Phosphorylation of Ribosomal Protein S6 Mediates Mammalian Target of Rapamycin Complex 1–Induced Parathyroid Cell Proliferation in Secondary Hyperparathyroidism

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ABSTRACT

Secondary hyperparathyroidism is characterized by increased serum parathyroid hormone (PTH) level and parathyroid cell proliferation. However, the molecular pathways mediating the increased parathyroid cell proliferation remain undefined. Here, we found that the mTOR pathway was activated in the parathyroid of rats with secondary hyperparathyroidism induced by either chronic hypocalcemia or uremia, which was measured by increased phosphorylation of ribosomal protein S6 (rpS6), a downstream target of the mTOR pathway. This activation correlated with increased parathyroid cell proliferation. Inhibition of mTOR complex 1 by rapamycin decreased or prevented parathyroid cell proliferation in secondary hyperparathyroidism rats and *in vitro* in uremic rat parathyroid glands in organ culture. Knockin rpS6$^{P-/-}$ mice, in which rpS6 cannot be phosphorylated because of substitution of all five phosphorylatable serines with alanines, had impaired PTH secretion after experimental uremia- or folic acid–induced AKI. Uremic rpS6$^{P-/-}$ mice had no increase in parathyroid cell proliferation compared with a marked increase in uremic wild–type mice. These results underscore the importance of mTOR activation and rpS6 phosphorylation for the pathogenesis of secondary hyperparathyroidism and indicate that mTORC1 is a significant regulator of parathyroid cell proliferation through rpS6.

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Secondary hyperparathyroidism (SHP) is a major complication of CKD and characterized by increases in parathyroid hormone (PTH) expression and parathyroid cell proliferation.[1–5] Decreased expression of the calcium, vitamin D, and fibroblast growth factor-23 receptors contributes to the increased parathyroid proliferation in uremia.[6–11] Expression of TGF-α and its receptor, EGF receptor (EGFR), is increased in uremic rats and patients.[12–14] TGF-α activation of EGFR decreases the CCAAT/enhancer-binding protein-β liver-enriched activator protein:liver-enriched inhibitory protein, inducing parathyroid growth in uremia. A dominant negative EGFR gene expressed specifically in the parathyroid glands prevented the activation of endogenous EGFR and the increase in parathyroid gland enlargement and serum PTH.[15,16] Parathyroid cell proliferation is increased in transgenic mice overexpressing cyclin D1 only in the parathyroid.[17]

The mammalian target of rapamycin (mTOR) integrates signaling pathways to regulate cell growth

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and proliferation.[18–20] mTOR is part of mammalian target of rapamycin complex 1 (mTORC1) and mTORC2. Rapamycin inhibits mTORC1 and proliferation.[20–24] Different stimuli activate mTORC1 through Akt phosphorylation. 4E binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase 1 (S6K1) are mTORC1 targets.[25,26] S6K phosphorylates ribosomal protein S6 (rpS6) on a cluster of five serine residues at the carboxy terminus.[27] Knockin (KI) mice encoding a mutant rpS6 harboring alanine substitutions at all five phosphorylation sites ($rpS6^{P-/-}$ mice) have reduced size, glucose intolerance, muscle weakness, and impaired renal hypertrophy after uninephrectomy.[28–30]

We show that the mTOR pathway is activated in SHP. Inhibition of mTORC1 decreased parathyroid cell proliferation *in vivo* and *in vitro*. Moreover, $rpS6^{P-/-}$ mice showed the essential role of mTORC1 activation in parathyroid cell proliferation in SHP.

RESULTS

The Parathyroid mTOR Pathway Is Activated in Hypocalcemic Rats

Dietary-induced hypocalcemia (Table 1) resulted in increased parathyroid–phosphorylated rpS6 (Ser240/244) and S6K1 (Thr389), reflecting activation of the mTORC1 pathway (Figure 1, A–D). mTORC1 inhibition by rapamycin reduced phosphorylated rpS6 in the parathyroids of the low calcium–fed rats (Figure 1, A–C) with no effect on serum biochemistry (Table 1). Therefore, parathyroid mTORC1 is activated in hypocalcemic rats and inhibited by rapamycin.

Rapamycin Decreases Parathyroid Cell Proliferation in Hypocalcemic Rats

Hypocalcemia led to the expected increase in parathyroid cell proliferation (Figure 1, E and F).[1] Importantly, rapamycin decreased parathyroid cell proliferation by 70% in hypocalcemic rats (Figure 1, E and F, Supplemental Figure 1). Of interest, both phosphorylated rpS6 and parathyroid cell proliferation were decreased by rapamycin in low-calcium rats to levels lower than controls. This may reflect the active proliferation of parathyroids in weanling rats used in the low-calcium experiments, unlike mature rats.[1] Therefore, mTORC1 activation is crucial for parathyroid cell proliferation caused by chronic hypocalcemia.

Table 1. Serum calcium, phosphate, and PTH levels in rats fed a control or low-calcium diet with and without rapamycin

Variable	Control	Low Calcium	Low Calcium and Rapamycin
Calcium (mg/dl)	10.0±0.1	7.5±0.3[a]	7.6±0.4[a]
Phosphate (mg/dl)	6.3±0.1	8.3±0.5[a]	7.3±0.2[a]
PTH (pg/ml)	266±18	1364±121[a]	1244±57[a]
Change in body weight (g)	ND	58.2±9.8	62.3±9.3

Results are mean±SEM (n=6–7). Results represent data from two experiments combined. Body weights: n=3–4. ND, not determined.
[a]$P<0.05$ compared with control diet.

The mTOR Pathway Is Activated in the Parathyroid Glands of Uremic Rats

We then studied the role of the mTORC1 pathway in uremic SHP induced by an adenine high–phosphorus diet (Figure 2A). This diet led to increased serum creatinine, phosphate, and PTH levels (Table 2).[31] Phosphorylated rpS6 (Ser240/244) was increased in the uremic rat parathyroids (Figure 2, B and C). Therefore, the parathyroid mTORC1 pathway is activated in SHP in uremic rats and in hypocalcemic rats as shown above.

Rapamycin Decreases Parathyroid Cell Proliferation in Uremic Rats

To study the effect of mTORC1 inhibition on uremia-induced SHP, rats were injected with rapamycin or vehicle for the last 3 days of the adenine high–phosphorus diet (Figure 2A). Rapamycin had no effect on serum creatinine and calcium levels but decreased serum PTH and hence, increased serum phosphate (Table 2). Rapamycin attenuated the increased parathyroid cell proliferation and serum PTH in uremic rats (Figure 2, D–F, Table 2).[1,3] The decrease in parathyroid cell proliferation was shown by both 5-bromo-2′-deoxyuridine (BrdU) (Figure 2, D and E) and Ki-67 staining (Supplemental Figure 2). BrdU staining was the same in small intestine from uremic rats with and without rapamycin (Supplemental Figure 3).

Phosphorylated rpS6 and parathyroid cell proliferation were also increased by immunofluorescence in the parathyroids of uremic rats and decreased by rapamycin (Figure 3A). Notably, staining for proliferation marker colocalized with phosphorylated rpS6 in many proliferating cells (Figure 3B). Therefore, rapamycin decreases parathyroid cell proliferation and serum PTH in CKD, showing a role for the parathyroid mTORC1 pathway in SHP.

Rapamycin Prevents Parathyroid Cell Proliferation in Uremic Rats

To study whether rapamycin not only corrects but also, prevents parathyroid cell proliferation, rats were fed a control or an adenine high–phosphorus diet for 3 or 7 days, and rapamycin was injected daily from the start of the adenine diet together with BrdU added to the drinking water (Figure 4, A and C). The addition of BrdU for the entire duration of the experiment represents the total number of cells proliferating. Serum creatinine levels were increased already at 3 days of the diet (control, 0.7±0.01 mg/dl; 3 days adenine, 1.02±0.04 mg/dl; adenine and rapamycin, 0.9±0.1 mg/dl [n=4]; $P<0.05$). Serum PTH did not change at day 3 (control, 375±40 pg/ml; adenine, 309±53 pg/ml; adenine and rapamycin, 360±60 pg/ml [n=4–7]; NS) but increased at day 7 (Table 3) as before.[31]

Parathyroid cell proliferation by BrdU staining was increased already at 3 days of the adenine diet (Figure 4B), which preceded the increase in serum PTH. Rapamycin decreased parathyroid cell proliferation at

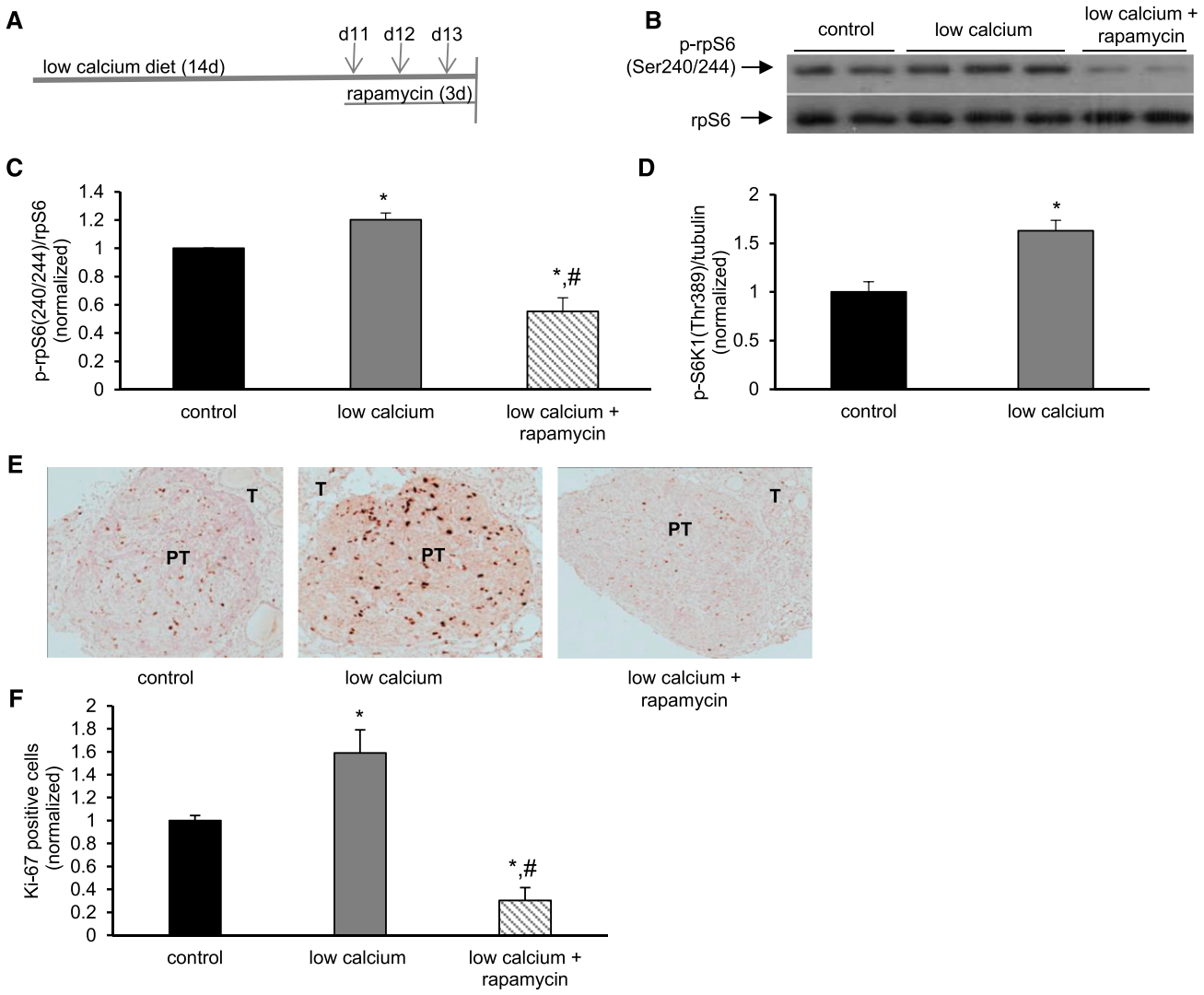

Figure 1. Activation of the parathyroid mTOR pathway and parathyroid cell proliferation in hypocalcemia induced SHP and inhibition by rapamycin. (A) Weanling rats were fed a control or low-calcium diet for 14 days. Rapamycin (0.2 mg/kg) or vehicle (DMSO) was injected intraperitoneally daily during the last 3 days of the low-calcium diet. (B) Representative Western blots for phosphorylated rpS6 (Ser240/244) and total rpS6 protein in protein extracts from single rat microdissected parathyroid glands in each lane. (C) Quantification of phosphorylated rpS6 corrected for total rpS6 (mean±SEM) from two experiments (n=5–6 rats). (D) Quantification of Western blot analysis for phosphorylated S6K1 (Thr389); mean±SEM; (n=6–7 rats). (E) Representative immunohistochemical staining for Ki-67. PT, parathyroid; T, thyroid. (F) Quantification of the number of Ki-67–positive cells presented as mean±SEM of Ki-67–stained nuclei per total nuclei. Combined data from two experiments are shown (n=5–9). *P<0.05 versus control; #P<0.05 versus low calcium.

both 3 and 7 days of the adenine diet (Figure 4, B and D). Similar results were shown using Ki-67 staining (Supplemental Figure 4). Therefore, the increased parathyroid cell proliferation caused by uremia is both reversed and prevented by rapamycin.

Rapamycin Decreases Parathyroid Cell Proliferation *In Vitro* in Organ Culture

The direct effect of rapamycin on parathyroid cell proliferation was shown in organ culture of parathyroids from rats fed an adenine diet. Microdissected parathyroid glands from single rats were incubated with BrdU and either rapamycin or vehicle (Figure 5A). BrdU immunostaining showed that the glands from the uremic rats continued to proliferate in culture. Importantly, rapamycin decreased parathyroid cell proliferation *in vitro*, showing the direct effect of rapamycin on parathyroid cell proliferation (Figure 5, B and C).

Uremic KI rpS6$^{P-/-}$ Mice Have Impaired PTH Secretion and No Parathyroid Cell Proliferation

To further study the role of the mTORC1 pathway on parathyroid cell proliferation, we used KI mice that are not susceptible to rpS6 phosphorylation. PTH and calcium levels

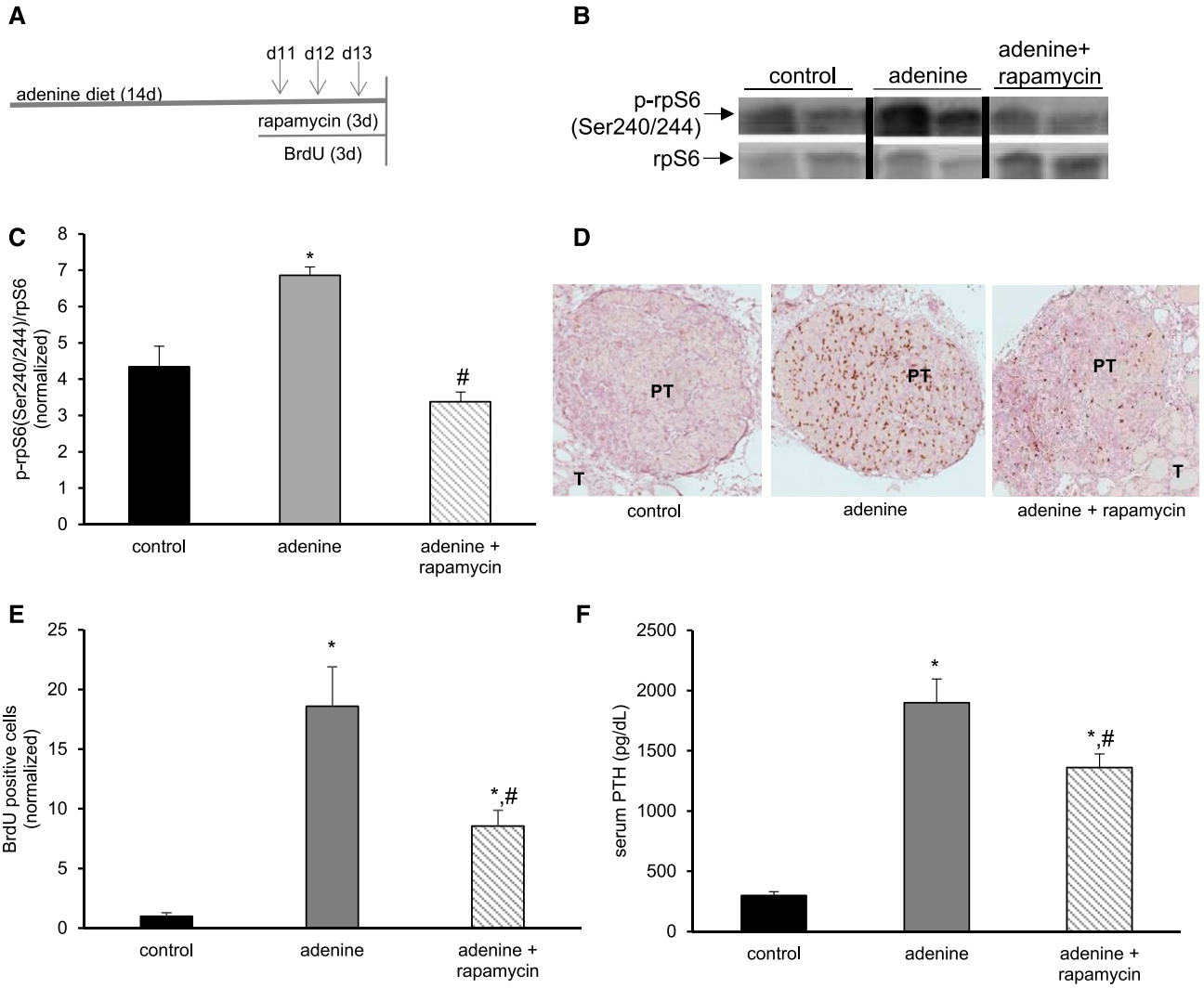

Figure 2. Activation of the parathyroid mTOR pathway and parathyroid cell proliferation in CKD induced SHP and inhibition by rapamycin. (A) Rats were fed a control or an adenine high–phosphorus diet for 2 weeks. Rapamycin (0.2 mg/kg) or vehicle was injected intraperitoneally daily during the last 3 days of the adenine diet when BrdU was added to the drinking water. (B) Western blot analysis of phosphorylated rpS6 (Ser240/244) in parathyroid extracts from rat parathyroids from individual rats. (C) Quantification of phosphorylated rpS6 levels presented as mean±SEM (n=4–5 rats). Similar results were obtained in a repeat experiment. (D and E) Parathyroid cell proliferation. (D) Representative immunohistochemical staining for BrdU. PT, parathyroid; T, thyroid. (E) Quantification of the number of proliferating cells presented as mean±SEM of BrdU-stained nuclei per total nuclei. (F) Serum PTH levels. Combined data from two experiments are shown (n=8–9).*P<0.05 versus control; $^{\#}P$<0.05 versus adenine.

were similar in both rpS6$^{P-/-}$ and wild-type (wt) mice (Figure 6). Adenine-induced uremia led to a decrease in serum calcium in the rpS6$^{P-/-}$ mice but not in the wt mice (Figure 6 B). Uremic mice decreased body weight similarly in both genotypes (wt adenine, 6.6±1.4 g; rpS6$^{P-/-}$ adenine, 6.2±1.5 g [n=5]; P=NS), such as in normal mice.[32] Serum PTH was increased 11-fold in the wt mice and only 3-fold in the KI mice, despite their lower serum calcium (Figure 6D). Therefore, rpS6 phosphorylation determines the increase in serum PTH in uremia.

There was increased rpS6 phosphorylation and parathyroid cell proliferation in uremic wt mice (Figure 6, E and F), such as in rats (Figure 3A). There was also an increase in proliferation of blood vessels in the uremic wt mice, such as shown in parathyroids from patients on dialysis (Figure 6E).[33] The rpS6$^{P-/-}$ mice, as expected, had no phosphorylated rpS6. Importantly, parathyroid cell proliferation was not increased in the uremic rpS6$^{P-/-}$ mice (Figure 6E and F, Supplemental Figure 5), indicating the critical role of rpS6 phosphorylation in uremic SHP.

rpS6$^{P-/-}$ Mice Have Impaired PTH Secretion after Folic Acid–Induced AKI

The rise of PTH level in folic acid–induced AKI was then studied in rpS6$^{P-/-}$ mice.[34–36] Folic acid induced uremia in

both genotypes with no change in serum calcium (not shown) and increases in serum phosphate and PTH levels that were smaller in rpS6$^{P-/-}$ mice (Figure 7, A–C). There was an increase in phosphorylated rpS6 in wt mice, showing activation of mTORC1 in the parathyroids of AKI wt mice (Figure 7D). In this acute model of renal injury, there was no increase in parathyroid cell proliferation (Figure 7D). Importantly, serum PTH levels increased less after folic acid administration to the rpS6$^{P-/-}$ mice compared with the wt mice (Figure 7C). Therefore, rpS6 phosphorylation is necessary for the increased serum PTH in adenine-induced uremia and AKI.

Table 2. Serum creatinine, calcium, phosphate, and PTH levels in uremic rats with and without rapamycin

Variable	Control	Adenine	Adenine and Rapamycin
Creatinine (mg/dl)	0.6±0.04	1.2±0.12[a]	1.4±0.2[a]
Calcium (mg/dl)	9.7±0.3	8.3±0.9	9.9±0.4
Phosphate (mg/dl)	7.7±0.2	10.4±0.7[a]	12.8±0.5[a,b]
PTH (pg/ml)	326±33	2064±213[a]	1479±122[a,b]
Change in body weight (g)	ND	−38±9	−35.7±11

Results are mean±SEM (n=9–12). Results represent data from three experiments combined. Body weights: n=5. ND, not determined.
[a]P<0.05 compared with control diet.
[b]P<0.05 compared with adenine diet.

DISCUSSION

We show that the mTORC1 pathway is essential for parathyroid cell proliferation in SHP of chronic hypocalcemia and experimental uremia. The parathyroid mTORC1 pathway was activated in both models as shown by rpS6 phosphorylation, correlating with increased parathyroid cell proliferation. Moreover, phosphorylated rpS6 colocalized with proliferation markers in uremic rats. Inhibition of the mTORC1 pathway decreased parathyroid cell proliferation in both hypocalcemic and uremic rats and *in vitro*. In uremic rats, rapamycin both corrected and prevented parathyroid cell hyperplasia. Interestingly, at 3 days of the adenine diet, the rats had a high serum urea with an increase in parathyroid cell proliferation, which preceded the increase in serum PTH that was seen only at 7 days.

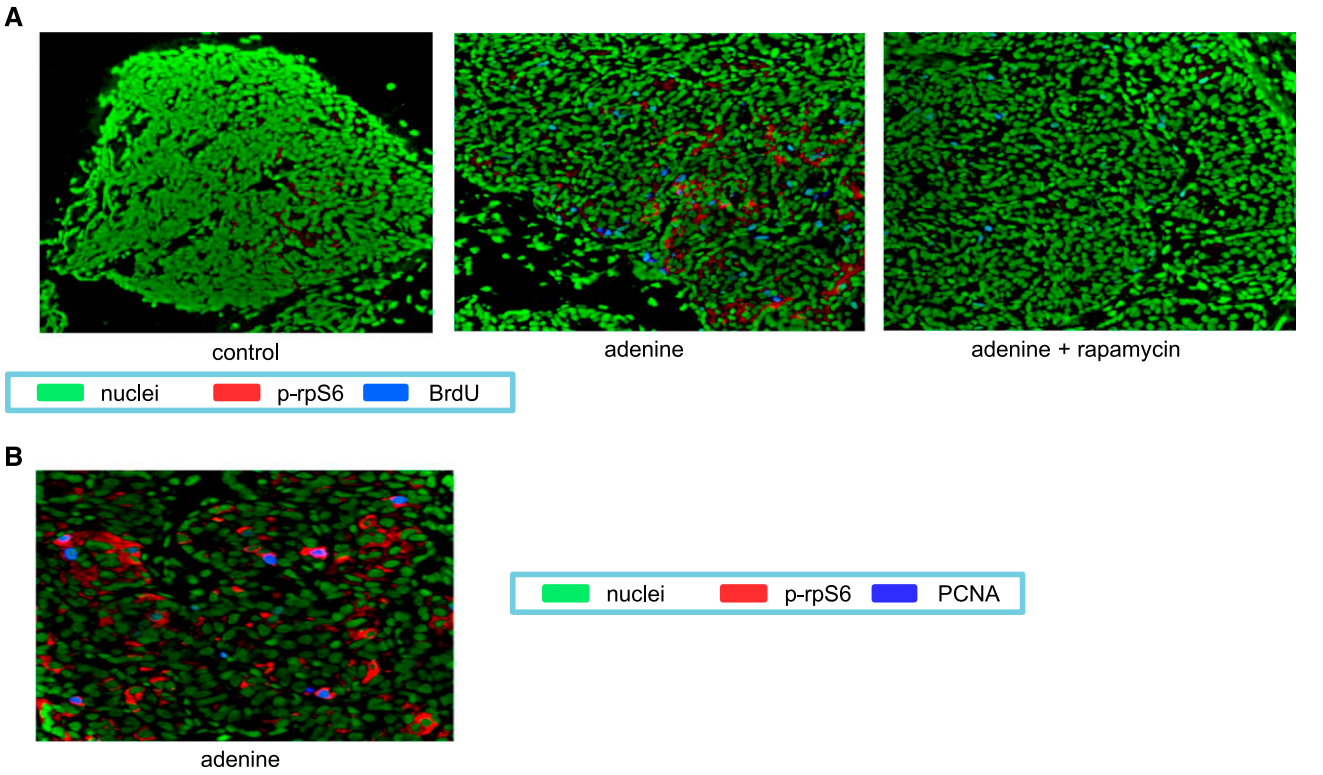

Figure 3. Parathyroid phosphorylated rpS6 (Ser240/244) expression and cell proliferation are increased in uremia and decreased by rapamycin. (A) Parathyroid sections from control and adenine high–phosphorus uremic rats injected with vehicle or rapamycin were stained for phosphorylated rpS6 and BrdU and examined by immunofluorescence. Nuclei were stained with sytox. (B) Representative parathyroid section from a uremic rat stained for phosphorylated rpS6 and PCNA shows colocalization of PCNA and phosphorylated rpS6. PCNA, proliferating cell nuclear antigen.

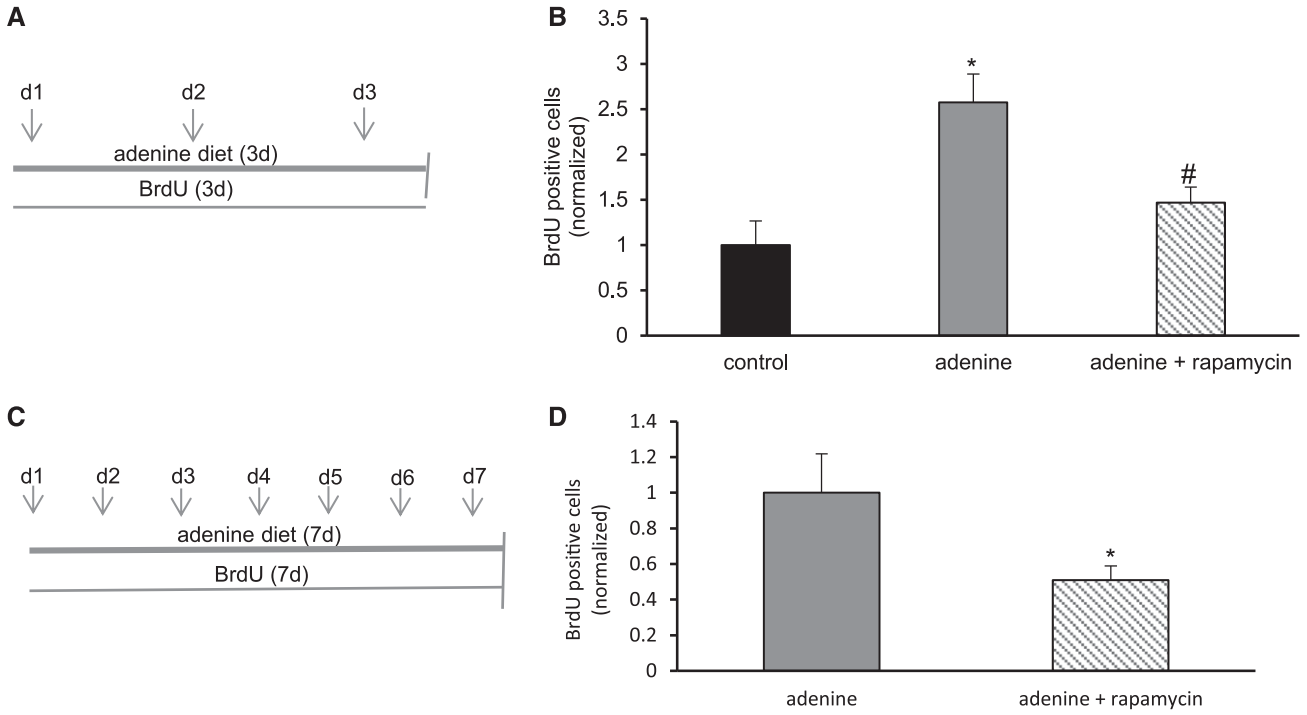

Figure 4. Rapamycin prevents the parathyroid cell proliferation of experimental uremia. Rats were fed a control or adenine high–phosphorus diet for (A and B) 3 or (C and D) 7 days. Rapamycin or vehicle was injected intraperitoneally daily, and BrdU was added to the drinking water of the adenine-fed rats for the entire duration of the experiment. Parathyroid sections were stained for BrdU. (B and D) Quantification of the number of proliferating cells presented as mean±SEM of BrdU-stained nuclei per total nuclei compared with (B) control diet or (D) adenine-fed rats. *$P<0.05$ versus control (n=7–9); #$P<0.05$ versus adenine (n=7–9); *$P<0.05$ versus adenine in D.

Table 3. Serum creatinine, calcium, phosphate, and PTH levels in uremic rats after 7 days of the adenine high–phosphorus diet with and without rapamycin given for 7 days

Variable	Control	Adenine	Adenine and Rapamycin
Creatinine (mg/dl)	0.45±0.1	1.3±0.21[a]	1.12±0.05[a]
Calcium (mg/dl)	7.9±1.3	11.9±1.8	9.0±0.4
Phosphate (mg/dl)	8.8±0.3	9.8±1	9.3±0.7
PTH (pg/ml)	442±43	867±264[a]	941±181[a]

Results are mean±SEM (n=3–6).
[a]$P<0.05$ compared with control diet.

In transgenic mice overexpressing cyclin D1 in the parathyroid, parathyroid cell proliferation also preceded the increase in serum PTH.[37] The direct effect of rapamycin on parathyroid cell proliferation was shown in parathyroids from uremic rats in organ culture. The glands in culture continued to proliferate, which was measured by BrdU incorporation, and this was inhibited by rapamycin. Therefore, the mTORC1 pathway is activated in SHP, and inhibition by rapamycin leads to decreased proliferation both *in vivo* and *in vitro*.

To further show the significance of the mTOR pathway in SHP, we studied $rpS6^{P-/-}$ KI mice, in which rpS6 cannot be phosphorylated.[28,29,38] The $rpS6^{P-/-}$ mice showed no difference in basal serum calcium and PTH and parathyroid cell proliferation and did not respond to two models of renal failure. Remarkably, the $rpS6^{P-/-}$ mice failed to increase parathyroid gland activity after the challenge of adenine-induced uremia, despite a decrease in serum calcium. Folic acid–induced AKI in wt mice led to an increase in serum PTH and parathyroid phosphorylated rpS6, indicating activation of the mTOR pathway (as shown in SHP rats) caused by chronic hypocalcemia and uremia. The $rpS6^{P-/-}$ mice had a muted increase in serum PTH after folic acid–induced AKI.

The failure of the uremic $rpS6^{P-/-}$ mice to increase parathyroid cell proliferation and the muted increase in serum PTH in acute and chronic models show that rpS6 phosphorylation is essential for these adaptive responses to uremia. However, the small increase in serum PTH in the uremic $rpS6^{P-/-}$ mice may be caused by mTORC1-independent pathway(s). Similarly, rapamycin given to uremic rats only partially decreased serum PTH, despite the abolition of parathyroid cell proliferation. Therefore, mTORC1 and rpS6 phosphorylation are crucial for parathyroid cell proliferation in SHP.

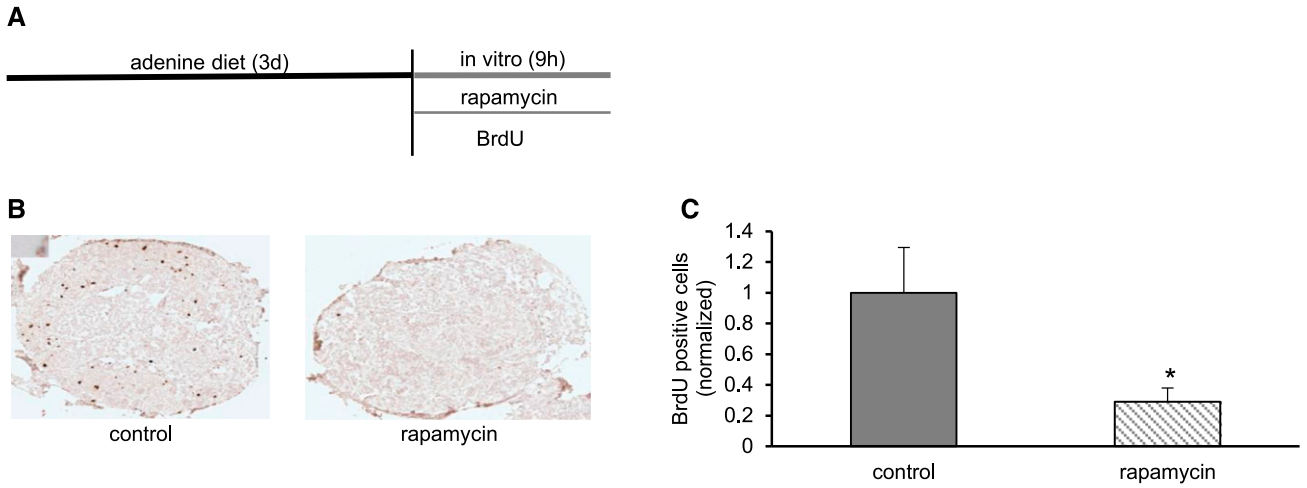

Figure 5. Rapamycin decreases parathyroid cell proliferation in parathyroid glands *in vitro*. (A) Rats were fed an adenine high–phosphorus diet for 3 days, and then, parathyroid glands from single rats were microdissected and incubated in culture with medium containing BrdU and rapamycin (50 nM) or vehicle (DMSO) for 9 hours. (B) Representative immunohistochemical staining for BrdU. (C) Quantification of the number of proliferating cells presented as mean±SEM of BrdU-stained nuclei per total nuclei. *$P<0.05$ versus control (n=7–8).

mTORC1 senses nutritional and growth factors necessary for cell growth and proliferation. Glucose or amino acid starvation results in the repression of mTORC1 activation and subsequently, the activation of downstream autophagy effector proteins.[20] We show that dietary calcium deprivation leads to SHP with activation of mTORC1 and SHP, which may imply an inhibitory effect on autophagy in the parathyroid of hypocalcemic rats. mTORC1 responds to growth factors, such as insulin, IGF-1, and EGF. Increased expression of TGF-α and its receptor, EGFR, was shown in patients with hyperplastic and adenomatous parathyroid glands and uremic rats.[12,15] On activation, EGFR signals through mitogen activated protein kinase activation.[15,39] mitogen activated protein kinase activation induces cyclin D1 and drives the cell cycle from G1 to S.[40] Overexpression of cyclin D1 in the parathyroids induces parathyroid cell hyperplasia.[17] EGFR also activates the AKT-mTORC1 pathway.[41] We show that parathyroid mTORC1 is activated in SHP of both uremia and hypocalcemia and essential for parathyroid cell proliferation. The mTORC1 and EGFR pathways act together to stimulate parathyroid cell proliferation in SHP (Figure 8).

CONCISE METHODS

Animals, Housing, and Diets

Weanling male Sprague–Dawley rats were fed a normal (1% calcium and 0.7% phosphorus) or calcium-depleted (0.02% calcium and 0.3% phosphorus) diet (Harlan Teklad) for 14 days.[42] Adult (150–175 g) male Sprague–Dawley rats were fed a normal or adenine high–phosphorus diet (0.75% adenine and 1.5% phosphorus; Harlan Teklad) for the indicated time periods of 3–14 days.[31]

$rpS6^{P-/-}$ mice were described previously.[28] Genotyping was conducted by PCR of ear or tail DNA using the following primers: 5′-GTCATCCAGCATGGGTGCTG-3′ and 5′-GGCTGATACCTTTTGGGACAG-3′. PCR products were digested with *Eco*RV to show one of five substitutions of serine to alanine.[28]

Uremia was induced by a modification of the casein–based adenine high–phosphorus diet described by Olauson and colleagues.[32] Mice were fed the diet (0.3% adenine, 0.6% calcium, and 1.2% phosphorus; TD.140214; Teklad, Madison, WI) for 21 days, with an interval of 7 days (days 10–15) when they were switched to a 0.2% adenine high–phosphorus diet (0.2% adenine, 0.6% calcium, and 1.2% phosphorus; TD.140213; Teklad). AKI was induced by a single intraperitoneal injection of folic acid (Sigma-Aldrich) at a dose of 240 mg/kg in 0.15 mol/L $NaHCO_3$ (pH 7.4) or vehicle.[36] Animals were euthanized at 20 hours when serum was collected.

Animals had free access to food and drinking water. Blood sampling was performed through the abdominal aorta at euthanasia. Serum was collected and analyzed for creatinine, urea, calcium, phosphate, and PTH levels. Parathyroid tissue was removed for immunohistochemistry, immunofluorescence, and Western blots. All animal experiments were approved by the Hadassah Hebrew University Animal Care and Use Committee.

Rapamycin and BrdU Administration

Rats were injected intraperitoneally with vehicle or rapamycin (LC Laboratories) at 0.2 mg/kg body wt dissolved in DMSO and diluted in normal saline. Rapamycin was injected for 1 or 3 consecutive days before euthanasia at day 14 of the low calcium– or adenine

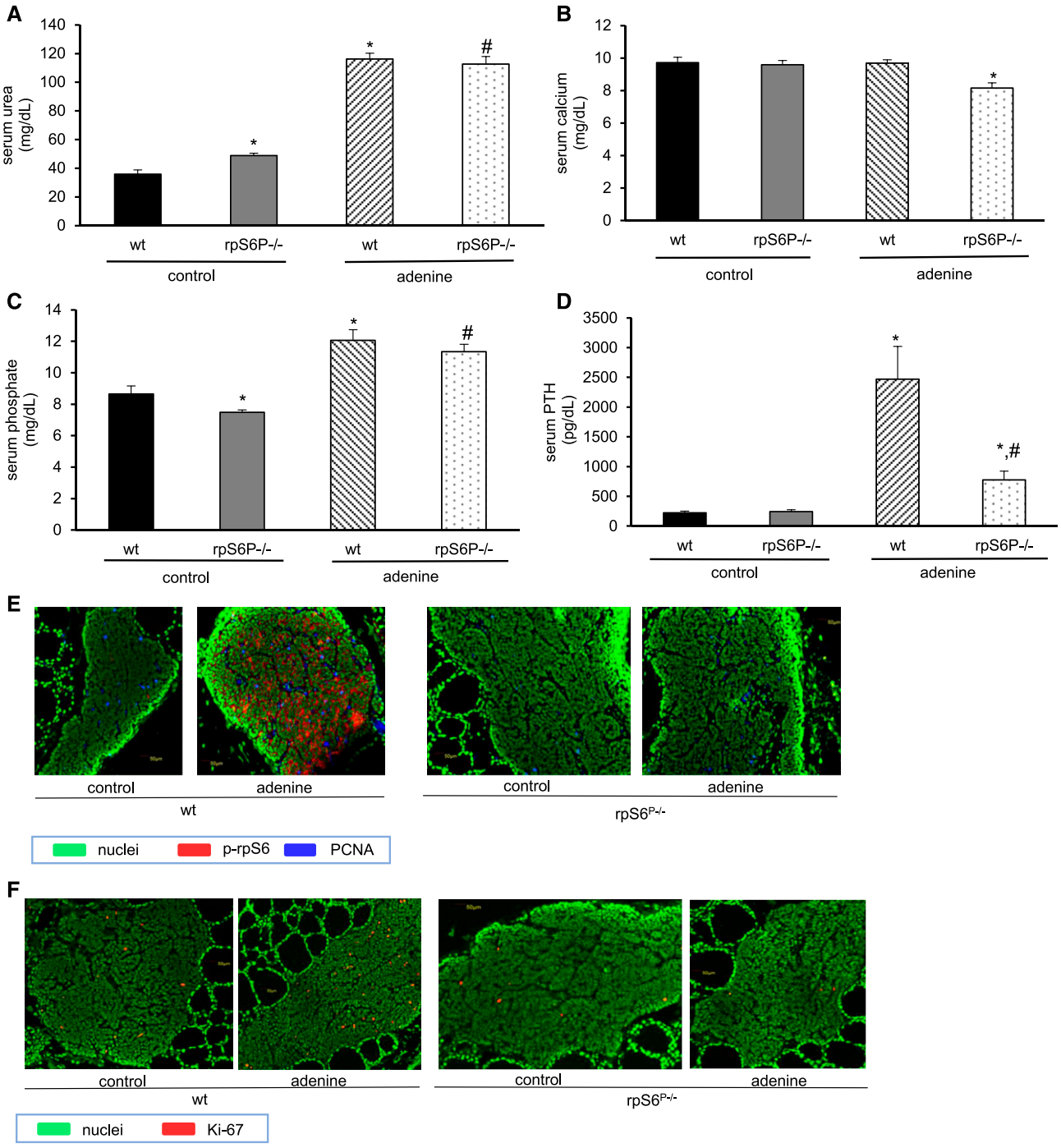

Figure 6. KI rpS6$^{P-/-}$ mice increase serum PTH levels less than wt mice in adenine high phosphorus–induced uremia; wt and KI rpS6$^{P-/-}$mice were fed a control or an adenine high–phosphorus diet for 3 weeks. (A) Serum urea, (B) calcium, (C) phosphate, and (D) PTH in control and uremic wt and rpS6$^{P-/-}$ mice. *P<0.05 versus wt control $^{\#}P$<0.05 versus control rpS6$^{P-/-}$ (n=5, control diet; n=7–8, adenine diet). (E) Representative immunostaining for phosphorylated rpS6 and PCNA, (F) immunostaining for Ki-67, which were both increased in the uremic wt mice and not in the rpS6$^{P-/-}$ mice. Nuclei were stained with sytox. PCNA, proliferating cell nuclear antigen.

high–phosphorus-fed rats. In some experiments, rapamycin was injected daily during the entire duration of the experiment of 3 or 7 days. BrdU (Sigma-Aldrich) was injected (5 mg/100 g body wt of rat) as a single injection or daily for 3 days before euthanasia. Insome experiments, BrdU was dissolved in the drinking water (0.8 mg/ml) for 3 or 7 days. Immunohistochemistry showing

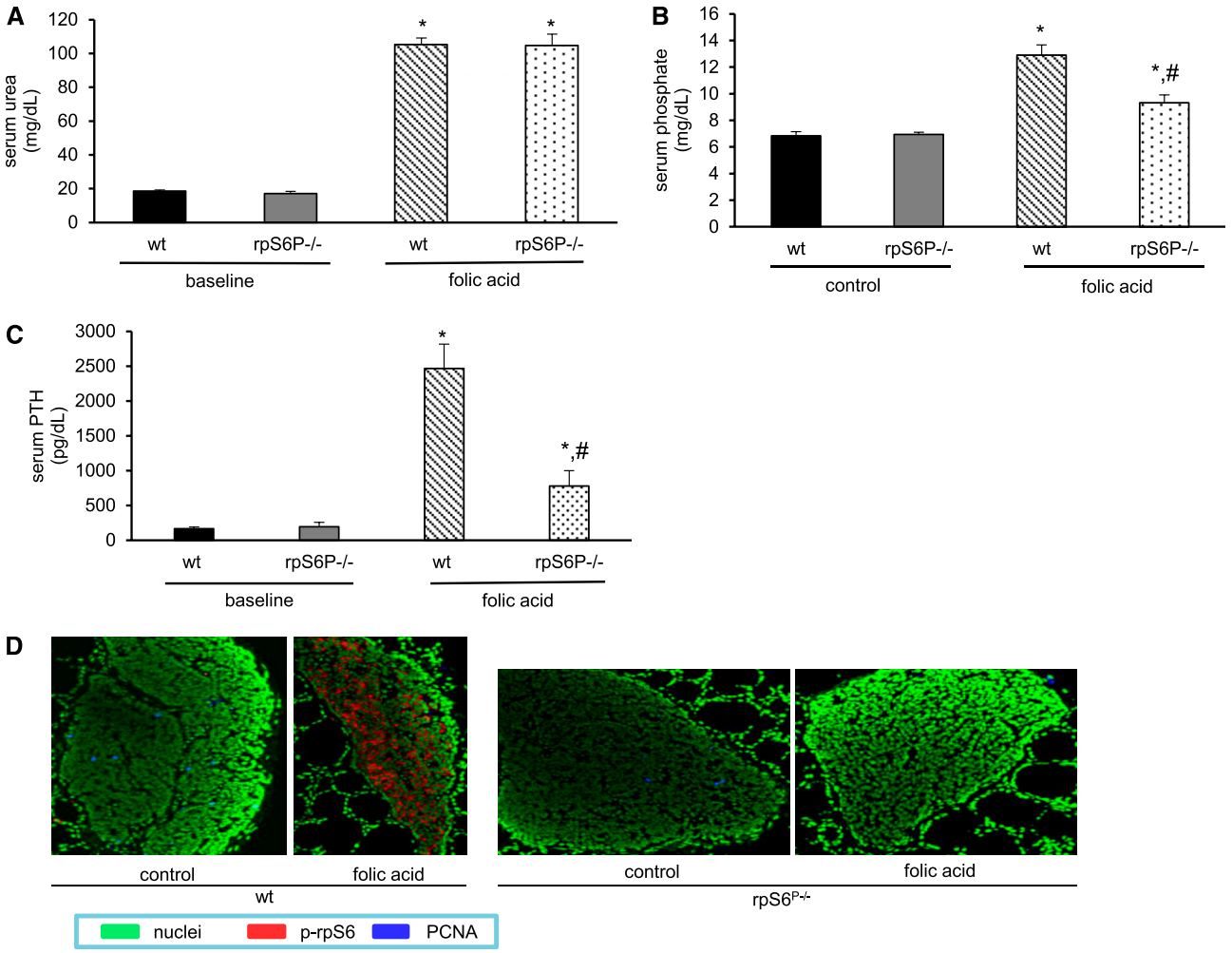

Figure 7. KI rpS6$^{P-/-}$ mice increase serum PTH level less than wt mice in folic acid–induced AKI. Folic acid (240 mg/kg) was injected intraperitoneally into rpS6$^{P-/-}$ and wt mice, and serum was collected 24 hours before (baseline) and at 20 hours after folic acid induction. (A) Serum urea, (B) phosphate, and (C) PTH in wt mice and rpS6$^{P-/-}$ mice. *P<0.05 versus baseline (n=4–6 mice); #P<0.05 versus folic acid wt (n=4–6 mice). (D) Representative immunostaining for phosphorylated rpS6 and PCNA. Nuclei were stained with sytox. PCNA, proliferating cell nuclear antigen.

BrdU incorporation in the intestinal villi served as a positive control (Supplemental Figure 3).

Serum Biochemistry and PTH Levels

Total calcium was measured using a calcium assay kit (QuantiChrom). Serum creatinine phosphorous and urea were measured by kits from Stanbio Laboratory. Serum PTH was measured using a rat or mouse intact PTH ELISA Kit (Immutopics).

Parathyroid Organ Cultures

Adult male Sprague–Dawley rats were fed an adenine high–phosphorus diet for 3 days to induce parathyroid cell proliferation. At day 3 of the diet, parathyroid glands were microdissected, and two glands from each rat were placed in 2-ml ventilated Eppendorf tubes and incubated in 0.5 ml Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1% L-glutamine, and 1% PenStrep (Gibco Life Technologies).[9] BrdU (30 nM) and rapamycin (50 nM) or vehicle (DMSO) were added to the growth medium. The tubes were placed in an incubator under rotation at 37°C for 9 hours; then, parathyroids were removed, and paraffin sections were prepared for immunohistochemistry.

Immunohistochemistry and Its Quantification

Microdissected rat parathyroid glands and mouse thyroparathyroid tissue were excised, fixed in 4% formaldehyde overnight, and embedded in paraffin, and 3 μM sections were prepared. After rehydration, antigens were retrieved by pressure cooker (121°C for 3 minutes) in 20 mM citric acid buffer (pH 6; Invitrogen) for BrdU and 100 mM glycine buffer (pH 9) for Ki-67. Immunostaining was performed overnight at 4°C using the following primary antibodies diluted in Cas block (Zymed Laboratories): BrdU (1:200; Neomarkers), Ki-67 (1:500; Biogenex). Slides were incubated with the appropriate horseradish peroxidase–conjugated

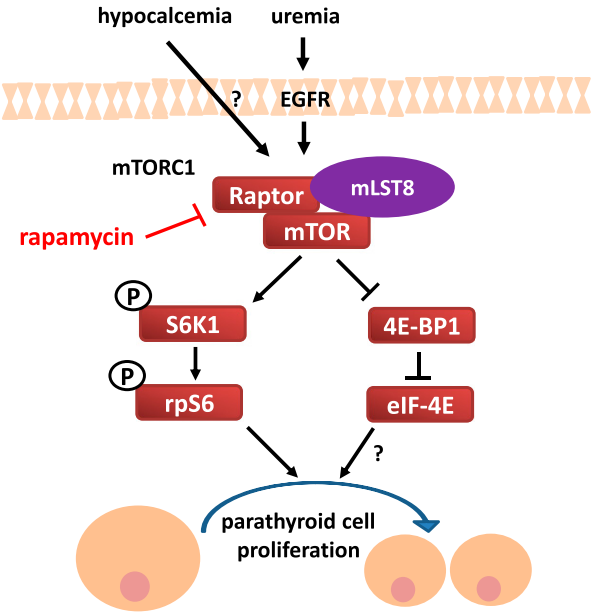

Figure 8. mTORC1 induces parathyroid cell proliferation in SHP through rpS6 phosphorylation. The mTOR pathway is activated in the parathyroids of rats and mice with SHP induced by either chronic hypocalcemia or uremia. On activation, mTORC1 phosphorylates and activates S6K1. Activated S6K1 phosphorylates rpS6. mTORC1 also disinhibits eukaryotic translation initiation factor-4E (eIF-4E) by inhibiting 4E- binding protein 1 (4E-BP1). We show that rpS6 phosphorylation is necessary for the increased parathyroid cell proliferation of SHP. Inhibition of mTORC1 by rapamycin decreased and prevented parathyroid cell proliferation in SHP. TGF-α and its receptor, EGFR, are increased in SHP. EGFR signals through mitogen activated protein kinase and mTORC1 activation.

secondary antibodies and stained by exposure to 3,3′-Diaminobenzidine (DAB) chromogen (Dako) followed by counterstaining with hematoxylin. All images are shown at a magnification of ×20. Immunostaining was quantified using an Ariol Automated System (Genetix). The number of stained nuclei was estimated by calculating the number of positive nuclei per total number of nuclei. This estimate was in excellent agreement with the number of positive nuclei per total area analyzed (not shown).

Immunofluorescence Staining

Slides were processed as above and then incubated overnight with the following primary antibodies: proliferating cell nuclear antigen (1:1000; Zymed Laboratories), p-rpS6 Ser240/244 (1:750; Cell Signaling Technology), and BrdU. Slides were incubated with fluorochrome–conjugated secondary antibodies (Cy3 and Cy5; Bethyl) diluted in 1% BSA in PBS for 2 hours followed by incubation with 0.02 mg/ml RNAse (Sigma-Aldrich) for 15 minutes and then, 25 nM Sytox (Life Technologies) for an additional 30 minute (all at the room temperature in the dark). Images were obtained using a Fluoview 1000 Olympus Fluorescence Microscope.

Western Blotting and Its Quantification

Protein extracts from the two parathyroid glands of each rat were prepared using TRIzol Reagent (Life Technologies). Protein extracts were dissolved in sample buffer (Tris [pH 6.8], glycerol, SDS, β-mercaptoethanol, and bromophenol blue) and run on SDS-PAGE, with each lane containing parathyroid extracts from a single rat. Immunostaining was performed overnight at 4°C using the following primary antibodies diluted in 5% BSA or 5% skim milk: p-rpS6 Ser240/244 (1:500; Cell Signaling Technology), rpS6 (1:1000; Cell Signaling Technology) and S6K1 thr389 (1:1000; Cell Signaling Technology). Membranes were then incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies, exposed to ECL (Santa Cruz Biotechnology), and analyzed by autoradiography. Protein bands were quantified with Quantity One (Bio-Rad).

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DISCLOSURES

J.S. serves on an Amgen advisory committee.

REFERENCES

1. Naveh-Many T, Rahamimov R, Livni N, Silver J: Parathyroid cell proliferation in normal and chronic renal failure rats. The effects of calcium, phosphate, and vitamin D. *J Clin Invest* 96: 1786–1793, 1995
2. Moallem E, Kilav R, Silver J, Naveh-Many T: RNA-Protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate. *J Biol Chem* 273: 5253–5259, 1998
3. Denda M, Finch J, Slatopolsky E: Phosphorus accelerates the development of parathyroid hyperplasia and secondary hyperparathyroidism in rats with renal failure. *Am J Kidney Dis* 28: 596–602, 1996
4. Dusso AS, Pavlopoulos T, Naumovich L, Lu Y, Finch J, Brown AJ, Morrissey J, Slatopolsky E: p21(WAF1) and transforming growth factor-alpha mediate dietary phosphate regulation of parathyroid cell growth. *Kidney Int* 59: 855–865, 2001
5. Wang Q, Palnitkar S, Parfitt AM: Parathyroid cell proliferation in the rat: Effect of age and of phosphate administration and recovery. *Endocrinology* 137: 4558–4562, 1996
6. Garfia B, Cañadillas S, Canalejo A, Luque F, Siendones E, Quesada M, Almadén Y, Aguilera-Tejero E, Rodríguez M: Regulation of parathyroid vitamin D receptor expression by extracellular calcium. *J Am Soc Nephrol* 13: 2945–2952, 2002
7. Lewin E, Garfia B, Recio FL, Rodriguez M, Olgaard K: Persistent downregulation of calcium-sensing receptor mRNA in rat parathyroids when severe secondary hyperparathyroidism is reversed by an isogenic kidney transplantation. *J Am Soc Nephrol* 13: 2110–2116, 2002
8. Gogusev J, Duchambon P, Hory B, Giovannini M, Goureau Y, Sarfati E, Drüeke TB: Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism. *Kidney Int* 51: 328–336, 1997
9. Galitzer H, Ben-Dov IZ, Silver J, Naveh-Many T: Parathyroid cell resistance to fibroblast growth factor 23 in secondary hyperparathyroidism of chronic kidney disease. *Kidney Int* 77: 211–218, 2010

10. Komaba H, Goto S, Fujii H, Hamada Y, Kobayashi A, Shibuya K, Tominaga Y, Otsuki N, Nibu K, Nakagawa K, Tsugawa N, Okano T, Kitazawa R, Fukagawa M, Kita T: Depressed expression of Klotho and FGF receptor 1 in hyperplastic parathyroid glands from uremic patients. *Kidney Int* 77: 232–238, 2010
11. Canalejo R, Canalejo A, Martinez-Moreno JM, Rodriguez-Ortiz ME, Estepa JC, Mendoza FJ, Munoz-Castaneda JR, Shalhoub V, Almaden Y, Rodriguez M: FGF23 fails to inhibit uremic parathyroid glands. *J Am Soc Nephrol* 21: 1125–1135, 2010
12. Gogusev J, Duchambon P, Stoermann-Chopard C, Giovannini M, Sarfati E, Drüeke TB: De novo expression of transforming growth factor-alpha in parathyroid gland tissue of patients with primary or secondary uraemic hyperparathyroidism. *Nephrol Dial Transplant* 11: 2155–2162, 1996
13. Drüeke TB: Cell biology of parathyroid gland hyperplasia in chronic renal failure. *J Am Soc Nephrol* 11: 1141–1152, 2000
14. Cozzolino M, Lu Y, Sato T, Yang J, Suarez IG, Brancaccio D, Slatopolsky E, Dusso AS: A critical role for enhanced TGF-alpha and EGFR expression in the initiation of parathyroid hyperplasia in experimental kidney disease. *Am J Physiol Renal Physiol* 289: F1096–F1102, 2005
15. Arcidiacono MV, Sato T, Alvarez-Hernandez D, Yang J, Tokumoto M, Gonzalez-Suarez I, Lu Y, Tominaga Y, Cannata-Andia J, Slatopolsky E, Dusso AS: EGFR activation increases parathyroid hyperplasia and calcitriol resistance in kidney disease. *J Am Soc Nephrol* 19: 310–320, 2008
16. Arcidiacono MV, Yang J, Fernandez E, Dusso A: Parathyroid-specific epidermal growth factor-receptor inactivation prevents uremia-induced parathyroid hyperplasia in mice. *Nephrol Dial Transplant* 30: 434–440, 2015
17. Imanishi Y, Hosokawa Y, Yoshimoto K, Schipani E, Mallya S, Papanikolaou A, Kifor O, Tokura T, Sablosky M, Ledgard F, Gronowicz G, Wang TC, Schmidt EV, Hall C, Brown EM, Bronson R, Arnold A: Primary hyperparathyroidism caused by parathyroid-targeted overexpression of cyclin D1 in transgenic mice. *J Clin Invest* 107: 1093–1102, 2001
18. Manning BD, Cantley LC: AKT/PKB signaling: Navigating downstream. *Cell* 129: 1261–1274, 2007
19. Wullschleger S, Loewith R, Hall MN: TOR signaling in growth and metabolism. *Cell* 124: 471–484, 2006
20. Shimobayashi M, Hall MN: Making new contacts: The mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol* 15: 155–162, 2014
21. Hay N, Sonenberg N: Upstream and downstream of mTOR. *Genes Dev* 18: 1926–1945, 2004
22. Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM: mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. *Science* 334: 678–683, 2011
23. Meyuhas O: Physiological roles of ribosomal protein S6: One of its kind. *Int Rev Cell Mol Biol* 268: 1–37, 2008
24. Sanders JA, Lakhani A, Phornphutkul C, Wu KY, Gruppuso PA: The effect of rapamycin on DNA synthesis in multiple tissues from late gestation fetal and postnatal rats. *Am J Physiol Cell Physiol* 295: C406–C413, 2008
25. Shahbazian D, Roux PP, Mieulet V, Cohen MS, Raught B, Taunton J, Hershey JWB, Blenis J, Pende M, Sonenberg N: The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. *EMBO J* 25: 2781–2791, 2006
26. Holz MK, Blenis J: Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. *J Biol Chem* 280: 26089–26093, 2005
27. Holz MK, Ballif BA, Gygi SP, Blenis J: mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* 123: 569–580, 2005
28. Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P, Meyuhas O: Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* 19: 2199–2211, 2005
29. Ruvinsky I, Katz M, Dreazen A, Gielchinsky Y, Saada A, Freedman N, Mishani E, Zimmerman G, Kasir J, Meyuhas O: Mice deficient in ribosomal protein S6 phosphorylation suffer from muscle weakness that reflects a growth defect and energy deficit. *PLoS One* 4: e5618, 2009
30. Xu J, Chen J, Dong Z, Meyuhas O, Chen JK: Phosphorylation of ribosomal protein S6 mediates compensatory renal hypertrophy. *Kidney Int* 87: 543–556, 2015
31. Levi R, Ben-Dov IZ, Lavi-Moshayoff V, Dinur M, Martin D, Naveh-Many T, Silver J: Increased parathyroid hormone gene expression in secondary hyperparathyroidism of experimental uremia is reversed by calcimimetics: Correlation with posttranslational modification of the trans acting factor AUF1. *J Am Soc Nephrol* 17: 107–112, 2006
32. Jia T, Olauson H, Lindberg K, Amin R, Edvardsson K, Lindholm B, Andersson G, Wernerson A, Sabbagh Y, Schiavi S, Larsson TE: A novel model of adenine-induced tubulointerstitial nephropathy in mice. *BMC Nephrol* 14: 116, 2013
33. Martins P, Schmitt F, Almeida H, Frazão JM: Evaluation of parathyroid gland angiogenesis in chronic kidney disease associated with secondary hyperparathyroidism. *Nephrol Dial Transplant* 23: 2889–2894, 2008
34. Husi H, Sanchez-Niño MD, Delles C, Mullen W, Vlahou A, Ortiz A, Mischak H: A combinatorial approach of Proteomics and Systems Biology in unravelling the mechanisms of acute kidney injury (AKI): Involvement of NMDA receptor GRIN1 in murine AKI. *BMC Syst Biol* 7: 110, 2013
35. Gupta A, Puri V, Sharma R, Puri S: Folic acid induces acute renal failure (ARF) by enhancing renal prooxidant state. *Exp Toxicol Pathol* 64: 225–232, 2012
36. Christov M, Waikar SS, Pereira RC, Havasi A, Leaf DE, Goltzman D, Pajevic PD, Wolf M, Jüppner H: Plasma FGF23 levels increase rapidly after acute kidney injury. *Kidney Int* 84: 776–785, 2013
37. Mallya SM, Gallagher JJ, Wild YK, Kifor O, Costa-Guda J, Saucier K, Brown EM, Arnold A: Abnormal parathyroid cell proliferation precedes biochemical abnormalities in a mouse model of primary hyperparathyroidism. *Mol Endocrinol* 19: 2603–2609, 2005
38. Harvey SJ, Jarad G, Cunningham J, Goldberg S, Schermer B, Harfe BD, McManus MT, Benzing T, Miner JH: Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. *J Am Soc Nephrol* 19: 2150–2158, 2008
39. Arcidiacono MV, Cozzolino M, Spiegel N, Tokumoto M, Yang J, Lu Y, Sato T, Lomonte C, Basile C, Slatopolsky E, Dusso AS: Activator protein 2alpha mediates parathyroid TGF-alpha self-induction in secondary hyperparathyroidism. *J Am Soc Nephrol* 19: 1919–1928, 2008
40. Terada Y, Inoshita S, Nakashima O, Kuwahara M, Sasaki S, Marumo F: Regulation of cyclin D1 expression and cell cycle progression by mitogen-activated protein kinase cascade. *Kidney Int* 56: 1258–1261, 1999
41. Huse JT, Holland EC: Targeting brain cancer: Advances in the molecular pathology of malignant glioma and medulloblastoma. *Nat Rev Cancer* 10: 319–331, 2010
42. Naveh-Many T, Silver J: Regulation of parathyroid hormone gene expression by hypocalcemia, hypercalcemia, and vitamin D in the rat. *J Clin Invest* 86: 1313–1319, 1990

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