MiR-135 suppresses glycolysis and promotes pancreatic cancer cell adaptation to metabolic stress by targeting phosphofructokinase-1

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancers. It thrives in a nutrient-poor environment; however, the mechanisms by which PDAC cells undergo metabolic reprogramming to adapt to metabolic stress are still poorly understood. Here, we show that microRNA-135 is significantly increased in PDAC patient samples compared to adjacent normal tissue. Mechanistically, miR-135 accumulates specifically in response to glutamine deprivation and requires ROS-dependent activation of mutant p53, which directly promotes miR-135 expression. Functionally, we found miR-135 targets phosphofructokinase-1 (PFK1) and inhibits aerobic glycolysis, thereby promoting the utilization of glucose to support the tricarboxylic acid (TCA) cycle. Consistently, miR-135 silencing sensitizes PDAC cells to glutamine deprivation and represses tumor growth in vivo. Together, these results identify a mechanism used by PDAC cells to survive the nutrient-poor tumor microenvironment, and also provide insight regarding the role of mutant p53 and miRNA in pancreatic cancer cell adaptation to metabolic stresses.
Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths in the United States, with a 5-year survival rate of 8%\(^1\). Since the pancreas has an anatomically inaccessible location that prevents routine examination\(^2\), this low survival rate is largely attributed to advanced stages diagnosis, when PDAC patients already exhibit metastasis; therefore, surgical or chemotherapeutic interventions have minimal impact\(^3,4\). Consequently, early-stage detection methods and effective preventive strategies are urgently needed for improving the death rates of this disease\(^5\). One obstacle underlying these clinical challenges is our limited understanding of how PDAC reprograms metabolism in the unique tumor microenvironment\(^6\). Unlike the more extensive understanding of the mutational mechanisms that initiate PDAC, the metabolic rewiring in this disease is still unclear.

Compared to other cancer types, PDAC is unique due to the notable extent of its desmoplastic reaction, which often forms dense stroma\(^7-8\). This dense tumor mass in PDAC leads to the generation of high levels of solid stress and fluid pressure in the tumors and compression of the vasculature, thereby creating a highly hypoxic and nutrient-poor microenvironment\(^9,10\). Thus, the lack of nutrients imposes major challenges for cells to maintain redox and metabolic homeostasis, as well as minimal support for macromolecular biosynthesis, which indicates that PDAC cells may reprogram metabolic pathways to support different energetic and biosynthetic demands in a state of constant nutrient deprivation\(^10,13,14\).

MicroRNAs, a class of 18–23 nucleotide noncoding RNAs, have gained much attention as a new family of molecules involved in mediating metabolic stress response in cancer\(^15,16\). For example, miRNAs can modulate critical signaling pathways such as LKB1/AMPK\(^16\), p53\(^17\), c-Myc\(^18\), PPAR\(^\gamma\)\(^19\), and ISCU1/220 that regulate metabolism indirectly. In this study, using RNA-seq analysis, we find miR-135 is upregulated in pancreatic cancer patient samples which is consistent with the report that miR-135b is a reported biomarker in pancreatic cancer patients\(^21\). Yet, the function of miR-135b in PDAC is unknown.

Here, compared to other metabolic stress, we show that both miR-135a and miR-135b are induced specifically under low glutamine conditions and are essential for PDAC cell survival upon glutamine deprivation in vitro and in vivo. We further demonstrate PFK1, a critical enzyme for glycolytic flux, is a miR-135 family target gene. Using metabolic tracer-labeling experiments, we show that miR-135 expression suppresses aerobic glycolysis and promotes glucose carbon contribution to the tricarboxylic acid (TCA) cycle, thus decreasing the glutamine dependence of PDAC cells. Consistently, we find PDAC patients express decreased PFK1 expression with inversely correlated higher levels of miR-135. This study delineates a previously unidentified pathway, in which PDAC senses glutamine levels and provides important evidence that miRNA is actively involved in pancreatic cancer cell adaptation to the nutrient-poor microenvironment.

**Results**

**miR-135 is induced upon glutamine deprivation in PDAC cells.** To identify the mechanism that mediates PDAC adaptation to metabolic stress, we first examined miRNA expression levels in seven pairs of human pancreatic cancer patient tumor tissue along with adjacent normal tissue by RNA-sequencing. miR-135b is the top significantly overexpressed miRNA in tumor tissues (P = 0.024, Student’s t test) (Fig. 1a). Since the mature forms of miR-135a and miR-135b differ by only one nucleotide and it is hard to distinguish miR-135a and miR-135b (Fig. 1b), we wondered whether this upregulation of both miR-135a and miR-135b exists in human PDAC tumors. To confirm this, we measured the expression of miR-135a and miR-135b in nine pairs of pancreatic patient tumors along with adjacent normal tissue by qPCR. Both miR-135a and miR-135b were highly expressed in PDAC tumors (Fig. 1b), indicating that the miR-135 family is induced in PDAC tumors.

To test if accumulation of miR-135 is due to the nutrient-poor environment of the PDAC tumors, we cultured PDAC cell line MIA PaCa-2 in medium lacking either glutamine, glucose, serine, glycine or cysteine, which were reported to be at low levels in pancreatic tumors\(^10\). We found the expression of miR-135a and miR-135b only increased in response to glutamine deprivation (Fig. 1c). Interestingly, the increased expression of miR-135b in response to glutamine deprivation was also observed in human fibrosarcoma, breast cancer, and melanoma cell lines (Supplementary Fig. 1). We further tested this using different PDAC cell lines, MIA PaCa-2, Panc-1, and BxPc-3, and found that expression of miR-135a and miR-135b significantly increased in all these cell lines tested upon glutamine deprivation (Fig. 1d). We also measured the expression of miR-135a and miR-135b in MIA PaCa-2 cells cultured in glutamine-free medium for 24 and 48 h. Both miRNAs expression was enhanced with prolonged periods of glutamine deprivation (Fig. 1e). Next, to test whether the increase of miR-135a and miR-135b is reversible, cells were cultured in glutamine-free medium for 24 h, after which glutamine was added back. We found miR-135a and miR-135b expression decreased following the addition of glutamine, suggesting the expression of the miR-135 family is responsive to glutamine stress signaling (Fig. 1f). Taken together, these data suggest that glutamine deprivation induces the expression of miR-135 family in PDAC cells.

miR-135 is induced by ROS-activated mutant p53. Glutamine has been shown to play an important role in controlling reactive oxidative species (ROS)\(^22\). We asked whether miR-135a and miR-135b are upregulated by the increased ROS experienced during glutamine deprivation. To test this, MIA PaCa-2 cells were cultured in glutamine-free medium supplemented with the antioxidant N-acetyl-l-Cysteine (NAC), which can restore the glutathione pool. NAC effectively prevented ROS increase upon glutamine deprivation (Fig. 2a). Glutamine deprivation-induced miR-135a and miR-135b expression were completely inhibited by NAC treatment (Fig. 2b), suggesting that miR-135 family induction is ROS-dependent. Moreover, when MIA PaCa-2 cells were supplemented with the antioxidant glutathione (GSH), the ROS level decreased (Fig. 2c), which was accompanied by inhibition in miR-135 family expression (Fig. 2d). Previously, we showed that glutamine deprivation-induced ROS activates mutant p53 (mutp53)\(^23\). Similarly, we found that glutamine deprivation-induced mutp53 phosphorylation in MIA PaCa-2 cells, which harbor mutated p53 (Fig. 2e). To assess whether ROS-activated mutp53 contributes to the regulation of the miR-135 family, we knocked down mutp53 in MIA PaCa-2 cells and found that the upregulation of miR-135a and miR-135b upon glutamine deprivation was largely attenuated in p53 knockdown cells (Fig. 2f, g), suggesting mutp53 in pancreatic cancer cells displays a gain-of-function in response to the metabolic stress. To further confirm this, we performed chromatin immunoprecipitation assays (ChIP) in MIA PaCa-2 cells cultured in complete or glutamine-free medium. We found that the binding of mutp53 to the promoters of CDKN1A, miR-135a and miR-135b were low in complete medium. However, following glutamine deprivation, mutp53 binding at these promoters was markedly increased (Fig. 2h, i). To further test if the expression of miR-135 is regulated by mutp53 in response to glutamine deprivation, we examined the expression of miR-135a and miR-135b in mouse
Fig. 1 miR-135 is induced upon glutamine deprivation in PDAC cells. a Heatmap of miRNAs expression in human pancreatic tumors compared with normal tissues measured by RNA-seq. b Alignment between mature miR-135b and miR-135a indicating one nucleotide difference; miR-135b and miR-135a expression in nine pairs of pancreatic tumors compared with adjacent normal tissues were measured by qPCR. Each value represents the mean ± SD in three independent experiments. **p < 0.01, ***p < 0.001, Student’s t test. c MIA PaCa-2 cells were cultured in medium without glutamine, glucose, serine, glycine or cysteine for 24 h. miR-135a and miR-135b relative expression were assessed by qPCR. Each value represents the mean ± SD in three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test. d MIA PaCa-2, PANC-1, and BxPc-3 cells were cultured in glutamine-free medium for 24 h. miR-135a and miR-135b relative expression were assessed by qPCR. Each value represents the mean ± SD in three independent experiments. *p < 0.05, **p < 0.01, Student’s t test. e MIA PaCa-2 cells were cultured in glutamine-free medium for 24 or 48 h. miR-135 expression was assessed by qPCR. Each value represents the mean ± SD in three independent experiments. **p < 0.01, ***p < 0.001, Student’s t test. f MIA PaCa-2 cells were cultured in glutamine-free medium for 24 h, then glutamine was added back, and cells were cultured an additional 24 h. miR-135a and miR-135b relative expression were assessed by qPCR. Each value represents the mean ± SD in three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test (Ctrl: complete medium; No Gln: glutamine-free medium). PDAC: pancreatic ductal adenocarcinoma.
Fig. 2 miR-135 is induced by ROS-activated mutant p53. a, c MIA PaCa-2 cells were cultured in complete medium or glutamine-free medium supplemented with 5 mM NAC or 10 mM GSH for 24 h. Then cells were stained with dihydroethidium and analyzed by flow cytometry or collected to assess relative miR-135a and miR-135b expression by qPCR (b, d). Each value represents the mean ± SD in three independent experiments. *p < 0.05, ***p < 0.001, Student’s t test. e MIA PaCa-2 cells were cultured in complete medium or glutamine-free medium supplemented with 5 mM NAC. Phospho-p53 (S15), total p53 and actin were assessed by western blotting. f p53 was knocked down by siRNA in MIA PaCa-2 cells. p53 protein was assessed by western blotting. g p53 knockdown and control MIA PaCa-2 cells were cultured in complete medium or glutamine-free medium for 24 h. miR-135a and miR-135b expression were assessed by qPCR. Each value represents the mean ± SD in five independent experiments. ***p < 0.001, Student’s t test. h Chromatin immunoprecipitation analysis was performed to determine p53 binding to the promoters of CDKN1A, miR-135a and miR-135b in response to glutamine deprivation. The binding was assessed by DNA gel. i The fold change of the p53 binding to the promoters of CDKN1A, miR-135a and miR-135b in response to glutamine deprivation were analyzed by qPCR. Each value represents the mean ± SD in three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test. j, k Kras/p53 mutant and Kras/p53 knockout mouse PDAC cells were cultured in complete medium or glutamine-free medium for 24 h. Total p53 protein was assessed by western blotting (j). miR-135 expression was measured by qPCR (k). Each value represents the mean ± SD in four independent experiments. **p < 0.01, ***p < 0.001, Student’s t test (Ctrl: complete medium; No Gln: glutamine-free medium). ROS: reactive oxidative species, NAC: N-acetyl-L-Cysteine, GSH: glutathione, PDAC: pancreatic ductal adenocarcinoma.
Kras/p53 mutant and Kras/p53 knockout PDAC cells cultured in glutamine-free medium. Consistent with our finding in human PDAC cells, we found that miR-135 expression was dramatically increased upon glutamine deprivation in Kras/p53 mutant cells. In contrast, this induction was blocked in Kras/p53 knockout cells (Fig. 2, k). Taken together, these results show glutamine deprivation-induced ROS activates mutp53 and enhances the binding between mutp53 and miR-135 family promoters to increase its expression.

miR-135 promotes PDAC survival upon glutamine deprivation. To determine if miR-135 plays an important role in PDAC cell survival during glutamine deprivation, we transiently transfected human miR-135 inhibitor in MIA PaCa-2, PANC-1 and BxPc-3 cells, and glutamine was removed for 24 h. miR-135a and miR-135b expression was effectively inhibited (Fig. 3a–c and Supplementary Fig. 2a–c). We measured cell viability over time using 4′,6-diamidino-2-phenylindole (DAPI) exclusion flow cytometry. All three PDAC cell lines treated with miR-135 inhibitor were more sensitive to glutamine deprivation (Fig. 3a). To further confirm miR-135’s role in survival, MIA PaCa-2 cells were transfected with control vector or an anti-miR-135 construct. Both miR-135a and miR-135b were inhibited in miR-135 knockdown cells (Fig. 3d and Supplementary Fig. 2d). We starved control and miR-135 knockdown cells of glutamine, and measured cell viability over time. miR-135 knockdown cells were more sensitive to glutamine deprivation, which is consistent with our previous results (Fig. 3e). Additionally, increased cleaved-caspase 3, a marker for apoptotic cell death, was observed in these miR-135 knockdown cells cultured in glutamine-free medium for 24 and 48 h compared to control cells (Fig. 3f). Moreover, we used MIA PaCa-2 cells engineered to stably express control vector or miR-135a (Fig. 3g) and starved these cells of glutamine over time. Both cell viability and western blot analysis of cleaved-caspase 3 indicated that miR-135a expressing cells were less sensitive to glutamine deprivation (Fig. 3h, i). Taken together, these data suggest miR-135 family promotes cell survival upon glutamine starvation.

miR-135 suppresses aerobic glycolysis. During periods of limited glutamine availability, we have previously demonstrated that a decrease in glycolysis is critical for cell survival24. We hypothesized, in pancreatic tumors, miR-135 plays a role in regulating glycolysis during glutamine deprivation. To test this, we cultured MIA PaCa-2, PANC-1, and BxPc-3 cells in complete and glutamine-free medium for 24 h and confirmed glutamine deprivation decreased glucose uptake and lactate production in PDAC cells (Fig. 4a–c). To test if glutamine deprivation-induced miR-135 inhibits aerobic glycolysis, we measured glucose and lactate in medium collected from MIA PaCa-2, PANC-1 and BxPc-3 cells expressing anti-NC or anti-miR-135. Accordingly, glycolysis was significantly upregulated in miR-135 knockdown cells (Fig. 4d–f). In addition, we assessed the glycolytic output during miR-135 inhibition in MIA PaCa-2 cells cultured in glutamine-free medium. In glutamine-deprived state, miR-135 inhibition still led to upregulation of glycolysis (Supplementary Fig. 3a). We next measured the extracellular acidification rate (ECAR), a readout of lactate production, in miR-135 knockdown MIA PaCa-2 cells and found increased glycolytic flux (Fig. 4g); however, miR-135 inhibition did not have a significant effect on oxidative phosphorylation (OXPHOS) (Supplementary Fig. 3b). Moreover, we found glucose uptake and lactate production were significantly decreased in miR-135 overexpressing MIA PaCa-2 cells, indicative of reduced glycolysis (Fig. 4h). In addition, we found that BxPc-3 cells, which endogenously express a higher level of miR-135 correlating with decreased glycolysis, compared to MIA PaCa-2 cells (Supplementary Fig. 3c).

To further test whether miR-135 promotes PDAC cell survival upon glutamine deprivation via glycolysis inhibition, MIA PaCa-2 cells were treated with the hexokinase inhibitor, 2-deoxy-D-glucose (2DG), which suppresses cellular glycolysis (Supplementary Fig. 3d). We found 2DG supplementation sufficiently rescued miR-135 knockdown cells, indicating that an inhibited glycolytic pathway is required for the survival of miR-135 knockdown cells (Fig. 4i and Supplementary Fig. 3e). To further confirm blockade of glycolysis rescues the effect of miR-135 inhibition, we cultured MIA PaCa-2 cells in medium with low glucose and glutamine-free medium. We found low glucose (5 mM) rescued the effect of miR-135 inhibition in glutamine-free conditions (Supplementary Fig. 3f). Taken together, these data suggest miR-135 downregulates glycolysis to promote PDAC cell survival during glutamine deprivation.

PFK1 is the direct target of miR-135. To identify downstream targets of miR-135, we checked previously validated targets in the DIANA TarBase 7.0 database. Interestingly, we found PFK1 was the only target gene predicted to play a role in the glycolytic pathway (Fig. 5a). To confirm whether PFK1 is a potential target of miR-135, the expression of PFK1 was measured over 48 h of glutamine deprivation in MIA PaCa-2 cells. As predicted, mRNA and protein levels of PFK1 decreased over time during glutamine deprivation (Fig. 5b, c). Two putative miR-135 binding sites exist in the PFK1 3’UTR. To verify whether these sites mediate the miR-135 regulatory effect on PFK1 expression, we cloned the wild-type PFK1 3’UTR (wt) or a miR-135 binding site mutant (mut) into a luciferase reporter plasmid (Fig. 5d and Supplementary Fig. 4a). We transfected this PFK1 3’UTR reporter construct into control and miR-135-expressing MIA PaCa-2 cells. miR-135 decreased the luciferase activity of the wt reporters. However, mutation of either miR-135 binding sites resulted in partial restoration of luciferase activity and complete restoration occurred when both sites were mutated (Fig. 5e). Therefore, these results suggest miR-135 binds to both putative target sites of the PFK1 3’UTR.

Furthermore, expression of miR-135 led to a decreased expression of PFK1, whereas inhibition of miR-135 resulted in an elevated PFK1 level, suggesting miR-135 regulates PFK1 expression (Fig. 5f, g). Consistently, glutamine deprivation decreased the protein expression of PFK1 in an miR-135-dependent manner (Fig. 5h). To confirm the survival effect of miR-135 is through downregulation of PFK1, we knocked out the PFK1 3’UTR region in MIA PaCa-2 cells using CRISPR-Cas9. In support of our conclusion, we found that deletion of the miR-135 target sites in PFK1 3’UTR led to more cell death upon glutamine deprivation (Fig. 5i). To determine whether the increased glycolysis observed in miR-135 knockdown cells (Fig. 4d) was due to increased PFK1 activity, we knocked down PFK1 by shRNA in these miR-135 knockdown cells (Fig. 5j). The increased glucose uptake and lactate production exhibited in the absence of miR-135 was abrogated by knockdown of PFK1 (Fig. 5k). To further confirm this, we measured ECAR in control, miR-135 knockdown and miR-135/PPK1 double knockdown cells. Glycolytic flux was substantially attenuated in the double knockdown cells (Fig. 5l). We previously showed that miR-135 induction upon glutamine deprivation is ROS and mutp53 dependent (Fig. 2b, d, g). To test if PFK1 expression is regulated by ROS, MIA PaCa-2 cells were cultured in glutamine-free medium supplemented with the antioxidants NAC and GSH. We found glutamine starvation led to a decrease in PFK1 level, which was reversed by antioxidant reagents, suggesting glutamine deprivation-induced ROS may regulate PFK1 expression via
Fig. 3 miR-135 promotes PDAC survival upon glutamine deprivation. a–c MIA PaCa-2 cells (a), PANC-1 (b), and BxPc-3 (c) were transiently transfected with inhibitor control or hsa-miR-135 inhibitor. 48 h posttransfection, cells were cultured in glutamine-free medium. Relative miR-135b expression was assessed by qPCR. Cell viability was assessed by DAPI exclusion flow cytometry at the indicated time points. d MIA PaCa-2 cells were transfected with microRNA inhibitor control clone (anti-NC) or miR-135 inhibitor clone (anti-miR-135). miR-135b expression was assessed by qPCR. e MIA PaCa-2 cells expressing anti-NC or anti-miR-135 were cultured in glutamine-free medium. Cell viability was assessed by DAPI exclusion flow cytometry at the indicated time points. Cells were also lysed and immunoblotting was performed with indicated antibodies (f). Each value represents the mean ± SD in three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test.
g MIA PaCa-2 cells were transfected with microRNA scramble control clone or miR-135a precursor clone. miR-135a relative expression was assessed by qPCR. Each value represents the mean ± SD in four independent experiments. ***p < 0.001, Student’s t test. h MIA PaCa-2 cells expressing scramble or miR-135a were cultured in glutamine-free medium. Cell viability was assessed by DAPI exclusion flow cytometry at the indicated time points. Each value represents the mean ± SD in three independent experiments. ***p < 0.001, Student’s t test. i Cells were also lysed and immunoblotting was performed with indicated antibodies (CM: complete medium; No Gln: glutamine-free medium).

PDAC: pancreatic ductal adenocarcinoma.
miR-135 knockdown leads to increased glutamine dependence. To elucidate how miR-135 affects glycolysis in PDAC cells, we performed stable isotope tracer-labeling experiments by culturing control, miR-135 knockdown, and miR-135/PFK1 double knockdown MIA PaCa-2 cells in complete medium containing universally labeled $^{13}$C-glucose (Fig. 6a). Metabolites from these cells were analyzed using the Nova Biopro analyzer. Metabolite levels per cell were normalized to control. Each value represents the mean ± SD in three independent experiments.

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cells were extracted and analyzed by LC-MS. The labeling experiments showed a significant increase in the enrichment of glucose into glycolytic metabolites in miR-135 knockdown cells (Fig. 6b), consistent with our previous data (Fig.4d–f). However, we observed a decline in the contribution of glucose carbon to the TCA cycle and TCA cycle derived amino acids, like aspartate, proline, and asparagine (Fig. 6b). Strikingly, PFK1 knockdown reversed the increase in labeled pyruvate, lactate, serine, and glycine observed in miR-135 knockdown cells (Fig.6c), suggesting miR-135 regulates glycolysis through PFK1. Interestingly, less glucose-derived pyruvate entered the TCA cycle since key intermediates, such as α-ketoglutarate, succinate, fumarate, and glutamate were significantly decreased in miR-135 knockdown cells (Fig. 6d). Meanwhile, miR-135 knockdown cells displayed an increased level of unlabeled α-ketoglutarate, succinate, fumarate, and glutamate (Fig. 6d), indicating these cells utilize glutamine to replenish these TCA cycle intermediates. To confirm this, we cultured control and miR-135 knockdown cells in complete medium containing universally labeled $^{13}$C-glutamine. We found $^{13}$C-glutamine-derived succinate, fumarate, malate, and aspartate...
were increased in miR-135 knockdown cells (Supplementary Fig. 5a). We next measured glutamine uptake in miR-135 knockdown and miR-135/PFK1 double knockdown cells. Consistently, miR-135 knockdown cells were more dependent on glutamine and this increased glutamine uptake is PFK1-dependent (Fig. 6e). Additionally, knockdown of miR-135 in PANC-1 and BxPc-3 increased glutamine uptake (Supplementary Fig. 5b, c). These results suggest the absence of miR-135 upregulates glycolysis, thereby allowing glutamine to replenish TCA intermediates, thus making them more sensitive to glutamine starvation (Fig. 6f). Taken together, these data show that miR-135 inhibits glycolysis directly by repressing PFK1 levels in response to glutamine deprivation.

Suppression of miR-135 in PDAC cells represses tumor growth. To test whether miR-135 is important for PDAC cell survival in vivo, miR-135 knockdown and control MIA PaCa-2 cells were injected subcutaneously into nude mice. A significant reduction in both tumor growth and weight was observed in miR-135 knockdown tumors (Fig. 7a–c). We checked miR-135 and PFK1 expression in tumors harvested from both groups. As expected, tumors with significantly reduced expression of miR-135 had enhanced levels of PFK1 protein (Fig. 7d and Supplementary Fig. 6a).

To further confirm whether glutamine deprivation induces miR-135 expression in vivo, we evaluated glutamine levels in tumors and serum. We found that compared to the circulating glutamine, PDAC tumors showed significantly decreased glutamine levels (Supplementary Fig. 6b). We dissected the core and periphery regions in control tumors and measured the glutamine concentration in these distinct regions. Levels of glutamine were significantly less in the core versus the periphery regions of these tumors (Fig. 7e), consistent with previous findings. Additionally, we assessed miR-135 expression level (Fig. 7f and Supplementary Fig. 6c) and noticed that miR-135 expression was significantly higher in the core region. Similarly, western blot analysis showed PFK1 was largely reduced in the core compared to the periphery region of the tumors (Fig. 7f). In addition, treatment with 6-Diazo-5-oxo-L-norleucine (DON), a glutamine analog, effectively inhibited tumor growth, confirming previously published data showing PDAC are highly glutamine dependent. Despite the dramatic effect of DON alone, miR-135 inhibition still significantly suppressed tumor growth (Supplementary Fig. 6d). Furthermore, we measured PFK1 expression in human PDAC patients’ normal and tumor tissues by immunohistochemistry. Consistent with miR-135 expression levels (Fig. 1b), PFK1 was decreased in pancreatic tumors (Fig. 7g). Taken together, these data show that inhibition of miR-135 represses pancreatic tumor growth.

Discussion
Pancreatic cancer is a devastating disease with extremely poor survival rates due to pancreatic tumors exhibiting differential dependency in metabolism. For example, it has been shown that PDAC cells have increased glutamine metabolism driven by Kras mutations. Moreover, PDAC cells activate salvage pathways such as macropinocytosis to sustain the intracellular requirement of glutamine. Thus, due to the selective usage of glutamine, PDAC cells may eventually encounter a glutamine poor environment, which has been supported by a few studies using patient samples. For example, metabolomic analysis of more than 49 patient PDAC samples versus adjacent benign tissues revealed that glutamine levels are significantly decreased in PDAC tumors. Interestingly, a recent effort to use glutaminase inhibitors to treat PDAC showed that pancreatic cancer cells have adaptive metabolic networks that sustain proliferation in vitro and in vivo upon inhibition of glutamine metabolism. Thus, it is important to study the adaptive pathways in PDAC cells when glutamine levels are low, or glutamine metabolism is inhibited.

We found PDAC cells are able to suppress aerobic glycolysis, in response to the low glutamine in the tumor microenvironment, by modulating the expression of miR-135. Recently, the coordination between cellular levels of glutamine metabolism and glycolysis was reported. For example, elevated glutamine levels support cell growth by stimulating aerobic glycolysis, but depletion of glutamine levels in the medium significantly reduces the glycolytic flux. In support of this, metabolomic analysis using PDAC patient samples indicate that glycolysis intermediates, including Glycerol-3-Phosphate, Glucose-6-Phosphate, and Fructose-6-Phosphate are significantly decreased in tumors compared with normal tissue. Despite the numerous reports that Kras mutation or hypoxia promote glycolysis, our data suggest that adaptive metabolic pathways in PDAC cells have an impact on tumor progression that might affect the therapeutic outcome in vivo.

Glutamine contributes to the biosynthesis of glutathione, which neutralizes ROS, and affects the production of NADPH via glutamate dehydrogenase (GLUD). Several studies indicate that glutamine deprivation triggers an increase in ROS levels. Our previous work and several other groups showed induced ROS led to the activation of mutp53, which displays a gain-of-function feature to adapt to the environment. Among these target genes of mutp53, miRNAs emerge as important regulators, and play a key role in response to environmental stress. Our ChIP assay showed that, under glutamine deprivation, mutp53 was bound directly to
135/PFK1 double knockdown MIA PaCa-2 cells were cultured in complete medium for 24 h. Glutamine uptake was measured using the Nova Biopro analyzer. Each value represents mean ± SD in three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test.

Labeled pyruvate, lactate, serine and glycine were analyzed in control (anti-NC), miR-135 knockdown (anti-miR-135) and miR-135/PFK1 double knockdown MIA PaCa-2 cells by using LC-MS. Fold change is calculated by the labeled metabolites normalized to control MIA PaCa-2 cells. Each value represents mean ± SD in three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test. Cells were lysed and immunoblotting was performed with indicated antibodies.
the miR-135 promoter region and induced its expression. Since p53 is frequently mutated in pancreatic cancer, it will be interesting to further investigate how the transactivation of mutp53 is modulated toward microRNAs in response to metabolic stress.

miR-135a and miR-135b are the two isoforms of miR-135. Although they are located at different chromosomes, the mature miR-135a and miR-135b have only one nucleotide difference, which is not in the miRNA binding region. Thus, it is reported

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**Fig. 7** Suppression of miR-135 in PDAC cells represses tumor growth. Nude mice were injected subcutaneously with Control (anti-NC) and miR-135 knockdown (anti-miR-135) MIA PaCa-2 cells. **a** Tumor size was measured over time (n = 9 mice per group). **b** Tumor weight was measured after tumors were harvested from mice (n = 9 mice per group). Each value represents the mean ± SD. **p < 0.01, ***p < 0.001, Student’s t test. **c** Representative tumors from each group. **d** miR-135b expression was determined in xenograft tumors by qPCR and PFK1 protein was assessed by western blotting. **e** Glutamine concentration in samples from peripheral or core regions of control tumors. **f** miR-135b was determined by qPCR and PFK1 protein was measured by western blotting analysis in the core and periphery regions. (C: core; P: periphery). Each value represents mean ± SD in three independent experiments. **p < 0.05, ***p < 0.01, ***p < 0.001, Student’s t test. **g** Pancreatic patients’ tumor and adjacent normal tissues were stained with DAPI, CK8/19 and PFK1 antibody. Scale bar = 100 µm. PDAC: pancreatic ductal adenocarcinoma.
that both miRNAs target the same genes, miR-135a and miR-135b were previously shown to be critical in cancer tumorigenesis, progression, and metastasis in lung cancer. Specifically, miR-135b was identified as an important biomarker in pancreatic cancer. These findings support our hypothesis that miR-135 is significant for pancreatic cancer cells’ ability to resist harsh changes in their microenvironment.

Recently, the coordination between cellular levels of glutamine and glucose was found to be a metabolic checkpoint. Low levels of glutamine, as well as glycolytic intermediates, were observed in pancreatic cancer patient samples compared with normal tissue [39, 40]. Consistently, our data showed that glutamine deprivation upregulated miR-135 expression, which decreased the expression of PFK1 to inhibit glycolysis. Through our metabolomics experiment, we showed that more glutamine entered the TCA cycle in miR-135 knockdown cells, indicating these cells are more dependent on glutamine metabolism. These findings suggest that inhibition of glycolytic flux may decrease glutamine dependence and contribute to PDAC cell survival under low glutamine conditions.

While altered metabolism has long been recognized as a central hallmark of cancer, we have only recently begun to elucidate a mechanistic understanding of PDAC metabolism. Since early attempts to detect or treat PDAC have met with minor success, it is urgent to reveal the features of rewired metabolism in pancreatic cancer with more tractable therapeutic targets. Our results highlight the importance of miR-135 in the posttranslational regulation of PFK1 activity to promote cell survival during glutamine deprivation. Although there is a possibility that miR-135 affects other pathways to promote PDAC cell survival, our data unequivocally establishes a key role for miR-135 in regulating glycolysis in vivo, which is important for PDAC cancer cell adaptation to low glutamine conditions. Interestingly, we found that PFK1 cells displayed a reduced proliferation rate, that suggests a putative role in survival (Supplementary Fig. 5d). Therefore, our data highlight the therapeutic potential of targeting miR-135 in combination with glutaminase inhibitors to enhance the treatment of pancreatic cancer.

**Methods**

**Reagents and plasmids.** Hs_miR-135a Primer (MS00008624), Hs_miR-135b Primer (MS00003470), Hsa_miR-135a Primer (MS0011130), Mm_miR-135b Primer (MS0001575) and Dual-luciferase reporter assay system (E1910) were purchased from Qiagen. siRNA to p53 (1-00339-00-0005) was purchased from Dharmacon. miRCURY LNA microRNA inhibitor control (199006-001) and hsa-miR-135 microRNA inhibitor (4109302-101) were purchased from Exiqon. Hsa-miR-135a precursor clone in lentiviral inhibitor control (199006-001) and hsa-miR-135 microRNA inhibitor (4103932-003329-00-0005) was purchased from Dharmacon. miRCURY LNA microRNA reporter assay system (E1910) were purchased from Qiagen. siRNA to p53 (L-Primer (MS00011130), Mm_miR-135b Primer (MS00001575) and Dual-luciferase reporter assay system (E1910) were purchased from Qiagen. siRNA to p53 (L-

**Cell culture and transfection.** MIA PaCa-2 (CRL-1420TM), Panc-1 (CRL-1646TM), BxPc-3 (CRL-1687TM), HT1080 (CCL-121TM), MDA-MB-231 (HTB-101) were purchased from ATCC. Patient-derived melanoma M229 was obtained from Roger Wilmut and the KrasG12D activation and biallelic p53 inactivation mouse model were obtained from Tyler Jacks.

**Quantitative real-time PCR.** Total RNA was extracted using Trizol reagent (15596026, Invitrogen). All libraries were prepared using the Illumina TruSeq Small RNA protocol with minor modification according to the manufacturer’s instructions with 12 cycles of PCR amplification after ligation of the 3′ adapters. Individual libraries were prepared for each primer in order to allow for pooling of multiple samples prior to sequencing. The library was quantified using qPCR. Sequencing was performed on a HiSeq2500 (Illumina Inc., San Diego, CA) and image processing and base calling were conducted using Illumina’s pipeline. Different miRNA expression analysis was performance on the filtered normalized miRNA count using paired Student’s t test. p values less than 0.05 were considered significant.

**Small RNA library preparation and sequencing.** Total RNA was extracted using Trizol reagent. Reverse transcription was performed using miScript II RT Kit (218161, Qiagen) for miRNAs and sScript cDNA Synthesis Kit (95047-100, Quant BioSciences) for mRNAs. qRT-PCR was performed in a CFX Connect Real-Time PCR Detection System (Bio-Rad) by using a reaction mixture with SYBR Green PCR Master Mix (95072) and NAC (A9165) were purchased from Sigma. Puromycin (P8833), Hygromycin B (H3274) and Lipofectamine RNAiMAX Transfection Reagent (15778075) and Lipofectamine 2000 transfection reagent (11668027) were purchased from Thermo Fisher Scientific.

**Immunoblotting.** Cells grown in 6 cm dishes were lysed on ice in lysis buffer (50 mM Tris-HCl [pH 7.4], 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM Ethylene glycol-bis-[β-aminoethyl ether]-N,N′,N′,N′-tetraacetic acid (EGTA), 250 mM mannitol, 1% [v/v] Triton X-100) containing protease inhibitors (complete 049519900, Roche). The protein concentrations of the lysates were measured using the BCA Assay kit (23223, Thermo). The lysates were boiled with NuPAGE LDS-PAGE sample buffer (177159, Invitrogen) supplemented with 5% β-mercaptoethanol (M3148, Sigma) for 5 min. Equal amounts of protein were loaded on precast NuPAGE Bis-Tris Gels (NP03210BOX, Life Technologies) followed by transfer onto nitrocellulose membrane (1620115, Bio-Rad). Immunoblotting was performed with the following antibodies: anti-PFK1 (1:1000) (ab154984, Abcam), anti-β-Actin (1:5000) (A1978, Sigma), anti-caspase-3 (1:1000) (9665a, Cell Signaling), anti-cleaved-caspase 3 (1:1000) (9665a, Cell

**Cell proliferation.** Cell proliferation was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (M2128, Sigma) according to the manufacturer’s instructions. Individual libraries were prepared for each primer in order to allow for pooling of multiple samples prior to sequencing. The library was quantified using qPCR. Sequencing was performed on a HiSeq2500 (Illumina Inc., San Diego, CA) and image processing and base calling were conducted using Illumina’s pipeline. Different miRNA expression analysis was performance on the filtered normalized miRNA count using paired Student’s t test. p values less than 0.05 were considered significant.
CRISPR-Cas9 genomic knockout. PKF1 3’ UTR was generated using lentiviral CRISPR/Cas9. Briefly, two oligos were designed to target the human PKF1 3’ UTR gene, using the online design tool at http://crispr.tefor.net/crispr.py. After lentiCRISPR v2 encoding Cas9 (25961, Addgene) was digested with BsmBI (R05805, Bios) at 37 °C for 30 min, plentiCRISPR was purified by DNA gel. The two oligos were phosphorylated and annealed at 70 °C for 5 min, then 40 ng BsmBI digested plasmid. MIA PaCa-2 cells were transfected with the lentiCRISPR v2-PKF1 3’ UTR-specific oligos via lentiviral-mediated transfection and selected by puromycin selection. The oligos to target PKF1 3’ UTR and primers for testing are listed in Supplementary Table 1.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed15. Briefly, cells were plated on complete medium or glutamine-free medium for 6 h and crosslinked by the addition of 1% formaldehyde solution (F8775, Sigma) at 30 °C for 10 min. The reaction was stopped by adding glycine (G8790, Sigma) at a final concentration of 125 mM. DNA was immunoprecipitated with p53 antibody and extracted using ChIP assay kit (17295, Millipore). Briefly, cells were rinsed twice with Phosphate-buffered saline (PBS), harvested by a silicon scraper, lysed in ice-old lysis buffer and sonicated to solubilize and shear crosslinked DNA to average size of 200–1000 bp fragments. After centrifugation, the supernatant was transferred to a new tube and diluted with ChIP Dilution Buffer containing protease inhibitors. To reduce nonspecific background, we preincubated the diluted solution with protein A agarose beads for 30 min at 4 °C with agitation. The supernatant fraction was collected, and mixed with p53 antibody (sc-126, Santa Cruz) and incubated overnight at 4 °C with rotation. Protein A agarose beads were added and rotated for 1 h at 4 °C. The agarose beads were pelleted by gentle centrifugation and washed with low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer and TE buffer. The histone complex from the antibody was eluted by elution buffer. 5 M NaCl were added to the eluates and heated at 65 °C for 4 h to reverse histone-DNA crosslinks, followed by addition of 0.5 M EDTA, 1 M Tris-HCl (pH 6.5), and 10 mg/ml Proteinase K. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. PCR was performed using Hotstart Taq DNA polymerase (203205, QiaGen). PCR products were detected using agarose gel electrophoresis. PCR primers for the ChIP assays are listed in Supplementary Table 1. qRT-PCR was also performed with SYBR Green PCR Master Mix (95072, Quanta Biosciences) using a CFX Connect Real-Time PCR Detection System (Bio-Rad). The primers for qPCR are the same as PCR experiment above. The PCR cycle parameters were as follows: 95 °C for 5 min; 40 cycles with denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s. All PCR amplifications were performed in triplicate and repeated in three independent experiments. The precipitated DNA fragments corresponding to specific genes were quantified by qPCR and expressed as percentages of their total input DNA fragments.

Luciferase assay. PKF1 3’ UTR wild-type clone in pMirTarget (sc209129) which is a reporter construct with firefly luciferase as a reporter was purchased from OriGene. PKF1 3’ UTR mutant clone was obtained by mutating PKF1 3’ UTR using Q5® site-directed mutagenesis kit (E05545, Bios). Both clones were cotransfected with Renilla luciferase gene as control to MIA PaCa-2 cells expressing pEGFP-C1 for 3 days with calipers over time and tumor volume was calculated using the formula l×w × 0.5. For DON treatment, nude mice were treated with PBS, or DON (10 mg/kg body weight) by intraperitoneal injection every other day for 2 weeks when control tumor volume was around 200 mm³. Tumor size was measured over time. At the end of the experiment, mice were euthanized and tumors were harvested. Samples from core and peripheral regions were dissected for further analysis.

Glucose/glutamine uptake and lactate production measurements. 2 × 10⁶ cells were plated on each well of a six-well plate and cultured overnight. For MIA PaCa-2 and PANC-1 cells, the medium was replaced by 1 ml DMEM supplemented with 10% FBS for 24 h, then 40 ng/ml puromycin selection. The oligos to target

Seahorse assays. The day before the seahorse assay, 7.5 × 10⁶ cells were seeded into seahorse XF24 microplates (102340, Agilent), and the XF24 cartridge (102341, Agilent) was preloaded in the seahorse stop station (Agilent). Before the assay, the medium was replaced by 0.5 ml XF base Medium (102353, Agilent) supplemented with 2 mM glucose (25030801, Gibco). Cells were incubated at 37 °C for 1 h in the seahorse prep station. 56 µl glucose (100 mM) (G8270, Sigma), 62 µl oligomycin (10 µM) (75351, Sigma) and 69 µl 2DG (1 M) (D6134, Sigma) were added into the cartridge wells. ECAR and OCR levels were determined using seahorse bioscience XF24 extracellular flux analyzer (Agilent) and each cycle of measurement involved mixing (3 min), waiting (2 min), and measuring (5 min) cycles.

Glutamine extraction and concentration measurement. Tissues were cut into less than 100 mg pieces and homogenized in 70% ethanol by a Precellys 24 homogenizer. After centrifugation at 13,000 rpm for 10 min in 4 °C, the pellets were collected, evaporated to dry and thoroughly extracted with distilled H₂O (1 l H₂O per mg of tissue). The concentration of glutamine was measured by the EnzChrom Glutamine Assay Kit (BioAssay Systems) following the manufacturer’s instructions. Briefly, for each standard and sample well (96-well plates), working reagents were prepared by mixing 65 µl Assay Buffer, 1 µl Enzyme A, 1 µl Enzyme B, 2.5 µl NAD and 14 µl MTT. 20 µl of standards or samples were transferred into each well and mixed thoroughly. The mixtures were incubated for 40 min at room temperature and added with 100 µl stop reagent. OD was read by a plate reader at 565 nm.

Immunohistochemistry of human pancreatic tumor samples. Nine pancreatic cancer patient tumor and adjacent normal samples were obtained from the Rambam biobank with ethical approval by the Institutional Review Board at the Rambam Health Care Campus and the institutional review board of the University of California, Irvine. The Institutional Review Board at the University of California, Irvine. We have complied with the relevant ethical considerations for animal research overseen by this committee. 2 × 10⁶ MIA PaCa-2 control or miR-135 knockdown cells were resuspended in 200 µl DMEM without PBS or antibiotics and were injected into six-week-old male NCr Nude mice (Taconic Bioscience) by subcutaneous injection. Tumor size was measured every 2–3 days with calipers over time and tumor volume was calculated using the formula 1/2(length × width × depth). For DON treatment, nude mice were treated with PBS, or DON (10 mg/kg body weight) by intraperitoneal injection every other day for 2 weeks when control tumor volume was around 200 mm³. Tumor size was measured over time. At the end of the experiment, mice were euthanized and tumors were harvested. Samples from core and peripheral regions were dissected for further analysis.

Mouse xenografts. All studies involving animals were performed according to the Institutional Animal Care and Use Committee (IACUC)-approved protocols at the University of California, Irvine. We have complied with the relevant ethical considerations for animal research overseen by this committee. 2 × 10⁶ MIA PaCa-2 cells were injected subcutaneously into the back of 12-week-old SCID Beige female mice (Bioserve) by subcutaneous injection. Tumor size was measured every 2–3 days with calipers over time and tumor volume was calculated using the formula 1/2(length × width × depth). For DON treatment, nude mice were treated with PBS, or DON (10 mg/kg body weight) by intraperitoneal injection every other day for 2 weeks when control tumor volume was around 200 mm³. Tumor size was measured over time. At the end of the experiment, mice were euthanized and tumors were harvested. Samples from core and peripheral regions were dissected for further analysis.

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Rambam Health Care Campus, Israel, and informed consents were obtained from patients. Experiments were performed in compliance with all relevant ethical regulations for work with human samples. The specimens were dissected to small sections and only those that were confirmed microscopically to be tumor or normal tissue were included in the analysis. All slides were verified by pathologists at the Rambam Medical Center Biobank specialized in pancreatic histopathology. Specimens that showed pancreatitis histology were not included in the analysis; thus, there were no specimens with an evidence of pancreatitis in this cohort. A distinction between cancerous, normal and pancreatitis tissue was performed by a standard H&E staining technique.

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Fluoroguard mounting medium (SCYTEK). Images were acquired using Zeiss Observer Z1 microscope using Axiovision program. Image analysis was performed discriminate healthy pancreas from PDAC.

As followed: High power fields of ≥20 magnification of healthy pancreas and tumorous pancreas tissues were captured. The cytoketerin staining was used to discriminate healthy pancreas from PDAC.

**Statistics.** For all these experiments, each was repeated independently at least two times with similar results. Results are shown as means; error bars represent standard deviation (SD). The unpaired two-tailed Student’s t-test was used to determine the statistical significance of differences between means (*p < 0.05, **p < 0.01) unless indicated separately.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this Article.

**Data availability.** The miRNA-seq data generated and analyzed in this study are available at the Gene Expression Omnibus (GEO) repository of the National Center for Biotechnology Information under accession code (GSE125538). The authors declare that all data generated from this study are included in this publication and its Supplementary Information or available from the corresponding author on request.

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Author contributions
Y.Y. performed most of the experiments, analyzed the data, prepared figures and wrote the manuscript. H.L. analyzed microRNA sequencing data. N.M. and Z.G. provided pancreatic patient samples data and performed IFIC experiment on patient samples. J.L., M.A.R. and J.W.L. conducted 13C-glucose tracing experiments. M.B.I.G., E.A.H., X.H.L. and T.Q.T. contributed to experimental design, data analysis, and manuscript writing. M.K. conceived and designed experiments and contributed to manuscript writing.

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