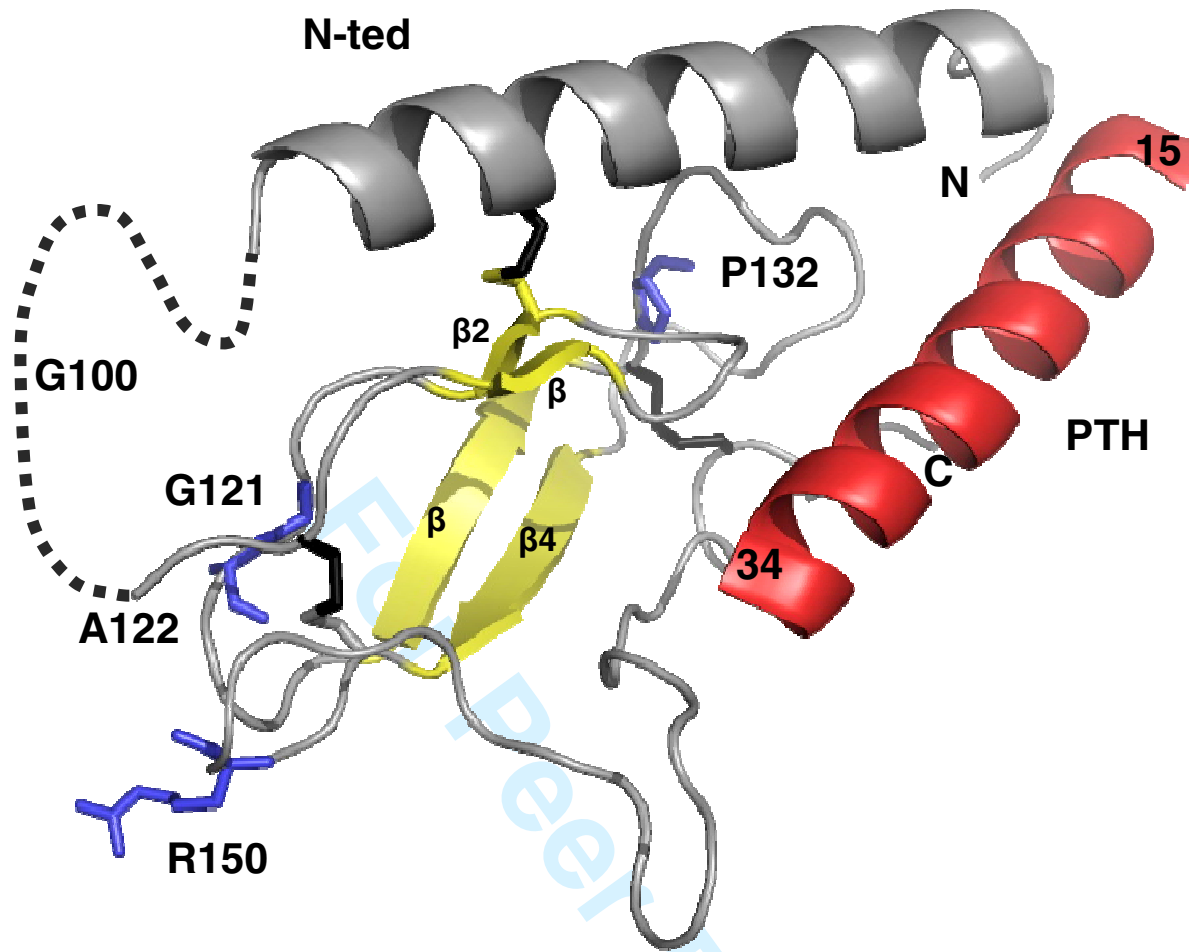


Figure 4