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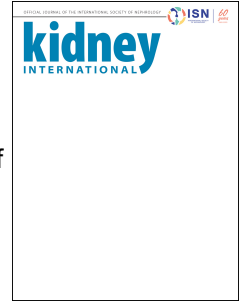
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Calpastatin prevents Angiotensin II-mediated podocyte injury through maintenance of autophagy

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Calpastatin prevents Angiotensin II-mediated podocyte injury through maintenance of autophagy

Wild-type

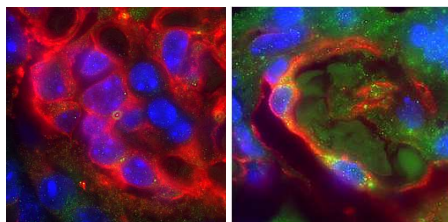


+ Angiotensin II

+ 3% NaCl Food

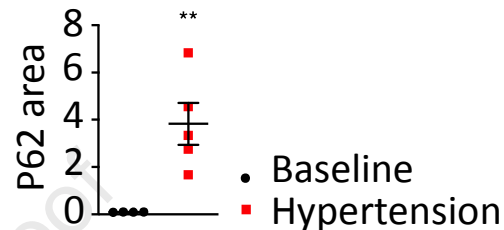


P62/PODXL



Baseline

Hypertension



AngII-mediated autophagy blockade

ATG5 Δ podocyte

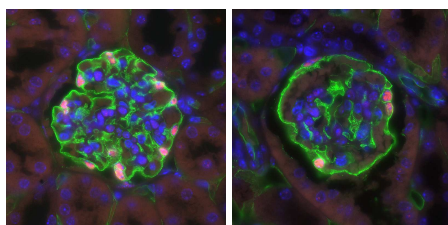


+ Angiotensin II

+ 3% NaCl Food

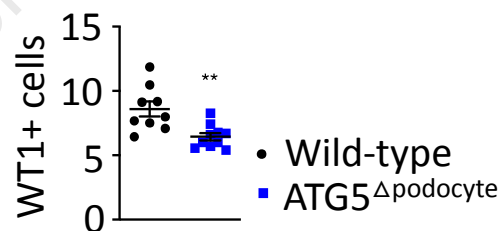


NPHS1/WT1



Wild-type

ATG5 Δ podocyte



Autophagy deficiency promotes AngII-mediated podocyte injury

Calpastatin^{Tg}

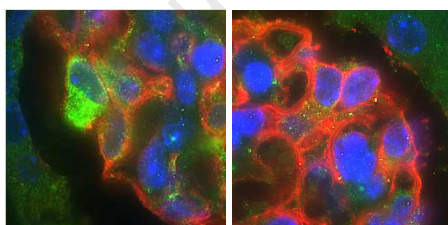


+ Angiotensin II

+ 3% NaCl Food

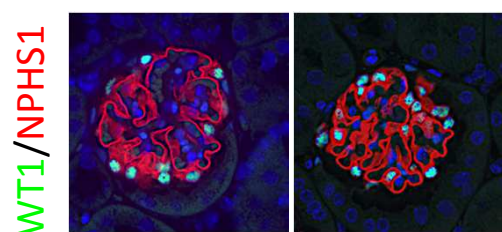


P62/PODXL



Wild-type

Calpastatin^{Tg}



Wild-type

Calpastatin^{Tg}

Calpastatin prevents AngII-mediated autophagy blockade and podocyte injury

CONCLUSION:

Calpastatin overexpression prevents angiotensin II-mediated podocyte injury through maintenance of autophagy

1 **[QUERY TO AUTHOR: title and abstract rewritten by Editorial Office – not subject to change]**

2 **Calpastatin prevents Angiotensin II-mediated podocyte injury through maintenance of autophagy**

3

4

5 **Running title: AngII-modulated autophagy in hypertensive nephropathy**

6

7

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15

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17

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19

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29

30

1 **ABSTRACT**

2

3 The strong predictive value of proteinuria in chronic glomerulopathies is firmly established as
4 well as the pathogenic role of angiotensin II promoting progression of glomerular disease
5 with an altered glomerular filtration barrier, podocyte injury and scarring of glomeruli. Here
6 we found that chronic angiotensin II-induced hypertension inhibited autophagy flux in mouse
7 glomeruli. Deletion of *Atg5* (a gene encoding a protein involved autophagy) specifically in the
8 podocyte resulted in accelerated angiotensin II-induced podocytopathy, accentuated
9 albuminuria and glomerulosclerosis. This indicates that autophagy is a key protective
10 mechanism in the podocyte in this condition. Angiotensin-II induced calpain activity in
11 podocytes inhibits autophagy flux. Podocytes from mice with transgenic expression of the
12 endogenous calpain inhibitor calpastatin displayed higher podocyte autophagy at baseline that
13 was resistant to angiotensin II-dependent inhibition. Also, sustained autophagy with
14 calpastatin limited podocyte damage and albuminuria. These findings suggest that
15 hypertension has pathogenic effects on the glomerular structure and function, in part through
16 activation of calpains leading to blockade of podocyte autophagy. These findings uncover an
17 original mechanism whereby angiotensin II-mediated hypertension inhibits autophagy via
18 calcium-induced recruitment of calpain with pathogenic consequences in case of imbalance
19 by calpastatin activity. Thus, preventing a calpain-mediated decrease in autophagy may be a
20 promising new therapeutic strategy for nephropathies associated with high renin- angiotensin
21 system activity.

22

23 **Keywords:** Angiotensin II, autophagy, podocyte, calpastatin, hypertension.

24

25

1 **TRANSLATIONAL STATEMENT**

2 Given the crucial role of autophagy in the development of kidney diseases, pharmacological
3 modulation of autophagy might be a promising strategy for the prevention and treatment of several
4 renal diseases. In parallel, overactivation of calpain activity in podocytes was found to play
5 detrimental effects on podocyte function whereas its deleterious mechanisms of action were not
6 identified. Here, we provide evidence that calpain links the deleterious action of angiotensin II to the
7 detrimental blockade of autophagy in podocyte and suggest that calpain inhibition could be a
8 promising therapeutic target for podocyte diseases partially through maintenance of podocyte
9 autophagy.

10

11 **INTRODUCTION**

12 Hypertension is second only to diabetes as a leading cause of progressive chronic kidney disease¹⁻³
13 and even modest elevation in blood pressure is an independent risk factor for end-stage kidney
14 disease⁴. An increasing number of experimental studies have highlighted the importance of podocyte
15 in the development of kidney injury. Progressive loss of podocytes and microvascular alterations
16 appear early with the functional renal decline in experimental hypertensive nephropathy⁵. In
17 patients, urinary excretion of viable podocytes was shown to be a sensitive and specific marker for
18 preeclampsia^{6, 7} and patients with nephrosclerosis had a significantly lower density of glomerular
19 podocytes than did kidney donors^{8, 9}. Furthermore, the pathogenic role of angiotensin II (AngII)
20 promoting progression of glomerular disease is well established, not only in hypertensive conditions
21 but also in several glomerular diseases¹⁰⁻²¹.

22 Glomerular hypertension results in glomerular capillary stretching, endothelial damage and elevated
23 glomerular protein filtration causing glomerular collapse and glomerulosclerosis. It also exerts a
24 direct action on glomerular structures, causing signaling regulatory responses aimed to compensate.
25 Activated systemic and local renin-angiotensin-aldosterone system (RAAS), fosters mesangial
26 hyperplasia and synthesis of vascular permeability factors. Concomitantly, podocytes display calcium

1 signaling^{22, 23} and modify their shape upon AngII type 1 receptor (AT1) dependent stimulation²⁴⁻²⁸.
2 These adaptive mechanisms become maladaptive in the long term, finally leading to
3 glomerulosclerosis. AT1 mediates prominent RAAS involvement for blood pressure and salt and
4 water homeostasis. Angiotensin-converting enzyme inhibitors (ACEI) and AT1 blockers are clinically
5 used for the treatment of hypertension and heart failure in patients. Interestingly, both blockers also
6 show a protective effect on kidney function.

7 Autophagy was demonstrated to be essential for the maintenance of cellular homeostasis,
8 particularly in post-mitotic cells^{29, 30}, and notably podocytes³¹⁻³⁴. Autophagy is a lysosomal-associated
9 degradation system for long-lived cytoplasmic proteins and dysfunctional organelles^{35, 36} and involves
10 sequestration of proteins and organelles in autophagosomes. The formation of autophagosomes is
11 dependent on the induction of several genes including *Map1lc3a/b*, *Beclin 1*, and *Atgs*³⁷. There is
12 growing evidence that dysregulation of the autophagic pathway is implicated in the pathogenesis of
13 kidney aging and several renal diseases such as acute kidney injury (AKI), polycystic kidney disease
14 (PKD), aging, and diabetic nephropathy (DN)^{31, 32, 38-41}.

15 Regulation of autophagy in podocytes, in physiological, and above all, in pathological context, is not
16 well known. We recently demonstrated that podocyte autophagy is independent of mTOR regulation
17 in physiological condition, making this cell type an exception⁴². AT1 activation stimulates protein
18 synthesis and protein turnover in cells. Thus, we reasoned that activation of the RAAS may also
19 influence protein turnover stimulation and proteostasis.

20 In the present study we focused on the role of AngII signaling in podocyte autophagy regulation. We
21 identified the calcium-activated proteases calpains mediating a chronic blocking effect of AngII on
22 podocyte autophagy. Further, we found that the endogenous calpain inhibitor calpastatin was able
23 to prevent AngII-dependent autophagy inhibition and podocyte injury during hypertension.

24 These findings uncover an original mechanism whereby AngII-mediated hypertension inhibits
25 autophagy *via* calcium-induced recruitment of calpain with pathogenic consequences in case of
26 imbalance by calpastatin activity.

1

2 **METHODS**

3 **Animals**

4 Calpastatin-Tg mice were kindly provided by Dr E. Letavernier⁴³. Mice with a podocyte-specific
5 disruption of the *Atg5* gene (*Nphs2.cre Atg5^{lox/lox}*), generated as previously described³¹ by crossing
6 *Nphs2.Cre* mice⁴⁴ with *Atg5^{lox/lox}* mice⁴⁵ on C57BL6/J background. *Nphs2.cre Atg5^{lox/lox}* mice and
7 control littermates males, aged 10–12 weeks, were used in this study. Hypertensive model was
8 induced by subcutaneous infusion of AngII (Sigma-Aldrich, A9525) at a dose of 1 µg/kg/min for 4 to 6
9 weeks *via* osmotic minipumps (Alzet Corp, Model 2006). Pumps were implanted subcutaneously on
10 the back between the shoulder blades and hips. Mice received salt supplementation (3% NaCl) in
11 food. *Atg5^{lox/lox}* (wild type, WT) mice were used as controls in all studies. For DOCA-salt with nephron
12 reduction model, adult male underwent unilateral left nephrectomy. 2 weeks after nephrectomy,
13 they received deoxycorticosterone acetate (DOCA) pellets with 21-day release (Innovative Research
14 of America) implanted sub-cutaneously. A second pellet was implanted 3 weeks after the first
15 implant. All mice received 0,9% NaCl in drinking water ad libitum and were sacrificed after 6 weeks of
16 DOCA administration⁴⁶. Experiments were conducted according to the French veterinary guidelines
17 and those formulated by the European Community for experimental animal use (L358-86/609EEC),
18 and were approved by the French Ministry of Research and local University Research Ethics
19 Committee (APAFIS-7646 and -22373).

20

21 **Primary podocyte culture experiment**

22 Culture of differentiated primary podocytes was performed as previously described^{47, 48}. Briefly,
23 freshly isolated renal cortex was mixed and digested by collagenase I (Gibco-17100-017) in RPMI
24 1640 (Life Technologies, 61870-044). Tissues were then passed through 70 µm and 40 µm cell
25 strainers (BD falcon-352340 and -352350). Glomeruli, which adhere to the 40 µm cell strainer, were
26 removed with phosphate-buffered saline (PBS; Life Technologies, 10010023) + 0.5% BSA (Eurobio-

1 HALALB07-65) injected under pressure, and were then washed twice in PBS. Freshly isolated
2 glomeruli were plated in 6-well dishes in RPMI 1640 (Gibco-61870036) supplemented with 10% fetal
3 calf serum and 1% penicillin/streptomycin (Life Technologies-15140122) to allow podocytes to exit
4 from glomeruli and grow. Podocyte enrichment was verified by western blot as previously
5 described^{31, 48, 49} (**Fig.S1**). Podocytes were cultured in the absence or presence of bafilomycin A1 (100
6 nmol/L, Sigma-Aldrich-B1793) for 4 hours. For immunofluorescence experiments, primary podocytes
7 were plated on 4 dishes labtek (Dutscher-055071). Podocytes were then fixed in PFA 4% for 10
8 minutes and processed for immunofluorescence.

9

10 **Calpain activity assay**

11 Intracellular calpain activity was determined in primary podocytes, as previously described⁵⁰⁻⁵². 100
12 000 cells were cultured in 24-well tissue culture dishes in RPMI 1640 supplemented with 10% fetal
13 calf serum and 1% penicillin/streptomycin. After the indicated culture period, the medium was
14 replaced with KRH solution (pH 7.4) containing 4 mM CaCl₂, with or without 10 μ M calpain inhibitor-
15 1, and incubated for 10 minutes before the addition of 50mM calpain substrate N-succinyl-Leu-Leu-
16 Val-Tyr-7-amino-4-methylcoumarin (Sigma-Aldrich-S6510). After a 90-minute incubation period,
17 calpain activity was determined as the difference between fluorescence (measured at 360 nm
18 excitation and 430 nm emission) with and without calpain inhibitor-1.

19

20 **Western blot**

21 Primary podocytes were scratched with 80 μ l of RIPA buffer containing phosphatase and protease
22 inhibitor. Protein concentration was measured with BCA Protein Assay Kit (Merck Biochemistry-
23 71285). Twenty micrograms of proteins were electrophoresed on Criterion TM XT precast gel (12%
24 Bis-tris, Biorad-3450124). The proteins were transferred into polyvinylidene difluoride membrane
25 (Thermofischer scientific-88518). After blocking in 5% milk in TBS-T, membranes were incubated with
26 rabbit polyclonal anti-LC3 (1:1000, cell signaling technology-#2575), rabbit polyclonal anti-ATG5

1 (1:2000, cell signaling technology-#2630), guinea-pig polyclonal anti-SQSTM1/P62 (1:10000, Progen-
2 GP62), rabbit polyclonal anti-calpain 1 domain IV, (1:1000, Abcam-ab39170), rabbit polyclonal anti-
3 calpain 2 amino-terminal end domain I (1:1000, Abcam -ab 39165) and mouse monoclonal IgG1 anti-
4 Calpain 4 (1:1000, Santa Cruz biotechnology-sc-32325), rabbit anti-podocin (1:1000, Abcam-
5 Ab50339), Guinea-pig anti-nephrin (1:500, Progen-GP-N2), rat monoclonal anti-TUBA/tubulin
6 antibody (1:5000, Abcam-ab6160). After washing, membranes were incubated with horseradish
7 peroxidase linked antibody (1:2000, cell signaling technology-7074,7076,7077). The detection of
8 specific signals was performed using the ECL Chemiluminescent Kit (Biorad-170-5070) on a LAS4000
9 device (Fuji). Densitometry analysis with Image J software (NIH) was used for quantification.

10

11 **Blood pressure measurements and physiological assessments**

12 Systolic blood pressure (SBP) of mice was recorded by the tail-cuff method (Visitech Systems Inc., BP-
13 2000). Ten measurements from each mouse were taken and then a mean value was determined. SBP
14 was measured at baseline (12-weeks of age) and then weekly until the end of the treatment period.
15 All mice were placed in metabolic cages with free access to water for 6-hour urine collection. Urinary
16 creatinine and plasma urea concentrations were analyzed spectrophotometrically by a colorimetric
17 method (Olympus, AU400). Urinary albumin excretion was measured using a specific ELISA assay for
18 the quantitative determination of albumin in mouse urine (Crystal chem-80630).

19

20 **Histology**

21 Kidneys were harvested and fixed in 4% PBS-buffered formalin. Paraffin-embedded sections (3 μ m
22 thick) were stained by Masson's trichrome to evaluate kidney morphology. Abnormalities in kidneys
23 were graded based on the presence and severity of component abnormalities, including
24 glomerulosclerosis, mesangial expansion, tubular atrophy or casts and fibrosis. The proportion of
25 sclerotic glomeruli was evaluated by a blind examination of at least 50 glomeruli per kidney section.

26

1 **Immunofluorescence staining of kidney sections and primary podocytes**

2 Fixed primary podocytes were blocked in TBS-T 3% BSA, and incubated overnight at 4°C with primary
3 antibodies guinea-pig anti-SQSTM1/P62 (1:1000, GP-62C, Progen) and rabbit anti-GFP (1:500, ab290,
4 Abcam). After TBS-T rinses, fluorophore-conjugated secondary antibodies, donkey anti-guinea-pig
5 IgG AF594-conjugated antibody (Jackson ImmunoResearch-706-585-148) and donkey anti-rabbit IgG
6 AF488-conjugated antibody (Invitrogen-A21206), were applied. Images were taken using a Zeiss 2
7 fluorescent microscope, AxioCam HRC camera and the Axiovision 4.3 software.

8 For FFPE kidneys, sections (3 µm) were deparaffinized, hydrated and antigen retrieval was performed
9 in heated citrate buffer pH=6. Sections were then permeabilized with Triton 0,1% and blocked in TBS-
10 T 3% BSA before overnight antibody incubation at 4°C. We used a goat anti-NPHS1/Nephrin antibody
11 (1:100, Progen-GP-N2), a guinea-pig anti-SQSTM1/P62 (1:1000, Progen- GP-62C), a rabbit anti-GFP
12 (1:1000, Abcam-ab290), a goat anti-PODXL (1:1000, Bio-techne-AF-1556) and a rabbit anti-WT1
13 (1:100, Abcam-ab192). Secondary antibodies were Alexa-488 and Alexa-568 conjugated antibodies
14 from Invitrogen. Nuclei were stained in blue using Hoechst. Slides were mounted using fluorescent
15 mounting medium (Dako-S3023). Photomicrographs were taken with an Axiophot Zeiss
16 photomicroscope (Jena) and the Axiovision software. Semi-automatic quantifications on Fiji were
17 used for quantifications of NPHS1+ and PODXL+ areas per glomerular section on at least 30 glomeruli
18 per mouse. Podocyte number was counted as the number of WT1+ nuclei per glomerular section on
19 at least 30 glomeruli per mouse.

20

21 **Transmission Electron Microscopy Procedure**

22 Small pieces of renal cortex (1 mm³) were fixed in 3% glutaraldehyde EM grade (Electron Microscopy
23 Science) for 1-to-30 days, and washed 3 times in PBS. Samples were postfixed in 1% osmium
24 tetroxide 0.1M (Electron Microscopy Science) in 0.1 M PBS (pH 7.4) and washed in water. Samples
25 were dehydrated in alcohol grades and 100% Propylene oxide (Electron Microscopy Science). Resin
26 infiltration was performed as following: mix 1:1 Epikote 812: propylene oxide 30 min followed by mix

1 1:2 Epikote 812: propylene oxide overnight room temperature. Samples were embedded in 4mm
2 gelatin capsules in 100% Epikote 812, and Polymerized in 60°C oven. Ultrathin sections were cut with
3 a Leica UFC7 ultramicrotome (Leica Microsystems GmbH) and deposited on Gilder grids 200 mesh
4 (Electron Microscopy Science). They were counterstained with uranyl acetate 7% (LFG) and Reynold's
5 lead citrate (LFG). Samples were examined in a JEOL 1011 transmission electron microscope (JEOL)
6 with an ORIUS 1000 CCD camera (GATAN), operated with Digital Micrograph software (GATAN) for
7 acquisition.

8

9 **qPCR array**

10 Freshly isolated glomeruli were frozen in Qiazol reagent at -80°C. Total RNA extraction using the
11 phenol-based method was processed according to the manufacturer's recommendations. cDNAs
12 were synthesized using the RT² first strand kit (Qiagen, 330401) and real-time PCR was performed
13 using Custom RT² profiler PCR Array (Qiagen, CLAM36771C) with RT² SYBR qPCR Mastermix (Qiagen,
14 330502). qPCR plates were run on an Applied Biosystems StepOnePlus cycler. Each array contains
15 quality control for reverse transcription efficiency and gDNA contamination. qPCR analysis was done
16 by the 2-DDCT method with help of the GeneGlobe Data Analysis Center
17 (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page) and expressed as
18 the Log₂ fold change in gene expression.

19

20 ***In silico* proteomic analysis**

21 *In silico* prediction of calpain cleavage site was done with DeepCalpainPredict
22 (<http://deepcalpain.cancerbio.info/help.php>), GPS-CCD (<http://ccd.biocuckoo.org>) and CaMPDB
23 (<http://calpain.org/>) online tools. Mouse protein amino acid sequence was obtained from Uniprot
24 (<https://www.uniprot.org>). Results are resumed in **Table S1** and **File S1**.

25

26 **Statistical analyses**

1 All graphs represent individual values and means +/-SEM. Statistical analyses were calculated using
2 GraphPad Prism software (La Jolla, CA). Comparison between two groups was performed by a
3 parametric Student t-test when samples passed the Anderson-Darling and D'Agostino normality tests
4 and F test for equality of variance. Otherwise a non-parametric Mann-Whitney test was used.
5 Comparison between multiple groups was performed by one-way or two-way ANOVA followed by
6 multiple comparison test with Sidak's correction. Values of $p < 0.05$ were considered significant. *
7 $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

8

9 RESULTS

10 ***Angiotensin II + HSD- induced hypertension inhibited podocyte autophagy***

11 Podocytes present a high level of autophagy *in vivo*, as shown by strong GFP expression in transgenic
12 mice with GFP fusion to LC3 (GFP-LC3 mice), a key marker of autophagy (**Fig.S2A**). Autophagy is a
13 dynamic process with constant formation of autophagosome and degradation of the
14 autophagolysosomes. Blocking autophagosomal degradation with chloroquine (CQ) resulted in
15 accumulation of GFP+ dots, indicating high autophagic flux in podocytes (**Fig.S2A,B**). Confirmation
16 that GFP+ dots were autophagosomes was shown by double immunofluorescence for GFP and
17 P62/SQSTM1, a chaperone protein degraded by autophagy (**Fig.S2C,D**). Again, CQ treatment induced
18 accumulation of GFP+ P62+ dots, demonstrating important autophagic flux in podocyte. Finally, high
19 autophagic flux was conserved *in vitro* as shown by GFP and P62 expression in primary podocyte
20 isolated from GFP-LC3 mice and strong accumulation of GFP+ and P62+ dots under Bafilomycin A1
21 treatment, another blocker of autophagosomal degradation (**Fig.S2E-H**).

22 The capacity of hypertension to modulate podocyte autophagic responses was then assessed in mice
23 infused with AngII with high salt diet (HSD) for 6 weeks and in non-hypertensive controls. As shown
24 in Figure 1, AngII +HSD induced P62 accumulation in glomeruli with strong accumulation in podocyte,
25 thus suggesting that AngII +HSD was responsible for podocyte autophagy blockade (**Fig.1A-D**).
26 Interestingly, P62 accumulation in podocyte was similar to the one observed in mice deficient for

1 podocyte autophagy (*Nphs2-Cre Atg5^{lox/lox}* mice). In another model of hypertension, the DOCA-salt
2 model, we also observed progressive P62 accumulation in podocytes along the time course of the
3 disease (**Fig.S3**).

4

5 **Deletion of *Atg5* specifically in podocytes results in increased albuminuria, podocyte loss and** 6 **glomerular injury in AngII + HSD model**

7 We then examined whether autophagy blockade only in podocytes (*Nphs2-cre Atg5^{lox/lox}* mice)
8 affects glomerular injury in AngII + HSD model. We first confirmed that *Nphs2-cre Atg5^{lox/lox}* mice had
9 normal blood pressure, normal kidney function and no glomerular histological lesions until 10
10 months of age, as previously reported (**Fig.S4**)³². Then, *Atg5^{lox/lox}* (WT) and *Nphs2-cre Atg5^{lox/lox}* mice
11 were infused with AngII with HSD for 6 weeks. Importantly, systolic blood pressure was similar in the
12 two groups after AngII infusion during the course of the study (**Fig.2A**), although the tail-cuff method
13 used to measure blood pressure might not have the ability to resolve small blood pressure
14 differences. AngII infusion with HSD markedly increased urinary albumin-to-creatinine ratio in WT
15 mice, and this effect was further significantly increased in *Nphs2-cre Atg5^{lox/lox}* mice (**Fig.2B**).
16 Hypertensive *Nphs2-cre Atg5^{lox/lox}* mice also displayed significantly increased glomerular sclerosis
17 when compared to WT littermates (**Fig.2C-E**). In agreement with the measured proteinuria,
18 proteinaceous casts and tubular dilatation were significantly more prevalent in mice with podocyte
19 deficiency in ATG5 (**Fig.2F-H**).

20 Podocyte number per glomerulus was significantly decreased in *Nphs2-cre Atg5^{lox/lox}* mice treated
21 with AngII + HSD (**Fig.2I-K**). Podocyte injury in *Nphs2-cre Atg5^{lox/lox}* mice with AngII infusion + HSD
22 even progressed to focal and segmental glomerulosclerosis (FSGS) as shown by expression of the PEC
23 activation marker CD44 in glomeruli (**Fig.2L,M**). Electron microscopy analysis identified significant
24 changes associated with ATG5 deficiency upon chronic AngII infusion with HSD, including foot
25 process effacement in hypertensive *Nphs2-cre Atg5^{lox/lox}* mice. By contrast, few ultrastructural
26 defects were found in podocytes from WT mice even after 10 weeks of AngII infusion with HSD

1 **(Fig.2N,O)**; indicating that the autophagic activity of podocytes is required for their resistance to
2 AngII + HSD-induced damage. Altogether, our results indicated that, in AngII + HSD model, autophagy
3 inhibition aggravates podocyte injury and loss and induces subsequent FSGS.

5 **AngII + HSD activates calpain activity in podocyte that contributes to autophagy blockade**

6 As calpain activity was found (1) to be activated by AngII in several cell types and (2) to cleave several
7 autophagy-related proteins⁵³, we wondered if AngII + HSD-mediated autophagy blockade could be
8 attributable to increased AngII-induced calpain activity. We first found that primary podocytes
9 expressed the three ubiquitous forms of calpains (**Fig.3A**). We then showed that AngII stimulated
10 calpain activity in primary podocytes. This rise in calpain activity was blocked by a selective calpain
11 inhibitor (**Fig.3B**). We used a knock-in mouse with extra calpastatin transgene expression (leading to
12 decreased calpain activity)⁴³, to assess the role of calpains in AngII + HSD-mediated renal injury and
13 autophagy blockade. Primary podocytes from calpastatin transgenic mice (CST^{Tg}) showed decreased
14 calpain activity in response to AngII when compared to podocytes from control mice (**Fig.3C**). Thus,
15 calpastatin overexpression in podocyte decreased AngII-mediated calpain activation.

16 We next assessed autophagy levels in podocyte from CST^{Tg} mice. We generated CST^{Tg} mice with GFP-
17 LC3 transgene. LC3-GFP reporter allowed counting of autophagosomes as GFP+/P62+ dots. P62 will
18 accumulate in aggregates in cells when autophagic flux is blocked. At basal state, we counted less
19 GFP+ P62+ dots (**Fig.4A,B,E**) and less P62 accumulation (**Fig.4C,D,F**) in podocytes of the CST^{Tg} GFP-LC3
20 mice than in podocytes of the normal GFP-LC3 mice, suggesting increased autophagic flux in
21 podocytes of mice with high calpastatin abundance.

22 Autophagic flux can be monitored by measurement of the conversion of the cytoplasmic form of LC3,
23 LC3-I, to the autophagosomal cleaved and phosphatidyl-ethanolamine (PE)-coupled form of LC3, LC3-
24 II, on Western blot. Podocytes from CST^{Tg} mice showed increased LC3-I to LC3-II conversion and
25 decreased P62 expression, both in presence and absence of Bafilomycin A₁ (**Fig.5A,B**), indicating
26 increased autophagic flux in podocytes with calpastatin overexpression. Finally, accumulation of

1 GFP+ P62+ dots in podocytes after chloroquine administration was more important in CST^{Tg} GFP-LC3
2 mice than in GFP-LC3 mice, thus confirming that calpastatin overexpression induces autophagic flux
3 in podocytes *in vivo* (**Fig.5C-E**). Altogether, our data suggest that AngII stimulates calpain activity in
4 podocytes, autophagy is inhibited by calpain in podocytes and that inhibition of the endogenous
5 calpain activity by calpastatin overexpression is sufficient to stimulate autophagic flux in podocytes.

7 **Calpastatin Tg mice are protected from AngII + HSD-induced podocyte injury**

8 CST^{Tg} mice did not show any renal alteration until at least 12 months-old (**Fig.S5**). We analyzed
9 podocyte injury in CST^{Tg} mice during Ang II + HSD treatment. While WT mice developed mild
10 glomerulosclerosis and podocyte injury after four weeks of hypertension, as shown by abnormal
11 expression of podocalyxin and nephrin; CST^{Tg} mice presented less sclerotic glomerular lesions
12 (**Fig.6A-B**) and preserved podocalyxin and nephrin expression (**Fig.6C-H**). Such differences in
13 podocyte phenotype were observed at a stage where the density of podocyte nuclei, was not
14 different between WT and CST^{Tg} hypertensive mice (**Fig.6I-K**).

15
16 Similarly, after 6 weeks of hypertension, GFP-LC3 and GFP-LC3 CST^{Tg} mice presented podocyte injury
17 but lesions were more prominent in the GFP-LC3 mice (**Fig.7**). Urine albumin-on-creatinine ratio was
18 higher in GFP-LC3 mice after 4 weeks of AngII + HSD (**Fig.7A**). Podocyte number was not different
19 between the two genotypes but nephrin expression was significantly more decreased in the GFP-LC3
20 control mice (**Fig.7B-E**). Correlating with CST-mediated protection, ultrastructural analysis mild and
21 focal podocyte foot process effacement in GFP-LC3 mice treated with AngII + HSD with better
22 preservation of the foot process in the CST^{Tg} mice, although the global quantification of the number
23 of foot process per GBM length was not statistically different, most likely because because podocyte
24 injury keeps focal and segmental in our model (**Fig.7F-H**). Of note, macrophages and T lymphocyte
25 infiltration in kidneys were not different between the groups (**Fig.S6**). Taken together these results
26 demonstrated that calpastatin prevented AngII + HSD-induced podocyte injury.

1

2 **Calpastatin overexpression restores autophagic flux in podocyte in mice after AngII + HSD** 3 **treatment**

4 Finally, we wondered if calpastatin-mediated glomerular protection in the AngII + HSD model
5 implicated autophagy maintenance in podocyte. Interestingly, AngII + HSD-induced P62 accumulation
6 in podocyte was prevented in CST^{Tg} and GFP-LC3 CST^{Tg} mice (**Fig.8A-D**), indicating that calpastatin
7 prevented blockade of podocyte autophagy. To note, the number of GFP+ P62+ dots labeling of
8 autophagosomes, were similar in podocyte from hypertensive GFP-LC3 and GFP-LC3 CST^{Tg} mice, thus
9 suggesting that AngII + HSD induced a slowdown of the podocyte autophagic flux rather than a
10 complete arrest (**Fig.8E-G**).

11

12 ***In silico* prediction of calpain cleavage site in podocyte proteins**

13 We used several *in silico* tools to predict potential calpain targets in podocyte (**Table S1 and File S1**).
14 Three online database were compared: GPS-CCD⁵⁴, CaMPDB⁵⁵ and DeepCalpain⁵⁶. *In silico* analysis
15 identified several podocyte proteins, nephrin and podocin among them, that could be cleaved by
16 calpains. Thus, calpastatin-mediated glomerular protection could be linked to a reduction of calpain
17 enzymatic activity leading to reduced degradation of some podocyte protein.

18 Furthermore, at least three autophagy-related proteins are direct targets of calpains. ATG5 is cleaved
19 by calpains, leading to a disturbance of the ATG12-ATG5 complex formation^{57,58}. Administration of
20 calpain inhibitors *in vivo* also prevented cleavage of the autophagy protein beclin-1⁵⁹. *In silico*
21 analysis of putative cleavage site on autophagy-related proteins support the hypothesis that calpain
22 could regulate autophagy through enzymatic cleavage of autophagy proteins.

23

24 **mRNA expression of ER and oxidative stress markers in glomeruli from mice treated with AngII +** 25 **HSD.**

26 We evaluated ER stress and oxidative stress by qPCR in glomeruli during AngII + HSD treatment. At

1 baseline, we did not observe any change in mRNA expression of analyzed genes in glomeruli from WT
2 and *Nphs2.cre Atg5^{lox/lox}* mice (**Fig.S7**). After 6 weeks of hypertension, glomeruli from *Nphs2.cre*
3 *Atg5^{lox/lox}* mice showed different mRNA profile of genes of the ER stress and oxidative stress
4 pathways with increased expression of *Sod1*, *Prdx1*, *Atf4*, *Gpx1* and *Hsp90b1* as compared to WT
5 glomeruli, thus suggesting that autophagy depletion in podocyte favoured AngII + HSD-induced ER
6 stress and oxidative stress. Conversely, glomeruli from *CST^{Tg}* mice presented downregulation of
7 several genes of the ER stress and oxidative stress pathways as well as decreased expression of some
8 pro-apoptotic genes (**Fig.9**).

9 Taken together, these results indicate that calpastatin overexpression could prevent glomerular
10 injury by reducing AngII + HSD-induced ER and oxidative stress.

11

12 DISCUSSION

13 In the present study, we demonstrated that, in AngII + HSD-induced hypertension, podocyte
14 autophagy is markedly downregulated. Furthermore, mice with podocyte-specific deletion of *Atg5*
15 were more prone to AngII + HSD-induced glomerulosclerosis and podocyte loss, thus showing that
16 autophagy in podocytes prevents development of hypertensive nephropathy, highlighting the critical
17 role of autophagy in AngII + HSD-induced podocyte injury. Little is known about the extracellular
18 stimuli that regulate cellular autophagy and these results shed light on the pathophysiological
19 regulation of podocyte autophagy by AngII.

20 In most human glomerulopathies, podocyte foot process effacement is a hallmark of glomerular
21 injury leading to proteinuria. Autophagy is likely to play an essential role in maintaining podocyte
22 function because these terminally differentiated cells display high rates of autophagy even in the
23 absence of stress. A previous study showed that AngII promotes autophagy through the generation
24 of ROS in a conditionally immortalized murine podocyte cell line⁶⁰. ROS production is indeed a
25 general inducer of autophagy in many cell types and the reasons for such discrepancy with our
26 findings are unclear. Unlike this latter study, we used murine primary cultured podocytes retaining

1 Podocin and Nephrin expression, and *in vivo* approaches and not murine cell line. We confirm
2 previous reports that postmitotic podocytes exhibit an unusually high level of constitutive
3 autophagy. Measurement of increased amount of LC3-II after AngII stimulation in the presence or
4 absence of lysosomal inhibitors is necessary to determine if the autophagic flux is increased or
5 blocked. Previous studies have neglected this phenomenon⁶⁰.

6 Here, we used a hypertensive model based on AngII perfusion and high salt diet. Podocyte exhibit
7 AT1 receptor and are exposed to freely filtered peptides such as AngII^{13, 16, 26, 61-65}. It is assumed that
8 the observed renoprotective effects of RAAS inhibition could be – at least partially – due to a
9 blockade of this podocyte-specific RAAS. Likewise, *in vivo* studies confirmed the effect of AngII on
10 podocyte injury and podocyte-specific gene targeting of AT1 demonstrated that activation of AT1
11 receptors in the glomerulus in experimental lupus nephritis is sufficient to accelerate renal injury in
12 the absence of hypertension^{66, 67}. Calpain-mediated autophagy dysregulation in our model could be
13 linked to a direct AngII-AT1 signaling on podocyte or to be a consequence of hypertension. We may
14 provide an early answer to this question. Indeed, in the DOCA-salt model, we also found P62
15 accumulation in podocyte from hypertensive mice (**Fig.S3**). This suggests that autophagy blockade
16 occurs in podocyte in this model, which is supposed to be independent of AngII⁶⁸. Further studies
17 evaluating podocyte injury pertaining to calpain activity and autophagic flux in mice deficient for AT1
18 in podocyte selectively would be required to delineate if such regulation of podocyte autophagy
19 depends on direct or indirect effects of AngII.

20

21 Calpains 1 and 2 are ubiquitous pro-inflammatory proteases, whose activity is controlled by
22 calpastatin, their specific inhibitor. Indeed, calpastatin inhibits selectively calpains and no other
23 proteases to date. Calpain activation has been linked recently to renal injury in several pathological
24 contexts^{69,70}. The calcium channel transporter Transient receptor potential channel C6 (TRPC6) was
25 found to activate calpain-1 in podocyte *via* a Ca²⁺/calcineurin activation. Kidneys of patients with
26 FSGS had increased TRPC6 expression, increased calpain and calcineurin activity, and reduced

1 expression of the calpain target Talin-1, which is critical for podocyte cytoskeletal stability⁷¹. TRPC6
2 also directly binds to calpain-1 and calpain-2. This interaction is crucial for the regulation of Talin-1
3 cleavage and control motility of podocytes⁷².

4 Transgenic mice over-expressing calpastatin are protected against vascular remodeling and AngII-
5 dependent inflammation⁷³, against inflammation in models of glomerulonephritis⁴³, sepsis⁷⁴ or
6 allograft rejection⁷⁵ and against aged-related inflammation⁷⁶. Podocyte injury in these mice has not
7 been explored. Letavernier et al. showed that overexpression of CST prevented AngII-dependent
8 perivascular inflammation in kidneys⁴³. Thus, renal protection in CST^{Tg} mice could be mediated, at
9 least partially, by an anti-inflammatory mechanism. We evaluate macrophages and lymphocytes
10 infiltration in our model and found no significant difference in renal inflammation between CST^{Tg} and
11 control mice when analyzing global renal leukocyte infiltration (**Fig.S6**).

12

13 Calpain were involved recently in autophagy regulation (reviewed recently in Weber 2019⁵³) with
14 interesting endothelio-protective properties of calpain inhibition in diabetic context through
15 restoration of autophagy⁷⁷. Here, we reported that calpain inhibition through calpastatin
16 overexpression prevented i) podocyte injury during hypertension and ii) restore autophagy in
17 podocytes, thus highlighting a novel deleterious role of calpain activation during hypertension
18 through the inhibition of autophagy.

19 Nearly all ATG proteins were shown to be cleaved by calpains *in vitro*⁷⁸. Here we postulated that
20 autophagy maintenance in hypertensive CST^{Tg} mice was mediated by calpain inhibition. To support
21 our hypothesis, we showed that podocytes from CST^{Tg} have a decreased calpain activity when
22 challenged with AngII *in vitro* (**Fig.3C**). We found increased ATG5 protein level in podocytes from
23 CST^{Tg} mice, which suggest that calpastatin overexpression prevented calpain-mediated ATG5
24 cleavage in this context (**Fig.5A**). On the other hand, it was demonstrated that CST-mediated calpains
25 inhibition could be independent of the inhibition of their protease activity⁷⁹, thus we could not
26 exclude a regulation of autophagy by calpastatin independent to calpain enzymatic activity.

1 In summary, these findings revealed a previously unrecognized role for calpastatin in the regulation
2 of podocyte autophagy and provide a lead for investigation of novel therapeutic strategies to
3 enhance podocyte survival during hypertensive nephropathies.

4

5 **DISCLOSURE**

6 The authors declare no competing financial interests.

7

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22

1 **SUPPLEMENTARY MATERIALS**

2 **Figure S1: Primary podocyte culture expresses podocyte markers.** Western blot analysis of the
3 expression of the podocyte markers NPHS1 and NPHS2 in primary podocyte culture from WT and
4 *CST^{Tg}* mice. Tubulin (TUBA) serves as a loading control. Representative of n=4 mice per genotype.

5
6 **Figure S2: High basal level of podocyte autophagy.** (A-B) Representative immunofluorescence
7 images of the expression of GFP (green) and NPHS1 (red) in glomeruli from *GFP-LC3* mice treated or
8 not with CQ (80mg/kg) 4 hours before to sacrifice. Arrowheads show GFP+ autophagosomes. (C-D)
9 Representative immunofluorescence images of the expression of GFP (green) and P62 (red) in
10 glomeruli from GFP-LC3 mice treated or not with CQ (80mg/kg) 4 hours before to sacrifice.
11 Arrowheads show GFP+ P62+ autophagosomes. (A-D) (') represent higher magnification. Nuclei were
12 stained with Hoechst (blue). Scale bar: 50µm. N=4 mice per condition (E-H) Representative
13 immunofluorescence images of the expression of GFP (green) and P62 (red) in primary podocyte
14 from *GFP-LC3* mice treated (F, H) or not (E, G) with Bafilomycin A1 (100nM) for 4 hours. N=5 mice per
15 condition.

16
17 **Figure S3: P62 accumulates in podocytes in the DOCA-salt model of hypertension.** (A-C)
18 Representative immunofluorescence images of the expression of P62 (green) and PODXL (red) in
19 glomeruli from WT mice after 2-to-6 weeks of DOCA-salt model. (') represent higher magnification.
20 Nuclei were stained with Hoechst (blue). Scale bar: 50µm. (D) Quantification of P62 area per
21 podocyte area (%). N=5-6 mice per condition. One-way ANOVA: Time p=0.0059, Sidak's multiple
22 comparison test: D42 vs. D14 ** p=0.0055, D28 vs. D14 p=0.8590

23
24 **Figure S4: Podocyte autophagy is dispensable for podocyte development.** (A) Systolic blood
25 pressure, (B) urine albumin-to-creatinine ratio and (C) blood urea nitrogen levels in *Atg5^{lox/lox}* and
26 *Nphs2.cre Atg5^{lox/lox}* mice. N= 5-6 mice per genotype. Values are presented as individual plots and

1 means +/- sem. Mann-Whitney test: p=0.7273 (A), p=0.4286 (B), p=0.6623 (C). (D-E) Representative
 2 images of Masson's trichrome-stained sections of glomeruli from *Atg5^{lox/lox}* and *Nphs2.cre Atg5^{lox/lox}*
 3 mice. (F-G) Representative immunofluorescence images of the expression of PODXL (green) and WT1
 4 (red) in *Atg5^{lox/lox}* and *Nphs2.cre Atg5^{lox/lox}* mice. Nuclei were stained with Hoechst (blue). (D-G) Scale
 5 bar: 50µm. N=6 mice per genotype (H-I) Representative photomicrographs of transmission electron
 6 microscopy sections of glomeruli from *Atg5^{lox/lox}* and *Nphs2.cre Atg5^{lox/lox}* mice. Scale bar: 1µm. N=3
 7 mice per genotype.

8
 9 **Figure S5: Calpastatin overexpression does not influence renal function at baseline.** (A) blood urea
 10 nitrogen and (B) plasma albumin levels in 12-weeks old *GFP-LC3* and *CST^{Tg} GFP-LC3* mice. N= 5 *GFP-*
 11 *LC3* and N=6 *CST^{Tg} GFP-LC3*. Values are presented as individual plots and means +/- sem. Mann-
 12 Whitney test: p=0.6623 (A) and p=0.9307 (B). (C-D) Representative images of Masson's trichrome-
 13 stained sections of glomeruli from *GFP-LC3* and *CST^{Tg} GFP-LC3* mice. (E-H) Representative
 14 immunofluorescence images of the expression of PODXL (E-F) and NPHS1 (G-H) in *GFP-LC3* and *CST^{Tg}*
 15 *GFP-LC3* mice. (C-H) Scale bar: 50µm. (I-J) Associated quantification of PODXL and NPHS1 area per
 16 glomerular section. N= 5 *GFP-LC3* and N=6 *CST^{Tg} GFP-LC3*. Values are presented as individual plots
 17 and means +/- sem. Mann-Whitney test: p= 0.1898 (A) and p= 0.8413 (B).

18
 19 **Figure S6: Calpastatin overexpression does not influence global renal inflammation.** Representative
 20 immunohistochemistry of the expression of F4/80 (A-B) and CD3 (D-E) in *GFP-LC3* and *CST^{Tg} GFP-LC3*
 21 mice after 6 weeks of Angiotensin II +HSD. Scale bar: 200µm. (C, F) Associated quantification of F4/80
 22 and CD3 area per kidney section. N=7 *CST^{Tg} GFP-LC3* and N=8 *GFP-LC3* mice. Values are presented as
 23 individual plots and means +/- sem. Mann-Whitney test: p=0.3969 (C) p=0.3357 (F).

24
 25 **Figure S7: Podocyte autophagy deficiency does not induce ER stress or oxidative stress in young**
 26 **adult at baseline.** qPCR analysis of the mRNA expression of genes of the ER stress, oxidative stress

1 and apoptosis pathway by Qiagen qPCR array in glomeruli from WT and *Nphs2.cre Atg5^{lox/lox}* mice (A)
2 and WT and *CST^{Tg}* mice (B). N=4 mice per genotype. For *Nox3*, Ct >33.

3

4 **Supplementary Table 1: *In silico* prevision of calpain cleavage sites.** Calpain cleavage sites were
5 predicted in podocyte-related and autophagy-related proteins with GPS-CCD
6 (<http://ccd.biocuckoo.org>), CaMPDB (<http://calpain.org>) and DeepCalpain Predict
7 (<http://deepcalpain.cancerbio.info/help.php>)

8

9 **Supplementary File 1: Full file of *in silico* prevision of calpain cleavage sites.** Calpain cleavage sites
10 were predicted in podocyte-related and autophagy-related proteins with GPS-CCD
11 (<http://ccd.biocuckoo.org>), CaMPDB (<http://calpain.org>) and DeepCalpain Predict
12 (<http://deepcalpain.cancerbio.info/help.php>). Prediction cleavage sites are resumed for each protein
13 for GPS-CCD and DeepCalpain Predict.

14

15 **FIGURE LEGENDS**

16

17 **Figure 1: Angiotensin II +HSD-induced hypertension inhibits glomerular autophagy**

18 (A-C) Immunofluorescence of PODXL (red) and P62 (green) in glomeruli from WT mice (A, A'), from
19 WT mice after 6 weeks of Angiotensin II +HSD (B, B') and from *Nphs2.cre Atg5^{lox/lox}* mice (C, C')
20 showing accumulation of P62 in podocyte during hypertension and in podocyte-specific ATG5
21 deficient mice. P62+ dots are shown with arrowheads in the WT in (A'). Nuclei were counterstained
22 with Hoechst (blue). (') represents higher magnification. Scale bar: 50 μ m. (D) Associated
23 quantification of the P62+ area expressed as the percentage of glomerular area. N= 4 WT and N=5
24 WT with angiotensin II +HSD and *Nphs2.cre Atg5^{lox/lox}* mice. Values are presented as individual plots
25 and mean +/- sem. * p=0.0159, Mann-Whitney test.

26

1 **Figure 2: Deletion of *Atg5* specifically in podocytes results in a significant increase in albuminuria,**
 2 **kidney injury and podocyte loss after 6 weeks of AngII infusion + HSD**

3 (A) Systolic blood pressure in *Atg5*^{lox/lox} and *Nphs2.cre Atg5*^{lox/lox} mice during 36 days of Angiotensin II
 4 +HSD. N=9 mice per genotype. Values are presented as mean +/- sem. Two-way ANOVA: ns. From (B)
 5 to (M), N= 10 mice per genotype. In (B, G, H, K) values are presented as individual plots and mean +/-
 6 sem. (B) Angiotensin II +HSD resulted in a dramatic increase of albuminuria in *Nphs2.cre Atg5*^{lox/lox}
 7 mice compared with *Atg5*^{lox/lox} control mice. Two-way ANOVA: Genotype p=0.013, Time p=0.0018. (C-
 8 F) Representative images of Masson's trichrome-stained sections of glomeruli from *Atg5*^{lox/lox} control
 9 and *Nphs2.cre Atg5*^{lox/lox} mice after 6 weeks of Angiotensin II +HSD. Scale bar : 50 μ m in (C-D) and 100
 10 μ m in (E-F). (G-H) Comparison of the proportion of sclerotic glomeruli (G) and of the number of
 11 tubular casts per microscope field (H). Mann-Whitney test: ** p=0.0026 in (H) *** p=0.0003 in (G). (I-
 12 J) Representative immunofluorescence images of the expression of WT1 (red) and PODXL (green) in
 13 glomeruli from *Atg5*^{lox/lox} control and *Nphs2.cre Atg5*^{lox/lox} mice after angiotensin II +HSD for 6 weeks.
 14 Nuclei were stained with Hoechst (blue). Scale bar: 50 μ m. (K) Quantification of the number of WT1-
 15 positive cells per glomerular sections. Mann-Whitney test: ** p=0.0029 (L-M) Representative
 16 immunofluorescence images of the expression of CD44 (green) in glomeruli from *Atg5*^{lox/lox} control
 17 and *Nphs2.cre Atg5*^{lox/lox} mice after angiotensin II +HSD for 6 weeks. Nuclei were stained with Hoechst
 18 (blue). Scale bar: 50 μ m. (N-O) Representative photomicrographs of transmission electron
 19 microscopy sections of glomeruli from *Atg5*^{lox/lox} control and *Nphs2.cre Atg5*^{lox/lox} mice after
 20 angiotensin II +HSD for 6 weeks; showing podocyte foot process effacement (arrowhead) in
 21 *Nphs2.cre Atg5*^{lox/lox} hypertensive mice. Scale bar: 1 μ m. N=3 mice per genotype.

22

23 **Figure 3: Calpain expression and activity in podocytes**

24 (A) Western blot analysis of the expression of Calpain 1, Calpain 2 and Calpain 4 in primary podocytes.
 25 Tubulin (TUBA) expression serves as normalization. (B) Calpain activity was measured on primary
 26 podocyte treated or not with AngII (100nM) for 24 hours with or without calpeptin (10 μ M). N=5

1 independent experiments. Values are presented as individual plots and mean +/- sem. One-way
 2 ANOVA: Treatment $p=0.0035$, Sidak's multiple comparison test: * $p=0.0128$ AngII vs. Baseline ##
 3 $p=0.0056$ AngII + calpeptin vs. AngII (C) Calpain activity was measured on primary podocyte from WT
 4 or CST^{Tg} mice treated or not with AngII (100nM) for 24 hours. $N=7$ independent experiments. Values
 5 are presented as individual plots and mean +/- sem. Two-way ANOVA paired for treatment:
 6 Genotype $p=0.0483$, Sidak's multiple comparison test: *** $p=0.0009$ for WT AngII vs. Baseline
 7 * $p=0.0420$ for CST^{Tg} AngII vs. Baseline. # $p=0.0424$ WT vs. CST^{Tg} AngII.

8

9 **Figure 4: Evaluation of the autophagic flux in podocyte of CST^{Tg} mice**

10 (A-B) Representative immunofluorescence images of the expression of GFP (green) and P62 (red) in
 11 glomeruli from 12 weeks-old *GFP-LC3* and *CST^{Tg} GFP-LC3* mice. Arrowheads show GFP+ P62+
 12 autophagosomes (C-D) Representative immunofluorescence images of the expression of P62 (green)
 13 and PODXL (red) in glomeruli from 12 weeks-old *GFP-LC3* and *CST^{Tg} GFP-LC3* mice. (') represents
 14 higher magnification. Nuclei were stained with Hoechst (blue). Scale bar: 50 μ m. (E) Quantification of
 15 the number of LC3+ P62+ dots per podocyte. Mann-Whitney test: ** $p=0.0065$ (F) Quantification of
 16 P62+ area per glomerular section. Mann-Whitney test: * $p=0.0420$. In (E, F) $N=5$ *GFP-LC3* and $N=8$
 17 *CST^{Tg} GFP-LC3* mice. Values are presented as individual plots and mean +/- sem.

18

19 **Figure 5: Blocking autophagosomal degradation confirmed increased podocyte autophagic flux in**
 20 **CST^{Tg} mice**

21 (A) Western blot analysis of the expression of LC3, SQSTM1/P62 and ATG5 in primary podocyte from
 22 WT or CST^{Tg} mice. Tubulin (TUBA) expression serves as normalization. Podocyte were treated or not
 23 with bafilomycin A1 (100nM) for 4 hours before culture arrest (B) Quantification of the LC3 II/
 24 Tubulin and P62/tubulin ratios. $N= 10$ WT and 8 CST^{Tg} mice. Values are presented as individual plots
 25 and mean +/- sem. Two-way ANOVA paired for treatment: for LC3 II/Tubulin Genotype $p=0.0008$
 26 Treatment $p<0.0001$ and for P62/Tubulin Genotype $p=0.0884$ Treatment $p<0.0001$. Sidak's multiple

1 comparison test: for LC3 II/Tubulin *** $p < 0.0001$ WT -BafA1 vs. WT +BafA1, *** $p < 0.0001$ CST^{Tg} -
 2 BafA1 vs. CST^{Tg} +BafA1, ## $p = 0.0028$ WT +BafA1 vs. CST^{Tg} +BafA1 and for P62/Tubulin *** $p < 0.0001$
 3 WT -BafA1 vs. WT +BafA1, *** $p < 0.0001$ CST^{Tg} -BafA1 vs. CST^{Tg} +BafA1. (C-D) Representative
 4 immunofluorescence images of the expression of GFP (green) and P62 (red) in glomeruli from 12
 5 weeks-old *GFP-LC3* and CST^{Tg} *GFP-LC3* mice. (') represents higher magnification. Nuclei were stained
 6 with Hoechst (blue). Scale bar: 50 μ m. Mice were treated with CQ (80mg/kg) 4 hours before sacrifice.
 7 Arrowheads show GFP+ P62+ autophagosomes (E) Quantification of the number of LC3+ P62+ dots
 8 per podocyte. N=5 mice per genotype. Values are presented as individual plots and mean +/- sem.
 9 Unpaired t-test with equal SD: * $p = 0.0302$

10

11 **Figure 6: Calpastatin overexpression prevents AngII + HSD-mediated podocyte injury**

12 (A-B) Representative images of Masson's trichrome-stained sections of glomeruli from WT and CST^{Tg}
 13 mice after 6 weeks of AngII +HSD. Scale bar: 50 μ m. (C-G) Representative immunofluorescence
 14 images of the expression of PODXL (C-D) and NPHS1 (F-G) in WT and CST^{Tg} mice after 6 weeks of
 15 AngII +HSD and (E, H) associated quantifications. Scale bar: 50 μ m. (I-J) Representative
 16 immunofluorescence images of the expression of WT1 (green) and PODXL (red) in glomeruli from WT
 17 and CST^{Tg} mice after 6 weeks of AngII +HSD and (K) associated quantification of the number of WT1+
 18 cells per glomerular section. Nuclei were stained with Hoechst (blue). Scale bar: 50 μ m. (E, H, K)
 19 Values are presented as individual plots and mean +/- sem. N= 9 WT and N=7 CST^{Tg} mice. Unpaired t-
 20 test with equal SD: ** $p = 0.0095$ (E), * $p = 0.0249$ (H), $p = 0.7891$ (K).

21

22 **Figure 7: Calpastatin overexpression prevents AngII + HSD-mediated podocyte injury in GFP-LC3** 23 **mice**

24 (A) AngII +HSD resulted in a dramatic increase of albuminuria in *GFP-LC3* mice compared with CST^{Tg}
 25 *GFP-LC3* mice. N=8-13 mice per genotype. Values are presented as individual plots and mean +/- sem.
 26 Two-way ANOVA Genotype * $p = 0.0429$ Time $p = 0.0165$. Sidak's multiple comparison test: * $p = 0.0453$

1 *GFP-LC3* vs. *CST^{Tg} GFP-LC3* at day 28. (B-C) Representative immunofluorescence images of the
 2 expression of WT1 (green) and nephrin (red) in glomeruli from 18 weeks-old *GFP-LC3* and *CST^{Tg} GFP-*
 3 *LC3* mice after 6 weeks of AngII infusion + HSD. Nuclei were stained with Hoechst (blue). Scale bar: 50
 4 μm . Quantification of nephrin (NPHS1) area per glomerular section (D) and of the number of WT1+
 5 cells per glomerular section (E) in 18 weeks-old *GFP-LC3* and *CST^{Tg} GFP-LC3* mice after 6 weeks of
 6 AngII infusion + HSD. N=19 *GFP-LC3* and N=25 *CST^{Tg} GFP-LC3* mice. Values are presented as individual
 7 plots and mean +/- sem. Unpaired t-test with equal SD: ** p=0.0010 (D) p=0.1158 (E). (F-G)
 8 Transmission electron microscopy images of glomeruli from *GFP-LC3* and *CST^{Tg} GFP-LC3* mice after 6
 9 weeks of AngII +HSD. Arrowheads show foot process effacement. Scale bar: 1 μm (H) Quantification
 10 of the number of foot process per μm of GBM. N=3 mice per genotype. Values are presented as
 11 individual plots and mean +/- sem. Each plot represents the mean number of podocyte per μm of
 12 GBM on one continuous length of GBM. Unpaired t-test with equal SD: p=0.7512.

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14 **Figure 8: Calpastatin overexpression prevents AngII + HSD-induced autophagy downregulation in**
 15 **podocytes**

16 (A-B) Representative immunofluorescence images of the expression of P62 (green) and PODXL (red)
 17 in glomeruli from *GFP-LC3* and *CST^{Tg} GFP-LC3* mice after 6 weeks of AngII +HSD. (') represents higher
 18 magnification. Nuclei were stained with Hoechst (blue). Scale bar: 50 μm . (C-D) Quantification of
 19 P62+ area per glomerular section. N=9 WT and N=7 *CST^{Tg}* mice in (C) and N=16 *GFP-LC3* and N=16
 20 *CST^{Tg} GFP-LC3* in (D). Values are presented as individual plots and mean +/- sem. Mann-Whitney test:
 21 ** p=0.0033 (C) and ** p=0.0011 (D). (E-F) Representative immunofluorescence images of the
 22 expression of GFP (green) and P62 (red) in glomeruli from *GFP-LC3* and *CST^{Tg} GFP-LC3* mice after 6
 23 weeks of AngII +HSD. (') represents higher magnification. Arrowheads show GFP+ P62+
 24 autophagosomes. (G) Quantification of the number of LC3+ P62+ dots per podocyte. N=11 *GFP-LC3*
 25 and N=16 *CST^{Tg} GFP-LC3*. Values are presented as individual plots and mean +/- sem. Unpaired t-test
 26 with equal SD: p= 0.1014

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Figure 9: Glomerular ER stress and oxidative stress

(A) qPCR analysis of the mRNA expression of genes of the ER stress, oxidative stress and apoptosis pathway by Qiagen qPCR array in glomeruli from WT, *Nphs2.cre Atg5^{lox/lox}* and *CST^{Tg}* mice after 6 weeks of AngII +HSD. Data are represented as a heatmap of the Log₂(fold change) in gene expression. N=5 mice per genotype (B) Representation of the genes significantly upregulated or downregulated in *Nphs2.cre Atg5^{lox/lox}* vs. WT mice. (C) Representation of the genes significantly upregulated or downregulated in *CST^{Tg}* vs. WT mice. (B,C) Unpaired t-test with equal SD p <0.05.

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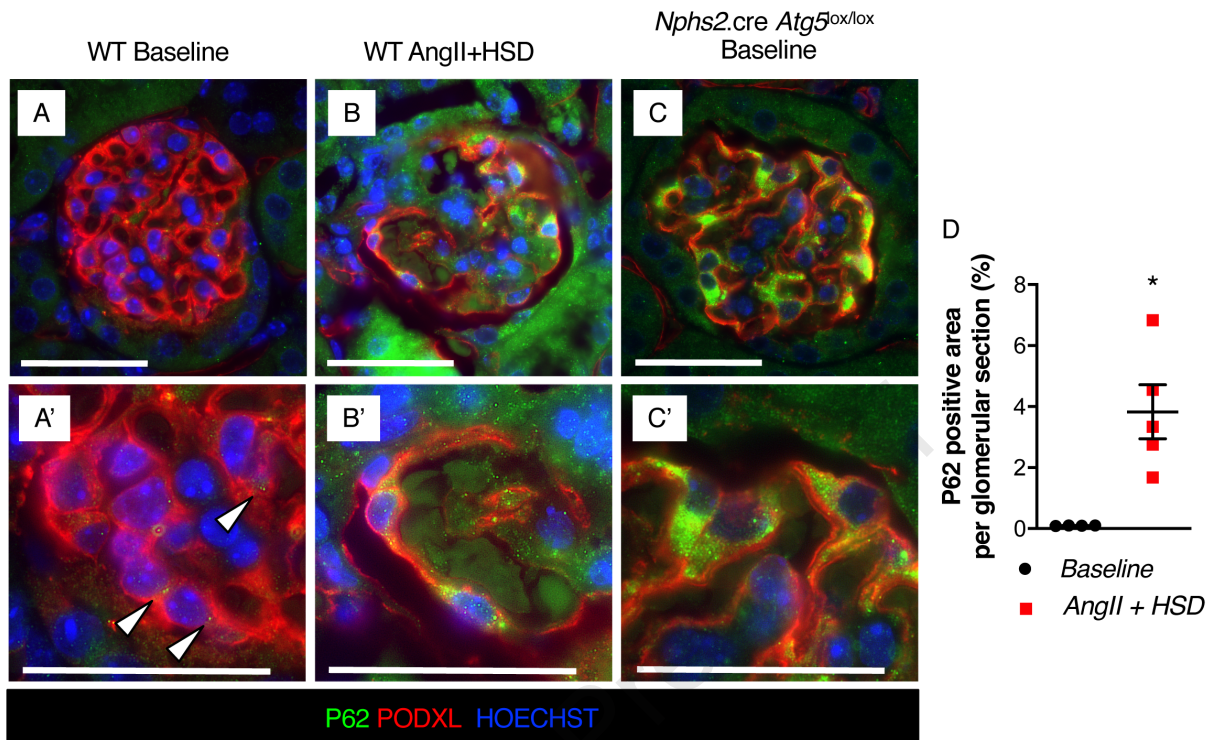
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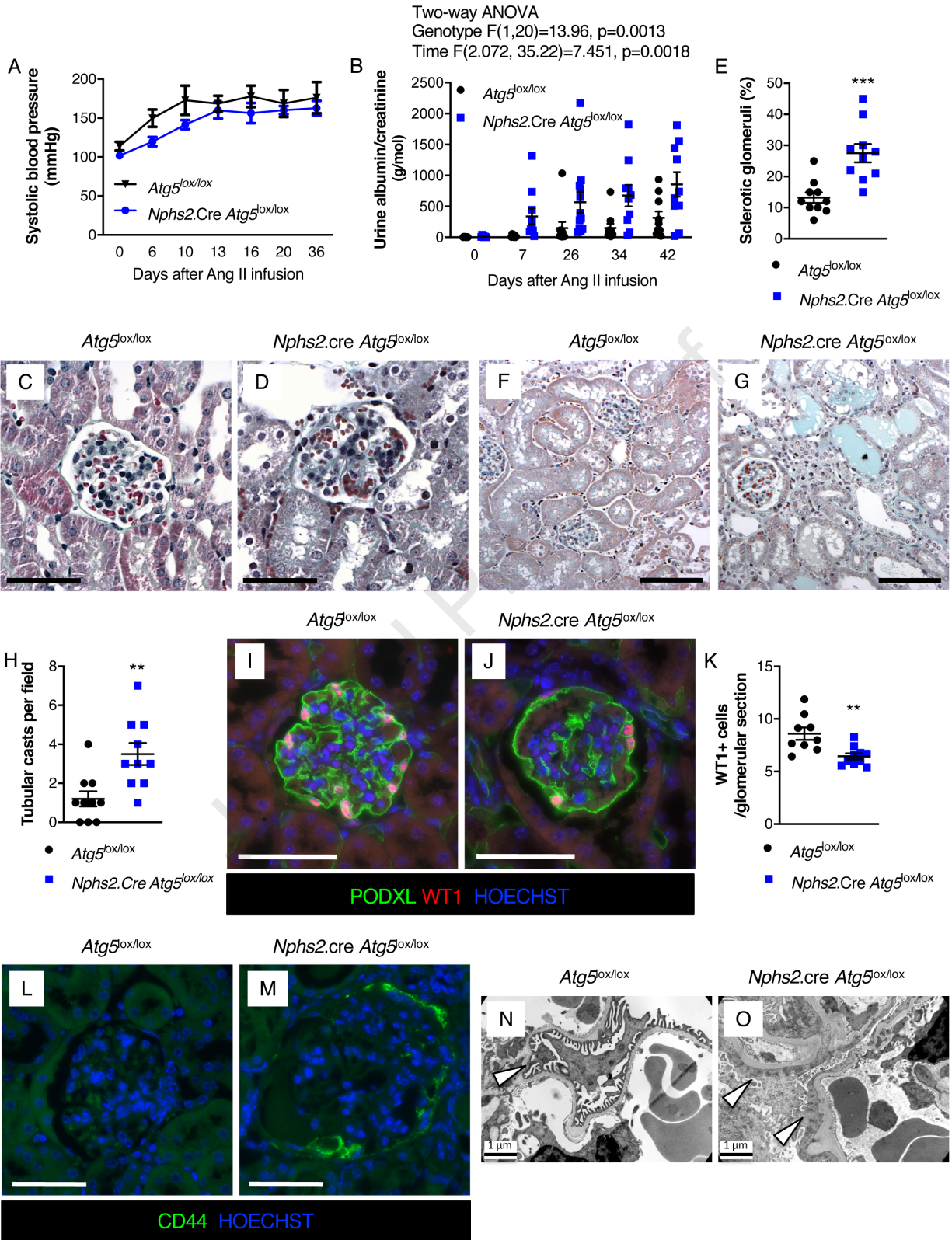
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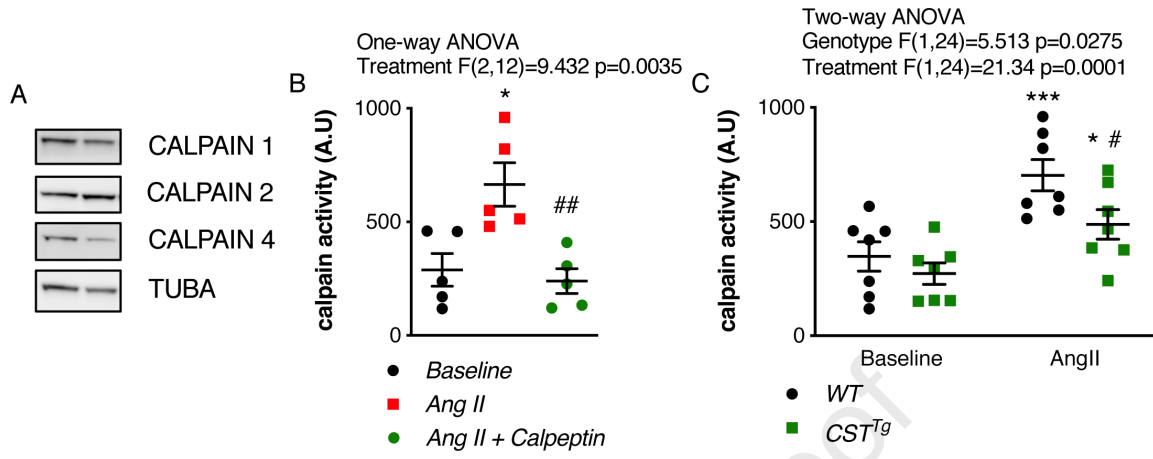
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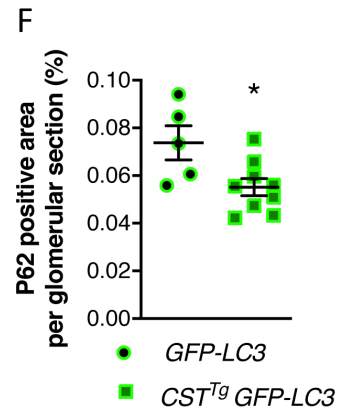
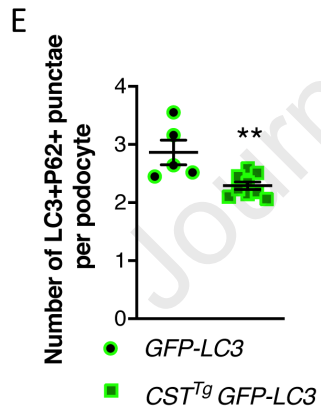
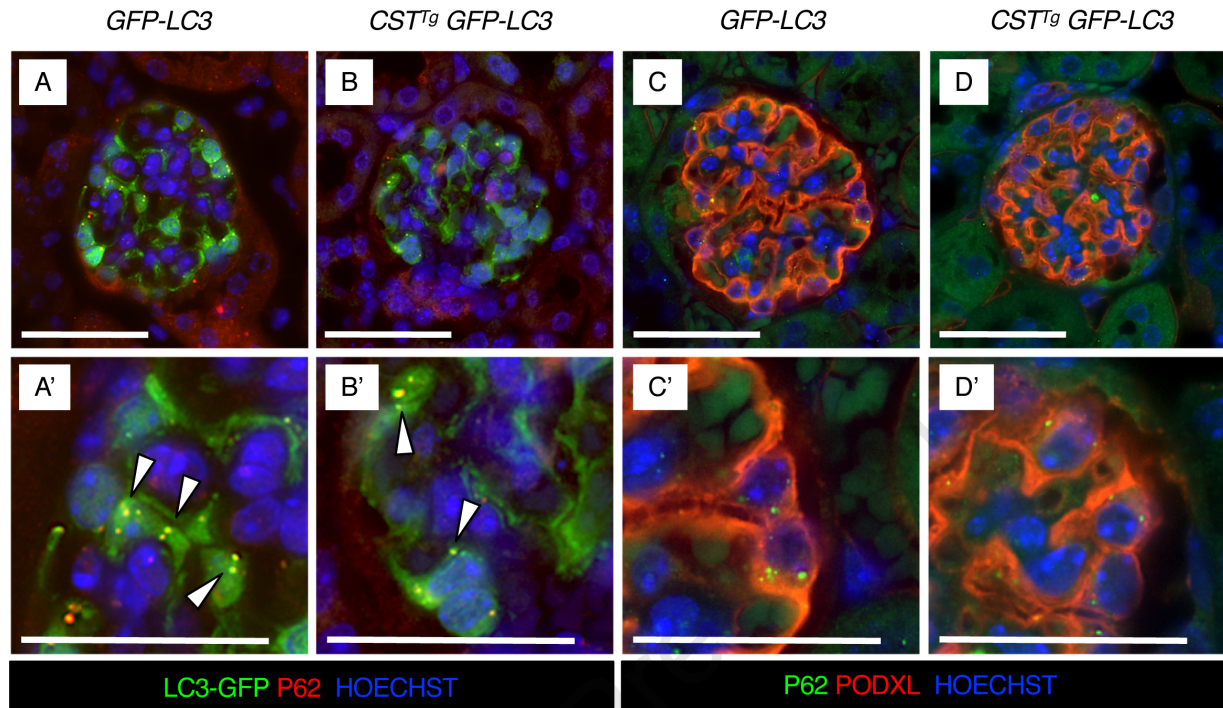
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4	NM_011631	HSP90B1	Heat shock protein 90, beta (Grp94), member 1	PPM05658A
5	NM_022310	HSP5A	Heat shock protein 5	PPM03586A
6	NM_007837	DDIT3	DNA-damage inducible transcript 3	PPM03736A
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8	NM_008929	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	PPM25697A
9	NM_007591	CALR	Calreticulin	PPM05020A
10	NM_022032	PERP	PERP, TP53 apoptosis effector	PPM04985A
11	NM_007522	BAD	BCL2-associated agonist of cell death	PPM02916A
12	NM_007527	BAX	Bcl2-associated X protein	PPM02917A
13	NM_009741	BCL2	B-cell leukemia/lymphoma 2	PPM02918A
14	NM_009761	BNIP3L	BCL2/adenovirus E1B interacting protein 3-like	PPM27647A
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16	NM_009812	CASP8	Caspase 8	PPM02923A
17	NM_011434	SOD1	Superoxide dismutase 1, soluble	PPM03582A
18	NM_198958	NOX3	NADPH oxidase 3	PPM40647A
19	NM_011198	PTGS2	Prostaglandin-endoperoxide synthase 2	PPM03647A
20	NM_011218	PTPRS	Protein tyrosine phosphatase, receptor type, S	PPM35547A
21	NM_023281	SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein	PPM31938A
22	NM_008706	NQO1	NAD(P)H dehydrogenase, quinone 1	PPM03466A
23	NM_010442	HMOX1	Heme oxygenase (decycling) 1	PPM04356A
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25	NM_011034	PRDX1	Peroxiredoxin 1	PPM04383A
26	NM_010357	GSTA4	Glutathione S-transferase, alpha 4	PPM03928A
27	NM_009735	B2M	Beta-2 microglobulin	PPM03562A
28	NM_008084	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	PPM02946A
29	NM_007393	ACTB	Actin, beta	PPM02945A
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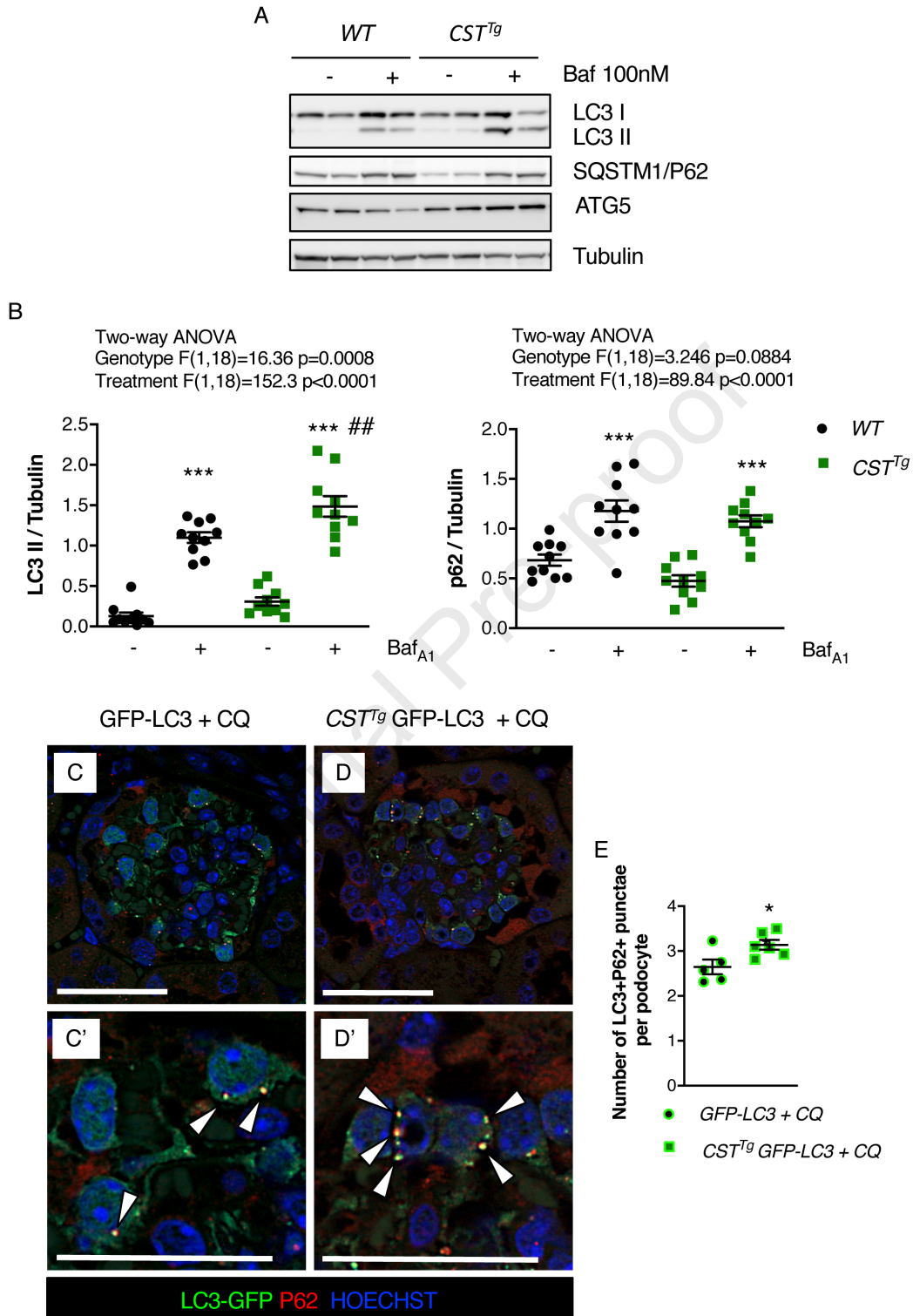
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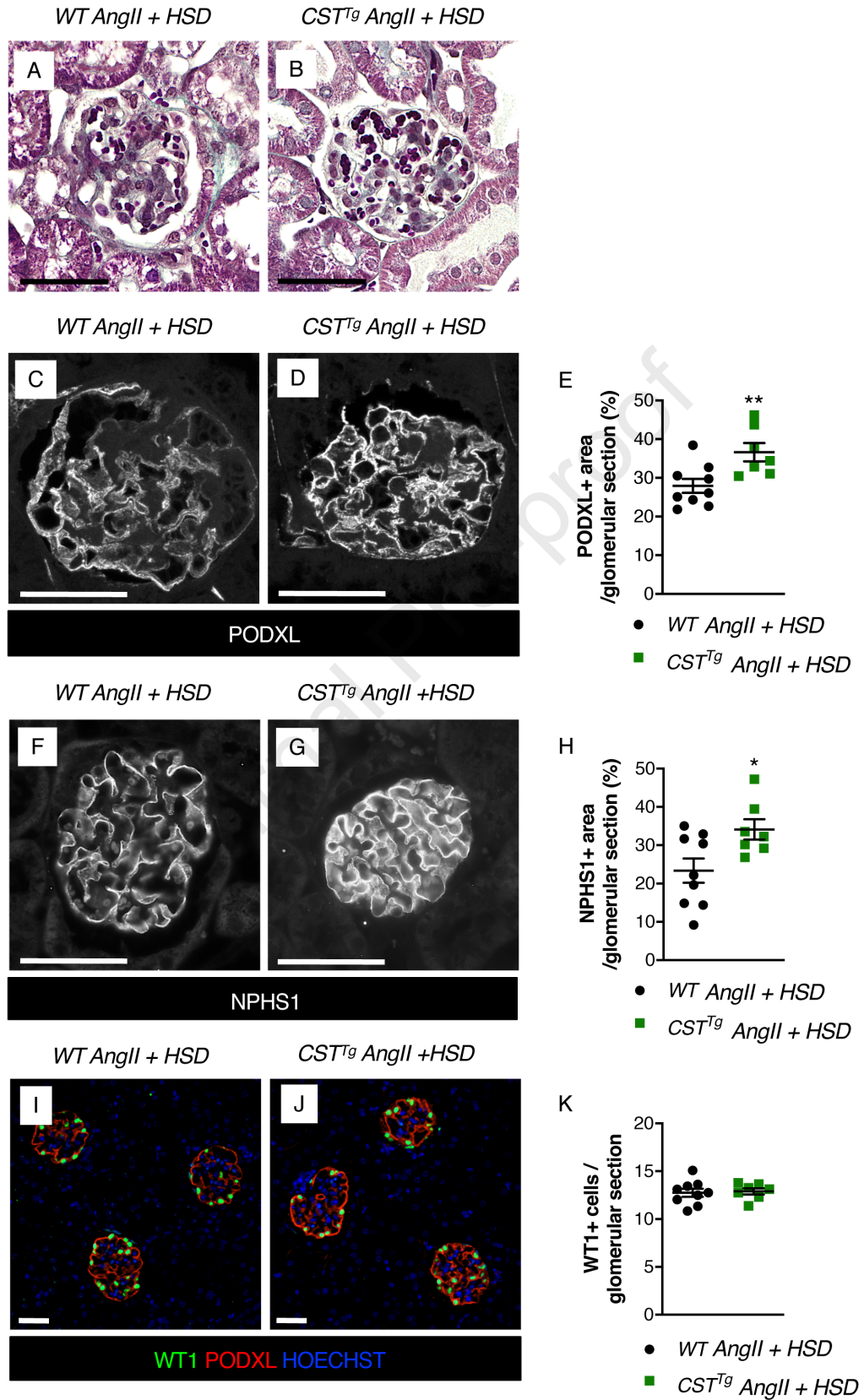






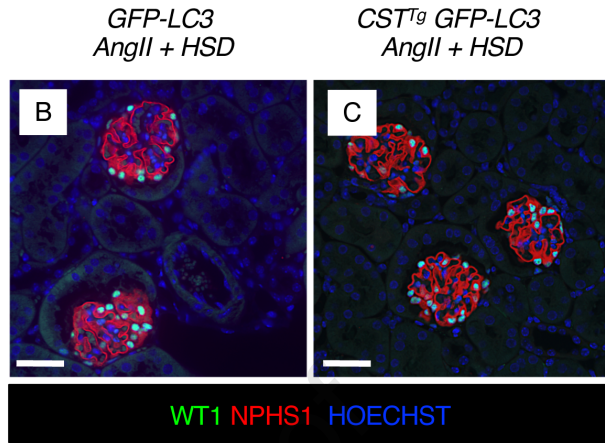
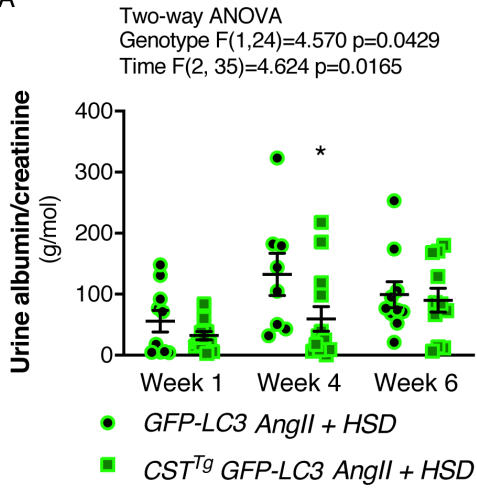




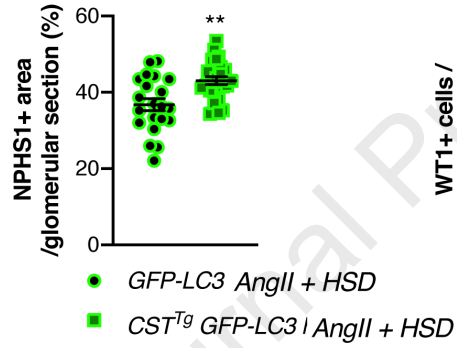


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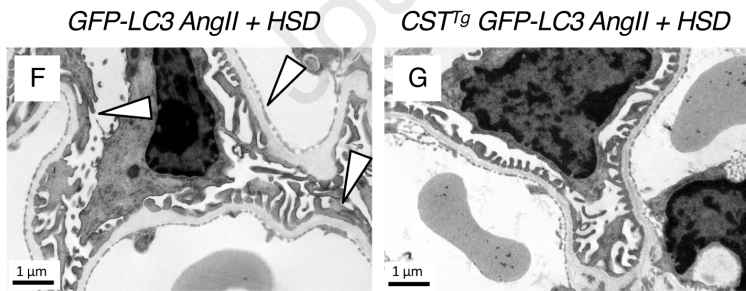
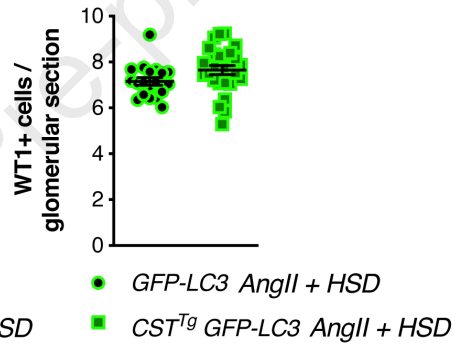
A



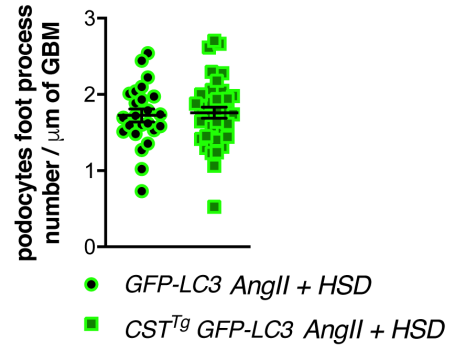
D

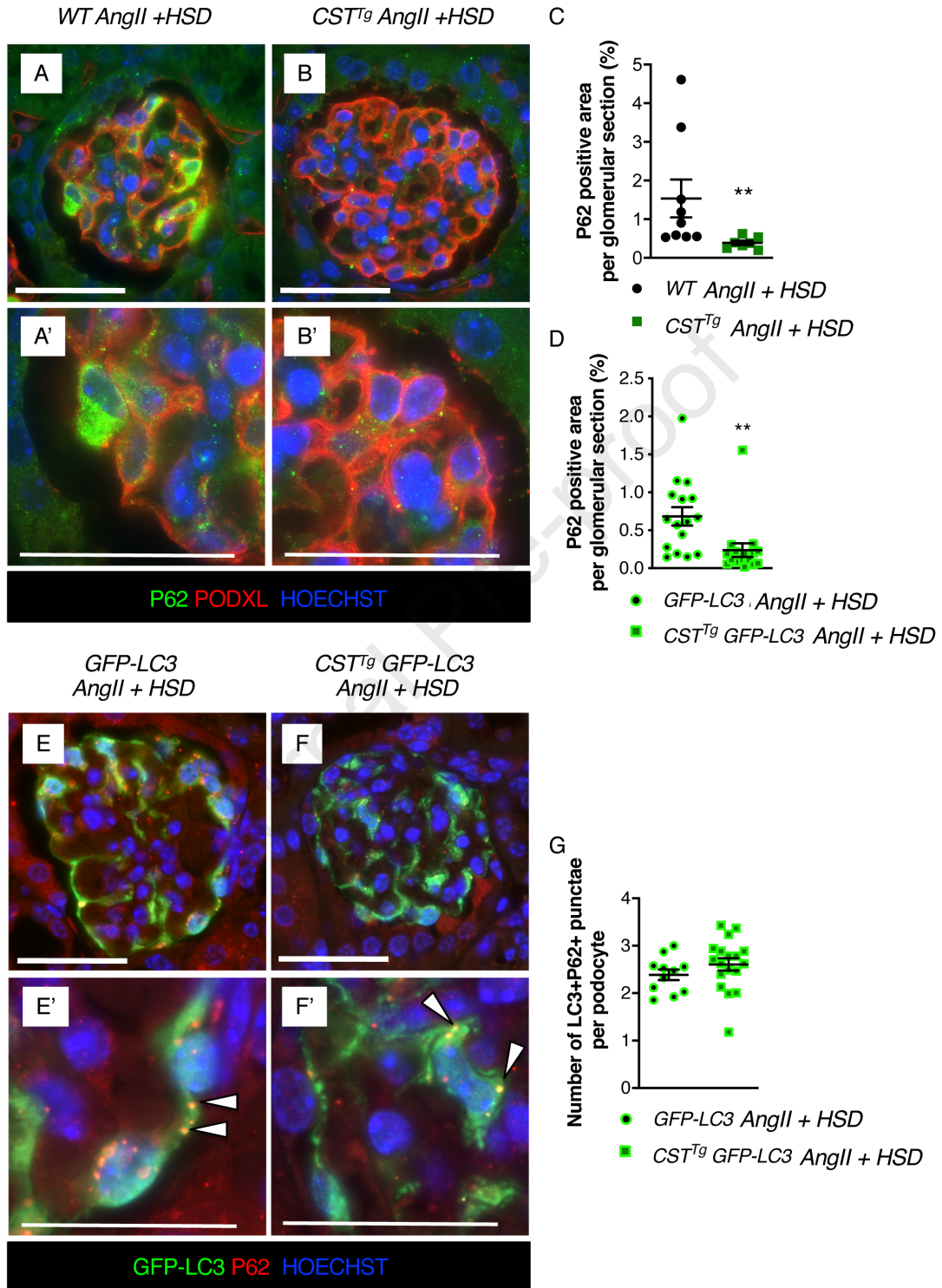


E

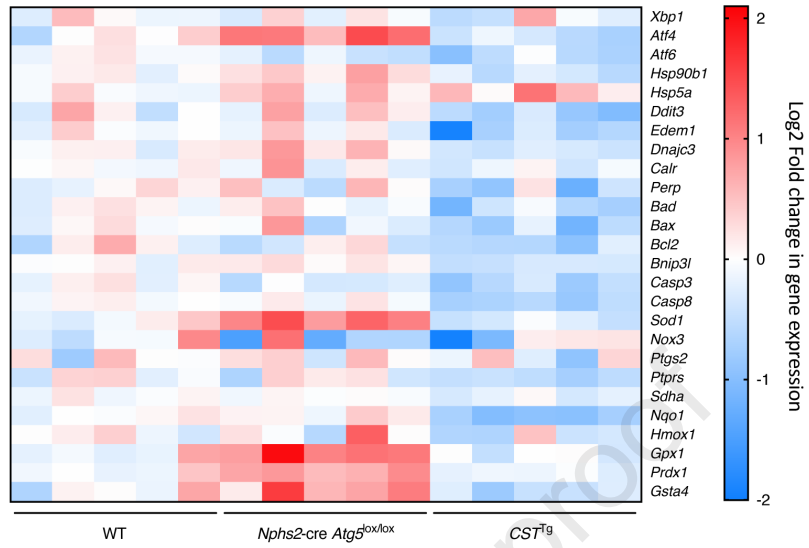


H





A



B

WT vs. <i>Nphs2-cre Atg5^{lox/lox}</i>	Log2 Fold change of gene expression	P value
<i>Sod1</i>	1,135	0,000414
<i>Prdx1</i>	0,751	0,000699
<i>Atf4</i>	1,118	0,001519
<i>Atf6</i>	-0,368	0,011152
<i>Gpx1</i>	1,286	0,013012
<i>Hsp90b1</i>	0,386	0,037785
<i>Casp3</i>	-0,304	0,04655

C

WT vs. <i>CST⁹</i>	Log2 Fold change of gene expression	P value
<i>Casp8</i>	-0,676	0,000017
<i>Nqo1</i>	-0,877	0,00005
<i>Bnip3l</i>	-0,396	0,001458
<i>Casp3</i>	-0,614	0,002444
<i>Dnajc3</i>	-0,371	0,004811
<i>Bax</i>	-0,638	0,005067
<i>Hsp90b1</i>	-0,380	0,007758
<i>Edem1</i>	-0,776	0,008106
<i>Atf6</i>	-0,533	0,01096
<i>Bad</i>	-0,554	0,015655
<i>Ptprs</i>	-0,545	0,019105
<i>Ddit3</i>	-0,689	0,042243