Endoplasmic reticulum-retained podocin mutants are massively degraded by the proteasome

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Running title: PodocinR138Q quality control and ERAD

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Keywords: genetic disease, kidney, intracellular trafficking, endoplasmic-reticulum-associated protein degradation (ERAD), proteasome, glycosylation, podocin, steroid-resistant nephrotic syndrome

ABSTRACT

Podocin is a key component of the slit diaphragm in the glomerular filtration barrier, and mutations in the podocin-encoding gene NPHS2 are a common cause of hereditary steroid-resistant nephrotic syndrome. A mutant allele encoding podocin with a p.R138Q amino acid substitution is the most frequent pathogenic variant in European and North American children, and the corresponding mutant protein is poorly expressed and retained in the endoplasmic reticulum (ER) both in vitro and in vivo. To better understand the defective trafficking and degradation of this mutant, we generated human podocyte cell lines stably expressing podocinwt or podocinR138Q. Although it has been proposed that podocin has a hairpin topology, we present evidence for podocinR138Q N-glycosylation, suggesting that most of the protein has a transmembrane topology. We find that N-glycosylated podocinR138Q has a longer half-life than non-glycosylated podocinR138Q, and that the latter is far more rapidly degraded than podocinwt. Consistent with its rapid degradation, podocinR138Q is exclusively degraded by the proteasome, whereas podocinwt is degraded by both the proteasomal and the lysosomal proteolytic machineries. In addition, we demonstrate an enhanced interaction of podocinR138Q with calnexin as the mechanism of ER retention. Calnexin knock-down enriches the podocinR138Q non-glycosylated fraction, whereas preventing exit from the calnexin cycle increases the glycosylated fraction. Altogether, we propose a model in which hairpin podocinR138Q is rapidly degraded by the proteasome, whereas transmembrane podocinR138Q degradation is delayed due to entry into the calnexin cycle.

Nephrotic syndrome (NS) is clinically characterized by proteinuria, edema, hypoalbuminemia and hyperlipidemia, and is a consequence of glomerular filtration barrier (GFB) dysfunction. The prognosis of steroid-resistant nephrotic syndrome (SRNS) is poor, with a high proportion of patients rapidly developing end-stage renal disease, requiring dialysis or transplantation (1,2). The GFB is comprised principally of podocytes, specialised epithelial cells which interdigitate at junctions known as slit diaphragms (SDs). Mutations in the NPHS2 gene, encoding the slit diaphragm (SD) protein podocin, are the most frequent monogenic cause of SRNS in childhood (3,4). Importantly, the missense mutation NPSH2: Arg138Gln (p.R138Q) accounts for 20 percent of all SRNS-causing alleles in Europe and

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North America (4), and is associated with an early-onset and rapidly progressing form of the disease (5). In accordance with the severe clinical phenotype, podocin p.R138Q (PodR138Q) is retained in the ER of podocytes and does not reach the SD, thus impairing correct functioning of the GFB (6,7).

Podocin belongs to the stomatin and prohibitin homology domain (PHB) protein family and is specifically expressed in podocytes. It has been proposed that podocin acts as a molecular scaffold for other SD proteins in lipid raft membrane subdomains. For example, podocin interacts with both nephrin and CD2AP through its carboxy (C)-terminus, and participates in various signaling events at the SD (8-10). Podocin has a predicted hairpin-loop topology, with both the amino (N)- and C-termini facing the cytoplasm, and its hydrophobic domain anchored either to the inner leaflet of the plasma membrane (PM) or to the outer leaflet of the ER-membrane (7). However, an alternative transmembrane topology has been described for stomatin and podocin, particularly when a conserved proline residue, critical for the kink of the hairpin topology, found at position 118 in podocin, is mutated, and has been linked to N-glycosylated forms of these proteins (11,12). In addition, although the stability and degradation of podocin has been associated with a short internalization motif located in its C-terminus and to an interaction with the ubiquitin ligase Ubr4 (13,14), the mechanisms of PodR138Q degradation are still unexplored. In this study, we report the degradation pathways followed by PodR138Q, which might aid to establish new therapeutic strategies.

RESULTS

PodocinR138Q is predominantly N-glycosylated - We generated two cell lines stably expressing two Human influenza hemagglutinin (HA) tags at the N-terminus of wild-type (WT) (Podwt) and PodR138Q podocin by lentiviral transduction of a human podocyte cell line. Although both cell lines expressed comparable levels of podocin mRNA (Figure 1A), we found significantly higher levels of Podwt protein in comparison to PodR138Q (Figure 1B). We then confirmed by immunofluorescence the expected subcellular localization at the PM for Podwt (co-localization with the wheat germ agglutinin, WGA) and at the ER for PodR138Q (a reticular pattern staining partially colocalizing with calnexin) (Figure 1C) (7). We demonstrated that neither Podwt nor PodR138Q stable overexpression caused ER stress as evidenced by the lack of upregulated levels of BiP protein (GRP78), an ER-chaperone widely used as an ER-stress indicator (Figure S1) (15-17). As positive controls of ER stress induction caused by misfolded mutant proteins, we generated human podocyte cell lines stably expressing V5-tagged nephrinwt (Nephwt) or nephrinS366R (NephS366R), since this latter mutant has been previously shown to induce ER stress (Figure S1A) (18).

Interestingly, we observed three specific bands for both Podwt and PodR138Q on podocin immunoblots. The fastest migrating band (lower band) was predominantly observed in Podwt protein extracts, whereas the slowest migrating band (upper band) was predominantly found in PodR138Q extracts (Figure 1B). To determine if the upper bands in the WT and mutant podocin triplets were N-glycosylated forms, we either inhibited N-glycosylation by treating cells with tunicamycin (Tm), or treated cell lysates with Peptide-N-Glycosidase F (PNGase F) to digest N-glycan groups. Upper and middle bands of the podocin triplets disappeared using both strategies (Figure 1D) allowing us to define the upper, the middle and the lower bands of the podocin triplets as double-glycosylated (dg), mono-glycosylated (mg) and non-glycosylated (ng) podocin. To test whether the difference in protein levels of Podwt and PodR138Q was due to these differential glycosylation patterns, we also created podocyte cell lines stably expressing HA-tagged Podwt and PodR138Q bearing mutations in the amino acid residues required for N-glycosylation, N199 and N355 (Podwt(N199Q,N355S) and PodR138Q(N199Q,N355S)). Immunoblots of protein extracts from these cells revealed that although the podocin triplet disappears with mutation of residues N199 and N355 (Figure S1B), glycosylation alone does not account for the differences in protein levels of Podwt and PodR138Q, as there remains increased amounts of Podwt(N199Q,N355S) compared to PodR138Q(N199Q,N355S).

Our results showed that the mutant PodR138Q was predominantly N-glycosylated, similarly to PodP118L (12), another ER-retained podocin
PodocinR138Q has a shorter half-life than Podwt and is rapidly degraded by the proteasome - To investigate whether the difference in protein levels of Podwt and PodR138Q was due to a higher degradation rate of the mutant protein, we determined the half-life (t1/2) of both proteins using a cycloheximide time course experiment, quantifying separately the glycosylated and non-glycosylated forms (Figure 2). No differences were observed between the t1/2 of the three Podwt forms, whereas a drastically reduced t1/2 was detected for ng-PodR138Q when compared to Podwt (7-fold). Consistent with this finding, we also demonstrated that the t1/2 of the glycosylation mutant PodR138Q(N199Q,N355S) was also reduced around 7 fold compared to Podwt(N199Q,N355S) (Figure S2B). Interestingly, ng-PodR138Q (i.e. hairpin-like topology) appeared to be degraded faster than the N-glycosylated forms of PodR138Q (i.e. those with transmembrane topology), implying that the hairpin-like structure is more exposed to intracellular degradative machinery (table in Figure 2 and Figure S2A). Since ng-PodR138Q is degraded very quickly, we hypothesized that the proteasome was mediating its clearance. We therefore used the reversible proteasome inhibitor bortezomib (Bz) to perform dose-response and time-course experiments (Figure 3). We observed that ng-PodR138Q accumulated in a dose-dependent manner, whereas levels of ng-Podwt increased significantly only at the highest Bz dose of 1 µM (Figure 3A). Consistent with their longer t1/2, both glycosylated Podwt and glycosylated PodR138Q were not increased at any dose of Bz after two hours (Figure 3A). Thus, we next performed an overnight time-course (16 h) using the lowest effective dose of Bz (0.1 µM) (Figure 3B). We found that only ng-PodR138Q accumulated with time, and, most interestingly, the balance between ng- and dg-PodR138Q was inverted, thus after 16 hrs the proportions of each PodR138Q band resembled the proportions of each Podwt band in podocin immunoblots (Figure 3B, colored graph). Levels of all forms of Podwt were increased only at the longest time-points. Similar findings were observed when we performed an overnight Bz time course on cells expressing the glycosylation mutants Podwt(N199Q,N355S) and PodR138Q(N199Q,N355S), with protein levels of the WT protein only significantly increased at the longest time point of 16 h, and a trend for levels of PodR138Q(N199Q,N355S) to accumulate in the presence of 0.1 µM bortezomib (Figure S3A). The increased amount of Podwt and PodR138Q after an overnight treatment with 0.1 µM Bz was confirmed by immunofluorescence analysis (Figure 3C). As expected, a sustained exposure to Bz led to ER stress, as indicated by BiP induction (Figure 3C), but to a higher extent in cells expressing PodR138Q (Figure S3B). Therefore, we concluded that the proteasome contributes to the degradation of both Podwt and PodR138Q, but more actively for ng-PodR138Q.

PodocinR138Q is not degraded by lysosomes - Since protein degradation proceeds via two major routes: the proteasomal and the autophagic-lysosomal pathways, we investigated whether lysosomal degradation contributes to Podwt and PodR138Q proteolysis. We therefore treated the cells with ammonium chloride (NH4Cl), a weak base known to inhibit lysosomal proteases. NH4Cl treatment significantly increased levels of the ng-Podwt protein fraction (Figure 4A) and levels of Podwt(N199Q,N355S), implying that lysosomal machinery is involved in Podwt degradation but not in PodR138Q proteolysis; indeed, we observed levels of both PodR138Q and PodR138Q(N199Q,N355S) to fall after a 16 h NH4Cl treatment (Figure 4A and Figure S4B). Then, by confocal microscopy, we confirmed the absence of PodR138Q in the late endosome/lysosome compartment labelled with CD63 (Figure 4B). Conversely, Podwt is predominantly present in this compartment, as already described (13). Taken together, our data suggest that Podwt is mainly degraded in lysosomes, in contrast to PodR138Q, which is exclusively degraded by the proteasome.

PodocinR138Q enhanced interaction with calnexin - Misfolded N-glycosylated proteins lo-
cated in the ER membrane are submitted to a strict quality control (QC) by the so-called calnexin (Cnx) cycle, being retained by the 90 kDa chaperone Cnx until they reach their native conformation or are otherwise sent for ER-associated degradation (ERAD). We hypothesized that if Pod\textsuperscript{R138Q} is mostly N-glycosylated and possesses a transmembrane topology, interaction with Cnx may be a mechanism of Pod\textsuperscript{R138Q} ER retention, as has already been described for some nephlin mutants (18). Immunoblots of HEK293T cells transfected with HA-tagged WT podocin and the two ER-retained mutants, Pod\textsuperscript{R138Q} and Pod\textsuperscript{R168C} (7) showed that similarly to our findings from podocyte cell lines stably expressing Pod\textsuperscript{wt} and Pod\textsuperscript{R138Q}, podocin was present as three differentially glycosylated species (Figure 5A). Co-immunoprecipitation studies revealed an interaction between Cnx and both WT and mutant podocin, with the mutant proteins interacting to a greater extent compared to the WT protein (Figure 5A). That we found Cnx also interacted with Pod\textsuperscript{wt} was not surprising, since HA immunoprecipitation also enriched a certain proportion of N-glycosylated Pod\textsuperscript{wt} (Figure 5A, upper panel). Surprisingly, ng-podocin, WT and mutants, also co-immunoprecipitated with Cnx, suggesting that hairpin podocin might interact with Cnx either indirectly, perhaps through oligomerization with the podocin transmembrane fraction via its N-terminus (19), or directly, through membrane or cytosolic domains. To test whether impairing the interaction of Pod\textsuperscript{R138Q} with Cnx would allow its ER exit and promote membrane localization, we first treated the cells with castanospermine (Cst), a specific inhibitor of glucosidases I and II that prevents the sugar trimming necessary for the recognition of the substrate protein N-glycan groups by the lectin domain of Cnx. Cst decreased Cnx interaction with Pod\textsuperscript{wt}, but not with Pod\textsuperscript{R138Q} (Figure 5B), suggesting that Pod\textsuperscript{R138Q} interaction with Cnx is N-glycan independent. Similar results were obtained when these experiments were performed using cells expressing the glycosylation mutants Pod\textsuperscript{wt}(N\textsuperscript{199Q},N\textsuperscript{355S}) and Pod\textsuperscript{R138Q}(N\textsuperscript{199Q},N\textsuperscript{355S}) (Figure 4C), supporting the idea that the interaction with Cnx is more lectin dependent for Pod\textsuperscript{wt} than for Pod\textsuperscript{R138Q}. Finally, we found that introduction of the p.N\textsuperscript{199Q} and p.N\textsuperscript{355S} into Pod\textsuperscript{R138Q} mutant in podocytes did not prevent its ER retention, implying that the suppression of the N-glycan dependent interaction with Cnx is not sufficient to bring Pod\textsuperscript{R138Q} to the PM (Figure S4A), and supporting the idea that podocin is capable of lectin-dependent binding to Cnx.

Glycosylated Pod\textsuperscript{R138Q} enters the calnexin cycle - Since we observed Pod\textsuperscript{R138Q}(N\textsuperscript{199Q},N\textsuperscript{355S}) is still able to interact with Cnx, we next knocked-down Cnx in the two stable podocyte cell lines using siRNA (Figure 6A and Figure S5A). Interestingly, we observed that levels of N-glycosylated forms of Pod\textsuperscript{R138Q} were decreased upon Cnx knock-down, in contrast to levels of ng-Pod\textsuperscript{R138Q} which were significantly increased (Figure 6A, color graph), suggesting that Cnx may play a role in stabilization of the transmembrane form of N-glycosylated Pod\textsuperscript{R138Q}, or indeed, that interaction with Cnx promotes glycosylation of Pod\textsuperscript{R138Q}. However, in support of the former statement, the total amount of Pod\textsuperscript{R138Q} was slightly decreased after Cnx knock-down, suggesting that interaction with Cnx might delay Pod\textsuperscript{R138Q} degradation (Figure 6A, lower graph). Next, we treated cells with kifunensine (Kif), a drug that inhibits the activity of alpha-mannosidase I, and thus the mannose trimming that tags Cnx substrates for ERAD (Figure 6B). Here, our results were precisely the opposite to those obtained upon Cnx knock-down, that is, levels of ng-Pod\textsuperscript{R138Q} were significantly reduced whereas levels of dg-Pod\textsuperscript{R138Q} were increased (Figure 6B, color graph). Furthermore, the total amount of Pod\textsuperscript{R138Q} was augmented after 4 h of treatment (Figure 6B, lower graph), demonstrating that Kif treatment partially prevented Pod\textsuperscript{R138Q} degradation. These last data support the idea that only N-glycosylated, and thus transmembrane Pod\textsuperscript{R138Q}, enters the Cnx cycle before being directed to ERAD. Finally, we observed by immunofluorescence that neither knock-down of Cnx nor blocking entry into the ERAD pathway with Kif were sufficient to target Pod\textsuperscript{R138Q} to the PM (data not shown). No significant changes on Pod\textsuperscript{wt} levels were observed upon Cnx knock-down, but 4 h of Kif treatment did increase levels of dg-Pod\textsuperscript{wt}, suggesting that glycosylated Pod\textsuperscript{wt} may also enter the Cnx cycle (Figure S5).

Bortezomib partially re-addresses Pod\textsuperscript{R138Q} to the plasma membrane - While studying the proteasomal degradation of Pod\textsuperscript{R138Q}, we
observed by immunofluorescence that a fraction of Pod\textsuperscript{R138Q} was localized, after Bz exposure, to thin filopodia PM protusions, similarly to Pod\textsuperscript{wt} in untreated cells, whereas Pod\textsuperscript{R138Q} was completely absent from these structures before treatment (Figure 7A). Targeting of Pod\textsuperscript{R138Q} to filopodia, quantified as the percentage of WGA-positive filopodia that were also positive for podocin, revealed that Bz treatment increased plasma membrane targeting of Pod\textsuperscript{R138Q}, as soon as 2 h after Bz treatment, although levels did not reach those of Pod\textsuperscript{wt} (Figure 7A, graph). We also observed a decreased interaction of od\textsuperscript{R138Q} with Cnx in HEK 293T cells treated with Bz, which may serve as an indicator of od\textsuperscript{R138Q} accumulation outside the ER (Figure 7B). Since we do not see Pod\textsuperscript{R138Q} at the PM (figure 7A) and the glycosylated forms of Pod\textsuperscript{R138Q} seem to be insensitive to Bz (Figure 3), we hypothesize that only ng-Pod\textsuperscript{R138Q} is reaching the PM upon Bz treatment. Taking into account all our data, we propose a schema depicting a dynamic interchange between the hairpin and the transmembrane topology at the different steps of ER QC and subsequent ERAD (Figure 7C). Briefly, hairpin Pod\textsuperscript{R138Q} is detected by the cytoplasmic QC machinery and rapidly degraded by the proteasome. In the meantime, N-glycosylated transmembrane Pod\textsuperscript{R138Q} enters the Cnx cycle to be eventually sent for ERAD. Since proteasomal degradation takes place in the cytosol, transmembrane Pod\textsuperscript{R138Q} would lose its N-glycan groups at some point between retrotranslocation and proteasomal degradation (20-22), possibly adopting a hairpin topology when exposed to the cytosol. Bz treatment would then inhibit the degradation of ng-Pod\textsuperscript{R138Q}, giving it the chance to follow the secretory pathway and reach the PM.

**DISCUSSION**

Despite p.R138Q being the most common podocin mutation causing SRNS in European and North American children (4), little is known about how this mutation affects podocin stability and degradation. In this study, we have found that human Pod\textsuperscript{R138Q}, when stably overexpressed in human podocyte cell lines, is clearly resolved as a triplet on immunoblots. The upper bands of these triplets correspond to N-glycosylated forms, which is particularly intriguing since it implies that the C-terminus of most Pod\textsuperscript{R138Q} is inside the ER lumen. N-glycosylation, and evidence of transmembrane topology, has been already described for stomatin, for the short isoform of podocin and for podocin\textsuperscript{P118L}, another ER-retained podocin mutant (11,12,23). Whereas Pro118, an amino acid which is highly conserved throughout the stomatin family, is located within the hydrophobic intramembrane region and is responsible for the kink of the hairpin topology (12), Arg138 and Arg168, which are also very well conserved, are located within the PHB domain, far away from this region. Nevertheless, we hypothesize that missense mutations in the PHB region may also destabilize podocin hairpin topology, the folding of which is already intrinsically inefficient (11). Indeed, most of the missense mutations affecting the PHB domain typically result in ER-retention (7), and only podocin mutants which are known to be retained in the ER present N-glycosylation levels comparable to Pod\textsuperscript{R138Q}. The switch in topology has important implications for the stability and the degradation of Pod\textsuperscript{R138Q}. We found the glycosylated Pod\textsuperscript{R138Q} forms to be more stable than ng-Pod\textsuperscript{R138Q}. The latter, with a hairpin structure, can be rapidly degraded by the proteasome. In contrast, we found evidence that the transmembrane isoform of Pod\textsuperscript{R138Q} enters the calcnexit cycle, which may explain its longer t\textsubscript{1/2}. Of course, it remains that glycosylation and deglycosylation events may be affecting the observed differences in stability of the different glycosylated podocin species, and undoubtedly these processes are also contributing to our results. That said, based on our experiments with Pod\textsuperscript{wt(N199Q,N355S)} and Pod\textsuperscript{R138Q(N199Q,N355S)}, which show these proteins behave in the same way as ng-Pod\textsuperscript{wt} and ng-Pod\textsuperscript{R138Q}, we believe that glycosylation of ng-podocin is not contributing to the observed increased stability of the glycosylated species, and in fact the longer t\textsubscript{1/2} of mg- and dg-Pod\textsuperscript{R138Q} are due to decreased exposure to the intracellular degradative machinery. According to our results, transmembrane Pod\textsuperscript{R138Q} would be exclusively located in the ER as an intermediary of Pod\textsuperscript{R138Q} QC and is there stabilized by interaction with Cnx. Indeed, in contrast to what has been shown for Pod\textsuperscript{P118L} (12), we did not find Pod\textsuperscript{R138Q} to be localized at the PM by immunofluorescence. This same model may apply for
misfolded Pod\textsuperscript{wt}, since it also interacts with Cnx and its glycosylated forms are enriched after Kif treatment. Nevertheless, the increased levels of Pod\textsuperscript{wt} following NH\textsubscript{4}Cl addition, together with the lack of response to Bz at short times, suggest that Pod\textsuperscript{wt} is mainly degraded in the endosome/lysosome compartment, a finding which is in accordance with results from other authors (13).

Cnx recognizes a complex code of glucose and mannose trimming in the N-glycan groups of its substrate proteins (21,24-26). The fact that Pod\textsuperscript{R138Q} topology and degradation is sensitive to mannose trimming implies that most probably there is an interaction with Cnx through its N-glycan groups. This result apparently contradicts the data obtained by the specific inhibition of the N-glycan dependent interaction of Cnx with Pod\textsuperscript{R138Q}, since the interaction with Cnx was not decreased. Nevertheless, there is an increasing amount of literature suggesting alternative sites of interaction to the lectin domain of Cnx (i.e. the Cnx transmembrane domain), especially in the case of mutant proteins (27-29). An interesting proposal is that there are at least two different types of interaction occurring sequentially or simultaneously; on carbohydrate based, through the N-glycan groups, and one peptide based, outside the Cnx lectin domain (30-32). This would explain why inhibiting lectin binding is insufficient to impair Pod\textsuperscript{R138Q} interaction with Cnx. In contrast, Cnx interaction with Pod\textsuperscript{wt} seemed to be more dependent on the N-glycan groups, even though Pod\textsuperscript{wt} is predominantly non-glycosylated. However, we cannot rule out a second Cnx interaction site also for Pod\textsuperscript{wt}, since deletion of N-glycan sites did not completely impair the interaction with Cnx and ng-Pod\textsuperscript{wt} also co-immunoprecipitated with Cnx. Finally, it is tempting to speculate that N-glycosylation and interaction with Cnx may be part of normal podocin biosynthesis and initial folding, and not only a mechanism of podocin quality control. In fact, Cnx is part of the ribosome-translocon complex and its initial association to nascent glycoproteins is usually co-translational (33,34).

The targeting of Pod\textsuperscript{R138Q} to the cell membrane using chemical chaperones as glycerol, trimethylamine-N-oxide and DMSO has already been reported in transiently transfected human podocytes (35). Nevertheless, there is still no treatment available for SRNS patients carrying the p.R138Q mutation. Here, we report the therapeutic potential of Bz (Velcade\textsuperscript{®}), a reversible inhibitor of the 26S proteasome that has been approved and successfully used in multiple myeloma therapy (36,37). Pod\textsuperscript{R140Q}, the mouse equivalent to Pod\textsuperscript{R138Q}, is poorly expressed in the glomeruli of the constitutive Nphs2\textsuperscript{R140Q/R140Q} knock-in mice (6), similarly to our findings in podocyte cell lines. By studying Pod\textsuperscript{R138Q} \textsubscript{1/2} and the degradation pathways it follows, we have found that low intracellular levels of Pod\textsuperscript{R138Q} are due to rapid proteasomal degradation. Bz not only increases Pod\textsuperscript{R138Q} protein levels, but allows a significant amount of Pod\textsuperscript{R138Q} to reach the PM, at least at filopodia. This could be simply caused by an overflow from the ER, since 2 h of Bz treatment already quadruples Pod\textsuperscript{R138Q} protein levels. Indeed, proteasomal inhibition has been found to inhibit the retrotranslocation of most ERAD substrates, thus giving them the chance to remain in the secretory pathway (21,38,39). Alternatively, since de-glycosylation by cytoplasmic peptide:N-glycanases (PNGases) is an additional step in glycoprotein ERAD (40), Bz would specifically rescue the ng- Pod\textsuperscript{R138Q} already targeted for proteasomal degradation. In support of this idea, a promising effect of Bz is that it enriches the ng-Pod\textsuperscript{R138Q} fraction corresponding to the potentially functional hairpin topology of Pod\textsuperscript{R138Q} at the SD. A successful therapy would need not only to bring Pod\textsuperscript{R138Q} to the PM, but also to promote its hairpin topology. Interestingly, Bz has been reported to rescue other misfolded mutant proteins back to the PM in vitro and in vivo (41,42). Additionally, second generation proteasomal inhibitors are currently being tested in clinical trials with less severe side effects than those of Bz, such as neurotoxicity or lymphopenia (43).

Altogether, although we lack in vivo studies, we propose that increasing ng-Pod\textsuperscript{R138Q} protein levels through the inhibition of the proteasomal degradation may be a reasonable strategy to treat patients with p.R138Q mutation and possibly with other ER retained podocin mutations.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cell Culture and Establishment of Lentiviral Cell Lines** - Human podocin-coding constructs were generated as described by Tory et
al. (44) Human wild-type \textit{NPHS1} cDNA, encoding nephrin, was amplified from the construct described by Philippe et al. (6) and subcloned into NotI and SpeI sites of LentiORF pLEX-MCS (Open Biosystems). Thus, the encoded proteins consist of podocin with two hemagglutinin tags (2HA) fused to its N-terminus, and nephrin with a V5 tag fused to its N-terminus. Site-directed mutagenesis (QuickChange kit, Stratagene) was used to generate the missense mutations used in this study: p.R138Q, p.R168C, p.N199Q, p.N355S for podocin, and p.S366R for nephrin. All constructs were verified by Sanger sequencing. A human immortalized podocyte cell line (AB8/13), obtained by transfection of the temperature-sensitive mutant tsA58 of the SV40-T-antigen-encoding gene, was kindly provided by M.A. Saleem (45). Stable podocyte cell lines were obtained by transduction of the above cell line with lentiviral vectors expressing either podocin (wt or mutants) or nephrin (wt or mutants) at a multiplicity of infection of one, and subsequently selected by puromycin (2 \( \mu \)g/ml). Podocytes were cultured at 33 \(^\circ\)C and 7 percent of CO\(_2\) in RPMI-1640 medium supplemented with 10 percent fetal bovine serum, insulin-transferrin-selenium, glutamine, and penicillin and streptomycin (all from Life Technologies). At this growth-permissive temperature, podocytes are proliferating and undifferentiated, and do not express either endogenous podocin or nephrin. HEK293T cells were maintained in DMEM medium supplemented with 10 percent fetal bovine serum, glutamine, and penicillin and streptomycin (all from Life Technologies). Cell lines used in this study were tested mycoplasma-free.

**Antibodies, enzymes and chemical compounds** - Peptide-N-Glycosidase F (PNGaseF) was purchased from New England BioLabs. Tunicamycin, cycloheximide, bortezomib, NH\(_4\)Cl, caspanspermine and kifunensine were purchased from Sigma-Aldrich. The commercial antibodies used were as follows: mouse anti-HA (HA.11 clone 16B12, Covance), mouse anti-calnexin (Cnx) (clone AF18, Enzo Life Sciences), mouse anti-CD63 (clone H5C6, DSHB) and mouse anti-calnexin (clone C5C9, Cell Signaling Technology). Rabbit anti-podocin AP-P35 (Pod) was described previously (46). Alexa Fluor 555–conjugated wheat germ agglutinin (WGA555) (W32464, Life Technologies) was used to stain the plasma membrane. Rat IgG2a (isotype control clone 2H3, MBL) for immunoprecipitation control experiments was purchased from MBL. Secondary antibodies for immunoblotting were sheep anti-mouse and donkey anti-rabbit HRP-conjugated antibodies (GE Healthcare UK). Secondary antibodies for immunofluorescence were donkey anti-rabbit and anti-mouse Alexa Fluor 488 and 647-conjugated antibodies (Life Sciences).

**siRNA Experiments** - The following siRNAs were used to transfect podocyte cell lines stably expressing podocin to specifically knock-down the human CNX (calnexin) gene: siRNA-1: 5’-AAGACGAUACCGAUGAUGAAA-3’ and siRNA-2: 5’-AAUGUGGGUGUGCCUAUGUGA-3’. siRNA against luciferase gene (Luc, 5’-GCCAUUCUAUCCUCUAGAGGAUG-3’) was used as a siRNA control. siRNAs were transfected using Lipofectamine® RNAiMAX Reagent (Invitrogen) at a concentration of 20 nM and the efficacy of siRNA was tested 3 days after transfection.

**Quantitative Real-Time PCR** - Total RNA was extracted from podocyte cell lines using the Qiagen extraction RNeasy® kit (Qiagen) and treated with DNase I. One \( \mu \)g of total RNA was reverse-transcribed using Superscript II according to the manufacturer’s protocol (Invitrogen). Relative expression levels of the studied mRNAs were determined by quantitative real-time PCR using Absolute SYBR Green ROX Mix (ABgene) with the following specific primers for human NPHS2: forward (929F) 5’-GGCTGAAGCGCAAAGACAAG-3’ and reverse (988R) 5’-GGCGTGAAGCCGAAGACAG-3’. Human hypoxanthine phosphoribosyltransferase (Hprt) was used as an internal standard. Data were analyzed according to the method described in Livak et al. (47).

**Immunofluorescence** - Podocytes were cultured on type I collagen-coated coverslips and either transiently transfected using FuGENE® HD (Promega) or treated with different drugs. When appropriate, cells were washed once with cold PBS, incubated with Alexa Fluor 555– conjugated WGA (1:600) for 15 min at 4\(^\circ\)C and then fixed with ice-cold ethanol for 5 min. Fixed cells were blocked with 1X PBS/1 percent BSA for 30 min before incubation with mouse anti-HA primary antibodies (1:500) or rabbit anti-podocin AP-P35 (1:300) when combined
with mouse anti-calnexin (1:500) or mouse anti-CD63 (1:200), followed by Alexa Fluor 488 or 647–conjugated secondary antibodies (1:200). Confocal images were captured using a 40x oil objective attached to a Leica SP8 confocal microscope. At least five random fields, with the only condition that filopodia were well visualized in the WGA channel, were considered for the quantification of the percentage area of WGA colocalization with podocin using ImageJ 1.48i software. Masks were carefully created to specifically quantify the labeling of the filopodia. Confocal settings and Image J thresholds were kept the same.

**Immunoblotting**- Proteins from podocyte cell lines were extracted in lysis buffer containing 150mM NaCl, 50mM Tris-HCL pH7, 0.5 percent of Triton-X100 with CompleteTM protease inhibitors (Roche). Protein dosage was then performed using the BCA protein assay kit (Thermo Scientific). Fifty micrograms of protein were loaded on a 10 percent SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5 percent skimmed milk in 1X Tris Buffered Saline/ 0.1 percent Tween-20 (TBST) for 1 hour and incubated with primary antibodies at a 1:1000 dilution. After washing, membranes were incubated with HRP-conjugated secondary antibodies diluted 1:10,000 in TBST/ 5 percent milk for 1 hour at room temperature. Signals were detected using ECL reagents (Amersham) and acquired in a Fusion Fx7 darkroom (Vilber Lourmat). Densitometry quantification was performed using Bio-1D software.

**Immunoprecipitation**- HEK293T cells were transiently transfected with HA-tagged wild-type (wt) podocin and mutants using calcium phosphate. Forty-eight hours post-transfection, cells were lysed in 150mM NaCl, 25mM Tris-HCL pH8, 0.5 percent Triton with protease inhibitors and HA-tagged podocin was immunoprecipitated using the μMACS Epitope Tag Protein Isolation Kit (Miltenyi Biotec). Briefly, fresh lysates (1-1.5 mg of protein) were incubated either with mouse anti-calnexin antibodies, followed by a 30-minutes incubation with magnetic beads-coupled to protein A, or directly with magnetic beads coupled to a HA antibody. A rat IgG2a isotype control was included to discard unspecific interactions. Immunoprecipitated proteins were isolated using μMACS Separation Columns in a magnetic μMACS separator and subsequently eluted with 1X Laemmli buffer. Lysates and immunoprecipitated samples were subjected to immunoblot.

**Statistical analyses** - All immunoblots were normalized to beta-actin and then to the corresponding control group or to immunoprecipitated protein in the control condition. Statistical analysis of at least three independent experiments was done using the one-sample two-tailed t-test (48) or a one-way ANOVA. P values: * P < 0,05; ** P < 0,01, *** P <0,001. GraphPad Prism 5 software was used to perform all statistical analyses (mean ± SD).

**Acknowledgments:** We thank Ana Maria Cuervo (Albert Einstein Colleg, NY) for helpful discussion and advices; Gisèle Froment, Didier Nègre and Caroline Costa from the lentivectors production facility / SFR BioSciences Gerland - Lyon Sud (UMS3444/US8) and the Confocal Platform at Imagine Institute.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

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**FOOTNOTES**

This work was supported by the European Union’s Seventh Framework Programme (FP7/2007-2013/n°305608-EURenOmics to C. Antignac), by State funding from the Agence Nationale de la Recherche under “Investissements d’avenir” program (ANR-10-IAHU-01 to C. Antignac) and by the Association des Malades d’un Syndrome Néphrotique (AMSN) (Programme Ambition Recherche 2008-2011 to G. Mollet).

The abbreviations used are: NS, nephrotic syndrome; GFB, glomerular filtration barrier; SRNS, steroid-resistant nephrotic syndrome; SD, slit diaphragm; PHB, prohibitin homology domain; ER, endoplasmic reticulum; HA, Human influenza hemagglutinin; wt, wild-type; WGA, wheat germ agglutinin; PNGase F, Peptide-N-Glycosidase F; Bz, bortezomib; QC, quality control; ERAD, endoplasmic reticulum-associated degradation; Cnx, calnexin; CHX, cycloheximide; Tm, tunicamycin; PM, plasma membrane; Cst, castanospermine; HEK293T, human embryonic kidney cells; Kif, kifunensine; DMSO, dimethylsulfoxide; dg, double-glycosylated; mg, mono- glycosylated; ng, non-glycosylated.

**FIGURE LEGENDS**

Figure 1. Stably over-expressed podocinR138Q is largely N-glycosylated, a feature shared only with other ER-retained podocin mutants. qRT-PCR (A), Western blot (B, D and E) and immunofluorescence analysis (C) comparing podocin expression levels, protein band distribution and subcellular localization, respectively,
Podocin\textsuperscript{R138Q} quality control and ERAD

in 2HA-Pod\textsuperscript{wt} and 2HA-Pod\textsuperscript{R138Q} expressing podocyte cell lines. (B) ng: non-glycosylated podocin; mg: mono-glycosylated podocin; dg: double-glycosylated podocin. Quantification of total podocin (ng, mg and dg) from three independent experiments are shown as mean with SD. *P <0.05. HA monoclonal antibody was used to identify podocin and beta-actin served as loading control. (C) Polyclonal anti-podocin AP-P35 (Pod) and monoclonal calnexin AF18 (Cnx) were used as primary antibodies. Cell membrane and nuclei were labeled with WGA and Hoechst (H), respectively. Scale bar = 30 \mu m. (D) Cells were treated overnight with 10 \mu g/ml tunicamycin (Tm) to impair N-glycosylation (upper immunoblot). Alternatively, cell lysates were treated with PNGase F, an enzyme that digests N-glycans from glycoproteins (lower immunoblot). Polyclonal AP-P35 or monoclonal anti-HA were used to immunoblot podocin. (E) Immunoblots of different podocin mutants known to possess different subcellular localizations. ER: endoplasmic reticulum-retained podocin mutants; V: vesicular podocin mutants; PM: plasma membrane localized podocin mutants.

Figure 2. Podocin\textsuperscript{R138Q} has a short half-life. Immunoblot analysis of the time-course of podocin degradation after inhibition of protein synthesis with cycloheximide (CHX) (25 \mu M) in podocyte cell lines. Densitometry data from three independent experiments are represented as mean with SD. Half-lives were estimated by fitting a one-phase exponential decay curve to the data, as in the graph shown for ng-Pod\textsuperscript{wt} and ng-Pod\textsuperscript{R138Q}, and are summarized in the table.

Figure 3. Podocin\textsuperscript{R138Q} is degraded by the proteasome. Podocyte cell lines were treated with the proteasome inhibitor bortezomib (Bz) or vehicle (DMSO) and processed for immunoblot (A and B) or immunofluorescence analysis of podocin (C). (A) Bz dose- response (0.02 to 1 \mu M) after 2 hours of treatment. (B) Time-course of podocin after addition of 0.1 \mu M Bz. (A and B) Podocin was identified using AP-P35 (Pod) primary antibody. BiP analysis was included to track ER stress (B). Quantification of ng bands is shown in the lower graphs in (A) and in the middle graphs in (B). Color graphs in (B) represent the percentage amount of each podocin band relative to total podocin. Quantitative results are shown as mean with SD (n=3). Asterisks refer to non-stimulated cells (ns or 0 h). *P <0.05 and **P <0.01. (C) Podocin was detected by incubation with monoclonal HA primary antibody (in green). Hoechst nuclei labeling was included (in blue). All images were taken using the same confocal microscope settings to allow comparison of the intensity of fluorescence. Scale bar = 40 \mu m.

Figure 4. Podocin\textsuperscript{R138Q} is not degraded in the lysosomal compartment. (A) Time-course immunoblot analysis of podocin content after impairment of lysosomal degradation with NH\textsubscript{4}Cl (50 mM). The polyclonal antibody AP-P35 (Pod) was used to immunolabel podocin. Graphs show densitometry quantification of ng-podocin in three independent experiments. Data were normalized to beta-actin and then to non-treated cells (0 h). Asterisks refer to 0 h. **P <0.01. (B) Double immunofluorescence staining of podocin (AP-P35; green) and the lysosome/late endosome marker CD63 (cyan). Nuclei labeling by Hoechst is included (blue). Scale bar = 10 \mu m.

Figure 5. Podocin\textsuperscript{R138Q} has an enhanced interaction with calnexin. (A-C) Co-immunoprecipitation analyses of podocin and calnexin (Cnx) in HEK293T cells. (A) Co-immunoprecipitation of HA-tagged Pod\textsuperscript{wt}, Pod\textsuperscript{R138Q}, Pod\textsuperscript{R168C} or an empty HA lentiviral vector (pLenti-HA) with Cnx. (B) Cells were treated with the glucosidase I and II inhibitor castanospermine (Cst; 500\mu M, 16 h) before performing HA-immunoprecipitation to study the lectin-dependent interaction of Cnx with Pod\textsuperscript{wt} and Pod\textsuperscript{R138Q}. (C) Co-immunoprecipitation of Cnx with podocin in cells overexpressing HA-tagged Pod\textsuperscript{wt} and Pod\textsuperscript{R138Q} with or without mutated N-glycosylation sites N199 and N355. (A-C) Monoclonal anti-HA and anti-Cnx AF18 were used to identify podocin and Cnx, respectively. Graphs represent the densitometry quantification of Cnx when podocin is immunoprecipitated (IP HA). Data are normalized to total immunoprecipitated podocin (HA triplet in IP HA) and represent at least three independent experiments. *P <0.05.
Figure 6. Podocin R$^{138}$Q enters the calnexin cycle. Immunoblot analysis of podocin (HA) after Cnx knock-down (siCnx) (A) or impairment of Cnx cycle exit through the inhibition of alpha-mannosidase I with kifunensine (Kif) (B) in HA WT and R138Q stably expressing podocyte cell lines. (A) Two siRNA oligonucleotides against Cnx were tested, alone (siCnx (1) and siCnx (2)) or in combination (siCnx (1+2)). Luciferase siRNA (siLuc) served as control. Cnx protein amount was quantified to confirm Cnx knock-down (upper left graph). (B) Effect of kifunensine on podocin protein content. ns: non-stimulated cells. (A and B) Podocin was quantified as the relative percentage amount of each band within the triplet (color graph) or as total protein (the three bands altogether; lower graph). *P <0.05 and **P <0.01.

Figure 7. Bortezomib partially re-addresses podocin R$^{138}$Q to the plasma membrane. (A) Immunofluorescence analysis showing Pod$^{R^{138}Q}$ (green) presence at filopodia, through colocalization with the PM marker WGA (red), after a 2 h treatment with bortezomib (Bz; 0.1 µM) or vehicle (DMSO). Pod$^{WT}$ co-localization with WGA is included as a positive control. Hoechst nuclei labeling (H) is also shown (blue). Scale bar = 10 µm. Image analysis of the WGA co-localization with podocin only at filopodia was obtained through the quantification of Regions Of Interest (ROIs) that carefully delimit cell perimeters. Graph corresponds to the quantification of one representative experiment. One-way ANOVA analysis of variance followed by Dunnet’s post-test was used as statistical analysis. **P <0.01 and ***P <0.001. (B) Co-immunoprecipitation (IP HA) of HA-podocin and calnexin (Cnx) after Bz addition (16 h at 0.1 µM). Graph represents the densitometry quantification of Cnx when podocin is immunoprecipitated. Data are normalized to total immunoprecipitated podocin (HA triplet) and represent at least three independent experiments. ***P <0.001. (C) Schematic summarizing the influence of different treatments on Pod$^{R^{138}Q}$ topology and subcellular localization. Red and green arrows indicate a dynamic change to the transmembrane “wrong” or hairpin “right” topology, respectively. Numbers circled in yellow: (1) there is an imbalance of hairpin Pod$^{R^{138}Q}$ towards a transmembrane topology, (2) decreasing Pod$^{R^{138}Q}$ interaction with Cnx through siCnx transfection favors the hairpin topology, (3) stabilization of Pod$^{R^{138}Q}$ interaction with Cnx, through inhibition of Cnx cycle exit with Kif, enhances podocin transmembrane topology and (4) inhibition of Pod$^{R^{138}Q}$ proteasomal degradation with Bz increases the proportion of hairpin Pod$^{R^{138}Q}$ and is the only treatment that allows partial relocalization to the PM. EC: extracellular matrix; PM: plasma membrane, C: cytosol, ERAD: endoplasmic-reticulum associated degradation, Kif: kifunensin.
Figure 2

Pod<sup>wt</sup>

| Time (h) | 0  | 0.5 | 1  | 2  | 4  | 8  | 16 |
|----------|----|-----|----|----|----|----|----|
| CHX      |    |     |    |    |    |    |    |

| Protein  | dg | mg | ng |
|----------|----|----|----|
| HA       |    |    |    |
| Actin    |    |    |    |

Pod<sup>R138Q</sup>

| Time (h) | 0  | 0.5 | 1  | 2  | 4  | 8  | 16 |
|----------|----|-----|----|----|----|----|----|
| CHX      |    |     |    |    |    |    |    |

| Protein  | dg | mg | ng |
|----------|----|----|----|
| HA       |    |    |    |
| Actin    |    |    |    |

Relative Podocin Amount (% Protein)

| Time (h) | 0  | 2  | 4  | 6  | 8  | 10 | 12 | 14 | 16 |
|----------|----|----|----|----|----|----|----|----|----|
| ng-Pod<sup>wt</sup> |    |    |    |    |    |    |    |    |    |
| ng Pod<sup>R138Q</sup> |    |    |    |    |    |    |    |    |    |

Table:

|                  | Pod<sup>wt</sup> | Pod<sup>R138Q</sup> |
|------------------|------------------|---------------------|
| ng               | 3.6              | 0.5                 |
| mg               | 3                | 1.7                 |
| dg               | 3.6              | 2.5                 |
Endoplasmic reticulum-retained podocin mutants are massively degraded by the proteasome
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J. Biol. Chem. published online January 30, 2018

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