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PODOCYTES MAINTAIN HIGH BASAL LEVELS OF AUTOPHAGY INDEPENDENT OF MTOR SIGNALING

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Abbreviations:
AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; ATG: autophagy related; AMPK: AMP-activated protein kinase; ER: endoplasmic reticulum; GFP: green fluorescent protein; LC3: microtubule-associated protein 1 light chain 3; mTOR: mammalian target of rapamycin; NPHS1: nephrin; NPHS2: podocin; PLA: proximity ligation assay; ULK1: unc-51-like autophagy activating kinase 1; RAPTOR: regulatory-associated protein of mTOR; RFP: red fluorescent protein; TSC1: tuberous sclerosis 1

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Abstract

Autophagy and mTOR mediated regulation of cytoplasmic homeostasis and podocyte size are two key cellular mechanisms that underpin the integrity of the renal filtration barrier. Investigations of various tissues and cell lines support the notion that mTOR suppresses autophagy. In a translational context it has been proposed that induction of autophagy through the inhibition of mTOR activity could be of clinical benefit. Unexpectedly, we report that genetic manipulation of mTOR activity does not influence the high levels of basal autophagy in podocytes either *in vitro* or *in vivo*. Instead we present data showing that autophagy in podocytes is primarily controlled by AMP-activated protein kinase (AMPK) and Unc-51-like kinase 1 (ULK1). Pharmacologic inhibition of mTOR further reveals that the uncoupling of mTOR activity and autophagy is time-dependent. Thus, our data reveal a novel cell-specific mechanism, which permits concurrent mTOR activity as well as high basal autophagy rates in podocytes. Therefore, we advocate manipulating the AMPK-ULK1 axis rather than inhibiting mTOR as intervention strategy to enhance autophagy for the treatment of podocyte diseases.

Introduction

Worldwide, glomerular diseases are the leading cause for end-stage renal disease (ESRD) requiring hemodialysis treatment.[1] Due to the increase of ESRD prevalence, understanding glomerular biology is critical for future targeted pharmacological treatments to maintain the glomerular filtration barrier - a unique structure characterized by a complex three-layered composition of podocytes, glomerular basement membrane and endothelial cells.[1,2] Podocytes are highly specialized epithelial cells with long interdigitating foot processes enclosing the glomerular capillaries and their progressive loss is associated with proteinuria and renal failure.[2,3,4,5]

Diminution in podocyte number due to cellular or tissue insult cannot be reconstituted by existing cells due to their post-mitotic nature. Their ability to survive depends on their capacity to endure cellular stress. Macroautophagy (later referred to as autophagy) has been implicated as the major cellular maintenance mechanism of podocytes characterized by removal of dysfunctional organelles and misfolded proteins by enclosure within double membrane vesicles called autophagosomes and their subsequent lysosomal fusion and degradation.[5-9] In brief, autophagy is initiated at an endoplasmic reticulum subdomain called the omegasome – a membrane compartment rich in phosphatidylinositol-3-phosphate.[10] After nucleation the phagophore, an isolation membrane and autophagosome precursor, is elongated using a lipid supply from various cellular membrane compartments.[11,12] At the same time, cytosolic microtubule-associated protein 1 light chain 3 (LC3-I) is conjugated to phosphatidylethanolamine and integrated into the autophagosomal membrane (the

lipidated form referred to as LC3-II, a key biochemical marker for autophagy).[13,14] Further phagophore elongation is driven by a complex interplay of WD repeat phosphoinositide interacting proteins as well as the autophagy-related (ATG) protein 8 and autophagy-related protein 12 conjugation system.[15-21]

Phagophore initiation, and thereby the initial step of autophagosome formation, is promoted by two complexes – the unc-51-like autophagy activating kinase 1 (ULK1) complex and the class III phosphoinositide 3-kinase – Beclin1 complex. In particular, the ULK1 complex serves as a hub integrating upstream signals to regulate autophagic activity.[22-26]

In podocytes, autophagy block by cell-specific deletion of *Atg5* (coding for a protein important for phagophore membrane elongation) leads to proteinuria and features of accelerated ageing as well as massively increased glomerular disease susceptibility (see ref. [5]) e.g. towards diabetic nephropathy (DN) and focal segmental glomerulosclerosis (FSGS).[6,27,28] Furthermore, podocyte maintenance is safeguarded by the mechanistic target of rapamycin (mTOR) pathway known to inhibit autophagy.[24,25,29] Podocytes utilize mTOR signaling for programs of cellular hypertrophy in response to cellular stress as observed in DN.[30] Both, impaired as well as hyperactive mTOR signaling results in proteinuria and podocyte loss.[29,31,32]

mTOR is a phosphatidylinositol 3-kinase (PI3K)-like serine/threonine protein kinase that is evolutionarily highly conserved and functions in two multiprotein complexes. mTOR complex 1 (mTORC1) mediates most of the known functions of the pathway like cellular growth and proliferation via substrates that include S6 kinase (p70S6k) and 4E-binding protein 1 (4E-BP1). Moreover, mTORC1 is the best characterized

repressor of autophagy (see ref. [33]), inhibiting this process by phosphorylating ULK1.[25]

That podocytes use both pathways – high basal autophagy levels as well as fluctuating mTOR activity – at the same time, appears to be paradox in the light of the known inhibitory function of mTOR on autophagy. To investigate podocyte-specific regulation of autophagy we applied in our present study genetic and pharmacological *in vitro* and *in vivo* models to comprehensively determine the impact of mTOR on podocyte autophagy and to elucidate time-dependent effects of mTOR kinase inhibition on autophagy regulation.

Results

Podocytes exhibit high levels of basal autophagy and autophagic flux

To assess basal autophagy, we analyzed autophagosome formation *in vivo* in adult (4-month-old) *GFP-LC3* transgenic mice using LC3 as a marker of autophagy. High numbers of autophagosomes were found in the glomeruli, but were rarely found in the tubular compartment **(Fig. 1A)**. Intravital two-photon microscopy confirmed high numbers of autophagosomes in podocytes under *in vivo* conditions **(Fig. 1B)**. Tandem fluorescent protein tagged LC3 (RFP-GFP-LC3) allows monitoring autophagic flux based on differential pH stability of RFP and GFP.[34] To determine the amount of *de novo* formed autophagosomes and the flux towards lysosomal degradation, we analyzed autophagosomes *in vivo* in 4-month-old transgenic *GFP/RFP-LC3* mice.[35] Higher numbers of GFP^+RFP^+ and RFP^+ puncta could be detected in podocytes compared to tubular cells **(Fig. 1C, 1D and S1A)**. The number of RFP^+ puncta was comparable with that found in liver tissue **(Fig. S1B and S1C)**, also known for high basal activity in autophagy (for reviews, see refs. [36] and [37]).

Autophagic flux in *GFP-LC3* mice was additionally determined by quantifying autophagosome numbers in the presence of inhibitors of lysosomal degradation.[38,39] *In vivo* assessment of autophagosomes revealed autophagosome accumulation in response to chloroquine indicating high autophagic flux toward lysosomal degradation and high levels of *de novo* autophagosome formation in podocytes compared to tubular cells **(Fig. 1E).** Furthermore, autophagic flux in podocytes was found to be even higher than in liver tissue **(Fig. S1D and S1E)**. *In vitro* podocytes showed higher levels of autophagy as compared to tubular cells indicated by higher levels of LC3-II and lower levels of the autophagy substrate SQSTM1 **(Fig. 1F and 1G)**. Also, autophagic flux was higher in podocytes than in tubular cells **(Fig. S1F and S1G)**. Interestingly**,** the activity of mTORC1 was significantly elevated in podocytes as determined by phosphorylation of the p70S6k substrate ribosomal protein S6 (pS6) and phosphorylated 4E-BP1 **(Fig. 1F and 1G)**. Together, these results indicate that podocyte exhibit an unusual concomitant autophagic flux and elevated mTORC1 activity.

Basal autophagy is independent of mTOR activity in podocytes

We next determined levels of autophagy in podocyte-specific genetic models of mTORC1 hypo- and hyperactivation. We generated mice bearing a podocyte-specific knockout for the gene coding for the regulatory-associated protein of mTOR *(Raptor)*, an essential component of mTORC1, by mating *Raptor*-floxed mice (*Raptor* $^{fl/fl}$) with mice expressing *Cre* recombinase under the control of the *Podocin (Nphs2)* promoter **(Fig. 2A).**[29,40] To hyperactivate mTORC1 in podocytes, we generated mice lacking *tuberous sclerosis 1* (*Tsc1,* coding for an upstream repressor of mTORC1) by using the same podocyte-specific deletion line (**Fig. 2A**). To monitor autophagosome formation, we crossed these mice to a *GFP-LC3* reporter strain **(Fig. S2A)**. [41]

Surprisingly, the two genetic models of mTORC1 hypo-and hyperactivation showed no difference in the number of autophagosomes compared to WT **(Fig. 2B – 2E)**. Next we assessed LC3-II and SQSTM1 levels in glomeruli harvested from these models using magnetic bead perfusion for high purity **(Fig. 2F and 2G)**. There was no significant difference in LC3-II or SQSTM1 levels indicating similar levels of basal autophagy in *Raptor* or *Tsc1* deficient podocytes. The podocyte-specific knockout for *Raptor* was confirmed in these glomerular lysates **(Fig. 2F)**. Since podocytes count for approximately 40% of all glomerular cells the low remaining levels of RAPTOR are caused neighboring mesangial cells not affected by *Nphs2-Cre*-mediated *Raptor* deletion.[42,43] As expected, *Raptor* deficiency lowered mTORC1 activity, which was indicated by lower levels of pS6 and lower levels p4E-BP1 **(Fig. 2F and 2G)**. Staining for pS6 revealed podocyte specific inhibition of mTORC1 **(Fig. S2B).** Correspondingly, mTORC1 hyperactivation due to deletion of *Tsc1* was indicated by increased phosphorylation of mTORC1 downstream targets S6 and 4E-BP1 **(Fig. 2F and 2G)**.

Assessment of autophagic flux in glomeruli obtained from mice with podocyte-specific *Raptor* or *Tsc1* deletion with and without chloroquine treatment revealed no difference compared to WT **(Fig. S2C)**. To exclude developmental effects of *Raptor* deficiency on podocyte autophagy, we assessed autophagy in an inducible model of podocyte-specific *Raptor* deletion by crossing *Raptor* $^{fl/fl}$ mice to *Nphs2-rtTA; tetO-Cre* mice **(Fig. S2D).** After induction of podocyte-specific *Raptor* deletion, we confirmed the knockout and determined LC3-II. Again, WT and *Raptor*-deficient glomeruli showed no significant difference in LC3-II **(Fig. S2E and S2F)**.

For primary podocyte analysis, we crossed *Raptor*-floxed mice (*Raptor* $^{fl/fl}$) and *Tsc1*-floxed mice (*Tsc1* $^{fl/fl}$) with podocyte- specific *Cre* expression to a *Tomato/EGFP*

reporter strain **(Fig. 3A and 3B)**. This permits the isolation of primary podocytes by fluorescence activated cell sorting (FACS) for *in vitro* culture **(Fig. S2G)**. Due to the suppression of mTORC1, primary *Raptor* deficient podocytes showed almost no proliferative capacity. However, they displayed comparable numbers of LC3 puncta in comparison to WT podocytes **(Fig. 3C and 3D)**. Profiling of key molecules that regulate autophagy through Western blotting was not possible due the paucity of *Raptor* deficient podocytes. Instead we applied the proximity ligation assay technique to monitor autophagy. Herein we assessed the association between LC3 and SQSTM1 which is critical to the formation of the autophagosome (see ref. [44]) **(Fig. 3E)**. Again, there was no detectable difference in autophagosome number between *Raptor* deficient cells and their control counterparts **(Fig. 3F and 3G)** - indicating no activation of autophagy despite mTORC1 suppression.

Primary podocytes with mTORC1 hyperactivation due to *Tsc1* deficiency showed similar numbers of LC3 puncta **(Fig. 3H and 3I)** and no difference in LC3-II or SQSTM1 levels compared to WT despite high mTORC1 activity (indicated by increased pS6/S6 ratio and p4E-BP1/4E-BP1 ratio) **(Fig. 3J and 3K)**. Autophagic flux was also comparable in WT versus *Tsc1* deficient podocytes **(Fig. S2H, S2I and S2J)**.

AMPK controls autophagy in podocytes

To investigate AMPK activity in podocytes we first assessed phosphorylation of AMPK at Thr172 (pAMPKThr172) and of its substrate acetyl-CoA carboxylase at Ser79 (pACCSer79). In tissue sections, the highest levels of pACC were found in glomeruli of mice with podocyte specific deletion of *Tsc1*, whereas *Raptor* deficient podocytes displayed low pACC levels **(Fig. 4A, quantification in S3A)**. Accordingly,

significantly increased levels of phosphorylated AMPK and pAMPK/AMPK ratio were found in glomerular lysates obtained from mice with podocyte specific *Tsc1* knockout **(Fig. 4B and 4C)**. Impaired mTORC1 formation (due to *Raptor* deficiency), however, was associated with significantly decreased pAMPK levels and lower pAMPK/AMPK ratio compared to WT **(Fig. 4B and 4C)**.

Next we assessed phosphorylation of the key autophagy regulator ULK1 by AMPK and mTORC1. While Ser757 is a target site of mTORC1 inhibiting ULK1 activity, Ser555 and Ser317 are target sites of AMPK with an activating effect on ULK1 and autophagy.[45-47] Glomeruli with podocyte specific *Tsc1* deficiency displayed the highest pULK1Ser757/ULK1 ratio, whereas impaired mTORC1 formation resulted in a significantly decreased pULK1Ser757/ULK1 ratio compared to WT **(Fig. 4B and 4D)**. pULK1Ser757/ULK1 ratio indicated enhanced or decreased mTORC1 activity, respectively, and was in line with our findings on pS6 and p4E-BP1 **(Fig. 2F)**. AMPK driven phosphorylation of ULK1 was significantly increased upon mTORC1 hyperactivity (pULK1Ser555, pULK1Ser317), whereas pULK1Ser317 was decreased in *Raptor* deficient podocytes compared to WT **(Fig. 4D)**.

To test if AMPK sustains autophagy in podocytes, autophagy was assessed *in vitro* in primary cultured podocytes treated with the AMPK acitvators 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and metformin. Both treatments enhanced autophagy as indicated by increased LC3-II and decreased SQSTM1 abundance **(Fig. 4E and 4F, Fig. S3B)**. Administration of chloroquine revealed an increase in autophagic flux **(Fig. S3C and S3D)**. As expected, AICAR activated AMPK, as assessed by pAMPKThr172 and ULK1 phosphorylation at the AMPK target site 555 **(Fig. 4E, 4G and 4H)**. Yet, mTORC1 activity was also suppressed by AICAR, as

shown by decreased phosphorylation of S6. mTORC1 inhibition might contribute to enhanced autophagy in AICAR treated podocytes.

AMPK stimulates autophagy independently of mTORC1 activity

To further test the role of mTORC1 in AMPK-induced autophagy, primary podocytes lacking *Tsc1* and corresponding WT podocytes were treated with AICAR *in vitro*. AICAR increased autophagy as determined by increased LC3-II and decreased SQSTM1 **(Fig. 5A and 5B)**. Chloroquine administration revealed increased autophagic flux **(Fig. S3D and S3E)**. Autophagy induction due to AICAR was comparable in *Tsc1* deficient and WT conditions **(Fig. 5A and 5B, S3D and S3E)**. Compared to WT, *Tsc1* deficient podocytes treated with vehicle showed at baseline higher levels of AMPK activation as indicated by increased pAMPK/AMPK ratio **(Fig. 5A and 5B)**. AICAR enhanced AMPK phosphorylation at Thr172, and ULK1 was phosphorylated at the known AMPK phosphorylation sites Ser317 and Ser555 **(Fig. 5A and 5B)**. The mTORC1- dependent inhibitory phosphorylation site Ser757 slightly decreased due to AICAR exposure in WT and *Tsc1* deficiency and mTORC1 activity was reduced as indicated by decreased levels of phosphorylated ribosomal protein S6 **(Fig. 5A)**.

Due to the limited proliferative capacity of *Raptor* deficient primary podocytes *in vitro*, we chose an *in vivo* approach to test for mTORC1 independent effects of AICAR on autophagy. Therefore, we performed AICAR treatment on mice with a podocyte-specific deletion of *Raptor*. Liver lysates revealed systemic AMPK activation **(Fig. S3F)**. In contrast to WT controls, where pS6 slightly decreased after AICAR administration, in *Raptor* deficient podocytes mTORC1 activity could not be further suppressed by AICAR **(Fig. S3G)**. Of note, when mTORC1 was inhibited by *Raptor*

knockout AICAR was able to enhance autophagy, as demonstrated by increased LC3 puncta formation **(Fig. 5C and 5E)** and reduced SQSTM1 abundance **(Fig. 5D and 5F)**. Furthermore, glomerular lysates of *Raptor* deficient mice showed increased levels of LC3 conversion compared to untreated mice **(Fig. 5G and 5H)**. We conclude that in podocytes AMPK can enhance autophagy when mTORC1 is inhibited.

To determine whether AMPK can also enhance autophagy under conditions of mTORC1 hyperactivation *in vivo* we performed AICAR treatment in mice with podocyte specific deletion of *Tsc1*. AICAR induced autophagy indicated by increased glomerular LC3 signal and decreased SQSTM1 **(Fig. S4A and S4B, quantification in S5A and S5B).** AMPK activation by AICAR was confirmed by glomerular pACC staining and pACC/ACC ratio obtained from glomerular lysates showing the highest pACC levels in *Tsc1* deficiency **(Fig. S5C, Fig. S5D and S5E)**. Autophagy induction by AICAR was comparable in mice with podocyte specific *Raptor* or *Tsc1* deficiency and WT controls as indicated by LC3 and SQSTM1 **(Fig. S4A and S4B, quantification in S5B)**. Thus, we conclude that AMPK enhances autophagy under conditions of mTORC1 inhibition or hyperactivation. Therefore, autophagy induction by AMPK is mTORC1-independent in podocytes.

To further confirm the role of AMPK on podocyte autophagy AMPK was inhibited using compound C (also known as 6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo [1,5-a]pyrimidine), a selective and reversible AMP-kinase inhibitor. Compound C inhibited autophagy and autophagic flux in WT and *Tsc1* deficiency **(Fig. S6A and S6B)**. Pharmacological inhibition of ULK1/2 by MRT68921 (as described in ref. [48]) decreased podocyte autophagic flux indicating an important role for ULK1 in maintaining podocyte autophagy **(Fig. S6C and S6D)**.

Effects of acute and long-term pharmacological inhibition of mTORC1 on autophagy

Since the independence of autophagy from genetic manipulation of mTORC1 activity was an unexpected finding and most previous data linking mTORC1 and autophagy have been based on pharmacological inhibition of mTORC1, we aimed next to compare short-term pharmacological effects to constitutive long-term genetic manipulation of mTORC1 activity. To investigate the short-term effect of pharmacological suppression of mTORC1, we treated transgenic *GFP-LC3* mice with rapamycin **(Fig. 6A)** and assessed autophagosome number. Hereby, autophagy levels were increased, indicated by increased numbers of GFP-LC3 puncta **(Fig. 6B and 6C)**, higher levels of LC3-II and a decrease in SQSTM1 **(Fig. 6D and 6E)**. Expectedly, S6 phosphorylation and pULK1Ser757/ULK1 ratio were reduced **(Fig. 6D and 6E)**. AMPK was activated as determined by increased pULKSer555 **(Fig. 6D and 6E)**. To control the robustness of our experimental readout, mice with podocyte-specific *Atg5*-deficiency underwent the same treatment regimen with rapamycin without showing any change in autophagy markers **(Fig. S6E)**. Also in mTORC1 hyperactive conditions (podocyte-specific *Tsc1* deletion) short-term treatment with rapamycin induced autophagy **(Fig. S6F)** and increased autophagy flux **(Fig. S6G and S6H)**, demonstrating the impact of mTOR signaling pathway in short-term regulation of autophagy. *In vivo* treatment of *Tsc1* deficient mice with rapamycin reduced glomerular pS6 levels **(Fig. S7A)** and activated autophagy (**Fig. S7B**, quantified in **Fig. S8A and S8B**). The effect of rapamycin on autophagy was slightly but not significantly lower in *Tsc1* deficiency compared to WT potentially due to incomplete mTORC1 inhibition.

To determine the effects of pharmacological long-term mTORC1 inhibition by rapamycin, we treated *GFP-LC3* mice with rapamycin for 3 weeks **(Fig. 6F)**. We confirmed the efficacy of the treatment through evidencing elevated serum levels of rapamycin in treated animals **(Fig. 6G)**. But when autophagy was assessed in glomerular lysates and compared to vehicle or short-term treatment we found that long-term treatment exhibited no effect on LC3-II or SQSTM1 levels **(Fig. 6H and 6I)**. Furthermore neither autophagosome number **(Fig. S8C and S8D)** nor SQSTM1 accumulation differed among the long-term treatment group and untreated controls **(Fig. S8E and S8F)**. Additionally, analysis of autophagosomes after lysosomal inhibition with chloroquine did not reveal any difference among the groups with regards to autophagosome number **(Fig. S9A and S9B)** or SQSTM1 accumulation **(Fig. S9C and S9D)**. Direct comparison of LC3 obtained from glomerular lysates of short-term and long-term treated mice with and without chloroquine revealed increased autophagic flux after short-term treatment but no change after long-term treatment compared to vehicle **(Fig. S9E, Fig. S9F and S9G)**. Of note, long-term and short-term treatment with rapamycin showed different impact on AMPK activation. AMPK was highly activated in the short-term regimen, whereas after long-term treatment AMPK activity was lowered compared short-term treated mice or to vehicle treated controls (**Fig. 6H and S9H**). mTORC1 inhibition decreased pULKSer757 levels in both treatment regimens. Activating phosphorylation of ULK1 at Ser555, however, was highly abundant after short-term treatment and lowest after long-term treatment (**Fig. 6H and S9H**).

To further compare time dependent effects of AMPK activation and mTORC1 inhibition on autophagy short-term and long-term treatments with AICAR and rapamycin were performed and analyzed head to head. Comparison of LC3-II and

SQSTM1 in glomerular lysates after 3 days of AICAR and rapamycin treatment, respectively, revealed increased autophagy in both treatment conditions **(Fig. S10A and Fig. S10B).** *In vitro*, AICAR and rapamycin treated primary murine podocytes showed active autophagy and increased autophagic flux after 3 days of treatment **(Fig. S10C).** Long-term administration of rapamycin, however, failed to activate autophagy and autophagic flux whereas AICAR maintained its pro-autophagic effect independent of treatment duration **(Fig. S10D).** Following rapamycin exposure AMPK activity increased after 24h, however, then decreased in a time-dependent manner in primary podocytes whereas mTORC1 signaling remains suppressed **(Fig. S10E and S10F)**. Long-term pretreatment with rapamycin and AICAR, respectively, followed by short-term AICAR or rapamycin exposure demonstrated maintained pro-autophagic effects of active AMPK signaling independent from mTORC1 activation state corresponding to our findings in *Raptor* of *Tsc1* deficient podocytes treated with AICAR **(Fig. S11A, S11B and C).** In conclusion, we propose that autophagy induction by short-term rapamycin treatment is mediated by both mTORC1 inhibition and AMPK activation. Both pathways synergistically activate ULK1 and thereby induce autophagy **(Fig. 7E)**. After long-term rapamycin treatment, however, AMPK is inactive and counteracts the activating signals of mTORC1. Autophagy levels remain unchanged **(Fig. 7F)**. Our data demonstrate that the impact of mTORC1 on podocyte autophagy is limited to short-term adaptive response and can be overridden by AMPK activity at any time suggesting AMPK-ULK1 signalling axis as the higher-ranking signaling network for podocyte autophagy control.

Discussion

ESRD is a global burden affecting over 2 million people worldwide relying on dialysis treatment or undergoing renal transplantation.[49,50] Glomerular diseases and diabetic nephropathy (DN) are the leading cause for ESRD in developed countries, indicating the importance of intact glomeruli and podocytes for maintaining renal integrity.[51] Autophagy has emerged as the key maintenance mechanism in podocytes as evidenced in various experimental and human proteinuric kidney diseases such as diabetic nephropathy, apolipoprotein L1 (APOL1) associated focal segmental glomerulosclerosis (FSGS), FSGS, membranous nephropathy, IgA nephropathy and M. Fabry.[5,6,28,52,53] While basal autophagy levels are already high,[5] it appears that further pharmacological activation of autophagy can provide additional beneficial effects, e.g. as short term treatment in situations of severe podocyte stress in acute inflammatory glomerular diseases. Safety concerns regarding further elevation of already high levels of autophagy in podocytes seem to be a minor issue since none of the published experimental models of constitutively upregulated autophagy exhibited a renal phenotype under basal conditions.[54,55]

However, a critical first step is to develop an understanding of the signaling cascades that regulate this process in podocytes.

The mTOR signaling pathway is recognized as the major regulator of autophagy, as demonstrated in yeast, drosophila and mammalians.[24-26,56,57] mTORC1 inhibits autophagy-initiating ULK1 complex by phosphorylating amongst others ULK1 at Ser757 in mice (Ser758 of human ULK1) leading to reduced ULK1 kinase activity and suppressed autophagy induction. [24-26,45,58] Since stress response of podocytes as observed in glomerular disease includes both upregulation of autophagy *and* increased mTORC1 activity, this indicates an uncoupling of mTOR and autophagy in these cells.[5,29] In line, we observed similar levels of autophagy in genetic models of

podocyte-specific mTORC1 hypo- and hyperactivation and under long-term pharmacological inhibition of mTORC1 formation, suggesting the existence of an additional signaling layer for regulating this important pathway for cellular maintenance. Previous studies propose different mechanisms allowing for this uncoupling of autophagy and mTORC1 activity or rapamycin action e.g. spatial coupling of mTOR and autophagy (see ref. [59]) or increased mTORC1 stabilization counteracting rapamycin effects under long-term exposure.[60] However, in podocytes, we identified the AMPK signaling pathway directly regulating ULK1 activity as a key regulatory axis governing autophagy in podocytes independent of mTORC1 activity and provide first *in vivo* evidence for this recently proposed mechanism.[45-47]

Crosstalk of AMPK and mTOR occurs at different levels of autophagy regulation (for reviews, see ref. [61-64]). AMPK signaling is proposed to act upstream of mTORC1 with low nutrient supply activating AMPK which then activates the TSC complex leading to mTORC1 inhibition and autophagy activation (for a review, see [65]). Additionally, AMPK can directly inhibit mTORC1 activity by phosphorylating RAPTOR.[66] More downstream, ULK1 acts as a signaling hub orchestrating signaling inputs from mTORC1 and AMPK. In response to starvation, ULK1 is rapidly dephosphorylated, in particular at the Ser757 mTORC1 target site, leading to autophagy induction.[45,58] Furthermore, phosphorylation of ULK1 at Ser317 and Ser777 by AMPK activates autophagy.[45] Besides initiating autophagy, the role of ULK1 is even more complex. In a feedback loop ULK1 is able to phosphorylate RAPTOR and thereby maintains inhibition of mTORC1 if nutrient availability is reduced and autophagy is activated.[67] Furthermore, ULK1/2 is able to phosphorylate all three subunits of AMPK inhibiting its activity and thereby acting as a negative feedback regulation of autophagy.[68] To unravel this complex interplay of AMPK, mTORC1 and ULK1 we combined genetic

and pharmacological approaches to identify the major regulatory signaling axis for podocyte autophagy regulation.

In our genetic models AMPK counteracts mTORC1 function in regard to autophagy regulation leading to maintained levels of basal autophagy **(Fig. 7A and 7B)**. In mTORC1 hypoactivation AMPK activity is found to be decreased **(Fig. 7A)**. Reduced inhibitory phosphorylation at ULK1 at Ser757 due to abolished mTORC1 formation is outbalanced by reduced activating signal at Ser555 and autophagy levels are maintained. These maintained levels of autophagy seem to be in contrast to previous studies describing impaired autophagy due to disturbed autophagolysosomal reformation in podocytes lacking *mTOR*.[69,70] Complete deletion of *mTOR* as performed in these studies additionally impacts mTORC2 which then might further disturb cellular lysosome homeostasis.[71,72] In our genetic models, however, lysosomal inhibition by chloroquine did not uncover differences in autophagic flux compared to WT suggesting limited differential impact of mTORC1 on lysosomal function and no critical effect on autophagy refreshment due to mTORC1 in podocytes.

In mTORC1 hyperactivation AMPK is activated thereby driving autophagy **(Figure 7B).** AMPK activation might potentially occur due to changes in the cellular metabolic state affecting AMP:ATP ratio if *Tsc1* is lacking as it has been reported in *Tsc1* deficient macrophages.[73] Activating phosphorylations of ULK1 by AMPK compensate for inhibitory mTORC1 signal and levels of autophagy finally remain unaffected.

The impact of AMPK on podocyte autophagy becomes most apparent when AMPK is activated by AICAR. Activating sites of ULK1 at Ser317 and Ser555 are phosphorylated and active AMPK concomitantly inhibits mTORC1 activity leading to

high levels of autophagy **(Fig. 7C)**. Interestingly, this concomitant mTORC1 inhibition turned out to be dispensable for autophagy activation as demonstrated in mice bearing a podocyte-specific deletion of *Raptor* **(Fig. 7D).** Although autophagy activation by AMPK dependent phosphorylation of ULK1 at Ser317 and Ser555 has been described *in vitro* (see ref.[45-47]), our data now provide the first *in vivo* evidence and point to the important role of ULK1 for autophagy regulation.

Furthermore, our findings in genetic models of mTORC1 hypo- and hypoactivation reflect a time dependent impact of mTORC1 on autophagy regulation **(Fig. 7E and 7D).** In contrast to short-term mTORC1 inhibition, long-term treatment with rapamycin failed to induce sustained autophagy activation due to reduced activating signals on ULK1 by AMPK as observed in mice with podocyte-specific deletion of *Raptor.* Again, AMPK counteracts mTORC1 function in regard to autophagy regulation.

Thus, our data highlight a tight control by upstream signaling events (i.e. mTORC1 and AMPK signaling) leading to persistent basal autophagy levels in podocytes. Seen at the level of cellular evolution this unique mode of autophagy regulation seems to be crucial for the establishment of the particularly highly differentiated and long-lived structures of the kidney filter. Next to the detailed genetic manipulation of mTOR future studies also including a genetic – and not only pharmacologic interference of AMPK signaling - would further deepen the understanding of the role of AMPK in this system independent from issues of compound specificity of AICAR or compound C or duration of treatment.

Inducing autophagy in podocytes might be a potential treatment approach to cure or at least prevent renal disease. mTOR inhibitors have known autophagy-inducing properties; however, there are several concerns about these compounds from a renal

perspective. Beside severe systemic effects on the immune system and fertility, mTOR inhibitors suppress mTORC1-driven adaptive hypertrophy in podocytes and thereby lead to podocyte loss and proteinuria and in some cases even to FSGS.[74-79] Furthermore, our genetic models and different treatment regimens showed that short-term effects targeting mTORC1 in podocytes failed to induce a sustained activation of autophagy. Instead we suggest that AMPK activating agents might provide an alternative treatment rationale to prevent podocyte disease, as they are effective in inducing autophagy even if mTOR signaling is impaired.

Due to the tight crosstalk of AMPK and mTORC1 activity in podocytes under physiological conditions, targeting downstream molecules like ULK1 might be a promising approach to induce autophagy to counteract various glomerular diseases without disturbing podocyte cell homeostasis.

Materials and Methods

Mice

Mice bearing *Tsc1* flox (exons 17 and 18) or *Raptor* flox alleles (exon 6) have been previously reported.[31,80,81] *Podocin* (*Nphs2*)-*Cre*$^{+}$ mice were provided by L. Holzman (Renal, Electrolyte and Hypertension Division, University of Pennsylvania School of Medicine, Philadelphia, PA, USA).[40] *Raptor*-floxed mice (*Raptor* $^{fl/fl}$) and *Tsc1*-floxed mice (*Tsc1* $^{fl/fl}$) were crossed with *Nphs2*-*Cre*$^{+}$ mice to generate podocyte-specific *Raptor* or *Tsc1* knockout mice (*Raptor* $^{fl/fl}$;*Nphs2*-*Cre*$^{+}$, Raptor$^{\Delta pod}$, *Tsc1* $^{fl/fl}$;*Nphs2*-

Cre^{+}, Tsc1$^{\Delta pod}$, respectively). WT;*Nphs2-Cre*$^{+}$ and *Tsc1* $^{fl/fl}$;*Nphs2-Cre*$^{-}$ or *Raptor* $^{fl/fl}$;*Nphs2-Cre*$^{-}$ littermates served as controls. *Tsc1*$^{\Delta pod}$ mice and *Raptor*$^{\Delta pod}$ mice were subsequently crossed to *GFP-LC3* transgenic mice, which have been previously reported.[41] All mice were crossed on a pure C57BL/6 background which is known to be relatively resistant to kidney injury. To focus on signaling effects on autophagy in intact podocytes *Raptor*$^{\Delta pod}$, *Tsc1*$^{\Delta pod}$ and WT mice were harvested at the age of 2 weeks, i.e. a time point prior to the development of proteinuria to avoid other severe phenotypic effects due to *Raptor* or *Tsc1* knockout secondarily affecting autophagy.

Nphs2-rtTA;tetO-Cre^{+} mice were provided by S. Quaggin (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Canada).[82] To generate doxycycline-inducible podocyte-specific *Raptor* knockout mice (*Raptor* $^{fl/fl}$;*Nphs2-rtTA;tetO-Cre*$^{+}$) *Raptor*-floxed mice (*Raptor* $^{fl/fl}$) were crossed with *Nphs2-rtTA;tetO-Cre*$^{+}$ mice. *TetO-Cre*$^{-}$ littermates served as a control. For the induction of *Raptor* deletion, 12-week-old *Raptor* $^{fl/fl}$;*Nphs2-rtTA;tetO-Cre*$^{+}$ mice received doxycycline (Sigma-Aldrich, D9891) via drinking water (2 mg/ml with 5% sucrose, protected from light) for a total of 14 days.

Mice bearing an *Atg5* flox allele (flanked exon 3) have been previously reported.[83] *Atg5* $^{fl/fl}$ mice were crossed with *Nphs2-Cre*$^{+}$ mice to generate podocyte-specific *Atg5* knockout mice (*Atg5* $^{fl/fl}$;*Nphs2-Cre*$^{+}$, *Atg5*$^{\Delta pod}$). WT;*Nphs2-Cre*$^{+}$ and *Atg5* $^{fl/fl}$;*Nphs2-Cre*$^{-}$ littermates served as controls.

To monitor autophagic flux, *GFP/RFP-LC3* mice were sacrificed after 16 weeks. For a review, see ref. [35]. To assess autophagic flux *in vivo*, *GFP-LC3* mice were harvested 4h after chloroquine (Cq) administration i.p. 100 mg Cq/kg body weight (BW) (Sigma-Aldrich, C6628).

AICAR treatments have been performed with 500 mg/kg BW 24h and 4h before harvest (see ref. [84]). For rapamycin treatment mice received either 4 or 10 mg/kg BW for long term (3 weeks) or short term (3 days) treatment, respectively. Different doses were used due to pharmacokinetics (loading dose for short term use).[85] Treatments have been performed in *Tsc1* $^{fl/fl}$;*Nphs2-Cre*$^{+}$, *Raptor* $^{fl/fl}$,*Nphs2-Cre*$^{+}$ and *ATG5* $^{fl/fl}$;*Nphs2-Cre*$^{+}$ mice and corresponding controls at the age of 2 weeks (no further phenotype at this time point). *GFP-LC3* and *GFP/RFP-LC3* mice were 4 months old if not mentioned otherwise. Rapamycin and AICAR were purchased from Sigma Aldrich and Santa Cruz, respectively, and both administered i.p.

To isolate primary podocytes *Raptor* $^{fl/fl}$;*Nphs2*-Cre^{+} and *Tsc1* $^{fl/fl}$;*Nphs2*-Cre^{+} were further crossed to *Tomato/EGFP* reporter mice. *Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J* mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Raptor* $^{fl/fl}$;*Tomato/EGFP*;*Nphs2*-Cre^{+} and *Tsc1* $^{fl/fl}$;*Tomato/EGFP*;*Nphs2*-Cre^{+} and WT;*Tomato/EGFP*; *Nphs2*-Cre^{+} were chosen for primary cell isolation. All animal studies were approved by the Committee on Research Animal Care Regierungspraesidium Freiburg and by INSERM according to the French veterinary guidelines respectively.

Primary Cell Isolation and Cultivation

Isolation of primary podocytes was performed as recently described.[86] To specifically label podocytes, conditional mouse lines were crossed to a well-established reporter mouse strain.[87] Expression of Cre-recombinase results in specific activation of EGFP in targeted cell populations.

To generate primary podocytes, first glomeruli were isolated from non-proteinuric 12 – 14 day old pups and cultivated in standard podocyte culture medium (RPMI 1640

supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and insulin/transferrin/selenite, all from Roche Diagnostics). After 5 days of expansion, cells were trypsinized and transferred to HBSS (Hanks Buffered Salt Solution, Gibco Life Technologies by Invitrogen, #14025092) supplemented with 0.1 % bovine serum albumin (Serva, #11930). Cell suspension was then sorted using FACS (MoFlo™ cell sorter, Beckmann Coulter) and only EGFP positive cells were further cultivated. Cultivation was performed using standard podocyte culture medium and cells were plated on collagen IV (Sigma-Aldrich) coated flasks or dishes (all Corning).

Primary podocytes were treated with AICAR 1mM for 24h. To inhibit mTORC1 *in vitro* podocytes were treated with rapamycin (Sigma-Aldrich) 1 µM for 6h. Compound C (Sigma-Aldrich) was applied at a dose of 5 µM for 2h. ULK1/2 inhibition was achieved by using MRT 689211 (Tocris) at 10µM for 12h (see ref. [48]).

Staining of primary podocytes

After FACS procedure, cells were seeded on collagen coated cover slips. 24 h later cells were fixed with paraformaldehyde and stained with mouse anti-LC3 (MBL, mAb M152-3).

Immunofluorescence staining

Kidneys frozen in Tissue-Tek OCT™ (Sakura) were cyrosectioned at 5 µm (Leica Kryostat). Sections were fixed with 4% paraformaldehyde. Non-specific protein binding was blocked with PBS containing 5% BSA. Sections were incubated for 1 hour with primary antibodies (rat anti-nidogen/entactin (Novus, NBP1-97001), rabbit anti-GFP (Biozol/MBL, #598)). After washing with PBS, fluorophore-conjugated secondary antibodies (Invitrogen) were applied for 30 min.

For paraffin sections, kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, and cut in 3 µm. After dehydration, antigen heat retrieval was performed at a pH of 6. Blocking was performed using PBS containing 5% BSA. Sections were incubated for 1 h with primary antibodies (rabbit anti-LC3B (Cell signaling, #2775), guinea pig anti-SQSTM1 (Progen, GP62-C), rabbit anti-pS6 Ser235/236 (Cell signaling, #4857), rabbit anti-phospho-acetyl-CoA carboxylase Ser79 (Cell signaling, #3661), rabbit anti-NPHS2 (Podocin, Sigma P0372), guinea pig anti-NPHS1 (Nephrin, Progen GP-N2). After washing with PBS, fluorophore-conjugated secondary antibodies (Invitrogen) were applied for 30 min. Images were taken using a Zeiss laser scan microscope or a Zeiss fluorescence microscope equipped with a 20 × and a 63 × water immersion objective. Signal was quantified using Image J out of 30 glomeruli per mouse if not mentioned otherwise. For the assessment of pACC signal integrated density was normalized to the intensity observed in WT glomeruli.

In vivo two-photon assessment of autophagy

4-month-old *GFP-LC3* mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). After placing a tracheal tube and cannulating the jugular vein 70kDa Texas red dextran was injected to label the vasculature. The left kidney was exposed by a flank incision and the animal then further placed on the microscope stage as described.[88,89] Images have been acquired using inverted Zeiss LSM710 NLO multiphoton confocal fluorescence microscope.

Glomerular isolation and antibodies used for Western blot

Glomeruli of non-proteinuric 12 – 14 days old mice were isolated using Dynabead™ perfusion (Thermo Fisher Scientific, described in refs. [90,91]) and were glass-glass homogenized in lysis buffer (RIPA buffer) [92]. After centrifugation (15,000 g for 15

minutes at 4°C), protein concentration was determined by BCA-Assay (Pierce). Equal amounts of protein were separated by SDS-PAGE. Following antibodies were used for Western blot: mouse anti-β-actin (Sigma-Aldrich, mAb A5441), mouse anti-α tubulin (Sigma Aldrich, mAb T6199), rabbit anti-ULK1 (Cell signaling, #8054), rabbit anti-pULK Ser757 (Cell signaling, #6888), rabbit anti-pULK1 Ser555 (Cell signaling, #5869), rabbit anti-pULK1 Ser317 (Cell signaling, #6887), rabbit anti-AMPKα (Cell signaling, #5381), rabbit anti-pAMPKα Thr172 (Cell signaling, #4188), guinea pig anti-SQSTM1 (Progen, GP62-C), rabbit anti-LC3B (Cell signaling, #2775), rabbit anti-S6 (Cell signaling, #2217), rabbit anti-pS6 Ser235/236 (Cell signaling, #4857), rabbit anti-Raptor (Cell signaling, #2280), rabbit anti-phospho-acetyl-CoA carboxylase Ser79 (Cell signaling, #3661), rabbit anti-4E-BP1 (Cell signaling, #9452), rabbit anti-phospho-4E-BP1 Thr37/46 (Cell signaling, #2855).

Immortalized human podocytes

Podocytes were kindly provided by Moin Saleem (Bristol, UK) and transduced to stably express *GFP-LC3* via lentiviral expression vector (kind gift of Noboru Mizushima, Department of Biochemistry and Molecular Biology, University of Tokyo, Japan). Podocytes were cultured as previously described (see ref. [93]) and seeded in RPMI 1640 supplemented with 10% FCS, insulin/transferrin/selenite, penicillin/streptomycin (all from Roche Diagnostics), pyruvate, 100x minimal essential medium and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (all Life technologies). When cells had grown to 60% confluence differentiation was induced at 37°C for 10–14 days. Cultured human podocytes were fully differentiated before any treatment.

Immortalized human proximal tubular cells

HK 2 cells were purchased from ATCC and transduced to stably express *GFP-LC3* via lentiviral expression vector (kind gift of Noboru Mizushima, University of Tokyo). Cells were cultured as described.[94]

Proximity Ligation Assay

Proximity ligation assay (PLA) was performed following the manufacturer`s instructions (Olink Bioscience). In brief, primary podocytes were seeded on Collagen IV coated diagnostic microscope slides (8 well, 6 mm, Thermo Scientific). After 24h cells were fixed with paraformaldehyde and stained with anti LC3 (MBL, mouse, mAb M152-3) and SQSTM1 (Santa Cruz, rabbit pAb, #5114). PLA was performed using Duolink® In Situ PLA Probe Anti-Mouse PLUS and using Duolink® In Situ PLA® Probe Anti-Rabbit MINUS following the manufacturer`s instructions. Imaging was performed using a Zeiss Axiovert 200M microscope, equipped with AxioCam MRc5 digital video camera and ApoTome immunofluorescence apparatus (Carl Zeiss SpA, Arese, Italy). Images were recorded using AxioVision software 4.3 and analyzed by Image J. Signal of 40 cells were analyzed per experiment; 3 experiments were performed.

Sirolimus serum levels

Serum levels of sirolimus (rapamycin) were obtained using LC-MS/MS. Analysis was kindly performed by Division of Clinical Chemistry, Department of Medicine, University Medical Center Freiburg.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). All experiments were performed at least 3 times. Statistical comparisons were performed with the program Excel using 2-tailed Student`s t test and with Graphpad Prism for ANOVA (Fig. S5F, Fig. S7A, Fig. S7B, Fig. S8F and S8G), respectively. Differences with $p \leq 0.05$ were considered significant and marked as *, $p \leq 0.01$ was marked as **.

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Figure legends:

Fig. 1: Podocytes exhibit high levels of basal autophagy and autophagic flux. **(A)** Representative images obtained from cryosections of 4-month old *GFP-LC3* mice stained for the basement membrane marker entactin/nidogen (red) and GFP-LC3

(green). **(B)** Two-photon images of glomeruli from *GFP-LC3* mice perfused with dextran as a marker for glomerular capillaries (in red) displaying autophagosomes *in vivo* (in green, marked with arrow). **(C)** Representative images obtained from cryosections of 4-month-old *GFP/RFP-LC3* mice stained for entactin/nidogen (purple). RFP-LC3 and GFP-LC3 fluorescence is endogenous. **(D)** Quantification of (C) out of 30 glomeruli from 3 mice each with surrounding tubular cells (** ≤ 0.01, * ≤ 0.05). **(E)** Cryosections displaying glomeruli (upper panel) and tubular system (lower panel) stained for entactin/nidogen (red) and GFP-LC3 (anti-GFP antibody, green) in 4-month-old WT mice with and without chloroquine (4h after chloroquine (Cq) administration i.p. 100 mg Cq/kg BW). **(F)** Western blot from immortalized human podocyte cell line and proximal tubular cell line (HK2) for mTORC1 downstream targets and LC3 abundance. **(G)** Densitometry obtained from (F) (** ≤ 0.01).

Fig. 2: Basal autophagy is independent of mTOR activity in podocytes *in vivo.* **(A)** Schematic of generating podocyte-specific deletion of *Raptor* or *Tsc1* using *Nphs2-Cre* mice and Cre-Lox technique. **(B)** Cryosections from 2-week-old mice bearing podocyte-specific knockout for *Raptor* and transgenic for *GFP-LC3* compared to *GFP-LC3* WT mice (entactin/nidogen in red, GFP-LC3 in green). **(C)** Quantification of GFP-LC3 autophagosomes per glomerular area out of 30 glomeruli each from 3 mice. **(D)** Cryosections from 2-week-old mice bearing podocyte-specific knockout for *Tsc1* and transgenic for *GFP-LC3* compared to *GFP-LC3* WT mice (entactin/nidogen in red, GFP-LC3 in green). **(E)** Quantification of GFP-LC3 autophagosomes per glomerular area out of 30 glomeruli each from 3 mice. **(F)** Western blot out of glomerular lysates obtained from 2- week-old mice for mTORC1 downstream targets and LC3 and SQSTM1 abundance. **(G)** Densitometry for LC3-II, SQSTM1 and pS6 obtained from 3 WT glomerular lysates and 3 glomerular lysates obtained from 2-

week-old mice bearing a podocyte-specific deletion of *Raptor* or *Tsc1*, respectively (** ≤ 0.01, * ≤ 0.05).

Fig. 3 Basal autophagy is independent of mTOR activity in podocytes *in vitro.* **(A)** Schematic of generating mice with a podocyte-specific knockout for *Tsc1* additionally harboring a *Tomato/EGFP* allele for primary cell isolation. **(B)** Schematic of generating mice with a podocyte-specific knockout for *Raptor* additionally harboring a *Tomato/EGFP* allele for primary cell isolation. **(C)** Primary podocytes with and without *Raptor* deficiency are stained for LC3 in red (green: endogenous fluorescence due to *Tomato/EGFP* reporter and *Nphs2*-Cre). **(D)** Quantification of LC3 signal out of 30 primary podocytes from 3 mice each. **(E)** Schematic illustrating proximity ligation assay (PLA) for SQSTM1 and LC3. **(F)** Representative image of primary podocytes with and without *Raptor* and LC3-SQSTM1-PLA signal in red (green: endogenous fluorescence due to *Tomato/EGFP* reporter and *NHPS2*-Cre). **(G)** Quantification of PLA signal from 30 podocytes obtained from 3 mice each. **(H)** Primary podocytes with and without *Tsc1* deficiency stained for LC3 in red (green: endogenous fluorescence due to *Tomato/EGFP* reporter and *Nphs2*-Cre). **(I)** Quantification of LC3 signal out of 30 primary podocytes from 3 mice each. **(J)** Western blot from primary podocytes for abundance of LC3, SQSTM1 and mTORC1 downstream targets. **(K)** Densitometry from (I) (** ≤ 0.01).

Fig. 4: AMPK controls autophagy in podocytes. **(A)** Representative sections from 2-week-old mice bearing podocyte-specific deletion of *Raptor* or *Tsc1* and controls stained for phosphorylated acetyl-CoA carboxylase Ser79 (pACC) in red and NPHS1 (green). **(B)** Western blot out of glomerular lysates obtained from 2- week-old mice

(genotype as indicated) for AMPKα and ULK1 and respective phosphorylation sites. **(C)** Densitometry for pAMPKαThr172 obtained from 3 WT glomerular lysates and 3 glomerular lysates obtained from 2-week-old mice bearing a podocyte-specific deletion of *Raptor* or *Tsc1*, respectively (** ≤ 0.01, * ≤ 0.05). **(D)** Densitometry for pULK1Ser757, pULK1Ser555 and pULK1Ser317 obtained from 3 WT glomerular lysates and 3 glomerular lysates obtained from 2-week-old mice bearing a podocyte-specific deletion of *Raptor* or *Tsc1*, respectively (** ≤ 0.01, * ≤ 0.05). **(E)** Western blot from primary podocytes treated with AICAR (1mM) for the time indicated (12h or 24h) to assess the abundance of pULK1Ser555, pAMPKαThr172 and pS6Ser235/236. **(F-H)** Densitometry from (E) (** ≤ 0.01, * ≤ 0.05).

Fig. 5: Activation of AMPK stimulates autophagy independently of mTORC1 activity. **(A)** Western blot from *Tsc1*-deficient podocytes and corresponding WT controls with and without AICAR treatment (1 mM, 24h) to assess the abundance of phosphorylated forms of ULK1 and AMPK as indicated. **(B)** Densitometry from (A) (** ≤ 0.01, * ≤ 0.05). **(C)** Representative section from 2-week-old mice bearing podocyte-specific deletion of *Raptor* with and without AICAR treatment (500 mg/kg BW 24h and 4h before harvest) stained with LC3 (red) and NPHS1/nephrin in green. **(D)** Representative section from 2-week-old mice bearing podocyte-specific deletion of *Raptor* with and without AICAR treatment (500 mg/kg BW 24h and 4h before harvest) stained with SQSTM1 (red) and NPHS1/nephrin in green. **(E)** Quantification of LC3 puncta per glomerular area (n = 3 each, 30 glomeruli counted per mouse, * ≤ 0.05). **(F)** Quantification of SQSTM1 puncta per glomerular area (n = 3 each, 30 glomeruli counted per mouse, * ≤ 0.05). **(G)** Western blot from glomerular lysates from 2-week-old mice bearing podocyte-specific deletion of *Raptor* with and without AICAR

treatment (500 mg/kg BW 24h and 4h before harvest) to assess the abundance of LC3-II. **(H)** Densitometry from (G) (* ≤ 0.05).

Fig. 6: Effects of acute and long-term pharmacological inhibition of mTORC1 activity on autophagy. **(A)** Schematic of the short-term treatment regimen (rapamycin vs. vehicle, n = 5 each, rapamycin dose: 10 mg/kg BW i.p. for 3 days). **(B)** Quantification of GFP-LC3 autophagosomes per glomerular area out of 30 glomeruli each from 5 mice per group (vehicle vs. rapamycin). **(C)** Representative cryosections from 16-week-old mice transgenic for GFP-LC3 with and without rapamycin treatment (entactin/nidogen in red, GFP-LC3 in green, rapamycin dose: 10 mg/kg BW i.p. for 3 days). **(D)** Representative Western blot from glomerular lysates from rapamycin and vehicle treated mice (WT mice, n = 3 each, rapamycin dose: 10 mg/kg BW i.p. for 3 days). **(E)** Densitometric quantification of (D) (** ≤ 0.01, * ≤ 0.05). **(F)** Schematic of the long-term treatment regimen (rapamycin dose: 4 mg/kg BW i.p. for 3 weeks). **(G)** Quantification of serum levels of rapamycin (n = 6 each) (*** ≤ 0.001). **(H)** Representative Western blot from glomerular lysates from rapamycin long-term, short-term and vehicle treated mice. **(I)** Densitometry from 3 glomerular lysates of each treatment and vehicle controls for LC3-II, SQSTM1 and pS6Ser235/236 (** ≤ 0.01).

Fig. 7: Activation of AMPK stimulates autophagy independently of mTOR activity. **(A)** AMPK activity is reduced if mTORC1 signaling is genetically impaired (activating phosphorylation sites at ULK1 are displayed in green, inhibitory sites are in red). **(B)** Inhibitory signals on ULK1 by genetically induced mTORC1 hyperactivity are compensated by active AMPK signaling. **(C)** Pharmacological activation of AMPK is able to induce autophagy in podocytes by activating ULK1. **(D)** mTORC1 can be bypassed in autophagy regulation as it occurs in the genetic model of mTORC1

inhibition by direct phosphorylation of ULK1 by AMPK. **(E)** Short-term treatment with rapamycin inhibits mTORC1 activity and activates AMPK leading to increased levels of autophagy. **(F)** Long-term treatment with rapamycin is compensated by reduced AMPK signaling in regard to autophagy regulation.

Fig. S1: Podocytes display high levels of basal autophagy. **(A)** Representative images from tubular system of 4-month-old *GFP/RFP-LC3* mice (endogenous fluorescence). **(B)** Representative images from the liver of 4-month-old *GFP/RFP-LC3* mice. **(C)** Quantification of (A), (B), and Fig.1C out of 50 cells obtained from 3 different mice (* ≤ 0.05). **(D)** Liver sections of *GFP-LC3* mice with and without chloroquine treatment (chloroquine i.p. 100 mg/kg BW for 4h as indicated in Method section) stained for GFP-LC3 (green) and nidogen/entactin (red). **(E)** Western blot analysis for the abundance of LC3 from tissue lysates from mice treated with and without chloroquine (100 mg/kg BW i.p. for 4h as indicated in Method section). **(F)** Autophagosome formation with and without chloroquine treatment (100 µM, 2 h) in an immortalized human proximal tubular cell line (HK2) and human podocytes stably expressing GFP-LC3 (in green). **(G)** Quantification of (F) (* ≤ 0.05).

Fig. S2: Genetic models of mTORC1 hypo- and hyperactivation and their impact on autophagy. **(A)** Schematic of generating mice with a podocyte-specific deletion of *Raptor* or *Tsc1* and transgenic for *GFP-LC3*. **(B)** Immunofluorescence staining for phosphorylated ribosomal protein S6 Ser235/236 in WT mice and mice bearing podocyte-specific deletion of *Raptor*. **(C)** Western blot analysis from glomerular lysates obtained from WT mice and mice bearing podocyte-specific deletion of *Raptor* or *Tsc1***,** respectively, with and without chloroquine (100 mg/kg BW i.p. for 4h)

for the abundance of LC3-I and LC3-II. **(D)** Schematic of generating mice with a doxycycline-inducible podocyte-specific deletion of *Raptor*. **(E)** Western blot analysis from glomerular lysates obtained from WT mice and mice after induction of podocyte-specific *Raptor* deletion. **(F)** Densitometry for LC3-II abundance from 3 samples each (induced *Raptor* deficiency vs. WT). **(G)** Schematic of obtaining pure primary podocyte culture by Tomato/EGFP labeling and cell sorting by FACS. **(H)** Western blot analysis for LC3-II abundance and mTORC1 downstream targets in primary cultured podocytes (WT vs. *Tsc1*-deficient) treated with chloroquine (100 µM, 2 h). **(I)** Western blot analysis for LC3-II abundance in primary cultured podocytes (WT vs. *Tsc1*-deficient) with and without chloroquine treatment (100 µM, 2 h). **(J)** Densitometry of 3 samples each as displayed in (H).

Fig. S3: Impact of AMPK activation of autophagy. **(A)** Integrated density of glomerular pACC signal obtained from 3 mice of each genotype (WT, *Raptor* and *Tsc1*-deficient, 20 glomeruli assessed per mouse, * ≤ 0.05). **(B)** Western blot for LC3-II abundance and AMPK activation in primary podocytes treated with metformin for 2 h with the indicated dose. **(C)** Western blot for LC3-II abundance in primary podocytes treated with metformin for 2 h with and without chloroquine (100 µM, 2 h). **(D)** Western blot from lysates obtained from primary podocytes (WT vs. *Tsc1*-deficient) treated with AICAR (1mM, 24h) or vehicle with or without chloroquine (100 µM, 2 h). **(E)** Densitometry for LC3-II from lysates obtained from primary podocytes (WT vs. *Tsc1*-deficient) treated with AICAR or vehicle with or without chloroquine (3 samples each, * ≤ 0.05, ** ≤ 0.01). **(F)** Western blot out of liver lysates for abundance of pAMPKαThr172 and pACCSer79 from mice treated with AICAR (500 mg/kg BW 24h and 4h before harvest) or vehicle. **(G)** Immunofluorescence staining for phosphorylated ribosomal protein S6 Ser235/236 in WT mice and mice bearing

podocyte-specific deletion of *Raptor* with and without AICAR treatment (500 mg/kg BW 24 h and 4 h before harvest).

S4: Effect of AMPK activation by AICAR on autophagy in genetic models of mTORC1 hypo- and hyperactivation. **(A)** Representative sections from 2-week-old mice bearing podocyte-specific deletion of *Raptor* or *Tsc1* and corresponding WT controls with and without AICAR treatment (500 mg/kg BW 24h and 4h before harvest) stained with LC3 (red) and NPHS1/nephrin in green. **(B)** Representative sections from 2-week-old mice bearing podocyte-specific deletion of *Raptor* or *Tsc1* and corresponding WT controls with and without AICAR treatment (500 mg/kg BW 24h and 4h before harvest) stained with SQSTM1 (red) and NPHS2 in green.

S5: Effect of AMPK activation by AICAR on autophagy in genetic models of mTORC1 hypo- and hyperactivation. **(A)** Quantification of Fig. S4A by assessing LC3 puncta per glomerular area (n = 3 each, 30 glomeruli counted per mouse) with and without AICAR treatment (* ≤ 0.05). **(B)** Quantification of Fig. S4B by assessing SQSTM1 puncta per glomerular area (n = 3 each, 30 glomeruli counted per mouse) with and without AICAR treatment. **(C)** Representative sections from 2-week-old mice bearing podocyte-specific deletion of *Raptor* or *Tsc1* and corresponding WT controls with and without AICAR treatment (500 mg/kg BW 24h and 4h before harvest) stained with pACCSer79 (red) and NPHS1/nephrin in green. **(D)** Western blot from glomerular lysates from 2-week-old mice bearing podocyte-specific deletion of *Raptor* or *Tsc1* and corresponding WT controls with and without AICAR treatment (500 mg/kg BW 24h and 4h before harvest) for the abundance of pACCSer79, ACC,

AMPK and pAMPKαThr172. **(E)** Quantification of (D) (3 samples each, ** ≤ 0.01, * ≤ 0.05).

S6: Effect of mTORC1 inhibition on autophagy. **(A)** Western blot from lysates obtained from primary podocytes (WT vs. *Tsc1*-deficient) treated with compound C (5 µM for 2h) or vehicle for AMPK activity and LC3. **(B)** Western blot from lysates obtained from primary podocytes (WT vs. *Tsc1*-deficient) treated with compound C (5 µM for 2h) with and without chloroquine (100 µM, 2 h). **(C)** Primary podocytes treated with the ULK1/2 inhibitor MRT68921 (10 µM for 12h) or vehicle with and without chloroquine (100 µM, 2 h). **(D)** Densitometry for LC3-II from lysates as displayed in (C) (3 samples each, ** ≤ 0.01, * ≤ 0.05). **(E)** Western blot from glomerular lysates for the abundance of LC3 and pS6Ser235/236 from rapamycin (10 mg/kg BW for 3 days) and vehicle treated mice bearing podocyte-specific deletion of *Atg5*. **(F)** Western blot from lysates obtained from primary podocytes (WT vs. *Tsc1*-deficient) treated with rapamycin (1 µM for 6h) or vehicle for mTORC1 downstream targets, phosphorylated AMPKα and LC3. **(G)** Western blot from lysates obtained from primary podocytes (WT vs. *Tsc1*-deficient) treated with rapamycin (1 µM for 6h) with and without chloroquine (100 µM, 2 h) for the abundance of LC3. **(H)** Densitometry for LC3-II from lysates as displayed in **(G)** (3 samples each, * ≤ 0.05, ** ≤ 0.01).

S7: Effect of short-term rapamycin treatment on autophagy in genetic models of mTORC1 hyperactivation and hypoactivation. **(A)** Representative sections from 2-week-old mice bearing podocyte-specific deletion of *Raptor* or *Tsc1* and corresponding WT controls with and without rapamycin treatment (10 mg/kg BW for 3 days) stained for pS6Ser235/236 (red) and NPHS1/nephrin in green. **(B)** Representative sections from 2-week-old mice bearing podocyte-specific deletion of *Raptor* or *Tsc1* and corresponding WT controls after rapamycin treatment (10 mg/kg

BW for 3 days) stained for LC3 (red) and NPHS1 in green (left column) or stained for SQSTM1 (red) and NPHS2 in green (right column).

S8: Effect of long-term rapamycin treatment on podocyte autophagy. **(A)** Quantification of Fig. S6A by assessing LC3 puncta per glomerular area (n = 3 each, 30 glomeruli counted per mouse, ** ≤ 0.01, * ≤ 0.05) after treatment with rapamycin (4 mg/kg BW for 3 weeks). **(B)** Quantification of Fig. S6B by assessing SQSTM1 puncta per glomerular area (n = 3 each, 30 glomeruli counted per mouse) after rapamycin treatment (** ≤ 0.01, * ≤ 0.05). **(C)** Immunofluorescence staining for GFP-LC3 (green) and NPHS2 (red) in sections obtained from mice treated with rapamycin or vehicle for 3 weeks. **(D)** Quantification for GFP-LC3 per glomerular area (rapamycin n = 3, vehicle n = 4). **(E)** Immunofluorescence staining for GFP-LC3 (green) and SQSTM1 (red) in sections obtained from mice treated with rapamycin or vehicle for 3 weeks. **(F)** Quantification for SQSTM1 per glomerular area (rapamycin n = 3, vehicle n = 4).

S9: Effect of long-term rapamycin treatment on podocyte autophagic flux. Mice were treated with rapamycin 4 mg/kg BW for 3 weeks receiving 100 mg/kg BW chloroquine 4 h before harvest. **(A)** Sections were stained for GFP-LC3 (green) and NPHS2 (red). **(B)** Quantification of LC3 signal per glomerular area (rapamycin + Cq n = 3, vehicle + Cq n = 4) **(C)** Sections were stained for GFP-LC3 (green) and SQSTM1 (red). **(D)** Quantification of SQSTM1 signal per glomerular area (rapamycin + Cq n = 3, vehicle + Cq n = 4). **(E)** Western blot from glomerular lysates obtained from mice treated with short-term rapamycin treatment (10 mg/kg BW for 3 days) or long-term rapamycin treatment (4 mg/kg BW for 3 weeks) or vehicle for the abundance of LC3 and SQSTM1 with and without chloroquine (100 mg/kg BW for 4h). **(F)** Densitometry for LC3-II to ACTIN ratio from 3 samples each (vehicle, rapamycin long-term and short-

term regimen with and without chloroquine) corresponding to Fig. S8E (** ≤ 0.01).). **(G)** Densitometry for SQSTM1 to ACTIN ratio from 3 samples each (vehicle, rapamycin long-term and short-term regimen with and without chloroquine) corresponding to Fig. S8E (** ≤ 0.01). **(H)** Densitometry for pAMPKαThr172 and pULK1Ser555 from 3 samples each (vehicle, rapamycin long-term and short-term regimen) corresponding to Fig. 6H (** ≤ 0.01, * ≤ 0.05).

S10: Time dependent effects of rapamycin and AICAR on podocyte autophagy. **(A)** Western blot from glomerular lysates obtained from mice treated with short-term rapamycin treatment (10 mg/kg BW for 3 days) or short-term AICAR treatment (500 mg/kg BW for 3 days) or vehicle for mTORC1 downstream targets, phosphorylated AMPKα and LC3. **(B)** Densitometry for pAMPKαThr172 and LC3 from 3 samples each as displayed in (A). **(C)** Western blot from primary murine podocytes treated with rapamycin (1 µM for 3 days) or AICAR (1mM for 3 days) with and without chloroquine (100 µM, 2 h) for the abundance of LC3, SQSTM1 and phosphorylated ULK1, S6 and AMPKα. **(D)** Western blot from primary murine podocytes treated with rapamycin (10 nM for 3 weeks) or AICAR (0.2 mM for 3 weeks) with and without chloroquine (100 µM, 2 h) for the abundance of LC3, SQSTM1 and phosphorylated ULK1, S6 and AMPKα. **(E)** Western blot from primary murine podocytes treated with rapamycin (1 µM for 3 days or 10 nM for 3 weeks) or AICAR (1mM for 3 days or 0.2 mM for 3 weeks) for the abundance of phosphorylated S6 and AMPKα. **(F)** Densitometry for pAMPKαThr172 as displayed in **(E)**.

S11: mTORC1 independent effects of AICAR on podocyte autophagy. **(A)** Western blot from primary murine podocytes pretreated with rapamycin (10 nM for 3 weeks) or AICAR (0.2 mM for 3 weeks) followed by rapamycin (1 µM for 3 days) or AICAR (1mM for 3 days) with and without chloroquine (100 µM, 2 h) for the abundance of

LC3, SQSTM1 and phosphorylated S6 and AMPKα. **(B)** Western blot from primary murine podocytes pretreated with rapamycin (10 nM for 3 weeks) or AICAR (0.2 mM for 3 weeks) followed by rapamycin (1 µM for 3 days) or AICAR (1mM for 3 days) for the abundance of phosphorylated S6 and AMPKα. **(C)** Densitometry for pAMPKαThr172 as displayed in (B).

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