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**Citation:** Noble, R. ORCID: 0000-0002-8057-4252, Walther, V., Roumestand, C., Hochberg, M. E., Hibner, U. and Lassus, P. (2021). Paracrine behaviors arbitrate parasite-like interactions between tumor subclones. *Frontiers Ecology and Evolution*, doi: 10.3389/fevo.2021.675638

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1 **Paracrine behaviors arbitrate parasite-like interactions between**  
2 **tumor subclones**

3

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## 24 **Abstract**

25 Explaining the emergence and maintenance of intratumor heterogeneity is an  
26 important question in cancer biology. Tumor cells can generate considerable subclonal  
27 diversity, which influences tumor growth rate, treatment resistance, and metastasis,  
28 yet we know remarkably little about how cells from different subclones interact. Here,  
29 we confronted two murine mammary cancer cell lines to determine both the nature and  
30 mechanisms of subclonal cellular interactions *in vitro*. Surprisingly, we found that,  
31 compared to monoculture, growth of the 'winner' was enhanced by the presence of the  
32 'loser' cell line, whereas growth of the latter was reduced. Mathematical modeling and  
33 laboratory assays indicated that these interactions are mediated by the production of  
34 paracrine metabolites resulting in the winner subclone effectively 'farming' the loser.  
35 Our findings add a new level of complexity to the mechanisms underlying subclonal  
36 growth dynamics.

37

## 38 **Introduction**

39 Considering tumors as complex ecosystems has led to the notion that diverse cancer  
40 cell subclones engage in heterotypic interactions reminiscent of those that operate in  
41 organismal communities (Heppner, 1984; Merlo et al., 2006; Axelrod et al., 2006;  
42 Tabassum and Polyak, 2015). Mutually negative interactions are thought to be  
43 ubiquitous in cancer (Nowell, 1976; Greaves & Maley, 2012). As in classic ecosystems,  
44 cancer cells compete for nutrients and space, and competition between emergent  
45 subclones gives rise to complex temporal and spatial dynamics of tumor composition  
46 and growth (Tabassum and Polyak, 2015). Positive ecological interactions (mutualism  
47 and commensalism) have been observed in cancer models in mice (Calbo et al., 2011;  
48 Cleary et al., 2014) and in drosophila (Ohsawa et al., 2012). In these cases, one  
49 subclone acquires new abilities, such as the capacity to grow or metastasize, only in  
50 the presence of another subclone, resulting in the tumor as a whole progressing  
51 towards a more aggressive phenotype. In contrast, the prevalence within tumors of  
52 asymmetric interactions such as amensalism, parasitism and facilitation remains an  
53 open question. Defining the mechanisms of tumor ecology is essential for a better  
54 understanding of cancer progression and may lead to novel therapeutic strategies  
55 (Gatenby and Brown, 2017; Maley et al., 2017).

56 To gain insight into molecular and cellular events related to ecological interactions  
57 between cancer subclones, we took advantage of a model described over three  
58 decades ago, based on two closely related murine cancer cell lines derived from a  
59 single spontaneous mouse mammary tumor (Dexter et al. 1978; Miller et al., 1988).  
60 When cultured separately, the two cell lines have similar growth rates, yet in co-culture  
61 one cell line (the 'winner') expands at the expense of the other (the 'loser'). Our careful  
62 re-examination of this model, combining experiments with mathematical modelling and  
63 parameter inference, indicated that the cellular behaviors of the two subclones are  
64 surprisingly sophisticated. Both cell lines produce paracrine metabolites that boost  
65 proliferation of the winner and also decrease the growth rate of the loser. Our results

66 thus unveil a type of facultative parasitic behavior of the winner subclone. We further  
67 identified beta-hydroxybutyrate and lactate as metabolites that contribute to these  
68 phenotypes and characterized their modes of action. We discuss our results in the  
69 context of how previously underappreciated ecological interactions may contribute to  
70 the complexity of tumor growth dynamics.

71

## 72 **Results**

### 73 *4T07 cells have a "winner" phenotype*

74 Two cell lines derived from a single mouse mammary carcinoma - 168 and 4T07 cells  
75 - have similar growth rates when cultured individually, yet the 4T07 clone displays a  
76 dominant phenotype when grown together, either in cell culture or in orthotopic  
77 allografts *in vivo* (Miller et al., 1988). Several hypotheses to account for this interesting  
78 behavior had been tested in the original work, but the precise mechanism behind these  
79 competitive interactions has so far not been identified.

80 We began by verifying that in our hands the lines maintain their competitive  
81 characteristics. To facilitate lineage tracing we first generated lines stably expressing  
82 GFP, the expression of which did not alter cell growth (Figure 1A). Next, we followed  
83 growth characteristics of 4T07 and 168FARN cells, the latter being a drug-resistant  
84 derivative of the original 168 clone (Aslakson et al., 1991), in a continuous culture for  
85 3 weeks. The cells were seeded as 1:1 mix at a density that allowed them to reach  
86 confluence within 3-4 days, at which point they were harvested and re-seeded in a new  
87 well at the original density. Remaining cells were analyzed by flow cytometry to  
88 determine the proportion of GFP expressing clones in the expanding population.

89 The homotypic co-culture (same line with and without GFP) confirmed that GFP has  
90 no impact on cellular proliferation (Figure 1B and Figure 3B). In contrast, heterotypic  
91 co-culture conditions (two different lines, one expressing GFP) revealed the  
92 dominance of the 4T07 clone (Figure 1B and Figure 3B).

93 These results confirm the originally described ecological interaction between the  
94 clones: 4T07 gradually dominates the culture while the 168FARN cells become scarce  
95 within 15-17 days. Importantly, the dominant phenotype is independent of the starting  
96 ratio between the two cell lines (Supplementary Figure 1A and B).

97

### 98 *Co-culture alters the proliferation rates of both "winner" and "loser" cells*

99 As originally discussed for the two clones under study (Miller et al., 1988), the  
100 expansion of a single clone in co-culture could be due to alterations in cell death or  
101 changes in the proliferation rates of either or both clones. We measured apoptosis in  
102 the loser 168FARN clone and found identical, very low levels of cell death under  
103 homotypic and heterotypic conditions (Supplementary Figure 2A). Next, we used time-  
104 lapse microscopy to assess the growth dynamics of both clones in continuous culture.  
105 The cells were seeded at a density that allowed reaching confluence in 4 days and

106 were photographed every 45 minutes for the last 3 days. We measured the overall  
107 pixel intensity for each frame (Figure 2A) as a proxy for the growth rate of the  
108 fluorescently tagged cell line. This analysis revealed that under co-culture conditions,  
109 the growth rate of 168FARN decreased, whereas that of 4T07 increased relative to  
110 mono-cultures. To test whether increased net growth of the winner population is due  
111 to the alteration of proliferation, we estimated the proportion of cells in the S phase of  
112 the cell cycle by performing pulse-chase EdU staining. The results presented in  
113 Supplementary Figure 2B confirmed that heterotypic co-culture gave rise to significant  
114 decrease in cells actively replicating DNA for the loser clone and a significant increase  
115 in the winner clone. Overall, these results suggest that the dominant phenotype  
116 displayed by the winner cells in co-culture can be explained by changes in proliferation  
117 that operate in opposing directions on the winner and the loser cells.

118

### 119 *Mathematical modelling and inference of evolutionary parameter values*

120 To gain further insight into the ecological interactions between the winner and loser  
121 cell types we turned to mathematical modelling. Examination of the growth curves  
122 revealed two distinct phases of evolutionary dynamics (Figure 2A and 2B). In phase  
123 1, from 0 to 45 hours, the two cell types grew exponentially in both homotypic and  
124 heterotypic cultures, and the growth rate of 168 was higher than that of 4T07. This  
125 first phase can be regarded as a transition period before the cells start altering and  
126 responding to their new environment. By contrast in phase 2, from 45 to 72 hours,  
127 the growth curves were strongly affected by interactions within and between the two  
128 cell types, and 4T07 grew faster than 168. **To enable us to determine the mode of the  
129 ecological dynamics in each phase, we opted for a parsimonious, piecewise  
130 mathematical model. Specifically, we** assumed a model with exponential growth in  
131 phase 1 and a transition to density-dependent competitive Lotka-Volterra-type  
132 dynamics in phase 2.

133

134 By fitting our model to the homotypic growth curves, we inferred the values of the phase  
135 1 and phase 2 growth rates and the within-type interaction parameters (Methods). To  
136 infer the between-type interactions, we used additional data from 72-hour competition  
137 assays, covering a wide range of initial ratios of the two cell types. Although this latter  
138 data set comprises only the initial and final proportions (at the beginning of phase 1  
139 and the end of phase 2), we were able to infer the proportions at the beginning of phase  
140 2 by adjusting for the exponential growth of both types during phase 1. We then used  
141 these inferred proportions and our previously inferred parameter values to estimate the  
142 remaining interaction parameters (Methods). The resulting model gives a good fit to  
143 the competition assay data (Figure 3A, first column) and is consistent with heterotypic  
144 time-lapse data not used for parameter inference (Figure 2; Supplementary figure 6).

145

146 The inferred parameter values (Table 1) imply that during phase 2, 4T07 has a large  
147 negative effect on both itself and on 168, consistent with 4T07 producing a harmful  
148 diffusible factor. The negative effect of 168 on itself is only about half as large, and 168

149 has approximately zero net effect on the growth of 4T07. This suggests that ubiquitous  
150 negative effects of 168 on 4T07 (e.g., likely due to waste products and competition for  
151 resources) are offset by positive effects, such as due to a beneficial diffusible factor.  
152 Also, during phase 2, the intrinsic growth rate of 168 (that is, the inferred growth rate  
153 before accounting for cell-cell interactions) is approximately 30% lower than that of  
154 4T07, consistent with the conventional hypothesis that producing beneficial factors is  
155 costly. This disadvantage is offset by 168 having an approximately 30% higher carrying  
156 capacity (defined as the upper limit of the homotypic population size). Over phase 2,  
157 or any longer period that includes phase 2, the inferred net growth rate of 4T07 (that  
158 is, the growth rate after accounting for cell-cell interactions) is invariably higher than  
159 that of 168, which means 4T07 will come to dominate numerically, no matter their initial  
160 frequency.

161  
162 Since we also conducted 96-hour competition assays, we were able to infer the  
163 population dynamics during a third phase (72-96 hours). For every initial ratio of the  
164 two cell types, the growth rate difference (also known as the gain function) was on  
165 average lower in the 96-hour than in 72-hour competition assays (Supplementary  
166 figure 5). Moreover, this difference did not depend on the initial ratio, which implies it  
167 was not caused by a change in interaction parameters. A parsimonious way to account  
168 for this effect is to assume a reduction in 4T07's intrinsic growth rate during phase 3,  
169 as would be expected to result from starvation and/or the build-up of toxic waste  
170 products. Making this adjustment to our model indeed produces a better fit to the  
171 competition assay data (Figure 3A, middle column; Figure 3B and 3C). The predicted  
172 dynamics are shown in Figure 2C and 2D.

173  
174 Finally, having inferred all the evolutionary parameter values, we calculated net growth  
175 rates of the two cell types, averaged over different time periods. Over any period that  
176 includes phase 2, our model predicts that the net growth rate of both cell types will  
177 decrease non-linearly with increasing initial 4T07 frequency (pink and blue curves in  
178 Figure 3A). However, the net growth rate of 4T07 decreases faster than that of 168,  
179 which is why the gain function (grey curve in Figure 3A) also decreases. In phase 3, if  
180 the initial proportion of 4T07 is high (above 70%), then 168 has a higher net growth  
181 rate than 4T07, but in this case both of the inferred net growth rates are negative.  
182 Overall, the interactions are effectively equivalent to those of a parasite and its host,  
183 such that the 'loser' 168 suffers from the presence of the 'winner' 4T07, while also  
184 enhancing the winner's fitness.

185  
186  *$\beta$ -hydroxybutyrate secreted by the loser clone stimulates winner clone proliferation*

187 To identify the molecular mechanisms at the basis of the altered growth of winners and  
188 losers when in co-culture, we first focused on the increase in proliferation rate of 4T07  
189 cells. Heterotypic culture experiments performed at low cell density suggested that the  
190 dominant effect did not require extensive cell-cell contacts (Supplementary Figure 3).

191 We reasoned that a soluble factor secreted by 168FARN could induce a proliferation  
192 boost in 4T07. To test this hypothesis, we collected conditioned media from each line  
193 cultured for three days and used each medium separately to grow 4T07 for an  
194 additional 24 hrs. As controls, we either left the 4T07 medium after the three days of  
195 conditioning or replaced it with fresh medium. The results shown in Figure 3A confirm  
196 our hypothesis: the medium conditioned by 168FARN induced a significant increase in  
197 4T07 proliferation. Importantly, this effect was not due to differences of medium  
198 exhaustion by the two cell lines, since the addition of fresh medium did not boost 4T07  
199 proliferation.

200 Since our data strongly suggested that a soluble factor originating from 168FARN  
201 accounted for the increase in 4T07 proliferation, we next sought to define its molecular  
202 nature. First, we separated the 168FARN-conditioned medium into high and low MW  
203 fractions with a 3 KDa molecular cutoff column. The low MW fraction contains mainly  
204 metabolites while the high one is enriched in proteins. After complementing each  
205 fraction, respectively, with 10% serum or with DMEM to obtain full media conditioned  
206 with either low or high MW secretomes, we used them in a proliferation assay as in  
207 Figure 4A. The results (Figure 4B) of this series of experiments unambiguously  
208 identified the low MW fraction of the 168FARN-conditioned medium as the source of  
209 the pro-proliferative factor. To further explore its identity, we employed nuclear  
210 magnetic resonance spectroscopy to compare the composition of low MW fractions  
211 prepared from fresh medium and from the 168FARN- and 4T07-conditioned ones  
212 (Henke et al., 1996). Two major peaks specific for the conditioned media corresponded  
213 to a very strong signal for lactate secreted by 4T07 cells, and a significant increase in  
214 a peak identified as  $\beta$ -hydroxybutyrate in the 168FARN-conditioned medium (Figure  
215 5A).  $\beta$ -hydroxybutyrate (BHB) is a ketone body mainly produced by the liver after long  
216 fasting periods and which is used by different tissues as a source of carbon to  
217 supplement the lack of glucose (Newman and Verdin, 2017). In addition,  $\beta$ -  
218 hydroxybutyrate is also produced by other cell types, such as adipocytes or cancer  
219 cells (Grabacka et al., 2016; Huang et al., 2017; Wang et al., 2017). To confirm the  
220 NMR-based identification of the  $\beta$ -hydroxybutyrate peak, we employed an enzymatic  
221 assay to measure  $\beta$ -hydroxybutyrate concentration in conditioned media from 4T07  
222 and 168FARN (Figure 5B). The results were in perfect agreement with the NMR  
223 analysis:  $\beta$ -hydroxybutyrate production is significantly higher in the loser than in the  
224 winner cell clone. To test whether this metabolite was indeed responsible for the  
225 increased proliferation of 4T07, we next complemented the medium of exponentially  
226 growing 4T07 cells with purified  $\beta$ -hydroxybutyrate. As shown in Figure 5C,  $\beta$ -  
227 hydroxybutyrate increased the 4T07 proliferation rate to a level comparable to that  
228 obtained with the 168-conditioned medium. We thus conclude that loser cells increase  
229 the winner's growth rate through the secretion of  $\beta$ -hydroxybutyrate.

230

231 *Presence of the winner clone stimulates  $\beta$ -hydroxybutyrate production by loser cells*



232 After assessing  $\beta$ -hydroxybutyrate production in homotypic cell culture, we evaluated  
233 its secretion under heterotypic conditions. We grew 168FARN alone or together with  
234 4T07 at a 1:1 ratio, maintaining the overall cell density constant. Surprisingly, despite  
235 the fact that under heterotypic conditions there are at least 50% fewer loser cells (which  
236 are the main producers of  $\beta$ -hydroxybutyrate, *cf.* Fig. 5B), the overall level of secreted  
237  $\beta$ -hydroxybutyrate was higher than in the homotypic culture (Figure 5D). This suggests  
238 that either the presence of 4T07 increased the production of the metabolite by  
239 168FARN or, alternatively, that it was 4T07 that produced more metabolite when grown  
240 in the presence of 168FARN. To distinguish between these hypotheses, we cultured  
241 both lines individually for three days, measured BHB concentration, and then  
242 exchanged the culture medium and quantified metabolite synthesis 24 hours later. The  
243 quantification of  $\beta$ -hydroxybutyrate produced over the last day (Day 4 BHB  
244 concentration minus Day 3 BHB concentration) shows that the 168FARN-conditioned  
245 medium had no effect on BHB secretion by 4T07 cells. In striking contrast, the  
246 production of the metabolite by 168FARN more than doubled under the influence of  
247 the 4T07-conditioned medium (Figure 5E). Thus, the winner cells stimulate the losers  
248 to produce a metabolite that boosts the former's proliferation

249

#### 250 *Mechanism of $\beta$ -hydroxybutyrate action*

251 We next asked about the mode of action of BHB on the 4T07 cells.  $\beta$ -hydroxybutyrate  
252 can be imported by four monocarboxylate transporters of the SLC16A gene family, the  
253 expression of which varies in different cell types. We assessed the expression of each  
254 transporter by RT-QPCR and found that MCT2, MCT3 and MCT4 were barely  
255 expressed while MCT1 was highly expressed (Figure 6A) in 4T07 cells. This result  
256 suggests that MCT1 is likely responsible for the import of BHB in this cell line.  
257 Interestingly, we found that MCT1 is three times more expressed in 4T07 than in 168  
258 cells (which, like 4T07, do not express the other MCTs - Supplementary figure 4A),  
259 suggesting that the winner cells are more efficient at taking up this metabolite than the  
260 losers (Supplementary Figure 4B). Finally, incubation of 4T07 with BHB upregulates  
261 MCT1, consistent with a positive feedback loop that could increase the transport of this  
262 ketone body into the dominant cell line (Supplementary figure 4C).

263  $\beta$ -hydroxybutyrate can be metabolized and used as a nutrient to replace glucose  
264 (Newman and Verdin, 2017). Experiments presented in Figure 3A show that fresh  
265 medium added at day 3 did not boost cell proliferation, suggesting that in this  
266 experimental setup the decrease in the carbon source is not a limiting factor for growth.  
267 It is thus unlikely that  $\beta$ -hydroxybutyrate is used as an energy resource to increase  
268 proliferation rate.  $\beta$ -hydroxybutyrate has previously been identified as an inhibitor of  
269 class I histone deacetylases (HDAC) that modulates the expression of genes involved  
270 in reactive oxygen species detoxification (Shimazu et al., 2013). Subsequently, another  
271 group found that adipocytes use  $\beta$ -hydroxybutyrate to modulate the expression of a  
272 subset of genes involved in the growth of breast cancer cells (Huang et al., 2017). We  
273 thus hypothesized that  $\beta$ -hydroxybutyrate might increase the growth rate of winners

274 through the inhibition of HDACs, thereby modulating the expression of genes involved  
275 either in ROS detoxification or in the induction of pro-proliferative factors. In support of  
276 this idea, incubation of 4T07 cells either with 168FARN-conditioned medium or with  
277 purified BHB increased H3K9 acetylation, albeit to a lesser extent than butyrate, a bona  
278 fide HDAC inhibitor (Figure 6B).

279 While we could not detect in 4T07 cells any modification of expression of ROS  
280 detoxification genes reported to be regulated by  $\beta$ -hydroxybutyrate in other cellular  
281 models (Shimazu et al., 2013), both  $\beta$ -hydroxybutyrate and 168-conditioned medium  
282 led to significant transcriptional activation of interleukine 11 (IL-11) and lipocalin 2  
283 (LCN2) (Figure 6C). Both genes have been previously described to promote cancer  
284 cell growth and to be regulated by  $\beta$ -hydroxybutyrate through its action on HDAC  
285 activity (Grivennikov, 2013; Huang et al., 2017; Yang and Moses, 2009). Thus, our  
286 data point to the molecular mechanisms involving direct proliferation signaling.

287

### 288 *Lactate secretion slows down loser cell proliferation*

289 In addition to the positive effect of the 168FARN cells on the proliferation rate of the  
290 4T07 clone, the data shown in Figure 2 indicate that the latter negatively influences the  
291 168FARN growth dynamics. The NMR analysis highlighted strong lactate production  
292 (see Figure 5A). This is consistent with our observation of the media color change  
293 during culture of the two lines, indicating that the winner clone has a glycolytic type of  
294 glucose metabolism leading to a rapid medium acidification in culture. Because  
295 extracellular acidification can be detrimental for cell growth, we next asked if 168FARN  
296 were particularly sensitive to such growth conditions. We quantified medium  
297 acidification by seeding cells at different densities and measuring the extracellular pH  
298 after 3 days of culture (Figure 7A). As expected, we found that 4T07 cells acidify the  
299 medium faster and attain a lower pH during culture compared to 168FARN cells.  
300 Indeed, pH ranged from 6.94 $\pm$ 0.005 (lowest density) to 6.79 $\pm$ 0.003 (highest density)  
301 for the winner line and from 7.38 $\pm$ 0.008 to 6.92 $\pm$ 0.006 for 168FARN. To test whether  
302 4T07-mediated extracellular acidification influenced 168FARN growth, we set up a  
303 proliferation assay for 168FARN cells grown in medium conditioned by the low and the  
304 high density grown 4T07 cells. To control for the effect of pH in the conditioned media,  
305 we included a treatment in which the medium from 4T07 was buffered at pH 7.0 by  
306 sodium bicarbonate. These experiments revealed that the medium from the low density  
307 4T07 cells (pH 6.94) had no effect on 168FARN proliferation. In contrast, the medium  
308 from the high density 4T07 (pH 6.79) drastically decreased the 168FARN growth rate.  
309 Moreover, buffering the same medium at pH 7.0 restored the proliferative capacity of  
310 168FARN culture (Figure 7B). We conclude that the loser clone is indeed highly  
311 sensitive to medium acidification. Taken together our data suggest that the decrease  
312 in the growth rate of 168FARN observed in heterotypic conditions is triggered by 4T07  
313 mediated extracellular acidification.

314

## 315 Discussion

316 Heterogeneity is a ubiquitous feature of tumors that influences growth and metastasis,  
317 and thus the potential for therapeutic success. Ecological interactions between  
318 subclones are key to the emergence of this heterogeneity, yet only few empirical  
319 studies have characterized the nature of these interactions or their underlying  
320 mechanisms. These include commensal (Kaznatcheev et al. 2019; Farrokhian et al.  
321 2020) and cooperative (Cleary et al. 2014) interactions *in vitro*, and how such  
322 interactions can drive tumor invasion (Chapman et al. 2014) and metastasis *in vivo*  
323 (Janiszewska et al. 2019; Naffar-Abu Amara et al. 2020).

324 Our study extends previous work (Robinson and Jordan 1989; Marusyk et al. 2014;  
325 Archetti et al. 2015) by demonstrating that two cell lines derived from the same tumor  
326 exhibit a sophisticated relationship, whereby one (the 'winner') effectively farms the  
327 population of the other (the 'loser'). We further identified key metabolites ( $\beta$ -  
328 hydroxybutyrate and lactate) that regulate these interactions between the winning and  
329 losing clones. Similar to Archetti et al (2015), we found that exploitative clonal  
330 interactions evolve through time, but whereas these authors observed a frequency-  
331 dependent change that could explain clonal coexistence, we were unable to detect this  
332 effect. Simple mathematical analysis within the framework of evolutionary game theory  
333 **nevertheless shows that, when accounting for microenvironmental heterogeneity, our**  
334 **inferred parameter values are plausibly consistent with** long-term clonal coexistence  
335 (Methods).

336 **Because our** *in vitro* experiments simplify the diverse, complex interrelationships that  
337 predominate in spatially complex microenvironments, **the parameter values we have**  
338 **inferred may not precisely translate to *in vivo* contexts.** For example, the scenario of  
339 our experimental model, which depends on microenvironmental acidification by the  
340 winner clone, may be less relevant to micrometastases that are small enough to  
341 maintain physiological pH (De Palma et al., 2017; Beckman et al., 2020). On the other  
342 hand, there is an overwhelming consensus that in larger tumors (both primary and  
343 metastatic), neoangiogenesis produces abnormal, leaky vessels that give rise to poor  
344 oxygenation and acidic conditions (De Bock et al., 2011), consistent with our  
345 experimental system. That paracrine signaling is responsible for the effects we  
346 observed between winner and loser cell lines suggests that the spatial arrangement of  
347 these cells could be crucial to their growth and relative frequencies *in situ* (Archetti et  
348 al 2015). The effect of spatial structure would depend on the typical distance that  
349 secreted molecules travel through the complex tumor microenvironment. Our results  
350 indicate that areas of contact or close proximity between the two subclones will grow  
351 faster and therefore come to dominate spatially isolated populations, producing what  
352 is effectively a mixed 4T07-168FARN 'phenotype'. The actual spatial arrangement of  
353 these two subclones in the original tumor is unknown, but the authors of the study  
354 originally isolating these cell lines note that they may represent only a small sample of  
355 the tumor's diversity (Dexter et al., 1978). A growing body of evidence suggests that  
356 single, site-specific biopsies may be of little use in quantifying spatial heterogeneity,  
357 due to the multiscale (local, regional, metastatic) nature of tumor evolution

358 (Amirouchene-Angelozzi et al., 2017). Computational modeling indicates that the  
359 range of cell-cell interaction and the mode of cell dispersal are crucial factors  
360 determining the pattern of intratumor heterogeneity and associated characteristics of  
361 tumor growth and evolutionary potential (Noble et al., 2020; Waclaw et al., 2015). While  
362 a comprehensive description of intra-tumoral ecological interactions is a daunting task,  
363 beyond the power of existing technology, a fuller understanding of their general  
364 features is essential for devising therapies aimed at rendering cancer a chronic,  
365 controllable disease (Gatenby & Brown, 2020; Viossat & Noble, 2021).

366 We find that the complex interactions between the 4T07 and 168FARN cells are  
367 governed by paracrine signaling emanating from both clones. This mechanistic  
368 conclusion differs from the original observations reported by Miller et al (Miller et al.,  
369 1988). Indeed, in the original publication the results concerning the inhibitory effect of  
370 4T07 conditioned media on 168 cells were inconclusive. This apparent discrepancy  
371 could be due to slightly different culture conditions used in the two sets of experiments.  
372 Indeed, the medium acidification due to the lactate release by the 4T07 that is  
373 responsible for slowing down the growth of 168 cells reaches the required threshold  
374 value only after prolonged culture (3-4 days under our experimental conditions). It is  
375 thus possible that in the original report the culture time and/or the cell density were  
376 insufficient for the clear visualization of the paracrine effect of the winners on the losers.  
377 Moreover, Miller et al. did not investigate the paracrine effect exerted by the 168 on  
378 the 4T07 cells. Our results are the first to show the reciprocal effects of both cell lines  
379 on each other, thus highlighting the complexity of their mutual interactions.

380 We have identified a ketone body,  $\beta$ -hydroxybutyrate, which is produced by loser cells  
381 and acts to increase the growth rate of winner cells. Mechanistically, the competitive  
382 advantage afforded by  $\beta$ -hydroxybutyrate to the winner clone appears to be mediated  
383 through the HDAC-controlled activation of a genetic program that boosts its  
384 proliferation. Ketone bodies are small lipid-derived molecules, physiologically  
385 produced by the liver and distributed via the circulation to metabolically active tissues,  
386 such as muscle or brain (Newman and Verdin, 2017), where they serve as a glucose-  
387 sparing energy source in times of fasting or prolonged exercise. Recently, several  
388 studies reported that cell types such as adipocytes, intestinal stem cells or cancer cells  
389 originating from colorectal carcinoma or melanoma can also produce  $\beta$ -  
390 hydroxybutyrate (Cheng et al., 2019; Grabacka et al., 2016; Huang et al., 2017;  
391 Shakery et al., 2018). Our results identifying  $\beta$ -hydroxybutyrate as a signaling molecule  
392 involved in intra-tumoral clonal interactions fall into the general category of these novel  
393 roles for ketone bodies in cell communication.

394 However, the link between ketone bodies and tumor development remains  
395 controversial. On the one hand, it was shown that ketonic diet slows down tumor  
396 development in brain cancer mice models (Poff et al., 2013, 2014). On the other hand,  
397 our results together with other recent data (Huang et al., 2017) suggest that  $\beta$ -  
398 hydroxybutyrate may favor breast cancer progression. One unexplored possibility to  
399 explain these contradictory observations is that this ketone body can be used

400 differently by different cancer cell types, for example as a carbohydrate supply or as a  
401 HDAC inhibitor, ultimately leading to cancer-type and context specific response.

402 In our experimental model,  $\beta$ -hydroxybutyrate increases winner cells proliferation by  
403 activating a genetic program through HDAC inhibition. Among the genes we  
404 discovered to be activated by the ketone body, IL-11 is an interleukin that displays a  
405 pro-proliferative activity (Grivennikov, 2013). Interestingly, in a distinct breast cancer  
406 cell cooperation model, sub-clonal expression of IL-11 favours the expansion not only  
407 of cells that express it, but also of other cellular sub-clones (Marusyk et al., 2014). This  
408 suggests that IL-11 acting in either paracrine or autocrine fashion could lead,  
409 respectively, to cooperation or to competition between subclones, thus participating  
410 actively in the selection and evolution of tumor heterogeneity.

411 Overall, our experimental data therefore suggest a model in which the winner line  
412 stimulates the production of and benefits from a compound delivered by the loser line  
413 and, conversely, the loser is negatively influenced by the presence of winners through  
414 secretion of another compound.

415 We note that while in artificially maintained conditions of non-constrained growth (in  
416 culture) the losers are eventually eliminated, many additional selective pressures that  
417 may affect clonal fitness operate *in vivo*. These involve cellular response to physical  
418 cues due to crowding (Vishwakarma and Piddini, 2020) and interactions with the  
419 extracellular matrix (Lu et al., 2012) as well as response to signaling from the stroma,  
420 including its inflammatory and immune components (Quail and Joyce, 2013). These  
421 elements are expected to influence the outcome of the direct interactions between the  
422 tumoral clones and may change the nature of their ecological interaction from net  
423 exploitation (*in vitro*) to mutual benefit (*in vivo*). Future study should evaluate whether  
424 parasitic effects are observed *in vivo*, and determine the extent to which these cell-cell  
425 interactions mediate important tumor characteristics, including growth, drug  
426 resistance, and metastatic behavior.

427

## 428 **Acknowledgements**

429 The authors wish to thank HTE ‘HetCoLi’ (HTE20161) and ITMO ‘Physique Cancer’  
430 (CanEvolve PC201306) for funding. RN acknowledges support from the National  
431 Cancer Institute of the National Institutes of Health under Award Number  
432 U54CA217376. The content is solely the responsibility of the authors and does not  
433 necessarily represent the official views of the National Institutes of Health. CR was  
434 supported by the French Infrastructure for Integrated Structural Biology (FRISBI) (grant  
435 No. ANR-10-INSB-05). MEH thanks the McDonnell Foundation for funding (Studying  
436 Complex Systems research award 220020294). We are grateful to Emie Quissac and  
437 Yasser Kerboua for their excellent technical assistance, and to Artem Kaznatcheev for  
438 helpful conversations.

439

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570 **Tables**

571

| Parameter          | Phase(s) | Inferred value | Interpretation  |
|--------------------|----------|----------------|---|
| $r_{L,1}$          | 1        | 0.044          | 168 growth rate in phase 1 (per hour)                       |
| $r_{W,1}$          | 1        | 0.031          | 4T07 growth rate in phase 1 (per hour)                      |
| $r_{L,2}$          | 2 and 3  | 0.073          | 168 intrinsic growth rate in phase 2 (per hour)             |
| $r_{W,2}$          | 2        | 0.102          | 4T07 intrinsic growth rate in phase 2 (per hour)            |
| $r_{W,3}$          | 3        | 0.04           | 4T07 intrinsic growth rate in phase 3 (per hour)            |
| $a$                | 2 and 3  | -0.004         | Density-dependent effect of 168 on 168                      |
| $b$                | 2 and 3  | -0.010         | Density-dependent effect of 4T07 on 168                     |
| $c$                | 2 and 3  | 0.000          | Density-dependent effect of 168 on 4T07                     |
| $d$                | 2 and 3  | -0.008         | Density-dependent effect of 4T07 on 4T07                    |
| $K_L = -r_{L,2}/a$ | 2 and 3  | 17             | 168 carrying capacity, relative to initial population size  |
| $K_W = -r_{W,2}/d$ | 2        | 13             | 4T07 carrying capacity, relative to initial population size |
| $\beta = b/a$      | 2 and 3  | 2.4            | Effect of 4T07 on 168, relative to effect of 168 on 168     |
| $\gamma = c/d$     | 2 and 3  | 0.0            | Effect of 168 on 4T07, relative to effect of 4T07 on 4T07   |

572 **Table 1. Mathematical model parameter values inferred from data.** The  
 573 interaction terms  $a, b, c$  and  $d$  are relative to population size, which is, in turn, relative  
 574 to initial population size.

575

576 **Figure Legends**

577

578 **Figure 1 Mutual impacts on subclonal growth**

579 **A:** 168FARN and 4T07 parental cells were transduced either with an empty retroviral  
 580 vector (168P and 4T07P) or with labelled with a GFP-encoding retrovirus (168G and  
 581 4T07G). Cells were seeded in triplicate in 6-well plates at a density of 50 000 cells/well  
 582 and cultured for the indicated times before harvesting and counting. **B:**  $10^5$  cells were  
 583 seeded at a 1:1ratio in homotypic (parental and GFP expressing derivative of the same  
 584 cell line) or heterotypic (different cell lines, one expressing GFP) co-cultures and  
 585 harvested and replated at the initial densities ( $10^5$  cells/plate) at indicated times. The  
 586 ratios of GFP-labelled to unlabelled cells were estimated by flow cytometry. The results  
 587 represent data from 3 independent experiments and are shown as mean +/- SEM.

588

589 **Figure 2. Normalized growth curves of homotypic and heterotypic mixes of**  
590 **subclones.**

591 **A:** The GFP fluorescence of the labeled subclone was measured by time-lapse  
592 microscopy. Cultures were seeded with  $10^5$  cells per well. Log-transformed data were  
593 normalized by fitting regression lines and dividing by the inferred value at 24 hours.  
594 Vertical dashed lines mark the start of phase 2 (45 hours) and phase 3 (72 hours). **B:**  
595 **Frequency dynamics.** Curves obtained by combining the results of two competition  
596 experiments: one with labelled 4T07 and the other with labelled 168. The initial 4T07  
597 proportion was 25% in both cases. The vertical axis is logit-transformed so that the  
598 slope of each curve is equal to the difference in net growth rates at the corresponding  
599 time (see Methods). Dotted regression lines are shown to draw attention to the change  
600 of slope. **C: Normalized growth curves according to mathematical model with**  
601 **parameter values inferred from data.** The model is described in Methods and  
602 parameter values are given in Table 1. **D: Frequency dynamics according to**  
603 **mathematical model with parameter values inferred from data.**

604  
605 **Figure 3. Mean net growth rate differences according to mathematical model and**  
606 **experimental data.**

607 **A: Inferred mean net growth rates and mean net growth rate differences (gain**  
608 **functions) over different time periods, corresponding to different phases within**  
609 **competition assays.** Columns correspond to different start times and rows to different  
610 end times of the phase(s) under consideration. For example, the centre panel labelled  
611 'Phase 2' corresponds to the period between 45 and 72 hours. The initial 4T07  
612 proportion (horizontal axis) is measured at the start of the respective period and the  
613 growth rate (vertical axis) is averaged over the period. Phase 1 data are from time-  
614 lapse microscopy. Other data points in the first column are from serial competition  
615 assays, such that each point corresponds to the slope of a thin grey line in **B**. Data  
616 points in the middle column are obtained from the competition assay data by adjusting  
617 for exponential growth during phase 1 (see Methods). Curves are the results of our  
618 mathematical model (Methods) with parameter values inferred from data (Table 1). **B:**  
619 **4T07 frequency dynamics across serial competition assays.** Thick solid lines are  
620 averaged data (means of replicates with similar initial 4T07 proportions) and thick  
621 dashed lines are results of our mathematical model with parameter values inferred  
622 from data. Thin grey lines are data for individual experiments. A total of  $10^5$  cells were  
623 seeded in co-cultures and harvested and replated as indicated. 4T07 parental cells  
624 were transduced either with an empty retroviral vector (4T07P) or labelled with a GFP-  
625 encoding retrovirus (4T07G). The ratios of GFP to unlabelled cells were estimated by  
626 flow cytometry. **C: Logit-transformed 4T07 frequency dynamics.** This panel shows  
627 the same data as **B** but with a logit-transformed vertical axis so that the slope of each  
628 curve is equal to the mean net growth rate difference (the gain function, as described  
629 in Methods and Supplementary figure 7).

630

631 **Figure 4. Soluble factor secreted by 168FARN cells accelerates proliferation of**  
632 **the 4T07 cells**

633 **A:** 4T07 cells were grown for 3 days at which point their medium was either left  
634 unchanged, or replaced by either 168FARN-conditioned medium or fresh medium, as  
635 indicated. Cells were collected 24 hrs later and counted. Cell numbers at day 3 were  
636 arbitrarily set at 1 in order to include the data from 3 independent experiments. **B:** The  
637 experiment was performed as in **A.** but the medium conditioned by 168FARN cells was  
638 fractionated by membrane ultrafiltration with a 3 KDa molecular cutoff. After  
639 complementing the low and the high MW fractions, respectively, with 10% serum and  
640 DMEM, the media were used to grow the 4T07 cells, as in **A.** The two fractions were  
641 also combined as a control. ns: not significant, \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , all  
642 compared to Day 4 point.

643

644 **Figure 5. Identification of soluble metabolites altering the heterotypic growth**  
645 **dynamics .**

646 **A:** Superimposition of the high-field region of representative 1D proton NMR spectra  
647 recorded at 700 MHz, 293 K and pH7 on samples of culture media collected after  
648 growing 40T7 cells (1) or 168FARN cells (2) for 3 days or of fresh cell culture medium  
649 (3). The arrows indicate the characteristic resonance of Lactate and  $\beta$ -hydroxybutyrate.  
650 The insert displays a zoom in this spectral region, revealing the H-alpha resonance of  
651 the  $\beta$ -hydroxybutyrate. For all spectra, peak intensities have been normalized on the  
652 intensity of the DSS resonance added as internal reference. **B:** Concentration of  $\beta$ -  
653 hydroxybutyrate from fresh medium and from conditioned medium from 168FARN or  
654 4T07 was quantified. **C:** Commercially available  $\beta$ -hydroxybutyrate at indicated  
655 concentrations was added to 4T07 cell culture at day 3 an the growth allowed to  
656 proceed for an additional 24 hrs. All points are compared to Day 4 point. **D:** 168FARN  
657 alone (homotypic) or in 1:1 co-culture with 4T01 cells were grown for 4 days and  
658 extracellular  $\beta$ -hydroxybutyrate was measured enzymatically as in 4B. **E:** 168FARN  
659 and 4T07 cells were cultured individually for 3 days. The medium was then replaced  
660 by the homotypic or heterotypic conditioned one, as indicated, and the culture allowed  
661 to continue for an additional 24 hrs. The  $\beta$ - hydroxybutyrate concentration was  
662 quantified at day 4. ns: not significant, \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

663

664 **Figure 6. Extracellular  $\beta$ -hydroxybutyrate leads to increased H3K9 histone**  
665 **acetylation and altered gene expression in 4T07 cells**

666 **A:** Expression levels of the slc16A family transporter genes in 4T07 were analyzed by  
667 RT-QPCR. Expression of HPRT served as normalization of the data. **B:** H3K9 histone  
668 acetylation was analyzed by immunblotting of extracts of 4T07 cells grown for 24 hrs  
669 in control, 168-conditioned medium or medium complemented with  $\beta$ -hydroxybutyrate  
670 or with butyrate, as indicated. Total histone 3 (H3) abundance served as normalization

671 control. **C:** 4T07 cells cultured for 3 days were incubated for 8 hours with 4T07- (Ctrl)  
672 or 168- conditioned medium or purified  $\beta$ -hydroxybutyrate (10mM) added to fresh  
673 medium. Total RNAs were purified and subjected to RT-QPCR with specific primers  
674 for LCN2 and IL-11. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

675

### 676 **Figure 7 Impact of extracellular pH on the loser clone growth.**

677 **A:** 168FARN and 4T07 cells were seeded at the indicated initial densities in 6-well  
678 plates and cultured for 3 days. Culture media were removed, immediately covered with  
679 a layer of mineral oil to prevent oxidation and the pH was measured. **B:**  $10^5$  168FARN  
680 cells were grown for 3 days. Medium was then replaced by conditioned media from  
681 cultures grown at low or high density, as indicated. Where indicated, 5mM  $\text{NaCO}_3$  was  
682 used to buffer the 4T07 conditioned medium to pH7. 24 hours later cells were  
683 harvested and counted. Data are from three independent experiments conducted in  
684 triplicates. ns: not significant, \*\*\* $p < 0.001$ .

685

### 686 **Supplementary Figure 1**

687 **A and B: Growth dynamics of subclones under homotypic and heterotypic**  
688 **conditions.**  $10^5$  cells were seeded at a 3:1 (A) or 1:4 (B) ratios in homotypic (parental  
689 and GFP expressing derivative of the same cell line) or heterotypic (different cell lines,  
690 one expressing GFP) co-cultures and harvested and replated at the initial densities  
691 ( $10^5$  cells/plate) at indicated times. The ratios of GFP-labelled to unlabelled cells were  
692 estimated by flow cytometry. The results represent data from 3 independent  
693 experiments and are shown as mean +/- SEM.

694

### 695 **Supplementary Figure 2**

696 **A: Apoptosis quantification of subclones under homotypic and heterotypic**  
697 **conditions.** A total of  $10^5$  cells were seeded. 168G cells were co-cultured with either  
698 the 168P (homotypic) or 4T07P (heterotypic) cells at a 1:1 ratio for 4 days and  
699 harvested. Apoptosis was quantified by flow cytometry following Annexin-V staining.  
700 ns: not significant. **B: S phase quantification of subclones under homotypic and**  
701 **heterotypic conditions.** A total of  $10^5$  cells were seeded. 168G cells were co-cultured  
702 with either the 168P (homotypic) or 4T07P (heterotypic) cells at a 1:1 ratio for 4 days.  
703 Before harvesting at day 4 cells were labelled by a 2hr pulse of EdU and the fraction  
704 of cells in the S phase was determined by flow cytometry. \* $p < 0.05$ , \*\* $p < 0.01$

705

### 706 **Supplementary Figure 3**

707 **A: Growth dynamics of subclones at low and high density.** Experiments were  
708 performed as in Figure 2B. Cells were grown in heterotypic conditions at a starting ratio  
709 of 1:1. Cells were seeded either at low density (50K) or high density (150k), diluted

710 and quantified every 3 days. At low density, cells do not reach confluence before  
711 replating. The results represent data from 3 independent experiments and are shown  
712 as mean +/- SEM.

713

#### 714 **Supplementary Figure 4**

715 **A: Expression levels of the *slc16A* family transporter genes in 168FARN .** RT-  
716 QPCR analysis was performed on 168FARN RNA for Mct2, Mct1, Mct3 and Mct4  
717 genes and normalized to HPRT. Relative expression levels were compared to Mct2.

718 **B: Slc16A1 expression in both subclones.** Slc16A1 RNA levels were monitored by  
719 RT-QPCR, normalized with HPRT and adjusted relative to levels in 168FARN cells  
720 cells. **C: Influence of Slc16A1 expression by  $\beta$ -hydroxybutyrate.** Experiment was  
721 performed as in Figure 5B. Slc16A1 RNA levels were quantified as in A and adjusted  
722 relative to levels in control condition. \*\*\* $p < 0.001$

723

#### 724 **Supplementary figure 5.**

725 **A: Mean net growth rate difference (gain function) versus initial 4T07 proportion**  
726 **in phases 1 and 2 (purple) and phases 1, 2 and 3 (green).** Each point corresponds  
727 to the outcome of a competition assay. Regression lines are shown with 95%  
728 confidence intervals. **B: Mean net growth rate difference versus initial 4T07**  
729 **proportion in phase 2 (purple) and phases 2 and 3 (green).** This data set was  
730 obtained from the data shown in **A** by adjusting for exponential growth in phase 1 (see  
731 Methods). **C: The same as A but including results for the first round of**  
732 **competition assays (days 0-3).** First-round measurements were excluded from  
733 analyses as they were unusually variable and unreliable due to an experimental  
734 artefact (see Methods). **D: The same as B but including results for the first round**  
735 **of competition assays (days 0-3).**

736

#### 737 **Supplementary figure 6. Relationship between population dynamics and net** 738 **growth rates.**

739 The net growth rate of each cell type (right column) is the derivative of its log-  
740 transformed growth curve (left column). **A: Mathematical model dynamics.** From the  
741 dynamical model, net growth rates can be found precisely by evaluating differential  
742 equation terms. The model was parameterized with values inferred from data (Table  
743 1) and initiated with a 3:1 ratio of 168 to 4T07. **B: Empirical dynamics.** From time-  
744 lapse data, net growth rates can be approximated as local gradients (difference  
745 quotients). In this example, we estimated net growth rates from smoothed growth  
746 curves by calculating difference quotients across a 5-hour span. Smoothed growth  
747 curves (not shown) were obtained by computing running medians with a 5-hour span.  
748 Since we did not use heterotypic time-lapse data for parameter inference, the

749 resemblance between the two rows of this figure contributes to validating our model.  
750 The data in **B** is the same as in Figure 2A and 2B.

751

## 752 **Supplementary figure 7. Mathematical relationships relevant to our methods.**

753 The diagram illustrates several equivalent ways of calculating the mean growth rate  
754 difference (gain function, blue) from the parameterized dynamical model (red). Also  
755 shown is our method of calculating the gain function from competition assay data  
756 (orange).

757

## 758 **Methods**

### 759 **Cell culture**

760 4T07 and 168FARN were a kind gift of Dr Robert Hipskind. All cell lines were cultured  
761 in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 ng/mL  
762 streptomycin, and 100 U/mL penicillin at 37 °C with 5% CO<sub>2</sub>.

763 For co-culture experiments a mixture of GFP-labelled and parental cells (empty-vector  
764 transduced) cells were seeded at the final density of 10<sup>5</sup> cells/well in 6-well plates,  
765 except where mentioned otherwise. Upon reaching confluence (3-4 days) they were  
766 harvested, diluted to the original density and replated. The remaining fraction was  
767 analyzed by flow cytometry.

768

### 769 **Immunoblot Analysis**

770 Cells were lysed in boiling Laemmli buffer supplemented with protease inhibitors, then  
771 sonicated and complemented with DTT. Protein concentration was determined by BCA  
772 (Thermo Scientific) assay. Fifteen to twenty micrograms of total protein were loaded  
773 onto SDS-PAGE gels and transferred onto nitrocellulose membranes. The membrane  
774 was blocked with TBST (1× TBS with 0.1% Tween 20) + 5% milk at room temperature  
775 for 1 h and incubated with primary antibody and then with horseradish peroxidase  
776 (HRP)-coupled secondary antibody (Amersham, Piscataway, NJ). Activity was  
777 visualized by electrochemiluminescence. Antibodies used in this study are anti Histone  
778 H3 (Cell signaling Technology #9717) and anti- Acetyl-Histone H3 (Lys9) (Cell  
779 signaling Technology #9649).

780

### 781 **Reverse Transcription and Real-Time PCR**

782 Total mRNA was isolated using a RNeasy mini kit (Qiagen, Germantown, MD, USA).  
783 Reverse transcription was performed with random hexamers and M-MLV Reverse  
784 Transcriptase (Invitrogen). Real-time PCR was performed in triplicates with LC  
785 FastStart DNA Master SYBR Green I on a LightCycler rapid thermal cycler system  
786 (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's

787 instructions. Housekeeping gene HPRT was used for normalization. Primers  
788 sequences are available upon request.

789

### 790 **Time-lapse microscopy**

791 Time-lapse microscopy was performed at 37 °C with 5% CO<sub>2</sub>, with images taken at  
792 45-minute intervals using an inverted Zeiss Axio-Observer microscope. The images  
793 were processed and analyzed using ImageJ software.

794

### 795 **EdU staining**

796 Cells were incubated with 10µM EdU for 2 hours, harvested and processed using the  
797 Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (ThermoFisher Scientific  
798 #C10424) following manufacturer instructions. Labeled cells were then analyzed on a  
799 FACSCalibur flow cytometer using CellQuestPro software (BD Biosciences).

800

### 801 **Apoptosis quantification**

802 To determine the percentage of apoptotic cells with externalized phosphatidylserine  
803 (PS), adherent and floating cells were collected and labeled with the Annexin V-Cy3  
804 Apoptosis Detection Kit (Abcam, Cambridge, UK, #ab14143) according to the  
805 manufacturer's instructions. Labeled cells were then analyzed on a FACSCalibur flow  
806 cytometer using CellQuestPro software (BD Biosciences).

807

### 808 **β-hydroxybutyrate quantification**

809 β-hydroxybutyrate concentration was measured by an enzymatic kit (Sigma-Aldrich  
810 MAK041) following the manufacturer instructions. Briefly, β-hydroxybutyrate present in  
811 the culture medium was determined by a coupled enzyme reaction, resulting in a  
812 colorimetric (450 nm) product, proportional to the β-hydroxybutyrate concentration.  
813 The absorbance was measured on a spectrophotometer.

814

### 815 **Medium fractionation**

816 In order to separate low molecular weight molecules from the conditioned culture  
817 medium, 5 to 10 ml were loaded on a Vivaspin Turbo 15 PES, 3,000 MWCO column  
818 (Sartorius VS15T91) and centrifuged at 4000G for 30 minutes following the  
819 manufacturer instructions. Both fractions were then used for subsequent experiments  
820 and RMN analysis.

821

### 822 **RMN analysis**



823 NMR experiments were recorded at 293K and pH 7 on an AVANCE III BRUKER  
824 spectrometer operating at 700 MHz (proton frequency), using a Z-gradient shielded TCI  
825 1H-13C-15N cryoprobe. Fully relaxed 1D 1H spectra were acquired with the regular 1D  
826 NOESY, using 5s as relaxation delay. The samples consisted on 1.5 mL of cell media  
827 (fresh or conditioned by cell culture), lyophilized and dissolved in 500  $\mu$ L of deuterated  
828 phosphate buffer (50 mM, pH 7). DSS (EURISOTOP<sup>®</sup>, final concentration: 0.5 mM)  
829 was added as internal reference for chemical shift referencing and as a concentration  
830 standard for spectra normalization. The assignment of the 1H resonances of the  
831 compound of interest in this study (Lactate,  $\beta$ -hydroxybutyrate) was based on chemical  
832 shifts reported on the literature (1) and further confirmed using 2D [1H,1H] (TOCSY)  
833 and [1H-13C] (HSQC, HSQC-TOCSY) NMR spectroscopy.

834

### 835 **Statistical analysis**

836 Experiments were repeated at least three times. Data are presented as mean  $\pm$  SEM.  
837 An Independent Student's t test was performed to analyze the assay results; a two-  
838 tailed Student's t test was used to compare the intergroup differences. Significance  
839 was accepted for values where  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*),  $P \leq 0.001$  (\*\*\*)).

### 840 **Overview of mathematical methods**

841 Our aim is to determine the general nature of the evolutionary dynamics in a form  
842 that can be readily compared to other **systems (as opposed to generating**  
843 **quantitative predictions for our particular system)**. Accordingly, we chose to fit a  
844 **simple, standard model to each distinct phase of the dynamics, such that the inferred**  
845 **parameter values have straightforward ecological interpretations. A key advantage of**  
846 **our method is that it is generic; in principle, the same method can be applied to any**  
847 **experimental evolution set-up with two competing populations of cancer cells,**  
848 **bacteria, or other entities.**

849

850 **This** mathematical approach is in the same vein as that of Kaznatcheev (2017) and  
851 Kaznatcheev et al. (2019) but with three important differences. First, our method can  
852 accommodate a smaller data set and is thus more economical because we mostly  
853 rely on measurements of initial and final proportions in competition assays, such as  
854 can be determined via flow cytometry, rather than extensive time-lapse image  
855 analysis. Second, whereas Kaznatcheev (2017) and Kaznatcheev et al. (2019)  
856 confine their analysis to exponential or logistic growth phases, we also examine  
857 phases in which cell populations exhibit non-logistic dynamics. Third, because we  
858 consider non-logistic growth phases, we use a density-dependent rather than a  
859 frequency-dependent model.

860

861 **We note that to make quantitative predictions of outcomes in different scenarios, we**  
862 **would require a different type of model with equations describing the dynamics of**  
863 **paracrine factors mediating clonal interactions. This more complicated model would**  
864 **include several more parameters and design choices (for example, how each**

865 paracrine factor's production rate and its effects vary with its concentration) and  
866 would thus be non-identifiable in the absence of detailed paracrine concentration  
867 measurements. Obtaining such measurements remains as a challenge for future  
868 studies.

869

### 870 **Definitions and mathematical relationships**

871 We define the intrinsic growth rate as the exponential growth rate in the absence of  
872 interactions. In the Lotka-Volterra differential equations, this parameter is multiplied  
873 by the population size of the respective type. The intrinsic growth rate is the limit of  
874 the net growth rate as the population sizes approach zero (when interaction terms  
875 are negligible).

876

877 We define the net growth rate as the actual rate of change of the population size (i.e.  
878 the time derivative), which is the sum of the basic growth rate and interaction terms.

879

880 Supplementary figures 6 and 7 illustrate some of the mathematical relationships  
881 relevant to our methods.

### 882 **Dynamical models and inference from homotypic growth curves**

883 We describe the exponential phase 1 dynamics as

884

$$885 \quad \frac{dL}{dt} = Lr_{L,1}, \quad \frac{dW}{dt} = Wr_{W,1},$$

886

887 where  $L$  (loser) and  $W$  (winner) are the population sizes of 168 and 4T07,  
888 respectively, and  $r_{L,1}$  and  $r_{W,1}$  are the respective growth rates.

889

890 In phase 2, we assume a density-dependent competitive Lotka-Volterra model,  
891 parameterized in terms of intrinsic growth rates  $r_{L,2}$  and  $r_{W,2}$  and interaction terms  $a$ ,  
892  $b$ ,  $c$  and  $d$ :

893

$$894 \quad \frac{dL}{dt} = L(r_{L,2} + aL + bW), \quad \frac{dW}{dt} = W(r_{W,2} + cL + dW).$$

895

896 In the homotypic case, terms  $bW$  and  $cL$  vanish and the phase 2 model is equivalent  
897 to logistic growth. We combine the two models and fit to the normalized time-lapse  
898 data for the homotypic growth curves using least-squares with R package deSolve  
899 (Soetaert et al., 2010) to infer the values of  $r_{L,1}$ ,  $r_{W,1}$ ,  $r_{L,2}$ ,  $r_{W,2}$ ,  $a$  and  $d$ .

900

901 In phase 3, we assume the same model as in phase 2 except we replace  $r_{W,2}$  by  $r_{W,3}$   
902 to account for the change in the 4T07 net growth rate (equivalent to adding a density-  
903 dependent death rate).

904

905 **Inferring between-type interaction terms**

906 To infer the interaction parameters  $b$  and  $c$  we need data that covers a wide range of  
907 proportions of the two cell types. Since our time-lapse data is limited to only a few  
908 initial conditions, we fit the model to the outcomes of serial competition assays, and  
909 we employ the heterotypic time-lapse data for validation only. First we define  
910

911 
$$l = \frac{L}{W + L}, \quad w = \frac{W}{W + L}, \quad s = \log \frac{w}{l} = \log \frac{w}{1 - w} = \text{logit}(w).$$

912  
913 The time derivative of the  $s$  is then equal to the net growth rate difference, which in  
914 phase 2 is  
915

916 
$$\frac{ds}{dt} = r_{W,2} - r_{L,2} + (d - b)W + (c - a)L.$$

917  
918 In the limit  $w \rightarrow 1$ , the final term  $(c - a)L$  is negligible and we can obtain  $b$  in terms of  
919  $\frac{ds}{dt}$ ,  $W$ , and parameters whose values we have already inferred, as follows:  
920

921 
$$\frac{ds}{dt} = r_{W,2} - r_{L,2} + (d - b)W$$
  
922 
$$\Rightarrow b = \frac{\frac{ds}{dt} - r_{W,2} + r_{L,2}}{W} + d.$$

923  
924 To obtain  $W$ , we note that in the limit  $w \rightarrow 1$ ,  
925

926 
$$\frac{dW}{dt} = W(r_{W,2} + dW),$$

927  
928 which is the logistic differential equation with solution  
929

930 
$$W(t) = \frac{W(t_1)re^{rt}}{r - W(t_1)(e^{rt} - 1)d'}$$

931  
932 where  $r = r_{W,2}$  and  $t_1$  is the time at which phase 2 begins. We can thus use our  
933 previously inferred parameter values to obtain  $W(t)$  at every time  $t$  in phase 2 (note  
934 that if there were not an analytical solution then we could have solved the equation  
935 numerically).  
936

937 Since  $W$  and  $\frac{ds}{dt}$  are linearly related, we can replace them by their mean values:  
938

939 
$$\frac{\text{mean}\left(\frac{ds}{dt}\right) - r_{W,2} + r_{L,2}}{\text{mean}(W)} + d = \frac{\text{mean}\left(\frac{ds}{dt}\right) - r_{W,2} + r_{L,2}}{\text{mean}\left(\frac{\frac{ds}{dt} - r_{W,2} + r_{L,2}}{b - d}\right)} + d = b.$$

940

941 Using the mean values to calculate  $b$  is convenient as our competition assays reveal  
 942 only the initial and final values of  $s$ . Specifically, we take the means in the interval  
 943  $[t_1, t_2]$ , where  $t_2$  is the time at which phase 2 ends and  
 944

945 
$$\text{mean}\left(\frac{ds}{dt}\right) = \frac{s(t_2) - s(t_1)}{t_2 - t_1} = \frac{\Delta s}{\Delta t}.$$

946

947 It remains only to obtain the value of the above expression – known as the gain  
 948 function – in the limit  $w(t_1) \rightarrow 1$ . From competition assay data, we can immediately  
 949 obtain  $s(t_2) = \log \frac{w(t_2)}{1-w(t_2)}$  for each value of  $s(0) = \log \frac{w(0)}{1-w(0)}$ . To infer  $w(t_1)$  and  $s(t_1)$ ,  
 950 we need to adjust for the exponential growth of both cell types during phase 1:  
 951

951

952 
$$s(t_1) = s(0) + t_1(r_{W,2} - r_{L,2})$$
  
 953 
$$\Rightarrow \text{logit}(w(t_1)) = \text{logit}(w(0)) + t_1(r_{W,2} - r_{L,2})$$
  
 954 
$$\Rightarrow w(t_1) = \text{logit}^{-1}(\text{logit}(w(0)) + t_1(r_{W,2} - r_{L,2})).$$

955

956 We thus obtain the values of  $s(t_1)$  and  $w(t_1)$  in each competition assay. Finally, we  
 957 determine by linear regression the relationship between  $\Delta s/\Delta t$  and  $w(t_1)$   
 958 (Supplementary figure 5B) and, from the equation of the regression line, infer the  
 959 value of  $\Delta s/\Delta t$  in the limit  $w(t_1) \rightarrow 1$ . We then have everything required to infer the  
 960 value of  $b$ . By an analogous method (switching  $L$  and  $W$ ,  $b$  and  $c$ , and  $a$  and  $d$ ) we  
 961 also infer the value of  $c$ .  
 962

962

### 963 **Excluding results of first-round competition assays**

964

965 In our regression to determine the relationship between  $\Delta s/\Delta t$  and  $w(t_1)$ , we  
 966 excluded data from the first round of competition assays (days 0 to 3 in Figures 3B  
 967 and 3C) because these measurements were unusually variable, and this variance  
 968 was most likely an experimental artefact. Specifically, setting up the initial experiment  
 969 took substantially longer than carrying out subsequent replatings as additional steps  
 970 were required before seeding the cells. Since cells were kept for longer in  
 971 suspension before the first round, they will have experienced more stress and  
 972 potentially mortality. This means that results of the first round of competition assays  
 973 are likely to be less reliable than results of subsequent rounds. For completeness,  
 974 Supplementary Figures 5C and 5D show linear regression applied to the entire data  
 975 set, including the first round.  
 976

976

977 **Carrying capacities**

978 To find carrying capacities, we note that the phase 2 model can alternatively be  
979 parameterized as

980

981 
$$\frac{dL}{dt} = Lr_{L,2} \left( 1 - \frac{L + \beta W}{K_L} \right), \quad \frac{dW}{dt} = Wr_{W,2} \left( 1 - \frac{\gamma L + W}{K_W} \right),$$

982

983 where the parameters are calculated as in Table 1. The carrying capacities  $K_W$  and  
984  $K_L$  are the upper limits approached by the population sizes of  $W$  and  $L$ , respectively,  
985 during phase 2.

986

987 **Potential for coexistence *in vivo***

988 In a growing tumor, we expect cell-cell competition to be less than in our *in vitro*  
989 experiments, because, in the former, resources are continually replenished and  
990 waste materials removed by the host circulatory system. The evolutionary dynamics  
991 will then mostly depend on the difference in intrinsic growth rates and interactions  
992 mediated by diffusible factors. Furthermore, during tumor growth, the dynamics may  
993 be better described by a frequency- rather than a density-dependent model. We can  
994 then describe the evolutionary dynamics within the framework of evolutionary game  
995 theory using the payoff matrix

996

997 
$$\begin{pmatrix} \beta_L - \gamma & \alpha_L - \gamma \\ \beta_W & \alpha_W \end{pmatrix},$$

998

999 where  $\alpha_L, \alpha_W < 0$  denote the harm inflicted by  $W$  on  $L$  and  $W$ , respectively;  $\beta_L, \beta_W >$   
1000  $0$  are the benefits bestowed by  $L$  to  $L$  and  $W$ , respectively; and  $\gamma > 0$  is the difference  
1001 between the intrinsic exponential growth rates. The relative values of the entries in  
1002 the payoff matrix determine which game (for example, prisoner's dilemma or hawk-  
1003 dove) is equivalent to the evolutionary dynamics.

1004

1005 The parameter values inferred for phase 2 of the competition assays imply

1006

1007 
$$\beta_W > \beta_L - \gamma > \alpha_W > \alpha_L - \gamma,$$

1008

1009 in which case the evolutionary dynamics are equivalent to a prisoner's dilemma  
1010 game for which  $W$  is the only evolutionarily stable strategy (ESS). This means that  $W$   
1011 (4T07) can invade and stably replace a population of  $L$  (168).

1012

1013 If instead  $\alpha_L - \gamma > \alpha_W$  then the payoff matrix defines a hawk-dove game that permits  
1014 coexistence. In this scenario,  $W$  harms itself more than it harms  $L$ , and this difference  
1015 outweighs  $W$ 's higher intrinsic growth rate. This could happen, for example, if harmful

1016 factors produced by  $W$  imperfectly diffuse, so that  $W$  cells experience a higher  
1017 concentration than  $L$  cells. At the mixed ESS, the  $W$  proportion is

1018

1019

$$\frac{\alpha_W - \alpha_L + \gamma}{\alpha_W - \alpha_L + \beta_L - \beta_W}.$$

1020

1021 However, if additionally  $\beta_L - \beta_W > \gamma$  (so that  $L$  benefits itself more than it benefits  $W$ ,  
1022 and this difference outweighs  $W$ 's higher intrinsic growth rate) then coexistence  
1023 again becomes impossible as the game again becomes a prisoner's dilemma but  
1024 with  $L$  as the ESS.

1025

1026 In a resource-poor environment, we might describe the evolutionary dynamics using  
1027 the payoff matrix

1028

1029

$$\begin{pmatrix} \beta_L - \gamma & \alpha_L - \gamma \\ \beta_W - \delta & \alpha_W - \delta \end{pmatrix},$$

1030

1031 where  $\delta$  is the reduction in  $W$ 's intrinsic growth rate due to the degraded environment  
1032 (as inferred for phase 3 of our 96-hour competition assays). This scenario favours  $L$   
1033 and suggests that  $L$  may be the ESS in a resource-poor environment, such as hypoxic  
1034 regions within a tumor.