Miriam Mergen¹, Christina Engel¹, Barbara Müller¹, Marie Follo², Tobias Schäfer¹, Manfred Jung³ and Gerd Walz¹

Correspondence and offprint requests to: Gerd Walz; E-mail: gerd.walz@uniklinik-freiburg.de

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Background. Nephronophthisis (NPH) is a rare recessive disease caused by several different gene mutations. Most gene products localize to the cilium, and thus, the various NPH manifestations including kidney cysts and situs inversus have been linked to ciliary defects.

Results. Here, we describe that targeted knockdown of NPHP2 significantly reduced the number of cilia on polarized MDCK cells. As one of the underlying molecular mechanisms, we identified a direct interaction between NPHP2 and Aurora

ABSTRACT

The nephronophthisis gene product NPHP2/Inversin interacts with Aurora A and interferes with HDAC6-mediated cilia disassembly


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INTRODUCTION

Nephronophthisis (NPH), although a rare autosomal recessive disorder, is the most common cause for hereditary end-stage renal disease in childhood and adolescence [1–4]. More than a dozen genes have been identified that cause NPH, or the related Joubert (JSRD) and Bardet-Biedl (BBS) syndromes [5, 6]. Most of the products encoded by NPH, JSRD or BBS genes localize to the cilium, a microtubular organelle present on most body cells. Hence, a dysfunction of the cilium is thought to cause cystic renal disease as well as the multiple extrarenal manifestations associated with these genetic syndromes. The non-motile primary cilium appears to function as a flow- and/or chemosensor that translates extracellular stimuli into complex cellular programmes required for tissue morphogenesis and organ development [7]. The cilium also functions as a secluded subcellular compartment that controls the levels of cellular and nuclear proteins. Prominent examples are the hedgehog and Wnt signalling pathways, where the levels of cellular and nuclear proteins. Prominent examples implicate NPH gene products in the control of ciliogenesis defects, and report that NPHP2 interferes with the Hef1/Aurora A pathway of ciliary disassembly.

MATERIAL AND METHODS

Establishment of transgenic cell lines

To generate inducible knockdown cell lines of canine NPHP2 and NPHP1, MDCK cells were stably transduced with a lentivirus, encoding the tetracycline-sensitive tTR-KRAB repressor. MDCK tTR-KRAB cells were subsequently transduced with a lentivirus, encoding the two shRNAs mediating NPHP2 or one shRNA mediating NPHP1 knockdown coupled to a GFP reporter (pLVTH vector). Transduction cycles were repeated up to three times. The target sequences were NPHP2/inv.i2 (5'–GGAAATGCTGATTTTGGCGAGAATCCAGGAGAAGTGGATA CA–3'), NPHP2/inv.i6 (5'–CCAGGAGAATGGGATA CAGGATA–3') and NPHP1/i1 (5'–GGTTTCAGTAGAC ATGTA–3'); published by Delous et al., Hum. Mol. Gen. 2009;18). The corresponding MDCK control cell line, tTR-KRAB luci.i., was obtained by transducing MDCK tTR-KRAB cells with shRNA specific for luciferase (target sequence: 5'–CGTACGCCAACTTCCGA–3').

Quantitative RT–PCR (qRT–PCR)

MDCK cells were grown until Day 9. Knockdown of NPHP2 was achieved by tetracycline at 5 µg/mL on Days 2, 3, 6, 7 and 8. Total RNA of tetracycline-induced and control cells was extracted using the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany), transcribed into cDNA by using the Reverse Transcription Super Script III first strand system (Invitrogen). Quantitative PCR was conducted with the MESA/SYBR® Assay (Eurogentech, Seraing, Belgium) according to the manufacturer's protocol. Samples were run on a Roche A, a cell cycle kinase that promotes ciliary disassembly after activation by Hef1. NPHP2 inhibited the phosphorylation and activation of Aurora A, and reduced its kinase activity in vitro. Aurora A and histone deacetylase inhibitors ameliorated the ciliogenesis defect in NPHP2-deficient MDCK cells, supporting our hypothesis that NPHP2 is involved in the control of ciliary disassembly. Furthermore, we observed that nephrocystin (NPHP1), an interaction partner of NPHP2, also binds Aurora A, exerting very similar inhibitory effects on Hef1-mediated Aurora A activation.

Conclusions. Taken together, these findings suggest that NPHP gene products can interfere with ciliary disassembly through interaction with the Hef1/Aurora A module, thereby modulating cell cycle control and cell proliferation.
LC480 and the results were quantified using the LC480 Software v.1.5.0. Transcript levels of target genes were normalized to transcript levels of HPRT, which was used as a reference gene. The following primers were used: NPHP2: forward primer (5′-CAGTGAAGGCAGTGATGGAA-3′), reverse primer (5′-GCCTGTTTGGTTTTTGTT-3′), and cHPRT: forward primer (5′-TTCCATCTATGACTGTAG-3′), reverse primer (5′-ATTATGCTCCTTGACCAA-3′).

### Wheat germ cell-free expression system for in vitro protein production

For cell-free expression of Flag-tagged human Aurora A, V5-tagged human NPHP2 and the control protein, V5. CD2AP, the RTS™ 100 Wheat Germ CECF Kit (5Prime GmbH, Hamburg/Germany) was used according to the manufacturer’s instructions. The pIVEX 1.4 WG vector was modified to include an N-terminal Flag- or V5-tag. The expression of the protein of interest was verified by western blot analysis.

### Antibodies and reagents

The following antibodies were used in the study: anti-acetylated tubulin (1:10,000; Sigma), anti-gamma tubulin (mouse, 1:3000; Sigma), anti-GFP (mouse, 1:3000; Santa Cruz), anti-Actin (mouse, 1:4000; Sigma), anti-AuroraA (rabbit, 1:500; BioLegend), anti-phospho AuroraA (C39D8) (rabbit, 1:500; Cell Signaling), polyclonal anti-NPHP1 (1:1000; a kind gift of T. Benzing, University of Cologne, Germany), anti-V5 (1:3000, mouse; Serotec), anti-M2 (1:3000, rabbit; Sigma-Aldrich), anti-V5 polyclonal (1:4000; Chemicon/Millipore) and anti-CEP164 (a kind gift of E. Nigg, Max Planck Institute, Martinsried/Germany). For immunofluorescence experiments, the primary antibodies were used in combination with Cy3- (1:1000, Jackson Immunoresearch) or Alexa-647-(1:1000, Invitrogen) labeled secondary antibodies. Hoechst 33342 was used for staining nuclei. Western blot analysis and immunoprecipitation assays were performed as previously described [36]. Quantification of non-saturated bands was performed using LabImage1D software. Aurora A inhibitor I (S1451; Selleck, Houston, TX, USA), suberoylanilide hydroxamic acid (SAHA), and the HDAC6 inhibitor ST80 [37] were used at concentrations as indicated.

### Ciliogenesis assay

To investigate ciliogenesis of MDCK cells, cells were seeded onto coverslips to reach full confluence the next day. Tetra-cycline was added at 5 µg/mL on Days 2, 3, 6, 7 and 8 of culture. Cells were fixed and ciliary stainings were performed on Day 9 of culture. To measure the number of ciliated cells, an automated microscopic assay was developed, using an Olympus Scan R Screening Station based on an IX81 inverse microscope stage. A ×40 UPLSAPO, N.A. 0.9 objective was used for image acquisition. For each sample, fluorescence images of at least one square area consisting of 4 × 4
neighbouring fields were recorded in an automated fashion. Each individual field, covering an area of 217 × 165 microns. For each field of view, one resulting maximal projection image derived from 5 z-levels spread over a total depth of 5 microns was saved for subsequent analysis. Data analysis was performed using the 'Scan R Analysis' Software v.1.2.0.6. Exploiting the fact that the shaft of a primary cilium emanates from the basal body, the samples were labelled with the basal body-specific anti-CEP164 antibody, whereas an anti-acetylated tubulin antibody was used to mark the ciliary shaft. The percentage of shRNA-expressing, knockdown cells was determined for each cell line by setting up a threshold level for GFP-fluorescence signal intensity to determine GFP-positive cells.

**Aurora A kinase assay**

To determine Aurora A kinase activity in vitro, the CycLex Aurora-A kinase Assay/Inhibitor Screening Kit (CycLex Ltd., Japan) was used according to the manufacturer’s instructions. To test for the effects of NPHP2 or the control protein GFP on Aurora A kinase activity in a cell-free system, HIS/Flag-tagged NPHP2 and GFP were produced with the wheat germ extracts, and proteins were purified by using a nickel resin. Samples were dialysed overnight against kinase buffer (10 mM Tris pH 7.5, 10 mM MgCl₂); Flag-tagged Aurora A was freshly produced. Protein levels were determined by western blot analysis. For each reaction, adjusted amounts of purified wheat germ proteins and 50 µl of supplied kinase buffer (75 µM ATP) were added to a final volume of 100 µl with kinase buffer. Samples were co-incubated for 30 min at 30°C on a shaker before performing a standard ELISA procedure.

**Statistical analysis**

Statistical analysis was carried out using IBM SPSS Statistics 19. P values were calculated by paired t-test from the mean values of ciliated cells of untreated versus tetracycline-induced transgenic cells. Significant differences were marked with asterisks (*P < 0.05; **P < 0.01; ***P < 0.001).

**FIGURE 2:** NPHP2 interacts weakly with Hef1. V5-tagged NPHP2 (V5.NPHP2) was co-expressed in HEK 293T cells with either Flag-tagged control protein CD2AP (F.CD2AP) or Flag-tagged Hef1 (F.Hef1). Only precipitation of Flag-tagged Hef1 immobilized NPHP2.

**FIGURE 3:** Aurora A interacts with Hef1 and NPHP2. V5-tagged NPHP2 (V5.NPHP2) and Hef1 (V5.Hef1) were co-expressed with Flag-tagged control protein CD2AP or Aurora A (F.AurA). Precipitation of F.AurA with anti-Flag M2 beads immobilized V5.Hef1 and V5.NPHP2, but not the control protein.
RESULTS

Knockdown of NPHP2 causes ciliogenesis defects

Normal ciliogenesis has been reported in the kidney of postnatal NPHP2-mutant (Inv) mice. Analysis of cilia at the ventral node suggested that the cilia of Inv mice are motile, but exhibit a polarization defect that causes an abnormal fluid movement and laterality defects. Knockdown of NPHP2 by shRNA in MDCK cells did not prevent ciliogenesis; however, these experiments were performed with transient transfection protocols. We decided to establish a robust, inducible shRNA system, and generated two different shRNAs targeting the expression of NPHP2 mRNA. The shRNA was expressed in a tetracycline-dependent fashion, using a lentiviral gene transfer system [22]. Treatment of MDCK cells with tetracycline reduced the NPHP2 mRNA, measured by quantitative RT–PCR (qRT–PCR), by >80% (Figure 1a). Automatized counting of cilia on ~50 000 cells in total showed a reduction of cilia between 45 and 50% (Figure 1b), suggesting that normal ciliogenesis in polarized MDCK cells is compromised in the absence of NPHP2.

NPHP2 interacts with Hef1 and Aurora A

Since the Hef1/Aurora A complex plays an important role in the balance between ciliogenesis and ciliary disassembly, we tested whether NPHP2 can affect the function of this protein complex. Co-immunoprecipitation assays revealed that NPHP2 weakly interacts with Hef1, when co-expressed in HEK 293T cells (Figure 2). A more robust interaction was observed between Hef1 and Aurora A, as recently reported (Figure 3). However, a comparable interaction was detected, when Aurora A was precipitated in the presence of NPHP2, indicating that NPHP2 can associate with the Hef1/Aurora A complex (Figure 3).

NPHP2 interferes with the activation of the Hef1/Aurora A ciliary disassembly module

Since activation of Hef1/Aurora A causes ciliary disassembly, we examined whether NPHP2 can inhibit the activation of

![Figure 4: NPHP2 interferes with Hef1 and Aurora A phosphorylation and activation. (a) Co-expression of Hef1 with Aurora A resulted in an increase of total Aurora A levels and increased its phosphorylation (lane 2), while co-expression of NPHP2 caused a decrease of Aurora A levels and phosphorylation (lane 3). Co-expression of NPHP2 (lane 4) decreased the phosphorylated form of Hef1. (b) NPHP2 reduced Aurora A phosphorylation, but did not reduce Hef1 expression levels in the presence of Hef1 (lane 6). (c) Quantification of Aurora A phosphorylation in the presence of a control protein (V5.CD2AP) or NPHP2 (V5.NPHP2), and the combination of NPHP2 or control protein and Hef1 [F.Hef1, V5.CD2AP, V5.NPHP2 (n = 3, P ≤ 0.01)], respectively. The level of phosphorylated Aurora A (p.AurA) was normalized for the total Aurora level (F.AurA).](https://academic.oup.com/ndt/article-abstract/28/11/2744/1823432)
Hef1/Aurora A. Co-expression of Hef1 resulted in increased levels of Aurora A with a proportional increase in activated (phosphorylated) Aurora A (Figure 4a). In contrast, co-expression of NPHP2 reduced Aurora A levels, and abolished detectable levels of phosphorylated Aurora A. NPHP2 also appeared to affect the level of phosphorylated Hef1, reducing the upper band of Hef1, which is thought to represent the active form responsible for the activation of Aurora A (Figure 4a). We next examined the effect of NPHP2 in the presence of Hef1, and found that NPHP2 was less effective inhibiting Hef1 phosphorylation in the presence of Aurora A, but reduced Aurora A phosphorylation in the presence of Hef1 despite an increase in overall levels of Aurora A (Figure 4b and c). These findings indicate that NPHP2 can directly antagonize Aurora A activation.

**NPHP2 binds and inhibits Aurora A in vitro**

To explore this possibility in more detail, we generated recombinant NPHP2 and Aurora A, using wheat germ extracts. This allowed us to address the question, whether NPHP2 can interact directly with Aurora, or needs additional components to form this complex. Precipitation of V5-tagged NPHP2, but not the V5-tagged control protein CD2AP immobilized Aurora A, confirming a direct interaction between both proteins (Figure 5a). We next examined whether NPHP2 interferes with Aurora A activity in a cell-free system. Recombinant NPHP2, GFP and Aurora A were again generated using the wheat germ extract. NPHP2 and GFP were His-tagged and purified on a nickel column. Incubation of Aurora A with NPHP2, but not with GFP resulted in a significant reduction of Aurora A activity, suggesting that the interaction of NPHP2

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**Figure 5:** NPHP2 directly interacts with Aurora A and inhibits the kinase activity of Aurora A. (a) Flag-tagged Aurora A (F.AurA), V5-tagged control protein (V5.CD2AP) and NPHP2 were expressed using wheat germ extracts. Immunoprecipitation of V5.NPHP2, but not the control protein, immobilized Aurora A. (b) Wheat germ lysates of F.AurA, F.NPHP2 and F.GFP were used to analyze the effect of the direct interaction of NPHP2 and AurA on AurA kinase activity. To determine AurA kinase activity, an ELISA-based approach was used. A dilution series of AurA wheat germ lysates was used to calculate a regression curve between relative AurA activity and AurA dilution factor, and was depicted on a half-logarithmic scale. For two representative experiments, the effect of NPHP2 on AurA kinase activity is shown in the diagram. NPHP2 co-incubation represents an AurA dilution of 2.5–3. (d) Co-incubation of F.NPHP2 reduces the relative AurA kinase activity significantly compared with co-incubation with the control protein F.GFP (t-test for combined samples, *P < 0.05). Depicted is the mean value ± SEM of the relative AurA activity (n = 3). (e) Enrichment of His-/Flag-tagged NPHP2 and GFP was confirmed by western blot.
negatively affects the ability of Aurora A to phosphorylate substrates.

**Histone deacetylase inhibitors rescue the ciliogenesis defect caused by NPHP2**

Since Aurora A is thought to trigger ciliary disassembly through activation of the histone deacetylase histone deacetylase inhibitor (HDAC) 6, we examined whether inhibition of HDAC6 can reverse the ciliogenesis defect caused by knockdown of NPHP2. Knockdown of NPHP2 through induction of NPHP2-specific shRNA reduced the presence of cilia by ∼20% (Figure 6b); in the presence of the selective Aurora A inhibitor, the reduction was ameliorated to ∼10%. Both SAHA, an unspecific HDAC inhibitor, and ST80, a specific small-molecule HDAC6 inhibitor, caused an increase in ciliogenesis, suggesting that in addition to antagonizing the effects of NPHP2, it stimulates ciliogenesis through stabilization of the microtubular axoneme (Figure 6b).

**Control of ciliary disassembly by NPHP gene products is not limited to NPHP2**

Inversin/NPHP2 has been shown to interact with nephrocytin/NPHP1 [23]. To substantiate our finding that knockdown of NPHP gene products interferes with a normal ciliogenesis in MDCK cells, we examined the effects of NPHP1 knockdown, speculating that NPHP1 might affect ciliogenesis through a similar mechanism. Using shRNA (i1) in combination with a tetracycline-inducible lentiviral transfer system, endogenous NPHP1 was not detectable after induction of the shRNA, revealed by simultaneous expression of GFP. While knockdown of NPHP2 yielded up to 50% reduction in cilia formation, the effect of NPHP1 was less extensive, yielding 29.5% reduction (Figure 7a). Co-immunoprecipitation experiments revealed that Aurora A precipitated with either NPHP1 or NPHP2 (Figure 7b). Further analysis demonstrated that the presence of Aurora A shifted Hef1 to the phosphorylated, higher molecular weight form; this shift was almost completely abrogated by either NPHP1 or NPHP2, suggesting a similar effect of both proteins on either Hef1 or Aurora A (Figure 7c). NPHP1 reduced the levels of activated phospho-Aurora A, and prevented its action by Hef1 almost as effectively as NPHP2 (Figure 7d). Taken together, these experiments support our hypothesis that NPHP gene products potentially interfere with cilia disassembly mediated by the Hef1/AurA module.

**FIGURE 6:** NPHP2 deficiency is rescued by Aurora A and HDAC inhibitors. (a) Ciliogenesis was quantified at Day 9 of culture after exposure to tetracycline and inhibitors as indicated. (b) For rescue experiments an Aurora A inhibitor (AurAi), SAHA or the HDAC6 inhibitor ST80 were administered on Days 6, 7 and 8 to ciliogenesis assays. In this series of experiments, NPHP2 knockdown reduced ciliation ∼20%. Administration of the Aurora A inhibitor, SAHA or the HDAC6 inhibitor ST80 alleviated ciliary loss. Means of three independent experiments ± SEM are depicted. Statistical analysis was performed using student’s t-test for combined samples. *P < 0.05; **P < 0.001.
DISCUSSION

Although patients with NPH reportedly have normal cilia, an increased frequency of upper respiratory airway infections as well as the occurrence of a situs inversus suggests that NPH gene products contribute to the function of motile cilia. Knockdown of NPHPs in animal models additionally supports a role of NPHPs in ciliogenesis. While hypomorphic NPHP4 is reportedly associated with defective sperm development and infertility in mice [21], deletion of NPHP3 extends the length of cilia [20]. Furthermore, the combined knockdown of

![FIGURE 7:](https://example.com/figure7.png)

NPHP1 depletion interferes with normal ciliogenesis in MDCK cells and antagonizes Hef1/Aurora A activation. (a) Flag-tagged NPHP1 (left lane) was utilized to correctly localize endogenous NPHP1 in MDCK cells, using an NPHP1 antiserum. An shRNA was selected that caused complete disappearance of the NPHP1 signal monitored by western blot analysis. Induction the shRNA by tetracycline, associated with the expression of GFP, reduced the number of ciliated cells by 29.5%. (b) Co-expression of Aurora A (F.AurA with NPHP1 and NPHP2 revealed that both NPHPs interacted with Aurora A. (c) While Aurora A increased the phosphorylated form of Hef1, associated with a shift to a higher molecular weight, this shift was completely prevented by either NPHP1 or NPHP2. (d) Both NPHP1 and NPHP2 blocked the Hef1-mediated activation of Aurora A.
Upon growth factor stimulation, cilia are disassembled in an activated state and prevent premature re-entry into the cell cycle involving the activation of Hef1 and Aurora A, activation of signalling pathways [30], cilia appear to retain cells in a differentiated state in all organs and tissues.

NPHPs in Caenorhabditis elegans causes notable structural changes of cilia, supporting their role in normal ciliogenesis and/or ciliary integrity [24–27].

We decided to examine the role of NPHP2 in ciliogenesis in a defined cell culture system. To avoid clonal artifacts, we used a lentiviral, tetracycline-dependent knockdown in MDCK cells, and compared the effects of two different NPHP2 shRNAs on the presence of primary cilia on the surface of polarized MDCK cells, using an unbiased, automated scoring system. Both shRNAs reduced the number of cilia by >40%, revealing that NPHP2 is involved in normal ciliogenesis and/or maintenance of cilia in MDCK cells. The requirement for cilia appears to change over time, and is tissue specific. For example, embryonic deletion of cilia in mouse kidneys results in severe cyst formation, while deletion of cilia in adulthood is tolerated without significant pathology [28]. In contrast, deletion of cilia in adult animals is associated with hyperphagia and obesity [29]. Thus, our findings are only indicative of a principal involvement of NPHP2 in ciliogenesis, but do not necessarily imply that this function is essential in all organs and tissues.

While functionally intact cilia can act as a platform for several signalling pathways [30], cilia appear to retain cells in a differentiated state and prevent premature re-entry into the cell cycle [31]. Upon growth factor stimulation, cilia are disassembled involving the activation of Hef1 and Aurora A, activation of HDAC6, and deacetylation of the ciliary microtubules [13, 32]. NPHP2 seems to interfere at several levels with this ciliary disassembly cascade, but appears to most prominently inhibit Aurora A through direct interaction with this kinase. We also observed a weak interaction between NPHP2 and Hef1, and a reduction of the phosphorylation status of Hef1 in the presence of NPHP2. Since NPHP2 is also present at the plasma membrane, NPHP2 could block Hef1/Aurora A-mediated ciliary disassembly at a very early activation step (Figure 8).

A shortcoming of our study is the reliance on ectopic expression of NPHP2 and Hef1/Aurora A in HEK 293T cells, an embryonic kidney cell line. Although we attempted to examine the interaction between endogenous NPHP2 and Aurora A in MDCK cells, protein levels were too low to be detected by western blot, using either self-produced or commercially available antibodies. To find additional support for our hypothesis that NPHP2 can control Aurora A activity, we resorted to two alternatives: we expressed both proteins in vitro, using a cell-free wheat germ extract, to demonstrate a direct interaction between both proteins, and purified His-tagged NPHP2 to demonstrate a direct inhibition of the Aurora A kinase activity by NPHP2. We further demonstrated that an Aurora A inhibitor can partially rescue the defective ciliogenesis which occurs after depletion of NPHP2. These observations suggest that NPHP2 can participate in cell cycle control through interaction with Aurora A. The cystic kidney disease in NPH differs from autosomal dominant polycystic kidney disease (ADPKD). Renal cysts are typically aligned along the cortico-medullary border, and the overall size and shape of the kidneys remain largely normal. Exceptions are the kidneys of patients with NPHP2 mutations that tend to be enlarged with irregular cysts similar to ADPKD kidneys [33, 34]. This particular manifestation might be the result of a defective Aurora A and cell cycle control. HDAC inhibitors were shown to ameliorate the progression of cystic kidney disease in several vertebrate models [35]. Our findings indicate that inhibition of Aurora A/HDAC6-mediated ciliary disassembly might be particularly beneficial in patients with type 2 nephronophthisis caused by mutations of NPHP2.

Since we failed to demonstrate the interaction between endogenous NPHP2 and Aurora A due to the lack of suitable antibodies, we extended our observation, speculating that other NPHP gene products may participate in the regulation of ciliary disassembly mediated by the Hef1/Aurora A module. We found that knockdown of nephrocystin/NPHP1 by shRNA resulted in a reduction of cilia formation in MDCK cells. Although interference with ciliogenesis was not quite as effective, NPHP1 co-immunoprecipitated with Aurora A and prevented activation of the Hef1/Aurora A module very similar to NPHP2.

**CONCLUSION**

This observation supports our hypothesis that NPHPs have the potential to prevent ciliary disassembly, perhaps to block cell cycle progression, maintaining cells in interphase. Future experiments need to address whether this capability is utilized during organ development and tissue homeostasis.

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CONFLICT OF INTEREST STATEMENT

None declared.

(See related article by Ong. Primary cilia and renal cysts: does length matter? Nephrol Dial Transplant 2013; 28: 2661–2663.)

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