Histone deacetylase 6 inhibition reduces cysts by decreasing cAMP and Ca\(^{2+}\) in knock-out mouse models of polycystic kidney disease

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Autosomal dominant polycystic kidney disease (ADPKD) is associated with progressive enlargement of multiple renal cysts, often leading to renal failure that cannot be prevented by a current treatment. Two proteins encoded by two genes are associated with ADPKD: PC1 (pkd1), primarily a signaling molecule, and PC2 (pkd2), a Ca\(^{2+}\) channel. Dysregulation of cAMP signaling is central to ADPKD, but the molecular mechanism is unresolved. Here, we studied the role of histone deacetylase 6 (HDAC6) in regulating cyst growth to test the possibility that inhibiting HDAC6 might help manage ADPKD. Chemical inhibition of HDAC6 reduced cyst growth in PC1-knock-out mice. In proximal tubule–derived, PC1-knock-out cells, adenyl cyclase 6 and 3 (AC6 and -3) are both expressed. AC6 protein expression was higher in cells lacking PC1, compared with control cells containing PC1. Intracellular Ca\(^{2+}\) was higher in PC1-knock-out cells than in control cells. HDAC inhibition caused a drop in intracellular Ca\(^{2+}\) and increased ATP-simulated Ca\(^{2+}\) release. HDAC6 inhibition reduced the release of Ca\(^{2+}\) from the endoplasmic reticulum induced by thapsigargin, an inhibitor of endoplasmic reticulum Ca\(^{2+}\)-ATPase. HDAC6 inhibition and treatment of cells with the intracellular Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N',N",N"'-tetraacetic acid tetrakis(acetoxyethyl ester) reduced cAMP levels in PC1-knock-out cells. Finally, the calmodulin inhibitors W-7 and W-13 reduced cAMP levels, and W-7 reduced cyst growth, suggesting that AC3 is involved in cyst growth regulated by HDAC6. We conclude that HDAC6 inhibition reduces cell growth primarily by reducing intracellular cAMP and Ca\(^{2+}\) levels. Our results provide potential therapeutic targets that may be useful as treatments for ADPKD.

ADPKD\(^{2}\) (1) is characterized by the progressive enlargement of multiple renal cysts, leading to hypertension, a decline in renal function, resulting in renal failure in 50% of patients (2). In ADPKD, there is no treatment regimen that reduces the need for renal transplants (3). Clearly, there is a critical need to develop treatments for ADPKD. Two genes are associated with ADPKD, pkd1 and -2, encoding the polycystins 1 and 2. Misfunction of either of the polycystins leads to cyst formation (4), a hallmark of the disease. Cysts develop in every nephron segment through a combination of aberrant epithelial cell proliferation and abnormal fluid secretion (4). ADPKD is associated with several abnormalities, including alterations in growth factor receptor distribution and activation (5), altered extracellular matrix, and mispolarization of essential membrane proteins (6 – 8). Cyst fluid is produced by a cAMP-dependent mechanism similar to that found in secretory epithelia.

Aberrant Ca\(^{2+}\) signaling in ADPKD

Misregulation of Ca\(^{2+}\) is associated with cyst formation in ADPKD (9), with some investigators reporting that disruption of Ca\(^{2+}\) signaling is the primary event that supports increased cyst growth (10). However, the precise details of how this misregulation of Ca\(^{2+}\) occurs are still very controversial. PCs play a key role in Ca\(^{2+}\) movement. For example, PC2 belongs to the TRP protein family, whose members conduct Ca\(^{2+}\) (11). Indeed, our early work was among the first to show that PC2 is involved in the movement of Ca\(^{2+}\) (12). PC1 and -2 operate in concert at three locations within the cell: in the ER to regulate Ca\(^{2+}\) entry; at the plasma membrane to regulate store-operated calcium entry (SOCE) via store-operated Ca\(^{2+}\) channels, and at the primary cilium (4) to perhaps sense fluid flow.

Several studies have shown that a reduction in the function of either PC1 or PC2 leads to dysregulation of Ca\(^{2+}\) signaling (see Ref. 13 for a review). In contrast, we have demonstrated that a reduction in PC1 function leads to enhanced ER Ca\(^{2+}\) release in response to the activation of P2Y purinergic receptors, as well as an increased entry of Ca\(^{2+}\) through the SOCE mechanism (14). We have shown that PC1 binds to the IP\(_{3}\)R and reduces the release of Ca\(^{2+}\) from the ER in response to signal transduction cascades. For example, some purinergic receptors (15) operate

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\(^{2}\)The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N',N",N"'-tetraacetic acid tetrakis(acetoxyethyl ester); ER, endoplasmic reticulum; IP\(_{3}\), inositol triphosphate; IP\(_{3}\)R, IP\(_{3}\) receptor; SOCE, store-operated calcium entry; AC, adenyl cyclase; HDAC, histone deacetylase; HDAC6i, histone deacetylase inhibition; IBMX, 3-isobutyl-1-methylxanthine; PN, Pkd1-null; PH, Pkd1-heterozygous; ANOVA, analysis of variance.

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by increasing IP₃, causing the release of Ca²⁺ by the ER via the IP₃R and thereby depleting ER Ca²⁺. PC1 reduces the release of Ca²⁺ from the ER and the influx of Ca²⁺ via SOCE during receptor-mediated Ca²⁺ signaling (14, 16). PC2, in the absence of PC1, has the opposite effect, enhancing Ca²⁺-dependent signal transduction. It is clear that Ca²⁺-dependent receptor signaling is defective in ADPKD, but its role in cyst formation is not fully established. Given that Ca²⁺ is a core function of the polycystins, it is a primary target for therapies designed to correct the basic defect in ADPKD.

**Aberrant cAMP signaling in ADPKD**

A key component of cyst formation is cAMP. One theory regarding why cysts grow in response to cAMP is that aberrant Ca²⁺ metabolism causes a switch to a proliferative cAMP-dependent phenotype in ADPKD (17). The thinking is that Ca²⁺ restriction in ADPKD cells causes cAMP-dependent activation of the B-Raf/MEK/ERK pathway, which results in increased cell growth (18). Likewise, increased Ca²⁺ influx into ADPKD cells restores more normal cAMP signaling, thereby reducing cell growth (19).

The link in ADPKD between Ca²⁺ and chronic increases in cAMP occurs through Ca²⁺-dependent adenyl cyclases (ACs) (20). Basically, there are two types of cyclases that respond to Ca²⁺. One type is activated by Ca²⁺ via calmodulin and is exemplified by AC3, and another type is inhibited by Ca²⁺ and exemplified by AC5/6 (21). AC6 is expressed at higher levels in ADPKD cells that lack PC1 (20) than in normal cells in which PC1 is functioning appropriately. In this scenario, Ca²⁺ restriction would be expected to increase AC6-mediated production of cAMP (20). In contrast to this prevailing theory, we have suggested that enhanced Ca²⁺ signaling via excessive release of Ca²⁺ from the ER occurs in ADPKD (14). In our case, the higher levels of Ca²⁺ would activate AC3. Others have supported the notion that elevated Ca²⁺ levels exist in ADPKD cells. For example, expression of the C-terminal fragment of PC1 can cause an increase in basal levels of intracellular Ca²⁺ and induce abnormal Ca²⁺ oscillations, which also result in increases in cell signaling (10). The results of these latter studies suggest an alternate hypothesis: that enhanced release of Ca²⁺ from the ER stimulates AC3 to elevate cAMP. AC3 is particularly relevant because it is associated with the primary cilium, particularly in the sensory system (22). Furthermore, it is normally expressed in renal epithelium (23), suggesting a possible role for this adenyl cyclase in ADPKD.

**Role of histone deacetylase 6 (HDAC6) in ADPKD**

HDAC6, a member of the HDAC family, is predominantly localized to the cytoplasm and has a unique substrate specificity for the deacylation of tubulin (24). Many important biological processes are regulated by HDAC6, including transcription, cell migration and proliferation, cell signaling, immune responses, and protein degradation. There are an increasing number of reports indicating that increased HDAC6 expression and activity are involved in a number of diseases, including cancer (25, 26). HDAC6 activity is clearly elevated in ADPKD cells (27), and there is growing evidence to suggest that HDAC6 plays a role in cyst formation in ADPKD. The goal of this report is to determine the mechanism by which HDAC6 reduces cyst growth. The ultimate objective is to lend credence to the idea that HDAC6 inhibitors may be useful as a therapeutic for ADPKD.

**Results**

**The HDAC6 inhibitor tubastatin slows renal cyst growth and improves renal function in Pkd1⁺/⁻;Pax8rtTA;TetO-cre mice**

In this study, we used the Pkd1⁺/⁻;Pax8rtTA;TetO-cre mouse model, which, when treated with doxycycline, allows the expression of Cre and ablation of PC1 (28). Mice were injected with doxycycline within the intraperitoneal space, a treatment that leads to the development of multiple large cysts and large polycystic kidneys at ~3 weeks of age (28, 29) (Fig. 1). Mice were injected daily with tubastatin (5 mg/kg) or DMSO from postnatal day 10 to 20, and kidneys were harvested on postnatal day 21 (Fig. 1). As we showed previously for tubacin (27), tubastatin also significantly slowed kidney growth, as assessed by kidney/body weight ratio. The average kidney/body weight ratio was lower in the tubastatin-treated group. In addition, the administration of tubastatin significantly decreased the cyst area when compared with the DMSO-treated mice. Administration of the HDAC6 inhibitor improved renal function, as evidenced by a lower serum urea nitrogen (BUN) in the tubastatin-treated group than in the DMSO-treated group (Fig. 1). Given that the results were equivalent to those we obtained in our previous studies using tubacin, we utilized both tubacin and tubastatin in the studies outlined below.

**Tubacin inhibits cyst growth Pkd1⁺/⁻ cells**

To study this inhibition further, we grew Pkd1-null (PN) cells in Matrigel culture to induce cyst formation. We conducted our experiments in a model ADPKD cell line that had been clonally isolated from single parental clones obtained from a Pkd1⁺/⁻ mouse manufactured in the ImmortoMouse containing the H-2Kb-tsA58 gene. The PN cells stably express the Cre recombinase, and the control cells (Pkd1-heterozygous; PH) are from the original clone, which is a heterozygote for the expression of PC1 (31, 32). Fig. 2 shows that large cysts developed, as observed after 16 days in culture. As expected, the cysts grew larger when treated with forskolin, indicating that the cyst growth is indeed CAMP-dependent, as shown previously (33). Tubacin applied every other day for 14 days inhibited cyst growth even in the presence of forskolin.

We then asked whether HDAC6 inhibition (HDAC6i) could reduce the size of already established cysts. Fig. 3 shows cysts treated with tubacin from day 9 to day 16 in the presence and absence of forskolin. In this case, the cyst size was much smaller in the tubacin-treated cells than in untreated cells. These data show that HDAC6 can both inhibit cyst formation and reduce the size of already established cysts.

**HDAC6i increases acetylated α-tubulin**

To determine the effect of tubacin, a known inhibitor of HDAC6 (34), on PN and PH cells, we measured the levels of acetylated α-tubulin. Fig. 4 (A and B) shows that there was indeed a large increase in acetylated α-tubulin when the cells...
were treated with tubacin, indicating that HDAC6i was indeed causing large increases in acetylated α-tubulin. Fig. 4 (C and D) shows that there is no effect of HDAC6i on the total levels of α-tubulin. Fig. 4C shows that tubacin is specific for the acetylation of α-tubulin compared with the acetylation of histone H3, as shown previously (35).

**HDAC6i down-regulates resting intracellular Ca\(^{2+}\) levels**

To explore the role of Ca\(^{2+}\), we measured intracellular Ca\(^{2+}\) using Fura2 as we have done previously (16). Interestingly, the PN cells had a higher resting Ca\(^{2+}\) than did the PH cells (Fig. 5, A and B).

Next, we treated the cells with ATP, which stimulates an increase in intracellular Ca\(^{2+}\) via purinergic receptors (16). As we observed previously in MDCK cells, the response to ATP was greater in the PN cells, in the absence of PC1, than in the PH cells, in which PC1 was present. These data add more support to our hypothesis that PC1, by binding to the IP3R, suppresses ER Ca\(^{2+}\) release (14). In its absence, Ca\(^{2+}\)-dependent signal transduction is enhanced.

The effect of HDAC6i was particularly interesting. HDAC6i sharply reduced the basal levels of intracellular Ca\(^{2+}\) in both PN and PH cells (Fig. 5, A and B). HDAC6i at the same time increased the ATP-induced increase in intracellular Ca\(^{2+}\) (Fig. 5C). Note that the ATP-induced increase in intracellular Ca\(^{2+}\) levels in PN and PH cells was greater following treatment with tubacin than before treatment. However, in PH cells, the tubacin effect was much higher compared with the PN cells.

**HDAC6i down-regulates the release of Ca\(^{2+}\) from the ER**

To address the effect of HDAC6i on ER Ca\(^{2+}\) release, we treated the cells with thapsigargin, a specific inhibitor of the ER Ca\(^{2+}\)-ATPase that, when applied, allows Ca\(^{2+}\) to leak out of the ER through independent Ca\(^{2+}\)-permeable pathways (36). The first observation of interest was that the magnitude of the thapsigargin-induced increase in intracellular Ca\(^{2+}\) was greater in PN than in PH cells (Fig. 6). This finding is consistent with our previous data showing that Ca\(^{2+}\) signaling is elevated in PC1-knock-out cells. Importantly, HDAC6i dramatically reduced thapsigargin-induced ER Ca\(^{2+}\) release to values identical to those observed in the PH cells.

**Adenylyl cyclase 6 and 3**

There are two classes of adenylyl cyclases that are regulated by intracellular Ca\(^{2+}\); one class is activated, and the other is inhibited. To address the role of adenylyl cyclases in ADPKD, we selected one from each class: AC6, whose activity is inhibited by Ca\(^{2+}\), and AC3, whose activity is enhanced in intracellular Ca\(^{2+}\) (23). AC6 is already known to play a role in ADPKD. AC6, which is inhibited by Ca\(^{2+}\), operates in the outer medullary collecting duct and the proximal tubule (37). AC3 is activated by calcium/calmodulin and is present in the proximal tubule (37, 38).
Fig. 7 clearly shows that the PN (Pkd1-knock-out) cells express large amounts of AC6 when compared with the heterozygous PH cells. Abundant expression of AC6 has been noted previously in cyst cells from another mouse model, in which PC1 levels were reduced specifically in collecting duct cells (20). In comparison, AC3 is expressed equally in PN and PH cells.

Treatment of the cells for 16 h with the HDAC6i tubacin did not alter the steady-state levels of either AC6 or AC3. Our data here are consistent with a role for AC6 in PC1-knock-out cells derived from proximal tubules. However, given that AC6 is inhibited by Ca\textsuperscript{2+}/H11001, it is likely that in these cells, the higher resting Ca\textsuperscript{2+}/H11001 levels point to AC3 having a major role in generating cAMP, particularly in PN cells.

**HDAC6i down-regulates cAMP levels**

We have shown previously that treatment of the Pkd1\textsuperscript{0/0}; Pax8\textsuperscript{KOE};TetO-cre mouse with tubacin reduces cyst growth and cAMP levels in vivo (27). Consistent with our in vivo observations, PN cells had higher resting levels of cAMP than did PH cells. Importantly, we found that administration of the HDAC6 inhibitor tubastatin (Fig. 7D) significantly decreased cAMP levels. These data suggest that one way that HDAC6i reduces cyst growth (as shown in Fig. 1) is most likely via a reduction in resting cAMP levels. To study this further, we treated cells with forskolin, which activates enzyme activity by binding to the cytoplasmic domain of the enzyme (39). Treating PN and PH cells with forskolin increased the cAMP activity 300–400-fold. Importantly, HDAC6i did not affect the forskolin-induced increase in adenylyl cyclase activity in PN cells (Fig. 8A) but had a small effect in PH cells.

Interestingly, the forskolin-induced increase in cAMP level was greater in PH compared with PN cells. We propose that the increase in the basal levels of cAMP in PN versus PH cells shown in Fig. 8A is most likely the result of higher levels of intracellular Ca\textsuperscript{2+} in PN versus PH cells.

Both the rate of production of via adenylyl cyclase and the rate of degradation by phosphodiesterase (40) determine the steady state levels of cAMP in cells. To evaluate the role of phosphodiesterase, we applied 3-isobutyl-1-methylxanthine (IBMX). IBMX by itself increased cAMP levels, which were further increased by adding forskolin plus IBMX. However, the stimulation by forskolin of cAMP was not significantly different in the presence or absence of IBMX, indicating that phosphodiesterases do not contribute to the magnitude of the cAMP levels activated by forskolin that we observe under our experimental conditions.

One may ask whether the decrease in cAMP levels induced by tubacin or tubastatin is a direct result of a reduction in HDAC6 activity. To address this, we silenced HDAC6 with siRNA (Fig. 8C). Note that silencing of HDAC6 reduces cAMP levels to the same extent as tubacin treatment (Fig. 8C), providing convincing evidence that the effect of tubacin is indeed caused by HDAC6i.
HDAC6 inhibition decreases cAMP and calcium in ADPKD

Figure 4. HDAC6i increases α-tubulin acetylation. Confluent PN/PH cells at 37 °C were treated with tubacin (10 μM) for 16 h. A, Western blot showing expression of acetylated α-tubulin in treated or control cells. B, columns represent averages ± S.E. (error bars) of the acetylated α-tubulin expression. C, comparison of acetylated α-tubulin with total α-tubulin. In the third panel from the top, the exposure time is increased to intensify the band in the PN cells. D, columns represent averages ± S.E. of the acetylated α-tubulin/total α-tubulin expression. *** p < 0.001; **** p < 0.0001. Data were analyzed by non-parametric t test. All experiments were repeated 4–7 times. E, acetylation of histone H3 (Lys-9) is included to show that tubacin specifically increases the acetylation of α-tubulin. Ezrin or β-actin was loading control.

Figure 3. Cyst growth in PN cells is stimulated by forskolin and inhibited by tubacin. Cysts were grown in Matrigel for 16 days. The cysts were treated with DMSO (control) or forskolin at 10 μM from day 9 to 16. Columns represent means ± S.E. (error bars) (n = 6–10). The average cyst area in the control group was normalized to 100%, and the rest of the cysts were compared in the presence or absence of tubacin. **** p < 0.0001. Note the almost 3-fold forskolin-dependent increase in cyst size. Asterisks indicate significance between the groups (ANOVA, Tukey multiple comparisons).
The strong decrease in intracellular Ca\(^{2+}\) induced by HDAC6i suggests that the reduction in cAMP induced by HDAC6i cannot be the result of Ca\(^{2+}\) regulation of AC6 but most likely occurs via a decrease in AC3 (20). Because the Ca\(^{2+}\) sensitivity of AC3 is modulated by calmodulin (3), we treated the cells with two known inhibitors of calmodulin (W-7 and W-13) to evaluate the role of AC3 (41, 42).

One can readily see (Fig. 8D) that W-7, when applied at 50 \(\mu\)M, reduced the cAMP activity by an extent similar to that of tubacin when applied at 10 \(\mu\)M. W-13 when applied at 50 \(\mu\)M has a greater effect compared with W-7 (\(p < 0.01\)). Increasing the W-13 concentration to 100 \(\mu\)M caused further decreases in cAMP.

Taken together, these data indicate that the elevated levels of cAMP in PN cells are most likely caused by the activation of AC3 via calmodulin. HDAC6i, by lowering intracellular Ca\(^{2+}\), would reduce AC3 activity and lower cAMP levels.

Lowering intracellular Ca\(^{2+}\) reduces the resting levels of cAMP

To study the role of Ca\(^{2+}\) in determining cAMP levels in PN cells, we treated the cells with 1,2-bis(2-aminophenoxy)
ethane-N,N',N''-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), the cell-permeant Ca\(^{2+}\)-chelator (43). Fig. 9A shows that BAPTA-AM, as expected, lowered intracellular Ca\(^{2+}\). Importantly, it also lowered resting levels of intracellular cAMP in a dose-dependent manner. Combined with the data on calmodulin inhibition in Fig. 8, the data in Fig. 9 demonstrate convincing a direct link between reduced intracellular Ca\(^{2+}\) and cAMP.

W-7 inhibits cyst growth in Pkd1\(^{-/-}\) cells

To explore further the role of calmodulin in cyst growth, we again grew PN cells in Matrigel. When evaluated at 15, 18, or 22 days, it was clear that W-7 was able to inhibit cyst growth to a degree similar to that seen for tubacin (Fig. 10). These data suggest that the elevated Ca\(^{2+}\) noted in PN cells leads to a stimulation of calmodulin, elevating cAMP via AC3 and promoting cyst growth. We do realize that calmodulin may affect other signal transduction pathways in addition to AC3, but these pathways would also be enhanced by the elevated Ca\(^{2+}\), increasing calmodulin activity. It is important to note that a role for calmodulin in regulating AC3 activity and potentially affecting other cell processes involved in cyst formation would be dependent on an elevation in Ca\(^{2+}\) similar to the one we observed in PN cells.

Discussion

**HDAC6i reduces cyst growth**

We have shown here that inhibiting HDAC6 activity and increasing the amount of acetylated tubulin via treatment with the HDAC6 inhibitor tubacin (27) or tubastatin (44) can inhibit cyst formation in a mouse model of polycystic kidney disease. To delve deeper into the mechanism by which the cysts form, we utilized an immortalized proximal tubule epithelial cell line derived from a PC1-knock-out animal (31, 32). The two cell lines, derived from a single proximal tubule clone, were either null for PC1 following stable transfection of Cre or expressed PC1 as a heterozygote (the parental cells). Using these cell lines as an *in vitro* model, we showed that HDAC6i reduces cyst growth and also shrinks already established cysts. HDAC6 is elevated in ADPKD cells, and thus inhibiting this enzyme has a beneficial effect in preserving renal function in ADPKD mouse models (27).

However, in view of all of the roles HDAC6i plays in cellular processes, one might wonder whether HDAC6i therapy will ultimately be safe. Interestingly, mice lacking HDAC6 are viable and fertile and have no gross morphological abnormalities (45); this observation has important implications for the safety of potential therapeutic inhibition of HDAC6. In light of the multitude of HDAC6 functions, why would inhibiting it be safe? The key point is that deacetylation activity is up-regulated in the disease state, and, as such, devising strategies to bring its activity down toward normal levels might prove therapeutic without causing adverse side effects.

The second question is whether tubacin or tubastatin will be therapeutic in patients. Both drugs are very specific inhibitors of HDAC6 enzymatic activity with a nanomolar IC\(_{50}\) level for inhibition (44). This is consistent with our experiments in mice,
where injection of tubastatin at 5 mg/kg (∼25 ng/mouse) reduces the pathological effects of ADPKD in the mouse model. Studies of specificity in A549 lung carcinoma cells and BSC-1 African green monkey kidney epithelial cells have found an EC50 of 2.5 M on tubulin acetylation and a maximum effect of 15 M (35). In our experiments, we showed that 10 M tubacin reduces cyst formation and growth in PN cells, which is below the maximum effective concentration noted in the A549 cells (35). In mouse embryonic stem cells that are highly sensitive to HDAC inhibitors, expression profiling did not detect alterations in gene expression profiles (35). Likewise, at 10 M, tubacin did not affect the acetylation of histone H3 (Lys-9 and Lys-14) (35). Histone H3 is one of the major proteins whose acetylation plays a role in the integrity of the nucleosome (46). These studies demonstrate that HDAC6 inhibitors, such as tubacin and tubastatin, are highly selective inhibitors of HDAC6. Finally, determination of whether they will be therapeutic in ADPKD patients will require further study. However, given their potency and selectivity and their propensity to inhibit cyst growth and preserve renal function in animal mod-

Figure 8. Forskolin activation of adenylyl cyclase activity. A, PN/PH confluent cells were treated with tubacin (10 μM) or DMSO for 16 h and then treated with forskolin (100 μM) and/or IBMX (100 μM) for 30 min before harvesting the cells for assay. B, PN cells were treated with three different HDAC6 siRNAs (see “Materials and methods”) or scrambled siRNA at 1 nm for 72 h. Western blotting confirmed the knockdown of HDAC6 protein. C, cAMP measured after HDAC6 silencing or after tubacin treatment. Ctnt, scrambled siRNA. D, confluent PN cells were treated with tubacin (10 μM) or W-7 (50 μM) or W-13 (50 or 100 μM) for 16 h. Columns represent averages ± S.E. (error bars). *, p < 0.05; **, p < 0.001. Statistical analysis was performed using ANOVA; Tukey multiple comparisons. Each set of data is from 6–7 individual wells; *, compared with PN cells. #, compared with PH cells. Note that in PN cells, tubacin did not have an effect on the increase in cAMP levels induced by forskolin. There was a small effect in PN cells. A similar pattern was evident in the presence of forskolin + IBMX, suggesting that IBMX was not having any effect in addition to forskolin. Note that W-7 had an effect similar to that of tubacin in reducing cAMP levels. W-13 had a more potent effect compared with W-7.

Figure 9. The Ca2+ chelator, BAPTA-AM, reduces intracellular Ca2+ and cAMP. A, intracellular Ca2+ (F340/F380) levels obtained by ratiometric Fura-2/AM analysis of PN cells treated with BAPTA (10 μM) or with tubacin (10 μM) for 16 h. B, PN confluent cells were treated with BAPTA (10 μM or 50 μM) for 16 h before harvesting the cells for assay. Cyclic AMP levels were measured with a direct cAMP enzyme immunoassay kit based on the manufacturer’s protocol. Results are expressed as pmol/ml. Columns represent averages ± S.E. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001. Statistical analysis was performed using ANOVA; Tukey multiple comparisons. Asterisks, significance between the two groups (n = 4–6).
els, HDAC6 inhibitors are good candidates for continued development.

Misregulation in Ca\textsuperscript{2+} is associated with cyst formation

A major finding from our work is that resting Ca\textsuperscript{2+} is higher in PN cells than in PH cells. Along with the higher levels of intracellular Ca\textsuperscript{2+}, we have shown that there is robust cyst growth that can be stimulated further by the application of forskolin. HDAC6i, on the other hand, inhibits cyst growth while causing a dramatic decrease in resting Ca\textsuperscript{2+}. Thus, our work suggests that the higher levels of resting Ca\textsuperscript{2+} are fueling cyst growth in PN cells derived from the proximal tubule. The question, then, is: What is the source of this elevated Ca\textsuperscript{2+}?

To understand more about the likely source of this elevated Ca\textsuperscript{2+}, we treated the cells with thapsigargin, a specific inhibitor of the ER Ca\textsuperscript{2+}-ATPase; when applied, this inhibitor allows Ca\textsuperscript{2+} to leak out of the ER through independent Ca\textsuperscript{2+}-permeable pathways (36, 47, 48). We noted that the magnitude of the thapsigargin-induced increase in intracellular Ca\textsuperscript{2+} was greater in the PN cells than in PH cells. These data suggest that the likely source fueling the increase in intracellular Ca\textsuperscript{2+} in PN cells is most likely the enhanced release of Ca\textsuperscript{2+} from the ER.

Our studies here also show that HDAC6i dramatically lowers ER Ca\textsuperscript{2+} release while at the same time increasing ATP-driven changes in intracellular Ca\textsuperscript{2+}. One way that HDAC6i may reduce ER Ca\textsuperscript{2+} release is via PC2, but more experiments will be needed to demonstrate this conclusively.

Application of ATP to the luminal membrane of cyst cells stimulates purinergic receptors, which in our cells leads to an increase in intracellular Ca\textsuperscript{2+} (49). There are several different kinds of purinergic receptors: those that are Ca\textsuperscript{2+} channels themselves and others that cause the release of Ca\textsuperscript{2+} from the ER via inositol triphosphate (IP\textsubscript{3}) (50). Given that HDAC6i inhibits ER Ca\textsuperscript{2+} release, the purinergic receptors activated in PN cells by ATP are most likely at the plasma membrane mediating Ca\textsuperscript{2+} entry there.

The notion that ADPKD is associated with increased ER Ca\textsuperscript{2+} release and elevated intracellular Ca\textsuperscript{2+} is consistent with our previous studies. We have shown that normally functioning PC1 binds to the IP\textsubscript{3}R (16, 51), reducing its ability to release Ca\textsuperscript{2+} upon stimulation by IP\textsubscript{3}. In the absence of functional PC1, as occurs in ADPKD, PC2, located in the ER, enhances Ca\textsuperscript{2+} release through the IP\textsubscript{3} receptor. We posit that the enhanced release results in higher levels of intracellular Ca\textsuperscript{2+}. The enhanced release of Ca\textsuperscript{2+} via the IP\textsubscript{3}R and PC2 drives cyst formation, a contention supported strongly by the results presented here.

**AC3 drives cyst growth in PN cells**

Interestingly, PN cells express ample amounts of AC6, which has previously been implicated in cyst development in the collecting duct. The recruitment of AC6 in this cystic model derived from proximal tubule suggests that although in ADPKD, cysts develop from all nephron segments (9), the cells may converge in a common phenotype that supports cyst growth. The cysts also express AC3, one of the adenylyl cyclases normally expressed in the proximal tubule (23). AC3 is particularly relevant because it is associated with the primary cilium, particularly in the sensory system (22). AC3 and AC6 are regulated in opposite ways by Ca\textsuperscript{2+}, with AC3 being activated and...
HDAC6 inhibition decreases cAMP and calcium in ADPKD

AC6 inhibited by high Ca\textsuperscript{2+}. Thus, the higher intracellular Ca\textsuperscript{2+} levels in PN cells than in PH cells would suggest that AC3 would be dominant in generating higher levels of cAMP in the PN cells as opposed to PH cells. Two pieces of evidence support this conclusion. Treatment of cells with the calmodulin inhibitors W-7 and W-13 or with the Ca\textsuperscript{2+} chelator BAPTA-AM, which reduces intracellular Ca\textsuperscript{2+}, lowers the resting levels of intracellular cAMP in PN cells. Thus, although AC6 is present in the cyst cells, it is not playing a dominant role in generating cAMP in PN cells at resting Ca\textsuperscript{2+} levels.

We have also shown here that inhibition of calmodulin by W-7 inhibits cyst growth. Calmodulin is a ubiquitous intracellular protein that participates in many cellular processes, including cell proliferation, programmed cell death, and autophagy. Aberrant calmodulin activity is known to play a role in cancer cell growth, metastasis, and angiogenesis. Thus, inhibition of cyst growth by W-7 indicates that calmodulin may function similarly in ADPKD cysts to promote cyst growth, particularly at the higher levels of Ca\textsuperscript{2+} that we observed in the PN cells.

**Targets of HDAC6**

A number of pathways in ADPKD in addition to what we show here either have been identified to be altered or are likely to be altered in a manner that ultimately reduces or inhibits cyst growth. For example, EGF receptor activity is increased, and the receptor is mislocalized to the apical membrane in Pkd1 KO mice. Interestingly, inhibition of HCAC6 activity in Pkd1 KO mice restored EGF localization to the basolateral cell membrane, suggesting that the deacetylation of α-tubulin caused a mislocalization of this receptor. It is known that abnormal activation of the Wnt/β-catenin pathway contributes to cyst formation in ADPKD. HDAC6 controls EGF-induced nuclear localization of β-catenin, suggesting that HDAC6 may also be involved in the misregulation of this pathway.

Acetylation of α-tubulin is important for the normal assembly of the primary cilium, where PC2 is found. HDAC6, via its deacetylase activity, plays a role in cell disassembly during cell division. In renal epithelia, the NAD\textsuperscript{+}-dependent deacetylase SIRT2, together with HDAC6, plays a role in the assembly and stability of the primary cilium. Both are up-regulated in ADPKD, potentially leading to aberrant centrosome amplification and polyploidy.

Finally, an additional target of HDAC6 is the acetylation of the heat shock protein Hsp90. It is known that Hsp 90 is hyperactive in ADPKD cysts and that inhibiting Hsp90 function with STA-2842 will reduce cyst growth. These studies suggest that, in addition to the pathways discovered here, HDAC6i may be beneficial in restoring receptor polarity and/or microtubule assembly via its effects on the acetylation of α-tubulin. Reducing Hsp90 acetylation via HDAC6i may also have the added benefit of reducing an ensemble of its client proteins that support cAMP formation.

**Conclusion**

We propose a model whereby HDAC6i reduces intracellular Ca\textsuperscript{2+} via inhibition of ER Ca\textsuperscript{2+} release. We propose that HDAC6 inhibits cell growth and proliferation primarily by dramatically reducing cAMP and Ca\textsuperscript{2+} levels. Our results provide therapeutic targets that may be useful as potential treatments for ADPKD.

**Materials and methods**

**Cell culture and reagents**

PN and PH cells were cultured as described previously (31, 32). PN and PH cells were obtained from the Mouse Genetics and Cell Line Core of the Yale O’Brien Center. On day 5, the cells were treated with tubacin (10 μM) or DMSO (vehicle for control cells) for 16 h. Tubacin (catalog no. SML0065), tubastatin A (catalog no. SML0044), and forskolin (catalog no. 11018) were purchased from Sigma; W-7 (catalog no. 0369) and W-13 (catalog no. 0361) were purchased from Tocris; BAPTA (catalog no. 57534) was purchased from Selleckchem; acetylated α-tubulin (SC23950), α-tubulin (SC8035), adenylate cyclase 3 (SC588), and ezrin were purchased from Santa Cruz Biotechnology, Inc.; and adenylyl cyclase 6 (GTX47798) was purchased from GeneTex. The Matrigel matrix used was from Sigma (catalog no. 354230).

Experiments were performed in a model ADPKD cell line that had been clonally isolated from single parental clones obtained from a Pkd1\textsuperscript{−/−} mouse manufactured in the Immorto-Mouse containing the H-2kb-tsa58 gene. The PN, pkd1, null cells stably express the Cre recombinase, and the control cells (PH) are from the original clone, which is a heterozygote for the expression of PC1 (31, 32). Both lines are epithelial cells derived from proximal tubule.

**Mouse strain and treatment**

All animal use complied with the guiding principles of the Johns Hopkins University institutional animal care and use committee. Pkd1\textsuperscript{−/−};Pax8\textsuperscript{−/−};TetO-cre mice on a C57BL/6 background were provided by the Baltimore PKD Center and used to test the role of tubastatin in cyst growth, as reported previously (27). Mice of both sexes were used in this study. Mice were injected i.p. with doxycycline resuspended in sterile water (4 μg of doxycycline/g body weight) on postnatal days 11, 12, and 13. Mice were injected daily with tubastatin (20 mg/kg) or DMSO from postnatal day 10 to 20. On postnatal day 21, the mice were euthanized. Serum was collected to measure serum urea nitrogen, and kidneys were harvested for histology (right kidney) and cyclic AMP assays (left kidney). Serum urea nitrogen levels were measured by the Molecular and Comparative Pathobiology Laboratory of the Johns Hopkins University. These methods were reported by us previously (27).

**Cyclic AMP assays**

PN/PH cells were maintained in DMEM/F-12 supplemented with 3% FBS and γ-interferon (5 units/ml; Sigma-Aldrich) at 33 °C and 5% CO\textsubscript{2}, and plated in a 6-well plate for 24 h. The cells were then changed to γ-interferon–free medium and maintained at 37 °C for 4 days before being used in the experiment. Confluent cells were treated with tubacin (10 μM) or DMSO for 16 h before the cells were harvested for the assay. Cyclic AMP levels were measured with a direct cAMP enzyme immunoassay kit (Sigma, catalog no. CA200) based on the manufacturer’s
protocol. Results are expressed as pmol/ml and were normalized to total cellular protein. Columns represent averages ± S.E. ***, p < 0.0005. **, p < 0.005. Statistical analysis was performed using a two-tailed Student’s t test.

siRNA knockdown of HDAC6

PN cells were cultured as mentioned under “Cell culture and reagents.” The cells were seeded on 6-well culture plates to 50–60% confluence in complete growth medium at 33 °C. Cells were then transferred to non-permissive conditions at 37 °C in γ-interferon and antibiotic-free culture media. Mouse HDAC6 siRNA or scramble siRNA (Origene catalog no. SR422236, which utilizes three unique 27-siRNA duplexes) was transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Each of these sequences was used individually or in combination. The 1 nM concentration and the 72-h time provided the best knockdown of HDAC6 protein expression. Target sequences are as follows: SR42236A, rGrArUrArGrArUrUrGrUrGrUrCrArGrUrUrGrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrCrArGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUUA; SR42236B, GrArUrGrArGrUrUrGrUrGrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrGrUrUrCrArGrUrUrGrUrUrGrUrUrGrUrUrG.

Fura-2 Ca2+ imaging assays

PN/PH cells were plated in 35 10-mm cell culture dishes for 24 h. The cells were then changed to γ-interferon–free medium and maintained at 37 °C for 4 days. Confluent cells were treated with tubacin (10 μM) or DMSO for 16 h before being used in the experiment. On day 5, the cells were washed in imaging buffer (20 mM HEPES, 126 mM NaCl, 4.5 mM KCl, 2 mM MgCl2, and 10 mM glucose at pH 7.4) three times. After washing, cells were loaded with the cell-permeant acetoxymethyl (AM) ester of the calcium indicator Fura-2 (Fura-2/AM) at 37 °C for 90 min. Fura-2/AM was first dissolved in 1 mg/ml Pluronic/DMSO and then diluted to 5 μM in imaging buffer containing 2 mM CaCl2. After the incubation, the cells were allowed to recover for 30 min in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium. They were then washed briefly in imaging buffer without calcium but with 0.15% EGTA. The cells were then washed twice in imaging buffer without calcium to wash away the residual EGTA and Ca2+. They were then placed on the stage of a Zeiss inverted microscope equipped with a Sutter Lambda 10-2 controller and filter wheel assembly. For ATP stimulation experiments, the cells were exposed to 100 μM ATP diluted in the imaging buffer. A Zeiss FluorArc mercury lamp was used to excite the cells at 340 and 380 nm, and the emission response was measured at 510 nm. Cell fluorescence was measured once every 4 s in response to excitation for 1000 ms at 340 nm and 200 ms at 380 nm. Image acquisition, image analysis, and filter wheel control were performed by IPLab software.

Author contributions—M. K. Y., Q. L., and L. C. were involved in conducting the experiments; L. C. made substantial contributions to conception and design, data analysis, and interpretation of data; V. C. provided helpful discussions regarding antibody selection; W. B. G. helped with editing and preparation of the manuscript; L. C. wrote the manuscript. All authors approved the manuscript.

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