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Compound heterozygous mutations in the luteinizing hormone receptor signal peptide causing 46,XY disorder of sex development

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Running Title: Novel LHCGR mutation causing 46,XY DSD

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Abstract

Testosterone production by the fetal testis depends on a functional relationship between hCG and the LH/chorionic gonadotrophin receptor (LHCGR). Failure of the receptor to correctly respond to its ligand leads to impaired sexual differentiation in males.

A phenotypically-female patient with pubertal delay, had a 46,XY karyotype and was diagnosed with 46X,Y disorder of sex development (DSD). Novel compound heterozygous LHCGR mutations were found in the signal peptide: a duplication p.L10_Q17dup of maternal origin, and a deletion (p.K12_L15del) and a p.L16Q missense mutation of paternal origin.

cAMP production was very low for both the deletion and duplication mutations and was halved for the missense mutant. The duplication and missense mutations were both expressed intracellularly, but at very low levels at the cell membrane; they were most likely retained in the endoplasmic reticulum. The deletion mutant had a very limited intracellular expression, indicating impaired biosynthesis. There was reduced expression of all three mutants, which was most marked for the deletion mutation. There was also decreased protein expression of all three mutant receptors. In the deletion mutation, the presence of a lower molecular weight band corresponding to LHCGR monomer, probably due to lack of glycosylation, and a lack of bands corresponding to dimers/oligomers suggests absent ER entry.

This novel case of 46X,Y DSD illustrates how three different LHCGR signal peptide mutations led to complete receptor inactivation by separate mechanisms. The study underlines the importance of specific regions of signal peptides and expands the spectrum of LHCGR mutations.

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Introduction

The luteinizing hormone/chorionic gonadotropin receptor (LHCGR) belongs to the family of gonadotropin hormone receptors (GPHRs) that also includes the follicle stimulating hormone receptor (FSHR) (1, 2). The stimulation of LHCGR in Leydig cells by its ligands, luteinizing hormone (LH) and its placental analog human chorionic gonadotropin (hCG), leads to activation of adenylyl cyclase via Gsα and generation of 3',5'-cyclic adenosine monophosphate (cAMP) with subsequent testosterone production in males (2, 3). This hCG-mediated testosterone production from fetal Leydig cells is essential for masculinization (4). Postnatally, LH is the physiological ligand for LHCGR. Functional alterations in this system due to mutations in both *LHB* and *LHCGR* genes have been reported. All reported clinical mutations in the *LHB* gene have been inactivating in nature (5, 6, 7, 8, 9, 10, 11). Clinical mutations in the *LHCGR* gene can, however, be inactivating or activating (12, 13, 14).

The earliest reports of constitutively active mutations in *LHCGR* were characterized by LH-independent activation of LHCGR leading to familial male-limited precocious puberty (also called testotoxicosis) (15, 16). On the other hand, the consequence of inactivating *LHCGR* mutations, depending upon the degree of inactivation, can range from a mild phenotype that is characterized by hypospadias, micropenis (Leydig cell hypoplasia type 2) to a female phenotype despite a male genotype (Leydig cell hypoplasia type 1) when the inactivation is complete (17).

We report three novel mutations in the putative signal peptide of the *LHCGR* found in a compound heterozygous state in a patient with a 46,XY disorder of sex development (DSD) and describe the clinical and molecular effects of this receptor dysfunction.

Patient and Methods

Case Report

A 16-year-old female Caucasian patient was referred to a pediatric endocrinologist for investigation of pubertal delay. She was otherwise well and was the second of four siblings (two brothers: one older, one younger and a younger sister). The family history was also unremarkable. The pubertal development of her older brother had been normal and the younger siblings were pre-pubertal. On examination, the patient was 178.8 cm in height (+2.3 SD; maternal height: 172 cm, paternal height: 183 cm) and she weighed 80kg. She had absent pubertal development with only sparse pubic hair (Tanner stage B1P1-2) and female external genitalia. There were no clinical signs of hyperandrogenism.

The hormonal workup found an elevated LH (32.6 UI/l; normal range: 2.4-12.6 U/l), a normal FSH (6.6 U/l, normal range: 3.5-12.5 U/l), undetectable estradiol (<5 pg/ml), normal progesterone (0.8 ng/ml, normal values for a female patient in the follicular phase: <1ng/ml), normal testosterone (0.17 ng/ml, normal female range: 0.10-0.70 ng/ml). Inhibin B was high 365.6 ng/l (normal range for a female patient in the follicular phase: <139pg/ml), while inhibin A was very low (<1 ng/l, normal range for a female patient in the follicular phase: 5.5-102 ng/l).

The karyotype was 46,XY. A pelvic ultrasound and MRI revealed absent ovaries and uterus. Testis-like structures were identified in the inguinal region bilaterally. A bilateral resection was performed and confirmed the presence of pre-pubertal testes with severe Leydig cell hypoplasia. Histologically, the seminiferous tubes were of various sizes, some entirely atrophic, others with a thickened basement membrane (Figure 1). A 1 mm gonadoblastoma was found in one testis. Germ cells could not be identified on anti-placental alkaline phosphatase immunohistochemistry.

Genetic studies

Following genetic counselling the patient and her family agreed to undergo genetic studies. DNA was extracted from peripheral blood leukocytes using the phenol-chloroform extraction. Sequencing of the *sex-determining region Y* (*SRY*) and the *androgen receptor (AR*) genes was normal. The *LHCGR* coding sequence was amplified by PCR followed by direct sequencing of PCR products. Exons 1 to 11 were amplified by one touchdown PCR. The PCR products were purified with ExoSAP-IT (USB Corporation) according to the manufacturer's instructions and then sequenced with the BigDye Terminator Ready Reaction Mix v.1.1 (Applied Biosystems). Informed consent has been obtained from the patient for publication of the case report and accompanying images.

Construction of plasmid vectors and mutagenesis

The wild-type (WT) LHCGR encoding plasmid (*N*-terminal HA tagged) has been previously described in (18). The construction of plasmid vectors coding for *N*-terminal HA-tagged LHCGR_L10_Q17dup, LHCGR_K12_L15del, and L16Q was achieved with REPLACR-mutagenesis as described in (19). mCherry

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sequence from pmR-mCherry Vector (Clontech #632542) were extracted by PCR using the following primers (forward primer: GCAATGGTGAGCAAGGGCGAGGAG and reverse primer: GGCGTTTCCGGACTTGTACAGCTC). The extracted mCherry DNA was subcloned into pcDNA 3.1/V5-His-TOPO vector using TOPO directional cloning kit (Clontech), using the manufacturers' instructions. The final construct was sequence verified and was named as mCherry-TOPO. This mCherry-TOPO construct was used as negative, mock transfection control in some of the experiments, as mentioned below.

Flow cytometry

HeLa cells were seeded in 6 well-plates at 0.3 X 10^6 cells per well in Dulbecco's Modified Eagle medium (DMEM)/F12 (Gibco) containing 10% fetal calf serum (FCS), 50 µg/mL streptomycin and 50 IU/mL penicillin. The cells were transfected with plasmids encoding either WT-LHCGR, LHCGR_L10_Q17dup, LHCGR_K12_L15del or p.L16Q mutants using JetPEI transfection reagent (Polyplus transfection). Post-transfection (48 h), cells were dissociated with Accutase (ThermoFisher Scientific) and fixed with 4 % paraformaldehyde. Cells were incubated first with Mouse Anti-HA antibody (Sigma-Aldrich #H9658) for 45 min and subsequently with Donkey Anti-Mouse Alexa-488 labelled secondary antibody (ThermoFisher Scientific # R37114) for 45 min. BS LSRFortessa (BD Biosciences) was used to read the fluorescence intensity at the cell surface. Mean fluorescence intensity (MFI) was plotted to compare relative expression levels at the cell surface of WT LHCGR and mutants.

Cell staining and Confocal Microscopy

HeLa cells were seeded and cultured in Lab-Tek II chamber slides (ThermoFisher Scientific) and were transiently transfected with plasmids encoding for LHCGR_K12_L15del, LHCGR_L10_Q17dup, L16Q or WT LHCGR. Cells were either fixed with 4 % PFA for cell surface staining or were fixed with methanol and 0.1 % Triton X-100 for cellular permeabilization. Cells were stained with Mouse Anti-HA primary antibody (Sigma-Aldrich #H9658) and Donkey Anti-Mouse Alexa 488 labelled secondary antibody (ThermoFisher Scientific # R37114). Slides were mounted with coverslips using Vectashield Antifade Mounting medium with DAPI (Vector laboratories #H-1200) for nuclear staining. The cells were imaged with Zeiss LSM 780. DAPI and Alexa 488 labelled antibodies were excited with 405 nm and 488 nm laser lines and emissions were collected at 498 nm and 562 nm, respectively. Imaris Software (Bitplane) was used for generating 3D Volume renders from the Z-stacks. For PFA stained (non-permeabilized) WT LHCGR and LHCGR_L10_Q17dup samples, contrast was uniformly increased for Alexa 488 channel to visualize faint cell surface expression of LHCGR_L10_Q17dup as compared to normal expression of WT LHCGR (Figure 2 B, C).

cAMP analysis

HEK293 cells stably expressing a luminescent cAMP sensor (Glosensor-22F) has been previously described and are referred to as HEK293-Glosensor (GS-293) (20). GS-293 cells were transiently transfected with LHCGR encoding plasmids (mutants or WT). To investigate the effect of one mutation on another, co-transfection with duplication and deletion mutants in a 1:1 ratio was performed. In order to control this experiment, GS-293 cells were co-transfected with plasmids encoding the WT receptor and empty vector in a 1:1 ratio. Cells were finally transfected with plasmids encoding each mutant and the WT receptor in a 1:1 ratio. After 48h, cell culture medium was replaced with assay medium. The assay medium consisted of equal amounts of DMEM-F12 and CO2-independent medium (Gibco) and supplemented with 2% GloSensor reagent (Promega) and 0.1% bovine serum albumin. Cells were equilibrated in assay medium for 1h at room temperature and were subsequently transferred to an EnSight plate reader (PerkinElmer) and were kept at a constant temperature of 25 °C. Baseline luminescence measurements were made for around 15 min and subsequently cells were stimulated with recombinant luteinizing hormone (rLH; 100ng/ml). cAMP production was then followed as a luminescent readout.

mRNA and protein expression

The expression levels of mRNA were measured using quantitative PCR (qPCR), while the expression of receptors at the protein level was measured by Western blotting. For both experiments, HEK293T cells were seeded in 12-well plates and transiently transfected with LHCGR encoding plasmids (WT or mutants) at 70% confluence. Further details on experimental conditions for qPCR and Western blots are available from the authors on request.

Statistics

Graphpad Prism 6 software was used to calculate P values, using one-way ANOVA to determine differences in multiple samples.

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Results

Genetic studies

Sequencing of the patient's DNA revealed a compound heterozygous mutation of the *LHCGR* gene that affected the signal peptide. A p.L10_Q17 dup mutation was found on one allele, with a p.K12_L15del mutation being present on the other allele. Family studies showed the L10_Q17dup mutation was of maternal origin, while the p.K12_L15del change was of paternal origin, with an additional c.47T>A mutation that leads to a missense change (p.L16Q). The older brother and the sister did not inherit either of the mutated alleles, whereas the younger brother inherited the L10_Q17dup mutation.

Cell surface expression

Since all mutations occurred in the signal peptide, we initially studied membrane expression of the mutant receptors. As compared with WT_LHCGR membrane expression, very low expression of the L10_Q17dup_LHCGR and L16Q_LHCGR was found. Both L10_Q17dup_LHCGR and L16Q_LHCGR surface expression levels were statistically lower than WT but higher than mock transfected cells. In the case of K12_L15del_LHCGR, negligible expression was observed and the median fluorescence intensity was equivalent to mock transfected cells (Figure 2A).

Cellular localization

We then determined the cellular localization of the WT LHCGR and mutants using confocal microscopy. HeLa cells transiently expressing the WT LHCGR, p.K12_L15del, p.L16Q and p.L10_Q17dup mutants were either permeabilized to determine intracellular localization or were non-permeabilized to visualize cell surface expression. WT LHCGR expression was mainly localized at the plasma membrane (Figure 3). L10_Q17dup_LHCGR and L16Q_LHCGR showed very weak plasma membrane expression as compared to WT LHCGR (Figure 2B), instead they were mainly found intracellularly, demonstrating a potential alteration of the normal cell surface trafficking via the ER (Figure 2C). The K12_L15del_LHCGR mutant, however, showed no cell surface expression and very weak intracellular expression (Figure 2 B, C).

cAMP analysis

Subsequently, we tested the ability of WT LHCGR, K12_L15del, L16Q and L10_Q17dup mutants to mediate cAMP production upon stimulation with rLH. WT LHCGR stimulated cAMP generation normally, as expected. cAMP production by L10_Q17dupLHCGR (AUC=14000) was 6.7-fold less than WT LHCGR (AUC=97000), while L16Q LHCGR (AUC=32000) generated 3.1-fold less cAMP than WT. K12_L15del LHCGR was unable to stimulate any cAMP production as the luminescence levels were similar to un-transfected or mock transfected negative control. Since the LHCGR_K12_L15del completely prevented the expression and cell membrane localization of the receptor, there was no reason to create a deletion mutant also carrying the p.L16Q missense mutation as found in the patient. However, to properly characterize the

potential effects of each mutant on the other, we co-transfected both L10_Q17dup and K12_L15del mutants and found that this resulted in cAMP generation that was only half of that generated by the L10_Q17dup_LHCGR mutant alone (Figure 3 A, B), which indicates an additive impairment of LHCGR function when the two mutants were co-expressed.

Co-expression of the mutant and the WT LHCGR

The duplication mutant did not have any effect on the WT, as the response was approximately the sum of WT plus mutant responses combined. In the case of deletion and the WT co-expression, 1.6-fold less cAMP production was observed, as compared to WT and empty vector co-transfection. In contrast, co-transfection of the missense mutation and the WT receptor resulted in negligible decrease in cAMP production versus WT LHCGR.

Quantitative Real-Time PCR

We then examined the expression of mutant receptors at the mRNA level and calculated relative gene expression by comparing the absolute expression of mutants and WT LHCGR. The expression of L10_Q17dup_LHCGR was 1.3-fold lower as compared to the WT receptor. On the contrary, the expression of K12_L15del_LHCGR and L16Q_LHCGR were 3.9- and 2.3-fold less, respectively (Figure 3C).

Western Blotting

Finally, Western blot analysis revealed reduced expression of all tested mutant receptors at the protein level. Interestingly, in the case of K12_L15del_LHCGR, the difference in the protein pattern was seen noticeable at the level corresponding to the monomeric LHCGR receptor. A band was located slightly lower down when compared to the WT LHCGR and to the other mutant receptors. Additionally, in the case of this particular K12_L15del_LHCGR mutant, no bands specific to dimers and oligomers were visible (Figure 4A, B). This suggests that K12_L15del_LHCGR exists only as a monomer. Taken together, these results suggest that the K12_L15del_LHCGR does not undergo glycosylation (21, 22).

In the case of L10_Q17dup_LHCGR, increased protein expression of bands corresponding to dimers and oligomers was observed in the cells treated with a proteasome inhibitor MG132 (Figure 4B) indicating that the receptor is degraded in the cytoplasm and this, in turn, results in reduced expression of dimers and oligomers on the cell membrane.

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Discussion

The clinical phenotype of patients with mutations of LHCGR depends on the degree of inactivation of the receptor, but it can be as severe as 46,XY DSD with female external genitalia in cases of complete LHCGR inactivation, as in the current case (17). These patients do not present signs of estrogenization or virilization as neither estrogens nor androgens are secreted correctly by the gonads; late diagnosis of this condition exposes patients to an increased risk of severe osteoporosis (23). Moreover, the case presented here illustrates the risk for gonadal malignancy as a gonadoblastoma was found in one of the resected gonads. The development of the gonadoblastoma is probably related to the presence of a dysgenetic gonad and the abnormal maturation of germ cells as well as to the undescended position of the gonads (24, 25).

The genetic mutations seen in the current case underline the importance of signal peptides in protein function. Signal peptides are involved in the intracellular trafficking of secretory and membrane proteins, targeting them to the endoplasmic reticulum in eukaryotic cells (26). Signal peptides dictate the targeting pathway, timing and mode of insertion into the translocon, and influence the cellular distribution of the protein and the downstream processes of protein maturation. Moreover, some signal sequences can accomplish cellular functions even after cleavage from their corresponding proteins (27). Most G protein-coupled receptors (GPCRs) contain uncleaved signal sequences, called signal anchor sequences, that usually represent the first transmembrane domain of the receptor (28). In the case of the glycoprotein hormone receptors, however, the signal peptide is cleavable, which is important for correct protein synthesis. The ligand-binding *N*-terminal region of glycoprotein hormone receptors is ample in size and complex in structure. As it contains regions that could become misfolded in the cytoplasm, the nascent protein is inserted into the translocon of the ER membrane co-translationally. This likely occurs to allow for the long *N*-terminal end of the receptor to be properly synthesized and folded in the ER lumen (28).

The patient described here had a compound heterozygous mutation of the *LHCGR* gene, one allele having a duplication (p.L10_Q17dup_LHCGR) and the other allele bearing a deletion (p.K12_L15del_LHCGR) plus a missense mutation (p.L16Q). All mutations were located in the signal peptide. When over-expressed, p.L10_Q17dup_LHCGR and p.L16Q were weakly expressed on the cell surface and most expression remained intracellular. The weak membrane expression of p.L10_Q17dup_LHCGR and p.L16Q is also responsible for the very low cAMP production upon stimulation with the ligand ,rLH. This is in stark contrast to WT LHCGR that showed high membrane expression and high cAMP production upon receptor activation with rLH. It is important to note that experimental receptor overexpression by transfection leads to receptor levels that are in excess of those that would be seen in the physiological setting. This indicates that the true *in vivo* expression of and signaling by the p.L10_Q17dup_LHCGR, or p.L16Q, would be virtually negligible. Further studies which included quantitative PCR and Western Blotting showed reduced expression of p.L10_Q17dup_LHCGR and p.L16Q at both mRNA and protein levels in comparison with the

WT LHCGR. Nonetheless, increased expression of p.L10_Q17dup_LHCGR was seen following proteasome inhibition in comparison with untreated cells. This suggests that the degradation of p.L10_Q17dup_LHCGR occurs within the ubiquitin-proteasome system.

The second mutant, K12_L15del_LHCGR, had no cell surface expression and very weak intracellular expression. In agreement with this, there was no detectable cAMP production following stimulation of K12_L15del_LHCGR with rLH. The mRNA and protein expression were also significantly decreased as compared to the WT LHCGR. K12_L15del _LHCGR probably does not enter the ER and is further degraded in the cytoplasm. Since this deletion renders the receptor completely inactive, no further detrimental effect would be added by the intermediately functional p.L16Q mutation.

It was shown by Karamyshev *et al*. that mutations in the signal sequence can lead to degradation of mRNA (29). This cellular quality control mechanism involves Argonaute 2 interacting with the nascent protein chain. It leads to specific mRNA degradation and thereby prevents the accumulation of an aberrant protein at an early stage in protein synthesis. That work showed that the more leucine residues that were deleted from the signal sequence's hydrophobic core, the less mature protein was produced (29). The p.K12_L15del_LHCGR mutation, found on the paternal allele in the current case, results in the deletion of three of the five leucine residues from the hydrophobic core of the signal peptide. This could plausibly induce the aforementioned quality control mechanism and lead to marked degradation of mRNA, which is supported by our *in vitro* results showing a very low mRNA level of this mutant receptor.

Several other mutations have already been described in the signal peptide of the *LHCGR*. Among these is a 33-bp duplication between nucleotides 54 and 55, corresponding to 11 amino acids inserted between residues 18 and 19 (30, 31). Wu *et al.* have shown that cells expressing that mutant receptor did not bind ligand on the cell surface or in cell lysates, which indicates either a binding or a trafficking defect occurred (30). The first group of duplicated amino acids are the same as those found duplicated on the maternal allele in the patient we describe in the current report. Our results show that the mutant is present in very small quantities at the cell membrane and can respond to hormonal stimulation to generate cAMP. The functional defect of the receptor is most likely due to incorrect protein trafficking as a result of protein misfolding or aggregation, leading to scant or no cell membrane expression. The last two amino acids (LQ) of the previously mentioned duplicated sequence correspond to a common polymorphism of the *LHCGR* (30, 31, 32, 33). Other recent work has shown that this polymorphic insertion (CTGCAG) seems to slightly modify the function of the signal peptide by more efficient translocation through the ER than the WT signal peptide, followed by an increased glycosylation of the receptor and better expression on the cellular membrane (34). At a clinical level, the presence of this LQ polymorphism seems to represent an independent prognostic factor for a shorter disease-free survival in breast cancer and it appears to be more prevalent in infertile patients with endometriosis (35). A small variation from this polymorphism (CTGCCG), leading to the

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insertion of a leucine and proline in the signal peptide was reported by Bentov *et al* in a female patient with secondary amenorrhea and infertility (36). The patient exhibited resistance to the hCG treatment during the IVF protocols with a poor oocyte recovery despite the good follicular development. Whether that *LHCGR* variant was the cause of the phenotype in the report of Bentov *et al* is still unclear, as in that case the patient was heterozygous for the mutation and no functional studies were performed.

A different compound heterozygous *LHCGR* mutation was reported in another infertile female patient (37). One of those mutations was found in the signal peptide and consisted in a 27bp deletion, corresponding to amino acids 12-20. That deletion, therefore, eliminated the leucine-rich area of the hydrophobic core and part of the *C*-terminal end of the signal peptide, most likely rendering the mutant incapable of translocation into the ER. Another mutation in the signal peptide of the *LHCGR* was recently described by Vezzoli *et al*. in a composite heterozygote Leydig cell hypoplasia type 2 patient (38). The mutation in the signal peptide was p.L10P, replacing one of the leucines of the hydrophobic core with a proline, that is known to induce kinks in the secondary protein structure (39). The authors showed that the mutant protein function was severely impaired with a defect in ER targeting or insertion of the nascent chain containing the mutant signal peptide. That mutation modified one leucine residue at position 10, leading to effects at the mRNA level and normal transcription and translation (38). We found that both mRNA and protein levels were significantly lower in our experiments with the p.L16Q mutant. This suggests that the leucine at position 16 plays an important role in governing LHCGR transcription and translation.

In summary, we report a new case of 46,XY DSD due to a complete inactivation of the LHCGR. This inactivation was due to a compound heterozygous mutation in *LHCGR* signal peptide. Our findings expand the current spectrum of *LHCGR* mutations and our understanding of the molecular mechanisms by which these mutant proteins are defective. We have illustrated that the duplication mutant is incapable of correct membrane expression and is most likely impeded in the endoplasmic reticulum. The deletion mutant, on the other hand, is barely expressed and is likely to be subject to an early quality control mechanism that degrades its mRNA before translation. The missense mutation alone, found in the same allele as the deletion, shows a decreased membrane expression and increased retention intracellularly.

Disclosure: The authors state that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions

AT, IP, KSz and ARM designed the experiments. AT, IP and AFD wrote the manuscript. IP, JF and ASP were responsible for clinical data acquisition. AT and ARM generated the plasmids constructs. AT performed confocal microscopy and functional cAMP analysis. KSz measured the cell surface expression levels via flow cytometry, gene expression using qPCR and protein expression with Western Blotting. IH,

AB, AFD, ASP and ARM supervised the research. All authors have read and approved the manuscript.

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Figure Legends

Figure 1. Histological section of resected testicular tissue: Hematoxylin and eosin staining (200X) showing the absence of Leydig cells and the presence of immature seminiferous tubules with a thickened basement membrane.

Figure 2. Panel A. Cell surface receptor expression. Flow cytometric analysis revealed that the surface expression of LHCGR mutants p.L10_Q17dup, p.L16Q and p.K12_L15del is statistically lower than WT LHCGR expression. The percentage median fluorescence intensity (MFI) of the duplication and that of the L16Q mutant are significantly lower than WT but statistically higher than the negative control. The deletion mutant surface expression is similar to mock transfected control cells. Data is expressed as MFI ± standard error of the mean (SEM) of three independent experiments. ***$p<0.0001$ and **$p<0.006$. **Panel B.** Cellular localization of receptors using confocal microscopy (non-permeabilized). Non-permeabilized HeLa cells expressing LHCGR WT or mutants shows cell surface localization of WT LHCGR. LHCGR_L10_Q17dup, and L16Q mutants are very weakly expressed at the cell surface while the LHCGR_K12_L15Del mutant shows no cell surface expression. **Panel C.** Cellular localization of receptors using confocal microscopy (permeabilized). HeLa cells expressing either WT LHCGR or LHCGR mutants, upon permeabilization, shows the intracellular localization of LHCGR_L10_Q17dup while WT LHCGR is mainly expressed at the cell membrane. The LHCGR_K12_L15del mutant shows very weak intracellular expression. HeLa cells expressing the L16Q missense mutation shows low surface expression and mostly intracellular retention.

Figure 3. Panel A. cAMP production following LHCGR stimulation by rLH. Panel **A** GS-293 sensor cells transiently expressing LHCGR (WT or mutants) were stimulated with rLH and cAMP production was followed as a luminescent readout (relative light units; RLU). The cAMP production following stimulation of LHCGR_L10_Q17dup mutant was dramatically lower (6.7-fold) than that mediated by WT LHCGR stimulation. L16Q responded at a level that was about 33% of that of WT LHCGR (2.4 fold). The LHCGR_K12_L15del mutant, however, did not lead to any cAMP production upon rLH stimulation. **Panel B** GS-293 sensor cells transiently co-expressing the WT LHCGR and mutant receptors. Co-expression of LHCGR_K12_L15del and WT LHCGR resulted in decreased cAMP production (1.6-fold) in comparison with the WT LHCGR and empty vector co-expression. When L16Q_LHCGR was co-expressed with WT LHCGR the production of cAMP was similar to that observed in the control (WT LHCGR and empty vector). In contrast, cAMP production by cells transfected with LHCGR_L10_Q17dup and WT LHCGR was the sum of mutant and WT LHCGR responses. Data is representative of the experiment performed in triplicate and was repeated independently at least three times. **Panel C.** mRNA expression of WT LHCGR and mutant receptors. The analysis of qPCR results revealed slightly decreased expression of L10_Q17dup_LHCGR mutant (1.3-fold) as compared to the WT LHCGR. On the contrary, expression of K12_L15del_LHCGR was 3.9-fold lower, whereas expression of L16Q_LHCGR - 2.3-fold lower. *GAPDH*

and *PPIA* housekeeping genes were used to normalize the expression of WT LHCGR and mutants. Data is representative of three independent experiments performed in triplicates.

Figure 4. Western Blot analysis of protein expression. **Panel A.** HEK293T cells transiently transfected with plasmids encoding either the WT LHCGR or mutant receptors. Twenty-four hours post-transfection. Cells were treated with either 1‰ DMSO (A) or 10 nM MG132 **Panel B**. After forty-eight hours, Western Blot was performed, which showed decreased protein expression of mutants as compared to the WT LHCGR. In the case of K12_L15del_LHCGR it revealed the lack of bands corresponding to dimers and oligomers as well as a band with lower molecular weight than the monomeric form of LHCGR. B-actin was used as internal control.

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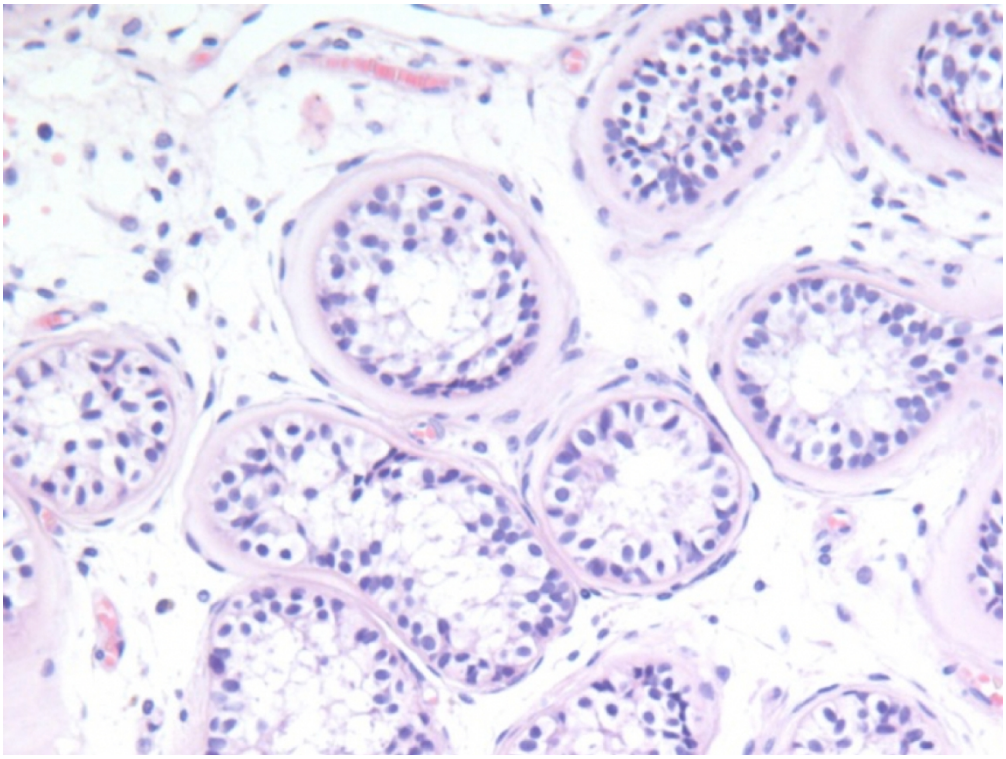

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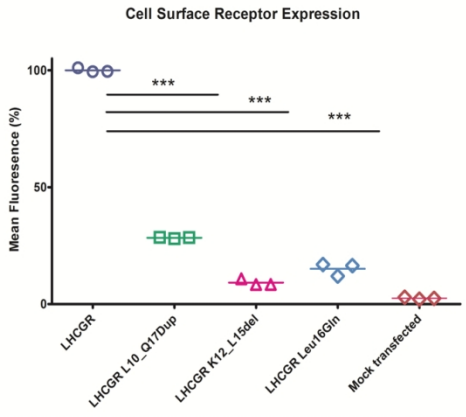

Figure 2A

2A

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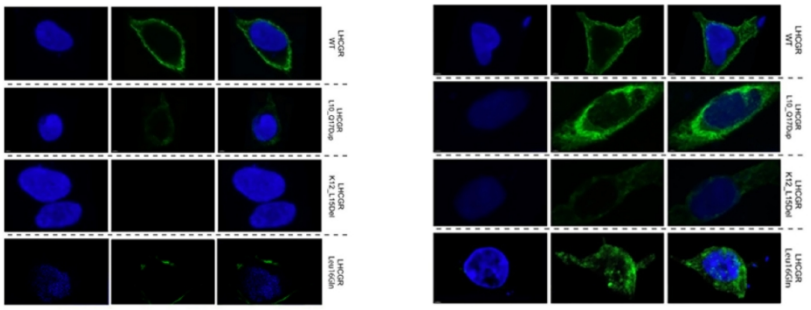

B. Non permeabilized cells C. Permeabilized cells

Figure 2 B, C

2BC

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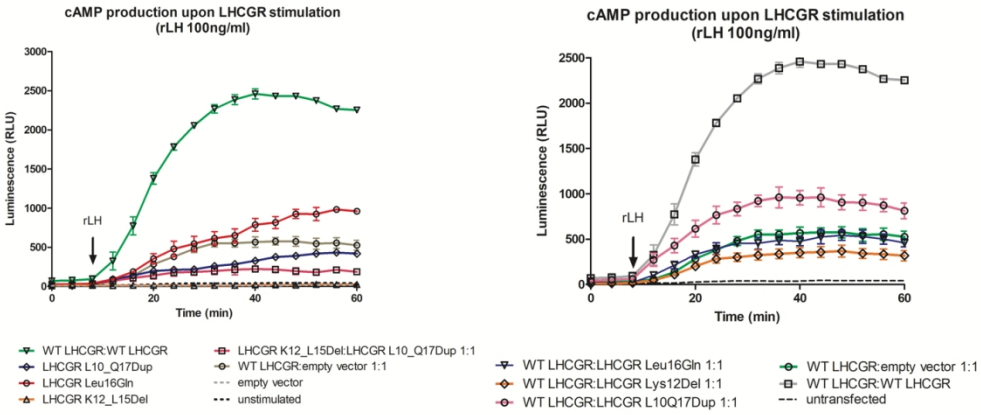

A B

Figure 3

3AB

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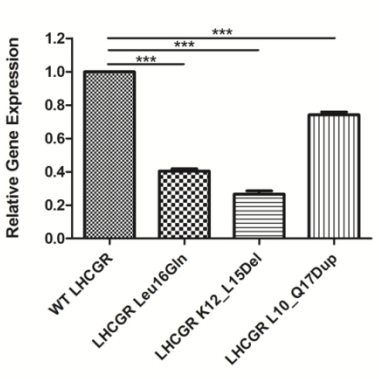
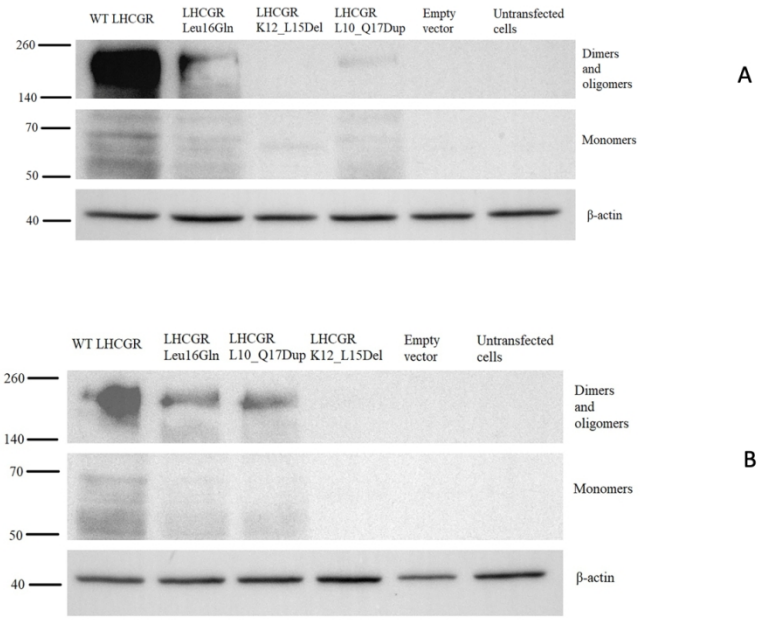


C

Figure 3

3C

254x190mm (144 x 144 DPI)



4AB

254x190mm (144 x 144 DPI)