

1 **Systemic gene therapy with thymosin β 4 alleviates glomerular injury in mice**

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Abstract

25 Plasma ultrafiltration in the kidney occurs across glomerular capillaries, which are
 26 surrounded by epithelial cells called podocytes. Podocytes have a unique shape maintained
 27 by a complex cytoskeleton, which becomes disrupted in glomerular disease resulting in
 28 defective filtration and albuminuria. Lack of endogenous thymosin β 4 (TB4), an actin
 29 sequestering peptide, exacerbates glomerular injury and disrupts the organisation of the
 30 podocyte actin cytoskeleton, however, the effect of exogenous TB4 therapy on
 31 podocytopathy is unknown. Here, through interrogating single-cell RNA-sequencing data of
 32 isolated glomeruli we demonstrate that Adriamycin, a toxin which injures podocytes and
 33 leads to leakage of albumin in the urine of mice, results in reduced levels of podocyte TB4.
 34 Systemic administration of an adeno-associated virus vector encoding TB4 prevented
 35 Adriamycin-induced podocyte loss and albuminuria. Adriamycin injury was associated with
 36 disorganisation of the actin cytoskeleton *in vitro*, which was ameliorated by exogenous TB4.
 37 Furthermore, Adriamycin administration in mice was associated with increased prevalence
 38 of podocyte vesicles, a mechanism by which albumin may leak into the urine, which was
 39 also prevented by TB4. Collectively, we propose that TB4 gene therapy prevents podocyte
 40 injury and maintains glomerular filtration via modulation of the podocyte cytoskeleton thus
 41 presenting a novel treatment strategy for glomerular disease.

Introduction

One in ten people worldwide have chronic kidney disease (CKD).¹ A subset of patients with CKD progress to end-stage kidney disease (ESKD), which requires dialysis or transplantation and is a risk factor for cardiovascular disease and all-cause mortality.¹ CKD progression is linked to breakdown of the glomerular filtration barrier, the site of ultrafiltration in the kidney, which consists of endothelial cells, the glomerular basement membrane (GBM) and epithelial podocytes.^{2,3} Podocytes have a unique architecture with foot processes that extend from their cell bodies, interdigitate and form slit diaphragms facilitating size and charge-selective filtration and preventing the loss of plasma proteins.^{4,5} In health, podocyte shape is maintained by a complex, highly regulated actin cytoskeleton, which supports the foot processes,^{6,7} and anchors the cell to the GBM.⁸ During glomerular disease, the podocyte cytoskeleton becomes disorganised often leading to podocyte loss accompanied by impaired filtration and leakage of plasma proteins, such as albumin, into the urine.^{6,9-11} Albuminuria is a hallmark of glomerular disease, irrespective of the underlying aetiology.¹² Therefore, therapies that protect the podocyte cytoskeleton could provide a novel strategy to preserve the integrity of the glomerular filtration barrier, prevent albuminuria and improve glomerular disease progression.

Thymosin β 4 (TB4) sequesters monomeric G-actin in mammalian cells^{13,14} and maintains high concentrations of G-actin available for polymerisation into actin filaments (F-actin).¹⁵ Our previous work has shown that endogenous TB4 is expressed in podocytes and plays a protective role in glomerular disease. We found that lack of endogenous TB4 worsens albuminuria, renal function and glomerular injury in a mouse model of glomerulonephritis, concomitant with redistribution of podocytes from the glomerular tuft, where they

64 contribute to filtration barrier integrity, to the Bowman's capsule. Furthermore, a direct role
65 for endogenous TB4 on the podocyte cytoskeleton was established *in vitro*, with a shift from
66 cortical actin to cytoplasmic actin stress fibres and enhanced migration observed in
67 podocytes lacking TB4.¹⁶

68 These findings raise the possibility that exogenous TB4 could be used as a therapy to protect
69 the podocyte cytoskeleton and slow the progression of glomerular disease. Exogenous TB4
70 has already shown promise as a treatment for a diverse range of conditions, such as
71 myocardial infarction,¹⁷ dry eye syndrome,¹⁸ stroke¹⁹ and inflammatory lung disease.²⁰
72 Furthermore, exogenous TB4 is protective in several animal models of kidney injury,²¹
73 including diabetic nephropathy,²² unilateral ureteral obstruction^{23,24} and acute ischaemia
74 reperfusion injury,²⁵ but none of these studies examined the specific effect of exogenous
75 TB4 on podocytes. Additionally, the studies above administered TB4 peptide, which has a
76 relatively short half-life with enhanced levels in the plasma evident for only 6 hours
77 following injection.²⁶ To overcome the rapid metabolic turnover of TB4, recombinant adeno-
78 associated virus (AAV)-mediated gene therapy provides a solution through its ability to
79 induce stable, long term transgene expression via a single injection.²⁷⁻²⁹ Indeed, TB4-
80 encoding AAV constructs have been successfully used for tissue-specific (muscle, heart) or
81 systemic upregulation of TB4 in mouse, rabbit and pig disease models with therapeutic
82 effects.³⁰⁻³²

83 In this study, we show that podocyte injury induced by a toxin (Adriamycin (ADR)) is
84 associated with reduced expression of endogenous TB4 through interrogating single-cell
85 RNA sequencing (scRNAseq) data of isolated glomeruli. Using systemic gene therapy,
86 upregulation of the plasma concentration of TB4 was able to prevent ADR-induced

87 albuminuria and podocyte loss. Further examination of the podocyte F-actin structures *in*
88 *vitro* revealed that exogenous TB4 prevented ADR-induced cytoskeletal disorganisation.
89 Collectively, our work shows that gene therapy-mediated systemic administration of TB4
90 presents a novel treatment strategy to protect podocytes from injury and preserve the
91 integrity of the glomerular filtration barrier.

Results

92 **ADR is associated with the downregulation of TB4 in podocytes**

93 To induce glomerular damage *in vivo*, we administered ADR, a chemotherapeutic drug, to
94 male BALB/c mice. In BALB/c mice ADR replicates some of the features of human focal
95 segmental glomerulosclerosis (FSGS)³³ and has toxic effects on podocytes *in vitro* and *in*
96 *vivo*, including cytoskeletal disorganisation, foot process effacement and a loss of podocyte
97 viability^{9,34,35}. Firstly, we examined if ADR administration altered glomerular TB4 expression.
98 To do this, we isolated glomeruli from ADR-administered BALB/c mice by Dynabead
99 perfusion³⁶ and using quantitative real-time PCR (qRT-PCR), we found that there was a
100 downregulation of the mRNA transcript for TB4, *Tmsb4x*, at 7 days post ADR administration
101 compared with saline injected mice (P=0.076) but not at 14 days (**Figure 1a**). To further
102 interrogate the expression of *Tmsb4x* in specific glomerular cells following ADR injury, we
103 analysed a published scRNAseq dataset obtained from glomeruli isolated by Dynabeads of
104 healthy or ADR-injured C57BL/6J mice³⁷. Using unsupervised clustering analysis we derived
105 ten transcriptionally distinct cell types (**Figure 1b**), which using established markers of
106 glomerular cell types, we identified to represent all the known glomerular cell types
107 (**Supplementary Figure 1a**). Confirming the identity of podocytes, the podocyte cluster
108 expressed both nephrin (*Nphs1*) and podocin (*Nphs2*), components of the podocyte slit

diaphragm (**Figure 1c**). Comparison of control and ADR-injured glomeruli showed representation of all cell types in both groups (**Supplementary Figure 1b**). As previously described³⁷, we found a reduction in the proportion of podocyte cells in the ADR-injured glomeruli compared with controls (**Supplementary Figure 1c**). ADR injury was associated with significant downregulation of *Tmsb4x* in the glomerular tuft, assessed by grouped analysis of glomerular endothelial, mesangial and podocyte cells (**Figure 1d**; 0.14 log fold change; $P < 0.05$). When podocyte cells were analysed individually, we found a larger 0.37 log fold reduction in *Tmsb4x* in those obtained from ADR-injured compared with healthy glomeruli (**Figure 1e**; $P < 0.05$). Collectively, these findings demonstrate that ADR injury is associated with reduced *Tmsb4x* expression in the glomerulus and specifically in podocytes.

Systemic upregulation of TB4 using gene therapy

We then assessed the effects of exogenous TB4 administration on ADR-induced glomerular disease. We took a preventative strategy and BALB/c mice were administered intravenously AAV serotype 2/7 expressing either *Tmsb4x* or the *LacZ* gene as a control.^{30,32} Three weeks after AAV injection ADR was administered (day 0). Fourteen days later the animals were euthanised and we compared mice administered either: (i) AAV-*Tmsb4x* and ADR (*Tmsb4x*/ADR); (ii) AAV-*LacZ* and ADR (*LacZ*/ADR) and (iii) AAV-*LacZ* and saline as our control group (*LacZ*/saline) (**Figure 2a**). Initially, we examined if AAV-*Tmsb4x* altered *Tmsb4x* mRNA levels in the liver, as this is the primary site of transduction following systemic injection of AAV 2/7.³⁸ We found a 10-fold increase in liver *Tmsb4x* mRNA levels in the *Tmsb4x*/ADR group compared with the *LacZ*/ADR group ($P < 0.001$) (**Figure 2b**). This corresponded with strong positive immunostaining for TB4 in the liver in *Tmsb4x*/ADR mice (**Figure 2c**). Additionally, as TB4 is a secreted peptide³⁹, we measured circulating levels using ELISA and

found a 2-fold increase in the *Tmsb4x*/ADR group compared with either the *LacZ*/ADR (P<0.05) or *LacZ*/saline group (P<0.01) (**Figure 2d**). Using qRT-PCR on whole kidney lysates we found no difference in *Tmsb4x* mRNA levels between the three experimental groups (**Figure 2e**) in agreement with previous reports that AAV 2/7 does not specifically target the kidney.³⁸

TB4 prevents ADR-induced albuminuria

Next, we examined the effect of TB4 administration on ADR injury *in vivo*. Firstly, as an overall measure of health, we weighed the mice throughout the time course of the experiment. All mice administered ADR lost a significantly greater proportion of their body weight 48 hours following ADR injection compared with *LacZ*/saline animals (**Figure 3a**). This difference was sustained at both day 7 and 14 after ADR injection. At all time-points there was no difference between ADR mice administered AAV-*LacZ* and AAV-*Tmsb4x*.

We then analysed the levels of albumin in the urine, a marker of glomerular filtration barrier integrity.⁴⁰ Prior to ADR injection, there was no difference in the levels of urinary albumin (*LacZ*/saline group: 95 ±16 µg/24 hours, *LacZ*/ADR group: 41 ±16 µg/24 hours, *Tmsb4x*/ADR group: 60 ±32 µg/24 hours). Similar levels were found two days after ADR administration with no differences between the three groups of mice (*LacZ*/saline group: 103 ±20 µg/24 hours, *LacZ*/ADR group: 65 ±11 µg/24 hours, *Tmsb4x*/ADR group: 73 ±7 µg/24 hours). Conversely, at 14 days post ADR injection, albuminuria significantly increased to 1448 ±115 µg/24 hours in the *LacZ*/ADR mice compared with 107 ±22 µg/24 hours in *LacZ*/saline animals (P<0.001). Strikingly, gene therapy with TB4 prevented this increase with a urinary albumin excretion of 214 ±43 µg/24 hours in the *Tmsb4x*/ADR group (P<0.01 versus *LacZ*/ADR group) (**Figure 3b**). At 14 days, the albumin to creatinine ratio of the *LacZ*/ADR

group was also increased 25-fold in comparison with *LacZ*/saline mice ($P < 0.001$). This increase was prevented in the ADR mice treated with TB4 ($P < 0.001$ versus *LacZ*/ADR group; **Figure 3c**). Urea is freely filtered through the glomerular filtration barrier and increased plasma blood urea nitrogen (BUN) concentration is an indicator of declining kidney function⁴¹. However, we found that there were no significant changes between any of the groups 14 days after ADR administration (**Figure 3d**).

Previously the protective effect of TB4 in CKD has been linked to its anti-inflammatory properties^{16,24,42}. Therefore, we quantified F4/80⁺ macrophage numbers within and around the glomerular tuft by immunohistochemistry (**Supplementary Figure 2a**). F4/80⁺ macrophages within the glomerular tuft were rare across all three groups (**Supplementary Figure 2b**). The number of macrophages outside the glomerular tuft was similar among the groups (**Supplementary Figure 2c**), excluding a role for inflammation in our model and suggesting that the protective effect of TB4 in ADR-induced glomerular injury occurs by other means.

TB4 prevents ADR-induced podocyte injury *in vivo*

Next, we assessed the effect of exogenous TB4 on podocytes in this model. As ADR has been shown to cause podocyte loss⁴³ we quantified the number of Wilms Tumour 1 positive (WT1⁺) podocytes in the glomerulus⁴⁴ (**Figure 4a**). When analysing the results obtained per mouse, we found lower podocyte number and density (WT1⁺ podocytes/glomerular area) in the *LacZ*/ADR group compared with the *LacZ*/saline and *Tmsb4x*/ADR groups, however this was not statistically significant (**Figure 4b, c**). Since one of the features of FSGS is that not all glomeruli are affected to the same extent⁴⁵, we also examined podocyte number at the level of individual glomeruli by assessing a total of at least 250 glomeruli from 5 different

mice in each group. Here, we found a significant decrease in podocyte number (8.85 ± 0.31 podocytes) and density ($5.31 \times 10^{-3} \pm 0.18 \times 10^{-3}$ podocytes/ μm^2) in the *LacZ*/ADR group compared with *LacZ*/saline (10.94 ± 0.25 podocytes and $6.59 \times 10^{-3} \pm 0.13 \times 10^{-3}$ podocytes/ μm^2 respectively) ($P < 0.001$ in both cases). TB4 treatment prevented the ADR-induced podocyte loss and preserved podocyte number (10.38 ± 0.34 podocytes) ($P < 0.001$ versus *LacZ*/ADR group) and podocyte density ($6.57 \times 10^{-3} \pm 0.20$ podocytes/ μm^2) ($P < 0.001$ versus *LacZ*/ADR group) (**Figure 4d, e**).

Alterations to podocyte F-actin have been associated with foot process effacement and albuminuria *in vivo*^{9,10}. We hypothesised that the protective effect of TB4 in ADR injury may be partly mediated by its ability to sequester G-actin and regulate F-actin polymerisation.^{13,14,46} Using synaptopodin as a podocyte marker,⁴⁷ and phalloidin to visualise F-actin filaments, we quantified F-actin in the synaptopodin-positive regions (**Figure 4f**). We found that there was no difference in the mean podocyte F-actin fluorescence between any of the groups (**Figure 4g**). There were also no changes to the total area (μm^2) or percentage of podocyte area covered by F-actin, which remained at approximately 65% (**Figure 4h, i**), indicating that neither ADR nor TB4 alter the amount of F-actin within podocytes.

Albumin leakage can also occur via transcellular transport mediated by endocytosis of albumin in podocyte vesicles followed by excretion to the urinary space.⁴⁸⁻⁵¹ Double staining of glomeruli with synaptopodin and F-actin revealed vesicle-like structures with a diameter of 1-1.3 μm within podocytes (**Figure 5a-c**). There was a significant increase in the number of podocyte vesicles per glomerulus in *LacZ*/ADR (7.17 ± 0.32) compared with *LacZ*/saline treated mice (5.33 ± 0.26 ; $P < 0.001$). TB4 treatment prevented this increase (5.58 ± 0.26

vesicles per glomerulus; $P < 0.01$) (**Figure 5d**). To account for changes in glomerular area, vesicle number was normalised to glomerular area (vesicle density). *LacZ*/saline glomeruli had a vesicle density of $2.29 \times 10^{-3} \pm 0.11 \times 10^{-3}$ vesicles per μm^2 which increased to $2.71 \times 10^{-3} \pm 0.11$ vesicles per μm^2 after ADR treatment ($P < 0.001$). *Tmsb4x*/ADR treated glomeruli had a significantly lower vesicle density than *LacZ*/ADR glomeruli ($2.24 \times 10^{-3} \pm 0.09 \times 10^{-3}$ vesicles per μm^2 ; $P < 0.05$) (**Figure 5e**).

TB4 prevents ADR-induced podocyte F-actin reorganisation *in vitro*

As we have previously shown that lack of endogenous TB4 affects the organisation of F-actin fibres in cultured podocytes¹⁶, we next sought to perform a detailed assessment of podocyte F-actin architecture *in vitro*. Cultured differentiated mouse podocytes, an established model to study the regulation of the podocyte cytoskeleton^{52,53} were treated with a low (0.0125 $\mu\text{g/ml}$) or high (0.125 $\mu\text{g/ml}$) dose of ADR and the potential of synthetic TB4 (100 ng/ml) to abrogate the effects of ADR was assessed (**Figure 6a**). ADR treatment reduced *Tmsb4x* expression in cultured podocytes which was significant at the high dose (60% decrease; $P < 0.01$; **Figure 6b**) mirroring our findings *in vivo*. Treatment with 0.125 $\mu\text{g/ml}$ of ADR led to a significant decrease in podocyte viability ($P < 0.05$) and podocyte cell area ($P < 0.01$) compared with podocytes treated with media alone, which was not prevented by co-administration of exogenous TB4 (**Figure 6c, d**). Next, we quantified podocyte F-actin. Treatment with ADR did not alter podocyte mean F-actin fluorescence, which remained unaffected by TB4 (**Figure 6e**), in agreement with our *in vivo* finding that neither ADR nor TB4 alter the amount of F-actin within podocytes.

Finally, to study podocyte F-actin organisation in more detail, we classified F-actin arrangements into cortical actin stress fibres, cytoplasmic stress fibres or unorganised fibres

(see examples in **Figure 6f**). Prior to ADR administration, the majority of podocytes ($61.2 \pm 2.2\%$) had a prevalence of cortical actin stress fibres, compared with $26.4 \pm 2.5\%$ podocytes with cytoplasmic stress fibres and $12.4 \pm 2.0\%$ podocytes with unorganised actin fibres. Treatment with $0.0125 \mu\text{g/ml}$ of ADR changed this distribution with significantly reduced cortical stress fibre prevalence to $33.2 \pm 2.3\%$ of podocytes ($P < 0.001$) and increased prevalence of unorganised actin fibres ($31.2 \pm 3.2\%$ of podocytes, $P < 0.01$) compared with untreated podocytes. Treatment with exogenous TB4 prevented the ADR-induced F-actin reorganisation and significantly increased the proportion of podocytes with cortical actin stress fibres ($62.8 \pm 1.4\%$, $P < 0.001$) and decreased the proportion of podocytes with unorganised actin fibres ($20 \pm 2.6\%$, $P < 0.05$) compared with the group treated with low dose ADR. Treatment with $0.125 \mu\text{g/ml}$ of ADR led to exacerbated cytoskeletal disorganisation, reducing cortical stress fibre prevalence to $25.2 \pm 2.1\%$ of podocytes ($P < 0.001$) and increasing unorganised actin fibre prevalence to $50.4 \pm 4.2\%$ of podocytes ($P < 0.001$) compared with the untreated group. Co-treatment with exogenous TB4 ameliorated the effects of ADR with cortical stress fibre frequency at $57.2 \pm 2.6\%$ ($P < 0.001$) and unorganised actin fibre frequency at $24 \pm 4.2\%$ of podocytes ($P < 0.001$) (**Figure 6g-i**).

Discussion

In this study we have shown that ADR injury results in reduced *Tmsb4x* mRNA levels in glomeruli and particularly in podocytes. Systemic upregulation of TB4 using AAV-mediated gene therapy prevents ADR-induced albuminuria and podocyte loss *in vivo* and treatment with synthetic TB4 prevents ADR-induced cytoskeletal disorganisation *in vitro*. Thus, we have provided the first evidence that exogenous TB4 can protect podocytes from injury and improve glomerular disease.

Previous studies have demonstrated the expression of endogenous *Tmsb4x* in mouse glomeruli predominately in podocytes.^{16,54,55} The effect of glomerular disease on TB4 levels, however, is less clear. A proteomic study using the rat kidney remnant model of renal fibrosis found that TB4 levels increased threefold in sclerotic *versus* normal glomeruli.⁵⁶ Our group previously demonstrated that *Tmsb4x* levels were not altered in whole kidneys obtained from mice with glomerulonephritis or in glomerular extracts obtained from human biopsy specimens from patients with rapidly progressive glomerulonephritis or lupus nephritis.¹⁶ These studies, however, did not assess *Tmsb4x* levels in a cell type-specific manner. Here, we have shown that ADR injury results in a transient reduction of *Tmsb4x* levels in glomeruli and we have performed analysis of a scRNAseq dataset³⁷ to demonstrate reduced *Tmsb4x* levels specifically in podocytes.

Since endogenous TB4 has a protective role in glomerular disease,^{16,42} we hypothesised that treatment with exogenous TB4 would be beneficial in ADR injury. Indeed, we found that TB4 administration prevented the onset of albuminuria in mice injured with ADR. Podocyte cells are a crucial component of the glomerular filtration barrier and they are the primary target of ADR injury in the kidney.³³ We demonstrated that TB4 prevented podocyte loss in ADR-injured mice. Loss of podocytes from the glomerular tuft may result from cell death or from injury that causes podocyte detachment.² The actin cytoskeleton is critical to maintain podocyte shape⁵⁷ and attachment to the GBM.⁸ We developed a novel method to quantify podocyte F-actin *in vivo* and found that neither ADR nor TB4 affected the amount of F-actin in the podocytes. However, detailed analysis of the F-actin cytoskeleton in cultured podocytes revealed that even though there were no changes in the amount of F-actin, ADR injury increased the proportion of podocytes with unorganised actin and this was prevented

by treatment with TB4. These findings demonstrate that TB4 protects the podocyte cytoskeleton, prevents podocyte injury and loss which is associated with an improvement in albuminuria following ADR injury. In the epidermis, lack of endogenous TB4 results in hindered eyelid closure and hair follicle angling and defects in planar cell polarity (PCP) with impaired stability of adherens junctions, aberrant F-actin distribution and changes in cell shape.⁵⁸ PCP is also implicated in podocyte health in development and disease.⁵⁹ Vangl-like 2 (Vangl2), a core PCP protein, is required for the normal differentiation of glomeruli^{60,61} and podocyte-specific deletion of *Vangl2* exacerbates experimental glomerulonephritis in mice.^{62,63} It is therefore possible that some of the effects of TB4 on podocyte cells might be mediated via PCP pathways.

Another mechanism that contributes to albuminuria is the endocytosis of albumin in podocyte vesicles and subsequent excretion to the urinary space. This mechanism has been demonstrated by confocal, electron and intravital multiphoton microscopy in rat models of glomerular injury⁴⁸⁻⁵¹ and has been estimated to contribute 10% of total albumin filtration.⁴⁹ We observed podocyte vesicle-like structures with a diameter of 1-1.3µm, consistent with the size of vesicles reported in the aforementioned studies. These vesicles were more prevalent in ADR-injured glomeruli and their occurrence was reduced by TB4 treatment. Local actin polymerisation is critical for the formation of endocytic vesicles.⁶⁴ TB4 gene therapy may reduce the formation of endocytic vesicles by preventing local actin polymerisation. Indeed, it has been demonstrated that excess concentration of TB4 inhibits endocytosis *in vitro*.⁶⁵ A recent study has demonstrated that TB4 binds to the endocytic receptor, low density lipoprotein receptor related protein 1 (LRP1), and regulates whether endocytosed protein complexes are recycled to the cell surface or targeted for lysosomal

degradation.⁶⁶ It remains to be investigated whether TB4 may interact with receptors that facilitate albumin endocytosis and downstream processing in the kidney. The regulation of vesicle formation and transcytosis of albumin by exogenous TB4 may thus represent a novel strategy to limit albuminuria in glomerular disease.

Previous studies have shown that endogenous and exogenous TB4 can improve inflammation in animal models of kidney injury including nephrotoxic nephritis,¹⁶ angiotensin-II induced hypertensive nephropathy⁴² and acute ischaemia reperfusion injury.²⁵ In our study, we assessed the effects of TB4 in the early stages of ADR injury when macrophage infiltration is not present, demonstrating that the protective effect of TB4 is likely independent of its anti-inflammatory properties in this case.

In this study, we used AAV-mediated systemic gene therapy to achieve long term transgene upregulation.³⁸ Previous studies have used administration of TB4 protein, which maintains enhanced circulating TB4 levels for only 6 hours.²⁶ Our strategy circumvents the quick turnover of TB4 thus achieving sustained levels of upregulation five weeks after AAV administration. Future studies could target TB4-encoding AAVs to the kidney, however, systemic administration of AAV serotypes 1-9 has shown no efficient transduction in the kidney.³⁸ Transcriptional targeting,⁶⁷ synthetic AAVs⁶⁸ and novel administration routes, such as administration by retrograde ureteral and subcapsular injections⁶⁹ or by injection into the renal vein,⁷⁰ have shown promise and they could be utilised to achieve kidney-specific overexpression of TB4. Inducible AAVs^{71,72} would enable regulation of the timing of TB4 upregulation to assess its ability to improve the progression of established glomerular disease.

In summary, we have shown that ADR injury results in reduced levels of endogenous TB4, podocyte loss and proteinuria. Systemic gene therapy with TB4 prevents proteinuria and podocyte loss, likely by protecting the actin cytoskeleton and limiting albumin transcytosis. These findings suggest that treatment with TB4 could be a potential novel therapeutic strategy to prevent podocyte injury and maintain filtration in glomerular disease.

Materials and methods

scRNAseq analysis

scRNAseq analysis was performed using RStudio for Macintosh (RStudio Inc., v1.2.5042) using R (v4.0.2). The complete annotated R code used to perform the analyses and generate plots used in the manuscript has been deposited in GitHub and can be accessed at https://github.com/davidlonglab/Mason_TB4_2021.

Data acquisition

The raw scRNAseq dataset used in this analysis was acquired from a study characterising the single-cell transcriptome of murine ADR nephropathy using the 10X Genomics platform³⁷. Matrices of gene counts per droplet, generated after alignment of reads to genes, were acquired from the National Center for Biotechnology Information Gene Expression Omnibus (GSE146912) and are available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146912>.

Quality control, data processing and integration

All the following analysis was performed using the Seurat toolkit⁷³. The count matrices from $n = 2$ control samples (8,412 cells) and $n = 2$ samples with Adriamycin nephropathy (8,296 cells) were merged into a single object. Genes expressed in two or fewer droplets were excluded and droplets with < 200 and > 4000 detected genes and $> 10\%$ of features

mapping to the mitochondrial genome were excluded. The counts were then normalized using the NormalizeData function and scaled by all detected genes using the ScaleData function before principal component analysis (PCA), using the top nine components for downstream analyses. Integration and matching of cell types between experimental conditions was performed using the Harmony package for R⁷⁴.

Clustering, cell type identification and counting

Shared nearest neighbor graphing was performed using the FindNeighbors function. Unsupervised clustering was performed with the FindClusters function using the Louvain algorithm and a resolution of 0.4, generating 14 transcriptionally distinct clusters, before dimension reduction using Uniform Manifold Approximation and Projection (UMAP). Cell type identification was performed by assessing the top ten differentially expressed genes per cluster calculated using the FindAllMarkers function and canonical markers for glomerular cell types were compared from previous scRNAseq studies.^{37,75,76} By grouping clusters with a common cell identity together, ten glomerular cell types were subsequently identified and assigned. The number of cell types by experimental condition was exported and graphed in Prism (GraphPad, v9.0.0)

Comparison of Tmsb4x expression

The FindAllMarkers function was used to compare the scaled expression of *Tmsb4x* between ADR injury and control datasets. The average log fold change was calculated for podocytes or all glomerular cell types (glomerular endothelial cells, mesangial cells and podocytes) between experimental conditions. Wilcoxon Rank Sum tests was used to assess statistical significance, with an adjusted P value of ≤ 0.05 .

Adeno-associated viral generation

The recombinant AAV-*Tmsb4x* and AAV-*LacZ* vectors were produced using triple transfection in HEK293 cells. Cells were harvested and virus was purified by iodixanol-gradient centrifugation. The virus was further purified using Sepharose G100 SF resin (Merck, Darmstadt, Germany) in Econopac columns (Bio-Rad, Watford, UK). Virus was concentrated in PBS using Amicon Ultra-15 Centrifugal Filter Units (Merck) and stored at 4°C.^{31,77,78} Viral titre was quantified by inverted terminal repeat probe quantitative polymerase chain reaction (PCR). Helper plasmid delta F6 was purchased from Puresyn (Malvern, PA).

Experimental animals and procedures

All experiments were carried out according to a UK Home Office project and were compliant with the UK Animals (Scientific Procedures) Act 1986. Male BALB/c mice aged between 7-10 weeks were administered with AAV (sub-serotype 2/7; 5×10^{12} viral particles per mouse) expressing *LacZ* (AAV-*LacZ*) or *Tmsb4x* (AAV-*Tmsb4x*) via the tail vein. To induce glomerular injury, mice were intravenously injected with 10 mg/kg of ADR (Merck) or vehicle (0.9 % saline) 21 days after AAV administration.

Renal function

Urine was collected from mice by housing them individually in metabolic cages overnight. Blood samples were collected from the lateral saphenous vein. Albumin concentrations were measured by enzyme-linked immunosorbent assay^{36,79} (Bethyl Laboratories, Montgomery, TX). Urinary creatinine concentrations were quantified using a commercially available kit (Cayman Chemicals, Ann Arbor, MI). Blood urea nitrogen was assessed using a commercially available assay kit validated in mice (BioAssay Systems, Hayward, CA).⁸⁰

381 **TB4 enzyme linked immunosorbent assay (ELISA)**

382 The plasma concentration of TB4 was determined by ELISA based on the protocol previously
383 described by Mora *et. al.*²⁶ Standards of 10,000 ng/ml, 5,000 ng/ml, 2,500 ng/ml, 1,250
384 ng/ml, 625 ng/ml, 312.5 ng/ml, 156 ng/ml, 78 ng/ml and 39 ng/ml TB4 were prepared using
385 synthetic TB4 (ReGeneRx Biopharmaceuticals Inc, Rockville, MD) diluted in incubation buffer
386 ((pH 7.4, Na₂HPO₄ (0.01M), NaCl (0.15M), Tween-20 (0.055 % v/v), BSA (1 % v/v)). Equal
387 volumes of standards or samples, incubation buffer and TB4 antibody prediluted at 1:4000
388 (AF6796; R&D Systems, Minneapolis, MN,) were added to sterile borosilicate tubes and
389 incubated overnight at 4 °C. A flat bottom 96 well plate was coated with 100 µl of 50 ng/ml
390 recombinant TB4 in carbonate-bicarbonate buffer and incubated overnight at 4 °C. Negative
391 control wells were coated with buffer only. The plate was washed with washing buffer ((pH
392 7.4, Na₂HPO₄ (0.01M), NaCl (0.15M), CaCl₂ (1 mM), MgCl₂ (0.5 mM), Tween-20 (0.55% v/v)),
393 blocked with 200 µl of blocking buffer (5 % dry fat milk in incubation buffer) and 100 µl of
394 each standard and sample were added to the appropriate wells and incubated for 2 hours.
395 Following washing, 100 µl of goat anti-sheep HRP-conjugated secondary antibody (61-8620,
396 Thermo Fisher Scientific) diluted 1:2000 in incubation buffer was added to each well and
397 incubated for 1 hour before washing. Substrate solution (100 µl per well) containing equal
398 parts stabilised H₂O₂ and stabilised tetramethylbenzidine was added and 15 minutes later
399 the reaction was stopped with the addition of 50 µl of 2M sulphuric acid per well and
400 absorbance was read at 450 nm using a plate reader (M200 Pro, Tecan, Männedorf
401 Switzerland).

Tissue processing and immunostaining

Tissues were fixed in 4% paraformaldehyde in PBS. To prepare wax sections, tissues were dehydrated, embedded in paraffin and 5 µm thick sections were prepared. To prepare cryosections, tissues were placed overnight in 30% sucrose in PBS, embedded in Tissue-Tek optimal cutting temperature compound (Agar Scientific, Stansted, UK) and 8 µm sections were prepared.

Immunohistochemistry was performed for TB4 (AF6796, R&D Systems) followed by secondary rabbit anti-sheep antibodies (Thermo Fisher Scientific, Waltham, MA) and ImmPRESS polymer anti-rabbit IgG reagent (Vector Laboratories, Burlingame, CA) conjugated to horseradish peroxidase and detected by 3,3'-diaminobenzidine. Images were obtained on a Leica DM5500 B brightfield microscope (Leica Biosystems, Milton Keynes, UK).

Immunofluorescence was performed⁸¹ using primary antibodies against WT1 (AB89901, Abcam, Cambridge, UK), synaptopodin (163-004-SY, Synaptic Systems, Goettingen, Germany) and F4/80 (MCA497R, Bio-Rad), followed by appropriate AlexaFluor594 and AlexaFluor488 (Thermo Fisher Scientific, Waltham, MA) secondary antibodies. Negative controls consisted of omission of primary antibodies. Acti-Stain 488TM Phalloidin (Cytoskeleton, Denver, CO) was used to visualise actin filaments. Images were acquired using a Zeiss Laser Scanning 880 confocal microscope with a 63x NA1.4 Oil Plan Apochromat objective (Carl Zeiss, Oberkochen, Germany).

The number of WT1⁺ cells found within the glomerular tuft was counted in 50 glomeruli per mouse. Podocyte density was calculated as the number of WT1⁺ cells in the glomerular tuft, normalized to the glomerular area (measured using ImageJ⁸²). F4/80⁺ cells within the

glomerular tuft and in the peri-glomerular area were counted in 50 glomeruli per mouse. Podocyte vesicles were identified as circular structures that were surrounded by F-actin and were between 1 and 1.3 μm in diameter. The number of vesicles and vesicle density (number of vesicles/glomerular area) was counted in 50 glomeruli per mouse. For automated quantification of podocyte F-actin, a macro was generated, which has been deposited in GitHub (<https://github.com/DaleMoulding/Fiji-Macros/blob/master/README.md#podocyte-f-actin--synaptopodin>). A Gaussian blur with a Sigma (radius) value of 2.0 was applied to each channel to create a solid mask of synaptopodin to outline the podocyte area. The mean fluorescence of F-actin within the synaptopodin positive area was measured along with the total area (μm^2) and percentage area of F-actin in the synaptopodin positive regions.

Cell culture

Mouse podocytes⁵² were cultured as described⁶⁰ and allowed to differentiate for 14 days. Cells were treated with 100 ng/ml synthetic TB4 (ReGeneRx Biopharmaceuticals Inc) and either a low (0.0125 $\mu\text{g}/\text{ml}$) or a high (0.125 $\mu\text{g}/\text{ml}$) dose of ADR for 24 hours. Cell viability was determined by the methyltetrazolium assay. To visualize F-actin filaments, podocytes were fixed in 4% paraformaldehyde and 4% sucrose and stained with Acti-stainTM 488 Phalloidin (Cytoskeleton) and 50 cells per condition were assessed. The area of each cell and the mean F-actin fluorescence were quantified using ImageJ. Actin filaments were classified as cortical stress fibers, cytoplasmic stress fibers or unorganised actin.

Quantitative real-time PCR

RNA extracted from mouse whole-kidney (500 ng), glomerular extracts (100 ng; isolated by Dynabeads³⁶ (Thermo Fisher Scientific) or cultured podocytes (100 ng) was used to prepare

cDNA (iScript kit, Bio-Rad), and quantitative real-time PCR was performed as described previously³⁶ with *GapDH* as a housekeeping gene. All measurements were performed in duplicate.

Statistical analysis

All samples were assessed by independent observers blinded to treatment group. Data are presented as mean $\pm$ SEM and were analysed using GraphPad Prism v9 (GraphPad Software, La Jolla, CA). Normal distribution was assessed by Shapiro-Wilk test. When differences between 2 groups were evaluated, data were analysed using a t test. When 3 or more groups were assessed, 1-way ANOVA with Tukey's multiple comparison post hoc tests were used. Data affected by 2 variables were analysed using 2-way ANOVA with Tukey's multiple comparison post hoc tests. For analysis of individual glomeruli, data were analysed by Kruskal-Wallis non-parametric test followed by Dunn's multiple comparisons. Statistical significance was accepted at $P \leq 0.05$.

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475 WJM, DJJ, CK, CP-W, EP, PRR, DAL and EV; Resources, CK; Supervision, EV, DAL and CP-W;
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Figure Legends

Figure 1. Podocyte-specific *Tmsb4x* is downregulated in murine Adriamycin nephropathy.

(a) Expression of *Tmsb4x* as assessed by quantitative reverse transcriptase polymerase chain reaction in glomeruli of mice administered with 10 mg/kg of Adriamycin (ADR). **(b)** Uniform manifold approximation and projection (UMAP) from single-cell RNA sequencing data of 8,412 glomerular cells from two wildtype (control) mice and 8,296 glomerular cells from mice with Adriamycin nephropathy (ADR). After analysis and cell type assignment, ten transcriptionally distinct cell populations were discriminated including arterial endothelial cells (AEC), glomerular endothelial cells (GEC), macrophages (MΦ), mesangial cells, monocytes (Mono), parietal epithelial cells (PEC), podocytes, smooth muscle cells (SMC), T cells, tubular epithelial cells (TEC). The markers used for cell type identification and assignment, and the numbers of cell types per condition, are shown in **Figure S1**. **(c)** Feature plots showing expression of nephrin (*Nphs1*) and podocin (*Nphs2*), canonical markers of podocytes, across the dataset. **(d)** Violin plot comparing the scaled expression of *Tmsb4x* of all glomerular cells (podocytes, GECs, mesangium) between ADR ($n = 5,602$ cells) and control ($n = 7,190$ cells). An average log fold decrease of 0.13 was detected in ADR compared to control (*: adjusted p value < 0.0001). **(e)** Violin plot comparing the scaled expression of *Tmsb4x* of podocytes between ADR ($n = 378$ cells) and control ($n = 1,486$ cells). An average log fold decrease of 0.36 was detected in ADR compared to control (*: adjusted p value < 0.0001).

Figure 2. AAV2/7 causes systemic upregulation of thymosin $\beta 4$ *in vivo*. **(a)** BALB/c mice

were injected with AAV-*LacZ* or AAV-*Tmsb4x* three weeks prior to 10 mg/kg ADR or vehicle (saline) administration and culled 14 days after ADR/saline injection. **(b)** Liver expression of

Tmsb4x mRNA. **(c)** Expression of TB4 peptide in the liver. Arrowheads refer to cells with particularly high expression of TB4. Scale bars = 50 μ m. **(d)** Plasma concentration of TB4. **(e)** Whole kidney expression of *Tmsb4x* gene. All experiments were repeated a minimum of 5 times. For gene analysis and plasma concentration, assays were performed in duplicate and the data are presented as the mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. TB4, *Tmsb4x*, thymosin β 4; ADR, Adriamycin; *LacZ*, β -Galactosidase; AAV, adeno-associated virus.

Figure 3. Renal function following ADR/TB4 treatment. (a) Mouse weight change 2, 7 and 14 days after ADR/vehicle injection. Statistical annotations using * refer to comparison between *LacZ*/saline & *LacZ*/ADR. Annotations using # refer to comparison between *LacZ*/saline & *Tmsb4x*/ADR. **(b)** Twenty-four hour urinary albumin excretion 0, 2 and 14 days after ADR/vehicle injection. **(c)** Urinary albumin to creatinine ratio 14 days after ADR/vehicle injection. **(d)** Blood urea nitrogen concentration 14 days after ADR/vehicle injection. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. TB4, *Tmsb4x*, thymosin β 4; ADR, Adriamycin; *LacZ*, β -Galactosidase.

Figure 4. Analysis of podocytes *in vivo* after ADR/TB4 treatment. (a) Representative images of WT1⁺ cells in glomeruli from *LacZ*/saline, *LacZ*/ADR and *Tmsb4x* treated mice. White arrowheads indicate podocyte nuclei. White dashed line indicates glomerular tuft boundary. Quantification of **(b)** number of WT1⁺ cells in glomeruli and **(c)** glomerular WT1⁺ density. 50 glomeruli were analysed per sample. Quantification of **(d)** WT1⁺ cell count and **(e)** glomerular WT1⁺ density when analysing individual glomeruli. At least 250 glomeruli were analysed per condition. **(f)** Representative images of glomeruli from *LacZ*/saline, *LacZ*/ADR and *Tmsb4x* treated mice immunostained to visualise synaptopodin and F-actin. White arrows indicate F-actin in synaptopodin⁺ areas. Images have been edited to crop out positive

staining outside of the glomerular tuft in aid of the macro used for analysis. Quantification of **(g)** mean synaptopodin⁺ F-actin fluorescence, **(h)** area of synaptopodin⁺ covered by F-actin (μm^2) and **(i)** percentage of synaptopodin⁺ area that was F-actin⁺. Scale bars = 20 μm and the white dashed line indicates glomerular tuft boundaries. Each group contained at least 5 mice and 50 glomeruli were analysed per mouse. Data are presented as mean $\pm$ SEM. *** $P \leq 0.001$. TB4, *Tmsb4x*, thymosin $\beta 4$; ADR, Adriamycin; WT1, Wilms tumour 1; *LacZ*, β -Galactosidase.

Figure 5. TB4 prevents ADR-induced podocyte vesicle formation. Representative images of **(a)** synaptopodin, **(b)** F-actin and **(c)** the composite including nuclei stained with Hoechst 33342 from a *LacZ*/ADR treated glomerulus. White arrows indicate vesicles. Scale bar = 5 μm . Quantification of **(d)** the number of vesicles per glomerulus and **(e)** vesicle density. Each group contained at least 5 mice and 50 glomeruli were analysed per mouse. Data are presented as mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. TB4, *Tmsb4x*, thymosin $\beta 4$; ADR, Adriamycin; *LacZ*, β -Galactosidase.

Figure 6. Effect of exogenous TB4 on ADR-injured podocytes *in vitro*. **(a)** Conditionally immortalised mouse podocytes were treated with RPMI-1640/ADR/TB4 and analysed after 24 hours. **(b)** Expression of podocyte *Tmsb4x* mRNA after ADR/TB4 treatment. Effect of ADR/TB4 on **(c)** cell viability, **(d)** podocyte cell area and **(e)** podocyte mean F-actin fluorescence. **(f)** Representative images of podocytes stained with Acti-StainTM 488 Phalloidin displaying cortical actin stress fibres, cytoplasmic stress fibres and unorganised actin fibres. Scale bar = 10 μm . Percentage of podocytes displaying **(h)** cortical actin stress fibres, **(i)** cytoplasmic stress fibres and **(j)** unorganised stress fibres. All experiments were repeated a minimum of 4 times. For F-actin analysis 50 cells were analysed per experiment

740 * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. TB4, *Tmsb4x*, thymosin $\beta 4$; ADR, Adriamycin; IFN- γ ,
 741 interferon-gamma; OD, optical density; *GapDH*, Glyceraldehyde 3-phosphate
 742 dehydrogenase; MTT, methyltetrazolium.

Figure 1

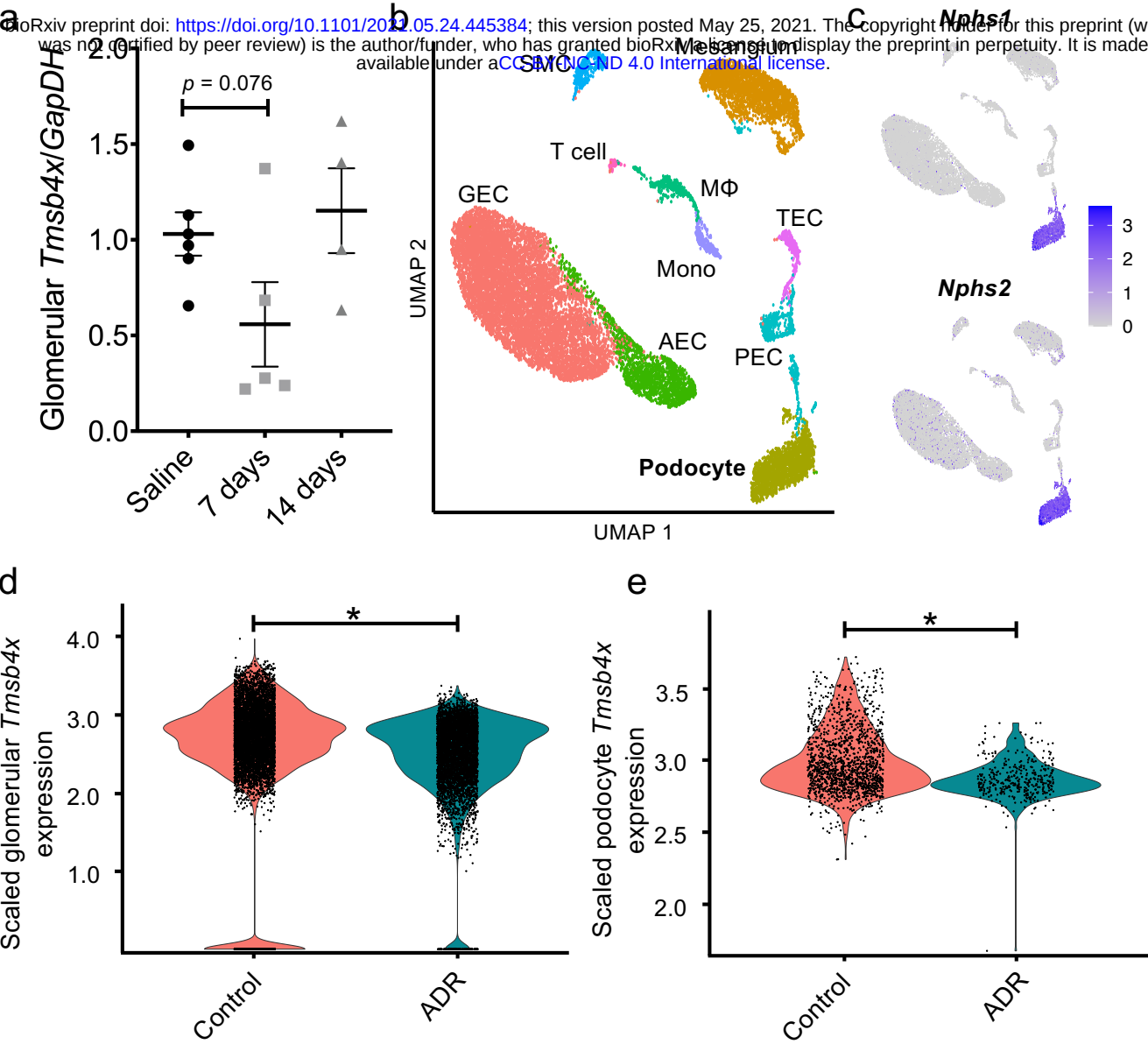


Figure 2

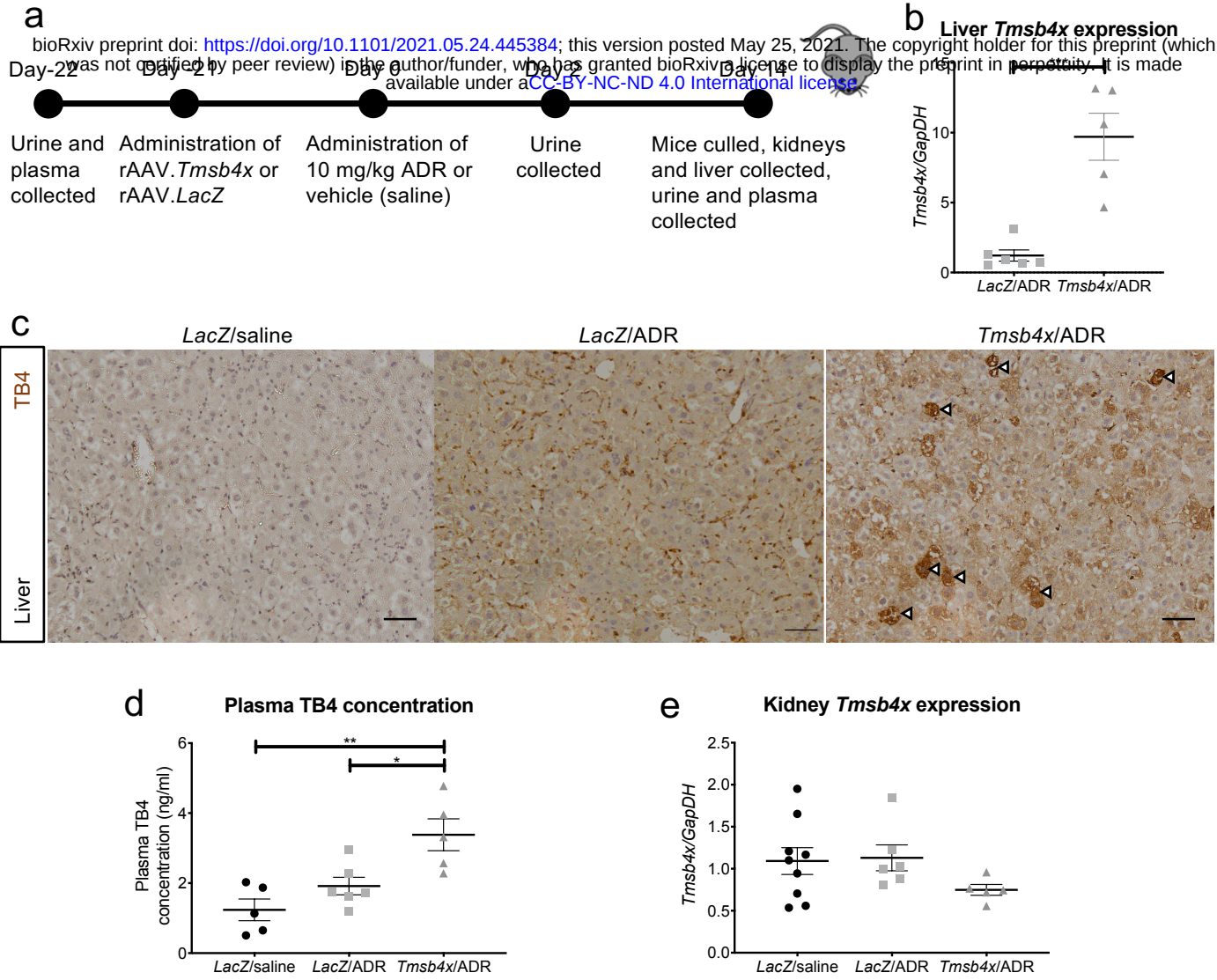
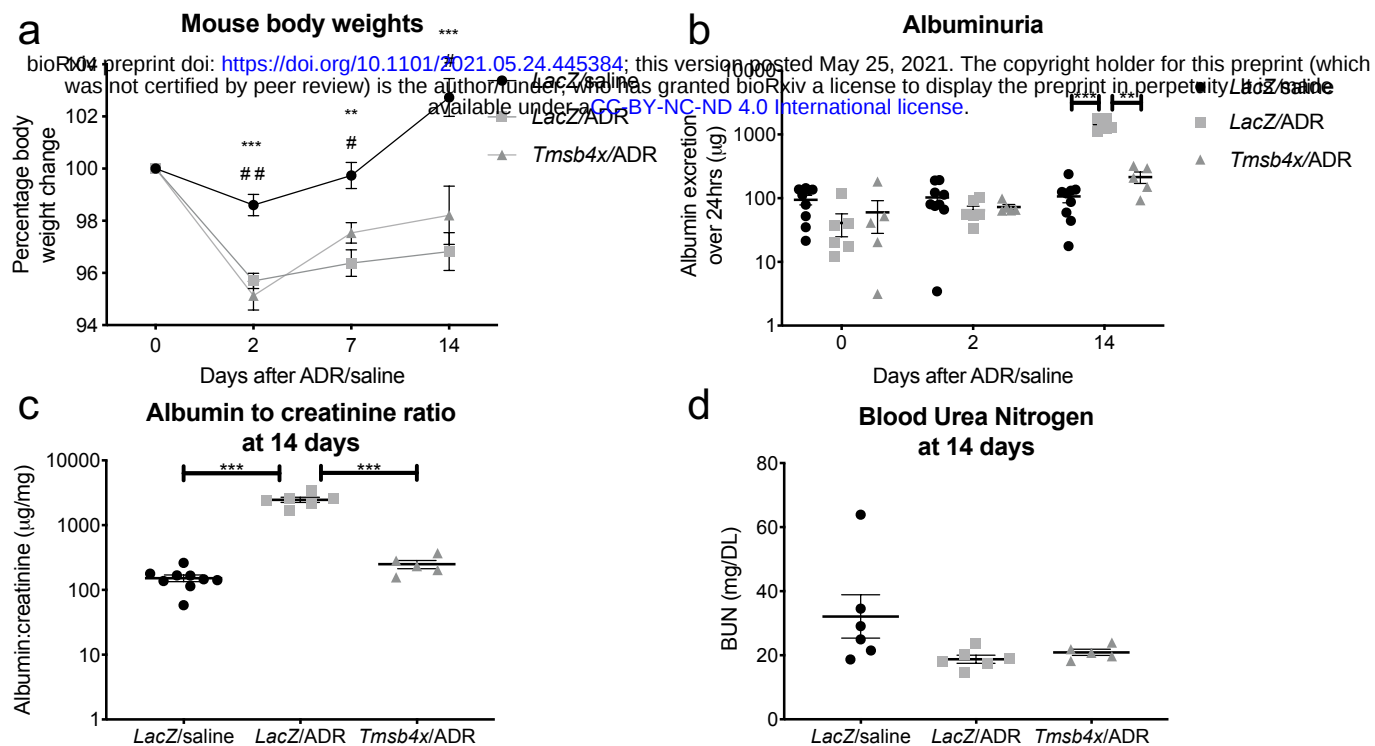


Figure 3



a

LacZ/saline *LacZ/ADR* *Tmsb4x/ADR*

WT1
Podocytes

b Podocyte number

WT1⁺ cell number

LacZ/saline *LacZ/ADR* *Tmsb4x/ADR*

c Podocyte density

WT1⁺ cells/ glomerular area (μm^2)

LacZ/saline *LacZ/ADR* *Tmsb4x/ADR*

d Podocyte count - individual glomeruli

WT1⁺ cell number

LacZ/saline *LacZ/ADR* *Tmsb4x/ADR*

e Podocyte density - individual glomeruli

WT1⁺ cells/area (μm^2)

LacZ/saline *LacZ/ADR* *Tmsb4x/ADR*

f

Synaptopodin F-actin Composite

LacZ/saline

LacZ/ADR

Tmsb4x/ADR

g Mean podocyte F-actin fluorescence

LacZ/saline *LacZ/ADR* *Tmsb4x/ADR*

h Area of podocytes covered by F-actin (μm^2)

LacZ/saline *LacZ/ADR* *Tmsb4x/ADR*

i Percentage of synaptopodin covered by F-actin

LacZ/saline *LacZ/ADR* *Tmsb4x/ADR*

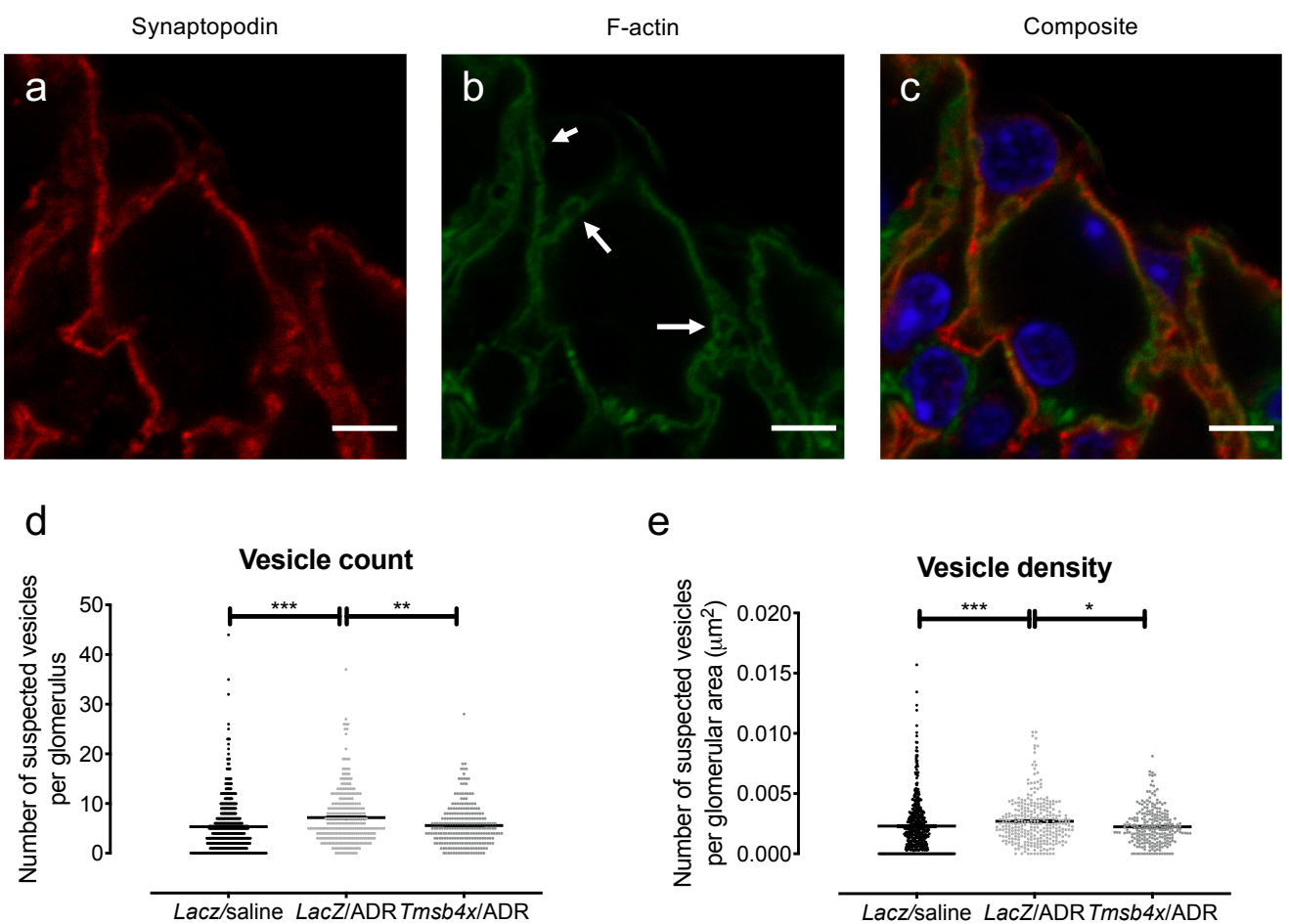


Figure 6

