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**“A disorder-to-order structural transition in the  
cytosolic tail of Fz4 is responsible for the  
misfolding of the L501fsX533 Fz4 mutant”**

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## **Abstract**

Frizzled 4 belongs to the superfamily of G protein coupled receptors. The unstructured cytosolic tail of the receptor is essential for the activation of different intracellular signalling pathway. Mutations in the *fr4* gene are responsible for the Familial exudative vitreoretinopathy (FEVR), among these the autosomal dominant frameshift mutation L501fsX533 that codes for a new carboxy terminal tail. Here we show that the new tail generated by the frameshift mutation acquires a helix-loop-helix conformation. The helices are amphipathic, display affinity for membranes and resemble the structure of Influenza Hemagglutinin fusion peptide at the pH of fusion. Shape and curvature of the mutant tail is proven essential for proper membrane interaction in vitro. The new fold of the tail is necessary for the aggregation and retention in the Endoplasmic Reticulum of the receptor via its interaction with the membrane and is sufficient to induce the misfolding and affect trafficking of a chimeric VSVG protein containing the mutated tail. Shifting the fold of the tail to a more disordered conformation relocates the receptor to the Plasma Membrane. Such disordered to ordered structural transition was never described in mammalian receptors and pinpoints the need to consider structural transition as possible outcome of mutations in unstructured regions of proteins.

# 1. Background

## 1.1 Overview on the secretory pathway

Eukaryotic cells are highly compartmentalised and each distinct membrane-bound organelle has a unique composition of proteins and lipids. This feature is essential for several cellular processes. To sort molecules to defined locations are required highly specific transport mechanisms which also ensure that the identity, and hence the function, of individual compartments are maintained. Proteins carry structural information that target them to their correct destination and many sorting signals have now been identified (Nakai 2000). Proteins that are transported within the secretory pathway are either secreted from the cell, destined to the plasma membrane, addressed to lysosomes, or are retained as “residents” in any of the organelles (Michelsen et al. 2005). The endoplasmic reticulum (ER) controls the correct fold of newly synthesised proteins, before being packaged into transport vesicles (Prydz et al. 2008). Secretory proteins are then transported through the Golgi cisternae to the trans-Golgi network (TGN), or Golgi exit site (Beckers and Rothman 1992).

At the TGN proteins are sorted according to their final destinations. The TGN and the early endosomal compartment represent the two major sorting stations of the cell. (De Matteis and Luini 2008). Proteins are internalised from the cell surface in endocytic vesicles and transported to the early endosomal compartment where sorting takes place. Thus, endocytosed proteins can then be recycled to the plasma membrane (such as recycling receptors), or transported to the TGN or to the lysosome via the late endosomes for degradation (Seaman 2007).

Protein transport in the secretory and endocytic pathways is a multi-step process involving the generation of transport carriers, the shipment of the transport carriers between compartments, and the specific fusion of these transport carriers with a target membrane (Derby and Gleeson 2007). During the last twenty years, much of the cellular machinery responsible for intracellular transport and protein sorting has been described. It is now clear that several human diseases are due to defects of intracellular trafficking, pinpointing the biological importance of these accurate membrane-mediated processes (Amara et al. 1992; Aridor and Hannan 2000; van Vliet et al. 2003; Olkkonen and Ikonen 2006).

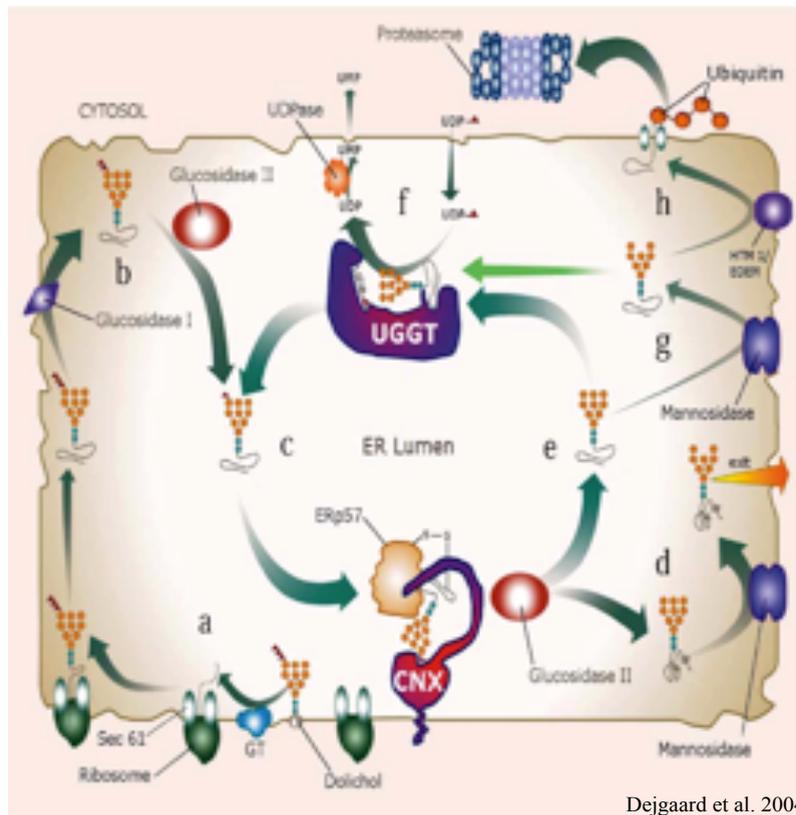
## 1.2 Visiting the ER

The ER provides for the fold and assembly of plasma membrane receptors, ion channels, secreted hormones, catabolic and metabolic enzymes. A quality control (QC) machinery co-ordinates ER activities in accord with cell and organism demands [Fig. 1 (Dejgaard et al. 2004)].

As a consequence, protein assembly and folding in the ER concern a broad range of essential biological process. Specific mistakes in protein handling in the ER can cause the development of different and seemingly unrelated diseases affecting different organs (Olkkonen and Ikonen 2000; Olkkonen and Ikonen 2006).

The first compartment of the secretory pathway is the ER (Aridor 2007), one of the largest intra-cellular organelles, responsible for more than 50% of the total membrane content in a secretory cell wherein takes up over 10% of a cell's volume (Chevet et al. 2001). The elaborate architecture correlates with the role of the ER in a variety of cellular processes including assembly of proteins, folding, biosynthesis of lipids and control of Ca<sup>2+</sup> signaling and homeostasis (Borgese et al. 2006). The elaborate activities of the ER are prone to mistakes that lead to development of diseases (Aridor and Balch 1999).

Upon translation, proteins destined for residence along the secretory pathway are targeted by N-terminal signal to the ER lumen (Walter and Johnson 1994). These proteins are synthesized by ER-associated ribosomes and co-translationally translocated across the membrane via interactions with the Sec61p complex and the signal recognition particle (SRP) receptor (Rapoport et al. 1996; Matlack et al. 1998). The polypeptide chain is cotranslationally translocated into the lumen of the ER through the Sec61p membrane channel, referred to as the translocon, with the assistance of the translocating-chain associating membrane (TRAM) protein (Gorlich and Rapoport 1993). The ER sorts proteins according their assembly and folding. Folded and assembled proteins are packaged in membrane carriers to be transported to the Golgi complex via vesicles formation mediated by cytosolic protein complexes, the COPII coat (Barlowe et al. 1994; Aridor et al. 1998; Bi et al. 2002; Mancias and Goldberg 2008). Misfolded and unstructured proteins are diverted from a folding pathway to proteasome-mediated degradation (Fig. 1) (Hoff et al. 2004; Hebert et al. 2010). Proteins are retro-translocate in the cytosol for proteasome-mediated degradation (Hiller et al. 1996; Pilon et al. 1997; Hebert et al. 2010).



**Figure 1.** CNX/CRT cycle. (a) The precursor glycan (Glc3Man5-9GlcNAc<sub>2</sub>) linked to the lipid molecule, dolichol (Burda and Aebi, 1999). (b) Glucosidase I and II successively trim two of the glucose residues leaving the Glc1Man9GlcNAc<sub>2</sub> core oligosaccharide. (c) Calnexin and its luminal paralogue calreticulin are lectins that specifically bind monoglucosylated oligosaccharides, and present them to the glycoprotein-specific thiol oxidoreductase, ERp57, which also binds to CNX/CRT. If the proteins are correctly folded (d) they proceed further into the secretory pathway, whereas incompletely folded proteins (e) are recognized by UGGT. (f) UGGT reglucosylates incompletely folded proteins by readdition of a single glucose residue from UDP-Glc. (g) The CNX/CRT cycle continues until the proteins are correctly folded or directed to ER-associated degradation (ERAD) after trimming by ER  $\alpha$ 1,2-mannosidases. (h) An enzymatically inactive member of this protein family, ER Degradation Enhancing  $\alpha$ -Mannosidase-like protein (EDEM) and the yeast homologue Mn11p (mannosidase-like protein) or Htm 1p (homologous to mannosidase I, shown as HTM 1) may participate as lectins and promote ERAD of incorrectly folded proteins that are then transported to the cytosol via the Sec61p translocon complex where they are proteolytically degraded by the proteasome system, in most cases following polyubiquitination.

### 1.3 ER processing disease

Mutations in proteins often hinders their proper folding and thus their activity. Generally, loss of function is caused by folding problems (Aridor 2007).

The scenario is much more complicated because sometimes mutation does not alter the function of protein. Despite its activity, the fold of the mutant is still recognized by QC that traps it in the ER. Defect not altering the function of the protein but still causing its miss-localization are called conformational disease.

A deletion of phenylalanine (F) at position 508 in cystic fibrosis transmembrane conductance regulator (CFTR) is the cause for disease in up to 80% of cystic fibrosis patients. The deletion in the cytosolic portion of CFTR causes the absence from the plasma membranes of epithelial cells from patients with  $\Delta F508$  allele. Unlike wild-type CFTR, which is transported to the plasma membrane along the secretory pathway,  $\Delta F508$  is retained and degraded in a pre-Golgi compartment. These observations have led to the hypothesis that the  $\Delta F508$  mutation leads to misfolding of CFTR and causing retention of the misfolded protein by the QC in the ER, resulting in its rapid degradation by the ubiquitin-proteasome pathway (Ward et al. 1995). Loss of CFTR function alters the hydration of the mucosal layer that lines the airway epithelia leading to persistent bacterial growth and development of lung fibrosis (Moskowitz et al. 2008).

When a protein fails to fold, recognition for export from the ER is prevented and also degradation is inhibited leading to accumulation and possible aggregation (Thomas et al. 2004). These events can induce the inflammatory responses and can also culminate in cellular apoptotic response, eliminating affected cells to preserve organ function. This solution may cause degenerative disease leading to organ decay. Therefore in addition to loss of function derived from the retention of the mutant protein intracellularly, apoptosis is observed due to the inability of the ER to degrade the mutant protein (Aridor 2007). Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a progressive decline in many cognitive processes that leads to dementia. Accumulation of  $\beta$ -amyloid plaques and neurofibrillary tangles are the well-known histopathological hallmarks of AD. It is recognized that increased production, oligomerization and aggregation of amyloid- $\beta$  peptides are the crucial reasons of the onset of AD. The toxic amyloid- $\beta$  peptides A $\beta$ 40 and A $\beta$ 42 are processed from the APP (amyloid- $\beta$  precursor protein) via cleavage by BACE1 ( $\beta$ -secretase) and  $\gamma$ -secretase complexes. APP is a transmembrane protein which is folded in the ER and transported through the Golgi complex to the plasma membrane, where APP can be endocytosed and transported to the lysosomal compartment. Several studies have demonstrated that amyloid- $\beta$  can be oligomerized and localized to intracellular compartments, in particular in AD, which can (i) alter the function of proteasomes and lysosomes, (ii) damage calcium homeostasis and (iii) enhance the formation of neurofibrillary tangles in neuron cells. Accumulation of unfolded or aggregated proteins, increased oxidative stress, and metabolic

disturbances are characteristic features of AD therefore it is clear that AD brains display many markers of ER stress. ER tries to defend the host by activating the UPR (unfolded protein response) including signaling pathways that recall the adaptive changes in metabolism and gene expression required to manage stress situations. If this condition becomes more prolonged or unbearable, the ER can then trigger the apoptotic program killing the cell but saving the tissue from necrotic injury (Salminen et al. 2009).

Variations on these conditions are observed in a number of seemingly unrelated degenerative and neurodegenerative diseases. ER-derived diseases also develop because of mutations in the machinery which senses, processes, exports or degrades proteins in the ER (Aridor 2007).

#### 1.4 Quality control: the folding in the ER

A number of chaperones in the lumen of the ER assists the protein to acquire its proper tertiary and quaternary structures (Chevet et al. 2001). They slow down folding and prevent aggregation, leading to the correct formation of disulphide bonds. The same chaperones are responsible for the identification of malformed proteins, sending them back through the translocon into the cytosol to be degraded by the proteasome (Lord et al. 2000).

Guided by the primary information encoded in the polypeptide sequence, proteins inserted in the ER acquire the energetically stable conformation. Therefore, hydrophobic side chains are packed within the protein and intramolecular interactions, as ionic and disulphide bonds, are formed to stabilize a folded configuration and reduce the free energy associated with the newly synthesized polypeptide (Anfinsen and Haber 1961). Recent work has demonstrated that stability of polypeptides in the chemical environment of the ER is sufficient for proteins to move forward in the secretory pathway (Sekijima et al. 2005). Soluble proteins are folded within the lumen of the ER whereas membrane proteins are folded in three environments; the cytosol of the cell, the lipid bilayer of the ER and the ER lumen. The basic chemical environment in the ER resembles that of the outside of the cell. ER lumen is oxidative and contains millimolar  $\text{Ca}^{2+}$  concentrations (Hwang et al. 1992; Schroder and Kaufman 2005). The lipid composition of the ER membrane also differs from that of the plasma membrane with low concentrations of cholesterol (Allan 1996). At the onset of the peptides translocation, folding is guided by interactions with components of the folding chaperones. During synthesis, the translocon pore permits the transfer of 70 amino acids, restricting the freedom of peptide folding during insertion within the ER (Matlack and Walter 1995; Ban et al. 2000). Once polypeptides emerge within the ER lumen are recognized and modified by the ER folding machinery and the signal peptide is removed. Oligosaccharyltransferase transfers high mannose oligosaccharides [Glc(3)Man(9)GlcNAc(2)] from dolichol linked precursors on the Asparagine residues within Asn-X-Ser sequences that extend from the translocon pore (Nilsson and von Heijne 1993). This reaction (N-glycosylation), which is typical of the ER, is not only orchestrated to attach big hydrophilic groups to the polypeptide but also generates recognition sites for folding chaperones (Helenius and Aebi 2004). Folding chaperones begin cycles of binding and release with polypeptides protruding from the translocon pore. These interactions hide non-folded peptide domains while the protein is folding to restrict the freedom of the peptide and drive it to adopt specific kinetically and energetically favorable conformations.

The ER contains high concentrations of several folding chaperones. The main chaperone set operating in the ER is the glucose-regulated protein (GRP) HSP70-like GRP78/Bip and its regulatory proteins. These include the HSP40-like J-domain containing ERdj1-5 (Shen et al. 2002; Dudek et al. 2005; Shen

and Hendershot 2005) and the nucleotide exchange factors BAP and GRP170 (Chung et al. 2002; Weitzmann et al. 2006). HSP90-like chaperones (GRP94) also contribute in ER folding. A typical aspect of the ER involves glycosylation-assisted folding which is mediated by resident lectins as calnexin and calreticulin (Helenius and Aebi 2004).

#### *PPI and PDI*

Enzymes that modify the topology of the synthesized protein belong to the basic set of chaperones. Peptidyl prolyl isomerases (Calebiro et al.) turn the peptide, which is blocked by the interaction of proline side chains with the peptide backbone (Barik 2006). Another typical aspect of the ER is its oxidative environment that permits disulphide bonds formation. Protein disulfide isomerase (PDI) enzymes containing paired cysteines (CXXC) motifs, use this paired residues to reduce and oxidize incorrect S-S bonds in client proteins. The oxidative potential of the ER is generated by ER oxidase 1 (Ero1) and Ero2 to balance the oxidized and reduced PDIs (Sevier and Kaiser 2002; Sevier and Kaiser 2006; Sevier et al. 2007).

In order to advance in the folding reaction, peptide domains (in particular hydrophobic domains that will be packed within the protein core) need to be stabilized in a protective environment. This environment is generated through transient chaperone binding to the client polypeptides.

#### *GRP78/Bip*

The ER chaperone GRP78/Bip interacts widely and with low affinity with hydrophobic peptide domains (Blond-Elguindi et al. 1993). J-domain containing ERdj1-5 proteins induce ATP hydrolysis on GRP78/Bip and the ADP-bound chaperone presents enhanced client-peptide binding affinity (Shen et al. 2002; Dudek et al. 2005; Shen and Hendershot 2005). Nucleotide exchange and ATP binding mediated by proteins such as BAP, promote peptide-client release to allow for the progression of folding (Chung et al. 2002).

#### *Lectin-chaperones*

Another mechanism is responsible of the on and off binding of lectin chaperones with client polypeptides by enzymatic modulation of the peptide-attached *N-glycan* (Helenius and Aebi 2004). Calnexin and its luminal soluble homolog Calreticulin are lectin-chaperones that interact with *N-glycosylated* polypeptides by binding to the monoglycosylated glycan Glc(1)Man(7-9)GlcNAc(2) present on glycoprotein-clients (Hebert et al. 1995; Cannon et al. 1996; Hebert et al. 1996). Calnexin displays a cytosolic tail, a transmembrane domain and an ER luminal domain containing a beta sandwich domain characteristic of lectins (Schrag et al. 2001; Williams 2006). Calreticulin is a soluble homolog of calnexin that resides in the lumen of the ER and contains a similar glycan-binding domain.

### *Glucosylase I and II*

The specific interaction of calnexin and calreticulin with monoglycosylated glycans regulates their binding to client proteins. To generate this glycan binding site, ER enzymes glucosylase I and II sequentially remove the two glucose residues from Glc(3)Man(9)GlcNAc(2) on the nascent polypeptides (Helenius and Aebi 2004; DePrez et al. 2005). Glucosylase II can further remove the last glucose residue and abolish the binding site for these lectin chaperones. The ER enzyme glucosyl transferase re-glucosylates Man(7-9)GlcNAc(2) to renovate the binding site for calnexin and calreticulin and allow for cyclic client-chaperone interactions that guide folding (Hebert et al. 1995; Cannon et al. 1996; Hebert et al. 1996; Helenius and Aebi 2004).

Several studies suggested that calnexin, calreticulin, GRP78/Bip and GRP94 assemble in high molecular weight complexes with a nascent polypeptide (Tatu and Helenius 1997). Therefore, chaperones in the ER form a network that propagate folding. Calnexin and calreticulin directly bind a glycoprotein-oxidoreductase ERp57 to permit the formation of disulphide bonds in the monoglycosylated unfolded proteins (Leach et al. 2002; Williams 2006). The cytosolic tail of calnexin, binding the ribosome complex, links the two sides of the ER membrane to couple protein synthesis and folding (Chevet et al. 1999; Delom and Chevet 2006; Aridor 2007).

## 1.5 Decision making in the ER

The ER contains high concentrations of unfolded peptides. Molecular QC is required to distinguish between proteins that acquire the correct fold and those that cannot achieve the correct structure required to progress through the secretory pathway. Non-folding proteins are removed by degradation to prevent accumulation and possible toxic aggregation. The crucial role of the ER in controlling the QC leads to the development of several ER-folding related diseases.

One molecular QC mechanism utilizes *N*-glycan to monitor the progression of folding. Removal and re-addition of the terminal glucose on Glc(1)Man(9)GlcNAc(2) by glucosidase II and glucosyltransferase (GT1) respectively, activate a loop of interactions between folded proteins and calnexin and calreticulin (Hebert et al. 1995; Schrag et al. 2001; Helenius and Aebi 2004; Williams 2006). Importantly, GT1 recognizes exposed hydrophobic domains in folding intermediates. Thus, GT1 selectively supports a productive folding pathway. The preferential re-glucosylation of partially folded intermediates directs the progression of the folding reaction (Caramelo et al. 2003; Caramelo et al. 2004). Furthermore, the cyclic bond between calnexin, calreticulin and client proteins are eventually broken by the removal of mannose residues from three branches of Man(9)GlcNAc(2) by ER mannosidases I and II, leading to the formation of Man(7-8)GlcNAc(2). These *N*-glycan, which are formed due to prolonged retention in the ER is inefficiently recognized by glucosidase II and GT1. Therefore the cycles of binding and release between client polypeptides and lectins are inhibited, interrupting protein-folding (Parodi 2000; Cabral et al. 2001; Totani et al. 2006). One of the mechanisms to recognize *N*-glycosylated clients for degradation involves the recognition of mannose trimmed *N*-glycans [Man(9)GlcNAc(2)] by ER lectins or enzymes of the ER degradation-enhancing alpha-mannosidase-like protein (EDEM) family (EDEM1-3). EDEM proteins were identified as a homologous of ER mannosidases that are up-regulated during ER stress and facilitate ER degradation (Oda et al. 2003; Olivari et al. 2005; Hirao et al. 2006). Moreover several evidences suggest that EDEM proteins not only bind but may also generate mannose trimmed *N*-glycans (Hirao et al. 2006; Olivari et al. 2006). The overall effect of EDEM recognition is to target client proteins for ER degradation (Olivari and Molinari 2007). EDEM proteins were shown to interact directly with components of the ER degradation machinery thus linking QC with ER degradation (Oda et al. 2006; Kabani et al. 2003).

Similarly, defects in protein folding located within cytoplasmic domains of membrane proteins are monitored through the interaction between cytosolic folding chaperones and client proteins. For example, degradation of CFTR is controlled by interactions with the cytosolic Hsc70 and HSP90, but modulation of these interactions can promote either folding or degradation (Meacham et al. 2001; Wang et al. 2006; Younger et al. 2006).

## 1.6 Quality Control: the folding out of the ER

QC pathways exist because proteins fold in an unfavorable cellular environment due to molecular crowding and relatively high temperatures (Ellis 2007). These two conditions favor aggregation inhibiting folding. Molecular chaperones favour folding preventing aggregation. This is achieved via weak interactions between the molecular chaperones and the exposed hydrophobic groups of unfolded or misfolded proteins. Under normal cellular conditions the expression level of molecular chaperones is fitted to the overall level of protein synthesis. Under stressful conditions, mature proteins unfold and surpass the capacity of chaperone machinery to prevent aggregation. This stress condition induces feedback response which results in increased expression level of molecular chaperones, due to the de-repression of the heat shock transcription factor Hsf1. Importantly, Hsp90 plays a role in Hsf1 repression via direct interaction. Under stress conditions, or inhibition with small molecules, Hsp90 dissociates from Hsf1 resulting in its activation (Zou et al. 1998). This stressful event is associated with the accumulation of aggregates containing damaged proteins. It seems clear, however, that aggregation represents the last attempts to avoid the degradation of misfolded proteins by the ubiquitin/proteasome system. It happens only when this system becomes insufficient that aggregation ensues.

The role of Hsp90 in the QC system was not appreciated until the benzoquinoid ansamycin, geldanamycin, was shown to be a specific inhibitor of the chaperone (Whitesell et al. 1994). Several studies revealed that client protein kinase and transcription factors were rapidly degraded via the ubiquitin/proteasome pathway. This evidence led to the development of more than 13 small molecules currently in clinical trials as chemotherapeutics (Trepel et al. 2010).

It is well established that Hsp90 sits at the distal end of the pathway of chaperone interactions for newly made transcription factors and protein kinases (Taipale et al. 2010). For example, newly made nuclear receptors such as the glucocorticoid receptor and the progesterone receptor interact with Hsp70 and Hsp40 chaperones before recruitment to Hsp90 by the participation of Hop, the Hsp organizing protein (Pratt and Toft 2003). The mechanism by which client protein folding is regulated by Hsp90's reaction cycle is still unclear. What is clear, however, is that many small molecule inhibitors act certainly by competitive inhibition mechanism of Hsp90's ATPase, which occurs in the N-domain of the chaperone (Prodromou et al. 1997). The inhibition of Hsp90 results in rapid degradation via ubiquitin/proteasome pathway for many, but not all, clients. It seems likely that N-terminal inhibitors promote degradation as a consequence of blocking the folding pathway. N-terminal Hsp90 inhibitors appear to promote the reorganization of chaperone complexes. For protein kinases, there is an increase in Hsp70 binding to the client with a reduction in Hsp90 binding (An et al. 2000). These findings correlate with the role of

Hsp70 in degradation processes (Kettern et al. 2010). This is coherent with Hsp70's ability to coordinate with different ubiquitin ligases, including CHIP and Ubr1 (Connell et al. 2001; Meacham et al. 2001; Nillegoda et al. 2010). Several molecular chaperones and co-chaperones function in both folding and degradation processes. Sse1, the Hsp110 chaperone that acts as a nucleotide exchange factor for Hsp70 (Shaner and Morano 2007) is required for the degradation of client protein kinases upon treatment with geldanamycin *in vivo*. (Mandal et al. 2010). This action requires direct interaction between Sse1 and Hsp70 and occurs before the protein is ubiquitylated. By contrast, loss of function of the Hsp40 co-chaperones Ydj1 leads to a rapid degradation of protein kinases, suggesting that it has a protective role for newly synthesized Hsp90 clients (Mandal et al. 2008). Ydj1 also functions as an Hsp70 co-chaperone, and stimulates Hsp70's ATPase, stabilizing Hsp70-client complexes (Cheetham and Caplan 1998). The distinct roles of Ydj1 and Sse1 as Hsp70 co-chaperones suggests a model in which the stable association of a client with Hsp70 is protective against degradation for client proteins. By contrast, complex dissociation is required for degradation when folding pathways are inhibited with geldanamycin.

There are several examples showing that Hsp90 is required for degradation of membrane proteins in the ER. These include cytochrome p450 2E1, mutant CFTR $\Delta$ F508 and Apolipoprotein B. In each of these cases, geldanamycin was found to inhibit degradation in cell free system when the protein was added to cytosolic extracts in the form of microsomes (Fuller and Cuthbert 2000; Goasduff and Cederbaum 2000; Gusarova et al. 2001). The example of CFTR $\Delta$ F508 is intriguing since this mutant form is stabilized upon Hsp90 inhibition *in vitro*, while the wild type counterpart is destabilized in cells treated with geldanamycin (Loo et al. 1998). This may suggest that the relative contribution of Hsp90 to the QC process strictly depends on the folding potential of the client.

These findings suggest that the chaperone machinery can be reprogrammed depending on the folding status of the client, and that clients whose folding potential is low eventually interact with a chaperone proteins responsible of degradation (Holmes et al. 2008; Theodoraki and Caplan 2012).

## 1.7 The Ubiquitin Proteasome System

When a protein fails to reach energetically stable conformation it is addressed to degradation. Importantly, degradation of proteins in the ER (ERAD; ER associated degradation) is a specific and finely regulated process. The cytosolic proteasome complex mediates ERAD. Unstable or misfolded proteins are retro-translocated from the ER to the cytosol for ubiquitylation and degradation. Therefore, the folding environment of the ER is physically distinct from the main site of protein degradation (Jensen et al. 1995; Ward et al. 1995; McCracken and Brodsky 1996; Romisch 2005).

The specificity of protein degradation in the ER is conferred by ubiquitylation. E3 ubiquitin ligases provide the recognition of targeted proteins or chaperones associated with target proteins, selecting ERAD substrates (Ciechanover 1994) for the ER-localized E2 enzymes action that transfers ubiquitin to the selected proteins, tagging those for proteasome-mediated degradation (Xu et al. 2006; Romisch 2005). For example, unstable folding intermediates at the N-terminal domain of CFTR is detected at the onset of synthesis and are recognized by a ubiquitin ligase complex assembled at the cytosolic face of the ER to mediated ER-retention, ubiquitylation and degradation.

Proteins selected for degradation (including luminal proteins) are dislocated from the ER to the cytosol (McCracken and Brodsky 1996; Wiertz et al. 1996; Romisch 2005) probably via Sec61 traslocon complex, which mediates peptide insertion into the ER, to mediate reverse traslocation to the cytosol. However, direct evidence for the role of the traslocon complex in ERAD is still lacking (Aridor 2007).

## 1.8 Frizzled receptors

Frizzled proteins (Fzs), are cell surface receptors involved in a variety biological processes during development as well as in adult life. Human as well as mouse genome has 10 different genes that codify for 10 different receptors. The primary structure of all ten mammalian Fzs displays many landmarks observed in virtually all G protein-coupled receptors, including an ectosolic N-terminus that is N-glycosylated, seven hydrophobic transmembrane segments predicted to form  $\alpha$ -helices, three intracellular and extracellular loops, and a cytosolic tail that harbor potential sites of protein phosphorylation [Fig. 2 (Schulte et al. 2007)].

Putative glycosylation sites on the extracellular loops of Fzs could be involved in ligand binding and have been shown to be important for receptor maturation in ER, which is regulated in a specific manner by the protein Shisa (Yamamoto et al. 2005). Sequence and mutational analyses revealed the presence of two PDZ (PSD-95/dics large/ZO-1 homologous) binding domains in the cytosolic tail, providing a protein-interacting interface for the receptor. The juxtamembrane KTxxxW motif is conserved among all 10 Fz receptors, while the second PDZ-binding motif, at the C-terminus of the receptors, is present only in Fz-1, -2, -4, -7 (Luyten et al. 2008)(Fig.10). The extracellular domain of Fzs can bind several secreted molecules and some are considered to be cognate ligands. The primary endogenous agonists are the WNT proteins, of which there are 19 mammalian forms. These proteins are able to trigger most of the Fz-mediated signaling pathways.

However, recent findings suggest that at least three other protein families can directly bind Fzs and activate downstream signaling: soluble Frizzled-related proteins (soluble FRPs), R-spondin and Norrin. R-spondin and Norrin were shown to interact with the CRD (cystein reach domain), while sFRPs were initially seen as WNT scavengers that prevented WNT binding to Fzs.

However, recent studies support the idea of direct binding of sFRPs to Fz-CRDs, probably by CRD dimerization followed by receptor activation, as in the case of sFRP1-mediated activation of Fz2 (Rodriguez et al. 2005).

The main signaling pathways [Fig. 3 (Wawrzak et al. 2009)] activated by all 10 Frizzled receptors are:

1. ***Fz/ $\beta$ -catenin pathway:*** agonist stimulation results in the activation of the phosphoprotein Dishevelled (DVL), leading to inhibition of a constitutively active glycogen-synthase kinase 3 within a destructive complex consisting of APC (adenomatous polyposis coli) and axin, which regulates the phosphorylation and destruction of b-catenin. The b-catenin thus spared is translocated to the nucleus, where it cooperates with TCF/LEF transcription factors to modify gene transcription (Gordon and Nusse 2006).

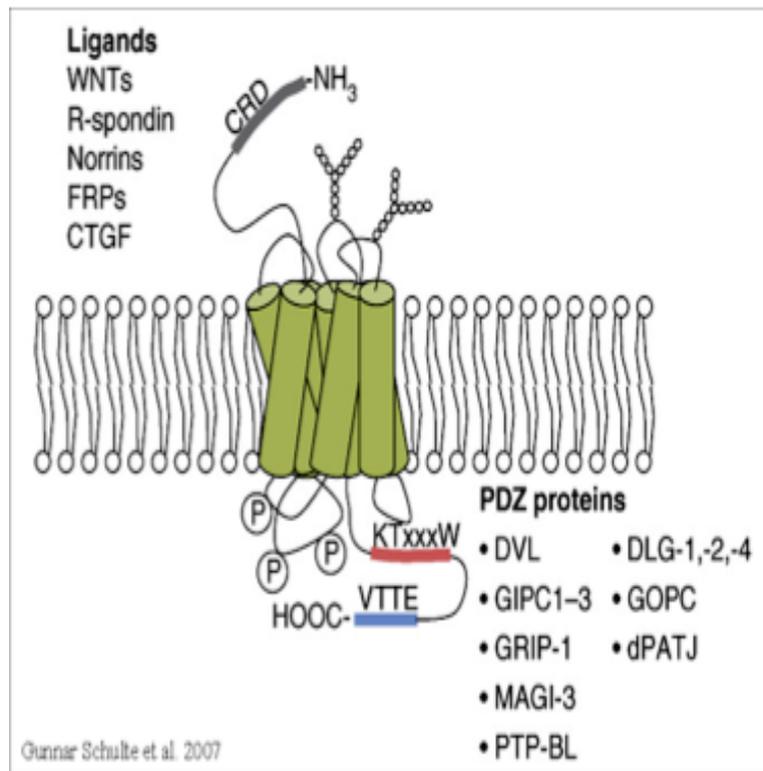
2. ***Fz/ $Ca^{2+}$  pathway:*** The agonists induce elevation of intracellular calcium levels in a G-protein-dependent manner either directly, through activation of phospholipases (Slusarski et al. 1997), or indirectly, via a decrease in intracellular cyclic GMP (Ma and Wang 2006), resulting in activation of

calcium-dependent kinases, such as calcium-dependent protein kinase (PKC) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase.

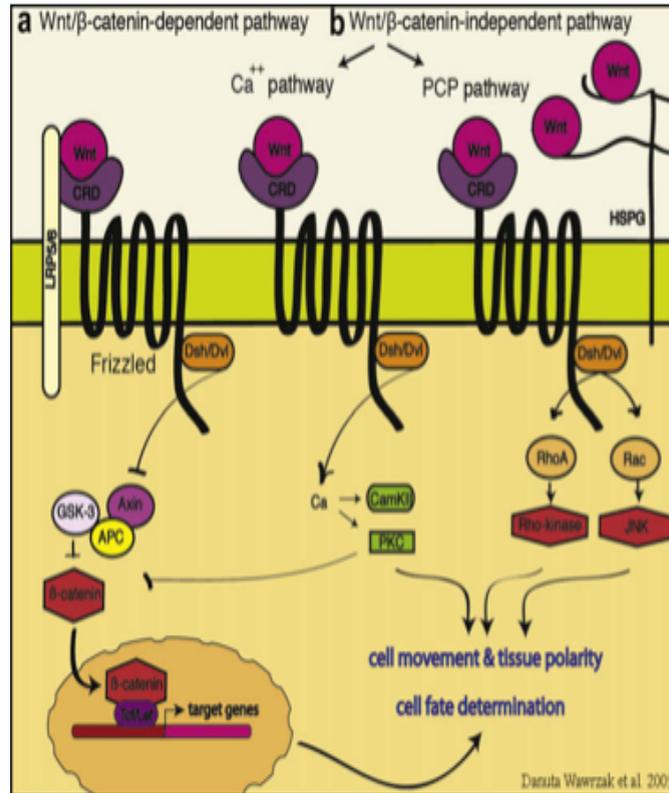
3. ***Fz/PCP (planar cell polarity) pathway:*** The information is believed to be transduced via DVL to the small GTPases RHO and RAC and their effectors, which are mainly ROCK (RHO kinase) and the c-jun- N-terminal kinase-c-jun-AP1 pathway (Seifert and Mlodzik 2007).

Mutagenesis has revealed that several residues in the intracellular loops and in the C-terminal domain of Fzs are crucial for signaling. Specifically, the mutation of the highly conserved internal KTxxxW motif in the C terminus, or single amino acid exchanges in the first (R340A) or the third (L524A) intracellular loops of human Fz5, completely abolished Fz signaling.

Importantly, identical mutations completely ablated the binding of DVL (DSH in *Xenopus* and *Drosophila*) and its membrane recruitment by Fzs (Cong et al. 2004).



**Figure 2: Schematic view of the assembly in the membrane of Fz receptors.** The model indicates extra and intracellular interacting proteins, putative glycosylation and phosphorylation sites. The N-terminal CRD is the primary binding site for ligands. The red stretch in the C terminus indicates the internal PDZ-interacting motif (KTxxxW), which is absolutely conserved in the different Fz isoforms and necessary for DVL binding and signaling. The blue stretch at the C-terminus indicates the presence of a classical, less well-conserved PDZ-ligand sequence present in a subset of Fz.



**Figure 3: Schematic representation of the Wnt signaling pathways.** a) The canonical, Wnt/β-catenin pathway. Binding of the Wnt ligand to the Fz and to the co-receptor of the LRP family leads to phosphorylation of Dishevelled (Dsh/Dvl), and to titration of Axin away from the β-catenin degradation complex, composed of APC, GSK-3 and Axin itself. As a consequence, the degradation complex cannot assemble, β-catenin is stabilized and translocates to the nucleus where, together with transcription factors of the Tcf/Lef family, it activates Wnt target genes, such as c-myc and cyclin D1. b) The non-canonical, β-catenin independent pathways: the PCP and the Ca<sup>2+</sup> pathway. A main branch of the PCP downstream of Dsh/Dvl involves the small GTPases of the Rho family, Rac and c-Jun N-terminal kinase (JNK). Dsh/Dvl can also stimulate calcium flux and protein kinase C (PKC) and calmodulin-dependent protein kinase II (CAMKII). The non-canonical Wnt signaling controls cell movement and tissue polarity. Heparan sulfate proteoglycans (HSPG) are known to bind and to concentrate Wnt at the cell surface and to influence Wnt signaling.

## 1.9 Familial exudative vitreoretinopathy (FEVR)

Familial exudative vitreoretinopathy (FEVR) is characterized by failure of peripheral retinal vascularization. The visual problems associated with FEVR result from secondary complications caused by retinal ischemia. The retinal avascularity is probably present from birth and generates sequelae that stabilize in early adult life or progress in later life. Expressivity may be asymmetric and is highly variable, ranging from mild or asymptomatic to severe (e.g., registered as blind) within the same family. The diagnosis of FEVR is based on bilateral peripheral retinal avascularity diagnosed by indirect ophthalmoscopy and fundus fluorescein angiography. Four genes are known to be associated with FEVR: FZD4, encoding for Fz4 receptor, LRP5, encoding for low-density lipoprotein receptor-related protein 5 (Robitaille et al. 2002), NDP, encoding for the secreted protein Norrin, that is one of the principal agonist Fz4 ligands (Sims 1993) and TSPAN12, a member of the tetraspanin superfamily (Poulter et al. 2010). In all cases, mutations affect the activation of the pathway promoted by Fz4 receptor.

It has been shown that Fz4 signaling regulates endothelial cells (EC)/mural cells (MC) interactions and is essential for vascular integrity in the retina and cerebellum (Ye et al. 2009). Furthermore, Fz4 is present throughout the developing and adult vasculature, and its inactivation results in severe vascular disorganization. It has been shown, cultured Fz4<sup>-/-</sup> retinal embryonic cells (Recsei et al.) are unable to form capillary-like structures but can integrate into these structures when they are formed by WT RECs. Moreover, Norrin/Fz4/Lrp signaling controls a transcriptional program in endothelial cells that is, at least partially, mediated by Sox17. These results establish Norrin/Fz4/Lrp signaling as a central regulator of REC development, and they indicate that the hypovascularization responsible for FEVR, Norrie disease or osteoporosis-pseudoglioma syndrome arises from the impairment of a specific transcriptional program. They also suggest that Fz4/Lrp signaling could play a role in vascular growth, remodeling, and maintenance in a variety of normal and pathologic contexts beyond the retina (Ye et al. 2009).

Among all described mutations associated to the FEVR, only the Fz4 L501fsX533 is associated with the autosomal dominant form (Kondo et al. 2003). This mutation consists in a deletion of a di-nucleotide (*ct*) in 1501-1502 position in exon2 of the FZD4 gene that results in a frameshift mutation that starts at the *leucine* in position 501 and finishes in position 533 where it introduces an early stop codon. In transfected cells, the effect of this mutation is the total absence of the mutant receptor on the plasma membrane (Robitaille et al. 2002). Most importantly, the mutant receptor acts as a dominant negative by trapping the wild-type counterpart in the ER by oligomerization (Kaykas et al. 2004).

## **2. Aim of the study**

The correct activity of a protein relates on its tridimensional fold. Functionality is achieved when each of the domains of a protein acquire the right structural conformation. Similarly, disordered regions need to remain as such. Genetic mutations alter the primary sequence of a protein interfering with the acquisition of a specific conformation or dictating a new fold. The intracellular destiny of the protein and its activity depends on the effect of these mutations. The protein may remain active otherwise a less active or, more drastically, a misfolded unfunctional protein may be generated. Many examples of the effect of genetic mutations on the structured regions of proteins are described (Illing et al. 2002; Kim et al. 2009; Hirota et al. 2010). However, less is known on the result of amino acid substitutions in their disordered parts.

Frizzled4 (Fz4), belongs to the family of Frizzled cell surface receptors involved in a variety of biological processes during development as well as in adult life (Schulte and Bryja 2007). The frameshift mutation L501fsX533 of Fz4 receptor is associated with a rare form of Familial exudative vitreoretinopathy (FEVR) (Robitaille et al. 2002). The mutation generates a different and shorter C-terminal cytosolic tail. The resulting mutant receptor fails to reach the Plasma Membrane (PM) of the cells and accumulates in the ER (Robitaille et al. 2002; Kaykas et al. 2004).

This misfolded mutant would trap wild type chains by improper heteroligomerization abolishing Fz4 signaling from the cell surface during retinal development by a dominant negative mechanism (Kaykas et al. 2004). This loss of function results in aberrant vascularization of the retina during development with severe consequences for the patients (Xu et al. 2004; Wang et al. 2012).

The aim of the present work was to elucidate the causes of misfolding and ER retention of Fz4 receptor induced by disease mutation L501fsX533, i.e. a first step to envisage therapeutic perspectives for the cure for adFEVR.

### 3. Materials and methods

All culture reagents were obtained from Sigma-Aldrich (Milan, Italy). Solid chemicals and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milan, Italy), Serva Feinbiochemica (Heidelberg, Germany), Delchimica (Naples, Italy) and BDH (Poole, United Kingdom). The radiochemicals were obtained from Perkin Elmer (Bruxelles, Belgium). Protein A-Sepharose CL-4B and the ECL reagents were from Amersham Biosciences (Milan, Italy). Protease Inhibitor Cocktail was obtained from Roche (Milan, Italy). ANAPOE-C<sub>10</sub>E<sub>6</sub> was obtained from Affymetrix (Santa Clara, California).

#### *Antibodies*

The following antibodies were used: mouse monoclonals anti-HA (Santa Cruz Biotechnology Inc.); rabbit polyclonal anti-HA (Sigma Aldrich); rabbit polyclonals anti-Calnexin (StressGene); Texas-Red-conjugated goat anti-mouse or rabbit IgG, FITC-conjugated goat anti-mouse or rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

#### *Peptides*

Synthetic peptides corresponding to the sequence of:

wt (CSAKTLHTWQKCSNRLVNSGKVKREKRGNGWVKPGKGSETVV),  
FEVR (CSAKTSHVAEVFQQIGEFWKGKEREERKWLGEAWKRQ),  
FEVR-6Ala (CSAKTSHVAEVFQQIGEFWKGKEAAAAAALGEAWKRQ)  
were purchase at GL Biochem (Shanghai).

The liophilized were risuspended in water at concentration of around 5mg/mL each, to be then further diluted in the appropriate buffer. Concentration were determined with NANODROP 2000 Spectrophotometer, Thermo Scientific, using extintion coefficient ( $\epsilon_{wt}=11125$ ,  $\epsilon_{FEVR} = 16500$ ,  $\epsilon_{6Ala}=11000$ ).

#### *Structure prediction*

Structure prediction of Fz4 wt, FEVR and all mutant tails were performed at server Protein Model Portal (PMP) (Arnold et al. 2009) and I-Tasser-Protein structure e functional prediction (Zhang 2008). Cartoon representations, r.m.s. deviation, dihedral and planar angle were generated in Pymol Molecular Graphics System, Version 1.5.0.4 Schrodinger, LLC.

#### *Disorder prediction*

Disorder predictions were generated in DisEMBL Intrinsic Protein Disorder Prediction 1.5 (Linding et al. 2003).

Predictions of Transmembrane regions were generated in HMMTOP Version 2.0 (Tusnady and Simon 1998).

### *Circular Dichroism*

CD spectra were recorded in a Jasco J-710 CD Spectrophotometer.

Peptides were diluted to the final concentration of  $10^{-4}$ M in NaCl 150mM and were indicated ANAPOE 2% final solution was included.

Data were recorded in wavelength range of 200nm to 320nm with a data pitch of 1nm, in a continuous scanning mode at speed of 50nm/min. 4 seconds of response in standard sensitivity e 20 accumulation were used to generate the averaged spectra.

### *Trp fluorescence quenching and polarization*

Peptides, 10  $\mu$ M (in Hepes 50mM pH 8, 150mM NaCl) were equilibrated with dilutions of the micellar agent ANAPOE C<sub>10</sub>E<sub>6</sub> one hour before fluorescence measurement. Equilibrium fluorescence and Polarization measurement were monitored using a PheraStar fluorescence plate reader in 96 well plates. Peptides were excited at 280nm, and emission intensity was monitored at 340nm with an emission slit of 8 nm. Triplicates of the data were normalized and fitting to a sigmoidal dose-response curve was done in GraphPad.

### *Cell culture, transfection and immunofluorescence*

Human HEK293T and Huh-7 cells were routinely grown at 37°C in Dulbecco's Modified Essential Medium (DMEM), containing 10% fetal bovine serum (FBS) and transfected by using FuGene 6.0 according to the manufacturer instruction (Standley et al.).

Indirect immunofluorescence was performed as previously described (Stornaiuolo et al. 2003). Single confocal images were acquired at 63x and 100x magnification on a LSM510 Zeiss Confocal Microscope (Carl Zeiss Jena, Germany).

To separately stain Fz4 proteins on either cell surface (extra) or intracellular membranes (Staub et al.), indirect immunofluorescence was performed as previously described (Mottola et al. 2002; D'Agostino et al. 2011).

### *Pulse-Chase & EndoH assay*

HEK293T cells, transiently transfected with HA-Fz4 and HA-Fz4-FEVR for 48hrs, were starved with a modified DMEM without CYS/MET aminoacids, containing 1% FBS. After 30 minutes the cells were incubated (PULSE) for 20 minutes with the same modified DMEM containing S<sup>35</sup>-CYS/MET (100 $\mu$ ci/ml). After washing with PBS1X the cells were incubated (CHASE) for 2 and 4hrs with the unmodified DMEM 10% FBS containing cycloheximide (inhibitor of protein biosynthesis). After washing with PBS1X, HA-Fz4 and HA-Fz4-FEVR proteins were immunoprecipitated by using a mouse monoclonal anti-HA antibody for 16hrs at 4°C followed by Protein A-Sepharose beads (Pharmacia). After washing with the buffer used for the lysis, the immunoprecipitated proteins were treated with EndoH enzyme, according

to the manufacturer's instructions, and analyzed by SDS-PAGE.

#### *Temperature shift assay*

Huh-7 cells were cultured on coverslips and transfected with different VSVG constructs. After 2hrs at 37°C, cells were incubated at the non permissive temperature of 40°C for 16hrs. After this period, the first coverslips were fixed in *formaldehyde* 3,7% while the others were cultured at 32°C for 20 minutes before the fixation.

#### *Velocity gradient*

Cell lisates, diluted in glycerol at the final concentration of 6%, were loaded on the top of discontinuous 20 to the 40% glycerol gradients (5ml final volume). After ultracentrifugation in the SW40.1 rotor at 45000 rpm of 16hrs, 14 fractions were collected from the bottom of the gradient. All fraction were precipitated in 15% TCA for 2hrs at 4°C. After 30 minutes of centrifugation, the pellets were washed two times with *ice cold acetone*, solubilized in Laemmli Buffer and analyzed by SDS-PAGE.

#### *cDNA cloning and plasmid construction*

The construct pCDNA5-HA-Fz4 was kindly provided by M. MacDonald and M.R. Hayden. The mutant L501fsX533 (Fz4-FEVR), was obtained by site direct mutagenesis using the following oligos:

Fz4-FEVR

Fw: 5'-TGGTCTGCCAAAACCTTCACACGTGGCAGAAG-3'

Rv: 5'-AGTTTTGGCAGACCAAATCCACATG-3'

To obtain all the mutants in Alanin of Fz4-FEVR the construct pCDNA5-HA-Fz4-FEVR was used as a template and the site direct mutagenesis assay was performed according to the manufacturer instruction (Standley et al.).

-HA-Fz4-FEVR1-525

Fw: 5'-GAGAGGAAATGGTTGTGAGAAGCCTGGAAAAGGC-3'

Rev: 5'-CAACCATTTCCTCTCTTCTC-3'

-HA-Fz4-FEVR1-515

Fw: 5'-GGTGAATTCTGGAAATGAAAAGAGAGAGAAGAG-3'

Rev: 5'-TTTCCAGAATTCACCAATCTGTTGG-3'

-HA-Fz4-FEVR1-509

Fw: 5'-GAAGTGTTCCAACAGTGAGGTGAATTCTGGAAAGG-3'

Rev: 5'-CTGTTGGAACACTTCTGCCACG-3'

-HA-Fz4-FEVR1-498

Fw: 5'-GCATGTGGATTTGGTCTTGAAAACTTCACACGTGG-3'

Rev: 5'-AGACCAAATCCACATGCCTGAAG-3'

-HA-Fz4-FEVR W<sub>514A</sub>

Fw: 5'-CAGATTGGTGAATTCGCGAAAGGTAAAGAGAGAGAAG-3'

Rev: 5'-GAATTCACCAATCTGTTGGAAC-3'

-HA-Fz4-FEVR K<sub>517</sub>A-E<sub>518</sub>A  
 Fw: 5'-GAATTCTGGAAAGGTGCAGCGAAAGAGAGAGAAGAG-3'  
 Rev: 5'-CCAGAATTCACCAATCTGTTG-3'

-HA-Fz4-FEVR R<sub>519</sub>A  
 Fw: 5'-TGGAAAGGTAAAGAGGCAGAAGAGAGGAAATGG-3'  
 Rev: 5'-CTCTTTACCTTTCCAGAATTC-3'

-HA-Fz4-FEVR R<sub>519</sub>AE<sub>520</sub>A  
 Fw: 5'-TGGAAAGGTAAAGAGGCAGCAGAGAGGAAATGGTTG-3'  
 Rev: 5'-CTCTTTACCTTTCCAGAATTC-3'

-HA-Fz4-FEVR E<sub>520</sub>AE<sub>521</sub>A  
 Fw: 5'-GAAAGGTAAAGAGAGAGCAGCGAGGAAATGGTTGGGTG-3'  
 Rev: 5'-TCTCTCTTTACCTTTCCAGA-3'

-HA-Fz4-FEVR R<sub>522</sub>AK<sub>523</sub>A  
 Fw: 5'-AAAGAGAGAGAAGAGGCGGCATGGTTGGGTGAAGCC-3'  
 Rev: 5'-CTCTTCTCTCTTTACCTTTC-3'

-HA-Fz4-FEVR K<sub>523</sub>A  
 Fw: 5'-GAGAGAGAAGAGAGGGCATGGTTGGGTGAAGCC-3'  
 Rev: 5'-GTAAAGAGAGAGAAGAGAGG-3'

-HA-Fz4-FEVR E<sub>521</sub>AR<sub>522</sub>A  
 Fw: 5'-GGTAAAGAGAGAGAAGCGGCGAAATGGTTGGGTGAA-3'  
 Rev: 5'-TTCTCTCTTTACCTTTCC-3'

-HA-Fz4-FEVR K<sub>523</sub>AW<sub>524</sub>A  
 Fw: 5'-GAGAGAGAAGAGAGGGCAGCGTTGGGTGAAGCCTGG-3'  
 Rev: 5'-CCTCTCTTCTCTCTTTAC-3'

-HA-Fz4-FEVR W<sub>524</sub>A  
 Fw: 5'-AGAGAAGAGAGGAAAGCGTGGTTGGGTGAAGCC-3'  
 Rev: 5'-GAATTCACCAATCTGTTGGAAC-3'

-HA-Fz4-FEVR G<sub>526</sub>AE<sub>527</sub>A  
 Fw: 5'-GAGAGGAAATGGTTGGCTGCAGCCTGGAAAAGGCAG-3'  
 Rev: 5'-CAACCATTTCCTCTCTTCTC-3'

A procedure in three steps was used to generate HA-Fz4-FEVR-6Ala cDNA, using for each step the following couple of primers:

1° step: see Materials and Methods of HA-Fz4-FEVR R<sub>519</sub>AE<sub>520</sub>A.

2° step: -HA-Fz4-FEVR E<sub>521</sub>AR<sub>522</sub>A (4Ala)

Fw: 5'-CTGGAAAGGTAAAGAGGCAGCAGCGGCGAAATGGTTGGGTGAAG-3'

Rev: 5'-CTCTTTACCTTTCCAGAATTC-3'

3° step: -HA-Fz4-FEVR K<sub>523</sub>AW<sub>524</sub>A (6Ala)

Fw: 5'-GAGGCAGCAGCGGCGGCAGCGTTGGGTGAAGCCTGG-3'

Rev: 5'-CGCCGCTGCTGCTCTTTAC-3'

The template for each step was the product of the previous one.

To obtain the constructs 3xFLAG-VSVGts045-Fz4wt tail and 3xFLAG-VSVGts045-Fz4-FEVR tail, the regions encoding for the ectodomain and transmembrane domain (LTM) of VSVG and the tails of Fz4wt or Fz4-FEVR

were isolated from the cDNAs of the respective proteins and subcloned in the 3xFLAG-CMV-7.1 expression vector by using the following oligos:

VSVG (LTM)

Fw (HindIII): 5'-AAGCTTAAGTTCACCATAGTTTTTCC-3'

Rv (BglII): 5'-AGATCTGAGAACCAAGAATAGTCC-3'

Fz4-wt tail

Fw (BglII): 5'-AGATCTATGGACATCGCCATCCACCACCC-3'

Rv (XbaI): 5'-TCTAGACTATTTCTTGGGGGCTGCGG-3'

Fz4-FEVR tail

Fw (BglII): 5'-AGATCTATGGACATCGCCATCCACCACCC-3'

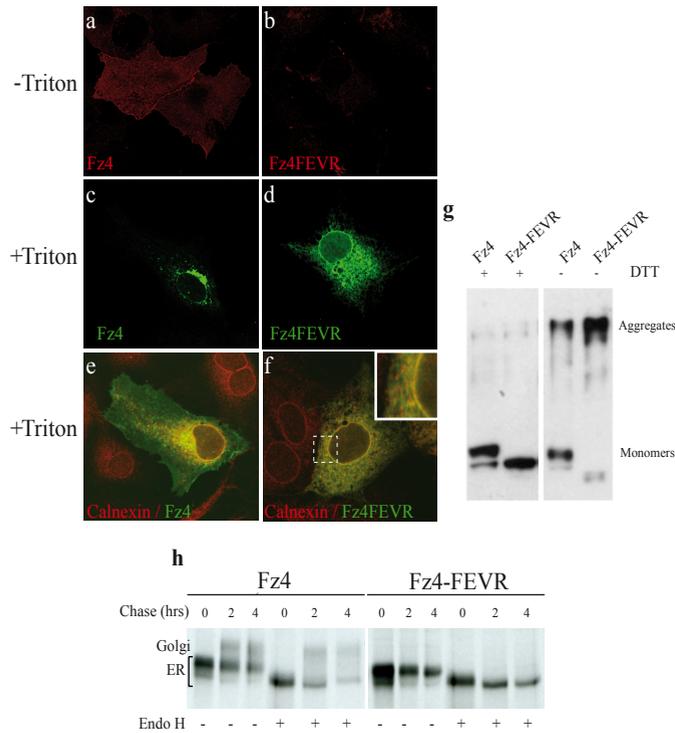
Rv (XbaI): 5'-TCTAGACTATTTCTTGGGGGCTGCGG-3'

Constructs were confirmed by sequencing.

## 4. Results

### 4.1 Intracellular destiny of Fz4-FEVR protein

HA-tagged Fz4 wt transiently overexpressed in Huh-7 (Fig. 4a) (as well as in HEK 293 and COS-7, data not shown) cells, localizes on the PM as shown by immunofluorescence staining performed on unpermeabilized cells. In contrast, HA-tagged Fz4-FEVR transiently expressed in the same cell lines (Fig. 4b) does not appear on the PM suggesting an intracellular localization of the mutant protein. The immunofluorescence performed after permeabilization reveals HA-Fz4 mainly in a pericentriolar region of the cells corresponding to the Golgi complex (Fig. 4c), while HA-Fz4-FEVR shows an ER localization (Fig. 4d). Double immunofluorescence of HA-Fz4 (Fig. 4e) or HA-Fz4-FEVR (Fig. 4f) with the ER marker calnexin confirmed the ER localization of the mutant protein. We know that Fz4-FEVR forms covalently linked aggregates trapped in the ER (personal communication). Comparison of the mobility on SDS-PAGE of non reduced samples confirmed that in Huh-7 (Fig. 4g) and in HEK 293 cells (data not shown) Fz4-FEVR fails to correctly fold forming covalently S-S linked aggregates. To further prove the ER localization of HA-Fz4-FEVR, endo-H sensitivity of the protein was tested in a pulse-chase assay. HA-Fz4 and HA-Fz4-FEVR were transiently expressed in HEK 293 cells. After 20 minutes of pulse in a  $^{35}\text{S}$  Cys and Met containing medium, cells were chased in unlabelled medium for 2 and 4 hours. Both HA-Fz4 and HA-Fz4-FEVR were sensitive to Endo H treatment immediately after the pulse (Fig. 4h). After 2 and 4 hours of chase a Endo H resistant band appeared for Fz4 but not for Fz4-FEVR confirming that the wild type protein but not the mutant acquired the Golgi specific sugar modification responsible for the resistance. The new tail of Fz4-FEVR compromises folding of the receptor and causing its retention in the ER.



**Figure 4: Fz4-FEVR fails to reach the Plasma Membrane and aggregates in the Endoplasmic Reticulum.** Surface (-Triton) and intracellular (+ Triton) localization of Fz4 (a, c) and Fz4-FEVR (b, d) and their colocalization with the ER marker Calnexin (e-f) after transient transfection in Huh-7 cells. The inset in f shows in yellow region of colocalization; g) SDS-PAGE of Fz4 and Fz4-FEVR run in reducing (+DTT) and not-reducing conditions (-DTT) (monomeric and aggregates forms are indicated); h) Endo-H resistance (Golgi) and sensitivity (ER) of newly synthesised Fz4 and Fz4-FEVR pulse-chased for the indicated time after transient transfection in HEK 293 cells.

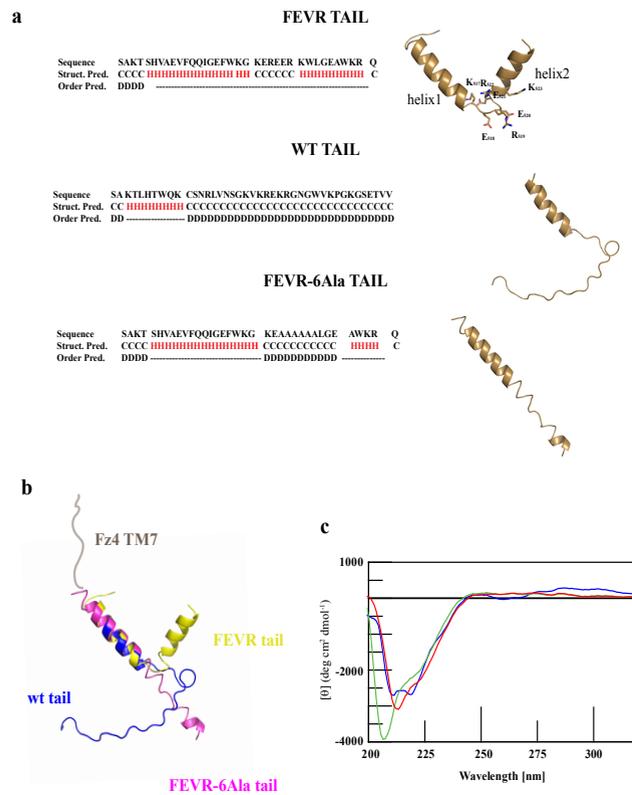
## 4.2 Conformation of Fz4-FEVR tail in solution

Search in protein databases finds no similarity between the aminoacidic sequence of the Fz4-FEVR tail (Fig. 5a) and any other sequence. Surprisingly, structure prediction softwares (see Materials and methods for details) suggested the Fz4-FEVR tail to be folded (Fig. 5a). The prediction indicates the mutated tail to acquire a helix-loop-helix conformation. Two consecutive helices (helix 1 Ser<sub>501</sub> to Gly<sub>516</sub> and helix 2 Lys<sub>523</sub> to Lys<sub>530</sub>), connected by a six amino acids long loop (Lys<sub>517</sub> to Arg<sub>522</sub>) define the predicted fold (Fig. 5a). The loop is predicted due to structure similarity with helix-loop-helix containing proteins like transcription factors and calcium binding proteins [PDB: 1R9D (O'Brien et al. 2004), 3FGH (Gangelhoff et al. 2009), 1GT0 (Remenyi et al. 2003)] (Jiang et al. 2010). Charged side chains are common in helix connecting loops and are responsible for the curvature, due to their interaction with water molecules or with ions (Sharma et al. 1997). The same prediction suggests only a smaller helical region for the wt sequence (Lys<sub>499</sub> to Lys<sub>506</sub>) (Fig. 5a). The existence of the helix in the wt was already postulated and shown to be responsible for the interaction of Fz4 with the effector protein Dishevelled (Wong et al. 2000; PUNCHIHEWA et al. 2009). The sequence Cys<sub>507</sub> to Val<sub>537</sub> of the wt tail was predicted disordered (PUNCHIHEWA et al. 2009). Softwares predicting disordered region in proteins support the random conformation of the C-terminal part of the wt tail but not of the Fz4-FEVR tail (Fig. 5a).

To confirm the predicted fold, a synthetic peptide corresponding to the Fz4-FEVR tail was analyzed by Circular Dichroism (Kelly et al. 2005). The spectrum was compared with that of a synthetic Fz4 wt tail and of a modified Fz4-FEVR mutant with 6 Alanine residues (Fz4-FEVR-6Ala) replacing the sequence between amino acid R<sub>519</sub> and W<sub>524</sub>. Since Alanine stretches have more structural flexibility, the carboxyl terminal part of the Fz4-FEVR-6Ala peptide was predicted more disordered. Indeed, the CD spectrum of the Fz4-FEVR tail peptide (Fig. 5c) shows two negative picks at around 208nm and 222nm proper of an helical fold with a molar ellipticity ratio  $\Theta[222\text{nm}]/\Theta[208\text{nm}]$  proximum to 1 (Kelly et al. 2005). The CD spectrum of the wt also shows two negative picks at the same wavelenghts. The ratio  $\Theta[222\text{nm}]/\Theta[208\text{nm}]$  now is less than 1 suggesting a less structured peptide as expected (Kelly et al. 2005). The decrease of molar ellipticity ratio reflects the contribution of random fold (minimum at 198nm and maximum at 210-230nm region) to the average spectrum (Kelly et al. 2005). In the Fz4-FEVR tail CD spectrum a low intensity positive peak around 280nm is also visible. This region of the CD spectrum is influenced by aromatic amino acid side chains (Trp, Tyr, Phe) only when these are part of folded domain (Heidebrecht et al. 2012) suggesting the two C-terminal Trp of the FEVR tail being part of a fold. The ratio  $\Theta[222\text{nm}]/\Theta[208\text{nm}]$  in the FEVR-6Ala CD spectrum is lower compared to the others confirming the disorder of the tail (Fig. 5c). Differently from the spectrum of the Fz4-FEVR tail, no positive signal was present in the

280 nm region despite of the presence of two Trp in Fz4-FEVR-6Ala tail. This absence would point to a less structured C-terminal part of the Fz4-FEVR-6Ala tail.

The L501fsX533 frameshift mutation induces structural transition in the Fz4 cytosolic tail. The sequence of Fz4-FEVR tail, but not the one of wt, acquires a stable alpha-helix conformation in solution. The region R<sub>519</sub>-W<sub>524</sub> confers stability to the fold.



**Figure 5: Fz4 wt tail and FEVR tail have a different fold.** a) Structural and disorder prediction of wt, FEVR and FEVR-6Ala tail (C: coil; H: helix; D: disordered region; – : ordered region) and cartoon representation of the predicted structure. Residues contributing to the loop of FEVR tail are shown in ball and sticks. b) Superposition of structural predictions of wt tail (cartoon blue), FEVR tail (cartoon yellow) and FEVR-6Ala tail (cartoon magenta). Gray line represent the seventh transmembrane segment of the Fz4 protein; c) Superposition of CD spectra of wt tail (red line), FEVR tail (blue line) and FEVR-6Ala tail (green line).

### 4.3 Affinity of Fz4-FEVR tail for membranes

The two predicted helices of the Fz4-FEVR peptide acquire a boomerang shape forming a planar angle of around  $90^\circ$  (Ala<sub>504</sub>-Lys<sub>517</sub>-Lys<sub>530</sub>) and a dihedral angle of  $23^\circ$  (Ala<sub>504</sub>-Lys<sub>517</sub>-Arg<sub>522</sub>-Lys<sub>530</sub>) (Fig. S1a, b). Hydrophobic and aromatic residues of the sequence all face the inner side of the boomerang making the Fz4-FEVR structure strongly amphipathic (Fig. 6a and Fig. S1c). In contrast, a more random distribution of polar and non polar amino acids is predicted for the wt tail (Fig. 6a) that on contrary appears non amphipathic.

While structure based similarity searches indicate structural homology to transcription factors, we noticed that Fz4-FEVR tail resembles in shape and hydrophobicity the fusion peptide of HA at the pH of fusion [PDB 1IBN (Han et al. 2001)] [root mean square (r.m.s.) deviation on alpha carbons 1.075 Å] (Fig. S1). The alignment between HA fusion peptide with wt tail gave no or partial similarity with higher r.m.s. scores (1.373 Å to wt tail). The boomerang shape formed by the two consecutive HA helices ( $102^\circ$  planar angle and  $17^\circ$  dihedral angle) (Fig. S1a, b) was shown to be essential to enable the fusion of the viral peptide to the membrane and mutation only marginally affecting the shape of the fold resulted in peptides with less fusogenic activity (Lai et al. 2006; Lai and Tamm 2007).

The topology of the Fz4-FEVR tail and its predicted amphipathicity would suggest proximity and even affinity of this region to the bilayer of the cytosolic side of the ER in the context of the full length Fz4-FEVR protein.

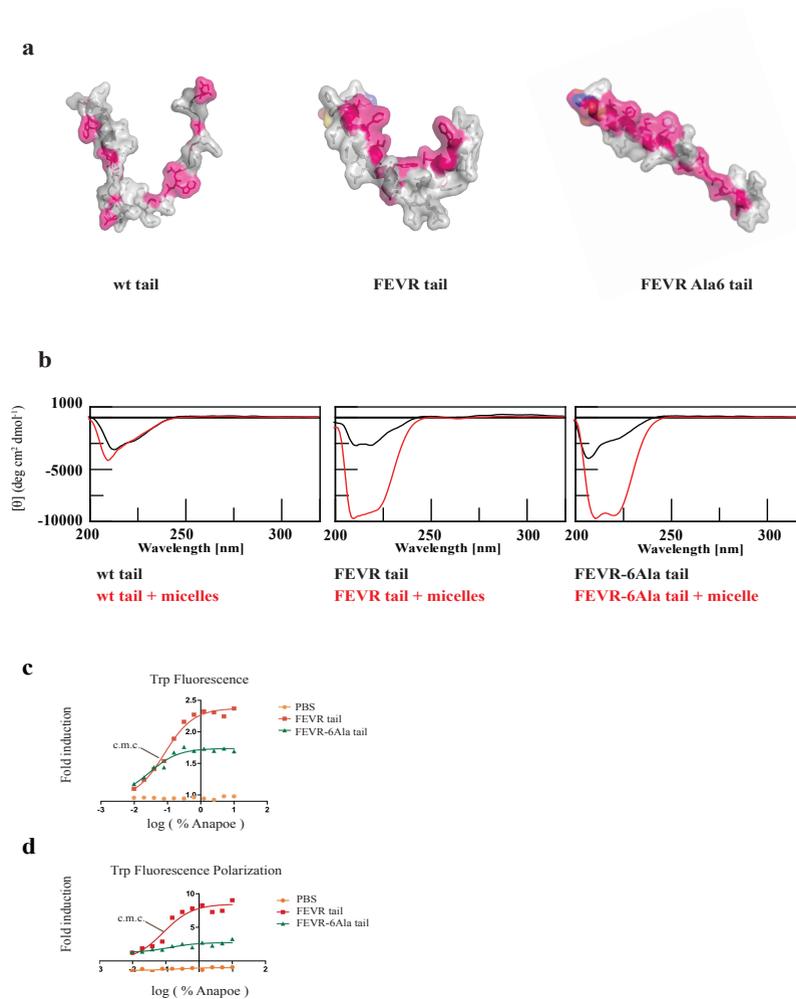
To test the affinity of the Fz4-FEVR tail for the membranes, the CD spectra of the peptides were recorded in presence of a micellar agent, the non ionic detergent ANAPOE-C<sub>10</sub>E<sub>6</sub> (Helenius et al. 1979) (Fig. 6b). In the presence of micelles, the  $\Theta$  at 222 and 208nm for the FEVR tail peptide increased four times, proving the peptide has affinity for membranes and that its fold gets stabilized by the lipids. The ratio  $\Theta[222\text{nm}]/\Theta[208\text{nm}]$  is almost unchanged suggesting a neutral effect of the micelles on the structure of the peptide. A minimal effect was visible when the micellar agent was added to the wt peptide suggesting minor propensity of this sequence to interact with the micelles. Surprisingly, ANAPOE-C<sub>10</sub>E<sub>6</sub> modified the CD spectrum of Fz4-FEVR-6Ala peptide. A clear increase of  $\Theta$  at 208 and 222nm was recorded and the ratio  $\Theta[222\text{nm}]/\Theta[208\text{nm}]$  went closer to 1 suggesting that despite its flexibility in solution, the C-terminal part of the peptide can acquire an alpha-helical fold when interacting with membranes (Fig. 6b).

To further prove interaction of the Fz4-FEVR tail and Fz4-FEVR-6Ala tail with membranes, we measured changes in Trp fluorescence intensity and Trp fluorescence polarization in the presence of micelles (Fig. 6c). Fz4-FEVR tail has one Trp located on helix 1 and two on helix 2. Trp fluorescence was measured in the presence of increasing concentration of ANAPOE-C<sub>10</sub>E<sub>6</sub>. In the presence of the detergent, a 2.2 fold increase in Trp fluorescence was measured suggesting a reduction in the number of the quenching water molecules around Trp and consistent with an interaction of the peptide with the

micelles. The increase in fluorescence is not visible at detergent concentration lower than the critical micellar concentration (c.m.c) confirming the need of formed micelles for the peptide to interact. Despite its affinity for membrane, a lower fluorescence increase was measured for the Fz4-FEVR-6Ala peptide above c.m.c. probably because the C-terminal end of the peptide is interacting differently with membrane.

Consistently, eight fold increase in Trp fluorescence polarization for Fz4-FEVR was measured at detergent concentration higher than the c.m.c. (Fig. 6c), suggesting a reduction in the speed of tumbling of the Trp when immersed in the micelles. No increase was measured for the Fz4-FEVR-6Ala mutant confirming the different way of insertion of the peptide in the membrane.

The two consecutive helices of the Fz4-FEVR tail forms a boomerang shape similar to the fusion peptide of HA. Fz4-FEVR tail, but not Fz4 wt tail, displays affinity for *in vitro* formed membranes. The helix 2 of Fz4-FEVR tail is stabilized by the membranes or, in absence of them, by the loop.



**Figure 6: FEVR tail has affinity for membranes.** a) Surface representation of wt tail, FEVR tail and FEVR-6Ala tail. Polar amino acids are indicated in gray, non polar in magenta; b) CD spectra of the indicated peptides in presence of micelles (red line). The spectra are superimposed with ones in absence of micelles (black line) shown in Fig. 2c; c) Trp fluorescence emission of FEVR peptide (red squares), FEVR-6Ala peptide (green triangles) in increasing concentration of micellar agent. Buffer and micellar agent fluorescence are shown (yellow circles); d) Trp fluorescence polarization of FEVR peptide and FEVR-6Ala peptide in increasing concentration of micelles (shown as in c). Critical micellar concentration (c. m. c.) is indicated.

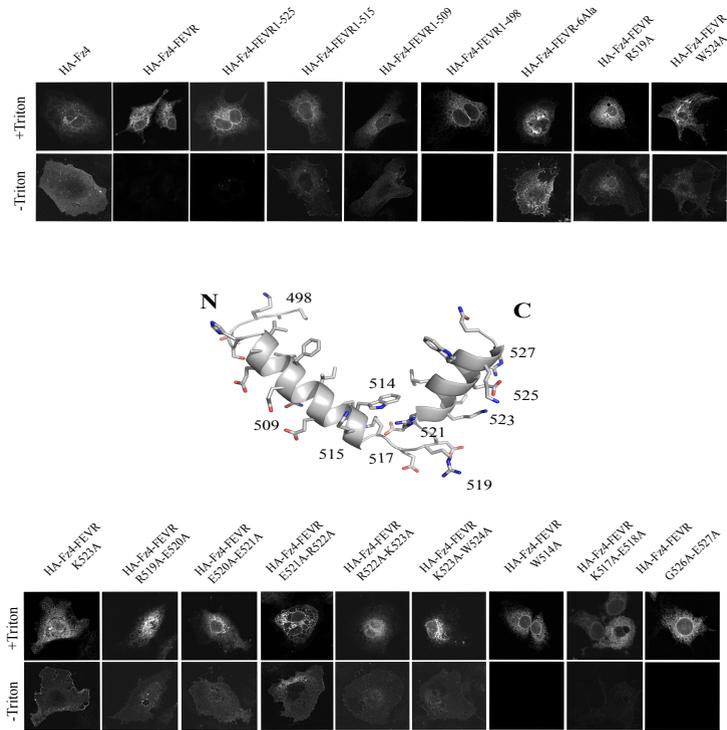
#### 4.4 Intracellular localization of Fz4-FEVR mutants

We envisaged that the new fold acquired by Fz4-FEVR tail and its affinity for membrane could explain Fz4-FEVR aggregation and ER retention.

Therefore, mutants disturbing shape and hydrophobicity of the tail were generated (structural prediction of some of them in Fig. S2) and their effect on the stability and intracellular localization of Fz4-FEVR was analysed in transfected cells. Constructs were transiently expressed in Huh-7 cells and their localization was visualized by confocal immunofluorescence (Fig. 7). Truncation of the last seven residues of the tail did not rescue the Fz4-FEVR1-525 protein that localizes intracellularly in the ER. In contrast, truncation of the last 17 (Fz4-FEVR1-515) and 23 (Fz4-FEVR1-509) residues rescued the phenotype localizing both receptors on the PM and in the Golgi complex, thus suggesting proper intracellular trafficking. The complete removal (Fz4-FEVR1-498) of the tail did not rescue the phenotype, proving the need of a cytosolic tail for the folding of the receptor.

Fz4-FEVR-6Ala clearly localizes on PM and in the Golgi complex. A partial recovery was also visible for mutant with single or double substitutions in the region of the tail closer to the helix connecting loop (residues R<sub>519</sub> to W<sub>524</sub>). Alanine scanning in this region (Fz4-FEVR-R<sub>519</sub>A, Fz4-FEVR-K<sub>523</sub>A and Fz4-FEVR-W<sub>524</sub>A) partially rescued the PM localization. Similar results were obtained when couple of residues were substituted in the same region (Fz4-FEVR-R<sub>519</sub>A-E<sub>520</sub>A, Fz4-FEVR-E<sub>520</sub>A-E<sub>521</sub>A, Fz4-FEVR-E<sub>521</sub>A-R<sub>522</sub>A, Fz4-FEVR-R<sub>522</sub>A-K<sub>523</sub>A and Fz4-FEVR-K<sub>523</sub>A-W<sub>524</sub>A). Single substitution performed outside the loop region did not rescue the localization of the receptor (Fz4-FEVR-W<sub>514</sub>A). The same results were obtained after double substitutions at the edge of the R<sub>519</sub>-W<sub>524</sub> region (Fz4-FEVR-K<sub>517</sub>A-E<sub>518</sub>A, Fz4-FEVR-G<sub>526</sub>A-E<sub>527</sub>A), that did not rescue the PM localization.

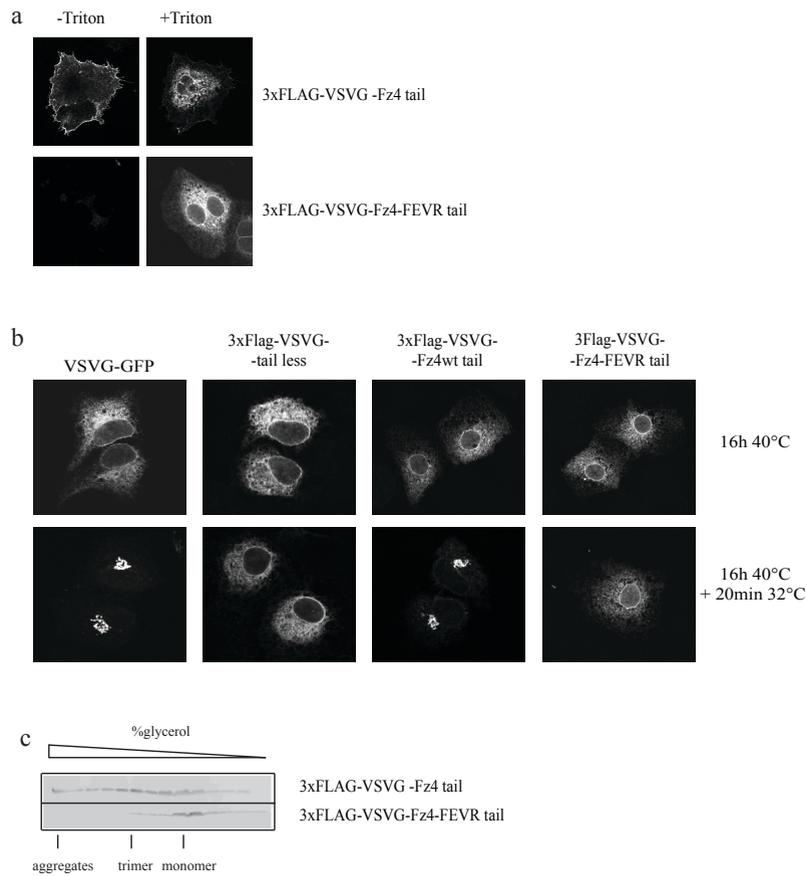
Disturbing the fold of the FEVR tail rescues the Fz4-FEVR localization at the cell surface. Removal of the helix 2, of the loop or affecting the curvature of helix-loop-helix conformation determines the export of the mutant receptor from the ER.



**Figure 7: Disturbing the fold of the FEVR tail induces the rescue of Fz4-FEVR to the PM.** Surface (-Triton) and intracellular (+ Triton) localization of Fz4, Fz4-FEVR and the indicated mutants transiently expressed in Huh-7 cells. Cartoon representation of FEVR tail with side chains in ball and sticks showing the position of some of the amino acids mutated in our study.

#### **4.5 Intracellular destiny of chimera VSVG-Fz4-FEVR tail**

In order to prove that the Fz4-FEVR tail is necessary and sufficient to induce misfolding and aggregation of Fz4-FEVR, a chimeric construct composed by the ectodomain and the transmembrane of the reporter VSVG glycoprotein coded by the ts-045 mutant strain fused to the cytosolic tail either of Fz4-FEVR or of Fz4 wt were generated (Fig. 8). VSVG ts-045 is a temperature sensitive mutant of VSVG that localizes as monomer in the ER at non permissive temperature (40° C). When the permissive temperature (32° C) is reestablished the protein trimerizes and moves to the PM (Doms et al. 1987). Both VSVG-Fz4wt tail and VSVG-Fz4-FEVR tail localizes in the ER at non permissive temperature but only VSVG-Fz4wt tail moved to the Golgi after the temperature shift (Fig. 8b). Analysed on a glycerol gradient, VSVG-Fz4wt tail appears trimeric as expected (Doms et al. 1987) (Fig. 8c) while VSVG-Fz4-FEVR tail appears monomeric. Despite the different context, Fz4-FEVR tail induces ER retention of VSVG. Differently from Fz4-FEVR, the tail does not cause aggregation of VSVG but blocks it in a monomeric but not trimerization prone fold.



**Figure 8: FEVR tail appended to the reporter molecule VSVG dictates ER retention of the chimera by misfolding.** a) Surface (-Triton) and intracellular (+ Triton) localization of VSVG-Fz4 wt tail and VSVG-Fz4-FEVR. b) Intracellular distribution of VSVG-Fz4 tail and VSVG-Fz4-FEVR tail at not permissive temperature (40 °C) and permissive one (32° C). c) Oligomeric state of VSVG-Fz4 tail and VSVG-Fz4-FEVR tail analyzed on glycerol gradient

## 5. Discussion

Until the early 1990s, a widely, almost exclusively accepted concept of protein function was the well-known *protein sequence-structure-function paradigm*. According to this concept, a protein can achieve its biological function only upon folding into a unique, structured state, which represents a kinetically accessible and an energetically favorable conformation (usually the global energy minimum for the whole protein) determined by its amino acid sequence. This specific conformation has been referred to as the native state of the protein or what we call ordered proteins. However, recent discoveries of intrinsically disordered proteins (IDPs) (Dunker and Obradovic 2001) [known also as natively disordered, natively unfolded and intrinsically unstructured proteins (Weinreb et al. 1996)] have significantly broadened the view of scientific community and increased the number of studies about these intriguing members of the protein world.

In contrast to ordered protein regions, ID regions are not characterized by the atom equilibrium positions and dihedral angle equilibrium values around which the residue spends most of the time. ID regions exist instead as dynamic ensembles in which atom positions and backbone Ramachandran angles vary significantly over time with no specific equilibrium values. The conformational change of ID regions are typically noncooperative and random. Thus, the view of disorder as dynamic ensembles does not exclude the temporary presence of local secondary structure that fluctuates in absence of stabilizing forces (Radivojac et al. 2007).

Disordered regions of over 30 residues in length are 5- to 15-fold enriched in higher eukaryotic proteomes (30%) relative to eubacteria (6%) and archaea (2%), respectively (Ward et al. 2004; Oldfield et al. 2005), which is believed to reflect the use of disordered domains in higher order eukaryotic signaling networks (Minde et al. 2011).

ID regions are often strongly modified by e.g. phosphorylation or acetylation that create changes in net charge of the binding region (Dunker et al. 2008). Such modifications can fine tune protein-protein interactions and activity of the protein complex in regulation, signaling and control pathways (Liu et al. 2006; Dahlberg et al. 2009).

IDPs differ from folded proteins by being able to easily adapt their shape to external influence.

In several reported cases, binding of interacting proteins induces (partial) folding or secondary structure formation of IDPs (Dyson and Wright 2005; Cortese et al. 2008; Marsh et al. 2010). Thus, disordered domains may provide significant binding plasticity. As binding of a disordered protein to a partner restricts its dynamics, the interaction will involve an entropic penalty, which folded proteins do not pay (Dyson and Wright 2005).

Long, unstructured regions are likely more apt to resist the effects of mutations than folded proteins.

It has been proposed several mechanism by which missense mutations can dysregulate IDPs function: i) altered protein interaction surface (Azzopardi et al. 2008); ii) changes in secondary structure formation (Nimura et al. 1997); iii) altered post-translational modifications (Ferrarese et al. 2007); iv) altered dynamics of conformational equilibrium (Minde et al. 2011).

### **5.1 Disorder to order transition in the fold of the Fz4-FEVR cytosolic tail**

Here we show that the mutation L501fsX533 induces an unfold to fold structural transition in the cytosolic tail of the Fz4 receptor. To our knowledge, this is the first example of a transition occurring in an intrinsically disordered region [IDRs (Radivojac et al. 2007)] of a mammalian protein experimentally analyzed. The effect is deleterious for the folding of the Fz4 protein, its intracellular localization and activity.

The L501fsX533 generates a very unusual frameshift mutation. In the mutant nucleotidic sequence the first stop codon is encountered 96 nucleotides after the deletion, that thus induces a new frame coding for a new 32 amino acids long sequence. The ratio between charged, polar and non polar amino acids is almost unaltered between wt and FEVR sequence. In contrast, Trp and Phe residues are present in higher number in the FEVR tail and increase its aromaticity, despite these amino acids rarely result from frameshift mutations due to the low degree of degeneration of their codons.

We prove that a synthetic peptide with the sequence of the Fz4-FEVR tail acquires an helical fold in solution. Specific negative peaks at around 208 and 222nm in the CD spectrum confirmed its alpha helical fold. This is also hypothesized by structure prediction servers that suppose for the peptide a boomerang shape formed by two consecutive helices connected by a loop. The first four amino acids of the wt tail are conserved in the mutant tail and this region was already shown to be in an alpha helical conformation (Punchihewa et al. 2009). The amino acids following the alpha helix in the wt tail have low helical propensity. Pro<sub>529</sub> disrupts the propensity of helical formation in the C-terminal region of the wt tail while the very high percentage of Glu and Lys in the corresponding part of the FEVR tail strongly favour the formation of the second helical fold.

## 5.2 FEVR tail has affinity for membranes

The helix-loop-helix shape of the FEVR tail would partially resemble the HA fusion peptide at the pH of fusion. This aminoacidic region in the sequence of HA undergoes a conformational change when the viral protein is in the endosomal compartment and has an unfold- to fold transition acquiring a boomerang shape similar to the one predicted for FEVR at physiological pH. The hydrophobicity of this HA segment was shown essential for the insertion of the peptide in the bilayer of the host cell and small deviation of the angle of the boomerang was proven to abolish the fusogenic activity. The two helices of the FEVR tail are strongly amphipatic due to the presence on the same side of the helix of hydrophobic and aromatic residues (Val<sub>503</sub>, Phe<sub>507</sub>, Ile<sub>510</sub>, Trp<sub>514</sub> on the first helix and Leu<sub>525</sub> and Trp<sub>529</sub> on the other one). In the wt tail the hydrophobicity is less anysotropic and thus the amphipaticity is lost.

We prove *in vitro* that, differently from the wt, the synthetic peptide of the Fz4-FEVR tail has affinity for lipids. Molar ellipticity, Trp fluorescence intensity and Trp fluorescence polarization of the FEVR tail increases in the presence of *in vitro* formed micelles. The reduction of the tumbling speed of the Trp when immersed in the micelles is in favour of an high affinity of the FEVR peptide for the hydrophobic environment present in the micelles. However, the charged residues on the peptide would exclude the FEVR tail be immersed completely in the bilayer of the Endoplasmic Reticulum. Moreover, transmembrane segments prediction software excludes the possibility that the FEVR tail would constitute an extra transmembrane of the receptor due to the highly charged Arg and Lys residues in the FEVR tail and supports the model of a peripheric interaction of the FEVR tail with the membrane.

Intriguing is the effect of the micelles on the FEVR-6Ala tail mutant. CD spectra indicate a structuring effect of the lipids on the peptide. Helices of FEVR-6Ala tail are probably unstable as seen by the CD spectrum of the peptide in absence of micelles (Fig. 2c) and get stabilized only by the presence of the detergent. In the FEVR tail the stabilizing effect on the helices is probably coming from the loop that allows long distance aromatic contact and Vanderwaals interaction. The lipids enforce the stability of the fold contributing with the hydrophobic interactions. Both helices get stabilized by the micelles.

### **5.3 Affecting the shape of the FEVR tail rescues Fz4, moving the receptor to the Plasma Membrane**

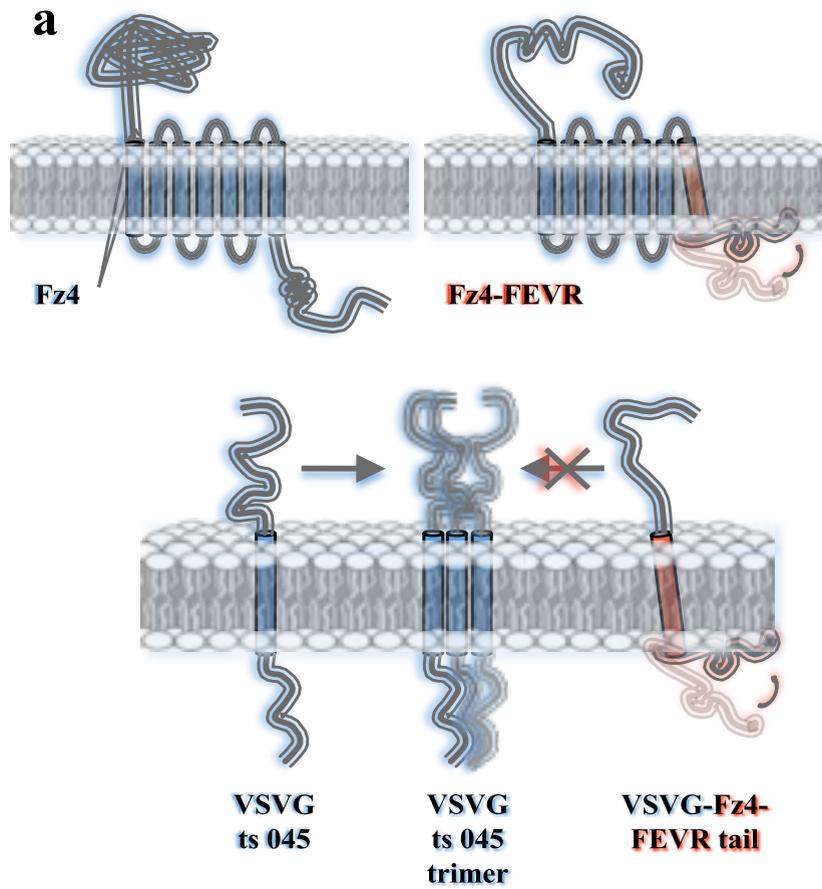
The need of the helix-loop-helix conformation for inducing the phenotype of Fz4-FEVR receptor was confirmed in transfected cells. When a Fz4-FEVR mutant missing the second helix was expressed in Huh-7 cells the receptor moved to the PM confirming the need of the second helical element. The same rescue effect was seen for mutants missing the loop confirming the need of the complete boomerang fold.

Six amino acids would form the kinked loop connecting the two helices in the FEVR fold. It is not possible to prove the presence and geometry of the loop *in vivo* but we proved that the sequence of the loop is essential for the Fz4-FEVR phenotype. When a synthetic peptide with six Ala residues substituting the sequence of the loop was analyzed by CD, its alpha-helical extent was reduced and it appears less structured. When a Fz4-FEVR mutant containing the above-mentioned substitution was expressed *in vivo*, the phenotype was rescued confirming the need of a structured tail for the FEVR induced effect on Fz4 receptor. The same rescue effect was seen for single or double substitution in this region of the FEVR tail. Different shape is predicted for the mutants (Fig. S2). *In vivo*, the PM localization of the mutants perfectly correlate with their structural divergence from the Fz4-FEVR. This was already shown for HA fusion peptide where single mutation in its sequence alters the fusogenic activity and affects infectivity of the virus.

Thus we hypothesize that the loop of the FEVR tail reduces the flexibility, stabilizes the boomerang fold of the tail favouring the interaction between the helix 1 and 2 and dictating the phenotype. This fold is a prerequisite for dictating the phenotype of the receptor.

#### 5.4 Effect of the FEVR tail on *in vivo* folding of Fz4 and VSVG

We here hypothesize that the FEVR tail is interacting with the cytosolic side of the ER membrane, hampering the correct folding of Fz4 (Fig. 6a, b). This is likely to happen *in vivo* because topologically, the tail is in close proximity with the ER bilayer, *in vivo*. The rigidity conferred to the cytoplasmic tail could reduce conformational freedom in the transmembrane segment of the Fz4 receptor. It was already shown that deletion of two amino acids in the last transmembrane of Fz4 ( $\Delta$ Met493Trp494) traps Fz4 in the ER and induces FEVR (Robitaille et al. 2002) suggesting the importance of the last transmembrane for a correct folding of Fz4. Our hypothesis could also explain the effect that we see of the FEVR tail in the context of the VSVG chimera (Fig. 9). As shown by the sedimentation profile, VSVG-Fz4-FEVR tail is trapped in a pre-trimeric state. The trimerization of VSVG is dependent by the ectodomain and not by the cytosolic tail. This is supporting a model where the orientation of the transmembrane of VSVG is altered by the cytosolic FEVR tail, and affects the proper interaction of the ectodomains of the glycoprotein probably by misalignment.



**Figure 9:** a) Schematic cartoon showing the proposed effect of the FEVR tail on the folding of the Fz4 and VSVG protein.

## 5.5 Considerations and Future Plans

We show that Fz4-FEVR tail acquires a helix-loop-helix conformation using *in silico*, *in vitro* and *in vivo* techniques and proving the new mutated fold being responsible for Fz4-FEVR aggregation. The two helices of the FEVR peptide are visible by CD, that on contrary is not able to give a clear indication of the existence of the interconnecting loop. Thus, a clear proof of its presence in the tail of Fz4-FEVR is still missing. An high resolution structure of the peptide in the micelles or even better the one of the full Fz4-FEVR receptor would be necessary and of great interest. Such structures would prove not only the existence of the loop and give geometrical details on its three-dimensional structure but they would clearly show the effect of the new tail on the misfolding of the full length Fz4-FEVR mutant.

Unfortunately, obtaining crystal structures of small peptides and even more of multispans protein cannot be easily achieved and it usually requires a great effort in time and money. On one hand the X-ray techniques, despite the continuous progress of the field, are struggling in obtaining high resolution structure of very flexible and heterogeneous samples like small peptides. On the other hand, solving structures of multispans proteins at high-resolution is just downing due to the instability of these proteins out of their cellular context. Moreover, X-ray crystallography and NMR would require structurally homogeneous samples to be successful, while Fz4-FEVR is in an aggregate form, that is usually very heterogeneous in its structure.

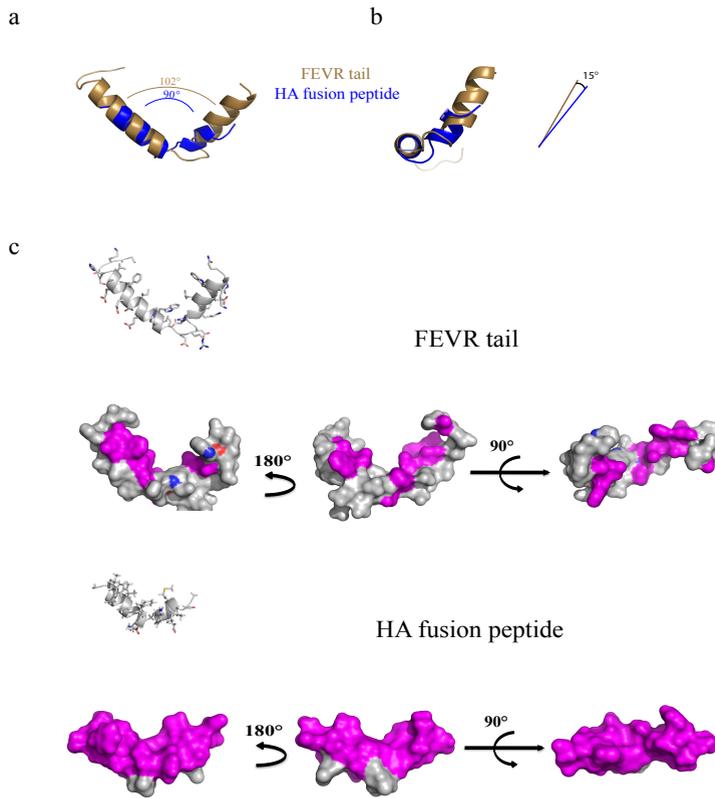
In the discussion we envisage that the high amphipathicity of the FEVR tail would be the reason of its interaction with membranes. We did test the affinity of the FEVR peptide for ANAPOE *in vitro* micelles.

In order to confirm the propensity of the peptide to interact with *in vivo* derived bilayers, we are planning to test its affinity for a crude homogenate of cells. This assay will also eventually measure the specificity of the peptide for membranes with a particular composition in phospholipids and cholesterol. This could for example support the idea of a specific interaction of the mutant tail with the cytosolic side of the ER vs the other ones is existing. On contrary, the absence of specificity could suggest a tendency of the tail to interact with membranes even upon rescue on PM.

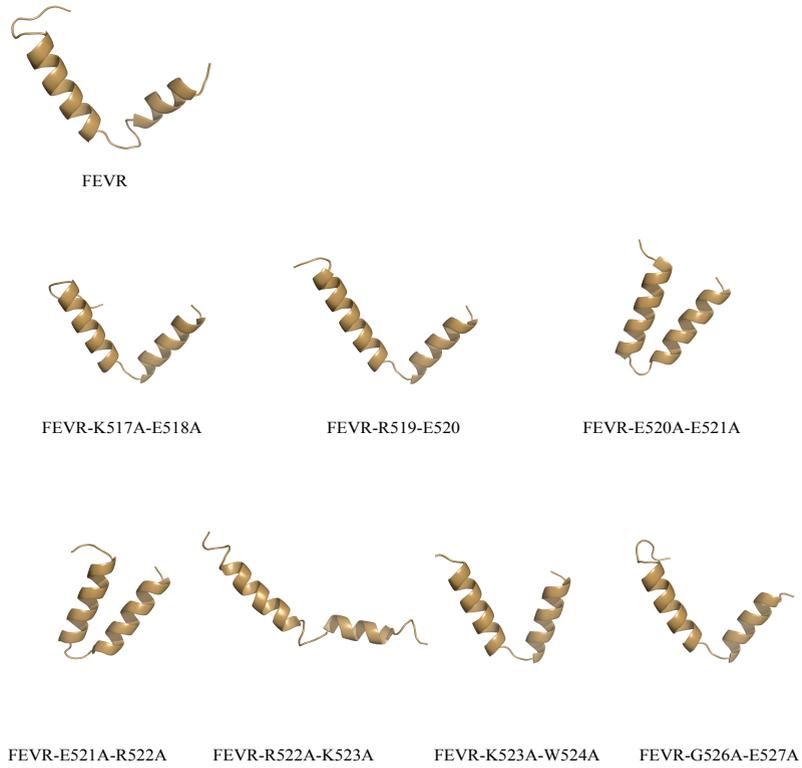
We hope this work will convince that the investigation of the effect of disease-mutations in disordered domains of other transmembrane receptors must be taken in account and it will suggest with the threefold approach we used a pipeline of experiments to demonstrate this effect experimentally. Finally, we think that the new insights into the mechanism of Fz4-FEVR retention and

misfolding could give important contribution to the understanding of the molecular basis of the conformational diseases.

## 6. Supplemental figures



**Figure S1: Comparison of HA fusion peptide and Fz4-FEVR tail.** a) Superposition of HA fusion peptide (cartoon representation, blue) and FEVR tail (cartoon representation, sand). Planar angles formed by the helices are indicated. b) Differences in dihedral angles between HA fusion peptide and FEVR tail represented as in a. c) Structure (cartoon with side chains in ball and sticks) and surface representation (polar amino acids in grey and non-polar in magenta) of FEVR tail and HA fusion peptide.



**Figure S2:** Structural prediction of FEVR tail compared with the ones predicted for the indicated mutans (cartoon representation sand).

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