

## A mild reduction of food intake slows disease progression in an orthologous mouse model of polycystic kidney disease

Kevin R. Kipp, Mina Rezaei, Louis Lin, Elyse C. Dewey, and  Thomas Weimbs

Department of Molecular, Cellular, and Developmental Biology and Neuroscience Research Institute, University of California, Santa Barbara, California

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**Kipp KR, Rezaei M, Lin L, Dewey EC, Weimbs T.** A mild reduction of food intake slows disease progression in an orthologous mouse model of polycystic kidney disease. *Am J Physiol Renal Physiol* 310: F726–F731, 2016. First published January 13, 2016; doi:10.1152/ajprenal.00551.2015.—Autosomal-dominant polycystic kidney disease (ADPKD) is a common cause of end-stage renal disease, and no approved treatment is available in the United States to slow disease progression. The mammalian target of rapamycin (mTOR) signaling pathway is aberrantly activated in renal cysts, and while mTOR inhibitors are highly effective in rodent models, clinical trials in ADPKD have been disappointing due to dose-limiting extrarenal side effects. Since mTOR is known to be regulated by nutrients and cellular energy status, we hypothesized that dietary restriction may affect renal cyst growth. Here, we show that reduced food intake (RFI) by 23% profoundly affects polycystic kidneys in an orthologous mouse model of ADPKD with a mosaic conditional knockout of PKD1. This mild level of RFI does not affect normal body weight gain, cause malnutrition, or have any other apparent side effects. RFI substantially slows disease progression: relative kidney weight increase was 41 vs. 151% in controls, and proliferation of cyst-lining cells was 7.7 vs. 15.9% in controls. Mice on an RFI diet maintained kidney function and did not progress to end-stage renal disease. The two major branches of mTORC1 signaling, S6 and 4EBP1, are both suppressed in cyst-lining cells by RFI, suggesting that this dietary regimen may be more broadly effective than pharmacological mTOR inhibition with rapalogs, which primarily affects the S6 branch. These results indicate that polycystic kidneys are exquisitely sensitive to minor reductions in nutrient supply or energy status. This study suggests that a mild decrease in food intake represents a potential therapeutic intervention to slow disease progression in ADPKD patients.

ADPKD; food restriction; mTOR; polycystic kidney disease

AUTOSOMAL-DOMINANT POLYCYSTIC kidney disease (ADPKD) is a very common inherited disease affecting the world's population with a frequency of ~1:500 (1, 10). Thousands of cysts develop in both kidneys due to excessive proliferation of tubule epithelial cells, leading to a gross organ size increase, fibrosis, and destruction of the normal renal parenchyma, with eventual progression to renal failure. Disease progression is typically relatively slow, and renal failure often occurs in the fifth or sixth decade of life, but the rate of progression can greatly vary from patient to patient.

No approved treatment to slow or halt disease progression is currently available in the United States. Recently, the vasopressin receptor (V2R) antagonist tolvaptan was approved for ADPKD in Japan, Canada, and Europe, but side effects, potential toxicity, and unfavorable cost effectiveness may limit

the usefulness of this drug (3). Besides V2R-mediated cAMP-signaling, numerous other aberrantly activated signaling pathways have been associated with renal cyst growth in PKD, including mammalian target of rapamycin (mTOR) signaling. The Ser/Thr kinase mTOR is strongly activated in human ADPKD and rodent models of PKD. Treatment of these rodent models with mTOR inhibitors such as rapamycin leads to very significant inhibition of renal cyst growth (14, 15, 18). These findings led to clinical trials with unfortunately disappointing results, suggesting no compelling benefit in patients (12, 21, 23). In hindsight, the discrepancy between the effects of mTOR inhibitors in PKD rodent models and ADPKD patients is most likely due to the fact that feasible drug doses in human patients are limited by the significant extrarenal side effects of mTOR inhibitors. This problem is particularly significant because of the slowly progressive nature of ADPKD that likely leads to the requirement for extremely long-term treatment on the order of years and decades. The realistic goal of an effective ADPKD therapy is not to achieve reversal of disease but to achieve slowing of further disease progression. Therefore, therapy will likely need to be initiated early in the course of disease progression and continue for the rest of a patient's life. This may be difficult to achieve with conventional drugs that inhibit the relatively ubiquitous signaling pathways associated with PKD. However, drug targeting to polycystic kidneys may circumvent this problem, as demonstrated in recently published approaches of targeting of small-molecular weight compounds (13) and antibodies (9) to PKD kidneys.

As an alternative to pharmacological intervention, we considered influencing mTOR activity in the kidney using a dietary strategy. mTOR is not only regulated by growth factor signaling but also by nutrient availability and the energy status of cells. For example, AMPK is activated under conditions of low ATP/AMP ratios that in turn lead to inhibition of mTOR (6). It has previously been shown that pharmacological activation of AMPK with metformin slows renal cyst growth in a PKD mouse model (17). mTOR activity is also exquisitely controlled by amino acids and insulin (8), which are directly influenced by food intake. Furthermore, recent results suggest metabolic alterations in cyst-lining cells in PKD that are akin to the Warburg effect in cancer cells and lead to increased glucose dependency (11).

We therefore hypothesized that mTOR activity in cyst-lining cells in PKD may be highly dependent on nutrient/energy supply and may be influenced by dietary modification alone, leading to decreased proliferation, slowing of disease progression, and prolongation of renal function. We tested this hypothesis by mildly reducing the food intake in a human-orthologous mouse model. In this model, the *Pkd1* gene is inactivated in a mosaic fashion to mimic the low frequency of

Address for reprint requests and other correspondence: T. Weimbs, Dept. of Molecular, Cellular, and Developmental Biology, Univ. of California Santa Barbara, CA 93106-9610 (e-mail: weimbs@lifesci.ucsb.edu).

loss of heterozygosity (LOH) mutations in human ADPKD (15). We previously showed that mTOR inhibition by rapamycin leads to a strong reduction of renal cyst growth in this model (15). We report here that reduced food intake (RFI) is indeed highly effective in suppressing disease progression in this model. A mild RFI that has no significant effect on the body weight of wild-type mice leads to significant suppression of renal cyst growth in PKD mice and preservation of renal function. During the completion of this manuscript, similar results were reported by another group using the  $Pkd1^{RC/RC}$  mouse model (22), which is homozygous for a hypomorphic mutation in  $Pkd1$  resulting in a misfolded polycystin-1 protein (5), and the  $Pkd2^{WS25/-}$  mouse model that develops PKD as a result of somatic inactivation of  $Pkd2$  (16, 24). This study is largely consistent with our results although there are potentially interesting differences in the analysis of the affected molecular pathways. Together, these independently derived findings make a strong case that RFI or caloric restriction may be beneficial in slowing disease progression in ADPKD patients.

## MATERIALS AND METHODS

**Animals.** All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California (Santa Barbara, CA) and adhered to the rules and regulations estab-

lished by the National Institutes of Health as described in *Guide for the Care and Use of Laboratory Animals*. The  $PKD^{cond/cond};Nes^{Cre}$  model has been described previously (15). Animals on the RFI vs. ad libitum (AL) regimen were individually caged to ensure accurate control of food intake. Animals on the RFI regimen were fed daily  $77.0 \pm 4.1\%$  of the measured average of feed consumed by age-matched AL-fed controls from postnatal day 35 until day 84. Animal feed was PicoLab Rodent Diet 20 from LabDiet (St. Louis, MO). Animal health was monitored daily for adverse events, and mice were weighed weekly. Blood urea nitrogen (BUN) was measured using a QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA) following the manufacturer's instructions. Progression to end-stage renal disease was assessed based on daily food intake, animal behavior, appearance, weight loss, and BUN levels.

**Immunoblotting.** Primary antibodies for pS6 (S240/244), p4EBP1 (T37/46), pAMPK (T172), and pLKB1 (S307) were from Cell Signaling Technology (Danvers, MA). Renal tissue samples were homogenized following flash freezing in liquid nitrogen on a mortar and pestle before lysing in RIPA buffer. Protein levels were normalized using a Promega BCA protein quantification kit. Whenever possible, samples were compared with loading controls from the same gel.

**Histology and immunofluorescence microscopy.** Standard paraffin-embedded sections (5  $\mu$ m) were stained with hematoxylin and eosin or Masson's trichrome. For immunofluorescence microscopy, sections were dewaxed in xylene, rehydrated through a series of graded alcohols, and then the antigens were retrieved in 10 mM sodium citrate, pH 6.0, in a pressure cooker for 5 min. The following antibodies were used for

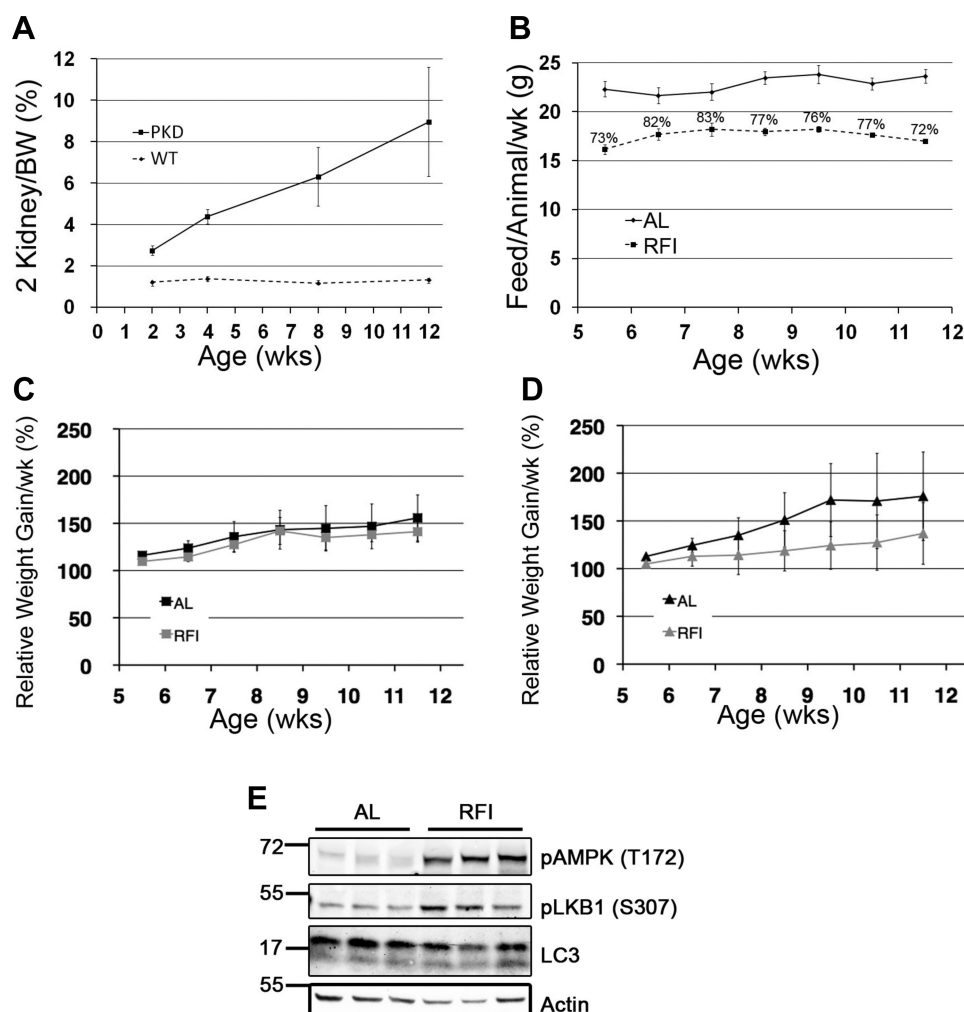


Fig. 1. Effects of a moderately reduced food intake (RFI) on control mice. **A**: disease progression as measured by 2-kidney-to-body weight ratios in untreated  $PKD1^{cond/cond};Nes^{Cre}$  mice (solid line) compared with wild-type mice (dashed line;  $n = 5$  animals/time point). **B**: average food intake per animal per week for mice on ad libitum (AL) diet (solid line) or RFI diet (dashed line). **C**: weight gain as a percentage of starting weight per week for wild-type mice on AL diet (black) or RFI diet (grey). **D**: weight gain for polycystic kidney disease (PKD) mice on the AL diet (black) or RFI diet (grey). **E**: immunoblot analysis of wild-type kidney lysates reveals an increase in overall LKB1 (S307) and AMPK (T172) phosphorylation in mice on the RFI diet compared with AL controls. The RFI diet does not increase LC3-II accumulation as a marker of autophagy.

immunostaining: pS6 S240/244 and S235/236 (2211 and 5364, respectively) and p4EBP1 T37/46 (2815) from Cell Signaling Technology and Ki-67 (AB9260) from EMD Millipore.

**Statistical analysis.** The statistical analysis was done using a Mann-Whitney unpaired one-tailed *t*-test.

## RESULTS

*A mild level of RFI does not significantly affect body weight in wild-type mice but activates renal LKB1/AMPK signaling.* *Pkd1*<sup>cond/cond</sup>:Nes<sup>Cre</sup> mice were used in this study and have previously been shown to replicate characteristic features of human PKD including aberrant mTOR activation, epithelial proliferation and apoptosis, and progressive fibrosis (15). To define a window of development of cystic disease in this model that may be amenable to intervention by food reduction, untreated animals were first assessed over time for severity of disease based on their two-kidney-to-body weight ratio and

health status. Renal size steadily increases postnatally (Fig. 1A), and the age of 12 wk was chosen as a humane end point due to deterioration of health and progression to end-stage renal disease after this time point. Based on this assessment, a regimen of mild food reduction was then devised for mice from postnatal week 5 to 12. All animals were individually caged, and daily food intake was determined in AL-fed mice. Animals on the RFI diet were fed on average 77% of the food consumed by the AL controls (Fig. 1B). In wild-type control animals (*PKD1*<sup>wt/wt</sup>:Nes<sup>Cre</sup>), this mild level of food intake reduction did not cause significant changes in body weight gain compared with AL-fed wild-type animals (Figs. 1C and 2D). The health of all animals was monitored daily, and no obvious negative effects were noticed.

Kidneys of wild-type animals on the AL vs. RFI diet were then assessed for changes in nutrient-dependent AMPK signal-

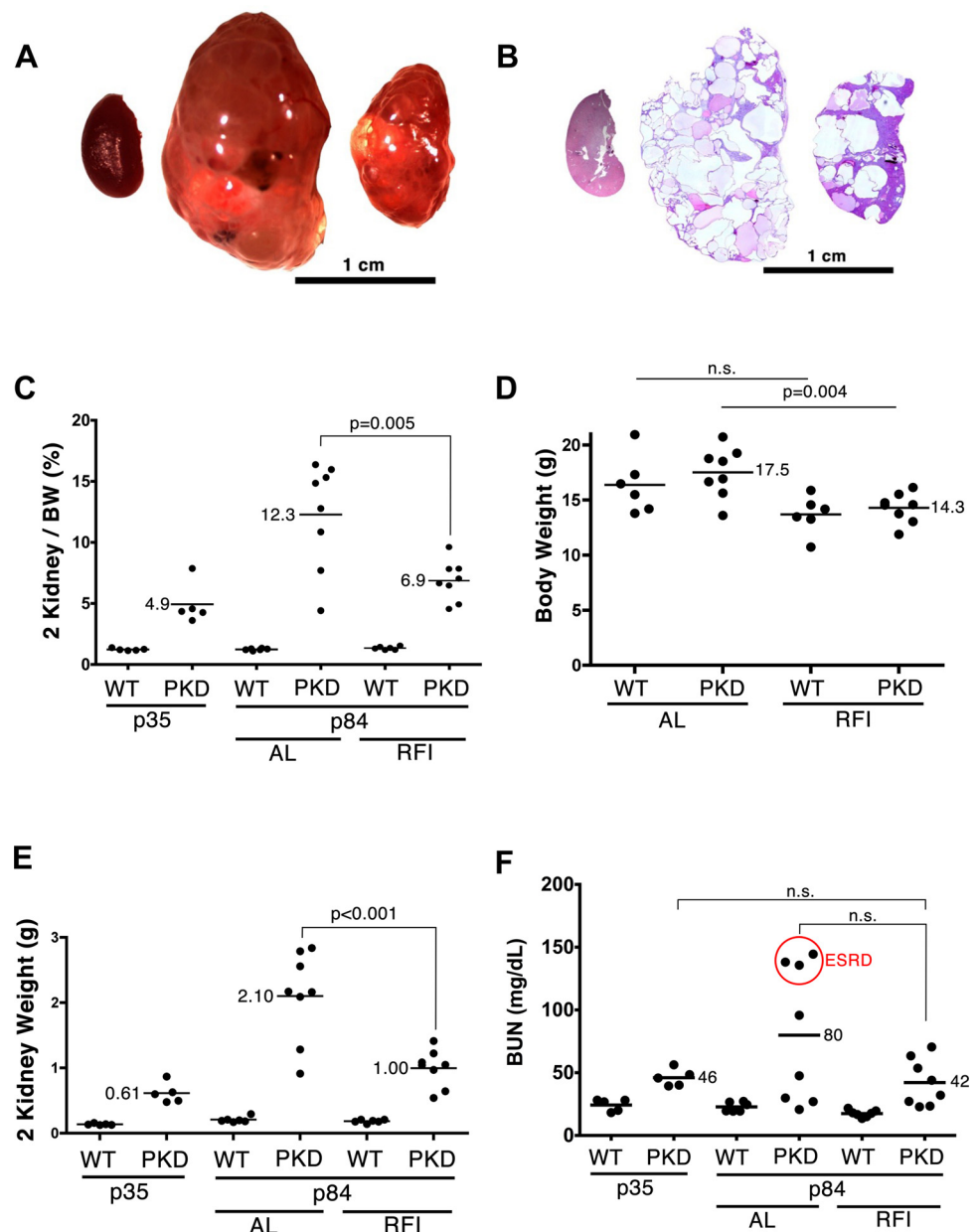


Fig. 2. RFI ameliorates disease progression in *PKD1*<sup>cond/cond</sup>:Nes<sup>Cre</sup> mice. Shown is a comparison of representative gross kidneys (A) and hematoxylin- and eosin- stained tissue sections (B) from AL wild-type (WT), PKD-AL, and PKD-RFI mice (left to right). C: 2-kidney-to-body weight ratios in untreated mice at the beginning of the treatment regimen (postnatal day 35; *p*35) and at the end (*p*84) of the AL or RFI dietary regimen. D: effect of dietary regimen on total body weights at *p*84. Two-kidney weights (E) and BUN (F) of mice are shown for the start (*p*35) or end (*p*84) of the trial. Red circle indicates animals that reached end-stage renal disease (ESRD) by the conclusion of the trial.



ing to test whether this mild level of RFI has any appreciable renal effect. The active, phosphorylated form of AMPK (T172) was increased in kidneys of RFI animals compared with AL animals (Fig. 1E); however, no change was seen in LC3-II levels as a readout of autophagy. A similar increase in phosphorylated LKB1 (S307) was observed, suggesting that LKB1, which is known to lead to AMPK phosphorylation (4), may be involved in AMPK activation under these conditions (Fig. 1E).

**RFI slows renal cyst growth in PKD mice.** To determine whether RFI can influence PKD disease progression, *Pkd1<sup>cond/cond</sup>*; *Nes<sup>cre</sup>* mice were fed either AL or with the same RFI regimen as above. PKD animals on the RFI regimen exhibited a strong reduction in renal and cyst growth compared with AL-fed mice. The two-kidney/body weight ratio increased 41% in

RFI-treated animals compared with 151% in AL-fed animals during the 7-wk treatment period (Fig. 2, A and B). RFI also resulted in a minor reduction of total body weight gain compared with the AL-diet in PKD mice (Figs. 1D and 2D). However, this reduction appears to be largely due to the significant suppression of renal mass (Fig. 2E). The RFI diet resulted in partial preservation of normal renal parenchyma compared with the AL diet (Figs. 2, A and B, and 3, A and B). Renal fibrosis and proliferation of cyst-lining epithelial cells were both reduced in PKD kidneys in the RFI cohort compared with the AL cohort (Fig. 3, C–G). BUN was measured as a surrogate marker of renal function. Due to the heterogeneity of renal function decline in PKD mice, the difference in the average BUN values between the AL and RFI groups was not

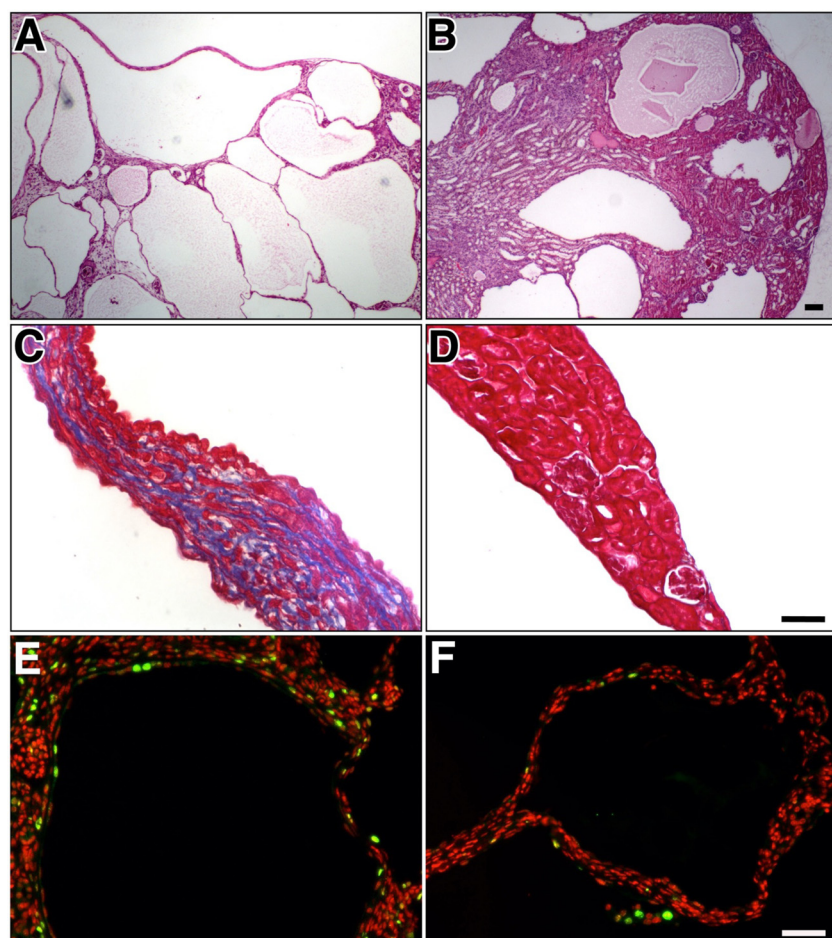


Fig. 3. RFI reduces interstitial fibrosis and proliferation of cyst-lining epithelial in PKD kidneys. A–F: representative images of kidney sections from PKD mice on AL diet (left) or RFI diet (right). A and B: hematoxylin- and eosin-stained tissue. C and D: Masson's trichrome staining to visualize collagen deposition. Intense interstitial collagen staining (blue) is apparent in kidneys of AL-fed PKD mice but is greatly reduced in kidneys of RFI-fed PKD mice. E and F: immunofluorescence microscopy for the proliferation marker Ki-67 (green). Nuclear counterstain is 4,6-diamidino-2-phenylindole (DAPI; red false color). Scale bars = 50  $\mu$ m. G: quantification of the percentage of Ki67-positive cyst-lining cells among the total number of DAPI-positive cyst-lining epithelial cells. Ki-67-positive cells and nuclei were counted in 5 random, low-magnification fields/kidney ( $n = 3$  mice/treatment condition).

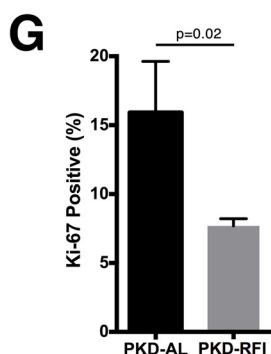
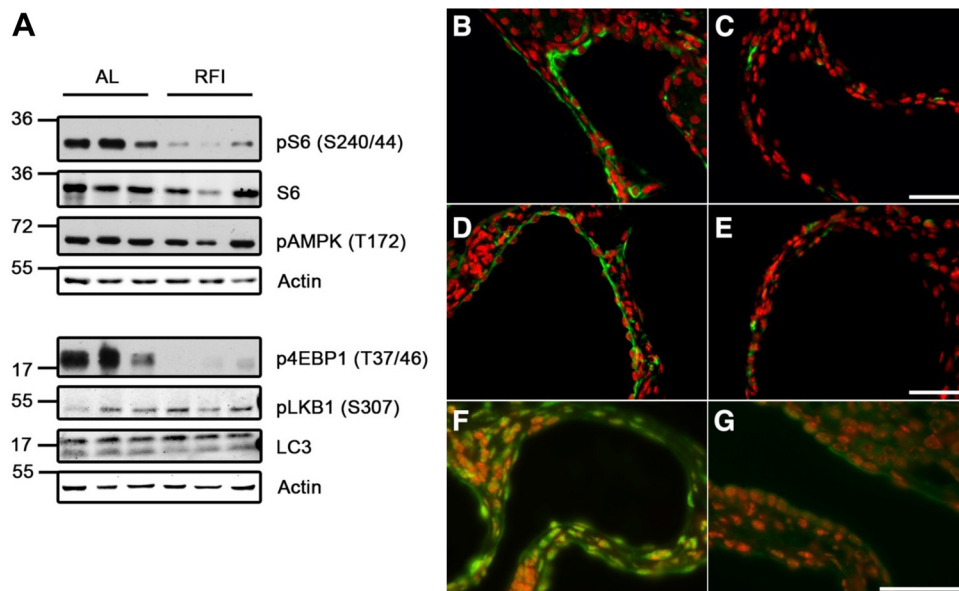


Fig. 4. RFI inhibits mTORC1 signaling in PKD kidneys. **A**: immunoblot analysis of total kidney lysates from PKD animals on either AL or RFI dietary regimen reveal a decrease in total phosphorylation of S6 (S240/244) and 4EBP1 (T37/46), and no significant change in the phosphorylation of LKB1 (S307) and AMPK (T172). Actin loading corresponds to the 3 blots loaded above them and each lane represents a single tissue lysate with  $n = 3$  analyzed/treatment group. **B–G**: localization of mTORC1 activity markers (green) by immunofluorescence microscopy in kidneys from PKD mice on AL diet (*left*) or RFI diet (*right*). **B** and **C**: pS6 (S240/44). **D** and **E**: pS6 (S235/236). **F** and **G**: p4E-BP1(T37/46). Nuclear counter-stain is DAPI (red false color). Scale bars = 50  $\mu$ m. All image acquisition and processing were done equivalently.



statistically significant (Fig. 2*F*). However, nearly half of the AL cohort reached end-stage renal failure by the end of the study period, with clear signs of deteriorating health and one animal reaching a humane end point several days (postnatal day 80) before the end of the trial (Fig. 2*F*). In contrast, none of the animals in the RFI cohort progressed to end-stage renal disease, suggesting that the RFI diet leads to preservation of renal function and increased survival.

To assess which of the relevant potential nutrient-dependent signaling pathways may be affected by RFI, kidneys were analyzed by immunoblotting and immunofluorescence microscopy. Phosphorylation of the downstream targets of mTORC1, ribosomal protein S6 and 4EBP1, respectively, were decreased in the RFI group with respect to the AL group both in total kidney lysates (Fig. 4*A*) and in cyst-lining cells (Fig. 4, *B–G*). Similar to the wild-type animals, the levels of the autophagy marker LC3-II were unchanged, but, unexpectedly, LKB1 and AMPK phosphorylation, while increased in the wild-type RFI group compared with the wild-type AL group (Fig. 1*D*), were not apparently influenced by diet in the PKD groups (Fig. 4*A*). These data demonstrate that an RFI regimen alone leads to strong inhibition of mTOR signaling in PKD cysts but this effect may be independent of LKB1/AMPK signaling.

## DISCUSSION

Our study suggests that the growth of polycystic kidneys is particularly dependent on the abundance of nutrients and/or energy status. A mild RFI that does not affect normal body weight gain still has a profound effect on polycystic kidneys, causing reduced cyst growth, fibrosis, proliferation, mTORC1 activation, and leads to preservation of renal function. The extent of the beneficial effects of RFI are similar to those of pharmacological mTOR inhibition with rapamycin that we previously reported in the same PKD mouse model (15). This suggests that the inhibition of mTORC1 signaling by RFI may be central to the observed inhibition of disease progression. The two major branches of signaling downstream of mTORC1 are the activation of S6 and 4EBP1, respectively (8). Interestingly, rapamycin has been shown to inhibit primarily

mTORC1's ability to phosphorylate S6 but not 4EBP1 (19). Our results indicate that RFI leads to strong inhibition of both S6 and 4EBP1 (Fig. 4), suggesting that RFI may be more broadly effective at inhibiting mTORC1 compared with rapamycin.

The extent of RFI by only 23% in our study is relatively mild and below the level that causes a significant decrease in body weight gain. Food was restricted as a whole, leaving the proportions of all nutrients unaltered. We speculate that the observed beneficial effects may be primarily due to caloric restriction. However, we cannot currently exclude the possibility that the reduction of any one particular nutrient, or nutrient group, may be more important than calorie content per se. Since mTOR activity is known to be regulated by amino acids, it is possible that reduced amino acid intake may inhibit renal mTOR in PKD. Previous work showed that reduced protein intake inhibits renal cyst growth in a PKD mouse model (20). However, protein intake was restricted by 76% in this study, much more than in our study, and a nonorthologous model was used. Furthermore, reducing protein intake by 78% failed to lead to a significant beneficial effect in a clinical study of ADPKD patients (7). mTOR activity was not investigated in these studies because they preceded the discovery of mTOR and its regulation. A possible effect of dietary amino acids on renal mTOR and PKD progression remains to be elucidated.

Other recent results suggest that cyst-lining cells may be particularly dependent on glucose as an energy source, and it was shown that treatment of PKD mice with the glucose analog 2-deoxyglucose reduced disease progression (2). These findings are more consistent with the interpretation that RFI is effective due to caloric restriction. It is also possible that RFI does not have a direct effect on cyst-lining cells but may rather act via hormone action such as insulin, which is known to affect mTOR. It will be important to determine in future studies the relative importance of individual nutrients on the progression of PKD.

Our results are consistent with those of an independent study that was published during the completion of our manuscript (22). Similarly strong beneficial effects of food restriction on

disease progression were reported in PKD mouse models. The only minor difference is that these investigators reported increased LKB1/AMPK signaling in PKD kidneys after food restriction, which was not observed in our study. This difference may potentially be due to differences in the mouse models used, and further investigation is warranted. The Pkd1<sup>RC/RC</sup> mouse model used in the other study (22) develops PKD due to a homozygous mutation in Pkd1, resulting in a hypomorphic, misfolded polycystin-1 protein (5). Therefore, every cell in this mouse model is potentially affected by the gene mutation. In contrast, expression of the Pkd1 gene is entirely ablated in the Pkd1<sup>cond/cond</sup>;Nes<sup>Cre</sup> mice used here but only in a fraction of the cells, presumably those that will become cystic. Other differences are the timing of disease progression and the extent of dietary treatment between these studies. Warner et al. (22) treated the Pkd1<sup>RC/RC</sup> mouse model with a more profound level of food reduction (40%) compared with our study (23%) and for a longer duration (from postnatal week 6 to 24) compared with our study (postnatal weeks 5–12). However, the fact that two independent studies come to very similar conclusions makes a compelling argument that RFI may be beneficial in ADPKD patients as well. Given that a relatively mild level of RFI already has a profound beneficial effect on PKD progression suggests that this effect is relatively kidney specific and can be achieved in the absence of malnutrition or other undesired effects.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

Author contributions: K.R.K. and T.W. provided conception and design of research; K.R.K., M.R., L.L., and E.C.D. performed experiments; K.R.K., M.R., L.L., E.C.D., and T.W. analyzed data; K.R.K., M.R., L.L., E.C.D., and T.W. interpreted results of experiments; K.R.K., M.R., L.L., and T.W. prepared figures; K.R.K. and T.W. drafted manuscript; K.R.K. and T.W. edited and revised manuscript; T.W. approved final version of manuscript.

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