1 Introduction

The balance of protein synthesis and degradation is a tightly regulated process to maintain normal cellular homeostasis and to ensure cell survival. Newly synthesized secretory proteins (e.g. hormones, receptors) have to obtain the correct conformation to be transported through the secretory pathway to their destinations and to achieve their specific biological functions. The rough endoplasmic reticulum (ER), the first station of the secretory pathway, is responsible for protein folding and the assembly of nascent, integral membrane and soluble, luminal proteins. Multiple ER-residing chaperones and enzymes contribute to this important process. Correctly folded secretory proteins are exported via the ER-Golgi intermediate compartment (ERGIC) to the Golgi apparatus, were they are sorted for transport to their final destinations. A quality control system operating in the secretory pathway determines correct folding, selectively recognizes non-native protein conformations and disposes them for turnover (Ellgaard, Molinari et al. 1999; Ellgaard and Helenius 2001). Terminally misfolded proteins are subjected to ER-associated degradation (ERAD) which includes retrotranslocation to the cytosol and proteasomal degradation (Werner, Brodsky et al. 1996). This process is an important part of the quality control mechanism to protect cells from damage and assures proper cell function.

Protein synthesis and the secretory pathway

Ribosomes in the cytoplasm catalyze the translation of mRNA into nascent polypeptide chains. N-terminal signal sequences at growing polypeptide chains define targets destined for the secretory pathway. The signal sequences are recognized and bound by signal recognition particles (SRPs) (Anderson, Walter et al. 1982; Nagai, Oubridge et al. 2003), which cause an intermission of the translation (Walter, Ibrahimi et al. 1981). This intermission leads to the transport of the ribosomes to SRP receptors at the ER membrane and to interaction with the heterotrimeric integral ER membrane channel Sec61 (Kalies, Gorlich et al. 1994). Nascent polypeptides are co-translationally translocated through the channel into the lumen of the ER (Musch, Wiedmann et al. 1992) and membrane proteins move laterally out of the channel into the membrane bilayer. The signal sequences of the growing polypeptides are usually cleaved off by signal peptidases during their passage through the translocation channel (Anderson, Walter et al. 1982; Rapoport 1991). Nascent proteins are folded into their native conformation with the assistance of an abundance of molecular chaperones and folding enzymes. All secretory proteins bind the key chaperone immunoglobulin heavy chain binding protein (BiP)

or glucose-regulated protein (GRP78) that assembles with extended hydrophobic peptide chains and assists in the folding process (Kabani, Kelley et al. 2003). In addition, disulfide bonds are introduced by the enzyme protein disulfide isomerase (PDI) (Freedman, Klappa et al. 2002). The ER is also the site for post-translational modifications. Proteins get core-glycosylated with high mannose sugar side chains on asparagine residues. All post-translational modifications contribute to the three-dimensional protein structure. The modified proteins are analyzed by a quality control system, which operates to ensure that only correctly folded or assembled proteins are allowed to exit the ER and traffic through the secretory pathway.

The newly synthesized proteins are transported in coat protein complex (COP) II-vesicles from ER-exit sites to the ERGIC (Aridor, Bannykh et al. 1995), whereas COPI-coated vesicles mediate the retrograde transport between both compartments (Figure 1). COPI and COPII are membrane coat complexes required for transport in the secretory pathway (Stephens, Lin-Marq et al. 2000). The ERGIC functions in both retrograde and anterograde sorting, but also in conformation-based quality control of nascent proteins (Hauri, Kappeler et al. 2000). The ERGIC is composed of tubulovesicular membrane clusters and is characterized by its membrane-marker protein ERGIC-53, which cycles between the ER and ERGIC (Klumperman, Schweizer et al. 1998). ERGIC-53 is a lectin and is involved in vesicular trafficking (Nyfeler, Zhang et al. 2006). Finally, secretory proteins are delivered from the ERGIC to the Golgi apparatus, which is the last known station with quality control function in the secretory pathway; thereby COPI vesicles derived from the ERGIC fuse with cis-Golgi membranes (Stephens, Lin-Marq et al. 2000).

The Golgi complex is composed of a stacked membrane cisternae; enzymes in the Golgi network process glycoproteins by replacing high mannose sugar side chains with complex carbohydrates. Besides N-linked glycosylation, some proteins can also be tagged with O-linked oligosaccharides. The fully processed, mature proteins are delivered to the trans-Golgi network, where they are sorted for transport to their site of action, e.g. to the plasma membrane, endosomes or lysosomes. Caldwell et al. demonstrated that two soluble ERAD substrates, which escaped the quality control system of the ER and were transported to the Golgi apparatus, were delivered back to the ER for degradation by a retrograde transport mechanism (Caldwell, Hill et al. 2001).

The endocytic pathway and protein turnover

Proteolysis regulates changes on protein levels important for development and function of cells and eliminates aberrant or misfolded proteins, which are potentially cytotoxic. Furthermore, it recycles cellular components and provides amino acids, which is essential for cell survival in circumstances like starvation. Moreover, proteolytic mechanisms

are implicated in cellular regulation and signal transduction processes (e.g. cell cycle control, DNA repair, apoptosis, activation of the transcription factor NF-κB). The main intracellular degradative pathways are lysosomes and the ubiquitin / proteasome system. Lysosomes degrade most cytosolic, long-lived proteins (macroautophagy), membrane vesicles carrying bulk flow, or proteins taken up by endocytosis from the plasma membrane (Terman, Gustafsson et al. 2007). The majority of cytosolic, short-lived polypeptides or misfolded, secretory proteins are targeted with a polyubiquitin chain for breakdown though the ubiquitin / proteasome pathway.

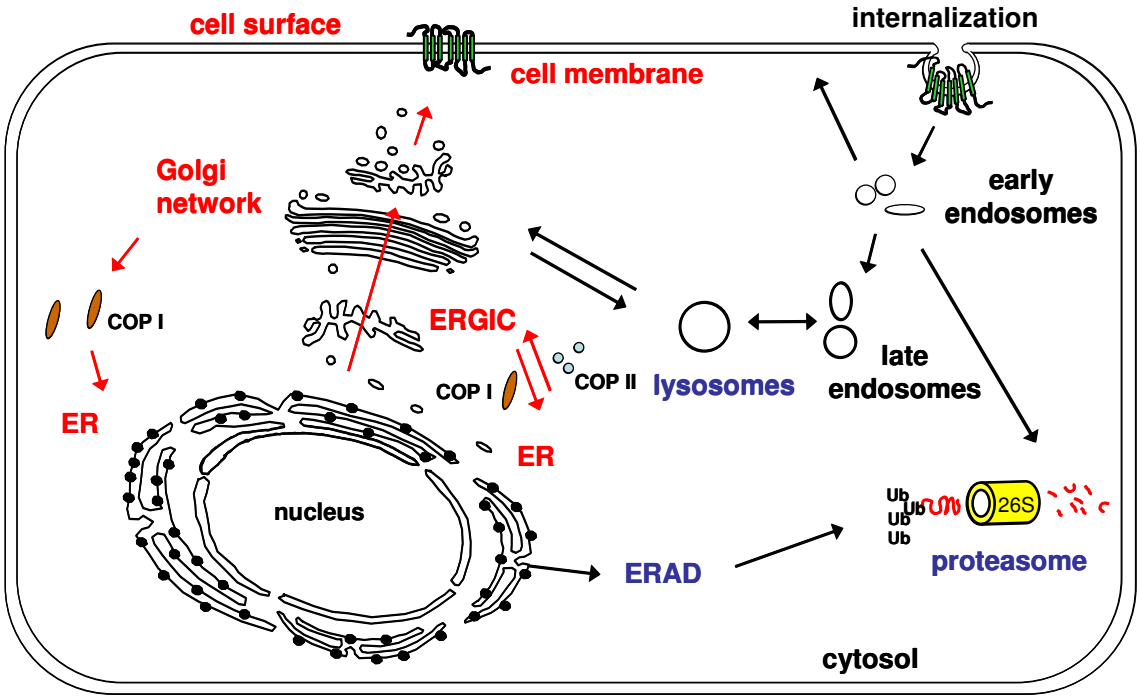

Figure 1: Intracellular trafficking of secretory proteins. *Red pathway*, newly synthesized ER-luminal- and transmembrane-localized proteins are packaged in COPII vesicles and are transported from the ER to the ERGIC and Golgi apparatus. In the Golgi network they are sorted for their final destination, e.g. the cell membrane. The ERGIC and Golgi apparatus constitute checkpoints of the conformation-based quality control system. Proteins may fail quality control and are transported in COPI vesicles back to the ER for degradation by the ERAD and the ubiquitin / proteasome system. *Black pathway*, membrane proteins internalize (constitutive or ligand-activated) into an endosomal compartment. Early endosomes provide ubiquitin-mediated cargo sorting and lead the endocytosed proteins to recycling or degradation pathways.

The endocytic pathway is used to deliver extracellular material from the environment and redundant or desensitized membrane proteins into the cells interior. Vesicles from the plasma membrane, e.g. internalized receptors or other endocytotic vesicles traffic through the early endocytic pathway (Figure 1) and fuse with endosomal structures. The first compartment of the endocytic pathway is constituted by early endosomes, which are a sorting compartment and provide various proteins required for vesicular transport. Some receptors that are internalized to early endosomes are able to recycle to the plasma membrane; others are sorted in multivesicular bodies which are transported to or

mature into late endosomes (Pillay, Elliott et al. 2002). Late endosomes fuse with lysosomes that function as storage organelles for many hydrolytic enzymes (acid hydrolases), like α-fucosidase, α-galactosidase, α-neuroaminidase and acid phosphatase, among others. Lysosomes are vesicular structures in the cytoplasm with a low internal pH. An impermeable membrane separates the lysosomal enzymes from other cellular components.

The driving force that controls sorting along the endocytic pathway is a pH gradient caused by a vacuolar adenosine triphosphatase (ATPase) proton pump in the endosomal membrane that is regulated by an Na^+ / K^+-ATPase (Fuchs, Schmid et al. 1989). The pH decreases from 6.6 to 4.5 from early endosomes to lysosomes and is necessary for efficient breaking of peptide bonds. Proteins are ultimately degraded in this specialized compartment by the action of hydrolyses at a low pH.

The ubiquitin / proteasome system

Ubiquitin is a 76 amino acid polypeptide that is highly conserved in eukaryotes and belongs to the post-translational modifications of substrate proteins. It is a "multilingual" marker and usually gets covalently attached to the ε-amino group on lysine or cysteine residues of target proteins. Depending on the type of ubiquitin, mono- or polyubiquitin, it can participate in different cellular processes. Polyubiquitin chains can be formed through different lysines within the ubiquitin molecules; K48- and K63-linked chains are common. They take part in many processes as cell cycle progression, protein transport, heat-shock response, DNA repair, signal transduction and endocytosis (Hershko and Ciechanover 1998).

The key role of K48-linked polyubiquitination is to target proteins for the highly selective degradation by the 26S proteasome (Chau, Tobias et al. 1989). Ubiquitination requires the action of three consecutively acting enzymes, E1, E2 and E3; the last of which determines substrate specificity (Figure 2) (Hershko and Ciechanover 1998). First, the ubiquitin-activating enzyme E1 activates the C terminus of ubiquitin and generates a reactive E1~ubiquitin thioester intermediate by coupling the ubiquitin C terminus (G 76) to a cysteine side chain in an ATP-dependent mechanism. The ubiquitin-conjugating enzyme (E2) accepts the activated ubiquitin from the E1 to a conserved cysteine. For ubiquitination of the target protein there are two possibilities: 1.) the ubiquitin is passed from the E2 to a cysteine of an ubiquitin ligase (E3) and the E3 transfers the ubiquitin to a lysine or cysteine side chain of a target protein, or 2.) the E2 ubiquitinates the target protein directly and the E3 functions as an adaptor (Hershko and Ciechanover 1998; Weissman 2001).

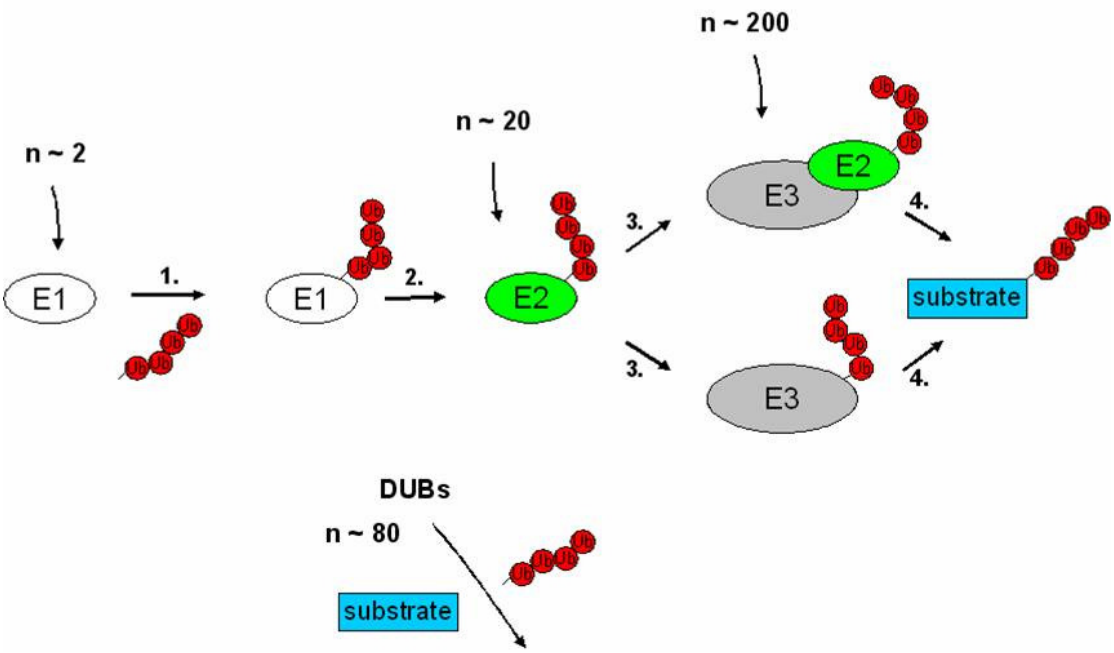

Figure 2: Mechanism of ubiquitination. Three enzymes act in concert to connect lysine or cysteine residues of proteins with the post-translational modification of mono- or polyubiquitin. First, the E1 enzyme (ubiquitin-activating enzyme) generates a reactive ubiquitin~E1 thioester binding the C terminus of ubiquitin to a cysteine residue in an ATP-dependent process. The activated ubiquitin is passed on a conserved cysteine of the E2 enzyme (ubiquitin-conjugating enzyme). Some E2 enzymes refer the ubiquitin directly to the E3 ligase, which attaches ubiquitin to the target protein. Others build a complex with the ubiquitin ligase for substrate ubiquitination. The specificity of the mechanism of ubiquitination is restricted to the E3 ligases that are implicated in substrate recognition. Deubiquitinating enzymes (DUBs) cleave ubiquitin or ubiquitin chains of substrate proteins and contribute to the diverse cellular signals arranged by ubiquitin modifications.

Deubiquitinating enzymes (DUBs) can remove ubiquitin moieties of target proteins indicating that the ubiquitination is a reversible process. Currently, the cleavage of ubiquitin chains is only incompletely understood due to a limited knowledge of substrate proteins. The DUB USP4 mediates the deubiquitination of the GPCR A_{2A}-adenosine receptor and enhances its cell surface expression (Milojevic, Reiterer et al. 2006). Therefore, DUBs control essential cellular activities regulated by the ubiquitin / proteasome system and may be important targets for pharmacological therapies.

The 26S proteasome

The 26S proteasome is a ATP-dependent protease of more then 65 subunits that degrades polyubiquitinated proteins into small peptides (Hershko and Ciechanover 1998). The gene is highly conserved throughout evolution between different species. Most proteasomes are located in the cytoplasm, but some also localize to the nucleus. Mammalian proteasomes are composed of two subcomplexes, the 19S regulatory particle (RP) and the 20S core particle (CP) (Figure 3). The 20S catalytic core is a barrel-shaped structure with an estimated size of ~700 kDa. It represents an ATP-independent protease containing chymotrypsin-like, trypsin-like and peptidylglutamyl peptide-

hydrolyzing (caspase-like) activities. The 20S catalytic core is composed of four axially positioned heteroheptameric rings, which face an interior chamber within the particle. It contains two identical outer α, non-catalytic rings and two inner β rings ($\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$), where proteolysis takes place (Groll, Ditzel et al. 1997). The α rings can adopt different confirmations and regulate the gating of the catalytic chamber (Whitby, Masters et al. 2000). Proteins are degraded into small peptides and are released into the cytoplasm where exopeptidases release single amino acids. The RPs (~900 kDa each) are attached to one or both ends of the 20S catalytic core like a cap and sequester the proteolytic active sites (Glickman, Rubin et al. 1998).

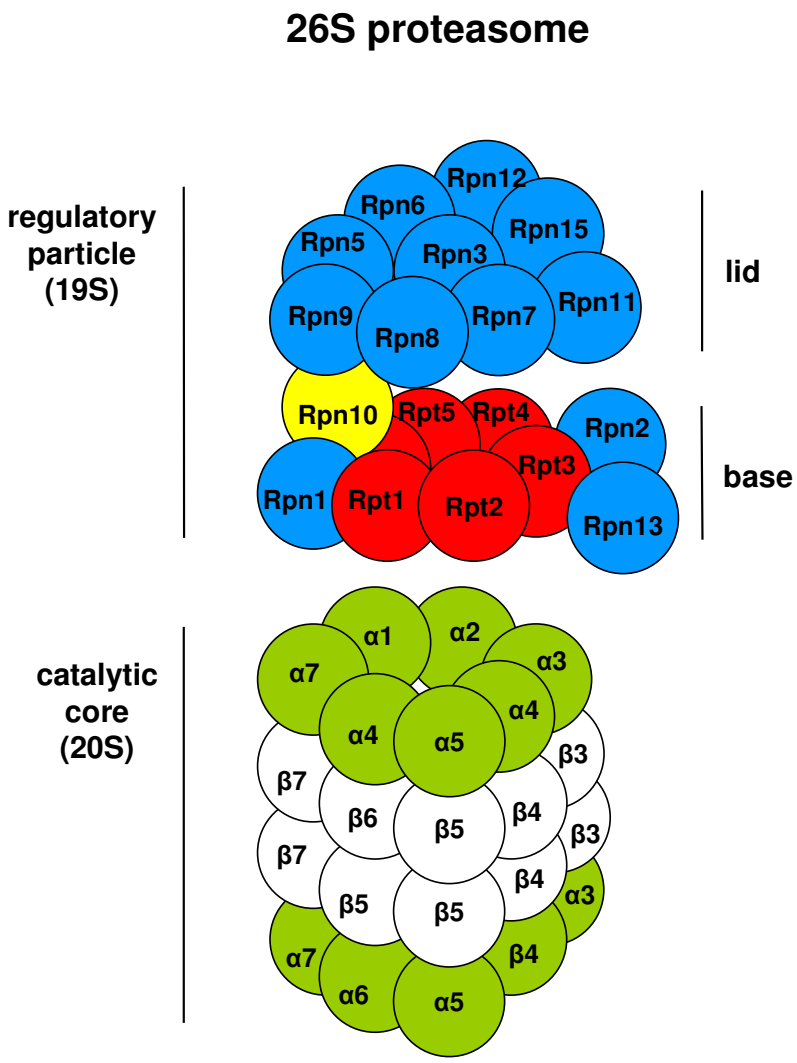

Figure 3: Multi-subunit composition of the mammalian 26S proteasome. The 20S catalytic core is a barrel-shaped structure and contains the proteolytic specifities. It is composed of two identical heteroheptameric inner β rings and two identical outer α rings. The catalytic core can be sequestered by one or two 19S regulatory subunits. The regulatory particles have functions in substrate recognition and gating of the catalytic core. The 19S particle is divided in base and lid, the base contains the six ATPases Rpt1 - 6 and three non-ATPase subunits Rpn1, Rpn2 and Rpn13. Rpn10 is located at the interface between base and lid, which exclusively consists of the non-ATPase subunits, Rpn3, Rpn5 - 9, Rpn11, Rpn12 and Rpn15.

Among the 19 subunits of the 19S RP are six subunits Rpt1 - 6 (regulatory particle AAA ATPase) (Finley, Tanaka et al. 1998) that build the ATPases of the AAA family (ATPases associated with various cellular activities) (DeMartino, Moomaw et al. 1994; Schmidt, Lupas et al. 1999). Recent studies indicate that the ATPases of the regulatory particle can interact with residues of α subunits from the CP and may facilitate the conformational changes which control substrate entry and substrate unfolding (Kohler, Cascio et al. 2001). The ATPase subunits Rpt1 – 6 and the three non-ATPase components Rpn1, Rpn2 and Rpn13 (regulatory particle non-ATPase) build the base of the RP complex, whereas the lid consists of nine non-ATPases, Rpn3, Rpn5 – 9, Rpn11, Rpn12 and Rpn15. Rpn10 has been reported as localized in the interface between the base and the lid. The RP has important functions in the recognition of polyubiquitinated substrates; the polyubiquitin chains are removed by the action of associated or cytoplasmic DUBs and the appropriate substrates are translocated to the catalytic core for turnover. The Rpn10 (Elsasser, Chandler-Militello et al. 2004), Rpt5 (Lam, Lawson et al. 2002) and Rpt1 subunits are described to bind polyubiquitinated proteins (Raasi and Wolf 2007), but the mechanisms of substrate recognition await further biochemical characterization. The Rpn11 subunit is a DUB (Yao and Cohen 2002) that is implicated in substrate deubiquitination. However, the deubiquitination process is not fully understood.

A variety of additional proteins that associate with the proteasome are able to modulate its function; for example, the translation elongation factor 1A (eEF1A) is thought to interact with Rpt1 and binds ubiquitinated, misfolded nascent polypeptide chains (Chuang, Chen et al. 2005). The authors raise the hypothesis that eEF1A may be responsible for transport of degradation substrates to the proteasome. It is also known that eEF1A plays a role in the translation process in delivering aminoacetyl-tRNAs to the translating ribosome. The attenuation of translation during the unfolded protein response (UPR) releases the factor for formation of a complex with the stress sensor heat-shock transcription factor 1 (Hirsch, Gauss et al. 2006). A protein associated with Rpn1 is Ubp6, a cysteine protease with a deubiquitinating function.

Despite an extensive body of evidence, the precise mechanisms for recognition, unfolding and translocation of proteins destined for proteasomal turnover and the interplay of the single subunits remains to be elucidated.

The proteolytic function of the CP can be blocked by proteasome inhibitors, which can affect the different proteolytic activities. The schedule shows a selection of important substances:

Proteasome inhibitors:

substances	chymotrypsin-like activity	trypsin-like activity	caspase-like activity
Epoxomicin	x	x	x
Lactacystin	x	x	
MG132 (Z-Leu-Leu-Leu-al)	x		x
PR11	x	x	x
Bortezomib			

ER stress and unfolded protein response (UPR)

Disturbance of the equilibrium of synthesis and degradation of proteins, e.g. through accumulation of misfolded proteins in the ER, usually results in cell stress. To avoid possible damage, the cell has to react to this situation. An accumulation of high amounts of unfolded or misfolded proteins may be toxic and lead to apoptosis. One reaction is the UPR, which elicits some events that can protect the cell from cell death (Schroder and Kaufman 2005). It inhibits general protein synthesis, which prevents further loading of newly synthesized proteins into the ER (Kaufman, Scheuner et al. 2002). Moreover, ER residing chaperones are up-regulated and facilitate protein folding (Kaufman, Scheuner et al. 2002). If this fails to relieve the ER stress, the ERAD machinery is up-regulated via the Ire1 pathway (Friedlander, Jarosch et al. 2000; Schroder, Clark et al. 2003) and aberrant proteins are degraded. Ire1 is a transmembrane ER-residing kinase that mediates the activation of the transcription factor XBP1, which induces many genes required for the ERAD. The degradation is usually mediated by the ubiquitin / proteasome system. Proteins, which are aggregation-prone or overextend the capacity of proteasomal turnover, may be transported to a cytoplasmic, perinuclear region for deposition in aggresomes. These inclusion bodies can contain UPR and ERAD components, like BiP, calnexin (Vattemi, Engel et al. 2004), the AAA ATPase valosin-containing protein (p97/VCP) or polyubiquitin (Liewluck, Hayashi et al. 2007), and are presumably eliminated by an autophagolysosomal mechanism. The second reaction to an excess of proteins in the ER can be the ER overload response. This leads to the activation of NF-κB, a transcription factor that mediates inflammation and apoptosis (Schroder and Kaufman 2006).

Conformation-based quality control of proteins in the secretory pathway

The quality control system of the secretory pathways represents a regulated process that ensures proper protein folding and export of newly synthesized proteins. It relies on ER

resident chaperones that assist in the folding process (Helenius, Marquardt et al. 1992). Proteins can fail quality control because of a variety of factors such as improper folding, alterations in post-translational modifications (disulfide bond formation, N/O-glycosylation) or truncations that alter the three-dimensional structure, and also by incorrect assembly of oligomeric proteins which influences the quaternary structure (Trombetta and Parodi 2003). Misfolded and unfolded proteins are retained and are eventually degraded by the ERAD.

Native glycoproteins are co-translationally glycosylated by the enzyme oligosacharyltransferase. During passage through and release of the Sec61 translocon they are bound by BiP and the N-glycan binding chaperones calnexin and calreticulin (Helenius and Aebi 2004).

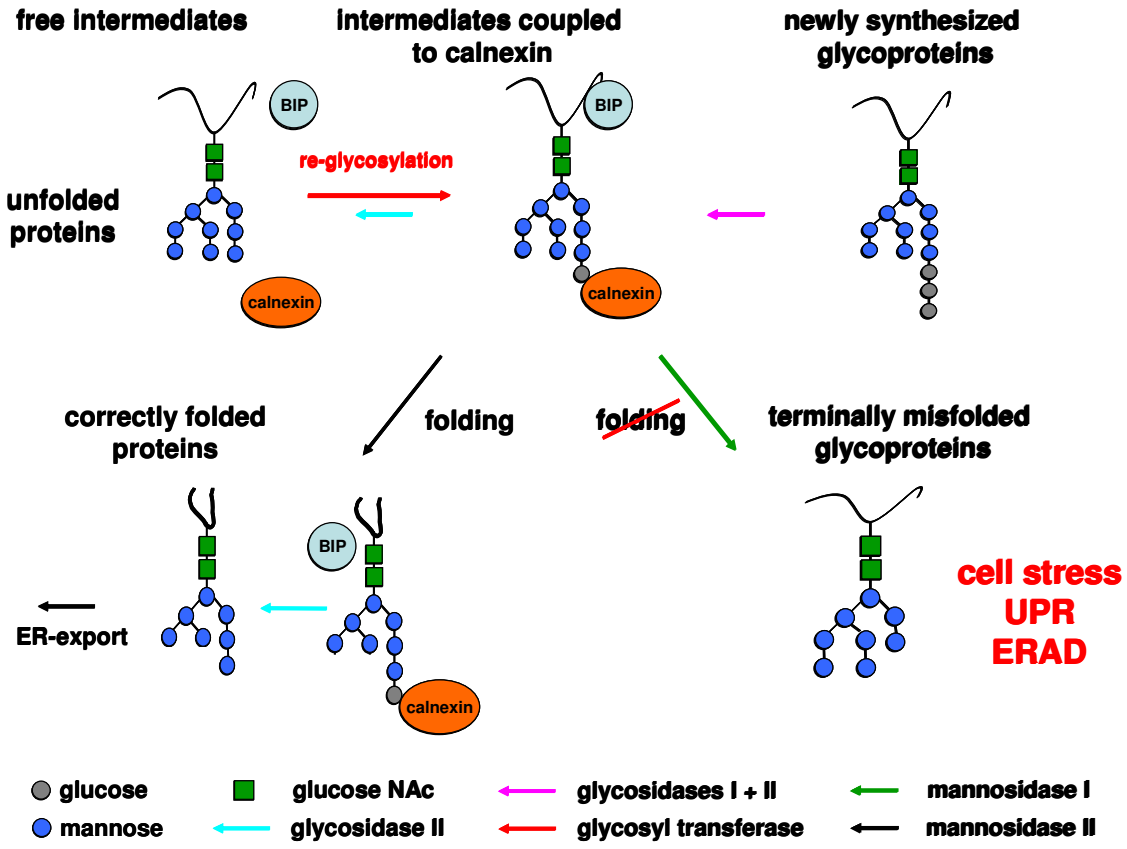

Figure 4: Quality control of N-glycans. Newly synthesized proteins are co-translationally core-glycosylated with high mannose and glucose residues. Calnexin or its soluble homolog calreticulin bind monoglycosylated folding intermediates generated by the enzymes glycosidase I and II. The chaperone BiP assists in the folding process by interaction with hydrophobic patches exposed by the folding intermediates. Nascent proteins undergo multiple cycles of cleavage of the terminally located glucose and release of calnexin / calreticulin including reglycosylation by glycosyl transferase and rebinding by the chaperones. Correctly folded N-glycans are divided from folding intermediates by an unknown mechanism and sorted for export to the secretory pathway by mannosidase II, which generates a Man7 structure. Terminally misfolded polypeptides are trimmed to Man8 structures by mannosidase I, they presumably associate with the lectin EDEM and are degraded by the ERAD. If large amounts of misfolded proteins accumulate, this may be accompanied by cell stress and activation of the UPR pathway.

Both chaperones are calcium-binding lectins that bind monoglycosylated proteins through N-linked carbohydrate structures and promote their retention and folding (Helenius and Aebi 2004). Calnexin is a membrane-bound chaperone and calreticulin is its soluble homolog. The nascent proteins undergo cycles of binding and release from calnexin or calreticulin during which they are trimmed by glycosidases, mannosidases and glycosyl transferases (GT) to achieve the correct conformation (Figure 4) (Hammond, Braakman et al. 1994). If properly folded and assembled proteins assume a transport-competent conformation, the ER mannosidase II generates a Man7 sugar chain and the protein is able to leave the ER and enter the secretory pathway (Trombetta and Parodi 2003).

All immature protein conformations are recognized and retained in an attempt to restore their transport-competent folding state. The reglycosylating enzyme GT can distinguish between non-native and native conformations (Caramelo, Castro et al. 2003). It generates monoglycosylated proteins recognized by calnexin / calreticulin. Thereby the reglycosylation is restricted to glycoproteins that cannot acquire their native confirmations. When the machinery is not able to fold proteins correctly, their sugar chains are trimmed to a Man8 form by the enzyme α-1,2-mannosidase I and the substrates cannot be reglycosylated by GT anymore (Trombetta and Parodi 2003). Man8 forms are recognized by the lectin EDEM which facilitates release from calnexin and are then exported from the ER by an unknown mechanism (Molinari, Calanca et al. 2003; Oda, Hosokawa et al. 2003). Finally proteins are eliminated by the ERAD pathway (Brodsky and McCracken 1999).

Subsequent work by Gauss et al. showed the connection between the quality control system and associated ER degradation machinery in yeast. The quality control lectin Yos9p directly interacts with Hrd3p that is associated with the Hrd1 (3-hydroxy-3-methylglutaryl-coenzymA reductase degradation) ligase, a central ERAD protein complex (Gauss, Jarosch et al. 2006). Yos9p is thought to recognize the Man8 structures (Bhamidipati, Denic et al. 2005) and plays a role in their delivery to the luminal domain of Hrd3p (Gauss, Jarosch et al. 2006). In mammals this interaction has yet to be elucidated, but a good candidate is the Yos9p functional equivalent Os-9.

ER-associated degradation (ERAD)

The ERAD is a highly regulated control mechanism whose main function is to eliminate high amounts of non-native or unassembled polypeptides of protein complexes that protect the ER from overload. The degradation requires a process called retrograde translocation to extract proteins from the ER; so far there is no known mechanism for turnover in the ER itself. Most of the proteins investigated in this field were identified from yeast, but there are mammalian homologs of ERAD components. However, the mam-

malian ERAD is more complex, the participation of the homologs has yet to be defined and the underlying mechanisms remain poorly understood.

The proteins that fail the quality control are unfolded and are subsequently targeted to retrotranslocation, ubiquitination and membrane extraction. In the cytosol degradation substrates are deglycosylated and eliminated by proteasomes (Brodsky and McCracken 1999). Major open questions in this field concern the different accessory proteins that are recruited to the substrate for each step of the ERAD pathway. Yos9p in yeast is thought to identify non-native Man8 structures (Bhamidipati, Denic et al. 2005). Recent results showed the participation of the mammalian counterpart Os-9 and the ER-luminal located chaperone GRP94 in the selection of misfolded substrates (Christianson, Shaler et al. 2008). Some evidence suggests that ERAD substrates may be transported through an unknown protein-conducting channel. The Sec61 translocon (Wiertz, Tortorella et al. 1996; Plemper, Bohmler et al. 1997; Tsai, Ye et al. 2002) and the Derlin-1 complex (Ye, Shibata et al. 2004) are thought to participate in this process. Studies addressing the role of the Sec61 translocon, the same complex used for ER import of proteins, have yielded conflicting results (Kalies, Allan et al. 2005; Carvalho, Goder et al. 2006). Derlin-1 is the mammalian homolog of yeast Der1p. It is associated with the ER membrane and is predicted to have four transmembrane-spanning domains with both the amino and carboxy termini in the cytosol. Derlin-1 forms a protein complex with valosin-containing protein (VCP)-interacting membrane protein (VIMP) and serves as a receptor for VCP in the US11-mediated retrotranslocation of major histocompatibility complex (MHC) class I heavy chains (Ye, Shibata et al. 2004). If the degradation substrates reach the cytoplasm, they are polyubiquitinated by ubiquitin ligases with K48-linked ubiquitin chains, which contain at least 4 ubiquitin molecules. The ligases that are known to participate in mammalian ERAD are Hrd1 (Bays, Gardner et al. 2001), gp78 (Fang, Ferrone et al. 2001), TEB4 (Hassink, Kikkert et al. 2005), Rma1 (Matsuda, Suzuki et al. 2001) and RFP2 (Lerner, Corcoran et al. 2007).

The polyubiquitinated proteins are extracted out of the ER membrane and are released into the cytoplasm by the AAA ATPase p97/VCP (Flierman, Ye et al. 2003; Ye, Meyer et al. 2003). p97/VCP, a key component of the ERAD pathway, is a cytosolic magnesium-dependent homohexameric protein complex. It is recruited to the ER membrane and interacts with its binary partner Ufd1/Np14 (Meyer, Shorter et al. 2000). It has been proposed that p97/VCP is involved in substrate ubiquitination, an idea consistent with studies showing interactions with VCP and different E3 ligases. The underlying molecular mechanisms have not yet been elucidated. Recent data also indicate that a complex of yeast Ufd2p/Dsk2p/Rad23p mediates the transfer of ERAD substrates from Cdc48p (yeast counterpart of VCP) to the proteasome. Herp, which is up-regulated under UPR conditions, is also described to associate with the ERAD complex of Hrd1, p97/VCP, Derlin-1 and VIMP (Schulze, Standera et al. 2005). In the cytosol, released

degradation substrates are recognized by the 19S regulatory particle of the 26S proteasome.

Recent data reveal that three different ERAD pathways exist in yeast and regulate the degradation of misfolded proteins depending on the position of the defect in the misfolded protein (Carvalho, Goder et al. 2006). These pathways use the same core machinery, but they differ in the components involved. The ERAD-L pathway eliminates target proteins with misfolded luminal domains; the key components are Hrd1p and Der1p. ERAD-M degrades proteins with misfolded transmembrane domains. Hrd1 is also central to this pathway, but it is supposed to be independent of Der1. The ERAD-C pathway facilitates turnover of proteins with mutations in cytosolic domains; it is independent of Der1 and Hrd1, and the Doa10 ligase is the key component. So far it is unknown if these pathways also operate in mammals.

Yeast and homolog mammalian ERAD components:

Yeast	**Mammals**
Hrd1	Hrd1
Doa10	TEB4
Hrd3	Sel1
Usa1	Herp
Der1	Derlin-1, -2, -3
Yos9	Os-9, XTP3-B
Cdc48	p97/VCP
Ubx2	VIMP
Np15/Ufd1	Np15/Ufd1
Htm1	EDEM

The retention of misfolded proteins leads to protein folding diseases

There are many diseases caused by the retention of proteins that failed the quality control and are deposited in aggresomes or are ultimately degraded by the ERAD pathway. These proteins do not reach their site of action and this leads to a loss of specific cell functions. Diseases like cystic fibrosis (CF), Alzheimer's disease, retinitis pigmentosa and X-linked nephrogenic diabetes insipidus (NDI) are important examples of protein folding diseases. The vast majority of CF cases are linked to the ΔF508 mutation, a deletion of a phenylalanine in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Kerem, Corey et al. 1990). Nearly 100 % of the mutated chloride channel gets stuck in the ER and is degraded by the ERAD pathway (Lukacs, Mohamed et al.

1994). Mutant CFTRs are also prone to aggregate and are deposited in aggresomes (Burnett and Pittman 2005). In the case of Alzheimer's disease, ER-retained presenilin enhances the levels of amyloid formation. Another hereditary folding disease, NDI, is caused by mutations in the *AVPR2* gene that encodes for the V2 vasopressin receptor (V2R). Until now, more than 170 different mutations of the V2R have been described that are spread over all receptor domains. Most of these mutant receptors are recognized by the quality control system and are retained in several compartments of the secretory pathway, namely the ER, ERGIC (Hermosilla, Oueslati et al. 2004) and Golgi network (this thesis). This distinguishes the V2R from other conformational misfolded and subsequently retained proteins as an excellent model protein to examine the interaction of intracellular quality control and degradation pathways.

G protein-coupled receptors

The V2R, the model protein used for the present study, belongs to the superfamily of G protein-coupled receptors (GPCR). GPCRs constitute the largest family of cell surface molecules involved in signal transduction and are the most important protein class of drug targets in modern pharmacology. 50 – 60 % of all current therapeutic agents influence biological functions by modulating GPCRs. The receptors share a characteristic structure of seven-transmembrane helices and three extra- and intracellular loops. GPCRs can be activated by a multiplicity of ligands such as amines (noradrenalin, dopamin, serotonine, histamine), peptides (angiotensin, vasopressin, opioids), amino acids (glutamate, GABA) or lipids (sphingosine-1-phosphate) regulating physiological key roles in cell function (Marinissen and Gutkind 2001). The receptors transduce these extracellular stimuli classically by a conformational change and the interaction with heterotrimeric G proteins that activate intracellular signaling pathways. Heterotrimeric G proteins are composed of an α-subunit and a β/γ-dimer; after activation by GPCRs they catalyze the exchange of GDP for GTP at the α-subunit. The β/γ-subunit dissociates from the α-subunit and both can stimulate a wide variety of downstream effectors. The kind of α-subunit determines the activated signaling pathway; four different families are described ($G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12/13}$). $G\alpha_s$ and $G\alpha_i$ modulate adenylyl cyclases and therefore cAMP levels, whereas $G\alpha_{q/11}$ activates the key enzyme phospholipase C which catalyzes the cleavage of phosphatidylinositol diphosphate into the second messengers diacylglycerol and inositol (1,4,5)-triphosphate (Marinissen and Gutkind 2001). $G\alpha_{12/13}$ mediates the activation of Rho family GTPases. The β/γ-subunits also alter cell function by controlling entry of ions through membrane channels or by modulating phospholipases and lipid kinases. The β/γ-dimers also activate downstream effectors, such as receptor tyrosine kinases and members of the mitogen-activated protein kinase (MAPK) pathway (Src, Ras); this also contributes to the diversity of signal transduction induced by GPCRs. Activation of GPCR additionally leads to G protein-independent

signaling, e.g. the phosphorylated receptors can be bound by β-arrestin for subsequent internalization. Finally, all activated signaling pathways lead to biological responses important for proper cellular function.

V2 vasopressin receptor

The *APVR2* gene is localized on the short arm of the X-chromosome; it encodes a GPCR of 371 amino acids (Rosenthal, Seibold et al. 1992; Oksche and Rosenthal 1998). The V2R is mainly expressed at the basolateral membrane, but also at the apical membrane of principal cells of the renal collecting duct (Nonoguchi, Owada et al. 1995; Schulein, Lorenz et al. 1998). Receptors are additionally present in the luminal epithelium of the endolymphatic sac (Taguchi, Takeda et al. 2007) and are presumed to be expressed in endothelial cells (Kaufmann, Oksche et al. 2000). In the kidney the V2R facilitates vasopressin- (AVP) mediated water reabsorption (Klussmann, Maric et al. 2000). The V2R shares the typical structure of GPCRs: seven transmembrane-spanning helices bridged by three intracellular and three extracellular loops. The extracellular N terminus contributes to ligand binding, whereas the intracellular C terminus and the third intracellular loop facilitate the signaling activity of the receptor. During synthesis the receptor is co-translationally inserted into the ER membrane. Post-translational sugar moieties at N22 (ER and Golgi apparatus) (Sadeghi, Innamorati et al. 1997) and C-terminal serines and / or threonines (Golgi apparatus) (Sadeghi and Birnbaumer 1999) as well as a disulfide bonds between C112 in the first extracellular loop and C192 in the second extracellular loop contribute to the three-dimensional receptor structure. The V2R is palmytoylated at cysteines C341 and C342, which leads to a stabilization of the protein in the membrane (Sadeghi, Innamorati et al. 1997). Correctly folded, transport-competent receptors transit through the secretory pathway (Thielen, Oueslati et al. 2005) and recent studies indicate that the ADP-ribosylation factor 6 (ARF6) is likely to be involved in the ER to Golgi trafficking of V2Rs (Madziva and Birnbaumer 2006).

The ligand AVP is released by an increase in plasma osmolarity (>2 %) or severe hypovolemia (>10 %) and initiates different signal transduction pathways, such as vasoconstriction by activation of V1a receptors or antidiuresis via the V2R. In detail, the hormone-stimulated V2R activates adenylyl cyclase and protein kinase A (PKA). This signaling cascade culminates with the fusion of aquaporin-2 (AQP2) carrying vesicles with the apical membrane, which induces an increase in water permeability (Oksche and Rosenthal 1998). Binding of AVP to the receptor results in a conformational change of the ligand-receptor complex which leads to the coupling of the heterotrimeric G protein $G\alpha_s$ (Figure 5). $G\alpha_s$ stimulates adenylyl cyclases (most probably V and VI) and GPCR kinases (GRKs). The adenylyl cyclases mediate the synthesis of the second messenger cAMP, which subsequently activates the cAMP-dependent PKA. Finally, PKA pho-

shorylates AQP2 associated with cytosolic transport vesicles; the AQP2-carrying vesicles are inserted mainly into the apical, but also in the basolateral membrane to facilitate water influx. The second reaction catalyzed by PKA is an increase in urea permeability of the inner medullary collecting duct mediated by the activation of a vasopressin-regulated urea transporter (Klein, Murrell et al. 2006). In addition, hormone binding of the V2R leads to an increase in Na^+ transport mediated by the Na^+- K^+- $2Cl^-$ symporter in the thick ascending limb and collecting duct. All reactions result in conservation of water. In addition, the small GTP-binding protein RhoA, which is involved in cytoskeleton organization, is recruited by PKA and participates in the AQP2 shuttle (Klussmann, Tamma et al. 2001).

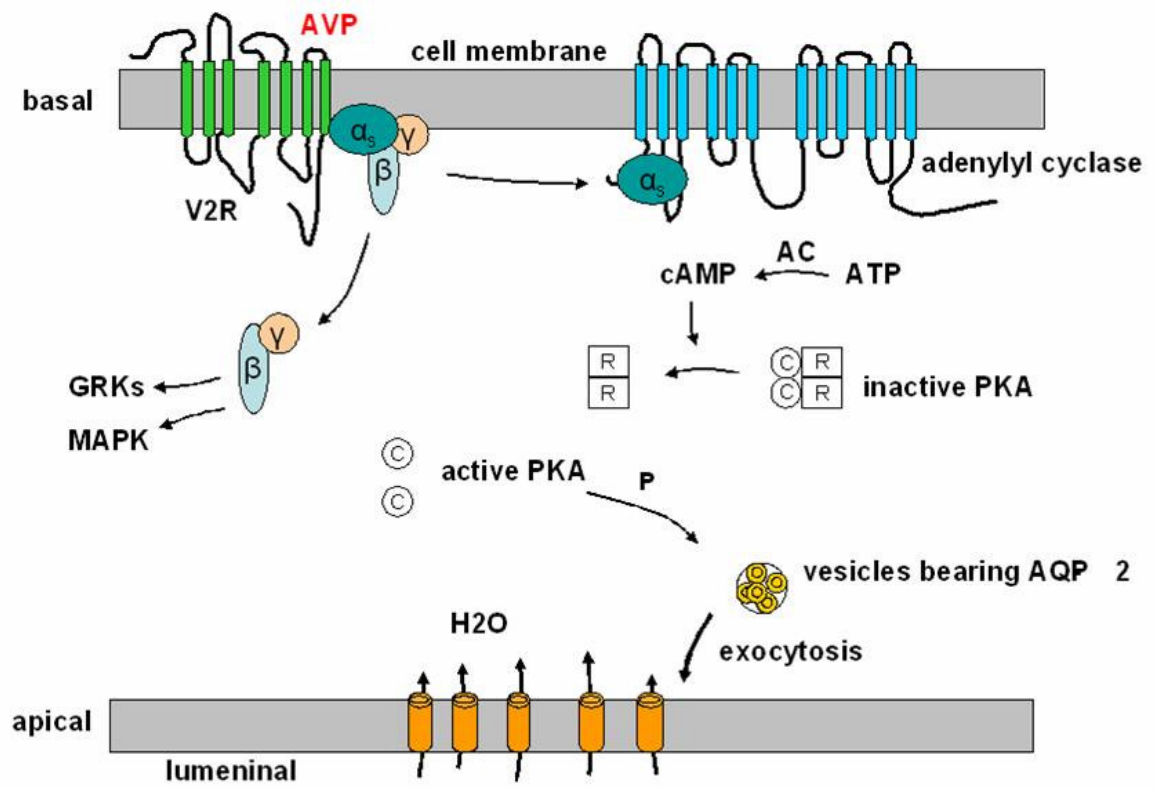

Figure 5: Signal transduction of V2Rs in a principal cell. The hormone AVP induces a conformation change of the V2R and activates the G protein G_S. The dimeric G_S dissociates in α- and βγ-subunits which influence different signaling pathways independently. The α-subunit mediates activation of a membrane-associated adenylyl cyclase that monitors the release of the second messenger cAMP. In presence of cAMP, protein kinase A (PKA) is activated by dissociation of regulatory (R) and catalytic (C) subunits. PKA-induced phosphorylation facilitates the exocytosis of AQP2-bearing vesicles into the luminal membrane including water influx. Beside the α-mediated signaling, the βγ-subunit activates GRKs which contribute to receptor desensitation and to activation of the MAPK pathway.

Beside ACs $G\alpha_s$ additionally stimulates GRKs. GRKs phosphorylate the receptor at its C terminus (Innamorati, Sadeghi et al. 1997) resulting in homologous desensitization (Birnbaumer, Antaramian et al. 1992) through recruitment and binding of cytosolic β-arrestin2. β-arrestins function as scaffolding proteins and prevent the receptors from

overstimulation through further G protein activation despite the continued activation by AVP. Martin et al. showed that the V2R is additionally ubiquitinated in an agonist-dependent manner (Martin, Lefkowitz et al. 2003). Finally, arrestins bind to clathrin and facilitate the internalization of ubiquitinated receptors in clathrin-coated pits to an endosomal compartment (Bowen-Pidgeon, Innamorati et al. 2001). In contrast to the β_2-adrenoceptor, the activated V2R stably associates with β-arrestin2 and ubiquitin and is sorted to lysosomes for degradation without recycling to the cell surface (Shenoy and Lefkowitz 2003; Bouley, Lin et al. 2005). Recent data indicate that β-arrestin can also be ubiquitinated, which leads to a special conformation of the receptor / β-arrestin complex and to the ultimate turnover of receptors (Shenoy and Lefkowitz 2005).

Moreover, the receptor / β-arrestin complex is involved in activating a G protein-independent signaling cascade. It induces the MAPK pathway including extracellular signal-regulated kinases (ERK1/2) (Charest, Oligny-Longpre et al. 2007). This pathway regulates the transcription of genes through phosphorylation of transcription factors and modulates critical signaling pathways like cell differentiation, proliferation and apoptosis.

At steady state, there is a balance of V2R synthesis and breakdown; newly synthesized, immature receptors are localized in ER and ERGIC, whereas mature receptors are in the Golgi network and at the plasma membrane. To maintain normal protein levels, redundant receptors from the plasma membrane undergo constitutive internalization and are degraded in lysosomes (Schmidt, Lautz et al., submitted 2008).

X-linked nephrogenic diabetes insipidus (NDI)

Inactivating mutations in the *APVR2* gene may lead to alterations in the three-dimensional structure of the V2R and therefore to a loss of function. Most frequent are point mutations that lead to the exchange of single amino acids, but frameshift mutations, deletions and insertions of amino acids also appear. Congenital X-linked NDI is characterized by the inability of the kidney to concentrate the urine in response to the hormone AVP. The mutant receptors may have a defect in their synthesis, processing or intracellular transport. The resulting defects can be divided into different groups: 1. mutants that are transported to the cell surface, but fail to interact with AVP or fail to activate the G protein / adenylyl cyclase system, 2. intracellular retained mutants or, 3. instable mRNA. Intracellular retained mutants resulting from missense mutations are used in this study to address the role of the mammalian ERAD. The V2R mutants are recognized by the cellular quality control system and are retained in different compartments of the secretory pathway dependent on their folding defect (Hermosilla, Oueslati et al. 2004). They only differ in single amino acids substituted in the cytoplasmic, transmembrane or ER-luminal / extracellular domains of the receptor (Figure 6).

The L62P mutation (leucine at position 62 is substituted by proline) is presumably localized at the interface between the first transmembrane domain and the first intracellular loop of the receptor. The mutant is reported to be retained in the ER exclusively (Hermosilla, Oueslati et al. 2004). The G201D mutation is in the third ER-luminal / extracellular loop and causes Golgi apparatus retention (this work). V226E is localized in the fifth transmembrane domain and mutant receptors are retained in the ERGIC (Hermosilla, Oueslati et al. 2004). S167L is located in the fourth transmembrane domain and the intracellular localization of the mutant is restricted to the ER (Wüller, Wiesner et al. 2004).

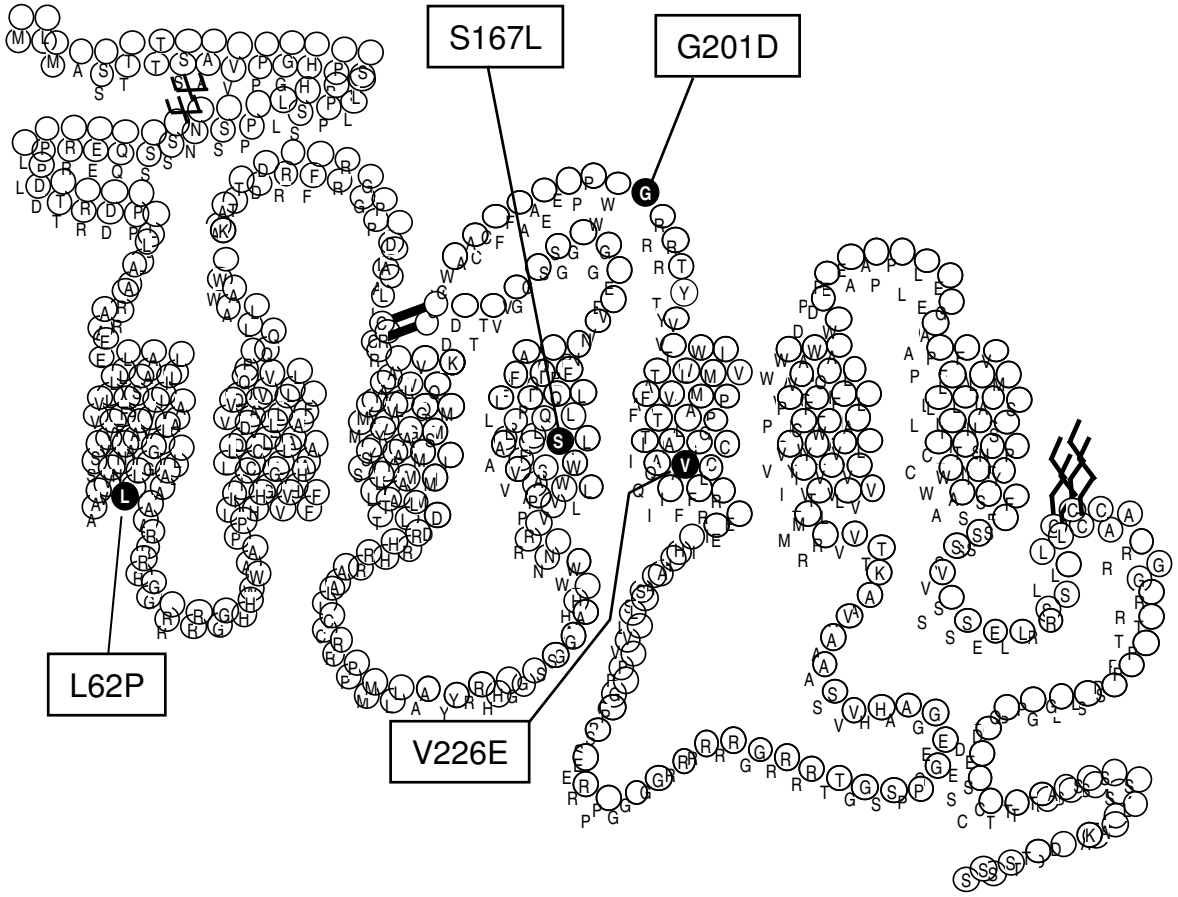

Figure 6: Two-dimensional model of the V2R and four NDI-causing mutants. The amino acid sequence of the receptor is shown in one letter code, N-glycosylation at position N22, disulfide bond between C112 and C192 and palmitoylation of residues C341 and C342 are depicted. Substituted amino acids of mutants L62P, S167L, V226E, and G201D are shown in black.

So far there is no specific treatment of X-linked NDI; patients have to adjust water intake to the amount of excreted urine. Indometacin, thiazide and amiloride are given as a concomitant therapy. In the future gene therapy may be an approach to restore V2R function, but at present all common methods to infiltrate somatic cells with intact genes lack safety and efficiency.