

Phospholipase D-dependent mTOR complex 1 (mTORC1) activation by glutamine

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Glutamine is a key nutrient required for sustaining cell proliferation, contributing to nucleotide, protein, and lipid synthesis. The mTOR complex 1 (mTORC1) is a highly conserved protein complex that acts as a sensor of nutrients, relaying signals for the shift from catabolic to anabolic metabolism. Although glutamine plays an important role in mTORC1 activation, the mechanism is not clear. Here we describe a leucine- and Rag-independent mechanism of mTORC1 activation by glutamine that depends on phospholipase D and the production of phosphatidic acid, which is required for the stability and activity of mTORC1. The phospholipase D-dependent activation of mTORC1 by glutamine depended on the GTPases ADP ribosylation factor 1 (Arf1), RalA, and Rheb. Glutamine deprivation could be rescued by α -ketoglutarate, a downstream metabolite of glutamine. This mechanism represents a distinct nutrient input to mTORC1 that is independent of Rag GTPases and leucine.

Glutamine is the most abundant amino acid in human serum and is consumed by cancer cells at a significantly higher rate than any other amino acid (1). Glutamine, originally thought to be a nonessential amino acid, is sometimes referred to as a “conditionally” essential amino acid because of its important role in providing carbon and/or nitrogen for protein, lipid, and nucleotide biosynthesis. Upon entering the cell, glutamine can be converted into glutamate by glutaminase or by enzymes involved in nucleotide biosynthesis, removing glutamine’s amide group as a nitrogen source for the production of nucleotides and nonessential amino acids (2). The resulting gluta-

mate can be converted into the citric acid cycle intermediate α -ketoglutarate (α KG)⁴ by transaminases such as glutamate-oxaloacetate transaminase (GOT), producing α KG and aspartate from oxaloacetate, or by glutamate dehydrogenase (GDH), generating α KG and free ammonia (2). In this way, glutamine is able to replenish the citric acid cycle in dividing cells, where citrate exits the mitochondria to be used for fatty acid and cholesterol biosynthesis (1).

Glutamine’s large role in amino acid biosynthesis makes it a useful sensor involved in the regulation of protein translation. mTOR is a central regulator of protein translation and, in particular, is responsive to glutamine levels (3–5). mTOR is a highly conserved serine/threonine kinase and part of mTOR complex 1 (mTORC1), which exerts many of its effects through its downstream effector, ribosomal subunit S6 kinase (S6K), in response to nutrients, mainly amino acids, cellular energy levels, and glucose (6). A key regulator of mTORC1 is the metabolite phosphatidic acid (PA), which stabilizes both mTORC1 and mTORC2 complexes (7). We and others have reported that PA, generated by phospholipase D (PLD)-catalyzed hydrolysis of phosphatidylcholine, is required for the activation of mTORC1 in response to both glucose and essential amino acids (EAAs) (8, 9).

Although mTORC1 is activated in response to amino acids, leucine produces the most significant response from mTOR, and glutamine is necessary for maximum mTORC1 activation (1, 4). Glutamine import occurs through the solute carrier family (SLC) 1A5 transporter (4). Some of this glutamine can be rapidly exported in exchange for the influx of EAAs, notably leucine, via SLC7A5/SLC3A2. Nicklin *et al.* (4) showed that glutamine uptake and subsequent efflux are necessary for mTORC1 activation. This study identified the importance of glutamine for mTORC1 activation by leucine in that leucine is incapable of stimulating mTORC1 in the absence of glutamine or when SLC1A5 or SLC7A5 is inhibited (4). Further, Durán

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⁴ The abbreviations used are: α KG, α -ketoglutarate; GOT, glutamate-oxaloacetate transaminase; GDH, glutamate dehydrogenase; PA, phosphatidic acid; 16:0–16:0 PA, dipalmitoyl-PA; 16:0–18:1 PA, 1-palmitoyl-2-oleoyl-PA; PLD, phospholipase D; EAA, essential amino acid; KO, knockout; DM α KG, dimethyl- α KG; HEK, human embryonic kidney; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; DFBS, dialyzed fetal bovine serum; PLDi, PLD inhibitors; CM, conditioned medium; PS, phosphatidylserine; Ctrl, control; AATC, aspartate aminotransferase cytosolic; AATM, aspartate aminotransferase mitochondrial.

et al. (5) showed that glutamine and leucine synergize to activate mTORC1 via enhanced glutaminolysis and production of α KG upstream of the Rag GTPases, which are necessary for mTORC1 activation by most amino acids. However, Jewell *et al.* (3) demonstrated that glutamine activates and promotes mTORC1 translocation to the lysosome independently of RagA/B and requires ADP ribosylation factor 1 (Arf1) GTPase and the vacuolar ATPase.

In sum, these studies have identified two different mechanisms for mTORC1 activation by glutamine: one that is Rag-dependent, whereby the production of α KG from glutamine stimulates Rag GTPase activation (5), and one that is Rag-independent and Arf1-dependent (3). It is unclear whether the Rag-independent mechanism relies on the amino acid itself or the metabolism of glutamine. Additionally, Arf1 has been identified previously as necessary for amino acid–induced activation of mTORC1 in an indirect manner (10). Arf6 has also been indicated as vital for mTORC1 activation, with Arf6 being required for mTORC1 stimulation by EAAs and glucose (8, 11). Importantly, the Arf GTPase family has been implicated in regulating PLD activity via recruitment of Arf into the complex consisting of PLD1 and the Ras family GTPase RalA (12, 13).

Because PLD is regulated by the Arf GTPases, we hypothesized that PLD is involved in glutamine-dependent mTORC1 activation in a Rag-independent manner. In this study, we provide evidence that PLD-derived PA is required for glutamine to activate mTORC1, even in the absence of leucine or RagA/B. This work describes a key component, PLD, in the Rag-independent pathway of glutamine activation of mTORC1. PLD possibly represents an important compensation mechanism for glutamine to keep mTORC1 active even in the absence of EAAs, particularly leucine.

Results

PLD activity is required for mTORC1 activation by glutamine

It is known that glutamine is required for mTORC1 activation (3–5). Previous work from our laboratory has indicated that PLD activity is required for mTORC1 activation by other nutrients, notably EAAs, and glucose (8). The nutrient-responsive role of PLD has led us to hypothesize that PLD activity is required for glutamine to stimulate mTORC1. To test this, we used two K-Ras–driven cancer cell lines: MDA-MB-231 and Calu-1, which are known to have elevated PLD activity (14). To measure mTORC1 activation, we looked at phosphorylation of the mTORC1 substrate ribosomal protein p70-S6 kinase (p70^{S6K}) at Thr-389. The MDA-MB-231 and Calu-1 cells were deprived of glutamine for 24 h to inactivate mTORC1, as described previously (5, 15). Consistent with the previous literature, although we did see a modest reduction in phosphorylation of p70^{S6K} by 6 h (data not shown), 24-h glutamine deprivation gave us the most robust inactivation of mTORC1 and was therefore the time point used throughout. Following 24-h glutamine starvation, cells were refed glutamine for 1 h to restimulate mTORC1 in the presence of either scrambled control siRNA or siRNA for the two PLD isoforms, PLD1 and PLD2. As shown in Fig. 1A, siRNA-mediated knockdown of PLD1 and PLD2 effectively blocked phosphorylation of p70^{S6K},

especially in MDA-MB-231 cells. Although suppression of PLD1 and PLD2 in Calu-1 cells did not suppress phosphorylation of p70^{S6K} to the 95% confidence level, there was still a clear trend in these cells. These data indicate a PLD requirement for glutamine to stimulate mTORC1. We next utilized inhibitors against PLD1 and PLD2 or DMSO vehicle control, and, as shown in Fig. 1B, PLD inhibitors also blocked phosphorylation of p70^{S6K} in both cell lines (Fig. 1B). We next treated the MDA-MB-231 and Calu-1 cells with 1-butanol (1-BtOH) to inhibit PA production via PLD. 1-BtOH acts as a more efficient nucleophile than water, and, as a consequence, PLD produces phosphatidylbutanol at the expense of PA (16). As shown in Fig. 1C, 1-BtOH blocked phosphorylation of p70^{S6K} in response to glutamine even more effectively than PLD inhibitors, an effect seen previously in response to EAAs and glucose (8). Tertiary butanol (*t*-BtOH), which is not an effective substrate for PLD, was used as a negative control and did not block glutamine-dependent mTORC1 activation in either cell line (Fig. 1C). These data indicate that mTORC1 activation by glutamine depends on PLD.

We next examined the effect of glutamine deprivation on PLD activity. MDA-MB-231 and Calu-1 cells were deprived of glutamine for 24 h, and the levels of PLD activity were determined relative to cells in complete medium. As shown in Fig. 1D, glutamine deprivation reduced the level of PLD activity in both the MDA-MB-231 and Calu-1 cells by 60% and 40%, respectively. These data indicate that a substantial amount of cellular PLD activity depends on the presence of glutamine.

To further establish a role for PLD and PA in the activation of mTOR by glutamine, we investigated whether mTORC1 activation following glutamine deprivation could be rescued with exogenously supplied PA. We used 1-palmitoyl-2-oleoyl-PA (16:0–18:1 PA), which, as we have reported previously, activates mTORC1 and mTORC2 following serum withdrawal (7, 17). Likewise, Chen and co-workers (18) have reported that PA with at least one monounsaturated fatty acid activates mTORC1. As shown in Fig. 1E, 16:0-18:1 PA rescued p70^{S6K} phosphorylation in both MDA-MB-231 and Calu-1 cells, whereas dipalmitoyl-PA (16:0-16:0 PA) or 1-palmitoyl-2-oleoyl-phosphatidylserine (16:0-18:1 PS) were incapable of rescuing mTORC1 activation. These data demonstrate a specificity for 16:0-18:1 PA in rescuing mTORC1 activation following glutamine deprivation. Collectively, the data in Fig. 1 reveal a role for PLD-derived PA in the activation of mTORC1 in response to glutamine.

Glutamine activation of PLD and mTORC1 occurs independently of leucine

Murphy and co-workers (4) showed previously that glutamine uptake and subsequent efflux through SLC7A5/SLC3A2 in exchange for leucine influx is necessary for mTORC1 activation (Fig. 2A). This work showed that leucine does not stimulate mTORC1 in the absence of glutamine. We therefore wanted to determine whether the PLD-dependent induction of mTORC1 depended on leucine. Consistent with work by Murphy and co-workers (4), leucine stimulated p70^{S6K} phosphorylation in MDA-MB-231 cells strongly in the presence of glutamine, whereas, in the absence of glutamine, leucine had only a modest

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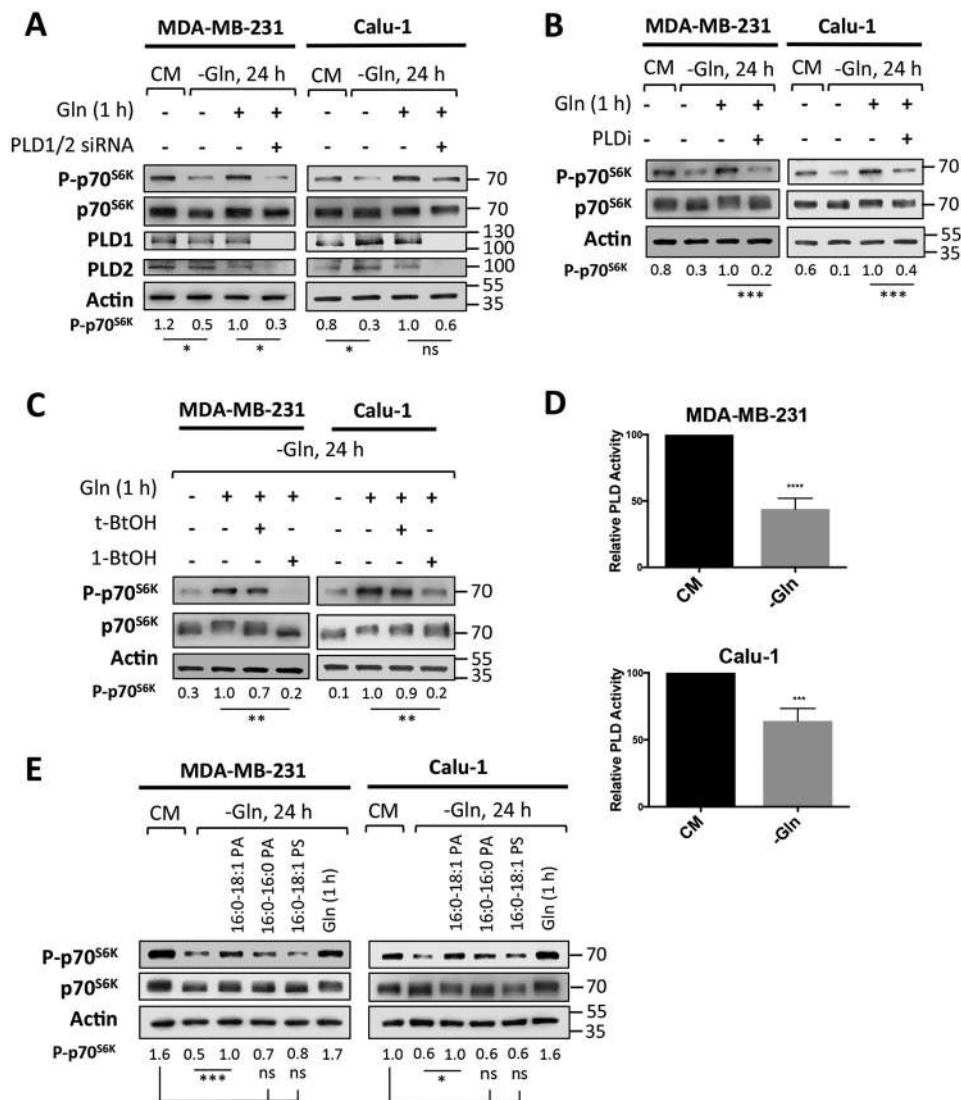


Figure 1. Glutamine-induced activation of mTORC1 depends on PLD-derived PA. *A*, MDA-MB-231 cells and Calu-1 cells were plated in 6-well plates at 60% confluence in CM containing 10% FBS overnight. The following day, cells were transfected either with a control or PLD1 and PLD2 (*PLD1/2*) siRNA for a total of 96 h. The cells were shifted to either fresh CM containing 10% DFBS and 8 mM Gln or Gln-free medium supplemented with 10% DFBS for the last 24 h of the experiment. Cells were refed with 8 mM Gln for 1 h. Lysates were analyzed for phosphorylated mTORC1 substrate p70^{S6K} at Thr-389 (*p-p70^{S6K}*) by Western blotting. *B*, MDA-MB-231 and Calu-1 cells were plated as in *A*, starved of Gln for 24 h, and refed with 8 mM Gln for 1 h in the presence or absence of 10 μ M PLD1 and 10 μ M PLD2 inhibitor (*PLDi*). All control wells were given DMSO, and cells were collected for Western blotting. *C*, MDA-MB-231 and Calu-1 cells were plated as in *A*, starved of Gln for 24 h, and refed 8 mM Gln for 1 h in the absence or presence of 0.8% *t*-BtOH or 0.8% 1-BtOH and collected for Western blotting. *D*, MDA-MB-231 cells ($n = 5$) and Calu-1 cells ($n = 4$) were plated as in *A*. The following day, cells were shifted to fresh medium with 8 mM Gln (CM) or without Gln (-Gln) for 24 h. Cells were then harvested and analyzed for PLD activity as described under "Experimental procedures." Relative PLD activity values are normalized to CM sample, which was given a value of 100%. Significance asterisks are as compared with the CM sample. Data are represented as mean \pm S.D. *E*, MDA-MB-231 and Calu-1 cells were plated as in *A* and then shifted to either fresh CM containing 8 mM Gln and 10% DFBS or Gln-free DMEM containing 10% DFBS for 24 h. Cells were then treated with two doses of 300 μ M 16:0-18:1 PA, 16:0-16:0 PA, or 16:0-18:1 PS at 30-min time intervals or one dose of 8 mM Gln for 1 h. Cells were then collected for Western blotting. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$; ns, not significant.

effect on p70^{S6K} phosphorylation (Fig. 2*B*, compare lanes 2 and 3 with lanes 6 and 7). However, glutamine was able to stimulate mTORC1 in both the presence and absence of leucine (Fig. 2*B*, lanes 5 and 8). In fact, glutamine was sufficient to fully reactivate mTORC1 in the absence of leucine, indicating that glutamine has its own mechanism to stimulate mTORC1 independent of leucine. Additionally, leucine and glutamine synergized to activate mTORC1 to a much fuller extent, with levels of P-p70^{S6K} even higher than that of the control lane (Fig. 2*B*, lanes 1 and 9).

We next examined the effect of leucine and glutamine deprivation on PLD activity. When cells were deprived of only leu-

cine, PLD activity was modestly decreased, whereas glutamine deprivation resulted in a much stronger decrease in PLD activity (Fig. 2*C*). No further decrease in PLD activity was observed upon deprivation of both glutamine and leucine (Fig. 2*C*). Thus, glutamine has a more significant role in regulating PLD activity than leucine. We also examined the effect of leucine and glutamine stimulation on PLD activity. Consistent with data from Murphy and co-workers (4), where the activation of mTORC1 by leucine depended on glutamine, and our data in Fig. 2*B*, leucine did not significantly stimulate PLD activity in the absence of glutamine (Fig. 2*D*). In contrast, glutamine activated PLD in the absence of leucine (Fig. 2*D*). Although this increase

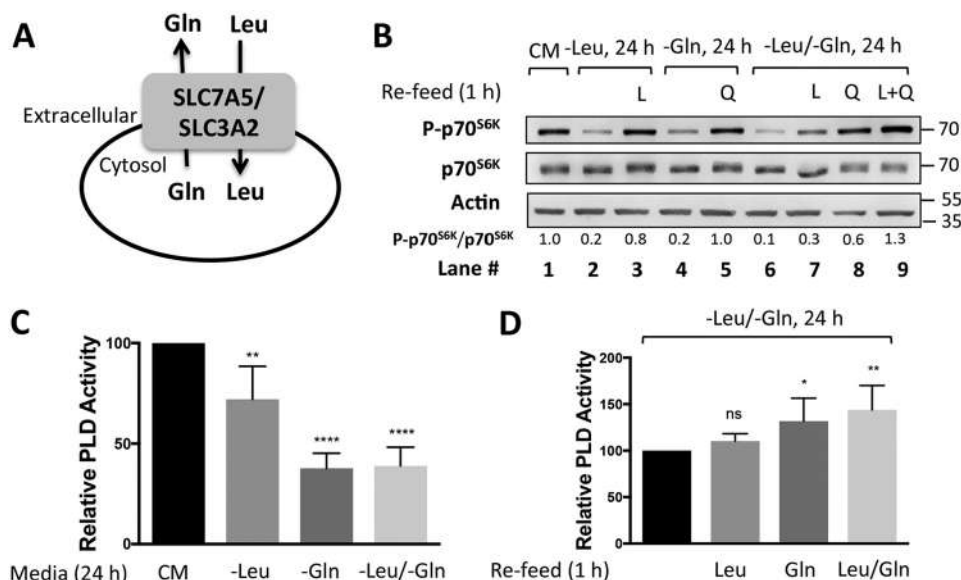


Figure 2. Glutamine stimulates mTORC1 and PLD in a leucine-independent manner. *A*, schematic showing the exchange of Gln for Leu through the bidirectional transporter SLC7A5/SLC3A2. *B*, MDA-MB-231 cells were plated in 6-well plates at 60% confluence in CM containing 10% FBS overnight. The following day, cells were shifted to either fresh DMEM containing 8 mM Gln and 0.8 mM Leu (CM), leucine-free DMEM (–Leu), Gln-free DMEM (–Gln), or DMEM lacking both Leu and Gln (–Leu/–Gln) for 24 h. All wells contained 10% DFBS. Cells were re-fed 8 mM Gln (Q) and/or 0.8 mM Leu (L) for 1 h and analyzed by Western blotting. The image shown is representative of at least three independent experiments. *C*, MDA-MB-231 cells were plated and shifted to CM ($n = 6$), –Leu ($n = 6$), –Gln ($n = 3$), or –Leu/–Gln ($n = 5$) for 24 h as in *B*, at which point cells were harvested and collected for a PLD activity assay. Relative PLD activity levels are normalized to the CM sample (first column), which has been given a value of 100%. Significance asterisks are compared with this first column. *D*, MDA-MB-231 cells were plated as in *B* and then shifted to Leu- and Gln-free DMEM containing 10% DFBS for 24 h and then re-fed with either Leu ($n = 5$), Gln ($n = 4$), or Leu and Gln ($n = 5$) together for 1 h. The cells were then harvested and analyzed for PLD activity. Relative PLD activity levels are normalized to the –Leu/–Gln sample ($n = 5$) without any refeed (first column), which has been given a value of 100%, and significance asterisks are compared with this first column. All relative PLD activity values in *C* and *D* are displayed as mean \pm S.D. *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$; ns, not significant.

was modest, it was found to be statistically significant whereas leucine stimulation was not (Fig. 2D). Furthermore, this modest increase in PLD activity is consistent with previous reports of PLD stimulation by amino acids (9), insulin (19), and serum (20). Treatment with leucine and glutamine simultaneously produced a somewhat larger increase in PLD activity (Fig. 2D). The further increase in PLD activity and mTORC1 activation with both leucine and glutamine is likely due to reactivation of the antiporter system (Fig. 2A), bringing leucine into the cell to further activate mTORC1. Collectively, the data in Fig. 2 further demonstrate a role for PLD in the glutamine induction of mTORC1 and establish that the effect of leucine on both mTORC1 and PLD activity depends on glutamine.

Arf1, RalA, and Rheb are required for glutamine to stimulate mTORC1 in a PA-dependent manner

Guan and co-workers (3) reported previously that, although all other amino acids require the Rag GTPases to activate mTORC1, glutamine does not. Intriguingly, activation of mTORC1 by glutamine depended on the GTPase Arf1 (3). Because several Arf GTPases have been implicated as activators of PLD activity (8, 12, 13, 21), we hypothesized that Arf1-dependent mTORC1 activation depends on PLD activity. As expected, knockdown of Arf1 decreased PLD activity in MDA-MB-231 cells (Fig. 3A). Further, knockdown of Arf1 resulted in a block of glutamine-dependent mTORC1 activation, as reported previously (3) (Fig. 3B, left panel). Exogenously supplied PA partially rescued p70^{S6K} phosphorylation caused by Arf1 inhibition, indicating that PA production is downstream of glutamine-Arf1 signaling (Fig. 3B, left panel). The same effect

was seen in the absence of leucine, indicating that this pathway is active even in the absence of leucine (Fig. 3B, right panel). This suggests that the Arf1–PLD pathway is a parallel pathway that can remain active even in the absence of EAAs such as leucine.

We previously reported that Arf6 is required for the elevation of PLD activity in response to serum deprivation and mTORC1 activation in response to EAAs and glucose (8). We therefore investigated whether Arf6 is required for glutamine-induced mTORC1 activation. siRNA-mediated knockdown of Arf6 did not result in a decrease in PLD activity in MDA-MB-231 cells (Fig. 3A) and only modestly decreased mTORC1 activation in response to glutamine (Fig. 3C). Furthermore, this decrease in mTORC1 activation upon Arf6 knockdown was not rescued with exogenous PA (Fig. 3C). The inhibition of mTORC1 activation produced from knockdown of Arf6 was not nearly as strong as that from the knockdown of Arf1, and because Arf6 knockdown did not affect basal levels of PLD activity and could not be rescued with PA, this suggests that this was not due to Arf6-dependent PLD activity. Thus, Arf6 is not required for glutamine-induced mTORC1 activation in a PLD-dependent manner. This represents a differential utilization of Arf GTPases for PLD/mTORC1 activation in response to different nutrient sources.

It has been reported previously that another small GTPase, RalA, can promote Arf1-dependent PLD activity by direct interaction with PLD1 (13). Similarly, we reported that, upon activation with GTP, RalA, which is constitutively bound to PLD1, recruits Arf GTPases to enhance PLD activity (12). We

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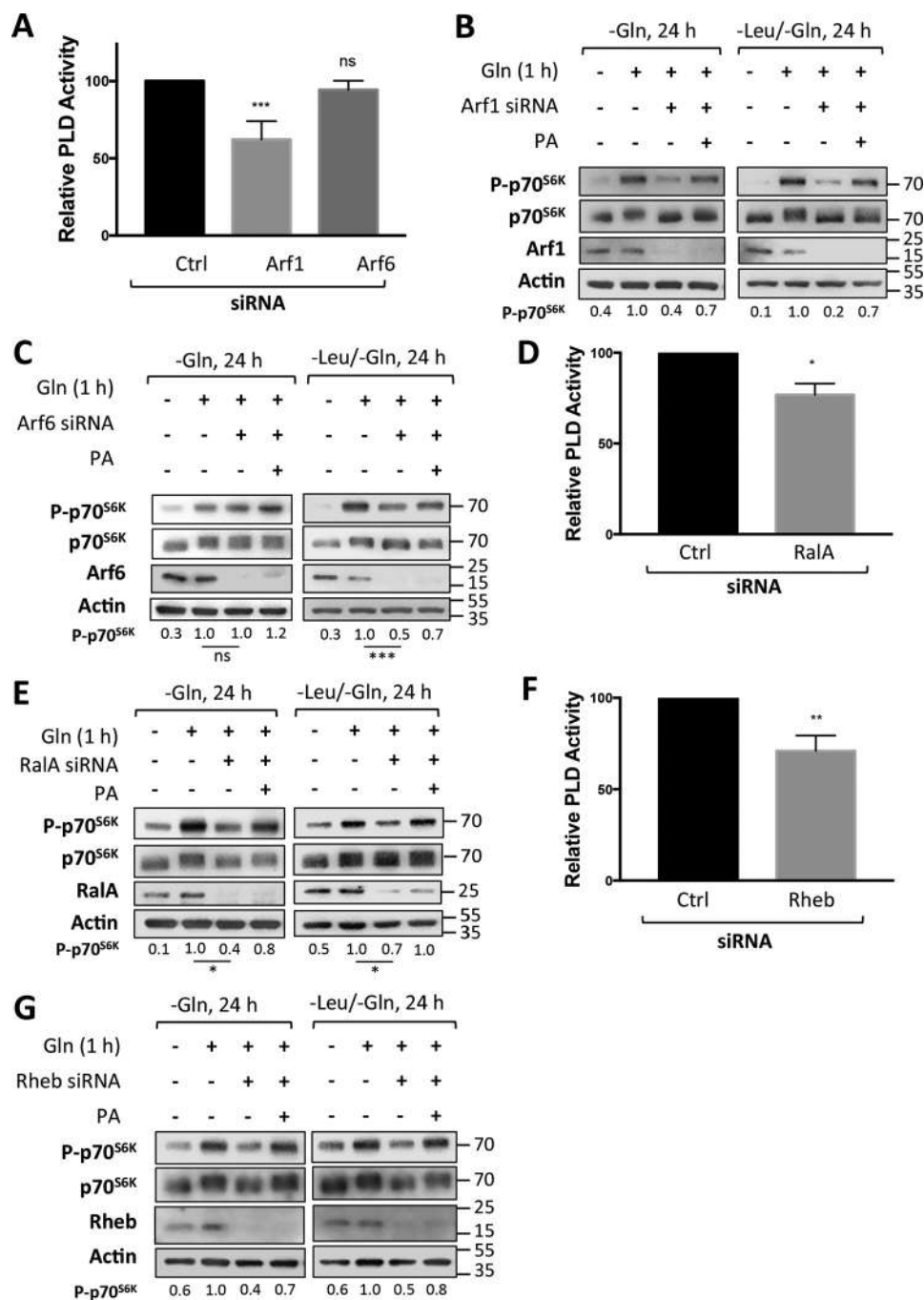


Figure 3. Glutamine stimulates mTORC1 and PLD in an Arf1/RalA/Rheb-dependent manner. *A*, MDA-MB-231 cells were plated in 6-well plates at 40% confluence in CM supplemented with 10% FBS. The following day, cells were transfected either with a control (Ctrl, $n = 4$), Arf1 ($n = 4$), or Arf6 ($n = 4$) siRNA. After 72 h, cells were harvested and collected for a PLD activity assay. *B*, MDA-MB-231 cells were plated and transfected with Ctrl or Arf1 siRNA as in *A*. For the last 24 h of the experiment, cells were starved of either Gln (-Gln, left panel) or both Leu and Gln (-Leu/-Gln, right panel). Cells were refed Gln for 1 h in the absence or presence of PA (two doses of 300 μ M 16:0-18:1 PA at 30-min intervals). Cells were then collected and analyzed by Western blotting. *C*, MDA-MB-231 cells were plated and transfected with Ctrl or Arf6 siRNA as in *A*. Cells were shifted to -Gln DMEM (left panel) or -Leu/-Gln DMEM (right panel) for the last 24 h of the experiment. Cells were refed Gln for 1 h in the presence or absence of 16:0-18:1 PA as in *B*. Cells were then collected and analyzed by Western blotting. *D*, MDA-MB-231 cells were plated in 6-well plates at 40% confluence in CM supplemented with 10% FBS. The following day, cells were transfected either with a control ($n = 4$) or RalA ($n = 4$) siRNA. After 48 h, cells were harvested and collected for a PLD activity assay. *E*, MDA-MB-231 cells were plated and transfected with Ctrl or RalA siRNA as in *D*. Cells were shifted to -Gln medium for 24 h and refed Gln for 1 h in the absence or presence of 16:0-18:1 PA as in *B*. Cells were then collected and analyzed by Western blotting. *F*, MDA-MB-231 cells were plated in 6-well plates at 40% confluence in CM supplemented with 10% FBS. The following day, cells were transfected either with a Ctrl ($n = 3$) or Rheb ($n = 3$) siRNA. After 48 h, cells were harvested and collected for a PLD activity assay. *G*, MDA-MB-231 cells were plated and transfected with Ctrl or Rheb siRNA as in *F*. Cells were shifted to -Gln medium for 24 h and refed Gln for 1 h in the absence or presence of 16:0-18:1 PA as in *B*. Cells were then collected and analyzed by Western blotting. All PLD activity values in *A*, *D*, and *F* are normalized to the Ctrl siRNA sample, which has been given a value of 100%, and significance asterisks are compared with the Ctrl sample. Relative PLD activity is represented as mean \pm S.D. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, not significant.

therefore examined whether RalA was required for the activation of mTORC1 by glutamine. Knockdown of RalA decreased PLD activity in MDA-MB-231 cells (Fig. 3D) and suppressed the glutamine induction of mTORC1 in the presence and absence of leucine (Fig. 3E). This effect could be rescued with the addition of exogenous PA (Fig. 3E), indicating that PA production via PLD is downstream of RalA.

We next looked into the dependence for Rheb in glutamine-induced mTORC1 activation. Rheb is typically required for mTORC1 activation at the lysosomal membrane (23). Further, Rheb associates with PLD in a GTP-dependent manner (20). Indeed, we saw that siRNA-mediated knockdown of Rheb decreased the basal levels of PLD activity (Fig. 3F). Next, we found that, in both the presence and absence of leucine, knockdown of Rheb blocked glutamine induction of phosphorylation of p70^{S6K}, and thus, mTORC1 activation (Fig. 3G). This effect was rescued with addition of exogenous PA, indicating that PLD activation is downstream of Rheb (Fig. 3G). These data indicate that, although Rheb is required for the activation of mTORC1 by glutamine, PLD lies downstream of Rheb, playing a role in this activation. Taken together, the data in Fig. 3 identify Arf1, RalA, and Rheb GTPases as crucial players in activating PLD for the subsequent activation of mTORC1.

Glutamine activates mTORC1 via PLD independently of the Rag GTPases

Although Rheb is required for activation of mTORC1 at lysosomes, the Rag GTPases are thought to recruit mTORC1 to the lysosomal membrane in response to amino acids, whereby Rheb can activate mTORC1. However, although Guan and co-workers (3) reported that glutamine does not require the Rag GTPases for the lysosomal localization or activation of mTORC1, Hall and co-workers (5) have shown that glutamine and leucine activate mTORC1 via α KG production upstream of the Rag GTPases. We were therefore interested in understanding the interplay of the two pathways, Rag-independent and Rag-dependent, glutamine uses to activate mTORC1. The leucine independence of the PLD-dependent pathway of mTORC1 activation by glutamine suggested that PLD is not dependent on the Rag pathway and, instead, works in parallel to the Rag GTPases because leucine (and all other amino acids besides glutamine) depend on the presence of the Rag GTPases (3). We hypothesized that if the Rag-dependent and Rag-independent pathways work in parallel, then the absence of RagA/B results in the activation of a compensatory pathway dependent on PLD activity.

The Rag GTPases exist as obligate heterodimers consisting of either RagA or RagB with RagC or RagD (24). Thus, RagC and RagD require either RagA or RagB for mTORC1 lysosomal localization. To study the effect of loss of Rag GTPase activity, we genetically ablated RagA and RagB to abolish any of the functional heterodimers that could be used by the cell. We knocked down RagA and RagB using siRNA in MDA-MB-231 cells and looked at basal PLD activity levels. As shown in Fig. 4A, knockdown of RagA/B resulted in a significant increase in PLD activity. Similarly, using HEK293A control or HEK293A RagA/B knockout (KO) cell line described previously (3), we

found that the RagA/B KO cell line had elevated basal PLD activity compared with control HEK293A cells (Fig. 4B). The data in Fig. 4, A and B, suggest that PLD compensates and increases its production of PA in the absence of the Rag GTPases to allow glutamine to keep mTORC1 active. Furthermore, as shown in Fig. 4C, PA was still able to rescue phosphorylation of p70^{S6K} in the absence of glutamine in RagA/B KO cells, indicating that PA does not require RagA/B to stimulate mTORC1 (Fig. 4C).

Because it has been reported previously that glutamine exerts much of its effects on mTORC1 via production of α KG, which goes on to stimulate mTORC1 via GTP loading and activation of the RagB GTPase (5), we next wanted to see whether α KG requires the presence of the Rag GTPases to activate mTORC1. We added dimethyl- α KG (DM α KG), a cell-permeable form of α KG, to HEK293A control and RagA/B KO cells to see whether it could rescue mTORC1 activation following 24 h of glutamine deprivation even in the absence of the Rags. We were surprised to see that DM α KG was able to activate mTORC1 in both control and RagA/B KO HEK293A cells (Fig. 4D). These data indicate that, although α KG can activate Rag B (5), α KG does not require the presence of RagA/B to stimulate mTORC1 (Fig. 4D). Collectively, the data shown in Fig. 4 suggest that the PLD/PA signaling pathway is separate but parallel to the Rag pathway for glutamine-dependent mTORC1 activation and compensates upon inhibition of the Rag GTPases. Furthermore, these data suggest that α KG also plays a role in this parallel, PLD-dependent pathway.

α KG stimulates mTORC1 in a PLD/PA-dependent manner

It remains unclear whether the Arf1-mediated pathway of Gln-dependent mTORC1 activation requires glutamine itself or a downstream metabolite of glutamine, such as α KG (3). However, the data in Fig. 4D suggest that α KG must activate mTORC1 in a manner that does not require the Rag GTPases. Thus, we next looked to see whether α KG was able to stimulate mTORC1 in the absence of PLD. As shown in Fig. 5, A–C, supplementation of DM α KG to glutamine-deprived MDA-MB-231 cells was sufficient to rescue p70^{S6K} phosphorylation (Fig. 5, A–C). Furthermore, DM α KG stimulation of mTORC1 was blocked by PLD inhibitors (Fig. 5A) and 1-butanol treatment (Fig. 5B), indicating that PLD activity and PLD-derived PA is required for α KG to activate mTORC1. Additionally, knockdown of Arf1 blocked DM α KG from activating mTORC1 and phosphorylating p70^{S6K}, indicating that Arf1 is also required for activation of mTORC1 by α KG (Fig. 5C). Taken together, these data indicate that α KG does not require RagA/B to activate mTORC1 and can utilize the Arf1/PLD signaling pathway for mTORC1 activation.

Glutamate derived from glutamine can be converted into α KG via GDH or GOT (Fig. 5D). Because GDH is allosterically activated by leucine (25), we hypothesized that the PLD pathway, which occurs independently of leucine, involves α KG production via GOT rather than GDH. Interestingly, we found that both knockdown of either GOT or GDH blocked glutamine from activating mTORC1 (Fig. 5E), indicating that either enzyme can be utilized for α KG production from glutamine and the subsequent activation of mTORC1 (Fig. 5E). Furthermore,

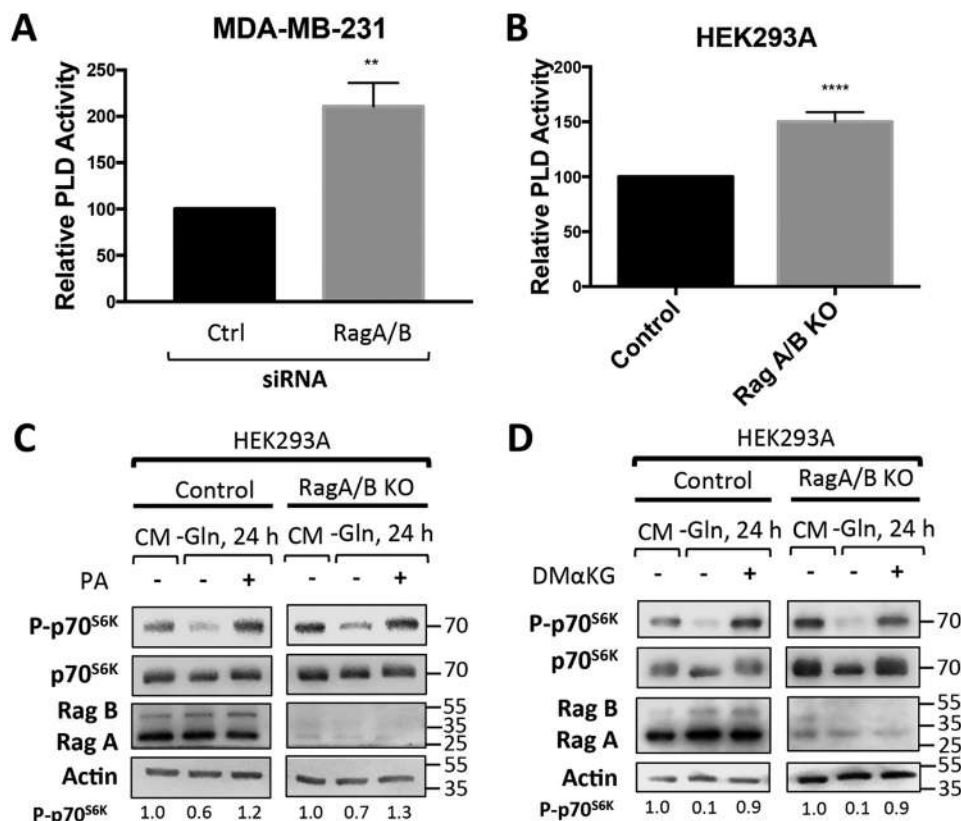


Figure 4. Glutamine stimulates mTORC1 in a Rag-independent manner via elevated PLD activity. *A*, MDA-MB-231 cells were plated in 6-well plates at 40% confluence in CM supplemented with 10% FBS. The following day, cells were transfected either with a control ($n = 3$) or RagA and Rag B (*RagA/B*, $n = 3$) siRNA. After 72 h, cells were harvested and collected for a PLD activity assay. *B*, HEK293A control ($n = 5$) and RagA/B KO ($n = 5$) cells were plated at 70% confluence in CM supplemented with 10% FBS. The following day, cells were harvested and analyzed for basal PLD activity levels. *C*, HEK293A control and RagA/B KO cells were plated in 6-well plates at 60% confluence in CM containing 10% FBS. The following day, cells were shifted to Gln-free medium supplemented with 10% DFBS (*-Gln*) for the last 24 h of the experiment, refed two doses of 300 μM 16:0-18:1 PA at 30-min intervals, and then collected for Western blotting. *D*, HEK293A control and RagA/B KO cells were plated in 6-well plates at 60% confluence in CM containing 10% FBS. The following day, cells were shifted to either fresh DMEM containing 8 mM Gln and 10% DFBS (*CM*) or glutamine-free DMEM supplemented with 10% DFBS for the last 24 h of the experiment. Cells were refed 4 mM DM α KG for the last 2 h of the experiment. Lysates were analyzed by Western blotting. All PLD activity values in *A* and *B* are normalized to the Ctrl siRNA sample, which has been given a value of 100%, and *significance asterisks* are compared with the Ctrl sample. Relative PLD activity is represented as mean \pm S.D. **, $p \leq 0.01$; ****, $p \leq 0.0001$.

the dependence on either enzyme did not change in the absence of leucine (Fig. 5E). PA was able to rescue the effects of knocking down both GOT or GDH in the presence and absence of leucine, indicating that PA production lies downstream of α KG production (Fig. 5E).

Although GDH results in the production of α KG, with ammonia as a byproduct, GOT results in the production of aspartate as well. Aspartate has been identified as an essential nutrient for K-Ras–driven cancer cell growth (26) and cell cycle progression through S phase following glutamine deprivation (26, 27). Although we have already shown that α KG can rescue mTORC1 activation following glutamine deprivation, we next wanted to see whether aspartate could as well. In fact, L-aspartic acid β -methyl ester hydrochloride, a cell-permeable analog of aspartate, was incapable of rescuing phosphorylation of p70^{S6K} (Fig. 5, F and G). This was the case both in the presence or absence of leucine (Fig. 5F) and indicates that, although GOT is required for production of α KG for mTORC1 activation, the production of aspartate is not required for mTORC1. This is interesting, and may imply a specific role for aspartate in cell survival (26, 27) rather than mTORC1 activation.

Discussion

In this report, we provided evidence that PLD activity is required for glutamine to activate mTORC1 (see schematic model in Fig. 6). This pathway involves Arf1 for the activation of PLD (Fig. 3, A and B), which is consistent with work from Guan and co-workers (3) identifying an Arf1 requirement in a Rag-independent mechanism by which glutamine activates mTORC1. Furthermore, we have also shown that PLD acts independently of the Rag GTPases in that PA can stimulate mTORC1 in the absence of RagA/B (Fig. 4C). Intriguingly, we saw that genetic ablation of RagA/B results in elevated basal levels of PLD activity (Fig. 4, A and B), suggesting that PLD represents a compensatory pathway for glutamine to activate mTORC1 by an alternate pathway. Although all other amino acids require the Rags for lysosomal localization and subsequent activation of mTORC1, glutamine does not (3). The work in our report demonstrates that glutamine utilizes PLD in the absence of the EAA leucine (Fig. 2). It has been thought previously that glutamine plays a large role in activating mTORC1 through its own efflux to bring leucine, a potent mTORC1 activator, into the cell (4). However, the pathway described

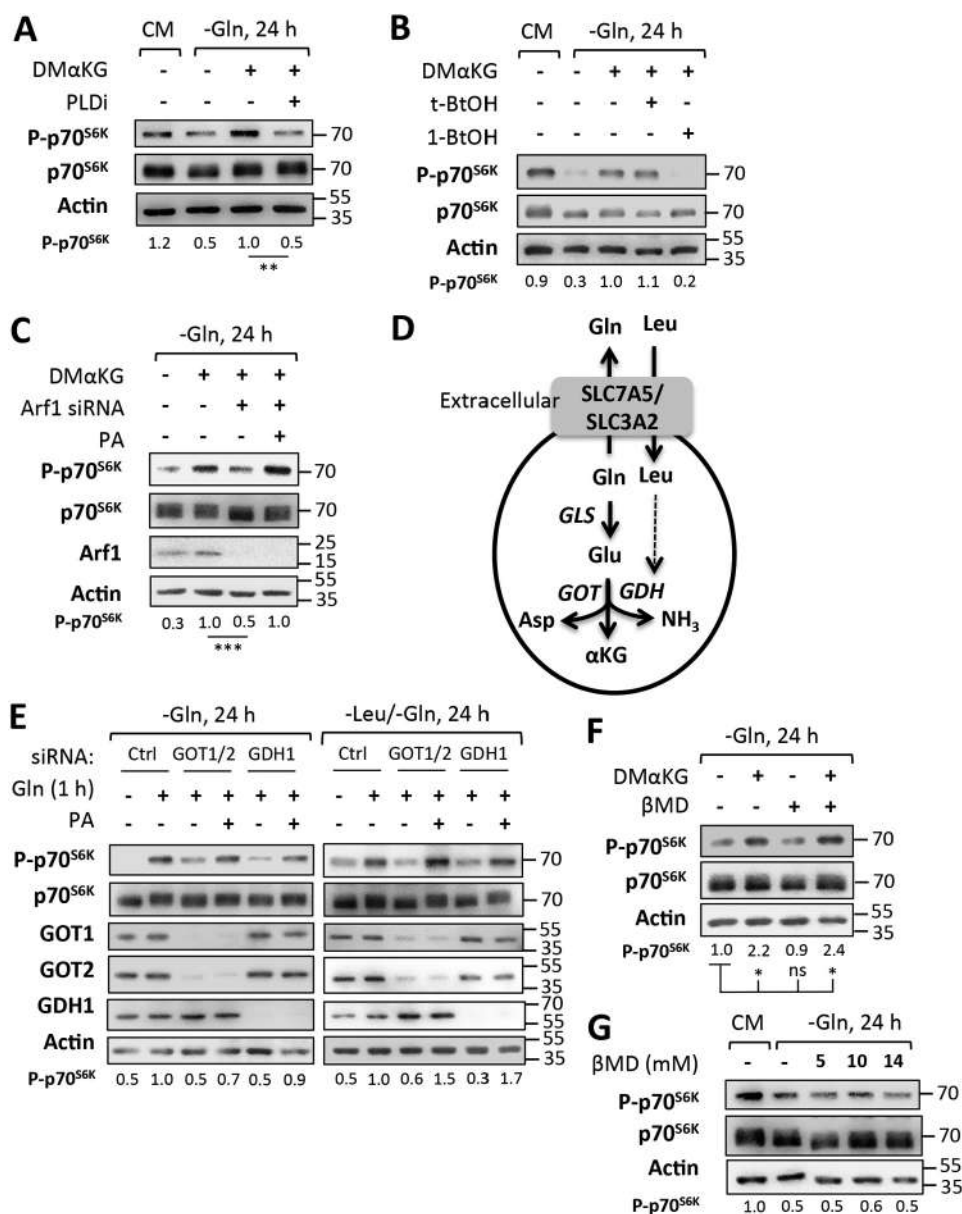


Figure 5. PLD/Arf1, but not RagA/B, are required for α KG to activate mTORC1. *A*, MDA-MB-231 cells were plated in 6-well plates at 60% confluence in CM containing 10% FBS overnight. The cells were then shifted to either fresh DMEM with 8 mM Gln and 10% DFBS (CM) or glutamine-free medium supplemented with 10% DFBS for the last 24 h of the experiment and refed 4 mM DM α KG for 2 h in the presence or absence of 10 μ M PLD1 and 10 μ M PLD2 inhibitors (PLDi). All control wells were given DMSO, and cells were collected for Western blotting. *B*, MDA-MB-231 cells were plated as in *A*. The cells were then shifted to either fresh CM or glutamine-free medium supplemented with 10% DFBS for the last 24 h of the experiment and refed 4 mM DM α KG for 2 h in the presence of either 0.8% t-BtOH or 0.8% 1-BtOH. Lysates were analyzed by Western blotting. *C*, MDA-MB-231 cells were plated in 6-well plates at 40% confluence in CM containing 10% FBS overnight. The following day, cells were transfected either with control or Arf1 siRNA for a total of 72 h as in Fig. 3*A*. For the last 24 h of the experiment, cells were starved of Gln and then refed DM α KG for 2 h in the absence or presence of PA (two doses of 300 μ M 16:0-18:1 PA at 30-min intervals). Cells were then collected, and lysates were analyzed by Western blotting. *D*, schematic of α KG production from Gln. Briefly, Gln is converted to glutamate (Glu) via glutaminase (GLS). Glutamate can be converted to α KG via GOT, with the concomitant production of Asp or via GDH, which is allosterically activated by Leu. Leu is brought into the cell in exchange for Gln via SLC7A5/SLC3A2. *E*, MDA-MB-231 cells were plated in 6-well plates at 40% confluence in CM containing 10% FBS. The following day, cells were transfected either with a Ctrl, GOT1/2, or GDH1 siRNA for a total of 72 h. For the last 24 h of the experiment, cells were starved of Gln (-Gln, left panel) or both Leu and Gln (-Leu/-Gln, right panel). Cells were refed Gln for 1 h in the absence or presence of 16:0-18:1 PA as in *C*. Cells were then collected and analyzed by Western blotting. *F*, MDA-MB-231 cells were plated in 6-well plates at 60% confluence in CM containing 10% FBS overnight. The cells were then shifted to either Gln-free medium (-Gln, left) or Leu and Gln-free medium (-Leu/-Gln), both supplemented with 10% DFBS, for 24 h. Cells were then refed either 4 mM DM α KG or 10 mM β MD for 2 h or 8 mM Gln for 1 h. Cells were then collected, and lysates were analyzed by Western blotting. *G*, MDA-MB-231 cells were plated in 6-well plates at 60% confluence in CM containing 10% FBS overnight. The cells were then shifted to Gln-free medium (-Gln) supplemented with 10% DFBS for 24 h. Cells were then refed the indicated concentrations of β MD for 2 h. Cells were then collected, and lysates were analyzed by Western blotting.

here demonstrates a more direct mechanism by which glutamine can activate mTORC1 in the absence of leucine or the traditional amino acid-induced Rag GTPase machinery (Fig. 6).

Knockdown of Arf1 prevented glutamine from inducing mTORC1 activity. Additionally, we demonstrated that another Arf isoform, Arf6, was not required for glutamine to activate mTORC1 (Fig. 3*C*). We previously reported that Arf6, but not

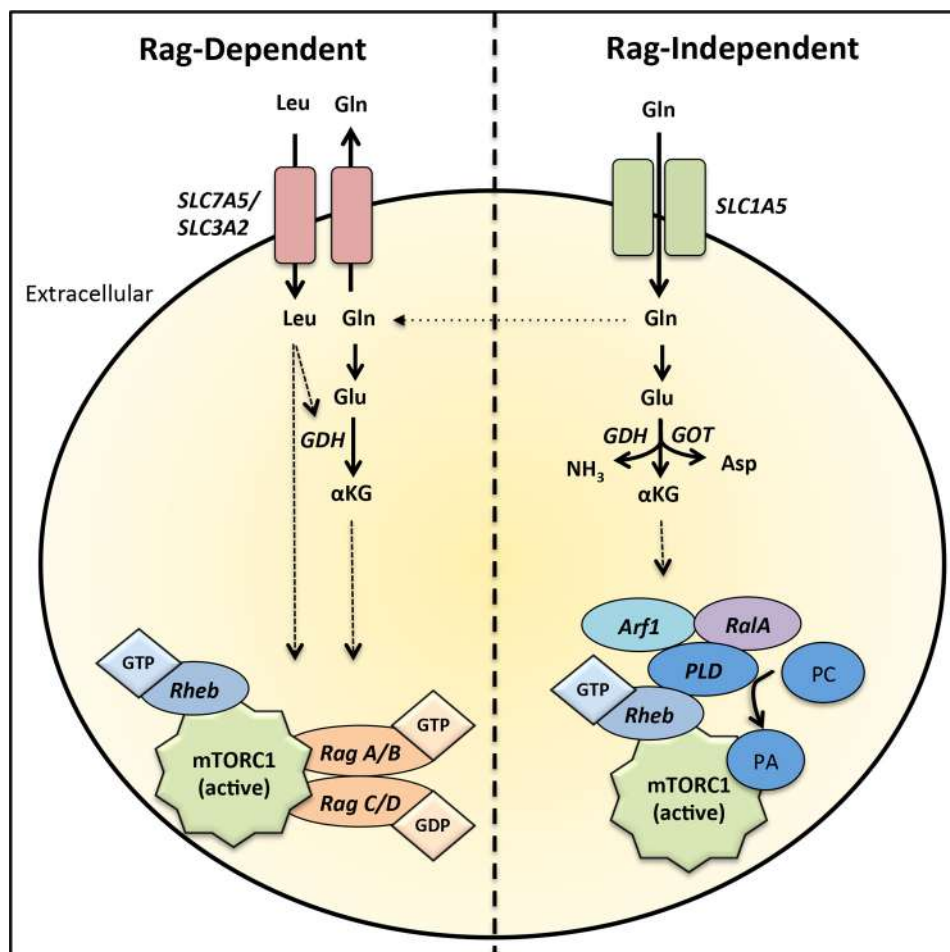


Figure 6. Schematic of Rag-independent Gln stimulation of mTORC1 via PLD-derived PA. We have shown that Gln, which enters the cell through SLC1A5, can be metabolized into α KG via either GOT or GDH in the presence or absence of Leu. Although α KG has been shown previously to activate mTORC1 by stimulating GTP loading of RagB for its subsequent activation (5), in this study, we have shown that α KG can activate mTORC1 independently of RagA/B. It remains unclear how α KG activates the Arf1-RalA-Rheb-PLD signaling apparatus independently of Rag GTPases; however, PA can activate mTORC1 in the absence of any of the GTPases and in the absence of Gln. The Rag-independent pathway for mTORC1 activation is also independent of Leu. However, Leu can be brought into the cell in exchange for Gln via SLC7A5/SLC3A2, is a potent activator of Rag signaling to mTORC1, and can stimulate glutaminolysis through the activation of GDH (3, 4, 25).

Arf1, was required for EAA- and glucose-induced mTORC1 activation (8). These data indicate a differential Arf requirement for Arf-dependent mTORC1 activation in response to distinct nutrient sources.

The involvement of RalA, Arf1, and PLD is of interest in that these enzymes are all involved in vesicle trafficking (29–31), suggesting a role for these enzymes in facilitating the localization of mTORC1 to the lysosome in the absence of Rag GTPases, whereby mTORC1 can become activated by Rheb, which is also required in this pathway (Fig. 3G). Overall, we have reinforced the idea that glutamine does not require the traditional Rag GTPase amino acid–signaling machinery to activate mTORC1. Although it remains unclear exactly what signal is driving mTORC1 to the lysosome in the absence of RagA/B, we have provided evidence for the requirement of the vesicle trafficking enzymes PLD, Arf1, and RalA.

Hall and co-workers (5) have suggested that glutamine-derived α KG stimulates activation of RagB and the subsequent activation of mTORC1. However, it was unclear whether the Rag-independent pathway described by Guan and co-workers (3) involved glutamine itself or a downstream metabolite of

glutamine. In this report, we have shown that α KG does not require RagA/B and is capable of activating mTORC1 even in RagA/B KO cells (Fig. 4D). Furthermore, our data indicate that α KG requires Arf1 and PLD to activate mTORC1 (Fig. 5, A–C). Blocking the production of α KG from glutamine by knocking down either GDH or GOT resulted in a block of mTORC1 activation that could be rescued by PA (Fig. 5E). This was interesting, as we expected that, because of the leucine independence of the pathway, the GOT reaction would be favorable, as leucine allosterically activates GDH (25) and stimulates glutaminolysis (4). Furthermore, we utilized K-Ras–driven cancer cells for these experiments, which have been thought previously to depend more on GOT for survival (26). Nonetheless, knockdown of GDH also inhibited mTORC1 and, thus, still retained a role in producing α KG for mTORC1 activation in the absence of leucine, suggesting that α KG production is more important for this activity rather than the production of aspartate via GOT (Fig. 5E). Indeed, although DM α KG activated mTORC1 following glutamine deprivation, the cell-permeable aspartate analog β MD did not (Fig. 5, F and G). We have shown previously that K-Ras cells arrest in S phase upon glutamine

deprivation and that aspartate, but not α KG, could rescue the arrest (27), suggesting that, although aspartate production by GOT is required for K-Ras cancer cell survival (26) and cell cycle progression (27), there is a differential utilization of α KG for mTORC1 activation (Fig. 5). Although it remains unclear how α KG is activating Arf1/RalA/PLD signaling, α KG is at the center of cellular metabolism and represents a useful sensor of metabolic sufficiency to activate mTORC1. Besides donating carbons for the citric acid cycle, α KG serves as a co-factor for α KG-dependent dioxygenases such as prolyl hydroxylases (32) as well as the several histone and DNA demethylases (33). In fact, Gottlieb and co-workers have shown previously that prolyl hydroxylases respond to α KG levels to activate mTORC1 (32). Thus, there are several α KG-sensors that could play a role in this pathway, and further studies are warranted to better understand the mechanism for the effects of α KG.

The work described in this report utilized the two K-Ras driven cell lines, MDA-MB-231 and Calu-1. Although these cell lines were chosen because of their elevated PLD activity (14), K-Ras–driven cancer cells have also been shown to have higher glutamine addictions than other cancer cells. K-Ras mutations have been associated with elevated expression of SLC1A5, resulting in increased glutamine uptake (34) as well as elevated expression of GOT (26). The metabolic rewiring of enzymes involved in glutamine metabolism results in K-Ras–driven cancer cells to be even more glutamine-dependent than other cancer cells. Although exploiting cancer's addiction to glucose has been utilized for years in the clinic (35), more recent data indicating cancer's requirement for glutamine is leading to new therapeutic agents to exploit glutamine dependence (1, 36, 37). The work shown here suggests that there may be a connection between elevated PLD activity and increased glutamine addiction in K-Ras cancers, providing a rationale for targeting PLD to shut off glutamine signaling in the hope to decrease cancer proliferation. Our laboratory has shown previously that phospholipase D activity is directly correlated with an increase in cancer aggressiveness (14, 16, 38). The significance of these findings lies in the fact that suppression of the PA–mTOR signaling pathway, including suppression of PLD activity in cancer cells, may be a novel strategy to control cancer cell proliferation, particularly in glutamine-addicted cancers. Going forward, we hope to find that, by interfering with the glutamine–PLD axis, we may be capable of controlling cancer cell proliferation. Recent literature suggests that tumor cell metabolism may be an Achilles heel for cancer cells (39). Thus, by controlling glutamine-dependent increases in PLD activity, we have the potential to exploit tumor cell metabolism and reveal novel therapeutic opportunities for controlling both PLD and mTOR activity that is responsive to glutamine. Although targeting mTOR has had very limited success (40), and mTOR knockout is embryonic lethal (28), PLD inhibitors seem to be well tolerated, and PLD1/PLD2 knockout mice are viable (22). The involvement of PLD in the signals emanating from glutamine to activate mTORC1 represents a therapeutic opportunity for targeting glutamine-addicted cancers.

Experimental procedures

Antibodies and reagents

The antibodies used were as follows: GDH1/2 (12793), pThr-389–p70^{S6K} (p-p70^{S6K}, 9234), p70^{S6K} (9202), RagA (4357), RalA (3526), Rheb (13879S), PLD1 (3832), and PLD2 (13904S) were obtained from Cell Signaling Technology; Actin (60008-1-Ig) from Proteintech Group; and Arf1 (sc-53168), Arf6 (sc-7971), GOT1/AATC (sc-46283), GOT2/AATM (sc-135181), and RagB (sc-169101) from Santa Cruz. Ultima Gold scintillation fluid (6013681) and [³H]myristic acid (NET830005MC) were obtained from PerkinElmer Life Sciences. The PLD1 inhibitor (VU0359595, 857371), PLD2 inhibitor (VU0285655-1, 857372), 18:1 phosphatidylbutanol standard solution (860203C), and 16:0-18:1 phosphatidic acid (POPA, 840857C) were obtained from Avanti Polar Lipids, Inc. 1-Butanol (B7906) and tertiary-butanol (47172) were obtained from Sigma-Aldrich. TLC plates (1.11798.0001) were obtained from Millipore Sigma. DMSO (25-950-CQC) was obtained from Fisher Scientific.

Cells and culture conditions

The MDA-MB-231 breast and Calu-1 lung cancer cell lines were obtained from the American Type Culture Collection. HEK293A control and RagA/B KO lines were a gift from the Kun-Liang Guan laboratory and have been described previously (3). Calu-1 cells were cultured in McCoy's 5A medium (Sigma, M8403) containing 10% fetal bovine serum (FBS) (F4135) and 4 mM L-glutamine (Sigma, G7513). All other cells were cultured in Dulbecco's modified Eagle's medium (Sigma, D6429) containing 10% FBS. All culture media were supplemented with 1× antibiotic/antimycotic solution (Sigma, A5955).

Amino acid starvation experiments

All starvation experiments were done by rinsing the plates with Hanks' balanced salt solution (Sigma H9394) twice and then placing the appropriate starvation medium in the wells as follows. For Gln starvation, cells were placed in Gln-free Dulbecco's modified Eagle's medium (DMEM) (Sigma, D5546) or Gln-free McCoy's 5A medium (for Calu-1 cells) (Sigma, M8403) supplemented with 10% dialyzed FBS (DFBS) (Sigma, F0392) for 24 h. Control wells (labeled CM) contained 8 mM Gln (Sigma, G7513) and 10% DFBS for the full 24 h. Cells simultaneously starved of leucine and glutamine were shifted to Leu/Gln-free DMEM (MP Biomedicals, 1642149) for 24 h, supplemented with 10% DFBS. Control wells (CM) contained 8 mM Gln and 0.8 mM Leu (Sigma, L8912) for the full 24 h.

Amino acid refeed experiments

Samples were refeed with 8 mM L-Gln or 0.8 mM L-Leu for the last hour of the experiment. Samples refeed with 4 mM D α KG (Sigma, 349631) or 10 mM L-aspartic acid β -methyl ester hydrochloride (Sigma, A8291) were added to cells for 2 h.

PLD inhibitor treatments

PLD inhibitor (PLDi)–treated samples were simultaneously given 10 μ M VU0359595 to inhibit PLD1 and 10 μ M VU0285655–

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1 to inhibit PLD2 for 1 h. Control lanes were treated with DMSO.

siRNA treatment

Transient siRNA transfections were carried out as described previously (27). Cells were plated in 6-well plates in complete medium containing 10% FBS. The following day, transfections with siRNA (100 nM) in Lipofectamine RNAiMAX (Thermo Fisher Scientific 137780) were performed in Opti-MEM reduced serum medium (Thermo Fisher Scientific, 31985070). After 6 h, cells were given 10% FBS, and the following day, the cells were shifted to complete medium with 10% FBS. Cells were then allowed to incubate for indicated times for each experiment as described in the figure legends. The following siRNAs were used in the study: Arf1 siRNA (Ambion, 4457298), GDH1 siRNA (Ambion, 4427038), RalA siRNA (Ambion, 4427037), and nontargeting siRNA (Ambion, 4390843) were obtained from Thermo Fisher Scientific; GOT1/AATC siRNA (sc-45602), GOT2/AATM siRNA (sc-60052), and Arf6 siRNA (sc-43619) were obtained from Santa Cruz Biotechnology; Rheb siRNA (M-009692-02), PLD1 siRNA (L-009413-00-0005), and PLD2 siRNA (L-005064-00-0005) were obtained from Dharmacon.

Western blot analysis

Extraction of proteins from cultured cells and Western blot analysis of extracted proteins was performed as described previously (27). Proteins were extracted from cultured cells in M-PER (Thermo Fisher Scientific 78501). Equal amounts of total proteins were separated using SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were then blocked using 5% nonfat dry milk in PBS containing 0.1% Tween 20. Membranes were incubated overnight with primary antibodies as described in the figure legends. Either anti-mouse, anti-rabbit, or anti-goat horseradish peroxidase-conjugated IgG (Protein Tech Group, SA00001-1, SA00001-2, or SA00001-3, respectively) was used for detection using the ECL reagents (Kindle Biosciences, LLC R1002 or R1003). Western blots were quantified using Image Studio Lite software, and all p-p70^{S6K} levels were normalized to the loading control actin. Western blotting images and quantifications for each figure are from at least two independent experiments. Significance is shown for relevant parameters and where differences were small.

Measurement of PLD activity

PLD activity was determined by quantifying the transphosphatidyl reaction in the presence of 0.8% 1-BtOH as described previously (33), with minor revisions as described below. Cells plated in 6-well dishes were labeled with [³H]myristic acid (3.0 μCi) for the last 4 h of the experiment. For the last 20 min of the experiment, 0.8% 1-BtOH was added to the cells to create the transphosphatidyl reaction, producing [³H]phosphatidylbutanol as a readout of PLD activity. Cells were collected by scraping in the medium and centrifuged to collect a pellet. The pellet was subsequently washed with PBS and lysed in 500 μl of ice-cold acidified methanol (methanol-6N HCl, 50:2). This was added to the first extraction buffer

(155 μl NaCl and 500 μl chloroform) and centrifuged at 4 °C at 13,300 rpm for 3 min. The lower organic layer was added to the second extraction buffer (350 μl of water, 115 μl of 1 M NaCl, and 115 μl of methanol) and centrifuged as above. The amount of radioactivity in the lower organic layer was quantified using a scintillation counter, and equal amounts of radioactivity of the total lipids from each sample were dried under nitrogen along with 10 μl of phosphatidylbutanol standard solution (10 mg/ml). However, to compare the HEK WT cells with the RagA/B KO cells, we dried equal volumes of the total lipids under nitrogen to be normalized to protein concentration later. All of the dried lipid samples were resuspended in 30 μl of spotting solution (chloroform:methanol, 9:1) and applied to a TLC plate. TLC plates were run in a chamber containing 100 ml of the upper mobile phase solution of ethyl acetate:isooctane:glacial acetic acid:water, 88:40:16:80, for 2.5 h. Plates were allowed to air-dry and then placed in an iodine chamber to detect the presence of lipids. The lipid band corresponding to phosphatidylbutanol was scraped and placed in scintillation fluid to be quantified as a measure of PLD activity. For the HEK cells, the amount of radioactivity scraped was divided by their respective protein concentrations to account for differences between cell proliferation between the WT and KO cell lines.

Preparation of lipid vesicles

Lipid vesicles were prepared as described previously (17). Lipids in chloroform (16:0-18:1 PA and 16:0-18:1 PS) were dried under nitrogen and resuspended in Dulbecco's PBS (Thermo Fisher Scientific, 14190). 16:0-16:0 PA powder was resuspended in Dulbecco's PBS. All lipid/DPBS mixtures were sonicated for 3 min. Cells were treated with vesicles for a final concentration of 300 μM for 30 min twice for a total treatment time of 1 h.

Statistical analysis

All statistical details (i.e., sample size, *p* value summaries) of experiments can be found in the figures and their corresponding figure legends. Statistical significance was analyzed using Graph Pad Prism 7 software. For comparisons between two sets of samples, an unpaired *t* test was performed. For multiple comparisons, an unpaired one-way analysis of variance was performed. All data are plotted as the mean with standard deviation. The *p* values are as follows: *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001; ****, *p* ≤ 0.0001. Not significant (ns) means *p* > 0.05.

Author contributions—E. B., D. M., V. V., M. A. F., and D. A. F. conceptualization; E. B., D. M., V. V., I. Z., P. F., and D. A. F. data curation; E. B. and D. A. F. formal analysis; E. B. and D. A. F. validation; E. B., D. M., V. V., and D. A. F. investigation; E. B., D. M., V. V., I. Z., P. F., and D. A. F. methodology; E. B. and D. A. F. writing-original draft; E. B. and D. A. F. writing-review and editing; D. A. F. resources; D. A. F. supervision; D. A. F. funding acquisition; D. A. F. project administration.

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