# Analysis of Wt1 function using a conditional knock-out mouse model

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## Zusammenfassung

Das Wilms Tumorsuppressorprotein 1, WT1, ist ein Zink-Finger Transkriptionsfaktor, der eine essentielle Rolle in der Entwicklung von Herz, Gonaden, Nieren und anderen Organen spielt. *WT1*-Mutationen stehen im Zusammenhang mit verschiedenen Erkrankungen des Menschen. Dazu gehören Wilms Tumorerkrankungen, das Denys-Drash- und das Frasier-Syndrom. Wilms Tumore gehören zu den häufigsten Nierentumoren bei Kindern. Die beiden genannten Syndrome sind gekennzeichnet durch urogenitale Fehlbildungen und den Verlust der Nierenfunktion. In Säugetieren wird *Wt1* während der gesamten Nierenentwicklung exprimiert und ist vor allem für die Einleitung der Nierenentwicklung von besonderer Bedeutung. *Wt1-knock-out*-Mäuse bilden keine Nieren und sterben, vermutlich aufgrund von Herzfehlern, kurz vor der Geburt. In der adulten Niere ist die Expression von *Wt1* begrenzt auf eine einzige Zellpopulation. Hierbei handelt es sich um die Podozyten, welche einen Hauptbestandteil des renalen Filtrationsapparates darstellen. Podozytendefekte führen zu schweren Nierenfunktionsstörungen. Im Rahmen dieser Arbeit wurde ein konditionales *Wt1-knock-out*-Mausmodell generiert, das es ermöglicht, *Wt1* ausschließlich in Podozyten-Vorläuferzellen der sich entwickelnden Niere zu inaktivieren. Entsprechende Mäuse starben spätestens 24 Stunden nach der Geburt; Harnausscheidung war zu keinem Zeitpunkt nachweisbar (Anurie). Zudem konnte eine signifikante Reduktion der Nierenkörperchen (Glomeruli) in neugeborenen Mäusen mit podozyten-spezifischer *Wt1* Inaktivierung festgestellt werden. Die Nierenkörperchen, in denen unter anderem auch die Podozyten lokalisiert sind, sind verantwortlich für die Ultrafiltration des Blutes und somit für die Primärharnbildung. Mithilfe elektronenmikroskopischer Untersuchungen an noch vorhandenen Nierenkörperchen konnte gezeigt werden, dass Podozyten starken strukturellen Veränderungen unterworfen waren. Dies führte zum Verlust von für die Filtrationsfunktion der Niere essentiellen Strukturen, den sogenannten Fußfortsätzen. Außerdem war eine ausgeprägte Degeneration der proximalen Tubuli evident. Aus diesen Befunden läßt sich schlussfolgern, dass Wt1 ein wichtiger Faktor für die Entwicklung und Funktion von Podozyten ist. *Wt1*-Deletion in sich entwickelnden Podozyten führt demnach zur Degeneration dieser Zellpopulation und dies wiederum zum Verlust der Nierenfunktion.

Ein weiteres Ziel dieser Arbeit war es, die Rolle von Wt1 in der Erhaltung und Funktion der Podozyten in adulten Mäusen zu untersuchen. Zu diesem Zweck wurde ein System angewendet, mit dem die Inaktivierung von *Wt1* über die Applikation des Antibiotikums

Doxyzyklin induziert werden kann. Podozyten-begrenzte *Wt1*-Deletion in adulten Mäusen führte zur Ausscheidung hoher Proteinmengen mit dem Urin (Proteinurie). Die Podozyten dieser Tiere wiesen eine starke Reduktion der Fußfortsätze auf. Letzteres war vermutlich die Ursache für die Proteinurie. Die Tiere waren über einen kurzen Zeitraum lebensfähig. Nach viermonatiger Doxyzyklin-Behandlung jedoch starben zwei von drei Tieren. Diese Studie konnte demnach zum ersten Mal belegen, dass Wt1 auch für die Erhaltung und funktionelle Integrität der Podozyten in adulten Tieren essentiell ist.

## Abstract

The Wilms tumor suppressor protein 1 Wt1 is a zinc-finger transcription factor and essential for the development of several organs including heart, gonads and kidneys. *WT1* mutations are associated with a variety of human diseases such as Wilms tumor, a frequent pediatric kidney cancer, as well as Denys-Drash and Frasier syndrome. The latter are characterized by genitourinary malformations and impairment of kidney function. In mammals *Wt1* is expressed throughout all stages of kidney development and is specifically required for the onset of kidney formation. *Wt1* knock-out mice do not form kidneys and die shortly before birth, most probably due to heart failure. In the adult kidney Wt1 is restricted to a single cell population termed podocytes. This particular cell type forms a major part of the renal filtration barrier. Podocyte defects result in severe kidney malfunction. In this work a conditional knock-out mouse model was generated allowing the deletion of *Wt1* specifically in podocyte precursor cells of the developing kidney. Mice with podocyte-specific Wt1 inactivation died 24 hours after birth and showed no signs of urine production (anuria). The number of glomeruli, being the basic filtration units of the kidney, was significantly reduced in newborn animals due to enhanced apoptosis. Electron microscopic analyses of remaining glomeruli revealed the degeneration of podocyte structures (foot-processes) essential for the renal filtration capacity. In addition, proximal tubuli were degenerated to a great extent. Thus, Wt1 is important for podocyte development and functionality. Degeneration of developing podocytes as a consequence of *Wt1* inactivation leads to the loss of integral parts of the renal filtration apparatus and to loss of kidney function.

To assess the role of Wt1 in the maintenance of podocyte and kidney function a system was used whereby *Wt1* deletion can be induced by administration of the drug doxycycline. Podocyte restricted inactivation of the gene in adult mice resulted in severe proteinuria, which was accompanied by a significant reduction in the number of foot-processes. In most cases mice were viable over a short period of time. After four months of continuous doxycycline treatment, however, two out of three mice had died. In this study it was demonstrated for the first time that Wt1 is essential for the maintenance and functional integrity of podocytes in adult mice.

## Abbreviations

| | |
|---|---|
| A | Adenine |
| AML | Acute myeloid leukemia |
| Bp | Base pairs |
| BSA | Bovine serum albumine |
| C | Cytosine |
| °C | Degrees Celsius |
| CIOMS | Council for International Organizations of Medical Sciences |
| cm | Centimeter |
| cpm | Counts per minute |
| $C_T$ | Threshold cycle |
| C-terminus | Carboxy-terminus |
| Cys | Cystein |
| d | day(s) |
| Da | Dalton |
| DAB | 3,3,'-diaminobenzidine |
| dCTP | Deoxycytosine triphosphate |
| DDS | Denys-Drash syndrome |
| DMS | Diffuse mesangial sclerosis |
| DMSO | Dimethyl sulphoxid |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| dT | Deoxythymidine |
| DTA | Diphteria Toxin A |
| E | Embryonic stage |
| *E. coli* | *Escherichia coli* |
| e.g. | *exempli gratia* |
| EDTA | Ethylendiamin-tetra-acetic acid |
| EM | Electron Microscopy |
| ES cells | Embryonic stem cells |
| ET | Endothelium |
| et al. | *et alii* |
| F | Filial generation |

| | |
|---|---|
| Fig | Figure |
| FLI | Leibniz Institute for Age Research - Fritz Lipmann Institute |
| FP | Foot processes |
| FS | Frasier syndrome |
| FSGS | Focal segmental glomerulosclerosis |
| g | Gram |
| G | Guanine |
| GBM | Glomerular basement membrane |
| GL | Glomerulus |
| Gln | Glycin |
| h | Hour |
| His | Histidine |
| ic | Inner renal cortex |
| i.e. | *id est* |
| IgG | Immunoglobulin G |
| J | Joule |
| K | Lysine |
| kb | Kilo base pairs |
| kDa | Kilo Dalton |
| kg | Kilogram |
| ko | knock-out |
| M | Molar |
| mA | Milliampere |
| mg | Milligram |
| min | Minute |
| ml | Millilitre |
| mM | Millimolar |
| mRNA | messenger RNA |
| µg | Microgram |
| µl | Microlitre |
| µm | Micrometer |
| µM | Micromolar |
| NEB | New England Biolabs |

| | |
|---|---|
| Neo | Neomycin |
| NGS | Normal goat serum |
| nM | Nanomolar |
| NMD | Nonsense mediated decay |
| N-terminus | Amino-terminus |
| oc | Outer renal cortex |
| P | Podocyte |
| PAA | Polyacryl amide |
| PAS | Periodic acid Schiff |
| PBND | PCR buffer with non-denaturing detergents |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PEC | Pariethal epithelial cells |
| PEI | Polyethyleneimine |
| PFA | Paraformaldehyde |
| pH | *potentium hydrogenium* |
| Pro | Proline |
| PT | Proximal convoluted tubulus |
| qRT-PCR | quantitative real-time RT-PCR |
| RNA | Ribonucleic acid |
| Rpm | Rotations per minute |
| RT | Reverse Transcriptase |
| RT-PCR | Reverse-Transcription-PCR |
| S | Serine |
| s | Second |
| SD | Slit diaphragm |
| SDS | Sodium dodecylsulfate |
| SM | Slit membrane |
| T | Thymine |
| T | Threonine |
| Taq | *Thermus aquaticus* |
| TdT | Terminal deoxynucleotidyl transferase |
| TEM | Transmission electron microscopy |

| | |
|---|---|
| Temp. | Temperature |
| Tris | Tris-hydroxymethyl-aminomethane |
| TUNEL | TdT-mediated dUTP nick end labelling |
| U | Uracile |
| UV | Ultra violet light |
| V | Volt |
| v/v | Volume to volume |
| w/v | Weight to volume |
| WAGR | Wilms tumor, aniridia, genitourinary malformations, mental retardation |
| X-Gal | 5-bromo-4-chloro-3-indolyl-β-D-galactosidase |
| YAC | Yeast artificial chromosome |
| Zn-finger | Zinc-finger |

# 1 Introduction

## 1.1 WT1

The Wilms tumor suppressor gene, *WT1,* was identified in a search for the genetic causes of Wilms Tumor, a frequent pediatric kidney cancer with an incidence of 1 in 10,000 children in North America[1, 2]. *WT1* was found to be inactivated in 10-15 % of cases[3, 4]. The human gene maps to chromosome 11p13 (REF. 2) and spans about 50 kb of genomic DNA encompassing 10 exons (Fig. 1 A)[5, 6]. It encodes a protein family of at least 24 different isoforms derived from alternative splicing, usage of alternative translation starts and RNA-editing. The major characteristics of WT1 proteins are those of typical transcription factors: they harbor a N-terminal proline/glutamine-rich transactivation domain and four C-terminal zinc fingers of the $Cys_2$-$His_2$ type (Fig. 1 B). Each zinc finger is encoded by a separate exon, exons 7-10; the transactivation domain is encoded by exon 1 alone[6, 7].

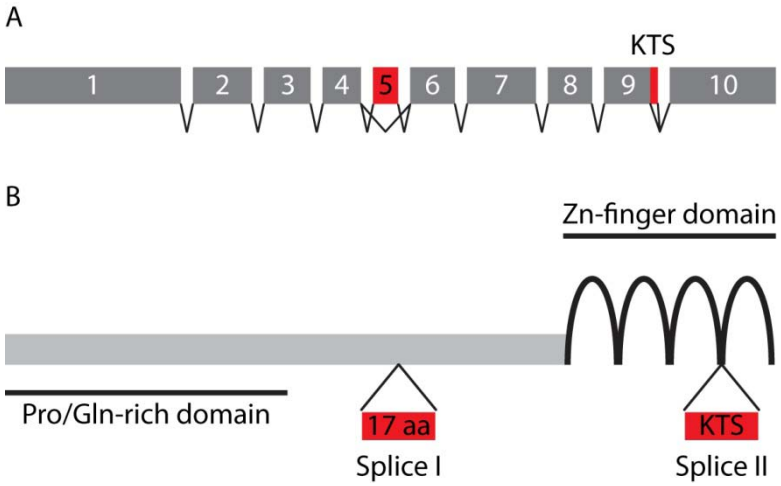


**Fig. 1 Wt1 – mRNA (A) and protein structure (B).** Wt1 mRNA consists of 10 exons. Two major alternative splicing events affect exon 5 (splice I), which codes for 17 amino acids in the central part of the protein and a 9 bp sequence at the end of exon 9 (splice II) encoding amino acids lysine (K), threonine (T) and serine (S) between zinc fingers 3 and 4. The protein harbors a N-terminal proline/glutamine rich *trans*-activation domain and a zinc-finger domain at its C-terminus. (Modified from Englert, 1998)

The four most abundant WT1 isoforms are derived from two alternative splicing events (Fig. 1 A, B): the first affects the mammalian specific exon 5, which codes for 17 amino acids located between transactivation and zinc finger domain[6, 8]. The functional properties of this amino acid sequence have not yet been determined; specific deletion, however, has no obvious pathological consequences in mice[9]. The second alternative splicing event gives rise to functionally very different WT1 isoforms. Alternative inclusion or omission of a 9 bp sequence at the end of exon 9 results in protein variants either containing or lacking

the three amino acids lysine (K), threonine (T) and serine (S) between zinc finger domains 3 and 4 (REF. 6). Corresponding isoforms are termed WT1(+KTS) and WT1(-KTS) and are expressed in a constant ratio of about 2:1 in humans[6].

The WT1(-KTS) isoform has been extensively studied and proven to act mainly as transcription factor with either activating or repressive function depending on the target gene[10-12]. In table 1 examples of proposed WT1 target genes are listed.

Since insertion of the KTS sequence negatively influences DNA binding capability[13, 14], the WT1(+KTS) variant was thought to fulfill other functions. As a matter of fact, WT1(+KTS) isoforms colocalize and interact with splice factors and pre-mRNA[15, 16]. This has provoked the assumption that it might be involved in RNA processing. Though, direct evidence is still not available.

**Table 1** Wt1 target genes

| Target Gene | Gene product | Activation/Repression | Reference |
|---|---|---|---|
| *EGR-1* | Early growth response protein 1 | Repression | 17 |
| *TGF-β1* | Transforming growth factor beta 1 | Repression | 18 |
| *IGF-2* | Insulin-like growth factor 2 | Repression | 19 |
| *Areg* | Amphiregulin | Activation | 20 |
| *Podxl* | Podocalyxin | Activation | 21 |
| *Nphs1* | Nephrin | Activation | 22 |
| *Pax-2* | Paired box protein 2 | Repression | 23 |
| *Sry* | Sex determining region of chr. Y | Activation | 24 |
| *PDGF-A* | Platelet-derived growth factor A | Repression | 25 |
| *VEGF* | Vascular endothelial growth factor | Activation | 26 |

*WT1* mutations are not only relevant for the formation of Wilms tumor, but also account for a number of other human diseases. One example is the WAGR (*Wilms tumor*, *aniridia*, *genitourinary malformations*, *mental retardation*) syndrome. It results from chromosomal deletions at locus 11p13 that encompass *WT1* and *PAX6*, among others[27-29]. PAX6, being a master regulator of eye development, is likely to cause the aniridia phenotype. Reduction in *WT1* gene dosage on the other hand is held responsible for the genitourinary malformations and predisposition to Wilms Tumor. WAGR patients with point mutations in the remaining *WT1* allele of renal precursor cells have a high risk to develop Wilms tumor[30, 31].

Another syndrome associated with *WT1* mutations is the Denys-Drash syndrome (DDS). It is characterized by severe urogenital defects, such as nephropathy and gonadal dysgenesis

with male pseudohermaphroditism. Furthermore, 70 – 80 % of DDS-patients develop Wilms tumor. DDS is caused by missense mutations in a *WT1* allele. The most common mutation results in an arginine to tryptophane conversion at position 394 within the third zinc finger[32-35]. DNA binding capability of WT1 is hereby abrogated. Considering the severity of the developmental defects and the unusually high incidence of Wilms tumor a dominant-negative effect of the mutation seems likely, especially, since WT1 normally functions by formation of homodimers through N-terminal interaction. Association of defective and wild-type protein might thus aggravate the effect[36-39].

Frasier syndrome (FS) is associated with male-to-female sex reversal and renal anomalies including glomerulopathy. However, patients show no pre-disposition to Wilms tumor. FS is a consequence of a heterozygous point mutation in the alternative donor splice site in intron 9, which leads to the loss of *WT1(+KTS)* expression from the affected allele and, hence, to a change in the WT1(+KTS)/WT1(-KTS) ratio[40, 41]. That a constant ratio of these WT1 isoforms is indispensable for proper development, especially of the renal and genital system, has been emphasized in a mouse model: mice expressing both *Wt1* isoforms from the wildtype allele, but only *Wt1(-KTS)* from the other develop renal dysfunctions comparable to those observed in human FS patients. Animals with total loss of *Wt1(+KTS)* expression show complete male-to-female sex reversal and severe kidney defects.

Specific inactivation of the Wt1(–KTS) isoform results in reduced kidney size and glomerular number, as well as retarded gonad development. Both total *Wt1(+KTS)* and *Wt1(–KTS)* knock-out mice die shortly after birth[42, 43].

Somatic *WT1* mutations have also been detected in acute myeloid leukemia (AML)[44], DDS-associated gonadoblastoma[32] and very rare cases of mesotheliomas[45]. All this has underlined the now widely accepted hypothesis that WT1 functions as tumor suppressor. However, growing evidence supports the possibility that, under certain conditions, *WT1* also acts as oncogene: in a number of adult tumors, including colorectal[46], desmoid[47], breast[48] and brain[49] tumors it is ectopically expressed and exhibits no mutations. Along this line, over-expression of wild-type, non-mutated *WT1* in some leukemias accounts for a poor prognosis[50, 51].

Studies of human syndromes and mouse models have revealed an essential role of WT1 in the development of kidneys and gonads. But, as learned from mice, *Wt1* expression is not at all restricted to the urogenital system. On the contrary, *Wt1* is found to be expressed in and relevant for a wealth of other developing organs and tissues. This includes spleen,

spinal cord, brain, retina, adrenal glands, hematopoietic system, olfactory system and mesothelial tissue lining heart, diaphragm, liver and gut[52-58]. Insight into Wt1 function in most of the mentioned tissues and organs has been gained predominantly by the study of conventional *Wt1* knock-out mice[59]. In contrast to human patients, heterozygous inactivation of *Wt1* has no pathological implications in mice. Instead, *Wt1*$^{-/-}$ animals die *in utero* between embryonic day E13.0 and E15.0, presumably due to malformations of heart and/or diaphragm. Kidney and gonad development commences, but ceases rapidly by apoptosis of renal and gonadal progenitor cells resulting in agenesis of both organs.

## 1.2 The mammalian kidney – Structure and function

In mammals the metanephros, or permanent kidney, is the major excretory and homeostatic organ. It performs two essential functions: first, metabolic end products, such as urea and ammonium are removed from the blood through passive filtration. Second, electrolyte concentration and pH of the body fluid are controlled through modifications of the ultrafiltrate, or primary urine. Moreover, the kidney is involved in blood pressure regulation via excretion or retention of salt and water leading to changes in blood volume and pressure. The enzyme renin is released from the kidneys in case of low blood pressure. It activates the renin-angiotensin-aldosterone system, which ultimately results in the elevation of blood pressure levels. Another important property of the kidney is the production of the hormone erythropoietin (Epo), which is essential for the control of red blood cell production, or erythropoiesis.[60]

The adult mammalian kidney is of bean-shape with a superior and an inferior pole. Its parenchyma can be grossly divided into a cortex and medulla. The basic structural and functional unit, the nephron, draws through both parts (Fig. 2). All essential kidney functions from blood filtration to re-absorption of salt and water are performed by the latter structure. Each adult human kidney contains 300.000 to 1.200.000 nephrons; in the mouse between 8.000 and 12.000 nephrons are present per kidney.[60]

The nephron consists basically of two parts, the renal corpuscle, or glomerulus, and the renal tubule. The renal corpuscle is formed by the Bowman's capsule, being the expanded proximal end of the nephron, and the capillary (vascular) tuft, which invades the Bowman's capsule. Afferent and efferent arterioles enter and exit at the capsule's vascular pole. At the opposite end, termed the urinary pole, the Bowman's capsule is connected to the renal tubule. The renal tubule itself is structured into the proximal tubule, with a convoluted part

directly extending from the Bowman's capsule, and a straight part, the loop of Henle and the distal tubule, including a straight and convoluted compartment. The connecting segment connects the nephron with the collecting duct (Fig. 2).[61]

Glomeruli, convoluted parts of proximal and distal tubules, as well as connecting segments are located within the renal cortex. The medulla contains the loop of Henle, the collecting ducts and most parts of the straight proximal and distal tubules.[61]

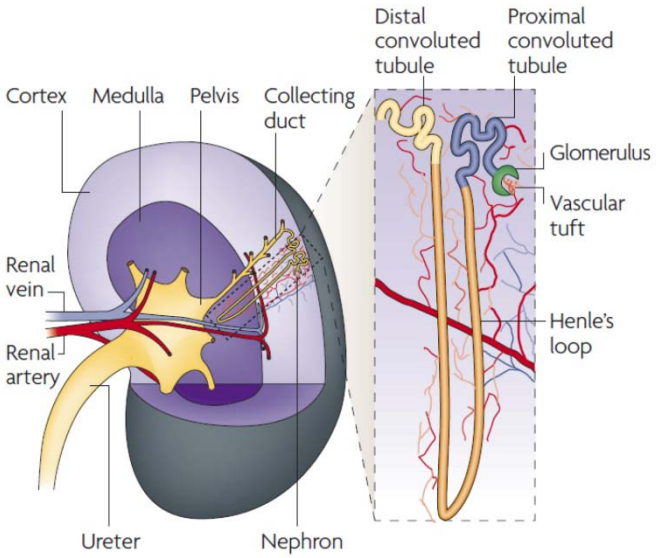


**Fig. 2 Gross anatomy of the adult kidney.** The kidney can roughly be divided into cortex and medulla. The major functional unit, the nephron, consists of the glomerulus with vascular tuft, proximal and distal tubules and the loop of Henle. Collecting ducts transport the urine to the renal pelvis, which is connected to the ureter. (Modified from Schedl, 2007)

Blood filtration takes place in the glomerulus. The filtration barrier is constituted essentially of three components: endothelial cells of the capillary tuft, a glomerular basement membrane (GBM) and the so called slit diaphragm formed by highly specialized cells of the visceral epithelial layer of the Bowman's capsule (Fig. 3 A, B). Glomerular endothelial cells are highly fenestrated, but do not have diaphragms. They serve as a barrier for cellular, but not sub-cellular components of the blood. Visceral epithelial cells, also termed podocytes, form long primary processes that entwine around glomerular capillary vessels. By extension of interdigitating secondary processes, the so called foot processes, the glomerular endothelium is entirely enwrapped and covered by podocytes. Interdigitating foot processes of neighboring primary processes are separated by a narrow filtration slit, which is bridged by a filamentous sheet referred to as the slit diaphragm (Fig. 3 A, B). The latter structure functions as efficient barrier for macromolecules exhibiting diameters larger than 8.0 nm. Endothelial and podocyte layer are separated from each other by the

glomerular basement membrane (GBM). It results from fusion of the basal laminae of both cell layers and forms a tight meshwork consisting predominantly of type IV collagen fibrils. Apart from being a mechanical filter excluding molecules larger than albumin (~69 kDa), the glomerular filtration barrier also has charge selective properties. Many of its components, including the GBM, are negatively charged and therefore repel most plasma proteins.[60-62]

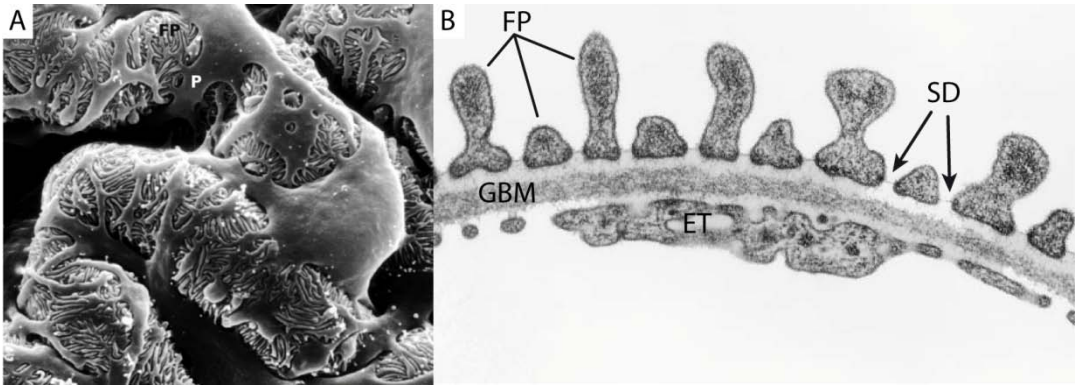


**Fig. 3 Structure of Podocytes (A) and glomerular filtration barrier (B).** Podocytes (or visceral epithelial cells) are characterized by long primary (P) and interdigitating secondary processes, or foot processes (FP), that completely enwrap glomerular capillary vessels. The space between interdigitating foot processes is bridged by the slit diaphragm (SD). Podocyte cell layer and endothelium (ET) are connected via the glomerular basement membrane (GBM). Together, podocytes, slit diaphragm, GBM and endothelium constitute the glomerular filtration barrier. (Modified from Pavenstädt et al., 2003)

Another cell population present in the glomerulus is no component of the filtration barrier, but has important structural functions: masangial cells are found in between glomerular capillaries. They form a tight mesangial matrix and are essential for maintaining the structural integrity of the glomerulus against strong hydrostatic forces within capillary vessels.[60, 61]

The outer surface of the Bowman's capsule is constituted by the pariethal epithelium, consisting of simple epithelial cells (pariethal epithelial cells, PEC). It is continuous with the visceral epithelium formed by podocytes. Both layers are separated by the Bowman's space into which the ultrafiltrate is forced. From there it is drained into the proximal convoluted tubule. The entire proximal tubule accounts for most reabsorption of proteins, amino acids, glucose, creatinin, water and $Cl^-$ as well as $Na^+$ ions. After further modifications in the remaining tubular sections, the filtrate is finally released from the kidney as highly concentrated urine.[61]

## 1.3 Kidney development and Wt1

The mammalian kidney develops from an epithelial component, namely the primary nephric duct or Wolffian duct, and a mesenchymal component, the metanephric blastema. Both derive from the intermediate mesoderm. For kidney development to commence reciprocal interactions between the two components are required[63]. Initially, the metanephric blastema is specified as renal precursor by processes involving transcription factors odd skipped related 1 (Osr1)[64, 65], paired-box proteins Pax2 and 8 (REF. 66), and eyes absent homologue 1 (Eya1)[67]. At around embryonic day E10.5 in mice (week 5 to 6 of gestation in humans) the specified metanephric blastema induces the primary nephric duct to form an outgrowth, the ureteric bud. This structure invades the blastema and undergoes branching morphogenesis. At the tips of newly formed branches adjacent mesenchymal cells are induced to aggregate and thereby form a cap structure. A proportion of these cells begins the process of mesenchymal-to-epithelial transition, resulting in the formation of the renal vesicle. Another proportion generates the interstitial stroma. As it progresses, the renal vesicle gradually forms two distinct clefts giving rise to the comma-shaped and S-shaped body, respectively. The S-shaped body further elongates and differentiates into the various segments of the nephron. On its distal end it fuses with the ureteric duct to form a continuous lumen. At the proximal end the glomerulus develops: signals released from podocyte precursor cells attract endothelial cells to invade and to form the vascular tuft. In turn, endothelial cells produce factors that support mesangial cell differentiation. At this stage, termed capillary loop stage, components of the renal filtration barrier begin to arise. Podocyte precursors start to form foot processes as well as slit diaphragms. Also, the glomerular basement membrane (GBM) takes shape. By embryonic day E15.5 maturing nephrons harboring all functionally relevant components and

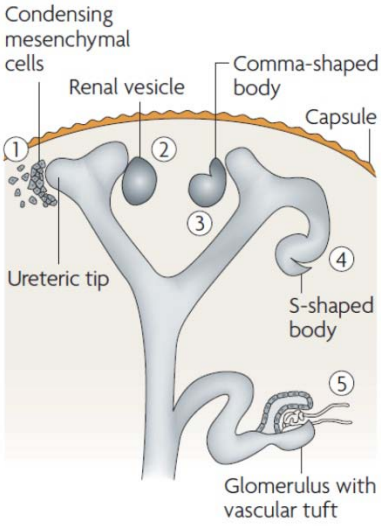


**Fig. 4 Kidney and nephron development.** At E15.5 nephrons of different developmental stages are present in the kidney. (1) Nephron development starts with condensing mesenchymal cells at ureteric tips. (2) The renal vesicle results from mesenchymal-to-epithelial transition. (3) Comma-shaped body. (4) S-shaped body. (5) Mature nephron with glomerulus and tubules. Nephron development is a continous process, progressing from outer to inner cortex. (Modified from Schedl, 2007)

structures can be observed in the embryonic kidney. The still extending and branching ureteric duct has by now formed a major part of the collecting duct system (Fig. 4)[63, 68, 69].

Kidney size is primarily defined by the overall number of nephrons generated during development. As described, nephron formation is a continuous process driven by ongoing ureteric duct branching. New nephrons are added at the outermost cortex, where undifferentiated mesenchymal precursor cells are located. The most advanced or differentiated nephrons thus reside close to the medulla in the innermost cortex. In humans kidney development is completed with birth. In mice, however, it continues for about 1 week after birth[70].

*Wt1* is highly expressed in condensing mesenchymal cells in the outer cortex (nephrogenic zone), in podocyte precursor cells of comma- and S-shaped bodies, and in fully differentiated podocytes[11]. In concordance with this expression pattern Wt1 has important functions in various stages of kidney development.

Essential for the first step in nephrogenesis, the induction of ureteric bud outgrowth, is the secretion of glial-derived neurotrophic factor (Gdnf) from the mesenchyme and binding to its respective receptor, Ret (Proto-oncogene tyrosine-protein kinase receptor), in the primary nephric duct[71]. The lack of Gdnf-Ret signaling in mutant mice leads to renal agenesis. Nevertheless, other yet unknown pathways independent of Gdnf-Ret signaling are assumed to play a role in ureteric bud initiation[68]. One might involve Wt1. In conventional *Wt1* knock-out mice ureteric bud outgrowth fails to occur and the metanephric blastema undergoes apoptosis[59], which also results in renal agenesis. However, Gdnf signaling is unaffected[72].

Wt1 seems to play a role in nephron induction as well. Nephron development or differentiation depends on initial signals released from the ureteric duct. This process is mainly regulated by the canonical Wnt-ß-catenin pathway, with Wnt9b (wingless-type MMTV integration site 9b) being the key player[73, 74]. *Wnt9b* is expressed in and released from the ureteric duct stalk, but is absent from the ureteric tips. In *Wnt9b* knock-out mice first-stage nephrogenic markers Lim homeobox protein 1 (Lim1), paired-box 8 (Pax8), Fibroblast growth factor 8 (Fgf8) and Wnt4 are not detectable in the mesenchyme[75]. Wnt4 has proven to be both necessary and sufficient for induction of nephron formation. The latter process does not take place in respective knock-out mice[76]. Together with Fgf8 (REFS 77, 78) and Pax2 (REF. 79), Wt1 (REF. 80) is part of a complex network to induce *Wnt4* expression and is therefore equally important for nephron induction. Recent findings from

kidney culture experiments suggest that reduction of *Wt1* expression levels not only prevents nephron initiation to take place, but also leads to abnormal proliferation of mesenchymal cells[81]. It is conceivable that loss of *Wt1* function at this stage arrests nephron precursor cells in a multipotent state. Subsequent transformation of these cells might contribute to Wilms Tumor formation.

Segmentation and maturation is the last step in nephron development. In this process the different compartments, proximal and distal tubules, Henle loop and glomerulus, differentiate along the proximal-distal axis. For glomerular differentiation and maturation podocytes and their respective precursor cells are of vital importance. Podocyte precursor cells secrete vascular endothelial growth factor (Vegf) that attracts endothelial cells[82], which themselves release platelet derived growth factor (Pdgf) necessary for the differentiation of mesangial cells[83]. *Wt1* is highly expressed in podocyte precursors and is involved in the regulation of *Vegf* expression[26, 82, 84]. Furthermore, it acts as repressor for *Pax2*[23]. This seems to be of particular importance for podocyte differentiation itself, since ectopic *Pax2* expression in mice causes severe glomerular disease[85, 86]. In the adult kidney *Wt1* expression remains restricted to podocytes.

## 1.4 Podocytes and Wt1

The podocyte (Fig. 3 A) fulfills important tasks in the developing glomerulus and is vital for function and maintenance of the renal filtration barrier (Fig. 3 B) in the adult organism. It's most prominent feature is the ability to form extending primary and secondary processes (foot processes) and the associated slit diaphragm. The latter resembles adherens-like intracellular junctions. Important factors constituting this unique structure include the transmembrane proteins nephrin (Nphs1)[87, 88], Neph1[89], P-cadherin[90] and Fat, a member of the cadherin superfamily[91]. Expression of the respective genes is restricted to late-stage and mature podocytes. Nephrin is the best studied component of the slit diaphragm; *NPHS1* has been identified as the gene mutated in congenital nephrotic syndrome of the Finish type, an autosomal-recessive disease with an incidence of 1 in 10,000 neonates in Finland[87]. It is characterized by defects of the renal filtration barrier resulting in massive proteinuria and, ultimately, in end stage renal failure, when untreated. *Nphs1* knock-out animals die 24 hours after birth and display massive proteinuria paralleled by effacement (loss) of foot processes and absence of the slit diaphragm[92]. *In vitro* experiments suggest that Wt1 is involved in *Nphs1* regulation[22].

The slit diaphragm is covered by a thick coat rich of sialoglycoproteins such as podocalyxin (Podxl), which contributes to the highly negative surface charge of podocytes[93]. *Podxl* knock-out mice die shortly after birth from anuric renal failure. Podocytes develop, but fail to form foot processes and slit diaphragms[94]. Studies on rat embryonic kidney cell precursors gained some evidence for direct activation of *Podxl* by Wt1[21].

A number of factors located at the cytoplasmatic side of the foot process membrane link the slit diaphragm and its constituents to the cytoskeleton. To this group of linkers belong podocin (Nphs2), CD2-associated protein (Cd2ap) and zona occludens protein 1 (Zo-1), among others. *NPHS2* is mutated in patients with autosomal-recessive steroid-resistant nephrotic syndrome, which is a rapidly progressing disease resulting in end-stage renal failure. A comparable phenotype was diagnosed in mice carrying an *Nphs2* mutation frequently found in nephrotic patients. Apart from strong proteinuria and glomerulosclerosis, podocyte anomalies including widespread foot process effacement were observed[95]. *Nphs2* deficient mice harbor similar phenotypical features, such as proteinuria and glomerulosclerosis. They die shortly after birth from renal failure[96]. *Cd2ap* knock-out mice have a comparable, but less severe phenotype[97]. Podocin is thought to form a hairpin-like structure associated with the foot process membrane directly at the insertion site of the slit diaphragm. It has also been shown to interact with Cd2ap and nephrin[98, 99].

Another important podocyte/glomerular marker is synaptopodin; the function of the latter is not yet clearly defined, but it is associated with actin molecules of the cytoskeleton and regulates the actin-bundling activity of alpha-actinin, another cytoskeleton-associated protein[100, 101]. Synaptopodin is distributed along the entire podocyte with highest concentration in foot processes.

Insights into Wt1 function at late stages of nephron development and podocyte differentiation have mostly been gained by studies of organ culture and cell culture systems. This includes findings about transcriptional targets *Wnt4*, *Pax2*, *Vegf*, *nephrin* and *podocalyxin*. Since kidney development does not proceed beyond initial stages in conventional *Wt1* knock-out mice, *in vivo* data are rare. However, some mouse models predominantly harboring mutant forms of *Wt1* are available and have served to underline the general importance of this gene for proper podocyte function and maintenance. One approach aimed to rescue the *Wt1* knock-out phenotype by introduction of a yeast artificial chromosome expressing human *WT1* into the genome of *Wt1-null* animals. WT1

expression could be restored to up to 70% of wild-type levels. However, mice developed severe glomerular defects and died rapidly from end stage renal failure[102].

Mice carrying different *Wt1* mutations observed in patients with Denys-Drash syndrome (DDS) have been generated: a mutation that truncates zinc finger 3 at codon 396 also induced the typical urogenital anomalies of DDS in mice. Expression of podocyte-specific genes was significantly lower in mutant animals and *Pax-2* was found to be misexpressed in some cases. But it was not clear, whether this was the cause or a result of disease progression and podocyte damage[103-105]. Introduction of the most frequently observed DDS mutation led to the development of some DDS symptoms in mice. Molecular changes including up-regulation of *Cd2ap* and *Podocin*, however, could not be directly linked to the reduction of Wt1 protein levels[106]. Thus, the molecular role of Wt1 in podocytes and associated diseases remains to be defined.

## 1.5 Aim of this work

The overall intention of this work was to gain *in vivo* information on Wt1-function in (1) late stages of kidney and podocyte development and (2) podocyte maintenance in the adult animal. For the first, developmental approach a mouse line was generated allowing podocyte-restricted *Wt1*-deletion from a late developmental stage on. In a second approach, an inducible podocyte-specific *Wt1*-knock-out mouse line was used to analyze Wt1 function in podocytes of adult animals.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Oligonucleotides

Oligonucleotides for PCR applications were designed using the freely accessible online programs Primer3 (http://frodo.wi.mit.edu/primer3/) and Metabion Oligonucleotide Properties Calculator (http://www.metabion.com/support/biocalc.php). All oligonucleotides utilized in this study were obtained from Metabion (Germany) or Eurofins/MWG (Germany) and are listed in table 2.

**Table 2** Oligonucleotides

| Type | Name | Sequence | Reference |
|---|---|---|---|
| *LoxP* amplification | Wt1flox-5'loxP-1F | ACTGCTGACGAGAGTTAGGACA | This work |
| | Wt1flox-5'loxP-1R | AGATGGAGGCATCCAGTAAAGA | This work |
| Southern Blot probe | ENG1-W1 | AAGAGCAGACTGCACTCGCAGCTGG | This work |
| | ENG1-W2 | GAGACCTTCTTGTGGCTGCTCAGCC | This work |
| Genotyping | Cre-RS-1F | TCGACCAGGTTCGTTCACTC | This work |
| | Cre-RS-1R | CATCCTTAGCGCCGTAAATC | This work |
| | rtTA-podoprobe-F | CGCACTTCAGTTACTTCAGGTCCTC | Shigehara et al.[107] |
| | rtTA-podoprobe-B | GCTTATGCCTGATGTTGATGATGC | Shigehara et al.[107] |
| | Wt1-CreX-1F | GACCGTTGAACTGGCTCAG | This work |
| | Wt1-CreX-1R | GTCCGGGCAAGTTAAAGGG | This work |
| | Wt1flox-1F | GCCCTACAGCAGGTAAGAAGG | This work |
| | Wt1flox-1R | CTGGAGACCTGAGACAAGCA | This work |
| qRT-PCR | Nephrin-1F | GTTCAGCTGGGAGAGACTGG | This work |
| | Nephrin-1R | GTCCACGATGCACTGGTAAG | This work |
| | Podocin-1F | GAGAGCGAGCGACCAGAG | This work |
| | Podocin-1R | TGCAGAACCAGATGGAAAAAG | This work |
| | Cd2ap-1F | TAAATGGGAGACGAGGGATG | This work |
| | Cd2ap-1R | GCTTTGGTTTGTGGATGTGG | This work |
| | Synpo-1F | GAGGCACGAGGTGGTTTC | This work |
| | Synpo-1R | GCCTCCTTCAGGTCGTTTTC | This work |
| | Tbp-1F | GGCCTCTCAGAAGCATCACTA | Klattig[108] |
| | Tbp-1R | GCCAAGCCCTGAGCATAA | Klattig[108] |
| | Wt1-1F | AGTTCCCCAACCATTCCTTC | Klattig et al.[108, 109] |
| | Wt1-5R | TTCAAGCTGGGAGGTCATTT | Klattig et al.[108, 109] |

### 2.1.2 Plasmids

The TA-cloning vectors pCRII-TOPO and pCR4-TOPO (both Invitrogen, USA) were used for cloning, sequencing and DNA-probe propagation.

### 2.1.3 Bacterial strains

Escherichia coli strains used for cloning, sub-cloning and plasmid propagation:

DH5α — Recombination defective, suppressive strain; genotype: *fhuA2* Δ*(argF-lacZ)U169 phoA glnV44 Φ80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

TOP10F' — Chemically competent strain for high efficiency transformation; genotype: F´ (*lac*Iq Tn*10* (TetR)) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G (Invitrogen, USA)

### 2.1.4 Antibodies

Anti-Wt1 (human, mouse, rat), (C-19), rabbit polyclonal (Santa Cruz Biotechnology, USA)

Anti-Synaptopodin (human, mouse, rat), (G1D4), mouse monoclonal (Progen Biotechnik, Germany)

Anti-Podocin (mouse), (G-20), goat polyclonal (Santa Cruz Biotechnology, USA)

Anti-rabbit IgG, goat, Alexa Fluor 546 labeled (Invitrogen, USA)

Anti-mouse IgG, goat, Alexa Fluor 488 labeled (Invitrogen, USA)

Anti-goat IgG, rabbit, Alexa Fluor 488 labeled (Invitrogen, USA)

### 2.1.5 Mice

The original transgenic mouse lines used in this work are listed in table 3. Mice were in C57BL/6J genetic background. Only animals from the *Wt1flox* strain or animals derived from crossings with *Wt1flox* mice were of mixed C57BL/6J/129/SvPas origin.

**Table 3** Transgenic mouse lines

| Line | Description | Provider | Reference |
|---|---|---|---|
| *Wt1flox* | Mouse line carrying two *loxP* sites flanking exons 2 and 3 of the *Wt1* gene | | This work |
| *Podocin-Cre* | Mouse line harboring a *Cre* transgene; the expression of which is controlled by a *NPHS2* promoter fragment | Ralph Witzgall, University of Regensburg | Moeller et al.[110, 111] |
| *Podocin-rtTA* | Mouse line, in which *rtTA* (reverse tetracycline inducible transactivator) expression is governed by a *NPHS2* promoter fragment | Ralph Witzgall, University of Regensburg | Shigehara et al.[107] |

| | | | |
|---|---|---|---|
| *LC-1* | Mouse line transgenic for *Cre recombinase* and *luciferase*. Expression of both is controlled by a bidirectional *rtTA*-responsive promoter element | Ralph Witzgall, University of Regensburg | Schonig et al.[112] |
| *Rosa26-LacZ* | Cre reporter mouse strain carrying a transgene that consists of a *CMV* promoter fragment, a stop cassette flanked by *loxP* sites and the *E. coli lacZ* gene | Carmen Birch-meier-Kohler, Max-Delbrück-Centrum Berlin | Soriano[113] |

All animal experiments were performed in the Animal Facility of the Leibniz Institute for Age Research – Fritz Lipmann Institute (FLI), Jena, Germany, according to the rules of the German Animal Welfare Law and were licensed by the local authorities. This is consistent with the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences (CIOMS). The animals were housed under specific pathogen-free or pathogen-reduced conditions in a 12 h light cycle and were provided with standard mouse chow and tap water *ad libitum*.

## 2.2 Methods

### 2.2.1 Standard techniques

Standard molecular biology techniques, such as plasmid propagation and isolation, preparation of DNA and RNA, enzymatic manipulation of DNA and RNA and polymerase chain reaction (PCR) were performed according to Sambrook et al.[114] and/or Ausubel et al.[115].

### 2.2.2 Generation of a conditional *Wt1* knock-out mouse line

*Wt1* conditional knock-out mice (*Wt1flox* mice) were generated by introduction of *loxP* sites upstream of exon 2 and downstream of exon 3 of the *Wt1* gene. The corresponding targeting construct was generated by genOway (Lyon, France) and is based on a PAC clone containing the murine *Wt1* genomic sequence[116]. Electroporation of 129/SvPas ES cells, microinjection into C57BL/6J blastocysts, production of germline transmitting chimeras and, eventually, $Wt1^{fl/+}$ animals were also performed by genOway. PCR and Southern Blot based screenings for correct targeting of and homologous recombination in ES cells and chimeric animals were accomplished as part of the PhD work. To confirm correct transgene integration in resulting *Wt1flox* mice, Southern Blot analysis (2.2.5) was performed. Furthermore, *loxP* sites were amplified and sequenced. The primers Wt1flox-5'loxP-1F and Wt1flox-5'loxP-1R were used to amplify the 5' *loxP* site, and the primers Wt1flox-1F and Wt1flox-1R served to amplify the 3' *loxP* site. Both PCR products were subsequently cloned

into the TA cloning vector pCR4-TOPO (Invitrogen, USA) and sent to Eurofins/MWG for sequencing.

### 2.2.3 Generation of podocyte-specific *Wt1* knock-out mice

2.2.3.1 Constitutive podocyte-specific *Wt1* knock-out mouse line (*Wt1flox;Podocin-Cre*)

For the generation of a constitutive podocyte-specific *Wt1* knock-out mouse line animals of the *Wt1flox* and *Podocin-Cre* strains were crossed according to the following procedure: $Wt1^{fl/fl}$ mice were mated with individuals hemizygous for the *Podocin-Cre* transgene to obtain $Wt1^{fl/+}$*;Pod-Cre* mice in the F1 generation. These double heterozygous F1 animals were again crossed with $Wt1^{fl/fl}$ mice to produce homozygously *floxed* and *Podocin-Cre* hemizygous animals ($Wt1^{fl/fl}$*;Pod-Cre*) in the F2 generation. $Wt1^{fl/fl}$ and $Wt1^{fl/+}$*;Pod-Cre* mice derived from this crossing (litter mates) were used as controls.

2.2.3.2 Inducible podocyte-specific *Wt1* knock-out mouse line (*iWt1-KO*)

$Wt1^{fl/fl}$ mice were mated with *LC-1/+* and *Podocin-rtTA/+* animals, respectively, to obtain $Wt1^{fl/+}$*;LC-1*, as well as $Wt1^{fl/+}$*;Podocin-rtTA* mice in the F1 generation. Double-heterozygous individuals from both newly generated lines, *Wt1flox;LC-1* and *Wt1flox;Podocin-rtTA*, were again crossed with $Wt1^{fl/fl}$ animals to produce in the F2 generation $Wt1^{fl/fl}$*;LC-1* and $Wt1^{fl/fl}$*;Podocin-rtTA* mice, respectively. Intercrossing of $Wt1^{fl/fl}$*;LC1* with $Wt1^{fl/+}$*;Podocin-rtTA* individuals yielded both, $Wt1^{fl/fl}$*;Podocin-rtTA;LC-1* mice (*iWt1-KO*) and $Wt1^{flox/+}$*;Podocin-rtTA;LC-1* control animals with a frequency of 12.5% each in the F3 generation. To induce *Cre* expression a solution of 5% sucrose and 2 mg/ml doxycycline hyclate (AppliChem, Germany) in tap water was administered.

### 2.2.4 Genotype determination of transgenic mice by PCR

For routine genotyping tail tips of 10 day old mice were clipped and DNA was isolated by tissue lysis with 10 mg/ml proteinase K (Roche, Switzerland) in PBND buffer at 55°C for 12 h. Proteinase K was inactivated by boiling at 95°C for 15 min. Then 1 µl sample was used for subsequent PCR based genotype analysis. To verify the presence of floxed *Wt1* alleles (*Wt1flox* PCR), primers Wt1flox-1F and Wt1flox-1R were applied. The *Podocin-Cre*, *LC-1* and *Rosa26-LacZ* transgenes were detected using *Cre*-specific primers Cre-RS-1F and Cre-RS-1R (*Cre* PCR). Primers rtTA-podoprobe-F and rtTA-podoprobe-B were utilized to determine whether or not the *Podocin-rtTA* transgene was present in the genome of analyzed mice.

For all PCR reactions the Qiagen Taq polymerase/Buffer system (Qiagen, Germany) was applied. Master mix compositions and PCR conditions are indicated below.

**Master mix *Wt1flox* PCR**

| Ingredient | Final conc | Volume [µl] |
|---|---|---|
| DNA | | 1 |
| Primer 1 | 0.25 µM | 0.5 |
| Primer 2 | 0.25 µM | 0.5 |
| 10x PCR buffer | 1x | 2 |
| dNTP mix | 200 µM | 0.4 |
| Polymerase | 0.0125 U/µl | 0.05 |
| $H_2O$ | | 15.55 |
| $Volume_{total}$ | | 20 |

**Cycler program *Wt1 flox* PCR**

| Cycles | Temp [°C] | Duration [s] | Gradient |
|---|---|---|---|
| 1 | 94 | 120 | |
| 10 | 94 | 20 | |
| | 65 | 20 | -0.7°C/cycle |
| | 72 | 35 | |
| 30 | 94 | 20 | |
| | 58 | 20 | |
| | 72 | 35 | |
| 1 | 4 | ∞ | |

**Master mix *Cre* PCR**

| Ingredient | Final conc | Volume [µl] |
|---|---|---|
| DNA | | 1 |
| Primer 1 | 0.4 µM | 0.8 |
| Primer 2 | 0.4 µM | 0.8 |
| 10x PCR buffer | 1x | 2 |
| dNTP mix | 200 µM | 0.4 |
| Polymerase | 0.0125 U/µl | 0.05 |
| $H_2O$ | | 14.95 |
| $Volume_{total}$ | | 20 |

**Cycler program *Cre* PCR**

| Cycles | Temp [°C] | Duration [s] | Gradient |
|---|---|---|---|
| 1 | 94 | 120 | |
| 10 | 94 | 20 | |
| | 65 | 20 | -0.7°C/cycle |
| | 72 | 30 | |
| 30 | 94 | 20 | |
| | 58 | 20 | |
| | 72 | 30 | |
| 1 | 4 | ∞ | |

**Master mix *Podocin-rtTA* PCR**

| Ingredient | Final conc | Volume [µl] |
|---|---|---|
| DNA | | 1 |
| Primer 1 | 0.25 µM | 0.5 |
| Primer 2 | 0.25 µM | 0.5 |
| 10x PCR buffer | 1x | 2 |
| dNTP mix | 250 µM | 0.5 |
| Polymerase | 0.025 U/µl | 0.1 |
| $H_2O$ | | 15.4 |
| $Volume_{total}$ | | 20 |

**Cycler program *Podocin-rtTA* PCR**

| Cycles | Temp [°C] | Duration [s] | Gradient |
|---|---|---|---|
| 1 | 95 | 120 | |
| 40 | 95 | 20 | |
| | 58 | 20 | |
| | 72 | 35 | |
| 1 | 4 | ∞ | |

To prove Cre activity and *Wt1* deletion, respectively, DNA was obtained from total embryonic and newborn kidneys as well as adult kidney samples. For this the QIAamp DNA Mini and Micro kit (Qiagen, Germany) were used, following the provided protocols for DNA isolation from tissues. DNA was eluted and stored in 10 mM Tris-HCl, pH 8.0. The PCR was then performed with 100 ng total kidney DNA as template, Dream Taq polymerase/Buffer system (Fermentas, Lithuania) and primers Wt1-CreX-1F and Wt1-CreX-1R. For master mix compositions and PCR conditions see tables below.

**Master mix *Wt1* deletion PCR**

| Ingredient | Final conc | Volume [µl] |
|---|---|---|
| DNA | | 2 |
| Primer 1 | 0.25 µM | 1.25 |
| Primer 2 | 0.25 µM | 1.25 |
| 10x PCR buffer | 1x | 5 |
| dNTP mix | 200 µM | 1 |
| Tween 20 | 0.5% | 0.25 |
| Polymerase | | 0.2 |
| $H_2O$ | | 39.05 |
| $Volume_{total}$ | | 50 |

**Cycler program *Wt1* deletion PCR**

| Cycles | Temp [°C] | Duration [s] | Gradient |
|---|---|---|---|
| 1 | 94 | 60 | |
| 10 | 94 | 20 | |
| | 65 | 20 | -0.7°C/cycle |
| | 72 | 60 | |
| 26 | 94 | 20 | |
| | 58 | 20 | |
| | 72 | 60 | |
| 1 | 72 | 5 | |
| 1 | 4 | ∞ | |

### 2.2.5 Southern Blot analysis

#### 2.2.5.1 Generation of radioactively labeled DNA probes

DNA probes for Southern Blot analyses were synthesized and radioactively labeled using the NEBlot Kit (New England Biolabs, USA). Briefly, probe synthesis was performed *in vitro* from a denatured double-stranded DNA template by the Klenow fragment of the *Escherichia coli* DNA polymerase I. Replacement of dCTP with [alpha-$^{32}$P]dCTP in the reaction mix allowed simultaneous radioactive labeling of the probe. Readily labeled probes were afterwards purified with ProbeQuant G-50 Micro Columns (GE Healthcare, USA).

The DNA template to generate the 406 bp *Wt1* specific probe applied in this study was PCR amplified utilizing primers ENG1-W1 and ENG1-W2 (See table 2 for primer sequences) and cloned into pCRII-TOPO with the TOPO TA Cloning Kit (Invitrogen, USA). Prior to probe synthesis and labeling the DNA fragment was released from the vector by *EcoRI* (Fermentas, Lithuania) restriction enzyme digestion and purified employing agarose gel extraction kits QiaExII (Qiagen, Germany) or PeqGold (Peqlab, Germany).

#### 2.2.5.2 Southern Blot analysis of mouse genomic tail DNA

Mice were anesthetized by inhalation of isoflurane (Deltaselect, Germany), before approximately 1 cm of the tail tip was clipped. Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Germany), according to the provided protocol for DNA purification from tissues. DNA was eluted with and stored in 10 mM Tris-HCl, pH 8.0. 5 to 10 µg of purified total genomic DNA were digested with 80 units of *BamHI* (Fermentas, Lithuania) for 12 to 16 h at 37°C. Following digestion, DNA was precipitated with 100% ethanol for 12 h at 4°C, washed with 70% ethanol and redissolved in 10 mM Tris-HCl, pH 8.0, for 12 to 24 h at 4°C. After addition of an appropriate volume of 10x DNA Loading Dye

the DNA was loaded onto a 1.0% TBE agarose gel supplemented with 1 µl/100 ml ethidium bromide (Stock 5 mg/ml; Roth, Germany). Hyperladder I (Bioline, Germany) served as marker. After electrophoresis in 1x TBE Buffer at 100 V the gel was treated with (1) 0.125 M HCl for 10 min to depurinate DNA and (2) 0.4 M NaOH for 30 min to denature DNA. After that DNA was transferred to a positively charged nylon membrane (Hybond-XL; GE Healthcare, USA) by alkaline transfer with 0.4 M NaOH using a self-made capillary blotting chamber. Following a transfer process of 12 to 16 h, DNA and nylon membrane were cross-linked by exposure to 0.12 J/cm$^2$ of UV light ($\lambda$=312 nm). Prior to hybridization the blotting membrane was pre-incubated with modified Church-Gilbert Hybridization Buffer for at least 30 min at 65°C. Then the hybridization buffer was replaced by a fresh volume of pre-heated buffer and the previously denatured radioactive probe (95°C, 5 min) was added according to a final activity of 2,000,000 cpm/ml. After hybridization for 16 h at 65°C in a rotation oven the membrane was washed at 65°C in pre-warmed Low-Stringency Washing Solution for 15 min and in High-Stringency Washing Solution for another 15 min. Radioactivity was detected and visualized with a phosphoimager system (FLA-7000, Fujifilm, Japan).

| | |
|---|---|
| 10x DNA Loading Dye | 0.25% bromophenol blue; 0.25% xylene cyanol; 67% (w/v) sucrose; in $dH_2O$ |
| 1x TBE Buffer | 90 mM Tris-HCl; 90 mM boric acid; 2.5 mM EDTA; pH 8.3 |
| Modified Church-Gilbert Hyb. Buffer | 1 mM EDTA; 500 mM $Na_2HPO_4$, pH 7.2; 7% SDS |
| Low Stringency Washing Solution | 3x SSC, pH 7.0; 0.1% SDS |
| High Stringency Washing Solution | 0.5x SSC, pH 7.0; 0.1% SDS |

### 2.2.6 Isolation, treatment and embedding of kidneys for paraffin and frozen sections

Newborn (P0) mice were sacrificed by decapitation. Kidneys were instantly isolated and either dehydrated in 10% and 30% sucrose for 10 min and 30 min, respectively, or fixed in a solution of 1x phosphate buffered saline (PBS, pH 7,2) and 4% paraformaldehyde (PFA; Merck, Germany) for 12 – 14 hours at 4 – 8°C. Adult mice were anaesthetized by combined intraperitoneal injection of ketamine (80 mg/kg bodyweight; Ketavet, Pfizer, USA) and xylazine (8 mg/kg bodyweight; Rompun, Bayer, Germany). Then animals were perfused with 40 ml ice-cold 1x PBS through the left ventricle of the heart. Kidneys were obtained, cut into two halves and kidney poles were removed. Kidney halves were subsequently dehydrated in 10% sucrose for 10 min and 30% sucrose for 30 min - 2 h, or else fixed for 24 h in 4% PFA/1x PBS at 4 – 8°C. For preparation of frozen sections dehydrated P0 kidneys

and adult kidney halves were embedded in Neg -50 Frozen Section Medium (Thermo-Fisher-Scientific, USA) and frozen at -80°C. Sections (10 µm) were prepared on a Microm HM 550 cryotome (Microm, Germany) and mounted on SuperFrost slides (Menzel, Germany). To obtain paraffin sections PFA-fixed P0 kidneys and adult kidney halves were dehydrated in graded series of isopropanol or ethanol, infiltrated with chloroform or xylene and embedded in Paraplast or Paraplast X-Tra (McCormick Scientific, USA) according to standard procedures. Paraffin sections (5 µm) were prepared on a Microm HM 340 E microtome (Microm, Germany) and mounted on poly-lysine coated slides (Menzel, Germany).

| | |
|---|---|
| 1x PBS | 137 mM NaCl; 2.7 mM KCl; 4.3 mM $Na_2HPO_4$; 1.47 mM $KH_2PO_4$; pH 7.2 |
| 4% PFA/1x PBS | 4% (w/v) paraformaldehyde in 1x PBS |

### 2.2.7 Periodic acid-Schiff staining (PAS)

Paraffin sections were dewaxed in xylene, rehydrated in a graded ethanol series and washed with $dH_2O$. Then sections were incubated in 0.5% Periodic acid (Roth, Germany) for 5 min at room temperature and washed 1 min with frequently exchanged $dH_2O$. After washing, sections were incubated in Schiff-Reagent (Roth, Germany) for 15 min at room temperature and rinsed with running tap water for at least 3 min. For counter staining Mayer's Hematoxylin (Roth, Germany) was applied for 1 min. Sections were subsequently rinsed with running tap water for at least 3 min or submerged in 1x PBS for 1 min, washed with $dH_2O$, dehydrated in 95% ethanol, 100% ethanol and two changes of xylene and finally mounted using Cytoseal XYL (Thermo Fisher Scientific, USA) or Roti-Histokitt II (Roth, Germany). Sections were analyzed using a Zeiss Axiovert 135 TV inverse microscope. Pictures were taken with a Zeiss AxioCam MRc digital color camera.

### 2.2.8 Immunohistochemistry

Cryo sections were fixed in cold (4°C) 4% PFA/1x PBS or 100% acetone for 10 min, washed three times in 1x PBS/0.01% Triton-X and blocked for 30 min in Blocking Solution. After washing with 1x PBS/0.01% Triton-X, sections were incubated with primary antibodies solved in 1x PBS for 12 – 14 h at 37°C. Primary antibodies were applied in the following dilutions: Wt1 antibody 1:100; Podocin antibody 1:100; Synaptopodin antibody 1:200. Sections were then washed three times with 1x PBS/0.01% Triton-X and incubated with 1:500 dilutions of secondary antibodies in 1x PBS for 1 h at room temperature. Before

mounting with Prolong Gold (Invitrogen, USA), sections were treated with a 1:100 dilution of Hoechst 33342 Staining (Invitrogen, USA) in 1x PBS for 10 min and briefly washed with 1x PBS. Alternatively, sections were mounted using Vectashield/DAPI (Vector Laboratories, USA). Sections were analyzed with a Zeiss Axioimager.Z1 microscope. Pictures were taken with a Zeiss AxioCam MRm digital camera.

| | |
|---|---|
| Blocking Solution | 1% (w/v) bovine serum albumin (BSA); 0.6% (w/v) normal goat serum (NGS); 1% (v/v) DMSO; 0.5% (v/v) Tween-20; in 1x PBS |

### 2.2.9 Apoptosis assay (TUNEL)

Apoptosis in newborn kidneys was analyzed using the ApopTag Plus *In Situ* Apoptosis Detection Kit (Chemicon, USA) according to the instructions of the manufacturer. Briefly, 5-µm paraffin kidney sections were dewaxed in xylene, rehydrated in a graded ethanol series, washed with 1x PBS, fixed in 4% PFA/1x PBS for 15 min, again washed in 1x PBS, permeabilized for 15 min with 10 µg/ml proteinase K (Roche, Switzerland), washed and refixed in 4% PFA/1x PBS for 5 min. After additional washing with 1x PBS, endogenous peroxidase activity was quenched by treatment of sections with 3% $H_2O_2$/1x PBS for 5 min. Sections were again washed with 1x PBS, excess liquid was carefully removed and 100 µl/slide Equilibration Buffer was applied for 10 min. After removal of excess liquid, 100 µl/slide TdT (Terminal Deoxynucleotidyl Transferase) reaction mixture, consisting of TdT Reaction Buffer and TdT enzyme, was added to sections. Slides were then covered with plastic-cover slips and incubated for 1 h at 37°C in a humidified chamber. The TdT reaction was stopped through treatment of sections with Stop/Wash Buffer (1:35 dilution of Stop/Wash Buffer in $dH_2O$) for 10 min and washing in three changes of 1x PBS. Excess liquid was removed, 100 µl/slide Anti-Digoxigenin-Peroxidase Antibody Solution was added, slides were again covered with plastic cover slips and incubated for 30 min at room temperature in a humidified chamber. Following washing with four changes of 1x PBS, 80 µl/slide Peroxidase Substrate, being a 1:50 dilution of DAB (3,3'-diaminobenzidine) substrate in DAB Dilution Buffer, was applied for 3 to 8 min until a light brown background staining was noticeable on sections. Then sections were immediately washed with four changes of $dH_2O$, counterstained with 0.5% Methyl-Green (Labvision, USA) for 3 min, dehydrated in 95% ethanol, 100% ethanol and xylene and mounted using Cytoseal XYL (Thermo Fisher Scientific, USA). Sections were examined on a Zeiss Axiovert 135 TV inverse microscope. Pictures were taken using a Zeiss AxioCam MRc digital color camera.

| | |
|---|---|
| Proteinase K Buffer | 20 mM Tris-HCl, pH 7.5; 1 mM EDTA |

### 2.2.10 Transmission electron microscopy (TEM)

Newborn (P0) mice were sacrificed by decaptitation, kidneys were harvested, washed in 1x PBS, fixed in a freshly prepared solution of 2% glutaraldehyde (Sigma, USA) in1x PBS for 2 d at 4°C and conserved for 2 – 4 d in 1x PBS-buffered 0,02% sodium azide (Merck, Germany) at 4°C. Kidneys were sent in 1x PBS to Prof. Dr. Ralph Witzgall, University of Regensburg. Adult mice were deeply anaesthetized with xylazine/ketamine (described in 2.2.4) and perfused with 40 ml freshly prepared 2% glutaraldehyde/1x PBS through the heart's left ventricle. After isolation, kidneys were cut into small pieces of approximately 1 $mm^3$ and post-fixed in 2% glutaraldehyde/1x PBS for 3 – 4 d at 4°C. Following this procedure, newborn and adult kidney pieces were extensively washed with 1x PBS, infiltrated with 2% osmium tetroxide ($OsO_4$)/1x PBS for contrasting, washed with 1x PBS, dehydrated in a graded ethanol series and embedded in EPON 812 (Shell, UK). Ultrathin sections were prepared on a Leica EM UC6 microtome and mounted on copper slot grids coated with polyvinyl butyral film (Pioloform; Wacker, Germany). Sections were analyzed using a Zeiss CEM 902 TEM. All TEM analyses were performed in the group of Prof. Dr. Ralph Witzgall, University of Regensburg.

#### 2.2.10.1 Polyehtylenimine (PEI) staining

Kidneys of sacrificed adult mice were isolated, cut into 1 $mm^3$ pieces, stained with 0.5% polyethyleneimine (PEI) in 0.9% NaCl, pH 7.3, for 30 min, washed with three changes of 50 mM sodium cacodylate for 10 min each, fixed in 2% phosphotungstic acid/0.1% glutaraldehyde for 1 h, washed with 50 mM sodium cacodylate buffer, treated for 2h with 1% $OsO_4$ in 25 mM sodium cacodylate for contrasting, dehydrated and embedded in EPON 812.

### 2.2.11 Localization of *Cre* expression

#### 2.2.11.1 Generation of *Rosa26-LacZ;Podocin-rtTA;LC-1* reporter mouse line

In order to generate triple heterozygous mice, the following mating regime was applied: heterozygous animals of the *Rosa26-lacZ* and *Podocin-rtTA* strain were intercrossed resulting in double-heterozygous *Rosa26-lacZ/+;Podocin-rtTA/+* mice (frequency 25%) in the F1 generation. Next, these double-heterozygous F1 animals were mated with *LC-1*

hemizygous individuals to obtain triple heterozygous mice in the F2 generation (frequency 12.5%).

2.2.11.2 Beta-galactosidase assay

*Cre* expression was induced by administration of a 5% sucrose solution supplemented with 2 mg/ml doxycycline (AppliChem, Germany) for 7 d. Mice were sacrificed, kidneys were immediately isolated, embedded in cryo matrix and sectioned, as described. 10 µm cryo sections were fixed in fixation solution for 10 min. After that, sections were washed three times with 1x PBS, stained in staining solution for 12 h at 37°C and washed again three times with 1x PBS. For counter staining sections were submerged in 0.5% eosin (Roth, Germany) for 1 min and washed once in $dH_2O$. Following this, sections were dehydrated in a graded ethanol series and xylene, and were then mounted with Cytoseal XYL (Thermo Fisher Scientific, USA). Sections were analyzed on a Zeiss Axioimager.Z1 microscope. Pictures were taken with a Zeiss AxioCam MRc5 digital color camera.

| | |
|---|---|
| Fixation solution | 1% formaldehyde; 0.2% glutaraldehyde; 2 mM $MgCl_2$; 5 mM EGTA; 0.02% NP-40 |
| Staining solution | 0.1% (w/v) X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside); 5 mM $K_3Fe(CN)_6$; 5 mM $K_4Fe(CN)_6$-3 H2O; 2 mM $MgCl_2$; 0.01% sodium deoxycholate; 0.02% NP-40 |

### 2.2.12 Proteinuria assay

Urine was gathered from living animals or directly removed from bladders of sacrificed mice. 1 µl urine and 1 µl BSA solution (3 µg/µl; Sigma, USA), that served as control, were each complemented with 9 µl $dH_2O$ and 10 µl 2x Laemmli Sample Buffer, boiled for 5 min at 95°C and then loaded onto a 10% polyacrylamide gel, together with 5 µl Page Ruler Prestained Protein Ladder (Fermentas, Lithuania). Following electrophoresis in 1x Laemmli Running Buffer at 180 V, 30 mA the gel was stained in Coomassie Brilliant Blue R250 according to standard procedures.

| | |
|---|---|
| 2x Laemmli Sample Buffer | 125 mM Tris-HCL, pH 6.8; 4% SDS; 20% glycerol; 0.004% bromophenol blue; 8% 2-mercaptoethanol |
| 1x Laemmli Running Buffer | 25 mM Tris, pH 8.6; 192 mM glycin; 1% SDS |

### 2.2.13 Real-time RT-PCR analyses (qRT-PCR)

#### 2.2.13.1 Glomerulus extraction, RNA isolation and *DNaseI* digestion

Glomeruli were extracted from adult mouse kidneys following a method described by Takemoto et al.[117] with modifications established by Blutke[118]. Briefly, adult mice were anesthetized with Ketamine/Xylazine as described in 2.2.6. Animals were perfused with 40 ml of a solution containing magnetic beads with a diameter of 450 nm (Dynabeads M-450 Epoxy, Invitrogen, USA) and 40°C 1x PBS through the heart. The perfusion pressure was kept constant at 60 mmHg. Kidneys were removed, minced into 1 $mm^3$ pieces and treated with 1 mg/ml *collagenase A* (Roche, Switzerland) in 1x HBSS buffer for 30 min. Glomeruli were isolated using a BD IMag magnet (BD Biosciences, USA). After several rounds of washing/isolation glomeruli were lysed and homogenized in Trizol Reagent (Invitrogen, USA). RNA was isolated/purified from the aqueous phase employing the RNeasy Mini Kit (Qiagen, Germany). Prior to reverse transcription 770 ng RNA were treated with RQ1 RNase-Free DNaseI (Promega, USA) for 30 min at 37°C.

#### 2.2.13.2 Reverse transcription

For reverse transcription with SuperScript II RNase $H^-$ Reverse Transcriptase (Invitrogen, USA) and oligo-dT primers (Roche, Switzerland) 350 ng glomerular RNA were used. With the same amount of RNA a control reaction without reverse transcriptase (RT-) was set up in order to verify possible genomic DNA contamination. 10 µl of 1:10 dilutions of both, cDNA and control samples were used for subsequent real-time RT-PCR analyses.

#### 2.2.13.3 Quantitative real-time RT-PCR (qRT-PCR)

Quantitative real-time RT-PCR analyses were performed in a 96-well format on a Biorad iCycler or iQ5 system. Gene expression levels of samples to be compared were measured in one plate simultaneously and were normalized to corresponding *tbp* (TATA-box binding protein) expression. Samples were measured in triplicates. Primers used for qRT-PCR were chosen to span at least one intron and are listed in table 2 (2.1.1). Master mix composition and basic PCR conditions are shown in tables below.

**Master mix qRT- PCR**

| Ingredient | Final conc | Volume [µl] |
|---|---|---|
| cDNA | | 10 |
| Primer 1 | 0.4 µM | 1 |
| Primer 2 | 0.4 µM | 1 |
| 10x PCR buffer | 1x | 2.5 |
| dNTP mix | 200 µM | 0.5 |
| $MgCl_2$ | 2 mM | 0.75 |
| SYBR Green I | | 0.75 |
| FITC | 400 pM | 0.25 |
| Polymerase | 0.03 U/µl | 0.15 |
| $H_2O$ | | 8.1 |
| $Volume_{total}$ | | 25 |

**Cycler program qRT-PCR**

| Cycles | Temp [°C] | Duration [s] |
|---|---|---|
| 1 | 94 | 60 |
| 35 | 94 | 30 |
| | 60 | 30 |
| | 72 | 30 |
| | 80 | 15 |
| 1 | 8 | ∞ |

SYBR Green I (Stock 10,000x; Sigma, USA) was used to detect DNA and was diluted 1:2,000 prior to application. Fluorescein (FITC; Biorad, USA) served as calibration dye. Fluorescence was detected at an 80°C step following elongation at 72°C to decrease signals emitted from short primer-dimer sequences. After amplification, a melt curve was generated to verify the amount of primer-dimers formed in the PCR reaction. Differences in gene expression were calculated according to the double-delta-$C_T$ method[119].

| | |
|---|---|
| 10x PCR buffer | 200 mM Tris-HCl, pH 8.4; 500 mM KCl |
| 1x HBSS | 1.26 mM $CaCl_2$-$2H_2O$, 0.5 mM $MgCl_2$-6 $H_2O$, 0.4 mM $MgSO_4$-7 $H_2O$, 5.33 mM KCl, 0.44 mM $KH_2PO_4$, 137.9 mM NaCl, 0.34 mM $NaHPO_4$-2 $H_2O$, 5.55 mM alpha-D-glucose-$1H_2O$ |

# 3 Results

In this study the function of Wt1 in developing and mature podocytes was addressed. To do so constitutive as well as inducible podocyte-specific *Wt1* knock-out mice were generated using the *loxP*/Cre system[120-123]. The latter is based on the recognition and subsequent recombination of neighboring *loxP* sites, 34 bp inverted repeats, by the bacteriophage-P1-derived enzyme Cre-recombinase. Sequences or genomic regions located between two similarly orientated *loxP* sites are thus deleted. The basis for both podocyte-specific *Wt1* knock-out mouse models was a conditional *Wt1* knock-out mouse line (*Wt1flox*), in which parts of the gene are flanked by *loxP* sites. Animals expressing *Cre* in a podocyte-restricted manner were crossed into this background.

## 3.1 The conditional *Wt1* knock-out mouse

### 3.1.1 The targeting strategy

The strategy chosen to generate the conditional *Wt1* knock-out mouse line comprised flanking of exons 2 and 3 of the genomic *Wt1* locus with *loxP* sites (Fig. 5 A). Upon Cre-mediated deletion joining of exons 1 (914 bp) and 4 (78 bp) during splicing of primary transcripts should result in a frame shift producing a pre-mature stop codon (Fig 5 B, C). The resulting mRNA molecules would probably be subject to nonsense mediated decay (NMD)[124] or translated into truncated, non-functional Wt1 protein.

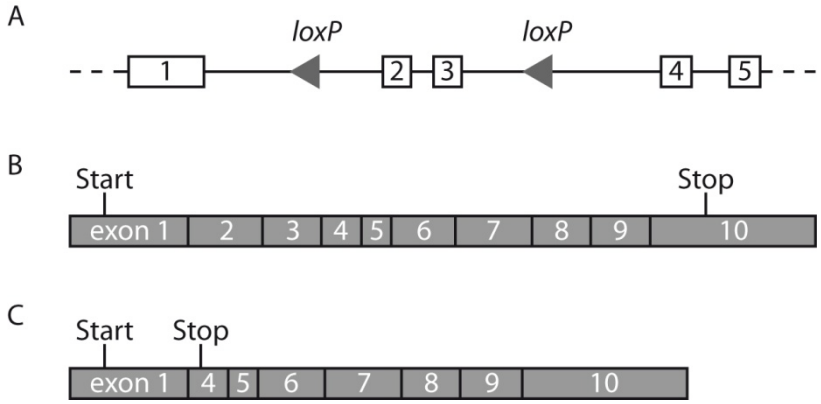


**Fig. 5. The conditional *Wt1* knock-out mouse targeting strategy.** (A) The targeting strategy is based on the insertion of *loxP* sites in the same orientation 5′ of exon 2 and 3′ of exon 3 of the *Wt1* gene. (B) Wild-type *Wt1* mRNA. (C) mRNA derived from the deleted *Wt1* locus as a consequence of Cre activity. The terms Start and Stop symbolize corresponding codons; *loxP*, Cre recombinase recognition sites.

### 3.1.2 Generation of conditional *Wt1* knock-out mice

*LoxP* sites were integrated into the *Wt1* locus of 129/SvPas embryonic stem cells (short ES cells) by homologous recombination with a targeting construct encompassing the following elements: the *Wt1* homology region including exons 1 to 5, as well as respective 5′ and 3′ flanking regions, *loxP* sites in introns 1 and 3, a neomycin-resistance cassette (neo) flanked by *FRT* sites for positive selection, and a diphtheria toxin A cassette (DTA) located outside the *Wt1* homologous region for negative selection (Fig. 6 A).

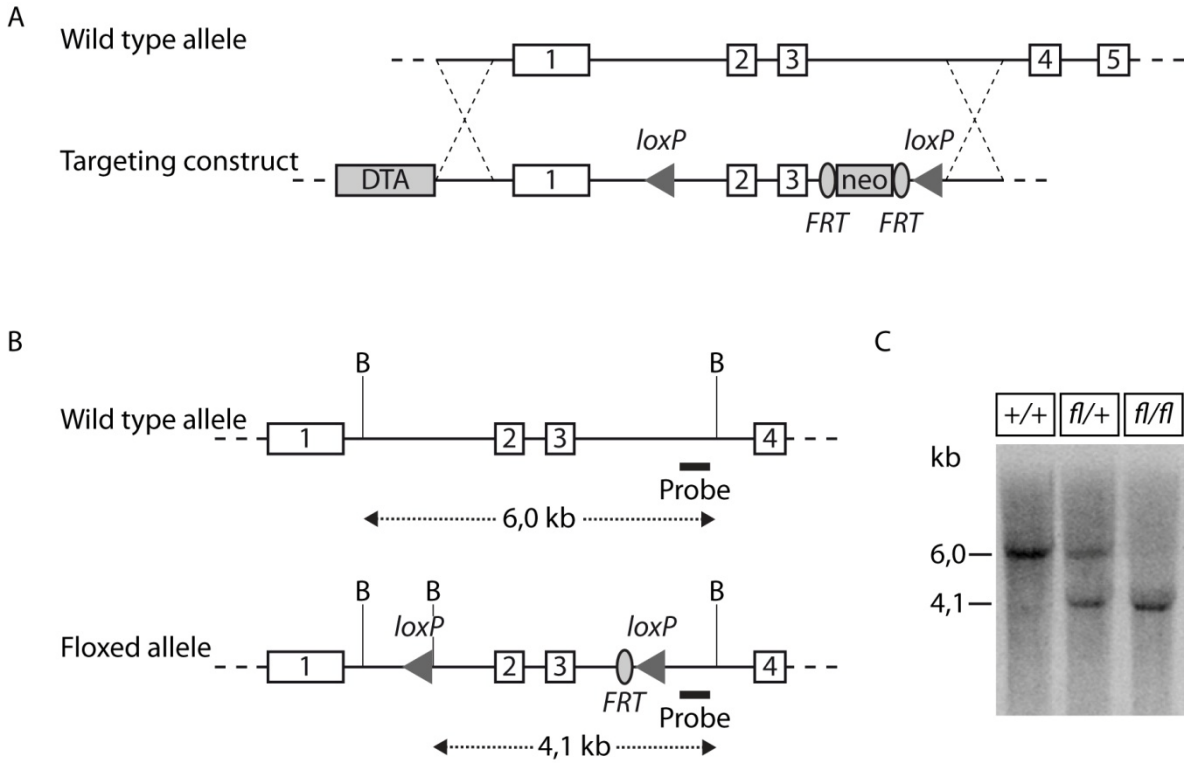


**Fig. 6. Generation and genotyping of conditional *Wt1* knock-out mice.** (A) The *Wt1flox* targeting construct used to replace the *Wt1* genomic sequence (wild type allele) by homologous recombination (B) Southern Blot strategy to genotype *Wt1flox* mice. *BamHI* sites are present in intron 1 and 3 of the wild type *Wt1* allele yielding a 6 kb fragment upon enzymatic digestion. The floxed *Wt1* allele contains an additional *BamHI* site integrated alongside with the 5′ *loxP* site. Restriction enzyme digestion results in a 4.1 kb fragment. (C) Southern Blot analysis of *BamHI* digested tail genomic DNA of $Wt^{+/+}$ (+/+), $Wt1^{fl/+}$ (*fl*/+) and $Wt1^{fl/fl}$ (*fl/fl*) mice showing presence and correct integration of the *Wt1flox* allele (4.1 kb fragment) only in the *fl*/+ and *fl/fl* mouse genome. B, *BamHI* restriction site; DTA, diphtheria toxin A cassette; *FRT*, Flp recombinase recognition site*; loxP*, Cre-recombinase recognition site; neo, neomycin resistance cassette.

Seven correctly targeted ES cells were injected into wild type C57BL/6J blastocysts. Two of these seven ES cell clones produced offspring with a high rate of chimerism between 50 % and 98 %. Male chimeras were then bred with wild type C57BL/6J females. One male and two female F1 mice carrying the *Wt1flox* transgene were then crossed with C57BL/6J *Flp* deleter mice to remove the neomycin-resistance cassette by recombination of *FRT* sites. Finally, two mice of different sex and mixed C57BL/6J/129/SvPas genetic background were

obtained, both being heterozygous for the *floxed Wt1* locus ($Wt1^{fl/+}$). Intercrossing allowed the generation of homozygous animals.

Correct targeting of *Wt1flox* mice was confirmed by Southern Blot based on a *BamHI* digestion strategy (Fig. 6 B and C). Additionally, 5' and 3' *loxP* sites were PCR-amplified, sub-cloned and sequenced. Both sites were present in the correct location within the intronic regions targeted and showed no sequence variation.

## 3.2 Constitutive podocyte-specific knock-out of *Wt1*

### 3.2.1 Generation of podocyte-specific *Wt1* knock-out mice

To generate a podocyte-specific *Wt1* knock-out mouse line *Wt1flox* animals were crossed with mice from a *Podocin-Cre* line (*Pod-Cre*, in short) expressing *Cre* under the control of a 2.5 kb promoter fragment of the human *podocin* gene (*NPHS2*)[110]. Transgene expression driven from this promoter is podocyte restricted and arises during late glomerular developmental stages (S-shaped body stage)[111]. Promoter activity is maintained in podocytes of mature glomeruli.

### 3.2.2 *Wt1* deletion is kidney specific and is first detectable at embryonic stage E14.5

In order to determine, whether the conditional *Wt1* knock-out is indeed kidney restricted, PCR on genomic DNA from different internal organs of newborn $Wt1^{fl/fl}$;*Pod-Cre* mice and kidneys of control animals ($Wt1^{fl/fl}$) was performed using primers flanking the *floxed Wt1* region. In case of *Wt1* deletion a DNA fragment of 735 bp ($Wt1^{\Delta}$) should be amplified. The *floxed* allele on the other hand yields an amplification product of 1694 bp ($Wt1^{flox}$). As shown in Fig. 7 A the $Wt1^{\Delta}$ fragment was exclusively detectable in kidneys of the examined $Wt1^{fl/fl}$;*Pod-Cre* mouse, not in other organs from the same animal, or in kidneys of a $Wt1^{fl/fl}$ litter mate control (termed 'C' in Fig. 7 A). This suggests that *Wt1* deletion is a) strictly dependent on *Cre* expression and b) kidney/podocyte restricted.

*Wt1* deletion could first be detected in embryonic kidneys of E14.5 $Wt1^{fl/fl}$;*Pod-Cre* mice (Fig. 7 B) confirming that *Cre* expression arises at late S-shaped body stage, during nephron patterning and maturation.

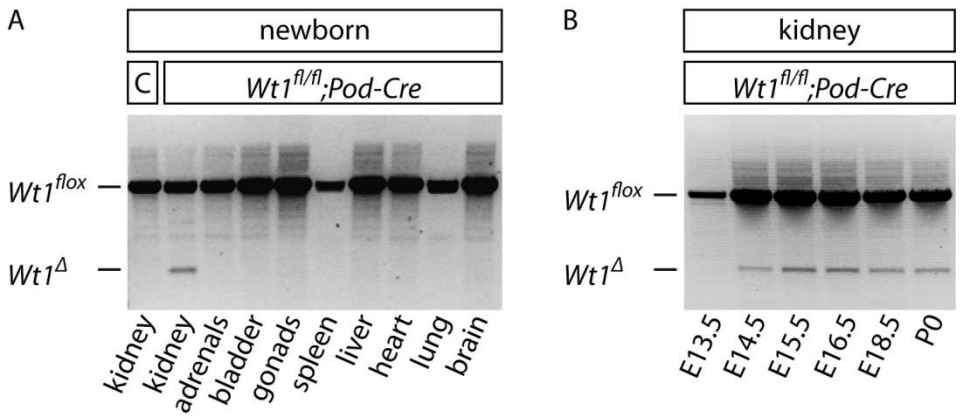


**Fig. 7. Detection of Cre-mediated *Wt1*-deletion by PCR** (A) Genomic DNA of kidneys and indicated internal organs of newborn *Wt1*$^{fl/fl}$*;Pod-Cre* mice was isolated and subjected to PCR using primers flanking the floxed *Wt1* sequence. As control (indicated as 'C') kidney DNA of *Wt1*$^{fl/fl}$ animals was used. The 735 bp amplification product of the deleted *Wt1* locus (*Wt1*$^{\Delta}$) is only yielded in PCR on *Wt1*$^{fl/fl}$*;Pod-Cre* kidney DNA. Since *Cre* is exclusively expressed in podocytes, the undeleted *Wt*$^{flox}$ allele is present in all organs (1694 bp PCR product). (B) PCR to determine the time point of first Cre-mediated *Wt1*-deletion on kidney DNA from *Wt1*$^{fl/fl}$*;Pod-Cre* mice of different embryonic stages (E13.5 to newborn, P0). *Wt1*-deletion (*Wt1*$^{\Delta}$ fragment) is first detectable in kidneys of E14.5 mice.

### 3.2.3 *Wt1*$^{fl/fl}$*;Pod-Cre* animals die shortly after birth and exhibit severe damages of the renal filtration apparatus

*Wt1*$^{fl/fl}$*;Pod-Cre* mice were born at the expected Mendelian ratio. In contrast to double heterozygous litter mates, however, these animals died rapidly, 12 to 24 hours after birth. The bladders of newborn *Wt1*$^{fl/fl}$*;Pod-Cre* animals were always found to be empty (anuria) and kidneys displayed red spots due to localized hemorrhages (Fig. 8 A). In *Wt1*$^{fl/+}$*;Pod-Cre* and *Wt1*$^{fl/fl}$ litter mates this was never observed. Signs for ascites or edema as a possible consequence of kidney failure could not be detected.

On the histological level the absence of urine could be explained by a strong reduction in the number of glomeruli (Fig. 8 C), as compared to *Wt1*$^{fl/+}$*;Pod-Cre* controls (Fig. 8 B). The remaining glomeruli were markedly smaller in size and cell number (Fig. 8 E, arrow) in relation to those of control animals (Fig. 8 D, arrows). Additionally, degenerating glomeruli could be identified (Fig. 8 E, arrowhead), which were entirely absent from control kidney sections. Forms of glomerulosclerosis, often characterized by mesangial matrix expansion and frequently found in patients and mice with *Wt1* mutations, were not observed in podocyte-specific *Wt1* knock-out kidneys. Instead, proximal convoluted tubules, the tubular parts directly connected with glomeruli, were strongly reduced in number and appeared to be collapsed, most probably due to the absence of primary urine (Fig. 8 C). In kidneys of *Wt1*$^{fl/+}$*;Pod-Cre* control animals proximal convoluted tubules appeared normal in

terms of number and shape (Fig. 8 B, dashed circle). In mice developing nephrons of different stages can be found in the outer cortex until approximately 7 days after birth. Microscopic analysis revealed no alteration in early nephrogenic stages in newborn *Wt1*$^{fl/fl}$*;Pod-Cre* mice.

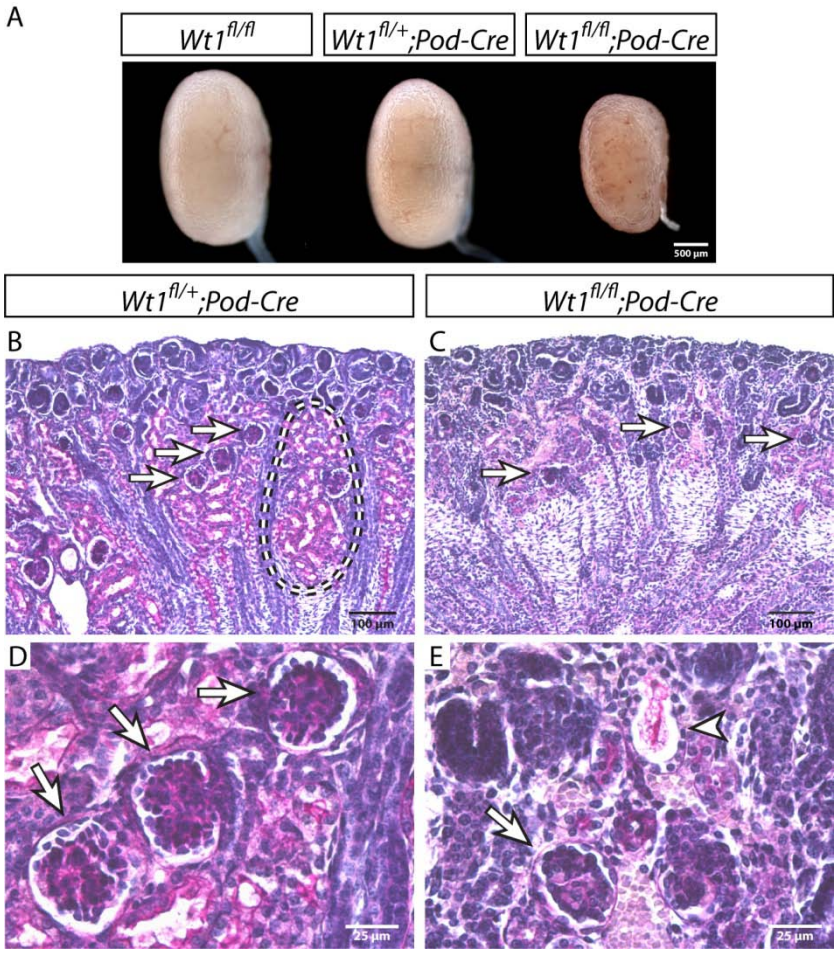


**Fig. 8. Macroscopic and histological analysis of newborn *Wt1*$^{fl/fl}$*;Pod-Cre* and control kidneys** (A) Kidneys obtained from newborn *Wt1*$^{fl/fl}$, *Wt1*$^{fl/+}$*;Pod-Cre* and *Wt1*$^{fl/fl}$*;Pod-Cre* litter mates. Superficial hemorrhages, indicated by red spots on the kidney surface, are present only in *Wt1*$^{fl/fl}$*;Pod-Cre* kidneys. (B) Low and (D) high magnification of a PAS stained 5 µm paraffin section of a newborn *Wt*$^{fl/+}$*;Pod-Cre* control kidney. Arrows indicate selected mature glomeruli, the dashed circle marks an exemplified zone of proximal convoluted tubules. (C) Low and (E) high magnification of a PAS stained 5 µm kidney paraffin section of a newborn *Wt1*$^{fl/fl}$*;Pod-Cre* mouse. Here arrows indicate mal-developed glomeruli. The number of proximal convoluted tubuli in (C) is strongly reduced. Arrowhead (E) points to a degenerated glomerulus.

### 3.2.4 Immunohistochemical analyses reveal significant loss of glomeruli

To support the histological findings the presence of Wt1 and different glomerular marker proteins within the cortex of conditional knock-out kidneys was studied by double-immunohistochemistry. As glomerular markers synaptopodin and podocin were used. Both factors are located in the cytoplasm of podocytes and are associated with the slit

diaphragm. In concordance with published data, expression of *Wt1* was strongest in developing nephrons of early developmental stages within the outer renal cortex (Fig. 9 A – D). These early nephrogenic structures do not yet exhibit synaptopodin or podocin synthesis. Extending into the inner cortex, *Wt1* expression becomes gradually weaker and is less broad as in the outer cortex. Co-localization with synaptopodin or podocin indicated that it remained to be expressed solely in podocytes of pre-mature (Fig. 9 A and C, arrowheads) and more mature glomeruli (Fig. 9 A and C, arrows) of newborn *Wt1$^{fl/+}$;Pod-Cre* kidneys. In conditional *Wt1* knock-out animals, however, neither Wt1, nor synaptopodin or podocin are detectable within the inner renal cortex (Fig. 9 B and D). Considering histological results, this might be due to the absence of glomeruli.

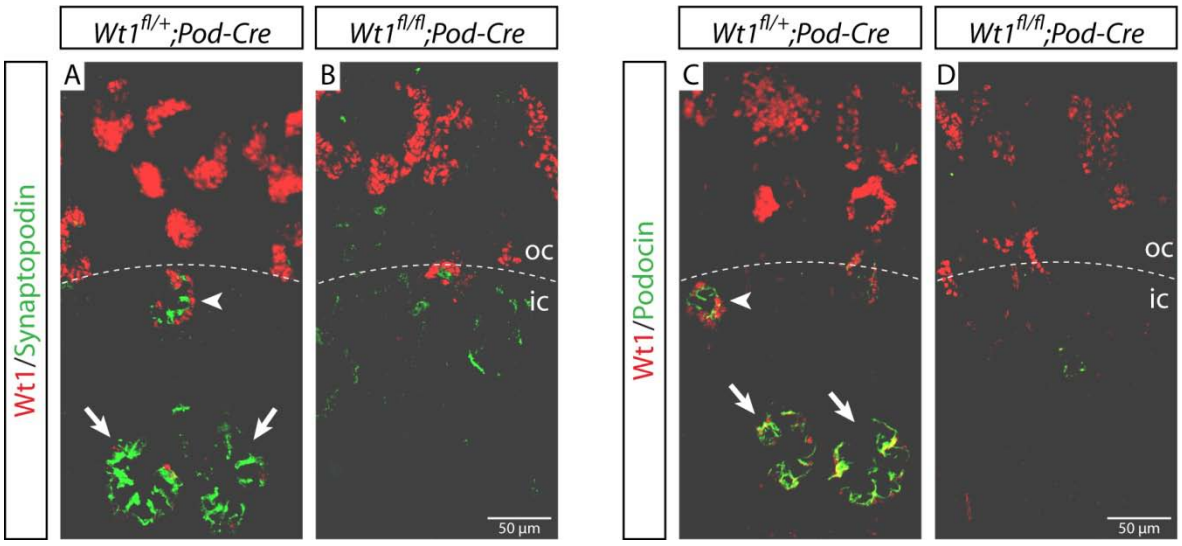


**Fig. 9. Presence of glomerular marker proteins and Wt1.** Immunohistochemistry on 10 µm cryo sections using antibodies against Wt1 (red) and mature podocyte markers synaptopodin and podocin (green). (A and C) In the inner renal cortex (ic) of newborn *Wt1$^{fl/+}$;Pod-Cre* kidneys Wt1, synaptopodin and podocin are detectable in pre-mature (arrowheads) and more mature glomeruli (arrows). However, none of the proteins are present in the inner renal cortex (ic) of *Wt1$^{fl/fl}$;Pod-Cre* kidneys indicating absence of glomeruli. (A, B, C, D) In the outer renal cortex (oc) of all animals early nephrogenic structures are located showing high levels of Wt1. oc, outer renal cortex; ic, inner renal cortex. The dashed line roughly determines the border between outer and inner renal cortex.

### 3.2.5 Loss of glomeruli is paralleled by enhanced apoptosis in the cortex of *Wt1$^{fl/fl}$;Pod-Cre* kidneys

Using TUNEL technique it could be demonstrated that loss of glomerular and tubular structures in newborn *Wt1$^{fl/fl}$;Pod-Cre* animals was paralleled by enhanced apoptosis along the renal cortex (Fig. 10 B). Higher magnification revealed that mostly detached podocytes of remaining glomeruli underwent programmed cell death (Fig. 10 D, arrows). *Wt1$^{fl/+}$;Pod-*

*Cre* control animals exhibited only sporadic apoptotic cells within the outermost cortex, and none in glomeruli or tubules (Fig. 10 A and C).

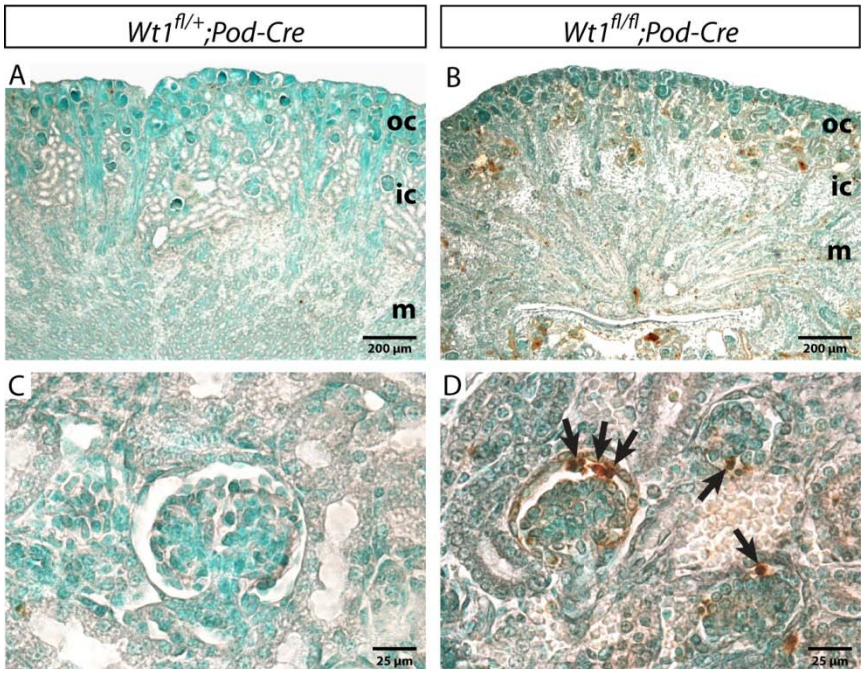


**Fig. 10. Analysis of apoptosis in newborn *Wt1$^{fl/fl}$;Pod-Cre* and control kidneys.** TUNEL assay on 5 µm kidney paraffin sections counterstained with methyl green nuclear staining. (A) Low and (C) high magnification of *Wt1$^{fl/+}$;Pod-Cre* control kidney sections. (A) Only sporadic apoptotic cells (brown staining) are present in the outer renal cortex (oc). (C) No apoptotic cells are observable in glomeruli. (B) Low and (D) high magnification *of Wt1$^{fl/fl}$;Pod-Cre* kidney sections. (B) Strongly enhanced apoptosis is detectable in the entire renal cortex. (D) Detached glomerular podocytes are mainly affected by apoptosis (arrows). oc, outer renal cortex; ic, inner renal cortex; m, medulla.

### 3.2.6 Ultrastructural studies reveal foot process effacement in podocytes of *Wt1$^{fl/fl}$;Pod-Cre* animals

Electron microscopic studies were performed to reveal possible structural changes in remaining glomeruli of newborn *Wt1$^{fl/fl}$;Pod-Cre* kidneys. *Wt1$^{fl/+}$;Pod-Cre* animals served as controls and exhibited normally developed glomeruli (Fig. 11 A). Podocytes of the latter were of normal appearance and had extensive foot processes (Fig. 11 C, arrows). In kidneys of podocyte-specific *Wt1* knock-out mice, however, severe structural damages could be identified. A considerable number of glomeruli displayed single capillary vessels not covered by podocytes (Fig. 11 B, arrow). As a consequence, high amounts of proteinaceous material emanated from these vessels (Fig. 11 B, asterisk). Additional protein casts could be observed in pathologically dilated tubules. Furthermore, podocytes displaying widespread foot process effacement were identified in a major number of glomeruli (Fig. 11 C, arrowheads). Although glomerular basement membrane (GBM) and endothelial cell layer

seemed to be intact; the loss of foot processes and associated slit diaphragms most probably rendered affected glomeruli incapable of filtration.

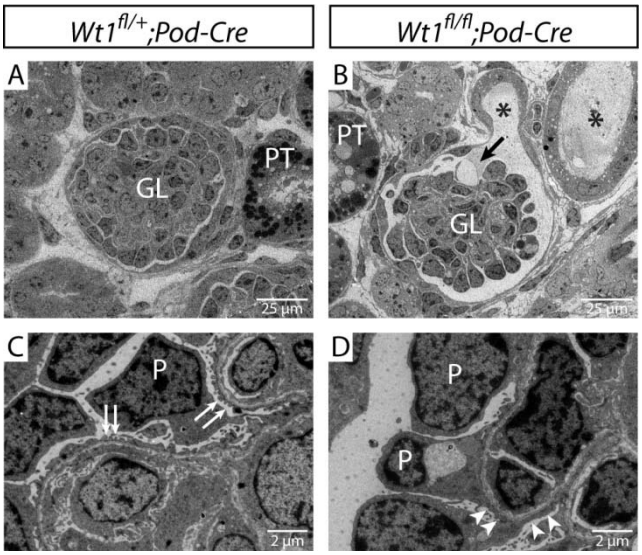


**Fig. 11. Ultrastructural analysis of newborn *Wt1*$^{fl/fl}$*;Pod-Cre* and control kidneys.** (A) Electron microscopy of a *Wt1*$^{fl/+}$*;Pod-Cre* kidney section showing single glomerulus (GL) and convoluted proximal tubulus (PT) of normal appearance. (C) High magnification indentifies podocytes (P) with normal foot processes (arrows). (B) Section of a *Wt1*$^{fl/fl}$*;Pod-Cre* kidney depicting a glomerulus in which one capillary vessel deprived of surrounding podocytes (arrow) can be seen. Proteinaceous material (asterisks) is emanating into the Bowman's space. Protein casts are also found in dilated tubulus adjacent to the glomerulus (GL). (D) High magnification of a *Wt1*$^{fl/fl}$*;Pod-Cre* kidney section reveals widespread foot process effacement (arrowheads). GL, glomerulus; PT, proximal convoluted tubulus.

### 3.2.7 Summary

The findings presented above suggest that *Wt1* deletion in developing podocytes of late developmental stages resulted primarily in structural changes in the form of widespread foot process effacement, which were followed by degeneration and apoptosis of podocytes. This was accompanied by the decay of entire glomeruli resulting in the dramatic reduction of overall glomerular number. Alongside with the degeneration of the glomerular filtration apparatus proximal convoluted tubules were strongly reduced as well. Since kidney function could no longer be maintained *Wt1*$^{fl/fl}$*;Pod-Cre* mice did not produce urine and died rapidly after birth.

## 3.3 Podocyte-specific *Wt1* knock-out in adult mice

### 3.3.1 Generation of inducible podocyte-specific *Wt1* knock-out mice

To generate the inducible podocyte-specific *Wt1* knock-out mouse the *loxP*/Cre system was used in combination with elements originally derived from the Tn10 tetracycline-

resistance operon of *Escherichia coli*. In principle, gene expression from this operon is controlled by an operator sequence (*tetO*) to which a transcriptional repressor (TetR) is constantly bound. Association of the drug tetracycline with TetR leads to the dissociation of the repressor from the operator sequence and, consequently, to the initiation of gene expression.[125]

Systems are now available, in which the Tet-repressor has been converted into a transcriptional activator (rtTA) by fusion to transcription activation domains. This transactivator can bind to and activate its cognate operator sequence exclusively in presence of the tetracycline-derivate doxycycline (anhydrotetracycline)[126,127]. A particular mouse line (*Podocin-rtTA*) allows podocyte-specific *rtTA* expression under control of the above described 2.5 kb *NPHS2* promoter fragment[107]. In addition, mice are available where *Cre* expression is governed by elements consisting of an array of *tetO* sequences fused to minimal promoters ($P_{tet}$). These regulatory elements are strictly responsive to doxycycline-activated transcriptional activators (rtTA)[128, 129]. The *LC-1* mouse line used here features a bi-directional $P_{tet}$ element ($P_{tetbi-1}$) controlling both *Cre* and *luciferase* expression at the same time. The transgene is integrated into the mouse *LC-1* locus allowing tight transcriptional control and low background expression[112].

The two mouse lines were crossed into the *Wt1flox* background to yield the inducible podocyte-specific *Wt1* knock-out mouse, *Wt1$^{fl/fl}$;Podocin-rtTA;LC-1* (hereafter termed *iWt1-KO*), and respective control animals, *Wt1$^{fl/+}$;Podocin-rtTA;LC-1* and *Wt1$^{fl/fl}$;LC-1*.

### 3.3.2 *Cre* expression controlled by the *Podocin-rtTA*/*LC-1* system is podocyte-specific

In order to prove that the *Podocin-rtTA*/*LC-1* system ensures podocyte restricted *Cre* expression a triple heterozygous mouse line was established that harbors a *Rosa26-LacZ* reporter allele in addition to the *Podocin-rtTA* and *LC-1* transgenes. Briefly, the *Rosa26-LacZ* reporter construct expresses the *E. coli lacZ* gene under control of a constitutively active promoter. However, transcription is inhibited by a stop cassette that is flanked by *loxP* sites. Only in cells synthesizing Cre-recombinase the stop cassette is removed by *loxP* recombination enabling *lacZ* expression to occur[113]. Presence of the respective gene product, beta-galactosidase, can be detected on tissue sections by adding the enzymes' substrate, X-Gal. The substrate is converted into a product of blue color.

To induce *Cre* expression 2 month old triple heterozygous mice (*Rosa26-LacZ;Podocin-rtTA;LC-1*) were provided with 2 mg/ml doxycycline in the drinking water for 7 days. Age

matched control mice lacking the *LC-1* transgene (*Rosa26-LacZ;Podocin-rtTA)* were treated accordingly. X-Gal staining of kidney sections revealed that Cre-recombinase was exclusively synthesized in glomerular podocytes of triple transgenic mice (Fig. 12 B, D) and entirely absent from control mice (Fig. 12 A, C). Thus, the *Podocin-rtTA/LC-1* system is indeed suitable to drive *Cre* expression in a podocyte-restricted and inducible manner.

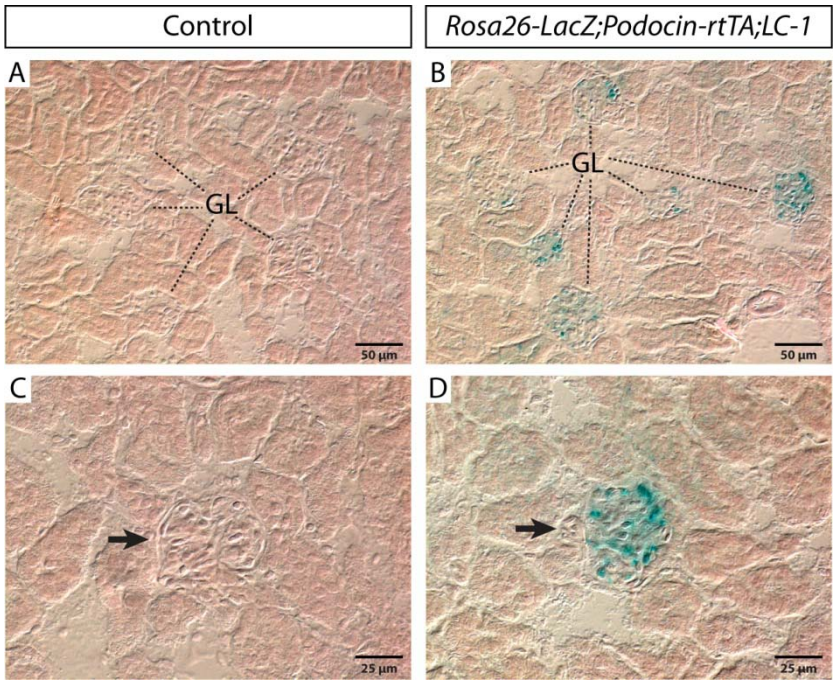


**Fig. 12. Analysis of Cre activity using a *Rosa26-LacZ* reporter mouse line.** Mice were supplied with doxycycline for 7 days before kidneys were harvested and sectioned. 10 µm cryo sections were X-Gal treated and counter stained with eosin. (A), (C) Kidneys of control mice lacking the *LC1* (*Cre*) transgene, but harboring both the *Rosa26-LacZ* reporter and the *Podocin-rtTA* construct exhibit no blue staining. (B), (C) In *Rosa26-LacZ;Podocin-rtTA;LC-1* animals Cre activity was only detectable in glomerular podocytes and not in any other renal cell population or structure. (C) and (D) being higher magnifications of (A) and (B), respectively. Glomeruli are termed GL or are otherwise indicated by arrows.

### 3.3.3 Adult doxycycline-induced *iWt1-KO* mice develop massive proteinuria, but display only subtle kidney pathologies

*iWt1-KO* and control mice (*Wt1*$^{fl/+}$*;Podocin-rtTA;LC-1*) with a minimum age of 2 months were provided with drinking water supplemented with 2mg/ml doxycycline. Proteinuria, being the first sign for damages in the kidney's filtration apparatus, was monitored. After five to seven days of continuous doxycycline application, *iWt1-KO* mice developed massive proteinuria. Control mice exhibited only physiological levels of urine protein content (Fig. 13 A) showing that doxycycline does not cause proteinuria by itself.

To determine the daily amount of protein excreted, urine of mice that had been induced for 7 days was gathered over 24 hours. Subsequent analyses revealed total protein

concentrations between 2.7 µg/µl ($V_{total/24\ h}$ 350 µl) and 18.9 µg/µl ($V_{total/24\ h}$ 400 µl) in the urine of *iWt1-KO* mice compared to concentrations between 0.1 µg/µl ($V_{total/24\ h}$ 750 µl) and 0.3 µg/µl ($V_{total/24\ h}$ 150 µl) of control animals. This effect was sex independent: the extent of proteinuria was similar in mice of both sexes.

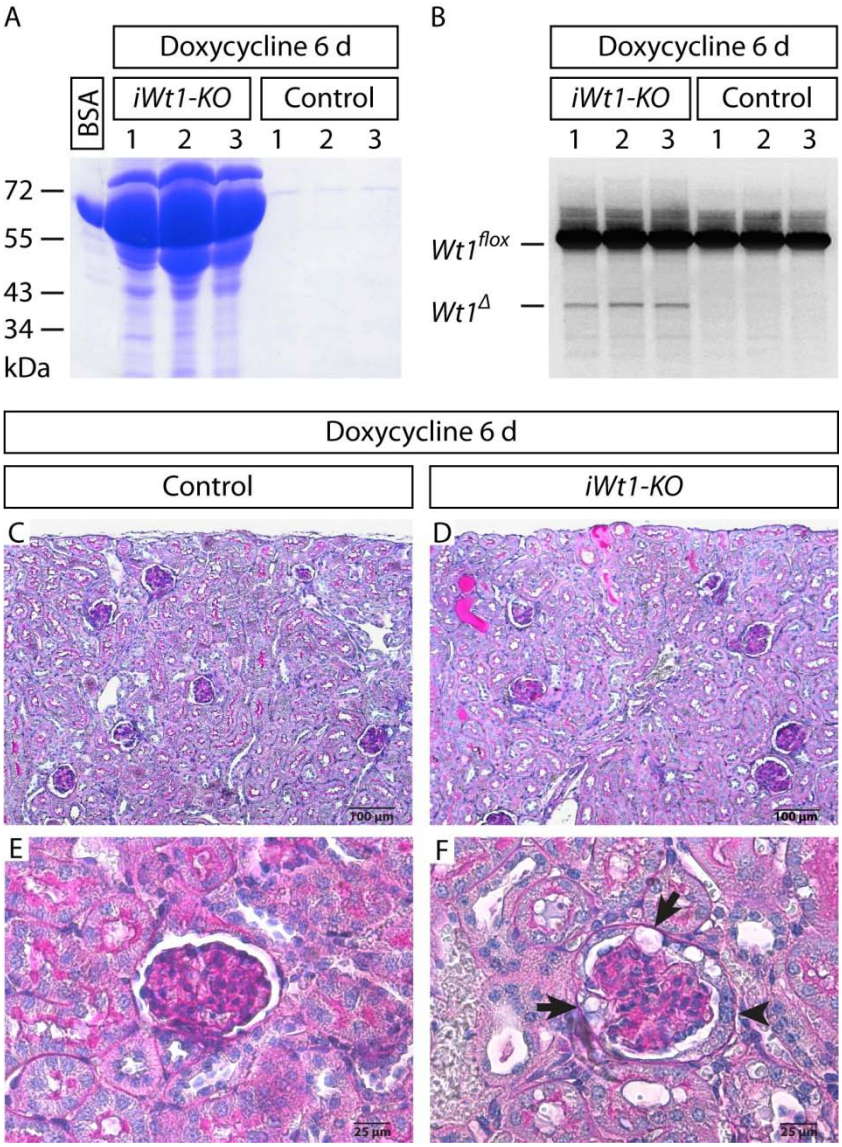


**Fig. 13. Phenotype analysis of adult *iWt1-KO* mice after podocyte-specific *Wt1* inactivation** (A) Denaturing polyacrylamide gel loaded with 1 µl urine of three different *iWt1-KO* mice and the same number of control (*Wt1$^{fl/+}$;Podocin-rtTA;LC-1*) animals treated with 2 mg/ml doxycycline for 6 days. 3 µg of bovine serum albumin (BSA) were loaded as standard. Coomassie Brilliant Blue staining revealed that *iWt1-KO* mice had developed proteinuria. (B) PCR on genomic DNA from kidneys of three different *iWT1-KO* and control (*Wt1$^{fl/fl}$;LC-1*) mice previously treated with 2 mg/ml doxycycline for 6 days. The PCR product specific for the deleted *Wt1* locus (*Wt1$^{\Delta}$*) was exclusively amplified from *iWt1-KO* kidney DNA. (C) PAS stained 5 µm paraffin section of a *Wt1$^{fl/+}$;Podocin-rtTA;LC-1* control kidney showing normal kidney tissue. (E) Normal glomerulus of the section depicted in (C). (D) *iWt1-KO* kidney paraffin section after PAS staining; the tissue appears normal except for dilated tubuli containing proteinaceous material (strong red staining). (F) Single glomerulus with hypertrophied Bowman's Capsule (arrowhead) and vacuolized pariethal epithelial cells (PEC, arrows). All mice used for histology had been induced with 2 mg/ml doxycycline until *iWt1-KO* animals developed proteinuria (6 days).

By PCR on total kidney DNA it was confirmed that *Wt1* was deleted in kidneys of doxycycline-treated *iWt1-KO* mice suffering from proteinuria (Fig. 13 B). In induced control animals lacking the *Podocin-rtTA* transgene (*Wt1$^{fl/fl}$;LC-1*) *Wt1* deletion could not be detected. This proves that the *Cre* expression system employed here is strictly regulated by doxycycline-activated rtTA and does not display background expression in absence of the transactivator (Fig. 13 B).

Apart from massive proteinuria, induced *iWT1-KO* mice appeared normal and healthy. The body cavity was free of fluid; signs of edema or ascites were not observed. Kidneys of knock-out and control animals were comparable in terms of appearance, size and weight.

In PAS stained kidney sections no gross abnormalities were found with one exception: dilated tubules filled with proteinaceous material were frequently present (Fig. 13 D, asterisks). The latter were never observed in control animals (Fig. 13 B, C).

A minor number of *iWt1-KO* glomeruli displayed phenotypic changes primarily affecting pariethal epithelial cells (PEC's) of the Bowman's Capsule. Here the entire PEC layer was thickened and interspersed with strongly vacuolized cells (Fig. 13 F, arrows). Also podocytes were sometimes hypertrophied and vacuolized in these cases.

### 3.3.4 The reduction of Wt1 protein levels varies strongly in kidneys of *iWt1-KO* mice

After having shown that *Wt1* was deleted upon induction of *Cre* expression in *iWt1-KO* kidneys it was necessary to analyze whether Wt1 protein levels were also affected in glomerular podocytes. Therefore, co-immunohistochemistry was performed using an antibody against the podocyte/glomerular marker podocin in combination with a Wt1 C-terminal antibody. Kidneys from *iWt1-KO* mice and *Wt1$^{fl/+}$;Podocin-rtTA;LC-1* controls were harvested when proteinuria was diagnosed in the *iWt1-KO* cohort. Interestingly, in *iWt1-KO* mice Wt1 protein was still detectable in a number of glomeruli and the extent of overall reduction varied strongly in kidneys of different individuals. In three *iWt1-KO* animals the number of Wt1-negative glomeruli in relation to the total glomerular number was determined on the basis of five kidney sections per individual. In 11.8%, 24.3% and 69% of all glomeruli Wt1 was not detectable. On sections of control animals no Wt1-negative glomeruli were observed. Obviously, the reduction of Wt1 protein levels in a small number of glomeruli is sufficient to cause proteinuria. In Fig. 14 the immunohistochemistry results are depicted showing representative examples for Wt1-positive and negative glomeruli in *iWt1-KO* kidneys.

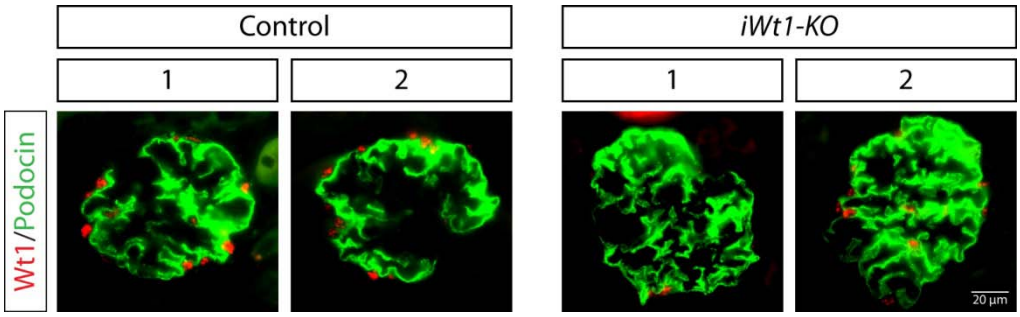


**Fig. 14. Presence of Wt1 protein after podocyte specific *Wt1* inactivation in adult mice.** Double-immunohistochemistry on 10 µm cryo sections using Wt1- and podocin-specific antibodies. Wt1 is located in the nuclei of podocytes. Podocin is found in the cytoplasm of podocytes and was utilized as glomerular marker. Control and *iWt1-KO* mice were treated with 2 mg/ml doxycycline until *iWt1-KO* animals developed proteinuria. In glomeruli of control kidneys Wt1 protein levels were comparable (two examples are shown). *iWt1-KO* mice exhibited a heterogeneous picture: glomeruli lacking Wt1 protein (example 1) as well as glomeruli with unchanged Wt1 levels were found (example 2) in the same kidney.

### 3.3.5 Podocytes of adult *iWt1-KO* mice display widespread loss of foot processes

Kidneys from induced, proteinuria-positive *iWt1-KO* mice were subjected to electron microscopy based ultra-structural analyses. It was found that podocytes of a considerable number of glomeruli exhibited widespread loss of foot processes and slit diaphragms, respectively (Fig. 15 B and D, arrows), as observed in constitutive podocyte-specific *Wt1* knock-out kidneys. Furthermore, the podocyte-layer as well as the endothelium appeared abnormally distant from the glomerular basement membrane (GBM) (Fig. 15 D). In rare cases also other structural anomalies, such as spider like foot processes, could be noticed in kidney sections of *iWt1-KO* animals. However, pathological changes were not present in all glomeruli or all podocytes of a given glomerulus. In a number of cases podocytes had wild type appearance. Nevertheless, the pathologies detected in *iWT1-KO* mice were never observed in *Wt1*$^{fl/+}$*;Podocin-rtTA;LC-1* control kidneys (Fig.15 A and C) and are thus likely to be the cause of proteinuria.

To analyze a possible role of Wt1 in the maintenance of the functional integrity of the glomerular basement membrane (GBM) polyethylenimine (PEI) staining[130] was performed. PEI is a strongly cationic agent that binds to anionic sites present in the GBM. Since charge selectivity is assumed to be an important functional feature of the GBM any deviation in terms of charge might affect the overall renal filtration capacity[62]. In electron microscopic analyses PEI stained sites appear as dark dots within the GBM. As depicted in Fig. 15 C and D, the number of anionic sites in GBM's of *iWT1-KO* (Fig. 15 D) and control mice (Fig. 15 C)

were comparable. Thus, GBM charge seemed unaffected by the podocyte-specific inactivation of *Wt1*.

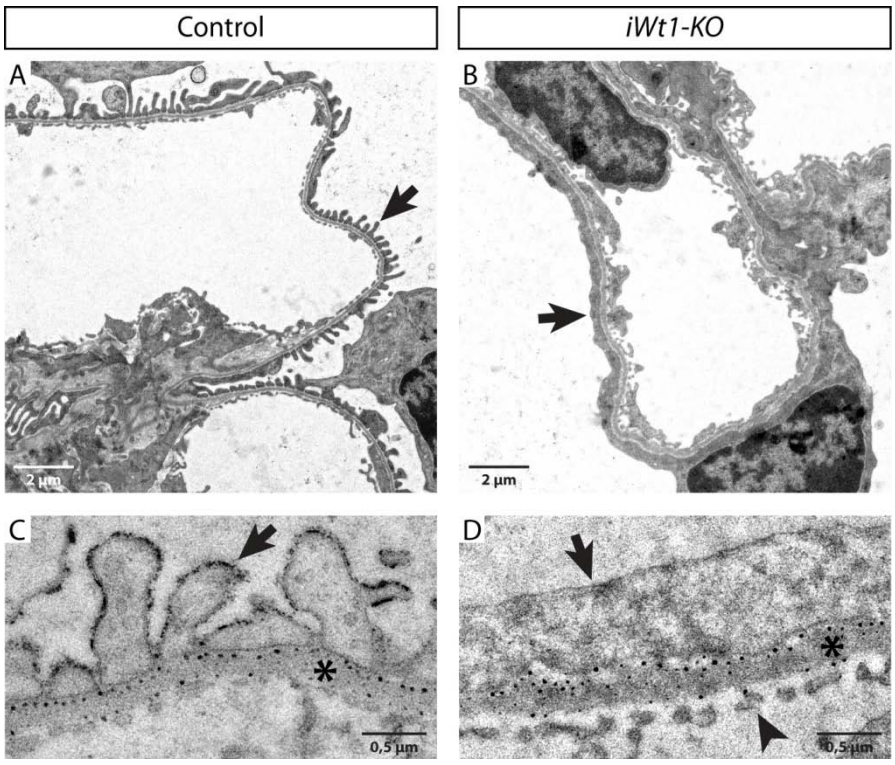


**Fig. 15. Ultrastructural analysis of adult induced *iWt1-KO* and control kidneys.** Mice were treated with 2 mg/ml doxycycline until *iWt1-KO* animals developed proteinuria; then kidneys were harvested. (A) Capillary vessel of a control glomerulus surrounded by podocytes with normally developed foot processes (arrow). (B) Podocytes loose foot processes after podocyte-specific inactivation of *Wt1* in *iWt1-KO* mice (arrow). (C and D) Polyethylenimine (PEI) staining to analyze the charge of the glomerular basement membrane (GBM). PEI marks anionic sites in the GBM (black spots). No obvious charge differences between the GBM's (asterisks) of induced *iWt1-KO* and control (*Wt1$^{fl/+}$;Podocin-rtTA;LC-1*) glomeruli are detectable. (D) The distribution of PEI stained anionic sites within the GBM appears different compared to (C), since the GBM is irregularly shaped and in parts detached from podocytes and endothelium (arrowhead). The arrow in (C) marks a foot process and in (D) foot process deprived podocytes.

### 3.3.6 *Wt1* expression levels are significantly reduced in glomeruli of long-time induced *iWT1-KO* mice

To explore the molecular causes of foot process effacement in *iWt1-KO* animals and to identify Wt1 target genes relevant for proper podocyte function microarray and real-time RT-PCR experiments were performed on the basis of both total kidney RNA and glomerular RNA of short-time induced (7 d) mice. However, no differences in *Wt1* and overall gene expression levels between *iWt1-KO*, *Wt1$^{fl/+}$;Podocin-rtTA;LC-1* and *Wt1$^{fl/fl}$;LC-1* animals were detected (data not shown). For this reason preliminary long-time induction studies were conducted: *iWt1-KO* mice (n=2) and *Wt1$^{fl/fl}$;LC-1* control animals (n=2) were treated with 2 mg/ml doxycycline for 2 months before glomeruli were isolated from the kidneys.

Subsequent real-time RT-PCR analyses on glomerular RNA/cDNA revealed that *Wt1* expression was significantly reduced by factor 8 in *iWt1-KO* glomeruli (Fig. 16). The expression of genes encoding foot process associated factors, *Nphs2* (*podocin*) and *Cd2ap*, and the major constituent of the slit diaphragm, *Nphs1* (*nephrin*), was found to be mildly reduced, between factor 1.5 and 2. Similarly, the podocyte marker gene *Synpo* (*synaptopodin*) was down-regulated by around factor 2 (Fig. 16).

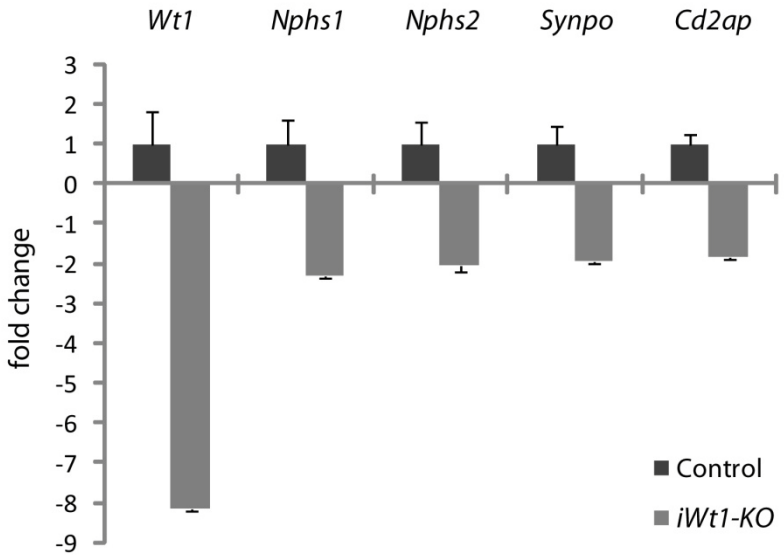


**Fig. 16. Analysis of gene expression in glomeruli of long-time induced *iWt1-KO* animals.** Both *iWT1-KO* (n=2) and *Wt1$^{fl/fl}$;LC-1* control animals (n=2) were treated with 2mg/ml doxycycline for 2 months. Expression of *Wt1*, *Nphs1*, *Nphs2*, *Synpo* and *Cd2ap* was measured by real-time RT-PCR on glomerular RNA/cDNA and was normalized to *tbp* (TATA box binding protein). Gene expression levels of *iWT1-KO* glomeruli were determined in relation to those of control glomeruli, which were set as 1. Differences in gene expression are depicted as fold difference. A negative prefix indicates down-regulation of genes in *iWt1-KO* glomeruli. Data represent the means and standard deviations of two independent experiments, measured in triplicates. *Wt1* was down-regulated by factor 8 in *iWt1-KO* glomeruli; expression of all other genes was slightly reduced by factor 1.5 to 2.

### 3.3.7 Two out of three *iWt1-KO* mice die after long-time induction; in surviving animals proteinuria decreases significantly

In order to examine the consequences of long-time induction *iWt1-KO* mice and *Wt1$^{fl/fl}$;LC-1* control animals were supplied with 2mg/ml doxycycline for 4 months. After 7 days of treatment, *iWt1-KO* mice developed proteinuria (Fig. 17, 7 d). The protein content, however, decreased significantly in the urine of all of these mice after 45 days of doxycycline administration (Fig. 17, 45 d). In one out of three *iWt1-KO* animals proteinuria could not be diagnosed after 125 days of treatment (Fig. 17, 125 d); in the two other mice the urine protein content increased again after this period of time (Fig. 17, 125 d, asterisks). Two days later both animals were found dead. The cause of death could not be elucidated.

Also, the processes leading to the temporal reduction and/or complete regression of proteinuria remain to be analyzed.

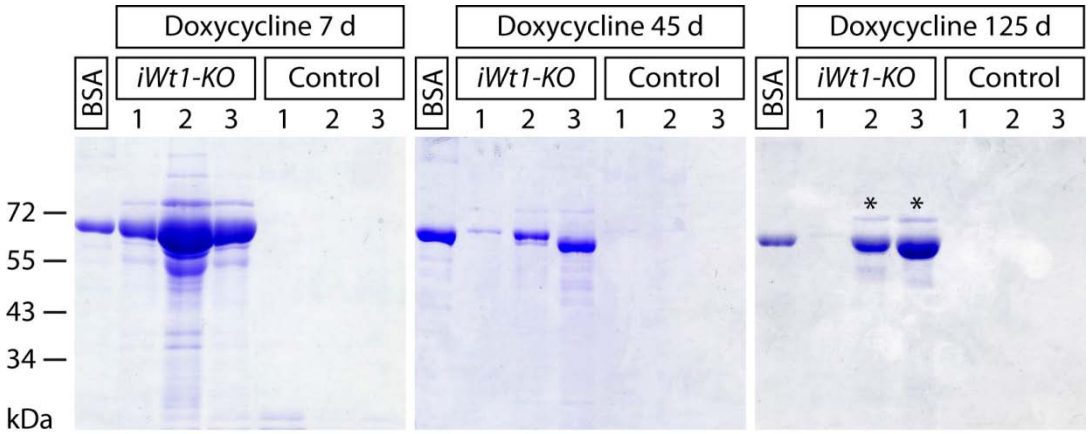


**Fig. 17. Proteinuria progression during long-time induction of *iWt1-KO* animals.** Mice were continuously supplied with 2 mg/ml doxycycline; after 7, 45 and 125 days of treatment 1 µl urine of each mouse was loaded onto a denaturing polyacrylamide gel. 3 µg BSA were used as standard. The gels were stained with Coomassie Brilliant Blue. After 7 days of treatment all three *iWt1-KO* animals had developed proteinuria, which decreased in intensity after 45 days of doxycycline administration. In one of these mice (animal 1) the urine was nearly free of protein, in the two other mice (animals 2 and 3) urine protein concentrations increased again after 125 days of induction. *Wt1*$^{fl/fl}$*;LC-1* control mice never developed proteinuria. Asterisks indicate mice that died after 127 days of induction.

### 3.3.8 Summary

It has been shown that the *Podocin-rtTA/LC-1* system used to generate the inducible podocyte-specific *Wt1* knock-out mouse (*iWt1-KO*) was suitable to ensure podocyte-restricted and inducible *Cre* expression. After five to nine days of doxycycline-mediated induction, adult *iWt1-KO* animals developed proteinuria, which was accompanied by *Wt1* deletion. Subtle histological changes, mostly in the form of protein casts within the renal tubular system, were observed. On the protein level Wt1 was absent or reduced in a varying number of glomeruli of different individuals. Accordingly, podocyte-associated ultrastructural changes, namely foot process effacement, were diagnosed in a varying number of podocytes and glomeruli, respectively. Changes in gene expression were only detectable after long-time doxycycline treatment. *Wt1* expression was clearly reduced; other podocyte marker genes were slightly down-regulated. After 4 months of continuous induction two out of three mice died, one mouse, however, recovered almost completely from proteinuria.

# 4 Discussion

## 4.1 Wt1 is required for podocyte and glomerulus differentiation

The general importance of Wt1 in kidney development and function is undoubted since mutations in the gene have been associated with renal glomerular pathologies found in patients with Denys-Drash (DDS), Frasier (FS) or WAGR syndrome[27-29, 32-35, 40, 41]. In addition, mice carrying DDS- or FS-associated *Wt1* mutations recapitulated the kidney phenotypes characteristic for the respective syndrome[42, 103, 104, 106]. Analyses of constitutive *Wt1* knock-out mice demonstrated that initiation of kidney development depends on Wt1 and that deletion of the gene results in renal agenesis[59]. The involvement of Wt1 in later renal developmental stages, such as nephron induction and glomerular differentiation, however, has not been supported by comparable *in vivo* data. A possible role of Wt1 in processes affecting the differentiation and maturation of the glomerulus has only been deduced from *in vitro* data suggesting a dependence of *Vegfa* expression and *Pax2* down-regulation on Wt1 (REFS [23, 26]). But this and other functions could not be addressed *in vivo* due to the lack of suitable animal models. For this reason a mouse model has been generated in this work that enabled the knock-out of *Wt1* specifically in podocyte precursor cells of the S-shaped body. Mice with the knock-out genotype (*Wt1*$^{fl/fl}$*;Pod-Cre*) were born, but died rapidly between 12 and 24 h after birth and did not produce urine (anuria). The kidneys were normal in size and weight, but displayed superficial hemorrhage. Kidney development was normal until around E18.5 and E19.5. From these stages on a gradual reduction in the number of glomeruli became evident. At the time of birth almost no glomeruli could be identified. The remaining glomeruli were markedly smaller and showed different levels of beginning disintegration. Capillary tufts, though accordingly smaller, exhibited no obvious structural defects or alterations. To confirm the absence of glomeruli, immunohistochemical analyses were performed using antibodies against Wt1 and two other marker proteins, all present in maturing podocytes. The two markers analyzed have not been reported to be targets of Wt1 and are differently distributed within the cell; synaptopodin is an actin-associated protein, localized in the cytoplasm of the podocyte cell body and in primary and secondary processes[100], whereas podocin is located at the cell membrane of podocyte foot processes and is coupled to the insertion site of the slit diaphragm. Wt1 was only detectable in the outer renal cortex, where mesenchymal cells and nephron pre-cursor structures of early developmental

stages reside. From the inner renal cortex, where glomeruli are normally located, Wt1 was absent. The same was true for the two additional podocyte marker proteins. These results, alone, only demonstrated the absence of podocytes from the inner renal cortex or loss of marker gene expression within these cells. In combination with the histological findings, however, these data clearly implicated a loss or strong reduction in the number of glomeruli as a consequence of *Wt1* deletion. Still, to fully prove that glomeruli are indeed absent from the kidney cortex it would be necessary to investigate the presence of the two other cell populations constituting the glomerulus, mesangial and endothelial cells.

The histological examinations revealed not only the loss of maturing glomeruli but also a significant reduction in the number of proximal tubuli. It is conceivable that this is a direct consequence of the degeneration and resulting physical absence of glomeruli, to which the proximal tubuli are directly connected. A rather hypothetical explanation would require the excretion of factors necessary for the differentiation or maintenance of the tubular epithelium by glomerular cells. In this case degeneration of glomeruli would also lead to the loss or de-differentiation of tubular structures. However, such paracrine interactions between these two nephron components, glomeruli and tubuli, have not yet been reported.

The remaining proximal tubuli were collapsed, most probably due to the absence of urine. Interestingly, other visible tubular compartments including distal tubuli seemed to be unaffected, except that in some cases they, too, were found to be collapsed. A detailed immunohistochemical analysis of the tubular apparatus using antibodies against marker proteins of the different tubular segments was not conducted, but would be necessary to determine precisely, which of the segments are lost or regressing.

Ultra structural analyses of residual glomeruli of *Wt1*$^{fl/fl}$*;Pod-Cre* kidneys revealed pathological changes predominantly affecting podocytes; the most obvious phenotype observed was global foot process effacement and loss of the slit diaphragm as a consequence. In general, these structural changes would result in severe glomerular filtration defects with proteinuria as the major symptom. However, it seems that the observed anomalies represent only a very early phenotype or the onset of a rapidly progressing degenerative process. A next step might involve the detachment of podocytes, which was sometimes observed in electron microscopy: capillary vessels were found to be deprived of podocytes and glomerular basement membrane. Histological analyses confirmed the presence of detached cells within the Bowman's space of residual

glomeruli and TUNEL assays revealed that these cells underwent programmed cell death. Thus, it appears that *Wt1* deletion leads to structural changes in podocytes, which, in turn, result in the detachment and degeneration of these cells. The loss of a critical number of podocytes seems to be followed by the disintegration of the entire glomerulus. The significant reduction in the number of glomeruli, as observed in newborn *Wt1*$^{fl/fl}$*;Pod-Cre* mice, is ultimately responsible for the impairment of kidney function. During embryonic development the fetal blood is filtered by the mother. At birth, however, the kidney defect becomes apparent and results in the early postnatal death of affected mice. Interestingly, none of the *Wt1*$^{fl/+}$*;Pod-Cre* animals have developed any signs of kidney pathologies so far. Even after a follow-up period of up to one year these mice were free of obvious symptoms or kidney related diseases. It appears that *Wt1* haploinsufficiency does not interfere with proper podocyte development and function.

Our findings are supported by a recent publication of Jia et al. The authors could show that podocyte ablation during murine nephrogenesis leads to severe abnormalities of glomeruli and proximal tubuli comparable to those observed in *Wt1*$^{fl/fl}$*;Pod-Cre* mice. To specifically disrupt developing podocytes *in vivo* a podocyte-specific Cre transgene (*Podocin-Cre*) was used to direct the production of diphtheria toxin A (DTA) directly within this cell population[131]. Transgenic mice phenocopied *Wt1*$^{fl/fl}$*;Pod-Cre* animals and provided further prove that podocytes are indeed essential for glomerulus and nephron differentiation. The study indirectly emphasizes the significance of Wt1 in these processes, since *Wt1* deletion results in the degeneration of podocytes as shown in this work.

## 4.2 Wt1-associated podocyte defects result in kidney failure in patients and mice

In this work it was demonstrated that Wt1 is an essential factor for podocyte differentiation and function. Moreover, evidence was provided that loss of podocytes leads to the degeneration of glomeruli, which, in turn, seems to interfere with nephron development and/or maintenance in general. Further prove for the important role of Wt1 in podocytes and glomeruli had been gained by analyses of patients and mice with heterozygous *WT1*/*Wt1* point mutations. Diseases caused by these mutations are the Denys-Drash (DDS)[32] and Frasier syndrome (FS)[40]. Patients suffering from Denys-Drash syndrome are normally free of symptoms at birth, but rapidly develop nephrotic syndrome before the

age of 2 years[132]. Nephrotic syndrome is characterized by strong proteinuria, hypoalbuminemia and peripheral edema. DDS patients commonly progress to end-stage renal failure before the age of 4. Their kidneys exhibit diffuse mesangial glomerulosclerosis, which is characterized by an expansion of the mesangial matrix and sometimes mesangial proliferation. The resulting sclerosis (or scarring) in combination with podocyte defects and podocyte loss leads to impairment of glomerular function. The term "diffuse" refers to the fact that all or most glomeruli of a given kidney are affected. At late stages of the disease sclerotic lesions are also observable in proximal tubuli of DDS kidneys. Podocytes are hypertrophied and vacuolized[61]. DDS mouse models greatly phenocopied patients: animals with a truncating *Wt1* mutation at codon 396 were asymptomatic at birth, but developed progressive focal segmental glomerulosclerosis 4 to 8 months later. In focal segmental glomerulosclerosis sclerotic lesions are only present in parts (segmental) of some (focal) glomeruli. The milder kidney defect was probably due to the use of chimeric mice with a low expression level of the mutant transgene. Only one heterozygous mouse was obtained; it was sterile and developed diffuse mesangial sclerosis 8 months after birth[103]. Introduction of the most common DDS-associated *WT1* mutation (R394W) in mice resulted in the severe kidney pathologies characteristic for the syndrome, but this greatly depended on the mouse genetic background. Heterozygous animals in an MF1 background died 5 months after birth from end stage renal failure caused by diffuse mesangial sclerosis. Podocytes displayed widespread foot processes effacement.[106] Homozygous DDS mutants were embryonically lethal. It is still discussed why the mutation in only one *Wt1* allele result in disease. DDS-associated *WT1* mutations are assumed to be dominant-negative: one affected allele would therefore be sufficient to reduce the amount of functional Wt1 protein significantly[36-39]. And this, most likely, causes the severe kidney phenotype observed in patients and mice. However, pathological changes can first be identified in the mature kidney. Thus, it seems that even a strong reduction in functional WT1 protein levels does not interfere with kidney development, but causes disease in the mature or maturing kidney.

In patients with Frasier syndrome the Wt1(+KTS)/(-KTS) ratio is imbalanced due to heterozygous point mutations in the alternative splice donor site of intron 9 (REF. 40). At birth those patients do not show any obvious kidney pathologies, but, in the majority of cases, develop proteinuria before 7 years of age. It increases progressively leading to nephrotic syndrome and slowly to end-stage renal failure. Renal biopsies mostly reveal

focal segmental glomerulosclerosis[61]. In a corresponding mouse model only the (–KTS) isoform is expressed from one allele, due to a mutation in the alternative splice donor site at the end of exon 9. Since both isoforms were still expressed from the remaining wild-type allele, the introduced mutation led to the FS typical imbalance of the (+KTS)/(-KTS) ratio. 70% of these mice developed proteinuria 2 to 3 months after birth and died from renal insufficiency. Kidneys displayed focal segmental or diffuse mesangial sclerosis; glomerular and tubular numbers were unaffected.[42] When both alleles were mutated and only the (-KTS) isoform was present defects comparable to those in *Wt1$^{fl/fl}$;Pod-Cre* animals were observed: mice were born, but died 24 h after birth. The bladders were found to be empty and kidneys were hemorrhagic. Podocytes lost foot processes. But the number of glomeruli and proximal tubuli remained unaffected. It was reasoned that the (+KTS) isoform is important for the development of the podocyte architecture.

Total ablation of the (–KTS) isoform, leaving only *(+KTS)* expression, resulted in a similarly severe kidney phenotype leading to early postnatal death. Interestingly, in this case also a strong reduction in glomerular number was observed and supported by the finding that synaptopodin was entirely absent from the renal cortex. In *Wt1$^{fl/fl}$;Pod-Cre* mice a similar defect was observed. One might speculate that Wt1(-KTS) is necessary for development and differentiation of podocytes and glomeruli, respectively, whereas the (+KTS) variant is needed to build up the unique podocyte specific structure. Ablation of both variants in late stage podocyte precursors, thus, leads to structural changes and de-differentiation of podocytes, as observed in podocyte-specific *Wt1*-null animals animals. In kidneys of (–KTS) ablated mice the decrease in glomerular number was accompanied by a reduction in the number of proximal convoluted tubuli[42]. A phenomenon also diagnosed in *Wt1$^{fl/fl}$;Pod-Cre* animals. Again, it is tempting to speculate that factors derived from the glomerulus might stimulate the growth or maintenance of the tubular epithelium.

That overall reduction in *Wt1* expression levels leads to comparable defects as observed in mutant mice or animals with Wt1(+KTS)/(-KTS) imbalance was demonstrated in a mouse model that aimed to "rescue" *Wt1* knock-out mice. By introduction of a YAC harboring the human *WT1* gene into the genome of *Wt1$^{-/-}$* mice wild type *WT1* expression could be restored by 62 to 70%. Mice were born and had developed kidneys. Between 10 days and 6 weeks proteinuria was detected that was caused by global mesangial sclerosis and, depending on the *WT1* expression level, crescentic glomerulonephritis[102]. The latter is caused by severe damages of the renal filtration barrier and characterized by the formation

of crescent shaped scars within the glomerulus. Crescent formation results from (1) the leakage of fibrin and other plasma proteins into the Bowman's space, (2) inflammatory processes and (3) the proliferation of pariethal epithelial cells. Both diffuse mesangial sclerosis and crescentic glomerulonephritis rapidly progress into end-stage renal failure from which the "rescued" animals eventually died[102].

As illustrated, *Wt1* mutations and reduced *Wt1* expression levels almost always result in glomerulosclerosis in humans and mice. Studies on the genetic basis of familial and sporadic focal segmental glomerulosclerosis (FSGS) have led to the identification of several factors in addition to *WT1* that might be responsible for the disease. These genes include *NPHS1* (*nephrin*) and *NPHS2* (*podocin*), both of which code for slit diaphragm proteins. Furthermore, the gene coding for the cytoskeletal protein alpha-actinin-4, *ACTN4*, and *TRPC6*, which encodes a cation channel, have been implicated in FSGS etiology. All these factors are expressed in podocytes and most of which are necessary for the maintenance of the renal filtration barrier. Thus, this form of glomerulosclerosis is a podocytopathy (for reviews see (REFS. 133-135). The causes of mesangial sclerosis are not well understood. But it seems likely that podocyte defects are also responsible for mesangial proliferation and mesangial matrix expansion: damages of podocytes might lead to the destabilization of the glomerulus against the strong hydrostatic forces in capillary vessels. As a response, mesangial cells might be stimulated to increase mesangial matrix synthesis and/or to proliferate.

Overall, these studies prove two points: (1) Podocytes are essential for glomerular integrity and function. (2) Wt1 is essential for the maintenance of podocyte structure and thereby indispensable for glomerular and kidney function in general. The podocyte-specific *Wt1* knock-out mouse model presented in this work provides further evidence for these findings. It also proved that total *Wt1* inactivation exclusively in podocytes results in a more severe phenotype than global reduction of functional Wt1. Instead of post-natal glomerulosclerosis *Wt1*$^{fl/fl}$*;Pod-Cre* animals had lost a high proportion of glomeruli already shortly before birth and died perinatally. Similarly, total ablation of the Wt1(-KTS) or Wt1(+KTS) isoforms accounted for a more severe phenotype than *Wt1* mutation or reduced expression.

## 4.3 Wt1 is a transcriptional regulator of both "structural" and "developmental" genes in podocytes

The analysis of Wt1 target genes has proven to be difficult in *Wt1$^{fl/fl}$;Pod-Cre* mice. Expression of glomerulus/podocyte specific genes, including *nephrin* and *synaptopodin*, appeared to be downregulated or lost simply because the structures they are normally expressed in were absent or reduced in number. On the other hand factors like *Pax2* and *Wt1* itself are highly expressed in the metanephric mesenchyme and early nephron precursor cells; changes of expression levels in podocytes, which represent a minor cell population within the kidney, might therefore not be detectable. However, several factors with implications in podocyte development and function have been discussed in the literature to be transcriptional targets of Wt1: (1) *Pax2* is highly expressed in the induced metanephric mesenchyme, but is abruptly downregulated in developing nephrons[136]. It is believed that Pax2 induces *Wt1* expression and that Wt1 in turn negatively regulates *Pax2* (REFS. [23, 137]). Failure to repress *Pax2* in developing nephrons leads to severe developmental defects, such as reduced nephron number[85]. Ectopic expression of the gene in podocytes resulted in end stage renal failure of affected animals[86]. Interestingly, activation of *Pax2* in adult mice led to the downregulation of *Wt1* and *nephrin* and ultimately to the dedifferentiation of podocytes[86]. On the other hand, *Pax2* was found to be upregulated in sclerotic glomeruli of Denys-Drash mice with a *Wt1* mutation in codon 396 (REF. 104). However, it was only upregulated in globally sclerotic kidneys; reexpression of *Pax2* might thus be a consequence of disease progression and not its cause. (2) *Vegfa* (*vascular endothelial growth factor a*) has been reported to be positively regulated by Wt1. It is expressed in podocyte precursor cells and has been shown to be necessary for the migration of endothelial cells into the S-shaped body; a process that is of vital importance for further nephron development[26, 84]. So far no *in vivo* evidence has been provided to support a possible role of Wt1 in the regulation of *Vegfa*. (3) Genes encoding growth factors: Wt1 is assumed to be a transcriptional repressor of growth factors[138]. In line with this hypothesis *PDGF-A* (platelet derived growth factor A) and *TGF-β1* (*transforming growth factor beta1*), both are discussed as direct Wt1 target genes, were upregulated in glomeruli of DDS patients[18, 25, 139]. In DDS mice with the most common *Wt1* point mutation in codon 394 *Tgf-β1* and *Igf-1* (*insulin-like growth factor 1*) expression was enhanced[106]. Yet, in none of the cases inappropriate proliferation was detectable in glomeruli. Whether growth factors are involved in or upregulated as a consequence of podocyte defects associated

with *Wt1* mutations remains to be proven. In *Wt1*$^{fl/fl}$*;Pod-Cre* glomeruli no indications for increased proliferation were observed. (4) *Nphs1* was shown to be a direct target of Wt1 *in vitro*[22]. Its gene product nephrin is the major slit diaphragm component[140]. Mutations in the gene are associated with focal segmental glomerulosclerosis[87]. Total knock-out of the gene led to severe podocyte defects including loss of slit-diaphragms and foot-processes[92]. Mice exhibited strong proteinuria and died 24 h after birth. Regression of glomeruli or tubular compartments, as seen in *Wt1*$^{fl/fl}$*;Pod-Cre* animals, was never observed. In mice with reduced *Wt1* expression *Nphs1* was also found to be downregulated[102]. (5) *Podxl* codes for podocalyxin, another component of the slit diaphragm, and has been reported to be directly activated by Wt1 (REFS. [21, 140]). Deletion of the gene in mice results in podocyte defects comparable to those caused by inactivation of nephrin or other slit diaphragm associated factors[94]. In mice with reduced *Wt1* expression *podxl* was also downregulated[102]. (6) Amphiregulin (*Areg*) is a member of the epidermal growth factor family. *Areg* is coexpressed with *Wt1* during kidney development and is positively regulated by Wt1 *in vitro*[20]. Interestingly, total knock-out of the gene produced no obvious kidney pathologies[141]. The role of amphiregulin in podocyte biology remains to be determined.

The expression of other podocyte-relevant factors such as podocin, alpha-actinin-4 and Cd2ap was altered in sclerotic glomeruli of DDS mouse models[104, 106]. But this could not be directly linked to Wt1. It is likely that glomerular defects result in a generally altered gene expression profile; the changes in expression of podocyte-associated genes might simply be a consequence of this possibility.

However, considering the severity of the kidney defects in *Wt1*$^{fl/fl}$*;Pod-Cre* animals and other mouse models it seems indeed likely that Wt1 is directly and/or indirectly involved in the regulation of several genes important for podocyte function. The wide spectrum of proposed Wt1 target genes, ranging from developmental factors such as *Pax2* to structural factors like *podocalyxin*, reflects the versatility of Wt1 function and complicates its analysis at the same time.

## 4.4 Wt1 is important for the maintenance of podocyte structure and function in adult mice

*Wt1* is expressed in podocytes throughout the life of mammalian organisms[10]. For this reason the gene has long been assumed to be important not only for development and differentiation, but also for the maintenance of this cell population. The fact that mutations

in *Wt1* can result in podocyte defects, as demonstrated above, has supported this assumption. But a podocyte restricted *Wt1* knock-out in adult mice has not yet been conducted. In the presented work an inducible Cre/*loxP* based strategy was followed to achieve exactly this. For that purpose, the doxycycline-inducible *Podocin-rtTA/LC-1* Cre-expression system was used in combination with the conditional *Wt1* knock-out mouse. To prove podocyte-specificity of the Cre expression system, a compound reporter mouse line was generated harboring the following transgenes: a *Rosa26-lacZ* reporter element, the Cre expression construct, *LC-1*, and the *Podocin-rtTA* transgene. After Cre expression was induced by doxycycline administration for 7 days in 2 months old triple transgenic mice (*Rosa26-lacZ/+;Podocin-rtTA/+;LC-1/+*) Cre activity was detected exclusively in glomerular podocytes, not in other structures within the kidney. Although almost all glomeruli per kidney section were Cre positive, it appeared that only a proportion of podocytes showed Cre activity. This might be due to limited efficiency of the doxycycline mediated induction. Treatment with the drug over a longer period would probably increase the percentage of Cre positive podocytes. Other organs, including liver, bladder, gut, brain, spleen, gonads, heart, lung and stomach, were examined additionally, but displayed no signs of Cre activity.

After having shown that the *Podocin-rtTA/LC-1* system allowed podocyte restricted Cre expression the two transgenes were introduced into the $Wt1^{fl/fl}$ background giving rise to *iWt1-KO* mice. For all conducted analyses animals of at least two months of age were used to exclude developmental processes taking place in the kidneys. Furthermore, only age and sex matched animals were compared, so that possible age or sex related anatomical differences could not be misinterpreted as pathological phenotype.

The most common indication of defects in the glomerular filtration apparatus is the appearance of high molecular proteins, such as albumin, in the urine[142]. Therefore, *iWt1-KO* and control mice were monitored for proteinuria after doxycycline treatment was started. By day 9 of treatment all examined *iWt1-KO* mice had developed massive proteinuria. In contrast, urine of $Wt1^{fl/+}$*;Podocin-rtTA;LC-1* animals remained free of protein, even after 6 months of continuous doxycycline supply. *Wt1* deletion was detected by PCR on whole kidney DNA in *iWT1-KO* mice and to a lower extend in heterozygous controls ($Wt1^{fl/+}$*;Podocin-rtTA;LC-1*). In $Wt1^{fl/fl}$*;LC-1* mice, lacking the *rtTA* transgene, *Wt1* was not deleted and proteinuria was not detected. These findings allowed the conclusion that proteinuria was a consequence of homozygous kidney/podocyte restricted *Wt1*

inactivation. Haploinsufficiency on the other hand seems not to interfere with normal kidney function. It has been reported recently that doxycycline itself can induce kidney defects[143]. The fact that *Wt1$^{fl/+}$;Podocin-rtTA;LC-1* control animals equally treated with doxycycline did not develop proteinuria or other obvious kidney defects excluded this possibility, however.

Despite the high amounts of excreted albumin the anomalies detected with histological methods were surprisingly subtle. Beside a high frequency of dilated tubuli filled with high amounts of proteinaceous material no gross anatomical changes were diagnosed. Rarely, glomeruli displayed thickening of the pariethal epithelium and sporadic vacuolization of podocytes, which seemed to adhere to the Bowman's capsule. Whether this was a sign for beginning glomerulosclerosis is not entirely clear. To address this, mice need to be induced and monitored for a longer time period. Overall, no obvious differences between kidney sections of doxycycline induced *iWt1-KO*, *Wt1$^{fl/+}$;Podocin-rtTA;LC-1* and *Wt1$^{fl/fl}$;LC-1* animals could be identified, with the exceptions mentioned above. On the ultra structural level, however, one striking difference was observed: podocytes of *iWt1-KO* mice displayed foot-process effacement and, as a consequence, loss of the slit diaphragm (SD) in a focal, segmental distribution. Furthermore, the podocyte cell layer seemed to be detached from the underlying glomerular basement membrane (GBM), which displayed normal size and charge. The endothelium appeared intact, but was abnormally distant from the GBM. This indicated a partial disruption of the glomerular filtration apparatus, which is in turn likely to cause proteinuria. It has been demonstrated many times in human and mouse, that foot process effacement results in proteinuria[61, 142].

The fact that only a relatively small proportion of podocytes displayed foot-process effacement might again be explained with the limited efficiency of Cre activation and is in line with findings in the *Rosa26-LacZ;Podocin-rtTA;LC-1* reporter mouse line. Doxycycline mediated induction for 6 to 8 days might be sufficient to cause proteinuria, but might not be enough to target the majority of podocytes. This was further supported by the observation that Wt1 protein was still detectable in a considerable, but varying number of glomeruli and podocytes, respectively. However, in all observed *iWt1-KO* mice Wt1 protein levels were at least moderately reduced. For this reason microarray studies were performed to identify Wt1 dependent factors important for the maintenance of the structural and functional features of podocytes. However, experiments using both total kidney RNA and RNA obtained from isolated glomeruli of induced *iWt1-KO* and *Wt1$^{fl/fl}$;LC-1*

control mice failed to identify differentially expressed genes. Even *Wt1* expression was comparable in knock-out and control animals. Thus, long-time experiments were conducted to induce *Wt1* deletion in a higher proportion of podocytes. For this, mice were continuously induced with doxycycline and glomerular gene expression was analyzed by real-time qRT-PCR. After 2 months of treatment *Wt1* was found to be down-regulated in glomeruli of *iWt1-KO* mice by factor 8 when compared to *Wt1*$^{fl/fl}$*;LC-1* controls. The expression of other podocyte specific factors including *podocin* (*Nphs2*), *Cd2ap*, *synaptopodin* and the known Wt1 target gene *nephrin* (*Nphs1*) was reduced between factor 1.5 and 2. Technically the study has to be considered preliminary, since mouse cohorts were too small (n=2) to draw statistically valid conclusions. Furthermore, only podocyte marker genes were analyzed; podocyte-independent factors were not included. However, from the data obtained one might assume that Wt1 is important for the regulation of several key constituents of the podocyte; due to the mild changes in gene expression it seems likely that other factors are also involved in their regulation. Considering the podocyte defects observed it is conceivable that the cumulative down-regulation, however mild, of a number of genes relevant for the formation and maintenance of foot processes might eventually result in the degeneration of these structures. On the other hand it seems likely that other Wt1 target genes, including so far unknown factors, are necessary for the maintenance of podocyte architecture and function. To identify such factors in a large scale directly in podocytes a diploma student of our group, Claudia Lück, is currently working on a method to induce *Wt1* deletion in isolated (primary) podocytes of adult conditional *Wt1* knock-out mice (*Wt1*$^{fl/fl}$). In collaboration with Frank Edenhofer in Bonn these primary podocytes will be transduced with a TAT-Cre fusion protein *in vitro*. Edenhofer and colleagues could show that Cre-recombinase fused to an HIV-TAT protein can be efficiently delivered into cells and that Cre-activity is not compromised by TAT[144]. Our own findings suggest high transduction rates and efficient Cre mediated *loxP* recombination when primary podocytes are used. Gene expression analyses will be performed with RNA sequencing techniques and might help to better understand the role of Wt1 in podocytes.

An interesting new aspect of the presented mouse model was revealed by long-time induction experiments. It is based on the finding that the urine protein content of *iWt1-KO* mice gradually decreased, the longer the animals were treated with doxycycline. In one

out of three long-time induced *iWt1-KO* animals proteinuria was hardly detectable after 4 months of doxycycline administration. In the two other mice urine protein levels dropped after induction for more than one month, but increased again to the initial levels. After 4 months those animals suddenly died. It is not clear what causes this phenomenon: one possibility is that defective glomeruli simply degenerate. In this case urine protein levels would drop as long as intact glomeruli are still present. However, since mice are continuously treated with doxycycline more and more glomeruli develop defects leading again to an increase in protein levels. In two of three mice this was observed after 4 months. Finally, constant glomerular degeneration would lead to kidney malfunction and most probably death. To confirm this hypothesis histological analyses have to be performed to prove glomerular degeneration, which was never observed in shortly induced animals. The model would not explain why one of the mice showed complete recovery.

Another possible explanation is that tubular reabsorption of protein is increased in response to elevated levels in the ultrafiltrate. However, it has been shown that protein retrieval by the epithelium of proximal tubuli is rather inefficient[142] and therefore unlikely to cope with the high amounts of protein in the urine of *iWt1-KO* mice.

A more tempting speculation implicates regenerative processes leading to the replacement of defective podocytes and thereby to a reduction of proteinuria. Podocytes in the adult are incapable of replicative cell proliferation and it was assumed for many years that lost podocytes cannot be replaced. Recently, two publications showed that (1) a type of progenitor cells is present in the Bowman's capsule with the potential to replace podocytes[145] and (2) that pariethal epithelial cells (PEC) can migrate from the Bowman's capsule into the vascular tuft and differentiate into podocytes[146]. However, these processes are rather slow, so that massive podocyte degeneration, as seen in nephrotic syndrome, cannot be compensated. But it is conceivable that the described regenerative mechanisms might mitigate the effects of podocyte loss to a certain extend and at least temporarily. It will be interesting to address these hypotheses in the future.

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# Lebenslauf

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# Publikationen

## Publikationen in wissenschaftlichen Journalen:

Klattig, J., **Sierig, R.**, Kruspe, D., Besenbeck, B., & Englert, C., Wilms' tumor protein Wt1 is an activator of the anti-Mullerian hormone receptor gene Amhr2. *Mol Cell Biol* 27 (12), 4355-4364 (2007).

Klattig, J., **Sierig, R.**, Kruspe, D., Makki, M.S., & Englert, C., WT1-mediated gene regulation in early urogenital ridge development. *Sex Dev* 1 (4), 238-254 (2007).

## Poster:

**Sierig, R.**, Kastner, J., Lück, C., Witzgall, R., & Englert, C., The Wilms Tumor protein is required for kidney function in adult mice. *16th International Society for Developmental Biology Congress, Edinburgh* (2009).

**Sierig, R.**, Kruspe, D., Kastner, J., Lück, C., Witzgall, R., & Englert, C., The Wilms Tumour Protein 1 in the developing and mature mouse kidney. *18th GfE Meeting, Hannover* (2009).

**Sierig, R.**, Kastner, J., Witzgall, R., & Englert, C., Wt1 in kidney development and homeostasis. *1st International WT1 Workshop, Manchester* (2008).

**Sierig, R.**, Kastner, J., Reinhardt, P., Witzgall, R., & Englert, C., Wt1 – A key factor in kidney development and maintenance. *20th International Congress of Genetics, Berlin* (2008).

Klattig, J., Makki An, M. S., **Sierig, R.**, & Englert, C., A comprehensive view on Wt1-mediated gene expression. *ELSO Meeting, Dresden* (2005).

## Selbständigkeitserklärung

Hiermit erkläre ich, die vorliegende Dissertation selbständig und nur unter Verwendung der angegebenen Hilfsmittel, Literatur und persönlichen Mitteilungen angefertigt zu haben. Ferner versichere ich, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad doctor rerum naturalium (Dr. rer. nat.) beworben habe und dass ich weder früher, noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des oben genannten akademischen Grades an einer anderen Hochschule beantragt habe. Die Hilfe eines Promotionsberaters wurde von mir nicht in Anspruch genommen. Dritte haben von mir weder mittelbar noch unmittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit der vorgelegten Dissertation stehen. Die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät ist mir bekannt.

Jena, den 28. September 2009

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