Critical role for PI3-kinase in regulating the use of proteins as an amino acid source

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Ras-transformed cells can grow in amino acid-poor environments by recovering amino acids through macropinocytosis and lysosomal catabolism of extracellular proteins. However, when studying non-transformed fibroblasts, we found that Ras GTPases are dispensable for growth-factor-stimulated macropinocytosis and lysosomal catabolism of extracellular proteins. Instead, we establish a critical role for phosphatidylinositol 3-kinase (PI3-kinase) signaling in cell proliferation that is supported by protein macropinocytosis. Downstream of PI3-kinase, distinct effectors have opposing roles in regulating uptake and catabolism of extracellular proteins. Rac1 and PLC are required for nutritional use of extracellular proteins. In contrast, Akt suppresses lysosomal catabolism of ingested proteins when free amino acids are abundant. The interplay between these pathways allows cells with oncogenic PIK3CA mutations or PTEN deletion to grow using diverse amino acid sources. Thus, the prevalence of PI3-kinase and PTEN mutations in cancer may result in part because they allow cells to cope with fluctuating nutrient availability.

PI3-kinase signaling pathway | cellular nutrient acquisition | cancer metabolism | macropinocytosis | lysosomal protein catabolism

The enclosure of cells by a plasma membrane necessitates dedicated transport systems that mediate nutrient uptake from the environment. Mammalian cells import low molecular weight nutrients, such as glucose and amino acids through cell-surface nutrient transporters, and internalize more complex nutrients such as proteins through endocytosis. Nutrient uptake in mammalian cells is stimulated by growth-factor signaling pathways. This principle prevents uncontrolled nutrient consumption and ensures that nutrient supply matches a cell's biosynthetic demands (1). Downstream of growth factors, receptor tyrosine kinases activate lipid kinases of the PI3-kinase class IA family, which stimulate cellular uptake of glucose and amino acids. PI3-kinase promotes glucose uptake through its effector, the serine/threonine kinase Akt, which induces surface translocation of the transporter GLUT4 in response to insulin and enhances expression of the ubiquitous transporter GLUT1 (2–4). Akt can also promote expression of CD98 heavy chain, which functions as the common subunit of several plasma membrane amino acid transporters, including the neutral amino acid transporter LAT1 (5).

When available extracellularly, glucose and amino acids are avidly taken up by mammalian cells to support energy production and macromolecular synthesis. However, extracellular proteins have emerged as an alternative nutrient source in nutrient-depleted environments such as poorly perfused tumors (6, 7). Mammalian cells can ingest proteins through a nonselective form of endocytosis referred to as macropinocytosis (8, 9). Although most cell types do not have any basal macropinocytic activity without external stimuli, constitutively activated Ras GTPases induce macropinocytosis cell autonomously (10, 11). As a consequence, Ras-transformed cells can recover amino acids from extracellular proteins to sustain proliferation when free amino acids become limiting extracellularly (12–14).

Although oncogenic Ras proteins trigger macropinosome formation, macropinocytosis in nontransformed cells is initiated by growth factors. In fact, actin-driven membrane ruffling and subsequent engulfment of extracellular solutes

into macropinosomes are among the earliest cellular responses to growth-factor stimulation (15, 16), both of which are blocked by inhibition of PI3-kinase (11, 17). However, although macropinocytosis can be initiated by growth-factor signaling, it is not clear which downstream effectors are required for the use of extracellular proteins as an amino acid source. Unlike glucose and free amino acids, proteins ingested through macropinocytosis only unlock their nutritional value upon hydrolysis into their monomeric constituents in the lysosome (6, 18). Lysosomal catabolism of extracellularly derived proteins has emerged as an important step at which amino acid recovery is regulated. The kinase mechanistic target of rapamycin complex 1 (mTORC1) suppresses lysosomal degradation of ingested proteins, and mTORC1 inactivation consequently promotes the proliferation of cells that rely on proteins as nutrients (14). mTORC1 is an intracellular nutrient sensor that becomes active under amino acid replete conditions, but also receives inputs from growth factors through its upstream activator Akt (19, 20). Growth-factor signaling therefore regulates both macropinocytosis and lysosomal proteolysis of extracellular proteins. However, how these activities are coordinated to allow cells to use proteins as an amino acid source has been unknown.

Here, we examine roles of Ras and PI3-kinase for growth-factor-initiated cell proliferation in the presence of different amino acid sources. Although oncogenic Ras has previously been demonstrated to induce macropinocytosis cell intrinsically, we show that PI3-kinase but not Ras is required for macropinocytosis in response to growth-factor stimulation. Therefore,

Significance

Mammalian cells can take up monomeric amino acids through cell-surface transporters or recover amino acids through macropinocytosis and lysosomal catabolism of extracellular proteins. In mammalian cells, nutrient uptake is regulated by growth factors. Yet how growth-factor signaling orchestrates different nutrient uptake routes is unclear. Here, we establish a central role for growth-factor-activated phosphatidylinositol 3-kinase (PI3-kinase) signaling in promoting cellular amino acid uptake and show that distinct effector branches regulate expression of amino acid transporters, and macropinocytosis and lysosomal catabolism of ingested proteins. Therefore, PI3-kinase signaling supports cell proliferation in the presence of various amino acid sources. These findings suggest that oncogenic PI3-kinase pathway activation is a selective advantage for tumor cells proliferating in fluctuating nutrient environments.

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PI3-kinase and its effectors Rac1 and PLC promote cell growth that uses extracellular proteins as an amino acid source. In contrast, the PI3-kinase effector Akt suppresses lysosomal catabolism of ingested proteins and enhances expression of plasma membrane amino acid transporters. Thus, activation of PI3-kinase initiates cellular pathways that promote acquisition of either free amino acids or extracellular proteins. As a result, PI3-kinase signaling can sustain cell proliferation in diverse nutritional environments.

Results

Roles of Growth-Factor Signaling Pathways in Supporting Cell Proliferation During Amino Acid Limitation. It remains unclear how growth-factor signaling regulates cellular adaptation to limiting availability of extracellular amino acids. Two arms of signaling downstream of growth-factor-activated receptor tyrosine kinases have been demonstrated to promote cellular nutrient uptake: Akt, which promotes expression and surface presentation of nutrient transporters (5), and Ras, which induces macropinocytosis of extracellular macromolecules as an alternative amino acid source (12). To study consequences of activating either signaling pathway, we generated immortalized mouse embryonic fibroblasts (MEFs) that harbor an oncogenic K-Ras^{G12D} allele or express myristoylated Akt1 (myr-Akt1), which is constitutively active. As Akt and Ras participate in signaling pathways by which PI3-kinase transduces growth-factor signals, we also examined MEFs in which the negative PI3-kinase pathway regulator, PTEN, was deleted by CRISPR/Cas9. When placed in medium containing 10% serum and high amino acid levels (complete medium), control cells proliferated well (Fig. 1A). Expression of myr-Akt1 or deletion of PTEN did not affect serum-stimulated cell proliferation, and K-Ras^{G12D} expression caused a slight enhancement of growth in complete medium. When serum-stimulated in medium containing low levels of all amino acids (10% of complete medium), wild-type and K-Ras^{G12D} MEFs were not able to proliferate (Fig. 1B). In contrast, myr-Akt1 expression or PTEN KO enabled cells to sustain robust proliferation in low amino acid medium for several consecutive days.

Besides importing amino acids in their monomeric form, cells can also acquire amino acids through macropinocytosis and lysosomal catabolism of extracellular proteins. Of note, standard tissue culture media formulations disfavor the nutritional utilization of extracellular proteins, because compared with mammalian plasma and interstitial fluids they contain supraphysiological amino acid levels, but strongly reduced protein levels. To shift the balance of amino acid availability in favor of extracellular proteins, low amino acid medium was thus supplemented with 3% albumin to mimic its physiological concentration (2–5% in human interstitial fluids and plasma). K-Ras^{G12D} MEFs could not proliferate in low amino acid medium; albumin supplementation caused a striking rescue of proliferation (Fig. 1C). These results confirm the ability of activating Ras mutations to support cell proliferation that uses extracellular proteins as an amino acid source (14). Myr-Akt1 cells proliferated in low amino acid medium, but their growth was not improved by albumin addition. Growth rates of PTEN KO cells were modestly increased when albumin was supplemented at physiological levels (Fig. 1C). Albumin supplementation also allowed wild-type controls to proliferate in low amino acid medium, but only to a minor extent (Fig. S1A).

Akt Activation or PTEN Deletion Results in Increased Levels of Amino Acid Transporters. As amino acid-deprived wild-type MEFs progressively lose viability, Akt might sustain cell proliferation during amino acid limitation through its ability to suppress apoptosis (21). To test this, we blocked apoptosis in MEFs by overexpression of antiapoptotic Bcl-xl or genetic ablation of the apoptosis mediators Bax and Bak. Over a range of amino acid concentrations, Bcl-xl overexpressing cells and controls proliferated at the same rate (Fig. S1B). Moreover, Bax/Bak deletion did not improve cell proliferation under amino acid limitation,

Fig. 1. Roles of growth-factor signaling pathways in supporting cell proliferation during amino acid (AA) limitation. (A and B) Growth curves of K-Ras^{G12D}, myr-Akt1, and PTEN KO MEFs and wild-type controls in medium with amino acids provided at (A) 100% of complete medium (high) or (B) 10% of complete medium (low). See Fig. 2A for Western blot (WB) of Akt signaling activity and PTEN deletion of cells used in Fig. 1. (C) Growth curves of K-Ras^{G12D}, myr-Akt1, and PTEN KO MEFs in medium with amino acids provided at 10% of complete medium (low) ± 3% albumin. Data are shown as mean ± SD (*n* = 3) and are representative of at least four independent experiments.

whereas myr-Akt1 expression restored proliferation of wild-type controls (Fig. S1C). Thus, myr-Akt1 does not sustain cell proliferation in low amino acid medium by blocking cell death. Another way by which growth-factor signaling could promote cellular amino acid acquisition is the induction of plasma membrane amino acid transporters such as CD98. Indeed, myr-Akt1 expression or PTEN KO, which increased phosphorylation of the Akt target tuberous sclerosis complex 2 (TSC2), caused a significant elevation of CD98 protein levels (Fig. 2A). In contrast, K-Ras^{G12D} MEFs had slightly decreased CD98 levels. Previous work had shown that myr-Akt sustains cell-surface levels of CD98 in growth-factor-deprived lymphocytes in an mTORC1-dependent manner (5). To ask whether mTORC1 was required for CD98 expression in MEFs, we inhibited mTORC1 by genetic ablation of raptor or treatment with the mTOR kinase inhibitor Torin 1. Raptor knockout or Torin 1 treatment caused a strong reduction in CD98 (Fig. 2B and C). The PI3-kinase class IA inhibitor GDC0941 and the pan-Akt inhibitor MK-2206, which moderately decreased mTORC1 activity, had less of an effect on CD98 levels. These data indicate that PTEN KO and myr-Akt1 increase CD98 levels through activation of mTORC1.

Rac1 and PLC Are Required for Extracellular Protein-Dependent Cell Proliferation. The small GTPase Rac1 and phospholipase C (PLC) are intracellular effectors downstream of growth-factor signaling that regulate actin-driven membrane ruffling and macropinosome

Fig. 2. Akt activation or loss of PTEN results in increased CD98 levels. (A) CD98 levels in myr-Akt1, PTEN KO, or K-Ras^{G12D} MEFs and controls, analyzed by Western blotting (WB). (B) CD98 levels in Raptor KO MEFs, analyzed by WB. (C) CD98 levels analyzed by WB in wild-type MEFs treated for 16 h with the following inhibitors: PI3-kinase (1 μ M GDC0941), Akt (2 μ M MK-2206), and mTOR (250 nM Torin 1, 100 nM rapamycin). Data are representative of three independent experiments.

closure (11, 22, 23). We therefore asked whether Rac1 and PLC were required for proliferation of growth-factor-stimulated cells that depended on extracellular proteins as nutrients. We first determined requirements for Rac1 and PLC in cellular albumin uptake in response to growth-factor stimulation. Serum-deprived MEFs were incubated for 30 min with fluorescently labeled albumin and concomitantly stimulated with platelet-derived growth factor (PDGF) in the presence of the Rac1 inhibitor NSC23766 or the

PLC γ inhibitor U-73122. Inhibiting either Rac1 or PLC γ caused a significant decrease in intracellular albumin (Fig. 3 A and B). Treating cells concomitantly with both inhibitors decreased albumin uptake even further. To ask if Rac1 or PLC γ inhibition affected lysosomal catabolism of internalized albumin, we used a self-quenched albumin that fluoresces upon degradation (DQ-BSA). NSC23766 and U-73122 significantly decreased DQ-BSA fluorescence, consistent with the effect of these inhibitors to suppress cellular albumin uptake (Fig. S2 A and B). While we cannot exclude the possibility that NSC23766 and U-73122 could have additional targets, these data indicate that inhibition of Rac1 and PLC γ limits ingestion of extracellular proteins, consistent with the role of these proteins in macropinosome formation.

We next asked whether Rac1- and PLC-mediated albumin uptake contributed to extracellular protein-dependent cell proliferation. To address this question, we turned to a nutritional setting where albumin is an obligatory amino acid source. When wild-type MEFs are placed in medium lacking the essential amino acid leucine, the most abundant amino acid in the mammalian proteome, they cease to proliferate and progressively lose viability, despite constant stimulation with serum-provided growth factors. Cell survival in leucine-free medium can be rescued by supplementing 3% albumin as an alternative source for leucine (14) (Fig. S2C). To examine the relevance of Rac1 and PLC γ for extracellular protein-dependent cell survival, increasing concentrations of NSC23766 or U-73122 were added to leucine-free medium + 3% albumin and cell numbers determined after 4 d. Cell viability was

Fig. 3. Rac1 and PLC are required for extracellular protein-dependent cell proliferation. (A) Albumin uptake in MEFs treated with inhibitors against Rac1 (100 μ M NSC23766) or PLC γ (4 μ M U-73122) alone or in combination. (B) Quantification of albumin internalization in cells shown in A. Data are shown as mean $\pm$ SD (n = 18 fields of view) and representative of two independent experiments. *** P < 0.001, **** P < 0.0001. (Scale bars, 20 μ m.) (C and D) Cell numbers of WT MEFs (C) at day 4 of culture in leucine-free medium + 3% albumin or (D) at day 3 of culture in leucine-containing medium + 3% albumin. Inhibitors were added at the onset of starvation as indicated. Data are shown as mean $\pm$ SD (n = 3) and are representative of three independent experiments.

decreased by either inhibitor in a concentration-dependent manner and almost completely lost when both inhibitors were added together (Fig. 3C). In contrast, inhibiting Rac1 or PLC γ did not impair cell proliferation when free leucine was added to the medium (Fig. 3D). Thus, Rac1 and PLC are specifically required when cell survival depends on extracellular proteins as a source for leucine.

PI3-Kinase but Not Ras Is Required for Growth-Factor–Induced Macropinocytosis. Oncogenic Ras variants induce macropinocytosis in mammalian cells independently of growth-factor stimulation (10, 11). However, lack of inhibitors and genetic redundancy between different Ras homologs have made it difficult to establish whether

Ras was required for growth-factor–induced macropinocytosis. The recent generation of Rasless MEFs, which are devoid of all Ras proteins, allowed us to address this question (24). Inducing Cre recombinase in *H-Ras*, *N-Ras* double-knockout MEFs harboring conditional *K-Ras* alleles reduced Ras protein levels below detection limit within a week (Fig. 4A). Rasless MEFs had strongly diminished MAP kinase signaling, as shown by loss of Erk phosphorylation, but had normal levels of Akt and mTORC1 activity (Fig. 4A and Fig. S3A). We also introduced inducible H-Ras^{G12V} to compare deletion and oncogenic activation of Ras. Macromolecular uptake was assessed by internalization of fluorescently labeled high molecular weight dextran, which marks macropinosomes. To determine cell-intrinsic macropinocytic activity, inputs from growth-factor signaling

Fig. 4. PI3-kinase but not Ras is required for growth-factor–stimulated macropinocytosis. (A) Generation of Rasless MEFs from *K-Ras*^{lox}, *H-Ras*^{-/-}, *N-Ras*^{-/-} MEFs by induction of Cre recombinase with 4-hydroxytamoxifen, analyzed by WB. (B) High molecular weight dextran uptake in Rasless, H-Ras^{G12V}-expressing, and control MEFs after 16 h serum starvation $\pm$ restimulation with PDGF. (Scale bars, 50 μ m.) (C) Quantification of dextran uptake of cells shown in B. (D) Dextran uptake in Rasless MEFs after PDGF stimulation $\pm$ PI3-kinase inhibitor (1 μ M GDC0941). (Scale bars, 50 μ m.) (E) Quantification of dextran uptake in cells shown in D. (F) Quantification of dextran uptake in wild-type MEFs after PDGF stimulation $\pm$ PI3-kinase inhibitor (1 μ M GDC0941). (G) Dextran uptake in K-Ras^{G12V} MEFs after 16 h serum starvation $\pm$ PI3-kinase inhibition (1 μ M GDC0941, 2.5 μ M BKM-120). (Scale bars, 25 μ m.) (H) Quantification of dextran uptake in cells shown in G. Data are shown as mean $\pm$ SD (n = 10–18 fields of view) and are representative of two to four independent experiments. * P < 0.05, **** P < 0.0001.

were reduced by placing cells in medium containing 0.1% serum. Under these conditions, dextran uptake was almost undetectable in Rasless MEFs and controls (Fig. 4*B* and *C*). In contrast, H-Ras^{G12V} MEFs displayed robust dextran uptake, consistent with the ability of oncogenic Ras mutants to trigger macropinocytosis in the absence of serum-provided growth factors. To examine growth-factor-induced macropinocytosis, dextran uptake was examined in the presence of PDGF. Stimulation with PDGF only slightly increased dextran uptake in H-Ras^{G12V} MEFs, presumably because it was already high in the absence of growth factors (Fig. 4*B* and *C*). Unexpectedly, Rasless MEFs responded to PDGF stimulation with avid dextran uptake. PDGF-induced dextran uptake was consistently higher in Rasless MEFs than in controls.

We next asked whether loss of Ras signaling affected lysosomal catabolism of internalized albumin. Dequenching of DQ-BSA fluorescence was similar in Rasless MEFs and controls, while being moderately elevated in H-Ras^{G12V} MEFs (Fig. S3*B*). Moreover, Rasless MEFs and controls displayed a comparable increase in DQ-BSA degradation in response to mTOR inhibition and decrease upon treatment with lysosomal protease inhibitors (Fig. S3*C*). Rasless MEFs also had normal levels of autophagy induction upon amino acid starvation, as assessed by lipidation and turnover of LC3 (Fig. S3*D*). Levels of processed cathepsins were even slightly elevated in Rasless MEFs (Fig. S3*A*). Thus, complete loss of all Ras proteins does not cause any overt defects in lysosomal proteolysis. Together, these data establish that Ras GTPases are dispensable for macropinocytosis and lysosomal catabolism of albumin in response to growth-factor stimulation. Whether Ras proteins were required for the cell proliferation that uses proteins as nutrients could not be determined, because Rasless MEFs do not proliferate (24).

Besides Ras, PI3-kinase is an important regulator of macropinosome formation, and pharmacological inhibition of PI3-kinase suppresses macropinocytosis (11, 17). Moreover, PI3-kinase can activate Rac1 and PLC (25, 26). We therefore wondered whether in the absence of Ras, PDGF induced macropinocytosis through activating PI3-kinase. Indeed, pharmacological inhibition of PI3-kinase strongly decreased PDGF-induced dextran uptake in Rasless MEFs (Fig. 4*D* and *E*) and MEFs harboring wild-type Ras proteins (Fig. 4*F* and Fig. S4*A*). Macropinocytosis induced by K-Ras^{G12V} expression in the absence of growth factors was also significantly decreased by PI3-kinase inhibition (Fig. 4*G* and *H* and Fig. S4*B*). Thus, PI3-kinase is required for macropinocytosis that is either induced by growth factors or triggered by oncogenic Ras independently of growth-factor stimulation.

Akt Suppresses Lysosomal Catabolism of Extracellular Proteins. The above data demonstrate that Rac1 and PLC are required when cell proliferation relies on macropinocytosis as an amino acid acquisition strategy. However, Akt is the PI3-kinase effector pathway with the best-established role in promoting nutrient acquisition and cell growth (1, 21). We therefore asked whether Akt regulated the utilization of extracellular proteins as nutrients. To address this, wild-type MEFs were placed in leucine-free medium supplemented with 3% albumin and treated with the Akt inhibitor MK-2206. Control cells maintained viability in leucine-free medium + 3% albumin, but could not proliferate. However, inhibiting Akt with MK-2206 enabled cells to proliferate over several consecutive days (Fig. 5*A*). Moreover, increasing MK-2206 concentrations in leucine-free medium + albumin caused cells to accumulate at progressively higher numbers (Fig. S5*A*). Conversely, in leucine-containing medium MK-2206 caused a concentration-dependent decrease of cell numbers (Fig. S5*B*). Therefore, Akt inhibition promotes serum-induced cell proliferation that relies on albumin as a source for leucine, but suppresses growth in complete medium. Concomitant addition of MK-2206 with the Rac1 inhibitor NSC23766 or the PLC γ inhibitor U-73122 blunted cell growth in leucine-free medium + 3% albu-

min (Fig. S5*A*). Thus, the ability of Akt inhibition to enhance growth that uses albumin as a source of leucine remains dependent on Rac1 and PLC γ .

We next asked whether Akt signaling suppressed albumin-dependent growth by regulating macropinocytosis or lysosomal proteolysis. Modulating Akt activity in MEFs via myr-Akt1 expression or MK-2206 treatment did not affect dextran uptake (Fig. S5*C* and *D*). However, inhibiting Akt with MK-2206 caused a strong increase in dequenching of DQ-BSA fluorescence (Fig. 5*B* and *C*). Thus, although Akt does not regulate macropinocytosis, it suppresses lysosomal proteolysis of internalized albumin. These phenotypes are reminiscent of previous findings that mTORC1 inhibition promotes cell proliferation that uses extracellular proteins as an amino acid source by enhancing their lysosomal catabolism. Consistently, MK-2206 and Torin 1 promoted DQ-BSA degradation to a similar extent (Fig. S5*E*). Besides MEFs, various cancer cell lines respond to Akt and mTOR inhibitors with increased DQ-BSA proteolysis and albumin-dependent cell proliferation (14) (Fig. S5*F*).

To study the effects of increased Akt signaling, we expressed wild-type Akt1 or myristoylated Akt1, Akt2, or Akt3 in K-Ras^{G12D} MEFs (Fig. S6*A*). While K-Ras^{G12D} MEFs are normally able to grow in leucine-free medium + 3% albumin, they ceased to proliferate and rapidly lost viability when overexpressing an activated Akt variant (Fig. 5*D*). In contrast, expression of Akt1 or the different myr-Akt variants did not have adverse effects on cell proliferation in leucine-containing medium (Fig. S6*B*). As mTORC1 is a central effector pathway of Akt signaling, we asked whether suppression of albumin-dependent cell proliferation by myr-Akt required mTORC1. Rapamycin completely rescued proliferation of K-Ras^{G12D} MEFs expressing myr-Akt variants in leucine-free medium + 3% albumin (Fig. 5*E*). Moreover, addition of MK-2206 or rapamycin alone or in combination caused a similar increase in numbers of wild-type MEFs in leucine-free medium + 3% albumin (Fig. 5*F*). Thus, the Akt/mTORC1 signaling axis opposes the action of other branches of PI3-kinase signaling, which promote albumin-dependent cell proliferation.

Loss of PTEN and Activating Mutations in PIK3CA Support Extracellular Protein-Dependent Cell Proliferation. Activation of the PI3-kinase pathway by loss of the negative regulator PTEN or activating mutations in the catalytic subunit PIK3CA are highly prevalent in cancer (27). Because extracellular proteins could function as important alternative nutrients in nutrient-deprived tumor microenvironments, we determined the consequences of increased PI3-kinase signaling on extracellular protein-dependent cell proliferation. First, PI3-kinase pathway activity was increased by CRISPR/Cas9-mediated deletion of PTEN. Lentiviral delivery of three different gRNAs in wild-type MEFs resulted in robust depletion of PTEN (Fig. S7*A*). PTEN KO increased PDGF-induced macropinocytosis, as assessed by dextran uptake, which was sensitive to the PI3-kinase inhibitor GDC0941 (Fig. 6*A* and *B*). Despite this, when placed in leucine-free medium + 3% albumin containing 10% serum, PTEN KO MEFs did not proliferate faster than controls (Fig. S7*B*). However, PTEN KO enhanced Akt signaling (Fig. S7*A*), which might antagonize the nutritional utilization of proteins. Thus, we asked whether PTEN KO could improve extracellular protein-dependent cell proliferation if Akt/mTORC1 signaling was suppressed. When placed in leucine-free medium + 3% albumin, serum-induced proliferation of control cells was slightly improved by rapamycin or Torin 1 (Fig. S7*B*). However, PTEN KO MEFs responded to mTOR inhibition with dramatically improved proliferation (Fig. S7*B*) and PTEN KO synergized with mTORC1 inhibition to promote albumin-dependent cell proliferation (Fig. 6*C* and *D*). To compare how Akt/mTORC1 signaling regulated proliferation of PTEN KO MEFs when using either free amino acids or proteins, cells were placed in leucine-containing or leucine-free medium + 3% albumin

Fig. 5. Akt suppresses lysosomal catabolism of internalized proteins. (A) Growth curve of wild-type MEFs in leucine-free medium + 3% albumin $\pm$ Akt inhibitor (2 μ M MK-2206). (B) DQ-BSA hydrolysis in wild-type MEFs treated $\pm$ 1 μ M MK-2206. (Scale bars, 20 μ m.) (C) Quantification of DQ-BSA hydrolysis in cells shown in B. Data are shown as mean $\pm$ SD (n = 18 fields of view) and representative of five independent experiments. **** P < 0.0001. (D and E) Growth curves of K-Ras^{G12D} MEFs expressing Akt1, or myr-Akt1-3 in (D) leucine-free medium + 3% albumin and (E) leucine-free medium + 3% albumin, 100 nM rapamycin. (F) Cell numbers of wild-type MEFs at day 4 of culture in leucine-free medium + 3% albumin treated with 2 μ M MK-2206 and 100 nM rapamycin alone or in combination. Data in A and D–F are shown as mean $\pm$ SD (n = 3) and are representative of at least five (A) or two (D–F) independent experiments.

and treated with MK-2206, rapamycin, or Torin 1. When free leucine was provided extracellularly, all these inhibitors suppressed serum-induced cell proliferation (Fig. 6E). In contrast, they increased cell proliferation in medium where albumin was the leucine source (Fig. 6F). Together, these data demonstrate that PTEN KO confers a growth advantage when leucine must be derived from extracellular amino acids, if the Akt/mTORC1 signaling axis is suppressed (Fig. 7A).

Lastly, we investigated the role of PI3-kinase directly. Inhibition of PI3-kinase prevented proliferation of K-Ras^{G12D} MEFs in leucine-free medium + 3% albumin (Fig. 7B). Thus, PI3-kinase signaling is required for extracellular protein-dependent growth, even in cells that harbor an activating Ras mutation. To determine effects of increased PI3-kinase activity, we generated MEFs expressing activated variants of PIK3CA, which were discovered in cancer genome sequencing efforts (PIK3CA^{E545K} and PIK3CA^{H1047R}) (28) as well as genetically engineered myr-PIK3CA. When placed in complete medium, cells expressing activated PIK3CA accumulated to higher numbers (Fig. 7C). Similarly, all activated PIK3CA variants increased cell numbers in leucine-free medium + 3% albumin and in response to Torin 1 treatment (Fig. 7D and E). Thus, activating PIK3CA mutations confer a proliferative advantage to cells when leucine is provided either in its free form or when it must be recovered from extracellular proteins.

Discussion

The present work establishes a role for PI3-kinase in regulating cellular amino acid acquisition through two distinct arms of the signaling pathway (Fig. 7A). PI3-kinase and its effectors Rac1 and PLC are required for macropinocytosis, which allows cells to use extracellular proteins as an amino acid source. Conversely, the PI3-kinase effector Akt activates mTORC1 to

suppress lysosomal catabolism of internalized proteins while increasing expression of amino acid transporters. The Akt/mTORC1 signaling axis therefore determines a physiological state in which free amino acids are preferred nutrients while endolysosomal nutrient acquisition pathways are suppressed. However, mTORC1 activation requires both inputs from Akt and sufficient levels of intracellular amino acids. This suggests that downstream of PI3-kinase, macropinocytosis and lysosomal degradation of extracellular proteins may dominate when a decline in intracellular amino acid levels leads to inactivation of mTORC1. These principles allow PI3-kinase activation to sustain cell proliferation in the presence of different amino acid sources.

Many survival and growth-promoting activities of PI3-kinase in response to growth-factor signals are transduced by Akt (21). The predominant mechanism by which Akt supports cell growth is activation of mTORC1, which increases anabolic metabolism and macromolecular synthesis. Akt also influences metabolism directly, for instance by up-regulating glucose transporters and potentiating the activity of hexokinase to increase flux through glycolysis. PI3-kinase/Akt-dependent activation of mTORC1 also increases expression and surface presentation of CD98 heavy chain, the common subunit of LAT1 and several other amino acid transporters (5) (the present study). By increasing the permeability of the plasma membrane for low molecular weight nutrients, PI3-kinase and its effectors Akt/mTORC1 thus promote cell growth when extracellular glucose and amino acids are plentiful.

Although Akt is a particularly well-understood node of the PI3-kinase signaling pathway, several other PI3-kinase effectors regulate important aspects of cell physiology. For instance, PI3-kinase-mediated activation of Rac1 regulates cell motility through remodeling of the actin cytoskeleton (29). Rac1 also increases glycolytic flux in response to growth-factor-mediated PI3-kinase

Fig. 7. Cancer-associated PI3-kinase mutations enhance extracellular protein-dependent growth. (A) Model for regulation of amino acid acquisition by the PI3-kinase signaling pathway. In response to growth-factor stimulation, PI3-kinase initiates cellular uptake of proteins through macropinocytosis, with Rac1 and PLC being required effectors. Akt/mTORC1 signaling suppresses lysosomal catabolism of endocytosed proteins while increasing amino acid transporter levels. (B) Growth curve of K-Ras^{G12D} MEFs in leucine-free medium + 3% albumin ± PI3-kinase inhibitors (2 μ M GDC0941, 25 μ M LY29002). (C–E) Change in cell numbers of MEFs expressing activating PIK3CA mutants in medium supplemented with 3% albumin (C) at day 3 in leucine-containing medium, (D) at day 4 in leucine-free medium, and (E) at day 4 in leucine-free medium + 250 nM Torin 1. Data in B–E are shown as mean $\pm$ SD ($n = 3$) and are representative of at least three independent experiments.

inactivating mutations in the negative mTORC1 regulators TSC1/2, which result in constitutively elevated mTORC1 signaling, predominantly occur in tumors that arise in highly perfused organs such as brain, heart, and kidney (33). It remains open to future experiments to test this model in vivo and in different tissues and cell types. Tumor-associated Rac1 mutations have been reported (34, 35), and while they were suggested to contribute to cellular motility and invasiveness, it might be interesting to ask whether Rac1 mutations could also promote cellular adaptation to nutrient deprivation.

In addition to PI3-kinase activation, cytoplasmic injection or ectopic expression of oncogenic H-Ras and K-Ras can also induce high levels of membrane ruffling and macropinosome formation (10, 11). Consistently, macropinocytosis of extracellular proteins allows Ras-transformed cells to sustain proliferation in media that have reduced glutamine levels or lack essential amino acids such as leucine (12–14). However, the loss of function analysis of mammalian Ras proteins presented here reveal that wild-type Ras proteins do not play an essential role in macropinocytosis regulation. Rasless MEFs, which lack K-Ras, H-Ras, and N-Ras, do not display defects in macropinocytosis induction in response to growth-factor stimulation. Therefore, Ras is dispensable for the cellular events that shape a macropinosome, including the F-actin remodeling that drives membrane ruffling. However, Rasless MEFs are immotile, indicating that Ras GTPases are essential for cytoskeletal rearrangements that cause cell migration (24). Of note, cell motility and macropinocytosis can be antagonistic processes (36, 37), which is consistent with loss of migratory activity but enhanced growth-factor-stimulated macropinocytosis in Rasless MEFs. Although growth-factor-induced macropinocytosis in

mammalian cells does not require Ras, deletion of several Ras proteins was shown to suppress macropinocytosis in *Dictyostelium* (38, 39). It has been suggested that the critical function of *Dictyostelium* Ras is membrane recruitment and activation of PI3-kinase, which then orchestrates the subsequent events leading to macropinosome formation. However, mammalian PI3-kinase can be activated directly by receptor tyrosine kinases. This may explain why mammalian PI3-kinase activates macropinocytosis in response to growth-factor stimulation independently of Ras.

It may well be that the endolysosomal system evolved in ancestral unicellular eukaryotes as a nutrient acquisition pathway. In support of this notion, macropinocytosis allows modern amoeboid eukaryotes such as *Dictyostelium* to proliferate by feeding on extracellular macromolecules (9). In *Dictyostelium*, PI3-kinases are central regulators of macropinocytosis as a cellular feeding strategy. In contrast, it has been assumed that mammalian PI3-kinase supports cellular metabolism by increasing uptake of low molecular weight nutrients through plasma membrane transporters (21). However, the above data reveal that mammalian PI3-kinase also promotes the utilization of extracellular proteins as nutrients. This suggests that growth factors activate PI3-kinase as a general signal for mammalian cells to engage in nutrient uptake through both nutrient transporters and macropinocytosis. A cell's preference for either nutrient acquisition strategy in response to PI3-kinase signaling may then be determined intracellularly through inputs from nutrient-sensing signaling networks.

Materials and Methods

Reagents. Antibodies were from the following suppliers: Cell Signaling (2280 Raptor, 2708 S6K1, 2920 Akt, 2983 mTOR, 3617 phospho-T1462 TSC2, 3965 pan-Ras, 4060 phospho-S473 Akt, 4308 TSC2, 4377 phospho-T389 S6K1, 9101 phospho-T202/Y204 Erk1/2, 9107 Erk1/2, 9552 PTEN, 13038 phospho-T308 Akt), R&D Systems (AF965 cathepsin B, AF1515 cathepsin L), Santa Cruz Biotechnology (CD98 M-20), and Sigma (A2228 β -actin). Secondary antibodies were from GE Healthcare (HRP-linked anti-rabbit, HRP-linked anti-mouse) and Thermo Fisher Scientific (HRP-linked anti-goat). Alexa Fluor 647 BSA, Oregon Green dextran (70 kDa), DQ Green BSA, Hoechst 33342, LysoTracker Red, and mouse PDGF-BB were from Thermo Fisher Scientific. Inhibitors were from Cayman Chemical (GDC0941, MK-2206), Cell Signaling (LY294002), EMD Chemicals (rapamycin), Selleck Chemicals (BKM-120), Sigma (bafilomycin A1, leupeptin, E-64, pepstatin A, U-73122), and Tocris Bioscience (NSC 23766, Torin 1). Amino acid starvation media were from Athena (DMEM/F12 0423) or prepared by the Memorial Sloan Kettering Cancer Center media core facility. Dialyzed FBS was from Gemini Biosystems and essential amino acids (M5550) and BSA (A1470) were from Sigma.

Cell Lines and cDNA and gRNA Constructs. K-Ras^{G12D} MEFs were described in ref. 14 and Bax^{-/-}; Bak^{-/-} MEFs in ref. 40. K-Ras^{Lox}; H-Ras^{-/-}; N-Ras^{-/-} MEFs were kindly provided by Barbacid and colleagues (24) and Raptor^{lox} MEFs by Hall and colleagues (41). Expression of CreERT2 was induced by adding 1 μ M 4'-hydroxytamoxifen to culture medium, and experiments were performed after 4 d (Raptor KO) or 7–9 d (Ras KO) after induction of Cre.

Retroviral plasmids for constitutive expression of Akt variants (pBabe-Akt, myr-Akt1, myr-Akt2, and myr-Akt3) were from Addgene (42, 43). cDNAs of activated PIK3CA^{E545K}, PIK3CA^{H1047R}, and myr-PIK3CA were from Addgene (44); for inducible expression, PIK3CA variants were subcloned into a modified version of the retroviral vector pTRE-Tight (Clontech) (45). pTRE-Tight-based plasmids for inducible expression of K-Ras^{G12V} and H-Ras^{G12V} are described in ref. 14. pBabe Bcl-xl is described in ref. 46. Ras expression was induced by addition of 100 ng/mL doxycycline (Sigma) to culture medium and PIK3CA expression was induced by addition of 1 μ M doxycycline. gRNAs targeting PTEN were cloned into LentiCRISPR v2 (47).

Plasmids were cotransfected with lentivirus or retrovirus packaging plasmids into HEK293T cells using Lipofectamine 2000 Transfection Reagent (Life Technologies), fresh media were added after 16 h, and viral supernatants were collected 2 d after infection. Target cells were infected by addition of viral supernatant and 8 μ g/mL polybrene. Twenty-four hours after infection, cells were selected with appropriate antibiotics.

Cell Culture and Nutrient Starvation Experiments. Cell culture experiments were performed at 37 $^{\circ}$ C and 5% CO₂ in DMEM/F12 with 10% dialyzed FBS

(molecular weight cutoff 10,000; Gemini Biosystems), 100 units/mL penicillin, 100 µg/mL streptomycin, 17.5 mM glucose, and 4 mM glutamine.

For proliferation assays, MEFs were plated in complete medium, 5 h later briefly rinsed, and then cultured in starvation medium as indicated. For amino acid (AA) titration experiments, AAs were supplemented at indicated percentages of 1× minimum essential medium (MEM) amino acid solution (M5550, Sigma) and 4 mM glutamine. Numbers of adherent cells were determined using a Multisizer 3 Coulter Counter (Beckman).

Western Blotting. Cells were rinsed with ice-cold PBS and detached from culture plates by incubation with ice-cold trypsin (0.05%). Trypsin was inactivated with ice-cold serum and cells were pelleted and rinsed with PBS. Cells were lysed in ice-cold lysis buffer (50 mM Hepes, pH 7.4, 40 mM NaCl₂, 2 mM EDTA, 1 mM Na orthovanadate, 50 mM NaF, 10 mM Na pyrophosphate, 10 mM Na glycerophosphate, 1% Triton X-100, 1× Halt protease and phosphatase inhibitor mixtures; Thermo Scientific) for 15 min, and soluble lysate fractions were isolated by centrifugation at 16,000 × g for 10 min. Protein concentrations were determined with the Pierce BCA Protein Assay (Thermo Scientific) and equal amounts of proteins were analyzed by SDS gel electrophoresis and Western blotting following standard protocols.

Fluorescence Microscopy. Fluid phase uptake of fluorescently labeled macromolecules was determined by incubating cells with 0.1 mg/mL 70 kDa dextran or 0.1 mg/mL BSA for 30 min. Subsequently, cells were washed three times with ice-cold PBS and fixed with 4% formaldehyde in PBS for 15 min. After fixation, cells were labeled for 5 min with PBS + 10 µg/mL Hoechst, then washed in PBS and imaged immediately.

For live imaging of DQ-BSA fluorescence dequenching, medium was supplemented with 0.1 mg/mL DQ Green BSA. One hour before imaging, 10 nM LysoTracker Red and 0.2 µg/mL Hoechst were added. Cells were imaged either immediately after addition of DQ-BSA or after a 6-h incubation with DQ-BSA.

Images were acquired with a Zeiss LSM 5 LIVE or a Zeiss LSM 880 confocal microscope. Cellular uptake/degradation of fluorescently labeled dextran or albumin was quantified using the particle analyzer function of Fiji (48). Mean fluorescence intensity was determined by calculating the integrated signal density per cell in randomly chosen fields of view. Due to large size differences between MEFs deficient for Ras or expressing activated Ras, integrated signal density was normalized by cell area in experiments concerning Rasless MEFs.

Data Analysis. Graphs were created using GraphPad Prism software, and statistical analysis was performed with GraphPad Prism. No statistical method was used to predetermine sample size.

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- Palm W, Thompson CB (2017) Nutrient acquisition strategies of mammalian cells. *Nature* 546:234–242.
- Cong LN, et al. (1997) Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Mol Endocrinol* 11:1881–1890.
- Kohn AD, Summers SA, Birnbaum MJ, Roth RA (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 271:31372–31378.
- Elstrom RL, et al. (2004) Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res* 64:3892–3899.
- Edinger AL, Thompson CB (2002) Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol Biol Cell* 13:2276–2288.
- Davidson SM, Vander Heiden MG (2017) Critical functions of the lysosome in cancer biology. *Annu Rev Pharmacol Toxicol* 57:481–507.
- Boroughs LK, DeBerardinis RJ (2015) Metabolic pathways promoting cancer cell survival and growth. *Nat Cell Biol* 17:351–359.
- Swanson JA (2008) Shaping cups into phagosomes and macropinosomes. *Nat Rev Mol Cell Biol* 9:639–649.
- Bloomfield G, Kay RR (2016) Uses and abuses of macropinocytosis. *J Cell Sci* 129:2697–2705.
- Bar-Sagi D, Feramisco JR (1986) Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by Ras proteins. *Science* 233:1061–1068.
- Amyere M, et al. (2000) Constitutive macropinocytosis in oncogene-transformed fibroblasts depends on sequential permanent activation of phosphoinositide 3-kinase and phospholipase C. *Mol Biol Cell* 11:3453–3467.
- Commisso C, et al. (2013) Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. *Nature* 497:633–637.
- Kamphorst JJ, et al. (2015) Human pancreatic cancer tumors are nutrient poor and tumor cells actively scavenge extracellular protein. *Cancer Res* 75:544–553.
- Palm W, et al. (2015) The utilization of extracellular proteins as nutrients is suppressed by mTORC1. *Cell* 162:259–270.
- Brunk U, Schellens J, Westermark B (1976) Influence of epidermal growth factor (EGF) on ruffling activity, pinocytosis and proliferation of cultivated human glioma cells. *Exp Cell Res* 103:295–302.
- Haiger HT, McKanna JA, Cohen S (1979) Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor. *J Cell Biol* 83:82–90.
- Araki N, Johnson MT, Swanson JA (1996) A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* 135:1249–1260.
- Perera RM, Zoncu R (2016) The lysosome as a regulatory hub. *Annu Rev Cell Dev Biol* 32:223–253.
- Laplane M, Sabatini DM (2012) mTOR signaling in growth control and disease. *Cell* 149:274–293.
- Shimobayashi M, Hall MN (2014) Making new contacts: The mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol* 15:155–162.
- Manning BD, Toker A (2017) AKT/PKB signaling: Navigating the network. *Cell* 169:381–405.
- Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 70:401–410.
- Yoshida S, et al. (2015) Differential signaling during macropinocytosis in response to M-CSF and PMA in macrophages. *Front Physiol* 6:8.
- Drosten M, et al. (2010) Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival. *EMBO J* 29:1091–1104.
- Falasca M, et al. (1998) Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J* 17:414–422.
- Nobes CD, Hawkins P, Stephens L, Hall A (1995) Activation of the small GTP-binding proteins Rho and Rac by growth factor receptors. *J Cell Sci* 108:225–233.
- Chalhoub N, Baker SJ (2009) PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* 4:127–150.
- Samuels Y, et al. (2004) High frequency of mutations of the PIK3CA gene in human cancers. *Science* 304:554.
- Hanna S, El-Sibai M (2013) Signaling networks of Rho GTPases in cell motility. *Cell Signal* 25:1955–1961.
- Hu H, et al. (2016) Phosphoinositide 3-kinase regulates glycolysis through mobilization of aldolase from the actin cytoskeleton. *Cell* 164:433–446.
- Choo AY, et al. (2010) Glucose addition of TSC null cells is caused by failed mTORC1-dependent balancing of metabolic demand with supply. *Mol Cell* 38:487–499.
- Demetriades C, Doumpas N, Teleman AA (2014) Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. *Cell* 156:786–799.
- Curatolo P, Bombardieri R, Jozwiak S (2008) Tuberous sclerosis. *Lancet* 372:657–668.
- Schneider A, et al. (2000) Rac1 in human breast cancer: Overexpression, mutation analysis, and characterization of a new isoform, Rac1b. *Oncogene* 19:3013–3020.
- Krauthammer M, et al. (2012) Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. *Nat Genet* 44:1006–1014.
- Veltman DM, Lemieux MG, Knecht DA, Insall RH (2014) PIP₃-dependent macropinocytosis is incompatible with chemotaxis. *J Cell Biol* 204:497–505.
- Chabaud M, et al. (2015) Cell migration and antigen capture are antagonistic processes coupled by myosin II in dendritic cells. *Nat Commun* 6:7526.
- Hoeller O, et al. (2013) Two distinct functions for PI3-kinases in macropinocytosis. *J Cell Sci* 126:4296–4307.
- Chubb JR, Wilkins A, Thomas GM, Insall RH (2000) The Dictyostelium RasS protein is required for macropinocytosis, phagocytosis and the control of cell movement. *J Cell Sci* 113:709–719.
- Wei MC, et al. (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science* 292:727–730.
- Cybulski N, Zinzalla V, Hall MN (2012) Inducible raptor and rictor knockout mouse embryonic fibroblasts. *Methods Mol Biol* 821:267–278.
- DiRenzo J, et al. (2002) Growth factor requirements and basal phenotype of an immortalized mammary epithelial cell line. *Cancer Res* 62:89–98.
- Boehm JS, et al. (2007) Integrative genomic approaches identify IKKε as a breast cancer oncogene. *Cell* 129:1065–1079.
- Zhao JJ, et al. (2005) The oncogenic properties of mutant p110α and p110β phosphatidylinositol 3-kinases in human mammary epithelial cells. *Proc Natl Acad Sci USA* 102:18443–18448.
- Zuber J, et al. (2011) Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. *Nat Biotechnol* 29:79–83.
- Plas DR, Talapatra S, Edinger AL, Rathmell JC, Thompson CB (2001) Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology. *J Biol Chem* 276:12041–12048.
- Sanjana NE, Shalem O, Zhang F (2014) Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 11:783–784.
- Schindelin J, et al. (2012) Fiji: An open-source platform for biological-image analysis. *Nat Methods* 9:676–682.