

# Food Restriction Ameliorates the Development of Polycystic Kidney Disease

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## ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder characterized by the accumulation of kidney cysts that ultimately leads to loss of renal function and kidney failure. At present, the treatment for ADPKD is largely supportive. Multiple studies have focused on pharmacologic approaches to slow the development of the cystic disease; however, little is known about the role of nutrition and dietary manipulation in PKD. Here, we show that food restriction (FR) effectively slows the course of the disease in mouse models of ADPKD. Mild to moderate (10%–40%) FR reduced cyst area, renal fibrosis, inflammation, and injury in a dose-dependent manner. Molecular and biochemical studies in these mice indicate that FR ameliorates ADPKD through a mechanism involving suppression of the mammalian target of the rapamycin pathway and activation of the liver kinase B1/AMP-activated protein kinase pathway. Our data suggest that dietary interventions such as FR, or treatment that mimics the effects of such interventions, may be potential and novel preventive and therapeutic options for patients with ADPKD.

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Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic diseases in humans, affecting more than 600,000 Americans and an estimated 12.5 million people worldwide.<sup>1,2</sup> Mutations to either *PKD1* or *PKD2* cause ADPKD. This disease is characterized by accumulation of kidney cysts that ultimately lead to loss of renal function and kidney failure.<sup>1,2</sup> At present, its treatment is largely supportive, including dialysis and renal transplantation for patients with ESRD.<sup>1,2</sup> Currently, there is no accepted FDA-approved treatment option for the prevention or treatment of patients with ADPKD.

The precise mechanisms that modulate cyst formation and progression of ADPKD have not been fully elucidated. However, recent studies point to the central role of metabolic sensors in the pathogenesis of ADPKD.<sup>3–6</sup> The metabolism is under tight control via several metabolic sensors, and cells have to constantly monitor and adapt to environmental metabolic changes. Several specific cellular pathways have evolved to monitor the levels of systemic nutrients,

and modulate cellular metabolism and cell fate.<sup>7</sup> Examples of these metabolic sensors are the proteins AMP-activated kinase (AMPK), mammalian target of rapamycin (mTOR), and SIRTUINS.<sup>3,5,6</sup> Interestingly, several studies indicate that these metabolic sensors play a key role in the pathogenesis of ADPKD.<sup>3,5,6</sup> It appears that overactivation of mTOR and its downstream target S6 Kinase (S6K) leads to cyst formation and progression of ADPKD.<sup>6</sup> Furthermore, activation of the AMPK pathway has

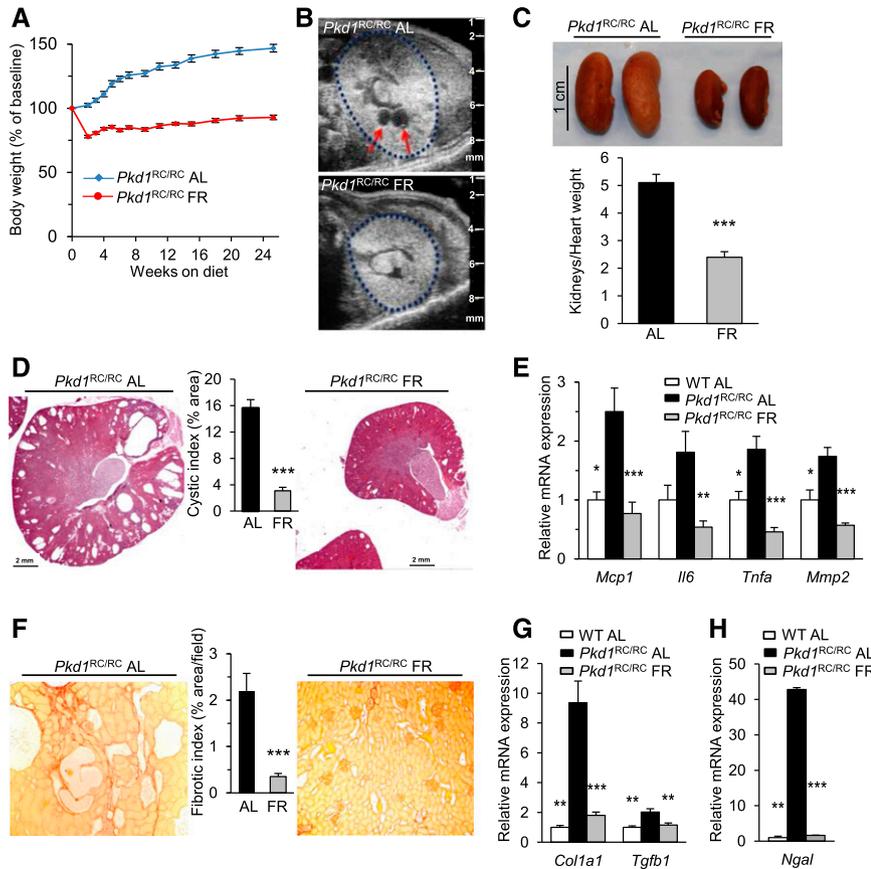
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**Figure 1.** Food restriction (FR) inhibits cystogenesis in *Pkd1<sup>RC/RC</sup>* mice. Six-week-old *Pkd1<sup>RC/RC</sup>* mice were fed a standard diet *ad libitum* (AL, *n*=12) or 40% FR (*n*=13) for 6 months. (A) Changes in body weight over time on diet (baseline weight at 6-week-old, *P*<0.001 for AL versus FR at all time points). (B–C) Kidney size is decreased by FR. (B) Representative ultrasound images showing larger kidney size (dotted outline) and prominence of echolucent cysts (red arrows) in AL compared with FR mice. (C) Upper panel, representative image of AL and FR kidney gross morphology. Lower panel, graph showing decreased total kidney weight, relative to heart weight, in FR-treated mice. (D) Cystic index is lower in FR. Representative renal histology images (hematoxylin and eosin stain) showing advanced cystic disease in AL compared with FR; inset graph shows quantitative analysis of cystic index, expressed as cyst area relative to kidney area (*n*=11 per group). (E) mRNA expression of inflammatory markers is lower in FR. RT-PCR data for *Mcp-1*, *Il-6*, *Tnf- $\alpha$* , and *Mmp-2* relative to *Gapdh* in kidneys of WT AL (*n*=4), and *Pkd1<sup>RC/RC</sup>* mice on AL and FR (*n*=8 per group). (F–G) Renal fibrosis is lower in FR. (F) Representative images of Sirius red-stained kidney sections; inset graph showing quantitative analysis of fibrotic index, expressed as proportion of cortical area with positive (red) staining per field (*n*=11 per group). (G) mRNA expression of *Col1 $\alpha$ 1* and *Tgf- $\beta$ 1*. (H) mRNA expression of the kidney injury marker *Ngal* is decreased by FR. Data are means $\pm$ SEM. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001, compared with *Pkd1<sup>RC/RC</sup>* AL group.

been proposed to prevent ADPKD.<sup>3</sup> Finally, overactivation of the NAD-dependent deacetylase SIRT1 was also implicated in the development of cysts, and inhibition of SIRT1 decreases cyst formation.<sup>5,8</sup> These findings strongly suggest that metabolic pressure plays a key role in the pathogenesis of ADPKD, and that dietary manipulations targeting metabolic sensors may have a role in the prevention or treatment of this disease.

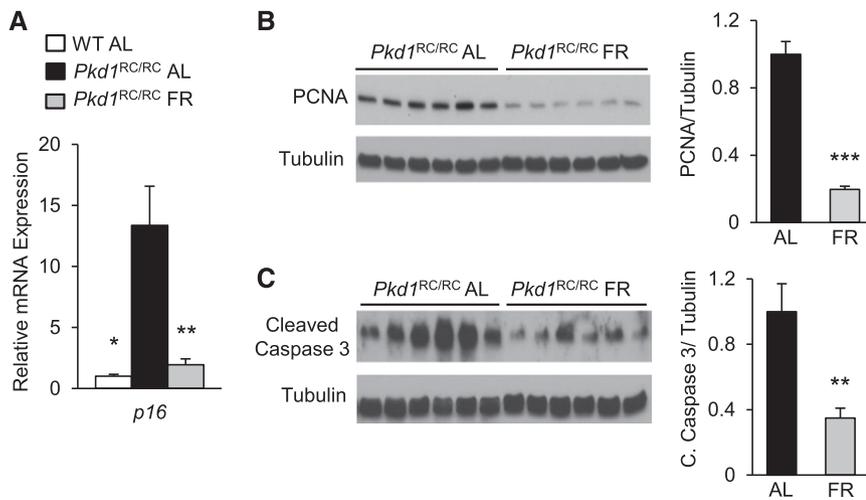
Food/caloric restriction (FR) without malnutrition (a decrease of 30%–50% of total caloric intake) has been shown to be the most reproducible and effective nongenetic manipulation that can slow aging and increase longevity in several organisms including mammals.<sup>9,10</sup> Interestingly, it has been proposed that the beneficial effects of FR are mediated by an interplay of several interacting metabolic sensors such as the AMPK, mTOR-S6K, and SIRT1 pathways.<sup>11</sup> During FR the mTOR-S6K pathway is inhibited and the AMPK pathway is stimulated.<sup>7</sup> Furthermore, a negative crosstalk loop exists between these pathways, where mTOR-S6K inhibits the AMPK pathway and *vice versa*.<sup>7</sup> Intervention with pharmacologic modulators of these pathways may mimic some features of FR.<sup>11</sup> However, no single pharmacologic FR mimetic can fully recapitulate the beneficial metabolic and physiologic consequences of FR.<sup>7,11,12</sup> Since the same metabolic sensors regulated by FR appear to be involved in the pathogenesis of ADPKD, we postulated that FR might prevent or delay the onset of ADPKD. In agreement with our hypothesis, we observed that FR protects against the development of ADPKD in animal models. Our data support the fact that metabolic adaptations have a key role in the development of ADPKD, and suggest that dietary interventions may have potential therapeutic and preventive roles in patients with ADPKD.

**RESULTS**

**FR Ameliorates the Development of ADPKD in Mouse Models**

FR without malnutrition (*i.e.*, 30%–50% of total caloric intake) provides a reproducible and effective nongenetic manipulation that delays aging and reduces cancer incidence in several organisms.<sup>11,13–15</sup> We introduced 40% FR in a progressive mouse model of ADPKD, *Pkd1<sup>R3277C/R3277C</sup>* (RC/RC),<sup>16</sup>

at 6 weeks of age and found an initial decrease in body weight that stabilized after 2 weeks (Figure 1A). Kidney size and cystogenesis were monitored during the course of study using ultrasonography, and at the end of the study (7.5 months of age) with gross anatomy and histology (Figure 1, B–D). The traditional way to express the effect of experimental manipulation on gross anatomic changes in animal



**Figure 2.** FR modulates cellular injury markers in *Pkd1*<sup>RC/RC</sup> mice. (A) *p16* relative mRNA levels in kidneys of WT mice fed AL ( $n=4$ ) and *Pkd1*<sup>RC/RC</sup> mice fed AL or 40% FR diet for 6 months ( $n=8$  per group). (B–C) Comparison of PCNA (B) and cleaved caspase 3 (C) by immunoblotting, employing tubulin as a loading control, in kidney extracts of *Pkd1*<sup>RC/RC</sup> AL and 40% FR mice. Each gel lane is obtained from an independent mouse ( $n=6$ ). Quantitative analysis of bands by densitometry for PCNA (B) and cleaved caspase 3 (C). Data are means  $\pm$  SEM. \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ , compared with *Pkd1*<sup>RC/RC</sup> AL group.

studies with ADPKD is the ratio of the two kidneys to body weight. However, since FR leads to a significant decrease in body weight (and would likely cause a false negative result), we express the changes as the ratio of two kidneys to an internal organ whose weight was not significantly changed by FR. In fact, we observed that the heart weight did not differ between *Pkd1*<sup>RC/RC</sup> mice on a standard *ad libitum* diet (AL) or FR (Supplemental Table 1A). Accordingly, in our studies we used the heart weight to normalize our results. However, in all the studies performed, even when the two kidneys to body weight ratio was used to determine the effect of FR, a statistically significant reduction of this ratio was observed in FR-treated *Pkd1*<sup>RC/RC</sup> mice (Supplemental Table 1B). Using these ratios and the histologic analysis of the cystic index, we observed that after 6 months of treatment FR almost completely inhibited the development of cysts (Figure 1, C and D, Supplemental Table 1, A and B). The effect of FR was observed in both male and female mice.

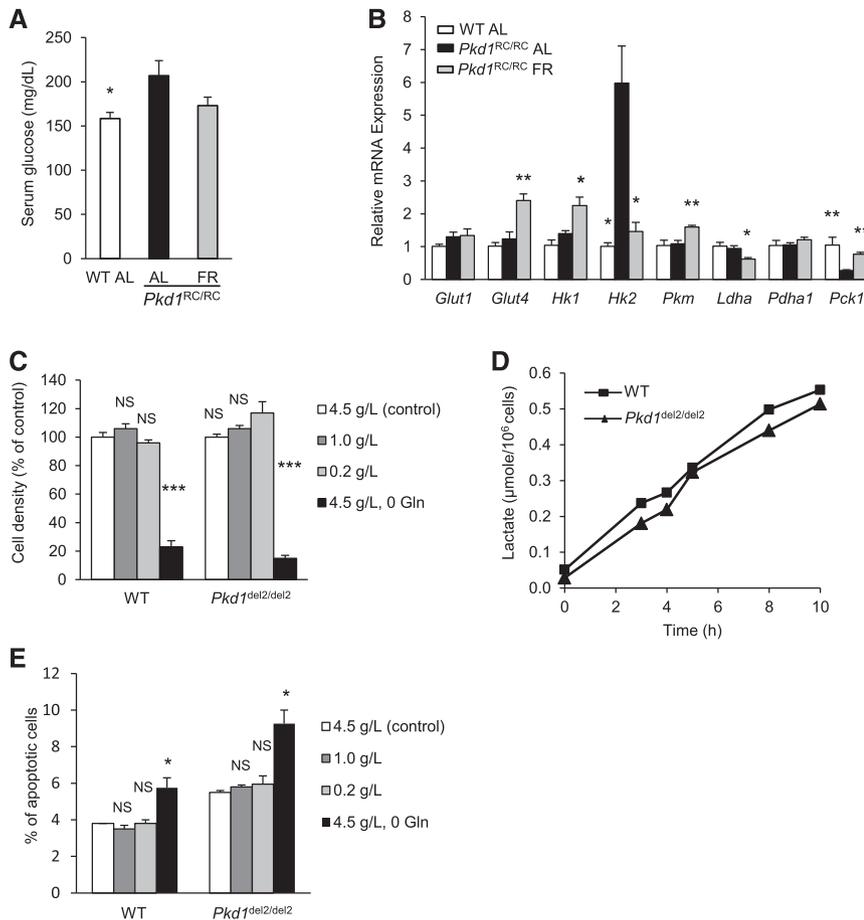
#### FR Decreases Markers of Inflammation, Fibrosis, Kidney Injury, Cell Proliferation, and Apoptosis

We further analyzed several pathologic aspects in the kidneys of *Pkd1*<sup>RC/RC</sup> animals, and observed that FR decreased kidney inflammation, fibrosis, and evidence of kidney injury (Figure 1, E–H). First we observed that, compared with age-matched wild-type (WT) controls (7.5-months-old), *Pkd1*<sup>RC/RC</sup> kidneys had higher levels of the inflammatory markers *Mcp-1*, *Il-6*, *Tnf- $\alpha$*  and matrix metalloproteinase 2 (*Mmp-2*), and expression of these markers was decreased to the levels of normal WT kidneys in FR-treated *Pkd1*<sup>RC/RC</sup> animals (Figure

1E). Histologic analysis revealed a nearly 10-fold decrease in renal fibrosis in FR-treated mice (Figure 1F). Consistent with this, RNA expression of fibrosis markers such as *Tgf- $\beta$ 1* and collagen 1 $\alpha$ 1 (*Col1 $\alpha$ 1*) was decreased by FR (Figure 1G). To determine if FR could prevent the development of kidney injury in ADPKD we further determined the expression of neutrophil gelatinase-associated lipocalin (*Ngal*), a marker of both AKI and CKI, in the kidneys of WT and *Pkd1*<sup>RC/RC</sup> mice on either AL or FR. We observed that, compatible with the development of kidney injury in the ADPKD animal model, *Ngal* was induced more than 40-fold in *Pkd1*<sup>RC/RC</sup> AL mice compared with WT controls (Figure 1H). This induction was completely abrogated by FR treatment (Figure 1H). These data together clearly demonstrate that FR can decrease not only the development of cysts, but also the development of inflammation, fibrosis, and kidney injury in an animal model of ADPKD. We also analyzed markers

of cellular stress, such as the cell cycle regulator *p16*. Interestingly, *p16* was dramatically increased in *Pkd1*<sup>RC/RC</sup> kidneys compared with WT controls; this increased expression was completely abrogated by FR (Figure 2A). Recently, expression of *p16* has been implicated in the development of a cellular phenotype characterized by a proinflammatory secretory profile, that may in turn play a key role in recruitment of macrophages and other inflammatory cells in the setting of tissue injury.<sup>17</sup> It is possible that *p16* activation in polycystic kidney disease (PKD) may play a significant role in its pathogenesis, and that its prevention by FR may have significant salutary effects. However, in the present study *p16* was solely used as a marker of response to cellular injury.

Other important mechanisms of cyst formation and kidney injury in ADPKD are cellular proliferation and apoptosis. In fact, cellular proliferation is considered a major factor leading to cyst formation in ADPKD.<sup>1,2</sup> In addition, increased apoptosis has been shown to play a role in cystic kidney disease-induced injury.<sup>18</sup> In this regard, we evaluated the effect of FR on expression of proliferating cell nuclear antigen (PCNA) as a marker of cellular proliferation, and activation of caspase-3 as a marker of apoptosis. PCNA levels and caspase-3 activation (assessed by relative abundance of the 17 kDa cleavage fragment) were both significantly decreased by FR in the kidneys of *Pkd1*<sup>RC/RC</sup> mice (Figure 2, B and C), indicating that suppression of cellular proliferation and apoptosis may play a key role in the effect of FR on ADPKD. To further evaluate the role of FR in cellular processes implicated on the pathogenesis of ADPKD, we determined the effect of FR on the autophagy marker LC3 (Supplemental Figure 1).<sup>4</sup> We observed no



**Figure 3.** FR modulates metabolic changes in ADPKD. (A) Comparison of serum glucose levels in WT AL mice ( $n=7$ ) and  $Pkd1^{RC/RC}$  mice fed AL or 40% FR diet for 6 months ( $n=11-13$  per group). (B) RT-PCR data showing the renal expression of glycolytic enzymes ( $n=4-6$  per group).  $*P<0.05$ ;  $**P<0.01$ , compared with  $Pkd1^{RC/RC}$  AL group. (C) Glucose needs of WT and  $Pkd1^{del2/del2}$  MEFs are not different for cell proliferation. Cells were cultured in the presence of various doses of glucose and glutamine, as indicated, for 72 hours. Cell density was determined by counting and expressed relative to the high glucose control for each cell type ( $n=4$  per group). (D) Lactate production is similar in WT and  $Pkd1^{del2/del2}$  MEFs. Aliquots of media were taken and assayed at the indicated time points of exposure to cells and assayed for lactate release. Data are normalized to cell counts. (E) Quantification of apoptosis by annexin V assay in WT and  $Pkd1^{del2/del2}$  cells exposed to varying glucose concentrations.  $*P<0.05$ ;  $***P<0.05$ , compared with high glucose control. *In vitro* studies were repeated at least four times. Gln, glutamine; NS, nonsignificant.

significant changes in LC3-II, indicating that FR may not change autophagy in ADPKD.

An increase in the cyclic-AMP (cAMP) pathway has been implicated in the pathogenesis of ADPKD, but here we found that FR treatment of  $Pkd1^{RC/RC}$  mice did not decrease levels of cAMP (Supplemental Figure 2). In contrast, we observed a trend toward increased levels, indicating that the mechanism by which FR protects against PKD is unlikely to be mediated by suppression of cAMP-mediated fluid secretion.

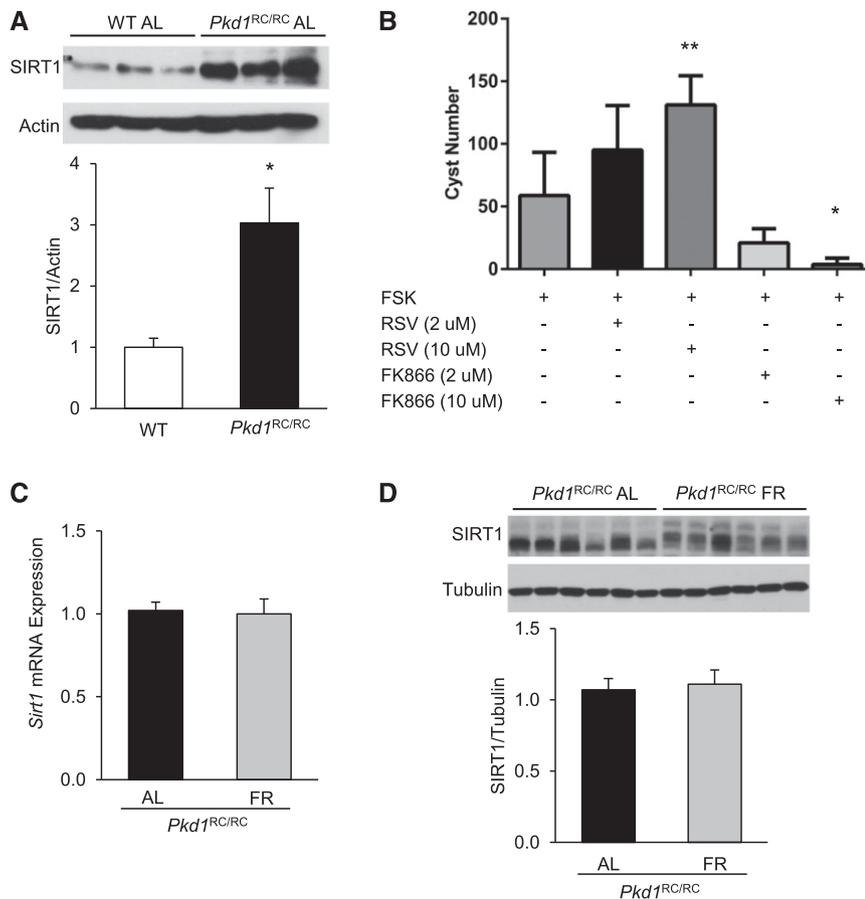
**FR Prevents Metabolic Adaptations in ADPKD**

Recently it has been proposed that, akin to cancer cells, PKD cells develop metabolic adaptations compatible with an increase in glycolytic activity.<sup>4</sup> Furthermore, it has been proposed that  $PKD1$  null cells are extremely sensitive to low glucose, and that short-term inhibition of glucose metabolism leads to apoptosis and slowing of cystic disease.<sup>4</sup> Here, we investigated whether FR decreases glucose to levels that could potentially cause glucose deprivation and cell death in cases of ADPKD. Firstly, although FR decreased serum glucose levels, it did not produce any evidence of severe hypoglycemia in these mice (Figure 3A, Supplemental Table 2). Serum glucose levels in FR-treated  $Pkd1^{RC/RC}$  mice were not significantly different from those in AL WT mice or AL  $Pkd1^{RC/RC}$  mice. Furthermore, levels of some markers of glucose transport to cells such as  $Glut4$ , and of gluconeogenesis such as phosphoenolpyruvate carboxykinase ( $Pck1$ ), were increased by FR (Figure 3B). These findings indicate that glucose availability to cells is unlikely to be decreased to deleterious levels in the setting of FR. In addition, untargeted global metabolomic analysis indicated no significant differences in kidney tissue glucose or lactate content between WT, AL  $Pkd1^{RC/RC}$ , and FR-treated  $Pkd1^{RC/RC}$  mice (data not shown).

We further evaluated the glucose sensitivity of several cellular models of ADPKD. In contrast with recently published data,<sup>4,19</sup> we observed that mouse embryonic fibroblasts (MEFs) derived from at least two different mouse models of ADPKD and one human cell line derived from a patient with ADPKD, were not more sensitive to changes in glucose availability and did not have higher lactate production than WT cells (Figure 3, C–E, Supplemental Figures 3–6). Glutamine needs were also not significantly different between

ADPKD cells and WT cells (Figure 3C, Supplemental Figures 3C, 5, and 6).

The effect of glucose deprivation on apoptosis was also evaluated in these cells. While the percentage of apoptotic cells observed in  $Pkd1$  MEFs and human  $PKD1$  null cyst epithelial lining cells was slightly higher than the respective WT controls, no changes in apoptosis were observed in response to 48 hours of glucose starvation, as assessed by annexin V and terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) (Figure 3E,



**Figure 4.** FR does not affect SIRT1 levels in *Pkd1<sup>RC/RC</sup>* mice. (A) Western blot analysis of SIRT1 in WT AL and *Pkd1<sup>RC/RC</sup>* AL mice (5-months-old,  $n=3$ ), and quantitative analysis of bands by densitometry.  $*P<0.05$ . (B) SIRT1 regulation of cyst formation in the MDCK cystogenic assay. Treatment with resveratrol (RSV), a SIRT1 activator, increased forskolin (FSK)-stimulated cyst formation, while FK866, a Nampt inhibitor, decreased cyst formation.  $*P<0.05$ ;  $**P<0.01$ , compared with FSK alone. (C) RT-PCR analysis of *Sirt1* expression in kidneys of *Pkd1<sup>RC/RC</sup>* mice fed AL or 40% FR diet for 6 months. (D) Western blot of SIRT1 protein levels in *Pkd1<sup>RC/RC</sup>* AL and FR kidneys; graph shows densitometry. Data for all are means  $\pm$  SEM.

Supplemental Figures 5 and 6). In summary, based on both these *in vitro* experiments and also on *in vivo* observations that demonstrated no significant level of systemic or tissue hypoglycemia in FR animals, it appears unlikely that the decrease in cystic disease caused by FR is mediated by metabolic collapse-induced cell death due to glucose starvation.

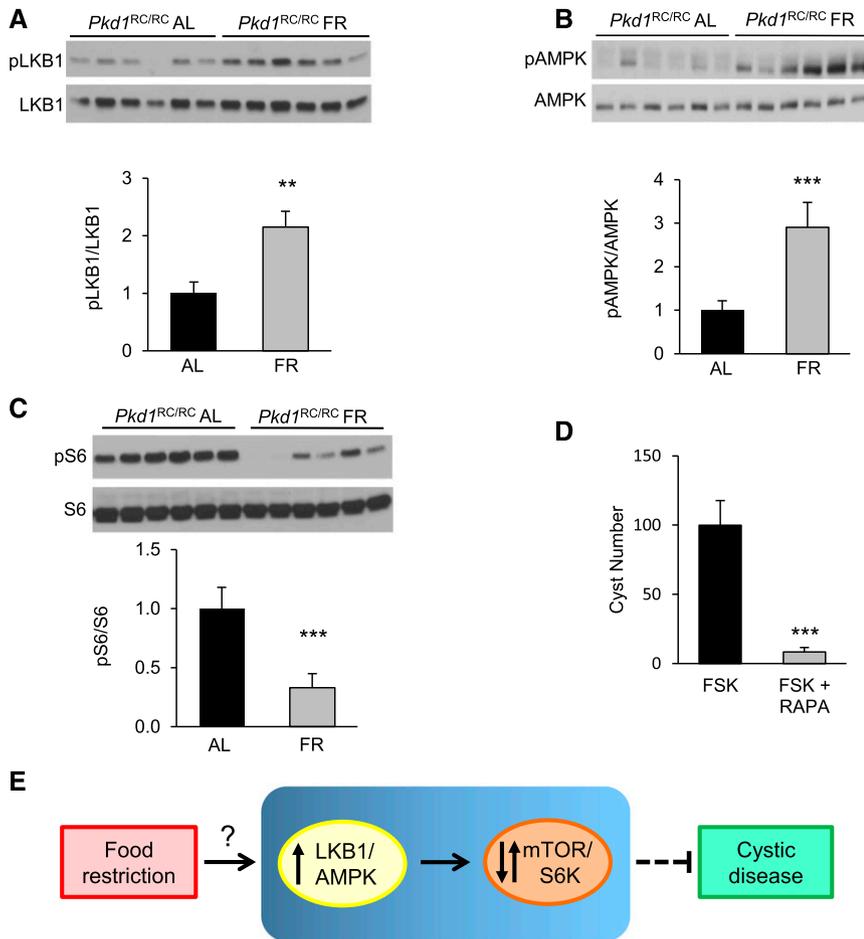
Although FR does not appear to decrease glucose availability, it could still prevent some of the metabolic adaptations observed in ADPKD. In fact, we observed that compared with WT mice, *Pkd1<sup>RC/RC</sup>* animals have important metabolic adaptations in their glycolytic pathway. In particular, ADPKD leads to an increase in the expression of hexokinase 2 (*Hk2*, Figure 3B). This metabolic change was completely reversed by FR. Whether the expression of *Hk2* is implicated in cystic disease or is a consequence of tissue injury is not known at this point. However, it supports the possibility that FR can promote metabolic reprogramming in ADPKD kidneys.

## FR Regulates Cellular Signaling Pathways in ADPKD that Provide Nutrient Sensing

To determine the signaling pathways involved in the protective effects of FR in ADPKD, we further investigated the effect of FR on cellular metabolic sensors in the *Pkd1<sup>RC/RC</sup>* mouse model and *in vitro*. Metabolic sensors have been shown to play an important role in both adaptation to FR and in ADPKD pathogenesis.<sup>3–6</sup> We first observed that, in *Pkd1<sup>RC/RC</sup>* mouse kidneys, expression of SIRT1 was increased several fold when compared with WT mouse kidney (Figure 4A). Additionally, treatment of Madin-Darby canine kidney (MDCK) cells in the cystogenic assay with the putative SIRT1 activator resveratrol led to an increase in cyst formation (Figure 4B). In fact, SIRT1 is a NAD-dependent deacetylase that has been implicated in cystogenesis in ADPKD models.<sup>5</sup> Inhibition of the NAD synthetic enzyme Nampt with FK866 in the MDCK cells led to a decrease in both the number and size of cysts (Figure 4B and data not shown). These data support the notion that SIRT1 is necessary for cyst formation. It has been previously proposed that FR may increase SIRT1 activity in tissues.<sup>11,12</sup> We observed that FR does not significantly change either *Sirt1* mRNA or SIRT1 protein levels in the kidneys of *Pkd1<sup>RC/RC</sup>* mice (Figure 4, C and D). These data together indicate that FR is unlikely to inhibit the development of ADPKD by modulation of the SIRT1 pathway. In this regard, we conclude that SIRT1 activation is involved in cyst formation,

and that the effect of FR in ADPKD cannot be explained by suppression of the SIRT1 pathway.

Two other metabolic sensors involved in the organismal response to FR have also been implicated in the pathogenesis of ADPKD, namely the mTOR-S6K and AMPK pathways. In ADPKD it has been shown that the mTOR pathway is activated, and that inhibition of mTOR with rapamycin decreases the development of ADPKD in mice.<sup>20</sup> It has been proposed that the AMPK pathway may also be involved in PKD pathogenesis.<sup>3</sup> In particular, it has been proposed that activation of AMPK can prevent cyst formation.<sup>3</sup> Compatible with other models of ADPKD, in the *Pkd1<sup>RC/RC</sup>* mouse model we found that the mTOR pathway is activated and the AMPK pathway is inhibited, as compared with WT controls (Supplemental Figure 7). These data indicate that the *Pkd1<sup>RC/RC</sup>* mouse model resembles other models of ADPKD in terms of its effect on metabolic sensors.



**Figure 5.** FR activates the LKB1-AMPK pathway and inhibits the mTOR pathway. (A–C) Comparison of phosphorylated (p) and total LKB1 (A), AMPK (B), and S6 (C) by immunoblotting in kidney extracts of *Pkd1<sup>RC/RC</sup>* mice on AL or 40% FR for 6 months. Each gel lane is obtained from an independent mouse. Quantitative analysis of bands by densitometry for p-LKB1/LKB1 (A), p-AMPK/AMPK (B), and p-S6/S6 (C). (D) Effect of rapamycin (Rapa), an mTOR inhibitor, on forskolin (FSK)-stimulated cyst formation in the MDCK cystogenic assay. Data are means±SEM. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001. (E) Tentative hypothesis for the mechanism of prevention of cystic disease by FR.

We next determined the effect of FR on the regulation of these metabolic sensors in the *Pkd1<sup>RC/RC</sup>* mice. We observed that 40% FR for 6 months positively regulates the LKB1-AMPK pathway, leading to phosphorylation and activation of these proteins (Figure 5, A and B). We also observed that FR decreases phosphorylation of S6, one of the key downstream substrates of the mTOR-S6K pathway (Figure 5C). To determine if activation of the AMPK pathway and inhibition of the mTOR-S6K pathway were early events during the course of FR, we studied the effect of short-term FR on the phosphorylation status of AMPK and S6. In fact, we observed early activation of AMPK when FR was instituted in *Pkd1<sup>RC/RC</sup>* mice for only 7 days, before any appreciable change in kidney size was observed, suggesting that changes in these pathways may precede the amelioration of cystic disease, and may be mechanistically involved in the protective effect of FR in ADPKD (Supplemental Figure 8).

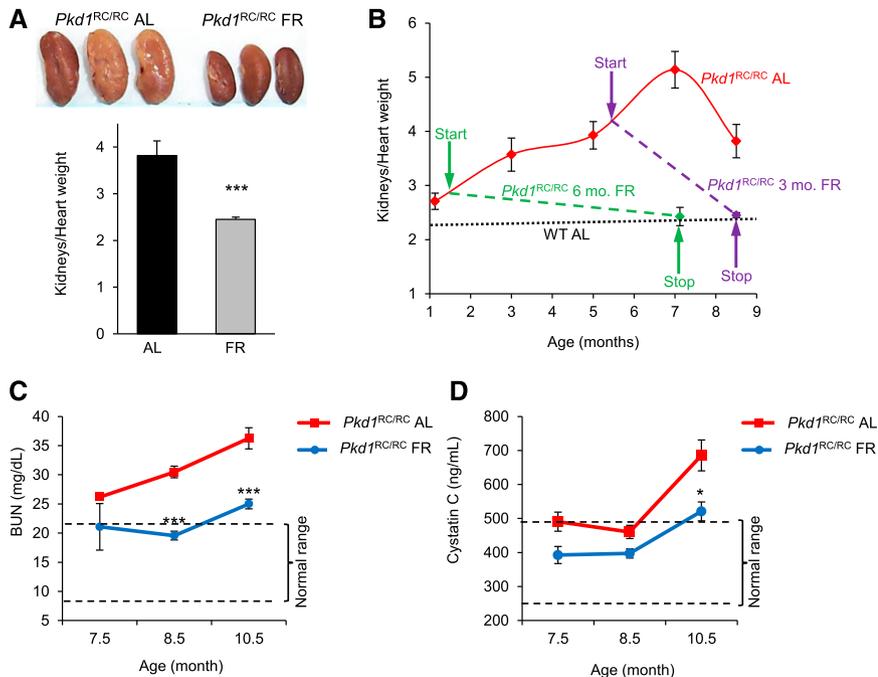
In addition, we further confirmed that inhibition of the mTOR pathway with rapamycin and activation of the AMPK pathway with metformin lead to decreased cystogenesis in MDCK and metanephric cultures (Figure 5D and data not shown). Although we do not have definitive experimental proof to support a mechanistic role of metabolic sensors in the amelioration of ADPKD by FR, we believe that our data indicate a likely modulation of the AMPK and mTOR pathways by FR in this model and may indicate that these factors are, at least in part, linked to the amelioration of cystic disease (Figure 5E).

Advances in the study of FR have determined that suppression of IGF-1 is central for the beneficial effects of FR.<sup>21</sup> Here we further explored the effect of FR on components of the IGF-1 pathway in the *Pkd1<sup>RC/RC</sup>* model. As shown in Supplemental Figure 9, we observed a very significant decrease in both the circulating levels and renal mRNA expression of IGF-1 in FR-treated *Pkd1<sup>RC/RC</sup>* mice. In contrast, we did not observe any effect of FR on expression of the *Igf-1r*, insulin levels, or AKT phosphorylation (Supplemental Figure 9). These data indicate that suppression of IGF-1 may also play a role in the inhibition of cystic disease in ADPKD.

### FR Prevents and Reverses Cystic Disease in *Pkd1<sup>RC/RC</sup>* Mice and Ameliorates Kidney Function

To determine if FR could not only prevent the development of the full extent of the cystic disease, but also reverse cystic disease, experiments were conducted in which FR was initiated after the cystic disease was already well established in the *Pkd1<sup>RC/RC</sup>* mouse model (Figure 6). In these experiments we observed that FR could decrease cystic burden when instituted in 5.5-month-old animals for 3 months (Figure 6, A and B). In addition, we also observed that FR, when started late in the course of the disease, could cause obvious beneficial effects in kidney function.

Here we determined the effect of FR in mice that already had developed enough cystic disease that would cause changes in markers of kidney function, such as BUN and cystatin C. We investigated the effect of FR in mice between the ages of 7.5 months and 10.5 months, when kidney dysfunction starts to develop in *Pkd1<sup>RC/RC</sup>* mice. When FR was started in mice at 9 months of age and continued for 1.5 months, we found that kidney function was improved as determined by both BUN and plasma cystatin C levels (Figure 6, C and D, Supplemental Figure 10).



**Figure 6.** The protective effect of food restriction (FR) in ADPKD is observed even when initiated later in disease course in *Pkd1<sup>RC/RC</sup>* mice. (A) *Pkd1<sup>RC/RC</sup>* mice (5.5-months-old) were fed a standard diet AL or 40% FR for 3 months. Upper panel, images of representative gross morphology for *Pkd1<sup>RC/RC</sup>* AL and FR kidneys. Lower panel, graph showing total kidney weight normalized to heart weight for AL and FR-treated animals, means $\pm$ SEM of seven to eight mice per group. \*\*\* $P$ <0.001. (B) Role of FR in prevention and regression of ADPKD. Graph summarizes the kidneys/heart weight data for *Pkd1<sup>RC/RC</sup>* mice on 40% FR for 6 months (green) and 3 months (purple) in relation to that of *Pkd1<sup>RC/RC</sup>* AL (red) and WT AL (black) mice. Red trend line demonstrates the normal progression of cystic disease in *Pkd1<sup>RC/RC</sup>* mice with no intervention. Black dotted line represents the trend observed in WT AL mice; ratios were consistently 2.3–2.5 at 3–10 months of age. The green and purple arrows indicate the ages at which FR was initiated and concluded in the respective studies; the green and purple dotted lines are extrapolations using *Pkd1<sup>RC/RC</sup>* AL data to estimate the kidney size at the beginning of the two studies. (C–D) Renal function in *Pkd1<sup>RC/RC</sup>* mice on AL or FR diet was determined by plasma BUN and cystatin C. Data were compared among different ages of mice. Means $\pm$ SEM of seven to eight mice per group. \* $P$ <0.05; \*\*\* $P$ <0.001. *Pkd1<sup>RC/RC</sup>* AL versus FR.

### FR has a Dose-Dependent Effect in ADPKD

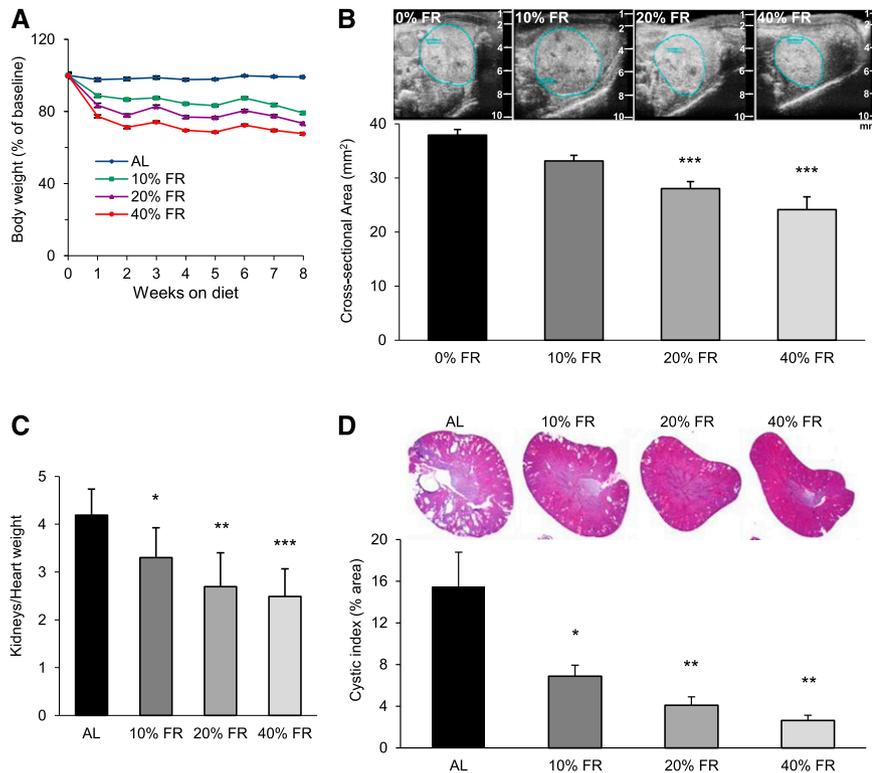
We further investigate the dosage effects of FR in ADPKD. We introduced 10%–40% FR for 2 months in *Pkd1<sup>RC/RC</sup>* mice and found dose-dependent decreases in body weight, kidneys to heart weight ratio, and cystogenesis (Figure 7, A–D). Surprisingly, even mild (10%) FR reduced cyst index by approximately 50% (Figure 7D), and 20% FR for 2 months gave a similar cyst reduction to those mice with 40% FR for 6 months (Figure 1, C and D, Figure 7, C and D). This suggests that even a small restriction of food intake decreases cystogenesis in ADPKD. Finally, to show the significance more widely in ADPKD we have confirmed the beneficial effects of FR in a second mouse model of PKD, *Pkd2<sup>WS25/-</sup>* (Figure 8).

### DISCUSSION

The precise mechanism by which kidney injury occurs in PKD has not been completely elucidated.<sup>1,2</sup> Development of cysts and kidney injury in PKD is mediated by at least two mechanisms, namely fluid secretion and cellular proliferation.<sup>1,2</sup> Cyst expansion in PKD involves progressive fluid accumulation, which is believed to require cAMP-mediated activation of chloride transport by the cystic fibrosis transmembrane conductance regulator protein, CFTR.<sup>22</sup> The effect of FR appears not to be mediated by changes in cAMP. In addition, it appears that the effect of FR is also not mediated by suppression of the SIRT1 pathway, or a decrease in glucose availability to the kidney. Very interestingly, we also describe for the first time that *Hk2* is upregulated in ADPKD, and that FR causes metabolic reprogramming. Whether the expression of *Hk2* is causative in cystic disease or a consequence of tissue injury is not known at this point. Furthermore, it will be imperative to determine which cells in the kidney express HK2 to further determine its role in ADPKD. In any case, our data support the possibility that FR can promote reprogramming of metabolism in ADPKD. Whether the changes in HK2 expression are the cause for the inhibition of the development of cystic disease, or a marker of amelioration of kidney injury is not known at the present time. Why our data differ from previously published data<sup>4–19</sup> on the role of aerobic glycolysis in ADPKD is not known. However, when comparing our model to previous studies, it could be due to the use of different animals, cellular models, culturing conditions, or the type of mutation carried by

the cells. In addition, one possibility is that HK2 expression and changes in glucose metabolism are not mediated by an epithelial cell autonomous process, and may be dependent on the interaction between invading inflammatory cells and kidney cells during the process of kidney injury.

Our data support the hypothesis that the effect of FR is mediated, at least in part, by a mechanism that involves the mTOR and the LKB1-AMPK pathways. It is also possible that since phosphorylated S6 was not significantly decreased after 1 week of FR, that the decrease in the mTOR-S6 pathway seen at the later time may be caused by a decrease in cystic disease itself. These possibilities will need to be further experimentally tested using appropriate animal models of ADPKD with genetically modified components of the AMPK and mTOR pathways.



**Figure 7.** The protective effect of FR is dose-dependent. Five-month-old *Pkd1<sup>RC/RC</sup>* mice were placed on AL, 10%, 20%, or 40% FR diet for 2 months ( $n=5$  per group). (A) Graph of body weights, showing decline in all FR groups over the first 2 weeks followed by stabilization. (B) Ultrasonography performed after 2 months on FR. Upper panel shows representative images. Lower panel shows the mean cross-sectional kidney area, derived from ultrasound imaging of all mice in each group ( $n=5$  per group). (C) Kidneys/heart weight ratio. (D) Representative histology images (hematoxylin and eosin stain) and quantitative analysis of cyst index are shown. Data are means  $\pm$  SEM. \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ , compared with *Pkd1<sup>RC/RC</sup>* AL group.

These animal models are being developed in our laboratory and will be the subject of future experiments. However, our data support a potential role for the regulation of metabolic sensors induced by FR in ADPKD.

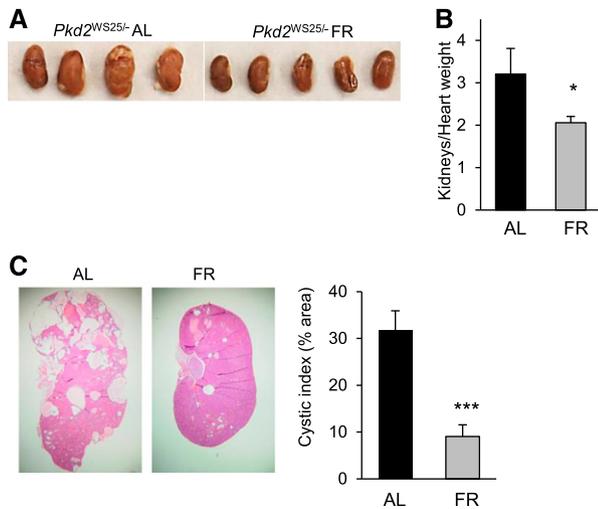
Very importantly, our data have significant translational potential and indicate a potential role for long-term dietary interventions in patients at risk for ADPKD. FR was first described by McCay and Crowell in 1934.<sup>23</sup> These investigators reported that laboratory rats fed a severely reduced calorie diet while maintaining micronutrient levels resulted in life spans of up to twice as long as otherwise expected. FR is well tolerated in laboratory animals and has salutary metabolic effects in several species, including mice, rats, monkeys, and humans.<sup>7,10,12</sup> Furthermore, FR has been shown to decrease oxidative stress and age-related renal dysfunction, and to prevent the development of ESRD in the rat remnant kidney model.<sup>10,13,24</sup>

Two main health-span studies have been published in FR in nonhuman primates (rhesus monkeys). In 2012, the group from the National Institute on Aging led by Dr. deCabo

published results indicating that FR confers health benefits in these animals.<sup>25</sup> A second study published in 2014 by the University of Wisconsin found that FR primates were only 36.4% as likely to die from age-related causes when compared with control animals, and had only 56.2% the rate of death from any cause compared with controls.<sup>26</sup> Furthermore, FR leads to a better metabolic profile in both animal models and humans.<sup>26,27</sup> For example, data from the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE) trials show that 20% FR for 12 months in overweight individuals resulted in a significant reduction in visceral fat mass, LDL-cholesterol, triglycerides, and C-reactive protein, and improved insulin sensitivity.<sup>27</sup> Long-term FR in humans results in a reduction of several metabolic and hormonal factors that have been associated with increased risk of some of the most common types of cancer in developed countries.<sup>13,15</sup> Individuals practicing FR without malnutrition have lower levels of total and abdominal fat, circulating insulin, and inflammatory cytokines.<sup>11,13</sup>

Unfortunately, long-term FR is unlikely to be well tolerated by most humans, and extremely difficult for patients to adhere to. Furthermore, extremes of FR may have deleterious effects, such as anemia, lower extremity edema, muscle wasting, weakness, neurologic deficits, dizziness, irritability, lethargy, decreased libido, and depression.<sup>11,13</sup> In this regard, further evaluation is needed to determine the proper timing and degree of FR that may provide beneficial effects in humans with ADPKD. Interestingly, we observed that even mild FR can significantly slow cyst progression in ADPKD.

The development of alternative modes of dietary restriction and the development of possible small molecule FR mimetics are of major pharmacologic interest. In fact, recent advances in the study of FR have determined that suppression of IGF-1 is central for the beneficial effects of FR.<sup>21,28</sup> Although IGF-1 has been proposed to be secreted in cystic fluid of ADPKD patients and may increase proliferation of PKD1 cystic cells, there is little information on the role of IGF-1 in cystogenesis in ADPKD.<sup>29,30</sup> In our model, we observed that FR leads to suppression of circulating IGF-1 levels and expression of IGF-1 in the kidney (Supplemental Figure 9). It is possible that FR may prevent ADPKD via suppression of the IGF-1/IGF-1R pathway. However, the precise mechanistic role of IGF-1 in ADPKD and its implications for the effect of FR remain to be studied.



**Figure 8.** Food restriction (FR) inhibits cystogenesis in *Pkd2*<sup>WS25/-</sup> mice. 8-week-old *Pkd2*<sup>WS25/-</sup> mice were fed AL or 40% FR for 2 months. (A) Images of representative gross morphology for *Pkd2*<sup>WS25/-</sup> AL and FR kidneys. (B) Graph showing total kidney weight normalized to heart weight for AL and FR-treated animals. (C) Hematoxylin and eosin images showing representative renal histology in AL and FR-treated animals. Graph showing quantitative analysis of cystic index. Data are means  $\pm$  SEM of seven to eight mice per group. \* $P < 0.05$ ; \*\*\* $P < 0.001$ , compared with *Pkd2*<sup>WS25/-</sup> AL.

It has also been shown that restriction of a single amino acid, namely methionine restriction (MR), can decrease IGF-1 levels and mimic several effects of FR.<sup>31</sup> In fact, MR extends life-span and metabolic health in several *in vitro* and animal models.<sup>28,32</sup> Furthermore, a form of MR and IGF-1 suppression can be achieved in humans with the so-called vegan diet.<sup>31</sup> Interestingly, there are studies implicating protein load as an important factor in the development of ADPKD in animal models; however, studies on protein restriction in patients with renal failure and advanced ADPKD failed to show benefits, and indeed demonstrated a trend for increased morbidity with low protein intake.<sup>33,34</sup> In this regard, it is important for future studies to determine the specific role of calories and macronutrients in the effect of nutritional manipulations described in here. In addition, it is possible that the timing of the dietary manipulations in ADPKD in humans may be very crucial, where dietary restriction may even have opposite effects before and after the disease is established. Furthermore, it is also possible that specific dietary manipulations such as FR may have a role in potentiating the effects of pharmacologic suppressors of the mTOR pathway like rapamycin.

## CONCISE METHODS

### Reagents, Antibodies, and Inhibitors

Except when specified, all reagents and chemicals were purchased from Sigma-Aldrich. Antibodies to cleaved caspase-3 (#9664), S6

(#2217), p-S6 (#4858), LKB1 (#3047), p-LKB1 (#3482), AMPK (#2532), p-AMPK (#2535), SirT1 (#2028), and LC3A/B (#4108) were purchased from Cell Signaling. PCNA (#sc-7907) antibody was purchased from Santa Cruz Biotechnology.

### Food Restriction Studies

The *Pkd1*<sup>RC/RC</sup> (*PKD1* p.R3277C) and *Pkd2*<sup>WS25/-</sup> mouse models of ADPKD were provided by Dr. Peter C. Harris.<sup>16</sup> Five animals per cage were housed in standard cages in a room maintained at constant temperature and humidity, and 12 hour light/dark cycles. FR was performed as described previously by others.<sup>35</sup> The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Mayo Clinic (Protocol no. A52112), and studies were conducted in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. At 1.5, 3, or 5 months of age, male and female mice were randomized in two groups: untreated control (AL) and FR.<sup>35</sup> Briefly, mice were placed in individual housing one week before experiments for acclimation. Known weight of food was provided to each mouse, and food intake in each mouse was determined every 24 hours for one week. Then, percentage of food restriction was calculated that was 10%–40% less than AL. Food was presented to pair-fed animals in the afternoon, about 2 hours before beginning of the dark cycle. Food intake for AL mice was monitored throughout the study, and the amount of food given to FR mice was adjusted accordingly. At the end of the study mice were killed, blood was taken for biochemical analysis, and organs were weighed. Portions of tissue were placed in formalin and processed for histologic studies, or snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for gene expression analysis.

### Ultrasound Imaging

Kidney size and extent of cyst formation in *Pkd1*<sup>RC/RC</sup> mice on AL and FR were monitored by ultrasonography using a VisualSonics Vevo 2100 Imaging System, as described previously.<sup>16</sup>

### Histologic Analysis

Formalin-fixed tissues were paraffin-embedded, cut into 5- $\mu\text{m}$  thick cross-sections, and stained by the Mayo Histology Core Laboratory with hematoxylin and eosin for analysis of cystic index, or with Sirius red for analysis of fibrosis. Cystic index (cyst area proportional to cross-sectional kidney area) was measured using ImageJ software. A cyst was defined as a dilated tubule with an area greater than 500  $\mu\text{m}^2$ . Analysis of renal fibrosis (proportion of positive staining area per field) was performed using Metamorph software (Universal Imaging).

### Western Blotting

Mouse kidney tissue and cultured cells were lysed in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) supplemented with 5 mM NaF, 50 mM 2-glycerophosphate, and a protease inhibitor cocktail (Roche Diagnostics). After 20 minutes of lysis, protein lysates were cleared by centrifugation at 12,000 rpm at  $4^{\circ}\text{C}$  for 10 minutes. The resulting supernatants were quantified for protein using a BioRad protein assay. The proteins were fractionated by SDS-PAGE, electro-transferred to Immobilon-P membranes

(EMD Millipore), blotted with the indicated primary antibodies followed by appropriate horseradish peroxidase-conjugated secondary antibodies, and detected using SuperSignal West Pico or Femto Chemiluminescence Substrate (Thermo Fisher Scientific). Membranes were stripped and probed with tubulin or  $\beta$ -actin antibody to control for equal gel loading and transfer. Films were scanned and densitometry was performed using ImageJ software.

### mRNA Isolation and PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). Synthesis of cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen). Commercially available TaqMan gene expression probes were obtained from Applied Biosystems (Supplemental Table 3). Quantitative RT-PCR was performed in triplicates using the TaqMan Gene Expression Master Mix (Applied Biosystems), according to the manufacturer's instructions, on a BioRad CFX384 thermal cycler. The relative mRNA abundance of target genes was calculated using the  $2^{-\text{ddCt}}$  method, with *Gapdh* as the internal reference.

### BUN and Cystatin C Measurements

Following death, blood was collected from the posterior vena cava into heparinized tubes, and plasma was separated from whole blood by centrifugation. The plasma urea concentration was determined using a QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA). BUN levels (mg/dL) were calculated by dividing the urea by 2.14. Plasma cystatin C levels were measured using a Mouse/Rat Cystatin C Quantikine ELISA Kit (R&D systems, Minneapolis, MN), according to the manufacturer's instructions.

### Cell Culture

WT MEFs, *Pkd1*<sup>del2/del2</sup> MEFs, *Pkd1*<sup>RC/RC</sup> MEFs, human renal cortical tubular epithelial cells, and cystic epithelial cells from ADPKD patients ( $n=912$ ) were provided by Dr. Harris. MDCK cells and MEFs were maintained in DMEM/F12 (Life Technologies) supplemented with 10% FBS and penicillin and streptomycin (Invitrogen). Cell proliferation was determined by seeding cells in six-well plates ( $3 \times 10^4$  per well) and counting at 24-hour intervals.

### Lactate Production

Cells were seeded in normal growth medium and allowed to attach for 24 hours. The medium was replaced by phenol red-free DMEM (4.5 g/l glucose) with 1% FBS, and aliquots of conditioned medium were collected at time intervals to evaluate lactate release. The assay was performed using hydrazine/glycine buffer (pH 9.2), 5 mg/ml  $\beta$ -NAD<sup>+</sup>, and 15 units/ml lactate dehydrogenase. NADH formation was monitored at 340 nm in a plate reader. A standard curve was generated and the amount of lactate per sample was normalized to the amount of cells.

### Annexin V/PI Assay

Cells were incubated for 48 hours in medium (DMEM and 10% FBS) containing various concentrations of glucose and glutamine. Staining was performed using Annexin V-FITC Early Apoptosis Detection Kit (Cell Signaling Technology, Beverly, MA), according to the manufacturer's

instructions. Briefly, cells were washed twice (1,200 rpm for 5 minutes) with ice-cold PBS and resuspended at  $10^6$  cells/ml with annexin V binding buffer. Annexin V-FITC and propidium iodide solution were added to each cell suspension. Flow cytometric evaluation was conducted immediately on a BD FACSCanto system (BD Biosciences, CA).

### Cystogenesis Assay

Cystogenesis studies with MDCK cells were performed essentially as previously described.<sup>36</sup> Briefly, cells were suspended in collagen I solution, seeded in 24-well plates at 4,000 cells per well, and incubated at 37°C for 30–45 minutes for collagen to polymerize. Cells were then given medium (DMEM/F12 and 1% FBS) containing the cAMP agonist forskolin (10  $\mu$ M). Resveratrol (2 or 5  $\mu$ M) and FK866 (2 or 5 nM) were added 4 days after forskolin. Rapamycin (20 nM) and metformin (1 mM) were given once cells were plated. Cells were cultured for 14 days, with media change every other day. At the end of the study, the number of cysts in each well was counted.

### Statistical Analyses

Data are expressed as means  $\pm$  SEM. Results are representative of four or more independent experiments for *in vitro* studies. Comparisons were made by unpaired *t* test and ANOVA. Nonparametric tests were used as dictated by data distribution. Significance was set at  $P < 0.05$ .

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### DISCLOSURES

None.

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See related editorial, “Slowing Polycystic Kidney Disease by Fasting,” on pages 1268–1270.

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