

1 **Title**

2  
3 Pancreatic tumors activate arginine biosynthesis to adapt to myeloid-driven amino acid stress

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5 **Running Title**

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7 Adaptive arginine biosynthesis in PDAC

8  
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20  
21 **Abstract**

22  
23 Nutrient stress in the tumor microenvironment requires cancer cells to adopt adaptive metabolic  
24 programs to maintain survival and proliferation. Therefore, knowledge of microenvironmental  
25 nutrient levels and how cancer cells cope with such nutrition is critical to understand the  
26 metabolism underpinning cancer cell biology. Previously, we performed quantitative  
27 metabolomics of the interstitial fluid (the local perfusate) of murine pancreatic ductal  
28 adenocarcinoma (PDAC) tumors to comprehensively characterize nutrient availability in the  
29 microenvironment of these tumors (Sullivan et al., 2019a). Here, we develop Tumor Interstitial  
30 Fluid Medium (TIFM), a cell culture medium that contains nutrient levels representative of the  
31 PDAC microenvironment, enabling study of PDAC metabolism under physiological nutrition. We  
32 show that PDAC cells cultured in TIFM, compared to standard laboratory models, adopt a cellular  
33 state more similar to PDAC cells in tumors. Further, using the TIFM model we identified arginine  
34 biosynthesis as a metabolic adaptation PDAC cells engage to cope with microenvironmental  
35 arginine starvation driven by myeloid cells in PDAC tumors. Altogether, these data show that  
36 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 Tumor Interstitial Fluid Medium (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<sup>G12D/+</sup>; Trp53<sup>fllox/fllox</sup>; Pdx-1-Cre (KP<sup>-/-</sup>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<sup>-/-</sup>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 were 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 2-5 $\mu$ M relative to 125 $\mu$ M 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  $^{13}\text{C}_5$ -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  $^{15}\text{N}_2$ -glutamine stable isotope tracing by multiple bolus intravenous injections of  $^{15}\text{N}_2$ -  
237 glutamine into mPDAC orthotopic tumor bearing mice and non-tumor bearing controls (Fig. 2I).  
238  $^{15}\text{N}_2$ -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  $^{15}\text{N}$  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%  $^{15}\text{N}_1$ -arginine and ~7%  $^{15}\text{N}_2$ -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%  $^{15}\text{N}_1$ -arginine and ~9%  $^{15}\text{N}_2$ -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  $^{15}\text{N}$  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 (~2.3 $\mu$ M) 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

### 314 **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<sup>+/-</sup>; Arg1<sup>fl/fl</sup>*)  
325 (Clausen et al., 1999; El Kasmi et al., 2008) and control animals (*Arg1<sup>fl/fl</sup>*). 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<sup>+/-</sup>; Arg1<sup>fl/fl</sup>* 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<sup>+/-</sup>; Arg1<sup>fl/fl</sup>* 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-CEBP $\beta$  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 2-5 $\mu$ M (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

## 426 **Stromal control of microenvironmental nutrient conditions**

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

446

## 447 **Figure Legends**

448 **Figure 1. Culturing PDAC cells in physiological nutrients levels better recapitulates the**  
449 **transcriptomic profile of cancer cells in the tumor microenvironment, including changes**  
450 **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<sup>-/-</sup>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<sup>-/-</sup>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  $\pm$  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 vivo*  
462 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<sub>2</sub> 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<sup>G12D</sup>*; *LSL-P53<sup>R172H</sup>*; *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<sup>-/-</sup>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<sub>2</sub> 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  $^{13}\text{C}_5$ -citrulline and  $^{15}\text{N}_2$ -

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

511 cells grown in TIFM with  $^{13}\text{C}_5$ -citrulline at PDAC IF concentration ( $67\mu\text{M}$ ) (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  $^{15}\text{N}_2$ -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  $^{15}\text{N}$ -labelled arginine isotopomers in tissues or plasma after  $^{15}\text{N}_2$ -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  $\pm$  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  $^{13}\text{C}$ -labelled arginine isotopomers in mPDAC2-TIFM and

534 mPDAC3-TIFM cells grown in TIFM with  $^{13}\text{C}_5$ -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  $\pm$  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  $\mu\text{g}/\text{mL}$  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 20 $\mu$ M 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  $\pm$  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  $\mu$ g/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 10 $\mu$ M 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  $\pm$  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: 100 $\mu$ m. (B) IHC staining for ARG1 in an  
583 advanced human PDAC tumor and adjacent untransformed pancreas. Scale bar: 500 $\mu$ m (top)  
584 and 100 $\mu$ m (bottom). (C) Schematic for crossing of LysM-Cre and Arg1<sup>fl/fl</sup>, tumor implantation on  
585 LysM-Cre<sup>+/+</sup>; Arg1<sup>fl/fl</sup> progeny and subsequent IF extraction. (D) IHC staining for ARG1 protein  
586 expression in orthotopic mPDAC3-TIFM tumors from LysM-Cre<sup>+/+</sup>; Arg1<sup>fl/fl</sup> and Arg1<sup>fl/fl</sup> littermate  
587 controls. Scale bar: 100 $\mu$ m. (E) Absolute concentration of arginine and ornithine in the IF of  
588 orthotopic mPDAC3-TIFM tumors from LysM-Cre<sup>+/+</sup>; Arg1<sup>fl/fl</sup> (n=7) and Arg1<sup>fl/fl</sup> 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<sup>+/+</sup>; Arg1<sup>fl/fl</sup> and Arg1<sup>fl/fl</sup> 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<sup>fl/fl</sup> 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*<sup>G12D/+</sup>; *Trp53*<sup>fllox/fllox</sup> *Pdx1-Cre* (KP<sup>-/-</sup>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

- $^{13}\text{C}$  labeled yeast extract (Cambridge Isotope Laboratory, Andover, MA, ISO1)
- $^2\text{H}_9$  choline (Cambridge Isotope Laboratory, Andover, MA, DLM-549)
- $^{13}\text{C}_4$  3-hydroxybutyrate (Cambridge Isotope Laboratory, Andover, MA, CLM-3853)
- $^{13}\text{C}_6^{15}\text{N}_2$  cystine (Cambridge Isotope Laboratory, Andover, MA, CNLM4244)
- $^{13}\text{C}_3$  lactate (Sigma Aldrich, Darmstadt, Germany, 485926)
- $^{13}\text{C}_6$  glucose (Cambridge Isotope Laboratory, Andover, MA, CLM-1396)
- $^{13}\text{C}_3$  serine (Cambridge Isotope Laboratory, Andover, MA, CLM-1574)
- $^{13}\text{C}_2$  glycine (Cambridge Isotope Laboratory, Andover, MA, CLM-1017)
- $^{13}\text{C}_5$  hypoxanthine (Cambridge Isotope Laboratory, Andover, MA, CLM8042)
- $^{13}\text{C}_2^{15}\text{N}$  taurine (Cambridge Isotope Laboratory, Andover, MA, CNLM-10253)
- $^{13}\text{C}_3$  glycerol (Cambridge Isotope Laboratory, Andover, MA, CLM-1510)
- $^2\text{H}_3$  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*<sup>G12D/+</sup>; *Trp53*<sup>flox/flox</sup>;  
680 *LSL-YFP*; *Pdx1-Cre* (KP<sup>-/-</sup>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 plating 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% CO<sub>2</sub>. 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<sup>-/-</sup>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  $\text{Doublings/day} = \log_2(\text{Final Day Abs}_{510} / \text{Initial Day Abs}_{510}) / \text{number of days elapsed in culture}$   
727  $\text{period}$

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  $^{13}\text{C}_6,^{15}\text{N}_4$  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-](https://gsea-msigdb.github.io/gseapreranked-gpmodule/v6/index.html)  
797 [msigdb.github.io/gseapreranked-gpmodule/v6/index.html](https://gsea-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 ( $\Delta Ct$ ) values was  
825 determined for each of the samples relative to *Gapdh* levels and compared with vehicle control  
826 ( $\Delta\Delta Ct$ ). 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 16 $\mu$ L MOX reagent (ThermoFisher) for 1h at 37°C and then with 20 $\mu$ L 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 <sup>13</sup>C<sub>5</sub>-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

## 887 **Animal Experiments**

888 Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC,  
889 Protocol #72587) and performed in strict accordance with the Guide for the Care and Use of  
890 Laboratory Animals of the National Institutes of Health (Bethesda, MD). Mice were housed in a  
891 pathogen-free animal facility at the University of Chicago with a 12 h light/12 h dark cycle, 30–  
892 70% humidity and 68–74°F temperatures maintained.

893

### 894 *Orthotopic tumor implantation and monitoring*

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

901

### 902 *In vivo Arg1 knockout*

903 LysM-Cre and Arg1<sup>fl/fl</sup> C57BL6J mice were bred to generate LysM-Cre<sup>+/+</sup>; Arg1<sup>fl/fl</sup> and litter mate  
904 control Arg1<sup>fl/fl</sup> mice. All mice were genotyped using primers described in Supplementary File 2.  
905 Animal husbandry was carried out in strict accordance with the University of Chicago Animal  
906 Resource Center guidelines. Tumor implantation as described above was performed in mice at  
907 8-12 weeks of age.

908

### 909 *In vivo arginase-1 pharmacological inhibition*

910 Orthotopic tumors were implanted in C57BL6J mice at 8-12 weeks of age. 4 weeks after induction,  
911 once tumors were close end stage disease, CB-1158 (MedChem Express) dissolved in sterile  
912 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* $^{15}\text{N}_2$ -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  $^{15}\text{N}_2$ -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  $-80^\circ\text{F}$  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 20 $\mu\text{m}$  nylon  
934 mesh filters (Spectrum Labs, Waltham, MA, 148134) on top of 50 mL conical tubes, and  
935 centrifuged for 10min. at  $4^\circ\text{C}$  at 400xg. IF was then collected, snap-frozen in liquid nitrogen and  
936 stored at  $-80^\circ\text{C}$  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  $1 \times 10^6$  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 5 $\mu$ L  
971 IF samples using 45 $\mu$ L 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  $\mu$ L of each extract was removed and dried under  
975 nitrogen gas and stored  $-80^{\circ}\text{C}$  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 5 $\mu$ L volume of the IF sample.

980

981 To measure amino acid amounts in intratumoral samples, intratumoral metabolites were extracted  
982 from  $\sim$ 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:

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

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

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1050

### 1051 **Competing interests**

1052 No competing interests declared

1053

### 1054 **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.

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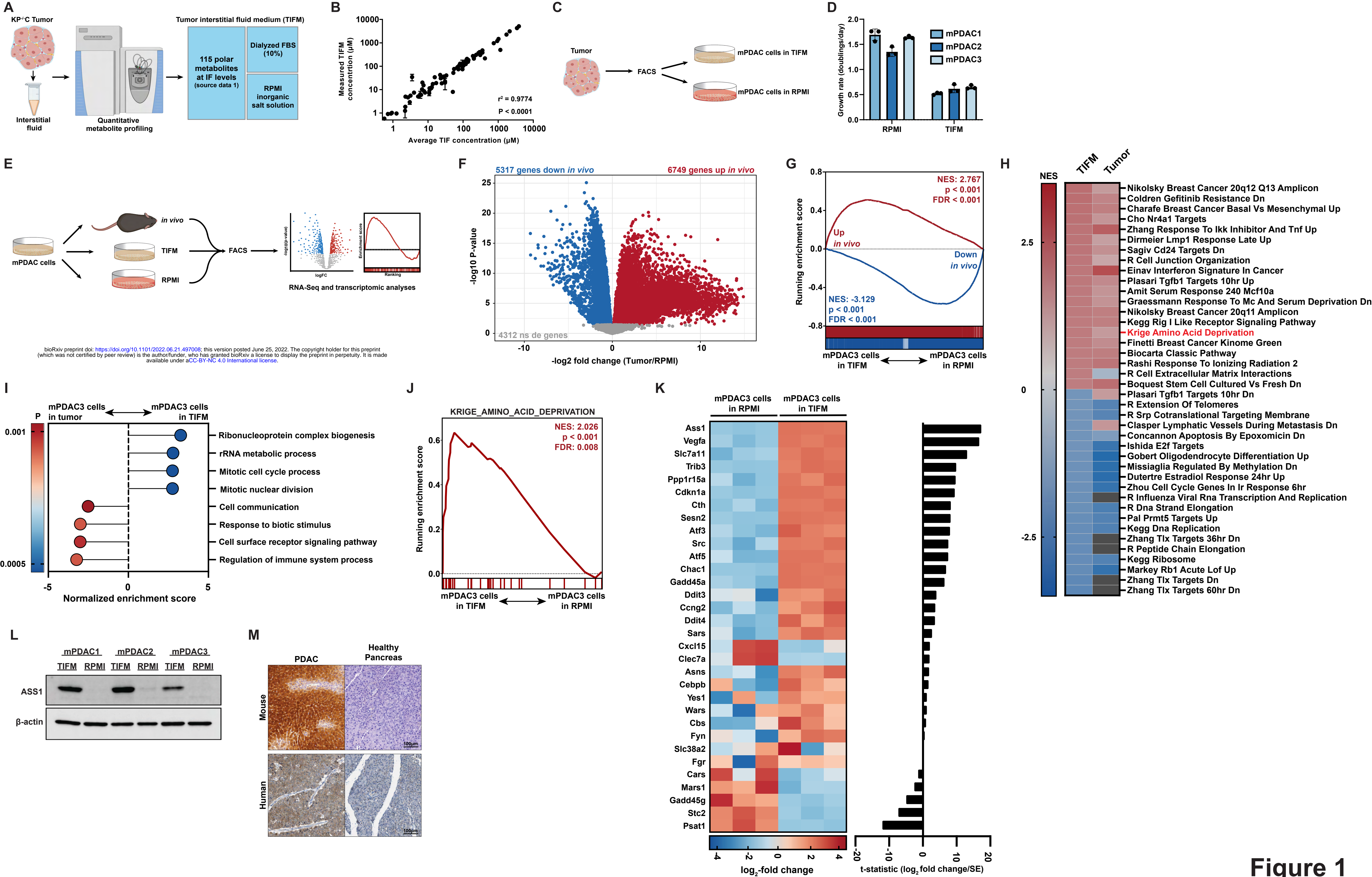
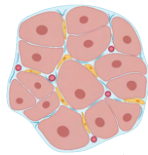
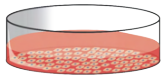


Figure 1

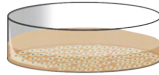
# KP<sup>-/-</sup>C Tumor



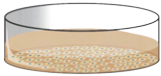
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in RPMI  $\geq 4$  weeks



mPDAC cells  
in TIFM  $\geq 4$  weeks



culture  
in TIFM



culture  
in RPMI

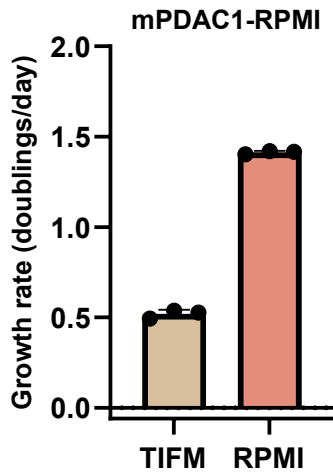
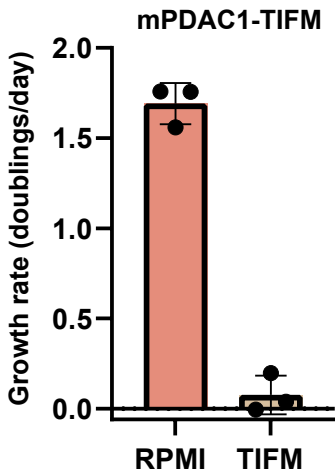
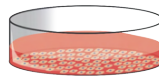


Figure 1 Supplement 1

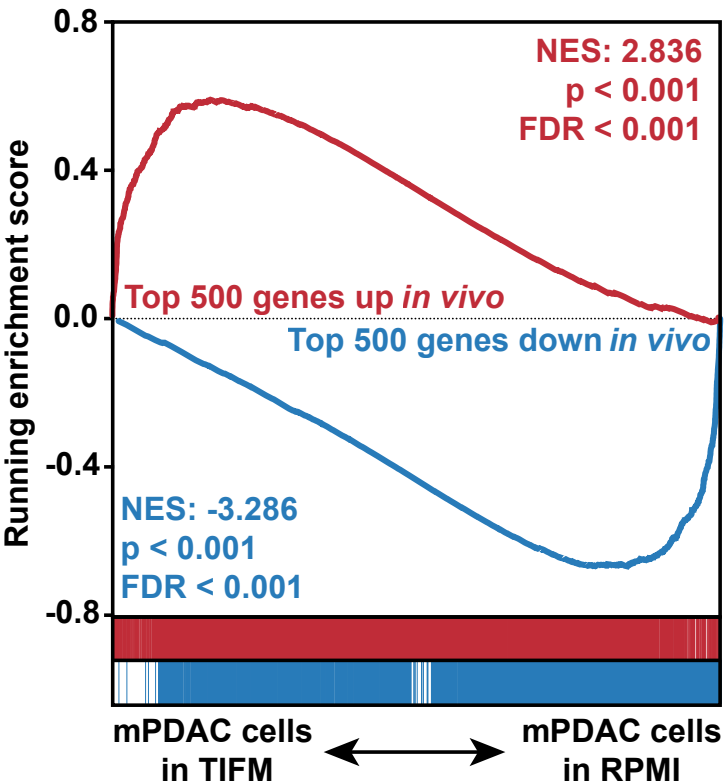


Figure 1 Supplement 2

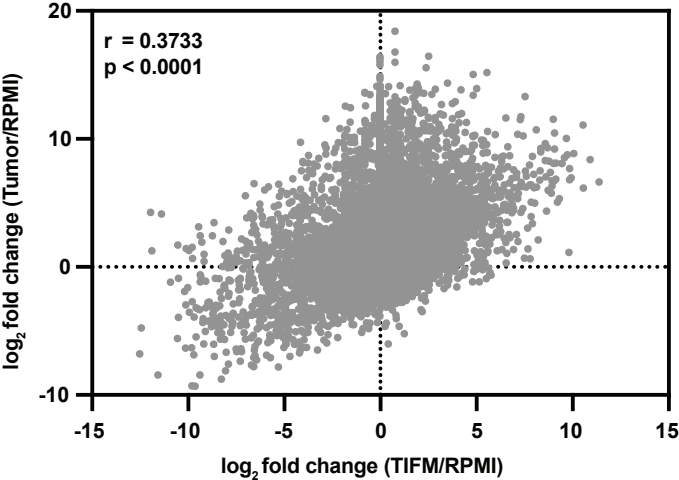
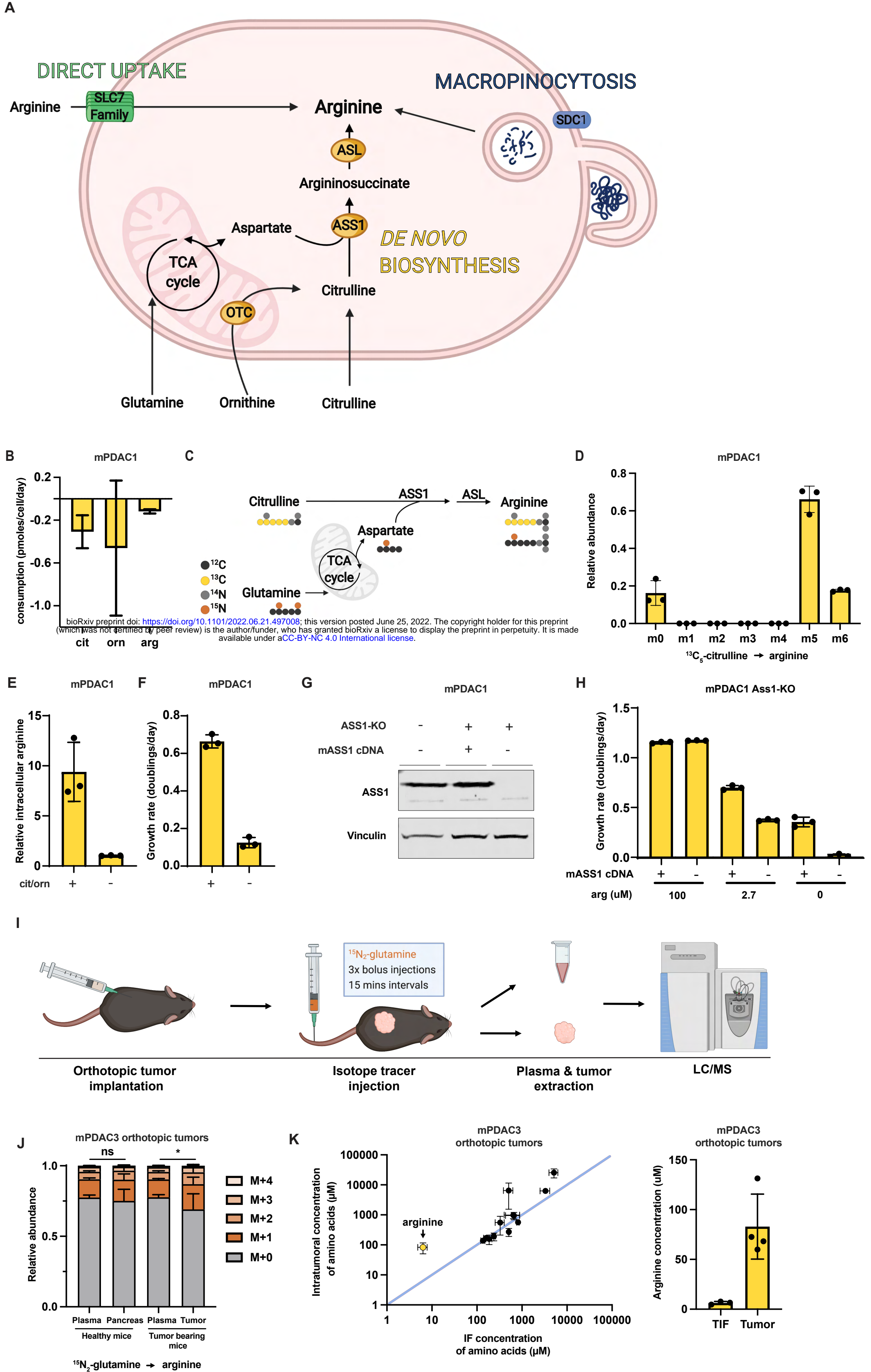


Figure 1 Supplement 3





**Figure 2**

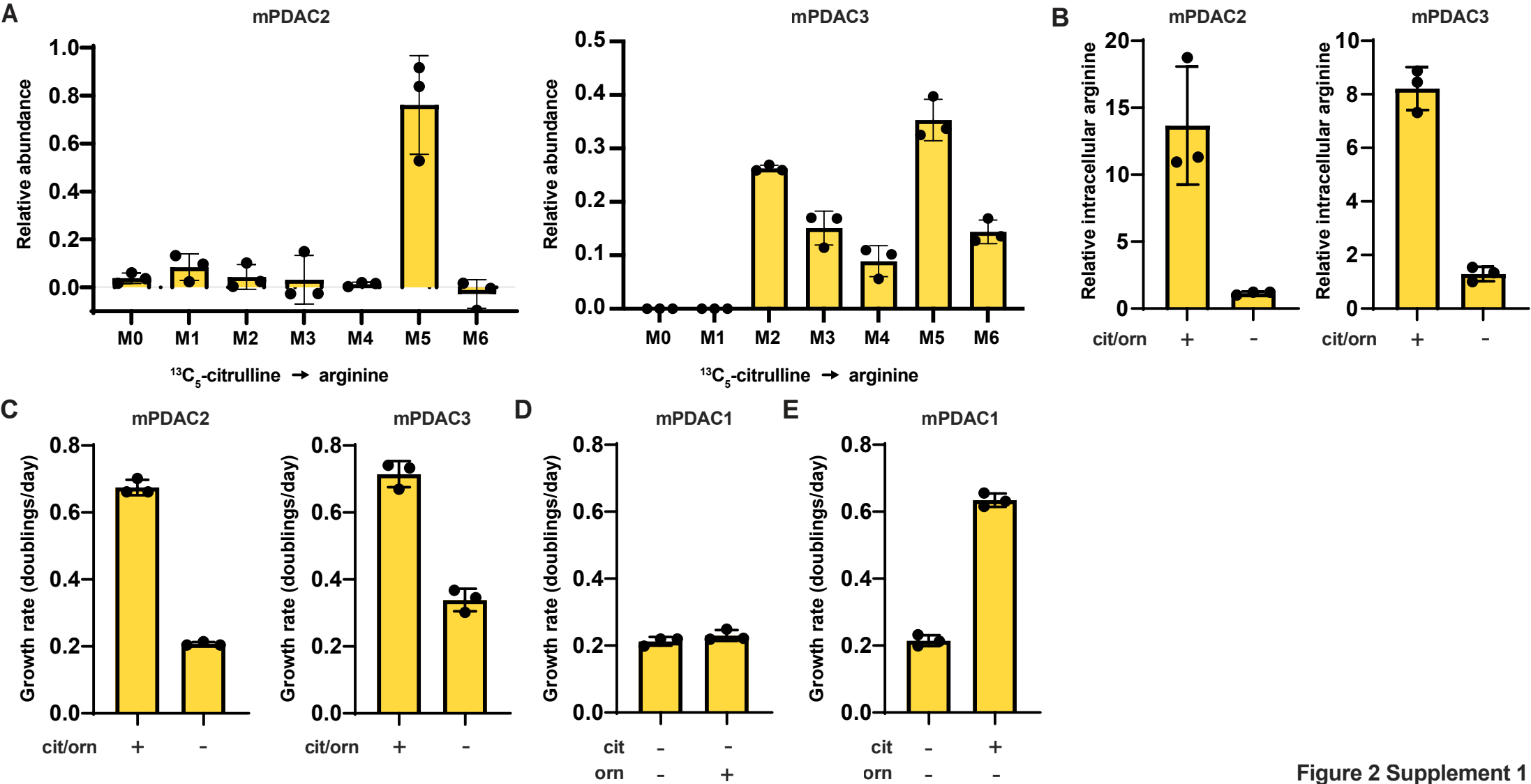


Figure 2 Supplement 1

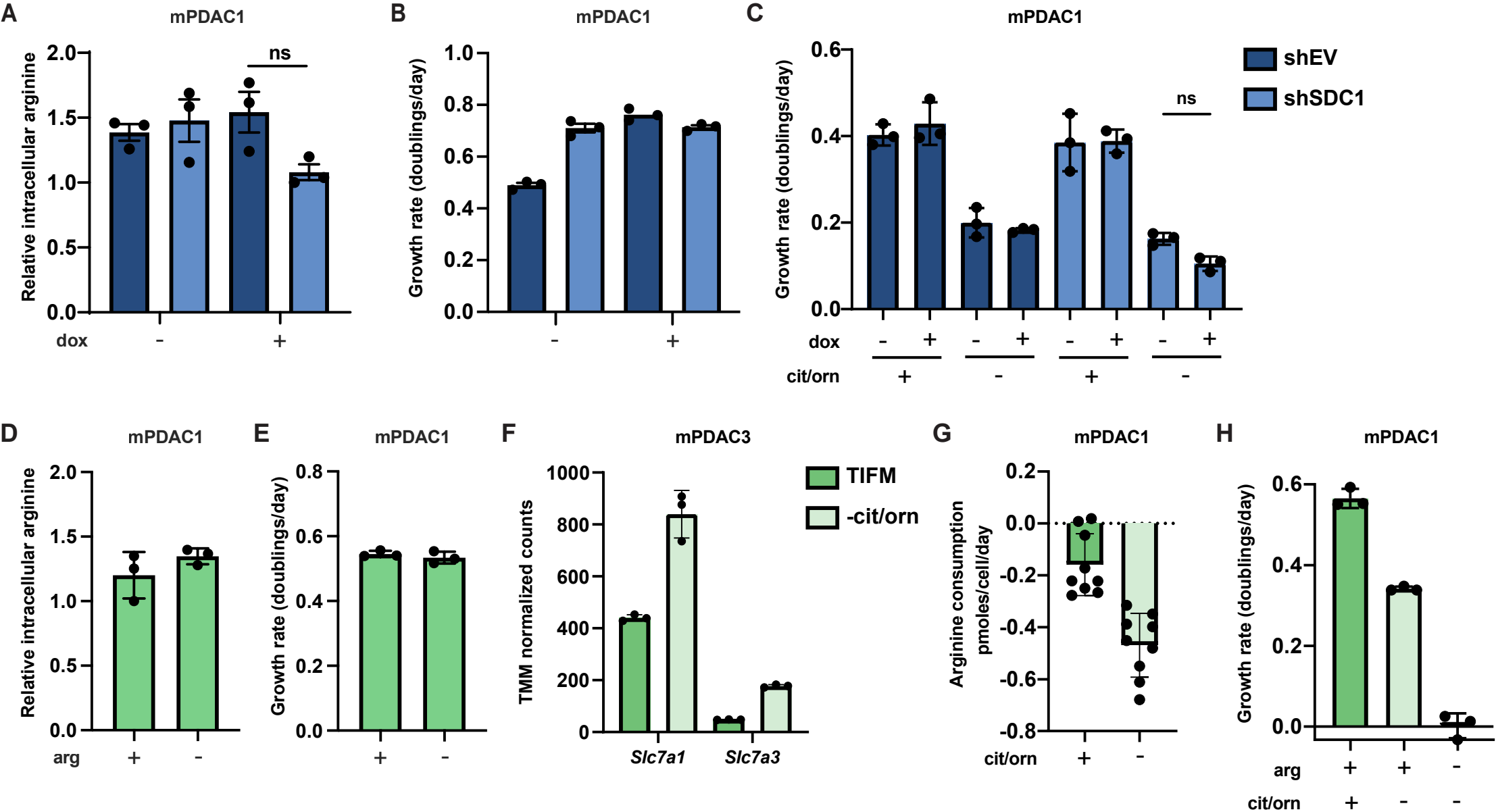


Figure 3



