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**An unbalanced translocation unmasks a recessive mutation in the follicle stimulating hormone receptor (FSHR) gene and causes FSH resistance**

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**Running title:** FSH resistance associated with a t(2;8)

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**Abstract**

Follicle stimulating hormone (FSH) mediated by its receptor (FSHR) is pivotal for normal gametogenesis. Inactivating *FSHR* mutations are known to cause hypergonadotropic hypogonadism with disturbed follicular maturation in females. So far, only very few recessive point mutations have been described. We report on a 17-year-old female with primary amenorrhoea, hypergonadotropic hypogonadism and disturbed folliculogenesis. Chromosome analysis detected a seemingly balanced translocation 46,XX,t(2;8)(p16.3or21;p23.1)mat. *FSHR* sequence analysis revealed a novel non-synonymous point mutation in exon 10 (c.1760C>A, p.Pro587His), but no wildtype allele. The mutation was also found in the father, but not in the mother. Further molecular-cytogenetic analyses of the breakpoint region on chromosome 2 showed the translocation to be unbalanced, containing a deletion with one breakpoint within the *FSHR* gene. The deletion size was narrowed down by array analysis to approximately 163 kb, involving exons 9 and 10 of the *FSHR* gene. Functional studies of the mutation revealed the complete lack of signal transduction presumably caused by a changed conformational structure of transmembrane helix 6. To our knowledge, this is the first description of a compound heterozygosity of an inactivating *FSHR* point mutation unmasked by a partial deletion. This coincidence of two rare changes caused the clinical signs consistent with FSH resistance.

**Keywords:** FSH receptor deficiency, FSHR mutation, chromosome 2p16.3, translocation, hypergonadotropic hypogonadism

## Introduction

Follicle stimulating hormone (FSH) and its corresponding receptor (FSHR) are essential factors for regular gonadal development, sexual maturation at puberty and gamete production during the fertile period in both sexes. Normal FSH function regulates maturation of follicles and estrogen production in females and supports spermatogenesis in males.<sup>1,2</sup> The FSHR (OMIM \*136435), a G-protein-coupled receptor, is expressed by granulosa cells in the ovary and Sertoli cells in the testis. The FSHR encoding gene was mapped to the short arm of chromosome 2 in 2p16.3.<sup>3</sup> It consists of 10 exons and spans about 54 kb of genomic DNA.<sup>4</sup> Mutations can be activating or inactivating. In females, inactivating mutations result clinically - depending on the degree of inactivation - in primary amenorrhoea, secondary amenorrhoea or premature ovarian failure with the histo-pathological finding of impaired follicular maturation (ovarian dysgenesis 1, OMIM #233300). Total FSHR inactivation is characterized by infertility with an early block in maturation and high numbers of primordial follicles as in prepubertal stage and hypoplastic ovaries. The first *FSHR* mutation, a homozygous missense mutation (566C>T, Ala189Val) was described in 1995 by Aittomäki et al. in several Finish patients with primary amenorrhoea, hypergonadotropic hypogonadism and blocked follicle maturation.<sup>5,6</sup> Since then, only 10 more *FSHR* inactivating mutations have been published.<sup>7-13</sup> The only activating mutation leading to a ligand-independent constitutive activation of the FSH receptor was identified in a hypophysectomized patient who had normal fertility under testosterone substitution alone without detectable serum gonadotropin levels.<sup>14</sup>

In addition, FSHR mutations causing ovarian hyperstimulation syndrome (OHSS) have recently been identified.<sup>15-18</sup> These mutations result in a decreased ligand specificity

allowing not only FSH but also hCG/TSH to stimulate the receptor. In gain-of-function mutations, the phenotypic changes are caused by heterozygosity and dominant inheritance whereas loss-of-function mutations are mostly transmitted recessively and need homozygosity or compound-heterozygosity to cause the clinical phenotype.<sup>19</sup>

Here, we describe for the first time the compound heterozygous combination of a novel inactivating point mutation that was unmasked by a small partial deletion of the *FSHR* locus resulting clinically in a hypergonadotropic hypogonadism with primary amenorrhoea.

## **Material and Methods**

### Conventional chromosomal analysis

After informed consent, peripheral blood was taken from the probanda and her parents and relatives. Lymphocytes were cultured with phytohemagglutinin and cells were harvested according to cytogenetic standard procedures of hypotonic treatment and methanol/acetic acid fixation (3:1). Chromosome analysis was performed using GTG-banding at the 500-550 band level (as determined by the ISCN 2009 standards).

### Fluorescence *in situ* hybridisation (FISH)

FISH was performed with whole chromosome painting probes (wcp) for chromosomes #2 and #8 (MetaSystems, Altlußheim, Germany), centromeric probes for chromosomes 8 and X (CEP8, CEPX), and LSI SRY (obtained from Abbott Molecular Inc., Downers Grove, IL, USA) and two BAC clones, both localized in 2p16.3 (RP11-19A8 and RP11-436L21, obtained from BlueGnome, Cambridge, UK). Hybridization and post-

hybridization washes were carried out according to the manufacturers' protocols. Chromosomes were counterstained with DAPI (4,6-diamino-2-phenylindole). Slides were analyzed with an epifluorescent microscope Axioplan (Zeiss, Oberkochen, Germany) and images were captured using Isis® software (MetaSystems, Altlußheim, Germany). FISH signals were examined both on metaphase chromosomes and interphase nuclei.

#### DNA isolation and sequencing

DNA was extracted from venous blood using the FlexiGene DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Each of the 10 exons of the FSHR gene (RefSeq NM\_000145) was amplified from genomic DNA by PCR and sequenced as described earlier.<sup>20</sup>

#### Microarray analysis

SNP array analysis was carried out using the GeneChip®HumanMapping 250 K Sty Array according to the protocol of the manufacturer (Affymetrix; Santa Clara, USA). Imaging of the microarrays was performed using the GCS3000-G7 scanner from Affymetrix. Genotype calls and probe-intensity data were extracted with the GTTYPE4.1 software (Affymetrix). Copy number analysis was performed using the CNAG v2.0 software (Copy Number Analyzer for GeneChip;<sup>21</sup> <http://www.genome.umin.jp>). The design of the 250 K Array and the interpretation of data was based on the March 2006 human genome sequence assembly (UCSC hg18). Conspicuous regions were compared with known CNVs, as provided by the Database of Genomic Variants (<http://projects.tcag.ca/variation/>).

### Functional characterization of the mutation

The identified nucleotide point mutation changing the codon for proline (CCC) into histidine (CAC) was introduced into the wt-FSHR cloned into the pSG5 plasmid by in-vitro mutagenesis as described before<sup>4</sup> using the quick-change-sited directed mutagenesis kit (Agilent Technologies, USA). Functional studies were performed by transient transfection of COS-7 cells (50,000 cells/well) by lipofection (Lipofectamine, GE Healthcare) using 2 µg plasmid DNA/well. 24 hours after transfection cells were stimulated for 3 h with increasing doses of human recFSH (Gonal F, Merck-Serono). cAMP was determined by an in-house assay. cAMP values were determined in duplicates and three independent transfections were performed.

### Molecular homology modelling

We generated homology models of the seven transmembrane helices, intracellular helix eight, and the helix-connecting loops for both wild-type FSHR and an inactive FSHR variant (P587H in TMH6). The models were based on rhodopsin (pdb code 2I35). FSHR-specific modifications relative to rhodopsin were made in the homology models.<sup>22</sup> In rhodopsin, interactions between the side-chains of two consecutive threonines and the helix backbone of the preceding residues cause a helical bulge in TMH2. In the FSHR, these consecutive threonines do not exist in TMH2 which suggests the presence of a regular  $\alpha$ -helix. At TMH5, a minor change in orientation of the N-terminal half of TMH5 (a twist of 10 to 15 degrees) was generated due to the lack of a proline as compared to rhodopsin. Gaps of missing residues in the loops of the template structure were closed by the 'Loop Search' tool implemented in Sybyl 7.3.5

(Tripos Inc., St. Louis, Missouri, 63144, USA). The generated homology models were initially subjected to conjugate gradient minimization (until converging at a termination gradient of 0.05 kcal/(mol\*Å)) and secondly to molecular dynamics (MD) simulation. The MD simulation of 2 nanoseconds (ns) was performed in two steps: i. 1 ns with fixed backbone atoms and ii. 1 ns with released backbone atoms. Images were produced using PyMOL software (DeLano WL, version 099, San Carlos, CA, USA).

## RESULTS

### Clinical report

A 17-year-old female was referred because of a primary amenorrhea and incomplete pubertal development. Breast and pubic development corresponded to Tanner stage 3 (B3, PH3). In addition, she had lichen sclerosus et atrophicus of the vulva. Her body measurements have always been normal [at birth: 49 cm length (-1.2 SDS), 3200 g weight (-0.6 SDS); at present: 159 cm height (-1.56 SDS), 48 kg weight (-0.83 SDS), 55.6 cm head circumference (+0.6 SDS) (according to <sup>23-25</sup>)]. She had a normal childhood development and did not show any dysmorphic signs. An X-ray of the left hand at age 17 years showed a bone age retarded by 3-4 years. Endocrinological investigations revealed a hypergonadotropic hypogonadism with prepubertal estradiol levels (results of two consecutive hormone measurements: FSH 105.9 IU/l and 121.8 IU/l (normal range, follicular phase 2.5-10.2 IU/l), LH 47.8 U/I and 46.7 U/I (normal range, follicular phase 1.9-12.5 IU/l), Estradiol 13.3 pg/ml and 10.7 pg/ml (normal range, follicular phase 11-165 pg/ml). At diagnostic laparoscopy, ovaries of normal size and a rather small uterus were seen. The histological examination of ovarian tissue showed a disturbed folliculogenesis with a high number of primordial follicles, but



complete absence of secondary or tertiary follicles. The lack of oocyte maturation combined with the hypergonadotropic hypogonadism led us to hypothesize a defective FSH action as the cause of the primary amenorrhea.

### Cytogenetic and molecular data

#### *Chromosome analysis*

Chromosome analysis was performed as first step to identify the cause of the primary amenorrhoea. This showed a female karyotype with two structurally normal X-chromosomes but a translocation between one chromosome #2 and one chromosome #8 [46,XX,t(2;8)(p16.3or21;p23.1)]. Wcp probes for chromosomes #2 and #8 confirmed the translocation partners and excluded a more complex involvement of other chromosomes (see Fig. 1A). The translocation appeared to be cytogenetically balanced. This was seemingly confirmed by the finding of the same translocation in the unaffected mother (normal male karyotype in the father). The translocation was absent in the maternal brother and sister as well as in the patient's brother. Maternal grand parents were not available for chromosome analysis. Since this translocation initially did not explain the primary amenorrhea, FISH with CEPX and SRY was performed and a numerical gonosomal mosaicism or an involvement of SRY was excluded in 100 cells (analysis of 40 metaphase spreads and 60 interphase nuclei, data not shown).

#### *FSHR mutation analysis*

Because of the clinical diagnosis of FSH resistance we performed sequence analysis of the *FSHR* gene. This revealed a single non-synonymous seemingly homozygous point mutation in exon 10 (CCC to CAC, Pro587His) but no wildtype allele (see Fig. 2A).

Screening the family members of the probanda revealed that the Pro587His *FSHR* mutation was transmitted by the father (heterozygous carrier), while the mother was not affected. The patient's brother was also found to be heterozygous for this mutation.

#### *Breakpoint characterization on chromosome 2p16.3*

Since the *FSHR* gene was mapped to the same chromosomal region as the cytogenetically defined translocation breakpoint (2p16.3or21),<sup>3</sup> the next step was to find out, whether the breakpoint affected the *FSHR* gene. We hybridized two BAC probes, RP11-436L21 containing exon 1, and RP11-19A8 containing exons 5-10 of the *FSHR* gene (for exact localization of BAC probes in relation to the *FSHR* gene see Fig. 1E). This showed two normal signals for RP11-436L21 on both the normal chromosome #2 and the derivative chromosome der(2), whereas clone RP11-19A8 only hybridized to the normal chromosome #2. The second signal was absent on the der(2) as well as on the der(8). This demonstrated the translocation t(2;8) to be associated with a partial deletion of the *FSHR* gene (see Fig. 1B) with one deletion breakpoint localized between the two BAC clones within the gene.

Hybridization of the two BAC clones to chromosomes of the proband's mother (also translocation carrier) revealed the same result of a deleted RP11-19A8 signal on the der(2) (see Fig. 1C). Hybridization to chromosomes of the father (Fig. 1D), the brother and the maternal aunt and uncle (all carry normal karyotypes) confirmed the expected normal signal constellation for both BACs on chromosome #2.

Microarray analysis showed a heterozygous deletion of about 162.7 kb in size in 2p16.3 containing approximately 30 SNPs. The proximal breakpoint was located between the

flanking SNP rs6729545 (position chr.2: 48,887,046) and the first deleted SNP rs9636432 (chr.2: 48,895,903 bp), the distal breakpoint between the last deleted SNP rs2268359 (chr.2: 49,058,614 bp) and the flanking SNP rs13396575 (chr.2: 49,065,417; UCSC hg18). The deleted region included exons 9 and 10 of the *FSHR* gene (see Figs. 1E and 1F).

#### *Functional characterization of the mutation*

Functional studies on transiently transfected COS-7 cells using the wt-FSHR and the Pro587His mutated FSHR revealed a normal increase of cAMP production upon FSH stimulation (up to 8-fold increase) in the wildtype, which was completely absent in the mutated FSHR (Fig. 2B). The functional data therefore strongly indicate that this mutation abolishes normal signal transduction.

#### *Structural consequences of the Pro587His FSHR mutation*

Pro587 is located in the 6th transmembrane helix (TMH6) and causes a kink-distortion, which is important for activation and signal transduction in class A G-protein-coupled receptor (GPCRs) (Fig.3). Based on molecular dynamic simulations, substitution of Pro to His changes the structure of TMH6. As a consequence the signalling associated interfaces between TMH5 and TMH6, or ECL2 and TMH6 are altered in spatial distance and in intramolecular side chain interactions.

## **DISCUSSION**

We report here on a compound heterozygosity of a novel non-synonymous Pro587His FSHR point mutation, unmasked by a partial deletion of the FSHR gene. Because of the

absence of a wild type allele a homozygous mutation was suspected initially, but mutation analysis in the parents failed to detect it in the mother. Therefore a deletion of the second allele became most likely, in particular because the breakpoint of the familial translocation seemed to be in close proximity to the *FSHR* locus. The mechanism of unmasking a recessive mutation by a deletion of the second allele was first described in 1993 by Rinchik et al.<sup>26</sup> in a patient with Prader–Willi syndrome and oculocutaneous albinism. Since then, it has been reported in several conditions but not in combination with a *FSHR* mutation.

We could prove our assumption by confirming the deletion by FISH and array analysis. According to the array results the deleted BAC clone (RP11-19A8) spans the breakpoint and is deleted for the most part. It can be assumed that the remaining portion is too small to generate a detectable fluorescence signal. Searching the Database of Genomic Variants (<http://projects.tcag.ca/variation/>) for known copy number variations in this region, one copy number loss was found (Variation\_8945, Pinto et al., 2007; one loss in 776 controls) that only in part overlapped with the deleted region in our patient.

It remains unknown whether the deletion in this family predisposed to the formation of the translocation or whether the deletion is a result of the translocation. This cannot be elucidated further since the grand parents are not available to investigate.

The deletion contains exons 9 and 10 of the *FSHR* gene. Exon 10 is its largest exon, constituting the entire transmembrane helix of the receptor, the intracellular domain and the C-terminal part of the extracellular domain. Exons 1-9 encode the extracellular domain.<sup>4</sup> Therefore, a deletion of these two exons should lead to a complete loss-of-function of this allele, if the mRNA is translated at all.

Up to now, only point mutations (either homozygous or compound heterozygous) have been published in patients with variable clinical phenotype and partial or complete FSH resistance.<sup>5-10,12,13</sup> Mutations were mainly identified in individuals from Finland<sup>5,6,8,10</sup> and from France.<sup>7,9,12</sup> To our knowledge, only ten different inactivating *FSHR* mutations have been published since 1995 up to now making this a very rare condition.

Beside mutations with complete FSH receptor inactivation and an early blocked follicular maturation as in prepubertal ovaries,<sup>5,6</sup> partially inactivating mutations were reported.<sup>7,9,19</sup> In these patients, ovaries were of normal size containing follicles developed up to the early antral stages. This observation suggested a limited FSH effect to be sufficient to promote follicular growth up to the small antral stage. This is in line with Meduri et al.,<sup>19</sup> who hypothesized the absolute requirement of FSH for follicular development starting from the primary stage in humans in contrast to the initial gonadotrophin-independent follicular growth prior to the preantral–early antral stages observed in rodents.

Apart from complete and partially inactivating mutations, a modifying effect of SNPs on *FSHR* function is discussed in the literature.<sup>27,28</sup> Nakamura et al.<sup>13</sup> reported on a patient with primary amenorrhea but normal sized ovaries containing some small antral follicles. These follicles reacted to high doses of hMG injection. Molecular analysis revealed only one heterozygote mutation in exon 8 (Val221Gly) and two SNPs in exon 10 (Ala307Thr and Ser680Asn). The authors hypothesize that the different phenotype might be caused by this constellation.

In 2003, Allen et al. published a novel inactivating mutation in exon 10 in a female patient with hypergonadotrophic ovarian failure and primary amenorrhoea.<sup>11</sup> The

mutation was diagnosed as homo- or hemizygous and characterized as complete loss-of-function. Parental analysis could identify the same mutation in the father but not in the mother. A possible uniparental disomy or large deletion was excluded by microsatellite analysis. A translocation was ruled out by normal conventional cytogenetic analysis and whole chromosome painting for chromosome 2. As the chosen microsatellites adjacent to the *FSHR* locus were 2.1 Mb and 1.5 Mb apart, there was a gap of about 3.6 Mb around *FSHR* gene so that a deletion seems to be possible. The authors suspected either a small deletion on the maternal allele or a gene conversion as two possible explanations but did not prove either hypothesis.

To find out whether there are additional patients with a deletion that might perhaps carry a possible compound heterozygote point mutation we checked the database for chromosomal imbalances (Decipher) for deletions in this region. At that time (July 2009), the database included 3 deletion patients. Two patients carry larger deletions including the *FSHR* gene (10.5 Mb and 1.55 Mb in size, respectively) without any clinical data that would fit with FSH resistance. A third patient carries a maternally inherited 120 kb deletion within the *FSHR* gene. He shows a congenital malformation/mental retardation syndrome with 36 clinical features (among them brain abnormalities, a congenital heart defect, deafness, craniofacial dysmorphism, macrocephaly, and mental retardation). We suppose that there must be a different, up to now unrecognized reason underlying his phenotype.

The *FSHR* is a member of the class A G-protein-coupled receptor (GPCR) family (rhodopsin-like), where a proline in TMH6 at corresponding position of Pro578 is highly conserved. The proline contributes to the formation of a kink in the helix and

divides the helix into segments (Fig. 3). It is known that such helix-kink distortions are locked by intramolecular side chain interactions between the tightly packed helices.<sup>29,30</sup> The kink in TMH6 is particularly important for activation and signal transduction in class A GPCRs. This general process is achieved by a shift in the spatial arrangement of the transmembrane helices.<sup>31</sup> In the activation-related scenario, TMH6 undergoes the most important movement where the kink functions as a pivot and finally leads to G-protein coupling and activation.<sup>32,33</sup> Substitution of the proline is expected to lead to modifications in the signalling properties by causing changes in the helix conformation, intramolecular interactions and thereby the helix arrangement. This is supported by recent findings regarding corresponding prolines in other glycoprotein hormone receptors (GPHRs), the thyrotropin receptor (hTSHR, Pro639)<sup>34</sup>, and the lutropin/choriogonadotropin receptor (rLHCGR, Pro588).<sup>35</sup> Our performed molecular homology models of the FSHR suggest slight changes in the helix 6 distortion caused by a Pro-His substitution (Fig. 3). In consequence, this FSHR variant lost the intrinsic signalling capability determined also by the arrangement of the helical bundle in the basal conformation.

In summary, inactivating *FSHR* mutations are a very rare cause of primary or early secondary amenorrhoea combined with arrested follicular maturation and anovulatory infertility. Depending on the mutation there is a broad spectrum of clinical phenotypes from complete FSH resistance to incomplete milder forms. Up to now, only point mutations have been found. To our knowledge, our patient presented in this paper is the primary one with a compound heterozygosity of a point mutation and a deletion in the *FSHR* gene. This deletion of one allele unmasks the mutation of the second allele. The

coincidence of two very rare findings - the novel point mutation and the familial unbalanced translocation - lead to an exceptional constellation causing the clinical signs consistent with FSH resistance. It should be kept in mind to search for deletions in patients with clinical FSH resistance in whom molecular analysis detected only one heterozygous mutation.

## **WEB RESOURCES**

CNAG V 2.0: <http://www.genome.umin.jp/>

Decipher: <https://decipher.sanger.ac.uk/>

<http://www.ensembl.org>;

<http://www.genome.ucsc.edu>

<http://projects.tcag.ca/variation/>

## **CONFLICT OF INTEREST STATEMENT**

The authors declare no conflict of interest.

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## FIGURE LEGENDS

### **Figure 1**

**A.** Partial karyotype of the patient

Chromosome analysis after GTG-banding revealed a seemingly balanced translocation between the short arms of one chromosome 2 and one chromosome 8 (shown on the lower left, marked by red arrows), resulting karyotype: 46,XX,t(2;8)(p16.3or21;p23.1). The translocation partners were confirmed and a more complex involvement of further chromosomes excluded by FISH using whole chromosome painting probes wcp2 (labelled in green) and wcp8 (labelled in red).

**B-D.** Breakpoint characterization on chromosome 2p16.3 by Fluorescence-*in situ*-hybridization

The cytogenetically defined translocation breakpoint in 2p16.3or21 was first characterized by two BAC probes: RP11-436L21 (labelled in red) containing exon 1, and RP11-19A8 (labelled in green) containing exons 5-10 of the *FSHR* (for localization of BAC clones related to the *FSHR* gene see **E**). A deletion of clone RP11-19A8 on the der(2) (marked by red arrowhead) was detected in the patient (**B**) and also in her mother who is also a translocation carrier (**C**) but not in the father (**D**). This demonstrated the translocation t(2;8) to be unbalanced and associated with a partial deletion of the *FSHR* gene.

**F.** Array analysis using GeneChip®HumanMapping 250 K Sty Array (Affymetrix)

The copy number plot (log<sub>2</sub> ratio) of the entire chromosome 2 shows a heterozygous deletion (marked with a black arrow) of about 162.7 kb in size in 2p16.3, including exons 9 and 10 of the *FSHR* gene.

**Figure 2**

**A.** Novel *FSHR* mutation Pro587His in Exon 10

Results of direct sequencing show the mutant allele (CAC) in the patient with absence of a wild type allele, the homozygous wild type allele in the patient's mother (CCC) and the heterozygous mutation (CNC) in the patient's father.

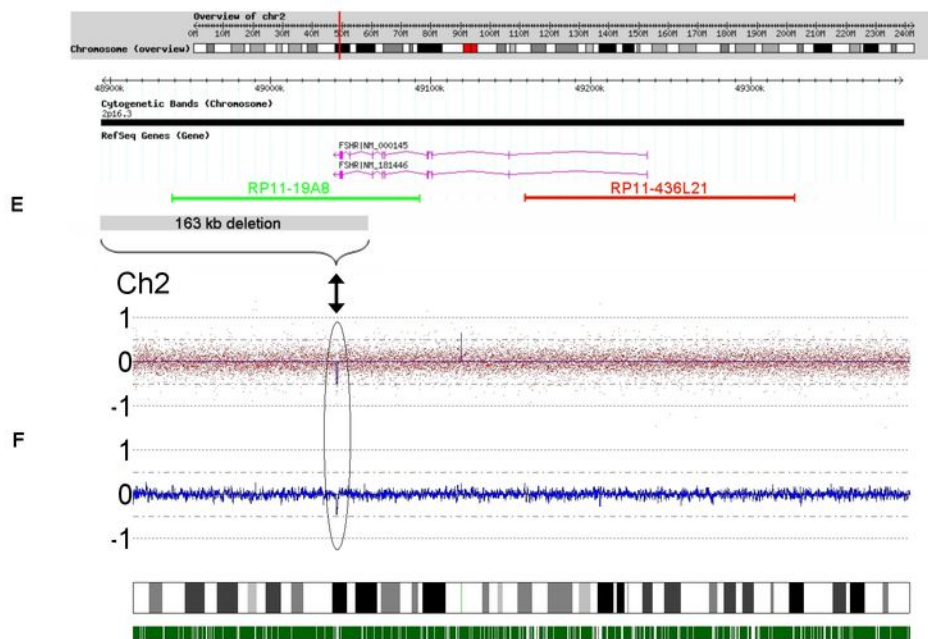
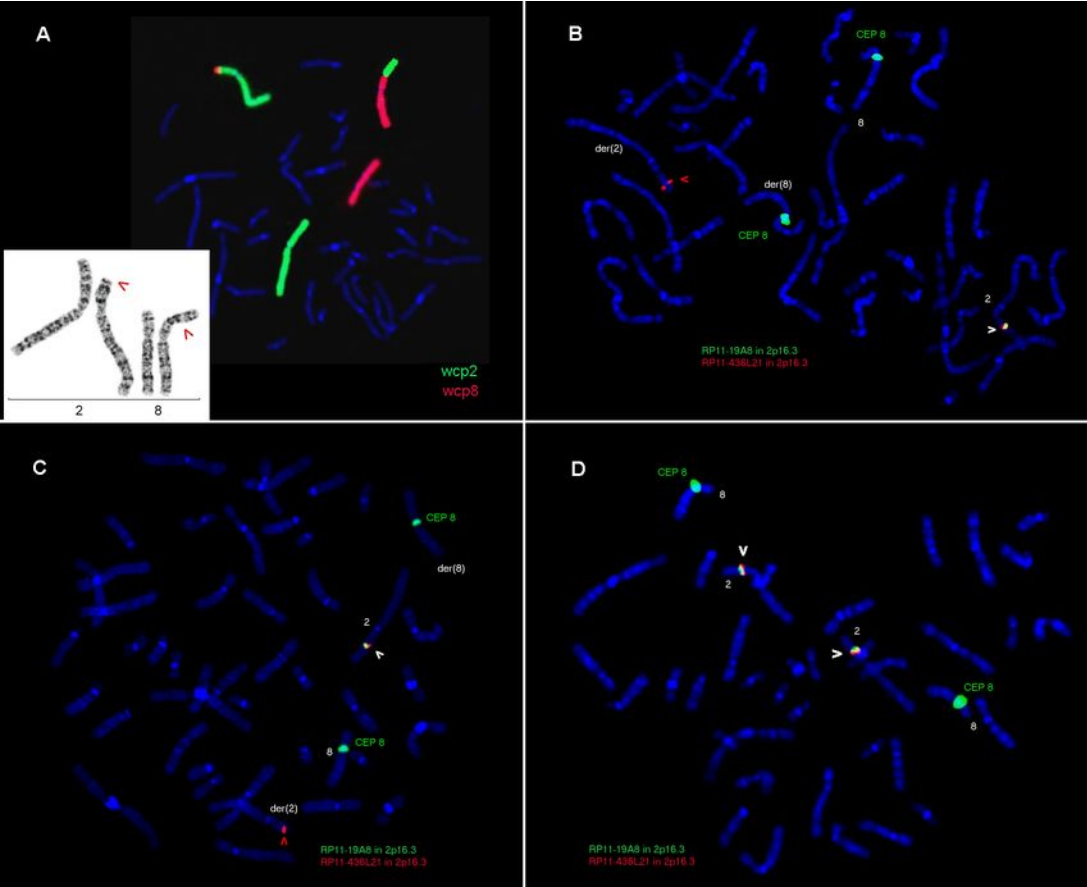
#### **B. Functional characterization of the Pro587His *FSHR* mutation**

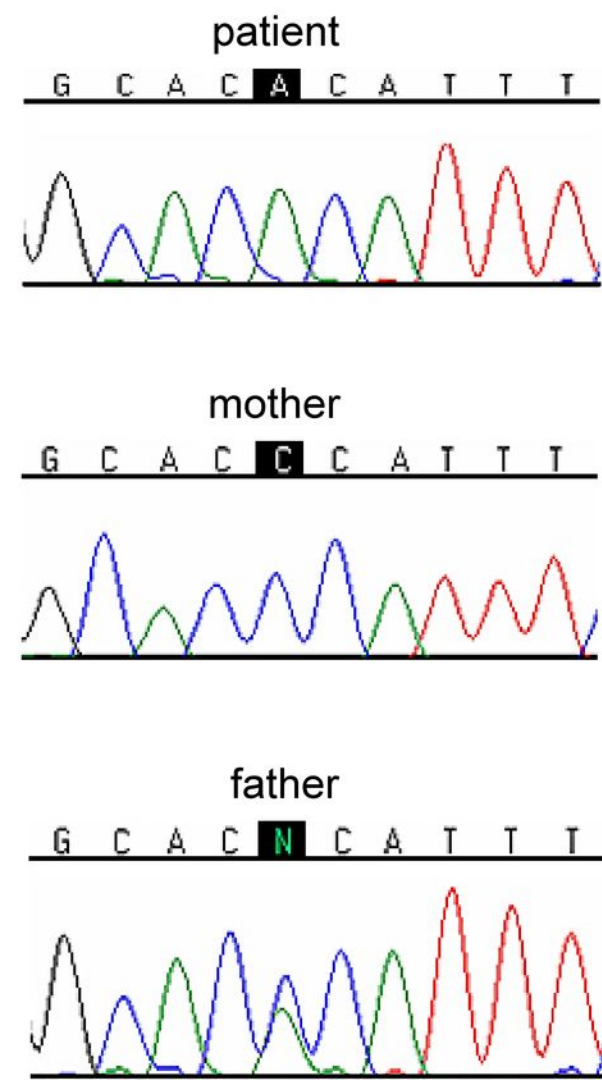
COS-7 cells were transiently transfected with either the wildtype (hFSHwt)- or the Pro587His (hFSHRmutPro587His)- FSH receptor. After transfection, cells were stimulated with increasing doses of human rec hFSH and cAMP production was determined. cAMP values were measured in duplicates and three independent transfections were performed.

#### **Figure 3**

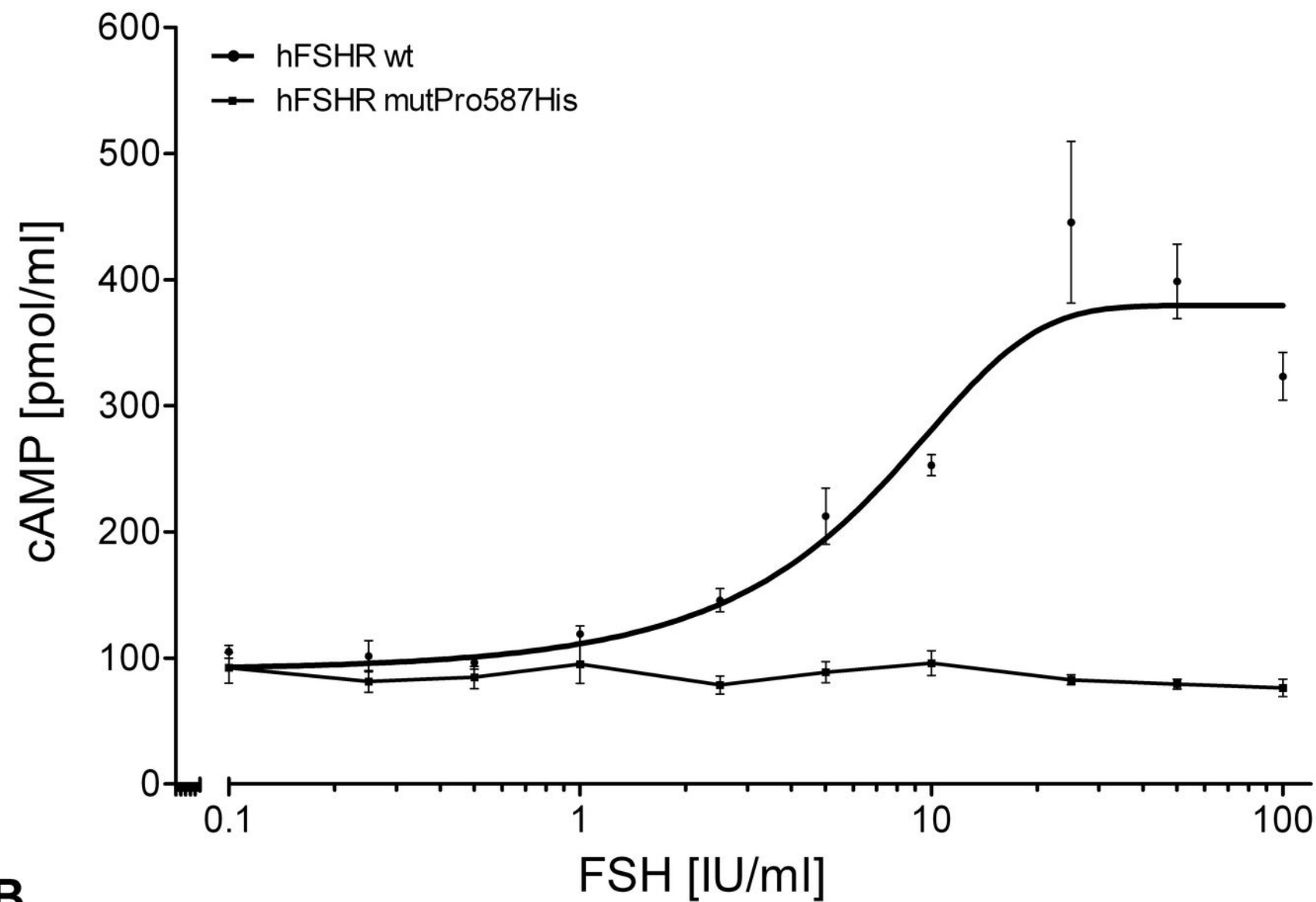
Molecular homology models of the wild type and the mutated *hFSHR*

The backbones of wild type (coloured rainbow) and the Pro-His mutated hFSHR (white) are overlaid. The wild type amino acid, Proline, is shown at position 587 as well as the mutated histidine side chain. The kink-distortion in helix 6 (orange) is caused by the proline and intramolecular side chain interaction between the specifically arranged helical conformations (e.g. TMH6 to TMH5 and TMH7). The kink is an important pivot during receptor activation. Substitution of Pro to His is likely to cause structural modifications in TMH6 and consequently also between structural components, e.g. the important interfaces between TMH5 and TMH6 or between TMH6 and ECL2. The evoked shift therefore changes stabilizing intramolecular interactions between the helices and leads to a loss of intrinsic signalling capability due to an altered basal conformation.





**A**



**B**

