Critical role for arginase 2 in obesity-associated pancreatic cancer

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Obesity is an established risk factor for pancreatic ductal adenocarcinoma (PDA). Despite recent identification of metabolic alterations in this lethal malignancy, the metabolic dependencies of obesity-associated PDA remain unknown. Here we show that obesity-driven PDA exhibits accelerated growth and a striking transcriptional enrichment for pathways regulating nitrogen metabolism. We find that the mitochondrial form of arginase (ARG2), which hydrolyzes arginine into ornithine and urea, is induced upon obesity, and silencing or loss of ARG2 markedly suppresses PDA. In vivo infusion of \textsuperscript{15}N-glutamine in obese mouse models of PDA demonstrates enhanced nitrogen flux into the urea cycle and infusion of \textsuperscript{15}N-arginine shows that Arg2 loss causes significant ammonia accumulation that results from the shunting of arginine catabolism into alternative nitrogen repositories. Furthermore, analysis of PDA patient tumors indicates that ARG2 levels correlate with body mass index (BMI). The specific dependency of PDA on ARG2 rather than the principal hepatic enzyme ARG1 opens a therapeutic window for obesity-associated pancreatic cancer.

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Pancreatic ductal adenocarcinoma (PDA) is a deadly malignancy with an incidence on the rise. Parallel to this rise has been a surge in obesity and the metabolic syndrome, established risk factors for PDA. Recent studies of tumor metabolism identified KRAS-driven alterations in nutrient scavenging and utilization that are critical for the maintenance of PDA tumors, the vast majority of which harbor activating KRAS mutations. However, whether these or other metabolic alterations occur in obesity-associated PDA remains unknown. Here we perform unbiased transcriptomic and metabolomic analyses on orthotopic transplant models of PDA, and identify striking alterations in nitrogen metabolism in PDA tumors of the obese. In particular, we find that arginase 2 (ARG2), the extrahepatic mitochondrial enzyme that catalyzes the conversion of arginine into ornithine and urea is induced upon obesity. This induction is accompanied by enhanced PDA growth and increased nitrogen flux from 15N-glutamine into the urea cycle, the principal mammalian pathway for ammonia detoxification, usually operating in the liver. We find that silencing or loss of ARG2 in human or murine tumors specifically, strongly suppresses PDA growth, particularly in obese hosts. Infusion of 15N-arginine in murine orthotopic transplant models of PDA demonstrates that ARG2 deficiency causes shunting of arginine catabolism away from the urea cycle and into creatine synthesis, resulting in significant accumulation of ammonia specifically in tumors of the obese. Our findings point to an essential in vivo role for ARG2 in pancreatic cancer, an insidious malignancy with enhanced protein breakdown and remodeling, and suggest a potential need for the channeling of nitrogen into the urea cycle in highly proliferative tumors. Importantly, because ARG1 and not ARG2 is the main hepatic ureagenic enzyme in mammals, specific targeting of ARG2 may provide a therapeutic opportunity for the treatment of pancreatic cancer patients, particularly those suffering from obesity and the metabolic syndrome.

**Results**

Nitrogen metabolism genes are induced in PDA of the obese. To identify metabolic dependencies in obesity-driven pancreatic cancer, KRAS-mutant human ASPC-1 cells expressing cerulean fluorescent protein (ASP-CFP) were used to generate lean and obese orthotopic xenograft models of PDA, as outlined in Fig. 1a. High fat diet-fed “obese” mice gained significantly more weight (P < 0.0001, two-way ANOVA followed by Tukey test) than their chow-fed “lean” counterparts (Fig. 1a and Supplementary Fig. 1a) and displayed features of the metabolic syndrome (Supplementary Fig. 1b–e). The lean and obese mice were then injected orthotopically into their pancreata with ASP-CFP cells and maintained on their corresponding diets for 6 weeks prior to tumor harvest (Fig. 1a). The resulting tumors were termed “Lean-CFP” and “Obese-CFP”, according to the metabolic state of the mouse. Consistent with a role for obesity in promoting PDA growth, Obese-CFP tumors grew significantly larger (1.5-fold, P = 0.002, t-test) than Lean-CFP (Fig. 1c). A mild stromal infiltration was observed in all PDA tumors, independent of obesity (Supplementary Fig. 2).

To identify obesity-associated transcriptional alterations in PDA, comparative microarray expression analysis was performed. Gene set enrichment analysis (GSEA) revealed a striking over-representation of KEGG-defined pathways involved in “Nitrogen Metabolism” (FDR q-value = 0.003; P < 0.001), “Alanine, Aspartate and Glutamate Metabolism” (FDR q-value = 0.172; P = 0.028), and “Arginine and Proline Metabolism” (FDR q-value = 0.245; P = 0.011) in Obese-CFP compared to Lean-CFP tumors (Fig. 1d). Notably, there was increased expression of glutaminase (GLS) and glutamate dehydrogenase (GLUD), which catalyze the deamination of glutamine and glutamate, respectively, generating ammonia. Expression of aspartate transaminase (GOT), which transfers the amino group from glutamate to oxaloacetate (OAA), generating aspartate was also upregulated (Fig. 1d). Moreover, there was induction in the rate-limiting enzyme in arginine biosynthesis, argininosuccinate synthetase (ASS1). In addition, mRNA levels of the mitochondrial, extrahepatic form of arginase (ARG2) were elevated. This is in contrast to its paralog (ARG1), which codes for the hepatic cytosolic form of arginine, the principal ureagenic enzyme that catalyzes the final step in nitrogen excretion and ammonia detoxification (Fig. 1d). Whereas ARG1 protein levels were faintly detected, those of ARG2 were induced in Obese-CFP compared to Lean-CFP tumors (Fig. 1e). These results imply a potential role for the urea cycle in PDA growth, as ninotgens from both ammonia and aspartate get incorporated into arginine and ultimately urea. Importantly, urea levels in the tumors were comparable to those in the liver, where the urea cycle largely operates, indicating that this pathway is indeed active in PDA (Fig. 1f).

ARG2 is critical for PDA growth particularly in the obese. To investigate its role in PDA tumor growth, ARG2 was knocked-down in ASPC-1 cells (Fig. 2a). Compared with control scramble knockdown (ASP-1-shScr), ARG2 silencing (ASP-1-shARG2) did not significantly affect PDA cell proliferation in vitro (Fig. 2b), nor did it lead to compensatory increases in ARG1 levels (Fig. 2a). This was consistent among other human PDA cells (Supplementary Fig. 3). To assess ARG2 activity, ASP-1-shScr and ASP-1-shARG2 cells were labeled with 13C6-arginine, followed by a measure of 13C-labeled ARG2 reaction products by liquid chromatography-mass spectrometry (LC–MS). Enrichment in 13C-labeled urea M1 (3.8%) and ornithine M5 (17.8%) was noted in the ASP-1-shScr control cells. These levels were strongly suppressed (urea 1.4%; ornithine 6.8%) upon ARG2 silencing (P ≤ 0.001, one-way ANOVA followed by Tukey test, Fig. 2c), indicating that ARG2 was indeed active in PDA cells. Surprisingly however, when the cells were labeled with 15N(amine)-glutamine, 15N-labeling was detected in glutamate, aspartate and carbamoyl aspartate (60–70%), the precursor of pyrimidine synthesis (Fig. 2d, e), but not in urea cycle metabolites citrulline, arginine or urea. These results indicate that although ARG2 is expressed and active in PDA cells in vitro, the amine nitrogen of glutamine is not disposed of through the urea cycle. Because ARG2 was induced in obesity-associated tumors (Fig. 1d, e), we asked whether its role in PDA growth was relevant in an in vivo, rather than in vitro setting. When PDA cells were grown as orthotopic xenografts in lean or obese mice (Fig. 1a and Supplementary Fig. 4a, b), a moderate decrease in tumor volume was noted in lean mice upon ARG2 knockdown (1.7-fold, Fig. 3a). However, this effect was more dramatic in obese mice, where only half of the ASP-1-shARG2 tumors grew to a measurable size, reaching volumes 2.1-fold smaller than those of the control shScr tumors (P ≤ 0.0001, one-way ANOVA followed by Tukey test), with the exception of one ASP-1-shARG2 tumor that was similar in size to the control knockdown (~150 mm3, black data point, Fig. 3a). Consistent with a strong dependence of obesity-associated PDA on ARG2, this tumor had re-expressed ARG2 in vivo (Fig. 3b, lane 11). Introduction of a short hairpin RNA-resistant form of human ARG2 (Fig. 3c) rescued the growth of these tumors compared to tumors overexpressing control GFP, where ARG2 knockdown caused a 2.2-fold suppression (P ≤ 0.05, one-way ANOVA followed by Tukey test, Fig. 3d, e).
**Fig. 1** Obesity leads to enhanced PDA growth and transcriptional induction of nitrogen and arginine metabolism. 

**a** Schematic timeline for the generation of lean or obese orthotopic xenograft models of PDA, using 4–6 week old male Rag1−/− mice. Arrow indicates orthotopic injection of AsPC1-CFP cells (105 per mouse).

**b** Growth curves of Lean-CFP (n = 15) or Obese-CFP (n = 14) mice. Arrow indicates orthotopic injection of AsPC1-CFP cells (105 per mouse).

**c** Volumes of orthotopic PDA tumors from mice in **b**.

**d** Heatmaps (left) of genes enriched in metabolic pathways in Lean-CFP compared to Obese-CFP tumors (n = 9 per group), with corresponding GSEA plots (right). Red indicates higher expression, and blue lower expression, relative to the mean expression level within each group. NES normalized enrichment scores, FDR false discovery rate, Nom. nominal.

**e** Immunoblots for ARG2 and ARG1 in representative orthotopic tumors from **c**.

**f** Graph demonstrating that the urea levels in PDA tumors are comparable to those in the liver of lean or obese mice (n = 5); data are representative of three independent experiments. In **b**, data represent the mean ± s.e.m. In **c**, data represent the mean ± s.d. *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001, two-way ANOVA followed by Tukey test in **b** and t-test in **c**.
To investigate the role of ARG2 in the metabolism of pancreatic tumors, we performed unbiased global metabolite profiling and found that nitrogen-centric pathways enriched in tumoral metabolites were highly impacted by ARG2 knockdown (Supplementary Fig. 4c). Indeed, ARG2 suppression led to a block in the urea cycle in PDA tumors, and indicate that this evidence for an in vivo nitrogen disposal route in the PDA tumors (Fig. 4d). This labeling is a result of glutamate-dependent transamination of α-KG, where glutamate-dependent transamination of α-KG, resulting in the generation of HCO₃⁻ and NH₄⁺. This process, known as the glutamine cycle, is a critical pathway in the metabolism of tumors, particularly in the PDA tumors. Furthermore, this labeling is a result of the accumulation of glutamine, aspartate, arginine, and ornithine in the tumors of the obese compared with the lean (P ≤ 0.01, one-way ANOVA followed by Tukey test, Fig. 4d). These results provide evidence for an in vivo nitrogen flow from glutamine into urea in PDA tumors, and indicate that this flow is further enhanced in an obese state.

Interestingly, although arginine donates its glutamine-derived nitrogens to urea, but not ornithine, upon arginase activity (Fig. 4b), significant ¹⁵N-labeling in ornithine M1 and M2 was detected in the PDA tumors (Fig. 4d). This labeling is a result of de novo ornithine biosynthesis, catalyzed by ornithine aminotransferase (OAT), where glutamate-dependent transamination of ornithine facilitates the formation of glutamate, which in turn is converted to α-KG by glutamate dehydrogenase (GHD), and subsequently to α-KG by glutamate dehydrogenase (GHD).

**Obesity enhances nitrogen flux into the urea cycle in PDA.** To trace the fate of nitrogen from glutamine into the urea cycle, lean, and obese mice bearing orthotopic PDA tumors were infused over a period of 3 h with ¹⁵N(amine)-glutamine, reaching steady-state plasma enrichment levels of ~60% within the first 30 min (Fig. 4a, b). This enrichment led to the ¹⁵N-labeling of glutamine and ornithine in the tumors of the obese compared with the lean (P ≤ 0.01, one-way ANOVA followed by Tukey test, Fig. 4d). These results provide evidence for an in vivo nitrogen flow from glutamine into urea in PDA tumors, and indicate that this flow is further enhanced in an obese state.
glutamate-γ-semialdehyde, produces ornithine M1 or M2 (Fig. 4b). As a consequence, M2- and M3-labeled citrulline, and to a lesser extent M3-labeled arginine, were also detected, and the abundance of M2- and M3-citrulline was significantly increased in PDA of the obese, compared to the lean (P ≤ 0.01, one-way ANOVA followed by Tukey test, Fig. 4b, d). Furthermore, ARG2 knockout strongly and specifically suppressed 15N-ornithine production in PDA tumors of obese, compared with lean mice (M1, P ≤ 0.05 and M2, P ≤ 0.01, one-way ANOVA followed by Tukey test, Fig. 4d). The enhanced incorporation of 15N from glutamine into the urea cycle through ornithine biosynthesis and its suppression upon ARG2 silencing provides further evidence for the need to dispose of excess nitrogen in tumors of the obese. Interestingly however, only a mild decrease in urea M1 and M2 levels was observed upon ARG2 knockout, likely due to the short duration of the infusions and the secretion of this highly soluble metabolite (Fig. 4d). Because the urea cycle mainly functions to prevent accumulation of toxic ammonia by converting excess nitrogen into ornithine and urea, we quantified 15N-ammonia levels derived from 15N-glutamine, following its chemical derivatization with dansyl chloride23. Consistent with an increase in nitrogen levels in obesity-associated PDA, we found a significant accumulation of 15N-ammonia in tumors of the obese, the levels of which tended to further increase upon ARG2 knockdown (P ≤ 0.05, one-way ANOVA followed by Tukey test, Fig. 4e).

**Arg2 loss causes ammonia accumulation in PDA of the obese.** To reinforce our findings and confirm a role for the urea cycle in PDA tumor growth, we sought to generate a PDA model with complete loss, rather than knockdown, of arginase 2. To that end, we bred the genetically engineered mouse model of PDA, *LSL-KrasG12D, p53fl/fl, pdx-1-Cre; (KPC mice)* to Arg2-deficient mice, which express an aberrant, inactive form of the protein21 (Supplementary Fig. 5a). Resultant tumors were used to generate KPC tumor cell lines with wild-type Arg2 expression (Arg2+/+) or Arg2 loss (Arg2−/−) as confirmed by genotyping21 (Fig. 5a). Arg2 loss caused a moderate but significant decrease in the proliferation of KPC cells in vitro (P ≤ 0.0001, one-way ANOVA followed by Tukey test, Fig. 5b). These cells were then grown as orthotopic PDA allografts in Kras- and Arg2-wild-type littermates. Consistent with the results from human PDA (Fig. 1c), mouse PDA tumor growth was accelerated by obesity (Fig. 5c). Importantly, when tumors were harvested at an early stage of progression (average size ~165 mm3), loss of Arg2 led to a more significant suppression of tumor growth in obese compared to lean mice (3.2-fold, P ≤ 0.05, one-way ANOVA, followed by Tukey test, Fig. 5c). This differential effect was strikingly more evident in more advanced tumors (~800 mm3), where a dramatic decrease in growth was observed in the obese (5.3-fold, P ≤ 0.01, one-way ANOVA, followed by Tukey test), but not in the lean (Supplementary Fig. 5b).

We then investigated the metabolic effect of Arg2 deficiency on nitrogen metabolism in KPC tumors. We reasoned that infusing heavy labeled arginine, the substrate of Arg2, would convey direct information concerning rapid accumulation of ammonia and alternative fates of excess nitrogen due to Arg2 loss. To that end, 15N4, 13C6-labeled arginine was infused for 3 h in lean and obese mice bearing tumors with or without Arg2 loss (KPC; Arg2−/− or KPC; Arg2+/+) and metabolic tracing of 15N and 13C into the urea cycle was performed (Fig. 5d–h). As expected, Arg2 deficiency led to mild non-significant increases in total levels of...
arginine and lower levels of ornithine (Fig. 5f). No significant changes in the relative abundance of intratumoral $^{15}$N-urea M2 were observed in Arg2$^{−/−}$ tumors (Fig. 5g), likely due to the short infusion period, and the secretion of urea from surrounding Arg2$^{+/−}$ cells. Nevertheless, a mild decrease in total levels of urea was noted in the Arg2$^{−/−}$ tumors of the obese (Fig. 5f).

Consistently, Arg2 loss resulted in significantly increased levels of $^{15}$N-arginine M4 and M2 in obesity-associated tumors ($P \leq 0.05$, one-way ANOVA, followed by Tukey test). This was accompanied by a notable shift in arginine catabolism towards creatine biosynthesis (Fig. 5e, g). The first step in this pathway is catalyzed by Gatm (glutamine amidotransferase, mitochondrial) and involves the transfer of a guanidino group from arginine to glycine, yielding ornithine and guanidinoacetate, the immediate precursor of creatine (Fig. 5e). Indeed, the relative abundance of $^{15}$N,13C-creatine (M2, M1) and creatinine (M2, M1) was significantly increased in tumors with Arg2 deficiency, particularly in the obese ($P \leq 0.05$ and $P \leq 0.01$, respectively, one-way ANOVA followed by Tukey test, Fig. 5g).

Notably, Arg2 deficiency caused a significant increase in labeled aspartate M1 and glutamine M2, specifically in tumors of the obese ($P \leq 0.05$, Fig. 5g), accompanied by a striking accumulation in $^{15}$N-ammonia labeled fraction derived from $^{15}$N$_4$,13C$_6$-arginine (4% in obese compared to 2.8% in lean, $P \leq 0.001$, one-way ANOVA followed by Tukey test, Fig. 5h). The specific accumulation of labeled glutamine, aspartate and ammonia from $^{15}$N$_4$,13C$_6$-arginine in Arg2-deficient tumors of the obese results from the shunting of arginine into ornithine by Gatm followed by a transamination reaction catalyzed by Oat to form glutamate-$\gamma$-semialdehyde M1 and $\alpha$-KG-derived glutamate M1 (Fig. 5e).
Fig. 5 Arg2 deficiency suppresses obesity-associated mouse PDA and results in ammonia accumulation. a DNA gel confirming the genotypes of KPC cells with wild-type (WT) expression or knockout (KO) of Arg2. b Proliferation curves of cells in a (n = 6). Data are representative of three independent experiments. c Volumes of orthotopic PDA tumors from cells in b injected (250,000 per mouse) in lean or obese 11-13 week old male C57BL/6J-129 svJae mice and grown for 2 weeks (n = 6 except for Obese-Arg2+/– n = 5). d $^{15}$N$_4$,13C$_6$ enrichment in plasma arginine in mice from c infused with $^{15}$N$_4$,13C$_6$-arginine (n = 3). e Schematic showing that Arg2 knockdown in obese PDA causes shunting of arginine away from the urea cycle and towards creatine biosynthesis, along with transfer of nitrogen into glutamate, aspartate, glutamine, and ammonia in Arg2+/– tumors of obese mice. Red arrows indicate increases in $^{15}$N-labeling, of indicated metabolites in Arg2+/– tumors of obese mice. Gmt, Guanidinoacetate N-Methyltransferase. f Relative abundance of total pool of the indicated metabolites from tumors in c. g Relative abundance of the indicated labeled isotopomers in tumors in c. h Relative abundance (left) and fractional labeling (right) of derivatized $^{15}$N-ammonia. Data represent the mean ± s.e.m. in b, or mean ± s.d. in c, d, f-h. * P ≤ 0.05, ** P ≤ 0.01 and *** P ≤ 0.001, **** P ≤ 0.0001, by two-way ANOVA in b, and one-way ANOVA in c, f-h, followed by Tukey test.

Labeled glutamate can also result from the conversion of $^{15}$N4,13C6-enriched arginine in tumors of Arg2+/– mice. Glutamine, aspartate, and ammonia upon Arg2 loss, along with the dramatic accumulation of ammonia specifically in tumors of the obese, emphasize a role for the urea cycle in nitrogen disposal in obesity-associated PDA.

Enhanced in vivo tumor growth induces ARG2 expression. The crucial role of ARG2 in PDA tumor growth in vivo, but not in vitro, led us to investigate whether candidate metabolites or growth factors that are elevated in obesity24 (Supplementary Fig. 1c–e), could lead to the induction of ARG2 expression. Interestingly, treatment of cultured human PDA cells (AsPC-1, HPAC and SUIT-2) with insulin (5–50 ng ml$^{-1}$), insulin-like growth factor-1 (IGF-1, 5–100 ng ml$^{-1}$), glucose (3–11 mM), or a chemically defined lipid mixture (0.1–2%) did not affect ARG2 levels (Supplementary Fig. 6a, b). These results, along with a recognized association of PDA tumors with enhanced protein catabolism8, 11, 15, 25, led us to hypothesize that obesity-induced PDA tumor growth could generate an in vivo dependency on the urea cycle for disposal of excess nitrogen.

We therefore sought to investigate the role of ARG2 in an obesity-independent model of PDA with enhanced tumor growth. To that end, we generated AsPC-1 PDA cells with constitutive activation of AKT, a kinase known to promote tumor growth and invasion26, 27. Unlike AsPC-1 parental and AsPC1-CFP cells, AsPC1-AKT exhibited AKT activation and a proliferative capacity that are independent of serum, insulin or IGF-1 (Fig. 6a and Supplementary Fig. 7a–c). These cells were used to generate “Lean-AKT” and “Obese-AKT” orthotopic tumors, in parallel to the Lean-CFP and Obese-CFP tumors generated from the isogenic AsPC1-CFP cells (Fig. 1a–c, Fig. 6b and Supplementary Fig. 8a–e). Notably, independent of obesity, the volumes of AsPC1-AKT tumors (Lean-AKT and Obese-AKT) were comparable to those of Obese-CFP tumors and 1.6-fold larger than those of Lean-CFP (~170 versus 105 mm$^3$, P ≤ 0.01 by one-way ANOVA followed by Tukey test, compare Figs. 6b and 1c).

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Although this suggests a potential role for AKT in mediating the obesity-driven increase in PDA growth, no significant changes in AKT phosphorylation (T308 or S473) were detected in Obese-CFP compared to Lean-CFP tumors (n = 12, Fig. 6c and Supplementary Fig. 9). These results indicate that obesity and AKT activation are likely to be independent contributors to enhanced in vivo PDA growth.

Strikingly, the transcriptional signature of AsPC1-AKT tumors originating from either lean or obese mice significantly overlapped with that of Obese-CFP, when compared to Lean-CFP tumors (Supplementary Fig. 8f), suggesting that enhanced in vivo tumor growth is a strong inducer of nitrogen metabolism and the urea cycle. It is also possible that these metabolic changes occurred as a result of acinar tissue disruption during AKT- or...
obesity-driven pancreatic tumor development. Consistently, ARG2 expression was markedly increased in the tumors harboring constitutively active AKT, independent of obesity ($P \leq 0.001$; one-way ANOVA followed by Tukey test, Fig. 6d, e and Supplementary Fig. 10a–c). Importantly, this increase was only observed in tumors, but not in AsPC1-AKT cells propagated in culture (Fig. 6f and Supplementary Fig. 10d), further highlighting the in vivo, rather than in vitro relevance of ARG2 in PDA tumor growth. It however remains to be investigated whether AKT activation results in an enhanced in vivo nitrogen flux into the urea cycle similar to that observed in obesity-driven PDA. Although the transcriptional mechanism leading to in vivo ARG2 induction is yet to be identified, these results are consistent with a possible reliance on the urea cycle for the disposal of excess nitrogen in highly proliferative hypovascular tumors. Such environment contrasts the in vitro setting, where cells are standardly cultured in copious amounts of media, which allows for the secretion and dilution of nitrogen-containing metabolites. Indeed, ARG2 suppression did not affect the growth of AsPC1-AKT cells, in vitro (Fig. 6f, g). However, when grown as orthotopic xenografts in lean immunodeficient mice, PDA tumors with AKT activation were larger than control CFP-expressing tumors (1.8-fold, $P \leq 0.05$), and displayed an enhanced dependency on ARG2 (3.3-fold, $P \leq 0.01$, one-way ANOVA followed by Tukey test, Fig. 6h).

ARG2 levels in PDA correlate with patient BMI. To extend the relevance of our findings, we immunostained PDA tissue microarrays from 92 patients with resected tumors, for ARG1, ARG2, and phospho-S473 AKT (pAKT). Because ARG2 levels increase in diabetic patients and are mitigated by insulin treatment, patients with diabetes were excluded from the study. Although PDA patients can lose weight as a result of cachexia, over a third of the patient pool (38%) was reported as overweight or obese at time of resection. Whereas significant ARG2 and pAKT staining was observed in the PDA tumors (Fig. 6, k), ARG1 was either undetected or weakly expressed. Using multivariable-adjusted logistic regression models, we found a significant positive association between BMI and ARG2 levels ($P = 0.039$, Fig. 6, l). Notably, adjusting for pAKT levels only mildly attenuated the significance but not the strength of this association ($P = 0.051$, Fig. 6l). In contrast, no significant association was found between BMI and pAKT. However, high pAKT levels were strongly associated with high ARG2 expression, independent of BMI ($P = 0.010$, Fig. 6k, l). These results corroborate our findings in the orthotopic PDA tumor models, where AKT and obesity independently induce ARG2 expression.

Discussion

Our study highlights a role for the urea cycle in maintaining the growth of pancreatic tumors, particularly in overweight or obese systemic metabolic states. The dependency of PDA tumors on ARG2, which catalyzes the final step of the urea cycle, suggests a need to alleviate an increased nitrogen burden, particularly in an obese host, where tumors exhibit enhanced growth in vivo. Pancreatic cancer is recognized for high-basal levels of autophagy, and macropinocytic uptake of extracellular proteins, both of which can lead to enhanced protein breakdown and remodeling to maintain tumor growth. We therefore propose that conditions that enhance PDA growth in vivo, such as obesity or AKT activation, might further exaggerate this process, generating excess levels of nitrogen and creating a dependency on ARG2. This dependency is specific to tumors but not cultured cancer cells, which lack the in vivo tumor microenvironment and are grown in a constantly replenished medium, preventing the intracellular accumulation of nitrogen-rich metabolites. Although our findings in orthotopic transplant models remain to be confirmed in an autochthonous mouse model of PDA with Arg2 loss, they are in line with recent reports highlighting the differential effects of in vitro versus in vivo environments on the growth and metabolism of tumors from different tissues. It is noteworthy that human disorders have not been associated with ARG2 deficiency. However, it remains to be investigated whether defects in its activity cause embryonic lethality. Nevertheless, whole body genetic deletion of Arg2 in mice does not lead to significant abnormalities, except for a mild increase in circulating levels of arginine. This is in contrast with reported deficiencies in the predominantly hepatic ARG1, which lead to robust hyperarginemia, neurological impairment, growth retardation and fatal episodes of hyperammonemia. Consequently, ARG2 provides an attractive therapeutic target in PDA patients, particularly those suffering from obesity or those with AKT-driven PDA tumors resulting from activating oncogenic mutations, independent of obesity.

Methods

Reagents. Antibodies for western blotting: AKT (#4691; 1:2000), pS473-AKT (4060; 1:2000), p308-AKT (#4404; 1:1000), PTEN (#9252; 1:1000), and obtained from Cell Signaling Technologies, ARG1 (#ab133543; 1:500), human ARG2 (#ab137069; 1:1000), mouse ARG2 (#ab39712; 1:2000), Bioss Antibodies and GAPDH (#sc-25778; 1:5000) from Santa Cruz. Antibodies for immunofluorescence were from Cell Signaling Technologies (pS473-AKT #4060; 1:50), abcam (α-SMA #ab5694; 1:200), Vector Laboratories (Biotinylated DAB Lectin #B-1035; 1:250), and Sigma-Aldrich (α-Enzyme #A8273; 1:1000). Human recombinant Insulin (Roche, 11376497001), D-Glucose (Sigma-Aldrich, G7021) and a chemically defined lipid mixture (Sigma Lipo288) were used to treat cultured cells.

Cell culture. Human PDA cell lines were from the American Type Culture Collection or ATCC (A-1PC-1, HPAC, MIA PaCa-2, PANC-1), Japanese Collection of Research Bioresources (SUIT-2), or the German Collection of Microorganisms and Cell Cultures (PA-TU-89887) and were treated with STR profiling at ATCC. All cell lines tested negative for mycoplasma using LookOut Mycoplasma PCR Kit (Sigma, MP0035). KPC-Ar2+ and KPC-Ar2- mouse PDA cell lines were dissociated from corresponding mouse tumors in Hanks buffered saline solution (HBSS, Invitrogen; calcium/magnesium-free) containing 0.025% trypsin-EDTA (Invitrogen) and 1 mg ml$^{-1}$ collagenase IV (Worthington Biochemicals). Following a 2-h incubation with rotation at 37 °C, the samples were triturated, resuspended in HBSS, and filtered through 40-μm cell strainers (BD Falcon). All cells were maintained at 37 °C in a humidified incubator with 5% CO$_2$ and were grown in RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum and penicillin-streptomycin (Gibco). For metabolic tracing studies in vitro, the culture medium was optimized to exclude arginase activity in 10% serum, as detected by LC-MS. The cells were instead incubated in media supplemented with 0.1% serum and 100 ng ml$^{-1}$ IGF-1. Unless otherwise indicated, all proliferation assays were also performed on cells cultured with 0.1% serum with 100 ng ml$^{-1}$ IGF-1, using the proliferation kit II (XTT; Roche).

Expression plasmids. pBabe-neo-nlsCFP and pBabe-neo-DPH-myrAKT1 retroviral vectors were a gift from J. Albeck (UC Davis) who had generated them as follows: For pBabe-neo-nlsCerulean, the sequence of Cerulean (a gift from David Piston) was subcloned into pBabe-neo with an SV40 nuclear localization sequence at the 5′ terminus. For pBabe-neo-myr-AKT1(deltaPH)-HA, the sequence for myr-AKT1(deltaPH) was subcloned into pBabe-neo from pWZLneo-myr-Akt(deltaPH)-ER, a gift from R. Roth. The IL15-neo-GFP was a gift from D.M. Sabatini (Whitehead Institute). The short hairpin (sh)RNA-resistant human ARG2 expression construct pLI.M15-neo-hARG2-sh1AR0 was generated as follows: human ARG2 cDNA (Clone ID: 5179861) sequence from pCMVSPORT6-ARG2 (GE-Dharmacon MHS6278-202800846) was subcloned into the lentiviral vector pLJM15-neo-polylinker (a gift from Dr. D.M. Sabatini) using the AgeI/XbaI restriction sites. This construct was subsequently modified by site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent 50521) using the following primers: shARG2-A10-resistance (5′-CGCTTACGTGGTTTTTGGTCAAGTGGAAAG-3′) and shARG2-A10-Resistant (5′-CTTCTACTCTCGATGATCGGT-3′). Retroviral and lentiviral
supernatants were generated by transfecting the above constructs into 293T cells and used to infect AsPC-1 cells. The infected cells were selected for at least 7 days. The intensity of ARG2 and pS473-AKT staining in PDA cases on tissue sections was evaluated using the following formula: intensity score = 0.5 × (% area of tumor with no staining) + 1 × (% tumor area with moderate staining) + 2 × (% tumor area with strong staining), where 200 would be the highest score. Analysis of human subjects included PDA tissue microarrays (TMAs) from 92 non-diabetic patients with surgically resected PDA (58 from Massachusetts General Hospital, 26 from Dana-Farber/Boston Children’s Hospital, and 34 from Drexel University). The TMAs were generated from discarded material following clinical diagnosis. For DF/BWCC patients, informed consent for use of clinical data and tissue specimens was obtained by the Dana-Farber/Boston Children’s Hospital Human Research Committee or IRB (Protocol Numbers 03-189 and 02-023). Patient height and weight were obtained from preoperative evaluation within 2 weeks before surgery and used to calculate BMI, which was modeled as a continuous variable. pAKT and ARG2 were evaluated as dichotomous categorical variables. Levels of each protein were dichotomized if ≥ 0.5 mg/ml. The intensity of ARG2 and pS473-AKT staining in PDA cases on tissue sections was standardized by the Institutional Review Board or IRB (Protocol Number 2009P001838). Analyses of the associations between BMI, PAKT, and ARG2 levels were conducted using binomial logistic regression, obtaining odds ratios (ORs) and 95% confidence intervals (95% CI). The ORs reflect the odds per unit increase in BMI (kg/m²) for a high ARG2 expressing tumor or the odds of high versus low AKT levels for a high-ARG2 expressing tumor. Regression models were adjusted for age at surgery, sex, and American Joint Committee on Cancer (AJCC) 7th edition pathological stage (IA-IIIA, IIB-III). All hypothesis tests were two-sided. Statistical significance was set at α = 0.05. Analyses were performed using SAS software (version 9.4, SAS Institute, Cary, NC).

**Gene expression profiling and GSEA.** Tumor tissues were homogenized and control AsPC1-CFP cultured cells were lysed in QAzo lysis reagent and total RNA was extracted using the RNAeasy Microarray Tissue Mini Kit (Qiagen). RNA quality was checked using the Bioanalyzer RNA Nano kit, and 325 ng were used for microarray labeling with the Agilent LowInput QuickAmp Labeling Kit Two-Color. Dye incorporation and yield were measured with a Nanodrop spectrophotometer. Equal amounts of differentially labeled control AsPC1-CFP cell line and tumor RNA was run on the Agilent_55_K_Catalog_Gena chip set and data was collected using the Agilent Scan 1.0 software at each stage (IA-IIA, IIB-III). All hypothesis tests were two-sided. Statistical significance was set at α = 0.05. Analyses were performed using SAS software (version 9.4, SAS Institute, Cary, NC).

**Metabolite extraction and quantification.** Tissues (10–30 mg) were homogenized using a Qiagen TissueLyzer II in 1:4 (w/v) water. 10 μl of each tissue homogenate was combined with 90 μl of extraction solvent (acetonitrile:methanol:acetic acid 75:25:0.2(w/v), 0.2 ng/ml d4-dphenylalanine and d4-valine, vortexed, centrifuged (10 min, 12 000 × g, 4 °C) and 10 μl of the supernatant was used for metabolite profiling. Data were acquired using a hydrophilic interaction liquid chromatography method (HILIC) with positive ion mode Mass Spectrometry (MS) operated on Nexera X2 UHPLC (Shimadzu Scientific Instruments, Marlborough, MA) coupled to a Q Exact orbitrap mass spectrometer (Thermo Fisher Scientific). The detector was operated in positive ion mode with HILIC separation over a 70–800 m/z range. For experiments measuring urea, data were collected in the 55–750 m/z range. The supernatants (10 μl) were mixed directly onto a 120 × 2 mm Atlantis HILIC column (Waters, Milford, MA). The column was eluted isocratically at a flow rate of 250 μl/min with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 1 min followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 min. The electrospray ionization voltage was 3.5 kV. LC-MS data were processed and visually inspected using TraceFinder 3.3 software. For global steady-state metabolite profiling, data were generated by Metaboanalyst 3.0, median- normalized, log-transformed, and dereplicated by Scaffold software. The in variable. In addition to LC-MS, urea levels were quantified using a urea assay kit (Sigma #MAK006). For metabolic tracing in cultured cells, dried extracts were suspended in 100 μl water. After centrifugation at top speed for 10 min, 2 μl of supernatant was injected for LC/MS analysis as described in ref. 49.

**Ammonia derivatization.** In total 10 μl of 1 mM ethanolic internal standard (in water) was directly added to tissue cultures, followed by addition of 250 μl of 0.5 mg/ml dansyl chloride (in acetonitrile) and homogenization on ice. The homogenates were centrifuged at top speed at 4 °C for 5 min, the supernatants collected and incubated at 70 °C for 5 h, followed by centrifugation at top speed at 4 °C for 5 min. The supernatants were then stored at −80 °C. In total 1 μl of supernatant was injected for LC-MS analysis. Chromatographic separation was performed on a Phenomenex Ultimate 3000 UPLC system using a Kinetex 50 × 2.1 mm C18 (100 Å, 2.6 μm particle size) column (Phenomenex). The mobile phases were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile.
orthotopic pancreatic xenografts were infused 3 weeks after the injection of tumor cells, with 15N-ammonia-glutamine (99% enrichment; Cambridge Isotope Laboratories, Andover, MA) as a bolus of 0.28 mg·kg⁻¹ body weight (0.3 mL) over 1 min, followed by a continuous infusion of 0.0015 mg·g⁻¹ body weight·min⁻¹ for 180 min. Alternatively, immune-proliferative Lewis lung carcinoma tumors were infused with 15N-ammonia divided by the raw peak area of dansylated ethylamine, whereas the fraction labeled is reported as the raw peak area of dansylated 15N-ammonia divided by the sum of raw peak areas of dansylated 15N- and unlabeled ammonia.

Infusion of labeled nutrients. Infusions were performed as previously described in ref. 38. Specifically, lean or obese immunodeficient mice bearing human orthotopic pancreatic xenografts were infused 3 weeks after the injection of tumor cells, with 15N-ammonia-glutamine (99% enrichment; Cambridge Isotope Laboratories, Andover, MA) as a bolus of 0.28 mg·kg⁻¹ body weight (0.3 mL) over 1 min, followed by a continuous infusion of 0.0015 mg·g⁻¹ body weight·min⁻¹ for 180 min. Alternatively, immune-proficient lean or obese mice bearing human orthotopic transplant tumors were infused 2 weeks after the injection of tumor cells, with 15N,13C2-NH2-glutamine.HCl (13C2, 99%; 15N4, 99% enrichment; Cambridge Isotope Laboratories, Andover, MA) as a bolus of 0.084 mg·g⁻¹ body weight (0.3 mL) over 1 min, followed by a continuous infusion of 0.0015 mg·g⁻¹ body weight·min⁻¹ for 180 min. In total 25 μL of tail blood was collected at 30 min intervals and used to quantify plasma enrichment of labeled nutrients by LC-MS. At the end of the infusions, mice were euthanized and the tumors rapidly harvested, weighed and snap-frozen in liquid nitrogen for LC-MS analysis.

Statistics. Statistical analyses are expressed as mean ± s.d., or s.e.m., unless otherwise indicated. No statistical methods were used to predetermine sample size. In comparing two groups, a tailed nonpaired Student t-test was conducted. For three or more groups, one-way ANOVA was conducted, except for growth curves (two-way ANOVA), followed by a post hoc Tukey test. P ≤ 0.05 was considered statistically significant. For GSEA and metabolic pathway analysis, a FDR q-value ≤ 0.05 was considered significant.

Data availability. The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE99289. The authors declare that all the other data supporting the findings of this study are available within the article, its Supplementary Information and from the corresponding author upon reasonable request.

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References
1. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2016. Cancer. J. Clin. 66, 7–30 (2016).
2. Rahib, L. et al. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreatic cancers in the United States. Cancer. Res. 74, 2913–2921 (2014).
3. Bracci, P. M. Obesity and pancreatic cancer: overview of epidemiologic evidence and biological mechanisms. Mol. Carcinog. 51, 53–63 (2012).
4. Calle, E. E., Rodriguez, C., Walker-Thurmond, K. & Thun, M. J. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N. Engl. J. Med. 348, 1625–1638 (2003).
5. Li, D. et al. Body mass index and risk, age of onset, and survival in patients with pancreatic cancer. Ann. Intern. Med. 130, 2553–2562 (2004).
6. Reboués, V. et al. Obesity and fatty pancreatic infiltration are risk factors for pancreatic precancerous lesions (PanIN). Clin. Cancer. Res. 21, 3522–3528 (2015).
7. Yuan, C. et al. Prediagnostic body mass index and pancreatic cancer survival. J. Clin. Oncol. 31, 4229–4236 (2013).
8. Perera, R. M. & Bardeesy, N. Pancreatic cancer metabolism: breaking it down to build it back up. Cancer Discov. 5, 1247–1261 (2015).
9. Son, J. et al. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. Nature 496, 101–103 (2013).
10. Ying, H. et al. Oncogenic Kras maintains pancreatic tumors through regulation of glycolytic glucose metabolism. Cell 150, 656–670 (2012).
11. Comimico, C. et al. Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. Nature 497, 633–637 (2013).
12. Guo, J. Y. et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev. 25, 460–470 (2011).
13. Kamphorst, J. J. et al. Human pancreatic cancer tumors are nutrient poor and tumor cells actively scavenge extracellular protein. Cancer Res. 75, 544–553 (2015).
14. Perera, R. M. et al. Transcriptional control of autophagy-lysosome function drives pancreatic cancer metabolism. Nature 524, 361–365 (2015).
15. Yang, S. et al. Pancreatic cancers require autophagy for tumor growth. Genes Dev. 25, 717–729 (2011).
16. Davidson, S. M. et al. Direct evidence for cancer-cell-autonomous extracellular protein catabolism in pancreatic tumors. Nat. Med. 23, 235–241 (2016).
17. Deignan, J. L., Cederbaum, S. D. & Grody, W. W. Contrasting features of urea cycle disorder in human patients and knockout mouse models. Mol. Genet. Metab. 93, 7–14 (2008).
18. Iyer, R. K. et al. Mouse model for human arginase deficiency. Mol. Cell Biol. 22, 4491–4498 (2002).
19. Kasten, J. et al. Lethal phenotype in conditional late-onset arginase deficiency in the mouse. Mol. Genet. Metab. 110, 222–230 (2013).
20. Sin, Y. Y. et al. Inducible arginase I deficiency in mice leads to hyperargininemia and altered amino acid metabolism. PLoS ONE 8, e80001 (2013).
21. Shi, O., Morris, S. M. Jr, Zoghbhi, H., Porter, C. W. & O’Brien, W. E. Generation of a mouse model for arginase II deficiency by targeted disruption of the arginase II gene. Mol. Cell. Biol. 21, 811–813 (2001).
22. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA 102, 15455–15459 (2005).
23. Mottier, N. & Jeanneret, F. Evaluation of two derivatization reagents for the determination by LC-MS/MS of ammonia in cigarette mainstream smoke. J. Agric. Food Chem. 59, 92–97 (2011).
24. Renehan, A. G., Зwahlen, M. & Egger, M. Adiposity and cancer risk: new mechanistic insights from epidemiology. Nat. Rev. Cancer 15, 484–498 (2015).
25. Davidson, S. M. et al. Environment impacts the metabolic dependencies of ras-driven non-small cell lung cancer. Cell Metab. 23, 517–528 (2016).
26. Albury, T. M. et al. Constitutively active Akt1 cooperates with KRas(G12D) to accelerate in vivo pancreatic tumor onset and progression. Neoplasia 17, 175–182 (2015).
27. Samuels, Y. et al. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. Cancer Cell. 7, 561–573 (2005).
28. Wolpin, B. M. et al. Hyperglycemia, insulin resistance, impaired pancreatic beta-cell function, and risk of pancreatic cancer. J. Natl. Cancer. Inst. 105, 1027–1035 (2013).
29. Li, D., Yeung, S. C., Hassan, M. M., Konovalova, M. & Abbruzzesse, J. L. Antidiabetic therapies affect risk of pancreatic cancer. Gastroenterology 137, 482–488, doi: 10.1053/j.gastro.2009.04.013 (2009).
30. Lashinger, L. M. et al. Dietary energy balance modulation of Kras- and Ink4a/Arf+/−/−driven pancreatic cancer: the role of insulin-like growth factor-1. Cancer Prev. Res. 6, 1046–1053 (2013).
31. Roberts, D. L., Dove, C. & Renehan, A. G. Biological mechanisms linking obesity and cancer risk: new perspectives. Annu. Rev. Med. 61, 301–316 (2010).
32. Kalaany, N. Y. & Sabatini, D. M. Tumours with PI3K activation are resistant to antidiabetic therapies affect risk of pancreatic cancer. Gastroenterology 137, 482–488, doi: 10.1053/j.gastro.2009.04.013 (2009).
33. Brien, W. E. Generation of stable knock-out models of murine Arginase-2. Mol. Genet. Metab. 93, 7–14 (2008).
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Author contributions
N.Y.K. conceived and designed the study. T.Z. performed all experiments. P.-Y.T. and C.D.D. assisted T.Z. in cell culture, proliferation assays, and western blots. D.S.H. performed and T.Z. and D.S.H. analyzed metabolite measurements. C.B.C. supervised the metabolite quantification and analyses. E.F. performed ammonia derivatization and quantification of urea and ammonia by mass spectrometry. P.J.L. performed the immunohistochemistry on tissue sections. M.M.-K. and B.M.W. provided tissue microarrays. M.M.-K. provided pathological assessment and scoring for immunostained human pancreatic tissue. V.M.-O. performed the statistical analysis on the human data. B.M.W. supervised the human analysis and along with V.M.-O., contributed to the discussion of the results. E.M.T. performed and analyzed the expression microarrays and was helped by T.Z. N.S. optimized and performed with the help of T.Z., the orthotopic PDA tumor cell injections. L.L. performed the in vivo infusions and was assisted by T.Z. N.Y.K. supervised all experimental analyses. N.Y.K. and T.Z. wrote the manuscript with feedback from all authors.

Additional information
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