The inositol Inpp5k 5-phosphatase affects osmoregulation through the vasopressin-aquaporin 2 pathway in the collecting system

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Abstract: Inositol Inpp5k (or Pps, SKIP) is a member of the inositol polyphosphate 5-phosphatases family with a poorly characterized function in vivo. In this study, we explored the function of this inositol 5-phosphatase in mice and cells overexpressing the 42-kDa mouse Inpp5k protein. Inpp5k transgenic mice present defects in water metabolism characterized by a reduced plasma osmolality at baseline, a delayed urinary water excretion following a water load, and an increased acute response to vasopressin. These defects are associated with the expression of the Inpp5k transgene in renal collecting ducts and with alterations in the arginine vasopressin/aquaporin-2 signalling pathway in this tubular segment. Analysis in a mouse collecting duct mCCD cell line revealed that Inpp5k overexpression leads to increased expression of the arginine vasopressin receptor type 2 and increased cAMP response to arginine vasopressin, providing a basis for increased aquaporin-2 expression and plasma membrane localization with increased osmotically induced water transport. Altogether, our results indicate that Inpp5k 5-phosphatase is important for the control of the arginine vasopressin/aquaporin-2 signalling pathway and water transport in kidney collecting ducts.

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The inositol Inpp5k 5-phosphatase affects osmoregulation through the vasopressin-aquaporin 2 pathway in the collecting system

Running title: Inpp5k and the control of renal water transport

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Abstract:

Inositol *Inpp5k* (or Pps, SKIP) is a member of the inositol polyphosphate 5-phosphatases family with a poorly characterized function *in vivo*. Here, we explored the function of this inositol 5-phosphatase in mice and cells overexpressing the 42 kDa mouse *Inpp5k* protein. *Inpp5k* transgenic mice present defects in water metabolism characterized by a reduced plasma osmolality at baseline, a delayed urinary water excretion following a water load, and an increased acute response to vasopressin. These defects are associated with the expression of the *Inpp5k* transgene in renal collecting ducts and with alterations in the arginine vasopressin/aquaporin-2 signalling pathway in this tubular segment. Analysis in a mouse collecting duct mCCD cell line revealed that *Inpp5k* overexpression leads to increased expression of the arginine vasopressin receptor type 2 and increased cAMP response to arginine vasopressin, providing a basis for increased aquaporin-2 expression and plasma membrane localization with increased osmotically-induced water transport. Altogether, our results indicate that *Inpp5k* 5-phosphatase is important for the control of the arginine vasopressin/aquaporin-2 signalling pathway and water transport in kidney collecting ducts.
Introduction:

Phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) demonstrate second messenger functions in multiple biological processes. PtdIns(4,5)P2 controls, among others, the dynamic turnover of actin cytoskeleton, assembly/disassembly of vesicular coats, secretion, gene expression and the fate of endocytic vesicles [13, 17, 29, 40]. It also regulates the activity of many ion channels and transporters [35, 36]. PtdIns(4,5)P2 is mainly localized in the inner leaflet of plasma membranes, but smaller pools have been detected in intracellular membranes, including Golgi, endoplasmic reticulum, endosomes and lamellipodia [13]. Mechanistically, a very large number of proteins are recruited to PtdIns(4,5)P2 via pleckstrin homology (PH), phagocyte oxidase (PX), Fab1p, YOTB, Vac1p and EEA1 (FYVE), epsin N-terminal homology (ENTH) domains or through small patches of basic amino acids [13, 18, 28]. PtdIns(4,5,P2) also serves as precursor of five essential signalling molecules: diacylglycerol, inositol (1,4,5)-trisphosphate, PtdIns4P, PtdIns5P and PtdIns(3,4,5)P3 [13, 17]. In non-stimulated cells, PtdIns(3,4,5)P3 levels are very low in the plasma membrane, reaching ~0.1% of the level of PtdIns(4,5)P2 [13, 17]. Upon cell activation, PtdIns(3,4,5)P3 levels are transiently increased by a factor ranging from 2- to 100-fold. This phosphoinositide is implicated in the control of cell survival, growth and proliferation, resistance to apoptosis, regulation of cytoskeleton dynamics, membrane trafficking, cell migration, and many of the metabolic responses to insulin [12, 13]. PtdIns(3,4,5)P3 regulates a wide variety of effector proteins primarily by binding and recruiting specific proteins.

In mammalian cells, PtdIns(4,5)P2 and/or PtdIns(3,4,5)P3 are substrates for several members of the phosphoinositide 5-phosphatases family, including Inppl1 (or
SHIP2), Inpp5e and Inpp5k (or SKIP, for Skeletal muscle and Kidney enriched Inositol Phosphatase, and Pps, for Putative Phosphatase) [2, 7, 30, 32, 33]. These enzymes tightly control the level of the above-mentioned phosphoinositides in a tissue-, cellular-, subcellular- and signalling cascade-specific way. Importantly, inactivation or overexpression of most members of this family has been associated with diseases in mouse and man [3, 30, 32, 33]. For example, Inpp5e is localized in the primary cilium of differentiated/quiescent cells; mice and man with genetic alterations in the Inpp5e gene develop ciliopathies [6, 22]. SHIP2 knock-out mice are resistant to high fat diet-induced obesity and have glucose metabolism alterations [10, 34]; polymorphisms in the \textit{SHIP2} gene have been identified in cohorts of type 2 diabetic patients, and overexpression of SHIP2 in transgenic mice is associated with glucose metabolism and insulin sensitivity defects [25, 26, 31].

Inpp5k is a PtdIns(4,5)P2 and PtdIns(3,4,5)P3 5-phosphatase. It is highly expressed in human skeletal muscles and kidney, hence its previous name SKIP [19]. In N1E-115 neuroblastoma cells, human Inpp5k regulates the actin cytoskeleton [19]. Human Inpp5k is translocated from the endoplasmic reticulum to plasma membrane ruffles following activation by EGF and insulin in Cos-7 and skeletal muscle C2C12 cell lines, respectively [16, 39]. Activation of PKB and p70 S6 kinases as well as GLUT4 glucose transporter translocation to the plasma membrane and membrane ruffles formation were significantly decreased in human Inpp5k-transfected and insulin-stimulated CHO cells [20]. Mice totally deficient for Inpp5k die during embryonic life for still unknown reason; Inpp5k\textsuperscript{+/−} mice have increased insulin sensitivity in skeletal muscles, confirming the results obtained in different cell lines [21]. However, the role of Inpp5k in the kidney, another tissue where the phosphatase has been reported to
be highly expressed, and its potential implication in a metabolic or a pathologic process in this tissue remain undefined.

In order to explore the function of Inpp5k in vivo, we generated and characterized a transgenic mouse line expressing the mouse Inpp5k 5-phosphatase in many tissues. Our in vivo and in vitro results reveal that overexpression of Inpp5k in kidney collecting duct cells is associated with defects in water handling paralleled by alterations in the arginine vasopressin (AVP)/aquaporin-2 (AQP2) signalling pathway resulting in impaired osmoregulation.
Materials and Methods:

**RNA analysis in mouse tissues and cells:** For Northern blot analysis, total RNA was extracted from mouse tissues using the TriPure Isolation reagent (Roche Applied Sciences) and loaded (10 µg/lane) on agarose gel. A 944 bp DNA fragment of the mouse Inpp5k cDNA served as a radiolabeled probe. A radiolabeled probe consisting in a 600 bp DNA fragment of the mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT) cDNA served as loading control.

For RT-PCR analysis on FACS-purified populations of dendritic cells, mast cells, macrophages, neutrophils, eosinophils, T and B lymphocytes, RNA was extracted and reverse-transcribed using random hexamers. cDNA was amplified by PCR using forward and reverse primers located in exon 8 and exon 12 of the mouse Inpp5k gene, respectively (see Suppl. Table 1 for the primers sequence; annealing temperature: 55.5°C; 30 cycles). For RT-PCR and real-time RT-PCR analysis on 6 month-old mouse kidney and brain and on mCCD cells, total RNA and cDNA were obtained as described [24]. The primers (Suppl. Table 1) were designed using Primer3. Changes in target gene mRNA levels were determined in duplicate by relative RT-qPCR with a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using iQ™ SYBR Green Supermix (Bio-Rad) detection of single PCR product accumulation. The PCR conditions, the negative controls, the preparation of the standard curves and the calculation of the reaction efficiencies were as previously described [1].

**Antibodies:** An affinity-purified rabbit antibody against the RSFLREDTLYEPEPQI carboxy-terminal mouse Inpp5k peptide was obtained from Eurogentec, Liège, Belgium. Pre-incubation of the anti-Inpp5k antibody with the immunogenic peptide resulted in the disappearance of the signal in Western blot, immunoprecipitation and
immunohistochemistry analyses (data not shown). Rabbit polyclonal antibodies raised against the AQP2 (Sigma-Aldrich), phosphorylated Ser256 AQP2 (p-AQP2) [8] and NKCC2 (Millipore), sheep anti-megalin (a gift from P. Verroust, Paris, France) [9], mouse monoclonal antibodies against the M2 clone of Flag® tag (FITC-conjugated, Sigma-Aldrich) and β-actin (Sigma-Aldrich), and Alexa633-labelled secondary anti-IgG antibodies (Invitrogen, Belgium) were also used.

**Protein analysis:** Tissues were homogenized in a buffer containing NP-40 (1%), Tris-HCl pH 8 (50 mM), NaCl (150 mM), and protease inhibitors (Complete™, Roche Diagnostics GmbH). Cell lysates were obtained by solubilizing frozen pellets in lysis buffer containing Complete Mini (Roche), followed by sonication and centrifugation at 16,000 g for 1 min at 4°C. When needed, total membrane and cytosolic fractions were separated by centrifugation (100,000 g for 2h). Protein concentrations were determined using BCA protein assay (Pierce, Aalst, Belgium). Cell and tissue samples were normalized for protein levels, diluted in Laemmlli sample buffer, separated by SDS-PAGE in reducing conditions, and blotted onto nitrocellulose or PVDF membranes. After blocking with non-fat milk, membranes were incubated overnight at 4°C with the primary antibody, washed, incubated with peroxidase-labeled secondary antibody, and visualized with enhanced chemiluminescence (Perkin Elmer).

When indicated, protein analysis was performed after immunoprecipitation of the Inpp5k protein either with the anti-Flag or the anti-Inpp5k rabbit polyclonal antibodies and protein A Sepharose CL-4B (Amersham). In these conditions, a HRP-coupled anti-Flag monoclonal antibody and a biotin-coupled anti-Inpp5k polyclonal antibody were used as primary antibodies in the Western blotting. For surface biotinylation assay, 10-day old confluent monolayers of mCCD cells were incubated with Sulfo-
NHS-biotine (1.5mg/ml, Pierce) at 4°C. Excess biotine was quenched by incubation with 100mM glycine and cells were lysed in Ripa buffer containing protease inhibitors. Lysate was centrifugated and its supernatant was incubated with streptavidine-conjugated agarose droplets (Pierce) to purify apical membranes from the cytosolic fraction. Proteins were extracted from renal cortex and medulla samples as described [24].

**Malachite phosphatase assay:** Inpp5k protein was immunoprecipitated from kidney extracts as described above. Inpp5k phosphoinositide phosphatase activity was measured with a phosphate release assay using an acidic malachite green. Briefly, Inpp5k protein immunoprecipitates were incubated with 100 µM of either PtdIns(3,4,5)P3 di-C16 or PtdIns(4,5)P2 di-C16 in mixed vesicles of Ptdserine for 10 min at 37°C. Reactions were stopped by addition of EDTA. After centrifugation, supernatants were incubated with malachite green reagent for 10 min. Absorbance was measured at 650 nm. As negative controls, we used the anti-Inpp5k antibody preincubated with the immunogenic peptide before adding the protein extract, and the anti-Flag antibody without addition of any kidney extract. Inorganic phosphate release was quantified by comparison to a standard curve of KH2PO4 in dH2O.

**Lentivirus construction and production:** pWPXLd, psPAX2 and pMD2.G plasmids were obtained from D. Trono (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland). The chicken β actin promoter/enhancer, the cytomegalovirus immediate-early enhancer (altogether referred as the CAGG promoter), a β globin intron, the eGFP cDNA flanked by loxP sites and the octapeptide Flag amino-terminal-tagged mouse Inpp5k cDNA corresponding to the 42 kDa isoform of the Inpp5k protein (NM-008916) were introduced between the 2 LTR sequences in the pWPXLd plasmid. This plasmid was named
A pWPXLd/GFP/Inpp5k plasmid was obtained after transformation of 294 Cre-expressing bacteria with the pWPXLd/GFP/Inpp5k plasmid. Recombinant lentiviruses were obtained after transfection of 293T cells with the pWPXLd/GFP/Inpp5k, psPAX2 and pMD2.G plasmids. After 2 days, the lentivirus-containing supernatant was harvested, purified and concentrated by ultracentrifugation, and resuspended in KSOM embryo culture medium (Specialty Media). The concentrated lentiviral suspension was titrated by infection of 293T cells for 2 days followed by GFP expression analysis using a flow cytometer.

Production of lentigenic mice: 2-4 cell embryos were recovered from superovulated (C57BL/6 x Balb/c)F1 female mice crossed with F1 males. Embryos were depellucided with tyrode acid (Sigma) and incubated with 5 to 10 x 10^3 lentiviral particles in a drop of KSOM medium under mineral oil (Sigma). After 2 days at 37°C and 5% CO2, viable embryos were rinsed several times with M2 embryo culture medium (Sigma) and reimplanted in the uterine horns of pseudogestant CD1 females (Harlan). Newborns were screened for GFP expression in the tail by fluorescent microscopy (Nikon Eclipse TE300). GFP/Inpp5k transgenic founders were crossed with PGK-Cre mice in order to remove the GFP cDNA from the inserted transgene and to obtain Inpp5k transgenic mice. GFP/Inpp5k and Inpp5k transgenic mice were backcrossed with C57BL/6 mice for 5 generations before analysis. No Cre transgene was left in the experimental mice. Non transgenic littermates resulting from the crossing between Inpp5k transgenic mice and C57BL/6 mice, as well as age-matched GFP/Inpp5k transgenic mice served as controls. All animal studies were authorized by The Animal Care Use and Review Committee of the Université Libre de Bruxelles.
**DNA analysis:** Genomic DNA was extracted from tail tips and analyzed by Southern blotting and PCR. DNA was digested with EcoRI or SpeI, loaded on an agarose gel and transferred onto a nitrocellulose membrane. A 950 bp DNA fragment of the mouse Inpp5k cDNA was used as a radiolabeled probe. For genotyping, PCR was used to amplify a DNA fragment of eGFP or Inpp5k cDNAs present in the transgene (see Suppl. Table 1 for primer sequences).

**Renal salt and water handling protocols:** Experiments were conducted on age- and gender-matched mice. Water handling at baseline and during various protocols was assessed in individual metabolic cages, after appropriate training, as described [1, 5]. Plasma samples and 24-h urine collections were obtained at baseline, and urinary concentrating ability was tested after 24-h water deprivation. The capacity to excrete a water load was tested after i.p. injection of 2 ml of sterile water; urine was collected under a plastic-wrapped container on an hourly basis for the next 6 hours as described [1]. The acute response to vasopressin was investigated by treating mice with an i.p. injection of 1ng/g body weight of dDAVP (desmopressin) (Minirin®, Ferring, Switzerland) in 100 µl of saline i.p. and collection of urine 5h later to measure osmolality.

**Plasma and urine analyses:** Plasma urea, glucose and creatinine were measured after appropriate sample dilution with a Synchron CX5 Analyzer (Beckman Coulter), and osmolality was measured using a Fiske Osmometer (Needham Heights, MA), as described [1, 5].

**Histology and immunohistochemistry:** Kidney samples were fixed in 4% paraformaldehyde (Boehringer Ingelheim, Heidelberg, Germany) in 0.1% mol/l phosphate buffer (pH 7.4) before embedding in paraffin, and processed for staining and antibodies incubation as described [1]. Sections were viewed under a Zeiss
fluorescence measurements were performed with a x63/1.4 Plan-Apochromat oil-immersion objective (Zeiss).

**mCCD cell culture, transfection, immunostaining, response to vasopressin and fluid transport measurement:** mCCD cells were cultured on collagen-coated PTFE filter membranes as described [14]. At 60 – 80% confluency, mCCD cells were transfected or not with a pWPXLd plasmid expressing either the wild type or the catalytically-inactive Inpp5k protein by the use of Fugene HD (Roche). The catalytically inactive 42 kDa mouse Inpp5k protein was obtained after mutation of the wild type Inpp5k cDNA, according to Communi and Wada [11, 38]: the P$^{283}$, D$^{287}$ and R$^{288}$ of the catalytic domain of the Inpp5k protein were respectively mutated in A, A and G. Six to ten-day old confluent monolayer cells were used in the experiments. For immunostaining, cells were fixed, permeabilized and incubated with a goat anti-rabbit AQP2 and a monoclonal anti-Flag® antibodies coupled with Alexa633 and FITC, respectively. Nuclei were stained with DAPI. Cells were analyzed with a Zeiss LSM510Meta Confocal microscope (Zeiss, Belgium). The response to vasopressin was tested by treating the filter-seeded cells (transfected or not, as described above) with 10nM dDAVP (Sigma) for 5min in the presence of 1mM 3-isobutyl-1-methylxanthine (IBMX, Sigma). After treatment, cells were washed, incubated with sample diluent for 10min at room temperature and centrifuged at 600g at 4°C for 15min. Fifty µl of the supernatant were used to assay cAMP, using a direct cAMP enzyme immunoassay kit (Arbor Assays, Ann Arbor, USA) according to the Manufacturer's instructions. Data are based on three independent experiments performed in duplicate.

**Cellular fluid transport:** Fluid transport was determined in confluent monolayers of mCCD cells at day 10 as described previously [15]. For baseline volume
measurement, cell culture medium was changed and replaced with fixed volume of media introduced in apical and basolateral sides. After an incubation time of 2h or 15h, the apical and basolateral medium were collected and measured on a precision balance. For the effect of an osmotic gradient on fluid transport, a fixed volume of diluted culture medium (2/5) was added to the apical side, while undiluted (5/5) culture medium was added to the basolateral side. After 2h, both apical and basolateral volumes were collected and measured. HgCl2 (1mM), the aquaporins inhibitor, was added to the apical side in the presence of a concentration gradient (2/5 apical and 5/5 basolateral). After 2h, apical and basolateral volumes were collected and measured. Protein levels from each cell culture were determined with a BCA kit (Pierce), and were found similar in all conditions.

**Nephron segments microdissection:** Eight week-old C57Bl/6 mice were used for microdissection of individual nephron segments. Briefly, thin coronal kidney slices were prepared, after which cortex and medulla were separated and digested with collagenase type II (1mg/ml) for 30 min (for proximal tubules) or 1h (for other tubules) at 37°C. Then, tubule suspension was washed and placed on a stage of an inverted microscope. A total of 50 proximal tubules (S1S2 and S3), thick ascending limbs of Henle’s loop (TAL), and collecting ducts (CD) were collected and placed in 300µl RLT-buffer (Qiagen). Total RNA was extracted immediately after microdissection using the RNAeasy Micro kit (Qiagen).

**Statistics:** All values are expressed as means ± standard error of the mean (SEM). Significance of differences between two means was calculated using the GraphPad InStat software (GraphPad Software, San Diego, USA). Significance level of the differences of the means (p-value) is indicated in the legend of each figure.
Results:

Production and characterization of Inpp5k transgenic mice:

In order to study the role of mouse Inpp5k phosphatase \textit{in vivo}, a transgenic mouse expressing a Flag-tagged 42 kDa Inpp5k protein was generated by infection of embryos with a recombinant lentivirus. Since our preliminary results indicated that Inpp5k expression is ubiquitous in mouse (Suppl. informations and Suppl. Figure 1), a transgene was constructed containing the chicken $\beta$ actin promoter/enhancer and the cytomegalovirus immediate-early enhancer (altogether referred as the CAGG promoter) which are known to direct expression of the transgene in many tissues in genetically-modified mouse (Figure 1). Downstream of the CAGG promoter, the transgene contained a $\beta$ globin intron, the GFP cDNA flanked by loxP sites, and the octapeptide Flag-tagged mouse Inpp5k cDNA. The transgene was inserted into the pWPXLD lentiviral plasmid and successfully tested for mutually exclusive expression of GFP or Inpp5k proteins in 293 cells (Suppl. informations and Suppl. Figure 2). Mouse embryos were infected with lentiviral particles and reimplanted in pseudogestant females. The presence of founders in the progeny was checked at birth by tail fluorescence analysis and confirmed by PCR analysis. Out of 21 newborns, two were fluorescent and both transmitted the transgene to their progeny (data not shown). Experiments were done to demonstrate that both lentiviral transgenic lines A and B had sole transgene integration in the genome, produced the Inpp5k transgene only after Cre recombination, and that the transgenic Inpp5k protein was ubiquitously expressed and catalytically active in both lines (Suppl. informations, Suppl. Figure 3 and data not shown). We also confirmed that both PtdIns(4,5)P2 and PtdIns(3,4,5)P3 are substrates for the murine Inpp5k protein (Suppl. informations and Suppl. Figure 3). Given the higher amount of Inpp5k protein
and 5-phosphatase activity detected in tissues of transgenic line B, this line was used in the following experiments. Age-matched non transgenic littermates and transgenic line B mice expressing the GFP protein served as controls. As no significant difference was observed between both groups of control mice, a potential effect of the transgene integration site on the observed phenotype was discarded, and these 2 groups were finally pooled.

**Altered osmoregulation in Inpp5k transgenic mice:**

Inpp5k transgenic mice were fertile and had a normal life span and general behaviour. No evident macroscopic nor microscopic alterations were detected in all tissues examined (data not shown). As Inpp5k mRNA and protein have been reported to be highly expressed in the kidney [19], renal function and osmoregulation parameters at baseline were investigated. Control and Inpp5k transgenic mice were similar in terms of body weight, water intake, urinary flow rate and renal function (plasma urea and creatinine) (Suppl. Table 2). However, transgenic Inpp5k mice had a significantly lower plasma osmolality than control mice (350 ± 3.7 and 335 ± 4.3 mosmol/kg H2O in control and transgenic mice, respectively; P<0.03, 5 to 6 mice per group). These data suggested an abnormal osmoregulation in Inpp5k transgenic mice and led us to analyze the renal expression of endogenous and transgenic Inpp5k as well as water handling by the kidney in these mice.

**Inpp5k expression in control and Inpp5k transgenic mice:**

Endogenous Inpp5k mRNA expression was investigated by RT-PCR in the different nephron segments of wild-type kidneys: endogenous Inpp5k mRNA was found in all segments, with the highest expression in the proximal tubule segments (Figure 2a and b). The presence of the 44 kDa Flag-tagged Inpp5k transgenic protein was identified in both cortical and medullary fractions of Inpp5k transgenic kidneys (Figure
2c). As expected, total Inpp5k protein expression was increased in transgenic kidneys, as compared to control kidneys. Kidney sections from control and Inpp5k transgenic mice were stained for total and transgenic Inpp5k proteins with an anti-Inpp5k and an anti-Flag antibody, respectively, and analyzed at the confocal microscope (Figure 3a). The Flag-tagged transgenic Inpp5k protein was detected in the proximal tubules and the medullary collecting ducts of transgenic kidneys, but not in control kidneys, as expected. As compared with endogenous Inpp5k expression in control kidneys, total Inpp5k protein expression was increased in transgenic kidneys. Cellular co-localization of the Flag-tagged Inpp5k transgenic protein with megalin (a proximal tubule marker) and AQP2 (a collecting duct marker) confirmed the expression of the transgenic Inpp5k protein in these specific nephron segments (Figure 3b). Interestingly, the expression of AQP2 was distinctly upregulated in the medullary collecting ducts of Inpp5k transgenic kidneys, whereas megalin and NKCC2 (a thick ascending limb of Henle’s loop marker) were found unchanged, as compared with control kidneys (Figure 3b and data not shown).

**Abnormal water handling in Inpp5k transgenic mice:**

The altered plasma osmolality associated with the expression of the transgenic Inpp5k protein in specific nephron segments known to control water and/or ions transport suggested abnormal osmoregulation in Inpp5k transgenic mice. In order to substantiate this defect, we first tested the urinary concentrating ability of Inpp5k transgenic mice following a water deprivation test. The water deprivation resulted in a similar weight loss in control and Inpp5k transgenic mice (averaging 14 ± 0.4% in control mice and 13 ± 0.8% in Inpp5k transgenic mice, n = 5 pairs) and, as expected, a significant reduction in the urine volume (Suppl. Figure 4). These changes were similar in both groups of mice, indicating that the mechanisms of solute accumulation...
in the medulla (i.e. transport of Na\(^+\) and urea) allowing a maximal urinary concentration were not affected by Inpp5k overexpression. The latter hypothesis was also supported by the similar response of the control and Inpp5k transgenic mice to the loop diuretic furosemide (data not shown).

We next evaluated the capacity of the transgenic kidney to eliminate a water load by diluting urine (Figure 4a). As compared with control mice, Inpp5k transgenic mice showed a significant delay in their ability to excrete the water load, pointing to a defective handling of water in the collecting duct. The acute response to vasopressin was also investigated in these mice, revealing that the maximal urine osmolality was significantly higher in Inpp5k transgenic mice as compared with controls (3815 ± 100 vs. 2561 ± 254 mOsm/kg H\(_2\)O, P < 0.005)(Figure 4b). Together, the lower plasma osmolality, impaired ability to excrete a water load and increased response to dDAVP indicated that lnpp5k transgenic mice had a significant defect in osmoregulation, which could be related to a specific effect of lnpp5k on the vasopressin/AQP2 axis in the kidneys.

**Abnormal arginine vasopressin (AVP) signalling pathway in lnpp5k transgenic mice:**

In order to investigate the mechanism of altered water handling in lnpp5k transgenic mice, real time RT-PCR was first used to test for the differential expression of transcripts that are primarily involved in the AVP signalling pathway, including AVPR2 receptor and AQP2 in the kidney, and AVP in the brain (Figure 4c). The expression of AVPR2 and AQP2 mRNA were significantly increased in the kidneys of the lnpp5k transgenic mice as compared with controls. By contrast, the kidney expression of transcripts corresponding to pathways involved in Na\(^+\) or K\(^+\) handling (e.g. Na\(^+\)/K\(^+\)-ATPase and the different ENaC subunits) was unchanged (Figure 4b).
In the brain, AVP mRNA expression was found significantly decreased in Inpp5k transgenic mice, as expected from a feedback response of the lower plasma osmolality values (Figure 4c). We next investigated AQP2 protein expression and phosphorylation in transgenic kidneys by Western blotting (Figure 4d and 4e). Immunoblot analyses essentially confirmed the immunostaining results presented in Figure 3b: a significant overexpression of AQP2 protein was detected in the transgenic kidney as compared with control kidney. The proportion of phosphorylated AQP2 protein was also significantly increased in Inpp5k transgenic kidney (Figure 4e).

Together, these results indicate that the osmoregulation defect detected in Inpp5k transgenic mice is caused by alteration in the AVPR2/AQP2 signalling pathway in kidney collecting ducts.

AQP2 and AVPR2 expression, cAMP production and water transport in mouse collecting duct cells overexpressing Inpp5k:

The mouse CCD (mCCD) cells represent a well established model to investigate the mechanisms of water reabsorption in the collecting duct. In order to confirm and substantiate the link between Inpp5k overexpression and the water handling defect observed in mice, mCCD cells were plated on filters and transfected or not with either a catalytically active or inactive Inpp5k expression vector. The cells were then cultured to confluency to develop a polarized monolayer. Transfection of mCCD cells with wild type Inpp5k resulted in higher AQP2 protein expression and plasma membrane localization than in non-transfected cells (Figure 5a-c). An intact Inpp5k enzymatic activity was required to observe the AQP2 expression and localization defects, since transfection of mCCD cells with a catalytically-inactive Inpp5k protein had no effect (Figure 5). Further studies demonstrated that cells transfected with
wild-type Inpp5k had a significantly higher mRNA expression of AVPR2 (Figure 5d), which was reflected by a significantly (> 2-fold) accentuated cAMP response to treatment with dDAVP (Figure 5e). Again, these changes were not observed in cells transfected with the catalytically inactive Inpp5k.

These AQP2 and AVPR2 studies were correlated with measurement of net water transport across polarized mCCD cell monolayers (Figure 6). Indeed, two and 15h after placing iso-osmotic media on both sides of the mCCD cell monolayer, an increased water transport was detected in wild-type Inpp5k-transfected cells, as compared with non transfected and catalytically inactive Inpp5k transfected cells. When a hypo-osmotic medium was placed at the apical side of the mCCD cell monolayer, water transport was increased in all three conditions tested. However, water transport was still significantly increased in wild type Inpp5k transfected cells (Figure 6). Addition of HgCl2, which blocks AQP2 by interacting with a critical cysteine residue in the water pore, nearly completely blocked the osmotically-induced water transport in all cells tested (Figure 6). Together, these results indicate that the active form of Inpp5k tightly controls AQP2 expression and plasma membrane localization, as well as the osmotically-induced water transport in mouse collecting duct cells.
Discussion:

We report here that transgenic mice overexpressing the phosphoinositide 5-phosphatase Inpp5k present an altered water metabolism characterized by a decreased plasma osmolality in basal conditions, a decreased ability to excrete a water load, and an increased urinary concentrating response to arginin vasopressin. These physiological alterations are associated with defects in the AVPR2/AQP2 signalling pathway in the collecting ducts of the kidney. Indeed, overexpression of wild type, but not that of catalytically-inactive Inpp5k in mouse collecting duct cells results in an increased water transport associated with increased AVPR2 expression and cAMP response to vasopressin, and with increased AQP2 expression and plasma membrane localization, providing a mechanism to explain the defects observed in Inpp5k transgenic mice. Together, we conclude that Inpp5k as an important regulator of the AVPR2/AQP2 signalling pathway and water metabolism.

Inpp5k is a member of the phosphoinositide 5-phosphatases family with catalytic activity directed towards PtdIns(4,5)P2 and PtdIns(3,4,5)P3 [19-21, 37]. Our Northern blot, RT-PCR and Western blot analyses have identified mouse Inpp5k as an ubiquitously expressed phosphatase. Several Inpp5k mRNA and protein molecular weight forms have been detected in mouse tissues, as in man [19]. In the kidney, Inpp5k mRNA is also differentially expressed along the nephron and the collecting duct, and 3 molecular forms were identified in the cortex and the medulla. This very complex pattern of expression is probably of significant relevance and must serve specific physiological function for the Inpp5k enzyme in the different regions of the kidney and other tissues.

Recently, PpsBrdm1 mice with a frameshift mutation in the Inpp5k/Pps coding sequence downstream of exon 7 have been generated [21]. The homozygous
Pps\textsuperscript{Brdm1} mutation caused embryonic lethality of unknown origin. In adult heterozygous Pps\textsuperscript{Brdm1/+} mice, a significant increase in insulin sensitivity was observed in skeletal muscles, and these mice were also resistant to a high fat diet-induced obesity. By contrast, no difference was detected between control and Pps\textsuperscript{Brdm1/+} mice for insulin signalling in the adipose tissue and for hepatic glucose production following insulin administration. Although our transgenic mice expressed high amount of catalytically active Inpp5k in skeletal muscles, no significant difference was found in insulin and glucose tolerance tests as compared to control mice (data not shown). This negative result is rather surprising, because overexpression of Inpp5k in insulin-stimulated CHO cells was reported to inhibit phosphoinositide 3-kinase signalling by reducing PtdIns(3,4,5)P3 concentrations and downstream signalling [20]. In addition, we expected to observe a frank insulin resistance in our transgenic mice which mirrored the increase in insulin sensitivity observed in Pps\textsuperscript{Brdm1/+} mice. The possible reason(s) of this discrepancy between our results and those of Takenawa are currently unknown. It could result from the use of different Inpp5k molecular weight forms with potentially different function and/or 5-phosphatase activities to express Inpp5k in our transgenic mice (42 kDa) and in CHO cells (approximately 50 kDa). Alternatively, the expression of the catalytically active Inpp5k protein in muscles of our transgenic mouse is may be not sufficient to reproduce the \textit{in vitro} effects or to mirror the phenotype detected in the Pps\textsuperscript{Brdm1/+} mice. Indeed, the presence of compensatory mechanisms involving other phosphoinositide phosphatases expressed in the muscles of our transgenic mice cannot be ruled out, similarly to the compensation of the absence of the OCRL protein by the 75 kDa protein [23].
Arginine vasopressin, AVPR2 and AQP2 are key components in the signalling pathway involved in the control of the final urine concentration by the principal cells lining the collecting ducts. Under basal conditions, AQP2-containing vesicles are maintained in the subapical region of the cells. When the organism is exposed to a loss of free water, plasma osmolality increases, stimulating the release of AVP by the posterior pituitary into the circulation. In turn, AVP binds to the AVPR2 on principal cells, leading to increased cAMP levels stimulating the expression of AQP2 as well as the phosphorylation and shuttle of AQP2 from subapical storage vesicles to the apical membrane, thereby increasing the osmotically-induced water transport. Our data demonstrate that AVPR2, AQP2 and p-AQP2 expression is upregulated in Inpp5k transgenic kidneys. The overexpression of these three factors in collecting duct cells would substantiate an increase in water reabsorption and the subsequent reduced basal osmolality and delayed ability to excrete a water load. The fact that this phenotype results from a primary renal deficit is confirmed by the physiological response of the brain, i.e. a decreased AVP mRNA expression secondary to lower plasma osmolality. It is noteworthy that the AQP2 mRNA and protein expressions are distinctly upregulated in the medullary collecting ducts of Inpp5k transgenic mice, whereas markers of other segments (i.e. megalin and NKCC2) are found unchanged. Furthermore, our studies in the mCCD cells demonstrate that Inpp5k overexpression alone, and not its catalytic mutant, is sufficient to upregulate AVPR2, increasing the cAMP response to dDAVP, which could then explain the observed increase in AQP2 expression and osmotic water transport. Unfortunately, we were unable to investigate the effects of a decreased Inpp5k protein expression on the AVP/AQP2 signalling pathway in this cellular system, because of the different kinetics of AQP2 expression and siRNA-induced Inpp5k downregulation in mCCD cells (data not shown).
Interestingly, no proximal tubule phenotype was detected in Inpp5k transgenic mice, despite expression of the transgene in this nephron segment. This may be related to the fact that the AQP1 water channels expressed in these tubules are constitutively inserted in the plasma membrane, without trafficking regulation nor response to AVP. The mechanism leading to increased expression of AVPR2 and AQP2 mRNA as well as to increased AQP2 plasma membrane localization in collecting duct cells overexpressing Inpp5k certainly requires an intact phosphoinositide 5-phosphatase activity. Phosphoinositides signalling occurs in many different intracellular membranes, including the inner surface of the plasma membrane and the membrane vesicles that move to the plasma membrane, providing a potential explanation for the increased AQP2 expression at the cell surface of collecting duct cells when catalytically active Inpp5k is overexpressed. It is noteworthy that a still poorly defined nuclear phosphoinositide signalling pathway, including PtdIns(4,5)P2 signalling, also occurs and has been shown to control gene expression, mRNA export as well as chromatin remodelling [4, 27]. The overexpression of the inositol Inpp5k 5-phosphatase in mCCD cells represents thus a new model to understand how phosphoinositides enter the nucleus and impact on nuclear processes.

In conclusion, we have here for the first time identified a role for the inositol 5-phosphatase Inpp5k in the mouse kidney: Inpp5k controls plasma osmolality via the regulation of the AVPR2/AQP-2 signalling pathway and of water transport in kidney collecting duct cells. Although the role of Inpp5k in the human kidney has not been investigated, our data suggest that Inpp5k could be important in patients with inappropriate water handling by the collecting duct.
References:


Inpp5b and Ocr1 may explain why deficiency of the murine ortholog for OCRL1 does not cause Lowe syndrome in mice. J Clin Invest 101:2042-2053.


Acknowledgments:

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Figure Legends:

**Figure 1: Inpp5k transgene structure.** Transgene structure before (a) and after (b) Cre recombination showing the CAGG promoter (including the CMV enhancer), the β globin intron, a first loxP site, the enhanced green fluorescent protein (GFP) cDNA, a second loxP site, and the Flag-tagged mouse Inpp5k cDNA. A viral LTR is present at both transgene extremities.

**Figure 2: Inpp5k expression in the kidney of control and Inpp5k transgenic mice.** (a) and (b) Comparative expression of endogenous Inpp5k mRNA in different nephron segments from wild-type kidney. The expression level of Inpp5k was analyzed by quantitative RT-PCR. Nephron segments are characterized by the enrichment for respective markers (b): podocin for glomeruli (Gl), AQP-1 for the S1S2 and S3 segments of the proximal tubule, NKCC2 for the thick ascending limb (TAL) and AQP-2 for the collecting duct (CD). (c) Representative immunoblot on cortical and medullary fractions of wild-type (ctrl) and Inpp5k transgenic (Tg) mice. Cytosolic proteins (20 µg) were separated by SDS-PAGE and blotted onto nitrocellulose membrane, before incubation with anti-Inpp5k (1/1000) or anti-Flag (1/10000) affinity-purified antibodies. Loading control was performed after membrane stripping and incubation with monoclonal anti-β-actin antibody (1/10 000).

**Figure 3: Inpp5k immunostaining on kidney sections from control and Inpp5k transgenic mice.** Control and Inpp5k transgenic (Tg) kidneys were stained with (a) anti-Inpp5k (1/200) and anti-Flag (1/100) antibodies, and with (b) anti-megalin (1/10000), anti-AQP2 (1/200) and anti-Flag (1/100) antibodies. The transgenic Inpp5k protein is located in the proximal tubules (cytoplasmic pattern, with expression of megalin in the apical membrane) and in the medullary collecting ducts (co-expression
with AQP2, which is distinctly upregulated in these profiles). Total Lnpp5k expression and AQP2 expression are increased in Lnpp5k transgenic kidneys. Bar: 10µm.

**Figure 4: Abnormal water handling, acute response to vasopressin and renal AQP2 expression in Lnpp5k transgenic mice:**

(a) The urine output was analyzed in control (white bars) and Lnpp5k transgenic (black bars) mice during 4 h in a test of acute water loading (2 ml intraperitoneally). Results are means ± SEM of 6 pairs of 5-month old male mice and are expressed as ml of urine excreted over the last 1h interval. Statistics: *: P<0.05.

(b) Effect of dDAVP treatment on urine osmolality in control and Lnpp5k transgenic mice. After adaptation and baseline collection, mice were treated with 1ng/g body weight of dDAVP (MinirinR) and urine was collected 5h later to measure osmolality (Uosmo). The two groups of mice show similar urine osmolality values at baseline, whereas treatment with dDAVP induces a significantly higher response in the Lnpp5k transgenic mice vs. controls. Data were obtained on 4 pairs of control vs. transgenic Lnpp5k mice. Statistics: **: P<0.005.

(c) Real time RT-PCR quantification of mRNA expression in the kidney and the brain. Messenger RNA levels were first adjusted to glyceraldehydes-3-phosphate dehydrogenase (GAPDH), then normalized to the control mice level set at 100% using the following formula: Ratio = 2^ΔCt(GAPDH – target Lnpp5k transgenic mice) - ΔCt(GAPDH – target control mice). The normalized values (mean ± SEM) are shown on the right of the panel. Eight pairs of kidney and brain from 5-month old male mice were analyzed. Statistics: *: P<0.05.

(d) Western blot analysis of AQP2, phosphorylated AQP2 (p-AQP2) and actin (as a loading control) in kidney extracts from control (C) and Lnpp5k transgenic mice (Lnpp5k). Five pairs of kidney from 5-month old male mice were analyzed. (e) Densitometry analyses (core and glycosylated bands) confirm that there is a significant increase of both AQP2 and p-AQP2 proteins in the kidney extracts from
Inpp5k transgenic (black bars) mice, as compared with control (white bars) mice. Results are means ± SEM and are expressed as a ratio between AQP2 or p-AQP2 signals and actin signal. Statistics: *: P<0.05; **: P<0.005.

Figure 5: Increased AQP2 and Avpr2 expression and dDAVP-induced cAMP production in Inpp5k-transfected mCCD cells:

(a) Non transfected, wild type or catalytically inactive Inpp5k-transfected mCCD cells were cultured to confluency on filters to obtain a polarized cell monolayer. Anti-AQP2 (red) and anti-Inpp5k (green) antibodies were incubated with permeabilized cells and analyzed by confocal microscopy. (b) Western blot analysis of non transfected and wild type or catalytically-inactive Inpp5k-transfected mCCD cell protein extracts with an anti-AQP2 antibody. Actin served as loading control. (c) Plasma membrane of 10-day old confluent mCCD cells under control conditions (c) or after transfection with either wild type Inpp5k (In) or catalytically-inactive Inpp5k (inIn) were obtained after biotinylation assay. Plasma membrane and cytosol fraction were analysed by Western blotting with an anti-AQP2 antibody. Equal amounts of protein (20 µg/lane) were charged. (d) Increased expression of Avpr2 in Inpp5k-transfected mCCD cells. Quantification (real-time PCR) of target mRNA in mCCD, expressed as relative expression over non transfected cells (n=4). Significant differences (*** p<0.001) between different conditions are indicated. (e) Effects of dDAVP (10nM) on cAMP production in mCCD cells transfected with Mock or inactive Inpp5k or Inpp5k plasmid. mCCD grown on filter membranes were pretreated with 1mM 3-isobutyl-1-methylxanthine (IBMX) for 10min. Where indicated (+), cells were stimulated with 10nM of dDAVP for 5min in the continuous presence of IBMX. Cells were lysed and subjected to cAMP measurements. The mean cAMP concentration (cAMP±/− SEM (in pmol/ml)) was determined from three independent experiments performed in
duplicate (6 filters per condition). Significant differences (* p<0.05; ** p<0.01; *** p<0.001) between different conditions are indicated. Data are mean ± SEM.

**Figure 6: Increased water transport in Inpp5k transfected mCCD cells:** Non transfected and wild type or catalytically inactive Inpp5k transfected mCCD cell monolayer were incubated with iso- (baseline) or hypo- (osmotic gradient) osmotic medium at the apical side. After 2 and 15h, medium volumes at the apical and basolateral sides of the cell monolayer were measured and the net water transport calculated. The aquaporins inhibitor HgCl2 (1 mM) was added to the medium at the apical side of the cell monolayer. For each condition, results are representative of 6 independent experiments performed in triplicate. Mean ± SEM are represented. Statistics: *: P<0.001 when comparing wild type Inpp5k-transfected cells with either non-transfected or catalytically inactive Inpp5k-transfected cells.
Supplementary Table 2: Baseline parameters, renal function and water metabolism in Inpp5k transgenic mice

<table>
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<th>Control mice</th>
<th>Inpp5k transgenic mice</th>
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<tr>
<td>Body weight (g)</td>
<td>31.0 ± 0.6 (16)</td>
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<td>Water Intake (ml/24h)</td>
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<td>Urine volume (µl/24h)</td>
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<td>Plasma Urea (mg/dl)</td>
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<tr>
<td>Plasma Creatinine (mg/dl)</td>
<td>0.098 ± 0.007 (5)</td>
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Data are means ± SEM. Numbers in parentheses refer to number of mice.
Supplementary information:

**Inpp5k expression in mouse tissues and cells:**

Tissue distribution of the mouse Inpp5k mRNA was investigated by Northern blotting and RT-PCR. As previously reported for human Inpp5k, 2 different transcripts of about 3.0 and 2.2 kb were detected in nearly all tissues tested (Suppl. Figure 1a). A very strong signal was observed in the testis, where an additional 2.4 kb transcript was detected. Expression was particularly important in eye and kidney, and nearly undetectable in the liver. RT-PCR performed on the same mRNA preparations confirmed the Northern blot results (data not shown). Inpp5k mRNA was also detected by PCR in cDNA preparations from hematopoietic cells, including resting and activated purified T and B lymphocytes, macrophages and neutrophils, as well as mature and immature dendritic cells, eosinophils and mast cells (Suppl. Figure 1B and data not shown). Next, tissue protein extracts were probed by Western blotting with an immunizing peptide affinity-purified rabbit antibody raised against the C-terminal 16 amino acids of the Inpp5k mouse protein. A total of four molecular weight forms were detected. In most of the tissues analyzed, polypeptides of 54 kDa and/or 42 kDa were clearly detected. Expression was very significant in testis, eye and spleen, and nearly undetectable in muscle and heart (Suppl. Figure 1C). However, prolonged exposure of the membrane confirmed the presence of a 42 kDa polypeptide in the two latter tissues (data not shown). A third 51 kDa immunoreactive signal was specifically observed in testis and brain. Finally, in the kidney, a 36 kDa polypeptide was also expressed in addition to the 42 kDa polypeptide (Suppl. Figure 1C). No signal was observed in Western blotting when the Inpp5k antibody was preincubated with the immunogenic peptide (data not shown).
GFP and Inpp5k proteins expression after pWPXLD lentiviral plasmid transfection in 293 cells:

293 cells were transfected either with the pWPXLD/GFP/Inpp5k plasmid containing both GFP and Inpp5k cDNAs, or with the pWPXLD/Inpp5k plasmid obtained after Cre recombination. As expected, cells transfected with the pWPXLD/GFP/Inpp5k plasmid were fluorescent but did not express the mouse Inpp5k protein, while pWPXLD/Inpp5k-transfected cells were not fluorescent but did express a 44 kDa Flag-tagged Inpp5k protein (Suppl. Figure 2).

Characterization of GFP- and Inpp5k-transgenic mouse lines:

Southern blot analysis using several restriction enzymes and a radiolabeled mouse Inpp5k cDNA probe revealed that, as compared to non transgenic control DNA, DNA extracted from both transgenic lines had an extra signal which corresponded to a unique transgene integration site in the genome (data not shown). In order to remove the GFP cDNA out of the genome and to induce the expression of the Flag-tagged Inpp5k protein, males from both lines were crossed with female PGK-Cre mice which express the Cre recombinase in all tissues early during embryogenesis. Immunoprecipitation followed by Western blotting revealed that no transgenic Inpp5k protein was expressed in the heart of GFP mice before Cre recombination, and that Cre recombination induced the production of a 44 kDa Flag-tagged Inpp5k protein only in Inpp5k mice (Suppl. Figure 3a). Similar results were obtained on kidney protein extracts (data not shown). Western blot analysis of the 44 kDa Flag-tagged transgenic Inpp5k protein in multiple tissues showed that the expression was rather ubiquitous (Suppl. Figure 3b). Finally, PtdIns(4,5)P2 and PtdIns(3,4,5)P3 phosphatase activities were analyzed in kidney protein extracts from non transgenic control and Inpp5k transgenic lines (Suppl. Figure 3c). Phosphatase activity was
significantly detected in the total kidney protein extracts from both Inpp5k transgenic lines after immunoprecipitation with the anti-Flag antibody. These data reflect thus the phosphoinositide phosphatase activity of the transgenic Inpp5k protein only. As expected, no significant phosphate release was detected in non transgenic control mice (Suppl. Figure 3c). In the anti-Inpp5k immunoprecipitates, phosphatase activity was detected with both substrates in non transgenic control mice, indicating the presence of the endogeneous Inpp5k protein. However, in both Inpp5k transgenic lines, activity measured with the two substrates was higher than in control mice, reaching significantly higher levels in transgenic line B kidney extracts (Suppl. Figure 3c). Preincubation of the anti-Inpp5k antibody with the immunogenic peptide before immunoprecipitation nearly completely prevented phosphatase activity from kidney extracts (data not shown).
Supplementary Figure Legends:

Supplementary Figure 1: Inpp5k mRNA and protein expression in mouse tissues and cells. (a, b) Inpp5k mRNA expression was analyzed by Northern blot in adult C57BL/6 mouse tissues using a Inpp5k cDNA probe (a), and by RT-PCR in purified mouse hematopoietic cells (b). T: thymus; B: brain; E: eye; H: heart; L: liver; AG: adrenal gland; K: kidney; Te: testis; O: ovary; AT: adipose tissue; TL: T lymphocyte; BL: B lymphocyte; M: macrophage; N: neutrophil; mDC and iDC: mature and immature dendritic cells.(-) and (+) represent resting and activated cells. C- and C+ are negative and positive controls for RT-PCR. Hprt: a cDNA probe encoding for hypoxanthine-guanine phosphoribosyltransferase served as loading control (c) Inpp5k protein expression was analyzed by Western blotting using an affinity-purified rabbit polyclonal anti-mouse Inpp5k antibody. An anti-actin antibody served as loading control. E: eye; H: heart; B: brain; Lu: lung; I: intestine; S: spleen; K: kidney; Te: testis; SM: skeletal muscle; O: ovary.

Supplementary Figure 2: Characterization of lentiviral vectors used for transgenic mouse production. Mutually exclusive expression of GFP and Flag-tagged Inpp5k proteins in 293 cells after transfection with either the pWPXLd/GFP/Inpp5k plasmid, or the pWPXLd/Inpp5k plasmid obtained after Cre recombination. Fluorescence analysis (left and middle panels) and Western blot analysis using an anti-Flag antibody (right panel). NT: not transfected.

Supplementary Figure 3: Characterization of Inpp5k transgenic mice: (a) Cre recombination induces the expression of the transgenic Inpp5k protein. Western blot analysis with an anti-Flag tag antibody shows that Inpp5k transgenic mice produce the Flag-tagged Inpp5k protein (44 kDa) in the heart. No signal is detected in GFP transgenic mice before Cre recombination nor in non transgenic control (C) mice. (b)
Expression of the Flag-tagged transgenic Inpp5k protein in tissues from Inpp5k transgenic mice by Western blotting. Loading control was checked with an anti-actin antibody. E: eye; T: thymus; H: heart; Lu: lung; L: liver; S: spleen; I: intestine; K: kidney; LN: lymph node; B: brain; Te: testis. (c) PtdIns(4,5)P2 and PtdIns(3,4,5)P3 5-phosphatase activities in anti-Flag or anti-Inpp5k immunoprecipitates from total kidney protein extracts isolated from transgenic lines A (LA) and B (LB), as well as from non transgenic control mice (C). Results are means ± SEM of 3 separate experiments. IP: immunoprecipitate. Statistics, as compared with non transgenic control mice: *: P<0.05; **: P<0.01; ***: P<0.005.

Supplementary Figure 4: Water deprivation test: Urine output was measured during a 24-h baseline (white columns) and a 24-h water deprivation (black columns) period in control (C) and Inpp5k transgenic (Inpp5k) mice. Results are means ± SEM of 5 pairs of 4-month old male mice and are expressed in µl of urine output per minute and per g of body weight.
Figure 1: Inpp5k transgene structure. Transgene structure before (a) and after (b) Cre recombination showing the CAGG promoter (including the CMV enhancer), the β globin intron, a first loxP site, the enhanced green fluorescent protein (GFP) cDNA, a second loxP site, and the Flag-tagged mouse Inpp5k cDNA. A viral LTR is present at both transgene extremities.
Inpp5k - Gl S1S2 S3 TAL CD H2O

Expression level (Inpp5k/GAPDH, % of S3)

Podocin - AQP1 - NKCC2

Expression level (Inpp5k/GAPDH, % of S3)

Cortex medulla

ctrl Tg ctrl Tg

Inpp5k Flag β-actin

kDa

- 55 - 33 - 44 - 45
Figure 2: Inpp5k expression in the kidney of control and Inpp5k transgenic mice. (a) and (b) Comparative expression of endogenous Inpp5k mRNA in different nephron segments from wild-type kidney. The expression level of Inpp5k was analyzed by quantitative RT-PCR. (b) Nephron segments are characterized by the enrichment for respective markers: podocin for glomeruli (Gl), AQP-1 for the S1S2 and S3 segments of the proximal tubule, NKCC2 for the thick ascending limb (TAL) and AQP-2 for the collecting duct (CD). (c) Representative immunoblot on cortical and medullary fractions of wild-type (ctrl) and Inpp5k transgenic (Tg) mice. Cytosolic proteins (20 µg) were separated by SDS-PAGE and blotted onto nitrocellulose membrane, before incubation with anti-Inpp5k (1/1000) or anti-Flag (1/1000) affinity-purified antibodies. Loading control was performed after membrane stripping and incubation with monoclonal antibodies anti-β-actin (1/10 000).
Cortex Medulla

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- **Cortex**:
  - Megalin: control (red), Tg (red)
  - Flag: control (green), Tg (green)

- **Medulla**:
  - Megalin: control (red), Tg (red)
  - Flag: control (green), Tg (green)
Figure 3: Inpp5k immunostaining on kidney sections from control and Inpp5k transgenic mice. Control and Inpp5k transgenic (Tg) kidneys were stained with (a) anti-Inpp5k (1/200) and anti-Flag (1/100) antibodies, and with (b) anti-megalin (1/10000), anti-AQP2 (1/200) and anti-Flag (1/100) antibodies. The transgenic Inpp5k protein is located in the proximal tubules (cytoplasmic pattern, with expression of megalin in the apical membrane) and in the medullary collecting ducts (co-expression with AQP2, which is distinctly upregulated in these profiles). Total Inpp5k expression and AQP2 expression are increased in Inpp5k transgenic kidneys. Bar: 10µm.
**a** Urine output (ml/last 1-h interval) after water load.

**b** Usmo (osmolality) in Control and Inpp5k conditions.

**c** Graph showing protein expression levels in different conditions.

**d** Western blot analysis of AQP-2 and p-AQP-2 proteins in C and Inpp5k conditions.

**e** Optical density/actin of AQP-2 and p-AQP-2 proteins.
Figure 4: Abnormal water handling, acute response to vasopressin and renal AQP2 expression in Inpp5k transgenic mice: (a) The urine output was analyzed in control (white bars) and Inpp5k transgenic (black bars) mice during 4 h in a test of acute water loading (2 ml intraperitoneally). Results are means ± SEM of 6 pairs of 5-month old male mice and are expressed as ml of urine excreted over the last 1h interval. Statistics: *: P<0.05. (b) Effect of dDAVP treatment on urine osmolality in control and Inpp5k transgenic mice. After adaptation and baseline collection, mice were treated with 1ng/g body weight of dDAVP (Minirin®) and urine was collected 5h later to measure osmolality (Uosmo). The two groups of mice show similar urine osmolality values at baseline, whereas treatment with dDAVP induces a significantly higher response in the Inpp5k transgenic mice vs. controls. Data were obtained on 4 pairs of control vs. transgenic Inpp5k mice. Statistics: **: P<0.005. (c) Real time RT-PCR quantification of mRNA expression in the kidney and the brain. Messenger RNA levels were first adjusted to glyceraldehydes-3-phosphate dehydrogenase (GAPDH), then normalized to the control mice level set at 100% using the following formula: Ratio = $2^{\Delta\text{Ct}(\text{GAPDH} - \text{target Inpp5k transgenic mice}) - \Delta\text{Ct}(\text{GAPDH} - \text{target control mice})}$. The normalized values (mean ± SEM) are shown on the right of the panel. Eight pairs of kidney and brain from 5-month old male mice were analyzed. Statistics: *: P<0.05. (d) Western blot analysis of AQP2, phosphorylated AQP2 (p-AQP2) and actin (as a loading control) in kidney extracts from control (C) and Inpp5k transgenic mice (Inpp5k). Five pairs of kidney from 5-month old male mice were analyzed. (e) Densitometry analyses (core and glycosylated bands) confirm that there is a significant increase of both AQP2 and p-AQP2 proteins in the kidney extracts from Inpp5k transgenic (black bars) mice, as compared with control (white bars) mice. Results are means ± SEM and are expressed as a ratio between AQP2 or p-AQP2 signals and actin signal. Statistics: *: P<0.05; **: P<0.005.
AQP-2 (red) Inpp5k (green)

AQP-2 (red)

Inpp5k (green)

- Actin

mCCD Inpp5k inactive

AQP-2 (red) Inpp5k

AQP-2 (red)

Inpp5k

mCCD mCCD + Inpp5k

Inactive Inpp5k

Expression levels (target gene / GAPDH, % of non-tranfected cells)

Non-tranfected

Inactive Inpp5k

Inpp5k

Non-tranfected

Inactive Inpp5k

Inpp5k

Avpr2

Avpr1a

Expression levels (target gene / GAPDH, % of non-tranfected cells)

AQP-2

Actin

mCCD Inpp5k

Inactive Inpp5k

0% 100% 200%

Expression levels (target gene / GAPDH, % of non-tranfected cells)

dDAVP (10nM) - + - + - +

dAMP +/- SEM (pmol/ml)

Mock Inactive Inpp5k Inpp5k

6 ± 2 82 ± 4 58 ± 9 127 ± 12
Figure 5: Increased AQP2 and Avpr2 expression and dDAVP-induced cAMP production in Inpp5k-transfected mCCD cells:

(a) Non transfected, wild type or catalytically inactive Inpp5k-transfected mCCD cells were cultured to confluency on filters to obtain a polarized cell monolayer. Anti-AQP2 (red) and anti-Inpp5k (green) antibodies were incubated with permeabilized cells and analyzed by confocal microscopy. (b) Western blot analysis of non transfected and wild type or catalytically-inactive Inpp5k-transfected mCCD cell protein extracts with an anti-AQP2 antibody. Actin served as loading control. (c) Plasma membrane of 10-day old confluent mCCD cells under control conditions (c) or after transfection with either wild type Inpp5k (In) or catalytically-inactive Inpp5k (inIn) were obtained after biotinylation assay. Plasma membrane and cytosol fraction were analysed by Western blotting with an anti-AQP2 antibody. Equal amounts of protein (20 µg/lane) were charged. (d) Increased expression of Avpr2 in Inpp5k-transfected mCCD cells. Quantification (real-time PCR) of target mRNA in mCCD, expressed as relative expression over non transfected cells (n=4). Significant differences (*** p<0.001) between different conditions are indicated. (e) Effects of dDAVP (10nM) on cAMP production in mCCD cells transfected with Mock or inactive Inpp5k or Inpp5k plasmid. mCCD grown on filter membranes were pretreated with 1mM 3-isobutyl-1-methylxanthine (IBMX) for 10min. Where indicated (+), cells were stimulated with 10nM of dDAVP for 5min in the continuous presence of IBMX. Cells were lysed and subjected to cAMP measurements. The mean cAMP concentration (cAMP+/− SEM (in pmol/ml)) was determined from three independent experiments performed in duplicate (6 filters per condition). Significant differences (* p<0.05; ** p<0.01; *** p<0.001) between different conditions are indicated. Data are mean ± SEM.
Non transfected mCCD cells

Inactive Inpp5k-transfected mCCD cells

µl/0.33 cm²)

Net water transport (µl/0.33 cm²)

Non transfected mCCD cells
Inpp5k-transfected mCCD cells
Inactive Inpp5k-transfected mCCD cells

+ HgCl₂

15h 2h
baseline osmotic gradient

Figure 6: Increased water transport in Inpp5k transfected mCCD cells: Non transfected and wild type or catalytically inactive Inpp5k transfected mCCD cell monolayer were incubated with iso- (baseline) or hypo- (osmotic gradient) osmotic medium at the apical side. After 2 and 15h, medium volumes at the apical and basolateral sides of the cell monolayer were measured and the net water transport calculated. The aquaporins inhibitor HgCl₂ (1 mM) was added to the medium at the apical side of the cell monolayer. For each condition, results are representative of 6 independent experiments performed in triplicate. Mean ± SEM are represented. Statistics: *: P<0.001 when comparing wild type Inpp5k-transfected cells with either non-transfected or catalytically inactive Inpp5k-transfected cells.
Supplementary Figure 1: Inpp5k mRNA and protein expression in mouse tissues and cells. (a, b) Inpp5k mRNA expression was analyzed by Northern blot in adult C57BL/6 mouse tissues using an Inpp5k cDNA probe (a), and by RT-PCR in purified mouse hematopoietic cells (b). T: thymus; B: brain; E: eye; H: heart; L: liver; AG: adrenal gland; K: kidney; Te: testis; O: ovary; AT: adipose tissue; TL: T lymphocyte; BL: B lymphocyte; M: macrophage; N: neutrophil; mDC and iDC: mature and immature dendritic cells. (-) and (+) represent resting and activated cells. C− and C+ are negative and positive controls for RT-PCR. Hprt: a cDNA probe encoding for hypoxanthine-guanine phosphoribosyltransferase served as loading control (c) Inpp5k protein expression was analyzed by Western blotting using an affinity-purified rabbit polyclonal anti-mouse Inpp5k antibody. An anti-actin antibody served as loading control. E: eye; H: heart; B: brain; Lu: lung; I: intestine; S: spleen; K: kidney; Te: testis; SM: skeletal muscle; O: ovary.
Supplementary Figure 2: Characterization of lentiviral vectors used for transgenic mouse production. Mutually exclusive expression of GFP and Flag-tagged Inpp5k proteins in 293 cells after transfection with either the pWPXLd/GFP-Inpp5k plasmid, or the pWPXLd/Inpp5k plasmid obtained after Cre recombination. Fluorescence analysis (left and middle panels) and Western blot analysis using an anti-Flag antibody (right panel). NT: not transfected.
Supplementary Figure 3: Characterization of Inpp5k transgenic mice: (a) Cre recombination induces the expression of the transgenic Inpp5k protein. Western blot analysis with an anti-Flag tag antibody shows that Inpp5k transgenic mice produce the Flag-tagged Inpp5k protein (44 kDa) in the heart. No signal is detected in GFP transgenic mice before Cre recombination nor in non transgenic control (C) mice. (b) Expression of the Flag-tagged transgenic Inpp5k protein in tissues from Inpp5k transgenic mice by Western blotting. Loading control was checked with an anti-actin antibody. E: eye; T: thymus; H: heart; Lu: lung; L: liver; S: spleen; I: intestine; K: kidney; LN: lymph node; B: brain; Te: testis. (c) PtdIns(4,5)P2 and PtdIns(3,4,5)P3 5-phosphatase activities in anti-Flag or anti-Inpp5k immunoprecipitates from total kidney protein extracts isolated from transgenic lines A (LA) and B (LB), as well as from non transgenic control mice (C). Results are means ± SEM of 3 separate experiments. IP: immunoprecipitate. Statistics, as compared with non transgenic control mice: *: P<0.05; **: P<0.01; ***: P<0.005.
Supplementary Figure 4: Water deprivation test: Urine output was measured during a 24-h baseline (white columns) and a 24-h water deprivation (black columns) period in control (C) and Inpp5k transgenic (Inpp5k) mice. Results are means ± SEM of 5 pairs of 4-month old male mice and are expressed in µl of urine output per minute and per g of body weight.