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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 *β -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 β -hydroxybutyrate in the 168FARN-conditioned medium (Figure
215 5A). β -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, β -
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 β -hydroxybutyrate peak, we employed an enzymatic
221 assay to measure β -hydroxybutyrate concentration in conditioned media from 4T07
222 and 168FARN (Figure 5B). The results were in perfect agreement with the NMR
223 analysis: β -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 β -hydroxybutyrate. As shown in Figure 5C, β -
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 β -hydroxybutyrate.

230

231 *Presence of the winner clone stimulates β -hydroxybutyrate production by loser cells*

232 After assessing β -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 β -hydroxybutyrate, *cf.* Fig. 5B), the overall level of secreted
237 β -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 β -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 β -hydroxybutyrate action*

251 We next asked about the mode of action of BHB on the 4T07 cells. β -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 β -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 β -hydroxybutyrate is used as an energy resource to increase
268 proliferation rate. β -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 β -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 β -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 β -hydroxybutyrate in other cellular
281 models (Shimazu et al., 2013), both β -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 β -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 (β -
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, β -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 β -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 β -
390 hydroxybutyrate (Cheng et al., 2019; Grabacka et al., 2016; Huang et al., 2017;
391 Shakery et al., 2018). Our results identifying β -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 β -
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, β -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

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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 β -hydroxybutyrate.
650 The insert displays a zoom in this spectral region, revealing the H-alpha resonance of
651 the β -hydroxybutyrate. For all spectra, peak intensities have been normalized on the
652 intensity of the DSS resonance added as internal reference. **B:** Concentration of β -
653 hydroxybutyrate from fresh medium and from conditioned medium from 168FARN or
654 4T07 was quantified. **C:** Commercially available β -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 β -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 β - 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 β -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 β -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 β -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 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 β -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% CO₂.

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 10⁵ 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% CO₂, 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 μ L of deuterated
828 phosphate buffer (50 mM, pH 7). DSS (EURISOTOP[®], 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, β -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

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

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

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 δ 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.