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# Sex-specific molecular signature of mouse podocytes in homeostasis and in response to pharmacological challenge with rapamycin

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## Abstract

**Background** Sex differences exist in the prevalence and progression of major glomerular diseases. Podocytes are the essential cell-type in the kidney which maintain the physiological blood-urine barrier, and pathological changes in podocyte homeostasis are critical accelerators of impairment of kidney function. However, sex-specific molecular signatures of podocytes under physiological and stress conditions remain unknown. This work aimed at identifying sexual dimorphic molecular signatures of podocytes under physiological condition and pharmacologically challenged homeostasis with mechanistic target of rapamycin (mTOR) inhibition. mTOR is a crucial regulator involved in a variety of physiological and pathological stress responses in the kidney and inhibition of this pathway may therefore serve as a general stress challenger to get fundamental insights into sex differences in podocytes.

**Methods** The genomic ROSAmT/mG-NPHS2 Cre mouse model was used which allows obtaining highly pure podocyte fractions for cell-specific molecular analyses, and vehicle or pharmacologic treatment with the mTOR inhibitor rapamycin was performed for 3 weeks. Subsequently, deep RNA sequencing and proteomics were performed of the isolated podocytes to identify intrinsic sex differences. Studies were supplemented with metabolomics from kidney cortex tissues.

**Results** Although kidney function and morphology remained normal in all experimental groups, RNA sequencing, proteomics and metabolomics revealed strong intrinsic sex differences in the expression levels of mitochondrial, translation and structural transcripts, protein abundances and regulation of metabolic pathways. Interestingly, rapamycin abolished prominent sex-specific clustering of podocyte gene expression and induced major changes only in male transcriptome. Several sex-biased transcription factors could be identified as possible upstream regulators of these sexually dimorphic responses. Concordant to transcriptomics, metabolomic changes were more prominent in males. Remarkably, high number of previously reported kidney disease genes showed intrinsic sexual dimorphism and/or different response patterns towards mTOR inhibition.

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**Conclusions** Our results highlight remarkable intrinsic sex-differences and sex-specific response patterns towards pharmacological challenged podocyte homeostasis which might fundamentally contribute to sex differences in kidney disease susceptibilities and progression. This work provides rationale and an in-depth database for novel targets to be tested in specific kidney disease models to advance with sex-specific treatment strategies.

## Highlights

- First study to describe sexually dimorphic molecular signature of podocytes, "gate-keepers" of the kidney glomerular filtration barrier.
- Large number of known kidney-disease related genes are newly identified for intrinsic sexual dimorphic expression as well as after pharmacological intervention with rapamycin.
- Male podocytes appear more susceptible to transcriptomic changes.
- Metabolomics supports higher functional consequences of mTOR inhibition on protein synthesis and impaired energy balance in male kidneys.
- Study provides an in-depth database for further research in the field of highly sexually dimorphic kidney diseases.

**Keywords** Podocytes, Sex differences, Transcriptome, Proteome, Metabolome, mTOR

## Plain Language Summary

The global burden of chronic kidney diseases is rapidly increasing and is projected to become the fifth most common cause of years of life lost worldwide by 2040. Sexual dimorphism in kidney diseases and transplantation is well known, yet sex-specific therapeutic strategies are still missing. One reason is the lack of knowledge due to the lack of inclusion of sex as a biological variable in study designs. This work aimed at identification of molecular signatures of male and female podocytes, gate-keepers of the glomerular filtration barrier. Like cardiomyocytes, podocytes are terminally differentiated cells which are highly susceptible towards pathological challenges. Podocytes are the decisive cell-type of the kidney to maintain the physiological blood-urine barrier, and disturbances of their homeostasis critically accelerate kidney function impairment. By help of a genomic mouse model, highly purified podocytes were obtained from male and female mice with and without pharmacological challenge of the mechanistic target of rapamycin (mTOR) signaling pathway which is known to be deregulated in major kidney diseases. Deep RNA sequencing, proteomics and metabolomics revealed strong intrinsic sex differences in the expression levels of mitochondrial, translation and structural transcripts, protein abundances and regulation of metabolic pathways which might fundamentally contribute to sex differences in kidney disease susceptibilities and progression. Remarkably, high number of previously reported kidney disease genes showed so far unknown intrinsic sexual dimorphism and/or different response patterns towards mTOR inhibition. Our work provides an in-depth database for novel targets to be tested in kidney disease models to advance with sex-specific treatment strategies.

## Background

The maintenance of podocyte homeostasis critically determines kidney function during glomerular disease development and progression [1]. Podocytes are terminally differentiated cells of the glomerulus and represent the essential cell-type to maintain the integrity of the glomerular filtration barrier. A large body of evidence demonstrates sex differences in protein filtration and sex-different susceptibilities towards ischemic injury [2–5]. Male gender has been associated with more rapid progression and worse outcome in major chronic kidney diseases [6–9]. Sex differences in podocyte biology might play a decisive role in these processes. The need for addressing sex differences in studies of health-related research has been requested for more than two decades [10, 11]. Now, discussing sexual aspects has become statutory in large grant applications [12–15] and requirement in clinical studies [11]. Despite these regulatory efforts and the well-known sex differences in kidney physiology and pathology, research addressing this issue on the molecular level of podocytes remains sparse [16]. Therefore, this study was designed to achieve deeper insights into sexual dimorphism of the molecular signature of

podocytes under normal homeostatic conditions and in response to inhibition of the mechanistic target of rapamycin (mTOR) signaling pathway with rapamycin.

The mTOR pathway is an important factor in the control of podocyte structure and function [17–19]. A well-balanced activation of this signaling pathway is crucial for compensatory mechanisms in diabetic nephropathy and focal segmental glomerulosclerosis [20]. In addition, mTOR inhibitors are commonly used as immunosuppressants [21]. Yet, it is still unknown why some kidney transplant patients develop de novo proteinuria while the majority of recipients of other solid organs improve their kidney function when on this medication. Furthermore, growing evidence suggests that mTOR inhibition improves physiological parameters associated with aging and aging-related disorders, thereby favoring longevity, interestingly to a higher extent in females compared to males [22–28]. We have recently reported sexual dimorphism in mTOR signaling in cardiomyocytes, with loss of cardioprotective phenotype in response to rapamycin restricted to females [29]. Like cardiomyocytes or neurons, podocytes have limited regenerative capacity. A deeper understanding of cell-type specific mechanisms and sex-specific responses to rapamycin might have important clinical consequences in the era of personalized medicine. Furthermore, disturbances in mTOR signaling occur in many kidney pathologies [17, 20, 30]. By using a pharmacological challenge targeting mTOR pathway with rapamycin we aimed to elucidate both, potential pathologically and physiologically relevant sex-specific responses. Using unbiased omics approaches, we provide detailed genomic data by deep sequencing of male and female podocytes under homeostatic condition and in response to mTOR inhibition. These studies were complemented with podocyte proteomics and metabolomics. The data reveal a so far unknown number of sexually dimorphic podocyte genes reported to be involved in the pathogenesis of kidney diseases and novel aspects of sexually dimorphic genomic and metabolic responses towards mTOR inhibition. These comprehensive data provide novel targets and rationale for future specific disease model studies in the field of sexually dimorphic glomerular disease manifestation and progression.

## Methods

### Mice

Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J mice were purchased from The Jackson Laboratory (Bar Harbour, Massachusetts, USA) and crossed with Tg(NPHS2-cre)295Lbh mice were bred and genotyped as previously reported [31]. Mice were housed in an SPF facility with free access to chow and water and a 12-h day/night cycle. All animal experiments were conducted according to standards and procedures approved by the local Animal Care and Use Committee (LaGeSo Berlin G0241/2015). Furthermore, the ARRIVE reporting guidelines were used [32].

### Experimental model

A total of 76 ROSAmT/mG-NPHS2 Cre mice (38 male and 38 female) aged 12–18 weeks were treated with either rapamycin (LC Laboratories, Woburn, Massachusetts; 1.5 mg rapamycin/kg BW administered intraperitoneally every third day) or vehicle over a period of 3 weeks. Urine, serum and cortex kidney tissue were harvested at day 21 of the treatment period.

Podocytes were isolated from 40 mice (male and female, 9 of each sex treated with rapamycin and 11 of each sex with vehicle) according to Boerries et al., [31] with slight modifications). Kidney cortex was digested and podocytes were isolated using fluorescence-activated cell sorting (FACS). For further details of the podocyte isolation procedure, see the Supplementary Methods.

The isolated podocytes from these 40 mice were allocated to RNA sequencing (podocytes from 22 mice), qPCR for validation of RNA sequencing (podocytes from 12 mice) and proteomics (podocytes from 6 mice). Podocytes from different mice were not pooled for those methods so that each isolated podocyte sample from one individual mouse represented one biological replicate. Due to restricted amount of podocyte material from one mouse each biological replicate could therefore only be used for one of those methods. The other 36 mice (from the 76 ROSAmT/mG-NPHS2 Cre mice) were used as follows: 8 mice were allocated to electron microscopy. From the remaining 28 mice, kidney cortex from one kidney was directly snap frozen in liquid nitrogen and later used for metabolomics and protein extraction, the other kidney was either perfused with paraformaldehyde and later used for immunohistochemistry or directly embedded for cryosections. Further details of mice sex and treatment for the different experiments including numbers and reasons of excluded samples if applicable are specified in the respective methods´ sections of the main manuscript and/or in the Supplementary Methods. Furthermore, numbers of biological replicates per analysis are indicated in the figure legends.

### RNA sequencing and transcriptomics analysis

For details regarding sample preparation of native podocytes for transcriptomics analysis, see the Supplementary Methods. Poly-A selected mRNA (NEB Next Ultra II Directional RNA Library Prep Kit) was used to generate cDNA libraries and deep RNA sequencing (2×75 bp,

paired-end) was carried out on an Illumina HiSeq4000 system. 4–6 biological replicates of each experimental group were analyzed. Sequencing performance provided 36–46 million paired-end reads per sample detecting 84% of all transcripts uniquely mapping sequences aligned to only one single gene within the genome.

For further details on differential expression computations and bioinformatic algorithms and packages, as well as validation experiments using qPCR, see Supplementary Methods and Supplementary Figure S3.

### Proteomics analysis

For details regarding sample preparation of native podocytes for proteomics analysis, see the Supplementary Methods. Proteomics data acquisition was performed on a quadrupole Orbitrap hybrid mass spectrometer (QExactive Plus, ThermoFischer) coupled to an easynLC exactly as previously described [33]. Quantitative analysis was performed using MaxQuant from individual podocyte isolations of 3 male and 3 female vehicle mice. For further details regarding the bioinformatics analysis see the Supplementary Methods.

Validation of proteomics was performed by western blotting of podocyte-specific proteins. See Supplementary Figure S4.

### Western blot and histological analyses, and electron microcopy

Kidney cortex tissue was used for examination of protein expression and phosphorylation using specific antibodies and immunoblotting. In addition, studies on kidney morphology and ultrastructure were performed using standard tissue preparations or as reported previously [34]. For used antibodies and stains and further preparative details please see Supplementary Methods.

### Metabolomics analysis

Metabolomics was performed in collaboration with Kirwan lab, BIH Metabolomics Platform, Berlin Institute of Health at Charité–Universitätsmedizin Berlin, as described previously [35]. For tissue extractions, flash frozen cortex-enriched kidney tissue from vehicle- and rapamycin-treated male and female mice were used (5–7 biological replicates for each experimental group). For details regarding sample preparation, metabolomics and bioinformatic analyses, see the Supplementary Methods.

### Statistics

For quantitative data, statistical tests (Wilcoxon ranked sum test, and univariate linear regression using R packages, R version 4.0.0) and Prism (v9.0, Graphpad) were performed as indicated. In general, $P$-value < 0.05 was considered significant. For large-scale data, correction for multiple testing was performed as described in the respective omics method sections. The number of biological replicates and statistical tests used for analysis are further indicated in the figure legends.

## Results

### Deep transcriptomic data of male and female podocytes

To characterize the sex-specific podocyte transcriptome under homeostatic conditions and in response to mTOR inhibition, male and female ROSAmT/mG-NPHS2 Cre mice were either treated with vehicle or rapamycin for

(See figure on next page.)

**Fig. 1** Experimental design, functional and histological model characterization. **a** Schematic representation of the experimental model. 76 male and female ROSAmT/mG-NPHS2 Cre mice were injected with rapamycin into the peritoneal cavity at a concentration of 1.5 mg/kg/BW or vehicle (DMSO) every third day during the experimental period. After three weeks, mice were sacrificed, and kidneys either flash frozen for histology and metabolomics (n = 28) or were perfused for electron microscopy (n = 8; 2 biological replicates per group) or used for podocyte isolation for qPCR and RNA sequencing (n = 34) and proteomics (n = 6). **b** Albumin/Creatinine ratio showed normal kidney function after three weeks of rapamycin in both, male and female mice (male vehicle n = 6; male rapamycin n = 11; female vehicle n = 6; female rapamycin n = 9). **c** Representative immunofluorescent images of cryo-sections of kidneys from ROSAmT/mG-NPHS2 Cre$^-$ and ROSAmT/mG-NPHS2 Cre$^+$ mice. Membrane-targeted GFP genetically labels NPHS2-expressing cells (podocytes) green, while all the other kidney cells are labelled red with TomatoRed (tdTomato). Scale bars, 150 µm. **d** Representative immunofluorescent images of cryosections of kidneys from ROSAmT/mG-NPHS2 Cre$^-$ and ROSAmT/mG-NPHS2 Cre$^+$ mice with/without indirect immunofluorescent co-staining for Synaptopodin (red). Membrane-targeted GFP genetically labels NPHS2-expressing cells (podocytes) green in Cre$^+$ mice. Nuclei were stained with DAPI. Scale bars, 150 µm.**e** Representative Periodic Acid-Schiff stainings (upper panel) and Sirius red stainings (middle panel) showing normal histomorphology with no increase in fibrosis after three weeks of rapamycin treatment in both, male and female kidney cortex tissues. Electron microscopy graphs (lower panel) displayed normal podocyte structure, foot processes, cell body, glomerular basement membrane and slit diaphragm in both sexes irrespective of treatment. Scale bars, 50 µm. **f** Representative western blots using protein extracts of podocyte-enriched kidney cortex tissues showing efficient mTORC1 inhibition in both, male and female kidneys after three weeks of rapamycin treatment as analyzed by mTORC1 downstream phosphorylation of p70S6K at Thr389 (pp70S6k) at Thr389 (n = 4–5 biological replicates per group). Bar graphs below display results of densitometric analysis with normalization of values to GAPDH and p70S6k. Mann–Whitney test was used to determine significant differences between vehicle and rapamycin treatment groups in each sex, * $P$-value < 0.05, ** $P$-value < 0.01

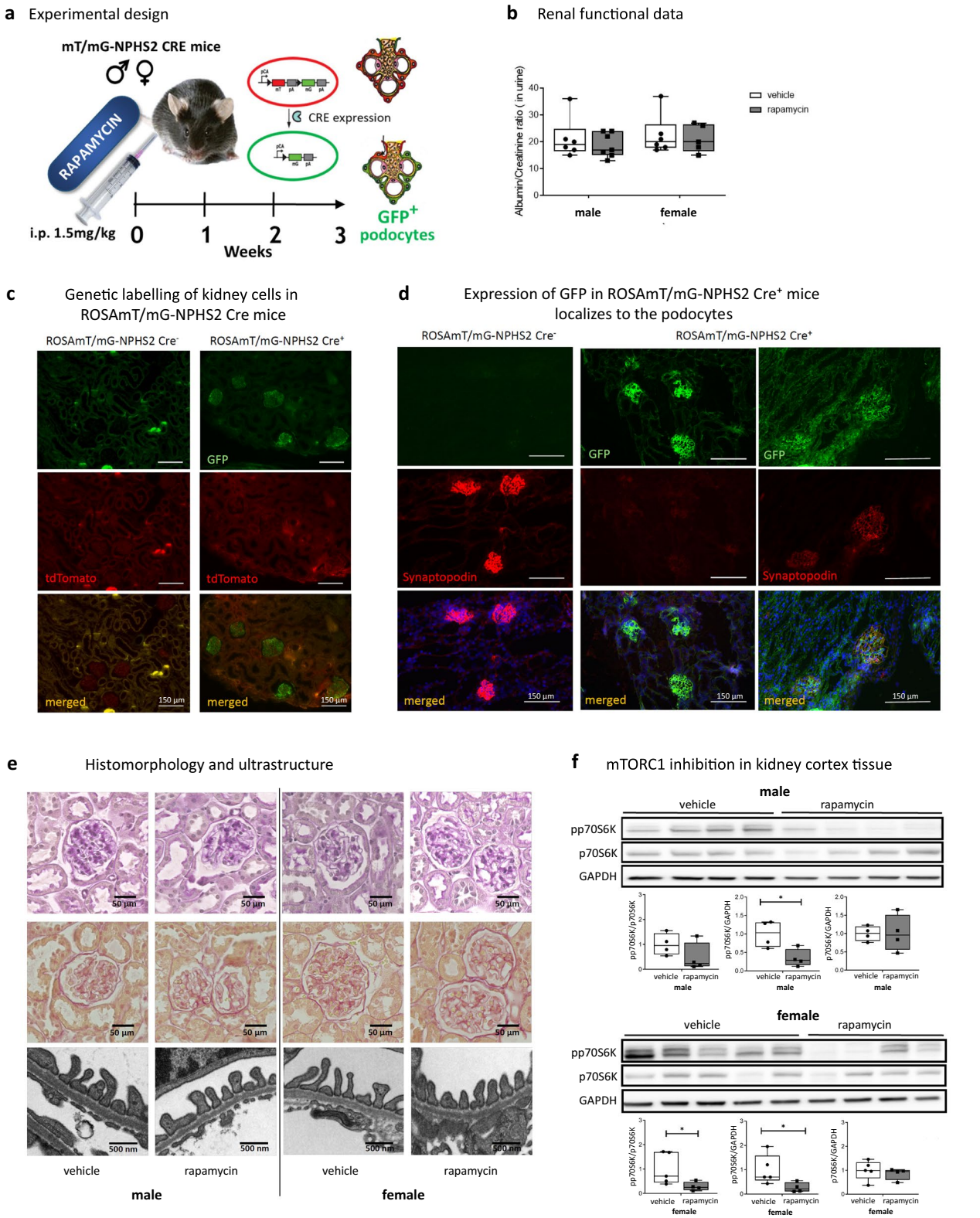



**Fig. 1** (See legend on previous page.)

three weeks and highly purified podocytes were obtained using fluorescence-activated cell sorting [31] (Fig. 1a, c, d). Trough concentrations of rapamycin were monitored and were within the range of clinically approved therapeutic levels [36] in all treated mice; 24.62 ± 3.45 and 21.6 ± 1.9 ng/ml, in male and female, respectively. Structural and functional kidney parameters are reported in Supplementary Table S1. Female mice displayed lower kidney and lower body weight compared to male mice. Rapamycin treatment induced slight non-significant body weight losses in both sexes. However, kidney/body weight ratio only significantly decreased in female rapamycin mice compared to female vehicle mice. Albumin and creatinine clearance were within the normal range in all experimental groups at the end of the experimental period (Supplementary Table S1, Fig. 1b).

Efficient mTORC1 inhibition was confirmed in kidney cortex tissues in both sexes (Fig. 1f). Interestingly, at the end of the treatment period none of the mice showed pathological alterations in kidney function, kidney cortex structure and podocyte morphology or ultrastructure (Supplementary Table S1, Fig. 1e).

In contrast to the unchanged physiological and structural findings, RNA sequencing revealed a significant separation of male and female transcriptomes under vehicle homeostatic conditions and remarkable sex-specific response patterns towards rapamycin treatment. Transcriptional responses to mTOR inhibition surprisingly abolished the clear separation by sex as demonstrated in principal component 1 (PC1) of principal component analysis (PCA) (Fig. 2a).

Overall, 1,569 genes were intrinsically significantly differentially expressed (DE) in male and female podocytes (adjusted *P*-value < 0.05) (Fig. 2b, c Supplementary Table S2), 886 genes were significantly higher expressed in female, 683 in male.

A heatmap of the top 100 of intrinsically sexually dimorphic genes (adjusted *P*-value < 0.01, expressed with Transcript per Million (TPM) > 1 in all biological replicates, therefore without the four only in male expressed Y-chromosomal genes *Kdm5d*, *Eif2s3y*, *Uty* and *Ddx3y*) is shown in Fig. 2d. Genes presented in the heatmap are listed in Supplementary Table S3, sheet Top100_DE_veh_p < 0.01_TPM > 1.

Concordant to visualization of loss of clear sex differences after rapamycin treatment in PCA (Fig. 2a), MA plot of distribution of sex-significantly DE genes in male and female rapamycin treated podocytes revealed only few sexually dimorphic genes, including mainly sex chromosomal genes (Fig. 2e).

To exclude contaminations with different cell types of the glomeruli during the purification process as a cause for the strong intrinsic sexual dimorphism, we compared our podocyte transcriptome with available kidney single cell data [37] (Supplementary Figure S2b). Furthermore, we matched our sequencing results to different podocyte specific RNAseq datasets [31, 38–40] and found high conformity between podocyte-specific transcripts further proving validity of the method (Supplementary Figure S2c). Additional experimental validation of transcriptomic data was performed by qPCR using independent cell isolations (Supplementary Figure S3).

Sexual dimorphism has been reported to be largely determined by the sex chromosomes genotype of the organism or sex hormonal regulations [41–43]. However, we could not relate the strong sexually dimorphic genes to specific positions on the sex chromosomes (apart from four genes uniquely expressed on male Y-chromosome). Interesting sub-clustering was observed, probably due to the mitochondrial expression which approximately split vehicle from rapamycin samples of each sex (Supplementary Figure S2d).

(See figure on next page.)

**Fig. 2** Characterization of the intrinsic sexually dimorphic podocyte transcriptome. **a** Principal component analysis of the top 500 most varying genes revealed sex-dependent separation of transcriptomes between vehicle mice (blue color indicates male podocytes, green color female podocytes; vehicle groups are represented by circles, rapamycin-treated by triangles), n = 4 male and female vehicle each; n = 5 male rapamycin treated and n = 6 female rapamycin treated). **b** Venn diagram demonstrating common and unique significantly sex-differently expressed genes between indicated comparisons (brown: male vs. female vehicle-treated group, grey: male vs. female rapamycin-treated group, green: female rapamycin vs. female vehicle, blue: male rapamycin vs. male vehicle). **c** Scatter plot showing distribution of gene copy numbers of podocytes from vehicle groups as base mean normalized expression over all samples versus log2FC male vehicle/female vehicle. Colors indicate significance levels (red: adjusted *P*-value < 0.01, orange: adjusted *P*-value between 0.01 and 0.05, dark grey: adjusted *P*-value between 0.05 and 0.1, medium/light grey: non-significantly differently expressed). **d** Heatmap of the top 100 of intrinsically sexually dimorphic genes in male versus female podocytes (adjusted *P*-value < 0.01 and expressed with TPM > 1 in all biological replicates) The four Y-chromosomal genes (*Kdm5d*, *Eif2s3y*, *Uty* and *Ddx3y*) are not shown in this heatmap. Blue indicates decreased expression level, white no regulation, red increased gene expression level. These genes are listed with respective Log2FC and TPMs in the Supplementary Table S3, sheet Top100_DE_p < 0.01_TPM > 1. **e** Scatter plot showing distribution of gene copy numbers of podocytes from rapamycin groups as normalized counts per transcript versus log2FC male/female. Colors indicate significance levels (red: adjusted *P*-value < 0.01, orange: adjusted *P*-value between 0.01 and 0.05, dark grey: adjusted *P*-value between 0.05 and 0.1, medium/light grey: non-significantly differently expressed)

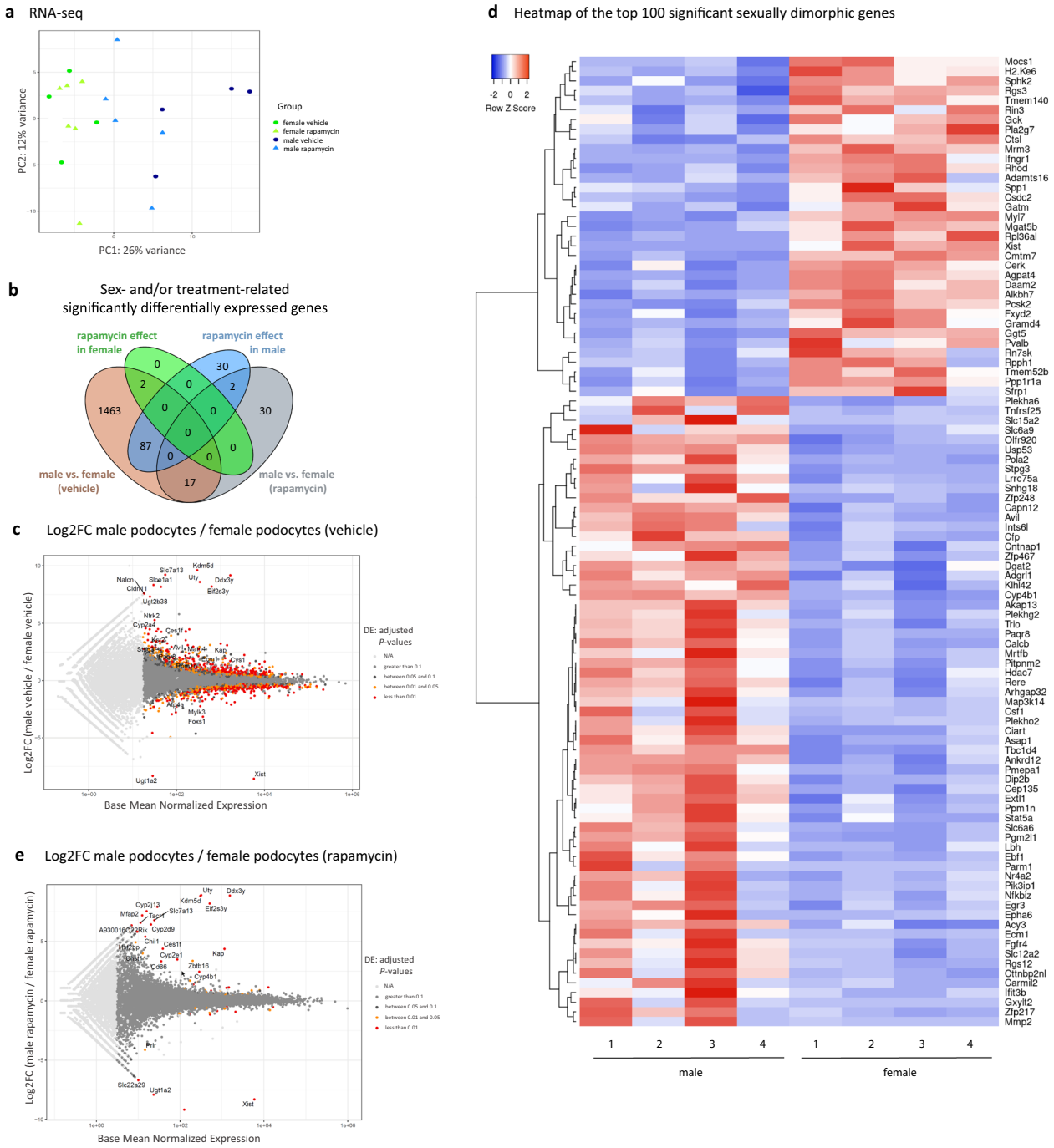



**Fig. 2** (See legend on previous page.)

To address possible sex hormonal regulations, we analyzed our intrinsically sexually dimorphic genes for known estrogen target genes [44, 45]. We found that out of more than 500 annotated genes corresponding to estrogen target genes pathways, 54 were intrinsically sexually dimorphic (37 up in male/17 up in female vehicle podocytes) (Supplementary Table S4), whereas the vast majority of these reported estrogen target genes were not significantly sex-differently expressed. Furthermore, only 6 estrogen target genes (*Daam2*, *Egf*, *Sgk3*, *Ago1*, *Il1r1*, *Tgfa*) were significantly differentially regulated by rapamycin treatment in males and only one in females (*Ankrd33b*). This indicates that a

large part of our observed sex-differences and sexually dimorphic responses to rapamycin in podocytes could not be attributed to direct genomic estrogen effects. Possible mechanism for sex-biased gene expression might also occur through sex-biased transcription factors (TFs) [43, 46]. We could identify 83 intrinsically sexually dimorphic transcriptional regulators (Supplementary Table S5) which are important candidates for regulation of sexual dimorphic gene expression.

### Functional characterization of the intrinsically sexually dimorphic podocyte transcriptome

Panther Protein Class categorization revealed that the GOs "Transporters", "cytoskeletal protein", "protein modifying enzymes/binding activity and transcriptional regulator" were the main significantly sexual dimorphic GOs. Further functional annotation of the DE genes ($P$-value < 0.05, ≥ 4 genes detected by panther gene ontology (GO) slim) revealed that distinct GOs were enriched in either female or male podocytes. Major gene sets overexpressed in females were related to "oxidation phosphorylation", "translation" and "ribosome", whereas in males "gene expression", "kinase activity", "cytoskeleton", "actin cytoskeleton organization" and "cell junction" were enriched (Fig. 3, Supplementary Table S6).

### Sexually dimorphic genes related to kidney diseases

Structural and functional integrity of the podocyte is strongly dependent on proper organization of its cytoskeleton and fulfillment of metabolic requirements, GO terms which appeared to be enriched in our sex DE analyses. To investigate clinical impact of these sexually dimorphic genes, we extended our analyses of intrinsically DE genes in podocytes to genes reported to be involved in the pathogenesis of kidney diseases. We used published data to determine any gene overlaps [47–58]. Out of 191 genes with described roles in kidney diseases, 161 were expressed with an abundance of at least 1 TPM in all vehicle podocytes, among them 47 genes with intrinsic significant sexual dimorphic expression (adjusted $P$-value < 0.05) (Fig. 4, Supplementary Table S7). Interestingly, only six out of these 47 intrinsically sexually dimorphic reported disease genes could be identified as estrogen target genes (Supplementary Table S4). A heatmap of the expression levels of these estrogen target genes (*Akt2, Ctsd, Egf, Igf1r, Kank1 and Daam2)*, as well as for other functional groups of intrinsically sex DE disease genes related to kidney disease are shown in Supplementary Figure S5. Respective genes are separately presented for transcriptional regulators, cytoskeleton-related genes, metabolism & protein turnover and signaling and kinases.

### Sex-specific transcriptional changes induced by mTOR inhibition

We first analyzed the effects of rapamycin treatment in each sex separately. Remarkably, male podocytes showed after exclusion of rhythmic genes DE of 119 genes (63 up/56 down, $P$-value < 0.05) and females only 2 DE genes (Fig. 5a). These data suggest that female podocytes may maintain a more stable transcriptome under the challenge of mTOR inhibition with rapamycin in contrast to male podocytes which underwent much more drastic shifts in expression level changes. Furthermore interestingly, 87% of genes significantly upregulated by rapamycin in males belonged to the female-biased genes and 66% of the significantly downregulated genes in males were male-biased genes (Fig. 5b). This suggests that interference with mTOR signaling affected intrinsically sex-biased gene expression to a greater extent than sex-independent gene expression.

Separate male and female subgroup analysis was undertaken of the intrinsically sex DE genes after rapamycin treatment. This revealed that the most prominent changes after treatment involved the intrinsically significantly different genes such as the sex different stress responses in genes related to "mTOR signaling", "protein anabolic and catabolic process", "oxidative phosphorylation", "organization and regulation of cytoskeleton", "cell adhesion", "exosome and exocytosis" and "transcription factors" (Fig. 5c).

Further Gene set enrichment analysis (GSEA) of the complete male and female transcriptomes revealed male-biased downregulation of gene sets related to many signaling pathways, inflammation and metabolism in response to rapamycin (Supplementary Table S8). In contrast, gene sets related to amino acid transport and metabolism appeared downregulated in both male and female podocytes, yet to a greater extent in males. We extended our analyses of effects of mTOR inhibition to canonical pathways´ activations in male and female podocytes with the use of QIAGEN IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA) [59]. Interestingly, many of the pathways showed significant changes in only one sex or in opposite directions, such as gene sets related to TCA cycle, Granzyme A, PDGF, Paxillin and RAAS and Sirtuin signaling pathways (Supplementary Table S9).

To get functional insight into these sex-different pathway and gene set activations induced by mTOR inhibition, further upstream regulator analyses were performed. With a Z-score of < |2| and $P$-value of overlap < 0.05, a total of 115 and 55 potential upstream regulators were identified in male and female, respectively (Supplementary Table S9). Among them were several genes known to be involved in kidney diseases, such

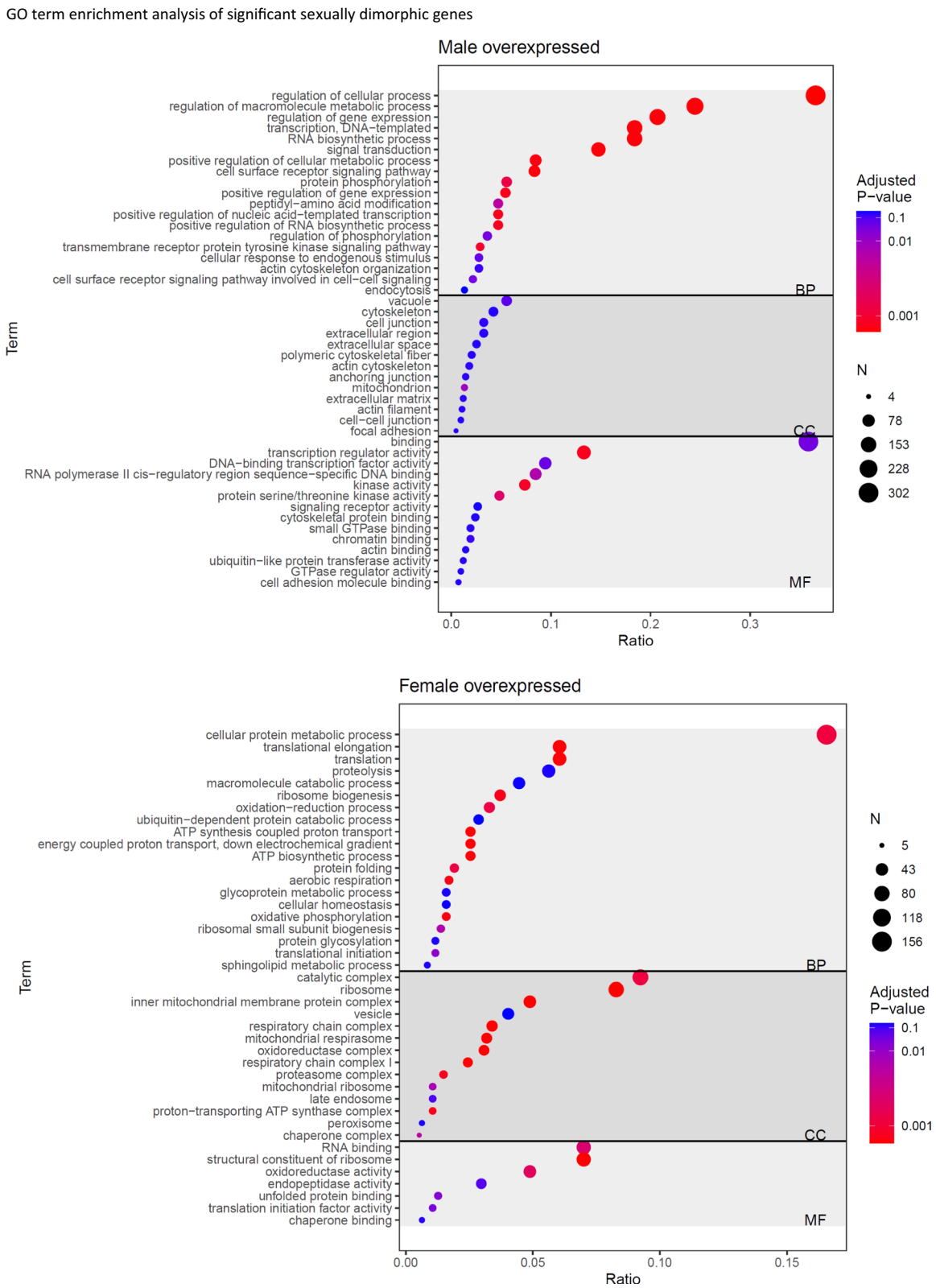



**Fig. 3** Functional characterization of significant sexually dimorphic genes. GO-slim Panther term enrichment analysis of the significantly overexpressed gene ontology terms of the 1,569 sex-DE genes for biological Process (upper in male, lower in female). (BP), molecular function (MF) and cellular component (CC). The size of the circles indicates the number of the significantly differently expressed genes in each GO (adjusted *P*-value < 0.05, FDR < 0.1)

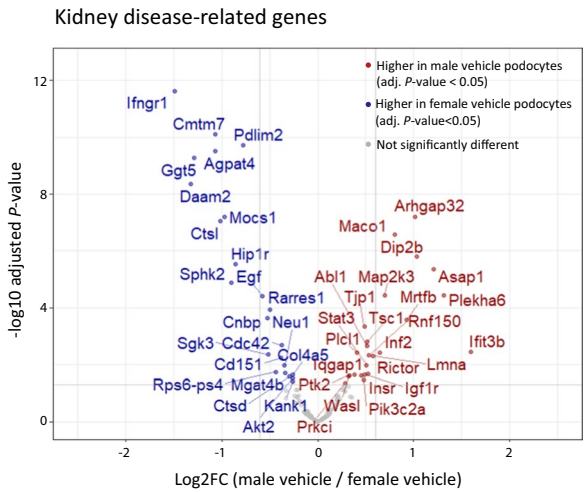



**Fig. 4** Kidney disease-related genes. Volcano plot displaying known kidney disease-related genes with gene names indicated for intrinsically sexually dimorphic genes (adjusted *P*-value < 0.05, TPM > 1 in all vehicle podocytes): the y-axis shows the statistical significance measured by –log10 adjusted *P*-value, and the x-axis shows the log2FC of male versus female vehicle podocytes (> 1 reflects higher than 2 FC in male podocytes; -1 means twofold lower in male podocytes). Each red point represents an individual gene that is intrinsically significantly higher expressed in male podocytes; each blue circle represents a specific gene intrinsically significantly lower expressed in male podocytes. (male vehicle n = 4, female vehicle n = 4, male rapamycin n = 5, female rapamycin n = 6)

as *Kdm5a*, *Rb1*, *Tead1*, *Xbp1* and *Ppargc1α* with so far unknown role as sexual dimorphism regulators [60–69].

Interaction effect analysis considering sex and treatment resulted in only 28 DE genes, most of them only significantly altered in males (Fig. 5d, Supplementary Table S2). Remarkably, 86% have been previously reported in relation to kidney pathologies and many of them are involved in cytoskeleton remodeling or represent transcriptional regulators.

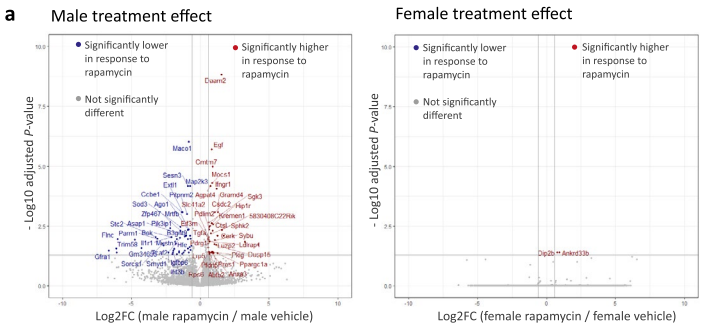
Considering the fact that mTOR has important effects on immunity [70–72], we additionally focused on inflammation and immune aspects of the podocytes. We did not induce any inflammatory condition or immune disease in our model, thereby we did neither expect nor found high gene expression levels of cytokines and immune-related receptors in both, control and rapamycin -treated mice. Along this line, we could not detect any lymphocyte infiltration in kidney cortex tissue in histological stainings of any of the four experimental groups. Nevertheless, in order to focus further on aspects of the "immune podocyte", we merged our gene expression lists with genes related to immunity in podocytes as reported in Bruno et al. [73] (see sex- and treatment related DE sublists of these genes presented in Supplementary Table S10). Under these genes, we only found complement 3 (*C3*) to be significantly sexually dimorphic with higher expression in male podocytes compared to female podocytes and a trend towards significant downregulation of *C3* in male in response to rapamycin treatment. Further, GSEA analysis showed that several GOs related to inflammation and immune response were regulated by rapamycin in both sexes, especially many of them significantly downregulated in male after rapamycin (see Supplementary Table 8, sheet "GOs related inflammatory and immune response").

### Characterization of the sexually dimorphic intrinsic podocyte proteome

Interestingly, proteomics analysis of isolated vehicle-treated male and female podocyte proteins confirmed significant enrichment of mitochondrial proteins in female podocytes (Fig. 6a, Supplementary Table S11). Proteins with sex-differential expression (FC > 2) were further analyzed using EnrichR. Concordant with the transcriptome, females had, in addition to enrichment in mitochondrial proteins, increased cytoskeletal and cytoskeleton-regulating proteins (Supplementary Fig. 4), whereas major hits in male podocytes were related to transcription and proteostasis (Fig. 6b). The higher number of cytoskeletal

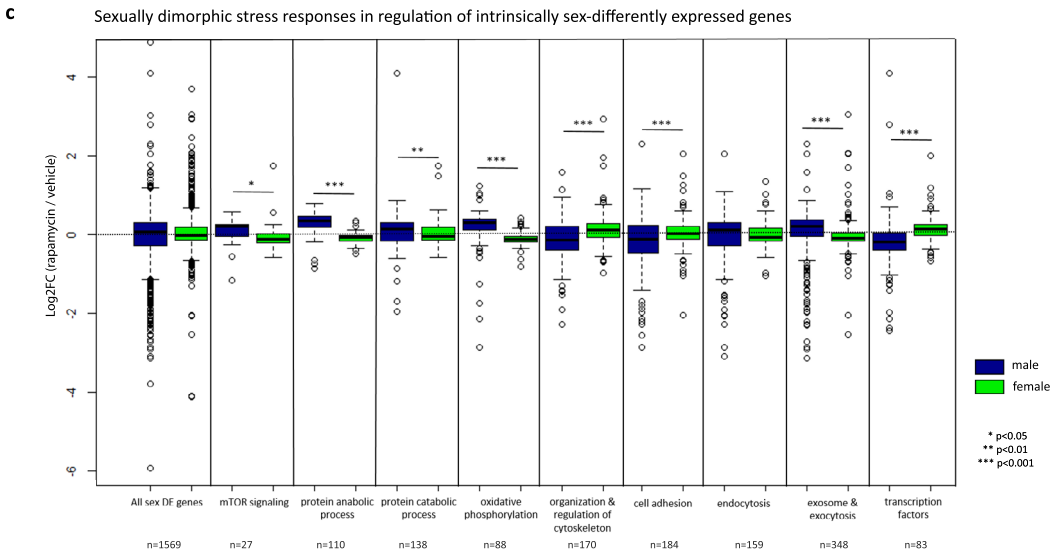
(See figure on next page.)

**Fig. 5** Sex-specific transcriptional changes induced by mTOR inhibition. **a** Volcano plots displaying rapamycin treatment effects in each sex separately (left graph representing results in male podocytes, right graph in female podocytes): the y-axis shows the statistical significance measured by –log10 adjusted *P*-value, and the x-axis shows the log2FC of rapamycin versus vehicle treated male, respectively rapamycin versus vehicle treated female podocytes (> 1 reflects higher 2 FC in rapamycin treated podocytes; -1 means twofold lower in rapamycin-treated podocytes). Each red point represents an individual gene that is significantly higher expressed in rapamycin-treated podocytes (adjusted *P*-value < 0.05); each blue circle represents a specific gene significantly lower expressed in rapamycin-treated podocytes. (male vehicle n = 4, female vehicle n = 4, male rapamycin n = 5, female rapamycin n = 6). **b** Table of by sex-bias grouped gene numbers significantly changed by rapamycin in each sex. **c** Graphs of sex-specific treatment changes of intrinsically sexually dimorphic genes for selected enriched GO terms. n = number of genes within specific term, significance levels * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001 as determined by Wilcoxon ranked sum test. (male vehicle n = 4, female vehicle n = 4, male rapamycin n = 5, female rapamycin n = 6). **d** Heatmap of the expression level and log2FC of significantly differently regulated genes by sex and treatment (interaction effect). Blue indicates decreased expression level, white no regulation, red increased gene expression level. (Each 4 biological replicates per group)




**b** Rapamycin-induced sex-specific transcriptional changes in male and female podocytes (adjusted *P*-value < 0.05)

| Intrinsic podocyte transcriptome | male rapa up (n=63) | male rapa down (n=56) | female rapa up (n=2) | female rapa down (n=0) |
|---|---|---|---|---|
| male-biased genes (n=683) | 0 | 37 | 1 | 0 |
| female-biased genes (n=886) | 55 | 0 | 1 | 0 |
| sex-independent genes (n=16569) | 8 | 19 | 0 | 0 |



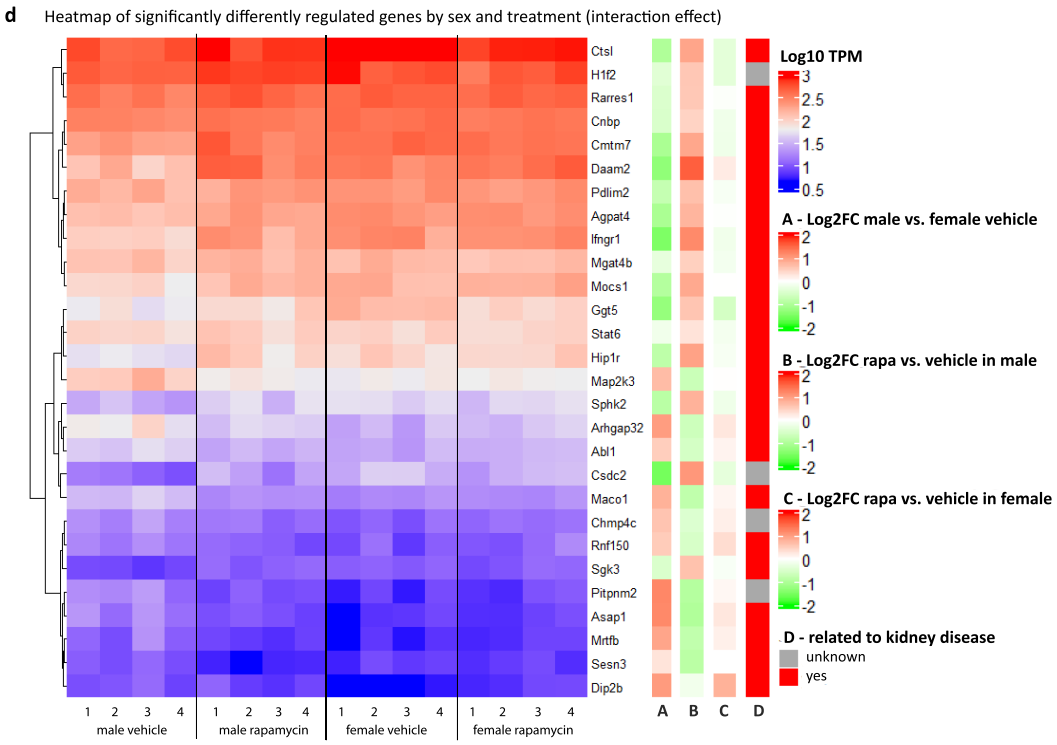




**Fig. 5** (See legend on previous page.)

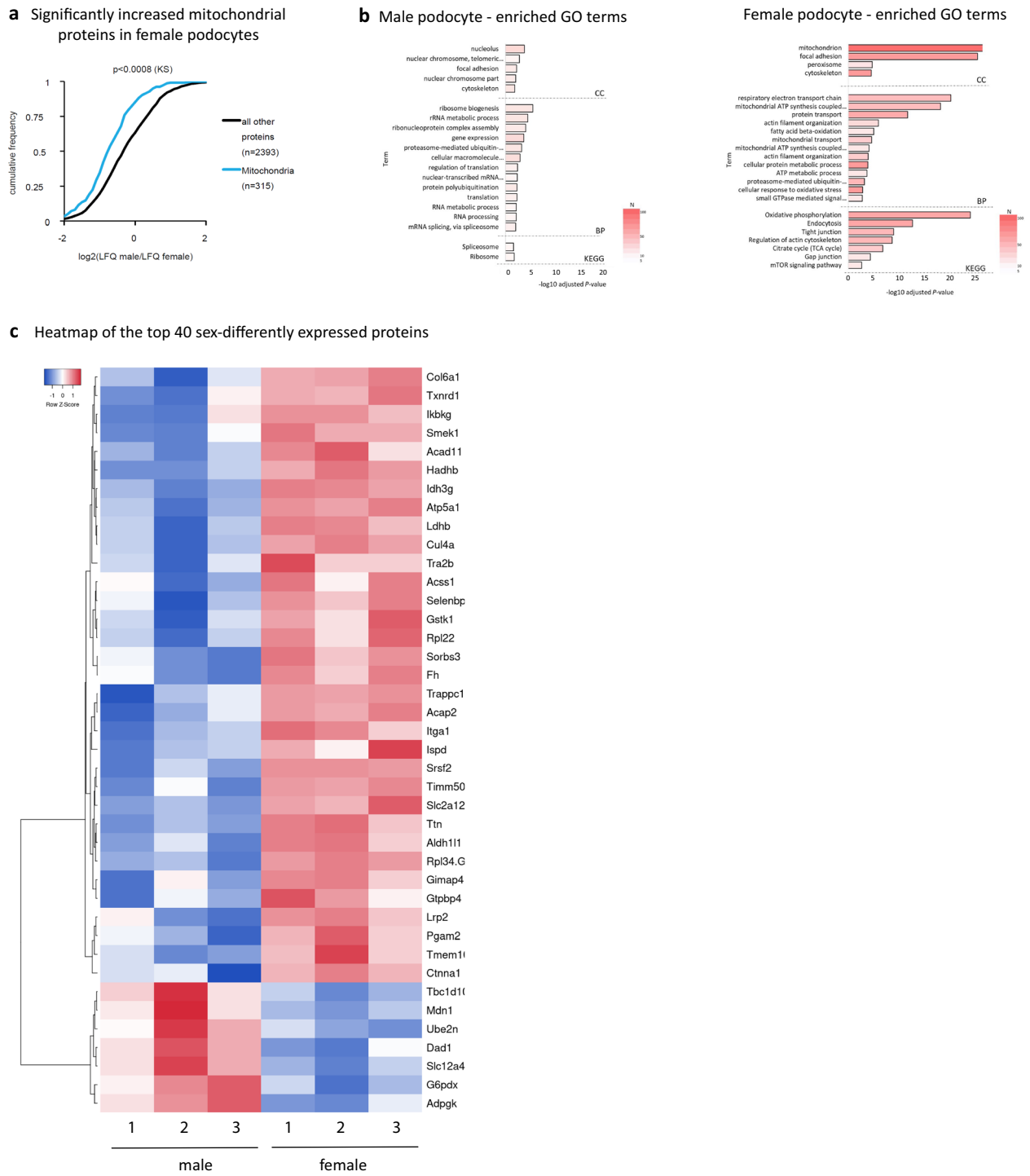



**Fig. 6** Characterization of the sexually dimorphic intrinsic podocyte proteome. **a** Podocyte-specific proteomics. Female podocytes showed significantly higher expression of total mitochondrial proteins compared to male podocytes (n = 3 biological replicates per each vehicle group). **b** EnrichR analysis of proteins with sex-differential expression (FC > 2) showed major hits in terms related to transcription and protein translation/turnover in male podocytes, whereas females had concordantly to the female transcriptome enrichment in mitochondrial proteins and increased cytoskeletal and cytoskeleton-regulating proteins. Left panel shows enriched terms for cellular component (CC), biological process (BP) and KEGG pathway (KEGG) in male podocytes, right panel respective enriched terms in female podocytes, adjusted *P*-value < 0.05, N = number of proteins within specific term. **c** Heatmap of the top 40 of intrinsically sexually dimorphic proteins in male versus female podocytes (adjusted *P*-value < 0.05) showed that a higher number of genes were enriched in female podocytes. Blue indicates decreased expression level, white no regulation, red increased gene expression level (n = 3 biological replicates per vehicle group)

and cytoskeleton-regulating proteins in female compared to male podocytes suggest a higher biophysical resilience to adapt to mechanical forces in their environment and therefore better maintenance of glomerular function in females under stress conditions [49, 74, 75].

Concordant with transcriptomics, GO terms "oxidative phosphorylation", "Glycolysis", "proteasome" and "mitochondria" were overrepresented in the dataset from female podocytes. Yet, as expected for comparisons of complex samples [76], the complete proteomes of male and female podocytes did not correlate strongly with their respective transcriptomes (Spearman rank coefficient in females: 0.43, in males: 0.39, p-values < 2.2e−16). Interestingly, the subsets of sexually dimorphic genes related to mitochondria/oxidative phosphorylation, correlated moderately with protein expressions in both, male and female podocytes (Spearman rank coefficient in females: 0.52, in males: 0.51, p-values < 2.1e−08). A heatmap of the top 40 significant sex-differently expressed proteins is presented in Fig. 6c.

### Sex differences in energy metabolism

GSEA of rapamycin effects on the whole male and female transcriptome had revealed that TCA cycle, carbohydrate derivative biosynthesis process and oxidation phosphorylation as well as several other metabolic processes decreased in females whereas males mostly responded conversely (Supplementary Table S8) suggesting sex-different stress responses. This, together with the high number of sexually dimorphic mitochondrial and translational genes and proteins prompted us to further study metabolic consequences of mTOR inhibition in male and female mice. Metabolomics was performed directly from podocyte enriched snap frozen kidney cortex tissue to ensure valid results of the in vivo changes induced by rapamycin. Data were analyzed to confirm correct metabolite annotation and for outliers (Supplementary Methods). Sixteen statistically significant ($p < 0.05$) outliers were removed. As expected, concordant with the well-known negative effect of mTOR inhibition on protein synthesis[77], significant accumulation of most amino acids occurred in both sexes, yet to a higher extent in males ($P$-value < 0.05) (Fig. 7a). In addition, metabolomics pointed to intrinsically increased glycolytic metabolites in female compared to male. Rapamycin reduced glycolysis and TCA cycle in both male and female. However, this reduction in response to rapamycin was only significant in females (Fig. 7b, Supplementary Figure S6). Phosphorylation of AMPK as indicator for the general energy status revealed a tendency towards higher levels in female compared to male, yet due to high interindividual variability especially in the female group this difference did not reach statistical significance (p-value = 0.07, Supplementary Figure S7). Both sexes

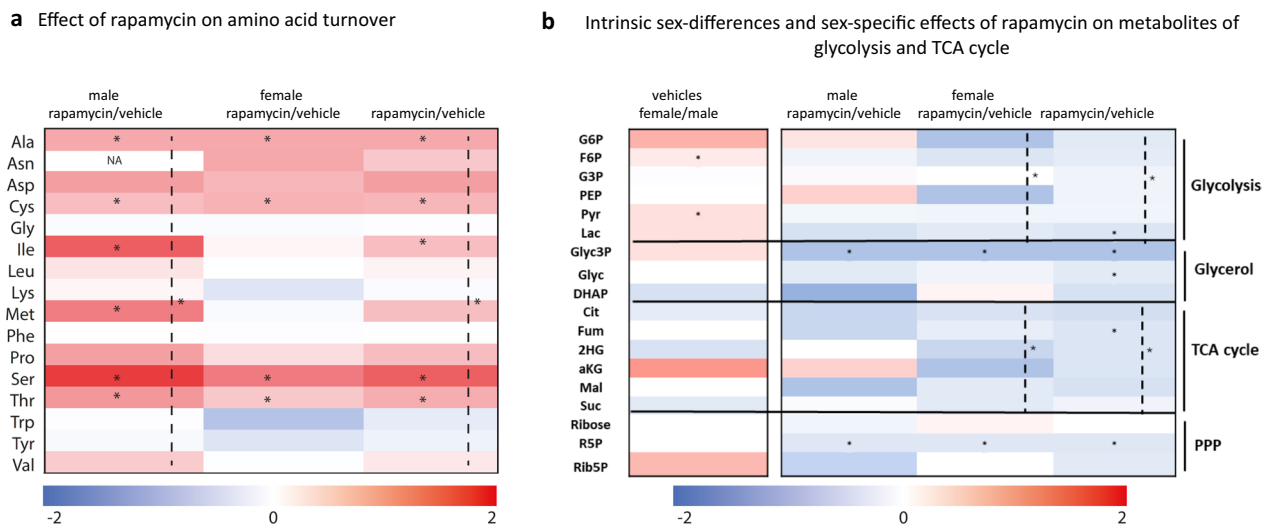



**Fig. 7** Sex differences in energy metabolism. **a** Heatmap of log2FC of the mean of the normalized peak areas of amino acids in male and female kidney cortex tissue in response to rapamycin in each sex separately and male and female combined (rapamycin/vehicle). Blue indicates decreased levels, white no regulation, red increased accumulation of metabolites (n = 6 male vehicle; n = 6 male rapamycin treated; n = 7 female vehicle and n = 5 female rapamycin treated n = 6), * $P$-value < 0.05. Dashed line represents significance for amino acids after univariate scaling. **b** Heatmap of log2FC of the mean of the normalized peak areas of glycolysis metabolites, TCA cycle, pentose phosphate pathways and others in male and female kidney cortex tissue in male and female vehicle, response to rapamycin in each sex separately and male and female combined (rapamycin/vehicle). Blue indicates decreased levels, white no regulation, red increased accumulation of metabolites. n = 6 biological replicates in each group, * $P$-value < 0.05. Dashed line represents significance for the group of glycolysis and TCA after univariate scaling

maintained pAMPK levels in response to rapamycin, yet male showed a tendence towards higher AMPK phosphorylation after rapamycin treatment. Total AMPK did not show intrinsic sex-differences and did not change by rapamycin treatment in both, male and female. (Supplementary Figure S7).

## Discussion

Podocytes are an essential part of the filtration barrier and any pathological alteration likely leads to proteinuria and serious pathologies [1, 74, 78]. Despite of high sexual dimorphism in many glomerular kidney disease prevalence and progression [2–4, 6], sex-specific molecular identity of podocytes remained so far unknown. A recent paper in Nature Methods called attention to that frequent limitation of molecular omics studies and requested sex annotation for fully accounting for the biology of sex differences [11]. Our study aimed at closing this knowledge gap in podocyte biology. We addressed intrinsic gene transcription and protein expression in murine male and female podocytes and determined sex-differences in podocyte responses towards pharmacologic intervention with rapamycin. Remarkably, many genes previously reported in podocytopathies showed so far unknown sexual dimorphic expression and transcriptional changes after rapamycin which might account for sexual dimorphic disease susceptibilities.

Our data suggest that female podocytes may have a more stable transcriptome considering the challenge of mTOR inhibition whereas males induce earlier molecular and metabolic changes. The mitochondrial energy providing machinery, endocytosis and exocytosis, and cytoskeletal integrity were major targets of transcriptional changes that were more strikingly affected in male podocytes. Interestingly, mTOR inhibition largely abrogated the clear separation of intrinsically sexually dimorphic genes in podocytes, mainly by changes in the male transcriptome.

Our stress model did not induce structural or kidney functional changes suggesting that the observed differences in podocytes might reflect early endogenous sex-specific compensatory mechanisms prior to disease development. The lack of functional changes contrasts with other reports where rapamycin induces proteinuria [20, 79]. This difference might be explained by the different mouse strains used in these studies. Other studies using C57BL/6 mice report lack of development of proteinuria by mTORC1 inhibition similar to our work [80]. Furthermore, a recent report shows that rapamycin might also delay development of proteinuria in specific disease models [81] suggesting that rapamycin effect on kidney functional changes is highly complex, mouse-strain- and context-dependent.

Female mice displayed lower kidney and lower body weight compared to male mice, which is considered physiological in these strains and moreover applies also to human [82–85]. Furthermore, rapamycin has already been shown to induce significant reductions in kidney/body weight ratio in several disease models [86–88] interestingly in some studies, similar to our study, to a higher degree in female compared to male mice [83]. Weight loss in response to rapamycin treatment has been attributed to central effects on mTORC1 inhibition in the hypothalamus which leads to decreased food intake [89, 90]. In addition, rapamycin regulates energy homeostasis by leading to enhanced insulin sensitivity [91]which causes less blood sugar increases, thereby reducing hunger and craving for food. Another reason for reduced food intake and subsequent weight loss under rapamycin treatment may be the regulation of gastric ghrelin production [92]. Unfortunately, we did not control for food intake in our mice that we were unable to report differences due to calorie input as possible cause for the reduction in body weight under rapamycin treatment. However, the weight losses were within the range of those reported previously in various mouse models and we did not observe any rapid weight losses that we do not consider that as a sign of rapamycin toxicity or over-inhibition of mTOR. The cause for the unproportional greater kidney weight loss compared to body weight loss in rapamycin-treated females remains elusive. One might speculate an impact of sex hormones [93, 94]. mTOR inhibition affected estrogen receptors and androgen receptor gene expression to different extents in our study which might have contributed directly or indirectly to these sex-differences in kidney weight loss.

Although major sex differences in gene expression have been generally attributed to sex chromosomes and sex-hormonal effects [42] most of the intrinsic sex differences in podocytes observed in this study could not be associated with their position on sex chromosomes or direct estrogen target effects. Previously, it has been reported in other species that autosomes house the majority of genes with sex-biased expression [95]. Furthermore, the male-predominant idiopathic nephrotic syndrome in children of 0–18 years of age supports the fact that factors other than hormonal effects contribute to at least some sexual dimorphic podocytopathies [50].

Recently, it has been reported that beyond chromosomes and sex-hormonal effects, several TF show directional agreement of the target genes between the activating or repressive effect of the TF, the sex bias of the TF, and the sex bias of the target gene and may account for about 27% of lineage-specific sex bias [42, 96, 97]. In our study, we could identify 83 transcriptional regulators of intrinsically sexually dimorphic genes in podocytes,

among them *Stat5a*, which has been recently demonstrated to regulate 23% of female-predominant genes in mouse liver [96]. Thus, regulation by sex-biased transcription factors most likely contributed essentially to our observed sex differences in podocytes.

Our differential expression analysis taking sex and treatment differences into account revealed further TF of interest, such as *Mrtfb, Cnbp* and *Stat6,* which appeared upregulated in males and downregulated in female podocytes in response to rapamycin. *Stat6* has previously been reported as regulator of cyst growth in polycystic kidney disease [98] and implicated in kidney fibrosis in a model of unilateral ureteral obstruction [99], yet further data on sex differences and functional role in podocytes are still lacking.

Other transcription factor candidates for sexual dimorphic gene expression patterns in our study identified by IPA were e.g. *Xbp1*, and *KDM5a*. *Kdm5a* mediates kidney failure in lipopolysaccharide-induced sepsis of mice [100], induces stem-like cancer cells and promotes renal cell carcinoma [62, 101]. Via activating or repressing transcription in demethylase-dependent or independent manners [101], it acts in both, homeostasis and disease [60, 61]. Kdm5a inhibitors are already tested in clinical trials [102]. Thereby, some of our observed intrinsic sex differences might have been mediated by epigenetic events [16, 41, 103] and might be an interesting future therapeutic target.

Recent evidence points to sex differences in circadian endocrine rhythms and oscillation of clock genes [104–106], which might be relevant for sexual dimorphic features of podocytopathies. E.g. *Arntl* has been shown to be critical for genes involved in podocyte integrity, metabolism and adhesion [107] and the transcription factor *Creb3l1* has recently been demonstrated in male podocyte injury and degeneration [108]. However, our study was not specifically designed to investigate such differences. Taking into consideration that sex differences do not only appear in expression levels at specific time points during the day but also might significantly peak earlier in one sex [106], future studies require investigating multiple different day times in addition to well-controlled times for sacrifice of mice.

Remarkably, many of the intrinsically sexually dimorphic and significantly deregulated genes after rapamycin treatment in this study were kidney disease-related. Yet, to our knowledge, none of the genes that were significantly differentially regulated considering sex and treatment have been investigated for sexual dimorphism in kidney cells so far. Interestingly, some of them were reported to be sexually dimorphic in other tissues, such as in microglia and skeletal muscle [76, 109]. Among them, *Daam2, Pdlim2*, *Asap1*, and *Sphk2* all of which have a known relationship to kidney diseases [110–114]. *Daam2*, a gene involved in actin remodeling with certain variants causing nephrotic syndrome [111], as well as *Cd151*, important for podocyte-basement membrane attachment [115–117] were intrinsically significantly higher expressed in female podocytes and significantly increased only in male podocytes after rapamycin treatment. Interestingly, *Daam2* and *Cd151* have recently been reported to be upregulated in a dataset of human focal glomerulosclerosis [118]. Beyond other genes in our study, sexually dimorphic response patterns were observed for *PGC-1α*, a master regulator of mitochondrial biogenesis and transcriptional factor relevant for podocyte homeostasis [119] and a known role in kidney diseases [65], and *Ctsl,* encoding a cysteine protease which promotes Cd2ap and synaptopodin proteolysis [57, 120]. It has been reported that podocyte pH modulation by glutamine supplementation reduces cytosolic cathepsin L protease activity and can reduce foot process effacement and proteinuria [121]. We did not measure pH in our isolated podocytes and were not able to determine cathepsin L levels in our podocyte-specific proteomics of vehicle groups. Furthermore, due to the possibility of conversion of glutamate during the autosampler and derivatization during LC–MS/MS measurements [122], trustable glutamine and glutamic acid levels could not be reported in our metabolomics study and we were therefore unable to make any statement about glutamine level in male and female vehicle and rapamycin groups. To approach this important question for functional relevance of rapamycin effects on cathepsin L further, future studies are warranted including pH and enzyme activity measurements into their study protocols.

Another important protein involved in actin-regulation of podocyte foot processes is Pdlim2 [113]. It has been recently validated as a podocyte-specific protein in stainings from the Human Protein Atlas [118]. By correlation of a set of podocyte-specific genes with GFR in a patient cohort with glomerular diseases the potential of these genes to be used as candidate markers for disease progression has been demonstrated [118]. A further gene of interest among the top 100 intrinsically sex-differently expressed podocyte genes that showed sex-different responses to rapamycin is *Sphk2*. Sphk2 has been shown to be involved in kidney fibrosis and diabetes-induced podocytopathy and has recently been suggested as pharmaceutical target to treat proteinuric kidney disease [123, 124].

Considering the sexual dimorphic expression of many of the genes identified in our study strengthens the need that further candidate marker studies should include sex as a biological variable in their design.

The significant transcriptional changes in male podocytes suggest a stronger dependence on intact mTOR signaling in males to maintain podocyte homeostasis. This was also observed at the metabolomic level, where genes involved in amino acid metabolism and transport were more suppressed in males. Furthermore, higher amino acid accumulation in male metabolomics pointed to higher reduction in protein synthesis in males compared to females. Sexual dimorphic responses to mTOR inhibition by rapamycin have also been reported on organismal level. Female mice, even when treated late in life, had a longer lifespan compared to male mice [125]. For this reason, our finding of sexual dimorphism in alpha-ketoglutarate levels is of interest. Alpha-ketoglutarate has been recently attributed a crucial role in delayed aging and improved longevity [126, 127].

Despite the fact that most studies about the effects on longevity haven´t been investigated in human, many people take rapamycin off-label [28]. Recently, Kaeberlein et al. performed an observational study comparing rapamycin users to non-rapamycin users for perceived health benefits and adverse events [128]. Mouth ulceration was the only self-reported significantly different adverse event in rapamycin users. In contrast, rapamycin users reported less abdominal cramps and pain, depression, muscle tightness, anxiety and eye pain. However, these domains except for mouth ulceration lost their significant difference when only female survey responders were included in the analysis. Although the study design was not double-blinded, placebo-controlled and no clinical laboratory results were evaluated it remains intriguing with regard to our findings to hypothesize that rapamycin effects display sexual dimorphic aspects in healthy and diseased people. Along this line, the results from an ongoing cross-sectional trial with rapamycin users versus non-users including clinical laboratory results, as well as molecular and metabolic signatures [28, 129] will be of specific interest for the translational value of our data. Yet, studies focusing on rapamycin effects in human kidneys are still scarce [25].

Concerning the treatment duration with mTOR inhibitors required to elicit functional effects, recent studies have shown rapamycin effects on longevity/aging already after a brief exposure to rapamycin of three months which was as effective as long-term treatment, despite the return of TORC1 levels to normal within 2 days of stopping rapamycin [27, 130]. These studies suggest that changes on downstream signaling induced by inhibition of mTORC1 such as induction of autophagy contribute to long lasting organismal changes and do not require persistent mTORC1 inhibition. Maintenance of autophagy or respective induction in kidney disease models with decreased activity has been shown to be cytoprotective for podocytes [131]. Therefore, time-restricted mTORC1 inhibition regimen might finally be clinically more beneficial compared to longtime treatments with mTORC1 inhibitors which still have high systemic and substantial side effects. Yet so far, we are not aware of any brief time-restricted intervention studies with rapamycin in the context of kidney diseases. Therefore translating our results to possible effects in kidney diseases we can only speculate that for the induction of a „rapamycin memory " 2 weeks of rapamycin treatment might be too short [132], whereas any time between the 3 month treatment period for the induction of longevity effects and the 25 weeks of treatment with relevant functional and structural effects [133] might be considered for conception of such proof of concept studies for the effectiveness of time-restricted administration of rapamycin in kidney disease models. Of note, dual mTORC1/2 inhibitors, such as AZD2014, do not maintain immunoregulatory effects after drug withdrawal and thus differ from the longer lasting effects of rapamycin [134]. For the induction of changes by rapamycin on protein translation, already 2 weeks of treatment appear to be enough [132].

Another approach to reduce negative clinical side effects of rapamycin treatment beyond changes in treatment time is the development of more specific mTORC1 inhibitors [28]. Rapamycin has tissue-dependent effects on mTORC2 and at least part of its negative effects e.g. on glucose and lipid metabolism have been attributed to inhibition of mTORC2 [135]. Therefore, more specific mTORC1 inhibitors might have the potential to reduce signaling network disturbances and compensatory up-/downregulation of mTORC2 in various tissues, thereby reducing negative side effects of rapamycin. Recently, other approaches to more specifically inhibit mTORC1 compared to rapamycin have been suggested as novel therapeutic option in podocyte diseases, such as indirect modulation of mTORC1 by ketone bodies [136]. Remarkably, part of the renoprotective effect of SGLT2 inhibitors might also rely on their effect to increase circulating ketone body concentration [137]. Reduction of mTORC1 activity can also be achieved by reducing the content of dietary protein or specific dietary amino acids [138]. Altogether, the development of specific mTORC1 inhibitors appears promising for health maintenance and treatment of a variety of diseases with mTOR-pathway-related pathologies.

In addition to variations in treatment times and specificities of mTOR inhibitors, sex differences in treatment results with different dosages have to be considered. Recently, Bitto et al. [130] reported that high-dose rapamycin treatment induced aggressive hematopoietic cancer development in female but not in male mice whereas lower dosage comparably increased lifespan in both

sexes. We induced mTORC1 inhibition in kidney cortex tissues of male and female mice applying the same dosage of rapamycin and treatment time and achieved comparable rapamycin trough levels. Nevertheless, molecular signatures in podocytes were sexually dimorphic.

We cannot rule out differential sex-specific mTORC1 inhibition in podocyte and non-podocyte kidney cortex cells by rapamycin. However, bioinformatic analysis of downstream target changes by rapamycin in each sex in of our RNASeq data pointed to significant mTORC1 inhibition in podocytes of both sexes. Beside direct effects of rapamycin on podocytes, indirect effects of rapamycin-induced changes in surrounding cells or more distal known effects of rapamycin, such as reported in gut bacteria [139], metabolism [140] and the immune system [141, 142] might have additionally shaped the sex-specific molecular signature of podocytes in response to systemic rapamycin treatment [28].

mTOR inhibitors have well-known effects on immunity [70–72]. Furthermore, immunity displays important sexual dimorphism [143–146]. Accumulating evidence suggests that podocytes are not only targets of immune responses but also play an active role in innate and adaptive immunity [1, 73]. They may aggravate immune and nonimmune glomerular injury through expression of receptors linked to pathways that induce proinflammatory molecules [147, 148]. Interestingly, we found *complement 3* to be significantly sexually dimorphic with higher gene expression in male podocytes compared to female podocytes and a trend towards significant downregulation of *C3* in male in response to rapamycin treatment. In human studies, C4 and its effector C3 were present at higher levels in cerebrospinal fluid and plasma in men compared to women. It was suggested that this sexual dimorphism contributed to women´s greater risk of systemic lupus erythematodes [149, 150]. Sexual dimorphism of genes involved in the complement system has been also reported in different immune cell types, such as macrophages [151].

Interestingly, mTOR inhibition affected estrogen receptors (*ESR1*, *ESR2*) and androgen receptor (*AR*) gene expression levels in male and female podocytes to different extents, which might constitute a further regulatory level for sex-biased gene expression. ERα, ERβ and AR are expressed in podocytes [152]. Downregulation of ERβ by rapamycin has previously been reported in other postmitotic cells, such as cardiomyocytes with sex-specific functional consequences [29]. Furthermore, it has recently been shown that female estrogen receptor knock-out mice developed glomerulosclerosis due to excessive ovarian testosterone production and secretion and extracellular signal-regulated kinase (ERK) pathway [152]. Therefore, balanced hormone receptor expression together with adequate sex hormone levels participate through genomic and non-genomic sex hormone effects, which needs to be considered when further investigating specific disease models.

Even if our metabolomics study and signal transduction investigations were performed only in kidney cortex tissue and we did not control for food intake and mitochondrial oxidative phosphorylation-related ATP production, our results on sex differences in metabolism are interesting especially in relation to recent studies performed in mice and human [9, 153, 154]. AMPK has been named the "guardian of metabolism and mitochondrial homeostasis" [155]. Under conditions of low energy, AMPK phosphorylates specific enzymes and growth control nodes to increase ATP generation and decrease ATP consumption [155].

In the kidney, AMPK activation may be protective against fibrosis, inflammation and mitochondrial dysfunction [156]. Furthermore, there is a close interaction between mTORC1 and AMPK signaling [157]. Concordant to our intrinsic sex difference in pAMPK levels, Lee et al. reported in a high-fat diet (HFD) mouse model decreased AMPK activity (indicated by increased phosphorylation) in male mice which were susceptible to kidney injury, whereas female mice did not display AMPK inactivation and were resistant to that obesity-induced HFD-induced kidney injury [158]. Considering the tendency of rapamycin-induced higher phosphorylation of AMPK in male in our study and their results in that disease model we may speculate that rapamycin might exert therapeutic effects in male mice.

Glycolysis and mitochondrial metabolism both contribute to podocyte energy supply. Cell environmental context and differentiation status impact on switches from fatty acid oxidation to glycolysis and vice versa [159–164].

On intrinsic transcriptional levels, we found overrepresentation of GO terms "oxidative phosphorylation", glycolysis" "proteasome" and "mitochondria" in functional GSEA analyses in the dataset of female podocytes. This is concordant to many studies reporting higher gene expression of these groups in various female cells [165, 166]. We are not able to directly relate our gene expression results from podocytes to our data on metabolomics without additional data as already mentioned above. Yet, metabolomics of kidney cortex comparing male and female vehicle pointed to significantly increased glycolysis in females (Fig. 7b). Increased pyruvate concentration in female proximal tubular epithelial cells has been suggested to be protective against diabetic kidney disease, whereas in male enhanced TCA metabolism could be deleterious [9]. Furthermore, in adults with chronic kidney disease, increased plasma levels of TCA cycle

metabolites positively associated with male sex, diabetes and all-cause mortality and negatively associated with estimated glomerular filtration rate (eGFR) [9]. In our model, rapamycin led to decreased glycolysis and TCA cycle in both, male and female kidney cortex, to a higher extent in females. The functional relevance of this finding requires further investigation in disease models.

## Limitations of the study

In this paper, we aimed to investigate the molecular signature of male and female podocytes and used a systemic pharmacological challenge with the mTOR inhibitor rapamycin to identify sex-specific response patterns in addition to podocyte cell intrinsically sex differences. Undoubtedly, there are also limitations associated with this approach. On one side, systemic drug administration reflects the frequent clinical situation where drugs affect not only the respective target cells but additionally other cell-types. However, by that, the molecular changes observed in podocytes might not be a direct drug effect, but also depend on the surrounding tissue or circulating factors which might themselves be sex-specific and therefore might have indirectly changed the male and female podocyte transcriptomes. Another limitation of our study is the cross-sectional design. Our work focuses on characterization of sex-differences on different molecular levels within one mouse strain at one age group with one specific rapamycin concentration for a fixed treatment time. Taking into account the fact that targeting mTOR signaling has different effects at various age groups [167–169], different age groups should be included in future studies when addressing therapeutic effects of mTOR inhibition.

In addition, isolation of primary podocytes from mouse tissues requires several preparative steps. This results in immediate early gene activation which might have further impacted on the molecular changes of the podocytes even if this stressor appeared to be comparable between all groups (Supplementary Table S12). This also prevented us from performing immunoblotting from isolated podocytes to assess phosphorylation of specific proteins in addition to the fact that isolated podocytes from one mouse do not yield enough material to apply such method. For that it is quite common in podocyte studies, that kidney cortex material is used instead [170–173]. Also, for metabolomics, it is important to directly harvest material after animal sacrifice. We therefore had to accept the limitation not to use the isolated podocytes, but instead kidney cortex tissue. On one side, this allowed us to obtain high quality material for metabolomics, but on the other side we had to accept that we thereby could not evaluate sexual dimorphism at the molecular level of the podocytes but only podocytes together with podocyte-adjacent cells in kidney cortex material. Moreover, to overcome at least partly this restriction, our studies were complemented with functional analyses of podocyte transcriptomes to indirectly assess podocyte-specific mTORC1 inhibitory effects and metabolic consequences of mTOR inhibition.

Furthermore, the fact that podocytes, even if crucially important for kidney function, belong to the low represented cells of the kidney make it difficult to compare our results with previous single cell studies in the kidney [37]. The correlation of our sexual dimorphic genes to a recently published single cell dataset from male and female mouse kidneys [174] only showed poor correlation (data not shown). Yet, podocytes were underrepresented in their study, underlining the fact that sexual dimorphism occurs cell-type specifically [118]. Interestingly, some of the intrinsically sexually dimorphic podocyte genes have been recently reported to be sex-differently expressed in cells of the renin lineage [175]. Univariate linear regression analysis revealed high correlation of the reported log2 fold change (FC) in renin lineage cells with our data (Supplementary Figure S1a).

## Perspectives and significance

Overall, this work provides a deep insight into sexual dimorphism of podocytes. Our study revealed that female podocytes show a more resistant profile to the effects of mTOR inhibition than male podocytes. Furthermore, important novel sex-biased transcription factors were identified in response to mTOR inhibition. This might contribute significantly to sex-different susceptibilities and clinical courses of a variety of sexual dimorphic glomerular kidney diseases.

More specific studies are urgently required, which is additionally supported by the fact that mTOR signaling varies not only with age and tissue, but also by sex, between mouse strains, and between different species [176]. A recent systematic review, targeting aging with rapamycin and its derivatives in humans summarized that positive effects of rapamycin had been shown on the immunological, cardiovascular, and integumentary systems, yet respiratory, digestive, renal and reproductive systems were still underassessed [25].

We do speculate that novel, more specific mTORC1 inhibitors which are already in the pharmaceutical pipeline might elicit beneficial effects in the treatment of chronic kidney diseases especially in male. Furthermore, sex-specific targeting of factors influencing immune functions of the podocyte may contribute to treat immune podocytopathies in the future.

Another interesting aspect is the well-known role of mTOR in aging. Concerning the sex-specific responses towards mTOR inhibition in our study, structural and

functional differences in the kidneys under stress might be expected between male and female patients and warrant further clinical investigation.

Future studies will be needed to specifically address pathological disease states or different induction mechanisms and investigate the effect of mTOR inhibition and targeting transcriptional factors to determine the pathophysiological impact of key molecular sex differences elucidated in this study and to move a critical step forward in the era of personalized medicine.

## Conclusions

Our results highlight remarkable intrinsic sex-differences and sex-specific response patterns towards pharmacological challenged podocytes. A large number of known kidney-disease related genes are newly identified to be sexually dimorphic and beyond classical sex hormonal effects further transcription factors were identified in sexually dimorphic response to mTOR inhibition with rapamycin. The female advantage in many kidney diseases` prevalence and disease progression might be strongly influenced by these genomic sex differences. This work can be used as a resource for specific genes to consider when trying to better understand sex differences in kidney health and disease.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13293-024-00647-7.

Additional file 1: Methods. Detailed descriptions of all methods used.

Additional file 2: Figure S1. Representative FACS cell sorting. Podocyte cell sorting showed a good separation on Fl1 with separation ratio of about 1:3 from the other glomerular cells.

Additional file 3: Figure S2. Comparing sequencing data to previously published data and exclusion cellular contamination and chromosomal effect on sex-specific gene expression. **a** Univariate linear regression analysis indicated a highly significant positive correlation of log2FC of male versus female commonly described genes of podocytes of this study and young cells of renin lineage (Wang Y et al., *Aging (Albany NY)*. 2018; https://doi.org/10.18632/aging.101416). Residual standard error: 2.175 on 19 degrees of freedom (DF), Multiple R-squared: 0.7219, Adjusted R-squared: 0.7072, F-statistic: 49.32 on 1 and 19 DF, *P* value: 1.097e-06. **b** Heatmaps of expression after variance stabilization for cell types marker genes from lists obtained from single cell data (Park J et al., Science 2018 (https://doi.org/10.1126/science.aar2131) indicating overrepresentation and enrichment for sex DE genes in podocytes, but not other major kidney cortex cells. Material from 20 mice were used to generate the heapmaps. **c** Venn diagram showing the number of common or unique genes detected in this and published podocyte datasets (Yellow: data from this study, brown: Borries M et al., *Kidney Int.* 2013 (https://doi.org/10.1038/ki.2012.487), green: Lu Y et al., *Kidney Int.* 2017 (https://doi.org/10.1016/j.kint.2017.04.022), Blue: Kann M et al., *J AM Soc Nephrol.* 2015 (https://doi.org/10.1681/ASN.2014090940), orange: Wang Y et al., *Kidney Int.* 2020 (https://doi.org/10.1016/j.kint.2020.05.052). **d** Heatmap of the average normalized expression by sample and by chromosome showing that sexual dimorphic gene expressions do not relate to sex chromosomes.

Additional file 4: Figure S3. Validation of sexual dimorphic transcriptional differences in podocyte. Transcriptomic data were confirmed with qRT-PCR including podocyte specific-genes from isolated podocytes of additional mice. Criteria for gene selection were intrinsically sexually dimorphic podocyte-specific genes with biological relevance for kidney physiology and disease such as *Slc6a6*, *Arhgap32* and *Tjp*1 as well as genes representing main sexual dimorphic GOs such as for "ribosomal proteins" *Rpl13* and "mitochondria/oxidative phosphorylation" *Cox7a2*. Further genes *Daam2*, *Ctsl* and *Arhgap32* were chosen for their relation to kidney diseases and the fact that they did not only show intrinsic sexual dimorphism but were additionally significantly differently regulated in male after rapamycin treatment. Other criteria for gene selection were transcript level expressions higher than 1000, log2FC differences between at least two experimental groups of 0.5–2.0. **a** qPCR results presented as mRNA expression changes from male and female vehicle podocytes (n = 4–8) in the left panel (*P*-values are indicated below each gene name), right panel shows expression level changes of RNA-seq for respective genes of vehicle groups, all genes were normalized to male vehicle, **b** qPCR results presented as mRNA expression changes from male rapamycin and male vehicle podocytes (n = 4–8) in the left panel (*P*-values are indicated below each gene name), right panel shows expression level changes of RNA-seq for respective genes of male rapamycin and vehicle groups, all genes were normalized to male vehicle (c) qPCR results presented as mRNA expression changes from female rapamycin and female vehicle podocytes (n = 5–9) in the left panel (*P*-values are indicated below each gene name), right panel shows expression level changes of RNA-seq for respective genes of female rapamycin and vehicle groups, all genes were normalized to female vehicle, Reference genes used for normalization were *Hdgf* and *Ywhaz* genes. Results are expressed as mean ± SEM, Mann-Whitney test was *used for statistical comparisons*.

Additional file 5: Figure S4. Validation of proteomics data. **a** Western blots of selected intrinsically sexually dimorphic podocyte-specific proteins (left panel) and quantification (right panel) (n = 4 in each group) of proteins isolated from male and female podocyte-enriched kidney cortex tissue. All proteins were normalized to GAPDH. Results are expressed as mean ± SEM, Mann-Whitney test was *used for statistical comparisons*. **b** Relative differential expression levels of selected proteins as determined by proteomics and immunoblotting.

Additional file 6: Figure S5. Functional grouping of intrinsic sexually dimorphic podocyte genes related to kidney diseases. Heatmap of the expression levels and log2FC of functional groups of sexually dimorphic genes related to kidney diseases. **a** estrogen target, **b** Transcriptional regulators, **c** Cytoskeleton-related genes, **d** metabolism and protein turnover and **e** signaling pathways/kinases related genes. Blue indicates decreased expression level, white no regulation, red increased gene expression level. (4 biological replicates in each group).

Additional file 7: Figure S6. Rapamycin treatment effect on metabolites in male and female podocyte-enriched kidney cortex tissue. Normalized peak areas of the biological replicates from male and female control and rapamycin-treated mice are individually plotted (male vehicle n = 6; male rapamycin n = 6; female vehicle n = 7; female rapamycin n = 5). The overall mean ± SEM is overlain. Median RSD = 11% for pooled QC, median RSD = 36, 31, 32 and 23 for male control, male rapamycin, female control and female rapamycin, respectively.

Additional file 8: Figure S7. pAMPK and AMPK expression in male and female kidney cortex tissue. Western blots indicating activation levels of AMPK by phosphorylation of AMPK at Thr172 and AMPK expression in podocyte-enriched kidney cortex tissue with densitometric quantifications normalized to AMPK for pAMPK and GAPDH for AMPK for **a** male (n = 7) and female (n = 7) vehicle groups and **b** vehicle (n = 6) and rapamycin (n = 5) in male in the left panel and vehicle (n = 6) and rapamycin (n = 5) in female in the right panel. No statistical differences were detected when all biological replicates shown here were included in the analyses. However, when considering the 4th vehicle replicate in the male vehicle and rapamycin groups as an outlier, mean value of pAMPK/AMPK for vehicle was less compared to rapamycin as visualized in the scatter blot below the western blots.

Additional file 9: Table S1. Kidney functional parameters and phenotypic characterization of male and female vehicle and treatment groups.

Additional file 10: Table S2. Podocyte-specific sequencing data – statistical analyses. Differential expression between male & female vehicle groups. Differential expression between male & female rapamycin-treated groups. Log2FC (before & after shrinkage) and *P*-values for the rapamycin effect differential expression in each sex. The combined *P*-value represents minus the logarithm (base 10) of the product between male & female *P*-values. List of genes that had a statistically significant interaction of rapamycin treatment effect between the sexes.

Additional file 11: Table S3. Podocyte-specific sequencing data – TPMs. Transcripts per million, for each sample. Top 100 intrinsically sexually dimorphic genes (y-chromosomal genes excluded) with DE parameters

Additional file 12: Table S4. Intrinsically sexually dimorphic estrogen target genes.

Additional file 13: Table S5. Intrinsically sexually dimorphic transcriptional regulator genes.

Additional file 14: Table S6. EnrichR analysis (KEGG, Wikipathway, MSigDB Hallmark) of intrinsic sex-DE genes for pathway enrichments in male and female podocytes.

Additional file 15: Table S7. Kidney disease-related genes.

Additional file 16: Table S8. Top hits of GSEA pathway analysis of rapamycin effect in male and female podocytes. Selective GSEA pathway related to inflammation and immune response of rapamycin effect in male and female podocytes.

Additional file 17: Table S9. Ingenuity pathway analysis of rapamycin effect in male and female podocytes.

Additional file 18: Table S10. Immune-related genes with intrinsic sexual dimorphism and regulation by rapamycin. Gene list for sublists preparation derived from Bruno et al., New insights into the immune functions of podocytes: the role of complement. Molecular and Cellular Pediatrics (2023) 10:3, https://doi.org/10.1186/s40348-023-00157-3. Sheet: "intrinsic sexual dimorphism"—DE genes related to immunity in male and female podocytes from vehicle treated mice. Sheet: "rapa effect in each sex" –DE genes related to immunity for rapamycin effect in each sex separately.

Additional file 19: Table S11. Podocyte-specific proteomics data.

Additional file 20: Table S12. Metabolomics data. List of metabolite derivatives and their biological group used for reference search. Example template – project QC sample reporting.

Additional file 21: Table S13. Podocyte isolation information and RNA quality control. Podocyte numbers, RNA quantities and RNA quality control. TPM of immediate early genes.

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**Author contributions**
DG and AK designed the study. OAD, DG, CS, RAC, PW, MIA, MMR, RFG, SB, AK, HZ carried out experiments. OAD, DG, CS, EB, PW, MMR, RFG, JK, FG, SB and AK analyzed the data. OAD, DG, EB, MMR, SB, RFG and AK created the figures. AK and OAD drafted the paper, OAD, DG, CS, RFG, EB, MMR, JK, NR, DB, TBH and AK revised the paper. All authors approved the final version of the manuscript.

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**Availability of data and materials**
Data generated and analyzed during this study are included in this published article and its supplementary information files. Further raw data supporting the findings of this study are openly available in the NCBI´s Gene Expression Omnibus repository [177], and are accessible through GEO Series accession number GSE276967 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE276967].

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The material reported in this manuscript is original. The submission contains material that has previously formed part of a PhD thesis and has been made publicly available according to the requirements of Charité Universitätsmedizin Berlin awarding the qualification (https://refubium.fu-berlin.de/handle/fub188/38211). All authors approve the manuscript and declare that they have no competing interests. JK is a consultant for Centogene GmbH.

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