

## 1 Hyaluronic Acid Fuels Pancreatic Cancer Growth

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21 **Abstract:** Rewired metabolism is a hallmark of pancreatic ductal adenocarcinomas (PDA).

22 Previously, we demonstrated that PDA cells enhance glycosylation precursor biogenesis  
23 through the hexosamine biosynthetic pathway (HBP) via activation of the rate limiting enzyme,  
24 glutamine-fructose 6-phosphate amidotransferase 1 (GFAT1). Here, we genetically ablated  
25 GFAT1 in PDA cell lines, which completely blocked proliferation in vitro and led to cell death. In  
26 contrast, GFAT1 knockout did not impair tumor growth, suggesting that cancer cells can  
27 maintain fidelity of glycosylation precursor pools by scavenging nutrients from the tumor  
28 microenvironment. Here, we show that hyaluronic acid (HA), an abundant carbohydrate polymer  
29 in pancreatic tumors composed of repeating N-acetyl-glucosamine (GlcNAc) and glucuronic  
30 acid sugars, can bypass GFAT1 to refuel the HBP via the GlcNAc salvage pathway.  
31 Furthermore, HA facilitates proliferation in nutrient-starved wild-type PDA. Together, these data  
32 show HA can serve as a nutrient fueling PDA metabolism beyond its previously appreciated  
33 structural and signaling roles.

34

35 **Keywords:** Pancreatic ductal adenocarcinoma, hexosamine biosynthetic pathway, GFAT1,  
36 NAGK, hyaluronic acid, GlcNAc, tumor microenvironment, cancer metabolism.

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37 **Introduction**

38 Pancreatic ductal adenocarcinoma (PDA) is one of the deadliest human cancers with no  
39 clinically effective treatment options (1). PDA is characterized by an intense fibroinflammatory  
40 stroma, poor vascularity, low nutrient levels, and rich deposition of extracellular matrix  
41 components. To survive and proliferate in this nutrient austere tumor microenvironment, the  
42 signature-driving oncogene in PDA, mutant Kras, facilitates the rewiring of PDA metabolism (2-  
43 4).

44 Among the rewired pathways, we previously demonstrated that mutant Kras promotes the  
45 activity of the hexosamine biosynthesis pathway (HBP) by upregulating expression of the rate-  
46 limiting enzyme glutamine-fructose 6-phosphate amidotransferase 1 (GFAT1) (5). The HBP is  
47 an evolutionarily conserved pathway that integrates glucose, glutamine, fatty acid, and  
48 nucleotide metabolism to generate the final product uridine diphosphate N-acetylglucosamine  
49 (UDP-GlcNAc). UDP-GlcNAc is a crucial donor molecule for glycosylation and O-GlcNAcylation,  
50 two essential post-translational modifications required for cellular structure, signaling, and  
51 survival (6). The HBP is the only way to generate UDP-GlcNAc *de novo*. Because the HBP  
52 integrates nutrients from several major macromolecular classes to produce UDP-GlcNAc,  
53 predictably it also acts a nutrient sensing mechanism for available energy within a cell (7).  
54 Indeed, numerous studies across cancer subtypes have demonstrated how HBP activity is  
55 enhanced to support tumor survival and growth (8-11) and even immune evasion through  
56 alteration of extracellular glycosylation content (12).

57 A compendium of studies during the last decade have revealed that PDA cells fuel their rewired  
58 metabolic programs through nutrient scavenging (5, 13-17). Mechanisms include sustained  
59 activation of intracellular recycling pathways (e.g. autophagy), the upregulation of nutrient  
60 transporter expression (e.g. carbohydrate, lipid, and amino acid transporters), and the activation  
61 of extracellular nutrient scavenging pathways (e.g. macropinocytosis). Further, PDA cells also  
62 participate in metabolic crosstalk and nutrient acquisition with non-cancerous cells in the tumor  
63 microenvironment (TME), such as cancer-associated fibroblasts (CAFs) and tumor-associated  
64 macrophages (TAMs) (18-22). A notable example is the observation that PDA cells can directly  
65 obtain nutrients from the CAF-derived extracellular matrix (ECM), such as collagen (17). Taken  
66 together, elucidating the interaction of PDA cells with different cell populations and ECM  
67 components will be instrumental for delineating deregulated PDA metabolism and improving  
68 therapeutic strategies.

69 A major structural component of the TME is hyaluronic acid (HA), a hydrophilic  
70 glycosaminoglycan. HA is ubiquitously present in human tissue, especially in skin, connective  
71 tissue, and joints, and it is richly abundant in pancreatic tumors (23). HA is primarily deposited  
72 by CAFs and, to some extent, by PDA cells (24, 25). HA avidly retains water, which is  
73 responsible for both its lubricating properties and, in PDA tumors, the supraphysiological  
74 pressure that impairs vascularity and limits drug penetrance (26, 27). An aspect of HA biology  
75 that has not previously been studied is its potential role as a nutrient. This is surprising given  
76 that HA is a carbohydrate polymer whose monomeric unit is a disaccharide of glucuronic acid  
77 and N-acetyl-glucosamine (GlcNAc).

78 Herein, we set forth to determine the utility of targeting the HBP in PDA. We found that GFAT1  
79 was required for cell survival *in vitro*. In marked contrast, GFAT1 knockout tumors readily grew  
80 *in vivo*. Based on this observation, we hypothesized that GlcNAc-containing components of the  
81 extracellular matrix could bypass the HBP *in vivo* by way of the GlcNAc salvage pathway. We  
82 demonstrate that HA can be metabolized by PDA cells to support growth by refilling the HBP. In  
83 sum, our study identifies HA as a novel nutrient source in PDA and contributes to a growing  
84 body of data illuminating the important role of the TME in cancer metabolism.

85 **Results**

86 **Pancreatic cancer cells require *de novo* HBP fidelity in vitro but not in vivo**

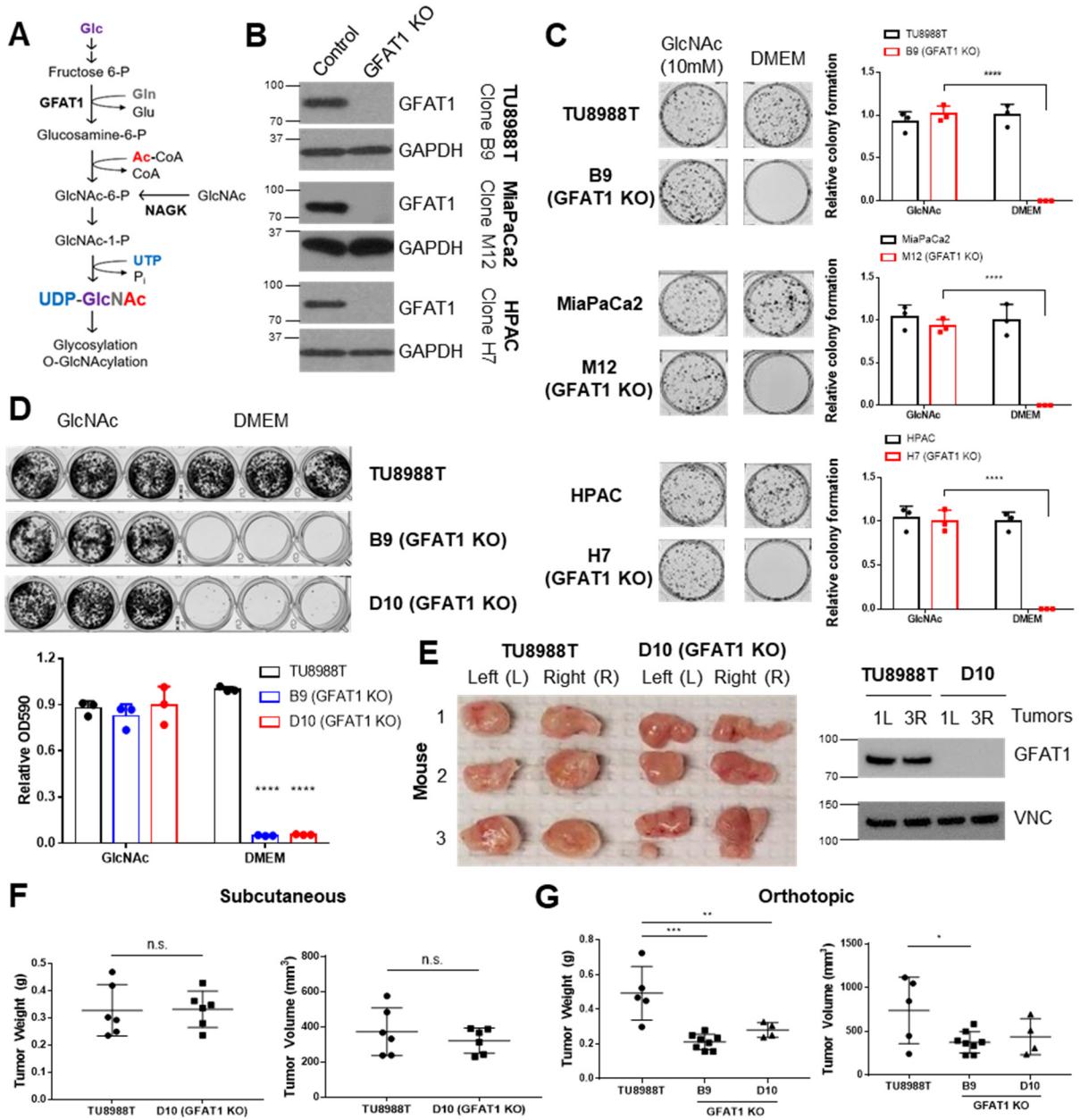
87 Previously, we found that mutant Kras transcriptionally activates GFAT1 expression  
88 downstream of MAPK signaling in a murine model of PDA to facilitate HBP activity (5). GFAT1  
89 catalyzes the reaction that generates glucosamine 6-phosphate and glutamate from fructose 6-  
90 phosphate and glutamine (**Figure 1A**). In another previous study we demonstrated that PDA  
91 cells are dependent on glutamine anaplerosis for proliferation (28). Thus, we hypothesized that  
92 inhibiting GFAT1 in PDA would have the simultaneous benefit of blocking two major metabolic  
93 pathways that support PDA proliferation, thereby providing a considerable therapeutic window.

94 Our previous results targeting GFAT1 in murine cells with shRNA yielded insufficient knockdown  
95 to draw a conclusion as to its necessity in PDA (5). Thus, here we used CRISPR/Cas9 to knock  
96 GFAT1 out from three established human PDA cell lines: HPAC, TU8988T, and MiaPaCa2.  
97 During selection, the pooled polyclonal populations were grown in GlcNAc, which bypasses  
98 GFAT1 via the GlcNAc salvage pathway (**Figure 1A**). This supplement was included to  
99 minimize metabolic rewiring within the selected populations.

100 The GFAT1 knockout lines had differential response to GlcNAc withdrawal. Among the three  
101 GFAT1 knockout cell lines, only the HPAC line exhibited a marked reduction in cell number,  
102 consistent with loss of viability, in the 4 days following GlcNAc withdrawal (**Supplementary**  
103 **Figure 1A**). The impact on proliferation was consistent with the decrease in the UDP-GlcNAc  
104 pool, which was analyzed using liquid chromatography-coupled tandem mass spectrometry (LC-  
105 MS/MS) (**Supplementary Figure 1B**). Consistent with the proliferative phenotypes across lines,  
106 the HPAC line also had a significantly smaller UDP-GlcNAc pool than that of either MiaPaCa2  
107 or 8988T cells (**Supplementary Figure 1C**). Cellular O-GlcNAc-ylation of the proteome was  
108 also measured by immunoblot three days after GlcNAc withdrawal. Again, consistent with the  
109 LC-MS/MS analysis, O-GlcNAc expression was significantly reduced in HPAC but was  
110 maintained in 8988T (**Supplementary Fig 1D**).

111 The data from TU8988T and MiaPaca2 were similar to those from our earlier studies (5), and  
112 thus we posited that knockout of GFAT1 was incomplete. As such, we subsequently generated  
113 clonal cell lines from the pooled lines. This analysis revealed that the degree of GFAT1  
114 knockout varied by cell line and by clone, and this correlated with their differential growth and  
115 sensitivity to GlcNAc withdrawal *in vitro* (**Supplementary Figure 1E,F**). Clones for each cell line  
116 without detectable GFAT1 expression (**Figure 1B**) were further validated by sequencing and  
117 were subsequently used to examine the role of the HBP more accurately.

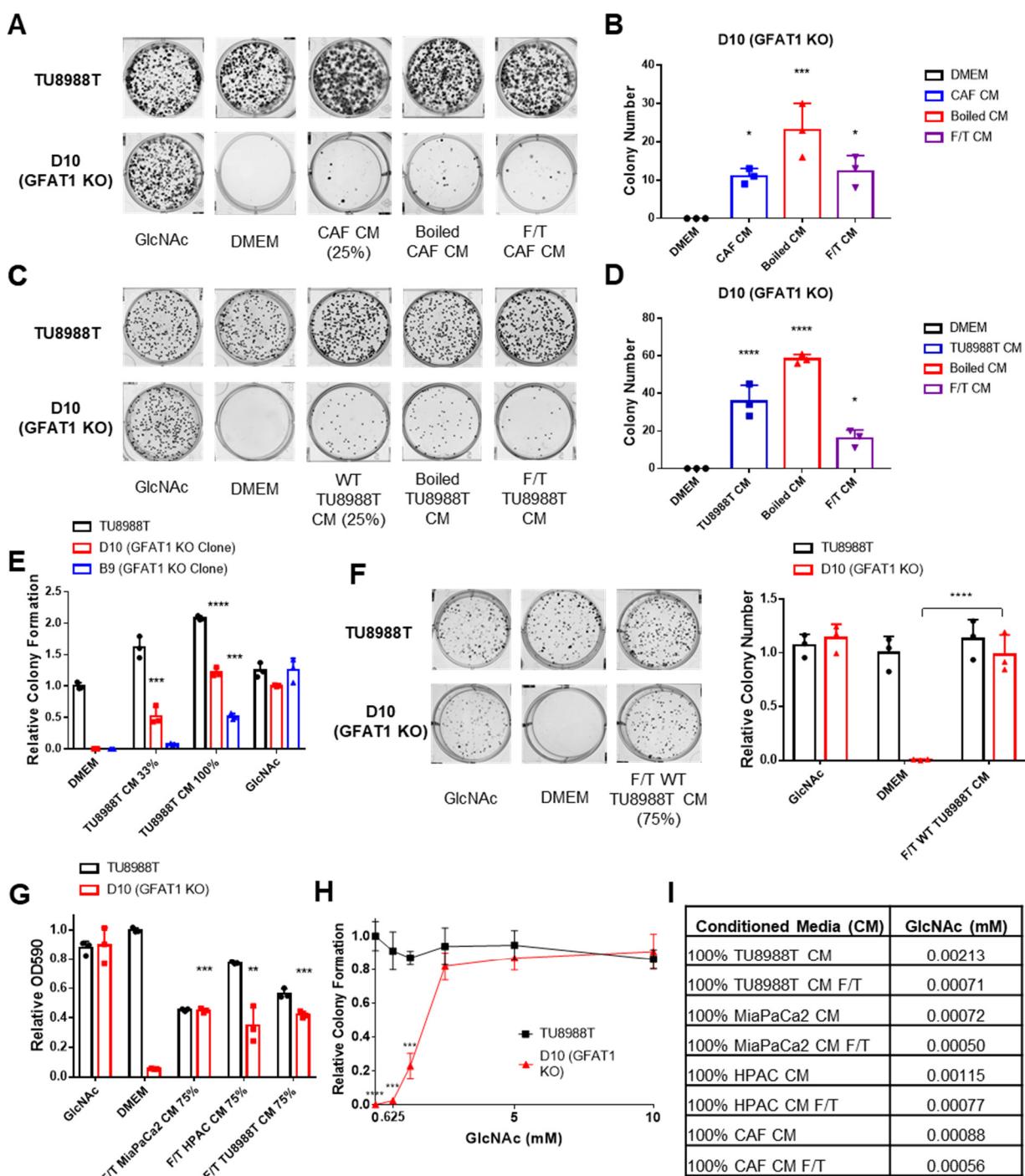
118 Using our genomically-sequenced and bona fide GFAT1 knockout clonal lines, we found that  
119 GFAT1 knockout led to an abolishment of colony formation (**Figure 1C**) and potently impaired  
120 proliferation (**Figure 1D, Supplementary Figure 1G**) in all three PDA cell lines *in vitro*. We then  
121 moved these cells into *in vivo* tumor models. Surprisingly, when either the pooled or the clonal  
122 knockout lines were implanted into the flanks of immunocompromised mice, they readily formed  
123 tumors that were comparable to their wild type counterparts in terms of weight and volume  
124 (**Figure 1E,F and Supplementary Figure 1H**). Similar results were obtained for GFAT1  
125 knockout clonal lines implanted orthotopically into the pancreas (**Figure 1G**). Of note, while  
126 clearly capable of forming tumors, the GFAT1 knockout clonal lines grown in the pancreas were  
127 smaller than the wild type tumors at endpoint. The marked discrepancy in phenotype between *in*  
128 *vitro* and *in vivo* settings led us to hypothesize that GFAT1 knockout clones were scavenging  
129 nutrients from the TME to refill the HBP, which enabled their survival and tumor growth.



**Figure 1. PDA requires *de novo* hexosamine biosynthetic pathway fidelity in vitro but not in vivo. (A)** Schematic overview of the hexosamine biosynthetic pathway (HBP) and the nutrient inputs. Ac-CoA, acetyl-coenzyme A; GFAT1, glutamine fructose 6-phosphate amidotransferase 1; Glc, glucose; GlcNAc, N-acetyl-glucosamine; Gln, glutamine; Glu, glutamate; NAGK, N-acetyl-glucosamine kinase; P<sub>i</sub>, inorganic phosphorus; UTP, uridine-triphosphate. **(B)** Western blot of GFAT1 and loading control GAPDH from validated CRISPR/Cas9 knockout TU8988T, MiaPaca2, and HPAC clones and their control (non-targeted sgRNA). **(C)** Representative wells from a colony-forming assay in parental and clonally-derived GFAT1 knockout cell lines grown in base media (DMEM) or base media supplemented with 10mM GlcNAc. Data quantitated at right, n=3. **(D)** Proliferation assay in parental and two GFAT1 knockout clonal TU8988T cell lines. Representative wells are presented above data quantitated by crystal violet extraction and measurement of optical density (OD) at 590nm, n=3. **(E)** Tumors from parental TU8988T (n=6) and GFAT1 knockout clone D10 (n=6) grown subcutaneously in immunocompromised mice. Accompanying western blot for GFAT1 and VINCULIN (VNC) loading control from representative tumor lysates. **(F)** Tumor volume and tumor weight from samples in **E**. **(G)** Tumor volume and tumor weight from parental TU8988T (n=5) and GFAT1 knockout clones B9 (n=8) and D10 (n=4) implanted and grown orthotopically in the pancreas of immunocompromised mice. Error bars represent mean  $\pm$  SD. n.s., non-significant; \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001.

131 **Conditioned media rescues proliferation of GFAT1 knockout PDA cells**

132 To test our scavenging hypothesis, we generated conditioned media (CM) from CAFs, the most  
 133 abundant stromal cell type in the pancreatic TME (29, 30). When GFAT1 KO clones were  
 134 incubated in patient-derived CAF CM, we observed a significant, albeit modest, rescue in colony  
 135 formation (**Figure 2A,B**). Unexpectedly, we observed a more robust, dose-dependent rescue of  
 136 colony formation in GFAT1 knockout cells with CM from wild type TU8988T cells (**Figure 2C-F**  
 137 and **Supplementary Figure 2A**). Similarly, CM from wild type HPAC and MiaPaCa2 cells was  
 138 also able to partially rescue proliferation of a subset of GFAT1 KO clones (**Figure 2G** and  
 139 **Supplementary Figure 2B,C**).



**Figure 2. Conditioned media from wild type PDA cells support proliferation of GFAT1 knockout cells. (A)**

Representative wells from a colony-forming assay in parental TU8988T and GFAT1 knockout clonal line D10 in 10mM GlcNAc, base media (DMEM), or base media supplemented 3:1 with cancer associated fibroblast (CAF) conditioned media (CM), boiled CAF CM, or CAF CM subject to freeze-thaw (F/T). **(B)** Quantitation of colonies from data in **A** (n=3). **(C)** Representative wells from a colony-forming assay in parental TU8988T and GFAT1 knockout clonal line D10 in 10mM GlcNAc, DMEM, or base media supplemented 3:1 with CM from wild type TU8988T cells, boiled TU8988T CM, or TU8988T CM subject to F/T. **(D)** Quantitation of colonies from data in **C** (n=3). **(E)** Quantitation of colony forming assay data of parental and GFAT1 knockout clonal TU8988T lines in base media, positive control GlcNAc, wild type TU8988T CM diluted 1:2 (33%) or used directly (100%) (n=3). **(F)** Representative wells and quantitation of colony forming assay data of parental and GFAT1 knockout clonal TU8988T lines in base media, positive control GlcNAc, and wild type TU8988T CM subject to F/T and diluted 3:1 (75%) (n=3). **(G)** Quantitation of colony forming assay data of parental and GFAT1 knockout clonal TU8988T lines in base media, positive control GlcNAc, or wild type TU8988T, HPAC, or MiaPaCa2 CM subject to F/T and diluted 3:1 (75%) (n=3). **(H)** GlcNAc dose response curve presented as relative colony number for parental and GFAT1 knockout TU8988T cells (n=3). **(I)** Absolute quantitation of GlcNAc in various CM by LC-MS/MS (n=3). Error bars represent mean  $\pm$  SD. n.s., non-significant; \*P < 0.05; \*\* P <0.01; \*\*\* P <0.001; \*\*\*\* P <0.0001.

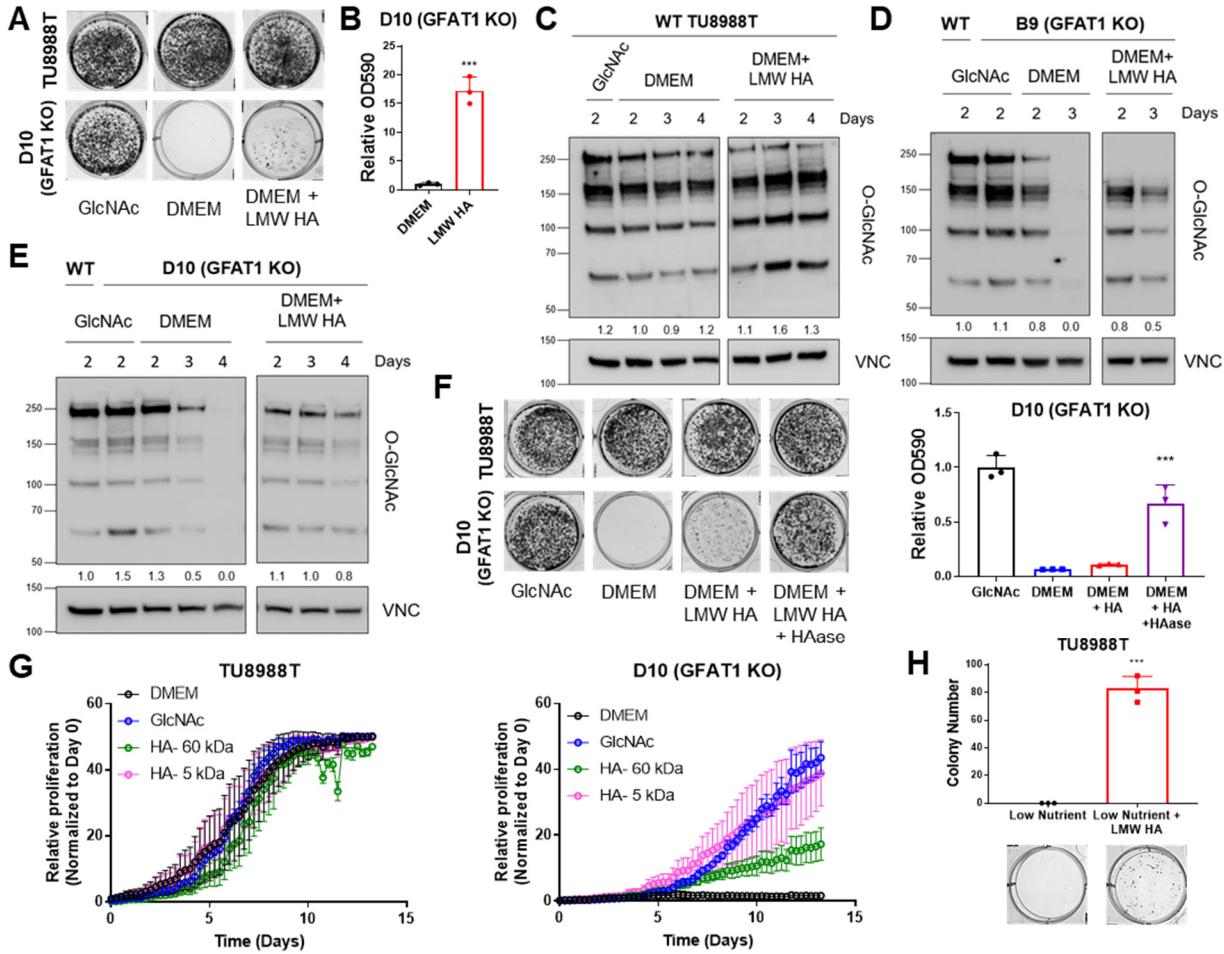
140 To begin to identify the rescue factors in the CM, we subjected the CM to boiling or repeated  
141 cycles of freezing and thawing (F/T). In each of these conditions, both the CAF and the PDA CM  
142 retained the ability to support colony formation in GFAT1 knockout cells (**Figure 2A,B**). These  
143 results suggested the relevant factor(s) did not require tertiary structure. Additionally, we  
144 observed that the rescue activity of the CM was dose dependent (**Figure 2E-G** and  
145 **Supplementary Figure 2A-C**).

146 As GlcNAc was used to establish our GFAT1 knockout lines, we first quantitated the GlcNAc  
147 concentration in the CM. GlcNAc dose response curves demonstrated that millimolar quantities  
148 of GlcNAc (>0.625mM) were required to rescue colony formation of GFAT1 knockout PDA cells  
149 (**Figure 2H** and **Supplementary Figure 2D**). By contrast, LC-MS/MS quantification of GlcNAc  
150 in the CM revealed that it was in the low micromolar range (**Figure 2I**), several orders of  
151 magnitude below the millimolar doses of exogenous GlcNAc required to maintain proliferation  
152 (**Figure 2H** and **Supplementary Figure 2D**). These results illustrated that free GlcNAc was not  
153 the relevant molecule in the CM mediating rescue. This led us to consider alternate possibilities,  
154 including GlcNAc-containing components of the TME.

155 **Hyaluronic acid facilitates proliferation in GFAT1 knockout PDA cells and nutrient-  
156 starved wild type PDA cells**

157 GlcNAc is a widely utilized molecule as a structural component of the extracellular matrix, a  
158 modification of various lipid species, and a post-translational modification on proteins (31, 32).  
159 Thus, we hypothesized that GlcNAc was released into CM as a component part of a lipid,  
160 protein, or glycosaminoglycan polymer, and that this mediated rescue of GFAT1 knockout. To  
161 test this, we first applied necrotic cellular debris from FL5.12 cells (33), which contains the full  
162 complement of biomolecules from dead cells, including GlcNAc-containing proteins and lipids, to  
163 GFAT1 knockout cells grown at clonal density. Necrotic cell debris was unable to rescue GFAT1  
164 knockout across our cell line panel (**Supplementary Figure 3A-F**). Next, we tested if  
165 glycosaminoglycan carbohydrate polymers could mediate rescue of GFAT1 knockout, in a  
166 matter akin to CM. High dose heparin was not able to rescue colony formation in GFAT1  
167 knockout cells (**Supplementary Figure 3A-F**), but 78 kDa HA provided a modest but significant  
168 rescue (**Figure 3A,B**).

169 HA is a carbohydrate polymer and an extracellular matrix component that is abundant in the  
170 PDA tumor microenvironment (23). The monomeric form of HA is a repeating disaccharide



**Figure 3. Hyaluronic acid rescues proliferation in GFAT1 knockout PDA cells and nutrient starved PDA cells.** (A) Representative wells from a colony-forming assay in parental and clonally-derived GFAT1 knockout TU8988T cell lines grown in base media (DMEM), positive control GlcNAc (10mM), or low molecular weight (LMW) hyaluronic acid (78kDa HA, 10mM). (B) Quantitation of data from A (n=3). (C) Western blot of proteome O-GlcNAc and loading control VINCULIN (VNC) in parental TU8988T cells grown in base media (DMEM) plus GlcNAc or LMW HA for the indicated time points. Band density was quantitated, normalized to control, and plotted below the blot. (D,E) Western blot of proteome O-GlcNAc and loading control VNC in GFAT1 knockout clonal lines (D) B9 and (E) D10 in base media (DMEM) plus GlcNAc or LMW HA for the indicated time points. Wild type (WT) TU8988T included as control. Band density was quantitated, normalized to control, and plotted below the blot. (F) Representative wells of a proliferation assay in parental TU8988T and GFAT1 knockout clonal line D10 grown in base media (DMEM), positive control GlcNAc (10mM), or base media supplemented 1:1 with boiled low molecular weight (LMW) hyaluronic acid (HA, 10mM) with and without pre-digestion with hyaluronidase (HAase). At endpoint, cells are stained with crystal violet, and the stain is then extracted and quantitated by OD at 590nm (n=3). (G) Proliferation time course, as measured on the Incucyte, of parental TU8988T and GFAT1 knockout cells in base media (DMEM), positive control (GlcNAc), 60 kDa HA (LMW HA), or 5 kDa HA (o-HA) (n=3). (H) Quantitated colony forming assay data and representative wells of parental TU8988T cells grown in low nutrient conditions (20-fold reduction in glucose, glutamine, and 10-fold reduction in serum) in the presence or absence of 10 mM LMW HA (n=3). Error bars represent mean  $\pm$  SD. \*\*\* P <0.001.

171 consisting of glucuronic acid and GlcNAc. HA polymer length, often described by its molecular  
 172 weight (MW), has important impacts on its biological activity. In non-pathological settings, newly  
 173 synthesized HA is predominantly high molecular weight (HMW; >1000kDa) (34). However, in  
 174 tumors and tumor interstitial fluid, there is a significantly elevated level of low molecular weight  
 175 (LMW; 10-250kDa) and oligo-HA (o-HA; <10kDa) (35, 36). Consistent with the rescue of colony

176 formation in GFAT1 knockout cells, LMW HA (78 kDa) was also able to rescue total cellular O-  
177 GlcNAc levels, as assessed by western blot (**Figure 3C-E**).

178 Cancer cells have been reported to uptake HA via macropinocytosis (37). Thus, a possible  
179 explanation for the modest rescue could be low macropinocytosis activity. However in PDA,  
180 mutant Kras drives high macropinocytosis (13), and quantitation of macropinocytotic activity with  
181 a fluorescent dextran-based assay revealed that our three PDA cell lines exhibited considerable  
182 macropinocytosis (**Supplementary Figure 3G**). This led us to hypothesize that HA entry into  
183 cells is not the rate limiting step, but rather the cleavage of HA into smaller fragments.  
184 Consistent with this, breaking down LMW HA with hyaluronidase enhanced the rescue of colony  
185 formation (**Figure 3F**). Of note, hyaluronidase was heat-inactivated before its application to  
186 GFAT1 knockout cells (**Supplementary Figure 3H**), as hyaluronidase has been reported to  
187 directly impact cellular metabolism (38). Next, we tracked the rescue of proliferation by LMW HA  
188 (60kDa) and o-HA (5kDa). This analysis revealed that HA-mediated rescue in proliferation was  
189 considerably higher for o-HA than for LMW HA (**Figure 3G**).

190 The studies detailed above were performed with GlcNAc auxotrophs. To determine the effect of  
191 HA in a more physiologically relevant setting, we provided HA to wild type PDA cells cultured in  
192 low-nutrient media (i.e. a 20-fold reduction in glucose and glutamine, and a 10-fold reduction in  
193 serum concentration). Here we found that LMW HA rescued colony formation (**Figure 3H**).  
194 Collectively, these data point to a novel role of HA to restore the HBP, promote survival and  
195 proliferation of GFAT1 null PDA and, moreover, also that of nutrient-starved wild type PDA cells.

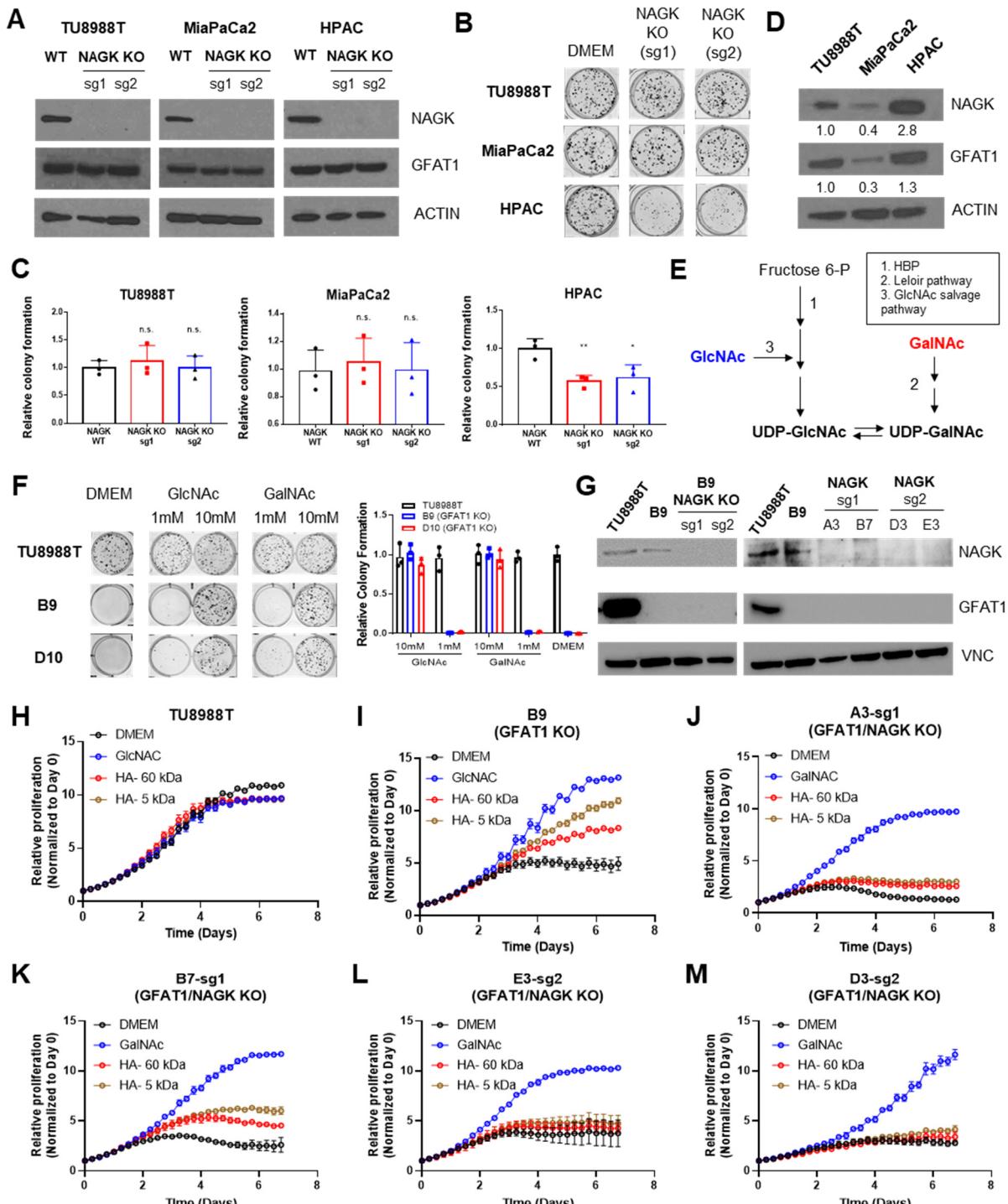
#### 196 **Hyaluronic acid rescues GFAT1 null PDA via the GlcNAc salvage pathway**

197 The GlcNAc salvage pathway bypasses GFAT1 by catalyzing the phosphorylation of GlcNAc to  
198 GlcNAc-6-phosphate, in a reaction mediated by N-acetyl-glucosamine kinase (NAGK). This  
199 GlcNAc-6-phosphate is subsequently converted into UDP-GlcNAc (**Figure 1A**). Therefore, we  
200 hypothesized that the carbohydrate polymer HA, which is 50% GlcNAc, fuels the HBP via the  
201 GlcNAc salvage pathway through NAGK. To test this, we employed the same CRISPR/Cas9  
202 strategy to target NAGK (**Figure 4A**). Knockout of NAGK in parental TU8988T and MiaPaCa2  
203 cell lines had no impact on colony formation, while reducing the colony forming capacity of  
204 HPAC cells (**Figure 4B,C**). These results were consistent with the elevated expression of NAGK  
205 in HPAC cells (**Figure 4D**). Of note, NAGK knockout did not result in up-regulation of GFAT1  
206 (**Figure 4A**), which could have suggested a compensatory metabolic rewiring.

207 Next, we targeted NAGK in our GFAT1 knockout clones. GFAT1/NAGK double knockout cells  
208 were generated in media containing N-acetyl-galactosamine (GalNAc), an isomer of GlcNAc.  
209 Supplementation with GalNAc enables bypass of both the *de novo* HBP and the GlcNAc  
210 salvage pathway, by way of the Leloir pathway (39), to support UDP-GalNAc and ultimately  
211 UDP-GlcNAc biogenesis (**Figure 4E**). In this way, we were again able to select viable lines  
212 while avoiding the selection of those with unpredictable metabolic adaptations.

213 The GalNAc dose response for GFAT1 knockout clones was comparable to that of GlcNAc  
214 (**Figure 2H**), demonstrating that they are indeed viable in GalNAc (**Figure 4F**). Although NAGK  
215 expression was efficiently knocked down in our pooled populations (**Figure 4G**), we again  
216 selected for clones in order to minimize the effect of NAGK-proficient clones persisting in the  
217 bulk population. From among these, we selected four GFAT1/NAGK clones and tracked their  
218 proliferation using the Incucyte upon rescue with varying sizes of HA or GalNAc (**Figure 4H-M**).  
219 These were compared relative to wild type TU8988T cells (**Figure 4H**) and the GFAT1 knockout

220 line (**Figure 4I**). In stark contrast to the GFAT1 knockout line, LMW HA- and o-HA was unable  
 221 to rescue GFAT1/NAGK double knockout lines (**Figure 4I-M**). These results illustrate that HA  
 222 rescue requires NAGK and the GlcNAc salvage pathway, consistent with the idea that HA-  
 223 derived GlcNAc fuels UDP-GlcNAc biosynthesis upon GFAT1 knockout. Altogether, our data  
 224 implicate HA as a novel nutrient for PDA. Mechanistically, HA regulates PDA metabolism by  
 225 refueling the HBP via the GlcNAc salvage pathway, supporting PDA survival and proliferation.



**Figure 4. Hyaluronic acid-derived GlcNAc rescues GFAT1 loss via the GlcNAc salvage pathway.** (A) Western blot of NAGK, GFAT1, and ACTIN loading control from TU8988T, MiaPaCa2, and HPAC parental (wild type, WT) and NAGK KO populations. NAGK was knocked out using two independent sgRNAs (sg1, sg2). (B) Representative wells from a colony forming assay for parental and NAGK knockout lines. (C) Quantitation of colony forming assay data in (B) (n=3). (D) Western blot for NAGK, GFAT1, and loading control ACTIN in parental PDA cell lines. Band density was quantitated, normalized to control, and plotted below the blot. (E) Schematic overview of the Leloir pathway of galactose catabolism integrated with the HBP and GlcNAc salvage pathway. (F) Representative wells from colony formation assays in parental and GFAT1 knockout clonal TU8988T cell lines in base media (DMEM), positive control GlcNAc, and N-acetyl-galactosamine (GalNAc). (G) Western blot for GFAT1, NAGK, and loading control VINCULIN (VNC) in parental TU8988T and HPAC, GFAT1 knockout clones, and GFAT1/NAGK double targeted lines. (H-M) Proliferation time course, as measured on the Incucyte, of parental (H,I) TU8988T and GFAT1 knockout line B9 in base media, GlcNAc positive control, 60 kDa HA, or 5 kDa HA; (J-M) GFAT1/NAGK double targeted clones in base media, GalNAc positive control, 60 kDa HA, or 5 kDa HA (n=3). Error bars represent mean  $\pm$  SD. n.s., non-significant; \*P < 0.05; \*\*P < 0.01.

## 226 Discussion

227 The HBP is activated in a Kras-dependent manner in PDA (5), and it is similarly elevated in  
228 numerous cancers to provide a diverse set of functions, including the regulation of proliferation,  
229 survival, angiogenesis, and metastasis (9). As such, we and others have proposed that the HBP  
230 may provide a selective vulnerability for cancer therapy, with GFAT1 as an attractive therapeutic  
231 target (5, 10, 40, 41). Indeed, several pan glutamine-deamidase inhibitors (e.g. azaserine and 6-  
232 diazo-5-oxo-L-norleucine), which potently suppress GFAT activity, have demonstrated anti-  
233 tumor activity *in vitro* and *in vivo* in PDA and other cancers (42, 43). However, because these  
234 drugs are not specific to the HBP, it has not been clear what impact GFAT-specific inhibition  
235 had on these phenotypes. As such, we took a genetic approach to knock out GFAT1 to  
236 elucidate the role of the HBP in PDA.

237 In the PDA models tested herein, GFAT1 knockout was not compatible with PDA cell  
238 proliferation *in vitro*, unless the media were supplemented with GlcNAc or GalNAc (**Figure 1C,D**  
239 and **Figure 4F**). However, these same cells readily formed tumors *in vivo* in subcutaneous and  
240 orthotopic models (**Figure 1E-G**). The stark discrepancy in phenotypes led us to hypothesize  
241 that the TME was providing the means to bypass GFAT1. Indeed, we found that denatured  
242 conditioned media from CAFs and wild type PDA cells were able to rescue viability in GFAT1  
243 knockout PDA cells, implicating a molecule(s) without tertiary structure (**Figure 2**). By examining  
244 several GlcNAc-containing candidates, we discovered a previously unknown role of HA as a  
245 nutrient source for PDA (**Figure 3**). We report that HA can refill the HBP via the GlcNAc salvage  
246 pathway (**Figure 4**) to support PDA survival and proliferation.

247 HA is traditionally regarded as a structural component in physiology (44). In addition to this role,  
248 a wealth of studies have ascribed additional functions to HA. For example, HA can activate cell-  
249 cell contact-mediated signal transduction through CD44 and/or receptor for HA-mediated  
250 motility (RHAMM) (45). The signaling activity/function of HA depends on its MW (44, 46).  
251 Similarly, a recent study illustrated that breakdown of the HA matrix with hyaluronidase enabled  
252 the interaction between growth factors and growth factor receptors (38). This promoted glucose  
253 metabolism, cellular proliferation, and migration. The role of HA in GFAT1 knockout and nutrient  
254 starved PDA cells described herein is likely independent of its structural and signaling functions,  
255 given that we observe considerably greater rescue with o-HA (**Figure 3F,G**), a form of HA that  
256 is not traditionally considered for these purposes.

257 Rather, our study introduces a novel role to HA as a fuel for PDA tumor growth (**Figure 3G,H**),  
258 further highlighting the significance and biological complexity of this predominant

259 glycosaminoglycan. Additionally, our study suggests that NAGK, through which HA-mediated  
260 GlcNAc presumably refuels the HBP in vivo, may be an attractive therapeutic target for PDA.  
261 Indeed, a recent study demonstrated that NAGK knockout in PDA impairs tumor growth in vivo,  
262 while only exhibiting a modest impact on cellular proliferation in vitro (*Wellen lab, co-submitted*  
263 *study*). These results are consistent with our observations on utilization of the GlcNAc salvage  
264 pathway to fuel UDP-GlcNAc pools from HA-derived GlcNAc (**Figure 4E**). Our study also  
265 contributes to a growing body of data illuminating unexpected nutrient sources in the TME that  
266 support cancer metabolism (13, 14, 16-21, 47), and this raises the possibility that other  
267 glycosaminoglycans may be similarly scavenged.

268 Due to its extremely hydrophilic nature, HA retains water and acts as a cushioning agent in  
269 tissue homeostasis and biomechanical integrity (44). In PDA, HA is a predominant component  
270 of the TME, and its water-retaining property is one of the main drivers of the supraphysiological  
271 intratumoral pressure (48). This pressure can exceed 10-fold that observed in the normal  
272 pancreas, and, as a result, tumor vasculature collapses (49-51). The limited access to  
273 circulation impairs nutrient and oxygen delivery, and it has been proposed that this is a critical  
274 impediment to tumoral drug delivery (52). Indeed, in animal models, breakdown of the HA matrix  
275 with a therapeutic hyaluronidase (PEGPH20) reduces intratumoral pressure, restores  
276 circulation, which facilitates drug delivery, and thereby improves response to chemotherapy (50,  
277 51). Based on these promising observations, PEGPH20 was tested in clinical trials alongside  
278 standard of care chemotherapy. Despite the successes in the preclinical models, PEGPH20 did  
279 not extend PDA patient survival (53).

280 The discrepancy between the clinical response to PEGPH20 and the preclinical data remains an  
281 active area of investigation and may concern the myriad additional roles of HA. For example,  
282 the HA matrix may be necessary to restrain tumor dissemination, as was shown for CAF  
283 depletion studies in PDA (54-57). Thus, the benefits afforded by enhanced drug penetration  
284 facilitated by PEGPH20 may be negated by this side effect. Along these lines, HA degradation  
285 may also enhance tumor metabolism and growth. This could occur through growth factor  
286 signaling-dependent (38) as well as signaling-independent pathways, like the GlcNAc salvage  
287 pathway described herein. In contrast, reduction in the HA content of tumors also facilitates T  
288 cell invasion (43), which may complement immunotherapy approaches, a concept that would be  
289 hindered by immunosuppressive chemotherapies. Given the conflicting roles of HA in tumor  
290 restraint and tumor growth, considerable work remains to be done to determine the most  
291 effective way to exploit this feature of pancreatic cancer.

## 292 **Materials and Methods**

### 293 **Cell Culture**

294 MiaPaCa2 and HPAC were obtained from ATCC. TU8988T was obtained from DSMZ. Patient-  
295 derived CAFs (58) were a generous gift from Rosa Hwang, and FL5.12 cells were a generous  
296 gift from Dr. Aimee Edinger. All cells were routinely checked for mycoplasma contamination with  
297 MycoAlert PLUS (Lonza) and validated for authenticity annually by STR profiling. Cells were  
298 maintained in standard high glucose DMEM without pyruvate (Gibco) supplemented with 10%  
299 fetal bovine serum (FBS; Corning). GFAT1 null PDA were cultured in standard media  
300 supplemented with 10mM GlcNAc (Sigma). GFAT1 null NAGK knockout PDA were cultured in  
301 standard media supplemented with 10mM GalNAc (Sigma). Low nutrient media was made with  
302 DMEM without glucose, glutamine and pyruvate (Gibco). Glucose, glutamine, and FBS were  
303 added to the final concentration of 1.25mM, 0.2mM and 1%, respectively. FL5.12 cells were  
304 maintained in RPMI 1640 (Gibco) supplemented with 10% FBS, 10mM HEPES (Sigma), 55μM

305 β-mercaptoethanol (Sigma), antibiotics, 2mM glutamine, and 500 pg/mL recombinant murine IL-  
306 3 (Peprotech 213-13).

307 **Generation of CRISPR/Cas9 knockout clones**

308 GFAT1 and NAGK knockout PDA cell lines were generated using CRISPR/Cas9 method  
309 described previously (22). Overlapping oligonucleotides (Feng Zhang lab human GeCKOv2  
310 CRISPR knockout pooled library; Addgene #1000000048) were annealed to generate sgRNA  
311 targeting GFAT1 or NAGK. sgRNA was cloned directly into the overhangs of PX459 V2.0 vector  
312 (Feng Zhang lab; Addgene plasmid #62988) that was digested with BbsI. The resulting  
313 CRISPR/Cas9 plasmid was transformed into chemically competent Stbl3 cells, miniprepped for  
314 plasmid DNA, and sequence-verified. sgRNA oligonucleotide pairs for GFAT1 (10) and NAGK  
315 are as follows: GFAT1 (sg1 Fwd 5'-CACCgCTTCAGAGACTGGAGTACAG-3'; sg1 Rev 5'-  
316 AACCTGTACTCCAGTCTGAAGC-3') and NAGK (sg1 Fwd 5'-  
317 CACCgTAGGGGAGGCACACGATCCG; sg1 Rev 5'-AACCGGATCGTGTGCCTCCCTAc-3';  
318 sg2 Fwd 5'-CACCgGCCTAGGGCCTATCTGAG-3'; sg2 Rev 5'-  
319 AACCTCAGAGATAGGCCCTAGGCC-3'). Human PDA were transiently transfected using  
320 Lipofectamine 3000 according to the manufacturer's instructions. Cells were selected with  
321 puromycin in the presence of GlcNAc (GFAT1 knockout bulk population) or GalNAc (GFAT1  
322 NAGK double knockout bulk population). To select clones, polyclonal pools were seeded into  
323 96-well plates at a density of 1 cell per well. Individual clones were expanded and verified via  
324 western blot and Sanger sequencing. TU8988T clone B9 has a 10 base pair (bp) and a 1bp  
325 deletion in GFAT1; TU8988T clone D10 has 2 different 1bp deletions in GFAT1; MiaPaCa2  
326 clone M12 has 2 different 1bp deletions in GFAT1; HPAC clone H1 has a 187bp deletion in  
327 GFAT1; HPAC clone H7 has a 187bp deletion in GFAT1.

328 **Conditioned media**

329 Conditioned media was generated by culturing cells in 15 cm<sup>2</sup> plates (25mL growth media/plate)  
330 for 72 hours at 37°C, 5% CO<sub>2</sub>, so that they reached ~90% confluence. The media were then  
331 filtered through a 0.45μm polyethersulfone membrane (VWR). Boiled conditioned media was  
332 warmed to 100°C for 15 minutes. Freeze-thaw conditioned media were initially stored at -80°C  
333 and were thawed in a 37°C water bath on the day of the experiment. As indicated, fresh growth  
334 media were added to the conditioned media at the ratios indicated to avoid nutrient/metabolite  
335 exhaustion.

336 **Colony formation and proliferation assays**

337 For colony formation assays, cells were plated in a 6-well plate in biological triplicate at 500  
338 cells/well in 2 mL of media and grown for 9-12 days. For proliferation assays, 5000 cells/well  
339 were plated. At end point, assays were fixed with 100% methanol for 10 minutes and stained  
340 with 0.5% crystal violet solution for 15 minutes. Relative colony formation was quantitated  
341 manually in a blinded fashion. Proliferation was quantified by removing the dye with 10% acetic  
342 acid and measuring OD595.

343 **CyQUANT viability assay**

344 Cells were seeded in 96-well black wall, clear bottom plates at 1000 cells/well in 50μL of media  
345 and incubated at 37°C, 5% CO<sub>2</sub> for indicated time points. At each time point, media was  
346 aspirated and plates were stored at -80°C. Proliferation was determined by CyQUANT  
347 (Invitrogen) according to the manufacturer's instructions. SpectraMax M3 Microplate reader  
348 (Molecular Devices) was used to measure fluorescence.

349 **IncuCyte S3: Real-time, live-cell proliferation assay**

350 1000 cells were seeded per well in a 96-well plate and incubated at 37°C, 5% CO<sub>2</sub> for cells to  
351 equilibrate. The next day, media were aspirated, washed once with PBS, and replaced with  
352 different media as indicated. Proliferation was measured on IncuCyte S3 using phase object  
353 confluence as a readout.

354 **Metabolite sample preparation**

355 Intracellular metabolite fractions were prepared from cells grown in 6-well plates. The media  
356 was aspirated, and cells were incubated with cold (-80°C) 80% methanol (1mL/well) on dry ice  
357 for 10 minutes. Then, the wells were scraped with a cell scraper and transferred to 1.5mL tubes  
358 on dry ice. To measure GlcNAc concentration in different conditioned media, 0.8mL of ice-cold  
359 100% methanol was added to 0.2mL of conditioned media, and the mixture was incubated on  
360 dry ice for 10 minutes.

361 After incubation of cell or media fractions on dry ice, the tubes were centrifuged at 15,000rpm  
362 for 10 minutes at 4°C to pellet the insoluble material, and the supernatant was collected in a  
363 fresh 1.5mL tube. Metabolite levels of intercellular fractions were normalized to the protein  
364 content of a parallel sample, and all samples were lyophilized on a SpeedVac, and re-  
365 suspended in a 50:50 mixture of methanol and water in HPLC vials for LC-MS/MS analysis.

366 **Liquid chromatography-coupled mass spectrometry**

367 To detect UDP-GlcNAc, the Shimadzu NEXERA integrated UHPLC system with a LC30AD  
368 pump, SIL30AC autosampler, CTO30A column oven, CBM20A controller was coupled with the  
369 AB Sciex TripleTOF 5600 MS system with DuoSpray ion source. All calibrations and operations  
370 were under control of Analyst TF 1.7.1. Calibrations of TOF-MS and TOF-MS/MS were  
371 achieved through reference APCI source of SCEIX calibration solution. A high throughput LC  
372 method of 8 min with flowrate of 0.4 ml/min with a Supelco Ascentis Express HILIC (75 mm X  
373 3.0 mm, 2.7 µm). Solvent A was made of 20 mM ammonium acetate of 95% water and 5%  
374 acetonitrile at pH 9.0. Solvent B was 95% acetonitrile and 5% water. LC gradient 0.0-0.5 min  
375 90% B, 3 min 50% B, 4.10 min 1% B, 5.5 min 1% B, 5.6 min 90% B, 6.5 min 90% B, 8 min  
376 stopping. Key parameters on the MS were the CE and CE spread of -35ev, 15ev. Data were  
377 compared to a reference standard. Data processing was performed by Sciex PeakView,  
378 MasterView, LibraryView and MQ software tools and ChemSpider database.

379 To measure GlcNAc concentration in the various conditioned media, we utilized an Agilent  
380 Technologies Triple Quad 6470 LC/MS system consisting of 1290 Infinity II LC Flexible Pump  
381 (Quaternary Pump), 1290 Infinity II Multisampler, 1290 Infinity II Multicolumn Thermostat with 6  
382 port valve and 6470 triple quad mass spectrometer. Agilent Masshunter Workstation Software  
383 LC/MS Data Acquisition for 6400 Series Triple Quadrupole MS with Version B.08.02 is used for  
384 compound optimization and sample data acquisition.

385 A GlcNAc standard was used to establish parameters, against which conditioned media were  
386 analyzed. For LC, an Agilent ZORBAX RRHD Extend-C18, 2.1 × 150 mm, 1.8 µm and ZORBAX  
387 Extend Fast Guards for UHPLC were used in the separation. LC gradient profile is: at 0.25  
388 ml/min, 0-2.5 min, 100% A; 7.5 min, 80% A and 20% C; 13 min 55% A and 45% C; 20 min, 1%  
389 A and 99% C; 24 min, 1% A and 99% C; 24.05 min, 1% A and 99% D; 27 min, 1% A and 99%  
390 D; at 0.8 ml/min, 27.5-31.35 min, 1% A and 99% D; at 0.6 ml/min, 31.50 min, 1% A and 99% D;  
391 at 0.4 ml/min, 32.25-39.9 min, 100% A; at 0.25 ml/min, 40 min, 100% A. Column temp is kept at  
392 35 °C, samples are at 4 °C, injection volume is 2 µL. Solvent A is 97% water and 3% methanol  
393 15 mM acetic acid and 10 mM tributylamine at pH of 5. Solvent C is 15 mM acetic acid and 10

394 mM tributylamine in methanol. Washing Solvent D is acetonitrile. LC system seal washing  
395 solvent 90% water and 10% isopropanol, needle wash solvent 75% methanol, 25% water.

396 6470 Triple Quad MS is calibrated with ESI-L Low concentration Tuning mix. Source parameters:  
397 Gas temp 150 °C, Gas flow 10 l/min, Nebulizer 45 psi, Sheath gas temp 325 °C, Sheath gas flow  
398 12 l/min, Capillary -2000 V, Delta EMV -200 V. Dynamic MRM scan type is used with 0.07 min  
399 peak width, acquisition time is 24 min. Delta retention time of plus and minus 1 min, fragmentor  
400 of 40 eV and cell accelerator of 5 eV are incorporated in the method.

#### 401 **Xenograft studies**

402 Animal experiments were conducted in accordance with the Office of Laboratory Animal Welfare  
403 and approved by the Institutional Animal Care and Use Committees of the University of  
404 Michigan. NOD-SCID gamma (NSG) mice (Jackson Laboratory), 6-10 weeks old of both sexes,  
405 were maintained in the facilities of the Unit for Laboratory Animal Medicine (ULAM) under  
406 specific pathogen-free conditions. Protocol#: PRO00008877.

407 Wild type TU8988T and two verified GFAT1 null clones (B9 and D10) were trypsinized and  
408 suspended at 1:1 ratio of DMEM (Gibco, 11965-092) cell suspension to Matrigel (Corning,  
409 354234). 150-200µL were used per injection. Orthotopic tumors were established by injecting  
410 0.5 x 10<sup>6</sup> cells in 50µL of 1:1 DMEM to Matrigel mixture. The experiment lasted five weeks. For  
411 subcutaneous xenograft studies with the pooled populations or validated clones, tumors were  
412 established with 5 x 10<sup>6</sup> cells in 200µL of 1:1 DMEM to Matrigel mixture.

413 Tumor size was measured with digital calipers two times per week. Tumor volume (V) was  
414 calculated as V = 1/2(length x width<sup>2</sup>). At endpoint, final tumor volume and mass were  
415 measured prior to processing. Tissue was snap-frozen in liquid nitrogen then stored at -80°C.

#### 416 **Western blot analysis**

417 After SDS-PAGE, proteins were transferred to PVDF membrane, blocked with 5% milk, and  
418 incubated with primary antibody overnight at 4°C. The membranes were washed with TBST,  
419 incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1hr  
420 and visualized on Bio-Rad imager with enhanced chemiluminescence detection system or  
421 exposed on radiographic film.

#### 422 **Antibodies**

423 The following antibodies were used in this study: VINCULIN (Cell Signaling 13901), ACTIN  
424 (Santa Cruz sc-47778), GAPDH (Cell Signaling 5174), GFAT1 (Abcam 125069), NAGK (Atlas  
425 Antibodies HPA035207), O-GlcNAc (Abcam 2735), secondary anti-mouse-HRP (Cell Signaling  
426 7076), and secondary anti-rabbit-HRP (Cell Signaling 7074).

#### 427 **Detection and quantification of macropinocytosis**

428 The macropinocytosis index was measured as previously described (59). In brief, cells were  
429 seeded on the coverslips in 24-well plate for 24 hours and serum-starved for 18 h. Cells were  
430 incubated with 1mg/ml high molecular weight TMR-dextran (Fina Biosolutions) in serum-free  
431 medium for 30 min at 37 °C. Cells were then washed 5 times with cold DPBS and fixed in 4%  
432 polyformaldehyde for 15 min. The coverslips were mounted onto slides using DAKO Mounting  
433 Medium (DAKO) in which nuclei were stained with DAPI. At least six images were captured for  
434 each sample using an Olympus FV1000 confocal microscope and analyzed using the particle  
435 analysis feature in ImageJ (NIH). The micropinocytosis index for each field was calculated as  
436 follow: Macropinocytosis Index = (total particle area/total cell area) × 100%.

437 **Hyaluronic acid, hyaluronidase, and heparin**

438 Heparin was obtained from Sigma (H3393). Oligo HA (5kDa) was obtained from Lifecore  
439 Biomedical. Two different LMW HA were used in this study: 78 kDa HA (Pure Health solutions)  
440 and 60kDa HA (Lifecore Biomedical). To make 10mM oligo- or LMW HA media, HA was added  
441 slowly into high glucose DMEM without pyruvate, stirred for two hours at room temperature, and  
442 filtered through 0.20 $\mu$ m polyethersulfone membrane. FBS was added to a final concentration of  
443 10%.

444 Hyaluronidase (Sigma H3506) treatment was performed as follows: 10mM LMW HA media and  
445 control media (DMEM + 10% FBS) were incubated with hyaluronidase, according to  
446 manufacturer's instructions, overnight in a 37°C water bath. The next day, media were boiled for  
447 15 minutes to denature hyaluronidase. The resulting media were mixed 1:1 with fresh growth  
448 media to avoid effects of nutrient/metabolite exhaustion.

449 **Preparation of necrotic FL5.12 cells**

450 Necrotic FL5.12 cells were prepared as described previously (33). Cells were washed three  
451 times with PBS, cultured in the FL5.12 media without IL-3 (100 million cells/mL) for 72 hours.  
452 The necrotic cells were spun down at 13,000 rpm for 10 minutes at 4°C, and the pellets were  
453 stored at -80°C until use.

454 **Statistical analysis**

455 Statistics were performed using GraphPad Prism 8. Groups of two were analyzed with two-  
456 tailed students t test. Groups of more than two were analyzed with one-way ANOVA Tukey  
457 post-hoc test. All error bars represent mean with standard deviation. A *P* value of less than 0.05  
458 was considered statistically significant. All group numbers and explanation of significant values  
459 are presented within the figure legends.

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473 **Competing Interests**

474 CAL is an inventor on patents pertaining to Kras regulated metabolic pathways, redox control  
475 pathways in pancreatic cancer, and targeting the GOT1-pathway as a therapeutic approach.  
476 KRK serves on the scientific advisory board of NVision Imaging Technologies.

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## SUPPLEMENTARY FIGURES

### Hyaluronic Acid Fuels Pancreatic Cancer Growth

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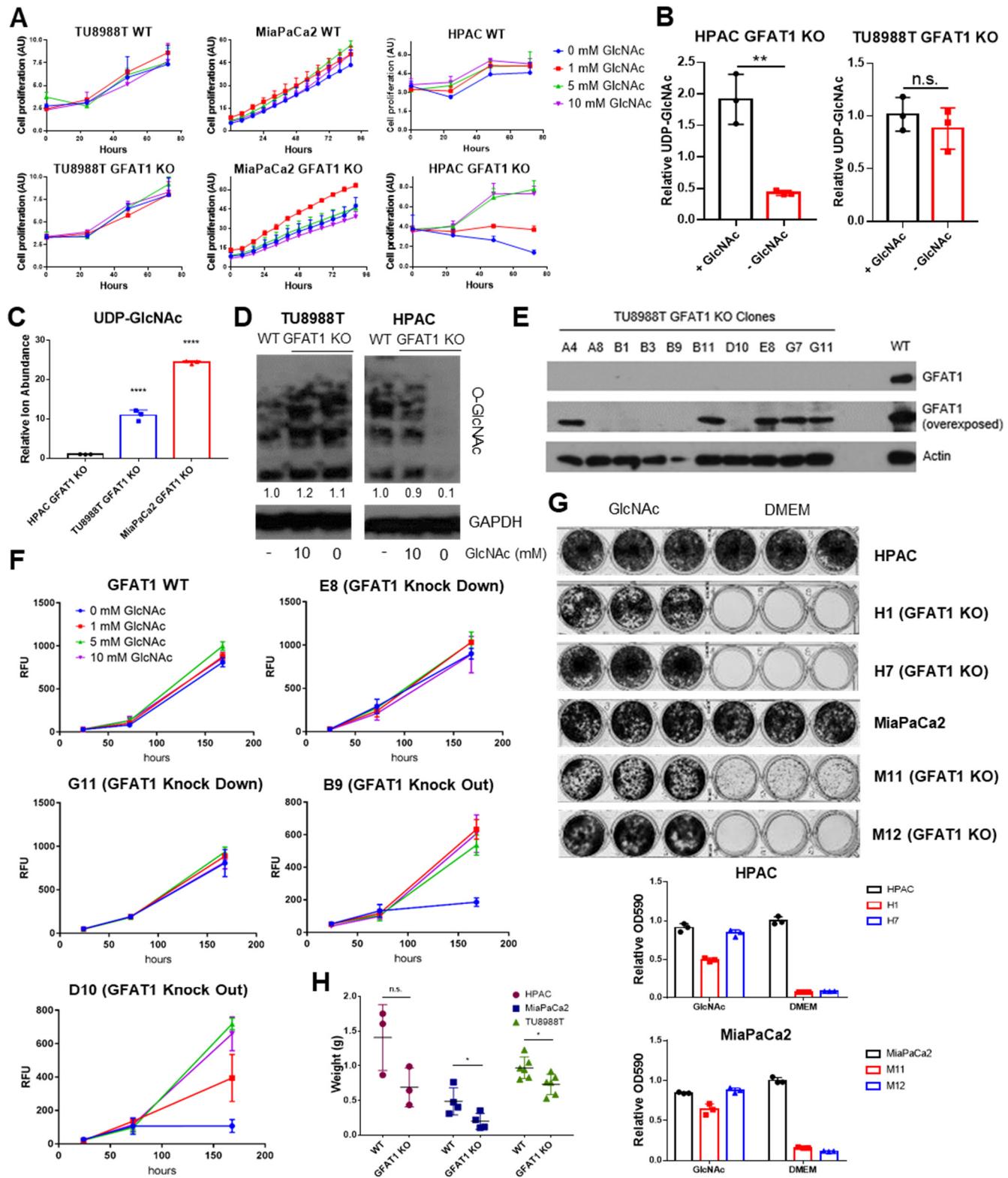
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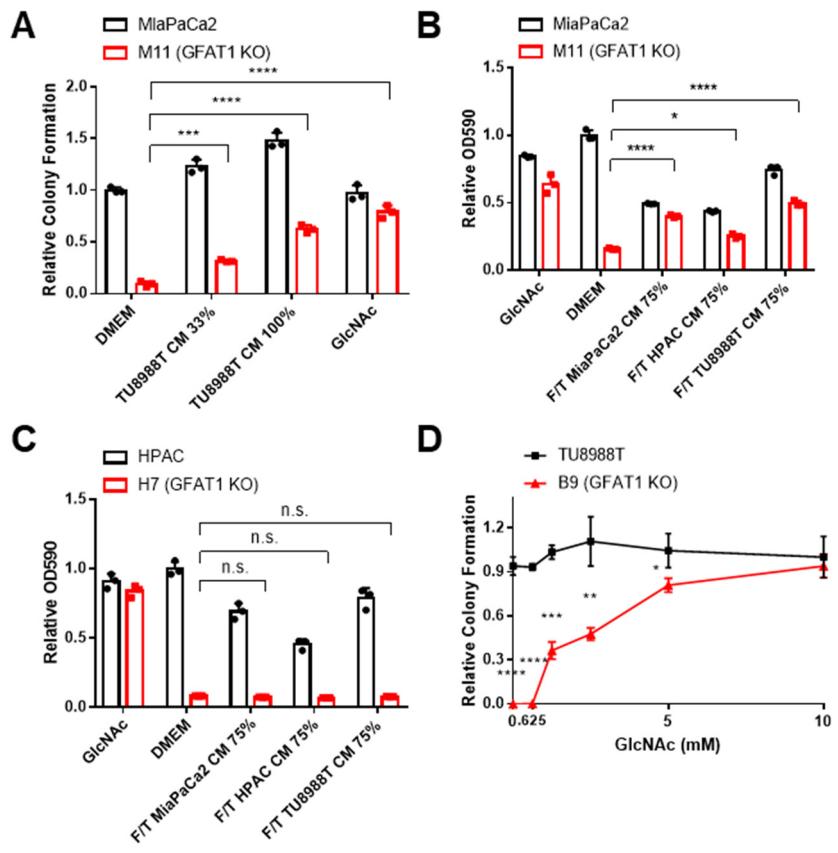
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\* Equal contribution

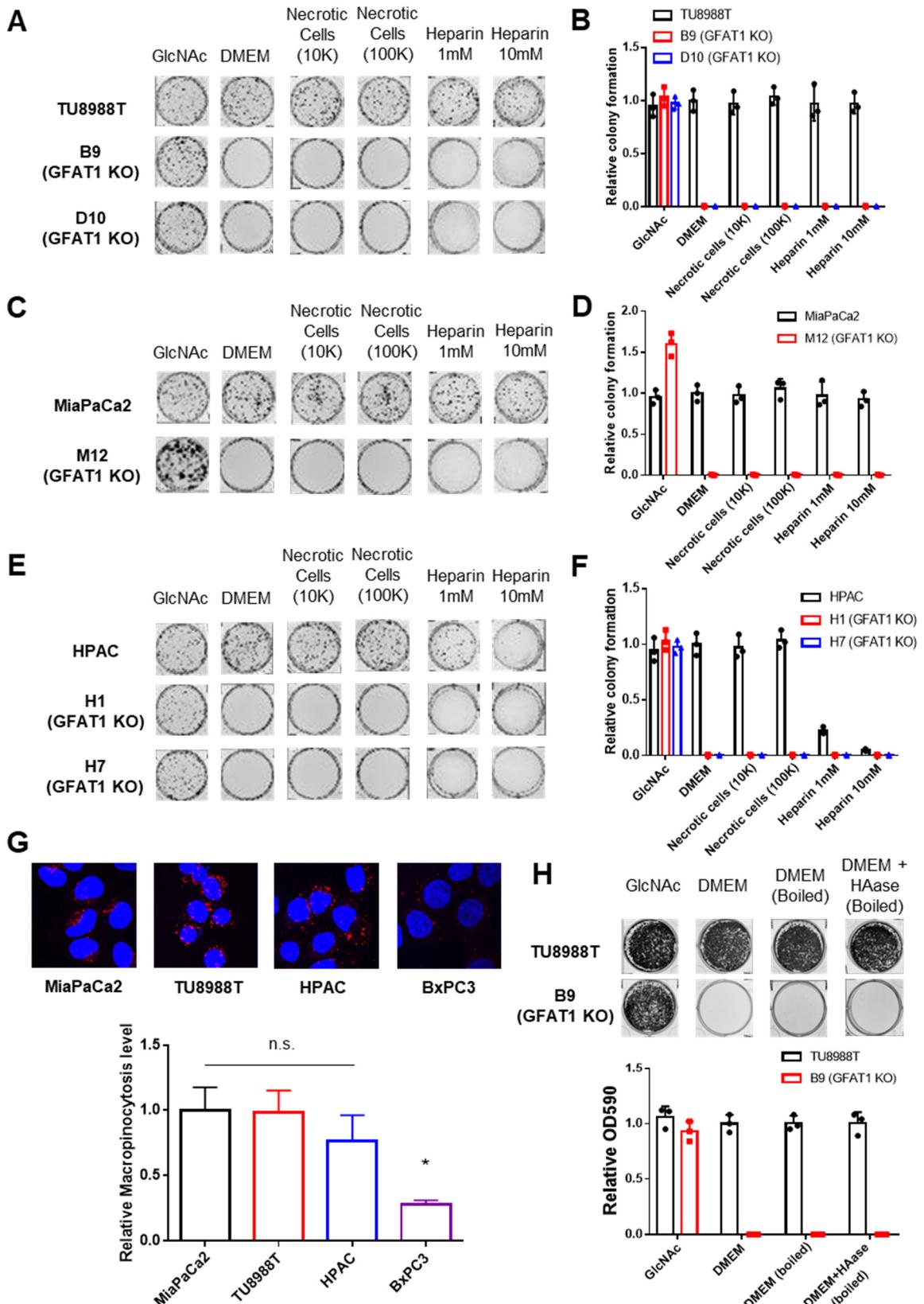
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**Supplementary Figure 1. Additional characterization of GFAT1 knockout PDA populations and clonal lines.** (A) Proliferation kinetics of parental PDA cell lines and corresponding pooled populations of GFAT1 knockout cells supplemented with varying concentrations of GlcNAc (n=3). Cell quantity was assessed by Cyquant (DNA intercalating dye) and plotted in absorbance units (AU). (B,C) UDP-GlcNAc levels measured by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) in (B) TU8988T and HPAC GFAT1 knockout lines in the presence or absence of 10 mM GlcNAc for 3 days, presented as relative abundance (n=3), and (C) TU8988T, HPAC, and MiaPaCa2 GFAT1 knockout cells grown without GlcNAc for 3 days (n=3), presented as relative ion abundance. (D) Western blot of proteome O-GlcNAc and loading control GAPDH in parental and GFAT1 knockout TU8988T and HPAC. GFAT1 knockout lines were grown in the presence or absence of 10mM GlcNAc for 3 days. (E) Western blot for GFAT1, at short and long exposure, and ACTIN loading control in a panel of clonal cell lines selected from the pooled population of GFAT1 knockout TU8988T cells. (F) Proliferation kinetics of parental TU8988T (GFAT1 WT) and clonal cell lines E8, G11, B9, and D10 selected from the pooled GFAT1 knockout population supplemented with varying concentrations of GlcNAc (n=3). Clones correspond to those in the western blot in E. Cell quantity was assessed by cell titer glo and plotted in relative fluorescent units (RFU). (G) Representative wells from proliferation assay in parental and two GFAT1 knockout clonal HPAC and MiaPaCa2 cell lines. At bottom, data are quantitated by crystal violet extraction and measurement of optical density (OD) at 590nm, n=3. (H) Tumors from parental (n=3) and GFAT1 knockout (n=3) HPAC; parental (n=4) and GFAT1 knockout (n=4) MiaPaCa2; and parental (n=6) and GFAT1 knockout (n=6) TU8988T cell lines grown subcutaneously in immunocompromised mice. Error bars represent mean  $\pm$  SD. n.s., non-significant; \*P < 0.05; \*\* P <0.01; \*\*\*\* P <0.0001.



**Supplementary Figure 2. Characterization of rescue activity of conditioned media and GlcNAc.** **(A)** Quantitation of colony forming assay data of parental MiaPaCa2 and GFAT1 knockout clonal line M11 in base media (DMEM), positive control GlcNAc, wild type TU8988T CM diluted 1:2 (33%) or used directly (100%) (n=3). **(B,C)** Quantitation of proliferation assay data of **(B)** parental MiaPaCa2 and GFAT1 knockout clonal line M11 and **(C)** parental HPAC and GFAT1 knockout clonal line H7 in base media (DMEM), positive control GlcNAc, or wild type TU8988T, HPAC, or MiaPaCa2 CM diluted 3:1 (75%) that was subjected to freeze-thaw (F/T) (n=3). Data represent crystal violet extracted from cells at endpoint and measured by optical density (OD) at 590nm. **(D)** GlcNAc dose response curve presented as relative colony number for parental TU8988T cells and GFAT1 knockout clonal line B9 (n=3). Error bars represent mean  $\pm$  SD. n.s., non-significant; \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*\*P < 0.0001.



**Supplementary Figure 3. Characterization of macropinocytosis and glycosaminoglycan rescue activity in PDA and GFAT1 knockout cells.** (A-F) Representative colony formation assays and their quantitation following treatment with two concentrations of heparin or necrotic cell debris that contain complete cellular contents, relative to base media (DMEM) and positive control GlcNAc in (A,B) parental and GFAT1 knockout TU8988T, (C,D) parental and GFAT1 knockout MiaPaCa2, and (E,F) parental and GFAT1 knockout HPAC. (G) Immunostaining images of intracellular fluorescently-tagged dextran (red) engulfed by macropinocytosis in PDA cell lines. Nuclear DAPI staining in blue. Quantitation of macropinocytotic index presented at bottom for n=6 wells per biological replicate (n=3). (H) Representative wells from a proliferation assay in parental TU8988T and GFAT1 knockout clone B9 in 10mM GlcNAc, base media (DMEM), base media supplemented 1:1 with boiled DMEM, or base media supplemented 1:1 with boiled HAase-treated DMEM. Data are quantitated below and represent crystal violet extracted from cells at endpoint and measured by optical density (OD) at 590nm (n=3). Error bars represent mean  $\pm$  SD. n.s., non-significant; \*P < 0.05.