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Abstract: This proposal will study novel mTORC1 and 2 signaling pathways that mediate ADPKD and investigate the effects of mTORC1 (Raptor) knockout, mTORC2 (Rictor) knockout or combined mTORC1 and 2 knockout on cyst growth and kidney function. The overall hypothesis is that there is increased mTORC1 (4E-BP1) and mTORC2 (AktSer473, PKCα and SGK1) signaling in PKD kidneys and that combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice will slow cyst growth and improve kidney function more than mTORC1 (Raptor) knockout or mTORC2 (Rictor) knockout alone. We have made significant progress in the fourth year: We have published the role 4E-BP1 signaling pathways in PKD kidneys and cells. We have preliminary data in Pkd1, Rictor double knockout mice. We have published: 1) The effect of trehalose on mTOR signaling and autophagy in PKD kidneys, 2) mTOR signaling and autophagy in the heart and kidney in PKD. We have continued to use FISP-MRI scanning to obtain precise measurements of kidney and cyst volume and the number of cysts in live PKD mice.					
15. SUBJECT TERMS Polycystic kidney disease, PKD, mTORC1, mTORC2, Raptor, Rictor.					
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1. INTRODUCTION:

ADPKD is the most common life threatening hereditary disease in the USA. ADPKD accounts for about 5-10% of end-stage renal failure in the USA requiring dialysis and renal transplantation. There is no effective treatment for ADPKD. This proposal will study novel mTORC1 (pS6 and 4E-BP1) and 2 (Akt, PKC alpha, SGK1) signaling pathways that mediate ADPKD and investigate the effects of mTORC1 (Raptor) knockout, or the mTORC1 inhibitor (sirolimus), mTORC2 (Rictor) knockout or combined mTORC1 and 2 knockout or the mTOR kinase inhibitor drugs (TORKs) on cyst growth and kidney function. The overall hypothesis is that there is increased mTORC1 (4E-BP1) and mTORC2 (AktSer473, PKC α and SGK1) signaling in PKD kidneys and that combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 $-/-$ mice will slow cyst growth and improve kidney function more than mTORC1 (Raptor) knockout or mTORC2 (Rictor) knockout alone or that the mTOR kinase inhibitors will be more effective than sirolimus in decreasing kidney cyst growth. These studies will unequivocally address whether the mTORC1 (4E-BP1) or mTORC2 pathway or both pathways is important in PKD and whether it is worthwhile performing therapeutic interventions using the novel mTORC1 and 2 inhibitors that are in clinical trials in humans

2. KEYWORDS:

Autosomal dominant polycystic kidney disease

mTORC1

Raptor

mTORC2

Rictor

4E-BP1

mTOR kinase inhibitor

3. ACCOMPLISHMENTS: What were the major goals of the project?

Major Task 1: To determine the effect of mTORC1 (Raptor) knockout in Pkd1 $-/-$ mice

Subtask 1: To determine whether Raptor knockout decreases the cystic phenotype

Subtask 2: To determine the effect of Raptor $-/-$ on 4E-BP1 signaling

Subtask 3: To determine the effect of 4E-BP inhibition on PKD

Major Task 2: To determine the effect of mTORC2 (Rictor) knockout in Pkd1 $-/-$ mice

Subtask 1: To determine whether Rictor knockout reduces the cystic phenotype in vivo

Subtask 2: AktSer473, SGK1 and PKC α phosphorylation in PKD in vivo

Subtask 3: AktSer473, SGK1 and PKC α phosphorylation in PKD in vitro

Major Task 3: To determine the effect of combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice

Subtask 1: To determine the effect of combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice on apoptosis and proliferation, cyst growth and kidney function.

SubTask 2: To determine the effect of TORCs versus sirolimus on PKD and kidney function

What was accomplished under these goals?

(1) Major activities:

Major task 1: 4E-BP1 signaling has been studied in ADPKD kidneys and PKD cells in vitro. This study has now been published in Hum Molec Genet.-see below.

We are continuing to breed Ksp1.3 mice with Pkd1^{fl/fl} and Raptor^{fl/fl} mice to develop Pkd1 -/-, Raptor -/- double knockout mice. These studies will continue as part of a no cost extension.

Major Task 2: Preliminary data on the development of Pkd1 -/- Rictor -/- double knockout mice using the tamoxifen-inducible ER2 Cre is presented below. These mice develop a slow form of PKD in 120 days.

We are continuing to breed Ksp1.3 mice with Pkd1^{fl/fl} and Rictor^{fl/fl} mice to develop Pkd1 -/-, Rictor -/- double knockout mice. See Table below. These studies will continue as part of a no cost extension.

Major task 3: Study of treatment of Pkd1 -/- mice with mTOR kinase inhibitors that inhibit mTORC1 and mTORC2 (TORCs) versus Sirolimus (mTORC1 inhibitor), has been published –see below.

Breeding of mice to develop triple knockout Pkd1-/-, Rictor -/-, Raptor -/- will continue as part of a no cost extension.

Major Tasks 1, 2 and 3: We are using FISP-MRI scans to determine polycystic kidney volume and cyst volume and number of cysts per kidney at different time points of the disease (Fig 2).

We have published data that mTOR signaling is activated in cardiac hypertrophy in PKD mice (See below)

We have published the effect of the autophagy inducer, trehalose, on mTOR signaling in the kidney and on PKD.

2) Specific objectives:

Major Task 1: To determine whether Raptor knockout decreases the cystic phenotype. To determine the effect of 4E-BP inhibition on PKD.

Major Task 2: To determine whether Rictor knockout reduces the cystic phenotype in vivo.

Major Task 3: To determine the effect of TORCs versus sirolimus on PKD and kidney function.

3) Major results:

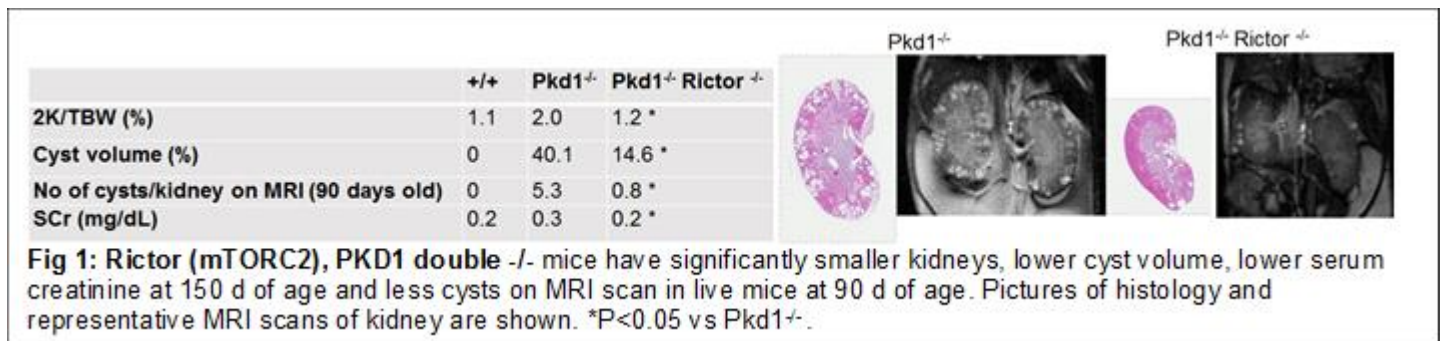
Major Task 1: To determine whether Raptor knockout decreases the cystic phenotype. To determine the effect of 4E-BP inhibition on PKD.

The consequences of increased 4E-BP1 in Polycystic kidney disease. Holditch SJ, Brown CN, Atwood D, Brown SE, Lombardi AM, Nguyen KN, Hill RC, Lanaspa M, Hopp K, Weiser-Evans M, Edelstein CL Hum. Molec Genet. 2019 Dec 15;28(24):4132-4147

Autosomal Dominant Polycystic kidney disease (ADPKD) is the most common hereditary renal disease, characterized by cyst formation and growth. Hyperproliferation is a major contributor to cyst growth. At the nexus of regulating proliferation, is 4E-BP1. We demonstrate that ADPKD mouse and rat models, ADPKD patient renal biopsies, and *PKD1*^{-/-} cells exhibited hyperphosphorylated 4E-BP1, a biomarker of increased translation and proliferation. We hypothesized that expression of constitutively active 4E-BP1 constructs (4E-BP1^{F113A} and 4E-BP1^{R13AF113A}) would decrease proliferation, and reduce cyst expansion. Utilizing the *Pkd1*^{RC/RC} mouse we determined the effect of 4E-BP1^{F113A} on PKD. Unexpectedly, 4E-BP1^{F113A} resulted in increased cyst burden and suppressed apoptosis markers, increased anti-apoptotic Bcl-2 protein and increased mitochondrial proteins. Exogenous 4E-BP1 enhanced proliferation, decreased apoptosis, increased anti-apoptotic Bcl-2 protein, impaired NADPH oxidoreductase activity, increased mitochondrial proteins and increased superoxide production in PKD patient derived renal epithelial cells. Reduced 4E-BP1 expression suppressed proliferation, restored apoptosis, and improved cellular metabolism. These findings provide insight into how cyst-lining cells respond to 4E-BP1.

Major Task 2: To determine whether Rictor knockout reduces the cystic phenotype in vivo

In new preliminary data, Rictor^{fl/fl} mice were bred with the *Pkd1*^{fl/fl}; KspCad-CreERT2 mice to develop Rictor^{fl/fl}, *Pkd1*^{fl/fl}; KspCad-CreERT2 mice that were treated with tamoxifen to develop kidney-specific Rictor, *Pkd1* double -/- mice that were evaluated at 90 and 150 days post tamoxifen (Fig1.). These mice develop slow PKD over a period of 150 days. gDNA was quantified by qPCR primers specific to PKD1 flox site and RICTOR flox site in Rnase treated DNA isolated from total kidney homogenates and showed that kidney specific PKD1 and Rictor genomic flox sites significantly reduced post Tamoxifen treatment.



We are breeding KSP 1.3 Cre mice with Pkd floxed and Rictor floxed mice to obtain KSP 1.3 Cre Pkd floxed mice and double knockout KSP 1.3 Cre Pkd floxed Rictor floxed mice. The mice develop rapid PKD and were sacrificed at days 16-18 of age. We have obtained mice of all these genotypes and results are shown in Table 1 below. In this model, heterozygous Rictor knockout in the polycystic kidney seems to make PKD worse. We will continue breeding these mice in a no cost extension and have 50 cages of mice for this breeding process.

Table 1	Wild type N=12	KSP1.3 Pkd1 -/- N=3	KSP1.3Pkd1-/- Rictor +/- N=4	KSP1.3Pkd1-/- Rictor +/- N=1
2K/TBW (%)	1.52 ± 0.03	38.9 ± 0.05 *	41.6 ± 1.1**	41.8

2K/TBW (%)= 2 kidney weight/total body weight as a % - a marker of cystic disease in the kidney

*P<0.001 vs. wild type, ** P<0.001 vs. wild type, P<0.01 vs KSP1.3 Pkd1 -/-.

Major Task 3: To determine the effect of TORCs versus sirolimus on PKD and kidney function.

We have found that in a mouse model of autosomal dominant polycystic kidney disease (ADPKD), both sirolimus and an mTOR kinase inhibitor decrease cyst volume and improve kidney function despite differential effects signaling and apoptosis. Study has been published:

Holditch S, Brown CN, Atwood DJ, Lombardi AM, Nguyen KN, Toll HW, Hopp K, Edelstein CL. A study of sirolimus and an mTOR kinase inhibitor (TORKi) in a hypomorphic Pkd1 mouse model of autosomal dominant polycystic kidney disease (ADPKD). Am J.Physiol Renal Physiol 2019 Jul 1;317(1):F187-F196

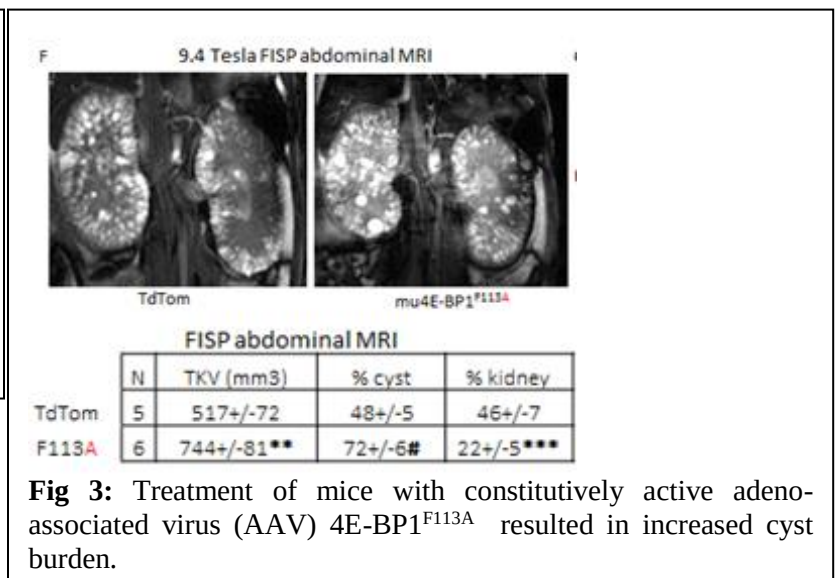
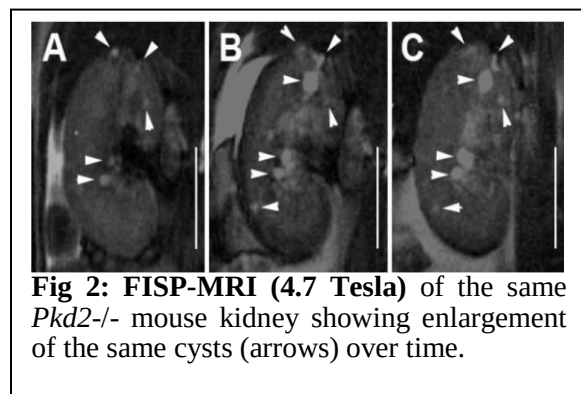
Autosomal dominant polycystic kidney disease (ADPKD) is characterized by cyst formation and growth, which is partially driven by abnormal proliferation of tubular cells. There is activation of the pro-proliferative

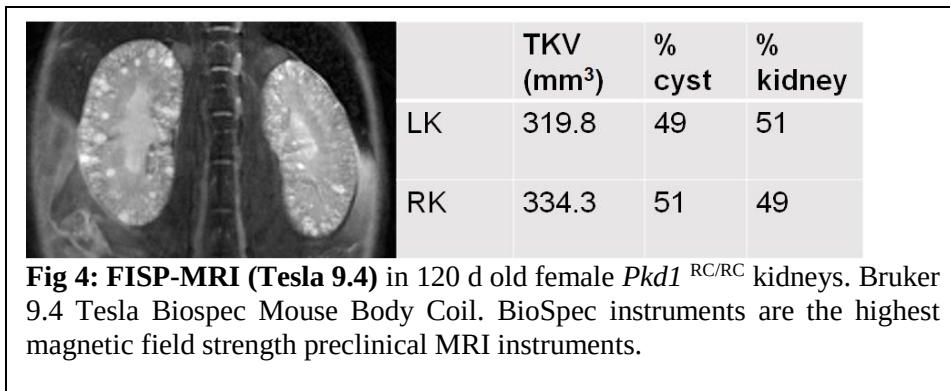
mechanistic target of rapamycin complex 1 and 2 (mTORC1 and 2) in PKD mouse kidneys. Sirolimus indirectly inhibits mTORC1. The novel mTOR kinase inhibitors (TORKi) directly inhibit mTOR kinase resulting in inhibition of both mTORC1 and 2. The aim of the present study was to determine the effects of sirolimus versus the TORKi, torin2, on cyst growth and kidney function in the *Pkd1* p.R3277C (*Pkd1*^{RC/RC}) mouse model, a hypomorphic *Pkd1* model orthologous to the human condition and to determine the effects of sirolimus versus torin2 on mTORC1 and 2 signaling in *PKD1*^{-/-} cells and in the kidneys of *Pkd1*^{RC/RC} mice. *In vitro*, both inhibitors reduced mTORC1 and mTORC2 phosphorylated substrates, and negatively impacted cellular metabolic activity as measured by the MTT assay. *Pkd1*^{RC/RC} mice were treated with sirolimus or torin2 from 50 to 120 days of age. Torin2 was as effective as sirolimus in decreasing cyst growth and improving loss of kidney function. Both sirolimus and torin2 decreased pS6, p4E-BP1, pAkt and proliferation in *Pkd1*^{RC/RC} kidneys. In conclusion, both torin2 and sirolimus were equally effective in decreasing cyst burden, improving kidney function, and mediated comparable effects on mTORC1 and 2 signaling and proliferation in the *Pkd1*^{RC/RC} kidney.

4) Other achievements: Major tasks 1, 2 and 3.

Use of FISP-MRI

We continue to use FISP-MRI to obtain measurements of kidney and cyst volume in live PKD (See Figs 2, 3 and 4).





We have published two papers in the last 12 months that include mTORC1/2 signaling in the heart and kidney in PKD.

mTORC1/2 signaling in the heart in PKD:

Atwood DJ, Pokhrel D, Brown CN, Holditch SJ, Bachu DM, Thorburn A, Hopp K, Edelstein CL. Increased mTOR and suppressed autophagic flux in the heart of a hypomorphic *Pkd1* mouse model of autosomal dominant polycystic kidney disease. Cell Signal. 2020 (in press)

Abstract:

Cardiac hypertrophy is common in autosomal dominant polycystic kidney disease (ADPKD) patients. We found increased heart weight in *Pkd1*^{RC/RC} and *Pkd2*^{WS25/+} mouse models of ADPKD. As there is a link between increased heart weight and mammalian target of rapamycin (mTOR), the aim of the study was to determine mTOR complex 1 and 2 signaling proteins in the heart in the *Pkd1*^{RC/RC} mouse model of PKD. In 70 day old *Pkd1*^{RC/RC} hearts, on immunoblot analysis, there was a large increase in p-AMPK^{Thr172}, a known autophagy inducer, and an increase in p-Akt^{Ser473} and p-Akt^{Thr308}, but no increase in other mTORC1/2 proteins (p-S6^{Ser240/244}, p-mTOR^{Ser2448}). In 150 day old *Pkd1*^{RC/RC} hearts, there was an increase in mTORC1 (p-S6^{Ser240/244}) and mTOR-related proteins (p-Akt^{Thr308}, p-GSK3β^{Ser9}, p-AMPK^{Thr172}). As the mTOR pathway is the master regulator of autophagy, autophagy proteins were measured. There was an increase in p-Bec1-1 (BECN1), an autophagy regulator and activating molecule in Beclin-1-regulated autophagy (AMBRA1), a regulator of Beclin that play a role in autophagosome formation, an early stage of autophagy. There was a defect in the later stage of autophagy, the fusion of the autophagosome with the lysosome, known as autophagic flux, as evidenced by the lack of an increase in LC3-II, a marker of autophagosomes, with the lysosomal inhibitor bafilomycin, in both 70 day old and 150 day old hearts. To determine the role of autophagy in causing increased heart weight, *Pkd1*^{RC/RC} were treated with 2-deoxyglucose (2-DG) or Tat-Bec1-1 peptide, agents known to induce autophagy. 2-DG treatment from 150 to 350 days of age, a time period when increased heart weight developed, did not reduce the increased heart weight. Unexpectedly, Tat-Bec1-1 peptide treatment from 70 to 120 days of age

resulted in increased heart weight. In summary, there is suppressed autophagic flux in the heart at an early age in *Pkd1*^{RC/RC} mice. Increased mTOR signaling in older mice is associated suppressed autophagic flux. There was a large increase in p-AMPK^{Thr172}, a known autophagy inducer, in both young and old mice. 2-DG treatment did not impact increased heart weight and Tat-Becn1 peptide increased heart weight.

mTOR signaling in the kidney in PKD:

Atwood DJ, Brown DN, Holditch SJ, Pokhrel D, Thorburn A, Hopp K and Edelstein CL. The effect of trehalose on autophagy-related proteins and cyst growth in a hypomorphic *Pkd1* mouse model of autosomal dominant polycystic kidney disease. Cell Signal. 2020 (in press).

Abstract:

Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited disorder characterized by kidney cyst growth often resulting in end-stage renal disease. There is growing attention on understanding the role of impaired autophagy in ADPKD. Trehalose (TRE) has been shown to increase both protein stability and aggregate clearance and induce autophagy in neurodegenerative diseases. TRE treatment in wild type mice compared to vehicle resulted in increased expression in the kidney of Atg12-5 complex and increased Rab9a, autophagy-related proteins that play a role in the formation of autophagosomes. Thus, the aim of the study was to determine the effect of TRE on cyst growth and autophagy-related proteins, in the hypomorphic *Pkd1*^{RC/RC} mouse model of ADPKD. *Pkd1*^{RC/RC} mice were treated 2% TRE in water from days 50 to 120 of age. TRE did not slow cyst growth or improve kidney function or affect proliferation and apoptosis in *Pkd1*^{RC/RC} kidneys. In *Pkd1*^{RC/RC} vs. wild type kidneys, expression of the Atg12-5 complex was inhibited by TRE resulting in increased free Atg12 and TRE was unable to rescue the deficiency of the Atg12-5 complex. Rab9a was decreased in *Pkd1*^{RC/RC} vs. wild type kidneys and unaffected by TRE. The TRE-induced increase in p62, a marker of autophagic cargo, that was seen in normal kidneys was blocked in *Pkd1*^{RC/RC} kidneys. In summary, the autophagy phenotype in *Pkd1*^{RC/RC} kidneys was characterized by decreases in crucial autophagy-related proteins (Atg12-5 complex, Atg5, Atg16L1), decreased Rab9a and increased mTORC1 (pS6, p-mTOR^{S2448}) proteins. TRE increased Atg12-5 complex, Rab9a and p62 in normal kidneys, but was unable to rescue the deficiency in autophagy proteins or suppress mTORC1 in *Pkd1*^{RC/RC} kidneys and did not protect against cyst growth.

What opportunities for training and professional development has the project provided?

The grant was not meant to provide training or professional development. However, during the summer there was 1 college student, Dheenu Bachu, working with our group as a volunteer to advance her professional skills in basic medical research.

How were the results disseminated to communities of interest?

Our results to date have been accepted as Poster Presentations at the annual American Society of Nephrology Meeting in Denver, Colorado in November, 2020. Due to Covid-19 the meeting will not be an in person meeting but a virtual meeting.

We have published a review paper that includes much of our recent research and will be of interest to the PKD community:

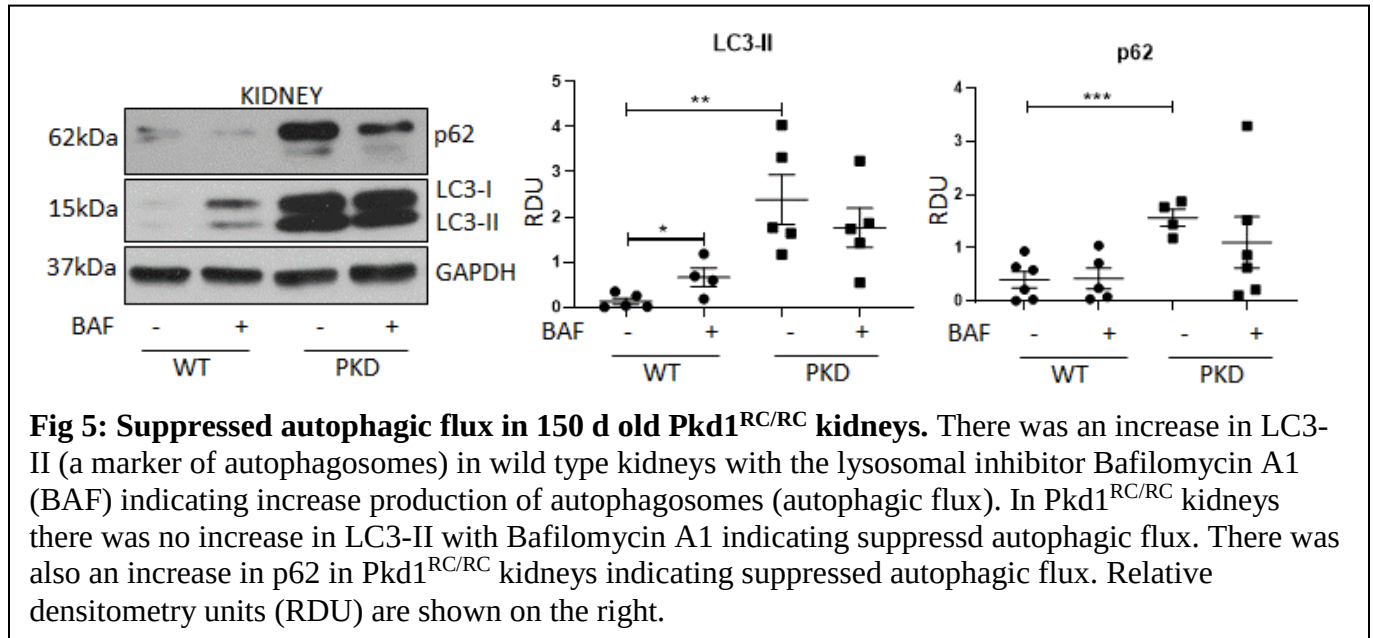
Nowak K, Edelstein CL Apoptosis and autophagy in PKD. Cell Signal 2020 Apr;68:109518. doi: 10.1016

Apoptosis in the cystic epithelium is observed in most rodent models of polycystic kidney disease (PKD) and in human autosomal dominant PKD (ADPKD). Apoptosis inhibition decreases cyst growth, whereas induction of apoptosis in the kidney of Bcl-2 deficient mice increases proliferation of the tubular epithelium and subsequent cyst formation. However, alternative evidence indicates that both induction of apoptosis as well as increased overall rates of apoptosis are associated with decreased cyst growth. Autophagic flux is suppressed in cell, zebra fish and mouse models of PKD and suppressed autophagy is known to be associated with increased apoptosis. There may be a link between apoptosis and autophagy in PKD. The mammalian target of rapamycin (mTOR), B-cell lymphoma 2 (Bcl-2) and caspase pathways that are known to be dysregulated in PKD, are also known to regulate both autophagy and apoptosis. Induction of autophagy in cell and zebrafish models of PKD results in suppression of apoptosis and reduced cyst growth supporting the hypothesis autophagy induction may have a therapeutic role in decreasing cyst growth, perhaps by decreasing apoptosis and proliferation in PKD. Future research is needed to evaluate the effects of direct autophagy inducers on apoptosis in rodent PKD models, as well as the cause and effect relationship between autophagy, apoptosis and cyst growth in PKD.

What do you plan to do during the next reporting period to accomplish the goals?

- 1) Continue breeding the Raptor^{fl/fl} mice with mice with a Ksp 1.3 Cre mice to develop Raptor^{-/-}, Pkd1^{-/-} double knockout mice.
- 2) We have developed Rictor^{-/-}, Pkd1^{-/-} double knockout mice Table 1. We will continue the breeding process to develop more single Pkd1^{-/-} and double Pkd1^{-/-}, Rictor^{-/-} mice

- 3) We plan to determine whether newer generation mTOR kinase inhibitors e.g. AZD2014 or antisense oligonucleotides (ASO) that target the mTOR system have the same protective effect as Torin 2 on PKD. We will also examine the heart for cardiac hypertrophy and mTOR signaling in these studies.
- 4) The mTOR pathway is integral to autophagy. There is suppressed autophagy in PKD kidneys (Fig 5). We will determine the role of mTORC1/2 in the suppressed autophagy in PKD kidneys and determine the effect of treatment of Pkd1^{RC/RC} mice with autophagy inducers e.g. 2 deoxy glucose, trehalose, Beclin-1 peptide, on mTORC1 and 2 signaling, autophagy and PKD.



- 5) We have hearts from Pkd1 ^{-/-} mice. We are studying mTORC1 and 2 signaling pathways in hearts from Pkd1 ^{-/-} mice. It will be novel to discover that mTORC1 or 2 signaling is activated in the hearts of Pkd1 ^{-/-} mice.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Major findings:

- 1) Reduced 4E-BP1 expression suppressed proliferation, restored apoptosis, and improved cellular metabolism in Pkd1^{RC/RC} mice and human Pkd1 cells. These findings provide insight into how cyst-lining cells respond to 4E-BP1. Interventions involving the 4E-BP1 system may provide a future rationale for treatment to decrease PKD (Published-see above).

2) The mTOR kinase inhibitor Torin2 is as effective as sirolimus to treat PKD in Pkd1 ^{-/-} mice despite different effects on mTOR signaling, proliferation and apoptosis. These findings provide a rationale for the testing of new generation mTOR kinase inhibitors e.g AZD 2014 in preclinical studies in animal models of PKD, These studies provide insights into the use of combined mTORC1 and 2 inhibitors (the mTOR kinase inhibitors or TORCs) as future treatments in PKD (Published-see above).

3) Activation of the mTORC1 and 2 pathway in Pkd1 ^{-/-} mice is associated with suppressed autophagy in the kidney. We will determine whether specific autophagy induction with Tat Beclin-1 peptide or autophagy induction with 2 deoxy glucose (2DG) inhibits mTORC1 or mTORC2 signaling and protects against PKD. These studies will provide novel insights on the potential use of autophagy inducers that are mTOR inhibitors as therapy for PKD.

4) There is suppressed autophagic flux in the heart at an early age in Pkd1^{RC/RC} mice. Increased mTOR signaling in older mice is associated suppressed autophagic flux. There was a large increase in p-AMPKThr172, a known autophagy inducer and mTORC1 inhibitor, in both young and old mice. 2-DG treatment did not impact increased heart weight and Tat-Beclin1 peptide increased heart weight (Published-see above).

5) The autophagy phenotype in Pkd1^{RC/RC} kidneys was characterized by decreases in crucial autophagy-related proteins (Atg12-5 complex, Atg5, Atg16L1), decreased Rab9a and increased mTORC1 (pS6, p-mTOR^{S2448}) proteins. TRE increased Atg12-5 complex, Rab9a and p62 in normal kidneys, but was unable to rescue the deficiency in autophagy proteins or suppress mTORC1 in Pkd1RC/RC kidneys and did not protect against cyst growth (Published-see above)

What was the impact on other disciplines?

Many cancers are driven by increased 4E-BP1 signaling. We determined that inhibition of the 4E-BP1 system in PKD has different effects compared to studies in cancer. Transformed hyperproliferative breast cancer cells, lymphocytes, and epithelial cells forced to shift from cap-dependent to IRES-dependent translation results in increased apoptosis, decreased proliferation, and decreased cell size, thus retarding the growth of transformed tissues. However in PKD, a disease known for being driven by abnormal epithelial proliferation, *we observed the opposite*. In our *in vitro* studies, a shift from cap-dependent to IRES-dependent translation mediated by constitutively active 4E-BP1 suppressed apoptosis, increased proliferation, stimulated mitochondrial biogenesis, and augmented superoxide production uniquely in a

PKD genotype, largely leaving non-PKD cell lines uninfluenced. *In vivo*, constitutively active 4E-BP1 resulted in mitochondrial dysregulation, and *worsened PKD disease*.

Many cancers are driven by increased mTORC2 signaling. Demonstration that mTOR kinase inhibitors e.g. Torin2, AZD2014 decrease proliferation and have an effect on apoptosis may provide a future rationale for the use of mTOR kinase inhibitors to certain cancers.

Suppressed autophagy and increased mTOR signaling plays a role in disease processes like cancer and aging. Our studies of the effect of autophagy inducers on mTOR signaling and PKD will help in the understanding of the pathogenesis and treatment of other processes like aging and cancer.

We demonstrated increased mTORC1/2 signaling and cardiac hypertrophy in Pkd1 RC/RC mice. These studies may provide insight into the mechanisms of cardiac hypertrophy.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

The study of mTOR kinase inhibitors like Torin2 or autophagy inducers to treat patients with ADPKD.

The genetic studies (Pkd1 ^{-/-} mice with additional knockout of mTORC1 or 2 or both) will offer mechanistic insights into novel mTORC1 and 2 signaling pathways in ADPKD.

The pharmacological studies (4E-BP1 inhibition, AktSer473, PKC α or SGK1 inhibition and TORKs) and gene therapy studies (AAV gene therapy against 4E-BP1) will provide insights into future therapies for PKD.

Discovery of new treatments for PKD could improve the quality of life and extend the life span of people with ADPKD.

Discovery of mechanisms and treatments for cardiac hypertrophy in ADPKD patients could impact life expectancy in PKD patients.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

The tamoxifen-inducible ER2 Cre did not always work leading to mice with a very variable form of the ADPKD disease. We are now using KSP 1.3 Cre mice that have an embryonic Cre that is spontaneous (does

not need tamoxifen) and the *Pkd1* ^{-/-} mice develop PKD and kidney failure at a much earlier age. This model will lead to a more reproducible and faster model of ADPKD.

Both polycystin (PC-1) and polycystin-2 (PC-2) are known to modulate the mTOR pathway. PC-1 deficiency upregulates the mTOR pathway via TSC-2. PC-2 modulates the mTOR pathway in human cardiomyocytes. On further consideration, we realized that PKD mice that also have the gene defect in the heart should have upregulation of mTORC1/2 in the heart. Our published data confirmed increased mTORC1/2 signaling in the heart in PKD mice. On examining our PKD mice further, we were intrigued by the degree of cardiac hypertrophy even in heterozygous *Pkd2*^{+/-} mice without PKD. **A critical barrier to progress on understanding the causes of cardiac hypertrophy in PKD is that it is often presumed that cardiac hypertrophy is due to the hypertension and that many pre-clinical studies in PKD do not study the heart or even measure the blood pressure.** Thus we are studying mTORC1/2 signaling in both the heart and kidneys in PKD

Actual or anticipated problems or delays and actions or plans to resolve them

Dr Holditch, the post-doctoral fellow/instructor who was performing the 4E-BP1 studies, resigned in July 2019 as her husband was transferred to another city. A new PRA, Deepak Pokhrel, was hired to continue the mTOR studies in PKD and has learnt all the necessary techniques related to mouse breeding and mTOR studies.

Laboratories were closed for 3 months, from March to end of May, 2020, at the start of the Covid-19 pandemic. Thus we were not able to perform experiments or provide the best breeding for our PKD mice. This loss of 3 months had an impact to severely slow our research and mouse breeding

Changes that had a significant impact on expenditures

Purchase of Ksp 1.3 Cre mice and increased number of breeding cages has increased expenses. Costs for FISP-MRI scans.

Less expenditures when lab was closed for 3 months due to Covid-19. Hence a second no cost extension was granted.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Publications, conference papers, and presentations

Nothing to report (yet)

Journal publications.

Nothing to report (yet)

Books or other non-periodical, one-time publications.

Nothing to report (yet)

Other publications, conference papers, and presentations.

Three abstracts accepted for presentation at the annual American Society of Nephrology virtual meeting from October 21-25, 2020.

- 1. On behalf of the American Society of Nephrology (ASN) and the Kidney Week Education Committee, thank you for submitting an abstract for Kidney Week, which will be a fully digital meeting. We are pleased to inform you that your abstract 3444404 "Mechanisms of suppressed autophagic flux in the kidney caused by sham surgery and unilateral nephrectomy" has been selected by the Committee for an ePoster presentation.**
- 2. On behalf of the American Society of Nephrology (ASN) and the Kidney Week Education Committee, thank you for submitting an abstract for Kidney Week, which will be a fully digital meeting. We are pleased to inform you that your abstract 3442415 "The effect of trehalose on autophagy-related proteins and cyst growth in a hypomorphic Pkd1 mouse model of autosomal dominant polycystic kidney disease (ADPKD)" has been selected by the Committee for an ePoster presentation.**

3. On behalf of the American Society of Nephrology (ASN) and the Kidney Week Education Committee, thank you for submitting an abstract for Kidney Week, which will be a fully digital meeting. We are pleased to inform you that your abstract 3442374 "Suppressed autophagic flux in the heart in a hypomorphic Pkd1 mouse model of ADPKD" has been selected by the Committee for an ePoster presentation.

4. Website(s) or other Internet site(s)

Nothing to report (yet)

Technologies or techniques

Nothing to report (yet)

Inventions, patent applications, and/or licenses

Nothing to report (yet)

Other Products

Nothing to report (yet)

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	Charles Edelstein
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12

Contribution to Project:	Dr. Edelstein has overseen the design, performance and analysis of the studies
Funding Support:	DOD funding. VA Merit award funding.
Name:	Deepak Pokhrel
Project Role:	PRA
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Creation of double knockout mic, Mouse treatment studies mTORC1/2 signaling pathways General Lab Manager duties
Funding Support:	50% DOD funding. 50% VA Merit Award funding
Name:	Dan Atwood
Project Role:	PRA
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Breeding of single and double knockout mice Genotyping of mice Performing immunohistochemistry Mouse treatment studies mTORC1/2 signaling pathways
Funding Support:	50% DOD funding. 50% VA Merit Award funding
Name:	Nicole Brown
Project Role:	PRA
Researcher Identifier (e.g.	

ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Autophagy studies. Genotyping animals, animal injections, Performing immunoblots,</i>
Funding Support:	50% DOD funding. 50% VA Merit Award funding

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

1) VA Merit Award VA Merit Award to Charles Edelstein. BX003803-01A1. Autophagy in polycystic kidney disease. (Edelstein) 07/01/18-07/01/22.

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable

QUAD CHARTS:

Not applicable

APPENDICES:

Copies of our 2 most recent publications are below:

1) Atwood DJ, Pokhrel D, Brown CN, Holditch SJ, Bachu DM, Thorburn A, Hopp K, Edelstein CL. Increased mTOR and suppressed autophagic flux in the heart of a hypomorphic Pkd1 mouse model of autosomal dominant polycystic kidney disease. Cell Signal. 2020 (in press)

2) Atwood DJ, Brown DN, Holditch SJ, Pokhrel D, Thorburn A, Hopp K and Edelstein CL. The effect of trehalose on autophagy-related proteins and cyst growth in a hypomorphic Pkd1 mouse model of autosomal dominant polycystic kidney disease. Cell Signal. 2020 (in press).

Increased mTOR and suppressed autophagic flux in the heart of a hypomorphic Pkd1 mouse model of autosomal dominant polycystic kidney disease

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Abstract

Cardiac hypertrophy is common in autosomal dominant polycystic kidney disease (ADPKD) patients. We found increased heart weight in *Pkd1*^{RC/RC} and *Pkd2*^{WS25/+} mouse models of ADPKD. As there is a link between increased heart weight and mammalian target of rapamycin (mTOR), the aim of the study was to determine mTOR complex 1 and 2 signaling proteins in the heart in the *Pkd1*^{RC/RC} mouse model of PKD. In 70 day old *Pkd1*^{RC/RC} hearts, on immunoblot analysis, there was a large increase in p-AMPK^{Thr172}, a known autophagy inducer, and an increase in p-Akt^{Ser473} and p-Akt^{Thr308}, but no increase in other mTORC1/2 proteins (p-S6^{Ser240/244}, p-mTOR^{Ser2448}). In 150 day old *Pkd1*^{RC/RC} hearts, there was an increase in mTORC1 (p-S6^{Ser240/244}) and mTOR-related proteins (p-Akt^{Thr308}, p-GSK3 β ^{Ser9}, p-AMPK^{Thr172}). As the mTOR pathway is the master regulator of autophagy, autophagy proteins were measured. There was an increase in p-Beclin-1 (BECN1), an autophagy regulator and activating molecule in Beclin-1-regulated autophagy (AMBRA1), a regulator of Beclin that play a role in autophagosome formation, an early stage of autophagy. There was a defect in the later stage of autophagy, the fusion of the autophagosome with the lysosome, known as autophagic flux, as evidenced by the lack of an increase in LC3-II, a marker of autophagosomes, with the lysosomal inhibitor bafilomycin, in both 70 day old and 150 day old hearts. To determine the role of autophagy in causing increased heart weight, *Pkd1*^{RC/RC} were treated with 2-deoxyglucose (2-DG) or Tat-Beclin1 peptide, agents known to induce autophagy. 2-DG treatment from 150 to 350 days of age, a time period when increased heart weight developed, did not reduce the increased heart weight. Unexpectedly, Tat-Beclin 1 peptide treatment from 70 to 120 days of age resulted in increased heart weight. In summary, there is suppressed autophagic flux in the heart at an early age in *Pkd1*^{RC/RC} mice. Increased mTOR signaling in older mice is associated suppressed autophagic flux. There was a large increase in p-AMPK^{Thr172}, a known autophagy inducer, in both young and old mice. 2-DG treatment did not impact increased heart weight and Tat-Beclin1 peptide increased heart weight.

Introduction

ADPKD is the most common life threatening genetic condition [1] [2]. Most cases of ADPKD are caused by mutations to either the *PKD1* (~80% of cases) or *PKD2* (~12% of cases) genes. The disease is characterized by slowly growing and persistent renal cysts that eventually cause end-stage renal disease [1]. The growth of cysts

in the kidney results in hypertension, chronic kidney disease and end stage kidney disease requiring dialysis and kidney transplantation [1] [2]. ADPKD is as common as 1 in 400 persons. Abnormalities of signaling pathways in PKD kidneys include mTORC1, cAMP, AMP-activated protein kinase (AMPK), signal transducer and activator of transcription (STAT), Wnt, G-protein, proto-oncogene tyrosine-protein kinase Src (c-Src), mitogen-activated protein kinase (MAPK), epidermal growth growth factor (EGFR), cyclin-dependent kinase (CDK), intracellular calcium, p53, Myc and calcium-sensing receptor signaling pathways [3]. Activation of the calcium-sensing receptor increases intracellular calcium and decreases cAMP and mTOR in PKD1 deficient proximal tubular epithelial cells [4]. Selective calcium-sensing receptor activation in PKD1 deficient proximal tubular epithelial cells restores altered mitochondrial function that is thought to play a role in cyst formation [5]. Hyperproliferation, increased fluid secretion, increased apoptosis and impaired autophagy in the cells lining the kidney cysts are all characteristic of ADPKD [6-9].

Cardiac disease is the commonest cause of death in ADPKD patients [10]. ADPKD patients have left ventricular hypertrophy (LVH) out of proportion to the degree of hypertension [11]. As complications of ADPKD like LVH are common, the health care burden of LVH in ADPKD patients is large. Thus, understanding the pathophysiology of cardiac hypertrophy in PKD and testing therapies to reduce cardiac hypertrophy in PKD are very important.

mTOR is as a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell survival, protein synthesis, autophagy, and transcription [12]. The mTOR pathway is a central regulator of mammalian metabolism and physiology [13]. mTOR exists in two distinct structural and functional complexes, mTORC1 and mTORC2, that are both known to regulate cardiac structure and function in models of cardiac hypertrophy. Studies show conflicting results on whether activation of mTORC1 and 2 in the heart promotes or improves cardiac hypertrophy [14]. While mTOR activation in the kidney is known to worsen PKD [15], mTOR activation in the heart in PKD has not been detailed. In the present study, we found increased heart weight in 180 day old *Pkd1*^{RC/RC} and 116 day old *Pkd2*^{WS25/-} mice with ADPKD and heart weight was decreased by an mTOR antisense oligonucleotide (ASO), that inhibits both mTORC1 and 2, in *Pkd2*^{WS25/-} mice. We have previously shown the successful impact of treatment with an mTOR ASO on kidney cyst growth in *Pkd2*^{WS25/-}

mice [16]. Thus we measured heart weight and mTORC1 and 2 proteins in the heart in *Pkd1^{RC/RC}* mice, a hypomorphic model of ADPKD.

ADPKD is caused by mutations in two genes, *PKD1* or *PKD2*, which encode polycystin-1 (PC-1) and -2 (PC-2), respectively. Both PC-1 and PC-2 are known to modulate the mTOR pathway. PC-1 deficiency causes upregulation of the mTOR pathway via tuberous sclerosis complex 2 (TSC2) [17] [18] [19] [20]. PC-2 modulates the mTOR pathway in human embryonic stem cell cardiomyocytes [21]. There is thought to be an emerging link between polycystins and the mTOR/4E-BP1 pathway in PKD [20]. As PC-1 and PC-2 are abnormal in both the kidneys and heart in ADPKD, we hypothesized that there would be mTOR activation in the heart in ADPKD mouse models that have a global gene defect that includes the heart.

In addition to being a master regulator of metabolism, mTORC1 is also a master regulator of autophagy [22] and mTOR activation inhibits autophagy [23]. Thus, we hypothesized that upregulation of mTOR would be associated with suppressed autophagy in the heart. Signaling pathways controlled by AMPK are also central to cellular metabolism [24] [25, 26]. AMPK is a universal regulator of autophagy [27]. Activation of AMPK is known to inhibit mTOR via unc-51- like autophagy activating kinase (ULK1) and activate autophagy. Because of the connection between AMPK, mTOR and autophagy, we also measured AMPK in the *Pkd1^{RC/RC}* hearts during a time course of PKD.

Methods:

In vivo model:

Pkd1^{RC/RC} mice have a hypomorphic *Pkd1* gene mutation orthologous of PKD patient disease variant, *PKD1* p.R3277C (*Pkd1*) [28]. *Pkd1^{RC/RC}* mice in the C57BL/6 background have cysts at birth that progressively enlarge from 1 month of age and older [29, 30]. At 120 days of age, the two kidney to total body weight ratio, a marker of kidney size, in *Pkd1^{RC/RC}* mouse kidneys is approximately double that of wild type controls and the percentage of kidney that is cystic is approximately 17% [31]. At 120 days of age, *Pkd1^{RC/RC}* mice have abnormal kidney function as indicated by increased BUN and serum creatinine compared to wild type controls [31]. There is an increase in markers of mTORC1 (pS6 and p4E-BP1) in non-cystic areas of *Pkd1^{RC/RC}* kidneys compared to wild type and pS6 and p4E-BP1 staining is present in cells lining kidney cysts [31]. Cyst

expansion and size correlates with increased tubular cell proliferation [28] and there is apoptosis in the cells lining the cysts in *Pkd1*^{RC/RC} mice [31]. Wild type C57BL/6J mice (#000664) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All experiments were conducted with adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado at Denver. Mice were maintained on a standard diet with standard pathogen-free housing conditions, and food and water were freely available.

Cardiac dimensions

Transverse heart sections were stained with hematoxylin-eosin and analyzed using Aperio ImageScope macros. Intra-ventricular septum (IVS) width and left ventricular wall (LVW) width were measured as described previously [32].

Experimental *in vivo* protocol:

2-Deoxyglucose (2-DG) has been shown to be an autophagy inducer in macrophages [33], cancer cells [34], endothelial cells [35] and hypothalamic cells via activation of AMPK [36]. Additionally, in patients with prostate cancer, administration of 2-DG for 14 days resulted in increased autophagy in peripheral blood mononuclear cells (PBMCs), as measured by decreased p62 [37]. 2-DG can stimulate autophagy by ER stress rather than ATP depletion [38]. 2-DG or vehicle (normal saline) was given via I.P. injection at a dose of 600mg/kg/day on weekdays from ages 70-120 days old or from 150-350 days old. 2-DG was purchased from Sigma Aldrich (Cat no. D8375).

Tat-Becn 1 peptide is a known autophagy inducer when given to mice [39] [40] [41]. Mice were treated with Tat-Becn 1 peptide or vehicle (normal saline) via I.P. injection at a dose of 20mg/kg/day on weekdays from 70-120 days of age. A cell permeable Tat-Becn 1 peptide was manufactured by the Peptide Core at the Univ. of Colorado Anschutz Medical Campus. The sequence of the Tat-Becn D11 peptide was RRRQRRKKRGYGGDHWIHFTANWV [40].

Pkd1^{RC/RC} mice were treated with the mTORC1 inhibitor, sirolimus or the mTOR kinase inhibitor, torin2, that inhibits both mTORC1 and 2, as we have previously described [31]. *Pkd2*^{WS25/+} mice with ADPKD were treated with an mTOR ASO, that inhibits both mTORC1 and 2, as we have previously described [16]. Han: SPRD (Cy/+) rats with ADPKD were treated with the mTOR kinase inhibitor, PP242, that inhibits both mTORC1 and 2, as we have described [42].

Measurement of autophagic flux

The number of autophagosomes in a cell can increase, either due to increased formation or due to decreased degradation by the lysosome [43]. To investigate whether there is increased autophagosome synthesis or decreased degradation by the lysosome, mice were injected intra peritoneal (I.P.) with the lysosomal proton pump inhibitor, bafilomycin (1.75 mg/kg) or vehicle 2 hours before sacrifice. After sacrifice LC3-II, a marker of autophagosomes and p62, a marker of autophagosome degradation was measured with bafilomycin or vehicle.

Immunoblot Analysis:

Protein was isolated from cells and tissues using RIPA, cOmplete protease and phoSTOP phosphatase inhibitor cocktails (Sigma). Homogenates were centrifuged and supernatant was taken for protein quantification by Bio-Rad (Hercules, CA, USA) DC Protein Assay as described by manufacturer. Samples were mixed with Laemmli Sample Buffer and boiled for 5 minutes. Samples were run on 8-15% polyacrylamide gels. Proteins were then transferred to 0.45µm PVDF membranes, blocked with 2.5% evaporated milk, and probed with antibodies listed in Supplemental Table 1. Blots were developed by chemiluminescence and analyzed for densitometry using ImageJ (National Institutes of Health).

Antibodies: Supplemental Table 1 lists the antibodies used in this study. The specificity of each of the antibodies used has been validated by the vendor (Cell Signaling Technology, Danvers, MA).

Statistical Analysis:

Data sets were analyzed by the non-parametric unpaired Mann Whitney test. Multiple group comparisons are performed using analysis of variance (ANOVA) with post-test according to Newman-Keuls. Single group comparisons were made using students T-test. A p-value of <0.05 was considered statistically significant. Values are expressed as means $\pm$ SEM.

Results:

Increased heart weight in rodent PKD models

Heart weight was analyzed from various published datasets of our group. Heart weight in *Pkd1*^{RC/RC} mice was not increased in 70, 90, 120 and 150 day old mice but increased in 180 day old and 270 day old mice compared to strain/age matched wild types. In 120 day old *Pkd1*^{RC/RC} mice we have demonstrated that the mTORC1 inhibitor, sirolimus and the mTOR kinase inhibitor, torin2, reduce PKD [31]. Heart weights were determined in mice from this published study [31]. In 120 day old *Pkd1*^{RC/RC} mice treated with sirolimus or the mTOR kinase inhibitor, torin 2, the increase in heart weight did not reach statistical significance (Table 1). In 116 day old *Pkd2*^{WS25/-} mice we have demonstrated that treatment with an mTOR antisense oligonucleotide (ASO) that inhibits both mTORC1 and 2, resulted in decreased PKD [44]. Heart weights were determined in *Pkd2*^{WS25/-} mice from this published study [44]. The increase in heart weight in 116 day old *Pkd2*^{WS25/-} mice was decreased by the mTOR ASO (Table 1). In 116 day old heterozygous *Pkd2*^{WS25/+} mice that do not have PKD, the heart weight was increased compared to age matched littermate controls (Table 1). We have demonstrated that the mTOR kinase inhibitor, PP242, decreased PKD at 8 weeks of age in the Cy/+ rat model [42]. Heart weights were determined in Cy/+ rats from this published study [42]. The increase in heart weight in Cy/+ rats was decreased by the mTOR kinase inhibitor, PP242, that inhibits both mTORC1 and 2 (Table 1). Thus, the effect of mTOR inhibition on heart weight is variable depending on the inhibitor used, the model of PKD and the timing of administration of mTOR inhibitors.

Cardiac hypertrophy in 180 and 270 day old *Pkd1*^{RC/RC} mice

IVS and LVW were measured in 90, 180 and 270 day old hearts. The increase in IVS in 80 day old mice did not reach statistical significance. The increase in heart weight in 180 and 270 day old *Pkd1*^{RC/RC} mice (Table 1) was associated with cardiac hypertrophy. There was an increase in both IVS and LVW in 180 and 270 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Figure 1).

p-Akt^{Thr308}, p-Akt^{Ser473} and p-AMPK^{Thr172}, but not other mTORC1 and 2 proteins, were increased in 70 day old *Pkd1*^{RC/RC} hearts

p-Akt^{Thr308}, that is directly upstream of mTORC1 and p-Akt^{Ser473}, a marker of mTORC2 activation, were increased in 70 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 2). p-S6^{Ser240/244} and p-mTOR^{Ser2448}, markers of mTORC1 activation were unchanged in 70 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 2). p-4E-BP1^{Thr37/46} that is immediately downstream of mTORC1 was decreased in 70 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 2). p-AMPK^{Thr172} was more than 3 -fold increased in 70 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 2). pULK1^{Ser757}, that is phosphorylated by mTOR, and pGSK3B^{Ser9}, that is a target of Akt, were unchanged in 70 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 2).

p-S6^{Ser240/244}, p-Akt^{Thr308}, p-AMPK^{Thr172} and p-GSK3β were increased in 150 day old *Pkd1*^{RC/RC} hearts

p-S6^{Ser240/244}, a marker of mTORC1 activation, and p-Akt^{Thr308}, that is directly upstream of mTORC1 were increased in 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 3). p-4E-BP1^{Thr37/46} was increased in 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls, but there was no increase when corrected for total 4E-BP1. p-mTOR^{Ser2448} and p-Akt^{Ser473}, a marker of mTORC2 activation, were unchanged in 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 3). As seen in 70 day old *Pkd1*^{RC/RC} hearts, p-AMPK^{Thr172} was much increased (more than 2-fold) in 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 3). p-GSK3β, that is directly phosphorylated by Akt was increased in 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 3). When nutrients are sufficient, mTORC1 phosphorylates ULK 1 at Ser⁷⁵⁷. p-ULK1^{Ser757} was unchanged in 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 3).

Autophagic flux is suppressed in hearts from both young and older *Pkd1*^{RC/RC} mice

In the present study, autophagic flux was measured by comparing the expression of LC3-II in animals treated with and without a lysosomal inhibitor, bafilomycin. If the expression of LC3-II increases in the presence of bafilomycin, it can be inferred that the animal is continually producing LC3-II. No increase in LC3-II with bafilomycin indicates a defect in autophagic flux, the fusion of autophagosomes with the lysosome. In 70 and 150 day old wild type hearts, there was a significant increase in LC3-II expression after treatment with bafilomycin, indicating autophagic flux (Fig 4). In 70 day old *Pkd1*^{RC/RC} hearts, there was a decrease in basal LC3-II compared to wild type (Fig 4). In 70 and 150 day old *Pkd1*^{RC/RC} hearts, there was no increase in LC3-II with bafilomycin indicating suppressed autophagic flux (Fig 4). P62/SQSTM1, a marker of autophagic cargo, is another method of determining flux as p62 is destroyed by the lysosome much like LC3-II. There was no change in p62 between 70 and 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 4). However, in 150 day old wild type hearts, bafilomycin treatment caused a larger build up of p62 than in *Pkd1*^{RC/RC} hearts (Fig 4). As p62 is a marker of autophagosomes, the smaller increase of p62 with bafilomycin in *Pkd1*^{RC/RC} hearts suggests suppressed autophagic flux.

Autophagy proteins pBECN1^{Ser15} and AMBRA1 were increased in 150 day old *Pkd1*^{RC/RC} hearts

pBECN1^{Ser15}, an important regulator of autophagosome formation [45] and AMBRA1 (activating molecule in Beclin1-regulated autophagy), a positive regulator of the BECN1 [46] were increased in 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 5). Autophagy related proteins Atg3, Atg12-5 complex and Rab9a, a marker of Atg5-independent alternative autophagy pathway was unchanged in 150 day old *Pkd1*^{RC/RC} hearts compared to wild type controls (Fig 5).

Treatment of mice with 2-DG does not decrease heart weight. Treatment of mice with Tat-Beclin 1 peptide results in increased heart weight.

Suppressed autophagic flux was seen in 70 and 120 day old *Pkd1*^{RC/RC} hearts. Thus the effect of autophagy inducers on heart weight was determined, with the idea that autophagy induction may impact heart weight. Mice

were treated with 2-DG from 70-120 days of age, a time before increased heart weight and from 150-350 days old, a time period when increased heart weight developed. 2-DG treatment from 70 to 120 days did not decrease heart weight (Fig 6A). 2-DG treatment from 150 to 350 days of age resulted in an increase in heart weight that did not reach statistical significance (Fig 6A). In support of an increase in heart weight with 2-DG treatment, the increase in heart weight between 150 and 350 days of age was significantly more with 2-DG than with vehicle (Fig 6B). Mice were treated with the autophagy-inducing Tat-Becn1 peptide from 70 to 120 days of age, a time period before there is increased heart weight. Tat-Becn1 treatment from 70 to 120 days of age resulted in increased heart weight (Fig 6C).

Discussion:

ADPKD patients, despite therapy with angiotensin converting enzyme (ACE) inhibitors, have higher left ventricular mass index (LVMI) than healthy controls [47]. In the HALT-PKD study, despite standard blood pressure control on an ACE inhibitor or angiotensin receptor blocker (ARB), few patients had increased LVMI on MRI scan, but many were in the upper range of normal (there was no specific comparison to the general population) [48]. In patients from the ALADIN-trial, left ventricle (LV) function determined by speckle-tracking echocardiography is impaired early and somatostatin-analogue therapy improves LV dysfunction in this population [49]. Thus, there is increased LVMI in ADPKD patients and describing the pattern and understanding the pathophysiology of cardiac disease in ADPKD in rodents is important in providing clues to develop future therapies to treat the cardiac disease in PKD patients.

There are reasons to suspect that mTOR would be activated in the enlarged heart in PKD. Firstly, mTOR is activated in the kidney [15] and liver [50] in ADPKD. Secondly, there is a known connection between abnormalities in PC-1 and PC-2 and mTOR, a known mediator of cardiac hypertrophy [20]. Thirdly, the present study shows that mTORC1/2 inhibition with an ASO that blocks both mTORC1 and 2, resulted in decreased heart weight in *Pkd2*^{-/-} mice (Table 1). However, in 120 day old *Pkd1*^{RC/RC} mice, that do not yet have increased heart weight, sirolimus and the mTOR kinase inhibitor, Torin2, had a tendency to increase heart weight (Table

1). Thus, the effect of mTOR inhibition on the heart may depend on the PKD model and whether the mTOR inhibitor is given before or after the onset of increased heart weight.

Studies show conflicting results on the role of mTORC1 and 2 in non PKD models of cardiac hypertrophy. Sirolimus, an mTORC1 inhibitor that blocks S6K attenuates cardiac hypertrophy [51], but mTORC1 (Raptor) or mTORC2 (Rictor) knockout in the heart worsens cardiac hypertrophy [52] [53]. In the present study, we show that pS6^{Ser240/244}, a marker of mTORC1, is activated in the heart at 150 days old, a time point before the onset of measurable increased heart weight at 180 day of age. At 70 days old there was a decrease in p4E-BP1 in *Pkd1*^{RC/RC} mouse kidneys. It is known that cardiac-specific 4E-BP1 knockout improves cardiac hypertrophy in a non PKD model [54]. Thus the decrease in p4E-BP1 in 70 day old mice seen in the present study may be an early compensatory mechanism to try to reduce cardiac hypertrophy. Thus while it is known that there are changes in mTOR proteins in the heart in non PKD models of cardiac hypertrophy, mTOR activation in the heart in PKD has not previously been described and is the focus of the present study.

Proteins that are known to be both upstream and downstream of mTORC1 were activated in *Pkd1*^{RC/RC} hearts. In 150 day old hearts there was an increase in p-Akt^{Thr308}, a protein kinase that is activated by insulin and various growth and survival factors and via growth factor-stimulated PI3K activity. Akt is known to directly phosphorylate mTORC1 (17) or phosphorylate and inactivate TSC-2, an inhibitor of mTOR [55] [56]. In the present study, there was increased p-Akt^{Thr308} and p-S6^{Ser240/244}, a marker of mTORC1, in 150 day old hearts. There was an increase in p-GSK3B in 150 day old hearts. GSK3 (GSK3 α and GSK3 β) regulate many cellular processes by directly phosphorylating substrates, that include metabolic enzymes, transcription factors, cell-cycle regulatory proteins, and cytoskeletal proteins [57] [58]. GSK3 is a well -characterized Akt target and Akt's phosphorylation of GSK3 β is inhibitory [59]. Therefore, in *Pkd1*^{RC/RC} hearts, Akt's phosphorylation of GSK3 β could result in inhibitory effects of GSK on cellular processes decreasing the direct phosphorylation of metabolic enzymes, transcription factors, cell-cycle regulatory proteins, and cytoskeletal proteins . mTOR signaling pathways in *Pkd1*^{RC/RC} hearts are demonstrated in Figure 7.

As mTOR activation is known to inhibit autophagy, we measured autophagic flux in the heart during a time-course of the PKD. In 70 and 120 day old *Pkd1*^{RC/RC} hearts there was suppressed autophagic flux. The

suppressed autophagic flux in 70 day old mice occurred before activation of mTORC1 or 2 suggesting that the suppressed autophagy was not related to mTORC1 or 2. We also measured other autophagy related proteins in PKD hearts. Autophagy related 5 (Atg5) is an important protein involved in the extension of the phagosome membrane in autophagic vesicles and is activated by Atg7. In the conventional autophagy pathway, two autophagy related proteins (Atg12 and Atg5) form a complex with other proteins such as LC3-II in order to elongate the phagosome and fully engulf autophagic cargo [60-63]. There is also an Atg5-independent mechanism of autophagy, called alternative autophagy, utilizing Rab9a, a small GTPase, that is recruited to autophagosome-like vacuoles after autophagosomal maturation resulting in autophagosome-like vacuole enlargement and eventual lysosomal fusion [64]. There was no change in Atg7, Atg12-5 complex, or Rab9a in PKD hearts, but a lack of increase of LC3-II with bafilomycin suggested that the autophagy defect in PKD hearts was related to a lysosomal defect.

Beclin1 (BECN1; coiled-coil, myosin-like BCL2-interacting protein) is an important regulator of early autophagosome formation [45]. BECN1 plays a role in autophagy initiation by regulating the autophagy-promoting activity of the Class III PI 3-kinase, Vps34, and BECN1 and is involved in the formation of autophagosome membranes. AMBRA1 (activating molecule in Beclin1-regulated autophagy) is required for BECN1 activity [46]. AMBRA1 interacts with BECN1 at the initiation of autophagy and promotes the binding between BECN 1 and its target kinase, Vps34. In the present study there was an increase in both AMBRA1 and pBECN1^{Ser15} in 150 day old hearts indicating that the proximal stages of autophagy were intact. There was decreased autophagic flux, the fusion of the autophagosome with the lysosome, a distal stage of autophagy. These findings suggest that the defect in autophagy in 150 day old PKD hearts may be due to a more distal lysosomal defect rather than a defect in the more proximal steps involving autophagy related proteins, BECN and AMBRA1.

There was a large increase in p-AMPK^{Thr172} in both young (70 day old) and older (150 day old) hearts at a time when there was suppressed autophagic flux but not yet increased heart weight. AMP activated protein kinase (AMPK), is a key energy sensor that regulates cellular metabolism to maintain energy homeostasis. There are a variety of physiological conditions that change the ATP to AMP ratio leading to the activation of AMPK,

including mitochondrial inhibition, nutrient starvation and exercise [65]. AMPK is known to inhibit mTORC1 and it has recently been demonstrated that mTORC1 also directly downregulates AMPK signaling [66]. One of the functions of AMPK is to promote autophagy by regulation of the mammalian autophagy-initiating kinase, ULK1, a homologue of yeast Atg1. In the present study, activation of AMPK was not associated with changes in ULK1 phosphorylation, suggesting that AMPK phosphorylation is not a mediator of autophagy via ULK1. AMPK has other autophagy-related functions in regulating mitochondrial homeostasis: biogenesis, fission and mitophagy. AMPK is activated in response to mitochondrial damage, as well as under other low ATP conditions to ensure that there is generation of new mitochondria [65]. In summary, the increase in AMPK in *Pkd1*^{RC/RC} hearts may be an attempt to repair a mitochondrial defect.

To determine the mechanistic effect of suppressed autophagy in causing increased heart weight, mice were treated with known autophagy inducers 2-DG or Tat-Becn1 peptide. 2-DG has been shown to induce autophagy and activate AMPK [67]. 2-DG treatment in mice has been shown to reduce cyst growth, but the effect on cardiac hypertrophy was not determined [68]. In the present study, 2-DG treatment, from 150 to 350 days of age, a time period when increased heart weight developed, did not reduce increased heart weight and had a tendency to increase heart weight. Tat-Becn1 peptide is a cell-penetrating autophagy-inducing peptide that induces autophagy in vivo in mice through interaction with the autophagy suppressor GAPR-1/GLIPR2 that promotes the release of BECN1 from the Golgi, resulting in enhanced early autophagosome formation [40] [41] [39]. Tat-Becn1 treatment from 70 to 120 days of age resulted in increased heart weight. Suppressed autophagic flux in 70 day old mice occurred before the onset of increased heart weight and the increased heart weight with Tat-Becn1 treatment at this time suggested that suppressed autophagy may be a mechanism to prevent cardiac hypertrophy in young PKD mice and that inducing autophagy may interfere with this process resulting in increased heart weight. The studies with 2-DG and Tat-Becn1 peptide suggest that autophagy induction in the heart does not improve increased heart weight.

There are reasons to suspect that the increased heart weight in mice is due to more than hypertension: The known expression of PC1 and PC2 in cardiac tissue suggests that there are direct effects of PC1 and PC2 on cardiac function [69]. There is evidence that PC-2 modulates intracellular calcium cycling, contributing to the

development of heart failure in *Pkd2* mutant zebrafish and in *Pkd2*^{+/-} mice that do not have PKD [70]. The present study confirmed increased heart weight in *Pkd2*^{WS25/-} mice that do not have PKD. Abnormalities in PC-1 have also been implicated in causing heart disease. In mice with a conditional silencing of PC-1 selectively in cardiomyocytes subjected to mechanical stress there was decreased cardiac function relative to littermate controls [71]. The hypothesis that cardiac hypertrophy in PKD is due to more than just hypertension is supported by human studies in which there is LVH in normotensive patients [72] [73].

In summary, there is mTORC1 activation in the heart in older *Pkd1*^{RC/RC} mice associated with suppressed autophagic flux. In younger mice suppressed autophagic flux in the heart was not associated with mTORC1 activation. There was a defect in autophagic flux, the fusion of phagosomes with the lysosome, rather than the more proximal autophagy steps involving autophagy related proteins. There was a large increase in p-AMPK^{Thr172}, a known autophagy inducer, in both young and old mice perhaps as a compensatory mechanism to suppressed autophagy. Treatment with the autophagy inducer, 2-DG, did not impact increased heart weight but treatment with Tat Beclin1 peptide increased heart weight.

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Table 1: Heart weight in rodent PKD models.

Model	Species	Age (days)	HW/BW (%)	Ref
+/+	Mouse	70	0.50 ± 0.03	
<i>Pkd1</i> ^{RC/RC} (B6)	Mouse	70	0.56 ± 0.04	
+/+	Mouse	90	0.64 ± 0.07	
<i>Pkd1</i> ^{RC/RC} (B6)	Mouse	90	0.63 ± 0.03	
+/+	Mouse	120	0.54 ± 0.07	
<i>Pkd1</i> ^{RC/RC} (B6)	Mouse	120	0.54 ± 0.07	
+/+	Mouse	150	0.49 ± 0.02	
<i>Pkd1</i> ^{RC/RC} (B6)	Mouse	150	0.52 ± 0.01	
+/+	Mouse	180	0.57 ± 0.08	
<i>Pkd1</i> ^{RC/RC} (B6)	Mouse	180	0.71 ± 0.09**	
+/+	Mouse	270	0.53 ± 0.06	
<i>Pkd1</i> ^{RC/RC} (B6)	Mouse	270	0.66 ± 0.07***	
<i>Pkd1</i> ^{RC/RC} (B6)+Veh	Mouse	120	0.47 ± 0.04	(34)
<i>Pkd1</i> ^{RC/RC} (B6)+Torin2	Mouse	120	0.58 ± 0.05	(34)
<i>Pkd1</i> ^{RC/RC} (B6)+Sirolimus	Mouse	120	0.55 ± 0.02	(34)
+/+	Mouse	116	0.52 ± 0.04	(9)
<i>Pkd2</i> ^{WS25/-} (B6) + Scr ASO	Mouse	116	0.69 ± 0.07*	(9)
<i>Pkd2</i> ^{WS25/-} (B6) + mTOR ASO	Mouse	116	0.55 ± 0.03*	(9)
+/+	Rat	56	0.43 ± 0.02	(35)
Cy/+ plus Veh	Rat	56	0.52 ± 0.02*	(35)
Cy/+ plus PP242	Rat	56	0.44 ± 0.01*	(35)

+/+=wild type, HW/BW (%) = heart weight to body weight ratio (%), *P<0.05, **P<0.01, ***P<0.001,

Cy/+ = Han:SPRD rat, Torin2 and PP242 = mTOR kinase inhibitors, Scr = scrambled, ASO = antisense oligonucleotide, B6 = C57BL/6 background, Veh = vehicle, Ref = reference

Figure legends

Figure 1: Cardiac hypertrophy in 180 and 270 day old *Pkd1*^{RC/RC} mice

Representative hematoxylin-eosin stained transverse sections of heart at the same magnification are demonstrated. There was no difference in size of intra-ventricular septum (IVS), left ventricular (LV) wall and right ventricle (RV) in 90 day old *Pkd1*^{RC/RC} (RC) hearts compared to wild type (WT) controls. There was an increase in both intra-ventricular septum (IVS) and LV wall size in 180 and 270 day old RC hearts compared to WT controls. Measurements (μm) of IVS, LVW and RV are shown in the table. n=4-5 per group. *P<0.05, **P<0.01. LVW=left ventricle wall. Scale bar=1000μm.

Figure 2: p-Akt^{Thr308}, p-Akt^{Ser473} and p-AMPK^{Thr172}, but not other mTORC1 and 2 proteins, were increased in 70 day old *Pkd1*^{RC/RC} hearts

Immunoblot analysis was performed for p-S6^{Ser240/244}, p-Akt^{Thr308}, p-Akt^{Ser473}, p-4E-BP1^{Thr37/46}, p-mTOR^{Ser 2448}, p-AMPK^{Thr172}, p-ULK1^{Ser 757} and p-GSK3B^{Ser9} in 70 day old *Pkd1*^{RC/RC} (RC) hearts compared to wild type (WT) controls. RDU=relative densitometry units corrected for GAPDH. Data is expressed as a mean +/- SEM, n= 6 per treatment group. *P < 0.05, **** P < 0.0001.

Figure 3: p-S6^{Ser240/244}, p-Akt^{Thr308}, p-AMPK^{Thr172} and p-GSK3β were increased in 150 day old *Pkd1*^{RC/RC} hearts

Immunoblot analysis was performed for p-S6, p-Akt^{Thr308}, p-4E-BP1^{Thr37/46}, p-mTOR^{Ser 2448}, p-Akt^{Ser 473}, p-AMPK^{Thr172}, p-ULK1^{Ser 757} and p-GSK3β in 150 day old *Pkd1*^{RC/RC} (RC) hearts compared to wild type (WT) controls. Both p-4E-BP1^{Thr37/46} corrected for the loading control vinculin (Vinc) and p-4E-BP1 corrected for total 4E-BP1 are shown. RDU=relative densitometry units corrected for GAPDH. Data is expressed as a mean +/- SEM, n= 6 per treatment group. *P < 0.05, ** P < 0.01.

Figure 4: Autophagic flux is suppressed in hearts from both young and older *Pkd1*^{RC/RC} mice

Mice were treated with bafilomycin (Baf) 1.75 mg/kg 2 hours before sacrifice. Immunoblot analysis for LC3-II and p62 in the heart was performed in Baf and vehicle treated 70 and 150 day old *Pkd1*^{RC/RC} mice (PKD) and

age matched wild type (WT) control mice. RDU=relative densitometry units corrected for GAPDH. Data is expressed as a mean +/- SEM, n= 6 per treatment group. *P < 0.05, ** P < 0.01.

Figure 5: Autophagy proteins p-BECN and AMBRA1 were increased in 150 day old *Pkd1*^{RC/RC} hearts

Immunoblot analysis was performed for autophagy related proteins Atg3, Atg12-5 complex and Rab9a, BECN, AMBRA1 in 150 day old *Pkd1*^{RC/RC} (RC) hearts compared to wild type (WT) controls. RDU=relative densitometry units corrected for GAPDH. Data is expressed as a mean +/- SEM, n= 6 per treatment group. *P < 0.05, ** P < 0.0001.

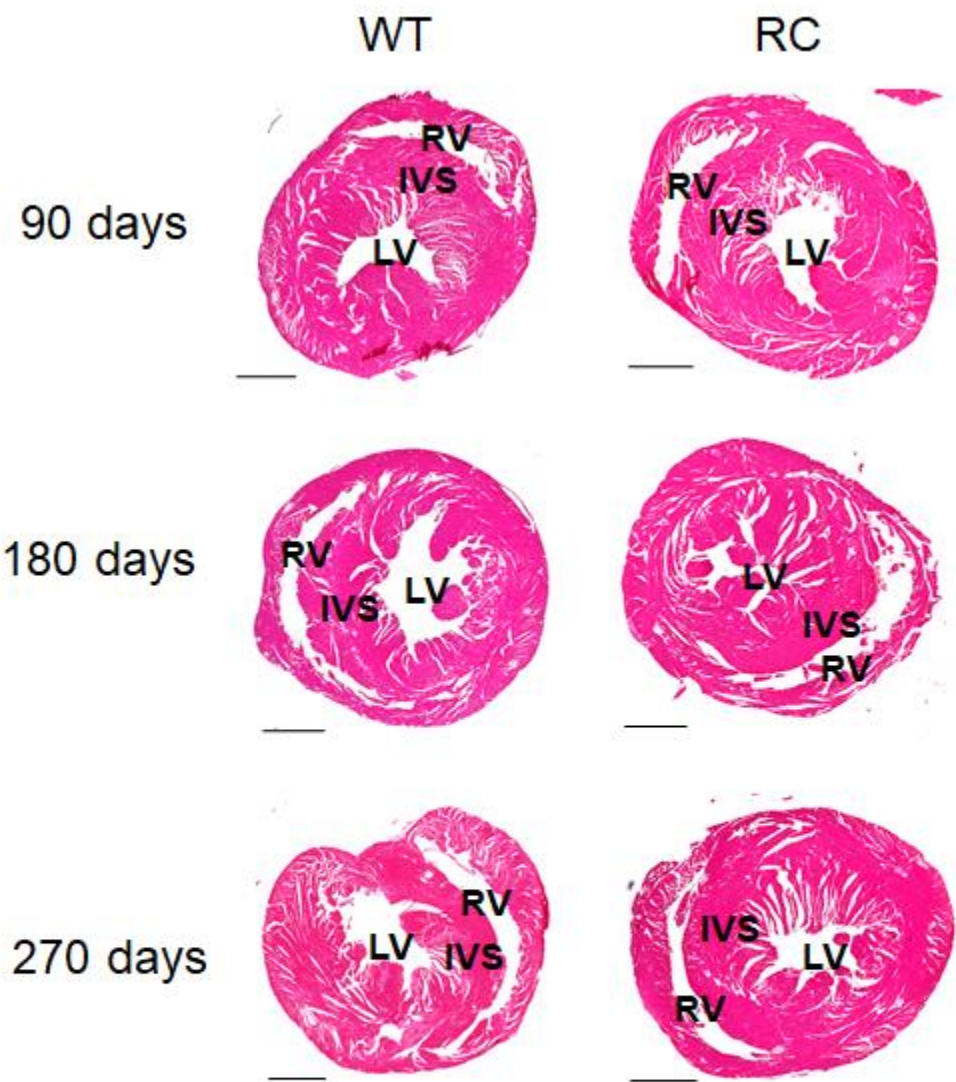
Fig 6: Treatment of *Pkd1*^{RC/RC} with 2-DG does not decrease heart weight. Treatment of *Pkd1*^{RC/RC} with Tat-Beclin 1 peptide results in increased heart weight.

Pkd1^{RC/RC} mice were treated with 2-deoxy glucose (2-DG) or vehicle (Veh) from 70 -120 days of age and from 150-350 days of age or TatBeclin1 peptide (Tat) or vehicle (Veh) from 70 to 120 days of age. (A) Heart weight/body weight (HW/BW) (%) in mice treated with Veh or 2-DG from 70-120 days of age and 150-350 days of age. (B) HW/BW (%) at 120 days vs. 350 days in mice treated with either Veh or 2-DG. (C) HW/BW (%) in mice treated with Veh or Tat.

Figure 7: mTOR signaling in *Pkd1*^{RC/RC} hearts

The potential connections between the mTOR signaling proteins that were increased in *Pkd1*^{RC/RC} hearts is demonstrated. In 70 day old hearts, there was an increase in p-AMPK^{Thr172}, p-Akt^{Ser473} and p-Akt^{Thr308}. In 150 day old hearts, there was an increase p-AMPK^{Thr172}, p-S6^{Ser240/244}, p-Akt^{Thr308} and p-GSK3 β ^{Ser9}. AMPK has been shown to be an upstream kinase necessary for activation of both p-Akt^{Ser473} and p-Akt^{Thr308} in metabolic stress, tumorigenesis and drug resistance [74]. However, AMPK is also known to inhibit p-Akt^{Thr308} [75]. Akt activation can also prevent phosphorylation of AMPK at Thr 172 [75]. While GSK3 β ^{Ser9} is usually inhibited by mTOR activation, GSK3 β ^{Ser9} can also activate mTORC1 [76]. GSK3 is a well -characterized Akt target and Akt's phosphorylation of GSK3 β is inhibitory [59]. p-S6^{Ser240/244} is a marker of mTORC1 and is usually inhibited by AMPK via ULK1 [23].

Figure 1



Age (days)	WT	RC	WT	RC
	IVS	IVS	LVW	LVW
90	372 ± 39	482 ± 88	558 ± 114	498 ± 48
180	372 ± 59	601 ± 46 *	438 ± 45	640 ± 24**
270	456 ± 41	817 ± 237*	634 ± 91	883 ± 53*

Figure 2

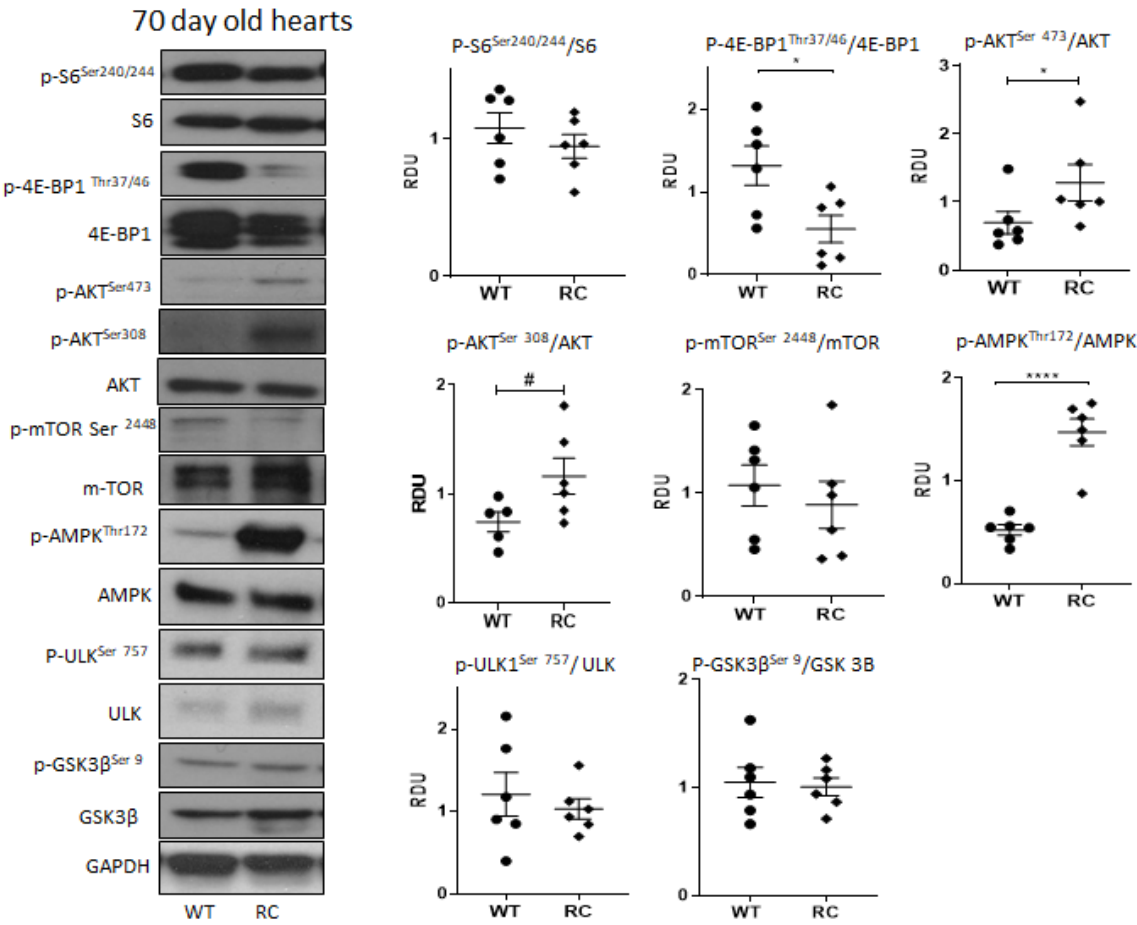


Figure 3

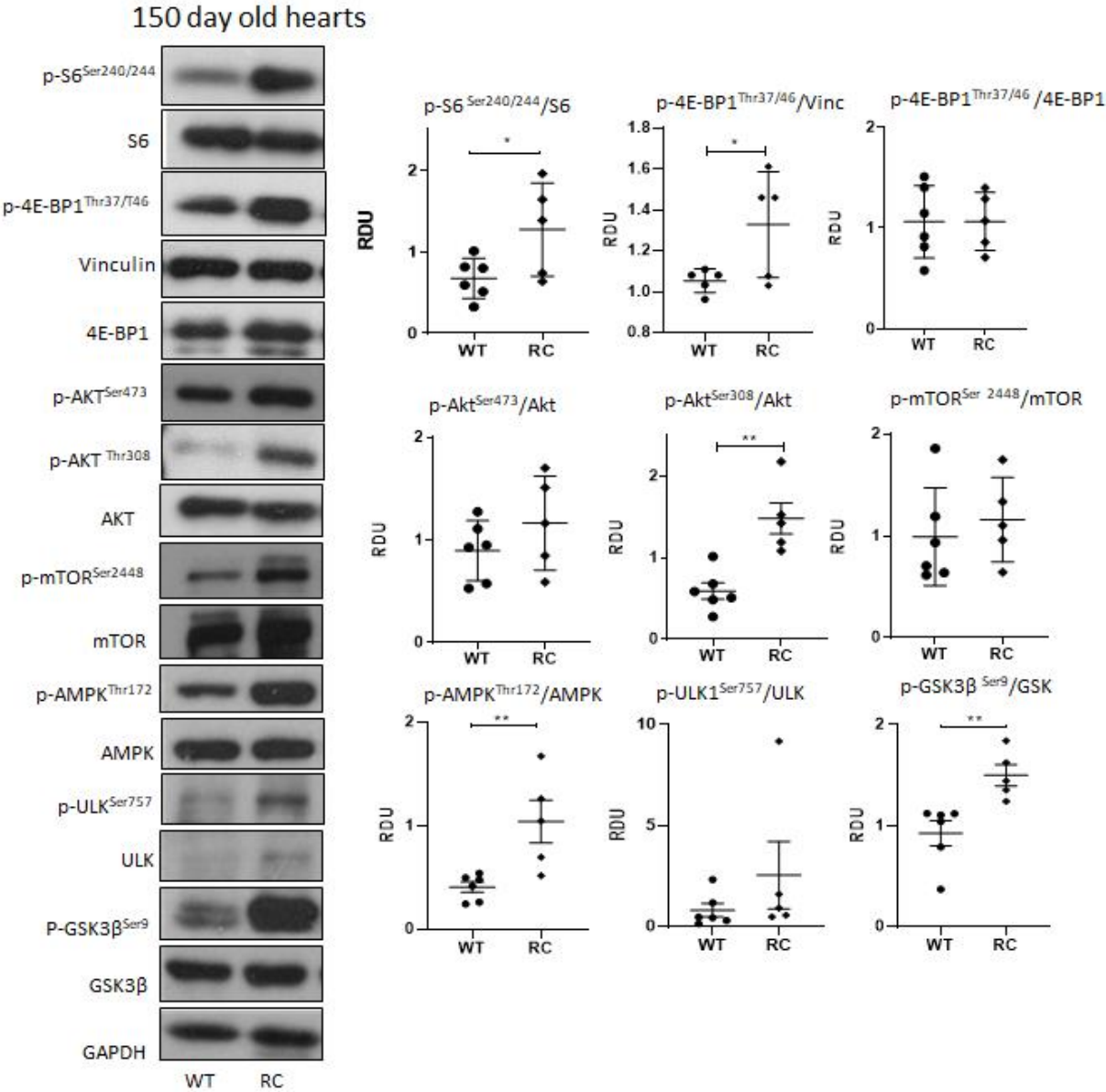


Figure 4

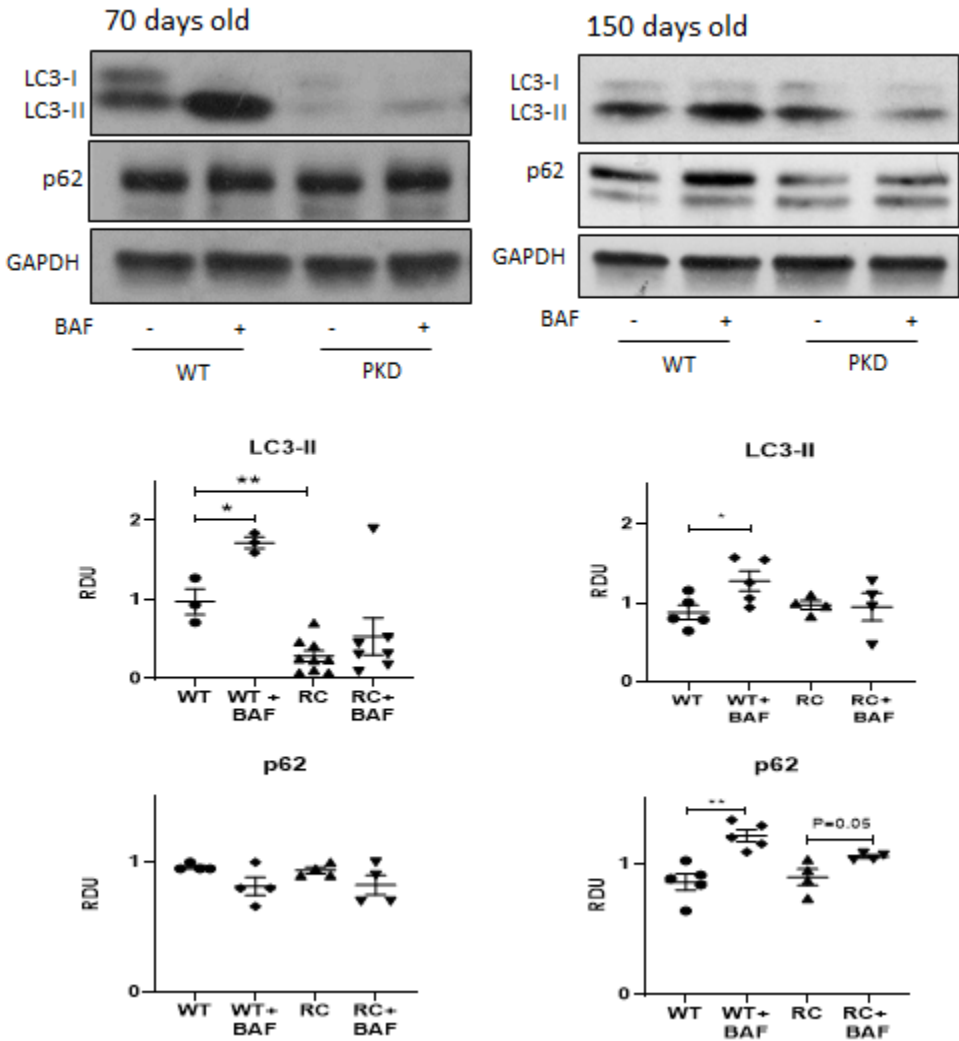


Figure 5

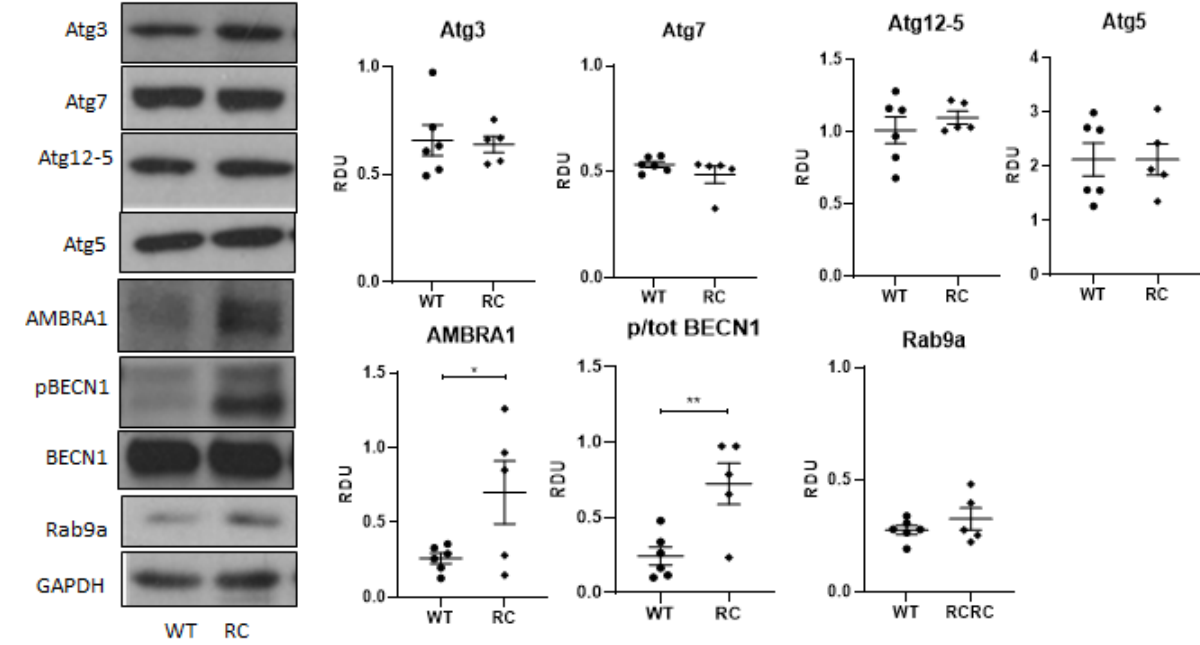


Figure 6

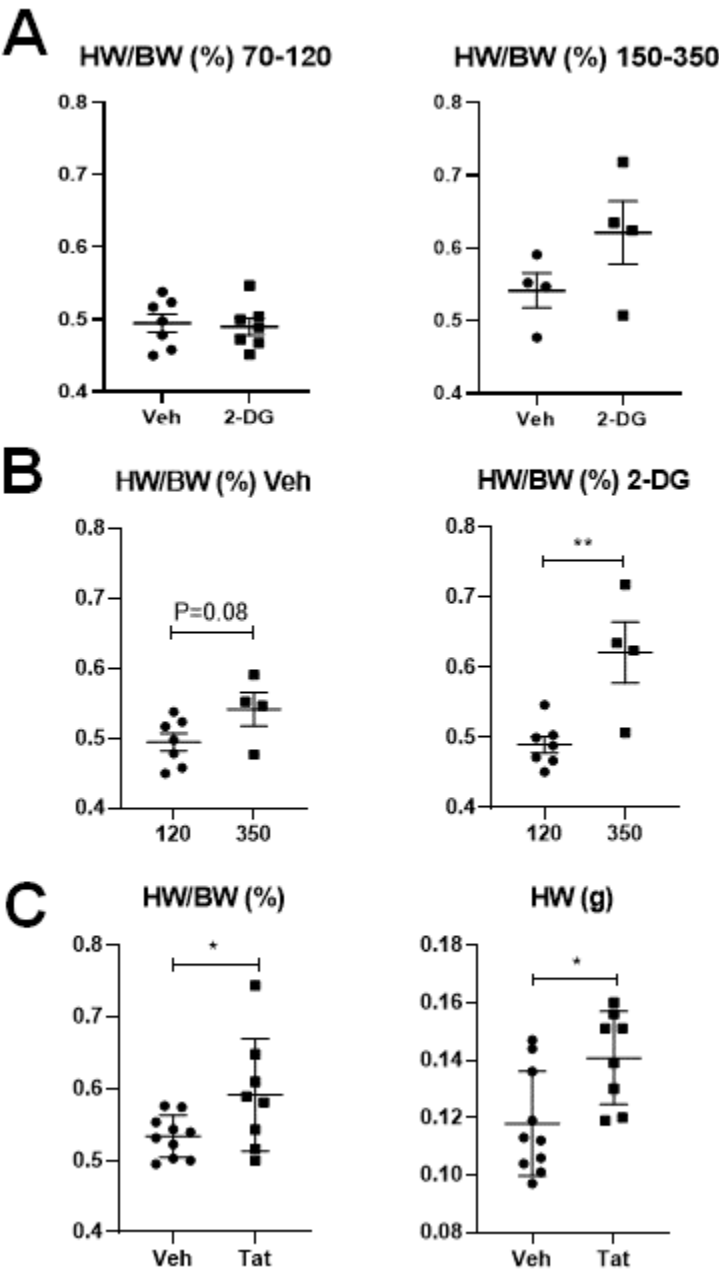
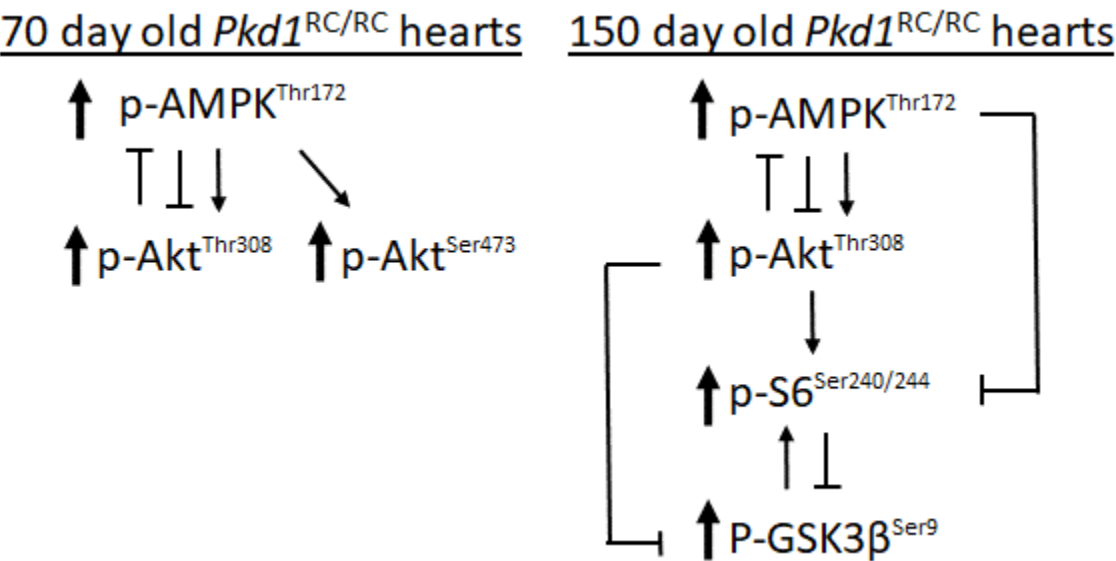


Figure 7



The effect of trehalose on autophagy-related proteins and cyst growth in a hypomorphic Pkd1 mouse model of autosomal dominant polycystic kidney disease

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Abstract:

Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited disorder characterized by kidney cyst growth often resulting in end-stage renal disease. There is growing attention on understanding the role of impaired autophagy in ADPKD. Trehalose (TRE) has been shown to increase both protein stability and aggregate clearance and induce autophagy in neurodegenerative diseases. TRE treatment in wild type mice compared to vehicle resulted in increased expression in the kidney of Atg12-5 complex and increased Rab9a, autophagy-related proteins that play a role in the formation of autophagosomes. Thus, the aim of the study was to determine the effect of TRE on cyst growth and autophagy-related proteins, in the hypomorphic *Pkd1*^{RC/RC} mouse model of ADPKD. *Pkd1*^{RC/RC} mice were treated 2% TRE in water from days 50 to 120 of age. TRE did not slow cyst growth or improve kidney function or affect proliferation and apoptosis in *Pkd1*^{RC/RC} kidneys. In *Pkd1*^{RC/RC} vs. wild type kidneys, expression of the Atg12-5 complex was inhibited by TRE resulting in increased free Atg12 and TRE was unable to rescue the deficiency of the Atg12-5 complex. Rab9a was decreased in *Pkd1*^{RC/RC} vs. wild type kidneys and unaffected by TRE. The TRE-induced increase in p62, a marker of autophagic cargo, that was seen in normal kidneys was blocked in *Pkd1*^{RC/RC} kidneys. In summary, the autophagy phenotype in *Pkd1*^{RC/RC} kidneys was characterized by decreases in crucial autophagy-related proteins (Atg12-5 complex, Atg5, Atg16L1), decreased Rab9a and increased mTORC1 (pS6, p-mTOR^{S2448}) proteins. TRE increased Atg12-5 complex, Rab9a and p62 in normal kidneys, but was unable to rescue the deficiency in autophagy proteins or suppress mTORC1 in *Pkd1*^{RC/RC} kidneys and did not protect against cyst growth.

Keywords: ADPKD, PKD, Atg12, Atg5, Rab9a, TRE, apoptosis, proliferation, polycystic

Introduction:

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is one of the most common genetic disorders in the world. The majority of ADPKD cases can be explained by mutations to either *PKD1* (~80% of cases) or *PKD2* (~12% of cases) and the disease is defined by slowly growing and persistent renal cysts that eventually cause

end-stage renal disease [1]. Hyper-proliferation, increased apoptosis and impaired autophagy in the kidney are all characteristic of ADPKD [2-5]. Autophagy is the process in which cellular components, such as damaged organelles and misfolded proteins, are degraded and recycled [6]. In conventional autophagy, two autophagy-related proteins (ATGs), Atg12 and Atg5 form a complex, recruiting other proteins such as microtubule-associated protein 1A/1B light chain 3B (LC3) to the growing autophagosome membrane in order to elongate and fully engulf autophagic cargo [6-9]. The Atg12-5 complex also binds to Atg16 which is required for the localization of Atg5 and Atg12 to the phagophore assembly site and for the elongation of isolation membranes (also called phagophores) during mammalian autophagy [10]. The association of the Atg12–5 complex with Atg16 unmasks a membrane-binding site in Atg5 and allows Atg5 to bind to phagophore precursors [11]. LC3-I is converted into the phosphatidylethanolamine (PE)-conjugated form, LC3-II, which allows conjugation to the phagosome membrane and LC3-II is the most reliable marker of completed autophagosomes [12]. The amount of LC3-II can increase in cells due to increased production or a block in autophagosome-lysosome fusion or a defect in lysosome function. If the basal increase in LC3-II is due to increased autophagosome production, then it is expected that lysosomal inhibition will further increase LC3-II (i.e. increased autophagic flux) [12]. The amount of sequestosome 1/p62 (p62) a marker of autophagic cargo, is another method of determining autophagic flux as p62 can be destroyed by the lysosome much like LC3. In general, an increase in p62 indicates suppressed autophagic flux [13].

Recent studies have also discovered an Atg5/7-independent mechanism of autophagy, called alternative autophagy, in which autophagosomes are generated in a Ras-related protein Rab9a (Rab9a)-dependent manner by the fusion of isolation membranes with vesicles derived from the trans-Golgi and late endosomes [14, 15]. Rab proteins are small GTPases that belong to the Ras-like GTPase superfamily and regulate vesicle trafficking [16]. Rabs are related to autophagy by regulating the transport and fusion of autophagosomes [16] [17]. Beclin1 is essential to the initiation of autophagy in both the canonical and alternative autophagy pathways [18]. Beclin1 plays a role in the initial steps in the assembly of autophagosomes from pre-autophagic structures by recruitment and activation of the class III phosphatidylinositol 3-kinase complex.

It has been demonstrated that there is suppressed autophagic flux in *Pkd1* ^{-/-} cells [3, 19] and in mouse PKD kidneys [20]. Stimulating autophagy has been shown to slow the progression of ADPKD in zebrafish [3]. Autophagy inducers like sirolimus, deoxy glucose and metformin are known to slow cyst growth in PKD [19, 21, 22]. In the present study, in normal kidneys both *ex vivo* and *in vivo*, we determined that the non-reducing glucose disaccharide, trehalose (TRE) had the potential to increase autophagy by increased expression of Atg12-5 complex and increased Rab9a. Thus, the aim of the study was to determine the effect of TRE on autophagy-related protein expression, especially Atg12-5 complex, Rab9a, and p62 as well as cyst growth in a *Pkd1*^{RC/RC} mice, a hypomorphic *Pkd1* mouse model.

TRE is found in most organisms other than vertebrates and in common food supplements [23-25]. TRE is a natural sugar often used to preserve the flavor, texture, and color of dehydrated and frozen foods by preventing protein degradation [26]. The cryo-protective properties of TRE are linked to its chemical chaperone properties, which influence protein folding through direct protein-TRE interactions resulting in protein stability and reduced accumulation of misfolded proteins [25]. The autophagy inducing effect of TRE in Huntington's Disease results in the stabilization of the toxic mutant huntingtin protein, resulting in less aggregation and enhanced clearance of soluble mutant huntingtin [27]. Thus, TRE has dual protective effects as both an inducer of autophagy and a chemical chaperone [27-31]. While TRE has been shown to increase both protein stability and aggregate clearance [27, 29, 32], it is unknown if this property increases the stability of Atg12-5 or its ability to form a complex. Also, *Pkd1*^{RC/RC} mice that were used in the present study, have a missense *pkd1* allele, matching a human disease variant, PKD1 p. R3277C (*Pkd1*^{RC/RC}) [33]. The PC1^{RC} protein is a temperature-sensitive folding mutant, with a ~65% reduction in PC1^{RC} protein levels relative to wild-type PC1 [33]. Given that *Pkd1*^{RC} is thought to be a folding mutant, we were interested to determine whether the PKD caused by *Pkd1*^{RC} could benefit from a chemical chaperone like TRE that increases protein stability.

Methods:

Trehalose assay: Trehalose was measured in serum and kidney per manufacturer's instructions using an enzymatic assay kit from Megazyme, Inc (Chicago, IL, USA) (Product code K-TREH). The Trehalose test kit is a method for the measurement and analysis of trehalose in foods, beverages and other materials. The principle

of the assay is that trehalose is hydrolysed to D-glucose by trehalase and the D-glucose released is phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP). In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP⁺) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose and with twice the amount of trehalose. NADPH is measured by the increase in absorbance at 340 nm. D-glucose and trehalose standard solutions were assayed as positive controls. A standard curve was determined for each assay performed.

In Vivo Models: *Pkd1*^{RC/RC} mice have a hypomorphic *Pkd1* gene mutation orthologous of PKD patient disease variant, PKD1 p. R3277C (*Pkd1*) [33]. *Pkd1*^{RC/RC} mice in the C57BL/6 background have small cysts as early as postnatal day 12 [34, 35]. Cyst expansion and size correlates with increased tubular cell proliferation [33]. Wild type C57BL/6J mice (#000664) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All experiments were conducted with adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Colorado at Denver. Mice were maintained on a standard diet with standard pathogen-free housing conditions, and food and water were freely available.

Experimental *in vivo* protocol: Glucose/sucrose, that is sweeter than TRE, in the drinking water has the potential to increase water intake, suppress ADH and slow cyst growth. However, it has been shown that mice fed drinking water supplemented with sucrose have a slightly increased cystic phenotype compared to untreated mice, supporting studies that sugar consumption has the potential to worsen ADPKD [36]. Thus we used tap water rather than glucose or sucrose in the drinking water as the control for TRE. A concentration of 2% TRE (mass/volume) in tap water was chosen for *in vivo* experiments, based on published studies [27-30, 37]. Male C57BL/6 *Pkd1*^{RC/RC} mice were treated with 2% TRE in water from days 64 to 70 of age (short-term study) or days 50 to 120 of age (long-term study). TRE was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Ex Vivo assay: Fresh kidney was homogenized on ice with a sonicator for 30 seconds in lysis buffer containing 10mM Tris-HCL (pH 7.5), 10mM NaCl, 0.1% Triton-X100, 10% Glycerol, cOmplete protease and PhosSTOP phosphatase inhibitor cocktails (Sigma), and 1mM PMSF. The homogenate was then centrifuged at 14,000RPM at 4°C. Supernatant was then immediately incubated with concentrations of 0, 10, 25, 100, and 250mM TRE, or 250mM Glucose or 250 mM sucrose in DMEM+10% FBS for 30min in an incubator at 37°C in 1.7 mL Eppendorf tubes. Protein quantification was then performed by Bio-Rad (Hercules, CA, USA) DC Protein Assay as described by manufacturer and immunoblot analysis was performed as described below.

Water Intake Study: 58-day old wild type mice were given either water or 2% TRE in water for 5 days. Starting the second to last day, the mice were placed in metabolic chambers for 16 hours and fluid intake was measured.

Measurement of Kidney Function: Blood urea nitrogen (BUN) was measured with a BioAssay Systems (Hayward, CA, USA) Urea Assay Kit according to manufacturer's instructions (DIUR-100).

Immunoblot Analysis: Protein was isolated from cells and tissues using RIPA, cOmplete protease and phoSTOP phosphatase inhibitor cocktails (Sigma). Homogenates were centrifuged and supernatant was taken for protein quantification by Bio-Rad (Hercules, CA, USA) DC Protein Assay as described by manufacturer. Samples were mixed with Laemmli Sample Buffer and beta-mercaptoethanol and boiled for 5 minutes. Samples were run on 4-15% polyacrylamide gels. Proteins were then transferred to 0.45µm PVDF membranes, blocked with 2.5% evaporated milk, and probed with antibodies listed in Supplemental Table 1. Blots were developed by chemiluminescence and analyzed for densitometry using ImageJ.

Antibodies: Supplemental Table 1 lists the antibodies used in this study. The specificity of each of the antibodies used has been validated by the vendor (Cell Signaling Technology, Danvers, MA).

Routine Histology: Tissues were fixed overnight in 10% formalin at 4°C. They were transferred to fresh 70% ethanol and left overnight at 4°C; this process was repeated twice more. Next, the tissues were processed and embedded in paraffin wax using Leica systems. Tissues were sectioned at 4µm and baked at 60°C for 2 hours. Kidneys were stained with hematoxylin-eosin and cystic index and number was quantified on two sections per mouse (left and right kidney) using a NIS Elements macro as previously published [35]. Cyst index or cyst number were expressed as the percentage of the cross-sectional area or number per cross sectional area,

respectively. Areas with tissue tears and bubbles that were identified with higher magnification (40X) were excluded from analysis.

Immunohistochemistry Protocol: Tissue sections were deparaffinized and rehydrated, then antigen unmasking was performed in sodium citrate buffer (pH 6.0) for 25 min at 100°C. After rinsing sections for 10 minutes in cold tap water, endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide for 10 minutes, followed by a 5-minute rinse in deionized water. Blocking was performed using Vectastain® Elite® ABC Kit blocking serum for 30 minutes at room temperature. Primary antibodies were diluted in tris-buffered saline with Tween20 (TBST) as indicated in Supplemental Table 1 and incubated overnight at 4°C in a humidified chamber. Immunoreactions were detected using the Vectastain® standard protocol with 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) counterstained with hematoxylin. Slides were subsequently dipped 1-3 times in 0.3% acid alcohol, then dehydrated and mounted. DAB positive staining was analyzed using Aperio ImageScope macros.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining protocol: TUNEL staining was performed on tissue sections using Promega DeadEnd™ Colorimetric Apoptosis Detection System Kit following the manufacturer's instructions.

Quantitation of immunohistochemical staining: The number of positive staining cells was counted using the Aperio ImageScope, Leica Biosystems, by an observer blinded to the treatment modality. Non-cystic tubules were defined as tubules less than 50µm in diameter. 15-20 fields of view (40X magnification) devoid of cysts in the cortex per sample were randomly selected for quantitation in non-cystic tubules. To avoid sensitivity and selection artifacts between non-cystic tubules and dilated possibly pre-cystic tubules, as well as potential changes in tubular epithelium lining massive cysts, positive staining was counted in cysts of approximately 75-200µm diameter. 50-75 cortical cysts per tissue section were randomly selected for histological analysis.

Immunofluorescence: Fixation of tissue, preparation of slides and immunofluorescence was performed as we have previously described [38]. Slides were imaged using an Olympus FV1000 confocal laser scanning microscope with a 100X oil objective. Images were taken of cortical tubular regions. Co-localization analysis

was performed using the Coloc2 plugin in ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA).

Statistical Analysis: Data sets were analyzed by the non-parametric unpaired Mann Whitney test. Multiple group comparisons are performed using analysis of variance (ANOVA) with post-test according to Newman-Keuls. Single comparisons were made using students T-test. A p-value of <0.05 was considered statistically significant. Values are expressed as means $\pm$ SEM.

Results:

TRE is detected in blood and kidney

TRE can be rapidly degraded into glucose by the brush border saccharidase, trehalase, which is present in the intestinal brush borders of most mammals including mice. Thus it was important to determine that TRE was absorbed into the blood. TRE was detected in serum and kidney in TRE-treated mice (Supplemental Fig. S1).

TRE supplementation did not increase water intake

In order to ensure that the any observed effects of TRE on cyst growth were not the result of increase water intake, which has been shown to slow cyst growth in rats [39], a preliminary water intake study was performed. Fifty eight day old wild type mice were given either vehicle (tap water) or 2% TRE in tap water for 5-days and then water intake, urinary arginine vasopressin (AVP), and serum copeptin were measured. While there was an upward trend in water intake with TRE treated mice, there were no significant differences in AVP or serum copeptin in vehicle or TRE –treated mice (Fig. 1).

Short term supplementation with TRE increased markers of autophagy

In order to determine the immediate effects of TRE treatment, wild type and *Pkd1*^{RC/RC} mice were given either VEH or 2% TRE in tap water for 6 days (64-70 days of age) and then sacrificed. After TRE treatment, Atg12-5 complex levels were significantly increased in wild type mice but remained unchanged with TRE in *Pkd1*^{RC/RC} mice which had higher baseline levels compared to wild type mice on immunoblot analysis (Fig. 2). However, no significant changes in free Atg12 expression were observed. As Atg12-5 complex formation is linked with autophagy stimulation, additional autophagy associated proteins were measured. In the canonical autophagy

pathway, LC3-II and p62, markers of autophagosomes and autophagic cargo respectively, were measured [9]. LC3-II and p62 expression was nearly 2-fold higher in *Pkd1*^{RC/RC} mice compared to wild type (Fig. 2). Treatment with TRE decreased LC3-II expression in *Pkd1*^{RC/RC} mice but had no effect on p62, suggesting decreased autophagosome production. Rab9a has been implicated in the formation of autophagosomes in the Atg5/7-independent alternative autophagy pathway [15]. TRE supplementation caused a significant increase in Rab9a in both wild type and *Pkd1*^{RC/RC} mice (Fig.2). Additionally, Beclin1 was examined as it is essential to the initiation of autophagy in both the canonical and alternative autophagy pathways [18]. *Pkd1*^{RC/RC} mice had a significant decrease in pBeclin1 compared to wild type mice and TRE supplementation caused non significant increases in pBeclin1 in both wild type and *Pkd1*^{RC/RC} mice compared to vehicle (Fig. 2B).

TRE increased Atg12-5 complex *ex vivo*

In order to confirm the effect of TRE to increase the Atg12-5 complex in wild type mice, *ex vivo* experiments on kidney homogenates from wild type mice were performed. Kidney homogenates from wild type mice were incubated with increasing concentrations of TRE in DMEM and with glucose at the highest concentration. Incubation with TRE at 10, 25 and 100mM concentrations significantly increased the levels of the Atg12-5 complex in homogenized wild type kidneys compared to vehicle and 250 nM TRE (Fig. 3). Additionally, incubation with 250mM glucose caused a non significant decrease in Atg12-5 complex expression. TRE also significantly increased LC3-II expression, independent of concentration used, with no increase with glucose supplementation. Only 25mM TRE increased P62 expression. Neither TRE nor glucose supplementation had an effect on Rab9a protein expression. In order to control that autophagic-stimulation was not a non-specific osmotic stress response, kidney homogenates were incubated with or without 250 mM sucrose. There was no difference in LC3, p62 or Atg12-5 in kidney homogenates treated with or without sucrose (Supplemental Figure S2)

Long term TRE supplementation did not slow cyst growth or improve kidney function

Next, the effect of long-term supplementation with TRE on cyst growth was determined in mice treated from 50-120 days of age (70 days of treatment). Water intake in TRE treated animals was significantly higher, approximately 15% more than with vehicle (Fig. 4A). However, previous studies have shown that a close to 7 – fold increase in water intake had no effect on plasma vasopressin levels or cyst burden in *Pkd1*^{RC/RC} mice [39] and as such this 15% increase in water intake with TRE was not expected to have any impact on the study. There was an increase in 2K/TBW ratio between 64 day old historical control mice and 120 day old mice treated with vehicle or TRE (Supplemental Fig S3). Long-term treatment with TRE from 50 to 120 days old had no impact on cyst growth or cyst number as determined by 2KW/TBW ratio, cyst number and cyst index (Fig. 4A). Long term treatment with TRE did not improve kidney function as measured by BUN (Fig. 4A). Representative kidney sections from vehicle and TRE-treated *Pkd1*^{RC/RC} mice are demonstrated in Fig 4A. There were no cysts present in wild type mice treated with TRE. Histological analysis of cystic tubules in age matched littermate *Pkd1*^{RC/RC} mice showed no significant differences in proliferation, as determined by PCNA staining, or apoptosis, as determined by TUNEL staining, in TRE vs. vehicle treated mice (Fig. 4B). Histological analysis of non cystic tubules in vehicle and TRE-treated wild type and age matched littermate *Pkd1*^{RC/RC} mice showed no significant differences in PCNA TUNEL staining, between groups (Fig. 4B)

TRE was unable to rescue the deficiency of the Atg12-5 complex or increase Rab9a in *Pkd1*^{RC/RC} kidneys.

Next, the effects of long-term supplementation with TRE on autophagy-related protein expression was determined in mice treated from 50-120 days of age (70 days of treatment). Immunoblot analysis revealed that TRE treatment significantly increased Atg12-5 complex expression in wild type kidneys but was unable to rescue the complex in *Pkd1*^{RC/RC} mice, which was effectively absent (Fig. 5). This was reflected in free Atg12 levels, which were unchanged between treatment groups but were 7-fold higher in *Pkd1*^{RC/RC} mice compared to wild type (Fig. 5). The increase in Atg12-5 complex in wild type mice treated with TRE was not caused by increased abundance of Atg12 and Atg5 as the levels of free protein was not changed. Additionally, Atg5 levels in *Pkd1*^{RC/RC} animals were approximately half that of wild type animals. This suggests that there is both decreased Atg5 available for Atg12 to bind to and that their ability to form a complex is also impaired. Without

the Atg12-5 complex, canonical autophagy cannot proceed so we examined the alternative autophagy pathway. Levels of the alternative autophagy pathway marker Rab9a were also significantly decreased in *Pkd1*^{RC/RC} compared to wild type mice and not changed between treatment groups, which is surprising as one would expect the Atg5-independent alternative autophagy pathway to ramp up activity in the absence of Atg5 as has been previously described [15, 40]. The canonical autophagy pathway-associated proteins LC3-II and p62 were both significantly increased with TRE treatment in wild type animals (Fig. 5), indicating a change in the autophagy pathway preventing turnover and degradation of LC3-II and p62 [7, 41, 42]. To determine whether the increased in LC3-II and p62 in wild type kidneys treated with TRE (Fig 5) was due to increased autophagic flux or due to inhibition of autophagic degradation, wild type mice were treated with TRE +vehicle vs. TRE+ bafilomycin A1 (a lysosomal inhibitor) 1.75 mg/kg IP at 2 hours before sacrifice. If the amount of LC3-II increases further in the presence of lysosomal protease inhibitors like bafilomycinA1, this indicates an increase in autophagic flux [41]. However, if LC3-II does not increase further with lysosomal inhibitors, this indicates that autophagosome accumulation occurred due to inhibition of autophagic degradation, for example, blockage of autophagosome-lysosome fusion [41]. There was no further increase in LC3-II or p62 in kidneys treated with TRE + bafilomycin A1 vs TRE alone suggesting that the increase in LC3-II and p62 was due to inhibition of autophagic degradation (Supplemental Fig S4). It is not recommended that the conversion of LC3-I to LC3-II be used as a marker of autophagy as LC3-I is technically less sensitive to detection by antibodies that also detect LC3-II [12]. LC3-I is labile and more sensitive to freeze-thawing and degradation in SDS buffer and the amount of LC3-I is cell and stress specific.

pBeclin1^{S15} and the alternative autophagy pathway marker Rab9a were not affected by TRE treatment in wild type mice, suggesting that TRE mainly acts on the canonical autophagy pathway in the long term. pBeclin1^{S15} was upregulated in vehicle treated *Pkd1*^{RC/RC} mice compared to wild type and was reduced to near wild type levels with TRE treatment.

On immunofluorescence staining for Rab9a and LAMP2 (lysosomal marker), TRE did not affect the co-localization of Rab9a and LAMP2 suggesting that TRE does not increase the localization of Rab9a in the lysosome (Supplemental Figure S5).

TRE increased pAMPK^{T117} and pGSK-3 β ^{S9} in wild type kidneys. TRE had no effect on mTORC1, mTORC2 and autophagy-related proteins (Atg3 and 7).

The effect of PKD and TRE on mTORC1/2 proteins, autophagy-related proteins, pAkt^{S308}, phospho AMP-activated protein kinase (pAMPK^{T117}) and phospho glycogen synthase kinase-3 β (pGSK-3 β) is demonstrated in Fig. 6. Atg16L1 was decreased in *Pkd1*^{RC/RC} vs. wild type kidneys. Markers of mTORC1 (p-mTOR^{S2448} and pS6^{S240/244}) were increased in *Pkd1*^{RC/RC} vs. wild type kidneys and unaffected by TRE. pAkt^{S473}, a marker of mTORC2 and pAkt^{S308} that is upstream of mTOR were decreased in *Pkd1*^{RC/RC} kidneys and unaffected by TRE. pAMPK^{T117} expression was increased in *Pkd1*^{RC/RC} vs. wild type kidneys, increased by TRE in wild type kidneys and unaffected by TRE in *Pkd1*^{RC/RC} kidneys. pGSK-3 β ^{S9} expression was increased by TRE in wild type kidneys. pGSK-3 β ^{S9} was decreased in *Pkd1*^{RC/RC} kidneys compared to wild type kidneys and unaffected by TRE in *Pkd1*^{RC/RC} kidneys. TRE had no effect on Atg 3 or Atg7.

Discussion:

Hyper-proliferation, increased apoptosis and impaired autophagy in the kidney are all characteristics of ADPKD [2-5]. Inhibition of proliferation with sirolimus or everolimus has been shown to decrease cyst growth and improve kidney function in mice [21, 43]. The mTOR inhibitors, sirolimus and everolimus, are known autophagy inducers [12]. Autophagy is an essential cellular process in which damaged organelles and misfolded proteins are sequestered by the autophagosome and then degraded after fusing with a lysosome [6]. Several studies show that this process is suppressed in ADPKD [2, 3, 36] and stimulating autophagy has been shown to slow the progression of ADPKD in zebrafish [3]. TRE is a non-reducing glucose disaccharide that is found in most organisms other than vertebrates and in common food supplements [23-25]. TRE is known to increase autophagy in several animal models of neurological disorders [27-29]. It is also known to stabilize proteins and cell membranes in response to freezing and dehydration [23]. Thus agents that induce autophagy, like the food additive TRE, have a potential therapeutic value to stabilize autophagy proteins and the *Pkd1*^{RC} mutant protein and decrease cyst growth in ADPKD.

In the present study, TRE did not decrease cyst growth. mTOR inhibitors and AMPK activators are known to decrease cyst growth in PKD [21] [22]. TRE did not decrease p-mTOR activation or further increase p-AMPK in *Pkd1^{RC/RC}* mice and this could partly explain why TRE was not protective against cyst growth. In this regard, TRE is known to be an mTOR-independent autophagy inducer by acting as a chemical chaperone [31]. In this study, TRE and mTOR inhibition with sirolimus have an additive effect on the clearance of aggregate-prone proteins because of increased autophagic activity in a model of Huntingtons disease.

In the conventional autophagy pathway, two autophagy-related proteins (Atg12 and Atg5) form a complex with other proteins such as LC3-II in order to elongate the phagosome and fully engulf autophagic cargo [6-9]. Deficiency in the Atg12-5 complex is known to impair autophagy leading to several diseases such as asthma, lupus erythematosus, and Crohn's disease, as well as several types of cancer [44-48]. There is also an Atg5-independent mechanism of autophagy, called alternative autophagy, utilizing Rab9a, a small GTPase, that is recruited to autophagosome-like vacuoles after autophagosomal maturation resulting in autophagosome-like vacuole enlargement and eventual lysosomal fusion [14, 15, 17]. TRE has been gaining attention recently regarding activating the autophagy pathway. TRE supplementation of the diets of mice with Parkinson's and Huntington's disease reduces disease severity and increases lifespan by increasing aggregate clearance via an mTOR-independent autophagy pathway [27-30]. The effect of TRE on Atg12-5 complex and Rab9a is not known. Thus, the first aim of the study was to determine the effect of TRE on autophagy-related proteins especially Atg12-5 complex, Rab9a and p62. Both in 70 and 120 day old wild type kidneys *in vivo* and in kidney homogenates from wild type mice *ex vivo*, TRE resulted in increased expression of the Atg12-5 complex and increased Rab9a. In the *ex vivo* experiments, the increase in Atg12-5 complex was accompanied by the increase in LC3-II and p62, markers of autophagic vesicles and cargo, respectively [7, 42]. These studies demonstrate for the first time that TRE was able to increase Atg12-5 and Rab9a, in wild type kidneys, reinforcing its potential as an autophagy inducer. Thus, we hypothesized that supplementing the diet with TRE would increase expression of the Atg12-5 complex and increase Rab9a in *Pkd1^{RC/RC}* kidneys.

In both *in vivo* and *ex vivo* experiments in wild type kidneys, TRE successfully increased the amount of Atg12-5 complex. This effect was not seen in *Pkd1^{RC/RC}* mice. 70 day old *Pkd1^{RC/RC}* kidneys had higher levels of

Atg12-5 complex than wild type mice while in older 120-day old mice, Atg12-5 complex was unexpectedly absent. Conversely, the opposite was found of pBeclin1^{S15}, whose levels in 70-day old *Pkd1*^{RC/RC} mice were slightly more than half the levels of wild type mice. This was reversed in 120-day old mice, with *Pkd1*^{RC/RC} mice having 3-fold higher levels of pBeclin1^{S15} compared to wild type, but this difference was eliminated with TRE treatment. Thus, while TRE had an effect on Atg12-5 complex and Rab9a in wild type kidneys, it did not affect these autophagy-related proteins in *Pkd1*^{RC/RC} kidneys.

Lack of an effect of TRE on autophagy-related proteins in *Pkd1*^{RC/RC} kidneys was associated with the lack of an effect on cyst growth and kidney function. Lack of a protective effect in PKD was associated with the inability of TRE to impact proliferation and apoptosis, processes that are crucial to increase cyst growth. Another study has also showed the lack of a therapeutic effect of TRE on ADPKD [36]. There are important differences between the published study and our study. In the published study, a different ADPKD model, the *Pkd1* miRNA transgenic mouse, was used. In the published study, TRE exhibited no effects on the mRNA levels of any of the measured autophagy proteins in either wild-type or diseased mice, while our study showed an effect of TRE on protein expression of Atg12-5 complex, Rab9a, p62 and LC3-II, pGSK3 β and pAMPK in normal kidneys and LC3-II, Rab9a and pBeclin1 in *Pkd1*^{RC/RC} kidneys. Tubular apoptosis was not described in the published study whereas we found that TRE had no effect on tubular apoptosis in both cystic and non cystic tubules in *Pkd1*^{RC/RC} kidneys. Pro-inflammatory or glycolysis related genes were studied in the published study, whereas we focused on the effect of TRE on autophagy-related proteins especially Atg12-5 complex.

A limitation of the current study is that treatment was started at 50 days of age, a time point when there was already a significant cyst burden [49]. In the younger (70-day old) mice, an increase in the alternative autophagy pathway protein, Rab9a, was seen with TRE. This effect was lost with long term treatment in older (120-day old) mice. If treatment was started at a younger age it is possible that the beneficial effect would have been maintained.

Finally, we noted a distinct autophagy-related phenotype in *Pkd1*^{RC/RC} mouse kidneys compared to wild type kidneys. There was a decrease in Atg12-5 complex, increased free Atg12 and decreased Rab9a in 120 day old *Pkd1*^{RC/RC} kidneys that was not present in 70 day old kidneys. There was an increase in LC3-II and p62 in 70

day old *Pkd1*^{RC/RC} kidneys that was not present in 120 day old kidneys. TRE increased Rab9a in 70 day old but not in 120 day old kidneys. The difference in autophagy proteins between 70 and 120 day old kidneys suggest that older age, worse polycystic kidney disease and worse kidney function in 120 day old mice vs. 70 day old mice [43] affects autophagy and the response to TRE. In this regard, aging is known to have significant effects on autophagy [50]. Atg3 and Atg16L1 are E2 enzymes essential for the LC3 lipidation process of autophagosomes. A large protein complex consisting of Atg5, Atg12 and Atg16L1 has recently been shown to be essential for the elongation of isolation membranes (also called phagophores) during mammalian autophagy [10]. Atg5 and Atg7 are key proteins involved in the extension/development of the autophagosomal membrane [12]. Atg16L1 and Atg5 were decreased in *Pkd1*^{RC/RC} vs. wild type kidneys confirming defects in autophagosome development and the autophagic process in PKD. mTORC1 is a known inhibitor of autophagy [12]. Markers of mTORC1 (pmTOR^{S2448} and pS6) were increased in *Pkd1*^{RC/RC} kidneys. TRE had no effect on these mTORC1 markers confirming its known mTOR-independent actions [31]. In contrast to mTORC1, mTORC2 is a known inducer of autophagy and knockout of mTORC2 results in suppressed autophagy [31]. pAkt^{S473}, a marker of mTORC2 was decreased in *Pkd1*^{RC/RC} kidneys consistent with suppressed autophagy. Akt is a serine/threonine-specific protein kinase activated by PI-3 kinase (PI3K) that is part of the PI3K/Akt/mTORC1 pathway [51]. Pharmacological inhibition of Akt promotes nuclear translocation of TFEB, lysosomal biogenesis and autophagy [52]. pAkt^{S308}, was decreased in *Pkd1*^{RC/RC} kidneys and unaffected by TRE. Phosphorylation of AMP-activated protein kinase (AMPK) that plays a role in cellular energy homeostasis and autophagy induction was increased in *Pkd1*^{RC/RC} kidneys, increased by TRE in wild type kidneys and unaffected by TRE in *Pkd1*^{RC/RC} kidneys. In this regard, the AMPK activator metformin is known to protect against PKD in mice [22]. The serine/threonine kinase glycogen synthase kinase-3 β (GSK-3 β) that functions in a wide range of cellular processes and is known to induce apoptosis and proliferation in cancer [53] was decreased in *Pkd1*^{RC/RC} kidneys and unaffected by TRE. In summary, the autophagy phenotype in *Pkd1*^{RC/RC} kidneys was characterized by decreases in crucial autophagy-related proteins (Atg12-5 complex, Atg5, Atg16L1), decreased Rab9a, increased mTORC1 (inhibitor of autophagy) and decreased mTORC2 (inducer of autophagy).

In conclusion, the present study shows a newly described effect of TRE to increase expression of Atg12-5 complex and increase Rab9a expression in normal kidneys. However, the effect of long term TRE treatment on Atg12-5, Rab9a and other autophagy proteins was not seen in *Pkd1*^{RC/RC} kidneys associated with a lack of protection against cyst growth.

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Figure Legends:

Figure 1: Trehalose (TRE) does not significantly affect water intake, urinary vasopressin (AVP), or serum copeptin.

Animals were given either tap water (VEH) or 2% TRE in tap water for 5-days from ages 58-63 days old and water intake was recorded. Then urinary vasopressin and serum copeptin were measured. Data is expressed as a mean +/- SEM. n=5-6 per group

Figure 2: Atg12-5, LC3-II, p62 were increased and pBeclin1^{Ser15} was decreased in *Pkd1*^{RC/RC} (PKD) vs. wild type (WT) kidneys. Short-term trehalose (TRE) supplementation increased Atg12-5 complex in WT but not PKD kidneys and increased Rab9a in both WT and PKD kidneys.

Animals were treated for 6 days from 64-70 day of age with TRE or vehicle (VEH). Immunoblot analysis was performed for LC3-II, p62, Atg12-5 complex, free Atg12, Rab9a and pBeclin1 (Becn1)^{S15}. RDU=relative densitometry units corrected for GAPDH. Data is expressed as a mean +/- SEM, n=4-6 per treatment group. *P < 0.05, ** P < 0.01.

Figure 3: Trehalose (TRE) increases autophagy proteins Atg12-5, p62, LC3-II ex vivo

Homogenized wild type mouse kidneys were incubated in a test tube with increasing concentrations of TRE or glucose (GLU) and were then immunoblotted for Atg12-5, p62, LC3-II and Rab9a proteins. RDU=relative densitometry units corrected for GAPDH. Data is expressed as a mean +/- SEM. n=4-6 per treatment group. P < 0.05 versus vehicle (VEH) and 250 nM TRE.

Figure 4: Long-term trehalose (TRE) supplementation did not improve kidney weight, cyst number, cyst index, BUN, tubular cell proliferation or tubular cell apoptosis

Animals were treated from 50-120 days of age with either vehicle (VEH) or TRE. (A) In older mice (120 days of age), water intake was slightly increased by TRE compared to no significant increase in water intake in younger mice (63 d old) (Figure 1). Kidney weight was measured by two kidney weight to body weight ratio (2KW/BW%). Cyst number and cyst index (percentage of the cross-sectional area of the kidney that was cystic) were determined. Renal function was assessed by BUN. Representative kidney cross sections at the same

magnification stained with hematoxylin-eosin are shown. Data is expressed as a mean $\pm$ SEM, n=3-6 per treatment group. *P < 0.05. **(B)** Immunohistochemistry for markers of proliferation (PCNA) and apoptosis (TUNEL) was performed and quantitated in cyst lining cells and non cystic tubules. Data is expressed as a mean $\pm$ SEM, n=4-6 per treatment group.

Figure 5: Long-term trehalose (TRE) supplementation resulted in an increase in Atg12-5, LC3-II, and p62 in wild type (WT) but not *Pkd1*^{RC/RC} kidneys. Atg12-5 complex was absent and free Atg12 was increased in *Pkd1*^{RC/RC} kidneys

Animals were treated for 70 days from 50-120 days of age with either vehicle (VEH) or TRE. Immunoblot analysis was performed for Atg12-5 complex, free Atg12, free Atg5, LC3-II, p62, Rab9a and pBeclin1 (Becn1)^{Ser15}. RDU=relative densitometry units corrected for GAPDH. Data is expressed as a mean $\pm$ SEM, n=4-6 per treatment group. *P < 0.05 ** P < 0.01, ***P < 0.001.

Figure 6: Autophagy phenotype in *Pkd1*^{RC/RC} kidneys: Increase in mTORC1 (pmTOR^{S2448}, pS6), decrease in mTORC2 (pAkt^{S473}), decrease in Atg16L1, pGSK3 β and pAkt^{S308} in *Pkd1*^{RC/RC} (PKD) vs. wild type (WT) kidneys that was not affected by trehalose (TRE).

Animals were treated for 70 days from 50-120 days of age with either vehicle (VEH) or TRE. The effect of PKD and TRE on mTORC1 (pmTOR^{S2448}, pS6), mTORC2 (pAkt^{S473}), pAkt^{S308}, pGSK3 β and autophagy-related proteins Atg3, Atg7 and Atg16L1 in WT and PKD kidneys was determined. RDU=relative densitometry units corrected for GAPDH. Data is expressed as a mean $\pm$ SEM, n=3-6 per treatment group. *P < 0.05 ** P < 0.01, ***P < 0.001, ****P<0.0001.

Fig 1

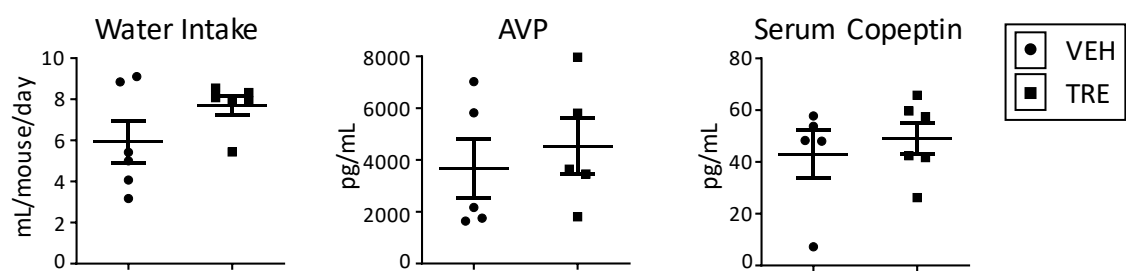


Fig 2

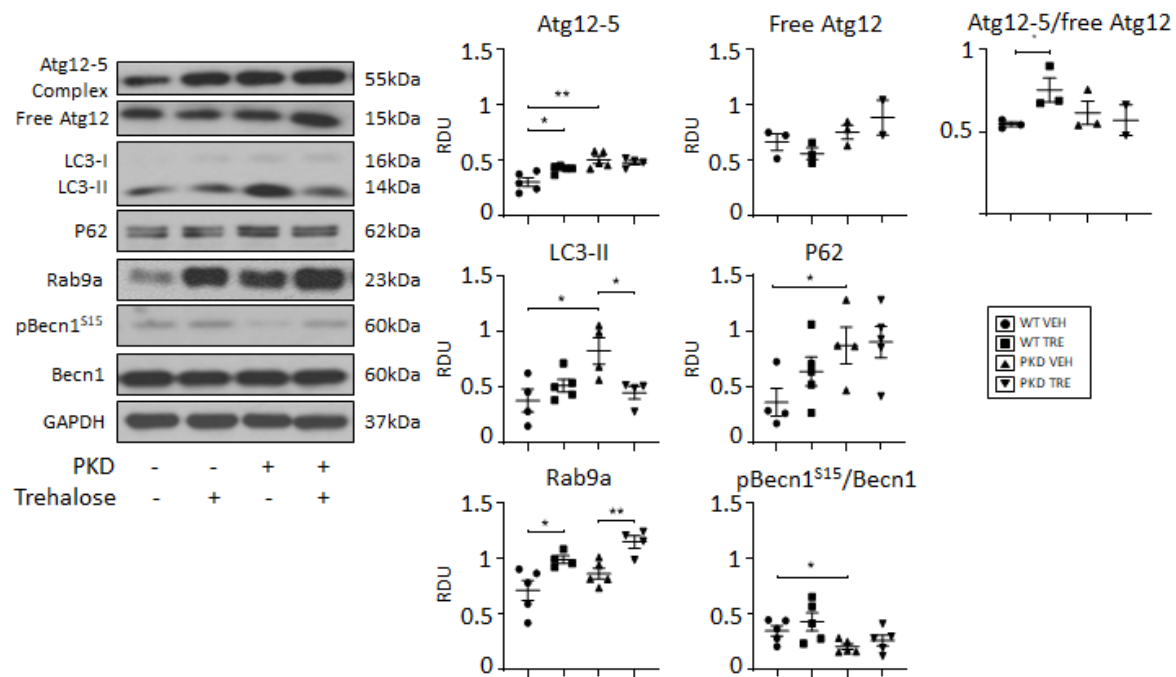


Fig. 3

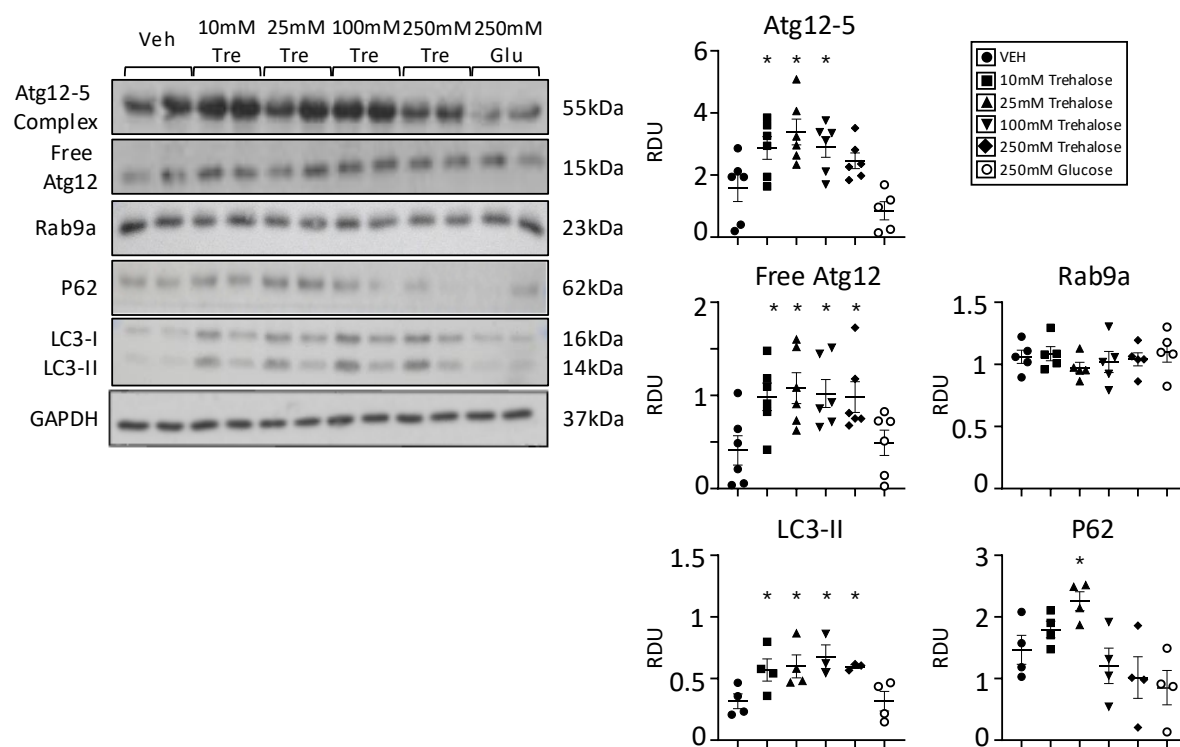


Fig 4

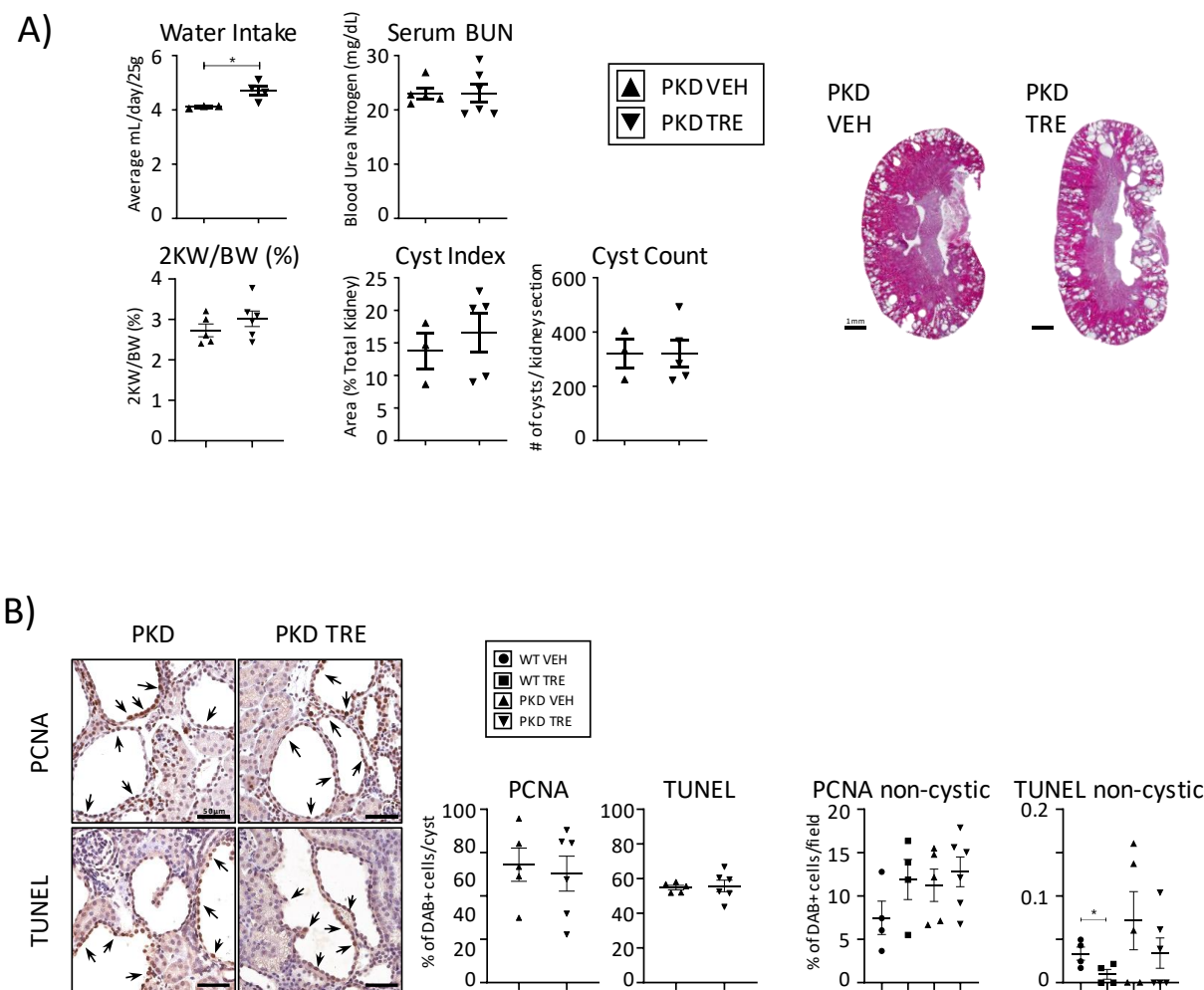


Fig 5

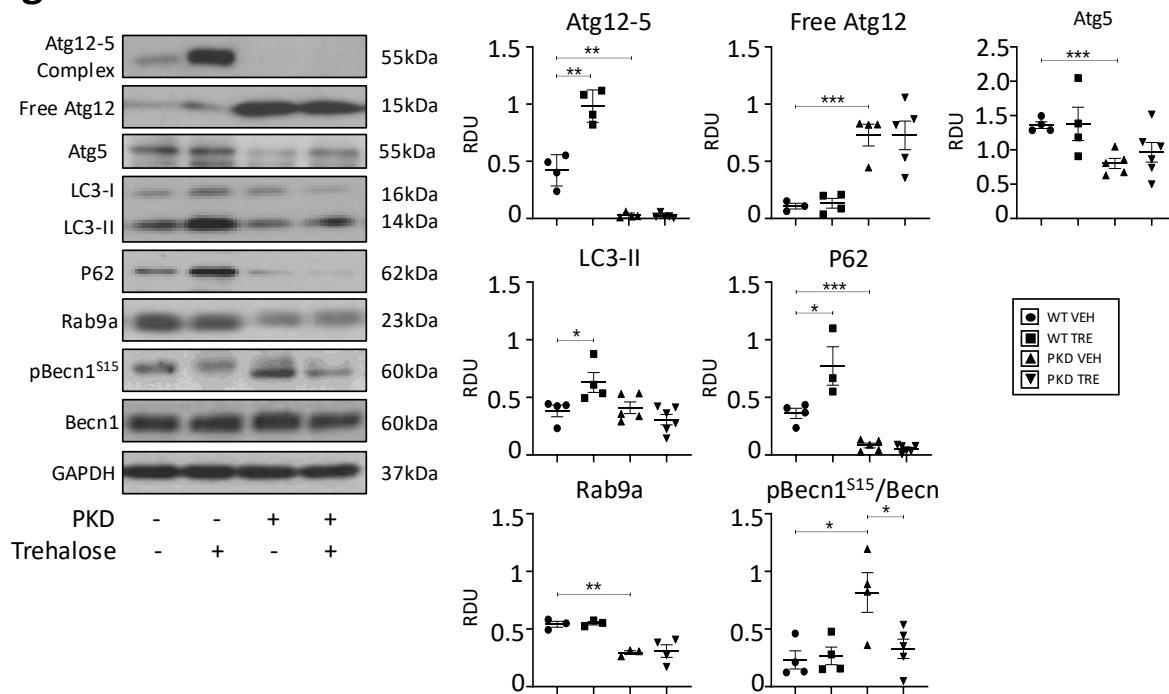


Fig 6

