Final report

Task 1. Identification of a novel gene implicated in steroid-resistant nephrotic syndrome

This work is currently under publication (not yet submitted) and therefore presented here in detail

We formerly identified an apparently **X-linked undescribed disorder in a four-generation family**. Affected males develop steroid-resistant nephrotic syndrome with cataract, hearing impairment, enteropathy, failure to thrive and bone marrow failure within the first years of life. All six affected males (in three generations) died before the age of 8 years, typically due to opportunistic infections. Females develop only cataract and hearing impairment during the second decade of life. By linkage analysis we identified the **causal locus (5.1Mb) at the telomeric end of the X long arm**, presuming that the mutation led to germline mosaicism in the first generation. Indeed, by sequencing the locus-specific coding regions of an unaffected and an affected haploidentical family member, we found a single **de novo mutation in *DKC1* (p.E206K)**. We considered this variant to be causal, as it segregated with the disorder even among the six haploidentical members of the IInd generation corresponding to germline mosaicism, affected a glutamine conserved in the Archaea, and the resulting change to lysine was predicted to be pathogenic and absent in both databases and in 555 alleles of 368 ethnically matched controls.

Dyskerin, the enzyme encoded by *DKC1*, functions in concert with three other protein members of a ribonucleoprotein complex, NOP10, NHP2 and GAR1 [1]. Two functions are attributed to them: telomere maintenance and pseudouridylation of RNAs. Mutations of *DKC1* are the most frequent causes of two bone marrow failure syndromes: dyskeratosis congenita (DKC) characterized by dyskeratosis, leukoplakia and nail dystrophy [2] and Hoyeraal-Hreidarsson (HH) syndrome characterized by in utero growth retardation, microcephaly, cerebellar hypoplasia, ataxia and developmental delay [3]. These disorders are related to telomere shortening: mutations of *TERT*, *TERC* and *TINF2*, implicated in telomere maintenance but not in pseudouridylation also result in DKC or HH syndromes [4-7]. Apart from bone marrow failure and diarrhoea we found **no overlap between the p.E206K-associated phenotype and the DKC or HH syndromes**. Furthermore, in contrast to the X-linked recessive transmission of *DKC1*-linked DKC and HH syndromes [8], females with p.E206K develop cataract and hearing impairment. The **clearly distinct phenotype** prompted us to examine the specific effect of the p.E206K mutation. DKC and HH syndromes are caused by 5' or 3' DKC1 mutations located partially in the PUA domain, implicated in the binding of (the RNA component of the complex,) the H/ACA guide RNA [9]. In contrast, **the p.E206K mutation affected the central catalytic site of pseudouridylation**, a region also implicated in the interaction with NOP10.

We first investigated the effect of the mutation on the localization of dyskerin. We generated dyskerin mutants by site-directed mutagenesis and studied in parallel the localization of A353V dyskerin, the most common mutant leading to dyskeratosis congenita. The wt and the two mutant forms **had a similar nuclear localization** (Fig. 1).

To assess the effect of the mutation on the NOP10-dyskerin interaction, we expressed V5-tagged wt NOP10 and Flag-tagged wt and mutant dyskerin separately in HEK293 cells, extracted them by immunoprecipitation and, in collaboration with Gusztáv Schay at the Department of Biophysics and Radiation Biology, Semmelweis University, studied the NOP10-dyskerin interaction by pressure-tuning fluorescence spectroscopy. We found the mutation to significantly **increase the strength of interaction**.

Next, we aimed to assess whether a telomer maintenance- or a pseudouridylation defect is responsible for the disorder. No affected boy was alive. The index girl, treated at the Ist Department of Pediatrics, is almost as severely affected as the males: she has been bearing a hearing device since the age of 1 year, has microophthalmia, was operated with cataract at the age of 5 years, and progressed to end-stage renal disease secondary to nephrotic syndrome at the age of 6 years. We found her severe phenotype to be explained by **a highly skewed X inactivation (90:10)**. We studied her telomere length by flow cytometry and FISH and found it to be similar to that of five age-matched controls (Fig. 2). This was in accordance with the lack of dyskeratosis congenital signs and symptoms (nail dystrophy, leukoplakia,

dyskeratosis) in the affected family members. This suggested that **telomere shortening does not play a role in this disorder**.

Furthermore, **we generated four mutant zebrafish lines by CRISPR/Cas9** system in collaboration with the group of Máté Varga, at the Genetic Department of Eötvös Loránd University. These mutants harboured inframe (n=2) or out-of-frame (n=2) indels. Despite several attempts we did not succeed to induce homologous recombination and to generate a knock-in line carrying the corresponding E201K mutation. Other zebrafish laboratories in Harvard or in the Imagine Institute, Paris, failed to generate knock-in models either (personal communication), indicating a general failure of homologous recombination induction in zebrafish. Nevertheless, the *dkc1* -/- embryos developed a phenotype reminiscent of the human phenotype after 36hpf with microophthalmia, cataract, inner ear and gut hypoplasia and died at 5dpf. We found an increased trans-endothelial filtration of FITC-dextran, recapitulating the nephrotic syndrome phenotype either. While wt human DKC1 mRNA rescued the phenotype, **the E206K mutant did not: no embryo survived beyond 5dpf.** This indicated that the **E206K mutation is a loss-of-function mutation**. In accordance with our findings in the affected girl, we found no telomere shortening in the *dkc1* -/- embryos at 4 dpf by flow-FISH. However, we found the **pseudouridylation of the 18S rRNA to be decreased by immuno-Northern and a decreased 18S/28S rRNA ratio.** The immuno-Northern was greatly helped by Dániel Silhavy from the Agricultural Biotechnology Center, Gödöllő. In accordance with the potential causality of the ribosomal dysgenesis, the zebrafish phenotype was recapitulated by cycloheximide treatment. **We thus suggest that ribosomal dysgenesis secondary to a decreased pseudouridylation of the 18S rRNA** and not telomere shortening **is responsible for this novel phenotype**. We are currently writing the article about these results, which are currently confidential.

Figure 1. Localization of Flag-tagged wt and mutant dyskerin in cultured podocytes. All the mutant and wt dyskerin showed a similar nucleolar localization.

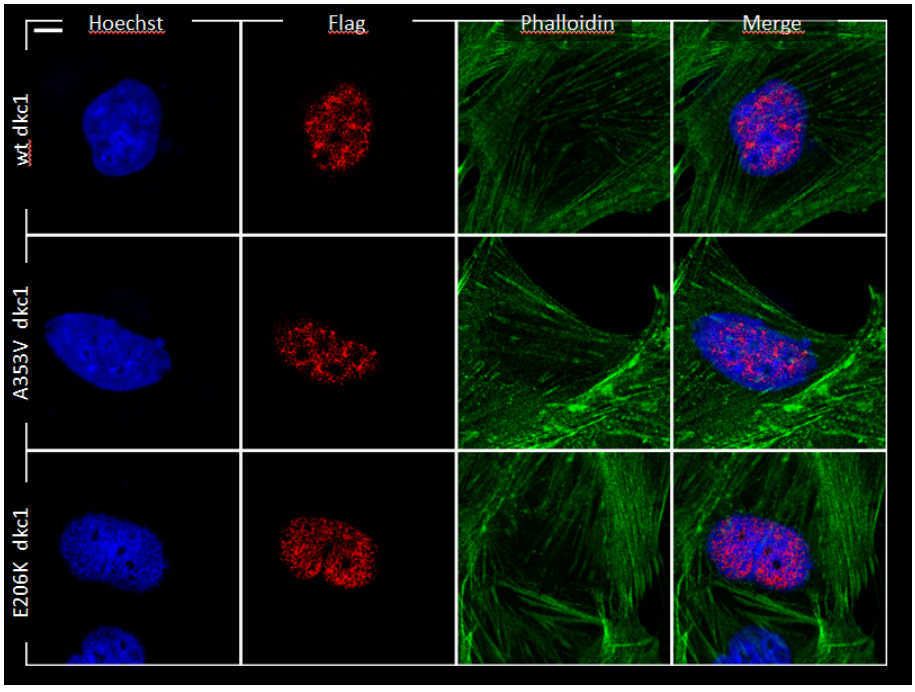


Figure 2. Telomer length of the index girl and five age-matched controls, two of whom suffered from steroid-resistant nephrotic syndrome. No difference was found.

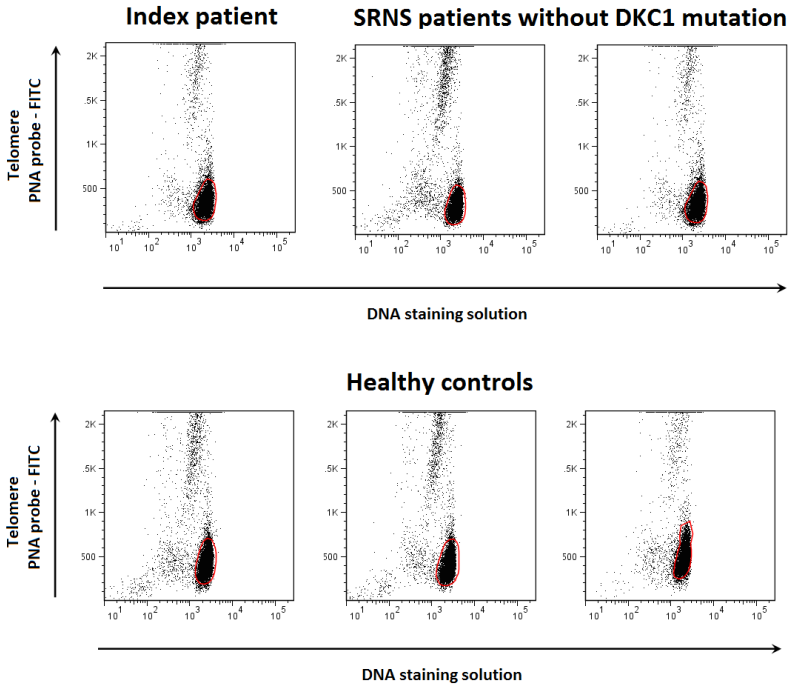
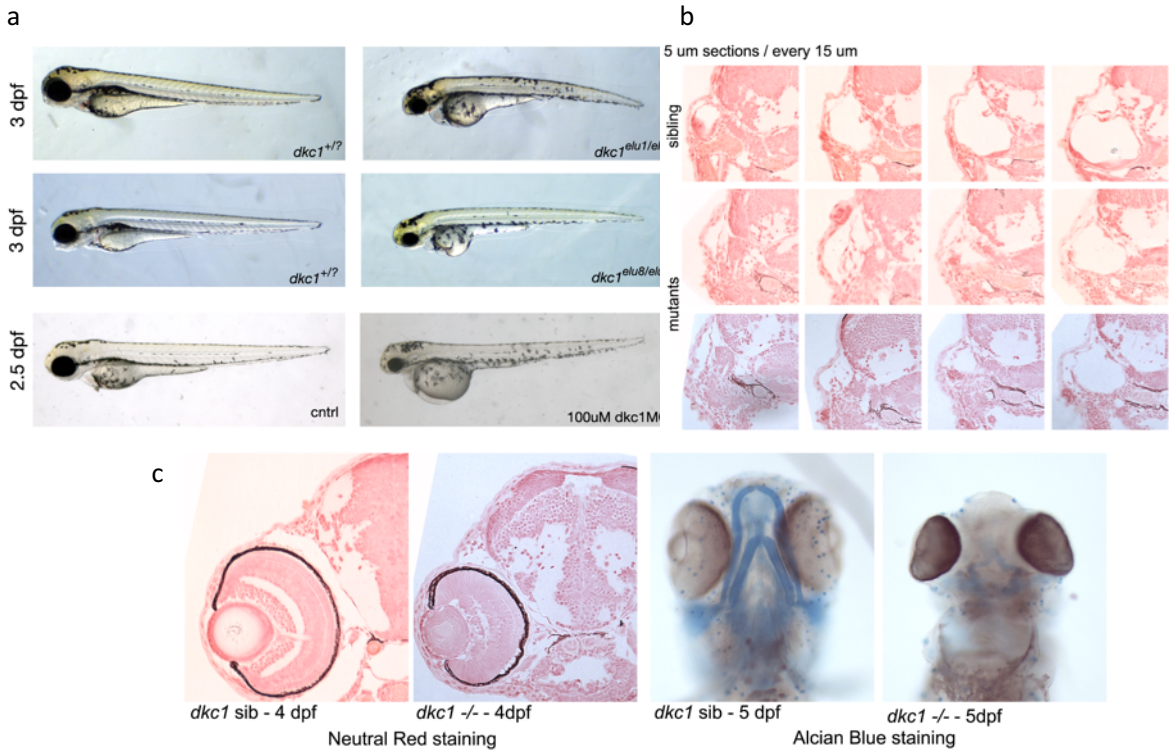


Figure 3. The *dkc1-/-* mutant zebrafish recapitulates the human phenotype with microophthalmia (a,c), inner ear hypoplasia (b) and cataract (c)



Task 2. Pathogenicity of *NPHS2* R229Q: the first variant in an autosomal recessive disorder, the pathogenicity of which depends on the trans-associated mutation

The most frequently mutated gene in hereditary (autosomal recessive) nephrotic syndrome is *NPHS2* [10]. It encodes podocin, a membrane-anchored component of the slit diaphragm. We aimed to understand the pathogenicity of its most frequent missense variant: the c.686G>A, p.R229Q. Its allele frequency in the European population is 3.6% (http://gnomad.broadinstitute.org). It has been considered to be a hypomorphic variant, that is pathogenic when trans-associated to *NPHS2* mutations [11-14]. Due to its high frequency, this variant had a special importance in the mutation screening: screening of this variant was the first step in the genetic diagnostic procedure in late-onset steroid-resistant nephrotic syndrome. Furthermore, spouses of *NPHS2* mutation carriers were advised to be screened for this relatively frequent polymorphism (carried by 1/14 individuals in Europe), to assess the risk of the couple of having an affected child. Its pathogenicity was in question only in the homozygous state.

We first showed that homozygous R229Q is not pathogenic in itself: we identified the first family in the literature with both affected and unaffected family members with homozygous R229Q. In contrast to all previous reports we carried on the mutation screening in second loci in the affected family member and identified the causal, de novo mutation in a second gene (*PAX2*, c.76dupG, p.V26Gfs*28). Furthermore, we showed that the unaffected family members with homozygous R229Q do not even have proteinuria at 40 and 60 years of age [15]. Thus, identification of homozygous R229Q in some patients is just a coincidence.

We next **questioned the pathogenicity of R229Q when trans-associated to *NPHS2* mutations** ([R229Q];[mutation]) on an epidemiological basis [11-14]. As the expected number of patients with [R229Q];[mutation] exceeded 100x the number of observed (published) patients in the medical literature, we reasoned that in the majority of the cases the [R229Q];[mutation] may not be pathogenic, in contrast to the general opinion. To prove this hypothesis, we needed to identify unaffected individuals with [R229Q];[mutation] in the general population. We therefore screened the obligate mutation carrier parents of affected children with *NPHS2* mutations for R229Q, and indeed, **found 6/129 healthy parents to carry [R229Q];[mutation]**, corresponding to the allele frequency of R229Q. Thus, we proved that the [R229Q];[mutation] is incompletely penetrant.

To understand the reason for the incomplete penetrance, we subsequently reasoned the potential role of the trans-associated mutation. Indeed, we found that while mutations of the two last (7-8.) exons are relatively rare (75/494, 15%) among the *NPHS2* mutations, these mutations were associated to R229Q in the vast majority of the affected individuals (63/71, 89%, $p=1.2x10^{-35}$). This showed that the associated mutations have a crucial role in determining the pathogenicity of **R229Q**, which **is only pathogenic when associated to specific 3' mutations**. All these 3' mutations led to amino acid substitutions in the C-terminal part of podocin. We expected them to exert a dominant negative effect on R229Q. To understand this effect, I moved to the laboratory of Hereditary Kidney Disorders in INSERM U983, Paris (Institut Imagine), where we studied the localization of R229Q podocin in function of the coexpressed podocin variant. We found R229Q podocin to be retained in cytoplasmic compartments when coexpressed in pathogenic associations and to be perfectly membranous in non-pathogenic associations. We also showed that the endogenous podocin in a Hungarian patient with a pathogenic association ([R229Q];[A284V]) is retained in podocytes cultured from urine. These results showed that **the dominant negative effect of 3' mutations is exerted through the blocking of membrane targeting of R229Q podocin**.

To understand the molecular basis of the dominant negative effect, Dóra K. Menyhárd in the MTA-ELTE Protein Modeling Research Group led by András Perczel modeled the podocin dimerization. According to their structural model, the dominant negative mutations as well as R229Q affect the structure of the C-terminal helical regions implicated in dimerization. **The pathogenic heterodimers have an altered overall structure** in contrast to the non-pathogenic ones.

We thus described the first variant in human genetics, the pathogenicity of which depends on the trans-associated mutation. The pathogenic associations result in an altered dimerization and thus block the membrane targeting of R229Q podocin. This has direct clinical relevance for several reasons:

1. The transmission pattern of mutation carriers may be dramatically different from the classical autosomal recessive transmission, leading us to introduce the '**mutation-dependent recessive inheritance**' term. According to this transmission, a couple of two affected and that of two unaffected individuals may similarly have 50% risk of having an affected child (in special genotype-associations).
2. Most of the spouses of *NPHS2* mutation carriers do not have to be screened for R229Q, as more than 90% of the *NPHS2* mutations are not pathogenic with R229Q. Accordingly, I had to call back several families with *NPHS2* (non-dominant) mutation carriers and patients to the genetic outpatient clinic and reinform them about this good news: **they are not at risk of having an affected child even if they marry an R229Q carrier**.
3. Given the high allele frequency of R229Q, patients with non-pathogenic [R229Q];[mutation] associations may be accidentally identified in patients with immune-mediated nephrotic syndrome. Accordingly, more than a dozen patients with non-pathogenic associations have been accidentally identified and reported in the medical literature. These patients were erroneously considered to suffer from a primary podocytopathy and were not treated with immunosuppressive agents. Based on our results, **these patients can now be properly treated**.
4. Interallelic interactions influencing the degree of pathogenicity of trans-associated pathogenic variants have been described in enzymopathies [16, 17]. The interactions of *NPHS2* are nevertheless the first in an autosomal recessive disorder which act through the membrane-trafficking and **can invert the non-pathogenic nature of a variant**. This observation pointed thus on a novel **mechanism how frequent polymorphisms can lead to rare monogenic disorders**, modifying the bioinformatical evaluation of sequence variants.

We presented these results at several national and international congresses (Kidney Week: congress of the American Society of Nephrology, Atlanta, 2013, plenary lecture at the Assises de Génétiques, Bordeaux, 2014, plenary lecture at the European Society of Paediatric Clinical Research congress in Budapest, 2015) and published in Nature Genetics [18].

Our subsequent experiments related to Task 2 are currently under publication (under review in PLOS Biology) and are therefore **presented here in detail**.

We subsequently set up a method in cooperation with the Department of Biophysics and Radiation Biology, Semmelweis University to assess the podocin heterooligomerization by measuring FRET efficiency in pairs of podocin mutants. This work has been co-funded by the MTA-SE Lendulet Research grant. We expressed six podocin mutants in HEK293 cells, extracted them by immunoprecipitation, stained with fluorescent (donor and acceptor) maleimides and, in the Department of Biophysics, measured FRET efficiency in the heterooligomers. Among the six podocin mutants we studied four different truncating mutants which all led to premature stop codon in the last exon (thus none of them leads to nonsense-mediated RNA decay), but differed in the modular build-up of their C-terminal tail domain: they lacked one, two or all three C-terminal helical regions. We expected these helical regions to play a primary role in dimerization (Fig. 1). Indeed, we showed by measuring FRET efficiency that the R286Tfs*17 podocin mutant, lacking all three helical regions, is unable to dimerize (Fig. 1). This **proved the exclusive role of the C-terminal tail in the dimerization**. This monomer-forming mutant is however membrane-localized and clinically hypomorphic, indicating that **lack of dimerization does not abolish neither the membrane-targeting, nor the function of podocin**. We also **proved the principal role of the oligomerization sites in mediating interallelic interactions**: while the monomer-forming podocin mutant (R286Tfs*17 podocin) remains membranous irrespective of the coexpressed podocin variant identity, all the other podocin variants with an intact H1 helical region (Fig. 1) significantly influenced each other's localization (r^2=0.68, P = 9.2 x 10^{-32}, Fig. 2 and 3).

Furthermore, we showed that a C-terminal **truncation affecting the third helical region induces internalization** (Fig. 4). Excitingly, these mutants became membranous when coexpressed with membranous podocin variants with an intact CTT. As such, the coexpression of R238S podocin prevented the endocytosis of F344Lfs*4 podocin providing the first example of a complementation between two pathogenic *NPHS2* mutations. It therefore seems to be **sufficient for one component of**

the oligomer to contain an intact C-terminal to prevent endocytosis, and besides the dominant negative effect, **interallelic complementation can also be mediated by podocin oligomerization**.

Figure 1. Podocin dimerizes through the C-terminal tail

a-b) Secondary structure prediction for the C-terminal fragment of stomatin (a) and podocin (b). Wild type C-terminal residues from E210 of stomatin and E264 of podocin are marked according to their loop- (blue), strand- (red) or helix-forming (green) propensity. Three helical regions are predicted in both stomatin (H1: 210-244, H2: 245-260, H3: 273-281) and podocin (H1: 283-313, H2: 317-330 and H3: 344-350).

c) Sequence of the studied truncated podocin mutants. The altered sequences following the frameshift sites are framed.

d-e) Förster resonance energy transfer (FRET) efficiency in the oligomers of wt (d) or R229Q podocin (e) and the truncated podocin variants. Wt (d) and R229Q podocin (e) are labeled with the donor dye, the second component by the acceptor dye. As compared with the F344* oligomers, the FRET efficiency increased in the non-pathogenic F344Lfs*4-wt oligomer ($P = 0.0003$) and decreased in the pathogenic F344Lfs*4-R229Q oligomer ($P = 0.024$), showing a different effect of the FDL344_346LTY substitutions on their conformation.

*$P \leq 0.0003$ as compared with the wt-negative control, $^{\#}P \leq 0.006$ as compared with the wt-wt homodimer, $^{\dagger}P = 0.0002$ as compared with the R229Q-negative control, $^{\S}P \leq 0.0003$ as compared with the R229Q-wt dimer, neg.: negative

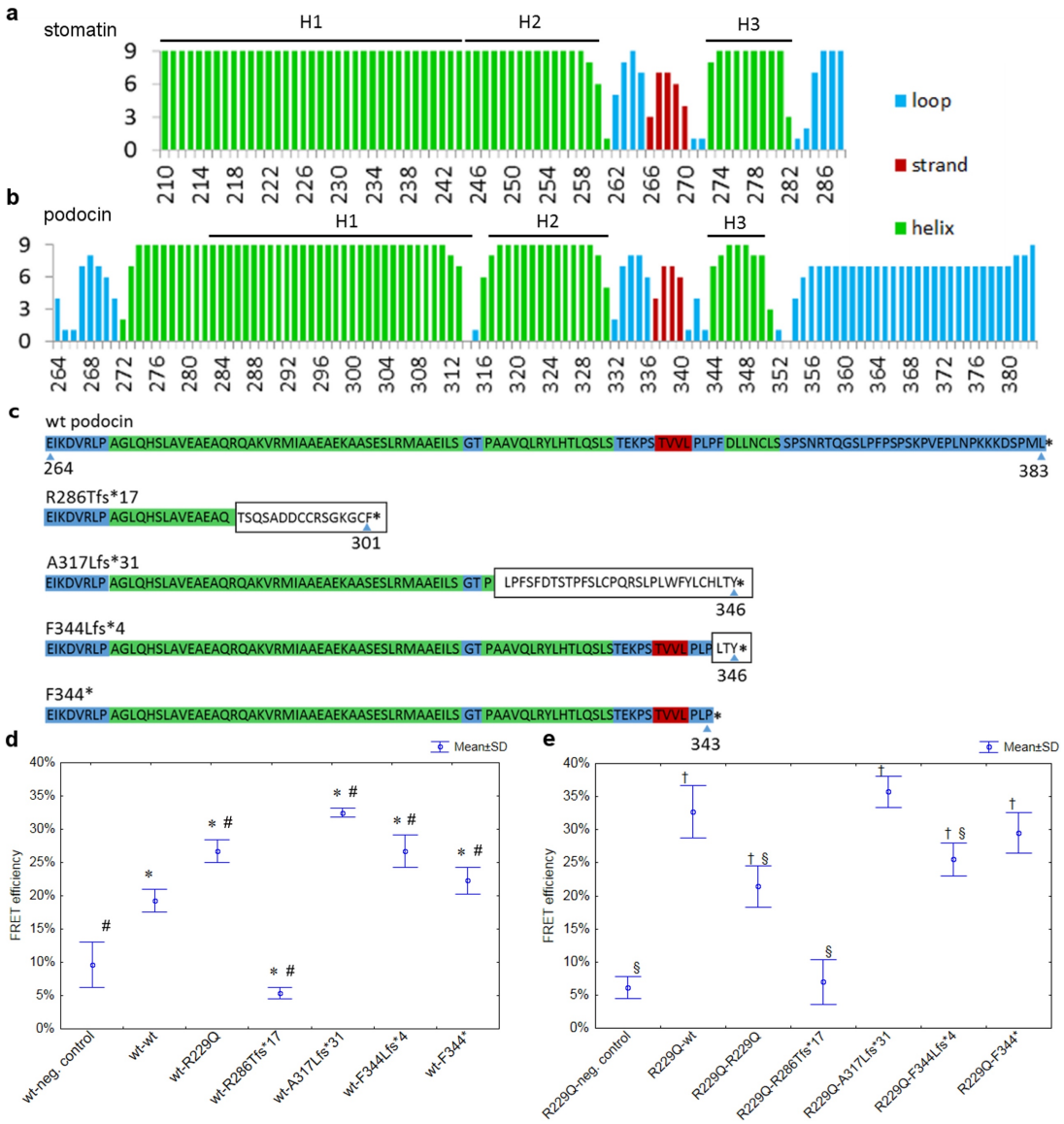


Figure 2. Membrane targeting of podocin variants as a function of the coexpressed podocin variant
a) Podocytes expressing GFP-tagged wt and missense podocin variants and/or HA-tagged wt and truncated podocin variants. The GFP-tagged podocin variants are shown in green, the HA-tagged podocin variants are in red. The plasma membrane is labeled with wheat germ agglutinin and shown in blue. The R286Tfs*17 podocin is perfectly membranous, does not colocalize with any of the coexpressed podocin variants, nor is influenced in its localization by them (c). In contrast, the A317Lfs*31 podocin is only partially membranous and the F344* and F344Lfs*4 podocin variants localize in vesicles. These three podocin variants colocalize with the coexpressed podocin variants and are strongly influenced in their localization by them (c, Fig. 3). Interestingly, the F344* and F344Lfs*4 podocin mutants become perfectly membranous when associated with membranous podocin variants, except for the pathogenic R229Q-F344Lfs*4 association. This latter shows a dispersed reticular localization, typical of pathogenic R229Q-associations. Scale bar, 20μm.
b-c) Membrane targeting of GFP-tagged missense and wt podocin variants (b) and HA-tagged truncated and wt podocin variants (c) as a function of the coexpressed podocin variants shown in parentheses, quantified in 10 cells per group. *P* values show the significance of Kruskal-Wallis tests.
*, **, *** levels of significance as compared with the membrane localization of the corresponding monoexpressed variant: $*P \leq 0.05$, $**P = 0.002$, $***P \leq 0.0001$

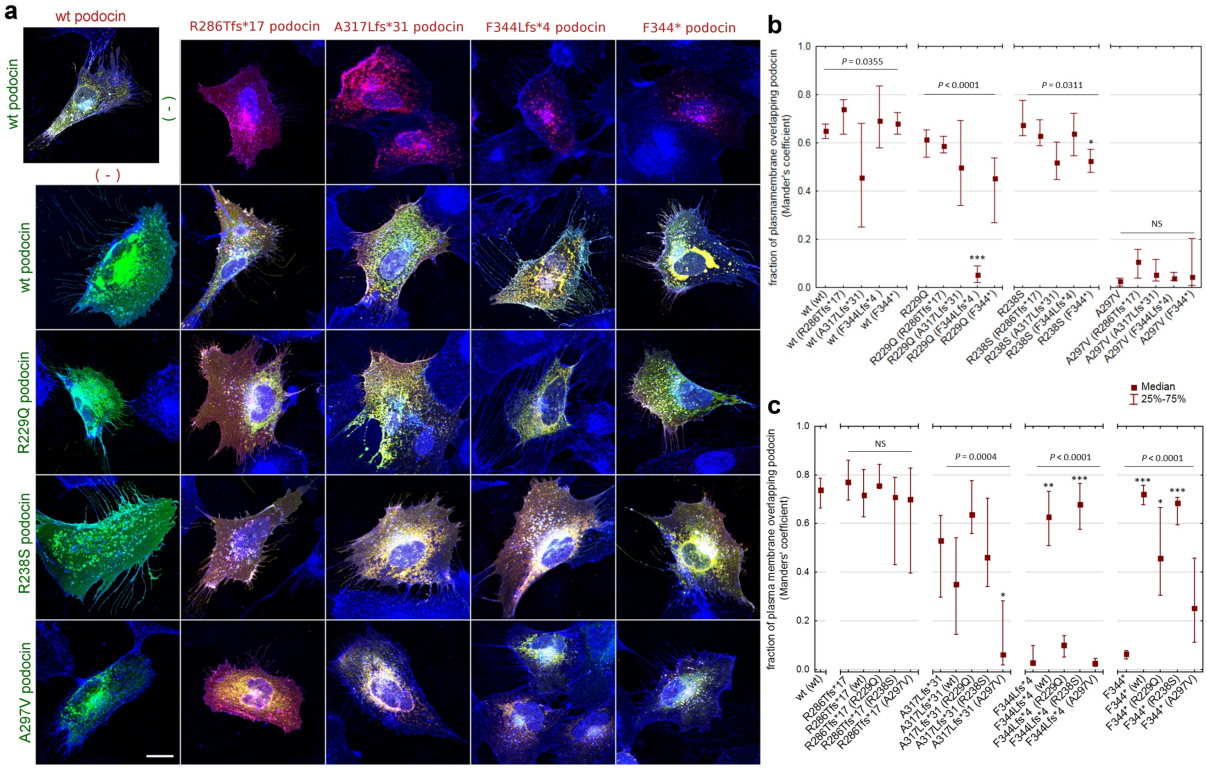
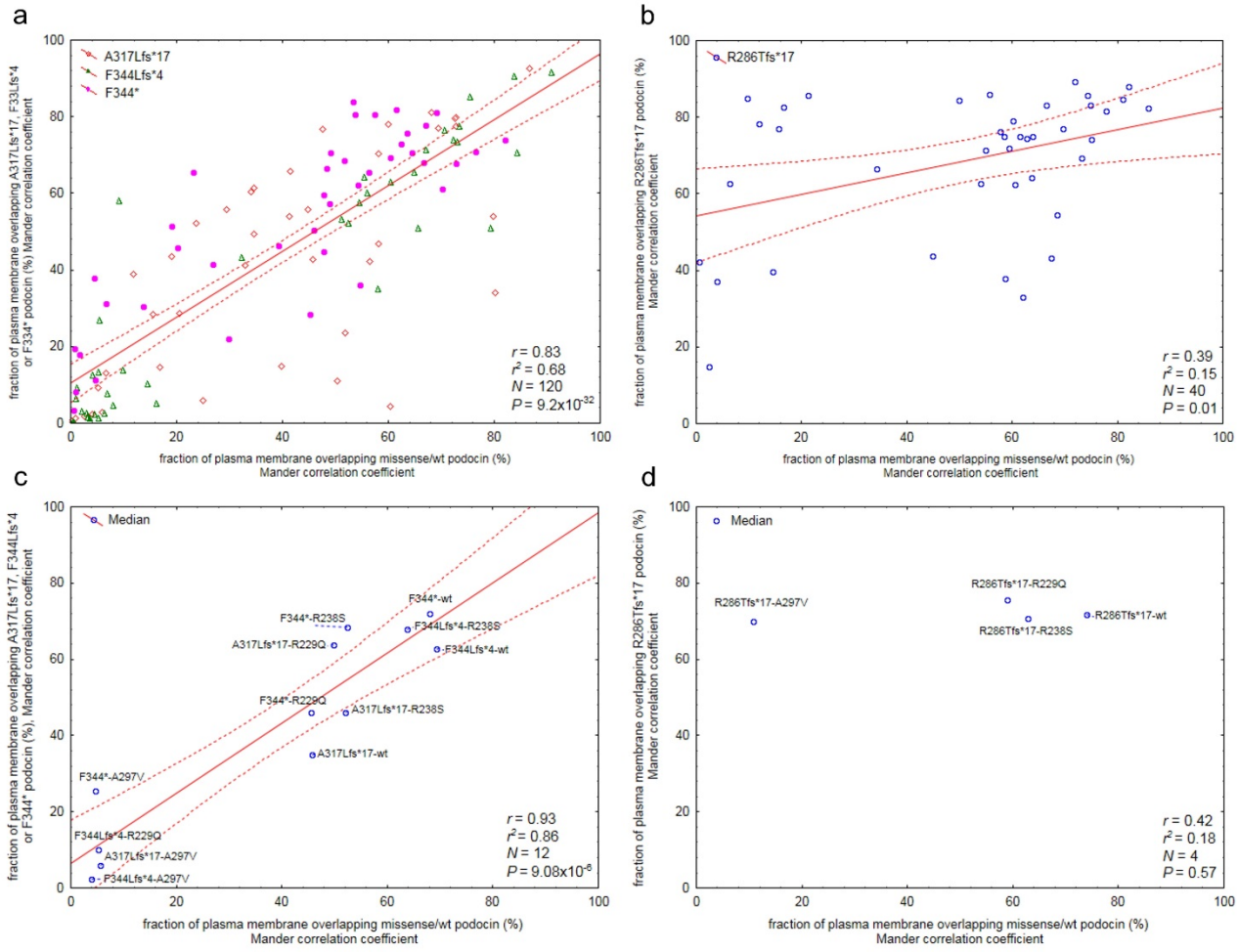


Figure 3. Correlation in the membrane localization of coexpressed podocin variants between variants with (a, c) and without (b, d) dimer-forming capacity.
Data are presented either as the values obtained in single cells (a,b) or as a median calculated in each group of 10 cells expressing the same podocin pairs, corresponding to Figure 2. While there is a strong correlation in the membrane-localization of the coexpressed podocin variants forming heterodimers or heterooligomers (a,c), the correlation in the membrane localization of podocin variants which do not form heterodimers is weak (b) or absent (d).



Task 2/b. Genotype-phenotype correlation in patients with *WT1* mutations

We carried out mutational screening of patients with nephrotic syndrome and have identified seven Hungarian families with *WT1* mutations. The first four of them have been included in a European cohort study in which we described the genotype-phenotype correlations in patients with *WT1* mutations. Renal progression, risk of Wilms' tumor, gonadoblastoma, intersex genitalia were assessed in function of the genotype. Results of this study greatly helps our clinical and genetic counseling practice [19].

Task 3. Gene hunting in Galloway-Mowat syndrome

Galloway-Mowat syndrome (OMIM 251300) is the association of microcephaly, severe developmental deficiency and steroid-resistant nephrotic syndrome. Genetically it is highly heterogeneous. Among the 91 patients with SRNS involved in the biobank, three children also suffer from microcephaly, severe mental retardation and epilepsy giving the clinical picture of Galloway-Mowat syndrome. Two of the three children were products of probably consanguineous marriages (parents originating from the same or neighboring small villages). No causal gene was known at the initiation of this study.

Our aim was to identify the causal mutation by whole exome sequencing in these patients and thus to identify novel genes implicated in this syndrome. In collaboration with the Laboratory of Hereditary Kidney Disorders in INSERM U983, Paris (Institut Imagine) we performed whole exome sequencing in these children, but failed to identify any mutation that could have been considered pathogenic. The first genes have been recently identified in Galloway-Mowat syndrome [20, 21], but the three Hungarian patients did not carry mutations in these genes. Even in large centers, one third of the whole exome sequencing procedures fails to identify the causal mutation.

References

1. Mitchell, J.R., E. Wood, and K. Collins, *A telomerase component is defective in the human disease dyskeratosis congenita.* Nature, 1999. **402**(6761): p. 551-5.
2. Heiss, N.S., et al., *X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions.* Nat Genet, 1998. **19**(1): p. 32-8.
3. Knight, S.W., et al., *Unexplained aplastic anaemia, immunodeficiency, and cerebellar hypoplasia (Hoyeraal-Hreidarsson syndrome) due to mutations in the dyskeratosis congenita gene, DKC1.* Br J Haematol, 1999. **107**(2): p. 335-9.
4. Vulliamy, T., et al., *The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita.* Nature, 2001. **413**(6854): p. 432-5.
5. Marrone, A., et al., *Telomerase reverse-transcriptase homozygous mutations in autosomal recessive dyskeratosis congenita and Hoyeraal-Hreidarsson syndrome.* Blood, 2007. **110**(13): p. 4198-205.
6. Savage, S.A., et al., *TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita.* Am J Hum Genet, 2008. **82**(2): p. 501-9.
7. Vulliamy, T., et al., *Telomere length measurement can distinguish pathogenic from non-pathogenic variants in the shelterin component, TIN2.* Clin Genet, 2012. **81**(1): p. 76-81.
8. Sirinavin, C. and A.A. Trowbridge, *Dyskeratosis congenita: clinical features and genetic aspects. Report of a family and review of the literature.* J Med Genet, 1975. **12**(4): p. 339-54.
9. Kiss, A.M., et al., *Human box H/ACA pseudouridylation guide RNA machinery.* Mol Cell Biol, 2004. **24**(13): p. 5797-807.
10. Sadowski, C.E., et al., *A single-gene cause in 29.5% of cases of steroid-resistant nephrotic syndrome.* J Am Soc Nephrol, 2015. **26**(6): p. 1279-89.
11. Tsukaguchi, H., et al., *NPHS2 mutations in late-onset focal segmental glomerulosclerosis: R229Q is a common disease-associated allele.* J Clin Invest, 2002. **110**(11): p. 1659-66.
12. Santin, S., et al., *Clinical value of NPHS2 analysis in early- and adult-onset steroid-resistant nephrotic syndrome.* Clin J Am Soc Nephrol, 2011. **6**(2): p. 344-54.
13. Machuca, E., et al., *Clinical and epidemiological assessment of steroid-resistant nephrotic syndrome associated with the NPHS2 R229Q variant.* Kidney Int, 2009. **75**(7): p. 727-35.
14. Hinkes, B., et al., *Specific podocin mutations correlate with age of onset in steroid-resistant nephrotic syndrome.* J Am Soc Nephrol, 2008. **19**(2): p. 365-71.
15. Kerti, A., et al., *NPHS2 homozygous p.R229Q variant: potential modifier instead of causal effect in focal segmental glomerulosclerosis.* Pediatr Nephrol, 2013. **28**(10): p. 2061-4.
16. Shen, N., et al., *Co-expression of phenylalanine hydroxylase variants and effects of interallelic complementation on in vitro enzyme activity and genotype-phenotype correlation.* Mol Genet Metab, 2016. **117**(3): p. 328-35.
17. Rodriguez-Pombo, P., et al., *Towards a model to explain the intragenic complementation in the heteromultimeric protein propionyl-CoA carboxylase.* Biochim Biophys Acta, 2005. **1740**(3): p. 489-98.
18. Tory, K., et al., *Mutation-dependent recessive inheritance of NPHS2-associated steroid-resistant nephrotic syndrome.* Nat Genet, 2014. **46**(3): p. 299-304.
19. Lipska, B.S., et al., *Genotype-phenotype associations in WT1 glomerulopathy.* Kidney Int, 2014. **85**(5): p. 1169-78.
20. Braun, D.A., et al., *Mutations in KEOPS-complex genes cause nephrotic syndrome with primary microcephaly.* Nat Genet, 2017. **49**(10): p. 1529-1538.
21. Colin, E., et al., *Loss-of-function mutations in WDR73 are responsible for microcephaly and steroid-resistant nephrotic syndrome: Galloway-Mowat syndrome.* Am J Hum Genet, 2014. **95**(6): p. 637-48.