Full Review

Protein trafficking defects in inherited kidney diseases

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ABSTRACT

The nephron, the basic structural and functional unit of the kidney, is lined by different, highly differentiated polarized epithelial cells. Their concerted action modifies the composition of the glomerular ultrafiltrate through reabsorption and secretion of essential solutes to finally produce urine. The highly specialized properties of the different epithelial cell types of the nephron are remarkable and rely on the regulated delivery of specific proteins to their final subcellular localization. Hence, mutations affecting sorting of individual proteins or inactivating the epithelial trafficking machinery have severe functional consequences causing disease. The presence of mutations leading to protein trafficking defect is indeed a mechanism of pathogenesis seen in an increasing number of disorders, including about one-third of monogenic diseases affecting the kidney. In this review, we focus on representative diseases to discuss different molecular mechanisms that primarily lead to defective protein transport, such as endoplasmic reticulum retention, mistargeting, defective endocytosis or degradation, eventually resulting in epithelial cell and kidney dysfunction. For each disease, we discuss the type of reported mutations, their molecular and cellular consequences and possible strategies for therapeutic intervention. Particular emphasis is given to new and prospective therapies aimed at rescuing the trafficking defect at the basis of these conformational diseases.

Keywords: inherited kidney disease, misfolded protein, molecular and pharmacological chaperone, protein homeostasis, protein trafficking

INTRODUCTION

The nephron, the functional unit within the kidney, contains several types of highly differentiated polarized epithelial cells that ensure barrier function and vectorial transport of ions, proteins and other molecules across the apical and basolateral plasma membranes. The sequential action of the different epithelial cell types lining the nephron, each with specific transport properties, modifies the composition of the primary ultrafiltrate through reabsorption and secretion of essential solutes, to eventually produce urine. The accurate delivery of specific proteins, such as channels or transporters, on the appropriate membrane domain ensures the complex and highly specialized functions of renal epithelia. In this context, mutations affecting intracellular sorting of individual proteins or inactivating the epithelial trafficking machinery can have severe functional consequences causing disease. To date, mutations in ~120 genes are known to be responsible for monogenic diseases affecting the kidney [1]. About one-third of them directly interfere with protein transport, affecting one or more segments of the nephron (Table 1). With few exceptions, protein products encoded by such genes enter the secretory pathway to reach their final destination. The first step of this journey occurs in the co-translational translocation in the endoplasmic reticulum (ER) where a quality control system ensures that only properly folded proteins can move forward. From the ER, proteins reach the Golgi where they undergo different modifications, the most common being the ones on N-linked glycans. At this stage, complex processes regulate anterograde and retrograde transport with the final goal of reaching the trans-Golgi network, where proteins are dispatched towards apical, basolateral or endolysosomal compartments (Figure 1a). Mutations in secretory proteins can affect their trafficking at any of these steps (Figure 1b–d). In this review, we ideally move along the secretory pathway and discuss examples of inherited kidney diseases characterized by different pathogenetic mechanisms. Storage disorders due to the accumulation of substrates of defective lysosomal enzymes are not specifically addressed and have recently been the focus of
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Continued
other reviews (as in [2]). For each disease, we discuss the type of reported mutations, their functional consequences and present and prospective strategies for therapeutic intervention.

### ER RETENTION

Retention in the ER is a mechanism shared by a wide range of conformational diseases and it is essentially due to mutations affecting protein folding (Figure 1b).

In some cases, misfolding mutations have a main loss-of-function effect, since the mutated protein is effectively degraded via ER-associated degradation (ERAD), as for example in nephrogenic diabetes insipidus (NDI).

#### Nephrogenic Diabetes Insipidus

NDI is a water balance disease characterized by the incapacity to concentrate urine resulting in severe polyuria and polydipsia. This may lead to dehydration, electrolyte imbalance (hypernatremia) and growth and mental retardation. Persistent polyuria can also result in urologic complications, from slight dilation of the urinary tract to severe hydronephrosis. The conventional therapy consists of adequate fluid supply to compensate for water loss and low sodium diet combined with thiazide and amiloride treatment. The most common forms in clinic are the acquired ones (mainly secondary to drug treatment, electrolyte imbalance and urinary tract obstruction) [3]. Congenital NDI is caused by mutations in the AVPR2 or AQP2 genes, responsible for X-linked (90%) and autosomal (10%; recessive and dominant) forms of the disease, respectively. These two genes are essential for the control of water balance mediated by the peptide hormone arginine vasopressin (AVP). AVP binds to vasopressin receptor type 2 (AVPR2), a G-protein-coupled receptor (GPCR) localized at the basolateral membrane of the principal cells of the kidney collecting duct. Upon activation by AVP, AVPR2 activates adenylate cyclases triggering a signalling cascade eventually resulting in activation of protein kinase A and phosphorylation of aquaporin 2 (AQP2) in the endosomal compartment. This leads to fusion of endosomal vesicles containing AQP2 with the apical plasma membrane where AQP2 allows water reabsorption. Once water balance is restored, AVP level decreases and AQP2 is removed from the plasma membrane by ubiquitylation-mediated endocytosis [4].

The X-linked form of congenital NDI is caused by loss-of-function mutations in the AVPR2 gene [5, 6], resulting in insensitivity of principal cells of the collecting duct to AVP. Mutations can be divided into four classes on the basis of their molecular mechanism of action [7]. Class 1 mutations (usually frameshift or nonsense) affect synthesis or processing of the mRNA (i.e. promoter alterations, aberrant splicing) and lead to absent (mRNA decay) or truncated protein. Class 2 mutations (missense, in-frame insertion/deletion) are the most common and cause misfolding of the protein, its retention in the ER and degradation. Class 3 mutations (missense and in-frame insertion/deletion) do not affect plasma membrane localization, but interfere with G protein or AVP binding. Class 4 mutations lead to protein missorting from the plasma membrane to different cellular compartments [8]. Some mutations can present features of different classes, as they can affect different stages of protein maturation and function. Class 2 AVPR2 mutations represent >50% of the reported mutations. Although ER retention and degradation are common features of all Class 2 mutants, the amount of retention differs between mutants, likely reflecting their degree of misfolding [9]. Some mutants are indeed partially trafficked to the plasma membrane where they can be functional or defective in AVP binding or G-protein coupling.

Partial trafficking to the basolateral membrane of functional protein could explain why some patients respond to high doses of the vasopressin synthetic analogue desmopressin [10]. Thus, a possible therapeutic strategy for mutants retaining functional activity is to induce their escape from ER retention and ERAD and their insertion at the plasma membrane. While the effect of non-selective chemical chaperones, as glycercer or dimethyl sulfoxide (DMSO), has been modest [11], interesting results were obtained with receptor antagonists (peptides or pharmacological chaperones). These molecules specifically bind to AVPR2 and aid the folding of mutated protein and its exit from the ER [12]. One such molecule (SR49059) had some beneficial effects in patients [13]. However, further clinical studies have been limited by the difficulty in identifying effective low-affinity ligands, able to rescue AVPR2 trafficking and to be efficiently displaced by AVP, and by the high concentrations required, raising significant safety issues. An alternative and more valid approach seems to be the use of non-peptide agonists [as OPC51, VA(9990)88 and VA (9990)89] that do not rescue trafficking of the receptor, but can directly activate intrinsically functional AVPR2 mutants.

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**Table 1.** Continued

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PM, plasma membrane; AM, apical plasma membrane; BM, basolateral plasma membrane; ER, endoplasmic reticulum; PT, proximal tubule; TAL, thick ascending limb; DCT, distal convoluted tubule; CD, collecting duct; Multiple, disease primarily affecting more than one nephron segments; Secondary, disease affecting the kidney via secondary effect.

*Disease nomenclature according to the Online Mendelian Inheritance in Man (OMIM) database.

°Loss of transcript/protein product is not indicated.
within the ER. This leads to GPCR activation and cyclic AMP increase, eventually resulting in increased AQP2 delivery to the plasma membrane [14]. Likely, this effect is due to ER-activated AVPR2 signalling to pre-formed receptor G-protein–adenylate cyclase complexes at the ER membrane, as observed for the activation of the ER-localized GPR30 by oestrogen [15] or for the β2-adrenergic receptor signalosome [16]. Further studies will be needed to assess the clinical applicability of this interesting class of molecules. A third strategy that could be beneficial for different AVPR2 mutants is to bypass AVPR2 function and induce AQP2 localization at the apical membrane, as recently demonstrated by treatment with agonists of E-prostanoid receptors EP2 and EP4 in rodent models [17,18].

AQP2 is the gene mutated in the autosomal dominant and recessive forms of NDI [19]. In the dominant form, representing 10% of autosomal NDI cases, mutations are found in the carboxy-terminal tail of the protein that is essential for proper trafficking. These mutations introduce missorting signals leading to mislocalization of AQP2 in the Golgi, late endosomes, lysosomes or basolateral membrane [20]. They can also affect apical membrane insertion by interfering with AVP-induced phosphorylation of AQP2 at serine 256 [21, 22]. These mutations are dominant since mutant protein can interact with wild-type AQP2 and affect its localization [23]. In the recessive form, the vast majority of mutations are missense changes in the region forming the aquaporin pore and they lead to protein misfolding, ER retention and proteasomal degradation. A different mechanism of recessive inheritance was discovered in two families, in which patients were compound heterozygotes for either A190T or R187C mutations, along with mutation P262L. While A190T and R187C AQP2 mutants are retained in the ER, the P262L isoform is retained in intracellular vesicles only in the absence of wild-type protein [24].

In the dominant forms, strategies aimed at increasing cyclic AMP and hence AQP2 phosphorylation could be effective, as successfully demonstrated by treatment with Rolipram, a phosphodiesterase 4 inhibitor, in a murine model of autosomal dominant NDI [25]. As for AVPR2 Class 2 mutants, modulating protein folding could be envisaged as a potential treatment for recessive AQP2 mutations. An Hsp90 inhibitor (17-AAG) has already been shown to partially rescue AQP2 trafficking in a conditional murine model of NDI [26]. Despite the interesting results in preclinical models, the applicability of molecules inhibiting phosphodiesterase 4 or Hsp90 in the therapy of NDI needs further studies addressing safety issues, given the abundance and composite biological roles of such proteins.

In some conformational diseases, ER-retained mutant protein is not effectively degraded and its accumulation exerts a gain-of-toxic-function effect. This mechanism is proved by the different phenotype of knock-out (KO) models and of models of expression of the mutant protein. An example is uromodulin-associated kidney disease (UAKD) that is caused by mutations of the UMOD gene encoding uromodulin (or Tamm–Horsfall protein) [27]. Studies in cellular models demonstrated that UMOD mutations lead to mutant protein retention and aggregation in the ER, likely due to protein misfolding [28]. This was also observed in patient renal biopsies and in the kidneys of mice expressing mutant uromodulin [29, 30]. These models recapitulate most of the features of the human disorder. Indeed, ER retention of
mutant uromodulin leads to progressive structural and functional damage of the thick ascending limb (TAL) of Henle’s loop, the nephron segment where uromodulin is expressed, to interstitial fibrosis and inflammation. On the contrary, Umod KO mice do not show any sign of tubulo-interstitial disease [31], demonstrating the gain-of-function effect of uromodulin mutations. Accumulation of mutant protein in the ER can impact on several cellular processes, ranging from protein homeostasis, redox balance and chronic induction of different cell stress pathways. Among these, the unfolded protein response (UPR) has been extensively studied. UPR is a complex adaptive pathway that counteracts protein folding overload in the ER by decreasing protein translation and by increasing the protein folding and degradation machinery. Such an adaptive pathway can turn into maladaptive when chronically induced, as in the case of accumulating misfolded mutant protein, and can eventually lead to apoptosis. Induction of the UPR has been detected in conformational renal diseases, as in nephrotic syndrome type I with nephrin mutations [32] and in Alport syndrome and Pierson syndrome, disorders of the glomerular basement membrane due to mutations in collagen IV [33] and laminin β2 [34], respectively. The pathogenic role of UPR and the effect of its modulation in renal disorders are still unclear and deserve further investigation.

**Protein trafficking defects in kidney diseases**

**MISTARGETING**

Protein targeting is the process by which proteins reach their final destination. It is controlled by information contained in the protein sequence and its dysregulation often leads to disease (Figure 1c). Mutations have a loss-of-function effect, due to the absence of the protein function in the physiological cell compartment. This can be associated with a gain-of-function component, due to the aberrant localization of functional mutant protein.

**Primary hyperoxaluria type I**

Mistargeting is among the causes of primary hyperoxaluria type I (PH1). PH1 is a rare autosomal recessive disease caused by mutations in the AGXT gene coding for the liver-specific peroxisomal enzyme alanine-glyoxylate aminotransferase (AGT) [35]. Deficiency in the activity of AGT causes accumulation of glyoxylate and consequently overproduction of the metabolic-end product oxalate. This leads to progressive deposition of insoluble calcium oxalate in the kidney and urinary tract. Calcium oxalate deposition eventually spreads throughout the body due to chronic kidney failure. Reported AGXT mutations are missense (50%), nonsense (13%), major or minor insertions or deletions (25%) or affect splicing (12%) [36]. These mutations can have different consequences altering protein function (loss of catalytic activity or co-factor binding), structure (increased degradation, reduced dimerization and aggregation) or subcellular localization (aberrant mitochondrial localization) [36]. The AGXT gene has a number of polymorphic variants in linkage disequilibrium forming ‘major’ and ‘minor’ (present in 15–20% of Caucasians) haplotypes. Main differences in the minor allele include an imperfect duplication in intron 1 and two coding non-synonymous polymorphisms (P11L and I340M). Interestingly, the frequency of AGXT minor allele raises to 46% in PH1 patients [36]. The substitution of a proline with a leucine at position 11 creates an N-terminal mitochondrial targeting sequence (MTS). This MTS is normally ineffective, as dimerization of the protein masks the binding motif for the mitochondrial import receptor TOM20 and it keeps AGT in a conformation that is incompatible with mitochondrial import. However, in conditions that affect protein dimerization, as for some AGT mutations, the MTS becomes accessible and AGT can be imported to the mitochondria [37]. This synergistic mechanism was first described for the most common mutation G170R [35], present in ~30% of PH1 cases. It was recently confirmed for three other mutants (I244T, F152I and G41R), showing different extent of protein mitochondrial re-localization [38]. These mutations do not completely abolish dimerization and binding to the peroxisomal import protein Pex5p and retain some catalytic activity, suggesting that they do not fully compromise protein folding and dimerization [38, 39]. Likely, they decrease the dimerization efficiency, and this is sufficient to allow the interaction of mutant protein MTS with TOM20. Delayed or defective folding is likely at the bases of the effect of other AGT mutations that are mainly associated with protein aggregation (in the cytosol or in peroxosomes) or with proteasome-mediated degradation [40]. Also in this case, AGT mutations show a synergistic effect with the minor allele and mutant proteins partially retain folding, transport and catalytic properties [39].

Patient treatment envisages high fluid intake and alkalization of the urine to reduce urinary Ca-oxalate saturation. Once renal function is lost, PH1 patients require liver and kidney transplantation. Administration of pyridoxine (vitamin B6) was proposed as a conservative PH1-specific measure, and about one-third of patients, mainly carrying G170R and F152I mutations, have shown to respond best [41]. A rational explanation for the therapeutic effect of this drug has been provided by a recent study in cell models stably expressing wild-type or mutant AGT. The authors proposed that treatment with pyridoxine, a metabolic precursor of pyridoxal phosphate (PLP), leads to increased concentration of PLP, an AGT co-factor. Increased intracellular PLP is able to act as a prosthetic group (increasing catalytic activity) and as a chemical chaperone (increasing expression and peroxisomal import) [42]. These promising findings demonstrate that AGT is a ‘druggable target’. The development of pharmacological compounds able to synergize with pyridoxine would thus seem an interesting strategy to ameliorate protein folding and stability, decrease aggregation and redirect mutant protein to peroxysomes. Potential targets are likely to be identified through further characterization of the complex molecular machinery that regulates AGT homeostasis, as the chaperones Hsp70, Hsp60 and Hsp90 [40].

In epithelial cells, mistargeting mutations can affect polarized protein trafficking to the apical or basolateral membranes, causing rerouting of the mutant protein to the incorrect membrane domain (Figure 1c), as for example in distal renal tubular acidosis (dRTA, also named Type 1 renal acidosis or classic RTA).

**Distal renal tubular acidosis**

dRTA is characterized by the inability to properly acidify the urine due to impaired secretion of hydrogen ions (H+) by
α-intercalated cells of the connecting tubules and cortical collecting ducts. This leads to chronic metabolic acidosis, which is responsible for growth retardation, bone disease and kidney stones [43]. Associated features include polyuria, nephrolithiasis, hypercalciuria and hypocitraturia. Alkali therapy allows the correction of biochemical abnormalities and also improves growth in children, kidney stones and bone abnormalities. The disease can be acquired, mainly attributed to immune disorders or drug-induced kidney damage, or can be hereditary. Hereditary forms are due to mutations in proteins involved in acid–base transport in the intercalated cells, i.e. the anion exchanger 1 (AE1), the H⁺-ATPase and the cytosolic carbonic anhydrase II (CA II). Mutations in the H⁺-ATPase and CA II are responsible for recessive forms of dRTA, due in both cases to loss-of-function mutations that lead to defective activity of the enzymes [44, 45]. Mutations (missense, small deletions and insertions) in AE1 lead to both autosomal dominant and recessive forms of dRTA [46, 47]. AE1, a chloride-bicarbonate exchanger, is encoded by the SLC4A1 gene. It is expressed at the plasma membrane of erythrocytes and, as a shorter isoform lacking the first N-terminal residues, at the basolateral membrane of alpha-intercalated cells [48]. In vitro studies performed in polarized Madin-Darby Canine Kidney (MDCK) cells have shown that mutations responsible for dominant dRTA can have two distinct mechanisms of action. A first group leads to ER retention and degradation of the mutant protein [49]. Interestingly, for one such mutation (S613F), AE1 intracellular retention was confirmed in patient kidney biopsy [50]. These mutant isoforms are partly trafficked to the plasma membrane and retain some activity in Xenopus oocytes, excluding a major effect on protein folding, suggesting the possibility of functional rescue [46, 51]. Another group of mutations is delivered to the membrane but with a reversal or loss of polarity. Indeed, mutant protein can be found either on both the basolateral and apical membrane or on the apical membrane only of polarized MDCK cells. Defective polarized trafficking of AE1 mutants is likely due to the disruption of basolateral targeting signals, as in the case of R901X mutation deleting the C-terminal signal sequence 904YDVE907 [52], and possibly to the introduction of apical targeting motifs, as reported for mutation M909T [53]. Mistargeted AE1 mutants were shown to be functional. Thus, their apical mislocalization could lead to the secretion of HCO₃⁻ into the collecting duct lumen resulting in highly alkaline urine. Mutations responsible for the recessive form of dRTA can lead to the production of partially functional or non-functional protein. Mutant isoforms are partly trafficked to the basolateral membrane (e.g. S773P), or retained in the Golgi compartment (G701D) or in the ER (S667F) of polarized MDCK cells [49, 54]. Dominant or recessive inheritance of AE1 mutations is determined by the interaction of mutant isoforms with co-expressed wild-type protein. Dominant mutants heterodimerize with the wild-type isoform and exert a negative effect on its trafficking [49]. On the contrary, trafficking of recessive mutants can actually be rescued by the presence of wild-type protein, thus explaining the lack of phenotype in heterozygous individuals [49, 54]. Interestingly, most of the dRTA-associated dominant mutations do not alter the trafficking and function of AE1 in erythrocytes and are not associated with haematological abnormalities. This suggests that these cells have a less stringent quality control system or express specific chaperones. One such molecule could be glycoporphin A (GPA), an erythroid-specific AE1-binding protein. Indeed, co-expression of GPA increases membrane localization of mutant AE1 kidney isoforms [47, 54] and its deficiency alters AE1 structure [55].

As trafficking defect of mutant AE1 is at the basis of the disease pathogenesis, several studies have been performed to find molecules able to rescue the delivery of active AE1 to the membrane. ER-retained dominant mutants (R589H and R901X) can be partially rescued to the membrane through inhibition of their interaction with the ER chaperone calnexin (by treatment with castanospermine) or with Hsc70 (by treatment with MAL3). Trafficking rescue is also observed after treatment with two small molecules (C3 and C4) that were already shown to increase folding efficiency of the ΔF508 CFTR mutant through a yet unclear mechanism [56]. Such treatments were not effective on the Golgi-retained mutant G701D. The trafficking of this mutant, and not the one of the ER-retained ones (R589H and C479W), could be rescued by low temperature and by treatment with chemical chaperones (DMSO and glycerol) and pharmaceutical agents (GX-809, another ΔF508 CFTR corrector). This effect could be explained by an increase in overall protein levels, possibly overcoming the cellular quality control systems. However, only DMSO was also able to restore mutant AE1 function [57]. These studies in cellular models lay the foundations for developing new therapeutic strategies in dRTA by rescuing mutant AE1 trafficking and function.

**DEFECTIVE ENDOCYTOSIS**

Endocytosis is a fundamental cellular process that involves internalization of extracellular molecules, plasma membrane proteins and lipids. The principal components of the mammalian endocytic pathways are early and late endosomes and lysosomes (Figure 1a). These membrane compartments allow the internalization of molecules from the plasma membrane, their recycling to the surface and their intracellular sorting or degradation. Defective endocytosis has been linked to several diseases (Figure 1d), such as Dent’s disease.

**Dent’s disease**

Dent’s disease is an X-linked recessive disorder that is characterized by dysfunction of the proximal tubule (renal Fanconi syndrome), resulting in low-molecular-weight proteinuria (LMWP), hypercalciuria, nephrocalcinosis, nephrolithiasis and progressive renal failure. The disease is genetically heterogeneous, being caused by mutations in the CLCN5 gene in 60% of patients (Dent’s disease 1) [58] and in the OCRL1 gene, also responsible for Lowe syndrome, in 15% of patients (Dent’s disease 2) [59]. Genes mutated in the remaining 25% of patients are still to be identified. The disease is essentially due to defective endocytosis in the proximal tubule. This process is of pivotal importance for reabsorbance of >80% of the proteins, as albumin and low-molecular-weight proteins (including vitamin-binding proteins and hormones), that are filtered by the glomeruli.

![Figure 1a](image-url)
CLCN5 encodes the chloride/proton (2Cl\(^-\)/H\(^+\)) exchanger CIC-5, functioning as a homodimer and expressed in renal and intestinal epithelia. In the kidney, CIC-5 is predominantly expressed in the proximal tubule and to a lesser extent in the TAL and in the α-intercalated cells of the collecting duct [60]. In the proximal tubule, CIC-5 is mainly found in subapical endosomes and a small fraction at the apical plasma membrane. Reported mutations in CLCN5 are nonsense (36%), missense (33%), frameshift (19%) or in-frame (2%) deletions or insertions, intragenic or complete deletions (3%) or affect splicing (7%) [61]. In the majority of the cases, they are predicted to lead to loss of protein function. This is confirmed in vivo by CIC-5 KO mice that faithfully recapitulate the disease features [62, 63]. It has been proposed that CIC-5 activity contributes to endosomal acidification, a condition indispensable for progression along the endocytic pathway, ligand–receptor dissociation, ligand processing and receptor degradation or recycling. As a chloride transporter, CIC-5 could provide the shunt conductance allowing the neutralization of H\(^+\) pumped into endosomes/vesicles by the v-type H\(^+\)-ATPase for acidification [64]. However, this hypothesis has been challenged. Indeed, a knock-in mouse model carrying a CIC-5 mutation (E211A) that converts the exchanger in an uncoupled Cl\(^-\) channel shows normal endosomal acidification. Still, it presents defective endocytosis and the same renal phenotype as CIC-5 KO mice and as patients with Dent’s disease. These results indicate that functional endocytosis requires not only a low endosomal pH but most importantly the endosomal accumulation of Cl\(^-\), and that this could be at the bases of the pathogenesis of Dent’s disease [65]. Moreover, CIC-5 deficiency has been associated with more general defects of protein trafficking in proximal tubule cells. Indeed, the levels of cubilin and megalin, the two multiligand receptors implicated in endocytosis, are reduced at the apical brush border of proximal tubules in CIC-5 KO mice. This contributes to LMWP and possibly to indirect downstream effects such as hyperphosphaturia and hypercalciuria, due to increased urinary levels of parathyroid hormone and vitamin D-binding protein [66, 67]. Defective exocytosis of Na\(^+\)-H\(^+\) exchanger isoform 3 (NHE3) and altered polarity of H\(^+\)-ATPase have also been reported in proximal tubules of CIC-5 KO mice [68] and in kidney biopsies of Dent’s disease patients [69], respectively. The mechanism by which the absence of CIC-5 leads to vesicular trafficking defect is still unknown. It could be correlated with the loss of key interactions of CIC-5 with components of the intracellular trafficking machinery such asSorting protein, an actin-depolymerizing protein, the Na\(^+\)-H\(^+\) exchange regulatory factor-2, Nedd-4, and the kinesin KIF3B [70].

In vitro studies of different missense CIC-5 mutations (~20% of the reported ones) have led to their classification into three main classes, according to the different functional and cell biological consequences [71]. About 60% of studied mutations belong to Class 1. They are mostly located at the interface between monomers and are predicted to affect the homodimer formation [72]. Few mutations of the same class are found away from the dimer interface and are thought to affect protein structure. Class 1 mutations lead to ER retention followed by proteasomal degradation of the mutant protein, with no induction of the UPR [73, 74]. Class 2 mutations (~20%) are mainly localized at the dimer interface. Mutant proteins are only partially retained in the ER, can acquire complex glycosylation and are present in the endosomes and at the plasma membrane. However, while the distribution of Class 2 mutants in early endosomes is normal, their plasma membrane levels are reduced due to delayed protein maturation, reduced protein stability and increased degradation. Class 3 mutations (20%) do not affect protein folding and stability, acquire complex glycosylation and are present at the plasma membrane and in the endosomes at levels comparable with the wild-type protein. However, they have reduced or no ion permeation. It is of note that these studies, providing important insights into the molecular mechanisms of CIC-5 mutations, were mostly conducted in non-polarized immortalized cell lines and in Xenopus oocytes. Since other studies already underline the different behaviour of mutant membrane proteins in non-polarized versus polarized cell models (e.g. AE1 missorting mutations [52, 75]), additional analysis in differentiated and polarized primary proximal tubule cells [76] may be needed to further characterize the trafficking effect of CIC-5 mutations.

Current treatment for Dent’s disease patients is only supportive and mainly involves increased water intake to decrease the risk of nephrolithiasis. However, no specific therapy aimed at correcting the molecular defect due to CIC-5 deficiency is available. In principle, one would expect that molecules restoring folding and stability of mutant CIC-5 could be effective in rescuing Class 1 and 2 mutants. In vitro studies have already shown that the use of the histone deacetylase (HDAC) inhibitor sodium butyrate (PBA) or low temperature conditions is not able to rescue the trafficking of Class 1 CIC-5 mutants, as opposed to what has been observed for misfolded ΔF508 CFTR [74]. These results suggest that CIC-5 mutations lead to severe protein misfolding, and that the development of pharmacological treatment able to restore proper folding will be the next challenge in the field. Molecules able to rescue CIC-5 mutations could be tested in vivo in the clinically relevant mouse models [62, 63], in vitro in murine primary proximal tubule cells [76] and possibly in immortalized proximal tubule epithelial cells from Dent’s disease patients [77].

OCRL1 is a lipid phosphatase controlling the pool of phosphatidylinositol 4,5 biphosphate (PIP\(_2\)), an intracellular messenger regulating membrane trafficking and cytoskeleton structure and function. OCRL1 is localized in the Golgi and early endosomes and may play a role in protein trafficking between these two compartments [78]. OCRL1 mutations in Dent’s disease 2 are mainly clustered in the S’ half region of the gene and hit the pleckstrin homology (exons 2–5) or the PIP-5-phosphatase (exons 9–15) domains. Nonsense, frameshift and splicing mutations, predicted to lead to premature termination, are localized in OCRL1 exons 2–7, whereas missense mutations are mainly clustered in exons 9–15 [79]. Different models have been proposed to explain the relatively mild phenotype of Dent’s disease 2 compared with Lowe syndrome, in which extra-renal symptoms affecting brain and eye are present. Dent’s missense mutations in the phosphatase domain might lead to a milder reduction in protein activity since they map to the domain surface. On the contrary, Lowe syndrome missense changes tend to
cluster in the domain core and are predicted to have a destabilizing effect [78]. Based on bioinformatics analysis, Shrimpton et al. [80] proposed that truncating mutations could be rescued in brain and eye tissues by the presence of an alternative OCRL1 isoform starting from exon 8.

DEFECTIVE DEGRADATION

Vesicular trafficking can modulate the function of plasma membrane proteins, since the rate of insertion and internalization modifies their amount at the plasma membrane (Figure 1a). Several studies have shown that ubiquitylation is an important signal for internalization and sorting of plasma membrane proteins. Its defects are associated with diseases (Figure 1d) such as Liddle syndrome and pseudohypoaldosteronism type 2 (PHAI2).

Liddle syndrome

Liddle syndrome is a rare autosomal dominant disease characterized by early onset salt-sensitive hypertension associated with low plasma renin activity, hypokalaemia and metabolic alkalosis. The disease is due to mutations (premature termination, frameshift or missense) in SCNN1B and SCNN1G genes coding for the β and γ subunit of the epithelial sodium channel (ENaC), respectively [81, 82]. ENaC is present in many epithelial cells of the body. In the kidney, it is found at the apical membrane of collecting duct principal cells. Mutations are responsible for high ENaC activity resulting in excessive sodium reabsorption in the distal nephron [83]. This increased activity is independent of the action of aldosterone, as patients have low levels of aldosterone secretion. Consistently, they are not responsive to aldosterone antagonists, but are responsive to low salt diet combined with treatment with ENaC inhibitors such as amiloride or triamterene [84]. With treatment, the prognosis is generally good. An early diagnosis is thus important to avoid cardiovascular and renal complications due to long-term undetected hypertension. Mutations are mainly located in the cytoplasmic C-terminal region and lead to its deletion or mutate a proline or tyrosine residue within a short proline-rich sequence called the PY motif (Pro-Pro-x-Tyr). This motif is highly conserved in all the ENaC subunits and acts as a binding site for NEDD4-2, an E3 ubiquitin ligase [85]. The WW domains of NEDD4-2 interact with the ENaC PY motif to induce its ubiquitylation. This process is required for ENaC endocytosis and degradation, thus reducing the number of active channels at the membrane. Mutations hence exert a gain-

![Diagram of protein folding and degradation](image)

**Figure 2:** Effect of the treatment with pharmacological chaperones or proteostasis regulators to counteract mutations affecting protein folding. In the absence of treatment, misfolded proteins are degraded or form intracellular aggregates. This not only leads to the absence of functional protein in its final compartment, but can also have a broader impact on other proteins sharing common proteostasis pathways. Pharmacological chaperones bind to their specific targets and stabilize them in a native or semi-native conformation rescuing protein trafficking and reducing degradation or aggregation. Proteostasis regulators, by acting on pathways controlling protein homeostasis as protein synthesis, folding, trafficking, aggregation and degradation (green shading), can increase the pool of proteins available for folding attempts and for pharmacological chaperones. The synergistic action of the two treatments can further increase the amount of functional protein in its physiological subcellular compartment. In some cases (not shown), functional rescue can also be obtained without restoring protein native localization, as discussed in the text for AVPR2 mutants.
of-function effect by inhibiting the binding of NEDD4-2 and consequently leading to an increased amount of active channel at the apical membrane [83]. To date, only two mutations (R563Q and N530S in the β and γ subunits, respectively) have been reported outside of the C-terminus in an extracellular loop of the ENAC subunit [86, 87]. It is also likely that these mutations lead to increased ENaC activity, although through a different mechanism. Mutation N530S was indeed shown to increase the channel open probability [87]. In order to counteract ENaC over-activation, effective treatments could be designed by developing more specific blockers. Since ENaC is activated through different proteolytic cleavages, the use of specific protease inhibitors could also be envisaged as an alternative therapeutic option [88].

**Pseudohypoaldosteronism type 2**

The importance of protein ubiquitylation in the regulation of renal Na⁺ transport has been extended by recent studies on the genetic bases of PHAII [89]. PHAII is due to increased activity of Na⁺–Cl⁻ co-transporter (NCC) at the apical membrane of the distal convoluted tubule leading to hypertensive, reduced distal Na⁺ delivery and consequent hyperkalaemia. Mutations were found in the two main regulators of NCC, the with-no-lysine kinases WNK1 and WNK4 [90]. Recently, mutations in the genes encoding the Kelch-like protein 3 (KLHL3)/Cullin 3 (CUL3) ubiquitin–ligase complex have been identified in PHAII patients [91, 92]. Such mutations result in defective NCC ubiquitylation and degradation. Interestingly, renal tubule-specific ablation of Nedd4L in the mouse results in a phenotype resembling PHAII, with increased expression and phosphorylation of NCC. This suggests that NEDD4-2 also plays a role in the regulation of NCC activity [93]. Targeting NEDD4-2 activity might thus be a possible strategy to treat hypertension in these diseases.

**PROSPECTIVE THERAPEUTIC STRATEGIES**

Identification of the genes responsible for monogenic diseases and the functional study of mutations and their consequences on the protein product are essential steps to understand disease pathophysiology, improve diagnosis and develop possible therapeutic strategies. In a growing number of genetic diseases associated with dysfunctional protein trafficking mutations affect protein folding, leading to gain- and/or loss-of-function effects. Often misfolded proteins retain their intrinsic activity, but their mutation results in disease because of protein mislocalization. For conformational diseases, the ideal therapeutic approach would be to identify small molecules that can bind to and stabilize the native state of mutant proteins, thereby increasing the level of folded protein while decreasing the amount of misfolded ones (Figure 2). Three different types of small molecules have been tested to this purpose: chemical chaperones, pharmacological chaperones and proteostasis regulators. Chemical chaperones [e.g. DMSO, trimethylamine N-oxide (TMAO), glycerol] are small molecules that do not directly bind to misfolded proteins, but stabilize them by reducing free movement and increasing hydration. Although chemical chaperones have been shown to be effective in rescuing transport and function of some misfolded proteins, as for example TMAO for ΔF508 CFTR [94], their pharmacological application seems limited by the lack of specificity and the high concentration required. This can be circumvented by the use of pharmacological chaperones, small molecules that specifically bind to a misfolded protein and can induce conformational stabilization. Their mode of action is still not well understood. These molecules could either bind and stabilize the native or native-like state of the target protein or they could bind to non-native folding intermediates, serving as a scaffold for subsequent folding attempts. Regardless of the molecular mechanism, the final effect is to increase the cellular availability of functional protein. Pharmacological chaperones bind with high affinity to their targets and they can thus be effective at low concentration. Their binding affinity should be taken into account when selecting new molecules, as binding at or near natural ligand-binding sites would require displacement of the chaperone to rescue the antagonistic effect and protein function. Due to their specificity, the use of pharmacological chaperones might fail to provide a comprehensive treatment for an individual conformational disease, as the variety of mutations could limit the application of a given molecule to a subset of mutations. However, the development of new powerful high-content screening methods could overcome this limitation [95]. Although only two pharmacological chaperones are currently approved for clinical use in conformational disorders (i.e. sapropterin dihydrochloride for phenylketonuria and tafamidis for transthyretin-related hereditary amyloidosis), several molecules are under preclinical and clinical trials [96]. Increasing interest is also given to modulators of protein homeostasis (or proteostasis). The maintenance of proteostasis is crucial for cellular and organismal health and is achieved by an integrated network of several hundred proteins. As our knowledge of these complex mechanisms steadily grows, we can identify targets and molecules to tune these processes, with the rationale that tipping the balance between protein synthesis, folding and degradation may have therapeutic relevance. Proteostasis modulators indeed act by increasing protein folding and/or enhancing protein degradation. Modulators of stress responses, as the heat shock response or the UPR, or of proteasomal or lysosomal/autophagic degradation pathways have already proved to be effective in cellular and animal models. This is the case of celastrol and guanabenz (UPR activators) in lysosomal storage diseases and in Type I diabetes, respectively, and of PBA (HDAC inhibitor) in cystic fibrosis [97]. Finally, interesting studies in cellular models of Gaucher and Tay-Sachs diseases demonstrated that combining more than one strategy, as acting on a stress response signalling pathway and using a specific pharmacological chaperone, could be the best approach to treat conformational diseases in the future [98].

Small molecules modulating protein homeostasis are being discovered at an impressive rate thanks to the development of improved screening strategies (high-throughput platforms and new compound libraries) and to the increasing knowledge of physiological and disease-associated mechanisms modulating protein folding and trafficking. The expansion of this relatively young class of pharmaceutical molecules may, in the near
future, improve current treatment or even offer treatment for yet incurable conformational diseases.

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CONFLICT OF INTEREST STATEMENT

None declared. The authors declare that the results presented in this paper have not been published previously in whole or part.

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