Autosomal-dominant polycystic kidney disease (ADPKD) is caused by mutations in either PKD1 or PKD2 and is characterized by the development of multiple bilateral renal cysts that replace normal kidney tissue. Here, we used Pkd1 mutant mouse models to demonstrate that the nicotinamide adenine dinucleotide-dependent (NAD-dependent) protein deacetylase sirtuin 1 (SIRT1) is involved in the pathophysiology of ADPKD. SIRT1 was upregulated through c-Myc in embryonic and postnatal Pkd1-mutant mouse renal epithelial cells and tissues and could be induced by TNF-α, which is present in cyst fluid during cyst development. Double conditional knockouts of Pkd1 and Sirt1 demonstrated delayed renal cyst formation in postnatal mouse kidneys compared with mice with single conditional knockout of Pkd1. Furthermore, treatment with a pan-sirtuin inhibitor (nicotinamide) or a SIRT1-specific inhibitor (EX-527) delayed cyst growth in Pkd1 knockout mouse embryonic kidneys, Pkd1 conditional knockout postnatal kidneys, and Pkd1 hypomorph kidneys. Increased SIRT1 expression in Pkd1 mutant renal epithelial cells regulated cyst epithelial cell proliferation through deacetylation and phosphorylation of Rb and regulated cyst epithelial cell death through deacetylation of p53. This newly identified role of SIRT1 signaling in cystic renal epithelial cells provides the opportunity to develop unique therapeutic strategies for ADPKD.
**Figure 1**

*Pkd1*-mutant renal epithelial cells and tissues demonstrated increased expression of SIRT1. (A) qRT-PCR analysis of relative Sirt1 mRNA expression in WT MEK (WT), Pkd1-null MEK (Null), PH2, and PN24 cells. (B) Top: Western blot analysis of SIRT1 and c-MYC expression from whole cell lysates. Bottom: Relative SIRT1 expression, quantified from 3 independent immunoblots and standardized to actin. (C) Top: Western blot analysis of SIRT1 expression in mouse IMCD3 cells with Pkd1 knockdown by 2 different lentivirus-mediated Pkd1 shRNAs, VIRHD/P/siPkd13297 (siPKD13297) and pGIPZ-siPkd1, compared with that in the cells transduced with the respective control vectors, VIRHD/P/siLuc and pGIPZ-NS. Bottom: Relative Pkd1 knockdown efficiency, evaluated by qRT-PCR, indicated that Pkd1 expression was reduced by more than 90% and 70% in VIRHD/P/siPKD13297 and pGIPZ-siPkd1–transduced mouse IMCD3 cells, respectively, compared with that in control vector–transduced cells. (D) Top: Western blot analysis of SIRT1 expression in kidneys from WT and Pkd1<sup>fl/fl</sup> mice collected at P7, P14, P21, and P28. Bottom: Relative SIRT1 expression in the kidneys, standardized to tubulin. (E and F) qRT-PCR analysis of Sirt1 mRNA expression (E) and Western blot analysis of SIRT1 and c-MYC expression (F) in P7 kidneys from Pkd1<sup>+/+<sup>:Ksp-Cre (WT) and Pkd1<sup>fl/fl</sup>:Ksp-Cre (Flox) neonates. (G) Western blot analysis of SIRT1 expression in primary human ADPKD and NHK cells. **P < 0.01.

_Pkd1_-null cell line PN24 compared with the postnatal _Pkd1_-heterozygous cell line PH2 (Figure 1, A and B). Knockdown of _Pkd1_ with 2 different lentivirus-mediated shRNAs in mouse inner medullary collecting duct (IMCD3) cells also resulted in upregulation of SIRT1 relative to appropriate controls (Figure 1C). SIRT1 expression was also increased in kidneys from well-characterized hypomorphic homozygous _Pkd1<sup>fl/fl</sup>_ mice (15) compared with that in age-matched WT kidneys at P7, P14, P21, and P28 (Figure 1D). In addition, mRNA and protein expression of SIRT1 increased in P7 kidneys of _Pkd1<sup>fl/fl</sup>:Ksp-Cre_ mice, as analyzed by quantitative RT-PCR (qRT-PCR), Western blot, and immunohistochemistry (Figure 1, E and F, and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI64401DS1). Furthermore, SIRT1 expression was upregulated in primary human ADPKD cells and ADPKD kidneys compared with primary normal human kidney (NHK) cells and normal kidneys, respectively (Figure 1G and Supplemental Figure 1B). These results suggest that the increased expression of SIRT1 in renal epithelial cells is caused by loss or mutation of _Pkd1_.

_PC1_ affects SIRT1 expression in renal epithelial cells through _c-MYC_. It has been reported that in ADPKD, renal _c-MYC_ expression is elevated up to 15-fold (16). _c-MYC_ has been shown to regulate SIRT1 expression in human cancer (HeLa) cells (17). Thus, _c-MYC_ may regulate SIRT1 expression in renal epithelial cells. In support of this notion, we found that (a) _c-MYC_ expression was increased in _Pkd1_-null MEK cells, PN24 cells, and kidney tissues from _Pkd1<sup>fl/fl</sup>:Ksp-Cre_ mice (Figure 1, A and B); (b) overexpression of _c-MYC_ increased mRNA and protein levels of SIRT1 in WT MEK cells and PH2 cells (Figure 2, A and B); (c) knockdown of _c-MYC_ with siRNA decreased mRNA and protein levels of SIRT1 in _Pkd1<sup>fl/fl</sup>_ MEK cells and PN24 cells (Figure 2, C and D); and (d) _c-MYC_ bound to 2 potential _c-MYC_-binding sites (E-boxes E1 and E2; ref. 18) of the SIRT1 promoter, as determined by ChIP assay with anti–_c-MYC_ antibody (Figure 2E). These results suggested that loss of _PC1_ mechanistically altered SIRT1 expression in renal epithelial cells through _c-MYC_.

SIRT1 expression can be further induced by TNF-α in _Pkd1_-mutant renal epithelial cells. TNF-α, which is detected in cyst fluid and promotes cyst formation (19), has been found to induce SIRT1 expres-
sion in vascular smooth muscle cells through the NF-κB p65/RelA subunit (20). We found that TNF-α induced mRNA and protein expression of SIRT1 in Pkd1-null MEK cells and PN24 cells (Figure 2, F and G). TNF-α also slightly induced SIRT1 expression in WT MEK cells, but had no effect in PH2 or mouse IMCD3 cells (Supplemental Figure 2). However, the NF-κB inhibitor SN50 efficiently blocked TNF-α–induced SIRT1 upregulation in Pkd1-null MEK cells and PN24 cells (Figure 2H), which suggests that TNF-α induces SIRT1 expression by activating the NF-κB pathway. Although it is unclear whether the cyst fluid TNF-α is initially secreted by immune cells or by cyst lining epithelial cells, these results suggest that the presence of TNF-α in cyst fluid during cyst development may serve as a secondary stimulus to further increase expression of SIRT1 in cyst lining epithelial cells in vivo.

Sirt1 and Pkd1 double conditional knockout delayed renal cyst formation. In order to explore the in vivo function of SIRT1 in a Pkd1-knockout mouse model, we crossed Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre female mice with Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre male mice, which have a kidney-specific Ksp-cadherin driving Cre expression. Cyst formation was significantly delayed in the absence of SIRT1 in Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre mice at P7 compared with that in age-matched Pkd1flox/flox:Sirt1+/+:Ksp-Cre and Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre mice (n = 10 per group; Figure 3, A–E). Kidney weight/body weight (KW/BW) ratios from Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre mice were dramatically reduced compared with Pkd1flox/flox:Sirt1+/+:Ksp-Cre mice (Figure 3F). In addition, blood urea nitrogen (BUN) levels were also significantly reduced in Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre mice compared with Pkd1flox/flox:Sirt1+/+:Ksp-Cre mice (Figure 3G), which indicates that renal function was normalized in Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre mice. At the same time, SIRT1 expression was not detected in cyst lining epithelial cells in kidneys from Pkd1 and Sirt1 double–conditional knockout mice, as analyzed by immunohistochemistry (Supplemental Figure 3). Proliferating cell nuclear antigen (PCNA) staining was used to determine the proliferation of cyst lining epithelial cells, which was significantly decreased in Pkd1 and Sirt1 double–conditional knockout versus Pkd1 single–conditional knockout (i.e., Sirt1+/+) renal epithelia (Figure 3H). Furthermore, we found that Pkd1 and Sirt1 double-
conditional knockout mice lived to a mean age of 21.9 ± 3.6 days, while Pkd1flox/flox:Sirt1+/+:Ksp-Cre mice died of polycystic kidney disease at 14.1 ± 0.9 days (P < 0.01; Figure 3I). Our in vivo data suggested that SIRT1 is involved in regulating renal cyst formation in Pkd1-knockout mice.

A pan-sirtuin inhibitor or a specific SIRT1 inhibitor delays cyst growth in Pkd1-mutant kidneys. To test whether inhibiting the activity of SIRT1 would suppress cyst formation in Pkd1–/– embryos, we injected nicotinamide into pregnant Pkd1+/– female mice from 7.5 dpc after mating with Pkd1+/– males, and analyzed MEKs at 15.5 dpc. We found that in all E15.5 Pkd1–/– embryos from nicotinamide-injected mothers, renal cyst formation was drastically reduced compared with kidneys of Pkd1–/– embryos from control DMSO-injected mothers (n = 10 per treatment group; P < 0.01; Figure 4, A–E). Furthermore, nicotinamide induced tubular epithelial cell apoptosis in kidneys from Pkd1–/– embryos, while apoptosis was rare and negligible in kidneys from DMSO-treated Pkd1–/– E15.5 embryos (Figure 4F). We also evaluated the effect of nicotinamide on renal cyst formation at 18.5 dpc; indeed, renal cyst growth was dramatically reduced in kidneys of E18.5 Pkd1–/– embryos from nicotinamide- versus DMSO-injected pregnant females (n = 10 per treatment group; Figure 4, G and H). Kidney weight was also significantly decreased in Pkd1–/– embryos from nicotinamide-injected pregnant females (Figure 4I). Again, tubular epithelial cell apoptosis was induced by nicotinamide in E18.5 kidneys from Pkd1–/– embryos, but was rare in E18.5 kidneys from DMSO-treated Pkd1–/– embryos (Figure 4J). Furthermore, we found that treatment with nicotinamide increased the survival of Pkd1–/– E18.5 embryos compared with those treated with DMSO (P < 0.01; Supplemental Table 1).

Next, we tested whether nicotinamide or EX-527, a specific SIRT1 inhibitor (21), could reduce cyst initiation or growth in Pkd1flox/flox:Ksp-Cre mice. Cyst progression is aggressive in the kidneys of Pkd1flox/flox:Ksp-Cre mice (22), which allowed us to examine the effect of nicotinamide on initiation and progressive enlargement of cyst formation. Pkd1flox/flox:Ksp-Cre pups were injected i.p. with...
nicotinamide (0.25 mg/g), EX-527 (2 mg/kg) or DMSO daily from P3 to P6, and kidneys were harvested and analyzed at P7. Administration of nicotinamide or EX-527 during this early phase delayed renal cyst growth ($P < 0.01$; Figure 5, A and B), inhibited cystic epithelial cell proliferation (PCNA staining; Figure 5E), and induced cystic epithelial cell apoptosis (TUNEL assay; Supplemental Figure 4A) in P7 kidneys from Pkd1flox/flox:Ksp-Cre mice compared with DMSO injection ($n = 10$ per treatment group). KW/BW ratios and BUN levels in Pkd1flox/flox:Ksp-Cre mice were dramatically reduced by nicotinamide or EX-527 treatment compared with DMSO or nicotinamide ($n = 10$ per treatment group). Gender did not influence cyst formation and progression, as determined by comparing 5 male mice and 5 female Pkd1flox/flox:Ksp-Cre mice per treatment group with respect to cystic index and KW/BW ratios. To further confirm that nicotinamide delayed cyst formation by specifically targeting SIRT1, we treated Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre mice with nicotinamide daily from P3 to P6 and collected the kidneys at P7. The rationale for this experiment was that if nicotinamide delays cyst growth in Pkd1 mutant mice by targeting SIRT1, then it will not affect cyst growth in Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre mice, which lack SIRT1. We found that nicotinamide treatment did not further delay cyst growth in P7 kidneys of Pkd1flox/flox:Sirt1flox/flox:Ksp-Cre mice ($n = 10$ per treatment group; Supplemental Figure 4, B–D). These results demonstrated that nicotinamide delayed cyst growth by specifically inhibiting SIRT1 in the Pkd1flox/flox:Ksp-Cre mice. Finally, we examined whether nicotinamide or EX-527 could delay cyst growth in the progressive hypomorphic Pkd1nl/nl mouse model (15). Pkd1nl/nl pups were injected i.p. with nicotinamide (0.25 mg/g), EX-527 (2 mg/kg), or DMSO daily from P5 to P27, and kidneys were harvested and analyzed at P28. Administration of nicotinamide or EX-527 delayed cyst progression (Figure 6, A and B), inhibited cystic epithelial cell proliferation (Figure 6E), and induced cystic epithelial cell apoptosis (Supplemental Figure 5) in P28 Pkd1nl/nl kidneys compared with kidneys of age-matched DMSO-injected Pkd1nl/nl mice ($n = 10$ per treatment group). Nicotinamide or EX-527 treatment also significantly decreased KW/BW ratios and BUN levels in Pkd1nl/nl mice compared with DMSO injection (Figure 6, C and D). We also found that gender did not affect cyst formation and progression in Pkd1nl/nl mice by compar-
ing 5 male mice and 5 female mice per group. These results further supported the notion that targeting SIRT1 with pharmacological inhibitors may delay cyst growth in ADPKD patients.

Silence or inhibition of SIRT1 decreases renal epithelial cell growth, but increases apoptosis. Our findings that genetic deletion of Sirt1 or inhibition of SIRT1 with nicotinamide or EX-527 in Pkd1-mutant background mice not only delayed cyst formation, but also decreased cystic epithelial cell proliferation and increased cystic epithelial cell apoptosis, suggested that SIRT1-mediated downstream pathways are involved in this process. To support this notion, we examined the effect of SIRT1 overexpression and SIRT1 depletion or inhibition on cell proliferation with a BrdU proliferation assay in mouse IMCD3 cells and Pkd1-null renal epithelial cells, respectively. We found that overexpressing HA-tagged WT SIRT1, but not the deacetylase catalytically inactive mutant SIRT1-H355A (23), increased BrdU incorporation in mouse IMCD3 cells (Figure 7A). In contrast, knockdown of SIRT1 with siRNA decreased BrdU incorporation in Pkd1-null MEK cells and PN24 cells (Figure 7, B and C). In addition, treatment with different concentrations of nicotinamide resulted in a dose-dependent decrease in BrdU incorporation in Pkd1-null MEK cells and PN24 cells (Figure 7, D and E). These results suggest that upregulation of SIRT1 increases S-phase entry in Pkd1-mutant renal epithelial cells.

Next, we examined whether nicotinamide had a proapoptotic effect on WT MEK, Pkd1-null MEK, PH2, and PN24 cells by TUNEL assay. Nicotinamide induced apoptosis in Pkd1-null MEK cells and PN24 cells, but not in WT MEK cells or PH2 cells (Supplemental Figure 6, A and B). Flow cytometry analysis demonstrated that apoptosis was significantly increased in Pkd1-null MEK cells and PN24 cells treated with nicotinamide compared with vehicle (Figure 7, F and G). We further found that treatment with nicotinamide markedly increased the level of active caspase-3 in Pkd1-null MEK cells and PN24 cells, but not that in WT MEK cells or PH2 cells (Figure 7H). Caspase-3 activation was confirmed by the appearance of cleaved poly(ADP-ribose) polymerase (PARP), a substrate of caspase-3 (Figure 7H), which suggests that caspase-3 is the downstream executor of nicotinamide-induced apoptosis in Pkd1-mutant renal epithelial cells.

SIRT1 regulates cystic epithelial cell proliferation by altering Rb acetylation and phosphorylation. Previous studies demonstrated that acetylation of Rb inhibits its phosphorylation by cyclin-dependent kinases and that SIRT1-mediated deacetylation of Rb increases its phosphorylation in vitro (12, 24). However, whether endogenous SIRT1 regulates Rb activity through this process is unknown. We demonstrated that knockdown of Pkd1 in mouse IMCD3 cells with 2 different lentiviruses expressing shRNAs increased not only SIRT1 expression (Figure 1C), but also Rb phosphorylation (Figure 8A), compared with
control mouse IMCD3 cells transduced with the respective control siLuc or pGIPZ-NS lentivectors. Phospho-Rb was also increased in Pkd1-null MEK cells and PN24 cells, as well as in kidney tissues from Pkd1null mice, compared with that seen in the respective WT MEK cells, PH2 cells, and control kidney tissues (Figure 8, B and C).

To support the functional relationship between SIRT1 and Rb in renal epithelial cells, we found that SIRT1 interacted with Rb by demonstrating that anti-Rb antibody could pull down SIRT1 (Figure 8D). Due to the lack of antibodies for acetyl-Rb, we used anti-Rb antibody to pull down Rb and subsequently used an anti–acetyl-α-lysine antibody to evaluate the acetylation of Rb, as performed by other laboratories (24, 25). We found that acetylated Rb was decreased in SIRT1 upregulating Pkd1-null MEK versus WT MEK cells (Figure 8D). In addition, we found that (a) overexpressing WT SIRT1, but not SIRT1-H355A, decreased p53 acetylation, but had no effect on phospho-Rb levels in mouse IMCD3 cells decreased p53 acetylation, but had no effect on phospho-Rb levels in mouse IMCD3 cells (Figure 9, A and B); (b) silencing SIRT1 with siRNA or inhibiting SIRT1 activity with nicotinamide increased phospho-Rb levels in Pkd1-null MEK cells compared with untreated control cells (Figure 9, C and D); and (c) overexpressing HA-tagged WT SIRT1, but not SIRT1-H355A, in mouse IMCD3 cells decreased p53 acetylation, but had no effect on phospho-Rb levels in Pkd1-null MEK cells compared with untreated control cells (Figure 9, C and D).

Rb regulates the cell cycle through its interaction with the E2F family of transcription factors, in that Rb dephosphorylation increases Rb-E2F1 complex formation, and Rb phosphorylation releases E2F1 from Rb–E2F complexes, enabling E2F-dependent transcription of genes that mediate S-phase entry (26, 27). We found that the expression of E2F1 downstream targets DHFR, cyclin D3, and cyclin E, which are involved in cell cycle regulation, was upregulated in PN24 cells compared with PH2 cells, while levels of these proteins decreased in nicotinamide-treated versus untreated PN24 cells (Supplemental Figure 7). These results suggested that SIRT1 regulates renal cystic epithelial cell proliferation through Rb-E2F signaling.

Nicotinamide induces cystic epithelial cell death through p53-mediated cell death pathway. Treatment with nicotinamide increased cystic epithelial cell death in Pkd1-knockout renal tissues. Previous studies demonstrated that SIRT1 protects cells from p53-mediated apoptosis through a deacetylation-dependent mechanism (10, 28, 29). Thus, we examined whether SIRT1-mediated p53 deacetylation was involved in nicotinamide-induced cystic epithelial cell death. We found that (a) SIRT1 interacted with p53 by demonstrating that anti-p53 antibody could pull down endogenous SIRT1 and that anti-SIRT1 antibody could pull down endogenous p53 in WT MEK cells and Pkd1-null MEK cells (Figure 10A); (b) p53 acetylation was decreased in Pkd1-null MEK cells versus WT MEK cells, while p53 expression exhibited no difference between these cells (Figure 10B); (c) overexpressing HA-tagged WT SIRT1, but not SIRT1-H355A, in mouse IMCD3 cells decreased p53 acetylation, but had no effect on phospho-Rb levels in Pkd1-null MEK cells compared with untreated control cells (Figure 10C).
on p53 expression (Supplemental Figure 8); (d) silencing SIRT1 with siRNA or inhibiting SIRT1 activity with its inhibitor, nicotinamide, increased the level of acetyl-p53 but had no effect on p53 expression in Pkd1-null MEK cells and PN24 cells compared with cells transfected with vector alone. (B–E) Silencing SIRT1 with siRNA (B and C) or inhibiting SIRT1 with the indicated concentrations of nicotinamide (D and E) decreased BrdU incorporation in (B and D) Pkd1-null MEK and (C and E) PN24 cells. BrdU incorporation index in the control cells was assigned as 100%. An average of 300 cells was counted for each experiment. n = 3. (F and G) Flow cytometry analysis indicated that apoptosis was induced in (F) Pkd1-null MEK cells treated with 10 mM nicotinamide for 24 hours and (G) PN24 cells treated with 40 mM nicotinamide for 48 hours. Nicotinamide-treated cells were labeled with annexin V and PI and analyzed by flow cytometry. Early and late apoptotic cells were represented by annexin V– and annexin V+PI+ cells, respectively, n = 3. (H) Caspase-3 activation was examined from whole cell lysates of WT MEK, Pkd1-null MEK, PH2, and PN24 cells treated or not with 10 mM nicotinamide for 24 hours. Nicotinamide treatment increased caspase-3 activation and caused the appearance of cleaved PARP (a substrate of caspase-3) in Pkd1-null MEK and PN24 cells. *P < 0.05; **P < 0.01.

Discussion
This study demonstrated a novel functional role of SIRT1 in ADPKD and provided a molecular basis for using nicotinamide (vitamin B3) to delay cyst formation. We found that SIRT1 expression was increased in Pkd1-mutant renal epithelial cells and tissues. Genetic deletion of Sirt1 in Pkd1–conditional knockout mice delayed renal cyst formation in postnatal kidneys. Inhibiting SIRT1 with nicotinamide (14) or the SIRT1-specific inhibitor EX-527 (21) delayed cyst formation in Pkd1-null MEKs, in Pkd1–conditional knockout postnatal kidneys, and in hypomorphic Pkd1nl/nl mouse kidneys, establishing an in vivo connection between SIRT1 and loss of PC1-mediated cyst formation (Figure 11D). In addition, we provided evidence that PC1 affects SIRT1 expression in renal epithelial cells through c-MYC. We conclude that increased SIRT1 in Pkd1-mutant renal epithelial cells (a) is a target of nicotinamide, which decreases proliferation and induces apoptosis of cystic epithelial cells; (b) regulates cyst epithelial cell proliferation through decreasing the acetylation and increasing the phosphorylation of Rb to regulate Rb-E2F1–mediated S-phase entry; (c) regulates p53 acetylation and p53-dependent apoptosis in response to nicotinamide; and (d) is regulated by c-MYC and can be further induced by TNF-α, which is present in cyst fluid during cyst development (Figure 11D). Since nicotinamide is a B3 vitamin with little toxicity reported, it has great therapeutic potential in ADPKD treatment.
SIRT1 is expressed abundantly in renal medullary interstitial cells, but at low levels in the renal cortex (31), which suggests that only low levels of SIRT1 may be detected in normal kidney epithelial cells. In the present study, we found that SIRT1 expression was markedly increased in DBA-positive Pkd1-null MEK cells and Pkd1-mutant postnatal proximal tubular–derived PN24 cells as well as in Pkd1-knockdown mouse IMCD3 cells and cyst lining epithelia of Pkd1-knockout kidney tissues (Figure 1). Our in vivo results demonstrating that cyst development was significantly delayed in ADPKD mice with a Sirt1-null background (Figure 3) strongly support an in vivo function of SIRT1 in ADPKD. A recent study reports that kidney-specific SIRT1 overexpression in proximal tubules does not appear to make mice susceptible to kidney cysts, but instead is protective against the consequence of ischemic and obstructive injury. These data suggest that overexpression of SIRT1 in proximal tubules, in the presence of WT PC1, may not result in renal cyst formation (32). Thus, the most plausible explanation for a pathological role of SIRT1 in renal cystogenesis is that PC1 mutations fundamentally change renal epithelial cells essential for cyst formation, and this process is modulated by SIRT1 activity.

Nicotinamide is a known inhibitor of SIRT1, and nicotinamide alters SIRT1-mediated downstream signaling pathways (14, 33). However, nicotinamide may inhibit the activity of other sirtuin proteins, including SIRT2–SIRT4 (14, 34, 35). To address the concern that nicotinamide might be targeting other sirtuin family members to delay renal cyst growth, we conducted several experiments and found that (a) in vivo administration of nicotinamide or EX-527 had similar effects on delaying cyst growth in Pkd1-knockout mouse models (Figures 5 and 6). We also found that knockdown of SIRT1 with siRNA and inhibition of SIRT1 with nicotinamide had similar effects on SIRT1-mediated Rb phosphorylation and p53 deacetylation in vitro. Thus, we attribute the effects of nicotinamide on delaying cyst formation to its inhibition of SIRT1-mediated signaling pathways in cystic epithelial cells.

We focused on nicotinamide due to the relative safety of its administration even at high doses for a variety of therapeutic applications (36). Our findings that nicotinamide delayed cyst growth not only in an aggressive Pkd1flox/flox:Ksp-Cre mouse model (Figure 5), but also in Pkd1 nl/nl mice (Figure 6), a progressive hypomorphic mouse model that has been recognized to closely resemble human ADPKD, support the potential clinical utility of nicotinamide in ADPKD patients. It has been reported that a 3-g/d dosage of nicotinamide is safe for adults (36). The dosage and route of administration of nicotinamide for treating ADPKD patients merits further investigation.

Increased proliferation is a crucial component of cystic expansion in ADPKD. There are several different signaling pathways that have been reported to regulate cystic epithelial cell proliferation (37, 38). In this study, we provided evidence that SIRT1-mediated deacetylation and phosphorylation of Rb, which inactivates Rb, regulated cystic epithelial cell proliferation (Figures 8 and 9). Rb is a central cell cycle regulator whose functions are in part regulated by diverse means, including posttranslational modifications such as phosphorylation and acetylation (12, 24). Active Rb is hypophosphorylated, and inactive Rb is hyperphosphorylated. Active Rb functions.
to repress the cell cycle through its interaction with the E2F family of transcription factors (26) and recruitment of chromatin-remodeling enzymes, such as histone deacetylases (HDACs), components of SWI2/SNF2 complex and methyltransferases, to E2F target gene promoters containing E2F sites (39, 40). However, phosphorylation of Rb can reverse this repression through dissociation of Rb–E2F complexes, enabling E2F-dependent transcription of genes that mediate S-phase entry (27). We found that SIRT1 regulated the acetylation and phosphorylation of Rb, since knockdown of SIRT1 with siRNA or inhibition of SIRT1 activity with nicotinamide increased Rb acetylation and decreased Rb phosphorylation (Figures 8 and 9). Treatment with nicotinamide also decreased the expression of the E2F1 targets DHFR, cyclin D3, and cyclin E, which were upregulated in Pkd1-null renal epithelial cells (Supplemental Figure 7). These data suggest that SIRT1 regulates (in part) cystic epithelial cell proliferation by altering the phosphorylation status of Rb and most likely through a Rb-mediated E2F pathway.

Increased levels of apoptosis are observed in human ADPKD as well as in rodent models of ADPKD and autosomal-recessive polycystic kidney disease (ARPKD) (41). However, apoptotic cells
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is stabilized and activated by DNA damage, stimulates the transcription of several genes in the apoptotic pathways (11). Whether FOXO3α and E2F1 are involved in nicotinamide-induced cystic epithelial cell death needs further investigation.

In addition, our present findings support a regulatory role for c-MYC and TNF-α on SIRT1 expression in Pkd1-mutant renal epithelial cells (Figure 2). Mutations of Pkd1 increase c-MYC expression, which as an initiating event increases SIRT1 expression. During cyst development, TNF-α is secreted into cyst fluid via an uncertain mechanism, which as a secondary event further stimulates the expression of SIRT1 through TNF-α-mediated NF-κB activation. SIRT1 has been shown to suppress NF-κB activity through deacetylating p65 in different cell lines, which inhibits the inflammation induced by NF-κB (45–47). Whether there is a feedback loop between SIRT1 expression and NF-κB activation in cystic epithelial cells and whether TNF-α signaling is able to override the inhibition of SIRT1 on NF-κB will require further investigation.

In sum, our present study identified SIRT1 as a novel regulator of cyst formation and provided the molecular mechanism and rationale for using nicotinamide (vitamin B3) as a novel therapeutic intervention in ADPKD to delay cyst formation. Our results that were rare in kidneys from models of conventional and conditional Pkd1 knockout (Figures 4–6), consistent with previous findings that the overall number of apoptotic nuclei in Pkd1lox/lox;Ksp-Cre or Pkd2-WS25 mouse kidneys is very low and is not significantly different between cystic and normal kidneys (42, 43). These observations are in contrast with the proposed role of apoptosis in mediating progressive loss of normal renal tissue during cyst development in a Pkd1 mutant mouse model. In this study, we provide evidence that SIRT1-mediated p53 deacetylation, which inactivates p53, may survive cystic epithelial cells from p53-mediated apoptosis during cyst development. We found that treatment with nicotinamide not only induced cystic epithelial cell apoptosis (Figure 7), but also increased p53 acetylation (Figure 10). Knockdown of p53 with siRNA or inhibition of SIRT1 with nicotinamide increased p53 acetylation, but did not affect p53 expression, in Pkd1-null MEK cells and PN24 cells that were (C) transfected with SIRT1 siRNA for 48 hours or (D) treated with 10 mM nicotinamide for 24 hours. *P < 0.05, **P < 0.01.

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Nicotinamide induces cystic epithelial cell death through p53. (A) Western blot analysis of p53 and active caspase-3 expression in Pkd1-null MEK cells transfected or not with p53 siRNA for 24 hours and then treated or not with 10 mM nicotinamide for another 24 hours. (B) Knockdown of p53 with siRNA prevented nicotinamide-induced apoptosis, as detected by TUNEL assay, in Pkd1-null MEK cells that were transfected or not with p53 siRNA for 24 hours and then treated or not with 10 mM nicotinamide for 24 hours. (C) Overexpression of WT p53, but not mutant p53-8KR (which is mutated at 8 acetylation sites), increased apoptosis in Pkd1-null MEK cells treated with nicotinamide. Pkd1-null MEK cells were transfected with WT p53, mutant p53-8KR, or empty vector together with or without nicotinamide for 24 hours, then analyzed by TUNEL assay. (D) SIRT1-mediated pathways in Pkd1-mutant renal epithelial cells. Pkd1 knockout or mutation upregulates SIRT1 through c-MYC. Upregulated SIRT1 in Pkd1-mutant renal epithelial cells (i) is a target of nicotinamide, which decreases proliferation and induces apoptosis of cystic epithelial cells to delay cyst growth in Pkd1-null mouse kidneys; (ii) regulates the acetylation and phosphorylation of Rb and further affects Rb-E2F–mediated S-phase entry; (iii) regulates the p53 acetylation and p53-dependent apoptosis in response to nicotinamide; and (iv) can be regulated by c-MYC and induced by TNF-α. Scale bars: 50 μm. **P < 0.01.
**IP and Western blot.** We performed IP and Western blotting on whole-cell lysates as previous described (38). Briefly, cells were lysed at 4°C with modified lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1% glycerol, 0.5 mM dithiothreitol, and 1 mM sodium vanadate plus protease inhibitor (Roche Applied Science). Cell extracts were clarified by centrifuging at 16,000 g for 15 minutes at 4°C, and the supernatants were subjected to IP with anti-SIRT1, anti-phospho-Rb, anti-angiogenin-ε-lysine, anti-p53, anti-acetyl-p53, anti-PARP, and anti-active caspase-3 antibodies (Cell Signaling Technologies; 1:1,000 dilution); anti-actin and anti-tubulin antibodies (Sigma-Aldrich; 1:5,000 dilution); and anti-Rb, anti-HA, anti-DHFR, anti-cyclin E, anti-c-MYC, and anti-cyclin D3 antibodies (Santa Cruz; 1:500 dilution). All primary antibodies were used in 1:50 dilution for IP and as indicated above for Western blotting. Donkey anti-rabbit IgG–horseradish peroxidase and donkey anti-mouse IgG–horseradish peroxidase (Santa Cruz; 1:8,000 dilution) were used as secondary antibodies.

**Immunohistochemistry.** Kidneys were fixed with 4% paraformaldehyde (pH 7.4). For PCNA staining, a monoclonal mouse anti-PCNA antibody (Cell Signaling Technologies; 1:1,000 dilution), a biotinylated secondary antibody (Sigma-Aldrich; 1:100 dilution), and DAB substrate system were used. For SIRT1 staining, a rabbit anti-SIRT1 antibody (Cell Signaling Technologies; 1:100 dilution) and a rabbit anti-bisulfite antibody (Epitomics; 1:100 dilution) were used. Kidney sections were counterstained with hematoxylin. Images were analyzed with a NIKON ECLIPSE 80i microscope.

**Phd1 knockdown by lentivirus carrying Phd1 shRNA.** HEK293T cells were transfected either with lentiviral plasmid pGIPZ-siPhd1 (Open Biosystems), carrying Phd1 shRNA, or with control empty vector pGIPZ-NS, plus psPAX2 packaging plasmid and pMD2.G envelope plasmid using calcium phosphate. After transfection for 12 hours, the medium containing the transfection reagent was removed and replaced with fresh complete DMEM plus 10% FBS and penicillin/streptomycin. The lentiviral particles were harvested from HEK293T cells after another 48 hours. Mouse IMCD3 cells were then infected with appropriate amounts of lentiviral particles together with 5 μg/ml polybrene (Sigma-Aldrich) for 24 hours, and then virus-containing medium was removed and replaced with fresh medium plus 10 μg/ml puromycin. After 48 hours of puromycin selection, all remaining cells were GFP positive, as detected by microscopy. Mouse IMCD3 cells were harvested after lentiviral particle infection for 5 days and analyzed by RT-PCR to examine the efficiency of Phd1 knockdown. We also used another lentiviral plasmid, VIRHD/P/siPhd1 (carrying Phd1 shRNA), to knock down Phd1 in mouse IMCD3 cells with the same protocol described above; the empty vector, VIRHD/P/siLuc, was used as control (both provided by G.L. Gussela, Mount Sinai School of Medicine, New York, New York, USA; ref. 50).

**qRT-PCR.** Total RNA was extracted using the RNeasy plus mini kit (Qiagen). 1 μg total RNA was used for RT reactions in a 20-μl reaction to synthesize cDNA using Iscript cDNA Synthesis Kit (BioRad). RNA expression profiles were analyzed by real-time PCR using iQ SYBER Green mix with ROX (BioRad) in a iCycler iQTM Real-time PCR detection system. Genes were amplified using the following primers: PKD1 forward, 5'-TCATTGCTCCTGGCCCGCTG-3'; PKD1 reverse, 5'-CCACGCTCT-GAAGTGATGTTGGG-3'; SIRT1 forward, 5'-CTCTGAAGATGTGACCGAGTTCAGT3'-; SIRT1 reverse, 5'-TGTAGAATGGCAACAAGTGTCCTC3'- actin forward, 5'-AAGAGCATATGAGCTGCTGA-3'; actin reverse, 5'-TACGGAGATGTCACACACAC-3'. The complete reactions were subjected to the following program of thermal cycling: 40 cycles of 10 seconds at 95°C and 20 seconds at 63°C. A melting curve was run after the PCR cycles, followed by a cooling step. Each sample was run in triplicate in each experiment, and each experiment was repeated 3 times. Expression levels of PKD1 and SIRT1 were normalized to the expression level of actin.

**RNA interference.** The RNA oligonucleotides that specifically targeted mouse SIRT1, mouse c-MYC, and mouse p53 were purchased from Thermoharmacon. The RNA oligonucleotides were transfected with DharmaFECT siRNA transfection reagent (Dharmacon). 48 hours after transfection, cells were harvested and analyzed by Western blotting.

**ChIP assay.** ChIP assay was performed according to the manufacturer’s protocol (EZ CHIP Chromatin Immunoprecipitation Kit; Upstate Biotechnology). Chromatin DNA was subjected to IP with anti-c-MYC antibody (SC-76; Santa Cruz) or normal rabbit IgG and then washed, after which the DNA-protein cross-links were reversed. The recovered DNA was analyzed by PCR for the presence of c-MYC binding motifs at mouse SIRT1 promoter between –1,009 and –850 bp (E1) and between –2,535 and –2,385 bp (E2) upstream of the SIRT1 ATG start codon. The PCR amplification for distant regions (~3,178 to –3,023 bp) was used as a negative control. The primers for E1 are 5'-AGACAGGAGGAGTGGATG-3' and 5'-AGCTCGGTGGCTGGCTCACT-3'; the primers for E2 are 5'-CTCTGACACGAG-3' and 5'-TCCGGTGGAAAGTGCTCCTG3'. A pair of distant primers (5'-AATTTCACACCCCTCCTC3' and 5'-GACAGGAGTGCTGC-3') was used as negative control.

**TUNEL assay.** TUNEL assay for nicotinamide-treated WT MEK, Phd1-null MEK, PH2, and PN24 cells and for nicotinamide- or EX-527–treated kidneys was performed according to the manufacturer’s protocols (In Situ Death Detection Kit; Roche). Prolong Gold Anti-fade reagent with DAPI (Invitrogen) was used. Immunofluorescence images were obtained with a NIKON ECLIPSE 80i microscope.

**Apoptosis assays.** Apoptosis was measured by flow cytometry with the FITC Annexin-V Apoptosis Detection Kit (BD Pharmingen) according to the manufacturer’s instruction. Annexin V^+^ and annexin V^-^ cells were considered early and late apoptotic, respectively.

**BrdU incorporation assay.** For BrdU incorporation assay in cells transfected with WT SIRT1 plasmid, SIRT1-H355A plasmid, or SIRT1 siRNA, after 12 hours of transfection, cells were induced to growth arrest by serum starvation for 24 hours, then cultured in regular media for another 12 hours. Subsequently, cells were pulse labeled with 10 μM BrdU for 1 hour, followed by a 12-hour chase, and then stained by anti-BrdU antibody (Sigma-Aldrich; 1:1,000 dilution). For BrdU incorporation assay in nicotinamide-treated cells, the cells were cultured with 5, 10, or 20 mM nicotinamide for 24 hours, then treated as described above. The percentage of BrdU-positive cells was counted; BrdU incorporation indices are shown relative to the control value (assigned as 100%).

**Mouse strains and treatment.** Phd1mut^−^ mice, generated as described previously (51), were used to examine the effect of nicotinamide on cyst growth during embryogenesis. In brief, Phd1-heterozygous mice (51) were paired, and pregnant females were injected i.p. daily, from 7.5 dpc to 14.75 or 17.5 dpc, with nicotinamide (0.5 mg/g body weight) or an equal volume of the vehicle DMSO. At the end of treatment, females were sacrificed, and MEKs were collected and fixed in 4% paraformaldehyde. Genomic DNA from the embryos was obtained (XNAT Extract-N-Amp Tissue PCR Kit; Sigma-Aldrich) and genotyped (JumpStart Kit; Sigma-Aldrich).

**Phd1^floxed/^Ksp-Cre mice were used to test the effect of nicotinamide or EX-527 on cyst progression at P7.** Phd1^floxed/^ mice and Ksp-Cre transgenic mice were generated as described previously (42, 52). Phd1^floxed/^ mice (B6; 129S4-Pkd1tm1Tsl/J; stock 010671; Jackson Laboratories) possess loxP sites on either side of exons 2–4 of Phd1 (42). Ksp-Cre mice express Cre recombinase under the control of the Ksp-cadherin promoter (52). Phd1^floxed/^Ksp-Cre mice
were generated by crossing-breed Pkd1floxflox female mice with Pkd1floxflox:Ksp-Cre male mice. Each neonate was injected i.p. daily with 0.25 mg/g nicotinamide, 2 mg/kg EX-527, or DMSO from P3 to P6. All animal protocols were approved and conducted in accordance with Laboratory Animal Resources of University of Kansas Medical Center and Institutional Animal Care and Use Committee regulations.

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