Title

Pancreatic tumors activate arginine biosynthesis to adapt to myeloid-driven amino acid stress

Running Title

Adaptive arginine biosynthesis in PDAC

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Abstract

Nutrient stress in the tumor microenvironment requires cancer cells to adopt adaptive metabolic programs to maintain survival and proliferation. Therefore, knowledge of microenvironmental nutrient levels and how cancer cells cope with such nutrition is critical to understand the metabolism underpinning cancer cell biology. Previously, we performed quantitative metabolomics of the interstitial fluid (the local perfusate) of murine pancreatic ductal adenocarcinoma (PDAC) tumors to comprehensively characterize nutrient availability in the microenvironment of these tumors (Sullivan et al., 2019a). Here, we develop T̲umor I̲nterstitial F̲luid M̲edium (TIFM), a cell culture medium that contains nutrient levels representative of the PDAC microenvironment, enabling study of PDAC metabolism under physiological nutrition. We show that PDAC cells cultured in TIFM, compared to standard laboratory models, adopt a cellular state more similar to PDAC cells in tumors. Further, using the TIFM model we identified arginine biosynthesis as a metabolic adaptation PDAC cells engage to cope with microenvironmental arginine starvation driven by myeloid cells in PDAC tumors. Altogether, these data show that nutrient availability in tumors is an important determinant of cancer cell metabolism and behavior,

37   and cell culture models that incorporate physiological nutrient availability have improved fidelity

38   and enable the discovery of novel cancer metabolic phenotypes.

39

40   **Introduction**

41   Altered cellular metabolism is common in cancers (DeBerardinis and Chandel, 2016) and enables

42   many pathological features of tumors (Faubert et al., 2020; Vander Heiden and DeBerardinis,

43   2017). This has led to substantial interest in identifying the metabolic properties of cancers and

44   the regulation underpinning these metabolic alterations to both delineate the basic biochemistry

45   underlying these diseases and to identify novel therapeutic targets. Recent work has led to the

46   understanding that tumor metabolic phenotypes are driven both by cancer cell-intrinsic factors,

47   such as oncogenic lesions and cellular epigenetic identity (Bi et al., 2018; Nagarajan et al., 2016),

48   and by cell-extrinsic factors in the tumor microenvironment (TME) (Altea-Manzano et al., 2020;

49   Gouirand et al., 2018; Lyssiotis and Kimmelman, 2017; Muir et al., 2018). In contrast to our

50   extensive knowledge of cell-intrinsic regulation of cancer metabolism, how the TME drives altered

51   metabolism in cancers and how such TME-driven metabolic phenotypes contribute to

52   tumorigenesis are poorly understood.

53

54   Nutrient availability is a key cell-extrinsic factor that influences cellular metabolism (Elia and

55   Fendt, 2016; Garcia-Bermudez et al., 2020; Muir et al., 2018). Many solid tumors have abnormal

56   vasculature that limits tumor perfusion (Goel et al., 2011; Olive et al., 2009; Provenzano et al.,

57   2012; Wiig and Swartz, 2012), which leads to abnormal nutrient availability in the TME (Gullino et

58   al., 1964; Ho et al., 2015; Vecchio et al., 2021). Thus, perturbed nutrient availability in the TME

59   has been postulated to be a critical driver of cancer metabolic phenotypes (Martínez-Reyes and

60   Chandel, 2021; Reid and Kong, 2013). However, the precise metabolic changes in tumors driven

61   by TME-nutrient cues are largely unknown due to a dearth of information on the nutrient milieu of

62   tumors and a lack of experimentally tractable model systems to study cellular metabolism under

63   such constraints (Ackermann and Tardito, 2019; Cantor, 2019).

64

65   To determine how nutrient availability in the TME influences cancer cell metabolism, we recently

66   performed quantitative metabolite profiling of >118 major nutrients and vitamins in the interstitial

67   fluid (the local perfusate of tissues and tumors; IF) in murine models of pancreatic

68   adenocarcinoma (PDAC), providing comprehensive and quantitative knowledge of how TME

69   nutrient availability is altered in PDAC (Sullivan et al., 2019a). Here, we sought to build upon these

70   findings by determining how the abnormal nutrient availability of the PDAC TME drives metabolic

71   phenotypes in PDAC. To do so, we leveraged our knowledge of PDAC IF metabolite

72   concentrations and recent techniques for generating cell culture media with physiological

73   concentrations of nutrients (Cantor et al., 2017; Vande Voorde et al., 2019) to formulate a novel

74   cell culture media termed Tumor Interstitial Fluid Medium (TIFM) that recapitulates the nutrient

75   composition of the PDAC TME. This novel cell culture system allows us to study PDAC cells in

76   an experimentally tractable *ex vivo* model while they metabolize substrates at physiological

77   concentrations and identify critical metabolic adaptations of PDAC cells and the TME nutrient

78   cues that drive these adaptations.

79

80   To identify such metabolic adaptations, we performed transcriptomic analysis of murine PDAC

81   cells growing in TIFM, standard culture and in orthotopic tumors. Through this analysis, we found

82   that many transcriptional features of PDAC cells growing *in vivo* are better recapitulated in TIFM

83   culture compared to standard culture models. This suggests that altered nutrient availability is a

84   major regulator of the cellular state of cancer cells in the TME and *ex vivo* models incorporating

85   physiological nutrition could improve the fidelity of cell culture models of cancer (Horvath et al.,

86   2016). A major metabolic signature we found in PDAC cells in TIFM and *in vivo* was activation of

87   the amino acid starvation transcriptional signature including increased expression of the *de novo*

88   arginine synthesis pathway. We further find that myeloid driven arginase activity deprives the

89   PDAC TME of arginine and that compensatory activation of the *de novo* arginine synthesis

90   pathway enables PDAC cells in TIFM and in tumors to acquire the arginine needed for amino acid

91   homeostasis despite TME arginine starvation. Collectively, this work identifies TME nutrient

92   availability as a key regulator of the *in vivo* cancer cell phenotype and demonstrates that analysis

93   of cancer cells under physiological nutrient conditions can identify key metabolic features of

94   tumors, such as the activation of *de novo* arginine in PDAC tumors needed to cope with myeloid

95   driven TME arginine starvation.

96

97   **Results**

98   **PDAC cells grown in tumor interstitial fluid based culture medium recapitulate the**

99   **transcriptomic behavior of PDAC cells growing *in vivo***

100 To study how the nutrient composition of the PDAC TME can influence cancer cell metabolism,

101 we developed a cell culture medium termed T̲umor I̲nterstitial F̲luid M̲edium (TIFM) based on

102 metabolite concentrations in PDAC IF (Sullivan et al., 2019a) using an approach similar to those

103 described for the generation of media with plasma levels of nutrients (Cantor et al., 2017; Vande

104 Voorde et al., 2019) (Fig. 1A). TIFM is composed of 115 metabolites at the average concentration

105 previously observed in the IF of *LSL-Kras^{G12D/+}; Trp53^{flox/flox}; Pdx-1-Cre* (KP$^{-/-}$C) (Bardeesy et al.,

106 2006; Sullivan et al., 2019a) murine PDAC tumors. These metabolites were selected on the

107 following bases: (1) commercial availability at high purity, (2) stability in aqueous solution, and (3)

108 presence in pancreatic TIF at a concentration > 0.5μM. To enable rapid identification of bio-active

109 nutrients, TIFM is composed of 9 pools of metabolites that are separately compounded (Cantor

110 et al., 2017; Vande Voorde et al., 2019). To generate the complete medium, the individual

111 metabolite powders are reconstituted in water along with salts at RPMI-1640 (RPMI)

112 concentrations and 10% dialyzed FBS (dFBS) to provide lipids, growth factors and any other

113 macromolecules necessary for cell growth. Sodium bicarbonate is also added at RPMI

114   concentrations to maintain physiological pH (Michl et al., 2019). The complete TIFM formulation

115   is described in Supplementary File 1. Importantly, quantitative metabolite profiling by liquid

116   chromatography-mass spectrometry (LC-MS) of TIFM confirmed that TIFM contained metabolites

117   at expected concentrations, confirming effective compounding of the TIFM metabolite mixture

118   (Fig. 1B). Thus, TIFM recapitulates the nutrient microenvironment of PDAC.

119

120   To determine if TIFM could sustain cancer cells, we isolated murine PDAC (mPDAC) cell lines

121   from three individual KP$^{-/-}$C tumors (the same mouse model used for TIF metabolomics analyses

122   and which formed the basis of TIFM composition (Sullivan et al., 2019a)) by fluorescence

123   activated cell sorting (FACS). We split the isolated cells from each tumor into two populations,

124   which were plated either in TIFM or standard culture conditions (RPMI-1640) to generate paired

125   mPDAC cell lines termed mPDAC-RPMI or mPDAC-TIFM. mPDAC-TIFM cells readily proliferate

126   in TIFM culture, albeit at a slower rate than in RPMI-1640 (Fig. 1D), suggesting that TIFM has the

127   necessary nutrients to sustain PDAC cell proliferation. Interestingly, while mPDAC-TIFM cells

128   continue proliferating when transitioned directly from culture in TIFM to RPMI-1640, transferring

129   mPDAC-RPMI cells directly to TIFM results in complete arrest of cell growth (Fig. 1 – Figure

130   supplement 1), suggesting that long term growth of mPDAC cells in standard cell culture media

131   results in loss of key adaptations to grow under TME nutrient stress. Thus, analysis of PDAC cell

132   metabolism in TIFM could identify novel metabolic adaptations required for growth under TME

133   conditions that would not be apparent from studying PDAC cells under standard culture

134   conditions.

135

136   To identify such adaptations, we performed transcriptomic profiling comparing gene expression

137   patterns of the same mPDAC cells (mPDAC3-TIFM) isolated by FACS: (1) after culture in TIFM,

138   (2) after culture in RPMI-1640 and (3) after growing as a syngeneic orthotopic murine tumor to

139   provide an *in vivo* reference (Fig. 1E). This experimental design allowed us to identify

140 transcriptionally-driven metabolic adaptations in TIFM and confirm these were operative *in vivo*.

141 Further, the *in vivo* transcriptomic data allows us to assess how the transcriptional state of PDAC

142 cells in different *ex vivo* models compares to the *bona fide in vivo* cell state. This analysis has

143 recently been suggested to be a critical benchmark for assessing *ex vivo* model fidelity (Raghavan

144 et al., 2021). We first established that compared to standard culture conditions, mPDAC cells in

145 orthotopic tumors substantially alter their transcriptional profile (Fig. 1F and Fig. 1 Source Data

146 1). In fact, the majority of detected transcripts (12,066/16,378) are differentially expressed in the

147 same mPDAC cells when grown *in vivo* compared to standard culture conditions. Next, we

148 generated sets of genes significantly upregulated and significantly downregulated *in vivo*

149 compared to RPMI*.* We then performed gene set enrichment analysis (GSEA) (Mootha et al.,

150 2003; Subramanian et al., 2005) on the transcriptional profiles of mPDAC3-TIFM cells growing in

151 TIFM and RPMI using the 'upregulated *in vivo*' and 'downregulated *in vivo*' gene sets. The gene

152 expression patterns of mPDAC cells growing in TIFM aligns more closely with the expression

153 pattern of mPDAC cells growing *in vivo* (Fig 1G). Smaller gene sets comprised of only the top 500

154 upregulated and top 500 downregulated genes show the same enrichment patterns with similar

155 enrichment scores confirming there is no enrichment score inflation due to the large gene set size

156 (Fig 1 – Figure supplement 2). We also found a strong correlation between gene expression

157 changes induced by culture in TIFM and growth *in vivo* compared to RPMI (Fig 1 – Figure

158 Supplement 3). Lastly, amongst the top 20 up- and downregulated curated gene signatures from

159 MSigDB (Mootha et al., 2003; Subramanian et al., 2005) in TIFM cultured mPDAC cells compared

160 to RPMI, most where similarly up or down-regulated *in vivo* compared to RPMI (Fig. 1H and Fig.

161 1 Source Data 2). Together, this transcriptional analysis suggests that mPDAC cells in TIFM

162 better recapitulate the cellular state of mPDAC cells *in vivo*.

163

164 We also sought to understand which aspects of the *in vivo* mPDAC cell state were not

165 recapitulated in TIFM. To identify the cellular processes that are differentially regulated between

166   cells growing in TIFM and cells *in vivo*, we performed differential gene expression analysis

167   between mPDAC3-TIFM cells *in vivo* and in TIFM and performed GSEA using Gene ontology

168   (GO) based gene sets. The main cellular processes that differentiate PDAC cells growing *in vivo*

169   from cell growing in TIFM are cell-cell communication, response to biotic stimuli, cell surface

170   receptor activated pathways and regulation of the immune system (Fig. 1I and Fig. 1 Source Data

171   3). These differences are likely due to the presence of the immune compartment and other

172   neighboring cell populations in PDAC tumors, an aspect of the TME not modeled in TIFM. On the

173   other hand, the main cellular processes positively enriched in PDAC cells in TIFM relative to *in*

174   *vivo* are ribosome complex biogenesis, rRNA processing and mitotic cell division (Fig. 1I and Fig.

175   1 Source Data 3), suggesting that, although the slower proliferation of mPDAC cells in TIFM (Fig.

176   1D) is more reminiscent of cells *in vivo*, cell cycle progression and translation are nevertheless

177   still higher in TIFM than *in vivo*. Altogether, these results show that mPDAC cells grown in TIFM

178   more closely recapitulate the transcriptomic profile of cells growing directly in the TME, suggesting

179   that TIFM is a useful system for the discovery and characterization of cancer cell adaptations to

180   physiological tumor nutrient stress in PDAC.

181

182   **Arginine biosynthesis allows PDAC cells to adapt to TME nutrient stress.**

183   We next sought to use the transcriptional profiles in mPDAC cells in TIFM and *in vivo,* to identify

184   metabolic adaptations cancer cells exhibit in response to tumor nutrient stress. We focused on

185   adaptation to amino acid deprivation, as this gene signature is one of highly enriched in TIFM

186   (Fig. 1J) and is similarly enriched in mPDAC cells *in vivo* (Fig. 1H). Leading edge analysis

187   (Subramanian et al., 2005) identified *Ass1* as the most differentially expressed gene driving this

188   signature (Fig. 1K). We further confirmed upregulation of ASS1 at the protein level by

189   immunoblotting in TIFM relative to RPMI (Fig. 1L). Immunohistological analysis of murine and

190   human PDAC tumors using data from the Human Protein Atlas (Uhlén et al., 2015) (Fig. 1M)

191   shows similarly robust expression of ASS1, especially when compared to the lack of expression

192   in the healthy exocrine pancreas. These data suggest that mPDAC cell upregulate ASS1 as part

193   of an adaptive response to amino acid deprivation to TME nutrient stress.

194

195   Argininosuccinate synthase 1 (ASS1) is the rate-limiting enzyme in the biosynthetic pathway of

196   the non-essential amino acid arginine (Haines et al., 2011). ASS1 catalyzes the synthesis of

197   argininosuccinate from citrulline and aspartate, which can then be converted to arginine and

198   fumarate by argininosuccinate lyase (Asl) (Fig. 2A). Thus, expression of ASS1 enables PDAC

199   cells to synthesize arginine *de novo*. Arginine is one of the most limiting nutrients in the murine

200   PDAC TME at 2-5µM relative to 125µM in plasma, a 20-50 fold decrease (Sullivan et al., 2019a).

201   The TME level of arginine is below the reported Km for arginine transport (Closs et al., 2004).

202   Thus, we hypothesized that mPDAC cells are starved of arginine in the TME and expression of

203   ASS1 allows mPDAC cells to adapt to such starvation by providing an alternative cellular arginine

204   source.

205

206   To test if mPDAC cells require *de novo* synthesis to maintain intracellular arginine pools, we first

207   asked if mPDAC cells in TIFM consume the precursors (citrulline or ornithine) used for arginine

208   synthesis. To do so, we used quantitative LC-MS metabolite profiling (Sullivan et al., 2019a) to

209   perform analysis of 108 metabolites that mPDAC1-TIFM and mPDAC1-RPMI cells consume or

210   release in their respective media (Hosios et al., 2016; Jain et al., 2012). We found that mPDAC1-

211   TIFM cells consumed citrulline and ornithine at levels higher than their uptake of arginine (Fig. 2B

212   and Fig. 2 Source Data 1), consistent with active arginine synthesis in TIFM cultured mPDAC

213   cells. We next used stable isotope tracing to measure intracellular arginine derived from *de novo*

214   synthesis. To do so, mPDAC cells were cultured in TIFM with $^{13}C_5$-citrulline and steady state

215   incorporation of citrulline carbon into arginine (Fig. 2C) was assessed by gas chromatography-

216   mass spectrometry (GC-MS). The vast majority of intracellular arginine (~85%) was labeled by

217   the citrulline *de novo* synthesis tracer (Fig. 2D) and this was consistent across multiple mPDAC

218   cell lines in TIFM (Fig. 2 – Figure supplement 1A). Consistent with the citrulline tracing data

219   indicating that *de novo* arginine synthesis contributes the majority of mPDAC arginine in TIFM,

220   inhibiting arginine synthesis by deprivation of the *de novo* synthesis precursors citrulline and

221   ornithine from TIFM results in a 10-fold decrease of intracellular arginine in mPDAC cells (Fig.

222   2E; Fig. 2 – Figure supplement 1B). This decrease in intracellular arginine is accompanied by a

223   significant decrease in cell proliferation (Fig. 2F; Fig. 2 – Figure supplement 1C). Importantly,

224   individual depletion of either citrulline or ornithine further shows that depletion of citrulline, but not

225   ornithine, is the key substrate mPDAC cells require for arginine synthesis (Fig. 2 – Figure

226   supplement 1D) (Fig. 2B). To confirm the finding that *de novo* arginine synthesis is critical for

227   mPDAC proliferation in TIFM, we used CRISPR-Cas9 to knockout (KO) *Ass1* in mPDAC cells

228   (Fig. 2G). Consistent with decreased mPDAC proliferation upon *de novo* arginine synthesis

229   inhibition by citrulline withdrawal, *Ass1* KO decreases mPDAC proliferation and this affect can be

230   rescued by supplying additional exogenous arginine in TIFM (Fig. 2H). Altogether, these findings

231   suggest that that *de novo* arginine synthesis is important to maintain intracellular arginine levels

232   and mPDAC cell proliferation in TIFM.

233

234   We next sought to determine if ASS1-mediated arginine synthesis contributes to arginine

235   homeostasis in murine PDAC tumors. To assess intratumoral PDAC arginine synthesis, we

236   performed $^{15}N_2$-glutamine stable isotope tracing by multiple bolus intravenous injections of $^{15}N_2$-

237   glutamine into mPDAC orthotopic tumor bearing mice and non-tumor bearing controls (Fig. 2I).

238    $^{15}N_2$-glutamine tracing can be used to monitor arginine synthesis and urea cycle activity in PDAC

239   (Zaytouni et al., 2017) by monitoring incorporation of labeled glutamine derived nitrogen into

240   arginine (Fig. 2C). After glutamine injection, healthy pancreas, tumor tissue and plasma samples

241   were collected and ^{15}N enrichment in arginine and relevant precursors was measured by LC-MS

242   (Fig. 2 – Source Data 2). Glutamine in plasma is also quickly metabolized by multiple organs and

243   reintroduced into the circulation as intermediate substrates that can contribute to intraorgan and

244   intratumoral labelling (Grima-Reyes et al., 2021). One of the main examples of these interorgan

245   exchange fluxes is the intestinal-renal axis, where glutamine metabolized by the small intestine

246   is released as citrulline and uptaken by the kidneys and further released as arginine for uptake

247   by different tissues (Boelens et al., 2005; Grima-Reyes et al., 2021). Consistent with this, we

248   observed an enrichment of ~14% $^{15}N_1$-arginine and ~7% $^{15}N_2$-arginine in the circulation of healthy

249   and tumor bearing mice (Fig. 2J). We would expect that, for tissues that rely on circulating arginine

250   and not on arginine production, the relative abundance of labelled arginine *in situ* would resemble

251   that of the circulation. In line with this, the relative abundance of arginine in non-ASS1 expressing

252   healthy pancreas resembles the arginine labeling distribution found in circulation (Fig. 2J). In

253   contrast, there is a greater amount of labelled arginine in PDAC tumor tissue compared to plasma,

254   with ~17% $^{15}N_1$-arginine and ~9% $^{15}N_2$-arginine in tumors (Fig. 2J). While these non-steady state

255   isotope labeling experiments cannot allow us to infer the fraction of intratumoral arginine that

256   arises from *de novo* synthesis in PDAC tumors (Buescher et al., 2015), the appearance of

257   additional ^{15}N enrichment in intratumoral arginine that cannot be explained by circulating labeled

258   arginine confirms active synthesis of arginine in PDAC tumors, consistent with previous results

259   (Fig. 1) that PDAC tumors highly express ASS1. Lastly, we measured the concentration of amino

260   acids including arginine in the interstitial fluid of orthotopic murine PDAC tumors and compared

261   this to the intratumoral arginine concentration (Fig. 2K and Fig. 2 Source Data 3). We observed

262   that for most amino acids the intratumoral concentration was similar to the IF concentration.

263   However, PDAC tumors had higher concentrations of free arginine than what is present in the

264   TIF. Thus, we conclude that PDAC tumors accumulate higher levels of arginine than available

265   from the local perfusate and that this is at least in part driven by *de novo* synthesis.

266

267   **Enhanced uptake of environmental arginine allows PDAC cells to cope with inhibition of**

268   ***de novo* arginine synthesis.**

269   Interestingly, while PDAC cell proliferative capacity is markedly reduced by inhibiting arginine

270   biosynthesis, this doesn't completely abrogate cell growth (Fig. 2F,H; Fig. 2 – Figure supplement

271   1C). Given the importance of arginine for cellular homeostasis and growth, we hypothesized that

272   PDAC cells must compensate with other mechanisms to acquire arginine when synthesis is

273   inhibited to maintain viability and proliferation, albeit at a reduced rate (Fig. 2A). In addition to *de*

274   *novo* synthesis, there are two other known mechanisms for arginine acquisition by PDAC cells:

275   macropinocytosis (Palm, 2019) and cationic amino acid transporter mediated uptake (Closs et al.,

276   2004). Therefore, we sought to determine how these pathways contribute to arginine homeostasis

277   in TIFM cultured mPDAC cells.

278

279   To test if macropinocytosis is important for arginine homeostasis in mPDAC cells, we generated

280   mPDAC1-TIFM cells with a doxycycline-inducible shRNA targeting glycoprotein syndecan-1

281   (SDC1), an important mediator of macropinocytosis in PDAC cells (Yao et al., 2019) (Fig. 3 –

282   Figure supplement 1A). Knockdown of *Sdc1* effectively reduced mPDAC1-TIFM

283   macropinocytosis rate as measured by uptake and catabolism of fluorogenic bovine serum

284   albumin (DQ-BSA), a model macropinocytosis substrate (Fig. 3 – Figure supplement 1B). *Sdc1*

285   knockdown had no effect on intracellular arginine pools nor cell proliferation in TIFM cultured

286   mPDAC cells (Fig. 3A, B). Consistent with this, pharmacological inhibition of lysosomal protein

287   breakdown with hydroxychloroquine (HQ) similarly impairs mPDAC1-TIFM macropinocytosis rate

288   without disrupting cell proliferation (Fig. 3 – Figure supplement 1C, D). Thus, in basal TIFM

289   conditions, macropinocytosis does not appear to be critical for cellular arginine homeostasis. We

290   next tested if macropinocytosis was critical for mPDAC cells upon inhibition of *de novo* arginine

291   synthesis. We measured DQ-BSA uptake and catabolism upon inhibition of arginine synthesis

292   and observed no change in macropinocytosis rate (Fig. 3 – Figure supplement 1E). Furthermore,

293   knockdown of *Sdc1* did not further impair mPDAC cell proliferation upon inhibition of arginine

294   synthesis (Fig. 3C). Thus, we conclude that macropinocytosis does not contribute to mPDAC

295 arginine homeostasis in TIFM, even as an adaptive mechanism upon inhibition of *de novo*

296 arginine synthesis.

297

298 We next tested if uptake of the small amount of free arginine in TIFM (~2.3µM) mediates the ability

299 of mPDAC cells to cope with inhibition of *de novo* arginine synthesis. In normal TIFM culture,

300 removal of arginine does not affect mPDAC intracellular arginine levels nor proliferative capacity

301 (Fig. 3D,E). Thus, as with macropinocytosis, arginine uptake is not critical for mPDAC arginine

302 homeostasis in TIFM conditions. We next tested if depriving mPDAC cells of the available

303 microenvironmental arginine after *de novo* biosynthesis is impaired would affect mPDAC arginine

304 homeostasis and growth. We found that inhibition of arginine synthesis in TIFM cultured mPDAC

305 cells leads to increased transcription of known arginine transporters (Fig. 3F) and leads to an

306 increased rate of arginine uptake by mPDAC cells (Fig. 3G). Furthermore, while we could not

307 detect decreases in mPDAC intracellular arginine levels after eliminating extracellular arginine

308 (Fig. 3 – Figure supplement 1F), eliminating TIFM extracellular arginine completely abrogates cell

309 growth in multiple mPDAC cell lines upon inhibition *of de* novo arginine synthesis (Fig. 3H – Figure

310 supplement 1G). Altogether, these data suggest that mPDAC cells upregulate the uptake of

311 extracellular arginine to cope with inhibition of arginine biosynthesis and that this could be in part

312 mediated by the selective upregulation of cationic amino acid transporters.

313

Myeloid arginase causes local arginine depletion in PDAC

315 Given that PDAC tumors preferentially upregulate arginine *de novo* synthesis to adapt to

316 microenvironmental depletion of arginine, we wanted to understand how arginine becomes

317 depleted in the PDAC TME. Human and murine PDAC tumors contain substantial myeloid

318 compartments (Lee et al., 2021; Zhu et al., 2017) including many arginase-1 expressing cells

319 (Trovato et al., 2019). We confirmed the presence of a robust myeloid and arginase-1 expressing

320 populations in both murine (Fig. 4A) and human PDAC (Fig. 4B) by immunohistochemical

321   analysis. Arginase-1 expressing cells are capable of metabolizing arginine into ornithine and urea

322   (Caldwell et al., 2018). Therefore, we hypothesized that myeloid arginase-1 activity could be

323   responsible for PDAC TME arginine starvation. To test this, we generated orthotopic allograft

324   mPDAC tumors in a mouse model with myeloid specific *Arg1* knockout (*LysM-Cre$^{+/+-}$; Arg1$^{fl/fl}$*)

325   (Clausen et al., 1999; El Kasmi et al., 2008) and control animals (*Arg1$^{fl/fl}$*). We then isolated IF

326   from these tumors at end-stage and measured the levels of amino acids including arginine and

327   ornithine in these samples (Fig. 4C). Compared to control animals, *LysM-Cre$^{+/+-}$; Arg1$^{fl/fl}$* tumors

328   show robust reduction of arginase-1 expression in tumors (Fig. 4D) confirming most arginase-1

329   in tumors is myeloid in origin. *LysM-Cre$^{+/+-}$; Arg1$^{fl/fl}$* tumors had ~9-fold increase in IF arginine

330   concentration and a roughly equimolar decrease in ornithine (Fig. 4E and Fig. 4 Source Data 1),

331   consistent with myeloid arginase-1 activity being responsible for TME arginine starvation in these

332   tumors. Pharmacological inhibition of arginase-1 with the small-molecule inhibitor CB-1158

333   (Steggerda et al., 2017) in mPDAC orthotopic tumors led to a similar increase in IF arginine

334   compared to control treated tumors (Fig. 4F and Fig. 4 Source Data 1). In summary, these results

335   show that arginase activity in the myeloid compartment of PDAC tumors is responsible for arginine

336   depletion in the TME, which results in activation of arginine biosynthesis by PDAC as an

337   adaptation to this metabolic stress in the PDAC TME.

338

339   **Discussion**

340   **TME nutrition is a major driver of PDAC cell state**

341   Cell-based models remain critical tools for mechanistic discovery and therapeutic target

342   identification in cancer biology. However, many biological findings and drug targets that arise from

343   these cell-based studies fail to translate to cancer cells *in vivo* or in clinical settings (Horvath et

344   al., 2016). That cancer cells behave so differently in *in vitro* cell-based systems than when in

345   tumors suggests that cancer cell behavior is not completely cell-intrinsically encoded. Rather, cell-

346   extrinsic cues in the TME are capable of dramatically influencing the cancer cell state and

347   impacting many aspects of cancer cell biology including therapy response (Hirata and Sahai,

348   2017). The importance of TME cues in regulating cancer cell behavior has prompted new efforts

349   to develop cell-based models that incorporate key microenvironmental influences to both improve

350   their disease relevance and fidelity (Horvath et al., 2016) and enable mechanistic studies

351   delineating how the microenvironment influences cancer cell biology.

352

353   We directly assessed the impact of the TME on the cellular state of murine PDAC cells by

354   transcriptomic analysis. We found that the TME does indeed induce massive changes in the

355   transcriptional state of PDAC cells compared to PDAC cells in standard culture, consistent with

356   the microenvironment dramatically changing the biology of cancer cells (Fig. 1F). We next

357   specifically asked how TME nutrition influences PDAC cell state and if better recapitulating

358   nutrient access in our cell-based PDAC models would leave PDAC cells in more *in vivo*-like state.

359   Given that metabolism is highly interconnected with epigenetic regulation of gene expression

360   (Chisolm and Weinmann, 2018; Dai et al., 2020; Diehl and Muir, 2020; Reid et al., 2017) and that

361   cellular metabolism is intricately tied to nutrient availability (Elia and Fendt, 2016; Muir et al.,

362   2018), we reasoned that physiological nutrient availability could have dramatic influences on

363   cellular state and be a key microenvironmental factor influencing cancer cell biology. Indeed, we

364   found that growth of PDAC cells in physiological nutrition caused substantial transcriptional

365   reprogramming of PDAC cells that pushed them towards the *in vivo* transcriptional state

366   compared to culture in standard non-physiological conditions (Fig. 1G, H). Thus, consistent with

367   recent studies that have employed cell culture models with physiological nutrient availability

368   (Ackermann and Tardito, 2019; Cantor, 2019; Cantor et al., 2017; Muir et al., 2017; Vande Voorde

369   et al., 2019), we have found that modeling physiological nutrient availability substantially improves

370   cell culture model fidelity. Thus, along with other efforts to improve the fidelity of cell culture

371   models by incorporating microenvironmental factors such as bio-scaffolds enabling three-

372   dimensional growth (Jensen and Teng, 2020; Pampaloni et al., 2007), we anticipate ensuring

373   proper nutrient availability will be critical in the developing more physiologically relevant *ex vivo*

374   cancer models, which will expand our ability to target cancer beyond cell-intrinsic dependencies

375   by allowing the identification and exploitation of microenvironmentally driven therapeutic targets

376   (Metcalf et al., 2021; Sela et al., 2022).

377

378   **Arginine is a limiting nutrient in the PDAC microenvironment**

379   Use of the nutrient microenvironment mimicking TIFM model uncovered arginine biosynthesis as

380   a metabolic adaptation that PDAC cells use under microenvironmental nutrient conditions. That

381   PDAC cells would synthesize arginine was initially surprising. Arginine biosynthesis is a

382   metabolically costly process that utilizes intracellular aspartate, a limiting nutrient for tumors

383   (Garcia-Bermudez et al., 2018; Sullivan et al., 2018). Aspartate limitation that arises from arginine

384   synthesis slows nucleotide synthesis and ultimately tumor growth (Rabinovich et al., 2015). Thus,

385   ASS1 acts as a metabolic tumor suppressor and is silenced in many tumor types (Lee et al.,

386   2018). Why would this tumor suppressive metabolic pathway be activated in PDAC?

387

388   Multiple studies have also shown that cancer cells can reactivate ASS1 expression and arginine

389   biosynthesis when extracellular arginine becomes limited to support tumor growth. For example,

390   ASS1-silenced tumors treated with arginine deiminase to eliminate extracellular arginine acquire

391   resistance to such therapy by reactivation of ASS1 expression (Rogers and Van Tine, 2019;

392   Rogers et al., 2021). In another example, reactivation of arginine biosynthesis was shown to be

393   necessary to support metastasis of clear cell renal cancers to the arginine limited lung

394   environment, whereas arginine biosynthesis was not necessary and inactive in the arginine-

395   replete primary tumor (Sciacovelli et al., 2021). Lastly, ATF4-CEBPβ mediated upregulation of

396   ASS1 upon amino acid stress has been shown to allow AML cells to adapt to low levels of

397   microenvironmental arginine (Crump et al., 2021). Altogether, these findings suggest that the

398   tumor suppressive role of arginine biosynthesis is context dependent. In the context of

399   microenvironmental arginine deprivation, ASS1 and arginine biosynthesis can switch their role to

400   become tumor supportive. One of the most depleted nutrients in the PDAC TME is the amino acid

401   arginine, which we previously observed was depleted ~20-50 fold from circulatory concentrations

402   to only 2-5µM (Sullivan et al., 2019a). Thus, given the TME context of PDAC tumors, ASS1 and

403   arginine biosynthesis enables PDAC cells to cope with these TME constraints rather than acting

404   as a tumor suppressor.

405

406   Aside from TME arginine restriction in PDAC triggering adaptive responses in the cancer cells,

407   the lack of arginine in the TME can have major impacts on stromal cells that may not have the

408   adaptive capabilities of PDAC cells. For example, anti-tumor lymphocytes require arginine for

409   functionality (Geiger et al., 2016), but are not able to upregulate arginine biosynthesis upon

410   arginine starvation (Crump et al., 2021). Thus, microenvironmental arginine availability is known

411   to limit immune responses in a variety of tumor types (Murray, 2016). This has led to many recent

412   efforts to develop pharmacological tools to increase TME arginine (Canale et al., 2021; Steggerda

413   et al., 2017), which have improved immunotherapeutic outcomes in a variety of murine tumor

414   models (Canale et al., 2021; Miret et al., 2019; Sosnowska et al., 2021; Steggerda et al., 2017).

415   Thus, the severe arginine restriction in the PDAC TME could be a major barrier to immunotherapy

416   in this disease, which is refractory to most immunotherapies (Hilmi et al., 2018). Consistent with

417   this hypothesis, Menjivar and colleagues found that low arginine availability does impair anti-

418   tumor immunity in PDAC and that raising TME arginine levels can improve tumor immune

419   surveillance and response to immunotherapy (Menjivar, co-submitted manuscript). Thus, arginine

420   starvation is a key nutrient limitation that both PDAC and stromal cells face in the TME. Arginine

421   starvation drives PDAC metabolic adaptation but other cell types without adaptive capacity, such

422   as lymphocytes, face arginine starvation driven dysfunction in the TME. Future studies delineating

423   how different cellular populations are affected by TME arginine starvation will prove critical to

424   better understanding how tumor physiology impacts cancer and stromal cell biology.

425

Stromal control of microenvironmental nutrient conditions

We previously had found that the TME is arginine depleted (Sullivan et al., 2019a). However, what drove arginine depletion in the TME was unknown. Here, we find that the large arginase-1 expressing myeloid compartment in PDAC tumors is largely responsible for TME arginine depletion (Fig. 4). Consistent with these findings, Menjivar and colleagues also found PDAC associated myeloid cells are critical for mediating TME arginine depletion (Menjivar, co-submitted manuscript). Thus, the most striking nutrient perturbation in the TME is not driven by abnormal cancer cell metabolism but is instead driven by stromal metabolic activity. This finding is in line with recent studies documenting the critical role that stromal cells have in influencing nutrient availability in the TME. For example, in addition to the role we have found for myeloid cells in limiting TME arginine, myeloid cells were found to be the major glucose consuming cell type in a variety of tumor types (Reinfeld et al., 2021). Thus, stromal myeloid cells are likely key regulators of glucose availability in the TME. Fibroblasts have been show to also regulate levels of key metabolites in the TME (Sherman et al., 2017), such as amino acids (Francescone et al., 2020; Sousa et al., 2016) and lipids (Auciello et al., 2019). Recently, tumor innervating neurons were also shown to regulate availability of amino acids in the TME (Banh et al., 2020). Thus, future studies delineating the complex metabolic interactions amongst tumor and stromal cells (Li and Simon, 2020) will be critical to understanding how nutrient availability is regulated in the tumor ecosystem and the resulting nutrient milieu impacts cancer and stromal cell metabolism and biology.

Figure Legends

Figure 1. Culturing PDAC cells in physiological nutrients levels better recapitulates the transcriptomic profile of cancer cells in the tumor microenvironment, including changes in expression of arginine metabolism genes. (A) Diagram of the Tumor Interstitial Fluid

451 Medium (TIFM) formulation. (B) Scatter plot of LC-MS measurements of metabolite

452 concentrations in TIFM (n=2) plotted against expected concentrations (average concentration of

453 the given metabolite in KP$^{-/-}$C IF). The values represent the mean of LC-MS measurements and

454 the error bars represent the range. (C) Diagram of the generation of paired PDAC cell lines grown

455 in TIFM and in RPMI isolated from KP$^{-/-}$C tumors, the same tumors used for IF measurements

456 and TIFM formulation. (D) Cell proliferation rate of paired mPDAC cell lines grown in TIFM or

457 RPMI (n=3). The values represent the mean and the error bars represent ± SD. (E) Diagram of

458 workflow for the transcriptomic comparison of mPDAC3-TIFM cells grown in TIFM (n=3), RPMI

459 (n=3) or as orthotopic allograft murine tumors (n=6). mPDAC cells from each condition were

460 isolated by FACS and RNA was isolated for transcriptomic analysis by next generation

461 sequencing. (F) Volcano plot of differentially expressed genes between mPDAC3-TIFM cells in *in*

462 *vivo* or cultured in RPMI. Blue: downregulated genes with adjusted p<0.05. Red: upregulated

463 genes with adjusted p<0.05. Gray: genes with adjusted p>0.05. Adjusted p-value was calculated

464 through Limma using the Benjamini and Hochberg false discovery rate method (Benjamini and

465 Hochberg, 1995) (G) GSEA of transcriptional differences between mPDAC3-TIFM cells cultured

466 in TIFM or RPMI using custom gene sets from Fig. 1F of those genes up and down *in vivo*. (H)

467 Top 40 enriched (red) or depleted (blue) gene sets from the MsigDB curated gene signature

468 collection mPDAC3-TIFM cells cultured in TIFM compared to culture in RPMI. Also shown are

469 enrichment scores of the same gene sets for mPDAC3-TIFM cells grown *in vivo* compared to

470 culture in RPMI. Grey boxes are gene sets not significantly differentially enriched in mPDAC3-

471 TIFM cells grown *in vivo* compared to cultured RPMI. (I) GSEA analysis using GO gene sets of

472 the transcriptional profile of mPDAC3-TIFM cells *in vivo* compared to mPDAC3-TIFM cells

473 cultured in TIFM. (J) GSEA analysis of the Krige_Amino_Acid_Deprivation signature in mPDAC3-

474 TIFM cells cultured in TIFM or RPMI. (K) Row scaled log$_2$ fold change of total gene counts

475 normalized by trimmed mean of M values (TMM) of GSEA plot in Fig. 1J (left) and t-statistic metric

476 for differential expression calculated with limma for these genes (right). (L) Immunoblot analysis

477   of Ass1 in mPDAC cell lines grown in TIFM or RPMI. (M) Immunohistochemical staining for ASS1

478   in *LSL-KRas^{G12D}; LSL-P53^{R172H}; Pdx1-Cre* murine PDAC tumors (Hingorani et al., 2005) and

479   healthy murine pancreas (top) as well as in human PDAC tumors and in healthy human pancreas

480   (bottom). Scale bar: 100µm. Human samples are from the Human Protein Atlas (Uhlén et al.,

481   2015).

482

483   **Figure 1 – Figure supplement 1. mPDAC cells cannot proliferate in TIFM after long term**

484   **culture in RPMI.** mPDAC cells were isolated from a KP$^{-/-}$C tumor and cultured in either TIFM or

485   RPMI directly. After long term culture (>1 month) in either media condition, cells were passaged

486   into the other media (i.e. cells grown in TIFM were subsequently cultured in RPMI and cells grown

487   to RPMI were subsequently grown in TIFM). The proliferation rate after each media swap was

488   measured (n=3). The values represent the mean and the error bars represent ± SD.

489

490   **Figure 1 – Figure supplement 2. GSEA using gene signatures of the top 500 upregulated**

491   **and top 500 downregulated genes *in vivo* vs. RPMI shows TIFM grown cells better**

492   **recapitulate the transcriptomic behavior of PDAC cells *in vivo*.** Custom gene sets of the top

493   500 genes up- and downregulated (by adjusted p-value) in mPDAC3-TIFM cells growing *in vivo*

494   compared to standard culture were generated from the data in Fig. 1E,F. GSEA analysis was

495   performed on transcriptional data of mPDAC3-TIFM cells cultured in TIFM and RPMI using these

496   gene sets.

497

498   **Figure 1 – Figure supplement 3. Correlation between gene expression changes in**

499   **mPDAC3-TIFM cells cultured in TIFM and *in vivo*.** Mean log$_2$ fold changes in gene expression

500   between mPDAC3-TIFM cells cultured in TIFM (n=3) versus RPMI standard culture reference

501   (n=3) and compared to mPDAC3-TIFM cells isolated from an orthotopic tumor (n=6) compared to

502   RPMI. Pearson correlation r = 0.3733, p<0.0001.

503

504   **Figure 2. Arginine biosynthesis allows PDAC cells to adapt to low microenvironmental**

505   **levels of arginine.** (A) Cells can acquire arginine by either of three routes: direct uptake of free

506   arginine from the microenvironment, *de novo* synthesis and uptake and breakdown of

507   extracellular protein (macropinocytosis). (B) Cellular consumption/release rate of citrulline,

508   ornithine and arginine by mPDAC1-TIFM cells cultured in TIFM (n=3). (C) Diagram showing

509   cellular metabolic pathways mediating isotopic label incorporation from $^{13}C_5$-citrulline and $^{15}N_2$-

510   glutamine into arginine. (D) Relative abundance of intracellular arginine isotopomers in mPDAC

511   cells grown in TIFM with $^{13}C_5$-citrulline at PDAC IF concentration (67μM) (n=3). (E) Relative

512   intracellular arginine levels of mPDAC cells grown in TIFM with (+) or without (-) TIF

513   concentrations of citrulline (cit) and ornithine (orn). (F) Cell proliferation rate of mPDAC cells in

514   same conditions as (E) (n=3). (G) mPDAC1-TIFM cells were infected with lentiviruses encoding

515   a *Ass1* targeting CRISPR vector. *Ass1* knockout cells were then infected with lentiviruses

516   expressing either CRISPR resistant *Ass1* cDNA or empty vector (E.V.) as indicated. Shown is an

517   immunoblot analysis of these modified cell lines for Ass1 protein expression with vinculin as a

518   loading control. (H) Cell proliferation rate of cells in (G) (n=3). Cells were grown in TIFM with

519   different arginine concentrations as indicated (n=3). (I) Diagram of stable isotope tracing by bolus

520   intravenous injections of $^{15}N_2$-glutamine in orthotopic mPDAC3-TIFM tumor bearing mice and non-

521   tumor-bearing controls followed by plasma sampling and tumor extraction for analysis of

522   intratumoral metabolite labeling during the period of kinetic labeling. (J) Relative abundance of

523   ^{15}N-labelled arginine isotopomers in tissues or plasma after $^{15}N_2$-glutamine tail-vein bolus

524   injections (n=7). (K) (*left*) Concentrations of amino acids in IF (n=3) and tumor samples (n=4) of

525   mPDAC3-RPMI orthotopic tumors measured by LC-MS. (*right*) Bar graph of intratumoral versus

526   IF samples arginine concentrations. For all panels, bar graphs represent mean and error bars

527   represent ± SD. P-value for (G) was calculated using an ordinary one-way anova test and in (K)

528   using a paired, one-tail student's t test. ns indicates p>0.05, * indicates p<0.05; ** indicates

529   p<0.01; *** indicates p<0.001.

530

531   **Figure 2—Figure supplement 1. Arginine biosynthesis allows for adaptation to low**

532   **physiological levels of microenvironmental arginine in multiple mPDAC cell lines.** (A)

533   Relative abundance of intracellular ^{13}C-labelled arginine isotopomers in mPDAC2-TIFM and

534   mPDAC3-TIFM cells grown in TIFM with ^{13}C$_5$-citrulline (n=3). (B) Relative intracellular arginine

535   levels of mPDAC2-TIFM and mPDAC3-TIFM cells grown in TIFM with (+) or without (-) PDAC IF

536   concentrations of citrulline (cit) and ornithine (orn) (n=3). (C) Cell proliferation rate of mPDAC2-

537   TIFM and mPDAC3-TIFM cells in same conditions as (B) (n=3). (D) Cell proliferation rate of

538   mPDAC1-TIFM cells with or without TIFM concentrations of ornithine (orn) (n=3). (E) Cell

539   proliferation rate of mPDAC1-TIFM cells with or without TIFM concentrations of citrulline (cit)

540   (n=3). For all panels, bars represent the mean and error bars represent the mean and the error

541   bars represent ± SD.

542

543

544   **Figure 3. Enhanced uptake of environmental arginine allows PDAC cells to cope with**

545   **inhibition of *de novo* arginine synthesis.** (A) Relative intracellular arginine levels of mPDAC1-

546   TIFM cells infected with lentiviruses encoding a doxycycline inducible *Sdc1* targeting shRNA or

547   an empty vector (EV) control treated with 1 μg/mL doxycycline or vehicle (n=3). (B) Cell

548   proliferation rate of mPDAC1-TIFM cells in same conditions as (A) (n=3). (C) Proliferation rate of

549   mPDAC1-TIFM cells as in (A) cultured in TIFM with or without TIFM concentrations of citrulline

550   (cit) and ornithine (orn) (n=3). (D) Relative intracellular arginine levels of mPDAC1-TIFM cells

551   grown in TIFM with or without TIFM concentrations of arginine (arg) (n=3). (E) Cell proliferation

552   rate of mPDAC1-TIFM cells in same conditions as (D) (n=3). (F) Trimmed mean of M values

553   (TMM)-normalized counts for *Slc7a1* and *Slc7a3,* two cationic amino acid transporters capable of

554   transporting arginine, from transcriptomic analysis (see **Methods**) of mPDAC3-TIFM cells grown

555   in either TIFM or TIFM without citrulline and ornithine (n=3). (G) Per-cell consumption rate of

556   arginine by mPDAC1-TIFM cells cultured in TIFM with or without TIFM concentrations of citrulline

557   and ornithine. Cells were supplemented with 20µM arginine to enable the consumption

558   measurements (n=9). (H) Proliferation rate of mPDAC1-TIFM cells grown with or without TIFM

559   concentrations of citrulline, ornithine, or arginine, as indicated (n=3). For all panels, bar graphs

560   represent the mean and the error bars represent ± SD. P-values for (A) and (C) were calculated

561   using an ordinary one-way anova test and ns indicates $p > 0.05$, * indicates $p < 0.05$; ** indicates

562    $p < 0.01$; *** indicates $p < 0.001$.

563

564   **Figure 3—Figure supplement 1. mPDAC cells do not upregulate macropinocytosis after**

565   **inhibition of arginine synthesis, but instead require arginine uptake to cope with inhibition**

566   **of *de novo* arginine synthesis** (A) RTqPCR analysis for *Sdc1* in mPDAC cells infected with

567   lentiviruses encoding a dox inducible *Sdc1* targeting shRNA or an empty vector (EV) control with

568   and without treatment with 1 µg/mL doxycycline or vehicle (n=2). (B) Macropinocytosis activity

569   measured by kinetic DQ-BSA uptake and catabolism in mPDAC1-TIFM cells from (A). (C)

570   Macropinocytosis activity measured by kinetic DQ-BSA uptake and catabolism in mPDAC1-TIFM

571   cells treated with 10µM hydroxychloroquine (HQ) or vehicle (water). (D) Cell proliferation rate of

572   mPDAC1-TIFM cells in same conditions as (C) (n=3). (E) Macropinocytosis activity measured by

573   DQ-BSA uptake and catabolism in mPDAC1-TIFM cells cultured in TIFM with or without TIF

574 concentrations of citrulline and ornithine. (F) Relative intracellular arginine levels of mPDAC1-

575 TIFM cells grown in TIFM with or without PDAC IF concentrations of citrulline, ornithine, or

576 arginine, as indicated (n=3). (G) Proliferation rate of mPDAC2-TIFM and mPDAC3-TIFM cells

577 grown in TIFM with or without PDAC IF concentrations of citrulline, ornithine, or arginine, as

578 indicated (n=3). For all panels, bar graphs represent the mean and the error bars represent ± SD.

579

580 **Figure 4. Myeloid arginase causes microenvironmental arginine depletion in PDAC tumors.**

581 (A) Immunohistochemical (IHC) staining for F4/80 and ARG1 in an orthotopic mPDAC1-TIFM

582 tumor and in healthy murine pancreas. Scale bars: 100µm. (B) IHC staining for ARG1 in an

583 advanced human PDAC tumor and adjacent untransformed pancreas. Scale bar: 500µm (top)

584 and 100µm (bottom). (C) Schematic for crossing of LysM-Cre and Arg1$^{fl/fl}$, tumor implantation on

585 LysM-Cre$^{+/+}$; Arg1$^{fl/fl}$ progeny and subsequent IF extraction. (D) IHC staining for ARG1 protein

586 expression in orthotopic mPDAC3-TIFM tumors from LysM-Cre$^{+/+}$; Arg1$^{fl/fl}$ and Arg1$^{fl/fl}$ littermate

587 controls. Scale bar: 100µm. (E) Absolute concentration of arginine and ornithine in the IF of

588 orthotopic mPDAC3-TIFM tumors from LysM-Cre$^{+/+}$; Arg1$^{fl/fl}$ (n=7) and Arg1$^{fl/fl}$ littermate controls

589 (n=4). (F) Absolute concentration of arginine and ornithine in the IF of mPDAC3-TIFM orthotopic

590 tumors after treatment with 100mg/kg of arginase inhibitor CB-1158 or vehicle (n=5). P-values for

591 (E) and (F) were calculated using a two-tailed Student's t test and ns indicates p>0.05, * indicates

592 p<0.05; ** indicates p<0.01; *** indicates p<0.001.

593

594 **Figure 1 Source Data 1** Table of differentially expressed genes between mPDAC3-TIFM cells

595 cultured in RPMI and isolated from orthotopic tumors generated by limma analysis of

596 transcriptomic data as show in Fig. 1F.

597

598 **Figure 1 Source Data 2** Table of the top 40 differentially enriched MSigDB curated gene sets

599 between mPDAC3-TIFM cells cultured in TIFM and RPMI as shown in Fig. 1H. Enrichment

600 scores and significance values for the same gene sets are also shown for transcriptomic data of

601 mPDAC3-TIFM cells isolated from orthotopic tumors and cultured in RPMI.

602

603 **Figure 1 Source Data 3** Table of GO gene set enrichment values comparing transcriptional

604 differences between mPDAC3-TIFM cells cultured in TIFM and isolated from orthotopic tumors

605 as show in Fig. 1I.

606

607 **Figure 2 Source Data 1** Table of metabolite consumption/release rates for mPDAC-TIFM

608 cultured in TIFM and mPDAC1-RPMI cells cultured in RPMI.

609

610 **Figure 2 Source Data 2** Mass isotopomer distributions for all metabolites analyzed by LC-MS in

611 Figure 2J.

612

613 **Figure 2 Source Data 3** All metabolite concentrations, tumor volumes, weights and densities

614 measured in Fig. 2K used to calculate IF and intratumoral concentrations of arginine.

615

616 **Figure 4 Source Data 1** All metabolite concentrations measured in the IF of orthotopic

617 mPDAC3-TIFM tumors from LysM-Cre$^{+/+}$; Arg1$^{fl/fl}$ and Arg1$^{fl/fl}$ littermate controls in Fig. 4E. All

618 metabolite concentrations measured in the IF of mPDAC3-TIFM orthotopic tumors after

619 treatment with 100mg/kg of arginase inhibitor CB-1158 or vehicle in Fig. 4F.

620

621 **Supplementary File 1** Table with complete formulation of TIFM including all commercial suppliers

622 and manufacturer part numbers for each metabolite included in TIFM.

623

624 **Supplementary File 2** Table of primers used for qPCR analyses and genotyping analysis of

625 LysM-Cre and Arg1$^{fl/fl}$ alleles. Table of shRNA hairpin and sgRNA sequences used in this study.

626

627 **Materials and Methods**

628 **Formulation of Tumor Interstitial Fluid Media**

629 TIFM is composed of 115 nutrients (Supplementary File 1) at levels that match the average of

630 measurements in the IF of murine *LSL-Kras$^{G12D/+}$; Trp53$^{flox/flox}$ Pdx1-Cre* (KP$^{-/-}$C) PDAC tumors

631 (Sullivan et al., 2019a). The medium is composed of 10 pools of metabolites each of which is

632 formulated by compounding dry powders of nutrients at appropriate ratios using a knife mill

633 homogenizer. To generate the complete medium, the 10 metabolite mixture powders are added

634 together and reconstituted in water with 10% dialyzed fetal bovine serum (FBS) to provide

635 essential lipids, proteins and growth factors. The electrolytes provided in pool 3 are adjusted so

636 that the electrolyte balance will be the same as RPMI-1640 medium, correcting for the sodium

637 chloride in the FBS and counter ions in the various metabolites in TIFM. We performed

638 quantitative LC-MS metabolite profiling (see **Quantification of metabolite levels in cell culture**

639 **media**) to ensure concentrations of nutrients in TIFM are reproducibly close to the formulated

640 concentration (Fig. 1B).

641

642 **Quantification of metabolite levels in cell culture media**

643 For quantification of metabolites in cell culture media, quantitative metabolite profiling of fluid

644 samples was performed on tissue culture media samples as previously described (Sullivan et al.,

645 2019a). Briefly, chemical standard libraries of 149 metabolites in seven pooled libraries were

646 prepared and serially diluted in HPLC grade water from in a dilution series from 5mM to 1µM to

647 generate 'external standard pools', which are used for calibration of isotopically labeled internal

648 standards and to quantitate concentrations of metabolites where internal standards were not

649 available.

650

651 We then extracted metabolites from 5µL of either cell culture media samples or external standard

652 pool dilutions using 45µL of a 75:25:0.1 HPLC grade acetonitrile:methanol:formic acid extraction

653 mix with the following labelled stable isotope internal standards:

654

- ^{13}C labeled yeast extract (Cambridge Isotope Laboratory, Andover, MA, ISO1)
- ^{2}H$_9$ choline   (Cambridge Isotope Laboratory,Andover, MA, DLM-549)
- ^{13}C$_4$ 3-hydroxybutyrate   (Cambridge Isotope Laboratory, Andover, MA, CLM-3853)
- ^{13}C$_6$^{15}N$_2$ cystine (Cambridge Isotope Laboratory, Andover, MA, CNLM4244)
- ^{13}C$_3$ lactate   (Sigma Aldrich, Darmstadt, Germany, 485926)
- ^{13}C$_6$ glucose   (Cambridge Isotope Laboratory, Andover, MA, CLM-1396)
- ^{13}C$_3$ serine   (Cam-bridge Isotope Laboratory, Andover, MA, CLM-1574)
- ^{13}C$_2$ glycine   (Cambridge Isotope Laboratory, Andover, MA, CLM-1017)
- ^{13}C$_5$ hypoxanthine   (Cambridge Isotope Laboratory, Andover, MA, CLM8042)
- ^{13}C$_2$15N taurine   (Cambridge Isotope Laboratory, Andover, MA, CNLM-10253)
- ^{13}C$_3$ glycerol   (Cambridge Isotope Laboratory, Andover, MA, CLM-1510)
- ^{2}H$_3$ creatinine   (Cambridge Isotope Laboratory, Andover, MA, DLM-3653)

655

656 Samples in extraction mix were vortexed for 10 min at 4°C and centrifugated at 15,000x rpm for

657 10 min at 4°C to pellet insoluble material. 20µL of the soluble polar metabolite supernatant was

658 moved to sample vials for analysis by LC-MS as previously described (Sullivan et al., 2019b,

659 2019a).

660

661 Once LC-MS analysis was performed, XCalibur 2.2 software (Thermo Fisher Scientific) was used

662 for metabolite identification. External standard libraries were used to confirm the m/z and retention

663 time for each metabolite. For quantitative analysis, when internal standards were available,

664 external standard libraries were used to quantitate concentrations of stable isotope labeled

665 internal standards in the extraction mix. Once internal standard concentrations were obtained,

666 peak area of an unlabeled metabolite in in the media samples was compared with the peak area

667 of the quantified internal standard to determine the concentration of the unlabeled metabolite.

668

669 For metabolites for which an internal standard was not present in the extraction mix, external

670 standard libraries were used to perform analysis of relevant metabolite concentrations. Briefly,

671 the peak area of the metabolite was normalized to the peak area of a labelled stable isotope

672 internal standard with the closest elution time, both in media samples and external standard library

673 dilutions. Using the external standard library dilutions, we created a standard curve based on the

674 linear relationship of the normalized peak area and the concentration of the metabolite, excluding

675 those metabolites with an $r^2 < 0.95$. This standard curve was then used to interpolate the

676 concentration of the metabolite in the media sample.

677

678 **Cell Isolation from tumors**

679 Murine cancer cell lines were derived from tumor bearing C57Bl6J *LSL-Kras$^{G12D/+}$; Trp53$^{flox/flox}$;*

680 *LSL-YFP; Pdx1-Cre* (KP$^{-/-}$CY) mice to allow for fluorescent lineage tracing and isolation of cancer

681 cells (Li et al., 2018). To isolate cancer cells from these tumors, the tumors were chopped finely

682 and digested with 30mg/mL dipase II (Roche 28405100), 10mg/mL collagenase I (Worthington

683 LS004194) and 10mg/mL DNase by constant rotation at 37C for 30 min. Digestion was quenched

684 with 0.5M Ethylenediaminetetraacetic acid (EDTA) and cells were passed through a 70µM filter

685 and rinsed with PBS before platting in RPMI-1640 (Corning 50–020-PC). YFP+ Cancer cells from

686 each tumor were sorted twice on a BD FACSAria II Cell Sorter.

687

688 **Cell lines and cell culture**

689 Use of cancer cell lines was approved by the Institutional Biosafety Committee (IBC no. 1560).

690 All cell lines were tested quarterly for mycoplasma using the MycoAlert Mycoplasma Detection

691 Kit (Lonza LT07-318). All cells were cultured in Heracell vios 160i incubators (Thermofisher) at

692 37°C and 5% CO2. Cell lines were routinely maintained in RPMI-1640 or TIFM supplemented

693 with 10% diaFBS (Gibco, #26400-044, Lot#2244935P).

694

695 All cell culture was performed in static culture conditions. TIFM contains substantially lower levels

696 of nutrients than most standard media formulations. Therefore, to ensure that there was not

697 nutrient deprivation in static cultures the following modifications to standard tissue culture

698 practices were made. Cells were cultured in larger volumes of media (8mL/35mm diameter well)

699 to prevent depletion of nutrients during the culture. Additionally, media were replaced every 24

700 hrs. We routinely measured concentration of the most rapidly consumed nutrient, glucose, using

701 a GlucCell glucometer (Bechard et al., 2020) to ensure that cultures used in experiments did not

702 experience a greater than 30% drop in glucose availability, which is within the range of KP$^{-/-}$C IF

703 glucose concentration measurements (Sullivan et al., 2019a). Lastly, passaging TIFM maintained

704 cells using standard trypsin (0.025%)/EDTA solution to detach cells leads to loss of viability upon

705 replating of cells. Therefore, cells were detached with a 1:1 mixture of 0.5% trypsin-EDTA

706 (Thermofisher) and serum free RPMI-1640 media (Thermofisher). This allowed routine passaging

707   and plating of cells with less loss of viability. These modifications were followed for both TIFM and

708   RPMI cultured cells.

709

710   **Determining cellular proliferation rate**

711   Quantification of cellular proliferation rate was performed by sulforhodamine B (SRB) assay as

712   described (Lien et al., 2021). Briefly, 10,000-15,000 cells were plated in 12-well plates in triplicates

713   for each condition and allowed to attach overnight. After attachment, one set of triplicate wells

714   were fixed by adding 10% trichloroacetic acid (TCA) to the media and incubating plates in 4°C to

715   provide an 'initial day' value. Media was changed on remaining cultures and were allowed to grow

716   for the indicated number of days. At the end of the growth period, cells were fixed by adding 10%

717   trichloroacetic acid (TCA) to the media and incubating plates in 4°C for at least one hour. All wells,

718   including the initial day wells were washed with deionized water, air-dried at room temperature,

719   and dyed with SRB in 1% acetic acid for 30 min. After, cells were washed with 1% acetic acid

720   three times and dried at 30°C for 15 minutes. SRB dye was solubilized with 10mM Tris pH 10.5

721   by gentle horizontal shaking for 5 min. Absorbance (abs) was measured at 510 nm in a clear 96-

722   well plate using a BioTek Cytation 1 Cell Imaging Multi-Mode Reader. After all measurements

723   were normalized to an averaged blank measurement (wells without cells but with media), growth

724   rate was calculated using the following equation:

725

726   Doublings/day = $\log_2$(Final Day Abs_{510}/Initial Day Abs_{510}) / number of days elapsed in culture

727   period

728

729   **Consumption/Release (Co/Re) analysis**

730   Measurement of cellular consumption and release of metabolites was performed according to

731   previous publications (Hosios et al., 2016; Jain et al., 2012; Muir et al., 2017). For the experimental

732   set up, 100,000-150,000 cells were seeded in 2mL of culture medium in six-well plates with 3

733   technical replicates per condition per time point and allowed to attach overnight. The following

734   day (day 1), cells were washed twice with 2mL PBS. They were then given 2mL of media, either

735   TIFM or RPMI. An unspent media sample was collected at this time as well and stored at -80 °C.

736   Cell number on day 1 was measured using a Vi-CELL XR Cell Viability Analyzer (Beckman

737   Coulter). 24h later (day 2), 1mL of spent media from cells was collected, centrifuged and stored

738   at -80 °C. Cell number was counted again. Quantification of metabolite levels in unspent (day 1)

739   and day 2 cell culture media samples was performed as described in **Quantification of**

740   **metabolite levels in cell culture media**.

741

742   To calculate Co/Re rates of a given metabolite, cell numbers on day 1 and day 2 were used to fit

743   an exponential growth function, which integrated yielded the number of (cell · days) the day 2

744   media was conditioned. Changes in nutrient concentration in cultures were then normalized to

745   this integrated growth curve to yield metabolite Co/Re per cell per unit of time.

746

747   **Experimental set up for consumption of arginine by GC-MS analysis**

748   Cells were plated as described for consumption/release (Co/Re) analysis as described in

749   **Consumption/Release (Co/Re) analysis**. The following day, cells were changed into either TIFM

750   or TIFM without citrulline and ornithine. Both media were supplemented with 20µM extracellular

751   arginine. Day 1 and day 2 media samples were collected and cell numbers were measured as in

752   **Consumption/Release (Co/Re) analysis**.

753

754   10µL of each media sample were mixed 10µL of water containing $^{13}C_6, ^{15}N_4$ arginine at 20µM and

755   600µL cold HPLC grade methanol. The solution was then vortexed for 10 min, and centrifuged at

756   21,000xg for 10 min. Finally, 450µL of each extract was aliquoted, dried under nitrogen gas and

757   stored at -80°C before further analysis. Sample derivatization GC-MS was then used to measure

758 the arginine concentration in each media sample as described below in **GC-MS analysis of**

759 **arginine**.

760

761 **RNA extraction, library preparation and transcriptomic analyses**

762 *Isolation of cultured and tumor cancer cell samples*

763 mPDAC3-TIFM cells were plated at 200,000 (TIFM) to 350,000 (RPMI) cells per 6cm plate in

764 triplicate cultures. RNA was extracted from cells 24 hrs later when the cells were proliferating

765 exponentially, the cells were trypsinized and isolated by fluorescence activated cell sorting

766 (FACS) for RNA extraction. For the *in vivo*, condition cells were isolated by FACS from end stage

767 orthotopic mPDAC3-TIFM tumors, as described in **Cell Isolation from tumors**.

768

769 *RNA extraction*

770 Cells from all conditions were sorted by FACS prior to RNA extraction to eliminate the FACS

771 sorting process as a confounder between cultured mPDAC3-TIFM cells and those isolated from

772 orthotopic tumors. For FACS sorting, cells were stained with DAPI (750 ng/mL) to separate

773 dead/dying cells from live cells, and live YFP+/DAPI- cells were sorted with a BD FACSAria II Cell

774 Sorter with a 100μm nozzle directly into Qiagen RLT RNA extraction buffer. The ratio of RNA

775 extraction buffer to sorted cellular volume was kept at 100μL of sorted sample per 350μL of RNA

776 extraction buffer. Total messenger RNA (mRNA) was extracted using the RNeasy Micro Kit

777 (Qiagen #74004) and RNA quality and quantity was assessed using the 2100 Bioanalyzer System

778 (Agilent).

779

780 *Library preparation and sequencing*

781 Strand-specific RNA-SEQ libraries were prepared using an TruSEQ mRNA RNA-SEQ library

782 protocol (Illumina). Library quality and quantity was assessed using the Agilent bio-analyzer and

783 libraries were sequenced using an Illumina NovaSEQ6000.

784

785   *Transcriptomic analyses*

786   Data processing and analysis was done using the R-based Galaxy platform

787   (https://usegalaxy.org/) (Afgan et al., 2018). Quality control was performed prior and after

788   concatenation of the raw data with the tools *MultiQC* and *FastQC* respectively. All samples

789   passed the quality check with most showing ~20% sequence duplication, sequence alignment

790   greater or equal to 80%, below and below 50% GC coverage, all of which is acceptable and/or

791   indicative of good quality for RNASeq samples (Dündar et al., 2015; Parekh et al., 2016). Samples

792   were then aligned, and counts were generated using the tools *HISAT2* (Galaxy Version

793   2.2.1+galaxy0, NCBI genome build GRCm38/mm10) and *featureCounts* (Galaxy Version

794   2.0.1+galaxy1), respectively. Differential expression analyses were performed with *limma* (Galaxy

795   Version 3.48.0+galaxy1) and Genome Set Enrichment Analysis (GSEA) with *fgsea* (Galaxy

796   Version 1.8.0+galaxy1) or GSEAPreranked (v6.0.12, https://gsea-

797   msigdb.github.io/gseapreranked-gpmodule/v6/index.html) (Jain et al., 2012; Reich et al., 2006;

798   Subramanian et al., 2005). GSEA plots were generated as previously described (Morris et al.,

799   2019).

800

801   **Immunoblot analysis**

802   For immunoblotting analysis, cells growing in log phase in a 6 well dish were washed with 2mL of

803   PBS and lysed in 100μL RIPA buffer [25 mM Tris-Cl, 150 mM NaCl, 0.5% sodium deoxycholate,

804   1% Triton X-100, 1x cOmplete protease inhibitor (Roche)]. Cells were scraped and the resulting

805   lysate was clarified by centrifugation at 21,000xg for 10 min. Protein concentration of the lysate

806   was determined by BCA assay (Thermofisher). Proteins (20–30μg) were resolved on SDS-PAGE,

807   4 to 12% Bis-Tris Gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane using

808   the iBlot 2 Dry Blotting System (Invitrogen). Membrane was blocked with Intercept Blocking Buffer

809   (Li-cor) at room temperature for 2h, stained with primary and secondary antibodies and then

810   visualized using a LI-COR imager with Image Studio software version 2.1.10.

811

812   The following primary antibodies were used: Ass1 (Atlas HPA020896; 1:200 dilution), Vinculin

813   (Proteintech 66305-1-Ig; 1:10000 dilution), Beta-Actin (Proteintech 660009-1-Ig; 1:10000 dilution).

814   The following secondary antibodies: IRDye 680LT Goat Anti-Mouse Ig (Li-cor G926-68020;

815   1:10000 dilution) IRDye 800CW Goat anti-Rabbit IgG (Li-cor 926-32211; 1:10000 dilution), IRDye

816   800CW Goat anti-Mouse IgG (Li-cor 926-32210; 1:10000 dilution)

817

818   **qRT-PCR analysis**

819   RNA was extracted using the RNeasy Mini Kit and optional on-the-column DNAse digestion

820   (Qiagen). Extracted RNA was converted to cDNA by reverse transcription using the High-Capacity

821   cDNA Reverse Transcription Kit (Applied Biosystems). Expression levels of *Sdc1* transcript were

822   amplified using PowerUp SYBR Green Master Mix (Invitrogen) and custom primers

823   (Supplementary File 2). Quantification was performed using a QuantStudio 3 Real-Time PCR

824   System (Applied Biosystems). The average change in threshold cycle (ΔCt) values was

825   determined for each of the samples relative to *Gapdh* levels and compared with vehicle control

826   (ΔΔCt). Finally relative gene expression was calculated as ($2^{-\Delta\Delta Ct}$). Experiments were performed

827   in with triplicate cultures and RNA extraction.

828

829   **GC-MS analysis of arginine**

830   Dry polar metabolites extracts from intracellular extracts or media samples were derivatized with

831   16μL MOX reagent (ThermoFisher) for 1h at 37°C and then with 20μL 1% tert-

832   butyldimethylchlorosilane in N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (Sigma Aldrich)

833   for 3h at 60°C. Derivatized samples were analyzed with an 8890 gas chromatograph system

834   (Agilent Technologies) with a HP-5ms Ultra Inert column (Agilent Technologies) coupled with an

835   5997B Mass Selective Detector (MSD) mass spectrometer (Agilent Technologies). Helium was

836   used as the carrier gas at a flow rate of 1.2 mL/min. One microliter of sample was injected in

837   splitless mode at 280°C. After injection, the GC oven was held at 100°C for 1 min. and increased

838   to 300°C at 3.5 °C/min. The oven was then ramped to 320°C at 20 °C/min. and held for 5 min. at

839   this 320°C. The MS system operated under electron impact ionization at 70 eV and the MS source

840   was held at 230 °C and quadrupole at 150 °C. Detector was set in scanning mode with a scanned

841   ion range of 100–650 m/z. Mass isotopomer distribution was determined using ion fragments

842   integration for each individual metabolite (Lewis et al., 2014) and natural abundance correction

843   was performed using IsoCor (Millard et al., 2019).

844

845   **Isotopic labeling experiments in cell culture and intracellular metabolite extraction**

846   To measure steady state labeling of polar metabolites by citrulline in cultured cells, triplicate

847   cultures of 150,000 cells/well were seeded in a 6 well dish in 2 mL of medium. Cells were allowed

848   to attach overnight. The following day cells were washed twice with PBS and then incubated with

849   8mL for 8 or 24h in TIFM with $^{13}C_5$-citrulline (Cambridge Isotope Laboratories, CLM-8653) added

850   at TIFM concentrations. Immediately after the labeling period, cells were quickly washed with

851   ~8mL of ice-cold blood bank saline. Cellular metabolites were extracted with addition of 600μL of

852   an ice-cold methanol followed by scraping the cells on ice. The solution was then vortexed for 10

853   min, and centrifuged at 21,000xg for 10 min. 450μL of each extract was aliquoted to fresh sample

854   tubes, dried under nitrogen gas and stored at -80°C before further analysis.

855

856   **CRISPR knockout and re-expression of *Ass1***

857   sgRNAs targeting *Ass1* (Supplementary File 2) were generated through the Broad Institute's

858   Genetic Perturbation Platform Web Portal (https://portals.broadinstitute.org/gpp/public/).

859   Oligonucleotide pairs were manufactured by Integrated DNA Technologies (IDT) and cloned into

860   lentiCRISPRv2 (Addgene: #52961) as previously described (Sanjana et al., 2014; Shalem et al.,

861   2014). HEK293T cells (Dharmacon) were transfected with the *Ass1* targeting lentiCRISPRv2

862   vectors and the lentiviral packing plasmids psPAX2 (Addgene: #12260) and pMD2.G (Addgene:

863   #12259). Medium was replaced after 24h, and lentivirus was harvested after 48h. Subconfluent

864   mPDAC3-TIFM cells were infected with lentivirus using 8µg/mL polybrene and infected cells were

865   selected in 2µg/mL puromycin and maintained with 100µM arginine. Single cell clones with

866   immunoblot-confirmed loss of Ass1 were selected. A single cell clone without detectable Ass1

867   expression was transformed with a lentivirus produced as above with a vector encoding CMV-

868   driven murine *Ass1* cDNA that would not be targeted by the *Ass1* sgRNA (VectorBuilder).

869

870   **shRNA knockdown of *Sdc1***

871   Hairpin sequences targeting *Sdc1* were obtained from (Fellmann et al., 2013). Oligonucleotide

872   pairs were manufactured by IDT and cloned into a lentiviral LT3GEPIR vector (Addgene:

873   #111177) to allow for doxycycline-inducible repression of gene expression. Lentiviral transfection

874   and transformation were performed as described in **CRISPR knockout and re-expression of**

875   ***Ass1*** and successfully transformed cells were selected and maintained with 2µg/mL puromycin.

876   Cells transformed with LT3GEPIR without an shRNA were used as a control.

877

878   **Analysis macropinocytic capacity by DQ-BSA**

879   The macropinocytic capacity of PDAC cells was assessed using a DQ Red BSA (Invitrogen)

880   uptake assay. Cells were seeded at either 15,000 cells/well for 12 wells or 50,000 cells/well for 6

881   wells and allowed to attach over night. The following day the media was replaced with fresh media

882   + 0.02mg/mL of the DQ Red BSA fluorogenic substrate and cells were harvested at different

883   timepoints for up to 6 hours. Cells were then washed with PBS, trypsinized, washed again with

884   PBS, fixed in 4% paraformaldehyde for 15 minutes at 4°C and DQ Red BSA fluorescence was

885   quantified by flow cytometry in at least 10,000 cells per sample.

886

Animal Experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC, Protocol #72587) and performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD). Mice were housed in a pathogen-free animal facility at the University of Chicago with a 12 h light/12 h dark cycle, 30–70% humidity and 68–74°F temperatures maintained.

Orthotopic tumor implantation and monitoring

C57BL6J mice 8-12 weeks of age were purchased from Jackson Laboratories. 250,000 cells/tumor were resuspended in 20µL of 5.6mg/mL Cultrex Reduced Growth Factor Basement Membrane Extract (RGF BME; R&D Biosystems #3433-010-01) and serum-free RPMI (SF-RPMI) solution. The BME:cellular mixture was injected into the splenic lobe of the pancreas of the mice as previously described (Erstad et al., 2018). After implantation mice, were monitored daily by abdominal palpation.

In vivo Arg1 knockout

LysM-Cre and $Arg1^{fl/fl}$ C57BL6J mice were bred to generate LysM-Cre $^{+/+}$; $Arg1^{fl/fl}$ and litter mate control $Arg1^{fl/fl}$ mice. All mice were genotyped using primes described in Supplementary File 2. Animal husbandry was carried out in strict accordance with the University of Chicago Animal Resource Center guidelines. Tumor implantation as described above was performed in mice at 8-12 weeks of age.

In vivo arginase-1 pharmacological inhibition

Orthotopic tumors were implanted in C57BL6J mice at 8-12 weeks of age. 4 weeks after induction, once tumors were close end stage disease, CB-1158 (MedChem Express) dissolved in sterile water was administered by oral gavage at 100mg/kg as previously described (Steggerda et al.,

913   2017). The acidity caused by the HCl in the drug solution was neutralized by adding equivalent

914   amount of NaOH and an equivalent NaCl in sterile water solution was prepared as vehicle. 2hrs

915   after treatment with CB-1158 or vehicle, mice were euthanized by cervical dislocation, and tumors

916   were harvested for TIF extraction.

917

918   *In vivo $^{15}N_2$-glutamine tracing*

919   Orthotopic tumors were implanted in C57BL6J mice at 8-12 weeks of age. 4 weeks after induction

920   tumor-bearing mice and healthy littermate controls were treated with $^{15}N_2$-glutamine (Cambridge

921   Isotope Laboratory #NLM-1328-PK) dissolved in sterile phosphate buffered saline at

922   7.2mg/animal by tail vein injection as previously described (Lane et al., 2015). Briefly, animals

923   were dosed three times at 15-minute intervals. 15 minutes after the final dose, ~100uL of blood

924   were be obtained by submandibular sampling as described previously (Parasuraman et al., 2010)

925   and animals were euthanized. The tumor or pancreas from each animal was then harvested and

926   immediately snap frozen using a BioSqueezer (BioSpec) cooled with liquid nitrogen and stored at

927   -80°F until further analysis.

928

929   *IF isolation from PDAC tumors*

930   IF was isolated from tumors as described before (Sullivan et al., 2019a). Briefly, tumors were

931   rapidly dissected after euthanizing animals. Tumors were weighed and rinsed in blood bank saline

932   solution (150 mM NaCl) and blotted on filter paper (VWR, Radnor, PA, 28298–020). The process

933   of dissection and tumor preparation took < 3min. Tumors were cut in half and put onto 20µm nylon

934   mesh filters (Spectrum Labs, Waltham, MA, 148134) on top of 50 mL conical tubes, and

935   centrifuged for 10min. at 4°C at 400xg. IF was then collected, snap-frozen in liquid nitrogen and

936   stored at -80°C until further analysis.

937

938   **HPLC-MS-MS analysis amino acid levels in PDAC IF samples upon arginase inhibition**

939   IF samples were analyzed by High-Performance Liquid Chromatography and Tandem Mass

940   Spectrometry (HPLC-MS-MS). Specifically, the system consisted of a Thermo Q-Exactive in line

941   with an electrospray source and an Ultimate3000 (Thermo) series HPLC consisting of a binary

942   pump, degasser, and auto-sampler outfitted with a Xbridge Amide column (Waters; dimensions

943   of 3.0 mm × 100 mm and a 3.5 μm particle size). The mobile phase A contained 95% (vol/vol)

944   water, 5% (vol/vol) acetonitrile, 10 mM ammonium hydroxide, 10 mM ammonium acetate, pH =

945   9.0; B was 100% Acetonitrile. The gradient was as following: 0 min, 15% A; 2.5 min, 64% A; 12.4

946   min, 40% A; 12.5 min, 30% A; 12.5-14 min, 30% A; 14-21 min, 15% A with a flow rate of 150

947   μL/min. The capillary of the ESI source was set to 275 °C, with sheath gas at 35 arbitrary units,

948   auxiliary gas at 5 arbitrary units and the spray voltage at 4.0 kV. In positive/negative polarity

949   switching mode, an m/z scan range from 60 to 900 was chosen and MS1 data was collected at a

950   resolution of 70,000. The automatic gain control (AGC) target was set at $1 × 10^6$ and the maximum

951   injection time was 200 ms. The targeted ions were subsequently fragmented, using the higher

952   energy collisional dissociation (HCD) cell set to 30% normalized collision energy in MS2 at a

953   resolution power of 17,500. Besides matching m/z, target metabolites are identified by matching

954   either retention time with analytical standards and/or MS2 fragmentation pattern. Data acquisition

955   and analysis were carried out by Xcalibur 4.1 software and Tracefinder 4.1 software, respectively

956   (both from Thermo Fisher Scientific).

957

958   **Measurement of intratumoral metabolite levels and metabolite isotope labeling patterns**

959   Cryogenically frozen tumor pieces were ground to a fine homogenous powder with a liquid

960   nitrogen cooled mortar and pestle. ~30mg of tissue powder was weighed into sample tubes, and

961   metabolites were extracted with 600μL HPLC grade methanol, 300μL HPLC grade water, and

962   400μL chloroform. Samples were vortexed for 10min at 4°C, centrifuged 21,000xg at 4°C for 10

963   min. 400μL of the aqueous top layer was removed into a new tube and dried under nitrogen. Dried

964   tumor extracts were resuspended in 100μL HPLC grade water and LC-MS analysis was

965   performed as described before(Sullivan et al., 2019b, 2019a). XCalibur 2.2 software (Thermo

966   Fisher Scientific) was used identification and relative quantification for metabolites. Natural

967   abundance correction was performed using the IsoCor (Millard et al., 2019).

968

969   **Measuring intratumor and IF concentrations of amino acids**

970   To quantitatively measure IF amino acid abundance, polar metabolites were extracted from 5μL

971   IF samples using 45μL 75:25:0.1 HPLC grade acetonitrile:methanol:formic acid extraction mix

972   into which a mixture of isotopically labeled amino acids of known concentrations (Cambridge

973   Isotope Laboratories, MSK-A2-1.2) was added. Samples were vortexed for 10 min, and

974   centrifuged at maximum speed for 10 min. 30 μL of each extract was removed and dried under

975   nitrogen gas and stored −80°C until further analysis by LC-MS as described in **Quantification of**

976   **metabolite levels in cell culture media**. Amino acids amounts in IF samples were determined

977   by comparison of peak areas of unlabeled amino acids with peak areas of labeled amino acids

978   that were present at known amounts. Concentrations were determined by dividing the amino acid

979   amount by the 5μL volume of the IF sample.

980

981   To measure amino acid amounts in intratumoral samples, intratumoral metabolites were extracted

982   from ~30mg and dried down in **Measurement of intratumoral metabolite levels and metabolite**

983   **isotope labeling patterns**. Dried samples were rehydrated with 2:1 methanol:water into which

984   a mixture of isotopically labeled amino acids of known concentrations (Cambridge Isotope

985   Laboratories, MSK-A2-1.2) was added. Samples were then analyzed by LC-MS as described in

986   **Quantification of metabolite levels in cell culture media**. Amino acid amounts per tumor

987   weight were determined by comparison of peak areas of unlabeled amino acids with peak areas

988   of labeled amino acids that were present at known amounts and dividing by the mass of tumor

989   extracted.

990

991   To compare metabolite concentrations between tumor and TIF samples, the density for orthotopic

992   mPDAC tumors was needed to convert amino acid amount per unit tumor mass into a

993   concentration (amino acid amount per unit volume). The density of freshly isolated mPDAC3-

994   RPMI tumors was measured by measuring tumor mass and calculating the volume (V) of the

995   tumors with the following formula:

996    $V = 4/3 * \pi * A * B * C$

997   where A, B, C are the lengths of the semi-axes of an ellipsoidal shape, which were measured

998   from tumors with an electronic caliper (Thermofisher). Tumor density was then calculated by

999   dividing the tumor mass by the calculated volume. Tumor density was then used to convert amino

1000   acid amount per tumor mass measurements into an intratumoral concentration.

1001

1002   **Human samples regulation**

1003   Human histology samples were obtained under approval by the Institutional Review Boards at the

1004   University of Chicago (IRB 17-0437).

1005

1006   **Immunohistochemistry**

1007   For ARG1 and ASS1 staining, the slides were stained using Leica Bond RX automatic stainer.

1008   Dewax (AR9222, Leica Microsystems) and rehydration procedure were performed in the system

1009   and a 20 min treatment of epitope retrieval solution I (Leica Biosystems, AR9961) was applied.

1010   anti-Arginase-1 (1:100, Cell Signaling #93668) or anti-Ass1 (1:100, Atlas HPA020896;) and were

1011   applied on tissue sections for 60min. Antigen-antibody binding was detected using Bond polymer

1012   refine detection (Leica Biosystems, DS9800). The tissue sections were counter stained with

1013   hematoxylin and covered with cover glasses.

1014

1015   For F4/80 staining, tissue sections were deparaffinized and rehydrated with xylenes and serial

1016   dilutions of EtOH to deionized water. They were incubated in antigen retrieval buffer (DAKO,

1017   S1699) and heated in steamer at 97°C for 20 minutes. Anti-mouse F4/80 antibody (1:200,

1018   MCA497GA, AbD Serotec) was applied on tissue sections for 1hr at room temperature. Tissue

1019   sections were washed with Tris buffered saline and then incubated with biotinylated anti-rat IgG

1020   (10 µg/ml, BA-4001, Vector laboratories) for 30 min at room temperature. Antigen-antibody

1021   binding was detected by Elite kit (PK-6100, Vector Laboratories) and DAB (DAKO, K3468)

1022   system.

1023

Acknowledgements

1050

Competing interests

1052   No competing interests declared

1053

Data availability

1055   Sequencing data from Figures 1 and 3 have been deposited in GEO under accession code

1056   GSE199163: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199163. Source data

1057   files with measured metabolite concentrations and isotopic labeling patterns are provided for

1058   Figures 2 and 4 and we are in the process of depositing raw mass spectra from these

1059   experiments in Metabolomics Workbench. Prior to upload, this data is available upon request.

1060

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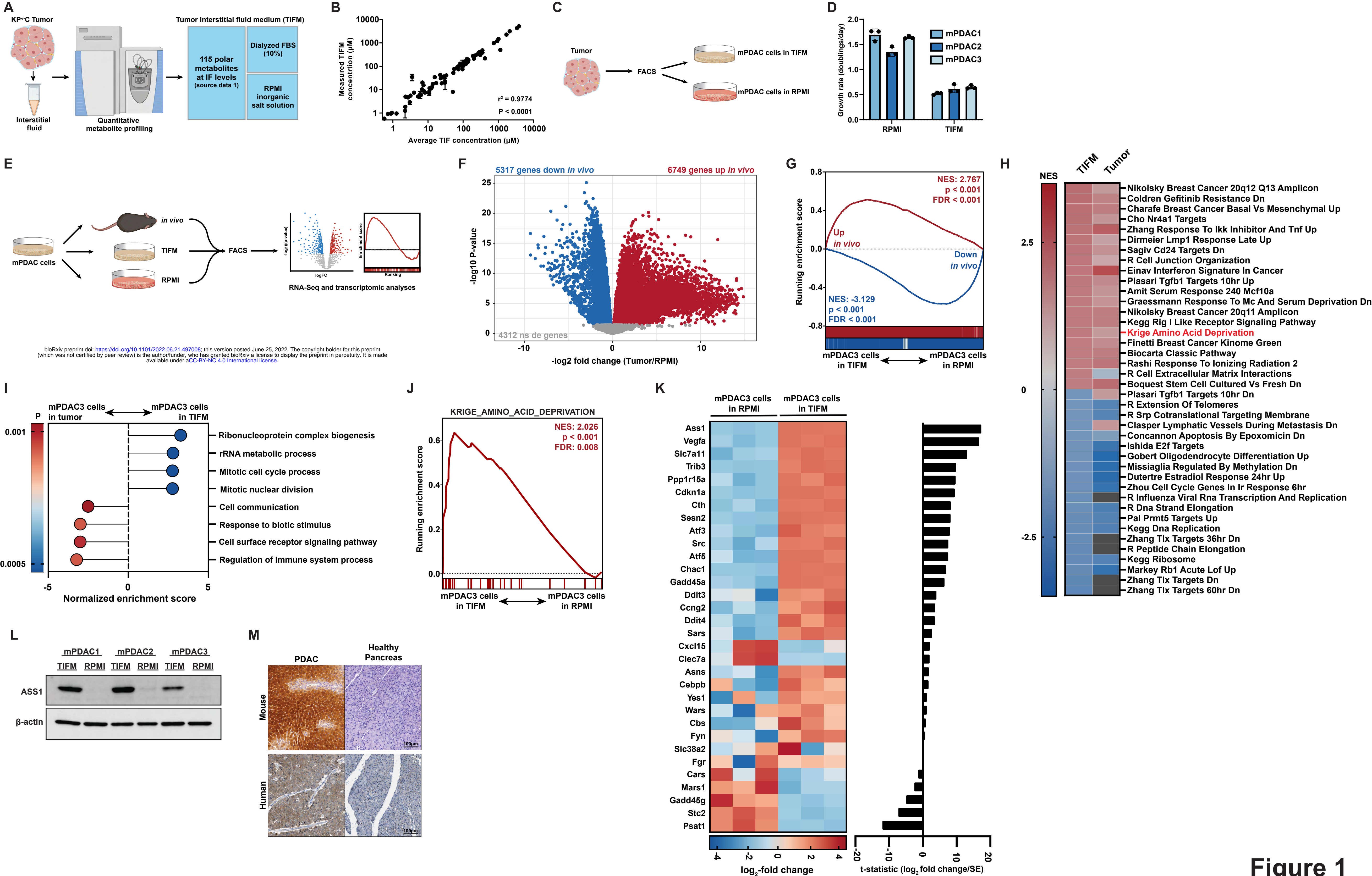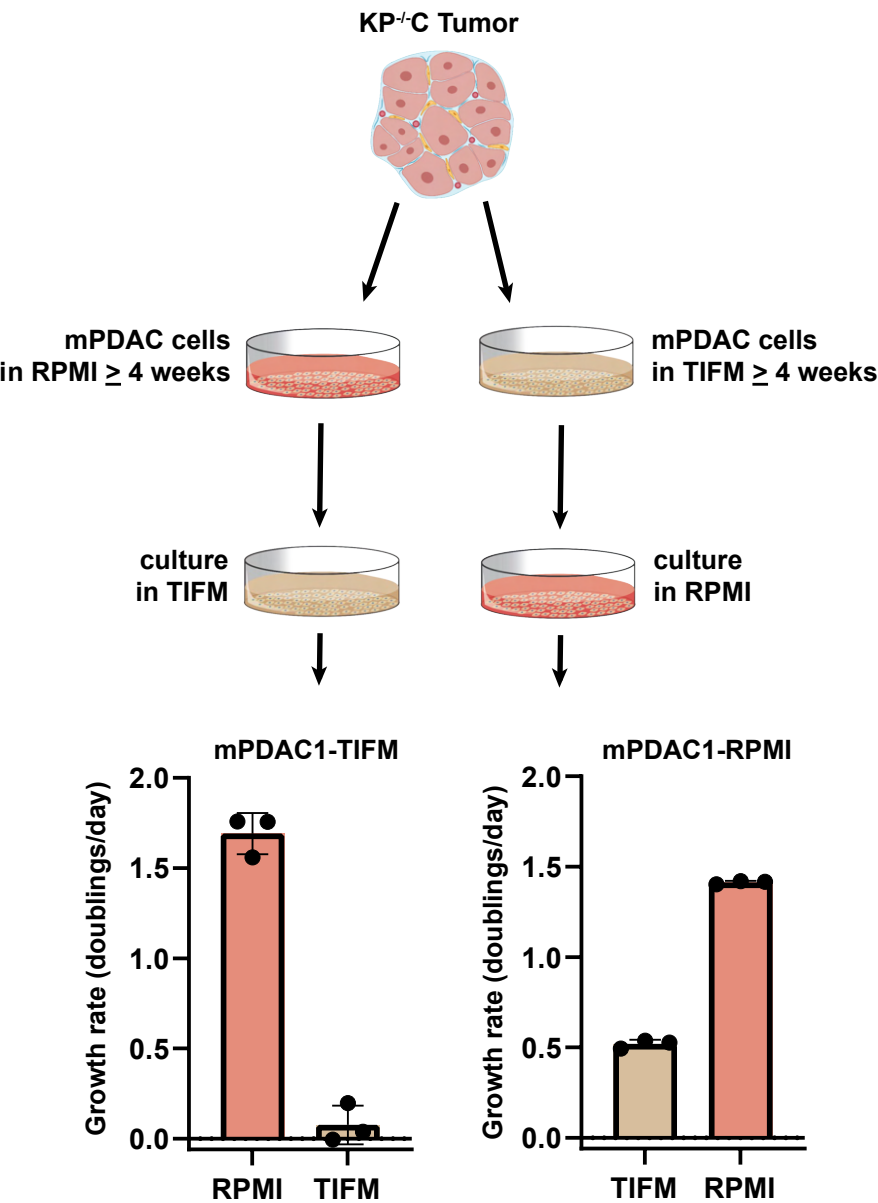1336 Immunity *47*, 597. .
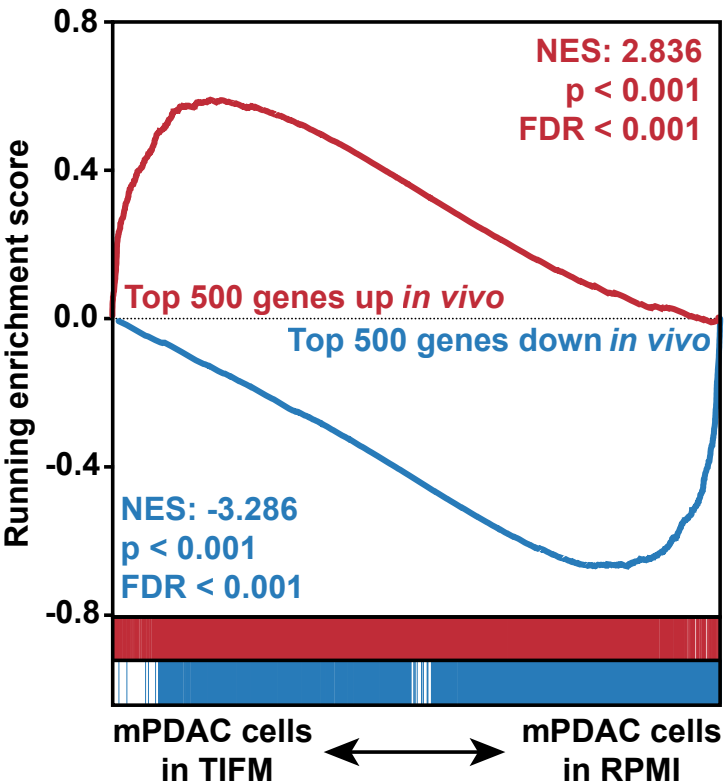
Figure 1

Figure 1 Supplement 1

NES: 2.836
p < 0.001
FDR < 0.001

Top 500 genes up *in vivo*

Top 500 genes down *in vivo*

NES: -3.286
p < 0.001
FDR < 0.001

mPDAC cells in TIFM

mPDAC cells in RPMI
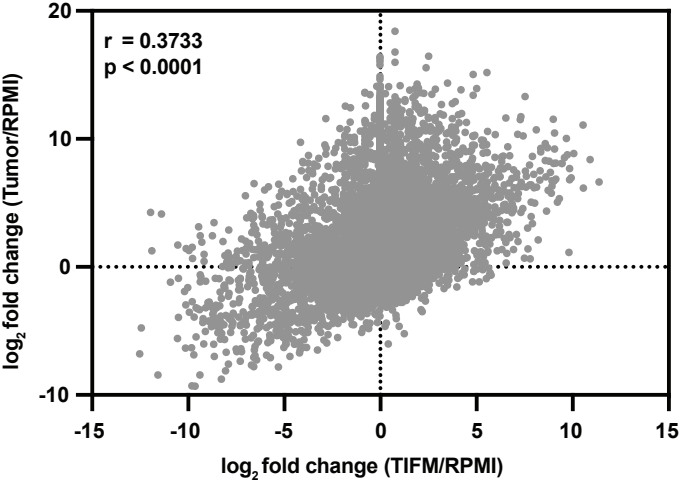
Figure 1 Supplement 2

r = 0.3733
p < 0.0001

Figure 1 Supplement 3
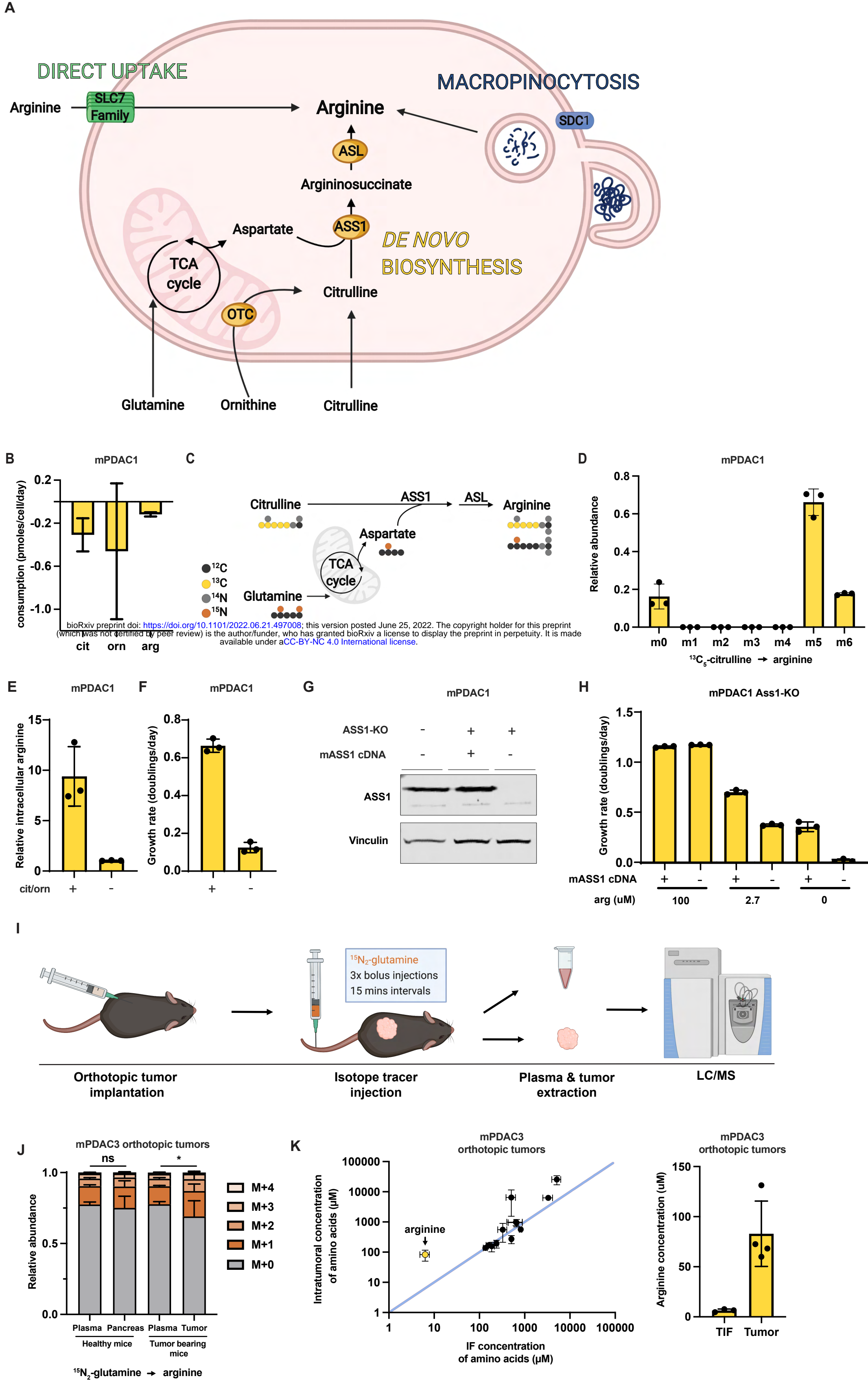
Figure 2
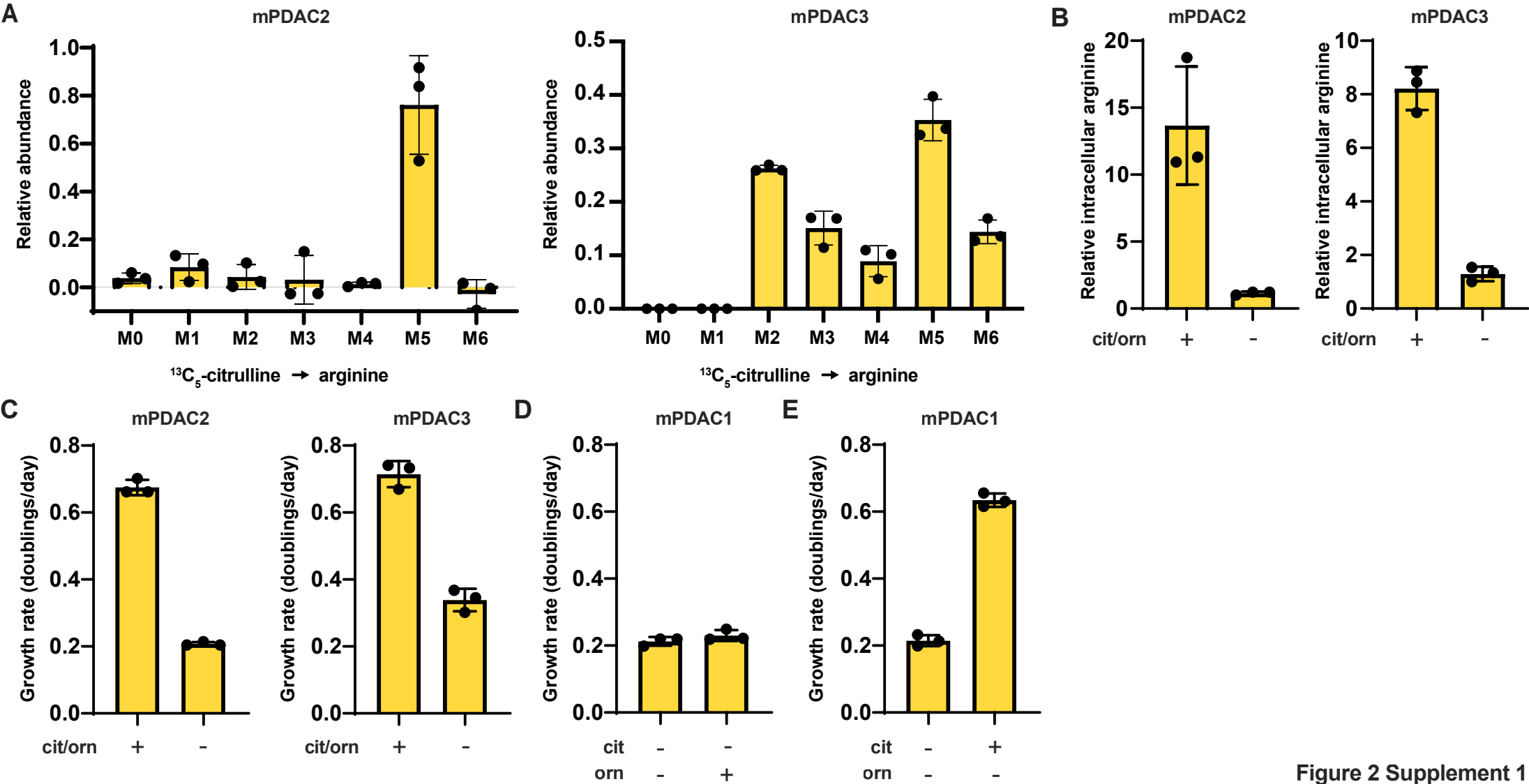
Figure 2 Supplement 1

Figure 3

Figure 3 Supplement 1

Figure 4