

ORIGINAL ARTICLE

HIF-independent role of prolyl hydroxylases in the cellular response to amino acids

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Hypoxia-inducible factor (HIF) prolyl hydroxylases (PHDs) are α -ketoglutarate (α KG)-dependent dioxygenases that function as cellular oxygen sensors. However, PHD activity also depends on factors other than oxygen, especially α KG, a key metabolic compound closely linked to amino-acid metabolism. We examined the connection between amino-acid availability and PHD activity. We found that amino-acid starvation leads to α KG depletion and to PHD inactivation but not to HIF stabilization. Furthermore, pharmacologic or genetic inhibition of PHDs induced autophagy and prevented mammalian target of rapamycin complex 1 (mTORC1) activation by amino acids in a HIF-independent manner. Therefore, PHDs sense not only oxygen but also respond to amino acids, constituting a broad intracellular nutrient-sensing network.

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INTRODUCTION

Prolyl hydroxylases (PHDs) are considered the oxygen sensors of cells. The cellular response to hypoxia is mediated by the transcription factor hypoxia-inducible factor (HIF)1, constituted by HIF1 α and HIF1 β . In the presence of molecular oxygen, PHD proteins hydroxylate HIF1 α in its oxygen-dependent degradation (ODD) domain allowing for the proteasomal degradation of HIF1 α .^{1–3} During hypoxia, the lack of molecular oxygen leads to the inhibition of PHDs, the consequent stabilization of HIF1 and the expression of its target genes.⁴ In humans, the PHD family comprises three different α -ketoglutarate (α KG)-dependent dioxygenases (PHD1, PHD2 and PHD3), requiring iron and ascorbate as cofactors. For the hydroxylation process, oxygen atoms are obtained from molecular oxygen while α KG provides electrons while being decarboxylated to succinate. First discovered in *Caenorhabditis elegans*, PHDs were later found in many other organisms including mammals, *Drosophila*, *Dictyostelium*, fission yeast and even photosynthetic organisms such as *Chlamydomonas reinhardtii*.^{5–9} The presence of PHD proteins in organisms lacking HIF raises the possibility PHD functions beyond HIF regulation.

It was previously discovered that PHDs are inhibited by their product, succinate, and that mutations in the tumor suppressor succinate dehydrogenase lead to the accumulation of succinate and consequently to HIF1 induction due to the inactivation of PHDs.^{10,11} This effect is reversed by artificially increasing intracellular α KG levels.¹² This finding pointed to the importance of α KG availability for PHD activity.

α KG is a central metabolic hub, involved in several crucial pathways. It participates in the tricarboxylic acid (TCA) cycle and in amino-acid transamination. Furthermore, α KG is the product of glutamine deamination (glutaminolysis), an anaplerotic process, which replenishes the TCA cycle. During glutaminolysis, glutamine is deaminated to α KG in a two steps process catalyzed by

glutaminase and glutamate dehydrogenase, the latter is positively regulated by leucine.¹³ The degradation of other amino acids, such as arginine, histidine or proline, also leads to the production of α KG. Thus, α KG is closely connected to amino-acid metabolism. Indeed, glutamine deprivation causes a marked drop in the levels of different TCA cycle intermediates.¹⁴ As PHDs are strictly dependent on α KG as an electron donor for prolyl hydroxylation, we hypothesized that PHDs may be dependent on amino-acid availability. Acting as α KG sensors, PHDs would thus provide not only oxygen-monitoring activity, but a broader metabolic-sensing system that also monitors the TCA cycle status. To date, it is not well understood how cells sense amino-acid availability, although it is known that, the serine/threonine kinase mammalian target of rapamycin complex 1 (mTORC1) is activated by amino acids.¹⁵

In this work, we demonstrated that both intracellular α KG levels and PHD activity are strictly dependent on amino-acid availability. We also demonstrated that PHD activity is required for mTORC1 activation by amino acids. We therefore linked amino-acid availability and mTORC1 activity via α KG production and PHD activation.

RESULTS

Amino-acid starvation causes depletion of α KG that leads to PHD inactivation

Using the U2OS cell line, we first determined the intracellular α KG levels and PHD activity in response to amino-acid availability. Amino-acid starvation caused a marked decrease in the intracellular levels of α KG (Figure 1a). Interestingly, glucose deprivation did not cause a comparable decrease in α KG (Figure 1a), supporting the notion that amino acids and not glucose are essential for maintaining TCA cycle components by sustaining the levels of α KG.

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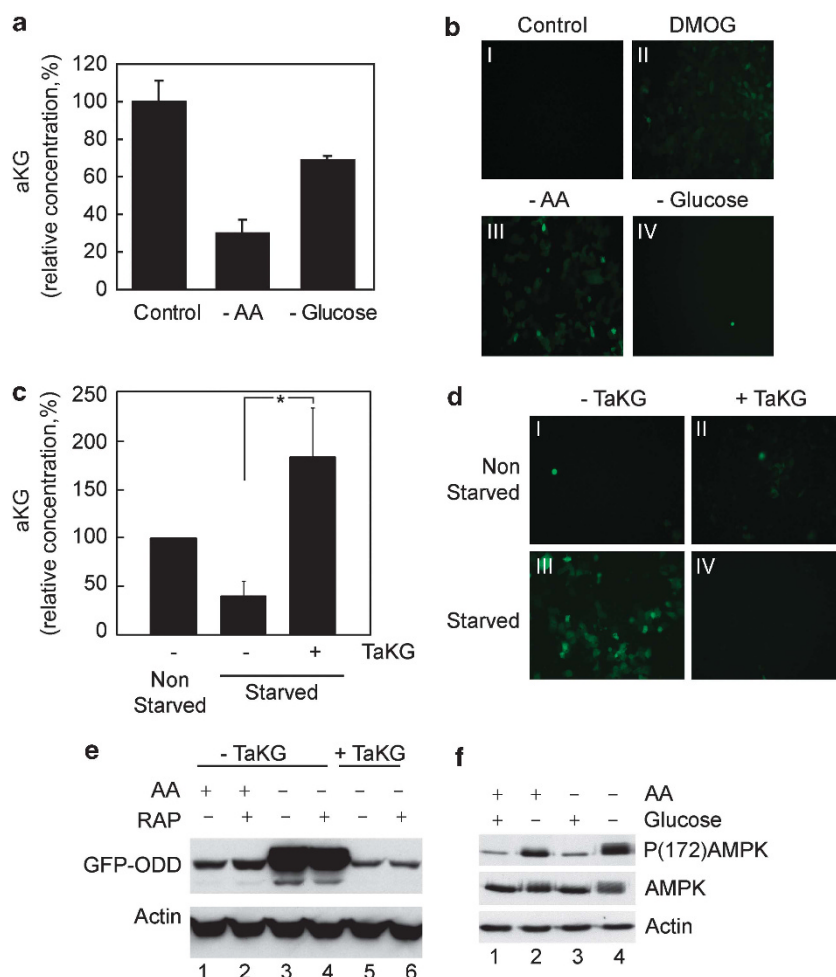


Figure 1. Amino-acid starvation decreases α KG levels and inhibits PHD activity. **(a)** Intracellular α KG levels of U2OS cells after incubation for 3 h under the indicated condition. **(b)** GFP-ODD accumulation in HCT116 under the indicated condition. **(c)** Intracellular α KG content of starved cells treated with T α KG as indicated. Non-starved cells were used as control. **(d, e)** GFP-ODD accumulation examined microscopically **(d)** or by western blot **(e)** in fed and starved cells treated with T α KG and rapamycin (RAP) as indicated. **(f)** Phosphorylation levels of AMPK in U2OS cells incubated in either the presence or absence of amino acids and glucose. Results represent the average and s.d. of three independent experiments (* $P < 0.05$).

As α KG is a co-substrate of PHDs, we next investigated whether amino-acid availability is required for PHD activity. Intracellular PHD activity was monitored using the HCT116 cell line that stably expresses a green fluorescent protein (GFP)-ODD fusion protein,¹² the levels of which inversely correlate with PHD activity. In the presence of amino acids, GFP-ODD levels were low, confirming high PHD activity under normal nutritional conditions (Figure 1b, i). After inhibition of PHDs using dimethyloxallyl glycine (DMOG) GFP-ODD levels accumulated (Figure 1b, ii). In accordance with the observed changes in α KG levels (Figure 1a), GFP accumulated only after amino acid but not glucose starvation (Figure 1b, iii–iv) in a dose-dependent manner (Supplementary Figures S1A, B). We therefore concluded that PHD inactivation correlates with α KG reduction during amino-acid starvation.

As amino acids control intracellular α KG levels, we investigated whether PHD inactivation observed after amino-acid withdrawal is a direct consequence of the drop in α KG. For this purpose, the 1-trifluoromethylbenzyl- α KG ester (T α KG), a cell permeable ester of α KG,¹² was utilized. The addition of T α KG to U2OS cells replenished α KG levels during amino-acid deprivation (Figure 1c) and restored low GFP-ODD levels (Figures 1d, e). This effect of T α KG was independent of mTORC1 activity, as rapamycin did not alter the GFP-ODD degradation upon T α KG addition (Figure 1e). Similar results were obtained in U2OS cells transiently expressing

GFP-ODD (Supplementary Figure S1C). These results confirmed that reduction of PHD activity after amino-acid withdrawal is indeed caused by the loss of α KG.

Amino-acid starvation may have an impact on mitochondrial functions causing changes in the energetic status of cells. Such a bioenergetic stress could signal to PHDs. Indeed, amino-acid-starved cells demonstrated reduced mitochondrial-dependent respiration and ATP levels, however, the addition of α KG to the amino-acid-starved cells did not rescue cells from this bioenergetic deficit (Supplementary Figure S2). Furthermore, unlike glucose, amino-acid starvation did not increase the phosphorylation status of AMP-activated protein kinase (AMPK) (Figure 1f), an energy-sensor enzyme, which is phosphorylated during bioenergetic failure.¹⁶ These results further confirmed that the inactivation of PHDs following amino-acid starvation was not mediated by the bioenergetic status of the cell.

Loss of PHD activity during amino-acid starvation does not activate HIF

The best characterized PHD targets are the HIF α proteins, which accumulates under hypoxia. Therefore, we studied whether inactivation of PHDs during amino-acid deprivation leads to the accumulation of HIF α and to an increase in HIF transcriptional

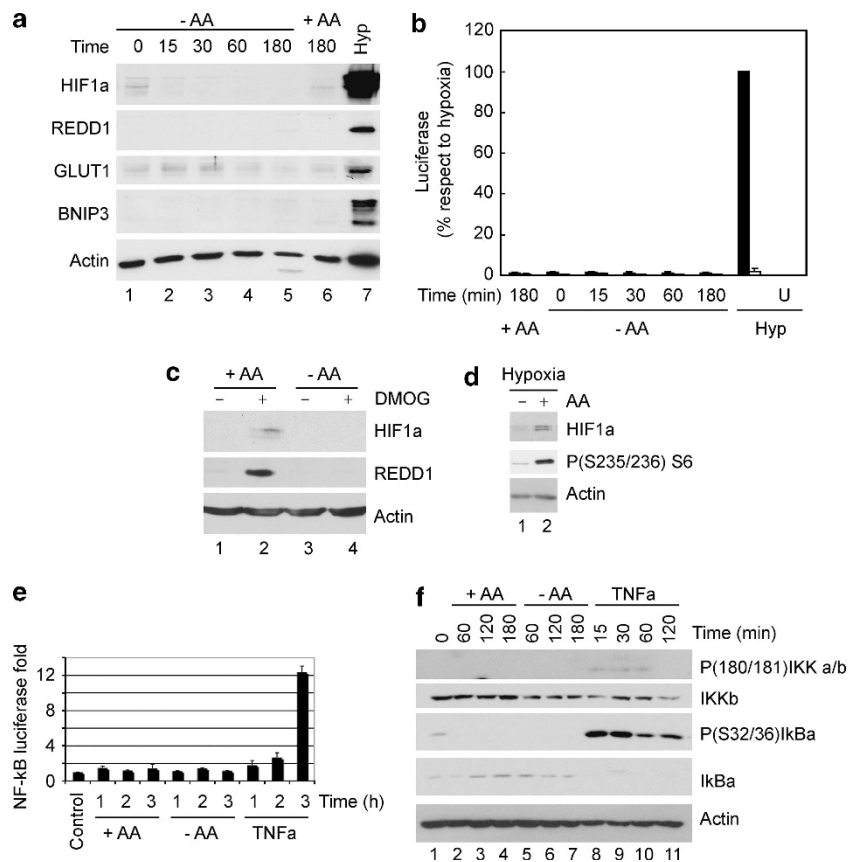


Figure 2. Inactivation of PHDs during amino-acid starvation does not activate HIF. **(a)** Protein levels of HIF1 α , REDD1, GLUT1 and BNIP3 in U2OS cells incubated in the presence or absence of amino acids for the indicated time. A positive control of cells incubated under hypoxic conditions for 12 h (Hyp) was included. **(b)** U2OS cells expressing a construct containing either a wild-type HRE-luciferase reporter (black bars) or a mutant HRE-luciferase reporter unable to respond to HIF1 α (white bars) were incubated in the presence or absence of amino acids for the indicated time and the luciferase intensity was analyzed using a luminometer (values represent average and s.e. of three independent experiments). A positive control of cells incubated in hypoxic conditions for 12 h was included (U = untransfected). **(c)** Protein levels of HIF1 α and REDD1 in U2OS cells incubated in fed or starving conditions for 2 h followed by 30 min of incubation in the presence or absence of DMOG. **(d)** U2OS cells where incubated in hypoxia (1% O₂) in starving conditions for 2 h followed by 1 extra hour of incubation in the presence or absence of amino acids. HIF1 α accumulation and S6 phosphorylation were then analyzed by western blot. **(e, f)** U2OS cells stably expressing NF- κ B-reporter construct pGL4.32[luc2P/NF- κ B-RE/Hygro] were incubated for the indicated time in KRBB either in the presence or absence of all amino acids or tumor necrosis factor- α (10 ng/ml). Activation of NF- κ B was analyzed by luminescence analysis of the NF- κ B-luciferase reporter **(e)** and by phosphorylation of I κ B kinase (IKK) and I κ B- α by western blot **(f)**. Graph depicts mean and s.d. of three independent experiments and represents fold activation compared with untreated samples.

activity. Despite the inactivation of PHDs (Figures 1a–e), no accumulation of HIF1 α was observed under amino-acid starvation (Figure 2a). Analysis of various HIF target genes (*REDD1*, *GLUT1* and *BNIP3*) as well as of HIF transcriptional activity (using a luciferase reporter) did not reveal changes in HIF activity (Figures 2a, b). This suggests that despite the fact that PHDs are inhibited after amino-acid starvation, HIF does not accumulate, likely due to a decrease in protein translation.¹⁷ Indeed, the inhibition of the proteasome using MG132 did not induce HIF1 α accumulation during amino-acid starvation (Supplementary Figure S3A). In support of this notion, HIF1 α and REDD1 levels were not increased by DMOG or hypoxia when cells were starved for amino acids (Figures 2c, d). The transcription factor nuclear factor (NF)- κ B is an additional target of PHDs.¹⁸ However, amino-acid withdrawal did not cause any change in NF- κ B signaling (Figures 2e, f). Therefore, we concluded that neither HIF nor NF- κ B is activated upon PHD inhibition in response to amino-acid starvation.

PHD inhibition induces autophagy independently of HIF

The role of PHDs in the biological response to nutrients deprivation was next analyzed. As amino-acid starvation is known

to induce autophagy,¹⁹ and the activity of PHDs is inhibited in the absence of amino acid, a relation between PHD inhibition and autophagy was investigated, using the aggregation of the LC3–GFP fusion protein in autophagosomes as a reporter. Indeed, inhibition of PHDs by DMOG for 5 h in U2OS cells was sufficient to markedly induce autophagy to a similar extent as amino-acid starvation (Figures 3a, b and Supplementary Figures S3B, C). As we did not observe HIF accumulation after PHD inhibition during amino-acid starvation, it is unlikely that the corresponding autophagy mediated by PHD inhibition is HIF dependent. Indeed, efficient silencing of HIF activity (Supplementary Figure S4) did not reduce the ability of the PHD inhibitor DMOG to induce autophagy (Figures 3a, b).

Reduction of intracellular α KG levels during amino-acid starvation correlates with mTORC1 inhibition

The data presented so far position the α KG-dependent activity of PHDs as a sensor of amino acids. Therefore, we next investigated whether the reduction in intracellular levels of α KG correlates with the well-characterized inhibition of mTORC1 during amino-acid starvation. As shown in Figures 4a, b, the

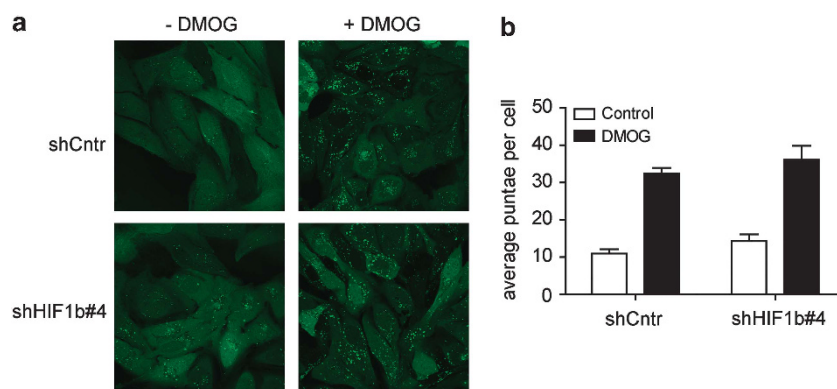


Figure 3. Inhibition of PHD by DMOG induces autophagy independently of HIF. U2OS cells stably expressing GFP-LC3 were transfected with a plasmid encoding control, nontargeting shRNA (shCntr) or transfected with a plasmid encoding shRNA for HIF1b (clone no. 4; shHIF1b no. 4). After 48 h, cells were incubated for 5 h in the presence of amino acids with or without DMOG as indicated. Aggregation of GFP-LC3 was analyzed by fluorescence microscopy (a) and quantified (b). Results represent the average and s.d. of three independent experiments.

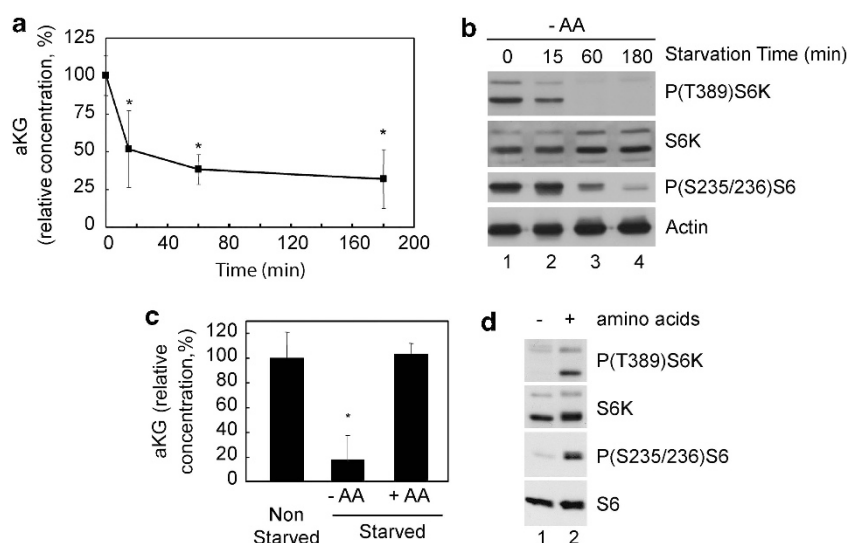


Figure 4. Reduction of intracellular α KG during amino-acid starvation correlates with mTORC1 inhibition. (a, b) Intracellular α KG levels (a) and phosphorylation status of S6K and S6 proteins (b) in U2OS cells at different time points after incubation without amino acids. (c, d) Intracellular α KG levels (c) and phosphorylation status of S6K and S6 proteins (d) in U2OS cells under normal (non-starved) conditions, under starvation for amino acids for 3 h (– AA) or under starvation for amino acid for 2 h followed by re-addition of amino acids for 1 h (+ AA).

kinetics of the reduction of intracellular α KG levels upon amino-acid withdrawal correlates with the inhibition of mTORC1 (measured by the phosphorylation status of its target proteins S6K and S6). Furthermore, re-addition of amino acids to amino-acid-starved cells restored both α KG levels and mTORC1 activity (Figures 4c, d). As leucine is a key amino acid in the regulation of mTORC1,²⁰ we also investigated the reduction of α KG levels after leucine starvation only. Similar to our observations with complete amino-acid starvation, leucine deprivation resulted in a reduction in α KG that correlated with mTORC1 inhibition (Supplementary Figures S5A, B). This result positioned leucine as a critical amino acid for sustaining α KG production. In support of this, the inhibition of α KG production by 6-diazo-5-oxo-L-norleucine, a glutaminase inhibitor, which blocks α KG production from glutamine, also impaired the activation of mTORC1 by leucine and glutamine (Supplementary Figure S5C). Furthermore, starving U2OS cells of glutamine reduced the intracellular levels glutamate and glutamine, but it had no effect on the levels of intracellular leucine or any other amino acid (Supplementary Figure S6). Therefore, at least in U2OS cells, glutamine is required to sustain

the intracellular levels of α KG, but not of leucine, contrary to a previous report.²¹

Inhibition of PHDs blocks the response of mTORC1 to amino acids. We next studied whether PHD activity is required for mTORC1 activation by amino acids. Inhibition of PHDs by DMOG blocked the ability of amino acids to re-activate mTORC1, as demonstrated by the lack of phosphorylation of the mTORC1 target proteins S6K, S6 and 4EBP1 (Figures 5a, b). Similar results were obtained using cobalt chloride (Figure 5b), a different inhibitor for PHDs. To further substantiate these results, PHDs were silenced with three different shRNAs, efficiently targeting each of the PHDs (Supplementary Figure S7). Although individual silencing of each of the PHDs did not block mTORC1 activation by amino acids (Supplementary Figures S8A–C), the silencing of all three PHDs simultaneously caused a marked inhibition in ribosomal S6 and S6K phosphorylation in response to amino acids (Figures 5c, d). Therefore, the results obtained with both chemical and genetic inhibition of PHDs strongly suggest that PHD activity is required

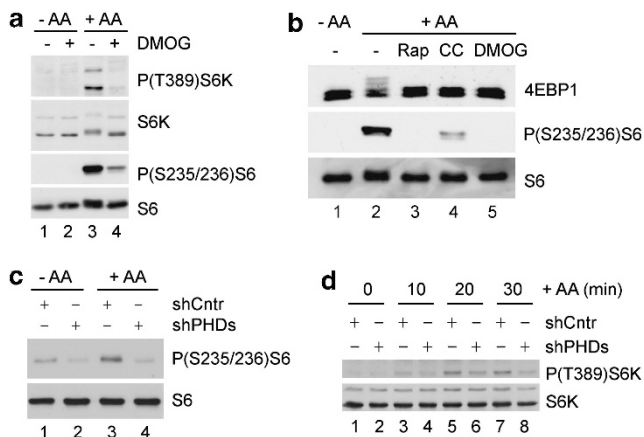


Figure 5. Inhibition of PHDs blocks the response of mTORC1 to amino acids. (a, b) Phosphorylation state of S6K, S6 and 4EBP1 in cells starved for 2 h and then re-fed with amino acids for one more hour in the presence or absence of DMOG, rapamycin (Rap) or cobalt chloride (CC). (c) U2OS cells were transfected with a plasmid encoding control, nontargeting shRNA (shCntr) or cotransfected with three different plasmids encoding shRNAs for each member of the PHD family (shPHDs). After 48 h, cells were starved for amino acids for 2 h and then incubated for 20 min with (+ AA) or without (– AA) amino acids. The phosphorylation state of S6 was analyzed by western blot. (d) Cells were transfected as in (c) and starved for amino acids for 2 h and then incubated for the indicated time with amino acids (+ AA). The phosphorylation state of S6K was analyzed by western blot.

for mTORC1 activation by amino acids. These results also propose a partial redundancy between all three PHDs in their response to amino acids and in mediating mTORC1 activation.

PHDs signal to mTORC1 via the Rag GTPases

As we demonstrated that HIF was not affected by PHD inhibition during amino-acid starvation, we hypothesized that mTORC1 downregulation during PHD inhibition is independent of HIF, which can inhibit mTORC1 via the activation of the TSC1/2 complex.²² To test this possibility HIF activity was efficiently silenced, as judged by the lack of REDD1 induction by DMOG in the presence of amino acids (Figure 6a) and as expected, HIF silencing did not prevent the inhibition of mTORC1 by DMOG (Figure 6a). Therefore, we concluded that PHDs are required to maintain mTORC1 in a HIF-independent manner. Furthermore, DMOG treatment prevented mTORC1 activation by amino acids in both TSC2^{+/+} and TSC2^{-/-} mouse embryonic fibroblasts (Figure 6b). In contrast, the expression of a constitutively active mutant of Rheb, which activates mTORC1 even in the absence of amino acids,²³ also induced S6K and S6 phosphorylation in the presence of DMOG (Figure 6c). These results suggest that PHDs signal toward mTORC1 through the amino-acid-signaling pathway, which involves the Rag small GTPases,²⁴ and not through the TSC1/2 pathway. Indeed, the expression of a constitutively active Rag also rescued mTORC1 activation in starved cells both in the presence or absence of DMOG (Figure 6d). Altogether, this work suggests that α KG-dependent activity of PHDs is necessary for mTORC1 activation by amino acids through the Rag GTPases and in a HIF/TSC-independent manner.

DISCUSSION

We demonstrate here that the ability of amino acids to induce mTORC1 signaling strictly requires PHD enzymatic activity. We show that α KG is an intracellular indicator of amino-acid

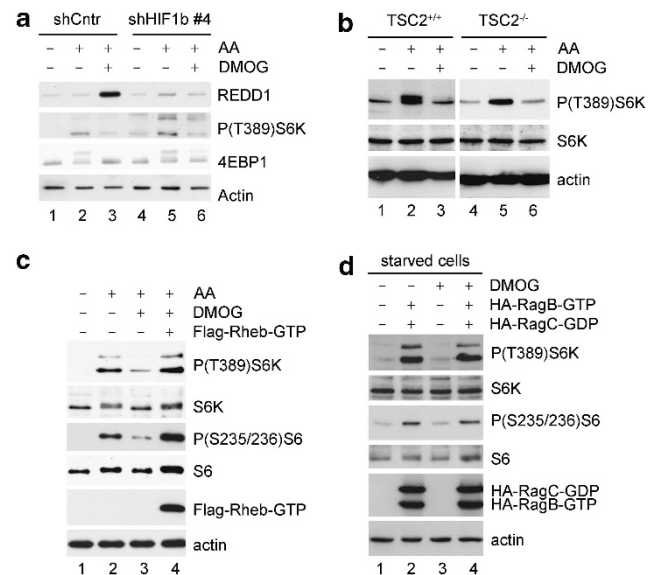


Figure 6. PHDs signal to mTORC1 in a HIF/TSC-independent manner. (a) U2OS cells were transfected with a plasmid encoding control, nontargeting shRNA (shCntr) or transfected with a plasmid encoding shRNA for HIF1b (clone no. 4; shHIF1b no. 4). After 48 h, cells were starved for amino acids for 2 h and then incubated for 20 min with (+ AA) or without (– AA) amino acids either in the presence or absence of DMOG. Accumulation of REDD1 and phosphorylation state of S6K and 4EBP1 were analyzed by western blot. (b) Phosphorylation status of S6K in TSC2^{+/+} and TSC2^{-/-} mouse embryonic fibroblasts starved for 2 h and then re-fed with amino acids for one more hour in the presence or absence of DMOG. (c) U2OS cells were transfected with either empty vector or Flag-Rheb-GTP, as indicated. After 48 h, cells were amino-acid starved for 2 h and re-stimulated for 1 h with amino acids (AA) either in the presence or absence of DMOG, as indicated. Phosphorylation of S6K and S6 was then measured by immunoblotting. (d) U2OS cells were transfected with either empty vector or with HA-RagB-GTP plus HA-RagC-GDP, as indicated. After 48 h, cells were amino-acid starved for 1 h where indicated, DMOG was added 30 min before the collection of the cells. Phosphorylation of S6K and S6 was then measured by immunoblotting.

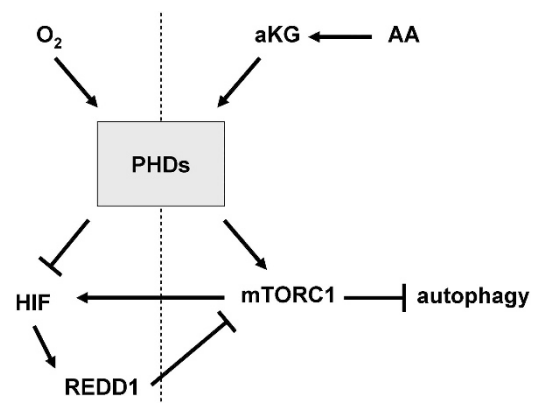


Figure 7. Model for oxygen- and nutrient-sensing mechanisms by PHDs. Left side: PHDs mediate the inhibition of HIF in the presence of oxygen. Under hypoxia, lack of oxygen inhibits PHD activity, resulting in the stabilization of HIF, which inhibits mTORC1 via REDD1 induction. Right side: PHDs are required for the activation of mTORC1 in the presence of amino acids. Under amino-acid starvation, lack of α KG inhibits PHD activity, resulting in the inhibition of mTORC1, hence a reduction in HIF expression. Loss of PHD activity can also mediate the induction of autophagy under amino-acid starvation conditions in a HIF-independent manner.

availability, and it transduces the information to PHDs, which in turn, are required for mTORC1 pathway activation. Importantly, in a recent report we also demonstrated that α KG production through glutaminolysis is a necessary step for the activation of mTORC1 by leucine and glutamine.²⁵ The results in this study confirm and extend this previous report. Of note, the estimated *in vitro* K_M of PHDs for α KG is $\sim 50 \mu\text{M}$,²⁶ while we previously calculated an intracellular α KG concentration in steady state in the range of $100 \mu\text{M}$,²⁷ indicating that PHDs are good candidates to sense α KG oscillations. In this model, PHD enzymes do not function solely as intracellular oxygen sensors, but also as nutrient sensors (Figure 7). As the effect of amino acids on mTORC1 was severely hindered by DMOG or PHDs silencing, it appears that PHD activity is necessary for a complete mTORC1 response to amino acids. Interestingly, PHD1 regulates energy metabolism in muscle²⁸ in a similar manner to mTORC1.²⁹ Furthermore, like dTOR, the *Drosophila* homolog of mTOR,³⁰ the *Drosophila* PHD is also required for cell growth.³¹ These observations genetically position PHDs and mTORC1 in a similar metabolic axis and together with our new data they propose a biological role of PHDs in turning nutrient availability or scarcity to signals that control cell growth or autophagy.

HIF α is the most-studied substrate of PHD enzymes, yet we have shown that HIF is not involved in the amino-acid-sensing process. The direct substrates of PHDs in the amino-acid-sensing pathway are yet to be identified. Nevertheless, the observation that PHDs clearly respond to amino acids indicates that these enzymes have important roles in metabolic control. The involvement of PHDs in the oxygen- and amino-acid-sensing mechanisms designates them as major cellular metabolic sensors. This dual role for PHDs raises the question how cells distinguish between oxygen- and amino-acid-limiting conditions. In fact, inhibition of PHDs under hypoxia leads to the accumulation of HIF1, which inhibits mTORC1 through REDD1 induction.³² On the other hand, PHD inhibition by amino-acid starvation leads to reduced mTORC1 activity, which prevents HIF1 α translation.³³ This feedback loop prevents HIF activation under amino-acid starvation, despite the fact that PHDs are inactivated.

In conclusion, we propose that PHD proteins function as general metabolic sensors in the cell, signaling scarcity not only of oxygen but also of amino acids and forming a link between amino-acid availability and the mTORC1 pathway.

MATERIALS AND METHODS

Reagents

Reagents were obtained from the following sources: Antibodies against ribosomal S6, phospho-S235/236 S6, S6K1, phospho-T389 S6K1, 4EBP1, AMP-activated protein kinase, phospho-T172 AMP-activated protein kinase, I κ B kinase β , phospho-S180/S181 I κ B kinase α/β , I κ B α and phospho-S32/36 I κ B α were obtained from Cell Signalling Technology, Beverly, MA, USA; anti-GFP and anti-HIF1 α were obtained from BD Biosciences, Franklin Lakes, NJ, USA; anti-actin, anti-tubulin and anti-BNIP3 were obtained from Sigma (Gillingham, UK); antibody against REDD1 was obtained from the ProteinTech Group, Chicago, IL, USA; anti-Glut1 was obtained from Abcam, Cambridge, UK; antibody against PHD1 was obtained from Novus, Littleton, CO, USA; PHD2 and PHD3 antibodies were kindly provided by Kevin Gatter; all horseradish peroxidase-conjugated secondary antibodies were from Cell Signalling Technology; every antibody was used at the manufacturer's recommended dilution. T α KG was obtained as described previously.¹² 6-Diazo-5-oxo-L-norleucine, CoCl₂, DMOG and rapamycin were from Sigma.

Plasmids, shRNAs and transfections

The plasmid pGL2/hypoxia responsive element (HRE)-luciferase was described elsewhere.¹⁰ pFLAG-PHD2 was obtained from Origene, Rockville, MD, USA. For knockdown experiments, pLKO.1-derived plasmids containing shRNA for PHD1 (RHS3979-9589735), PHD2 (RHS3979-9569473), PHD3 (RHS3979-9569477) and HIF1 β /ARNT (RHS4533-NM_178427) were purchased from

Open Biosystems, Lafayette, CO, USA. pLKO.1 plasmid containing control shRNA (Addgene plasmid 1864, Cambridge, MA, USA), plasmid expressing HA-RagB-Q99L (RagB-GTP; Addgene plasmid 19303) and plasmid expressing HA-RagC-S75L (RagC-GDP; Addgene plasmid 19305) were kindly provided by David M Sabatini.³⁴ Plasmid expressing FLAG-Rheb-N153T (RhebGTP; Addgene plasmid 19997) was kindly provided by Fuyuhiko Tamanoi. Cells were transfected with lipofectamine (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Cell culture

U2OS, HeLa and mouse embryonic fibroblasts (both TSC2+/+ and TSC2-/-) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. HCT116 cells stably expressing GFP-ODD (CL18 cells) were described previously.²⁷ CL18 cells were cultured in RPMI medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. For amino-acid starvation, cells were incubated in Krebs-Ringer Bicarbonate Buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 25 mM, NaCl 111 mM, NaHCO₃ 25 mM, KCl 5 mM, CaCl₂ 2.5 mM and MgCl₂ 1 mM) supplemented with glucose (4.5 g/l). Standard starvation time was 2 h, unless otherwise indicated. When required, cells were re-fed with a solution with all essential and nonessential amino acids (Invitrogen) during the indicated time. For T α KG re-stimulation purposes, these compounds were added to a final concentration of 1 mM for the indicated time. Inhibitors such as DMOG (1 mM final concentration), CoCl₂ (0.5 mM final concentration), 6-diazo-5-oxo-L-norleucine (40 μM final concentration) or rapamycin (50 nM final concentration) were added 1 h before the re-stimulation with amino acids. Hypoxia was achieved using a humidified, Invivo2 500 hypoxic chamber (Biotrace, Bridgend, UK) and Ruskin hypoxic gas mixer at 37 °C with 1% O₂, 5% CO₂ and 94% N₂. For U2OS cells stably expressing the NF- κ B-luciferase reporter, U2OS cells were transfected with the 2 μg of NF- κ B-reporter construct pGL4.32[luc2P/NF- κ B-RE/Hygro] (Promega, Madison, WI, USA) using genejuice (Merck Biosciences, Darmstadt, Germany). After 48 h, cells were split and cultivated under 300 $\mu\text{g}/\text{ml}$ Hygromycin B (Roche, Indianapolis, IN, USA). Individual clones were picked and selected based on their response to tumor necrosis factor- α .

Protein analysis

For western blot analysis, cells were extracted in Laemmli sample buffer. Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis, proteins were blotted onto nitrocellulose and analyzed with the appropriate antibody, using the ECL kit (GE Healthcare, Piscataway, NJ, USA) for detection.

Measurement of α -ketoglutarate

Intracellular levels of alpha-ketoglutarate were determined by following the method described previously.¹² U2OS cells were washed with phosphate-buffered saline and lysed with RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X 100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate supplemented with protease inhibitor cocktail (Invitrogen)). Cell lysates were collected from the plate, vortexed vigorously and centrifuged for 5 min at $15\,000 \times g$ at 4 °C. Aliquots of the extracts were analyzed immediately for alpha-ketoglutarate. The assay solution consisted of 100 mM KH₂PO₄ (pH 7.2), 10 mM NH₄Cl, 5 mM MgCl₂ and 0.15 mM nicotinamide adenine dinucleotide (Roche). Following equilibration at 37 °C with extract, the reaction was started by the addition of 5 units of glutamate dehydrogenase (Sigma). The absorbance decrease was monitored at 340 nm. The intracellular level of α -ketoglutarate was determined from the absorbance decrease in nicotinamide adenine dinucleotide (extinction coefficient, 6.22/mm/cm).

Measurement of oxygen consumption rate

A total of 3×10^4 cells were plated onto XF24 plates (Seahorse Bioscience, North Billerica, MA, USA) and incubated at 37 °C, 5% CO₂ for 24 h. The medium was then replaced with 675 μl of unbuffered Dulbecco's modified Eagle's medium (Seahorse Bioscience) supplemented with 2 mmol glutamine, 25 mmol glucose and 2% fetal bovine serum (pH was adjusted to 7.4 using sodium hydroxide 0.5 M) and cells were incubated at 37 °C in a CO₂-free incubator. Basal oxygen consumption rate was then recorded using the XF24 plate reader (Seahorse Bioscience). Every cycle of measurement consisted of 3 min mix, 3 min wait and 4 min measure. Oxygen consumption rate was normalized to cell number calculated

at the end of the experiments. Homogeneous plating of the cells and cell count was obtained by fixing the cells with trichloroacetic acid 10% for 1 h at 4 °C and then staining the fixed cells with 0.47% solution of Sulforhodamine B (Sigma).

Measurement of intracellular ATP levels

A total of 2×10^5 cells were grown on six well plates and treated as indicated. ATP was measured from the cell lysates of the same samples using a luciferase-based assay as per manufacturer's instructions (Sigma). Data were normalized to total protein contents of the cell lysate determined by Bicinchoninic Acid Assay (ThermoScientific, Rockford, IL, USA) using bovine serum albumin as standard.

Luciferase assay

For HRE-luciferase assays, U2OS cells were transfected with either wild type or mutant pGL2/HRE-luciferase 48 h before start of the experiment. Luciferase intensity was determined using the Luciferase Assay System (Promega) by following manufacturer's instruction and using a VeritasMicroplateLuminometer (Turner Biosystems, Madison, WI, USA). For NF- κ B-luciferase assay, cells were processed as described previously³⁵ using the Luciferase Assay System (Promega).

Measurement of intracellular content of amino acids

U2OS cells were plated in six wells plates (8×10^5 cells per well). After 24 h, cells were rapidly washed with phosphate-buffered saline and incubated for the indicated times in Krebs-Ringer Bicarbonate Buffer supplemented with glucose, essential and nonessential amino acids (Invitrogen), in the presence or absence of 2 mM glutamine. At the end of the treatments, the intracellular content of amino acids was determined as described previously³⁶. Briefly, cells were washed twice with ice cold phosphate-buffered saline, extracted for 5 min on ice with 1 ml of a 6.6-mM acetic acid solution in ethanol, and analyzed with a Biochrom 20 Amino Acid Analyzer (Biochrom, Cambridge, UK) using a High-Resolution Column Bio 20 Peek Lithium and the Physiological Fluid Chemical Kit (Biochrom) for elution. The column effluent was mixed with ninhydrin reagent, passed through the high temperature reaction coil and read by the photometer unit. Data are normalized on a standard curve and expressed as nmol/mg of protein.

Autophagy measurements

U2OS cells stably expressing LC3-GFP were cultured in 12-well glass-bottomed dishes. After 5 h, treatment in starving conditions either in the presence or absence of DMOG, GFP localization was detected using a confocal microscopy using a SP2 DMIRBE laser scanning confocal microscope with SP2 software (Leica, Wetzlar, Germany). GFP aggregation was then quantified.

GFP-ODD quantification

Fluorescence in HCT116 cell line stably expressing a GFP-ODD fusion protein was analyzed directly in culture using an Olympus CX41 Fluorescent Tissue Culture Microscope (Olympus, Tokyo, Japan).

Statistical analyses

Data were expressed as average \pm s.d. of at least three independent experiments. Student's *t*-test was used to determine differences among two groups. Significance was judged when $P < 0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

- Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* 2004; **5**: 343–354.
- Kaelin WG. Proline hydroxylation and gene expression. *Annu Rev Biochem* 2005; **74**: 115–128.
- Berra E, Ginouves A, Pouyssegur J. The hypoxia-inducible-factor hydroxylases bring fresh air into hypoxia signalling. *EMBO Rep* 2006; **7**: 41–45.
- Kaelin Jr. WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* 2008; **30**: 393–402.
- Taylor MS. Characterization and comparative analysis of the EGLN gene family. *Gene* 2001; **275**: 125–132.
- Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR *et al*. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 2001; **107**: 43–54.
- West CM, van der Wel H, Wang ZA. Prolyl 4-hydroxylase-1 mediates O₂ signaling during development of Dictyostelium. *Development* 2007; **134**: 3349–3358.
- Hughes BT, Espenshade PJ. Oxygen-regulated degradation of fission yeast SREBP by Ofd1, a prolyl hydroxylase family member. *EMBO J* 2008; **27**: 1491–1501.
- Keskiaho K, Hieta R, Sormunen J, Myllyharju J. *Chlamydomonas reinhardtii* has multiple prolyl 4-hydroxylases, one of which is essential for proper cell wall assembly. *Plant Cell* 2007; **19**: 256–269.
- Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD *et al*. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- α prolyl hydroxylase. *Cancer Cell* 2005; **7**: 77–85.
- Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ *et al*. Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. *Mol Cell Biol* 2007; **27**: 912–925.
- MacKenzie ED, Selak MA, Tennant DA, Payne LJ, Crosby S, Frederiksen CM *et al*. Cell-permeating α -ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells. *Mol Cell Biol* 2007; **27**: 3282–3289.
- Sener A, Malaisse WJ. L-leucine and a nonmetabolized analogue activate pancreatic islet glutamate dehydrogenase. *Nature* 1980; **288**: 187–189.
- Yuneva M, Zamboni N, Oefner P, Sachidanandam R, Lazebnik Y. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol* 2007; **178**: 93–105.
- Kim J, Guan KL. Amino acid signaling in TOR activation. *Annu Rev Biochem* 2011; **80**: 1001–1032.
- Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* 2011; **13**: 1016–1023.
- Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM *et al*. Modulation of hypoxia-inducible factor 1 α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* 2000; **60**: 1541–1545.
- Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F *et al*. Prolyl hydroxylase-1 negatively regulates I κ B kinase- β , giving insight into hypoxia-induced NF κ B activity. *Proc Natl Acad Sci USA* 2006; **103**: 18154–18159.
- Scott RC, Schuldiner O, Neufeld TP. Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev Cell* 2004; **7**: 167–178.
- Avruch J, Long X, Ortiz-Vega S, Rapley J, Papageorgiou A, Dai N. Amino acid regulation of TOR complex 1. *Am J Physiol Endocrinol Metab* 2009; **296**: E592–E602.
- Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B *et al*. Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell* 2009; **136**: 521–534.
- Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell* 2006; **124**: 471–484.
- Urano J, Sato T, Matsuo T, Otsubo Y, Yamamoto M, Tamanoi F. Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells. *Proc Natl Acad Sci USA* 2007; **104**: 3514–3519.
- Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L *et al*. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 2008; **320**: 1496–1501.
- Duran RV, Oppliger W, Robitaille AM, Heiserich L, Skendaj R, Gottlieb E *et al*. Glutaminolysis activates Rag-mTORC1 signaling. *Mol Cell* 2012; **47**: 349–358.
- Hirsila M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J Biol Chem* 2003; **278**: 30772–30780.
- Tennant DA, Frezza C, MacKenzie ED, Nguyen QD, Zheng L, Selak MA *et al*. Reactivating HIF prolyl hydroxylases under hypoxia results in metabolic catastrophe and cell death. *Oncogene* 2009; **28**: 4009–4021.
- Aragones J, Schneider M, Van Geyte K, Fraisl P, Dresselaers T, Mazzone M *et al*. Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nat Genet* 2008; **40**: 170–180.

- 29 Bentzinger CF, Romanino K, Cloetta D, Lin S, Mascarenhas JB, Oliveri F *et al*. Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. *Cell Metab* 2008; **8**: 411–424.
- 30 Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP. Regulation of cellular growth by the *Drosophila* target of rapamycin dTOR. *Genes Dev* 2000; **14**: 2712–2724.
- 31 Frei C, Edgar BA. *Drosophila* cyclin D/Cdk4 requires Hif-1 prolyl hydroxylase to drive cell growth. *Dev Cell* 2004; **6**: 241–251.
- 32 Brugarolas J, Lei K, Hurley RL, Manning BD, Reiling JH, Hafen E *et al*. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev* 2004; **18**: 2893–2904.
- 33 Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F *et al*. Regulation of hypoxia-inducible factor 1 α expression and function by the mammalian target of rapamycin. *Mol Cell Biol* 2002; **22**: 7004–7014.
- 34 Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005; **307**: 1098–1101.
- 35 Rocha S, Martin AM, Meek DW, Perkins ND. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. *Mol Cell Biol* 2003; **23**: 4713–4727.
- 36 Dall'Asta V, Bussolati O, Sala R, Parolari A, Alamanni F, Biglioli P *et al*. Amino acids are compatible osmolytes for volume recovery after hypertonic shrinkage in vascular endothelial cells. *Am J Physiol* 1999; **276**: C865–C872.



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