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Development of constructs for recombinant expression of human follicle-stimulating hormone receptor in rod cells of the zebrafish

Master's degree in Biochemistry

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All contents in this thesis are from exclusive responsibility of the author.

(Joana Carolina Quintela Carrola)

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Abstract

The follicle-stimulating hormone (FSH) is involved in the regulation of reproduction, by acting through a G protein-coupled receptor (GPCR) on the surface of target cells. Like most of the GPCRs, not much is known about the structure of the follicle-stimulating hormone receptor (FSHR). It is relatively difficult to purify the FSHR protein from gonadal tissues, due to its low abundance on its native cells, and therefore, to study its structure. For these reasons it is necessary to come up with a strategy that allows the production of large quantities of protein in order to use it in studies to obtain detailed structural information on full-length human FSHR.

Via polymerase chain reactions, site-directed mutagenesis and other molecular biological methods, we were able to construct mutated receptors with different signal sequences and different tags, including the last fifteen amino acids of human rhodopsin which allow the receptor to be expressed on retinal rod cells of zebrafish. Mutations were designed in a manner that the receptor becomes inactive, *i.e.* incapable of signal transduction but still expressed at cell surface. The receptor constructs were first tested in human embryonic kidney cells (HEK 293-T) and their inserts will be used for transgenetic studies on zebrafish in the future.

Key words: G protein-coupled receptors, follicle-stimulating hormone receptor, rhodopsin, mutations, zebrafish.

Resumo

A hormona estimulante do folículo (FSH) está envolvida na regulação da reprodução, actuando através de um receptor acoplado a proteínas G (GPCR) localizado à superfície das células alvo. Tal como a maioria dos GPCRs, pouco se sabe acerca da estrutura do receptor da hormona estimulante do folículo (FSHR). Este receptor é relativamente difícil de purificar de tecidos gonadais, devido à pouca abundância com que se encontra nas suas células nativas, e, portanto, de estudar a sua estrutura. Por estas razões é necessário criar uma estratégia que permita a produção de grandes quantidades de proteína de modo a poder usá-la em estudos dirigidos à obtenção de informação detalhada da estrutura completa do FSHR.

Através de reacções em cadeia da polimerase, mutagénese dirigida e outros métodos biomoleculares foi possível construir receptores mutantes com diferentes sequências sinal e diferentes *tags*, incluindo os últimos quinze aminoácidos da rodopsina humana que permitem a expressão do receptor em bastonetes da retina de peixe zebra. As mutações foram criadas de modo a que o receptor se torne inactivo, *i.e.* incapaz de realizar transdução de sinal mas que ainda é expresso à superfície da célula. Os receptores foram primeiro testados em células embriónicas humanas do rim (HEK 293-T) e os insertos serão usados em estudos de transgénese em peixe zebra.

Palavras-chave: Receptores acoplados a proteínas G, receptor da hormona estimulante do folículo, rodopsina, mutações, peixe zebra.

Abbreviations List

7-TM = 7-transmembrane domain

ATP = Adenosine triphosphate

cAMP = Cyclic adenosine monophosphate

CCR = C-terminal cysteine-rich

cDNA = Complementary deoxyribonucleic acid

CG = Chorionic gonadotropin

DMEM = Dulbecco's modified Eagle's medium

DNA = Deoxyribonucleic acid

(d)dNTPs = (di)deoxyribonucleotide triphophates

EC_{50} = Effect concentration 50%

ECD = Extracellular domain

EDTA = Ethylene-diamine-tetra-acetic acid

ELx = Extracellular loop n.º x

ELISA = Enzyme- linked immunosorbent assay

FSH = Follicle-stimulating hormone

FSHR = Follicle-stimulating hormone receptor

$FSHR_{HB}$ = hormone-binding domain of the human follicle-stimulating hormone receptor

GDP = Guanosine diphosphate

GFP = Green fluorescent protein

GnRH = Gonadotropin-releasing hormone

GnRHR = Gonadotropin-releasing hormone receptor

GPCR = G protein-coupled receptor

GpH = Glycoprotein hormone

GpHR = Glycoprotein hormone receptor

GTP = Guanosine triphosphate

HEK = Human embryonic kidney

hFSH = Human follicle-stimulating hormone

hFSHR = Human follicle-stimulating hormone receptor

LB = Luria-Bertani

LH = Luteinizing hormone

LHR = Luteinizing hormone receptor

LRRs = Leucine-rich repeats

MCS = Multiple cloning site

mRNA = Messenger ribonucleic acid

NCR = N-terminal cysteine rich

NTED = N-terminal exodomain

ONPG = *o*-nitrophenyl-β-D-galactopyranoside

PBS = Phosphate saline buffer

PCR = Polymerase chain reaction

PRL = Prolactin

PEI = Polyethylenimine

RHO_{15} = Last 15 amino acids of human rhodopsin

ROS = Rod outer segment

TMx = Transmembrane domain n.° x

TSH = Thyroid-stimulating hormone

TSHR = Thyroid-stimulating hormone receptor

wt-hFSHR = Wild type human follicle-stimulating hormone receptor

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1. Introduction

1.1 The endocrinology of reproduction

In mammals, reproduction is controlled by the pituitary hormones – luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which then reaches the pituitary, through the hypothalamo-pituitary portal vessels, where it stimulates the release of the gonadotropins FSH and LH. These two hormones will then act on either the ovaries in females or the testes in males, regulating the two most important functions of the gonads: the production of gametes and the secretion of sex hormones (Figure 1). (Vander, Sherman and Luciano, 2001)
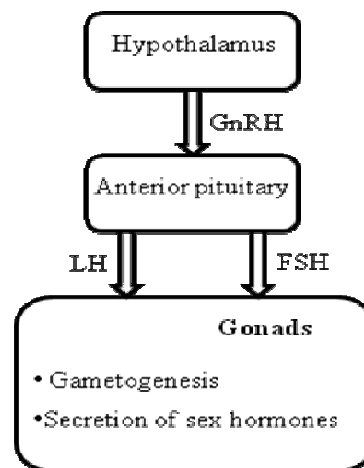
Figure 1: Schematic representation of the control of reproduction in both males and females.

FSH and LH belong to the glycoprotein hormone family, which also includes chorionic gonadotropin (CG) and thyroid-stimulating hormone (TSH). These hormones are disulphide-rich heterodimers consisting of a common α-subunit and a unique β-subunit, within a given species, that are non-covalently associated. They act on target cell surfaces by binding to specific G protein-coupled receptors (FSH and TSH bind to FSHR and TSHR, respectively; LH and CG bind to LHR), which contain an extracellular domain with multiple leucine-rich repeats and a transmembrane domain consisting of seven helices (Fan & Hendrickson, 2005).

1.2 The G protein-coupled receptors

G protein-coupled receptors (GPCRs) are the largest family of integral membrane proteins and are found in most eukaryotic organisms, where they modulate a wide variety of biological processes, through diverse extracellular stimuli. In fact, almost all known physiological processes are regulated by GPCRs, which means that various dysfunctions or diseases may be caused by defects in the signaling pathways. Therefore, a large fraction of drugs exert their action via interaction with these receptors. However, less than 10% of all known GPCRs are targeted which makes it very important to understand them. This is yet a difficult task since there is a serious lack of structural and functional data (Tao, 2006; McCusker *et al.*, 2007 and Park *et al.*, 2008).

There are several factors that make it difficult to obtain high resolution diffraction data for GPCRs in order to solve their structures. One of those factors is that GPCRs are usually expressed at very low levels in their native cells and it is very difficult to produce high amounts of recombinant receptor protein. Also the purification step(s) present(s) some difficulties because detergents need to be used to solubilize the GPCRs, reducing the yield of the procedure. In addition, degradation can occur, affecting the stability of the purified receptor. Furthermore, GPCRs have flexible domains that reduce the contact sites needed for crystal formation. Another factor is that many GPCRs exhibit basal activity, *i.e.* they can be in an activated state without being stimulated by an agonist, which has been associated with structural instability (Lundstrom *et al.*, 2006; Rasmussen *et al.*, 2007).

GPCRs, like all proteins, have their mRNA being transcribed in the nucleus and, then, at the ribosome, the polypeptide sequence is synthesized. In the endoplasmic reticulum (ER) immature carbohydrates are added onto the polypeptides, forming glycoproteins. The maturation of the carbohydrates occurs in the Golgi apparatus, after initial folding, and the receptor is transported to the cell surface (Tao, 2006).

Like the name says, GPCRs are coupled to a G protein, which is composed of three protein subunits, α, β, and γ. When the receptor is activated by a ligand, there is a conformational change in the receptor that leads a conformational change in the G

protein that leads to the conversion of GDP to GTP on the Gα subunit, causing the latter to be dissociated from Gβγ. One of the possible signaling pathways is initiated by the activation of adenylil cyclase by the Gα, if this protein is a so-called stimulatory Gα (Gα$_s$), by catalyzing the synthesis of cyclic AMP (cAMP) from ATP (Figure 2) (Alberts *et al.*, 2002).

Activated GPCRs can be desensitized by either phosphorylation or internalization. When internalized, they can be recycled back to the cell surface, where they can undergo another cycle of activation, or degraded in the lysosome (Tao, 2006).
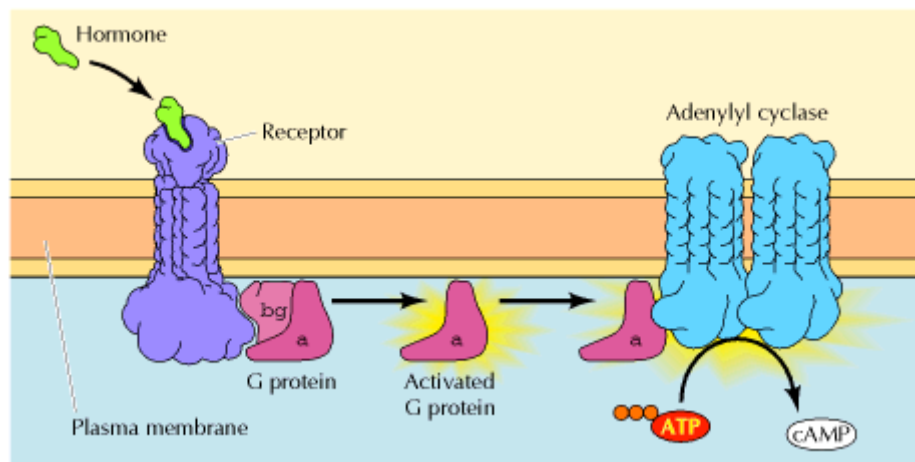
Figure 2: Hormonal activation of adenylyl cyclase. Binding of hormone promotes the interaction of the receptor with a G protein. The activated G protein α subunit then dissociates from the receptor and stimulates adenylyl cyclase, which catalyzes the conversion of ATP to cAMP (Cooper, 2000).

1.2.1 The G protein-coupled receptors structure

According to sequence homology, the GPCRs can be divided into classes: class A, or rhodopsin/β2 adrenergic receptor-like GPCRs; class B, or receptors related to the glucagon receptor; class C, which corresponds to receptors related to the metabotropic neurotransmitter receptors and two more minor classes. One feature that all these classes have in common is a transmembrane domain consisting of seven α-helices connected by three extracellular and three intracellular loops, with the amino terminus

located on the extracellular side and the carboxy terminus in the cytoplasm (Figure 3) (Gether, 2000 and Durme *et al.*, 2006).
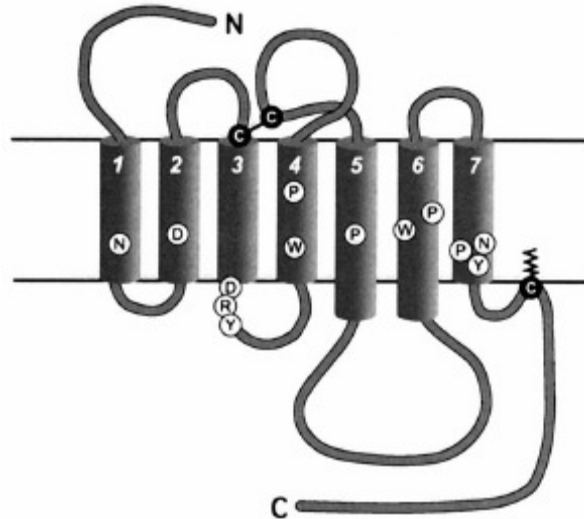
Figure 3: Schematic representation of the structure of a GPCR. This scheme represents a class A, or rhodopsin/β2 adrenergic receptor-like GPCR. These receptors contain some highly conserved residues, depicted by the black letters in white circles. The white letters in black circles show a disulfide bridge that connects the second (EL2) and third extracellular loop (EL3) and a palmitoylated cysteine in the carboxy-terminal tail that forms a putative fourth intracellular loop (Gether, 2000).

1.2.2 Rhodopsin

The first crystal structure available for a GPCR was the one of rhodopsin (Palczewski *et al*, 2000). This receptor responds to light by starting the signal transduction that induces vision. It can be isolated from retina, where it exists in large quantities, and it is very stable in the absence of light, which makes it relatively easy to purify and, therefore, to be studied.

Previous studies involving electron microscopy and cryoelectron microscopy (Schertler, 1993) had showed, two-dimensionally, the presence of the 7-TM domain (Figure 4) and, through homology-modelling studies, provided structural information about other GPCRs. These studies together with the information of the high resolution

structure obtained by X-ray crystallography (Figure 5) can help understanding how ligands bind to GPCRs and how the signaling pathways proceed since it contains highly conserved residues among GPCRs (Okada *et al.*, 2001 and Sakmar, 2002).
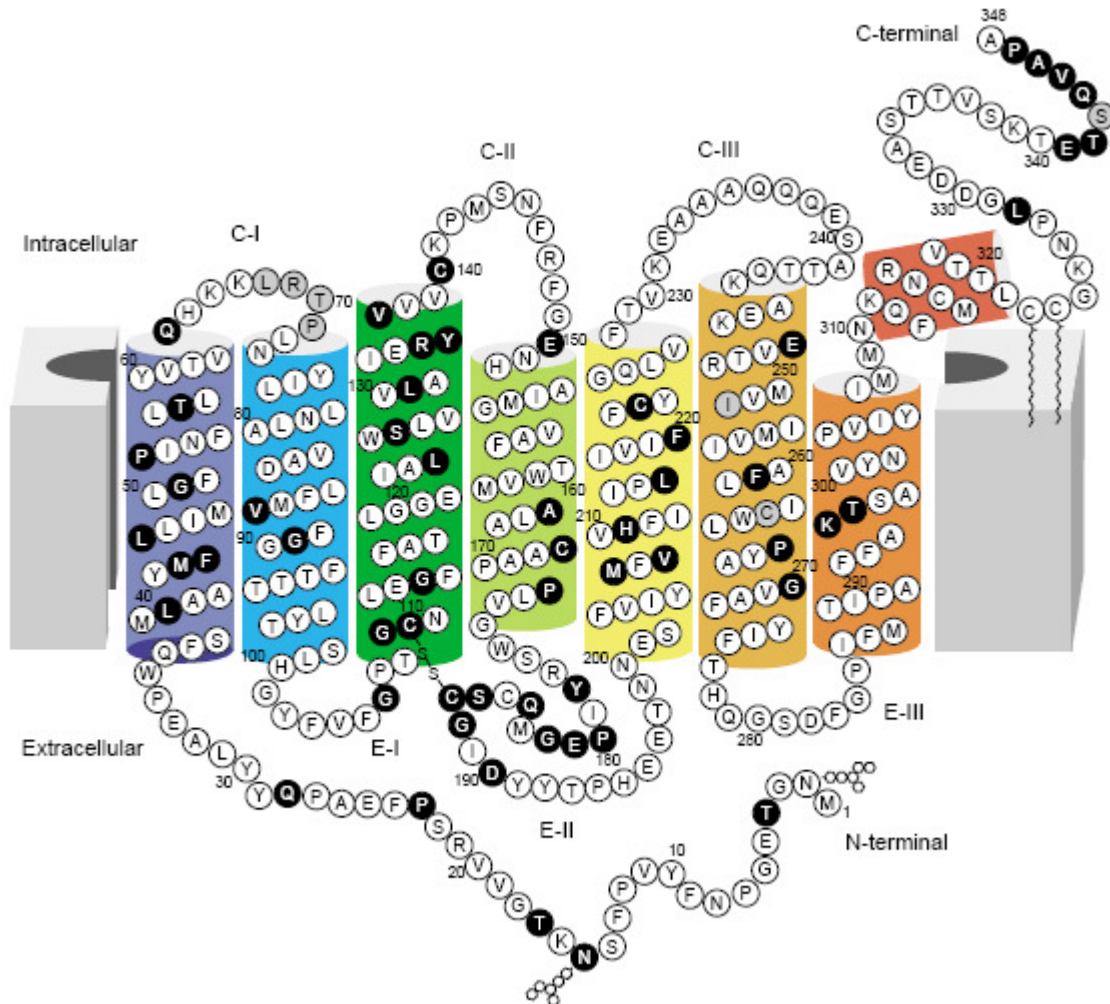
Figure 4: Two-dimensional model of bovine rhodopsin. Residues shown in black circles indicate the position of mutations associated with retinitis pigmentosa. In rhodopsin, extracellular corresponds to the intradiscal space (Okada *et al.*, 2001).
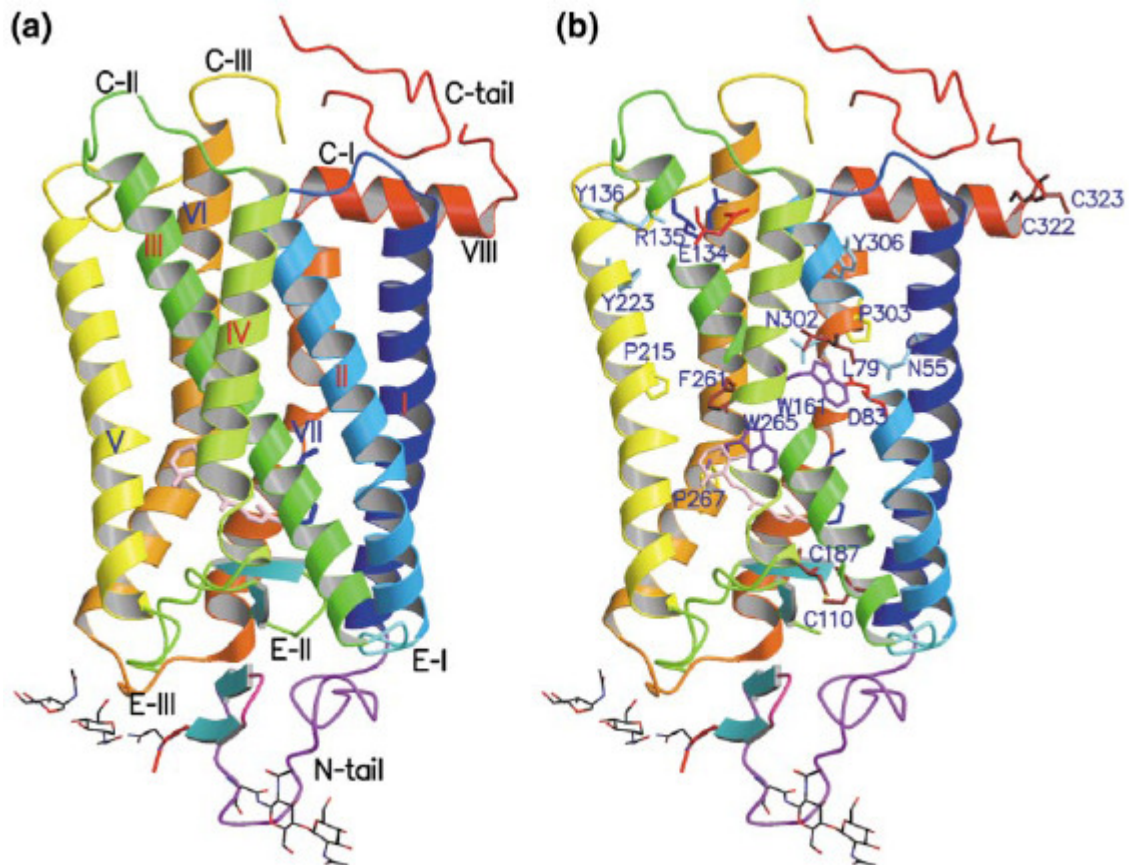
Figure 5: Crystal structure of bovine rhodopsin. (a) I to VII correspond to the transmembrane helices; VIII is a short helix found between H-VII and the C-tail; C-I, C-II and C-III are the intracellular loops; C-tail is the cytoplasmic tail; E-I, E-II and E-III are the extracellular loops and N-tail is the extracellular tail. Cys^{322} and Cys^{323} are palmitoylated and anchor the C-tail to the membrane. (b) Most conserved residues among G-protein-coupled receptor, including Cys^{110} and Cys^{187} that form a disulfide bridge between H-III and E-II, and Cys^{322} and Cys^{323} that are palmitoylated and anchor the C-tail to the membrane (Okada *et al.*, 2001).

The bovine rhodopsin protein shown in Figures 4 and 5 is 348 amino acid long, which is an intermediate number amongst GPCRs. It is expected that the lengths of the transmembrane helices and of the extracellular loops are approximately the same for these receptors. Since the majority of the conserved residues are found in that area, it means that the other areas are responsible for the ligand specificity of each receptor (Palczewski *et al.*, 2000).

Rhodopsin is expressed in retinal rod photoreceptor cells (Figure 6). These are specialized neurons that recognize the presence of light by means of the rhodopsin protein, which undergoes conformational changes leading to phototransduction. They

are highly differentiated cells consisting of a synapse or cell body, an inner segment and an outer segment (ROS). The inner segment contains the mitochondria, the ER and the Golgi apparatus and its where the rhodopsin is synthesized. The molecules are then transported to the ROS in post-Golgi membrane vesicles, where they are incorporated and accumulated in the ROS disk membranes (Filipek *et al.*, 2003; Tai *et al.*, 1999 and Zhang *et al.*, 2005).
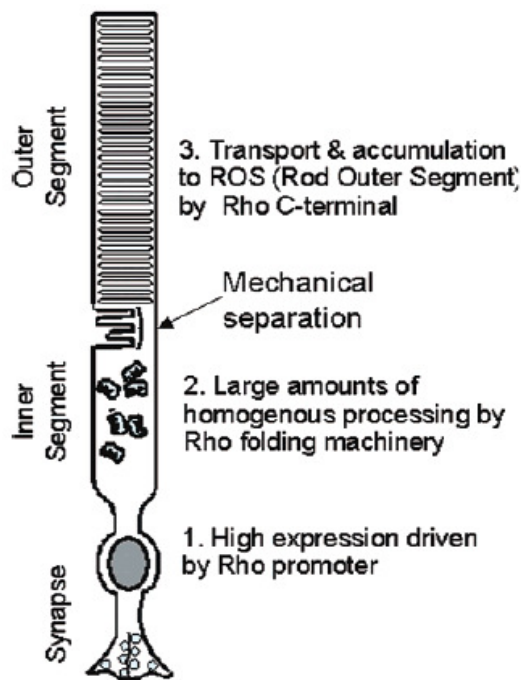
Figure 6: Morphology and characteristics of a highly differentiated vertebrate rod photoreceptor cell (Zhang *et al.*, 2005).

As said before, defects on GPCRs may cause several diseases and so rhodopsin mutants have been shown to be involved in eye diseases such as retinitis pigmentosa. Mutations in the C-terminal tail impair the trafficking of rhodopsin to the ROS, meaning that this is the region that contains the necessary signals for proper sorting and transport of rhodopsin (Perkins *et al.*, 2002).

1.2.3 Glycoprotein hormone receptors and their general structure

Within the class A of GPCRs, there is a subfamily consisting of the glycoprotein hormone receptors (GpHR) that includes the FSHR, the LHR and the TSHR. Like all GPCRs, these receptors have a seven transmembrane (7-TM) α-helical structure and a G protein coupled on their cytoplasmic surface, but unlike the rest of the rhodopsin-like receptors they have a large N-terminal exodomain (NTED) with multiple leucine-rich repeats (LRR) (Figure 7) (Fan and Hendrickson, 2005; Vassart *et al.*, 2004 and Durme *et al.*, 2006).
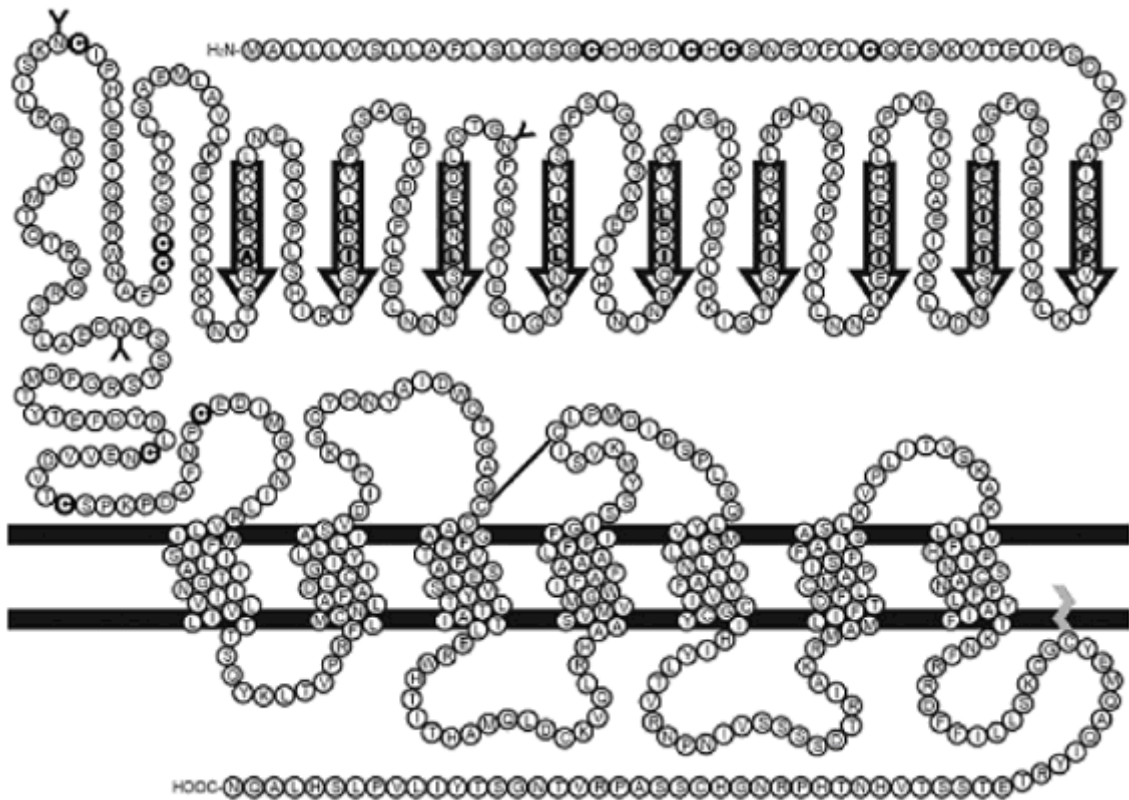
Figure 7: Schematic representation of the human FSHR protein, illustrating the NTED, the 7-TM helices and the C-terminal intracellular tail. The β-strand in each of the consecutive LRRs is indicated by an arrow, and the consecutive hydrophobic residues that form the hydrophobic core of the LRR domain are indicated by circles with a grey background. Putative N-linked carbohydrates are indicated by a "Y". The Cys residues present in the NCR and CCR subdomains are indicated by circles with a black background. The disulfide bond between the conserved Cys residues in EL1 and 2 is indicated by a black line. The cell membrane is represented by two black bars, and a palmitoylated Cys residue anchoring the C-terminal tail to the cell membrane is indicated by a grey zigzagging bar (Bogerd *et al*, 2005).

The three-dimensional structure of the porcine ribonuclease inhibitor (Kobe and Deisenhofer, 1993) helped to generate structural models of the NTED of the GpHRs, showing that the exodomain is composed of an N-terminal cysteine-rich cluster (NCR), nine imperfect LRR motifs and a C-terminal cysteine-rich cluster (CCR) (Figure 8a).

The LRR motifs consist of 20-25 amino acids that form a short β-strand and an α-helix positioned antiparallel to each other, forming repetitive right-handed hairpin-like units that are connected by short loops (Figure 8b). The β-strands make a concave inner surface that gives the molecule a horseshoe-shaped structure, where the ligand binds. Figure 7c shows a single LRR unit that is composed by seven residues – X_1-X_2-L-X_3-L-X_4-X_5 – where X is any amino acid and L can be leucine, isoleucine or other hydrophobic residues. The side chains of the L amino acids are directed towards the helix and form the hydrophobic core of this structure, giving it stability, whereas the side chains of the X residues point outwards, contributing to the hormone-binding interface. Therefore, the specificity and high affinity of the GpHRs for their respective ligands is determined by the NTED (Vassart *et al.*, 2004 and Bogerd, 2007).
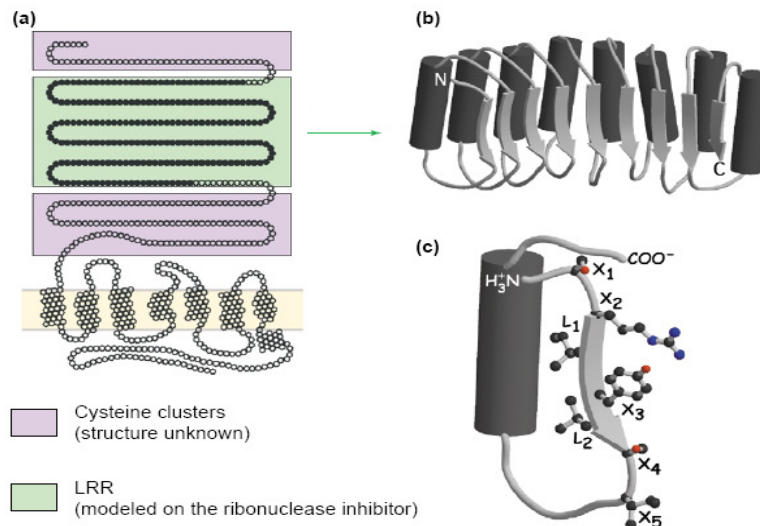
Figure 8: Schematic representation of the structures composing glycoprotein hormone receptors. (a) Two-dimensional representation of the glycoprotein hormone receptors' NTED, where the purple boxes correspond to N-terminal and C-terminal cysteine-rich regions of the ectodomain and the green box shows the nine leucine-rich repeats (LRR). (b) Repeats are made of β-strands (arrows) followed by α-helices (cylinders). (c) Single LRR unit composed by seven residues: X_1-X_2-L-X_3-L-X_4-X_5 (Vassart *et al.*, 2004).

When the hormone binds to the NTED, some changes not yet known occur in the 7-TM domain leading to the exchange of GDP for GTP on the Gα subunit of the G protein. This means that the receptor activation and signal transduction through G protein is determined by the 7-TM domain (Fan and Hendrickson, 2005).

1.2.4 The follicle-stimulating hormone receptor

In 1992, Kelton *et al.* cloned the human follicle-stimulating hormone receptor (FSHR) and found that it has an open reading frame of 2088 nucleotides, encoding for a 695 amino acid long protein. The first 17 amino acids encode for a hydrophobic signal peptide that precedes the N-terminal domain of 349 amino acids. The next 264 amino acids correspond to the transmembrane domain which is followed by the intracellular cytoplasmic domain (65 amino acids). The full sequence of the FSHR can be found in Appendix 6.1 and the schematic representation of its structure is shown in Figure 7.

In 2005, Fan and Hendrickson were able to determine the crystal structure of the N-terminal hormone-binding domain of the human follicle-stimulating hormone receptor (FSHR$_{HB}$) bound to the follicle-stimulating hormone (FSH) (Figure 9). In this structure, the FSHR$_{HB}$ appears in a truncated form, *i.e.* NCR and LRR without the CCR, meaning that this last portion is not implicated in the binding. In Figure 9b it is possible to see that the FSHR$_{HB}$ is shaped as a tube with a slight concave bend, where the FSH binds in a hand clasp manner – FSHR and FSH wrap around one another.
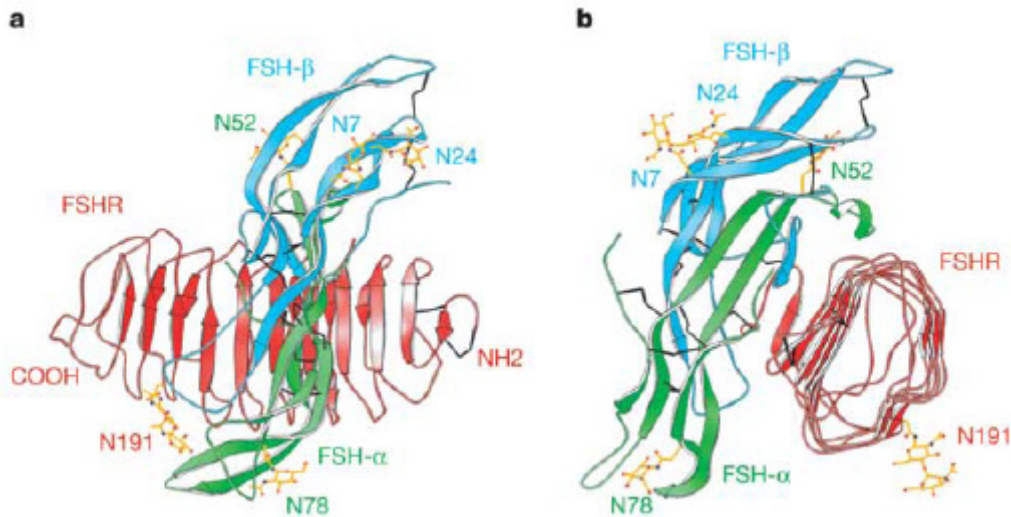
Figure 9: Crystal structure of human FSH bound to $FSHR_{HB}$. a) and b) Ribbon diagram of the complex structure shown in two views related by a 90° rotation about the vertical axis. FSH α-chains and β-chains are in green and cyan, respectively. $FSHR_{HB}$ is in red. The observed N-linked carbohydrates at N^{52} and N^{78} of FSH-α, N^7 and N^{24} of FSH-β, and N^{191} of $FSHR_{HB}$ are in yellow. Disulphide bonds are in black (adapted from Fan and Hendrickson, 2005).

1.2.5 Mutations

Inactivating mutations in the FSHR can help understand its physiological role and also the functioning of its domains, since some studies revealed that they cause ovarian dysgenesis, amenorrhea and infertility in women (Tao, 2006).

The first naturally occurring mutation was found in several Finnish patients with primary amenorrhea due to ovarian failure and refers to the substitution of alanine for valine in position 189 ($Ala^{189}Val$) in the extracellular domain (ECD) (Aittomäki *et al.*, 1995). This mutation has been shown to result in a decrease of the binding capacity and, consequently, of the signalling response. However, further studies on the cell surface expression of the receptor showed that only a weak expression was observed, meaning that the receptor is trapped inside the cell. This alanine is part of a highly conserved sequence of five amino acids (^{189}Ala-Phe-Asn-Gly-Thr^{193} in FSHR) and appears to be of extreme importance for folding and trafficking of the receptor (Rannikko *et al.*, 2002).

Some other mutations in the ECD include $Ile^{160}Thr$ (isoleucine → threonine), $Asn^{191}Ile$ (asparagine → isoleucine), $Asp^{224}Val$ (aspartic acid → valine) and $Pro^{348}Arg$

(proline → arginine) (Tao, 2006). Like $Ala^{189}Val$, $Ile^{160}Thr$, $Asn^{191}Ile$ and $Asp^{224}Val$ cause the receptor to be retained intracellularly and, as a consequence, reduce the production of cAMP (Gromoll *et al.*, 2002 and Meduri *et al.*, 2008). $Pro^{348}Arg$ results in a mutant FSHR that has complete loss of function in *in vitro* studies of receptor activity and is unable to bind hormone (Allen *et al.*, 2003).

In the second α-helix of the transmembrane domain (TM2), a mutation of alanine on position 419 by a threonine ($Ala^{419}Thr$) allows the receptor to have normal ligand-binding affinity and capacity but makes it defective in signalling (Doherty *et al.*, 2002). Other substitutions in the transmembrane domain include $Pro^{519}Thr$ (proline → threonine) in EL2, $Arg^{573}Cys$ (arginine → cysteine) in IL3 and $Leu^{601}Val$ (leucine → valine) in EL3: the first results in total loss of function and the other two are expressed normally at cell surface but exhibit low signalling.

In summary, the location of the amino acid (Figure 10) is more important than its nature since the mutations located in the extracellular domain of the receptor cause a defect in targeting the protein to the cell surface, whereas mutations in the transmembrane domain impair the signal transduction.
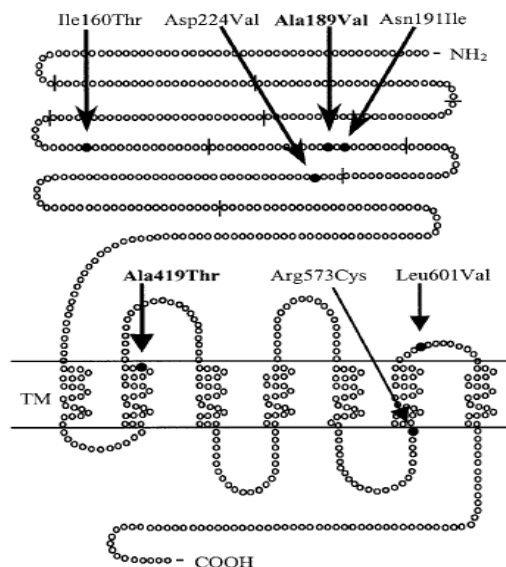
Figure 10: Schematic presentation of the FSHR with 7 of the known inactivating mutations (Doherty *et al.*, 2002).

1.3 Transgenesis

Transgenesis is a technique that allows the insertion of foreign DNA into the genome of a (model) organism in order to express specific gene products in a controlled manner. One such model is the zebrafish (*Danio rerio*) due to its easy maintenance and breeding. Another advantage of this organism is its transparent nature during embryonic and larval stages that makes it possible to, for example, observe green fluorescence in animals that express the GFP reporter gene (Perkins *et al.*, 2002; Laan *et a.l*, 2002; Kawakami *et al.*, 2004).

The use of transposable elements is one of the most efficient transgenetic methods. Discovered by Barbara McClintock in 1950, the transposons are sequences of DNA that are integrated in a host genome, without causing enormous DNA rearrangements.

Tol2 is a naturally occurring transposable element found in the genome of the Japanese medaka fish (*Oryzias latipes*). This element has been used for transgenesis of zebrafish: a plasmid containing the *Tol2* element, which lacked part of the transposase gene, in combination with the transposase mRNA, which was synthesized *in vitro* using cloned *Tol2* cDNA as a template, were co-injected into fertilized eggs. The transposase mRNA is then used by the egg to produce the transposase enzyme that catalyzes the excision of the vector sequences (*i.e.* the foreign DNA fragment to be used for transgenesis) from the plasmid and its integration into the host genome. The transposon-mediated DNA insertions can then be transmitted to the next generation through the germ lineage (Figure 11) (Kawakami, 2005).

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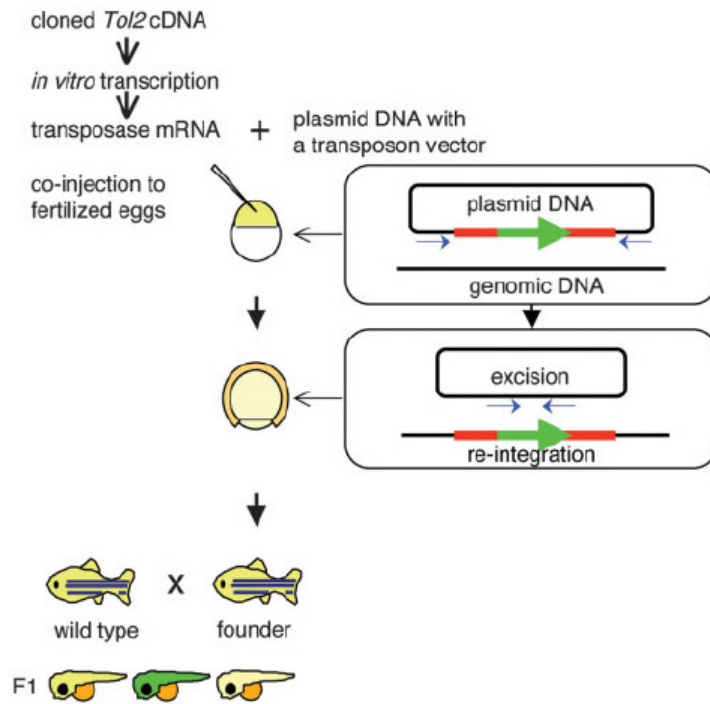
Figure 11: Transposition of the Tol2 transposon vector in zebrafish. A plasmid DNA containing a transposon vector (with the GFP gene) is co-injected with the transposase mRNA synthesized in vitro. The transposon vector is excised from the plasmid DNA, integrated in the genome and transmitted to the F1 generation. GFP is expressed by embryos carrying the insertions (adapted from Kawakami, 2005).

2. Material and Methods

2.1 Overall strategy

2.1.1 Part 1 – Construction of hFSHR with different signal sequences and different tags.

The general structure of the different hFSHR constructs is represented on Figure 12 with GPCR being the wild type human FSHR (wt-hFSHR) or the human FSHR with a catfish GnRHR C-terminal tail and the last 15 amino acids of the human rhodopsin sequence (hFSHR/cf-tail/RHO_{15}). The different signal sequences and different tags are described in Table 1.

Signal sequence	Tag	GPCR
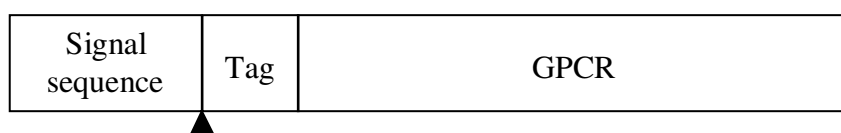
Figure 12: Basic scheme of the FSHR constructs.

Table 1: Description of the different signal sequences and different tags, according to the construct code, where X corresponds to either A (wt-hFSHR) or B (hFSHR/cf-tail/RHO_{15}), giving 22 constructs in total.

Construct code	Signal sequence	Tag	Extra tag
X-1			FLAG
X-5			---
X-9		FLAG	8xHis
X-13			STREP
X-17	Prolactin (PRL)		---
X-21			8xHis
X-25		STREP	FLAG
X-29			STREP
X-33			---
X-37	hGPα	STREP	8xHis
X-41			FLAG

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Using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) it is possible to predict the presence and location of signal peptide cleavage sites at the N terminus of amino acid sequences from different organisms, based on a combination of several artificial neural networks and hidden Markov models. Appendix 6.2 shows the prediction results for a prolactin-FLAG sequence.

The constructs with the different tags and the different signal sequences will be inserted into the *Bgl* II/ *Bam* HI sites of the multiple cloning site (MCS) of the pIRES2-EGFP vector (Figure 13) where the internal *Not* I site has been previously knocked out, through site-directed mutagenesis.
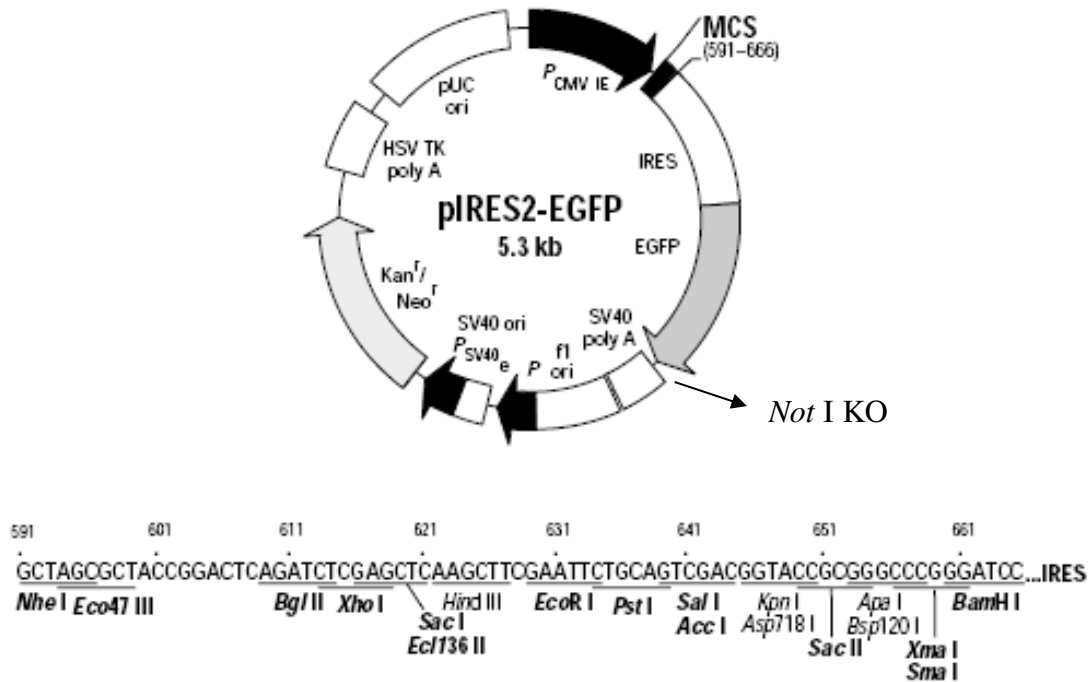
Figure 13: Restriction map and multiple cloning site (MCS) of pIRES2-EGFP Vector.

The plasmids will then be ready to be transfected into HEK 293-T cells to check for cell surface expression of the receptors through ELISA and to detect ligand-induced cAMP production through the β-galactosidase assay.

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2.1.2 Part 2 – Construction of mutated hFSHRs.

Part 1 will determine which constructs to use next, *i.e.* the constructs with best results for the ligand-induced cAMP production and the cell surface expression.

On these constructs, site-directed mutagenesis will be performed in order to create constructs that are expressed on cell surface but that are deficient in signaling.

Table 2: Site-directed mutagenesis to create signaling-inactive mutants.

	Mutations		
Single Mutant	Ala^{419}Thr	Arg^{573}Cys	Leu^{601}Val
Double Mutant	Ala^{419}Thr/Arg^{573}Cys	Arg^{573}Cys/Leu^{601}Val	Leu^{601}Val/Ala^{419}Thr
Triple Mutant	Ala^{419}Thr/Arg^{573}Cys/Leu^{601}Val		

2.2 Optmization of FSHR constructs

2.2.1 Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique that allows the production of millions of copies of a specific DNA fragment. The reaction normally consists of 25 to 35 cycles of basic steps where the temperature is shifted between 3 values:

- the mixture is heated to 94 °C, which is the denaturation temperature, and the hydrogen bonds that hold the DNA strands together are broken;
- then the temperature is lowered to 50-60 °C allowing the annealing of the primers to the single-stranded DNA template;
- finally, in the elongation step, the temperature is raised to 72 °C and this enables the *Taq* DNA polymerase to synthesize new strands complementary to the DNA template by attaching at the end of each primer.

One of the most important variables in a PCR reaction is the primer design. The melting temperature (T_m) of each primer should be approximately 70 °C and it is used to estimate the annealing temperature (5 °C below the lowest, calculated T_m). Also the length and the sequence of the primers must be accounted for: they should be at least 22

nucleotides long, have a G-C content of 45–60% and their 3'-terminal ends should not be complementary to each other and should contain a low G-C content (Brown, 2001 and Advantage® 2 PCR Enzyme System User Manual, Clontech, 2008).

> Construction of hFSHR/cf-tail/RHO$_{15}$

It is possible to construct a human FSHR with a catfish GnRHR C-terminal tail and the last 15 amino acids of human rhodopsin (RHO$_{15}$) by means of a fusion PCR.

A PCR was performed on a cfGnRHR/RHO$_{15}$, in order to obtain only the C-terminal tail with the RHO$_{15}$ (B on Figure 14). This portion partially overlaps with the hFSHR (A on Figure 14), allowing the two fragments to hybridize in a new PCR reaction, where the hybrid is elongated by the polymerase.
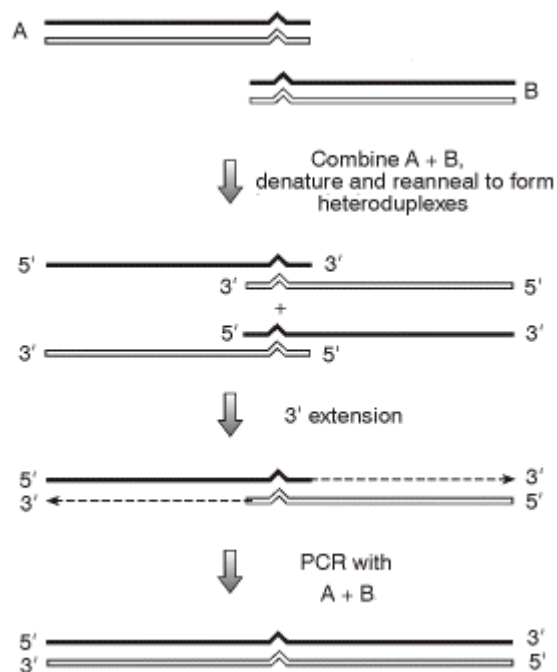
Figure 14: Example of a fusion PCR (adapted from Strachan and Read, 1999).

> Construction of hFSHR with different tags

In order to build constructs of the hFSHR with different tags, the PCR reaction mixtures had the composition described in Table 3 and Table 4 shows the primers used for the construction of receptors with a FLAG tag.

Table 3: Composition of the PCR reaction mixture using an Advantage 2 kit.

Component	Volume (μL)
H_2O	29
10x Adv2 reaction buffer	5
10x dNTPs	1
10 μM Forward primer	2
10 μM Reverse primer	2
1 ng/μL hFSHR	10
50x Adv2 DNA polymerase	1

Table 4: Sequence of the primers used to construct receptors with a FLAG tag (the codon sequence for the FLAG tag amino acid sequence DYKDDDDV is shown in bold); fw – forward; rv – reverse.

Primer	Sequence of primer
2919 (fw)	5'-CGCGGATCC**GATTACAAAGATGATGATGATGTC**GGCTGTCATCAT CGGATCTGTCACTGCTCTAA-3'
2916 (rv)	5'-GCTAGGCAGGGAATGAATTCTTGTTCTTGAAATATCT-3'

The reaction conditions were as follows:

Table 5: PCR program used to construct receptors with a FLAG tag.

Cycles	Temperature (°C)	Time (s)
1	94	10
10	94	10
	64	10
	72	65
25	94	10
	65	10
	72	65
1	72	60
1	4	∞

2.2.2 Agarose-gel electrophoresis and gel extraction

After the PCR reaction, the DNA needs to be separated from the rest of the PCR 'ingredients' and, in order to do this, agarose-gel electrophoresis is used.

DNA molecules carry a negative electric charge and, therefore, migrate towards the positive pole, when placed in an electric field. If the electrophoresis is performed in an agarose gel, the molecules can be separated according to their size, with optimal results depending on the gel concentration, for example, a 0.7% concentration allows the separation of fragments in a range from 0.8 to 12 kb, while a 2.0% is used to separate smaller fragments (0.05 to 2 kb). A DNA molecular weight marker is also loaded in the gel to allow a comparison with the sizes of these marker molecules. In order to visualize the result of the gel electrophoresis, the gel needs to be stained with ethidium bromide (EtBr); this compound binds to DNA molecules, by intercalating between adjacent base pairs, making them visible under UV light (Brown, 2001 and QIAEX II Handbook, 2008).

The DNA fragments extracted from the gel can be purified using the QIAEX II system from QIAGEN that is based on solubilization of agarose using iodine and selective adsorption of nucleic acids onto QIAEX II silica-gel particles in the presence of chaotropic salt; according to the manufacturer's instructions, QIAEX II silica-gel particles are added to the solubilized gel slice, the particles are collected by a brief centrifugation step, washed and the pure DNA fragment is eluted in Tris buffer or water (Figure 15). (QIAEX II Handbook, 2008)

Figure 15: QIAEX II Gel extraction procedure (adapted from QIAEX II Handbook, 2008).

2.3 Cloning

The next step is to insert the purified DNA fragment into a vector, a circular DNA molecule that will act as a vehicle, transporting the gene into a host cell where it is going to be multiplied. As the cell divides into its progeny, a colony of identical cells is formed, all carrying the recombinant DNA.

2.3.1 TOPO cloning

The pcDNA3.1/V5-His© TOPO® TA Expression Kit from Invitrogen provides a cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector (Figure 16) because *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products and the pcDNA3.1/V5-His-TOPO® has single, overhanging 3´ deoxythymidine (T) residues, allowing PCR inserts to ligate efficiently with the vector. The plasmid vector is supplied with a Topoisomerase I from *Vaccinia* virus covalently bound to it. This enzyme cleaves the phosphodiester bond after 5′-CCCTT and forms a covalent bond between a tyrosyl residue (Tyr-274) and the 3′ phosphate of the cleaved strand. The *Taq* polymerase-amplified PCR product is then ligated to the vector (Figure 17), releasing the topoisomerase (pcDNA3.1/V5-His© TOPO® TA Expression Kit, 2004).
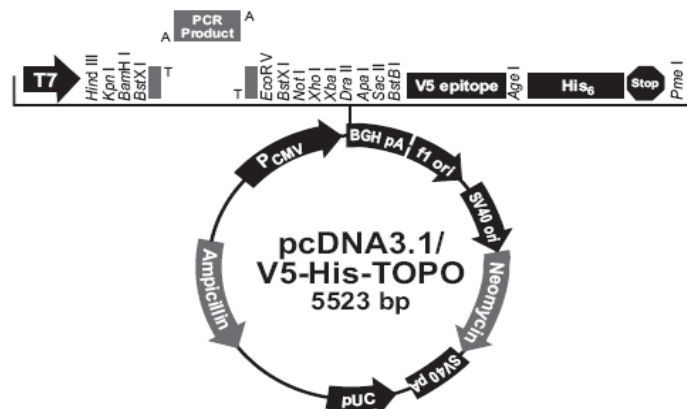
Figure 16: Features of the pcDNA3.1/V5-His-TOPO© vector. The TOPO® Cloning Site is located base pairs 953 and 954 (pcDNA3.1/V5-His© TOPO® TA Expression Kit, 2004).
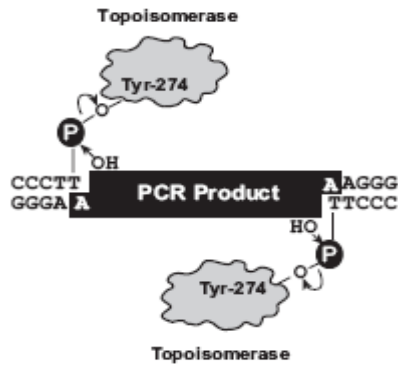
Figure 17: PCR product inserted in the the pcDNA3.1/V5-His-TOPO© vector with the topoisomerase covalently bound to it (pcDNA3.1/V5-His© TOPO® TA Expression Kit, 2004).

2.3.2 Transformation

Transformation is the term used to describe the uptake of a DNA molecule by a bacterial, fungal, animal or plant cell. The most used cells are the chemically competent *Escherichia coli* (*E. coli*) cells; a $CaCl_2$ treatment enables the cells to uptake a bigger amount of DNA. The pcDNA3.1/V5-His© TOPO® TA Expression Kit from Invitrogen includes One Shot® TOP10 chemically competent *E. coli* and the transformation is done according to the manufacturer's instructions, regardless if the recombinant DNA comes from a TOPO® Cloning reaction or from a rapid DNA ligation. The plates used are prepared from Luria-Bertani (LB) medium (25 g of LB broth per L of millipore water) with agarose (1,5g/100 mL of LB) and the intended antibiotic (50 μg of ampicilline or 30 μg of kanamycin per mL of LB) (Brown, 2001).

2.4 Colony/Orientation Check

Although only the cells that have taken up the recombinant DNA grow due to their antibiotic resistance, there is a possibility that this DNA is not correct, either because the vector ligated on itself, or because the PCR construct was inserted in the wrong way (*i.e.* not in the desired orientation). Hence, a colony/orientation check is necessary. This is done by picking a few colonies with a sterile toothpick, resuspend them in 50 μL of water and use 1 μL of this as template for a PCR. The primers used are

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described in Table 7 and the typical composition of the PCR mixture is represented on table 6.

Table 6: Composition of the colony check reaction mixture.

Component	Volume (μL)
H_2O	29
10x Super *Taq* PCR reaction buffer	2,5
10x dNTPs	2
25 μM Forward primer	1
25 μM Reverse primer	1
Template	1
0,25 U/μL Super *Taq*	1

Table 7: Sequence of the primers used to perform colony check on a pcDNA3.1/V5-His[©] TOPO[®] vector; fw – forward; rv – reverse.

Primer	Sequence of primer
1045 (fw)	5'-GGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAG-3'
1046 (rv)	5'-GATGGCTGGCAACTAGAAGGCACAGTCGAAA-3'

Table 8: Example of a PCR program used to perform colony check.

Cycles	Temperature (°C)	Time (s)
1	94	10
	94	10
35	65	10
	72	50
1	72	60
1	4	∞

After the PCR reaction, the mixtures are loaded in an agarose gel to check which ones have the expected length. The remainder of the resuspended colony mixture (49 μL) are then inoculated into 5.5 mL of LB medium with either 11 μL of ampicillin or 14

µL of kanamycin, depending on the vector used, and incubated overnight in a shaking incubator, at 37 °C, 200 rpm.

2.5 Plasmid DNA purification

The QIAprep Spin MiniPrep Kit from QIAGEN can be used to obtain high-purity plasmid DNA by following a simple bind-wash-elute procedure (Figure 18) – bacterial cultures are lysed, centrifuged and then applied to the QIAprep spin column that contains a silica gel membrane where plasmid DNA adsorbs in the presence of a high concentration of chaotropic salt and, finally, impurities are washed away and pure DNA is eluted in a small volume of elution buffer or water. (QIAprep MiniPrep Handbook, 2006)

Figure 18: QIAprep Spin MiniPrep plasmid DNA purification procedure (adapted from QIAprep MiniPrep Handbook, 2006).

2.6 DNA sequencing

The concentration of the plasmid DNA is then measured in a spectrophotometer (Pharmacia Biotech, GeneQuant II) and sent for sequencing at BaseClear, an accredited service laboratory for DNA-based research, quality assurance and forensics in Leiden, The Netherlands. Automated DNA sequencing determines the precise order of nucleotides and works in the following way: a primer is annealed on the single-stranded molecule to synthesize a new DNA strand; for this synthesis to occur, DNA polymerase needs the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP); together with the dNTPs, a small amount of dideoxynucleotides (*e.g.* ddATP) is added to the reaction; in this way, the elongation is blocked because the ddNTPs lack the 3'-hydroxyl group, resulting in a set of new chains, all of different lengths; the ddNTPs are

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labelled with a different fluorolabel for each ddNTP (Figure 19), which allows the detector to recognize the last base of the chains. At BaseClear, the data analysis is performed automatically by PHRED, the international standard software for superior base calling and sequence data interpretation. (Brown, 1999)
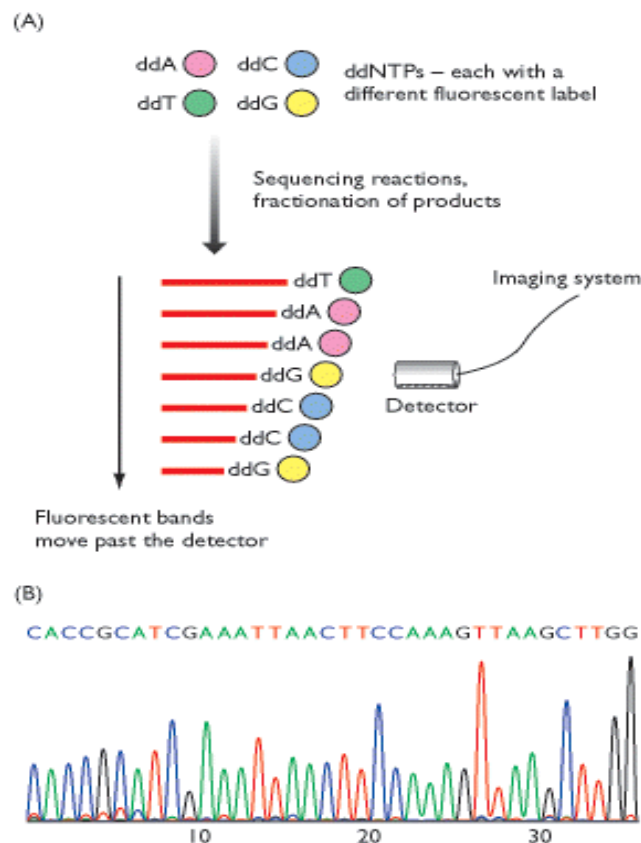
Figure 19: Automated DNA sequencing with fluorescently labeled dideoxynucleotides. (A) The chain termination reactions are carried out in a single tube, with each dideoxynucleotide labeled with a different fluorophore. In the automated sequencer, the bands in the electrophoresis gel move past a fluorescence detector, which identifies which dideoxynucleotide is present in each band. The information is passed to the imaging system. (B) The printout from an automated sequencer. The sequence is represented by a series of peaks, one for each nucleotide position. In this example, a green peak is an 'A', blue is 'C', black is 'G', and red is 'T' (Brown, 1999).

Appendix 6.3 shows an example of the sequencing results.

2.7 Insertion of the hFSHR with different tags in pIRES2-EGFP

2.7.1 Site-directed mutagenesis on pIRES2-EGFP

Site-directed mutagenesis is a method used to make precise alterations in gene sequences. The QuikChange site-directed mutagenesis kit from Stratagene is used to make point mutations and switch, delete or insert amino acids, by using *PfuTurbo*® DNA polymerase and a temperature cycler. This enzyme extends oligonucleotide primers that contain the desired mutation and are complementary to opposite strands of a vector, resulting in the generation of a mutated plasmid (Figure 20). After that, a treatment with 1μL of *Dpn* I endonuclease (20 u/μL), which target sequence is 5'-G_{m6}ATC-3', and incubation of the mixture at 37° for one hour, digests the parental DNA template and leaves the mutated DNA intact. In this way, and after cloning as explained before, only the cells that contain the mutated plasmid will grow, which is verified by DNA sequencing (Brown, 1999 and QuikChange® Site-Directed Mutagenesis Kit instruction manual, 2007).
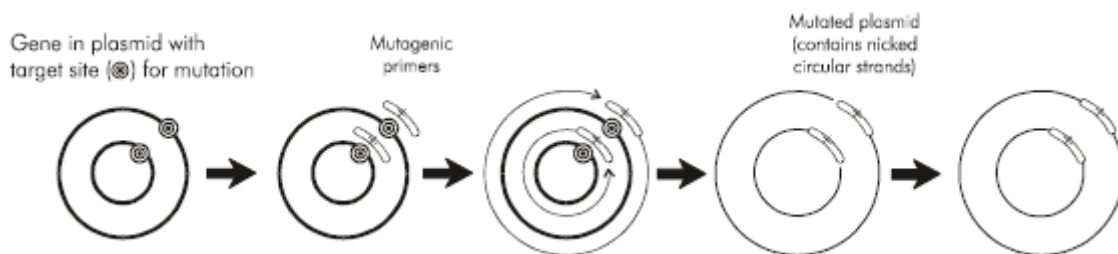
Figure 20: Overview of the QuikChange® site-directed mutagenesis method (QuikChange® Site-Directed Mutagenesis Kit instruction manual, 2007).

The parameters used to perform site-directed mutagenesis on the pIRES2-EGFP vector, in order to knock out the internal *Not* I site indicated in Figure 13, are described in Tables 9, 10 and 11.

This technique has also been used to change to internal *Bam* HI site of the human FSHR, for purposes of posterior procedures, into an *Eco* RI site.

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Table 9: Composition of the reaction mixture used to perform site-directed mutagenesis on the pIRES2-EGFP vector.

Component	Volume (µL)
H$_2$O	33
10x *Pfu* buffer + MgSO$_4$	5
10x dNTPs	4
125 ng/µL Forward primer	2
125 ng/µL Reverse primer	2
25 ng/µL template	3
Pfu enzyme	1

Table 10: Sequence of the primers used to perform site-directed mutagenesis on the pIRES2-EGFP vector (2888 and 2889 – *Not* I site knocked out in bold) and to change to internal *Bam* HI site of the human FSHR into an *Eco* RI site (2915 and 2916 – *Eco* RI site in bold); fw – forward; rv – reverse.

Primer	Sequence of primer
2888 (fw)	5'-AGCTGTACAAGTAAA**GCGGACGC**GACTCTAGATCATAATC-3'
2889 (rv)	5'-GATTATGATCTAGAGTC**GCGTCCGC**TTTACTTGTACAGCT-3'
2915 (fw)	5'-AGATATTTCAAGAACAA**GAATTC**ATTCCCTGCCTAGC-3'
2916 (rv)	5'-GCTAGGCAGGGAAT**GAATTC**TTGTTCTTGAAATATCT-3'

Table 11: PCR program used to perform site-directed mutagenesis on the pIRES2-EGFP vector.

Cycles	Temperature (°C)	Time (s)
1	94	30
	94	15
14	65	15
	72	360
1	72	60
1	4	∞

2.7.2 Construction of pJC1, pJC2 and pJC3

To facilitate the construction of the receptors, the signal sequences and the other tags were cloned into the MCS of the pIRES2-EGFP (Figure 13), by means of PCR reactions, resulting in the following vectors:

➢ pJC1: pIRES2-EGFP with *Bgl* II – Prl s.s.- FLAG tag – *Bam* HI - *Not* I – *Bgl* II
➢ pJC2 : pIRES2-EGFP with *Bgl* II – Prl s.s.- STREP tag – *Bam* HI - *Not* I – *Bgl* II
➢ pJC3 : pIRES2-EGFP with *Bgl* II – hGPα s.s.- STREP tag – *Bam* HI - *Not* I – *Bgl* II

2.7.2 Restriction endonuclease digestion

Restriction endonucleases are enzymes that recognize a specific sequence on a DNA molecule, cleaving it. In this work we needed to insert the constructed hFSHRs into pJC1, pJC2 and pJC3, and for that, digestion with restriction endonucleases was needed. Table 12 shows the enzymes used and table 13 describes an example of the composition of a reaction mixture.

Table 12: Restriction enzymes used in this work, the organism where they come from and the sequence they recognize.

Restriction enzymes	Organism	Recognition sequence
Bgl II	*Bacillus globigii*	AGATCT
Bam HI	*Bacillus amyloliquefaciens*	GGATCC
Eco RI	*Escherichia coli*	GAATTC
Not I	*Nocardia otitidis-caviarum*	GCGGCCGC

First, the wt hFSHR was cloned into the *Bam* HI/ *Not* I sites of pJC1, pJC2 and pJC3. Then, the hFSHR with the different tags were cut with *Bam* HI and *Eco* RI to be inserted in this portion of the vectors. Later, the hFSHR/cf-tail/RHO$_{15}$ was cut with *Eco* RI and *Not* I to be cloned into those sites of the vectors.

For example, pJC1, pJC2 and pJC3 were cut with *Bam* HI and *Not* I, to allow the insertion of *Bam* HI/ *Not* I cut wt hFSHR:

Table 13: Composition of a digestion reaction mixture – x is the volume that corresponds to 5 μg of plasmid in 50 μL of solution and y is the volume of H_2O necessary to complete the total volume.

Composition	Volumes (μL)
5 μg plasmid	x
H_2O	y
NEB3	5
BSA	5
Bam HI	2,5
Not I	2,5

2.7.3 Rapid DNA ligation

In order to ligate the cut fragments into pJC1, pJC2 and pJC3, the Rapid DNA Ligation Kit from Roche can be used, according to the manufacturer's instructions. This kit is supplied with a T4 DNA Ligase, which has the function of repairing single-stranded breaks or discontinuities in double-stranded DNA molecules or, in this case, joining together two individual fragments of double-stranded DNA; it forms a phosphodiester bond between the 3' hydroxyl end of one nucleotide with the 5' phosphate end of another. (Brown, 2001)

Cloning proceeded following steps 2.3.2, 2.4, 2.5 and 2.6.

2.8 Transient expression of constructed receptors in HEK 293-T cells

In order to express the constructed receptors in HEK 293-T cells, a transfection system was used. Transfection is a technique that enables the incorporation of exogenous DNA into cells growing in culture; transcription in these cells allows the DNA to be expressed (Lewin, 2008).

If the FSHR is expressed in a cell, and next stimulated with FSH, it will induce a signal-transduction pathway that leads to the activation of adenylyl cyclase and subsequent production of cAMP. In a ligand-induced cAMP production assay, it is possible to know how much hormone is necessary to activate the receptor.

A technique to test if the receptors are expressed at the cell surface of the cells transfected is the enzyme-linked immunosorbent assay (ELISA). This is a direct binding assay for antibodies or antigens. There is an enzyme that is chemically linked to the antibody, in this case, an antibody recognizing the receptor or a tag attached to the receptor; the unlabeled antigen is attached to the wells of a plastic multi-well microplate. The labeled antibody binds to the unlabeled antigen and any unbound antibody and other proteins are washed away. This binding is detected by a reaction that converts a colorless substrate into a colored reaction product – the color change can be read in a spectrophotometer.

2.9 Construction of mutated receptors

According to the results obtained for the ligand-induced cAMP production assay and for the ELISA, the most optimal construct was chosen. On this construct some mutations were made through site-directed mutagenesis, as described on 2.7.1. The primers used are depicted on Table 14 according to the desired mutation.

Table 14: Primers used for the site-directed mutagenesis; fw – forward; rv – reverse.

Mutation	Primer	Sequence of primer
Ala[419]Thr	3032 (fw)	5'-GGAATCTACCTGCTGCTCATT**A**CATCAGTTGATATCCATACC-3'
	3033 (rv)	5'-GGTATGGATATCAACTGATG**T**AATGAGCAGCAGGTAGATTCC-3'
Arg[573]Cys	3034 (fw)	5'-GACACCAGGATCGCCAAG**T**GCATGGCCATGCTCATCTTC-3'
	3035 (rv)	5'-GAAGATGAGCATGGCCATGC**A**CTTGGCGATCCTGGTGTC-3'
Leu[601]Val	3036 (fw)	5'-GCCTCCCTCAAGGTGCCC**G**TCATCACTGTGTCCAAAGC-3'
	3037 (rv)	5'-GCTTTGGACACAGTGATGA**C**GGGCACCTTGAGGGAGGC-3'

Insertion of the mutated constructs in pIRES2-EGFP, cloning and testing, proceeded according to 2.7.2, 2.7.3 and 2.8.

3. Results and Discussion

3.1 Part 1 – Construction of hFSHRs with different signal sequences and different tags

The FSHR constructs are composed of a signal sequence, one or more tags and the receptor itself, with its own C-terminal tail or with a catfish GnRHR C-terminal tail followed by the last 15 amino acids of human rhodopsin.

The presence of different signal sequences and different tags will be tested to reveal which combination is the most advantageous in the way that enables the best FSHR expression in HEK 293-T cells, and which will be easiest to purify.

The RHO_{15} sequence can act as purification tag as well. However, it is not only useful in purification due to the existence of 1D4, a monoclonal antibody (mAb) that recognizes the last 7 amino acids of human rhodopsin (TSQVAPA), but it is also necessary to get the receptor to be expressed in rod cells, since the RHO_{15} tag of rhodopsin is normally used as a quality control sequence for receptor trafficking to the rod outer segment, because this C-terminal portion is somehow recognized intracellularly in the rod cells. The last 7 amino acids of the human RHO_{15} sequence (STTVSKTA<u>TSQVAPA</u>) are similar to the last 7 amino acids of the zebrafish RHO_{15} sequence (KTEASSVS<u>SSSVSPA</u>) which led us to think that the human sequence can also be recognized in the rod cells of the zebrafish.

In 2007, Thomas *et al.* discovered that hFSHR undergoes proteolytic clipping of its C-terminal tail – mature FSHR was not detected by a mAb directed to an epitope in its C-terminus. Conversely, it is known that the C-terminal of the catfish GnRHR is not clipped and can function to bind G proteins, leading to signal transduction (Navratil *et al.*, 2006).

Once all the FSHR constructs were prepared, they were tested for hFSH-induced cAMP production and cell surface expression in transiently transfected HEK 293-T cells. The aim was to find which construct had a signaling and expression behavior similar to the wild type receptor.

Looking at Figures 21 and 22 it is evident that a few constructs yielded receptor proteins that were not expressed at the cell surface. These results were expected for constructs without a FLAG-tag (17, 21, 29, 33 and 37, either A or B), since the ELISA in this experiment was performed with an antibody against the FLAG tag. However, constructs A5 and B5 (with a single FLAG-tag) were also not expressed at cell surface either, leading us to believe that a single tag would somehow impair the trafficking of the receptor towards the cell surface.

The rest of the constructs seem to function in a proper way with expressions between 60 and 100%.
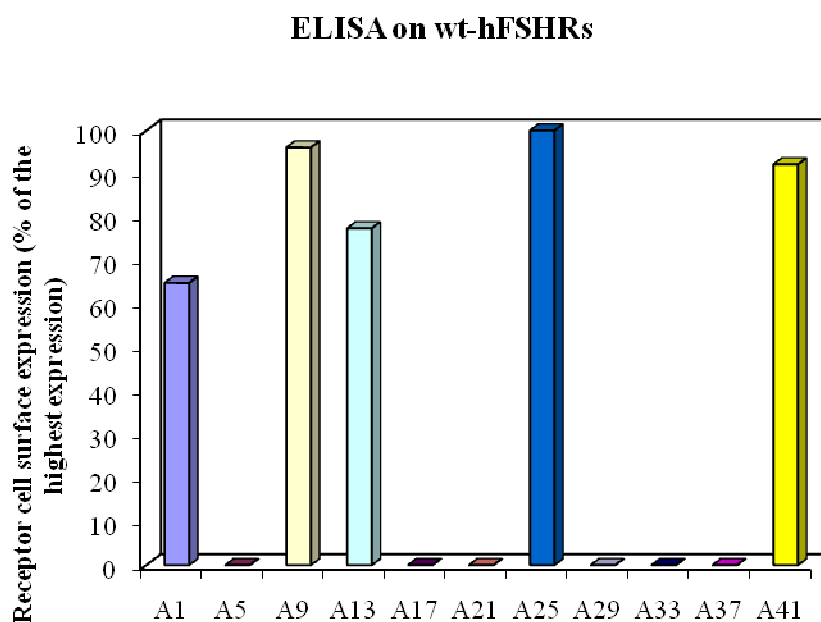
ELISA on wt-hFSHRs

Figure 21: Cell surface expression of various wt-hFSHR constructs with different signal sequences and tags, as determined by FLAG-tag ELISA.

ELISA on hFSHRs/cf-tail/RHO$_{15}$
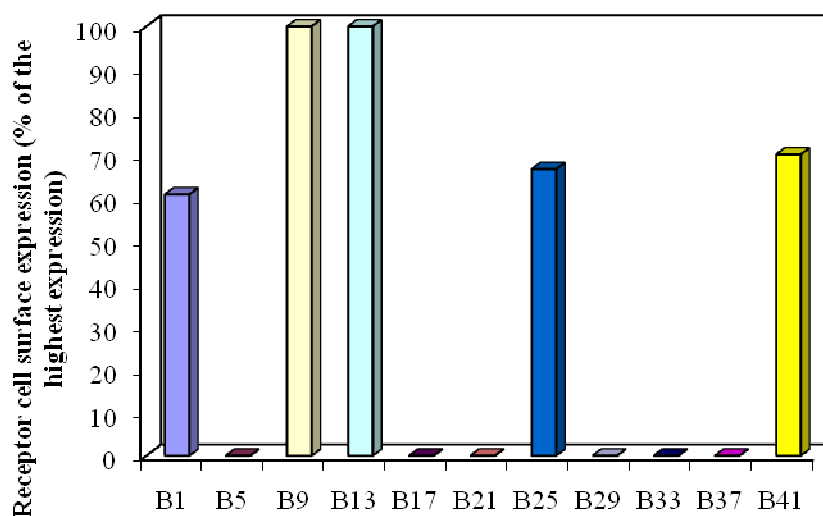
Figure 22: Cell surface expression of various hFSHR/cf-tail/RHO$_{15}$ constructs with different signal sequences and tags, as determined by FLAG-tag ELISA.

The ligand-induced cAMP production revealed that the responsiveness of A/B-5, A/B-17 and A/B-33 to hFSH was decreased, as shown in Table 15. Table 15 presents the EC$_{50}$ values of hFSH for the different receptors and when comparing these (ranging from 0.4821 to 170.3 ng/mL) with the expected value for the wt hFSHR (0.106 ng/mL; Vischer *et al.*, 2003), one can conclude that their signaling is impaired. This was confirmed by their response curves (Figures 23-3/4, 24-1/2 and 25-1/2).

Consistent with the ELISA results, receptors A/B-1, A/B-9, A/B-13, A/B-25 and A/B-41 seem to induce the production of cAMP (Figures 23-1/2/5/6/7/8, 24-5/6 and 25-5/6). However, while the EC$_{50}$ for the A constructs (*i.e.* wt-hFSHR constructs) are relatively close to the EC$_{50}$ of the wt hFSHR, for the B constructs (*i.e.* hFSHR constructs with the cf-tail/RHO$_{15}$ at their C-terminal end) these values are rather high. One possible explanation is that the normal FSHR C-terminal tail is somehow intimately involved in signaling given that in these constructs this portion is replaced by a cfGnRHR-tail/RHO$_{15}$ sequence.

Table 15: Summary of the ligand-induced cAMP production in HEK 293-T cells transiently transfected with wt-hFSHR and hFSHR/cf-tail/RHO_{15} with different signal sequences and different tags. ND – not detectable.

Receptor (wt-hFSHR)	EC_{50} (ng/mL)	Receptor (hFSHR/cf-tail/RHO_{15})	EC_{50} (ng/mL)
A1 - PRL-FLAG-FLAG	0.1160	B1 - PRL-FLAG-FLAG	29.33
A5 - PRL-FLAG	ND	B5 - PRL-FLAG	0.4821
A9 - PRL-FLAG-8xHis	0.1005	B9 - PRL-FLAG-8xHis	1.395
A13 - PRL-FLAG-STREP	0.0749	B13 - PRL-FLAG-STREP	5.440
A17 - PRL-STREP	0.7342	B17- PRL-STREP	35.08
A21- PRL-STREP-8xHis	0.05656	B21- PRL-STREP-8xHis	2.590
A25- PRL-STREP-FLAG	0.1794	B25- PRL-STREP-FLAG	5.683
A29- PRL-STREP-STREP	0.1860	B29- PRL-STREP-STREP	3.567
A33 - hGPα-STREP	3.828	B33- hGPα-STREP	170.3
A37- hGPα-STREP-8xHis	0.1878	B37- hGPα-STREP-8xHis	6.447
A41- hGPα-STREP-FLAG	0.5950	B41- hGPα-STREP-FLAG	4.013

A general analysis of the response curves and the EC_{50} values leads to the conclusion that receptors A9 and B9 (PRL-FLAG-8xHis-wt-hFSHR and PRL-FLAG-8xHis-hFSHR/cf-tail/RHO_{15}, respectively) are the ones most reliably resembling the signaling and expression behavior of the wt hFSHR. Therefore, they were used for the second part of this work.
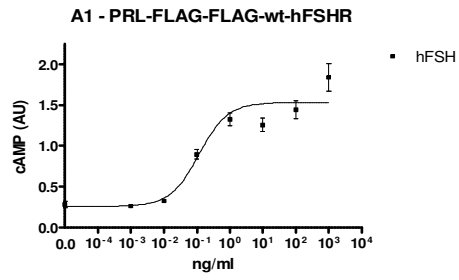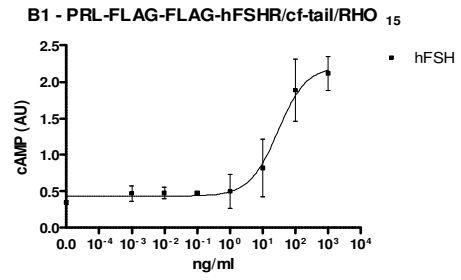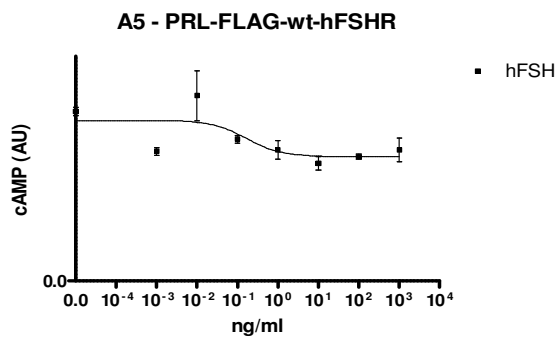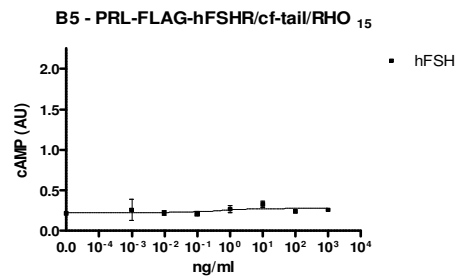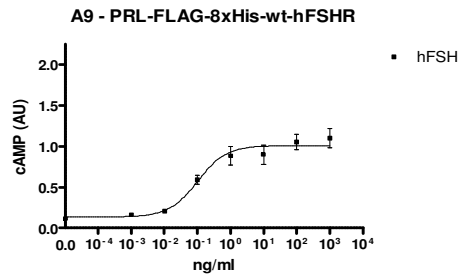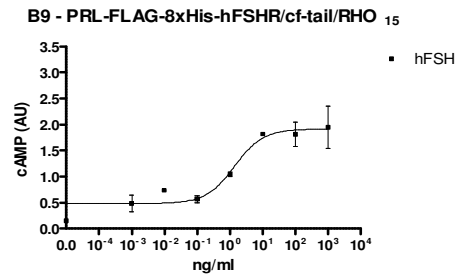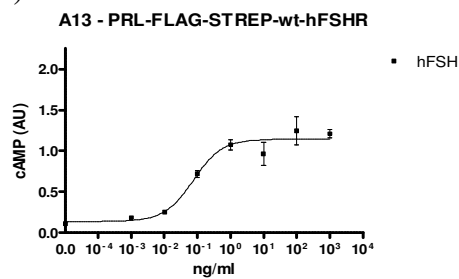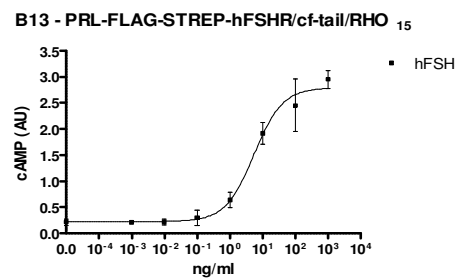
Figure 23: Ligand-induced cAMP production of: 1) A1; 2) B1; 3) A5; 4) B5; 5) A9; 6) B9; 7) A13; 8) B13.

1)

A17 - PRL-STREP-wt-hFSHR

2)

B17 - PRL-STREP-hFSHR/cf-tail/RHO $_{15}$

3)

A21 - PRL-STREP-8xHis-wt-hFSHR

4)

B21 - PRL-STREP-8xHis-hFSHR/cf-tail/RHO $_{15}$

5)

A25 - PRL-STREP-FLAG-wt-hFSHR

6)

B25 - PRL-STREP-FLAG-hFSHR/cf-tail/RHO $_{15}$

7)

9 - PRL-STREP-STREP-wt-hFSHR

8)

B29 - PRL-STREP-STREP-hFSHR/cf-tail/RHO $_{15}$
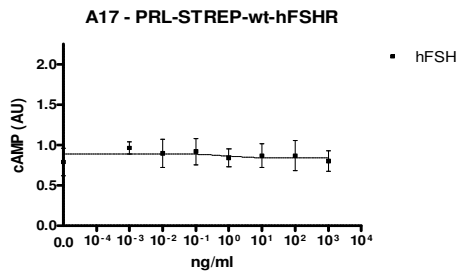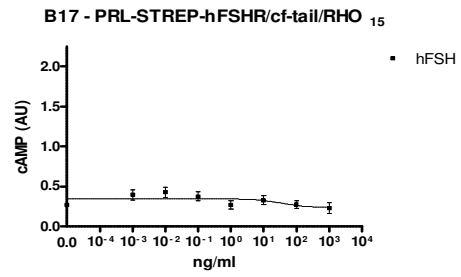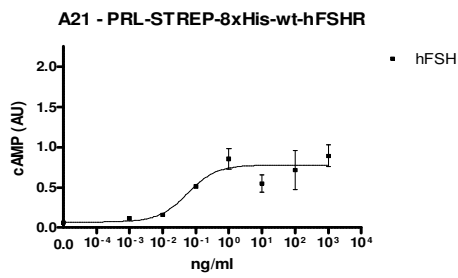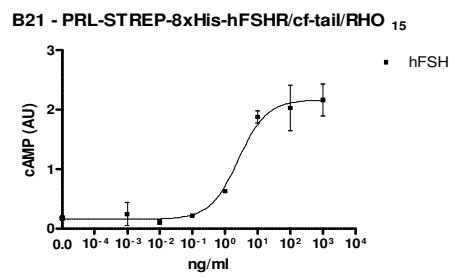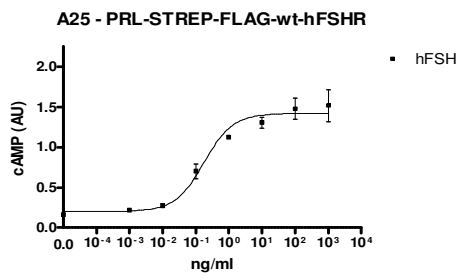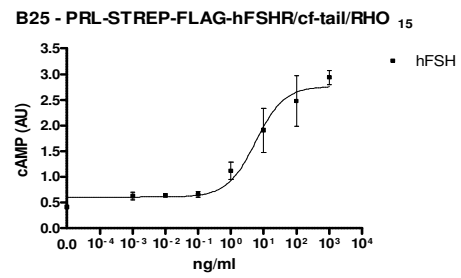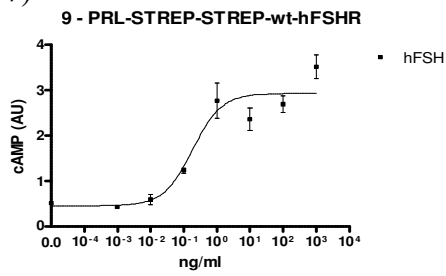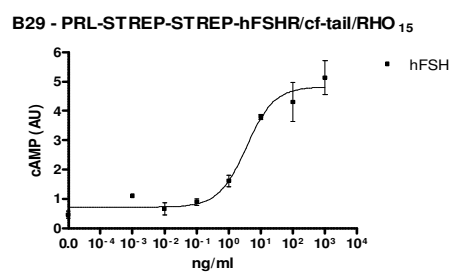
Figure 24: Ligand-induced cAMP production of: 1) A17; 2) B17; 3) A21; 4) B21; 5) A25; 6) B25; 7) A29; 8) B29.
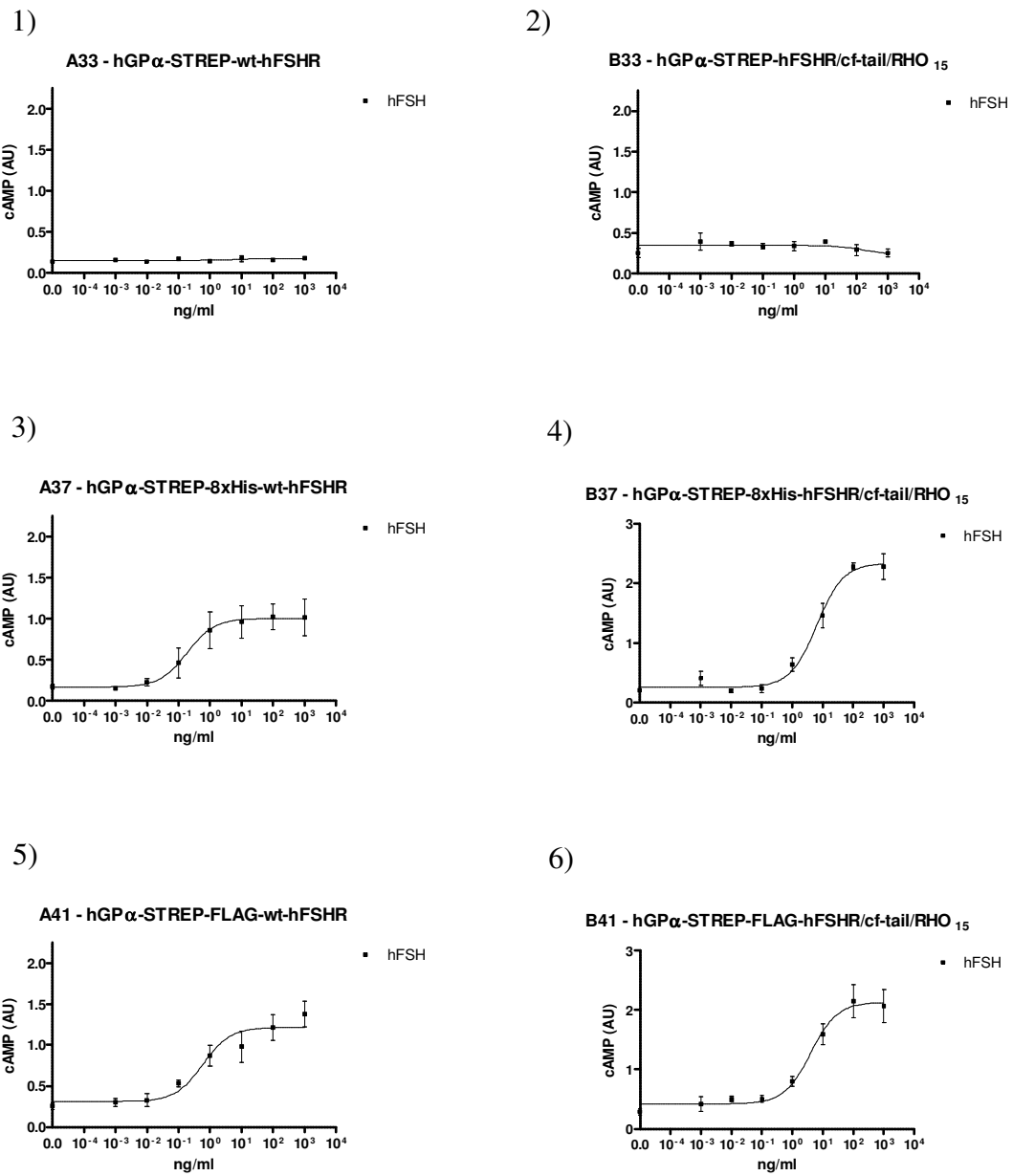
Figure 25: Ligand-induced cAMP production of: 1) A33; 2) B33; 3) A37; 4) B37; 5) A41; 6) B41.

3.2 Part 2 – Construction of mutant hFSHRs

The purpose of generating mutations in the receptors chosen in Part 1 was to create receptor constructs that are not capable of an FSH-induced production of cAMP, but that are well expressed at the cell surface or, in other words, produce receptor constructs that create inactive receptors. The proposed mutations are located in the transmembrane domain, which is known to be involved in signaling.

Unfortunately, we came to the conclusion that the ELISA was not trustworthy since the procedure that has been used in this laboratory was optimized for another type of tag. Due to lack of time, it was not possible to develop a new protocol and so the experiment could not be repeated. Nonetheless, the results obtained are shown in Figures 26 and 27.
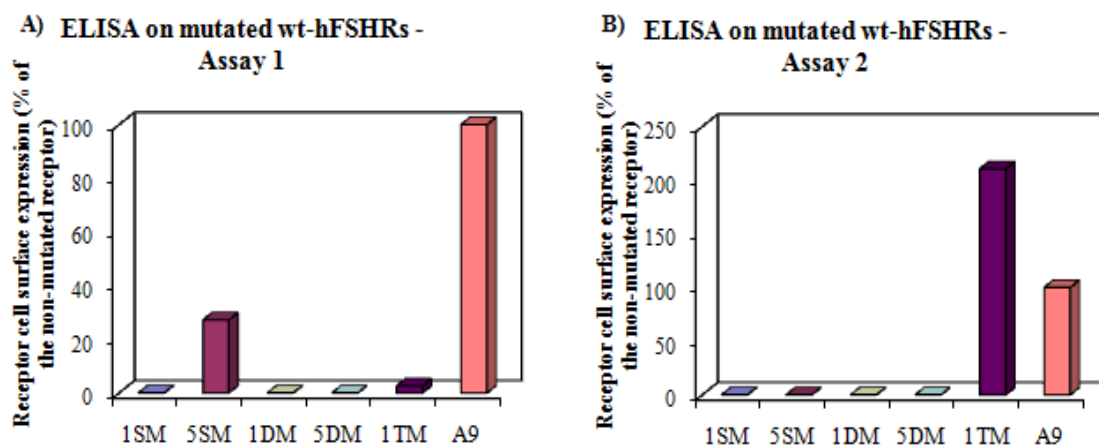
Figure 26: Cell surface expression of FLAG-tagged wt-hFSHR with the different mutations, as determined by FLAG-tag ELISA. A) and B) are duplicates, performed under the same conditions.
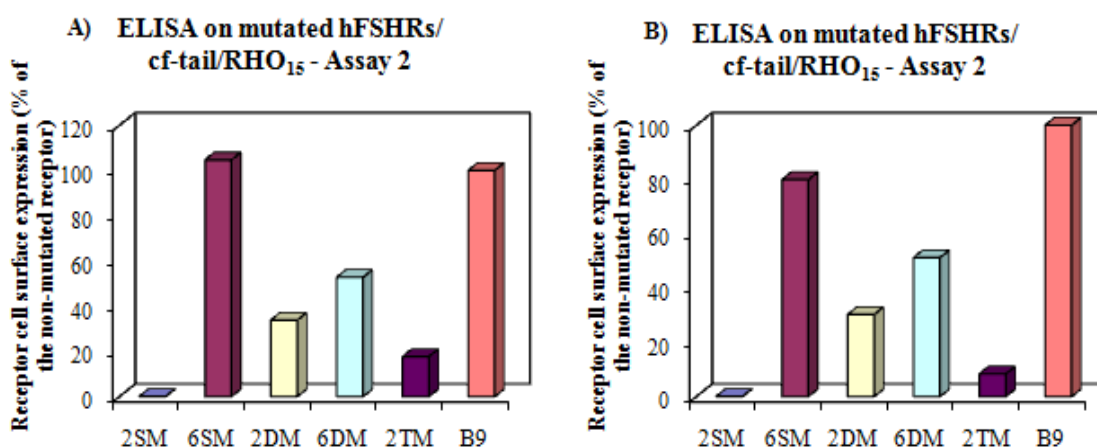
Figure 27: Cell surface expression of FLAG-tagged hFSHR/cf-tail/RHO$_{15}$ with the different mutations, as determined by FLAG-tag ELISA. A) and B) are duplicates, performed under the same conditions.

Although the mutants with Arg^{573}Cys (3SM and 4SM) and Arg^{573}Cys/Leu^{601}Val (3DM and 4DM) could not be tested because they contained sequence mistakes, it can be said that according to their EC$_{50}$ values (Table 16) and their response curves (Figures 28 and 29), one mutation is not enough to fully inactivate a receptor. But when two or three mutations are introduced, there is a drastic change in the receptor's response and the signaling is either decreased or even completely impaired. However, this can be due to the absence of the receptor at the cell surface (which could not be trustworthy determined; see above) or to the fact that the receptor is indeed inactive. The only way to confirm one of these hypotheses is through an ELISA. In case this procedure could have been repeated and if it showed that for example the PRL-FLAG-8xHis-hFSHR/cf-tail/RHO$_{15}$ with the three mutations (Ala^{419}Thr/Arg^{573}Cys/Leu^{601}Val) was expressed at the cell surface, we would have created an inactive receptor construct that could be used for receptor expression in zebrafish rod cells, followed by protein purification from zebrafish eyes/rod cells, and next protein crystallization trials. The latter studies are extremely difficult to perform with GPCRs because one needs a single protein species as input material for crystallization trials. Each GPCR is thought to exist in different states of activation (*i.e.* a single protein species), even in the absence of ligand. Therefore such a purified batch of GPCRs may contain different protein species, each with a different level of intrinsic signalling activity. Introducing the above mentioned

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mutations aimed at producing a batch of "silent receptor" proteins, each in the same state of receptor activation.

Table 16: Summary of the ligand-induced cAMP production in HEK 293-T cells transiently transfected with PRL-FLAG-8xHis-wt-hFSHR and PRL-FLAG-8xHis-hFSHR/cf-tail/RHO$_{15}$ with different mutations. ND – not detectable. NA – not applicable.

Construct code	Receptor	Mutations	EC$_{50}$ (ng/mL)
1SM		Ala^{419}Thr	3.160
5SM		Leu^{601}Val	0.1108
1DM	PRL-FLAG-8xHis-wt-hFSHR	Ala^{419}Thr/Arg^{573}Cys	0.0002
5DM		Leu^{601}Val/Ala^{419}Thr	147.8
1TM		Ala^{419}Thr/Arg^{573}Cys/Leu^{601}Val	0.0201
A9		NA	0.2268
2SM		Ala^{419}Thr	2.927
6SM		Leu^{601}Val	1.071
2DM	PRL-FLAG-8xHis-hFSHR/cf-tail/RHO$_{15}$	Ala^{419}Thr/Arg^{573}Cys	ND
6DM		Leu^{601}Val/Ala^{419}Thr	25.92
2TM		Ala^{419}Thr/Arg^{573}Cys/Leu^{601}Val	ND
B9		NA	1.332
B21 (mock)	PRL-STREP-8xHis-hFSHR/cf-tail/RHO$_{15}$	NA	1.433
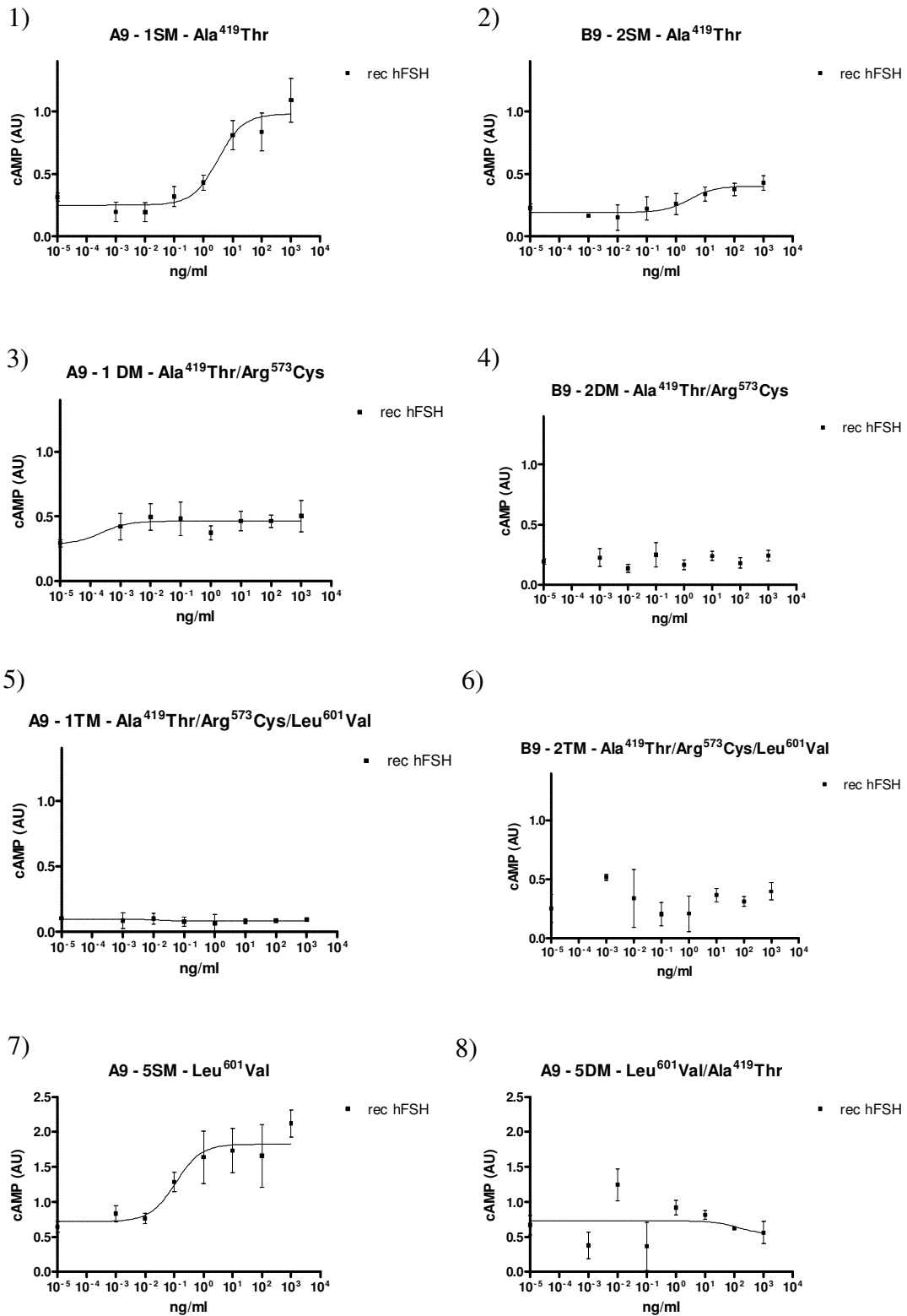
Figure 28: Ligand-induced cAMP production of mutant receptors when stimulated by hFSH: 1) 1SM; 2) 2SM; 3) 1DM; 4) 2DM; 5) 1TM; 6) 2TM; 7) 5SM and 8) 5DM.
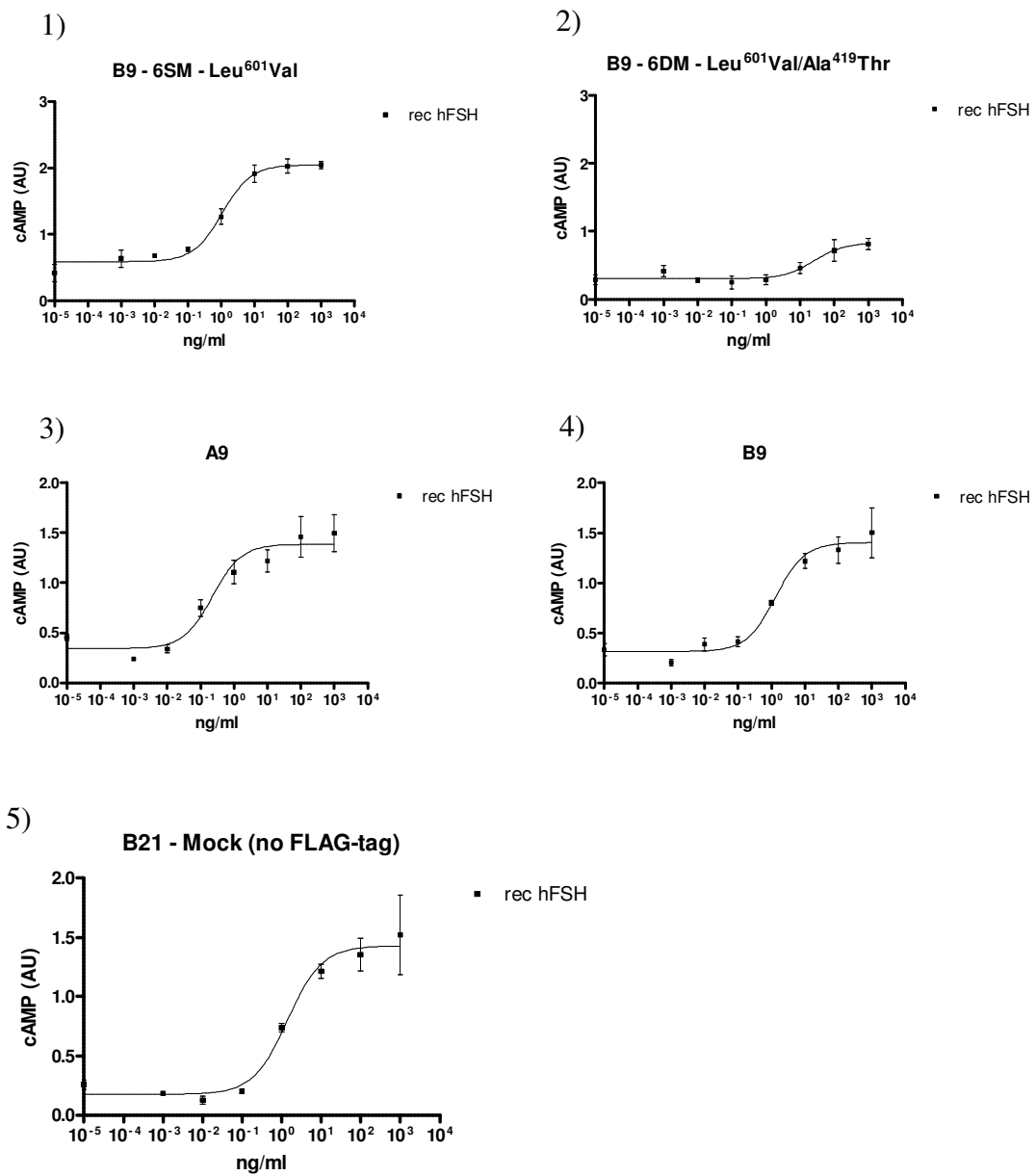
Figure 29: Ligand-induced cAMP production of: 1) 6SM; 2) 6DM; 3) A9; 4) B9 and 5) B21 (mock), when stimulated by hFSH.

4. Conclusion and Future Perspectives

The studies described in this thesis had the objective of identifying a hFSHR construct that is expressed in HEK 293-T cells. Next, such a construct will be expressed in rod cells of the zebrafish to try to isolate this protein for purification. Some signal sequences and tags were used and mentioned here, but receptors with other such sequences, like Cystatin signal sequence, a T7-tag and MYC-tag, have also been constructed and await testing.

Had the ELISA procedure worked well, we would possibly now have receptors ready to be used for the transgenesis studies. Because the receptors were constructed with a RHO_{15} sequence, the zebrafish will produce the mutant hFSHR as if it was rhodopsin and hopefully large quantities of protein will be present in their retinas.

Several potential steps of purification will then to be tested to see which one is useful:

➢ ROS purification as described by Liang *et al.*, 2003;

➢ Sucrose gradient (Hargrave, 1982);

➢ Concanavalin A affinity chromatography (Litman, 1982);

➢ Hydroxyapatite chromatography (Hong, 1982);

➢ 1D4 affinity (Molday and MacKenzie, 1983);

➢ Solubilization with mild detergents and divalent cations.

These purifications steps are expected to yield sufficient amount of protein that will be used towards obtaining detailed structural information on full-length hFSHR.

5. References

Aitomäki K, Lucena JLD, Pakarinen P, Sistonen P, Tapanainen J, Gromoll J, Kaskikari R, Sankila EM, Lehväslaiho H, Engel AR, Nieschlag E, Huhtaniemi I and Chapelle A **1995**. Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. Cell 82: 959-968.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P **2002**. Molecular Biology of the Cell, 4th edition, Garland Science, USA.

Allen LA, Achermann JC, Pakarinen P, Kotlar TJ, Huhtaniemi IT, Jameson JL, Cheetham TD and Ball SG **2003**. A novel loss of function mutation in exon 10 of the FSH receptor gene causing hypergonadotrophic hypogonadism: clinical and molecular characteristics. Human Reproduction 18 (2): 251-256.

Amsterdam A and Becker TS **2005**. Transgenes as screening tools to probe and manipulate the zebrafish genome. Developmental Dynamics 234: 255-268.

Ballesteros J and Palczewski K **2001**. G protein-coupled receptor drug discovery: Implications from the crystal structure of rhodopsin. Current Opinion in Drug Discovery and Development 4 (5): 561-574.

Beau I, Touraine P, Meduri G, Gougeon A, Desroches A, Matuchansky C, Milgrom E, Kuttenn F and Misrahi M 1998. A novel phenotype related to partial loss of function mutations of the follicle stimulating hormone receptor. The Journal of Clinical Investigation 102 (7): 1352-1359.

Bogerd J, Granneman JC, Schulz RW, Vischer HF **2005**. Fish FSH receptors bind LH: how to make the human FSH receptor to be more fishy? General Comparative Endocrinology 142(1-2): 34-43.

Bogerd J 2007. Ligand-selective determinants in gonadotropin receptors. Molecular and Cellular Endocrinology 260–262: 144-152.

Brown TA 1999 Genomes, 1st edition, BIOS Scientific Publishers Ltd, UK.

Brown TA 2001 Gene cloning and DNA analysis – An introduction, 4th edition, Blackwell Science Ltd, UK.

Cooper GM 2000. The Cell: A Molecular Approach, 2nd edition, Sinauer Associates, Inc., USA.

Dias JA and Roey P **2001**. Structural biology of human follitropin and its receptor. Archives of Medical Research 32: 510-519.

Doherty E, Pakarinen P, Tiitinen A, kiilavuori A, Huhtaniemi I, Forrest S and Aittomäki K **2002**. A novel mutation in the FSH receptor inhibiting signal transduction and causing primary ovarian failure. The Journal of Clinical Endocrinology & Metabolism 87 (3): 1151-1155.

Durme J, Horn F, Costagliola S, Vriend G and Vassart G **2006**. GRIS: Glycoprotein-hormone receptor information system. Molecular Endocrinology 20 (9): 2247-2255.

Fan QR and Hendrickson WA 2005. Structure of human follicle-stimulating hormone in complex with its receptor. Nature 433: 269-277.

Fan QR and Hendrickson WA 2007. Assembly and structural characterization of an authentic complex between human follicle stimulating hormone and a hormone-binding ectodomain of its receptor. Molecular and Cellular Endocrinology 260–262: 73-82.

Filipek S, Stenkamp RE, Teller DC and Palczewski K **2003**. G protein-coupled receptor rhodopsin: a prospectus. Annual Review of Physiology 65: 851-879.

Fox KM, Dias JA and Roey P **2001**. Three-dimensional structure of human follicle-stimulating hormone. Molecular Endocrinology 15 (3): 378-389.

Gether U 2000. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocrine Reviews 21 (1): 90-113.

Gromoll J, Schulz A, Borta H, Gudermann T, Teerds KJ, Greschniok A, Nieschlag E and Seif FJ **2002**. Homozygous mutation within the conserved Ala-Phe-Asn-Glu-Thr motif of exon 7 of the LH receptor causes male pseudohermaphroditism. European Journal of Endocrinology 147: 597-608.

Hargrave PA 1982. Rhodopsin chemistry, structure and topography. Prog. Retinal Res. (1): 1-51.

Hong K, Knudsen PJ, Hubbell WL **1982**. Purification of rhodopsin on hydroxyapatite columns, detergent exchange, and recombination with phospholipids. Methods in Enzymology (81): 144-50.

Kawakami K, Takeda H, Kawakami N, Kobayashi M, Matsuda N and Mishina M **2004**. A transposon-mediated gene trap technique approach identifies developmentally regulated genes in zebrafish. Developmental Cell 7: 133-144.

Kawakami K 2005. Transposon tools and methods in zebrafish. Developmental Dynamics 234: 244-254.

Kelton CA, Cheng SVY, Nugent NP, Schweickhardt RL, Rosenthal JL, Overton SA, Wands GD, Kuzeja JB, Luchette CA and Chappel SC **1992**. The cloning of the human follicle stimulating hormone receptor and its expression in COS-7, CHO, and Y-l cells. Molecular and Cellular Endocrinology 89: 141-151.

Kobe B and Deisenhofer J 1993. Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. Nature 366 (6457): 751-756.

Kobilka BK 1995. Amino and carboxyl terminal modifications to facilitate the production and purification of a G protein-coupled receptor. Analytical Biochemistry 231: 269-271.

Kobilka BK and Deupi X **2007**. Conformational complexity of G-protein-coupled receptors. Trends in Pharmacological Sciences 28 (8): 397-406.

Kobilka B and Schertler GFX **2007**. New G-protein-coupled receptor crystal structures: insights and limitations. Trends in Pharmacological Sciences 29 (2): 79-83.

Kodama T, Imai H, Doi T, Chisaka O, Shichida Y and Fujiyoshi Y **2005**. Expression and localization of an exogenous G protein-coupled receptor fused with the rhodopsin C-terminal sequence in the retinal rod cells of knockin mice. Experimental Eye Research 80: 859-869.

Laan M, Richmond H, He C and Campbell RK **2002**. Zebrafish as a model for vertebrate reproduction: characterization of the first functional zebrafish (*Danio rerio*) gonadotropin receptor. General and Comparative Endocrinology 125: 349-364.

Lewin B 2008 Genes IX, international edition, Jones and Bartlett Publishers, Inc, USA.

Li N, Salom D, Zhang L, Harris T, Ballesteros JA, Golczak M, Jastrzebska B, Palczewski K, Kurahara C, Juan T, Jordan S and Salon JA **2007**. Heterologous Expression of the Adenosine A1 Receptor in Transgenic Mouse Retina. Biochemistry 46 (28): 8350-8359.

Liang Y, Fotiadis D, Filipek S, Saperstein DA, Palczewski K and Engel A **2003**. Organization of the G protein-coupled receptors rhodopsin and opsin in native membranes. The Journal of Biological Chemistry 278 (24): 21655-21662.

Litman BJ 1982. Purification of rhodopsin by concanavalin A affinity chromatography. Methods in Enzymology 81:150-153.

Lundstrom K, Wagner R, Reinhart C, Desmyter A, Cherouati N, Magnin T, Zeder-Lutz G, Courtot M, Prual C, André N, Hassaine G, Michel H, Cambillau C and Pattus F **2006**. Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems. Journal of Structural and Functional Genomics 7: 71-91.

McClintock B 1950. The origin and behavior of mutable *loci* in maize. Genetics 36: 344-355.

McCusker EC, Bane SE, O'Malley MA and Robinson AS **2007**. Heterologous GPCR Expression: A Bottleneck to Obtaining Crystal Structures Biotechnology Progress 23: 540-547.

Meduri G, Bachelot A, Cocca MP, Vasseur C, Rodien P, Kuttenn F, Touraine P, Misrahi M **2008**. Molecular pathology of the FSH receptor: new insights into FSH physiology. Molecular and Cellular Endocrinology 282: 130-142.

Molday RS, MacKenzie D **1983**. Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes. Biochemistry 22 (3): 653-660.

Navratil A, Farmerie TA, Bogerd J, Nett TM amd Clay CM **2006**. Differential impact of intracellular carboxyl terminal domains on lipid raft localization of the murine gonadotropin-releasing hormone receptor. Biology of Reproduction 74: 788-797.

Okada T, Ernst OP, Palczewski K and Hofmann KP **2001**. Activation of rhodopsin: new insights from structural and biochemical studies. Trends in Biochemical Sciences 26: 318-324.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M **2000**. Crystal structure of rhodopsin: a G protein-coupled receptor. Science 289: 739-745.

Park SH, Lodowski DT and Palczewski K **2008**. Activation of G protein–coupled receptors: beyond two-state models and tertiary conformational changes. Annual Review of Pharmacology and Toxicology 48: 107-141.

Perkins BD, Kainz PM, O'Malley DM and Dowling JE **2002**. Transgenic expression of a GFP-rhodopsin COOH-terminal fusion protein in zebrafish rod photoreceptors. Visual Neuroscience 19: 257-264.

Rannikko A, Pakarinen P, Manna PR, Beau I, Misrahi M, Aittomäki K and Huhtaniemi I **2002**. Functional characterization of the human FSH receptor with an inactivating Ala189Val mutation. Molecular Human Reproduction 8 (4): 311-317.

Rasmussen SGF, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VRP, Sanishvili R, Fischetti RF, Schertler GFX, Weis WI and Kobilka BK **2007**. Crystal structure of the human β2-adrenergic G-protein-coupled receptor. Nature 450: 383-388.

Ridge KD and Palczewski K **2007**. Visual rhodopsin sees the light: structure and mechanism of G protein signaling. The Journal of Biological Chemistry 282 (13): 9297-9301.

Sakmar TP 2002. Structure of rhodopsin and the superfamily of seven-helical receptors: the same and not the same. Current Opinion in Cell Biology 14: 189-195.

Salom D, Wu N, Sun W, Dong Z, Palczewski K, Jordan S and Salon JA **2008**. Heterologous expression and purification of the serotonin type 4 receptor from transgenic mouse retina. Biochemistry 47 (50): 13296-13307.

Salom D, Trong I, Pohl E, Ballesteros JA, Stenkamp RE, Palczewski K and Lodowski DT **2006**. Improvements in G protein-coupled receptor purification yield light stable rhodopsin crystals. Journal of Structural Biology 156: 497-504.

Schertler GFX 2005. Structure of rhodopsin and the metarhodopsin I photointermediate. Current Opinion in Structural Biology 15: 408-415.

Simoni M, Gromoll J and Nieschlag E **1997**. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocrine Reviews 18 (6): 739-773.

Smits G, Campillo M, Govaerts C, Janssens V, Richteri C, Vassart G, Pardo L and Costagliola S **2003**. Glycoprotein hormone receptors: determinants in leucine-rich repeats responsible for ligand specificity. The EMBO Journal 22 (11): 2692-2703.

Stenkamp RE, Teller DC and Palczewski K **2002**. Crystal structure of rhodopsin: a G-protein-coupled receptor. ChemBioChem 3: 963-967.

Strachan T and Read AP **1999**. Human Molecular Genetics 2, 2nd edition, BIOS Scientific Publishers Ltd., USA.

Tai AW, Chuang JZ, Bode C, Wolfrum U and Sung CH **1999**. Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. Cell 97: 877-887.

Tam BM, Moritz OL, Hurd LB and Papermaster DS **2000**. Identification of an outer segment targeting signal in the COOH terminus of rhodopsin using transgenic *Xenopus laevis*. The Journal of Cell Biology 151 (7): 1369-1380.

Tao YX 2006. Inactivating mutations of G protein-coupled receptors and diseases: structure-function insights and therapeutic implications. Pharmacology & Therapeutics 111: 949-973.

Thomas RM, Nechamen CA, Mazurkiewicz JE, Muda M, Palmer S and Dias JA **2007**. Follice-stimulating hormone receptor forms oligomers and shows evidence of carboxyl-terminal proteolytic processing. Endocrinology 148 (5): 1987-1995.

Touraine P, Beau I, Gougeon A, Meduri G, Desroches A, Pichard C, Detoeuf M, Paniel B, Prieur M, Zorn JR, Milgrom E, Kuttenn F and Miseahi M **1999**. New natural inactivating mutations of the follicle-stimulating hormone receptor: correlations between receptor function and phenotype. Molecular Endocrinology 13 (11): 1884-1854.

Vander A, Sherman J and Luciano D **2001**. Human Physiology, 8th edition, McGraw-Hill, USA, pp. 638-648.

Vassart G, Pardo L and Costagliola S **2004**. A molecular dissection of the glycoprotein hormone receptors. Trends in Biochemical Sciences 29 (3): 119-126.

Vischer HF, Granneman JCM, Noordam MJ, Mosselman S and Bogerd J **2003**. Ligand Selectivity of Gonadotropin Receptors. Role of the β-strands of extracellular leucine-rich repeats 3 and 6 of the human luteinizing hormone receptor. The Journal of Biological Chemistry 278 (18): 15505-15513.

Zhang L, Salom D, He J, Okun A, Ballesteros J, Palczweski K and Li N **2005**. Expression of functional G protein-coupled receptors in photoreceptors of transgenic *Xenopus laevis*. Biochemistry 44: 14509-14518.

6.1 Sequence of the human FSHR

```
                              Kozak
            CGC XXX XXX  GCC ACC  ATG GCC CTG CTC CTG GTC TCT TT->
                                  ATG GCC CTG CTC CTG GTC TCT TTG CTG GCA
                                   M   A   L   L   L   V   S   L   L   A      10
                                  tac cgg gac gag gac cag aga aac gac cgt
```

```
            TTC CTG AGC TTG GGC TCA GGA  TAC CCC TAC GAC GTG CCC GAC TAC GCC
             F   L   S   L   G   S   G    Y   P   Y   D   V   P   D   Y   A       17
            aag gac tcg aac ccg agt cct  atg ggg atg ctg cac ggg ctg atg cgg
```

primer 2918 (hFSHR [ORF]-Fw primer; initial 69.4°C; *Bam* HI site; NNNNNN=GGA'TCC'CAC'CAC'CAC'CAC'CAT'CAT'CAT'CAC'GGC; **8 X HIS** plus Glycine)
primer 2919 (hFSHR [ORF]-Fw primer; initial 69.4°C; *Bam* HI site; NNNNNN=GGA'TCC'GAT'TAC'AAA'GAT'GAT'GAT'GAT'GTC'GGC; **Flag tag** plus Glycine)
primer 2920 (hFSHR [ORF]-Fw primer; initial 69.4°C; *Bam* HI site; NNNNNN=GGA'TCC'TGG'TCC'CAT'CCC'CAG'TTT'GAG'AAG'GGC; **Strep tag** plus Glycine)
CGC**NNNNNN** TGT CAT CAT CGG ATC TGT CAC TGC TCT AA->
         primer 2917 (hFSHR [ORF]-Fw primer; initial 69.4°C; *Bam* HI site)
CGC**GGATCC** TGT CAT CAT CGG ATC TGT CAC TGC TCT AA->

         primer 1540 (hFSHR [ECD]-Fw primer; initial 66.6°C, later 78.6°C; *Bam* HI site)
GGC**GGATCC** TGT CAT CAT CGG ATC TGT CAC TGC TCT AA->

```
            TGT CAT CAT CGG ATC TGT CAC TGC TCT AAC AGG GTT TTT CTC TGC CAA
             C   H   H   R   I   C   H   C   S   N   R   V   F   L   C   Q       33
            aca gta gta gcc tag aca gtg ac gaga ttg tcc caa aaa gag acg gtt
```

```
            GAG AGC AAG GTG ACA GAG ATT CCT TCT GAC CTC CCG AGG AAT GCC ATT
             E   S   K   V   T   E   I   P   S   D   L   P   R   N   A   I       49
            ctc tcg ttc cac tgt ctc taa gga aga ctg gag ggc tcc tta cgg taa
```

```
            GAA CTG AGG TTT GTC CTC ACC AAG CTT CGA GTC ATC CAA AAA GGT GCA
     β1      E   L   R   F   V   L   T   K   L   R   V   I   Q   K   G   A       65
            ctt gac tcc aaa cag gag tgg ttc gaa gct cag tag gtt ttt cca cgt
```

```
            TTT TCA GGA TTT GGG GAC CTG GAG AAA ATA GAG ATC TCT CAG AAT GAT
     β2      F   S   G   F   G   D   L   E   K   I   E   I   S   Q   N   D       81
            aaa agt cct aaa ccc ctg gac ctc ttt tat ctc tag aga gtc tta cta
```

```
            GTC TTG GAG GTG ATA GAG GCA GAT GTG TTC TCC AAC CTT CCC AAA TTA
             V   L   E   V   I   E   A   D   V   F   S   N   L   P   K   L       97
            cag aac ctc cac tat ctc cgt cta cac aag agg ttg gaa ggg ttt aat
```

```
            CAT GAA ATT AGA ATT GAA AAG GCC AAC AAC CTG CTC TAC ATC AAC CCT
     β3      H   E   I   R   I   E   K   A   N   N   L   L   Y   I   N   P       113
            gta ctt taa tct taa ctt ttc cgg ttg ttg gac gag atg tag ttg gga
```

```
            GAG GCC TTC CAG AAC CTT CCC AAC CTT CAA TAT CTG TTA ATA TCC AAC
     β4      E   A   F   Q   N   L   P   N   L   Q   Y   L   L   I   S   N       129
            ctc cgg aag gtc ttg gaa ggg ttg gaa gtt ata gac aat tat agg ttg
```

```
            ACA GGT ATT AAG CAC CTT CCA GAT GTT CAC AAG ATT CAT TCT CTC CAA
             T   G   I   K   H   L   P   D   V   H   K   I   H   S   L   Q       145
            tgt cca taa ttc gtg gaa ggt cta caa gtg ttc taa gta aga gag gtt
```

```
            AAA GTT TTA CTT GAC ATT CAA GAT AAC ATA AAC ATC CAC ACA ATT GAA
     β5      K   V   L   L   D   I   Q   D   N   I   N   I   H   T   I   E       161
            ttt caa aat gaa ctg taa gtt cta ttg tat ttg tag gtg tgt taa ctt
```

```
       AGA AAT TCT TTC GTG GGG CTG AGC TTT GAA AGT GTG ATT CTA TGG CTG
β6      R   N   S   F   V   G   L   S   F   E   S   V   I   L   W   L        177
       tct tta aga aag cac ccc gac tcg aaa ctt tca cac taa gat acc gac


       AAT AAG AAT GGG ATT CAA GAA ATA CAC AAC TGT GCA TTC AAT GGA ACC
β6     N   K   N   G   I   Q   E   I   H   N   C   A   F   N   G   T       193
       tta ttc tta ccc taa gtt ctt tat gtg ttg aca cgt aag tta cct tgg


       CAA CTA GAT GAG CTG AAT CTA AGC GAT AAT AAT AAT TTA GAA GAA TTG
β7     Q   L   D   E   L   N   L   S   D   N   N   N   L   E   E   L       209
       gtt gat cta ctc gac tta gat tcg cta tta tta tta aat ctt ctt aac


       CCT AAT GAT GTT TTC CAC GGA GCC TCT GGA CCA GTC ATT CTA GAT ATT
β8     P   N   D   V   F   H   G   A   S   G   P   V   I   L   D   I       225
       gga tta cta caa aag gtg cct cgg aga cct ggt cag taa gat cta taa
```

                          primer 1543 (*Bam* HI knock out-Fw; 72.0°C; overlap = 61.4°C)
                   AGA ACA AGG ATT CAT TCC CTG CCT AGC TAT GGC TT–>
                    primer 2915 (*Bam* HI to *Eco* RI; Fw; 69.5°C)
             TCA AGA ACA AGA ATT CAT TCC CTG CCT AGC–>

```
       TCA AGA ACA AGG ATC CAT TCC CTG CCT AGC TAT GGC TTA GAA AAT CTT
β8     S   R   T   R   I   H   S   L   P   S   Y   G   L   E   N   L       241
       agt tct tgt tcc tag gta agg gac gga tcg ata ccg aat ctt tta gaa
       AGT TCT TGT TCT TAA GTA AGG GAC GGA TCG–5′
             primer 2916 (Bam HI to Eco RI; Rv; 69.5°C)
       agt tct tgt tcc taa gta agg gac gg
           primer 1542 (Bam HI knock out-Rv; 68.3°C; overlap = 61.4°C)
```

```
                                                                      ACT
       AAG AAG CTG AGG GCC AGG TCG ACT TAC AAC TTA AAA AAG CTG CCT ACT
β9     K   K   L   R   A   R   S   T   Y   N   L   K   K   L   P   T       257
       ttc ttc gac tcc cgg tcc agc tga atg ttg aat ttt ttc gac gga tga


       CTG GAA AAG CTT GTC GCC CTC ATG GAA GCC AGC CTC ACC TAT CCC AGC
       L   E   K   L   V   A   L   M   E   A   S   L   T   Y   P   S       273
       gac ctt ttc gaa cag cgg gag tac ctt cgg tcg gag tgg ata ggg tcg


       CAT TGC TGT GCC TTT GCA AAC TGG AGA CGG CAA ATC TCT GAG CTT CAT
       H   C   C   A   F   A   N   W   R   R   Q   I   S   E   L   H       289
       gta acg aca cgg aaa cgt ttg acc tct gcc gtt tag aga ctc gaa gta


       CCA ATT TGC AAC AAA TCT ATT TTA AGG CAA GAA GTT GAT TAT ATG ACT
       P   I   C   N   K   S   I   L   R   Q   E   V   D   Y   M   T       305
       ggt taa acg ttg ttt aga taa aat tcc gtt ctt caa cta ata tac tga


       CAG GCT AGG GGT CAG AGA TCC TCT CTG GCA GAA GAC AAT GAG TCC AGC
       Q   A   R   G   Q   R   S   S   L   A   E   D   N   E   S   S       321
       gtc cga tcc cca gtc tct agg aga gac cgt ctt ctg tta ctc agg tcg


       TAC AGC AGA GGA TTT GAC ATG ACG TAC ACT GAG TTT GAC TAT GAC TTA
       Y   S   R   G   F   D   M   T   Y   T   E   F   D   Y   D   L       337
       atg tcg tct cct aaa ctg tac tgc atg tga ctc aaa ctg ata ctg aat


       TGC AAT GAA GTG GTT GAC GTG ACC TGC TCC CCT AAG CCA GAT GCA TTC
       C   N   E   V   V   D   V   T   C   S   P   K   P   D   A   F       353
       acg tta ctt cac caa ctg cac tgg acg agg gga ttc ggt cta cgt aag
```

```
AAC CCA TGT GAA GAT ATC ATG GGG TAC AAC ATC CTC AGA GTC CTG ATA
 N   P   C   E   D   I   M   G   Y   N   I   L   R   V   L   I     369
ttg ggt aca ctt cta tag tac ccc atg ttg tag gag tct cag gac tat


TGG TTT ATC AGC ATC CTG GCC ATC ACT GGG AAC ATC ATA GTG CTA GTG
 W   F   I   S   I   L   A   I   T   G   N   I   I   V   L   V     385
acc aaa tag tcg tag gac cgg tag tga ccc ttg tag tat cac gat cac


ATC CTA ACT ACC AGC CAA TAT AAA CTC ACA GTC CCC AGG TTC CTT ATG
 I   L   T   T   S   Q   Y   K   L   T   V   P   R   F   L   M     401
tag gat tga tgg tcg gtt ata ttt gag tgt cag ggg tcc aag gaa tac
TGC AAC CTG GCC TTT GCT GAT CTC TGC ATT GGA ATC TAC CTG CTG CTC
 C   N   L   A   F   A   D   L   C   I   G   I   Y   L   L   L     417
acg ttg gac cgg aaa cga cta gag acg taa cct tag atg gac gac gag


ATT GCA TCA GTT GAT ATC CAT ACC AAG AGC CAA TAT CAC AAC TAT GCC
 I   A   S   V   D   I   H   T   K   S   Q   Y   H   N   Y   A     433
taa cgt agt caa cta tag gta tgg ttc tcg gtt ata gtg ttg ata cgg


ATT GAC TGG CAA ACT GGG GCA GGC TGT GAT GCT GCT GGC TTT TTC ACT
 I   D   W   Q   T   G   A   G   C   D   A   A   G   F   F   T     449
taa ctg acc gtt tga ccc cgt ccg aca cta cga cga ccg aaa aag tga


GTC TTT GCC AGT GAG CTG TCA GTC TAC ACT CTG ACA GCT ATC ACC TTG
 V   F   A   S   E   L   S   V   Y   T   L   T   A   I   T   L     465
cag aaa cgg tca ctc gac agt cag atg tga gac tgt cga tag tgg aac


GAA AGA TGG CAT ACC ATC ACG CAT GCC ATG CAG CTG GAC TGC AAG GTG
 E   R   W   H   T   I   T   H   A   M   Q   L   D   C   K   V     481
ctt tct acc gta tgg tag tgc gta cgg tac gtc gac ctg acg ttc cac


CAG CTC CGC CAT GCT GCC AGT GTC ATG GTG ATG GGC TGG ATT TTT GCT
 Q   L   R   H   A   A   S   V   M   V   M   G   W   I   F   A     497
gtc gag gcg gta cga cgg tca cag tac cac tac ccg acc taa aaa cga


TTT GCA GCT GCC CTC TTT CCC ATC TTT GGC ATC AGC AGC TAC ATG AAG
 F   A   A   A   L   F   P   I   F   G   I   S   S   Y   M   K     513
aaa cgt cga cgg gag aaa ggg tag aaa ccg tag tcg tcg atg tac ttc


GTG AGC ATC TGC CTG CCC ATG GAT ATT GAC AGC CCT TTG TCA CAG CTG
 V   S   I   C   L   P   M   D   I   D   S   P   L   S   Q   L     529
cac tcg tag acg gac ggg tac cta taa cgt tcg gga aac agt gtc gac


TAT GTC ATG TCC CTC CTT GTG CTC AAT GTC CTG GCC TTT GTG GTC ATC
 Y   V   M   S   L   L   V   L   N   V   L   A   F   V   V   I     545
ata cag tac agg gag gaa cac gag tta cag gac cgg aaa cac cag tag


TGT GGC TGC TAT ATC CAC ATC TAC CTC ACA GTG CGG AAC CCC AAC ATC
 C   G   C   Y   I   H   I   Y   L   T   V   R   N   P   N   I     561
aca ccg acg ata tag gtg tag atg gag tgt cac gcc ttg ggg ttg tag


GTG TCC TCC TCT AGT GAC ACC AGG ATC GCC AAG CGC ATG GCC ATG CTC
 V   S   S   S   S   D   T   R   I   A   K   R   M   A   M   L     577
cac agg agg aga tca ctg tgg tcc tag cgg ttc gcg tac cgg tac gag
```

54

```
ATC TTC ACT GAC TTC CTC TGC ATG GCA CCC ATT TCT TTC TTT GCC ATT
 I   F   T   D   F   L   C   M   A   P   I   S   F   F   A   I      593
tag aag tga ctg aag gag acg tac cgt ggg taa aga aag aaa cgg taa


TCT GCC TCC CTC AAG GTG CCC CTC ATC ACT GTG TCC AAA GCA AAG ATT
 S   A   S   L   K   V   P   L   I   T   V   S   K   A   K   I      609
aga cgg agg gag ttc cac ggg gag tag tga cac agg ttt cgt ttc taa


CTG CTG GTT CTG TTT CAC CCC ATC AAC TCC TGT GCC AAC CCC TTC CTC
 L   L   V   L   F   H   P   I   N   S   C   A   N   P   F   L      625
gac gac caa gac aaa gtg ggg tag ttg agg aca cgg ttg ggg aag gag


TAT GCC ATC TTT ACC AAA AAC TTT CGC AGA GAT TTC TTC ATT CTG CTG
 Y   A   I   F   T   K   N   F   R   R   D   F   F   I   L   L      641
ata cgg tag aaa tgg ttt ttg aaa gcg tct cta aag aag taa gac gac


AGC AAG TGT GGC TGC TAT GAA ATG CAA GCC CAA ATT TAT AGG ACA GAA
 S   K   C   G   C   Y   E   M   Q   A   Q   I   Y   R   T   E      657
tcg ttc aca ccg acg ata ctt tac gtt cgg gtt taa ata tcc tgt ctt


ACT TCA TCC ACT GTC CAC AAC ACC CAT CCA AGG AAT GGC CAC TGC TCT
 T   S   S   T   V   H   N   T   H   P   R   N   G   H   C   S      673
tga agt agg tga cag gtg ttg tgg gta ggt tcc tta ccg gtg acg aga


TCA GCT CCC AGA GTC ACC AGT GGT TCC ACT TAC ATA CTT GTC CCT CTA
 S   A   P   R   V   T   S   G   S   T   Y   I   L   V   P   L      689
agt cga ggg tct cag tgg tca cca agg tga atg tat gaa cag gga gat
                                        <-A ATG TAT GAA CAG GGA GAT
                                                    <-g gga gat


AGT CAT TTA GCC CAA AAC [TGA]
 S   H   L   A   Q   N
tca gta aat cgg gtt ttg
TCA GTA AAT CGG GTT TTG ACT CGCCGGCG GC-5′
primer 2921 (hFSHR-Rv primer; initial 69.9ºC; Not I site)
tca gta aat cgg gtt ttg ACT CGCCGGCG T
primer 1577 (hFSHR-Rv primer; initial 59.4ºC, later 79.1ºC; Not I site)
```

55

6.2 Prediction of a signal peptide cleavage site

Prolactin ss – Flag tag

MDSKGSSQKGSRLLLLLVVSNLLLCQGVVSDYKDDDDV

(http://www.cbs.dtu.dk/services/SignalP/)

SignalP-NN result:
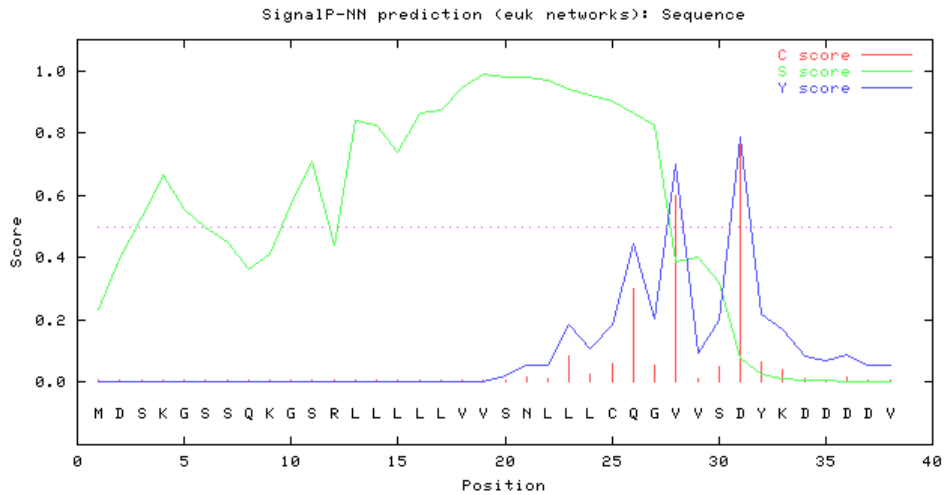

```
>Sequence                length = 38
# Measure   Position   Value   Cutoff   signal peptide?
  max. C     31        0.780   0.32     YES
  max. Y     31        0.788   0.33     YES
  max. S     19        0.988   0.87     YES
  mean S    1-30       0.680   0.48     YES
       D    1-30       0.734   0.43     YES
# Most likely cleavage site between pos. 30 and 31: VVS-DY
```

SignalP-HMM result:
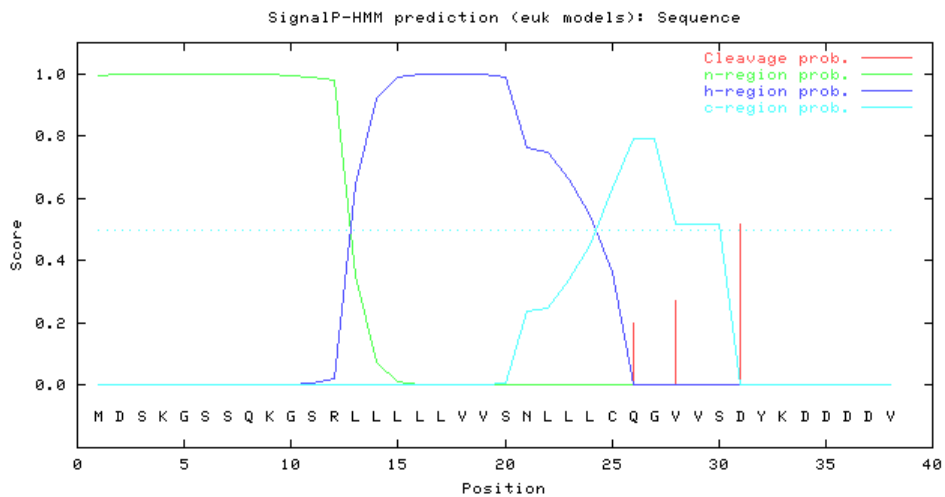

```
>Sequence
Prediction: Signal peptide
Signal peptide probability: 0.994
Signal anchor probability: 0.005
Max cleavage site probability: 0.519 between pos. 30 and 31
```

6.3 Sequencing results for the mutagenesis of the hFSHR internal **Bam HI** *site*

ACCTTCCAGATGTTCACAAGATTCATTCTCTCCAAAAAGTTTTACTTGACATTCAAGATAACATAAACATCCACACAATTGAAAGAAATTCTTTCGTGGGGCTGAGCTTTGAAAG

GTGTGATTCTATGGCTGAATAAGAATGGGATTCAAGAAATACACAACTGTGCATTCAATGGAACCCAACTAGATGAGCTGAATCTAAGCGATAATAATAATTTAGAAGAATTC

GCCTAATGATGTTTTCCACGGAGCCTCTGGACCAGTCATTCTAGATATTTCAAGAACAAGAATTCATTCCCTGCCTAGCTATGGCTTAGAAAATCTTAAGAAGCTGAGGGCCAG

6.4 Transient expression of constructed receptors in HEK 293-T cells

6.4.1 Cell-culture

Stock media/solutions for cell culturing (stored at 4°C, used at 37°C)

Materials:
- Culture medium:

> 500 mL Dulbecco's Modified Eagle's medium (DMEM) {Gibco 41965-039}
>
> 50 mL Fetal Bovine serum (Gibco 10270-106 /South American))
>
> 5 mL antibiotic-Antimyotic (100x) {Gibco 15240-062}
>
> 5 mL L-Glutamin (100x) {Gibco 25030-024}

- Wash-medium: 500 mL DMEM

- Trypsin/EDTA {Gibco 25300-054}
- D-PBS {Sigma D-8537}

6.4.2 PEI-Transfection + receptor expression

- Day 1: Seed cells

1. Place PBS, Culture medium and Trypsin/EDTA at 37°C.
2. Use HEK 293-T cells at 60-80% confluence in a 75 cm□ culture flask. Aspirate culture medium and gently rinse the cells with 3 mL PBS.
3. Resuspend the cells in 1 mL trypsin/EDTA for maximal 5 min, add 11 mL culture medium, resuspend and transfer suspension to 50 ml-tube
4. Mix 10 μL with 10 μL trypan-blue, count cells in 25 (inner) squares.
   (#cells/25) x 25 x 2 x 10^4 = n cells/ml stock
5. Dilute suspension with culture medium so that 1 mL contains 375000 cells.
6. Seed 8 ml (=3 x 10^6cells) in a 10 cm-costar culture dish {430167}and incubate overnight at 37°C under 5% CO2.

- Day 2: PEI-transfection

(Polyethylenimine; Polysciences Inc. Warrington PA, Cat.nr. 23966) with pCre/b-Gal Plasmid (our Glyc. Stock 6564) and receptor /pcDNA3.1 expression vector.

1. Place wash-medium and PBS at 37°C.
2. Prepare 800 μL transfection mix for each dish in 14 mL-transfection tubes {Falcon polystyrene round-bottom tubes 2057, 81780 Emergo):
   - 5 μg pCre/β-Gal
   - 500 ng receptor/pcDNA3.1
   - 33 μg PEI (1 μg/μL, stored frozen)
   - Fill up to 800 μL with sterile double-distilled water
   - Mix gently; incubate this transfection Mix for at least 15 min.
3. Meanwhile: aspirate culture medium and wash cells gently once with 3 mL PBS.
4. Add first 8 mL Wash-Medium and then 800 μL Transfection Mix to each dish. Resuspend the transfection Mix once (carefully) and add droplets homogenously.
5. Incubate for 3 hours at 37°C under 5% CO2.
6. Aspirate transfection medium.
7. Rinse cells carefully 2 times with 3 mL PBS.
8. Add 8 mL culture medium and incubate overnight at 37°C under 5% CO2.

- Day 3: Transfer transfected HEK 293-T cells to 24 and 96-wells plates.

1. Place culture medium, D-PBS and trypsin/EDTA at 37°C.
2. Aspirate culture medium and wash cells gently with PBS.
3. Resuspend cells in 1 mL trypsin/EDTA for maximal 5min and bring the 1 mL cells in 'X' mL culture medium (volume X is dependent of amount of stimulations).
4. Resuspend cells with a repeat pipet.
5. Functional assay: Fill enough wells of a 96-well Costar plate (Corning Costar – 3595} with 200 μL/well
6. ELISA: 1.0 mL aliquots in 24-well plate {Corning Costar – 3524}*, 1 or 2 wells/construct

7.   ELISA: 5 mL Mock cells in 6 cm Petri dish* (for pre-absorption)

* Wells must be coated before use with poly-D-Lysine {Sigma P-7280} before cells are applied;

       -750 μL/ 6cm dish; 250 μL/ 24-wells-plate

       -10 min at room temperature

       -aspirate poly-D-Lysine

       -rinse with 0.5 mL sterile double distilled water

       -aspirate water, "store" at room temperature

Poly-D-Lysine:

       - Dissolve 5 mg in 50 mL sterile double distilled water (0.1 mg/mL)

       - Filter through Millipore (0.22 μm)

       - Store aliquots at -20°C.

6.4.3 Ligand Stimulation + ELISA

- Day 4

Stimulation of transfected HEK-T 293 cells with ligands.

Lysis buffer: 0.25 M Tris/HCl pH 7.8

              0.5% NP 40 (0.5 mL/100 mL)

1.   Prepare Stimulation medium:

   # DMEM (HEPES-modified; {Sigma D-6171})

   # 0.1 mM IBMX (Sigma I-5879; Stock = 10 mM in DMSO) (=100x dil)

   # 0.01% BSA (Sigma A-9647)   (=10 mg/100 mL)

2.   Make serial dilutions of the ligand in stimulation medium.

3.   Aspirate culture medium from cells in 96-wells plates.

4.   Add 25 μL stimulation medium containing the desired (increasing) ligand concentration* to each well. Add stimulation medium without ligand (-) or

containing 10 μM Forskolin (+) (500 x from stock Sigma F-6886) in order to measure respectively basal and standard stimulation.

5. Incubate for 6 hours at 37°C under 5% CO_2.

6. Aspirate stimulation medium and freeze cells at -80°C for 15 min.

7. Thaw cells (15 min at 37°C) and add 50 μL lysis buffer to each well.

8. Vortex the plates gently and freeze overnight at -20°C.

* Prepare from the desired ligands the following concentrations in stimulation buffer by serial dilutions. Calculate the volume of ligand and stimulation buffer needed for each dilution step. Note that you'll need at least 3 x 25 μL of each concentration (for 1 construct).

For rhFSH (gift from Organon –Oss, Netherlands): 103 IU/vial (=10.3 μg)

10^4 ng/mL=………………………………………………….

10^3 ng/mL=………………………………………………….

10^2 ng/mL=………………………………………………….

10^1 ng/mL=………………………………………………….

10^0 ng/mL=………………………………………………….

10^{-1} ng/mL=………………………………………………….

10^{-2} ng/mL=………………………………………………….

10^{-3} ng/mL=………………………………………………….

ELISA

Materials:

- PBS (10 mM Na-phosphate pH 7.0; 150 mM NaCl)
- 4% Formaldehyde: make out of 37% (Merck 1.04003.2500)
- TBS (30 mL 5M NaCl, 50 mL 1 M Tris-HCl pH 7.5, filled up to 1 liter with double distilled water.
- Blocking buffer (0.1 M $NaHCO_3$, pH 8.6 adjusted with NaOH, 1% Milkpowder)
- Milk powder (Regilait)

- 1st Antibody: Anti-FLAG high affinity; rat monoclonal antibody; 50 µg in 0.5 ml double distilled water (stock) aliquots in -20°C.

1. Prepare 4% fresh FA (out of 37%).

2. Aspirate medium.

3. Wash with 0.5 mL pre-warmed TBS.

4. Fixate with 0.5 mL/well 4% PFA, 30 min, room temperature.

5. Prepare blocking buffer (0.1 M NaHCO$_3$ pH 8.6 adjusted with NaOH, 1% Milkpowder) = 0.5 gr milk powder + 50 mL NaHCO$_3$.

6. Aspirate FA.

7. Wash 3 times with 0.5 mL TBS.

8. Block at least 4 h (at room temperature) on a shaker with 0.5 mL blocking buffer/well.

9. 1.5 h before the blocking stops:

- Preabsorption antiserum:

10. Use spare HEK 293-T cells in a 6 cm dish.

11. Aspirate medium.

12. Wash once with 5 mL pre-warmed TBS.

13. 30 min fixation at room temperature with 4% PFA.

14. Wash 3 times with 5 mL TBS.

15. Dilute 1st antibody (anti-FLAG) 200 x in TBS/0.1%BSA (0.01 gr BSA/10 mL)

16. Incubate 5 mL diluted antibodies on fixated, washed HEK 293-T cells ('Mock") for 1 h at 37 °C. (5 mL = enough for 21 samples)

----

17. 5 min before preabsorption stops:

18. Aspirate blocking buffer from wells.

19. Add the preabsorbed 1st Antibody (anti-FLAG) = 0.23 mL/well

20. Incubate o/n at 4°C.

6.4.4 β-galactosidase-assay + ELISA

Substrate Buffer:

- 100 mM sodium phosphate, pH 7.5

- 10 mM KCl

- 1 mM $MgSO_4$

- 50 mM β-Mercapto-ethanol (add just prior to use; 1 M Stock)

Prepare as follows:

- Solution 1: 27.6 gr/L $NaH_2PO_4.H_2O$ (M=137.99)

- Solution 2: 35.59 gr/L $Na_2HPO_4.H_2O$ (M=177.99)

Make a 200 mM sodium phosphate solution: ≈ 16 mL solution 1 + ≈ 84 mL solution 2, adjust to pH 7.5 using these solutions.

Take 100 mL of this solution and add 2 mL KCL (1M), 200 μL $MgSO_4$ (1M) and 98 mL sterile double distilled water (do not autoclave! → precipitation!).

Substrate: 4 mg onpg /mL (Sigma N-1127) in 100 mM sodium phosphate buffer (without KCl and $MgSO_4$!).Aliquots can be stored in -20.

1. Pool substrate (50 μL/well) and substrate buffer (110 μL/well).
2. So add 160 μL of the mix to each well.
3. Incubate plates at room temperature depending on how long coloration develops.
4. Measure OD 405 nm (OD 655 nm as a reference) in a 96-well-microplate-reader, until Forskolin ≈ 1.0.
5. Divide all values by the forskolin value (use PrismGraph 4.0) after correction with Mock.
6. Make concentration-response-curves (Prism Graph) and draw conclusions.

ELISA

Materials:

- 2nd Antibody: anti-rat IgG (whole molecule) peroxidase conjugate (Sigma A-9037) (aliquots 16 μL)

- TMB (Sigma # T-8665)

- 0.5 M Sulphoric Acid (H_2SO_4, M=98.08 gr/mol; 1 liter = 1.84 gram)

1. Place TMB at room temperature.

2. Aspirate 1st Ab.

3. Wash 3 times with 0.5 mL TBS.

4. Dilute 2nd Antibody 1: 500 in fresh blocking buffer (0.1 gr milk /10 mL $NaHCO_3$) and add 0.25 mL to cells.

5. Incubate for 2 h at room temperature on shaker.

6. Aspirate 2nd Ab.

7. Wash 3 times with 0.5 mL TBS.

8. Add 0.4 mL TMB.

9. Stain on shaker at room temperature for 10-30 min. Stop reaction when a clear difference is seen between mock and FLAG-tagged receptors.

10. Stop reaction with 0.2 mL 0.5 M Sulphoric acid.

11. Transfer 0.1 mL to 96-wells plate (duplo).

12. Measure OD at 450 nm (use 655 nm as reference; Dual wavelength).

13. Calculate and make a drawing of the expression-levels (in Excel).