Intracellular Trafficking of Gonadotropin Receptors in Health and Disease

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Abstract

Gonadotropin receptors belong to the highly conserved subfamily of the G protein-coupled receptor (GPCR) superfamily, the so-called Rhodopsin-like family (class A), which is the largest class of GPCRs and currently a major drug target. Both the follicle-stimulating hormone receptor (FSHR) and the luteinizing hormone/chorionic gonadotropin hormone receptor (LHCGR) are

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mainly located in the gonads where they play key functions associated to essential reproductive functions. As any other protein, gonadotropin receptors must be properly folded into a mature tertiary conformation compatible with quaternary assembly and endoplasmic reticulum export to the cell surface plasma membrane. Several primary and secondary structural features, including presence of particular amino acid residues and short motifs and in addition, posttranslational modifications, regulate intracellular trafficking of gonadotropin receptors to the plasma membrane as well as internalization and recycling of the receptor back to the cell surface after activation by agonist. Inactivating mutations of gonadotropin receptors may derive from receptor misfolding and lead to absent or reduced plasma membrane expression of the altered receptor, thereby manifesting an array of phenotypical abnormalities mostly characterized by reproductive failure and/or abnormal or absence of development of secondary sex characteristics. In this chapter we review the structural requirements necessary for intracellular trafficking of the gonadotropin receptors, and describe how mutations in these receptors may lead to receptor misfolding and disease in humans.

Keywords
G protein-coupled receptors (GPCR) • Gonadotropin receptors • Gonadotropins • Intracellular traffic • Quality control system

1 Introduction

The pituitary gonadotropic hormones, follicle-stimulating hormone or follitropin (FSH) and luteinizing hormone or lutropin (LH), as well as placental chorionic gonadotropin (hCG), are glycoprotein hormones that play a pivotal role in reproduction. Their cognate receptors (FSHR and LHCGR -*the LH receptor binds both, LH and hCG*-) belong, together with the thyroid-stimulating hormone receptor (TSHR) expressed by thyroid follicular cells, to a highly conserved subfamily of the G protein-coupled receptor (GPCR) superfamily, the so-called Rhodopsin-like family (class A), which is the largest class of GPCRs and currently a major drug target. The FSHR and LHCGR are mainly expressed by specific cells in the gonads (Ascoli et al. 2002; Richards and Pangas 2010b; Simoni et al. 1997). The FSHR is expressed in ovarian granulosa cells and the testicular Sertoli cells of the seminiferous tubules. Here, the FSHR is essential for FSH-stimulated maturation of ovarian follicles and granulosa cell progesterone and estrogen production (Richards and Pangas 2010a), whereas in the testis, activation of the FSHR supports Sertoli cell growth and metabolism, promoting spermatogenesis (Huhtaniemi 2015). In males, LHCGR is expressed in the Leydig cells which comprise nests located between the seminiferous tubules, where LH stimulates androgen production, mainly testosterone which in addition to its effect on male secondary sex characteristics, is converted by Sertoli cells to estrogen (Haider 2004; Saez 1994). In females, the LHCGR is expressed in the ovarian theca cells lining the developing

follicle, where its cognate ligand induces production of aromatizable androgens, which are subsequently converted to estrogens in the granulosa cell layer (Richards and Pangas 2010b).

G protein-coupled receptors are membrane receptors that vary considerably in molecular size. Nevertheless, they share a common molecular topology consisting of a single polypeptide chain of variable length that traverses the lipid bilayer forming seven characteristic transmembrane hydrophobic α-helices [transmembrane domains (TMD)], connected by alternating extracellular and intracellular sequences or loops (EL and IL, respectively), with an extracellular NH_2-terminus and an intracellular carboxyl-terminal domain (Ctail) (Gershengorn and Osman 2001; Ulloa-Aguirre and Conn 1998). These receptors characteristically bind one or more heterotrimeric G proteins that become activated upon agonist binding, which in turn act as mediators of effector activation and intracellular signaling (Oldham and Hamm 2008). In particular, a large NH_2-terminal extracellular domain or ectodomain (ECD), where recognition and binding of their cognate ligands occurs, is characteristic of glycoprotein hormone receptors. This ECD is comprised of a central structural motif of imperfect leucine-rich repeats (LRR), a motif that is shared with a number of other membrane receptors involved in ligand selectivity and specific protein–protein interactions (Bogerd 2007). The carboxyl-terminal end of the large ECD displays the signal specificity subdomain (also called "hinge" region), which is an integral part of the ectodomain and that structurally links the leucine-rich ECD with the serpentine 7TMD of the receptor, where activation of the receptor occurs following conformational changes provoked by agonist interaction with the ECD (Chen et al. 2009; Krause et al. 2012; Majumdar and Dighe 2012). The hinge region has been structurally characterized for the human (h) FSHR (hFSHR) (Jiang et al. 2012) and evidence has linked this region to signaling functionality (Jiang et al. 2014).

The FSHR and the LHCGR exhibit a high degree of primary sequence homology. Whereas the ECD amino acid sequences of the gonadotropin receptors are approximately 46% identical, the 7TMD sequence portion of the receptors share nearly 72% homology (Dias and Van Roey 2001; Kleinau and Krause 2009). This high similarity between the 7TMD of the gonadotropin receptors might suggest similar mechanisms of receptor activation; however, it is noticeable that gain-of-function mutations in the 7TMD of the hFSHR are extremely rare when compared to the hLHCGR (Ulloa-Aguirre et al. 2014). A higher relative stability of the 7TMD of the hFSHR in the inactive state compared with that of the hLHCGR could explain this difference between gonadotropin receptors. Among the three domains, the intracellular regions have the lowest FSHR-LHCGR amino acid sequence homology (approximately 27% identity), with the exception of the NH_2-terminal end of the Ctail, which bear Cys residues for palmitoylation and a primary sequence motif ($F(x)_6LL$) that markedly influences trafficking from the endoplasmic reticulum (ER) to the cell surface plasma membrane (PM) (Duvernay et al. 2004, 2005; Timossi et al. 2004) (see Sects. 3.1.1 and 3.1.3).

Upon gonadotropin binding, the activated FSH and LHCG receptors trigger a number of intracellular signaling cascades. Although the classical $G\alpha_S$/cAMP/PKA

signaling pathway has been accepted as the main effector mechanism of gonadotropin biological action for a long time, it is currently clear that gonadotropin receptors (and the TSHR as well) may couple to other G protein subtypes and activate a number of distinct signaling pathways (Gloaguen et al. 2011; Ulloa-Aguirre et al. 2011), depending on the cell context and developmental stage of the host cells (Musnier et al. 2009).

It is well known that mutations resulting in changes in protein sequence may lead to misfolding, defined as a defect in protein folding due to a sufficient and persistent number of non-native interactions that significantly affect the overall architecture or conformation of the protein and/or its properties (Dobson 2003). Frequently, misfolding results in loss-of-function of the conformationally defective protein (Dobson 2004; Ulloa-Aguirre et al. 2004a) that may be transcribed and translated at normal levels, but is unable to reach its functional destination in the cell or to engage the secretory pathway (Dobson 2003, 2004). A number of mutations in several GPCRs associated with endocrine functions that lead to misfolding of the receptor protein and to partial or complete inability of the abnormal receptor to express at the PM level and interact with agonist have been described (Conn and Ulloa-Aguirre 2010). Several endocrine diseases caused by mutations in GPCRs that provoke protein misfolding and impaired traffic of the mutant to the PM include nephrogenic diabetes insipidus (which involves the vasopressin V2 Receptor; V2R) (Bichet 2006; Conn et al. 2007), familial hypocalciuric hypercalcemia (calcium-sensing receptor; CaSR) (Huang and Breitwieser 2007), congenital hypothyroidism (TSHR) (Calebiro et al. 2005), obesity (melanocortin-3 and -4 receptor; MC3R and MC4R, respectively) (Huang et al. 2017; Tao 2010; Tao and Conn 2014), and familial glucocorticoid deficiency (melanocortin-2 receptor; MC2R) (Clark et al. 2005). Mutation-provoked misfolding of GPCRs involved in the regulation of reproductive function may also occur and lead to distinct abnormalities, including hypogonadotropic hypogonadism [due to mutations in the gonadotropin-releasing hormone receptor or GnRHR (Ulloa-Aguirre et al. 2004b), neurokinin-3 receptor, prokineticin receptor-2, or kisspeptin receptor-1 (Francou et al. 2011; Monnier et al. 2009; Nimri et al. 2011)], male pseudohermaphroditism (hLHCGR), and ovarian failure (hFSHR) (Ulloa-Aguirre et al. 2014).

Before discussing the structural determinants involved in trafficking of the gonadotropin receptors and their pathogenic mutations, we will briefly review some of the general mechanisms dictating the intracellular trafficking of GPCRs, particularly of the rhodopsin-like receptors.

2 Endoplasmic Reticulum Quality Control System, Molecular Chaperones, and Regulation of Intracellular Trafficking

As any other protein produced by the cell, GPCRs begin their life cycle at the ER. Here, synthesis, folding, and assembly of proteins occurs (Fig. 1), and properly folded receptors that have reached a conformation compatible with ER export, are

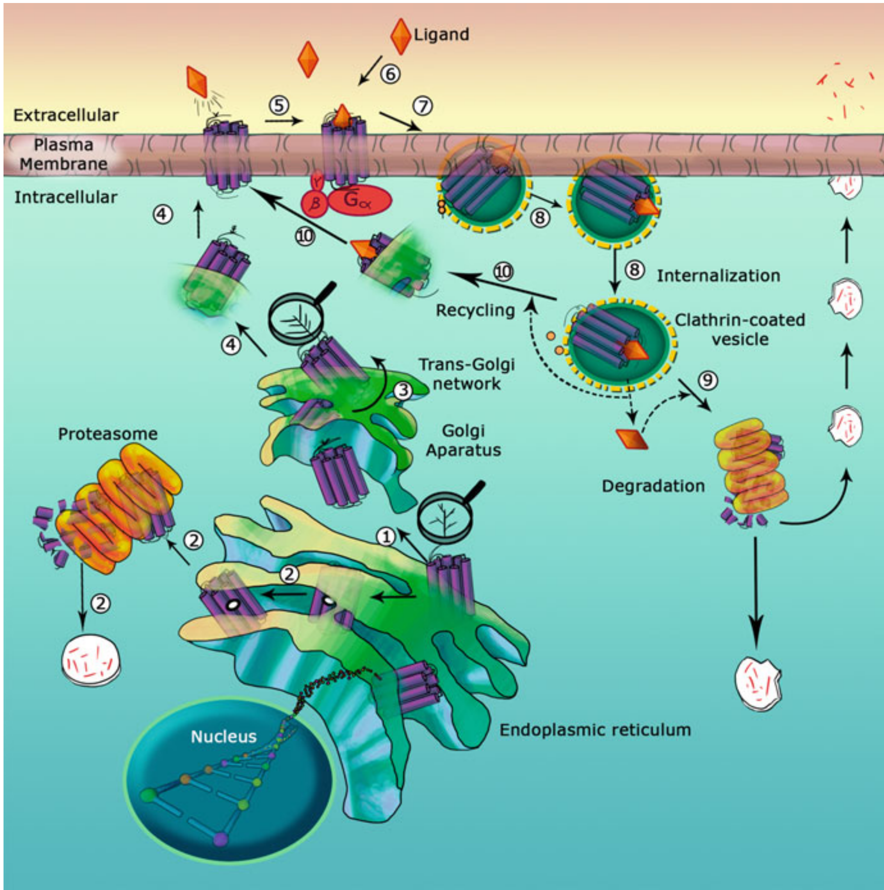

Fig. 1 Trafficking of rhodopsin-like G protein-coupled receptors. Newly synthesized proteins fold in the endoplasmic reticulum (step 1). Here, misfolded and misassambled products are retained and exposed to resident chaperones (oval structures) which attempt to correct folding and stabilize the protein in a conformation compatible with endoplasmic reticulum export. When correct folding fails, the misfolded protein is dislocated into the cytoplasm for proteosomal degradation (step 2). Correctly folded proteins are then translocated to the Golgi apparatus to complete processing such as glycosylation (magnifiers) (steps 1 and 3). Mature receptors are then exported to the plasma membrane (step 4) where they interact with cognate ligands (steps 5 and 6). Ligand activation of the receptor (step 6) is followed by phosphorylation (*orange circles*) of the receptor and recruitment of β-arrestins, which promote endocytosis (step 7) and internalization of the receptor-ligand complex (step 8). The receptor-ligand complex embedded in clathrin-coated vesicles may be either targeted to lysosomes for degradation, dissociate in the endosomal compartment with subsequent sorting of the ligand to lysosomal degradation (step 9) and the receptor to the recycling pathway, or recycled back to the plasma membrane (step 10) where agonist dissociates from the receptor to interact with agonist (Ascoli 1984; Sorkin and Von Zastrow 2002; Krishnamurthy et al. 2003; Melo-Nava et al. 2016)

then targeted to the ER-Golgi intermediate complex and thereafter to the Golgi apparatus and trans-Golgi network, where processing is completed and the receptor in transit is ready to continue their outward trafficking to the PM and become accessible to agonist (Broadley and Hartl 2009). Interaction between GPCRs and cognate agonists at the PM then stimulates downward trafficking which begins with internalization of the receptor through a series of distinct posttranslational modifications that include phosphorylation (which terminates G protein-mediated signaling) and β-arrestin recruitment, albeit in certain cell contexts GPCRs do not require β-arrestins for internalization (van Koppen and Jakobs 2004). β-arrestin recruitment by the phosphorylated receptor then allows interaction with clathrin and the clathrin adaptor AP2 to drive receptor internalization into endosomes, and either recycling of the receptor back to the PM or targeting to the lysosomes and/or proteasomes for degradation (Pavlos and Friedman 2017) (Fig. 1). Thus, a balance between synthesis and subsequent trafficking from the ER to the PM and the endocytosis-recycling/degradation pathway determine the net amount or density of functional receptor protein at the PM available to interact with agonist and provoke a biological response. Nonetheless, before reaching their final destination (e.g., the cell surface PM), newly synthesized GPCRs must be subjected to conformational screening by a strict quality control system (QCS) that monitors, and corrects if necessary, the folding of the nascent receptor into a three-dimensional structure compatible with ER export (Ulloa-Aguirre and Conn 2009). Monitoring the structural and conformational correctness of newly synthesized proteins by the QCS then determines the protein pools that must either be retained at the ER and eventually degraded in proteasomes or sorted to the Golgi apparatus and thereafter to the cell surface PM (Ulloa-Aguirre et al. 2004a). Thus, the QCS prevents accumulation of misfolded proteins that may aggregate and interfere with cell function. G protein-coupled receptor export from the ER to the Golgi is modulated by the interaction of the trafficking proteins with specialized folding factors, escort proteins, retention factors, enzymes, and members of the molecular chaperone families, which belong to the ER QCS and the so-called proteostasis network (Hartl et al. 2011; Hartl and Hayer-Hartl 2002; Hutt et al. 2009; Ron and Walter 2007). Specifically, molecular chaperones are key components of the ER QCS that screen native receptor conformation and promote delivery from the ER (Ellgaard et al. 2016; Ulloa-Aguirre et al. 2004a). Molecular chaperones not only recognize, but also retain and target misfolded, non-native protein conformers to degradation via the polyubiquitination/proteasome pathway (Chevet et al. 2001; Klausner and Sitia 1990; Schubert et al. 2000; Werner et al. 1996). Molecular chaperones may also disassemble protein aggregates and guard nascent polypeptides against unproductive and potentially toxic interactions that may occur during the various stages of folding (Duennwald et al. 2012). Surveillance of the QCS for correct folding and assembly of newly synthesized proteins relies more on some general structural features of the client protein (unpaired cysteines, exposure to hydrophobic shapes, immature glycans and specific sequence motifs), and thus possess the ability to recognize misfolded proteins when they expose hidden hydrophobic domains or particular sequences. For example, removal of the conserved ALAAALAAAA

hydrophobic sequence present in the extracellular NH_2-terminus of the α_{2C}-adrenergic receptor (AR) subtype significantly increases PM expression and reduces ER retention (Angelotti et al. 2010).

Because of the critical role of molecular chaperones in correcting folding and promoting the intracellular trafficking of proteins, the concept of molecular chaperoning has been applied to therapeutics to correct folding of defective proteins that provoke disease. Several examples of both in vitro and in vivo models where pharmacological chaperones (or pharmacoperones) [defined as small cell surface PM-permeable molecules that specifically bind to the abnormal, misfolded receptor protein, stabilizing it in a more native conformation, allowing the protein to pass the QCS and traffic from the ER to the PM where it may function normally or near normally (Conn et al. 2014b)] rescued function of misfolded GPCRs, including misfolded V2Rs (e.g., satavaptan, relcovaptan, VPA-985, tolvaptan, SR49059) (Albright et al. 1998; Bernier et al. 2006; Hawtin 2006; Robben et al. 2006, 2007) and rhodopsin (e.g., retinoids, 11-cis-7-ring retinal) (Li et al. 1998; Noorwez et al. 2004, 2008), the CaSR (e.g., NPS R-568) (Huang and Breitwieser 2007; Riccardi and Martin 2008), the MC3- and 4Rs (e.g., ML00253764, Ipsen 17) (Huang et al. 2017; Tao and Conn 2014), the melanin-concentrating hormone receptor-1 (e.g., NBI-A) (Fan et al. 2005), the gonadotropin receptors (e.g., Org 42599, Org 41841) (Janovick et al. 2009; Newton et al. 2011) (see the Chapter by Newton et al. for details), and the GnRHR (e.g., IN3, Q89, A177775, TAK-013) (Conn et al. 2014a; Conn and Ulloa-Aguirre 2010, 2011; Janovick et al. 2013; Ulloa-Aguirre and Conn 2016). Thus, the possibility that intracellularly retained misfolded GPCRs may be rescued by pharmacoperone drugs that emulate endogenous chaperones represents a unique opportunity for therapeutic intervention and design of new molecules potentially useful to treat an array of diseases.

2.1 Sequence Motifs That Promote/Prevent Outward Trafficking of GPCRs to the PM

Several sequence motifs regulate the exit of correctly folded GPCRs from the ER and the Golgi; some of these sequences associate with components of the COPII transport machinery and small GTPases, such as members of Rab (e.g., Rab1) and Sar1/ARF subfamilies (which are molecules involved in vesicle-mediated transport), to exit the ER (Wang and Wu 2012). These motifs include the dileucin [E$(x)_3$LL] and FN$(x)_2$LL(x)3L motifs, identified in the human V2R and V3R, respectively (Robert et al. 2005; Thielen et al. 2005), the F$(x)_6$LL motif identified in the carboxyl-terminus of several GPCRs (Duvernay et al. 2004, 2005), including the gonadotropin receptors, and the triple phenylalanine F$(x)_3$F$(x)_3$F sequence identified in the Ctail of the angiotensin II AT_1 receptor, the dopamine D_1 receptor, and the M_2-muscarinic receptor (Leclerc et al. 2002). Alteration of these motifs markedly reduces receptor export to the PM due to intracellular retention of the protein. These motifs are also used by some receptors en route for anterograde

trafficking from the trans-Golgi network to the PM via binding to the small GTPase Rab8 (Wang and Wu 2012).

By contrast, export motifs present in the NH_2-terminus of GPCRs are rather scarce; nevertheless, a distinct YS motif in α_{2A} and α_{2B} -ARs has been identified as involved for export from the Golgi (Dong and Wu 2006). Residues or sequences present in regions other than the NH_2- and carboxyl-termini of GPCRs and involved in anterograde receptor trafficking have also been identified; in the IL1, a single leucine residue located in the center of this loop (which is a highly conserved residue among class A members of GPCRs) appears to play an important role in ER export in several adrenergic receptors and the angiotensin II AT_1 receptor (Duvernay et al. 2009), and in the IL3, a triple arginine (3R) motif mediates interaction of the α_{2B}-AR with protein transport Sec24C/D isoforms (Dong et al. 2012).

In some GPCRs, mutations in two highly conserved motifs, the E/DRY motif at the boundary of the TMD3 and the IL2 and the N/DPxxY motif at the TMD7 near the cytoplasmic face of the PM, may lead to different functional outcomes, including defective intracellular trafficking, depending on the specific receptor [e.g., the E/DRY motif in the V_2R and the GnRHR, and the N/DpxxY motif in the V_2R, GnRHR, endothelin-B receptor, MC4R, and the chemokine receptor (CCR) 5 (Bernier et al. 2006; Leanos-Miranda et al. 2002; Topaloglu et al. 2009)]. Another important structural feature of GPCRs belonging to rhodopsin-like GPCRs is a disulfide bridge between the first and second extracellular loops, which is critical for stabilization of the 7TMD, as mutations in or at the vicinity of this bridge usually result in complete loss of activity which is associated with intracellular retention of the mutant receptor. Mutations at this particular location markedly modify the three-dimensional structure of the protein, making the misfolded receptor particularly difficult for stabilization with pharmacoperones (Leanos-Miranda et al. 2002; Mendes et al. 2005).

Retention motifs that restrict trafficking of the protein to the ER for further processing have also been identified and it has been shown that trafficking of a cargo protein markedly improves upon modification of these motifs. Some of these retention signals are the penta-arginine (RRRRR) sequence identified in the α_{2C}-AR (Ma et al. 2001), the RSRR sequence in the GABA-B1 receptor (Pagano et al. 2001), and the conserved ALAAALAAAAA hydrophobic sequence present in the NH_2-termini of α_2-ARs (Angelotti et al. 2010). Although the regulatory role of these retention signals is still unclear, they might restrict trafficking of receptors that have failed to heterodimerize or fold, processes that may hide those retention motifs, thereby facilitating trafficking of the protein to the PM. In the case of the GABA-B1 and GABA-B2 receptors, heterodimerization masks the RSRR retention signal present at the carboxyl-terminus of the GABA-B1 receptor, preventing retrograde transport from the Golgi to the ER via COP-I vesicles, thereby promoting forward transport and trafficking of the obligatory heterodimer to the PM (Benke et al. 2012). Thus, the presence of export and/or retention signals ensures that only correctly folded and assembled receptor proteins can be exported to the PM.

2.2 Posttranslational Modifications in GPCRs and Intracellular Trafficking

Four posttranslational modifications (PTM) are potentially important regulators of GPCR trafficking: glycosylation, palmitoylation, phosphorylation, and ubiquitination, the last two particularly important for desensitization and internalization of the receptor in response to activation by agonist. A common posttranslational modification is *N*-linked glycosylation at the consensus sequence Asn-*X*-Ser/Thr (Ulloa-Aguirre and Conn 1998). This posttranslational modification facilitates protein folding by increasing its solubility and stabilizing protein conformation (Caramelo and Parodi 2015; Helenius and Aebi 2004; Lamriben et al. 2016). A number of GPCRs interact with the chaperone calnexin, an ER lectin, and with its soluble homolog calreticulin, whose cycle is predominantly centered on substrate N-glycans present on the newly synthesized protein chain, adding hydrophobicity to the folding protein. The calnexin/calreticulin cycle depends on the concerted action of carbohydrate-modifying enzymes (glycosidases I and II), which yield monoglycosylated oligosaccharide structures that interact with the chaperones and remove the remaining glucose residue from the oligosaccharide, terminating the association with the chaperones. When *N*-linked glycosylation or early glycan processing fails, glycoproteins that misfold are detected by the QCS, and their anterograde traffic to the PM is limited (Morello et al. 2001). Glycosylation at the ECD and/or extracellular loops is absolutely required for cell surface expression of some, but not all GPCRs, as mutation or changes in positioning of the glycosylation sites may lead to decreased PM expression (Fukushima et al. 1995; Lanctot et al. 2005, 2006; Nakagawa et al. 2017; Sawutz et al. 1987; van Koppen and Nathanson 1990).

S-acylation of GPCRs with palmitic acid at conserved cysteine residues in the Ctail is another PTM occurring at the ER-Golgi intermediate compartment (Chini and Parenti 2009). This posttranslational modification may potentially influence the local conformation of the GPCR at the Ctail and thus regulate several functions of the receptor including targeting to the PM and G protein coupling (Blanpain et al. 2001; Fukushima et al. 2001; Menon et al. 2005; O'Dowd et al. 1989; Percherancier et al. 2001; Qanbar and Bouvier 2003; Resh 2006; Uribe et al. 2008). Palmitoylation also provides an additional site for anchoring of the receptor to the PM, creating a fourth intracellular loop (Chini and Parenti 2009); in some GPCRs, this modification also is important for internalization, efficiency of recycling, β-arrestin recruitment, endocytosis, and degradation (Chini and Parenti 2009; Melo-Nava et al. 2016; Munshi et al. 2005; Resh 2006).

Several studies support the concept that association among cell surface membrane-expressed receptors is a critical process for receptor activity. In the case of GPCRs, some receptors constitutively form multi-unit complexes during their synthesis in the ER or processing in the Golgi (Milligan 2007). Constitutive oligomerization has been demonstrated for a number of GPCRs (Angers et al. 2000, 2002; Ayoub et al. 2002; Guan et al. 2009; Guo et al. 2003; Herrick-Davis et al. 2004; Mazurkiewicz et al. 2015; McVey et al. 2001; Mercier et al. 2002; Salahpour

et al. 2004; Terrillon et al. 2004; Thomas et al. 2007), and homo- and hetero-oligomerization at the ER has been shown to be an effective quality control of protein folding prior to export to the PM (Milligan 2007). For example, in the case of the GABA-B receptor, heterodimerization between GABA-B receptor-1 and GABA-B receptor-2 is an obligatory prerequisite for cell surface expression of a functional receptor; formation of a coil–coil domain between the Ctail of the GABA-B receptor subtypes masks the R*x*R ER retention signal at the Ctail of the GABA-B1 receptor, thereby promoting the ER export of the heterodimer to the PM (Margeta-Mitrovic et al. 2000). A similar role in receptor anterograde trafficking has been observed for the α_{1D}-, α_{1B}- (Hague et al. 2004) and β_2-ARs (Salahpour et al. 2004; Uberti et al. 2005). It has also been shown that mutations in GPCRs may provoke dominant-negative effects on WT receptor species and interfere with trafficking of the latter or both the WT and mutant receptors to the PM (Salahpour et al. 2004) (Fig. 4). This dominant-negative effect of mutant receptors on anterograde receptor trafficking, which has been demonstrated for a number of GPCRs (Benkirane et al. 1997; Brothers et al. 2004; Karpa et al. 2000; Leanos-Miranda et al. 2003; Lee et al. 2000; Zarinan et al. 2010; Zhu and Wess 1998), might play a role in the phenotypic expression of diseases in individuals bearing simple heterozygous mutations. The mechanisms subserving these protein--protein associations as well as the mechanistic basis for the general need for oligomerization of these membrane proteins is poorly understood. However the importance of this process is underscored by the observation that oligomerization chaperones such as 14-3-3ε and 14-3-3ζ bind correctly assembled multimers and mediate release of the complex from the ER, representing a checkpoint for forward trafficking of maturing multimeric proteins (Yuan et al. 2003).

Ubiquitination plays important roles in both outward and downward trafficking of GPCRs (Dores and Trejo 2014; Jean-Charles et al. 2016). Many GPCRs are posttranslationally modified with ubiquitin, including the FSHR (Cohen et al. 2003); ubiquitination then regulates their transport from the ER to *trans*-Golgi network and the PM as well as the post-endocytic fate of the receptor after ligand-induced internalization. In both cases, ubiquitination functions as a sorting mechanism to target GPCRs to proteasomes or lysosomes, although not all GPCRs require ubiquitination to engage degradation. Further, in some GPCRs (e.g., the CCR7) ubiquitination seems necessary for recycling of the receptor after agonist-induced internalization (Canals et al. 2012; Wolfe et al. 2007). Ubiquitination at the ER during GPCR synthesis occurs when misfolding cannot be corrected by molecular chaperones (see Sect. 2), and thus is a mechanism by which misfolded receptors are tagged for clearance. Therefore, the ubiquitination/deubiquitination balance represents one of the means used by the cell to define the net density of functional receptors expressed at the PM to interact with their cognate ligand.

The last PTM discussed in this section, phosphorylation, is the most frequent PTM in GPCRs (Norskov-Lauritsen and Brauner-Osborne 2015; Tobin 2008; Tobin et al. 2008). Phosphorylation of GPCRs occur predominanty on serine and threonine residues (and less frequently at tyrosine residues) across the IL3 and/or the Ctail (Oakley et al. 2000, 2001; Tobin 2008). Agonist-activated receptors are

rapidly phosphorylated by G protein-coupled receptor kinases (GRKs), and this phophorylation promotes β-arrestin binding, G protein uncoupling and receptor (or receptor-ligand complex) endocytosis (Pitcher et al. 1998). In the case of GPCRs bearing multiple and clustered Ser and Thr residues (e.g., the AT_1 receptor, V2R, neurokinin-1 receptor, and FSHR), β-arrestins are co-internalized with the phosphorylated receptor associated with clathrin and the clathrin adaptor AP2, both components of the endocytic machinery (Kara et al. 2006; Oakley et al. 2000, 2001). Receptor-containing clathrin-coated pits then form, via the action of the GTPase dynamin, vesicles, recycling endosomes, and/or early endosomes and multivesicular bodies which will eventually target the internalized GPCR to lysosomal degradation (Magalhaes et al. 2012). In these GPCRs the association and co-internalization of the phosphorylated receptor with arrestins facilitates arrestin-mediated, G protein-independent activation of distinct signaling pathways (e.g., ERK1/2) (Lefkowitz and Shenoy 2005; Luttrell et al. 2001; Wei et al. 2003). Nevertheless, in some GPCRs, phosphorylation is not an absolute requirement for arrestin recruitment, uncoupling and internalization (Galliera et al. 2004; Jala et al. 2005; Kishi et al. 2002; Mukherjee et al. 2002; Tobin 2008); for example, alanine replacement of residues of the D2-dopamine receptor known to be phosphorylated did not block receptor-G protein uncoupling (Celver et al. 2013). The relative ratio of receptor molecules that either recycle back to the plasma membrane or are degraded in lysosomes varies depending on the GPCR. This suggests that there are differences among GPCRs in the mechanisms and/or the kinetics of the interactions between the receptor and its interacting proteins that regulate the post-endocytic fate of the internalized receptor. In aggregate, the combination of agonist-induced conformational changes in the cytosolic domains of the receptor and PTM (e.g., phosphorylation and/or ubiquitination) allows the receptor to interact with distinct molecules that turn-on the endocytic machinery involved in the control of downward trafficking and post-endocytic fate (degradation vs recycling) of the activated, internalized receptor, and, in some particular GPCRs, arrestin-mediated activation of signaling pathways.

In summary, intracellular trafficking of GPCRs relies on several factors, including: (a) the QCS of the cell, mainly molecular chaperones, which monitor the conformational features of newly synthesized proteins, and define which proteins must be retained at the ER and eventually degraded or routed to the Golgi apparatus and thereafter to the PM; (b) Short export and retention sequences embedded within the newly synthesized protein that regulate export of the protein from the ER and the Golgi; (c) PTM (e.g., glycosylation, palmitoylation, ubiquitination, and phosphorylation), which are involved in ER export to the PM, desensitization, internalization, and post-endocytic fate of the internalized receptor; (d) interaction of the GPCRs and other secretory proteins with the microtubule networks to control their cell surface movement (Duvernay et al. 2011); and (e) a well-organized association (i.e., homo- or heterodimerization) between GPCRs, which promotes proper folding-assembly and targeting to the PM. The next section discusses the structural requirements that control the intracellular traffic of gonadotropin receptors, and the

functional impact of structural alterations in these two GPCRs on PM expression and disease.

3 Trafficking of Gonadotropin Receptors in Health and Disease

Several factors regulate folding and intracellular traffic of gonadotropin receptors, including interactions with distinct molecular chaperones and presence of specific sequences, amino acid residues, and posttranslational modifications.

3.1 Trafficking of Gonadotropin Receptors from the ER to the Cell Surface Plasma Membrane

3.1.1 Sequence Motifs

As described in Sect. 2.1, several sequence motifs present in the GPCRs are involved in the exit of the receptors from the ER and the Golgi. In addition to the above described E/DRY motif (ERW in the gonadotropin receptors) at the boundary of the TMD3 and the IL2 and the N/DPxxY motif (NPFLY in the gonadotropin receptors) at the TMD7 near the cytoplasmic face of the PM and that are fundamental for the structure and function of GPCRs, the gonadotropin receptors also bear the $F(X)_6LL$ sequence in their Ctail, a motif closely involved in trafficking of several GPCRs to the PM (Duvernay et al. 2004). In the hFSHR, this export motif is located between amino acid residues 633 and 641, whereas in the hLHCGR it is located between residues 630 and 638 (Ascoli et al. 2002; Dias et al. 2002). The Ctail peptide of the hFSHR also contains the minimal BBXXB motif reversed in its juxtamembrane region (residues 631–635) (Timossi et al. 2004); the last two residues of this motif (R634 and R635) and the preceding F633 constitute the NH_2-terminal end of the highly conserved $F(X)_6LL$ motif, and thus mutations in these residues impair receptor trafficking and PM localization of the receptor (Timossi et al. 2004; Zarinan et al. 2010). The IL3 of the hFSH and hLHCG receptors also contains this BXXBB motif (residues 569–573 in the hFSHR and 566–570 in the hLHCGR) and either deletion or replacement of the basic residues of this motif with alanine impairs PM expression of the modified receptors (Schulz et al. 1999; Timossi et al. 2004). Another motif that influences gonadotropin receptor folding and trafficking is the AFNGT sequence motif (amino acid residues 193–197 in the hLHCGR and 189–193 in the hFSHR), which contains a potential glycosylation site [N195GT and N191GT, in the hLHCGR and hFSHR, respectively]. As will be discussed later, mutations in this motif influence receptor folding and trafficking to the PM, and thereby cause diseases due to resistance to gonadotropins (Gromoll et al. 2002; Tapanainen et al. 1998).

3.1.2 Glycosylation

Glycosylation plays an important role not only in folding, but also in the maturation and intracellular trafficking of the receptors from the ER to the cell surface PM (Helenius and Aebi 2004). The ECD of gonadotropin receptors contains several putative glycosylation sites (sequence Asn-*X*-Ser/Thr, where *X* is any amino acid except proline), six in the hLHCGR and three in the hFSHR (Dias et al. 2002). Evidence exists as to which sites are glycosylated in the hFSHR, which comes from the crystal structures of the FSHR ECD residues 25–250 in complex with FSH (Fan and Hendrickson 2005; Jiang et al. 2012). The structures show that carbohydrate is attached at residue Asn191 which protrudes into solvent, whereas no carbohydrate is attached at residue Asn199, which projects from the flat β-sheet into the hormone-receptor binding interface. Although no structural information is yet available on whether residues 293 and 318 are glycosylated, studies have suggested that the hFSHR receptor might be glycosylated at two of three (at positions 191, 199, 293) glycosylation consensus sequences, whereas in the hLHCGR at least five, or perhaps all six of the glycosylation sites may be glycosylated (Ascoli et al. 2002; Davis et al. 1995) (Fig. 2). Naturally occurring mutations in the ECD of the hFSHR (Huhtaniemi and Themmen 2005; Tapanainen et al. 1998) and the hLHCGR (Guan et al. 2009; Tao et al. 2004), near or at putative glycosylation sites (Fig. 2a, b), emphasize on the critical role of glycosylation in targeting of the gonadotropin receptors to the cell surface plasma membrane. Mutations at the NH_2-terminal end of the hFSHR ectodomain also affect expression of the receptor. Alanine scanning mutagenesis of this region has identified two regions encompassing amino acids 29–31 and 39–47 whose primary sequence is important for receptor trafficking (Nechamen and Dias 2000, 2003). In particular, mutations at Phe30, Ile40, Asp43, Leu44, Arg46, and Asn47 considerably reduced cell surface expression due to impaired intracellular trafficking (Nechamen and Dias 2003). Mutations at these sites impair proper glycosylation of the receptor but this is likely due to inappropriate amino terminal folding and trapping of these intermediates by surveillance proteins which then block appropriate glycosylation processing of endoglycosidase H (an enzyme that cleaves asparagine-linked mannose rich oligosaccharides, but not highly processed complex oligosaccharides from glycoproteins)-sensitive molecules in the ER-Golgi (Nechamen and Dias 2003).

It has been shown that the rat FSHR is glycosylated at two glycosylation consensus sequences and that the presence of carbohydrates at either one of these sites (Asn191 or Asn293) is sufficient for receptor folding and trafficking to the PM (Davis et al. 1995). Thus, in the rat FSHR, at least one glycosylation site at the FSHR ECD is required for receptor folding and efficient trafficking to the PM. Lack of glycosylation of the mature rat FSHR does not affect binding or affinity, indicating that this particular structure does not participate in ligand interaction. In the rat LHCGR, mutations that prevented glycosylation at the first three consensus sequences (positions 99, 174, and 195) (Nunez Miguel et al. 2017) did not affect receptor synthesis or ligand binding but decreased the efficiency of receptor folding, leading to reduced maturation, increased degradation of the precursor protein, and marked reduction in PM expression (Clouser and Menon 2005).

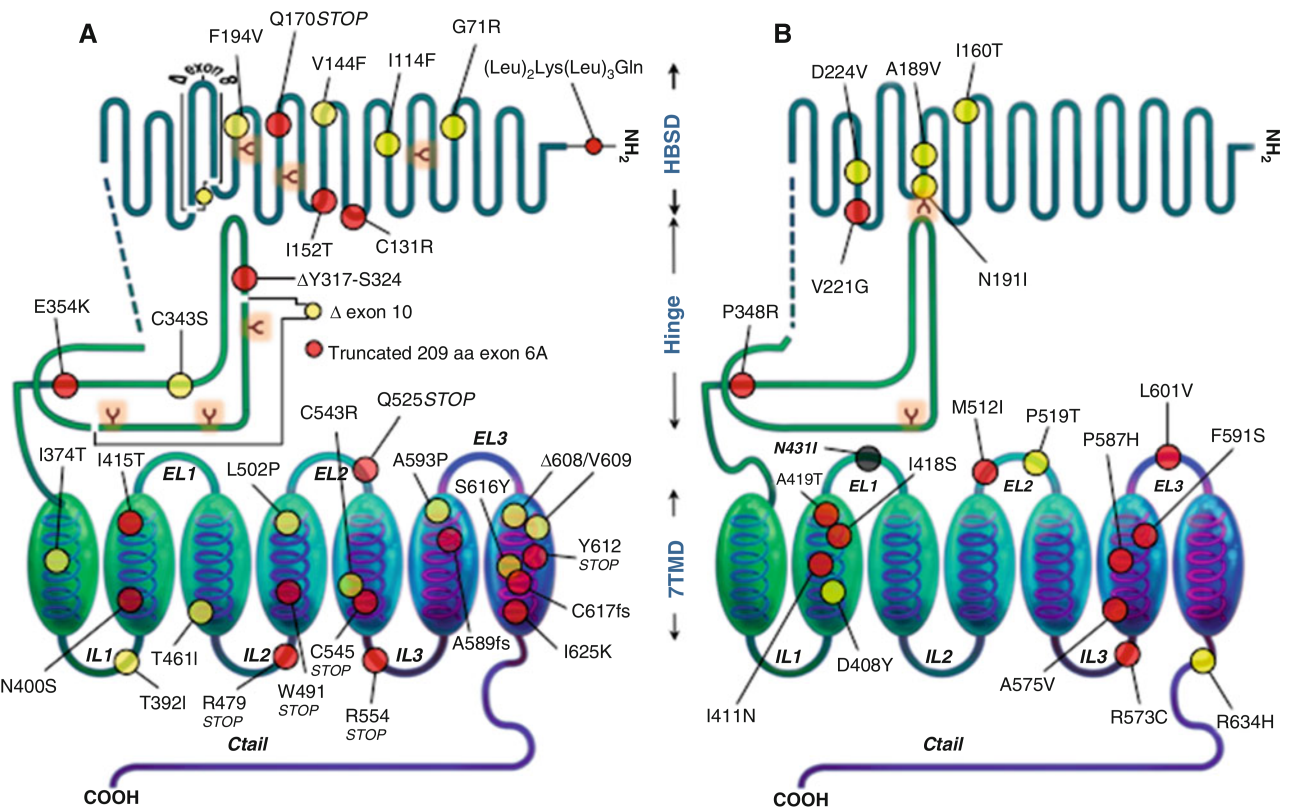

Fig. 2 Schematic representation of the human gonadotropin receptors, showing the location of inactivating, loss-of-function mutations (*orange* and *yellow circles*). The mutations represented by *yellow circles* result in misfolding and intracellular trapping of the mutant receptor, leading to reduced or absent cell surface plasma membrane expression. Glycosylation sites are shown as brown arbor-like structures. (**a**) hLHCGR; (**b**) hFSHR. *HBSD* hormone binding subdomain

Thus, glycans in both gonadotropin receptors are not involved in hormone binding but are important structures for the maturation process of the newly synthesized receptors, promoting its correct folding, conformational stability, and its trafficking to the PM.

Co-immunoprecipitation studies have identified some interacting molecular chaperones that support folding of the gonadotropin receptors during their residency at the ER. These studies have shown that the folding process of the rat FSH and LHCG receptor precursors (i.e., co-translationally glycosylated), involves interactions with the previously described chaperones calnexin and calreticulin (Sect. 2.2), which facilitate proper folding of intermediate glycoprotein molecules (Mizrachi and Segaloff 2004; Rozell et al. 1998). Another chaperone that interacts with immature gonadotropin receptors is the protein disulfide isomerase PDI (Mizrachi and Segaloff 2004), which is an ER-resident enzyme involved in disulfide bond formation of folding intermediates, and that probably acts as a co-chaperone with calnexin and calreticulin during their association with these receptors. Interestingly, particular mutants associate differentially with distinct chaperones; for example, the misfolded hLHCGR mutant Ser616Tyr, which leads to Leydig cell hypoplasia, associates with calnexin, calreticulin, and PDI, whereas the mutant Ala593Pro (which also leads to disease) does not apparently associate with the latter chaperone (Mizrachi and Segaloff 2004). Two other chaperones that promote protein folding, BiP and Grp94 [which do not interact with the gonadotropin receptors, but strongly associate with misfolded polypeptide chains to mediate retrotranslocation and proteosomal degradation (Gething 1999; Weekes et al. 2012)] also appeared to differentially interact with these mutants; BiP associated with both Ala593Pro and Ser616Tyr, and Grp94, although not detected as associated with Ser616YTyr, appeared associated with Ala593Pro (Mizrachi and Segaloff 2004). Thus, it seems that depending of the folding defect, distinct chaperones may interact with immature, misfolded gonadotropin receptor precursors, in an attempt to correct folding or to promote degradation in the proteasomes.

3.1.3 Palmitoylation

As discussed previously, for some GPCRs S-acylation with palmitic acid is often required for efficient delivery of the protein to the cell membrane, where it facilitates anchoring of the receptor Ctail to the PM. The hFSHR exhibits in its Ctail two conserved cysteine residues (at positions 646 and 672) and one non-conserved cysteine residue at position 644. Although the hFSHR is palmitoylated at all cysteine residues, regardless of their location in the Ctail of the receptor (Uribe et al. 2008), S-acylation at C644 and C672 is not essential for efficient hFSHR PM localization, whereas at C646 it is, as replacement of this residue with glycine or alanine reduced detection of the mature form of the receptor by ~40–70% (Melo-Nava et al. 2016; Uribe et al. 2008). Further, when all palmitoylation sites were removed from the hFSHR, cell surface PM expression was reduced to ~10–30% of that shown by the WT receptor (Melo-Nava et al. 2016; Uribe et al. 2008) (Fig. 3a). The hLHCGR is palmitoylated at two conserved

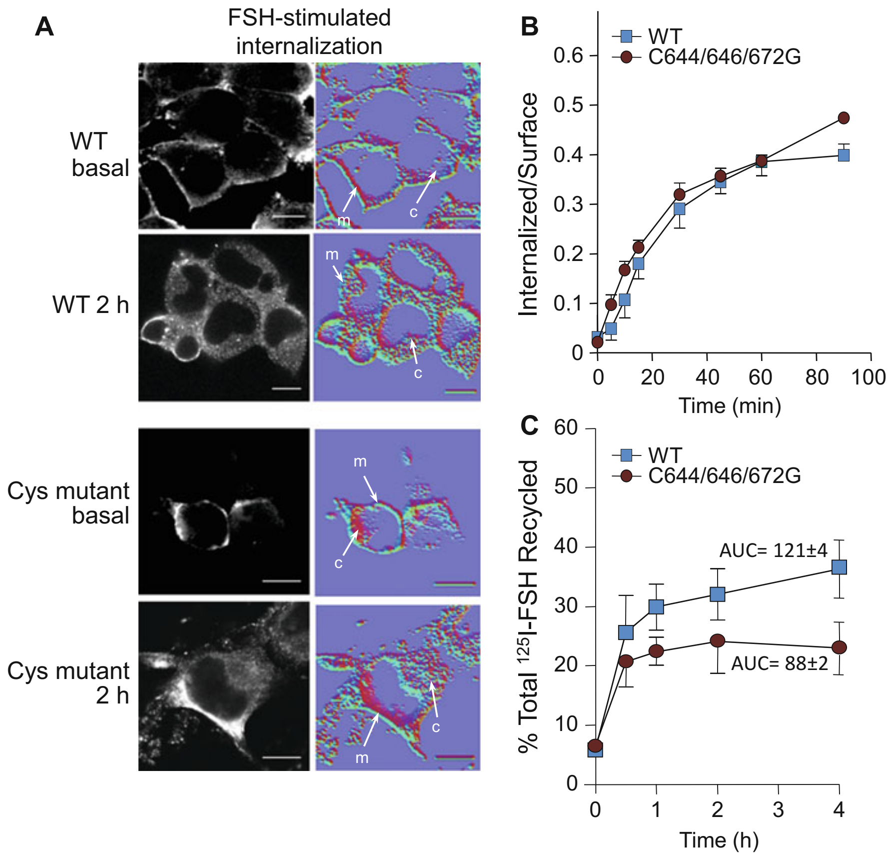

Fig. 3 FSH-stimulated internalization and recycling of the WT hFSHR and a triple mutant hFSHR in which potential palmitoylation sites (Cys644, 646, and 672) were replaced with glycine. (**a**) Confocal microscopy of HEK293 cells expressing the WT or triple mutant hFSHR before (basal) and after 2-h exposure to agonist (*left panels*). The spectral pixel-density analysis of the confocal images is shown in the *right panels*, which indicate the distribution of the WT and triple mutant hFSHR (*green-red* clusters against a *light blue* background), before and after 2 h exposure to FSH. The relocalization of the FSHR to the cytoplasm (*white arrows* in each spectral representation) after hFSH stimulation is evident in both WT and mutant hFSHR-transfected cells [for details see (Melo-Nava et al. 2016)]. *m* cell surface plasma membrane, *c* cytoplasm. (**b**) FSH-stimulated internalization of ^{125}I-FSH under nonequilibrium conditions. The graphs represent the internalized hormone (cell associated/surface ^{125}I-FSH ratio) in HEK293 cells transiently expressing either the WT of triple Cys mutant hFSHR as a function of time. The internalization kinetics were similar between the two hFSHRs, confirming the results obtained by confocal microscopy. (**c**) Total ^{125}I-FSH/hFSHR complex recycled back to the plasma membrane, following exposure of HEK293 cells transiently expressing the WT or triple Cys mutant hFSHR to FSH. Total recycled FSH/mutant hFSHR complex was decreased compared to the FSH/WT hFSHR complex. For details see Melo-Nava et al. (2016). The methods employed in (**b**) and (**c**) are described in detail in Ulloa-Aguirre et al. (2013)

cysteine residues (643 and 644) (Kawate and Menon 1994; Munshi et al. 2005; Zhu et al. 1995), but in contrast to the hFSHR, palmitoylation of this receptor is not important for trafficking to the PM, as abrogation of palmitoylation did not appear to affect PM expression and agonist binding (Kawate and Menon 1994; Munshi et al. 2005).

In both gonadotropin receptors, palmitoylation appears to influence agonist-stimulated internalization and the post-endocytic fate of the receptor, as will be discussed in Sect. 3.2

3.1.4 Homo- and Heterodimerization of Gonadotropin Receptors and Trafficking

A number of in vitro studies have shown that the glycoprotein hormone receptors self-associate (Horvat et al. 1999; Jonas et al. 2015; Kleinau et al. 2016; Latif et al. 2001; Mazurkiewicz et al. 2015; Tao et al. 2004; Thomas et al. 2007). Although the crystal structure of the hormone binding domain of the human FSHR in complex with FSH (Fan and Hendrickson 2005) indicated that the FSHR ectodomain may form weakly associated dimers, further studies employing combined biochemical and biophysical approaches directly demonstrated that the hFSHR self-associates early during receptor biosynthesis and that it can be identified as FSHR/FSHR homodimers or FSHR/LHCGR heterodimers in the cell surface PM of HEK293 cells (Mazurkiewicz et al. 2015; Thomas et al. 2007). Further, biochemical studies have found that the carboxyl-terminus of the hFSHR is clipped before trafficking to the PM, although it is not known whether clipping is necessary for efficient targeting of the receptor to the PM (Thomas et al. 2007). Although the mechanism and extent of FSHR self-association is not known, it seems reasonable to assume that multiple contacts occurring via the transmembrane domains and/or the Ctail play an important role (Guan et al. 2010; Jiang et al. 2014; Zarinan et al. 2010). In this vein, experiments employing interfering peptides to disrupt the dominant-negative effects of FSHR mutants on WT receptor PM expression support the idea that FSHR monomers may associate during trafficking from the ER to the PM via multiple contacts (Zarinan et al. 2010) (Fig. 4b). In this study, co-transfection of constant amounts of WT and increasing quantities of mutant [Arg573Ala (at the boundary of the IL3 and the TMD6) or Arg635Ala (within the F $(X)_6$LL motif at the Ctail of the receptor)] FSHR cDNAs in HEK293 cells progressively decreased agonist-stimulated cAMP accumulation, agonist binding, and PM expression of the mature WT hFSHR species. Co-transfection of short WT FSHR fragments bearing the site of the mutation (Leu526-Val599 fragment spanning the TMD5 and 6 and the IL3 WT sequence, or the Ala607-Asn695 fragment which included the TMD7and the Ctail) with the mutant receptors, specifically rescued WT FSHR PM expression from the transdominant inhibition by the mutants (Fig. 4b), suggesting that the fragments bound either the mutant or the WT hFSHR preventing their association as well as the intracellular retention and eventual degradation of the mutant:WT receptor complexes (Zarinan et al. 2010). Thus mutations causing misfolding of the receptor may lead to defective

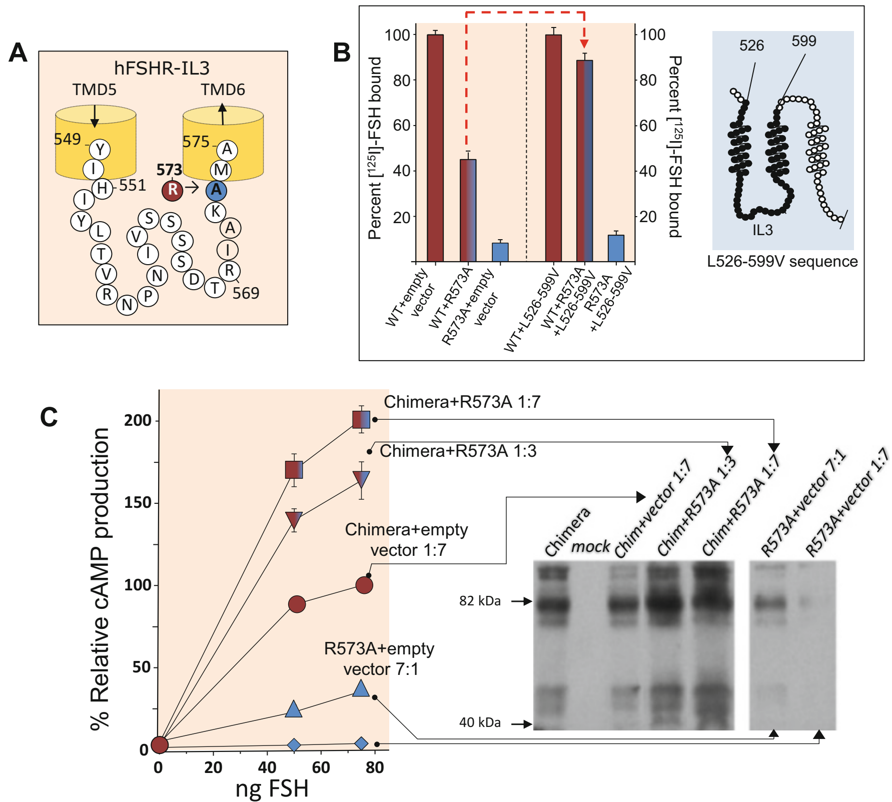

Fig. 4 Effects of the intracellular association of the loss-of-function, laboratory-manufactured Arg573Ala mutant hFSHR with the WT receptor or a WT FSHR in which the carboxyl-terminus (Ctail) was replaced with the Ctail of the hLHCGR ("Chimera"). (**a**) The location of the Arg573Ala mutation at the junction of the intracellular loop 3 (IL3) and the TMD6 of the hFSHR. (**b**) Dominant-negative effect of the Arg573Ala hFSHR mutant on WT receptor plasma membrane expression as assessed by a radioligand binding assay in HEK293 cells (Zarinan et al. 2010). The cell surface plasma membrane expression of the WT was significantly reduced when the cDNA WT hFSHR was co-transfected with the Arg573Ala mutant cDNA at a 1:7 WT/mutant receptor ratio (*left panel*), and recovered almost completely when a cDNA fragment of the WT hFSHR bearing the TMD5 and TMD6 [Leu526-Val599 sequence (*black circles* in the schematics within the *blue rectangle*)] was included in the co-transfection mix (*right panel*). (**c**) The Arg573Ala mutant hFSHR associated with the WT hFSHR/hLHCGR Ctail chimera to increase plasma membrane expression of the chimera (82 kDa in the western blot shown in the *right panel*); the corresponding cDNAs were co-transfected at either 1:3 or 1:7 chimera/mutant receptor ratio. In the experiments shown in (**b**) and (**c**), empty vector was added to the transfection mix so that the total amount of DNA transfected was always the same. For details see Zarinan et al. (2010)

intracellular transport and/or interference with proper maturation of the WT, functional receptor.

The LHCGR also form oligomers in the endoplasmic reticulum and at the PM, in a process that is unrelated to receptor activation (Guan et al. 2009). Interestingly, as

with the hFSHR, co-expression of misfolded hLHCGR mutants with its WT counterpart impaired PM expression of the WT receptor and attenuated signaling (Guan et al. 2009; Tao et al. 2004; Zhang et al. 2009). Co-expression of splice variants of the hLHCGR may also regulate the expression of the WT hLHCG and hFSH receptors by forming intracellular complexes that prevent proper processing of the intracellular LHCGR precursor. For example, hLHCGR transcripts lacking exon 9 are prevalent in normal human ovaries but the resulting protein is not able to bind ligand or to be adequately processed for allowing its expression at the cell surface (Nakamura et al. 2004; Yamashita et al. 2005). When co-expressed with the WT hLHCGR or hFSHR, the mutant lacking exon 9 associated with the immature forms of these receptors and exerted dominant-negative effects by decreasing their cell surface expression (Nakamura et al. 2004; Yamashita et al. 2005).

The dominant-negative effect of mutant receptors on WT anterograde trafficking and PM expression has also been demonstrated for several other GPCRs, including the human GnRHR (Brothers et al. 2004; Leanos-Miranda et al. 2003), the V2R (Zhu and Wess 1998), the D2- and D3-dopamine receptors (Karpa et al. 2000; Lee et al. 2000), and the CCR5 (Benkirane et al. 1997). The dominant-negative action that the mutant GnRH receptors have on the WT receptor appears to be due to ER retention of aggregates formed by WT and mutant proteins as revealed by confocal micoscopy (Brothers et al. 2004). This effect of mutant receptors on WT receptor expression might play a role in the phenotypic expression of diseases in individuals bearing simple heterozygous mutations. In this vein, it is interesting that heterozygous subjects bearing misfolded TSHR mutants (e.g., Cys41Ser, Leu467Pro, and Cys600Arg TSHR mutants) express clinical phenotypes of thyrotropin resistance presumably due to the dominant-negative effect of the mutants on WT receptor PM expression (Calebiro et al. 2005). The fact that individuals who are heterozygous for misfolded mutations in the gonadotropin receptors do not exhibit detectable reproductive abnormalities suggests that the attenuation in PM expression of the WT receptor that results from the dominant-negative effect of the misfolded mutant is not decreased enough to impact on cell function, given that occupancy of only a low fraction of gonadotropin receptors per cell is sufficient to elicit normal responses (Huhtaniemi et al. 1982). Conversely, misfolded trafficking-defective receptors could alternatively increase the amount of functional receptors at the PM via complexing with the WT receptor as has been found in some in vitro studies (Osuga et al. 1997; Zarinan et al. 2010) (Fig. 4c). Thus, it seems that association between mutant misfolded and WT receptors may either limit or favor ER export of functional receptors to the PM, depending on the nature of the folding defect of the mutant, the particular association between the misfolded and correctly folded receptor species, and the final conformation attained by the mutant/WT protein complex.

3.2 Downward Trafficking of PM-Expressed Gonadotropin Receptors: Agonist-Stimulated Internalization and Post-Endocytic Fate

As described in Sect. 2.2, agonist stimulation of GPCRs are followed by a series of structural modifications and associations with scaffold proteins that eventually lead to effector uncoupling, internalization, and either recycling back to the PM or degradation in lysosomes and/or proteasomes. In several experimental models, the hFSHR has been reported to be phosphorylated by second messenger-dependent kinases PKA and PKC, and more importantly by GRKs 2, 3, 5, and 6 (Kara et al. 2006; Krishnamurthy et al. 2003; Lazari et al. 1999; Troispoux et al. 1999). Although PKA and PKC contribute to both agonist-dependent (homologous) and agonist-independent (heterologous) desensitization of the hFSHR, GRK-mediated phosphorylation leads to more complex effects as they are centrally involved in homologous desensitization, regulation of β-arrestin recruitment, receptor internalization, and G protein-independent signaling (Kara et al. 2006). A cluster of five serine and threonine residues has been identified in the Ctail of the hFSHR, which is involved in FSH-stimulated FSHR phosphorylation as a result of GRK2 action (Kara et al. 2006). β-arrestins recruited to the agonist-occupied, GRK2- or GRK5/6-phosphorylated FSHR appear to exert distinct intracellular functions: GRK2-phosphorylated hFSHR predominates in the β-arrestin-mediated desensitization process, whereas GRK5- and GRK6-stimulated phosphorylation of the activated FSHR is necessary for β-arrestin-dependent activation of the MAPK-ERK1/2-signaling pathway (Kara et al. 2006; Marion et al. 2006; Reiter and Lefkowitz 2006). It is well established that β-arrestin 1/2 recruitment to GRK-phosphorylated FSHR leads to internalization and recycling of the receptor (Kara et al. 2006; Lazari et al. 1999; Piketty et al. 2006). In contrast to the hFSHR, the hLHCGR does not recruit GRKs to promote its desensitization; in this receptor, this effect is instead mediated by the interaction of the receptor with ADP ribosylation factor nucleotide-binding site opener (ARNO), which is an exchange factor for ADP ribosylation factor 6 (ARF6) that recruit β-arrestins when bound to GTP (Mukherjee et al. 2000, 2002). In the case of the hLHCGR, β-arrestins are not apparently involved in MAPK-ERK1/2 signaling, and thus this receptor does not demonstrate biased signaling through this particular signaling cascade (Landomiel et al. 2014). Although it has been recently demonstrated that hCG and hLH may exert differential (biased) ß-arrestin recruitment and downstream effects (e.g., progesterone synthesis) on the hLHCGR, it is still unknown whether receptor activation provoked by these closely related hormones (which bind the same receptor) also leads to differential internalization kinetics and post-endocytic sorting (Riccetti et al. 2017).

Most of the internalized hFSHR is recycled back to the PM (Fig. 1), whereas in the case of the hLHCGR only 30% of the internalized receptor recycles back to the cell surface (Krishnamurthy et al. 2003; Menon et al. 2005). Among factors involved in the post-endocytic processing and fate (recycling vs degradation) of gonadotropin receptors after internalization, palmitoylation seems to play an

important role (Kawate et al. 1997; Melo-Nava et al. 2016; Munshi et al. 2001, 2005; Uribe et al. 2008). The importance of this posttranslational modification in both internalization and post-endocytic processing of the PM-expressed receptor following formation of the hormone-receptor complex will depend on the particular receptor. It has been shown that abrogation of palmitoylation by replacement of Ctail Cys residues with glycine does not play any role in internalization of the hormone/FSHR complex (Fig. 3a, b), whereas in the hLHCGR prevention of palmitoylation increased the rate of agonist-stimulated internalization probably by regulating the accessibility of the receptor to the arrestin-mediated internalization pathway, which is not observed in the hFSHR (Kawate and Menon 1994; Melo-Nava et al. 2016; Munshi et al. 2001, 2005). Nevertheless, in both receptors the absence of palmitoylation impaired receptor recycling to the PM and increased the fraction of receptor/hormone complex sorted to degradation via the proteasome/lysosome pathway (Fig. 3c) (Melo-Nava et al. 2016; Munshi et al. 2005). Further studies in HEK293 cells have recently shown that the unpalmitoylated hFSHR is targeted for degradation predominantly through the proteasome pathway, since degradation of the altered receptor was counteracted by proteosomal but not lysosomal inhibition (Melo-Nava et al. 2016). In fact, it has been shown that the WT hFSHR is ubiquitinated in the IL3 and that proteasomal inhibitors increase cell surface residency of this receptor (Cohen et al. 2003). Thus in both gonadotropin receptors, palmitoylation plays an important role in intracellular trafficking, albeit with some differences between receptors: palmitoylation seems to be involved in both trafficking of the hFSHR from the ER to the PM as well as in the post-endocytic sorting of the internalized receptor, while in the hLHCGR, S-acylation appears to affect only the latter process.

In addition to palmitoylation, post-endocytic trafficking is also influenced by particular amino acid residues located in the Ctail of the gonadotropin receptors. Progressive truncations on the Ctail of the hLHCGR or replacement of distinct amino acid residues on this domain revealed that Cys699 and Leu683 are both required to route most of the internalized ligand/hLHCGR complex to the recycling pathway (Galet et al. 2004). In fact, replacement of these residues by mutagenesis re-routed the internalized complex to the degradation pathway, and conversely, when grafted to the rat LHR (which is preferentially routed to a degradation pathway), re-routed the internalized rat receptor to the recycling pathway (Galet et al. 2004). Other residues that may also be involved (although at a lesser extent than Cys699 and Leu683) in the preferential routing of the internalized hLHCGR to the PM are Gly687 and Thr688 (Galet et al. 2003, 2004). The recent finding that hCG and hLH, which when bound to the hLHCGR behave as biased agonists and differentially affect the conformation of β-arrestin 2 (Riccetti et al. 2017) suggests that the kinetics of both internalization and post endocytic sorting of the hCG/hLHCGR and hLH/hLHCGR complexes may also differ, a possibility that deserves further investigation. Similar to the hLHCGR, it has been shown that truncations affecting the last 8 amino acid residues of the hFSHR re-route a substantial portion of the internalized FSH-FSHR complex to a degradation pathway (Krishnamurthy et al. 2003).

In summary, several structural determinants are involved in the regulation of the intracellular trafficking of gonadotropin receptors. Failure to correctly express these determinants or mutations in the amino acid sequence may provoke distinct functional abnormalities, including failure of the receptor to express properly at the PM and thus in its capability to interact with and become activated by agonist. The next section briefly addresses how mutations in the gonadotropin receptors may affect export of the gonadotropin receptors from the ER to the PM and lead to disease.

3.3 Trafficking of Gonadotropin Receptors in Disease

Inactivating or loss-of-function mutations in the gonadotropin receptor genes may lead to disease, whenever both alleles are affected by the mutation, as occurs in individuals who are homozygous or compound heterozygous for mutations in the hFSHR or hLHCGR genes. Several inactivating mutations distributed throughout the polypeptide chain of the hLHCGR and hFSHR (which include point mutations, amino acid insertions or deletions, and premature truncations) have been described (Arnhold et al. 2009; Ben Hadj Hmida et al. 2016; Desai et al. 2013; Newton et al. 2016; Tao 2006; Ulloa-Aguirre and Zarinan 2016; Ulloa-Aguirre et al. 2014). The location of these alterations across the gonadotropin receptors is shown in Fig. 2. Most inactivating mutations of the gonadotropin receptors are germ-line, missense mutations that lead to single amino acid substitutions in the receptor protein. Due to the scattered distribution of the mutations along the primary sequence of the receptor, the mutations may alter domains or motifs associated with distinct functions including agonist binding, receptor activation, internalization or coupling to effectors. However, the mutations also frequently lead to receptor misfolding, yielding trafficking-defective proteins unable to transport from the ER to the PM. These functional defects are not mutually exclusive since one mutation may lead to functional alterations on both intracellular traffic and any other function. For example, the Ile625Lys hLHCGR pathogenic mutant, caused in vitro decreased PM expression and deficient coupling to effectors (Martens et al. 1998; Newton et al. 2016; Richter-Unruh et al. 2002). The delLeu608/Val609 misfolded hLHCGR mutant is expressed at very low levels at the PM and exhibits normal binding affinity, but it is unable to trigger intracellular signaling at the G_s protein level upon exposure to agonist (Latronico et al. 1998). These observations suggest that when mutations lead to both misfolding and intracellular retention of the protein, in addition to an altered intrinsic function of the receptor (e.g., signal transduction), the benefit of treatment with pharmacological chaperones to correct folding and targetting to the PM is expected to be limited (see Sects. 2 and 4).

Clinically, inactivating mutations in the *LHCGR* lead to distinct phenotypes, depending on the severity of the functional deficit. In males, phenotypes range from severe genital ambiguity to cryptorchidism and micropenis, whereas affected women may show pubertal development, but frequently present primary or secondary amenorrhea and infertility. In men, inactivating mutations in the hFSHR gene lead to impaired quality of spermatogenesis, with nearly normal testosterone

production which probably accounts for fertility preservation (Tapanainen et al. 1997), while in women the panorama is completely different and comprises an array of phenotypes ranging from lack of pubertal development and primary amenorrhea, with arrest of follicular maturation between primordial and preantral stage and complete resistance to FSH stimulation, to secondary amenorrhea and premature ovarian failure (Aittomaki et al. 1996; Huhtaniemi and Alevizaki 2007). In either case, the level of residual, functional receptors expressed at the PM, has been shown to correlate with the severity of the clinical phenotype presented by the patients, which is an important determinant for the response to exogenous gonadotropins (Vaskivuo et al. 2002).

Among the 34 or so hLHCGR mutants described so far (Fig. 2a), at least 15 are trafficking-defective receptors in which the net amount of functional receptors expressed at the PM is decreased to a variable extent (Newton et al. 2016; Ulloa-Aguirre et al. 2014). These trafficking-defective/misfolded receptors bear mutations either immediately upstream of the signal peptide cleavage site, in the ECD (Gromoll et al. 2002; Martens et al. 1998; Richter-Unruh et al. 2002, 2004; Wu et al. 1998) or in TMDs 1 and 3 to 7 (Kremer et al. 1995; Latronico et al. 1996, 1998; Laue et al. 1996; Martens et al. 1998, 2002; Newton et al. 2016; Richter-Unruh et al. 2002; Toledo et al. 1996) (Fig. 2a). Trafficking-defective mutants of the hLHCGR and their response to functional rescue by pharmacological chaperones are extensively discussed in Chapter 5. Nevertheless, considering the above discussion on the factors involved in folding and intracellular traffic of gonadotropin receptors, including interactions with molecular chaperones and posttranslational modifications, the particular structural and functional features of some of these mutants are worthy of mention here. In the Phe194Val mutant, the amino acid substitution affects the previously described highly conserved motif present in the gonadotropin receptors (193AFNGT197 at the hLHCGR ECD) that bears the Asn195-Gly-Thr glycosylation sequence (see Sect. 3.1.2). This mutation severely impairs trafficking of the mutant receptor to the PM without significantly altering agonist affinity (Gromoll et al. 2002). Meanwhile, substitution of Cys343 with Ser may affect formation of the putative disulfide bridge between this cysteine residue and Cys279, leading to disruption of the three-dimensional structure of the receptor, particularly at the hinge region, and to protein misfolding. In the case of the inactivating Ala593Pro and Ser616Tyr mutant hLHCGRs, the structurally abnormal proteins are conformationally distinct and exhibit different folding conformations during their maturation process, as suggested by their differential association with molecular chaperones at the ER (Mizrachi and Segaloff 2004) (see Sect. 3.1.2). These mutants exhibited normal ligand binding affinity but the response to agonist was absent or severely impaired due to intracellular retention. On the other hand, extensive deletions, as those occurring in exons 8 and 10 (located in the putative LRR and hinge regions of the hLHCGR, respectively) may severely compromise the conformation of the protein leading not only to misfolding but also to impaired ability of the receptor to interact with its cognate ligand and become activated. In this vein, the recently reported 1850delG mutation in exon 11 is interesting; this is a frameshift mutation that results in replacement of the last

83 amino acid residues of the receptor by a 21 amino acids sequence that lacks the traffic-regulating $F(X)_6LL$ motif at the NH_2-end of the Ctail (see Sect. 3.1.1). Although this mutant is not a misfolded protein but rather a receptor devoid of a sequence critical for intracellular traffic of the receptor to the PM, it was refractory to functional rescue by pharmacological chaperones (Rivero-Muller et al. 2015) demonstrating that not all trafficking defects can be rescued.

On the other hand, naturally occurring mutations in the hFSHR gene are fewer in number than those detected in the hLHCGR (Fig. 2b). Among the 18 or so mutant hFSHRs reported to date, at least seven are trafficking-defective proteins, many of which have been identified as intracellularly retained molecules [Ile160Thr, Ala189Val, Asn191Ile, Asp224Val, at the ECD (Beau et al. 1998; Gromoll et al. 1996; Touraine et al. 1999); Asp408Tyr at the TMD2 (Bramble et al. 2016); Pro519Thr at the EL2 (Meduri et al. 2003), and Arg634His at the NH_2-terminal end of the Ctail (Hugon-Rodin et al. 2017)] by in vitro studies. Similar to the loss-of-function hLHCGR mutants, a good general correlation between residual activity exhibited by the mutant hFSHRs in vitro and the severity of the clinical phenotype showed by patients bearing the mutation(s) also holds true (Aittomaki et al. 1996; Huhtaniemi and Themmen 2005). The most severe phenotype is exhibited by females homozygous for the trafficking-defective loss-of-function hFSHR mutations Ala189Val and Pro519Thr, and who presented with hypergonadotropic hypogonadism, arrest of follicular maturation beyond the primary stage and complete lack of responsiveness to FSH (Aittomaki et al. 1996; Huhtaniemi and Themmen 2005; Meduri et al. 2003). Less severe phenotypes were observed in women who were homozygous or compound heterozygous for other mutations and the phenotypes included secondary amenorrhea, gonadotropin resistance, and follicular development up to the antral stage (Beau et al. 1998; Huhtaniemi and Themmen 2005). The phenotype in homozygous males is not clinically obvious given that although sperm quality is altered fertility is preserved (Tapanainen et al. 1997), probably accounting for the rare detection of inactivating mutations in the hFSHR in males. The naturally occurring mutation Ala189Val causes a profound defect in targeting the receptor protein to the PM (Rannikko et al. 2002), as it compromises integrity of the 189AFNGT193 motif (Sect. 3.1.2). Valine in position 189 as well as isoleucine 191 may interfere with the structural integrity of the LRRs, which host the glycosylation site, and perturbation of this structure likely impairs proper receptor LRR formation, particularly its α-helical portion. Although the loss of a putative glycosylation site may affect folding and trafficking of the mutant receptor to the PM, it is not known whether the Ala189Val mutant is glycosylated or not at the Asn191 site. When the Ala189Val mutant is overexpressed in vitro, a negligible amount of the mutated receptor is present at the PM and most of the receptor protein is sequestered and retained inside the cell (Rannikko et al. 2002). Interestingly, the reduced level of PM expression of the Ala189Val hFSHR confers preferential coupling to the β-arrestin-mediated ERK 1/2 signaling pathway, similar to that observed when the WT receptor is expressed at low PM levels (Tranchant et al. 2011), indicating that the selective signaling observed is due to the low density of PM expression of the receptor rather than

because of the mutation causing a functional defect. This observation might explain why mutations of the FSHβ gene are more deleterious to male fertility than the hFSHR Ala189Val mutation (Layman et al. 2002; Lindstedt et al. 1998), which allows preservation of a fraction of the hFSHR-mediated signaling repertoire. In the case of the Asn191Ile mutant (Gromoll et al. 1996) it is also possible that its limited PM expression may be due to alterations in the structural integrity of the ectodomain at the 189AFNGT193 glycosylation motif, rather than to the absence of glycosylation at this particular site.

As with other GPCRs, the location of the mutation and the nature of the amino acid substitution define the functional features exhibited by mutant hFSHRs. The hFSHR Pro519Thr mutation in the center of the EL2 leads to complete failure to bind agonist and trigger intracellular signaling. It seems that the loss of a proline at position 519 provokes a severe conformational defect that leads to trapping of the receptor at the ER (Meduri et al. 2003). Because the peptide backbone of proline is constrained in a ring structure, occurrence of this amino acid is associated with a forced turn in the protein sequence, which is likely lost by the substitution with the more reactive threonine; it is thus possible that the abrupt turn at the middle of the EL2 is probably a requisite not only for activity (Dupakuntla et al. 2012) but also for routing. The effects of this particular mutation contrast with those provoked by other mutations in the serpentine region of the hFSHR (e.g., Ala419Thr and Leu601Val), which usually result in partial receptor inactivation, with minimal effects on FSH binding (Beau et al. 1998; Doherty et al. 2002; Touraine et al. 1999). Another example that emphasizes on the importance of the nature of the amino acid residue substitution is the Arg573Cys hFSHR mutant (Beau et al. 1998). This naturally occurring mutant binds agonist almost normally, whereas the laboratory-manufactured Arg573Ala mutant is expressed at the PM at very low levels (Zarinan et al. 2010) (Fig. 4b). The Arg634His trafficking-defective mutant of the hFSHR is also interesting (Hugon-Rodin et al. 2017); this mutation is located within the $F(x)_6LL$ motif critical for PM targeting (Duvernay et al. 2004), thus explaining the reduced PM expression of the mutant receptor. The functional defects of the Val221Gly (at the ectodomain) (Nakamura et al. 2008) and Ala575Val (at the TMD6) (Achrekar et al. 2010) mutant hFSHRs have not been studied in detail, whereas in the case of the Pro348Arg hFSHR (located at the hinge region of the receptor), both ligand binding and agonist-stimulated signaling were severely impaired (Allen et al. 2003). Whether these three hFSHR mutations interfere with proper trafficking of the receptor from the ER to the PM is still unknown.

One other mutant that is worth mentioning here is the Asn431Ile hFSHR because of its effects on PM expression and agonist-stimulated internalization (Casas-Gonzalez et al. 2012). This mutation (located in the middle of the EL1 Fig. 2b) was detected in a heterozygous man with completely normal spermatogenesis and no obvious clinical phenotype, but that showed undetectable serum FSH concentrations and increased serum antimüllerian hormone levels (a biochemical marker of FSH activity). In contrast with mutants of other GPCRs that promote constitutive receptor endocytosis as a result of increased phosphorylation and

β-arrestin recruitment (Barak et al. 2001; Shi et al. 1998; Wilbanks et al. 2002), the Asn431Ile hFSHR mutant showed decreased PM expression. However, the low level of constitutive activity and markedly reduced agonist-stimulated desensitization and internalization of this form of hFSHR detected when expressed in HEK293 likely explains the physiological phenotype detected in this subject (Casas-Gonzalez et al. 2012). Thus, this particular mutation altered both outward and downward trafficking of the receptor, the latter biochemical phenotype most likely related to the disengagement of the receptor from the mechanisms which normally prevent prolonged exposure and response to agonist.

4 Conclusions

As discussed in this chapter, misfolding and defective intracellular trafficking of gonadotropin receptors is a common abnormality provoked by naturally occurring pathogenic mutations. The success of treatment of misfolded, gonadotropin receptors (and other misfolded GPCRs leading to disease as well) with pharmacoperones depends on several factors, including the partial or complete integrity of domains involved in ligand binding, receptor activation and/or coupling to effectors. As demonstrated in vitro and in vivo for the hGnRHR and other GPCR mutants (Bernier et al. 2006; Conn and Ulloa-Aguirre 2010, 2011; Ulloa-Aguirre and Conn 2016), misfolded hFSH and hLHCG receptors (see Chapter 5) may be also rescued in vitro by pharmacoperone treatment that ameliorates the folding defect thereby promoting trafficking of the intracellularly trapped receptors from the ER to the cell surface PM. In the case of the misfolded Ala189Val hFSHR mutant, the effect of Org41841 [which is a thienopyr(im)idine molecule reported to bind a conserved region of the hLHCGR and that behaves as an allosteric modulator) (van Straten et al. 2002)], increased almost by twofold PM expression and FSH-stimulated cAMP production of the mutant hFSHR, without significantly altering mRNA expression of the receptor nor its ligand binding affinity (Janovick et al. 2009). This and other similar, cell permeant compounds might be potentially useful to treat patients with gonadotropin mutations who express mild clinical phenotypes. This is the case, for example of men bearing the Ala189Val mutation, in which specific allosteric modulators might improve the quality of the sperm parameters (Tapanainen et al. 1997). Validation of high throughput screening assays will undoubtedly allow identification of new, highly specific molecules that do not interfere with endogenous agonist binding or activation of the misfolded receptor and that may function well in vivo to rescue function of mutant gonadotropin receptors, as it has been achieved for other GPCRs that cause disease in humans (Conn et al. 2014a; Janovick et al. 2011; Ulloa-Aguirre et al. 2015).

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