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Loss of endogenous thymosin-β4 accelerates glomerular disease

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ABSTRACT

Glomerular disease is characterised by morphological changes in podocyte cells accompanied by inflammation and fibrosis. Thymosin-β4 regulates cell morphology, inflammation and fibrosis in several organs and administration of exogenous thymosin-β4 improves animal models of unilateral ureteral obstruction and diabetic nephropathy. However, the role of endogenous thymosin-β4 in the kidney is unknown. We demonstrate thymosin-β4 is expressed prominently in podocytes of developing and adult mouse glomeruli. Global loss of thymosin-β4 did not affect healthy glomeruli, but accelerated the severity of immune-mediated nephrotoxic nephritis with worse renal function, peri-glomerular inflammation and fibrosis. Lack of thymosin-β4 in nephrotoxic nephritis led to the redistribution of podocytes from the glomerular tuft towards the Bowman’s capsule suggesting a role for thymosin-β4 in the migration of these cells. Thymosin-β4 knock-down in cultured podocytes also increased migration in a wound-healing assay; accompanied by F-actin rearrangement and increased RhoA activity. We propose that endogenous thymosin-β4 is a modifier of glomerular injury, likely having a protective role acting as a brake to slow disease progression.

Keywords: cytoskeleton, fibrosis, glomerulus, inflammation, podocyte
INTRODUCTION

End-stage renal failure is a devastating condition, requiring life-long dialysis and transplantation and a risk factor for all-cause mortality and cardiovascular disease. Many cases are due to disruption of the glomerular filtration barrier, consisting of epithelial podocytes, endothelium, mesangium and glomerular basement membrane. Podocytes have a unique shape maintained by a complex cytoskeleton with branched foot process extensions which abut each other at slit diaphragms. During glomerular injury, podocyte architecture is perturbed resulting in defective filtration and proteinuria, often with inflammatory components characterised by leukocyte infiltration followed by glomerulosclerosis and tubulointerstitial fibrosis.

Thymosin-β4 (Tmsbx4) is a naturally-occurring peptide. It is the major G-actin sequestering protein in mammalian cells with critical roles in maintaining the cell cytoskeleton. In animal models, exogenous Tmsbx4 has beneficial effects in diverse pathologies including myocardial infarction, stroke, dry eye and inflammatory lung disease, and there are clinical trials assessing Tmsbx4 treatment in wound healing and cardioprotection. The utility of Tmsbx4 in these pathologies has been attributed to modulation of several cellular functions including cell motility, differentiation, survival, angiogenesis, inflammation and fibrosis.

Recent studies investigated exogenous Tmsbx4 as a treatment for kidney disease. Tmsbx4 reduced renal tubulointerstitial fibrosis following unilateral ureteral obstruction (UUO) in mice, potentially through decreasing plasminogen activator inhibitor-1 (PAI-1) expression and dampening transforming growth factor-β1 signalling. In KK Cg-Ay/J mice, a model of type II diabetes mellitus, daily Tmsbx4 treatment for three months reduced albuminuria and attenuated renal pathology. Furthermore, N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), the N-terminal tetrapeptide generated by Tmsbx4 cleavage, has
beneficial effects on fibrosis and inflammation in UUO, remnant kidneys, diabetic nephropathy and glomerulonephritis.\textsuperscript{18, 21-23}

*Tmsbx4* transcripts are detectable by *in-situ* hybridisation in developing and adult glomeruli\textsuperscript{24} with strong expression in podocytes\textsuperscript{24, 25}. Furthermore, in rat remnant kidneys, proteomic analysis of laser-capture dissected glomeruli demonstrated significantly increased Tmsbx4 in sclerotic versus normal glomeruli\textsuperscript{26}. Despite such evidence of expression and beneficial renal effects, the functional importance of endogenous Tmsbx4 in the kidney during health and disease is completely unknown.

In this study, we confirmed Tmsbx4 is highly expressed in the kidney glomerulus, predominately in podocytes. Using global *Tmsbx4* knock-out mice\textsuperscript{27} we demonstrated that endogenous Tmsbx4 was dispensable in healthy glomeruli. Furthermore, in an experimental model of glomerular damage, lack of *Tmsbx4* worsened disease progression by (i) enhancing podocyte migration facilitating their redistribution from glomerular tuft to Bowman’s capsule, and (ii) increasing peri-glomerular inflammation and interstitial fibrosis. Thus we provide the first evidence that endogenous Tmsbx4 is critical in the progression of glomerular disease.
RESULTS

**Tmsb4x is expressed in mouse glomerular podocytes**

*Tmsb4x* mRNA levels were assessed in spleen, liver, heart and the whole kidney of healthy adult mice. The highest transcript levels were found in the spleen with approximately 10 times less Tmsb4x in the kidney (Figure 1A). Using Dynabead perfusion we isolated glomeruli and found *Tmsb4x* levels were significantly enriched in glomeruli compared with the rest of the kidney (Figure 1A). *In-situ* hybridisation detected *Tmsb4x* expression in immature glomeruli of embryonic day (E)16.5 developing kidneys, predominately in podocytes (Figure 1B-C). The protein expression of Tmsb4x was also assessed by immunohistochemistry and we found strong localisation in glomerular podocytes at E18 (Figure 1D). This expression pattern was maintained in one-week-old postnatal (Figure 1E) and eight-week-old adult kidneys (Figure 1F). Tmsb4x podocyte expression was further confirmed by co-localisation of Tmsb4x with nephrin (Nphs1), a slit diaphragm component (Figure 1G-I) and nestin, an intermediate filament protein expressed in mature podocytes (Supplementary Figure 1A-C). In contrast, Tmsbx4 did not co-localise with the pan-endothelial marker, Cd31 (Supplementary Figure 1D-F).

**Lack of endogenous Tmsb4x has no effect on healthy glomeruli**

We examined mice with a global loss of *Tmsb4x* to assess the importance of endogenous *Tmsb4x* in healthy glomeruli. As Tmsb4x is mapped to the X chromosome, we crossed hemizygous null male mice (*Tmsb4x*<sup>−/y</sup>) with heterozygous *Tmsb4x*<sup>+/−</sup> adult females (Figure 2A). We found no lethal developmental abnormalities with the offspring conforming to Mendelian ratios (Supplementary Table 1). *Tmsb4x<sup>−/y</sup>* mice had similar albumin excretion (Figure 2B) and blood urea nitrogen (BUN) levels (Figure 2C) as male wild-type *Tmsb4x<sup>+/y</sup>* mice at the ages of 1, 3 and 6 months. There was no difference in body weight at 1 (Tmsb4x<sup>+/y</sup>=23.8±0.7g; Tmsb4x<sup>−/y</sup>=23.4±0.4g), 3 (Tmsb4x<sup>+/y</sup>=30.0±0.5g; Tmsb4x<sup>−/y</sup>=29.2±0.7g), or 6 months of age (Tmsb4x<sup>+/y</sup>=35.8±1.0g; Tmsb4x<sup>−/y</sup>=35.3±1.6g). Using semi-quantitative analysis of light microscopy images, we found no differences in glomerular
morphology between six-month-old Tmsb4x<sup>+/y</sup> and Tmsb4x<sup>-/y</sup> mice (Figure 2D-F). This was confirmed by transmission electron microscopy with normal foot process architecture, laminar structure of the basement membrane and the presence of endothelial fenestrae in both Tmsb4x<sup>+/y</sup> and Tmsb4x<sup>-/y</sup> mice (Figure 2G-H). We demonstrated the loss of Tmsb4x protein in glomeruli of Tmsb4x<sup>-/y</sup> mice compared with Tmsb4x<sup>+/y</sup> (Figure 2I-J); along with undetectable Tmsb4x mRNA levels in whole kidneys (Figure 2K). We examined whether there was any compensation for the lack of Tmsb4x by other β-thymosins, but found no changes in the renal mRNA levels of Tmsb10, Tmsb15a, Tmsb15b and Tmsb15l (Figure 2L-O). We also assessed mRNA levels of genes involved in actin polymerisation and found no differences in profilin (Pfn)-1 and -2 and destrin (Dstn) between Tmsb4x<sup>+/y</sup> and Tmsb4x<sup>-/y</sup> mice (Supplementary Figure 2A-C). In contrast, cofilin 1 (Cfl1) mRNA levels were significantly increased in Tmsb4x<sup>-/y</sup> kidneys by approximately 30% compared with Tmsb4x<sup>+/y</sup> mice (Supplementary Figure 2D). We specifically examined Cfl1 mRNA levels in podocytes and found no change following knockdown of endogenous Tmsb4x by small interfering RNA (siRNA) (Supplementary Figure 2E). Furthermore, there was no difference in Cfl1 mRNA levels in primary podocytes isolated from Tmsb4x<sup>+/y</sup> and Tmsb4x<sup>-/y</sup> mice (Supplementary Figure 2F). Finally, the mRNA levels of genes important for podocyte function, (Nphs1, Nphs2, Synpo, Cd2ap and Wt1) were unchanged between Tmsb4x<sup>-/y</sup> and Tmsb4x<sup>+/y</sup> kidneys (Supplementary Figure 3A-E).

Lack of endogenous Tmsbx4 worsens renal function and glomerular injury in NTS nephritis

Our results suggest lack of Tmsbx4 does not affect the function and morphology of healthy glomeruli. Therefore, we investigated whether Tmsbx4 has a role in glomerular disease. We utilised the NTS nephritis model, which replicates some of the pathological features of human crescentic glomerulonephritis. NTS nephritis involves the injury of intrinsic glomerular cells, including podocytes, as well as leukocyte infiltration, glomerulosclerosis and tubulointerstitial fibrosis processes in which Tmsbx4 has been implicated. We
predicted that lack of global Tmsbx4 may exacerbate NTS nephritis severity and examined this in three month old Tmsb4x+/y and Tmsb4x−/y mice (Figure 3A). Albuminuria and albumin/creatinine ratio was significantly increased in Tmsb4x+/y mice twenty-one days after NTS administration compared with the levels prior to immunisation (p<0.001 in both cases, Figure 3B-C). Strikingly, both albuminuria and albumin/creatinine ratio was further enhanced by approximately 5- and 7-fold respectively when NTS was injected to Tmsb4x+/y compared with Tmsb4x−/y mice (p<0.01 in both cases, Figure 3B-C). Administration of NTS to Tmsb4x−/y mice also significantly elevated plasma creatinine (p<0.01, Figure 3D), impaired creatinine clearance (p<0.01; Figure 3E) and raised BUN (p<0.05, Figure 3F) compared with Tmsb4x−/y mice with nephrotoxic nephritis. We found no difference in Tmsb4x levels in whole kidneys obtained from Tmsb4x−/y mice without disease or administered NTS (Supplementary Figure 4A). Furthermore, in kidney biopsies obtained from patients with either rapidly progressive glomerulonephritis (RPGN) or lupus nephritis (SLE), there was no change in glomerular or tubulointerstitial TMSB4X mRNA levels compared with living donor (LD) control kidneys (Supplementary Figure 4B-C).

Seven days after NTS administration, we observed mild glomerular injury in Tmsb4x−/y and Tmsb4x−/y mice with some glomeruli containing hyaline deposits, increased mesangial matrix and occasional adhesion of the glomerular tuft to Bowman’s capsule (Supplementary Figure 5A-C). After twenty-one days, there was a range of abnormalities in Tmsb4x−/y and Tmsb4x−/y mice injected with NTS including collapse of capillary loops, segmental or global glomerulosclerosis, adhesion of the tuft to Bowman’s capsule and glomerular epithelial hyperplasia lesions, a feature of early crescent formation in this model. Semi-quantitative histological scoring (Supplementary Figure 6A-E) by two blinded observers revealed that Tmsb4x−/y mice injected with NTS had significantly raised mean glomerular score compared with Tmsb4x−/y mice without disease (p<0.001, Figure 3G-J). Glomeruli of Tmsb4x−/y administered NTS had an even higher glomerular mean score which was significantly greater than Tmsb4x−/y mice with nephrotoxic nephritis (p<0.05). This was associated with
an increased proportion of sclerotic glomeruli and incidence of epithelial hyperplasia lesions in \( Tmsb4x^{-/y} \) compared with \( Tmsb4x^{+/y} \) mice administered NTS (Supplementary Figure 6F).

We examined whether the difference in disease severity between \( Tmsb4x^{-/y} \) and \( Tmsb4x^{+/y} \) mice with NTS could be due to a decrease in binding of the anti-glomerular antibody but found no difference in the amount of sheep IgG deposited within the glomerulus (Supplementary Figure 7A-C). We assessed whether lack of \( Tmsb4x \) changes the humoral immune response to sheep IgG. NTS injection led to significantly increased production of circulating murine IgG1 and IgG2a, but not IgG2b or IgG3 against sheep IgG compared with \( Tmsb4x^{+/y} \) mice without disease. However there was no difference in the plasma titres of any of the IgG subclasses between \( Tmsb4x^{-/y} \) and \( Tmsb4x^{+/y} \) mice administered NTS (Supplementary Figure 7D-G).

Changes in podocyte distribution in \( Tmsb4x^{-/y} \) glomeruli following NTS nephritis

Following NTS nephritis, we found that Tmsb4x still co-localised with Nphs1 (Figure 4A-C) and subsequently examined the effect a lack of Tmsb4x had on podocytes in this model. Firstly, we quantified WT1\(^+\) podocyte numbers in and outside of the glomerular tuft (Figure 4D-F). The number of glomerular tuft WT1\(^+\) cells was unchanged in \( Tmsb4x^{+/y} \) mice following NTS injection compared with mice without disease. However, NTS administration to \( Tmsb4x^{-/y} \) mice significantly reduced the number of WT1\(^+\) glomerular tuft cells compared with \( Tmsb4x^{+/y} \) mice with nephrotoxic nephritis (p<0.001; Figure 4G). This finding persisted after normalising the WT1\(^+\) cell number to glomerular tuft area (p<0.05; Figure 4H). In contrast, there was an increased number of WT1\(^+\) cells outside the glomerular tuft in both \( Tmsb4x^{+/y} \) and \( Tmsb4x^{-/y} \) mice following NTS administration, this was more prominent and significantly different in the \( Tmsb4x^{-/y} \) animals (p<0.05 compared with \( Tmsb4x^{+/y} \) without disease; Figure 4I). The total number of podocytes in the whole glomerulus did not differ between any of the groups (Figure 4J), suggesting that lack of Tmsb4x leads to podocyte redistribution from the glomerular tuft towards the Bowman’s capsule rather than affecting podocyte cell death. To
support this, we assessed podocyte apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in combination with WT1 staining (Supplementary Figure 8A-C). Administration of NTS increased the number of glomerular apoptotic cells compared with healthy mice and this effect was significant in Tmsb4-/- mice with glomerular disease (p<0.05; Supplementary Figure 8D). However, the number of TUNEL+/WT1+ cells was not significantly different between any of the groups (Supplementary Figure 8E).

Lack of Tmsb4x induces migration and modulates the cytoskeleton of podocytes in vitro

We postulated that the redistribution of podocytes in nephrotoxic nephritis may be due to changes in cell migration driven by lack of Tmsb4x. Therefore, we transfected cultured differentiated mouse podocytes with siRNA against Tmsb4x (Figure 5A); this resulted in >90% knockdown in Tmsb4x levels (Figure 5B; p<0.001). Knockdown of endogenous Tmsb4x did not affect podocyte viability (Figure 5C) but increased the number of cells that migrated into the wound area in a wound-healing assay (p<0.05; Figure 5D-E). Since the cytoskeleton is essential for cell movement, we visualised podocyte actin by phalloidin staining and classified the filament organisation as either cytoplasmic stress fibres (Figure 5F) or cortical actin (Figure 5G). Knockdown of endogenous Tmsb4x significantly increased the percentage of cells with stress actin fibre organisation (p<0.001; Figure 5H). Finally, we assessed the effects of Tmsb4x knockdown on the activation of RhoA and Cdc42, which regulate actin dynamics and cell migration. There was increased RhoA activity in podocytes transfected with Tmsb4x siRNA compared to control siRNA (p<0.05; Figure 5I) whereas Cdc42 activity was unaffected (Figure 5J).

Macrophage accumulation and increased fibrosis in Tmsb4x-/- glomeruli following NTS nephritis

Tmsb4x is expressed in macrophages and reduces inflammation in several disease settings. As immune cell infiltration plays a critical role in the initiation and
progression of crescentic glomerulonephritis. We found expression of Tmsb4x in F4/80+ macrophages surrounding the glomeruli and occasionally within the glomerular tuft (Figure 6A-C) and went on to examine the effect of Tmsb4x loss on glomerular inflammation in our experimental model.

We measured the number of Cd3+ (T cells) and F4/80+ cells in Tmsb4x<sup>-/-</sup> and Tmsb4x<sup>+/+</sup> glomeruli. Twenty-one days after NTS injection there was a significant increase in Cd3+ cells in the glomerular tuft of Tmsb4x<sup>-/-</sup> mice compared with Tmsb4x<sup>+/+</sup> without disease (p<0.05) but no significant difference when comparing Tmsb4x<sup>-/-</sup> and Tmsb4x<sup>+/+</sup> mice with nephrotoxic nephritis (Figure 6D-G). There was also no difference in the number of peri-glomerular Cd3+ cells between experimental groups (Figure 6H). Seven days following NTS administration, the number of F4/80+ glomerular tuft cells was similar in all experimental groups, but were significantly elevated in the peri-glomerular area of both NTS-injected Tmsb4x<sup>-/-</sup> and Tmsb4x<sup>+/+</sup> mice compared with healthy mice (p<0.01; Supplementary Figure 9A-B). The accumulation of F4/80+ cells persisted in Tmsb4x<sup>-/-</sup> mice twenty-one days after NTS administration, with increased numbers in both the glomerular tuft and peri-glomerular area compared with Tmsb4x<sup>-/-</sup> mice with or without disease (p<0.01, Figure 6I-M). mRNA levels of the pan-macrophage marker, Cd68, were also significantly higher twenty-one days following NTS injection in whole kidney homogenates obtained from Tmsb4x<sup>-/-</sup> compared with Tmsb4x<sup>+/+</sup> mice (Supplementary Figure 10A). Cd68 is expressed by all macrophages, but these comprise a diverse group which includes a broad spectrum of cellular phenotypes, often characterised as pro-inflammatory (M1-type) and tissue repair (M2-type) macrophages.

We quantified the mRNA levels of M1 (Mcp1, Cd86) and M2 markers (Cd206, Arg1) and found all of these genes were significantly upregulated in Tmsb4x<sup>-/-</sup> mice compared with Tmsb4x<sup>+/+</sup> following NTS (Supplementary Figure 10B-E). This suggests that there is a global increase in macrophages in Tmsb4x<sup>-/-</sup> kidneys following NTS rather than a shift towards a M1 or M2 phenotype.
Finally, the increased accumulation of macrophages in the peri-glomerular area in \textit{Tmsb4x}^{-/-} mice with NTS was associated with increased peri-glomerular fibrosis as shown by increased staining for both collagen IV (\textbf{Figure 7A-C}) and alpha smooth muscle actin (\textit{a-SMA}) (\textbf{Figure 7E-G}) in sections from \textit{Tmsb4x}^{-/-} compared with \textit{Tmsb4x}^{+/+} mice injected with NTS along with increased whole kidney mRNA levels of \textit{Col4a1} (p<0.05; \textbf{Figure 7D}) and \textit{Acta2} (p<0.05; \textbf{Figure 7H}).
DISCUSSION

In this study we found endogenous Tmsb4x was not required to maintain glomerular structure and function in healthy adult mice. However, in an experimental model of NTS nephritis, glomerular disease was exacerbated in mice lacking Tmsb4x accompanied by changes in the distribution of podocytes within the glomerulus, increased peri-glomerular macrophage accumulation and enhanced fibrosis. These findings provide the first evidence that endogenous Tmsb4x modifies glomerular injury, likely having a protective role acting as a brake to slow disease progression.

We showed that Tmsb4x is expressed in developing and adult mouse glomeruli, predominantly localised to podocytes. Prior studies have also found Tmsb4x in glomerular podocytes, but others have reported complete absence of Tmsb4x in the glomeruli of human fetal and adult kidneys and rat kidneys. These discrepancies may be due to differences in the antibodies and fixation methods used. Importantly, we obtained similar results for both Tmsb4x mRNA and protein and confirmed the specificity of antibody staining using tissues from Tmsb4x−/− mice as an additional negative control.

Given that Tmsb4x plays a role in actin binding, we initially hypothesised that lack of endogenous Tmsb4x might disrupt the highly-branched architecture of glomerular podocytes and impair renal function. However, lack of Tmsb4x did not affect glomerular morphology or podocyte architecture of normal healthy mice in vivo. We found upregulation of Cfn1, which severs actin filaments, in whole Tmsb4x−/− kidneys. This could partly compensate for the lack of Tmsb4x and maintain actin dynamics, however, Cfn1 was not specifically altered in podocytes lacking Tmsb4x thus making this unlikely.

A significant finding of our study was that severity of glomerular disease induced by NTS was greater in Tmsb4x−/− mice compared with wild-type littermates. We postulate endogenous Tmsb4x has a protective role in the setting of NTS, a prediction supported by a
study showing exogenous administration of Ac-SDKP ameliorated rat glomerulonephritis. However, we found whole mouse kidney Tmsb4x mRNA levels were unchanged with NTS nephritis and this was mirrored when we assessed TMSB4X mRNA levels in glomerular and tubulointerstitial extracts from human kidneys affected by RPGN or SLE. In contrast, a previous study in rat remnant kidneys where nephron loss results in focal segmental glomerulosclerosis showed Tmsbx4 protein levels were significantly increased in sclerotic versus normal glomeruli. The discrepancy between these findings may be due to the different renal injury models and time-points examined.

There are likely to be multiple mechanisms by which lack of endogenous Tmsb4x results in increased glomerular injury in our experimental model. During nephrotoxic nephritis, podocytes switch from a terminally-differentiated cell to a migratory cell that forms bridges between the glomerular tuft and the Bowman’s capsule and populates glomerular crescents. Lack of Tmsbx4 increased the number of glomeruli with adhesion of the tuft to the Bowman’s capsule and glomerular epithelial hyperplasia lesions, a feature of early crescent formation in this model. We also found there was a redistribution of podocytes from the glomerular tuft, where they contribute to filtration barrier integrity, towards the Bowman’s capsule. Our in-vitro data demonstrates that downregulation of endogenous Tmsbx4 in podocytes increases migration and we predict that this may promote their redistribution in the nephrotoxic nephritis model. The increased podocyte migration was associated with increased actin stress fibres and activation of RhoA, which has been linked to podocyte stress fibre formation. Moreover, podocyte-specific overexpression of RhoA induces proteinuria whereas RhoA inhibition improves renal injury in mouse models of nephrectomy and nephrotoxic nephritis, demonstrating the functional importance of this pathway in glomerular function. Other studies have shown RhoA activation inhibits podocyte migration, but these experiments used a constitutively active form of RhoA permanently in the GTP-bound state. This would result in a high degree of RhoA permanently in the GTP-bound state. In contrast, Tmsbx4 knockdown led to a 2-fold
upregulation of RhoA activity in podocytes. It has been postulated that this lesser degree of
RhoA activation promotes contractile stress fibre formation facilitating cell detachment in
migrating cells and enhancing lamellipodia formation driving cell motility. It has been
previously reported that activation of other Rho GTPases, Cdc42 and Rac1, may increase
podocyte migration. However, we found that downregulation of endogenous Tmsb4x did
not affect Cdc42 activation in podocytes. Rac1 activity was not assessed in this study and it
would be interesting to explore its involvement in the future.

Tmsb4x is also expressed in macrophages including in our nephrotoxic nephritis model, but its precise function is yet to be determined. It could be postulated that loss of
macrophage Tmsbx4 may regulate the actin cytoskeleton which has been implicated in both
phenotypic polarisation and migration. In our study, loss of Tmsbx4 did not alter
macrophage polarisation or the number of activated macrophages found in the glomerular
area in the early stages of nephrotoxic nephritis. However the number of activated
macrophages in the peri-glomerular area at the late stage of the disease was increased in
Tmsb4x−/− mice suggesting a deficiency in the resolution of inflammation resulting in
persistent macrophage accumulation. Macrophage accumulation may result from an
absence of the Tmsb4x-derivative thymosin-β4-sulfoxide, which has been shown to disperse
inflammatory macrophages at the injury site in zebrafish and mouse models of heart injury.
We also found that peri-glomerular fibrosis was enhanced in Tmsb4x−/− mice following NTS
administration compared with wild-type mice. This may represent a secondary effect of
enhanced inflammation and glomerular damage. However, prior studies have shown that
Tmsbx4 can alter both PAI-1 and TGF-β1, both of which are drivers of fibrosis and play
important roles in the progression of nephrotoxic nephritis.

In summary, we have provided the first evidence that lack of endogenous Tmsb4x does not
affect healthy glomeruli but exacerbates renal function impairment, peri-glomerular
inflammation and fibrosis in the context of nephrotoxic nephritis. These findings suggest that
modulating Tmsbx4 could be a potential therapeutic target in immune-mediated glomerular disease.
METHODS

Experimental animals and procedures

C57Bl/6 hemizygous null male mice (Tmsb4x<sup>-/-</sup>) were bred with heterozygous Tmsb4x<sup>+/-</sup> adult females to generate male wild-type (Tmsb4x<sup>+/y</sup>) and null mice. For the induction of glomerular disease Tmsb4<sup>x</sup>-<sup>-/y</sup> and Tmsb4x<sup>-/-</sup> mice were pre-immunised by subcutaneous injection of sheep IgG (250µg) in complete Freund’s adjuvant, followed by intravenous administration of sheep NTS (250µl) five days later. All procedures were approved by the UK Home Office.

Renal function

Urine was collected from mice by housing them individually in metabolic cages. Blood samples were collected from the lateral saphenous vein. Albumin concentrations were measured by enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX). Urinary and plasma creatinine concentration was measured using isotope dilution electrospray mass spectrometry. Creatinine clearance (µl/min per g of body weight) was derived from the formula:

\[ \text{Creatinine clearance} = \frac{\text{urinary creatinine} \times \text{urine volume} \times 1440}{\text{plasma creatinine} \times 1440} \times \frac{1}{\text{body weight} (\text{g})} \]

BUN was assessed using a commercially available assay kit, validated in mice (BioAssay Systems, Hayward, CA).

Histological Analysis and Immunohistochemistry

Kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, 5µm sections cut and stained with periodic acid–Schiff reagent. 50 glomeruli/sample were scored by two blinded observers using the following system: 0=normal glomerular structure; 1=increased mesangial matrix deposition and hypercellularity, some loss of capillary loops; 2=increased matrix deposition and focal areas of sclerosis; 3=>50% of glomerulus sclerotic, very few capillary loops; 4=>75% of glomerulus sclerotic and presence of glomerular epithelial hyperplasia lesions (Supplementary Figure 5). An average score was obtained for each kidney.
Immunohistochemistry or immunofluorescence was performed using antibodies against Tmsb4x (A9520, Immundiagnostik, Bensheim, Germany), Collagen IV (ab19808, Abcam, Cambridge, UK), Cd3 (ab16669, Abcam), α-SMA (M0851, Dako, Ely, UK), F4/80 (MCA497R, AbD Serotec, Oxford, UK), Nphs1 (GP-N2, Progen, Heidelberg, Germany), Nestin (NB100-1604, Novus Biologicals, Littleton, CO), WT1 (AP15857PU-S, Acris Antibodies, Herford, Germany), Cd31 (MA3105, Thermo Fisher Scientific, Waltham, MA) and Sheep IgG (A11016, Thermo Fisher Scientific). CD3+ and F4/80+ cells were counted in 50 glomeruli/sample. To assess glomerular sheep IgG deposition, mean fluorescence intensity was measured using ImageJ (30 glomeruli/sample). The number of WT1+ cells found within or outside (in glomerular crescents or lining the Bowman’s capsule) the glomerular tuft was counted in 50 consecutive glomeruli/sample. To account for any changes in glomerular tuft area, the number of WT1+ cells in the glomerular tuft was normalised to the glomerular area (measured using ImageJ in 15 glomeruli/sample. Apoptosis was identified using TUNEL (Roche, Burgess Hill, UK). The number of TUNEL+ and WT1+/TUNEL+ cells was counted in 50 glomeruli/sample.

**Measurement of murine IgG subclasses specific for sheep IgG**

The titres of murine IgG subclasses specific for sheep IgG were measured by ELISA in plasma as described using alkaline phosphatase subclass-specific antibodies for IgG1, IgG2b and IgG3 (SouthernBiotech, Birmingham, AL) and IgG2a (Bethyl Laboratories).

**In-situ hybridisation**

*In-situ* hybridisation on paraffin sections was performed as described using a digoxigenin-labelled antisense riboprobe specific for the 3'UTR of Tmsb4x, alongside a sense control.

**Electron microscopy**
For transmission electron microscopy, kidney cortex specimens (1mm$^3$) were post-fixed in osmium tetroxide, dehydrated in acetone, and embedded in epoxy resin. Ultrathin sections were stained with uranyl-acetate and lead citrate and examined.

**Quantitative real-time PCR**

RNA was extracted from mouse whole kidney or glomerular extracts (isolated by Dynabeads$^{28}$). 500ng was used to prepare cDNA (iScript kit, Bio-Rad, Hemel Hempstead, UK) and qRT-PCR performed as described$^{28}$ with *Hprt* as a housekeeping gene. All measurements were performed in duplicate. Renal biopsies from patients with RPGN (n=12), SLE (n=12) and LD controls (n=7) were collected within the framework of the European Renal cDNA Bank - Kroener–Fresenius Biopsy Bank$^{69}$ after informed consent and local ethical approval. Unfixed tissue was transferred to RNase inhibitor and manually micro-dissected into glomerular and tubulointerstitial compartments. Total RNA was isolated and qRT-PCR performed as reported$^{69}$ with 18S rRNA (Applied Biosystems) as the reference gene. Primer details are available on request.

**Cell culture**

Mouse podocytes$^{37}$ were cultured as described$^{70}$ and allowed to differentiate for 14 days. Cells were transfected with 10nM siRNA specific for *Tmsb4x* or with a non-targeting control (both from Santa Cruz Biotechnology, Dallas, TX) using the transfection reagent, Lipofectamine® RNAiMAX (Thermo Fisher Scientific) according to the manufacturer’s instructions.

Cell viability was assessed 24, 48 and 72 hours post-transfection using the methyltetrazolium assay. To assess migration podocytes were plated to confluence and a scratch created using a pipette tip. Images (four fields/condition) were taken 0, 6 and 24 hours later and the number of cells that migrated into the wound area counted. To visualise F-actin filaments 48 hours post-transfection podocytes were fixed in 4% paraformaldehyde,
4% sucrose and stained with AlexaFluor-594 phalloidin (Thermo Fisher Scientific). The arrangement of actin filaments (either cytoplasmic stress fibres or cortical actin) was assessed in thirty cells/condition. RhoA and Cdc42 activity were quantified in podocyte lysates by G-LISA® Small G-protein Activation Assays (Cytoskeleton, Denver, CO).

Glomeruli from \(Tmsb4x^{+/y}\) and \(Tmsb4x^{-/-}\) mice were isolated by Dynabeads® and cultured in Matrigel-coated plates (Corning, Tewksbury, MA) in DMEM:F12 with 10% FCS, 1% ITS, 100\(\mu\)g/ml penicillin (Thermo Fisher Scientific). On day 7, when podocytes had grown out of the glomeruli, they were detached using trypsin-EDTA and separated from glomeruli using 40\(\mu\)m cell strainers (Corning). Primary podocytes obtained with this method were >90% pure as judged by cell morphology and staining using podocyte (nephrin, nestin) markers.

**Statistical Methods**

All samples were assessed blinded to treatment. Data is presented as means±SEM and was analysed using GraphPad Prism (GraphPad Software, La Jolla, CA). When differences between two groups were evaluated data was analysed by t-test. When three or more groups were assessed one-way ANOVA with Bonferroni’s multiple comparison post-hoc tests was used. Data affected by two variables was analysed using two-way ANOVA with Bonferroni’s multiple comparison post-hoc tests. Statistical significance was accepted at \(p\leq0.05\).
DISCLOSURE

The authors have no competing interests to declare
FIGURE LEGENDS

Figure 1. Tmsb4x expression in the mouse glomerulus.

(A) Quantification of thymosin-β4 (Tmsb4x) mRNA levels in mouse adult spleen, liver, heart and kidney by qRT-PCR. Tmsb4x expression was also quantified in glomeruli-depleted [glom (-)] and glomeruli-enriched [glom (+)] kidney homogenates. The bars represent the mean of three samples ±SEM. (B-C) In situ hybridisation for Tmsb4x on E16.5 mouse kidney sections. Cells positive for Tmsb4x are indicated by arrows. (D-F) Immunohistochemistry for Tmsb4x in the mouse glomerulus at E18 (D), 1 week (E) and 8 weeks of age (F). Cells positive for Tmsb4x are indicated by arrows. (G-I) Representative pictures for Tmsb4x (G) and nephrin (Nphs1) (H) staining in the mouse adult wild-type glomerulus visualised by fluorescent microscopy. (I) Merged image showing Nphs1 (red) and Tmsb4x (green) staining; areas of co-localisation are indicated by arrows. Scale bar = 20μm; **p ≤ 0.01.

Figure 2. Renal function and glomerular morphology in Tmsb4x+/y and Tmsb4x−/y mice.

(A) Breeding scheme: Heterozygous female mice (Tmsb4x+/−) were bred with hemizygous null male mice (Tmsb4x−/y). Male wild-type (Tmsb4x+/y) and Tmsb4x−/y mice were compared in all subsequent experiments. (B) Twenty-four hour albumin excretion in urine of Tmsb4x+/y (n=14-15) and Tmsb4x−/y (n=9-10) mice collected at 1, 3, and 6 months of age. Data was log-transformed before analysis. (C) Blood urea nitrogen (BUN) concentration in Tmsb4x+/y (n=9-15) and Tmsb4x−/y mice (n=7-9) at 1, 3, and 6 months of age. Data was log-transformed before analysis. (D-E) Representative pictures of PAS staining of paraffin-embedded sections from Tmsb4x+/y (D) and Tmsb4x−/y (E) kidneys. Scale bar = 20μm. The glomerular score (F) was quantified as explained in the methods (Tmsb4x+/y: n=4; Tmsb4x−/y: n=5). (G-H) Representative images of the glomerular architecture of Tmsb4x+/y (G) and Tmsb4x−/y (H) kidneys assessed by transmission electron microscopy. An average of five glomeruli was examined per animal (Tmsb4x+/y, n=4; Tmsb4x−/y, n=3). (I-J) Representative pictures of
immunohistochemistry for thymosin-β4 on paraffin-embedded sections from \( Tmsb4x^{+/y} \) (I) and \( Tmsb4x^{-/-} \) (J) kidneys from 6 month old mice. Thymosin-β4-positive cells are indicated by arrows. Note: non-specific staining in tubules in \( Tmsb4x^{+/y} \) mice and \( Tmsb4x^{-/-} \) mice. Quantification of \( Tmsb4x \) (K), \( Tmsb10 \) (L), \( Tmsb15a \) (M), \( Tmsb15b \) (N) and \( Tmsb15l \) (O) mRNA levels in whole kidney homogenates of \( Tmsb4x^{+/y} \) (n=8) and \( Tmsb4x^{-/-} \) (n=4) mice by qRT-PCR. Data is presented as mean±SEM. ***p ≤ 0.001. Scale bar = 20µm.

**Figure 3. Renal function in \( Tmsb4x^{+/y} \) and \( Tmsb4x^{-/-} \) mice following the induction of nephrotoxic nephritis.**

(A) Outline of experimental strategy. Twenty-four hour albumin excretion in urine (B), urinary albumin to urinary creatinine ratio (C), plasma creatinine concentration (D), creatinine clearance (E) and blood urea nitrogen concentration (F) of \( Tmsb4x^{+/y} \) and \( Tmsb4x^{-/-} \) mice. Samples were collected prior to immunisation with Freund's adjuvant (\( Tmsb4x^{+/y} \); control group) and 21 days after administration of nephrotoxic serum (\( Tmsb4x^{+/y} +NTS \) and \( Tmsb4x^{-/-} +NTS \)); n=12 for each group. Data was log-transformed before analysis and is presented as mean±SEM. (G) Glomerular score was quantified as described in the methods in \( Tmsb4x^{+/y} \) (n=5), \( Tmsb4x^{+/y} +NTS \) (n=9) and \( Tmsb4x^{-/-} +NTS \) (n=6) mice. Data is presented as mean±SEM. Representative pictures of PAS staining in glomeruli from control mice (\( Tmsb4x^{+/y} \); H), and mice administered nephrotoxic serum (NTS) \( Tmsb4x^{+/y} +NTS \) (I) and \( Tmsb4x^{-/-} +NTS \) (J) are shown. Scale bar = 20µm. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**Figure 4. Podocyte assessment in \( Tmsb4x^{+/y} \) and \( Tmsb4x^{-/-} \) mice following the induction of nephrotoxic nephritis.**

(A-C) Representative picture for nephrin (Nphs1; podocytes) (A) and Tmsb4x (B) staining in the mouse adult wild-type glomerulus 21 days after injection with nephrotoxic serum. Cells positive for both Nphs1 (green) and Tmsb4x (red) are indicated by arrows in the merged
picture (C). (D-F) Representative pictures of glomeruli from $Tmsb4x^{+/-}$ (D), $Tmsb4x^{+/-} + NTS$ (E) and $Tmsb4x^{-/-} + NTS$ (F) mice stained for WT1. The glomerular tuft area is annotated by a dotted line. (G-J) Graphs showing the number of WT1-positive cells in the glomerular tuft (G), the number of WT1-positive cells in the glomerular tuft normalised to glomerular area (H), the number of WT1-positive cells in the area of the glomerulus surrounding the glomerular tuft (I) and the number of WT1-positive cells in the whole glomerulus (J). Cells were counted in 50 glomeruli per sample, except for D, where cells were counted and normalised to the glomerular area in 15 glomeruli per sample. Data is presented as mean±SEM. $Tmsb4x^{+/-}$ (n=5), $Tmsb4x^{+/-} + NTS$ (n=9) and $Tmsb4x^{-/-} + NTS$ (n=6) mice. Scale bar = 20μm.

**Figure 5: Effects of downregulating endogenous $Tmsb4x$ expression in podocytes in vitro.**

(A) Podocytes grown in vitro under permissive conditions were differentiated for 14 days before transfecting them with control siRNA or siRNA targeting $Tmsb4x$. (B) Quantification of $Tmsb4x$ mRNA levels in podocytes 48 hours after transfection. (C) Cell viability following knockdown of endogenous $Tmsb4x$ was assessed by MTT assay. (D) Podocyte migration following knockdown of endogenous $Tmsb4x$ was assessed by a wound-healing assay and the number of cells that migrated into the wound area was counted. (E) Representative pictures of podocytes transfected with control or $Tmsb4x$ siRNA 0, 6 and 24 hours after wound formation. (F-G) Representative pictures showing a podocyte with cytoplasmic stress fibre F-actin distribution (F) or cortical F-actin distribution (G). The percentage of cells with predominantly cytoplasmic stress fibres or cortical actin formation was quantified 48 hours after transfection (H). (I-J) Quantification of active RhoA (I) and active Cdc42 (J) 48 hours after transfection. All experiments were repeated three to four times and the data is presented as mean±SEM. *p ≤ 0.05, ***p ≤ 0.001.
Figure 6. Assessment of inflammation in nephrotoxic nephritis.

(A-C) Representative picture for F4/80 (macrophages; A) and Tmsb4x staining (B). Cells positive for both F4/80 (green) and Tmsb4x (red) are shown in the merged picture (C) and are indicated by arrows. Images were taken by fluorescent microscopy. Scale bar = 20µm.

(D-F) Representative pictures showing Cd3 (T-cell marker) staining in the glomerular tuft (arrows) and in the peri-glomerular area (arrowheads) of Tmsb4x$^{+/y}$ controls (D) and Tmsb4x$^{-/-}$ (E) and Tmsb4x$^{-/-}$ (F) mice 21 days following administration of nephrotoxic serum (NTS). The number of Cd3+ cells in the glomerular tuft (G) and in the peri-glomerular area (H) was counted in 50 glomeruli per sample and the average number was calculated (Tmsb4x$^{+/-}$, n=5, Tmsb4x$^{+/-}$+NTS, n=9, Tmsb4x$^{-/-}$+NTS, n=6). (I-K) Representative pictures showing F4/80 (activated macrophage marker) staining in the glomerular tuft (arrows) and the peri-glomerular area (arrowheads) of Tmsb4x$^{+/-}$ controls (I) Tmsb4x$^{+/-}$+NTS (J) and Tmsb4x$^{-/-}$+NTS (K) mice. The number of F4/80+ cells in the glomerular tuft (L) and in the peri-glomerular area (M) was counted in 50 glomeruli per sample and the average number was calculated (Tmsb4x$^{+/-}$, n=5, Tmsb4x$^{+/-}$+NTS, n=8, Tmsb4x$^{-/-}$+NTS, n=6). Data is presented as mean±SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Scale bar = 20µm.

Figure 7. Assessment of fibrosis in nephrotoxic nephritis.

(A-C) Representative images showing collagen IV staining in glomeruli of Tmsb4x$^{+/-}$ (A) Tmsb4x$^{+/-}$+NTS (B) and Tmsb4x$^{-/-}$+NTS (C) mice. (N) Quantification of collagen IV (Col4a1) mRNA levels in whole kidney homogenates of Tmsb4x$^{+/-}$ (n=8), Tmsb4x$^{+/-}$+NTS (n=9) and Tmsb4x$^{-/-}$+NTS (n=6) mice. (E-G) Representative pictures showing alpha smooth muscle actin (α-SMA) staining in glomeruli of Tmsb4x$^{+/-}$ (E) Tmsb4x$^{+/-}$+NTS (F) and Tmsb4x$^{-/-}$+NTS (G) mice. (H) Quantification of alpha smooth muscle actin (Acta2) mRNA levels in whole kidney homogenates of Tmsb4x$^{+/-}$ (n=8), Tmsb4x$^{+/-}$+NTS (n=8) and Tmsb4x$^{-/-}$
+NTS (n=5) mice. Data is presented as mean±SEM. *p ≤ 0.05, **p ≤ 0.01. Scale bar = 20µm.
ACKNOWLEDGMENTS

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REFERENCES


Figure 2

A

Tmsb4x<sup>−/−</sup> Female × Tmsb4x<sup>−/−</sup> Male

Tmsb4x<sup>−/−</sup> Tmsb4x<sup>−/−</sup> Tmsb4x<sup>−/−</sup> Tmsb4x<sup>−/−</sup>

B

Log albumin (µg per 24 h urine)

Age (months)

C

Log BUN (mg/dl)

Age (months)

D

E

F

Average glomerular score

G

H

I

J

K

L

M

N

O

Tmsb4x<sup>−/−</sup> Tmsb4x<sup>−/−</sup>

Tmsb4x<sup>−/−</sup> Tmsb4x<sup>−/−</sup> Tmsb4x<sup>−/−</sup> Tmsb4x<sup>−/−</sup>
Figure 3

A

Freund’s adjuvant s/c

Nephrotoxic serum IV

Day -6

Day -5

Day 0

Day 7

Day 21

Collect urine, plasma

Collect kidneys

Collect urine, plasma, kidneys

B

Log Albumin

(µg/ml of 24 hours)

10^2

10^3

10^4

10^5

10^6

Tmsb4x^+/y

Tmsb4x^+/y +NTS

Tmsb4x^−/y

Tmsb4x^−/y +NTS

***

**

C

Log albumin/mg creatinine

10^2

10^3

10^4

10^5

10^6

Tmsb4x^+/y

Tmsb4x^+/y +NTS

Tmsb4x^−/y

Tmsb4x^−/y +NTS

***

**

D

Log plasma creatinine (µA)

10^2

10^3

10^4

10^5

10^6

Tmsb4x^+/y

Tmsb4x^+/y +NTS

Tmsb4x^−/y

Tmsb4x^−/y +NTS

**

E

Log creatinine clearance

(µmol/min/1.73 m²)

10^2

10^3

10^4

10^5

10^6

Tmsb4x^+/y

Tmsb4x^+/y +NTS

Tmsb4x^−/y

Tmsb4x^−/y +NTS

**

F

Log BUN (mg/dL)

10^2

10^3

10^4

10^5

10^6

Tmsb4x^+/y

Tmsb4x^+/y +NTS

Tmsb4x^−/y

Tmsb4x^−/y +NTS

*

G

Score per glomerulus

1

2

3

4

Tmsb4x^+/y

Tmsb4x^+/y +NTS

Tmsb4x^−/y

Tmsb4x^−/y +NTS

***

*

H

Tmsb4x^+/y

I

Tmsb4x^+/y +NTS

J

Tmsb4x^−/y +NTS
Figure 4

A. Nphs1
B. Tmsb4x
C. Merged
D. WT1
E. Tmsb4x<sup>+/y</sup> + NTS
F. Tmsb4x<sup>+/y</sup> + NTS

G. Mean number of WT1+ cells in glomerular tuft
H. Mean number of WT1+ cells per glomerular tuft area

I. Mean number of WT1+ cells outside the glomerular tuft
J. Mean number of WT1+ cells in the glomerulus
Figure 5

A

Cells growing at 33°C + IFN-γ → Transfect with siRNA → End-points

B

Tmsb4x/hprt

C

Cell viability

D

Cell migration

E

0 hours

6 hours

24 hours

Control siRNA

Tmsb4x siRNA

F

G

H

F-actin distribution

I

RhoA activity

J

Cdc42 activity
Figure 6

(A) F4/80
(B) Tmsb4x
(C) Merged

(D) Cd3

(E) Tmsb4x+/y
(F) Tmsb4x+/y +NTS

(G) Average number of Cd3+ cells in glomerular tuft

(H) Average number of Cd3+ cells in peril glomerular area

(I) F4/80

(J) Tmsb4x+/y +NTS

(K) Tmsb4x+/y +NTS

(L) Average number of F4/80+ cells in glomerular tuft

(M) Average number of F4/80+ cells in peril glomerular area
Figure 7

Collagen IV

A  B  C

Tmsb4x<sup>+/y</sup>  Tmsb4x<sup>+/y</sup> +NTS  Tmsb4x<sup>y</sup> +NTS

α-SMA

D  E  F  G

Tmsb4x<sup>+/y</sup>  Tmsb4x<sup>+/y</sup> +NTS  Tmsb4x<sup>y</sup> +NTS

H

Col4/αHort

Tmsb4x<sup>+/y</sup>  Tmsb4x<sup>+/y</sup> +NTS  Tmsb4x<sup>y</sup> +NTS

Acta2/αHort

Tmsb4x<sup>+/y</sup>  Tmsb4x<sup>+/y</sup> +NTS  Tmsb4x<sup>y</sup> +NTS
Supplementary Data

Loss of endogenous thymosin-β4 accelerates glomerular disease
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**Supplementary Table 1** Proportions of $Tmsb4x^{+/-}$, $Tmsb4x^{-/-}$, $Tmsb4x^{+/-y}$ and $Tmsb4x^{-/-y}$ mice born after crossing adult male $Tmsb4x^{+/-y}$ mice with $Tmsb4x^{+/-}$ adult females.

<table>
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<th>$Tmsb4x^{-/-}$</th>
<th>$Tmsb4x^{+/-y}$</th>
<th>$Tmsb4x^{-/-y}$</th>
<th>Total</th>
<th>$X^2$</th>
<th>$P$ value</th>
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<td>Expected</td>
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<td>25%</td>
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<tr>
<td>Observed</td>
<td>92 (28%)</td>
<td>74 (23%)</td>
<td>85 (26%)</td>
<td>76 (23%)</td>
<td>327</td>
<td>2.554</td>
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</tbody>
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Supplementary Figure 1. (A-C) Representative pictures for Nestin (A) and Tmsb4x (B) staining in the mouse adult wild-type glomerulus visualised by fluorescent microscopy. (C) Merged image showing Tmsb4x (red) and Nestin (green) staining; areas of co-localisation are indicated by arrows. (D-F) Representative pictures for Tmsb4x (D) and Cd31 (E) staining in the mouse adult wild-type glomerulus visualised by fluorescent microscopy. (F) Merged image showing Tmsb4x (green) and Cd31 (red) staining. Scale bar = 20μm.
Supplementary Figure 2. Quantification of profilin 1 (Pfn1; A), profilin 2 (Pfn2; B), destrin (Dstr; C) and cofilin 1 (Cfl1; D) mRNA in whole kidney homogenates of Tmsb4x<sup>+/y</sup> (n=8) and Tmsb4x<sup>-/y</sup> (n=4) mice by qRT-PCR. Quantification of cofilin 1 (Cfl1) mRNA in podocytes 48 hours after transfection with control siRNA or siRNA targeting Tmsb4x (E, n=4) and in podocytes isolated from Tmsb4x<sup>+/y</sup> or Tmsb4x<sup>-/y</sup> mice (F, n=6). Data is presented as mean±SEM. *p<0.05
Supplementary Figure 3. Quantification of nephrin (*Nphs1; A*), podocin (*Nphs2; B*), synaptopodin (*Synpo; C*), cd2 associated protein (*Cd2ap; D*) and wilms tumor 1 (*Wt1; E*) mRNA in whole kidney homogenates of *Tmsb4x<sup>+/y</sup>* (n=8) and *Tmsb4x<sup>-/y</sup>* (n=4) mice by qRT-PCR. Data is presented as mean±SEM.
Supplementary Figure 4. (A) Quantification of Tmsb4x mRNA levels in whole kidney homogenates of Tmsb4x<sup>+/y</sup> (n=8) and Tmsb4x<sup>+/y</sup>+NTS (n=9) mice by qRT-PCR. (B-C) Quantification of TMSB4X mRNA levels in homogenates from human glomeruli (B) or tubulointerstitium (C). LD, living donor; RPGN, rapidly progressive glomerulonephritis; SLE, Lupus nephritis. Data is presented as mean±SEM.
Supplementary Figure 5. Representative pictures of the histology observed 7 days after NTS administration showing a normal glomerulus (A), a glomerulus with hyaline deposits indicated by arrowheads (B) and a glomerulus with a bridge formed between the tuft and Bowman’s capsule indicated by an arrow (C). Scale bar = 20µm.
Supplementary Figure 6. (A-C) Representative pictures for the different scoring categories for glomerular injury showing (A) a normal glomerulus, score 0; (B) a glomerulus with increased mesangial matrix deposition and some loss of capillary loops, score 1; (C) a glomerulus with increased mesangial matrix deposition and focal areas of sclerosis, score 2; (D) a glomerulus with >50% sclerotic area and very few capillary loops, score 3 and (E) a sclerotic glomerulus with an epithelial hyperplastic lesion (*) and a bridge formed between the tuft and Bowman's capsule indicated by an arrow, score 4. Scale bar = 20µm. (F) Proportions of glomeruli in the different scoring categories of glomerular injury in Tmsb4x^+/y (n=5), Tmsb4x^+/y +NTS (n=9) and Tmsb4x^−/− +NTS (n=6) mice. Bars represent the mean±SEM.
Supplementary Figure 7. (A-B) Representative pictures showing sheep IgG immunoreactivity in glomeruli of Tmsb4x

+/

(y) (A) and Tmsb4x

−/

(y) (B) mice injected with NTS. (C) Quantification of mean fluorescence in 30 glomeruli per sample (Tmsb4x

+/

+NTS, n=9; Tmsb4x

−/

+NTS, n=6). (D-G) Plasma titres of murine IgG subclasses (IgG1, D; IgG2a, E; IgG2b, F; IgG3, G) against Sheep IgG in control not immunised Tmsb4x

+/

mice (n=5) and in Tmsb4x

+/

(n=9) and Tmsb4x

−/

(n=6) mice 21 days after injection with nephrotoxic serum (+NTS). Data is presented as mean±SEM. Scale bar = 20µm, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Supplementary Figure 8. (A-C) Representative picture showing staining for TUNEL and WT1 in the glomerulus. A TUNEL-positive podocyte is identified by an arrowhead (C). The number of TUNEL-positive cells (D) and TUNEL-positive WT-1 positive cells per glomerulus was counted in 50 glomeruli per sample and the average number was calculated ($Tmsb4x^{+/y}$, n=4; $Tmsb4x^{+/y}$ +NTS, n=9; $Tmsb4x^{-/-y}$ +NTS, n=6). Data is presented as mean±SEM. Scale bar = 20µm, *p ≤ 0.05.
Supplementary Figure 9. The number of F4/80+ cells in the glomerular tuft (A) and in the peri-glomerular area (B) 7 days following administration of nephrotoxic serum (NTS) was counted in 50 glomeruli per sample and the average number was calculated ($Tmsb4x^{+/y}$, n=5, $Tmsb4x^{−/−}$ +NTS, n=6, $Tmsb4x^{+/−}$ +NTS, n=8). Data is presented as mean±SEM. **p ≤ 0.01.
Supplementary Figure 10. Quantification of Cd68 (A), MCP-1 (B), Cd86 (C), Cd206 (D) and Arg1 (E) mRNA in whole kidney homogenates of Tmsb4x<sup>+/y</sup> +NTS (n=9) and Tmsb4x<sup>-/y</sup> +NTS (n=6) mice.