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1 **Differential Metabolic Sensitivity of Insulin-like-response- and TORC1-Dependent**
2 **Overgrowth in *Drosophila* Fat Cells**

3
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32 **ABSTRACT**

33 Glycolysis and fatty acid (FA) synthesis directs the production of energy-carrying
34 molecules and building blocks necessary to support cell growth, although the absolute
35 requirement of these metabolic pathways must be deeply investigated. Here, we used
36 *Drosophila* genetics and focus on the TOR (Target Of Rapamycin) signaling network
37 that controls cell growth and homeostasis. In mammals, mTOR (mechanistic-TOR) is
38 present in two distinct complexes, mTORC1 and mTORC2; the former directly responds
39 to amino acids and energy levels, whereas the latter sustains insulin-like-peptide (Iip)
40 response. The TORC1 and Iip signaling branches can be independently modulated in
41 most *Drosophila* tissues. We show that TORC1 and Iip-dependent overgrowth can
42 operate independently in fat cells and that ubiquitous over-activation of TORC1 or Iip
43 signaling affects basal metabolism, supporting the use of *Drosophila* as a powerful
44 model to study the link between growth and metabolism. We show that cell-autonomous
45 restriction of glycolysis or FA synthesis in fat cells retrains overgrowth dependent on Iip-
46 but not TORC1-signaling. Additionally, the mutation of *FASN* (Fatty acid synthase)
47 results in a drop in TORC1 but not Iip signaling, whereas, at the cell-autonomous level,
48 this mutation affects none of these signals in fat cells. These findings thus, reveal
49 differential metabolic sensitivity of TORC1- and Iip-dependent growth and suggest that
50 cell-autonomous metabolic defects might elicit local compensatory pathways.
51 Conversely, enzyme knockdown in the whole organism results in animal death.
52 Importantly, our study weakens the use of single inhibitors to fight mTOR-related
53 diseases and strengthens the use of drug combination and selective tissue-targeting.

54

55

56 INTRODUCTION

57 The mTOR (mechanistic Target Of Rapamycin) regulatory network orchestrates
58 organismal growth in response to growth factor signaling and nutritional status
59 (LAPLANTE AND SABATINI 2012; HOWELL *et al.* 2013; LAMMING AND SABATINI 2013;
60 SHIMOBAYASHI AND HALL 2014; CARON *et al.* 2015; SAXTON AND SABATINI 2017;
61 MOSSMANN *et al.* 2018). Activation of this network promotes basal cellular functions,
62 thereby providing building blocks to sustain cellular growth. However, despite a plethora
63 of studies on the mTOR signaling network, the requirement of basal metabolism—
64 glycolysis and FA synthesis— for cell growth has not been systematically investigated.
65 The *Drosophila* model provides a powerful genetic system to address these issues
66 (UGUR *et al.* 2016), since both the intermediates of this signaling network and the basal
67 metabolic pathways are conserved in the fruit fly (MONTAGNE *et al.* 2001; HAY AND
68 SONENBERG 2004; PADMANABHA AND BAKER 2014; ANTIKAINEN *et al.* 2017; WANGLER *et al.*
69 2017; LEHMANN 2018).

70 The mTOR protein kinase is present in two distinct complexes, mTORC1 and mTORC2
71 that comprise raptor and rictor, respectively (KIM *et al.* 2002; SARBASSOV *et al.* 2005).
72 ATP and amino acid levels control the recruitment of an mTORC1 homodimer at the
73 lysosomal membrane in the vicinity of the small GTPase Rheb (Ras homologue
74 enriched in brain) (GOBERDHAN *et al.* 2009; MA AND BLENIS 2009; DIBBLE AND MANNING
75 2013; GROENEWOUD AND ZWARTKRUIS 2013; MONTAGNE 2016). Rheb stimulates
76 mTORC1 activity (YANG *et al.* 2017), which in turn regulates several downstream
77 targets. S6Kinase1 (S6K1) is one such kinase, sequentially activated through the
78 phosphorylation of its T389 and T229 residues by mTORC1 and by PDK1
79 (Phosphoinositide-dependent protein kinase 1), respectively (MONTAGNE AND THOMAS
80 2004; MAGNUSON *et al.* 2012). Rheb activation of mTORC1 is repressed by the tumor

81 suppressor TSC (Tuberous sclerosis complex) that comprises subunits TSC1 and TSC2
82 (RADIMERSKI *et al.* 2002a; GARAMI *et al.* 2003; INOKI *et al.* 2003a; DIBBLE *et al.* 2012).
83 The integrity of mTORC2 is required to sustain the downstream insulin-signaling
84 response (SARBASSOV *et al.* 2005). Binding of insulin or related peptides (I_{lps}) to their
85 cognate receptors results in recruitment of class I PI3K (Phosphoinositide 3-kinase) to
86 the membrane. PI3K phosphorylates inositol lipids to phosphatidylinositol-3,4,5-
87 triphosphate (PIP3) (ENGELMAN *et al.* 2006; HAEUSLER *et al.* 2018), while the tumor
88 suppressor PTEN counteracts this process (CULLY *et al.* 2006; GOBERDHAN *et al.* 2009).
89 PIP3 constitutes a membrane docking site for the protein kinase Akt whose activity
90 requires the subsequent phosphorylation of its S473 and T308 residues by mTORC2
91 and PDK1, respectively (LIEN *et al.* 2017).

92 Constitutive activation of mTORC1 in MEFs (Mouse embryonic fibroblasts) represses
93 the tricarboxylic acid (TCA) cycle and stimulates anaerobic glycolysis and biosynthesis
94 of FAs and cholesterol (DUVEL *et al.* 2010). Conversely, adipose-specific knockout of
95 raptor to impede mTORC1 formation, results in enhanced uncoupling of mitochondrial
96 activity (POLAK *et al.* 2008). The increased lipogenesis observed in mTORC1 stimulated
97 cells depends on the cofactor Lipin 1 and on a SREBP (Sterol responsive element
98 binding-protein) family member (DUVEL *et al.* 2010; PETERSON *et al.* 2011). Congruently,
99 TSC2 mutant cells become addicted to glucose as a result of mTORC1 hyper-activity
100 (INOKI *et al.* 2003b) and, in the context of mTORC1 inhibition, promote glutamine
101 anaplerosis (CHOO *et al.* 2010; CSIBI *et al.* 2013; CSIBI *et al.* 2014). I_{lp}-signaling also
102 impinges on basal metabolism. Intracellular activation of Akt increases ATP levels
103 (HAHN-WINDGASSEN *et al.* 2005; ROBEY AND HAY 2009) through the stimulation of
104 GLUT4-mediated glucose uptake (JALDIN-FINCATI *et al.* 2017) and the enhancement of
105 the expression and activity of glycolytic enzymes (GOTTLOB *et al.* 2001; HOUDANE *et al.*

106 2017). Akt also dampens glucose production by suppressing PEPCK
107 (gluconeogenesis), glucose-6-phosphatase (glycogenolysis) and the glycogen synthesis
108 repressor GSK3 (NAKAE *et al.* 2001; McMANUS *et al.* 2005). In addition, Akt promotes
109 mitochondrial metabolism and oxidative phosphorylations (GOTTLOB *et al.* 2001;
110 MAJEWSKI *et al.* 2004). Conversely, hepatic knockout of the mTORC2 specific-subunit
111 rictor results in constitutive gluconeogenesis and impaired glycolysis and lipogenesis
112 (HAGIWARA *et al.* 2012; YUAN *et al.* 2012). Taken together, these studies strongly
113 emphasize the role of mTOR in metabolic-related diseases and in adjusting metabolism
114 to the nutritional and energy status (MOSSMANN *et al.* 2018).

115 Despite a plethora of studies, the net requirement of these metabolic changes to sustain
116 overgrowth induced by over-activation of mTOR signaling has not been formally
117 addressed. This signaling network is conserved in *Drosophila* (hereafter called as TOR),
118 providing a powerful genetic system to address these issues. In the present study, we
119 investigated the requirement of glycolysis and FA synthesis for the cellular growth
120 induced by hyper-activation of TORC1 signaling and Iip response in *Drosophila*.
121 Consistent with previous studies (RADIMERSKI *et al.* 2002a; RADIMERSKI *et al.* 2002b;
122 DONG AND PAN 2004; MONTAGNE *et al.* 2010; PALLARES-CARTES *et al.* 2012), we show
123 that TORC1- and Iip-dependent overgrowth can be independently modulated in the
124 *Drosophila* fat body (FB), the organ that fulfils hepatic and adipose functions
125 (PADMANABHA AND BAKER 2014; ANTIKAINEN *et al.* 2017; LEHMANN 2018). We show that
126 ubiquitous over-activation of TORC1 or Iip signaling affects basal metabolism and that
127 metabolic restriction at the organismal level has dramatic consequences on animal
128 survival, but minor effect at the cell-autonomous level. This suggests that within an
129 organism, alternative strategies may operate to compensate local metabolic defects.
130 Finally, at the cell-autonomous level, metabolic restriction can partially restrain

131 overgrowth dependent on hyper-activation of Ilp- but not TORC1-signaling, indicating
132 that the potential compensatory metabolic pathways do not fully operate in the context
133 of Ilp-signaling stimulation.

134

135 **MATERIAL & METHODS**

136 **Genetics and fly handling**

137 Fly strains: *P[w[+mC]=tubP-GAL80]LL10,P[ry[+t7.2]=neoFRT]40A*, *daughterless(da)-*
138 *gal4*, *tub-gal80^{ts}*, *UAS-Dcr-2* (Bloomington Stock Center); *hs-flp,tub-gal4,UAS-nls-*
139 *GFP/FM7*, *FASN¹⁻²* (GARRIDO *et al.* 2015); *Tor^{AP}* (ZHANG *et al.* 2000); *Tor^{2L1}*, *Tor^{2L19}* and
140 *PTEN* (OLDHAM *et al.* 2000); *EP(UAS)-Rheb* (STOCKER *et al.* 2003); inducible interfering
141 RNA (*UAS-RNAi*) lines to *PTEN* (NIG 5671R-2), *FASN1* (VDRC 29349), *PFK1* (VDRC
142 3017), *PK* (VDRC 49533) *PDH* (VDRC 40410), *LDH* (VDRC 31192) (DIETZL *et al.* 2007).
143 To generate MARCM clones in a *Minute* background, the *FRT40/P(arm-LacZ w⁺)*
144 chromosome (BOHNI *et al.* 1999) was recombined with the *P[w[+mC]=tubP-*
145 *GAL80]LL10,P[ry[+t7.2]=neoFRT]40A* chromosome (LEE AND LUO 2001). Standard
146 media: agar (1g), polenta (6g) and yeast (4g) for 100ml. Lipid- (*beySD*) and sugar-
147 complemented media were as described (GARRIDO *et al.* 2015). *FASN¹⁻²* mutant larvae
148 were collected at L1 hatching on grape juice plates following the lack a GFP-labelled
149 balancer and transferred to fresh tubes. Prepupae were collected once a day to
150 evaluate developmental delay and to measure body weight and size.

151

152 **Molecular biology and Biochemistry**

153 RNAi-knockdown efficacy (Figure S1): *UAS-Dcr-2;da-gal4,tub-gal80^{ts}* females mated
154 with *UAS-RNAi* males were let to lay eggs overnight. tubes were kept at 19°C for two

155 days, then transferred at 29°C; two days later, larvae of roughly the same size were
156 collected for quantitative PCR as described (PARVY *et al.* 2012). For western-blotting,
157 protein extracts were prepared as described and antibodies were previously described
158 for dS6K and phospho-S6 (MONTAGNE *et al.* 2010; ROMERO-POZUELO *et al.* 2017) or
159 commercially provided for phospho-dS6K, Akt and ERK (Cell signaling 9209, 9272,
160 4054, 4695, 4370).

161 For metabolic measurements, tubes containing 24-hr egg collection were maintained at
162 29°C to strengthen the gal4/UAS effect. Larvae were selected prior to L2/L3 transition
163 and transferred on either a standard diet or a 20%-SSD. Collection of prepupae and
164 metabolic measurements were performed as described (GARRIDO *et al.* 2015). For
165 protein and TAG measurements, prepupae were weighted and crushed in 400 µl PBT
166 (PBS, 0.1% Triton X-100) and centrifuged for 5 mn at 12000 rpm. For proteins, 10 µl of
167 extract mixed with 1 ml of 5X-diluted reagent (Bio-Rad Protein Assay Kit) were
168 incubated 15 mn in dark at room temperature; OD was measured at 595 nm. For TAGs,
169 20 µl of extract mixed with 500 µl of reagent (Kit STANBIO Triglyceride LiquiColor) were
170 incubated 15 mn at 37°C; OD was measured at 500 nm. For trehalose and glycogen,
171 prepupae were crushed in 350 µl Na₂CO₃⁻ (0,25 M) (PBS 0.1% Triton X-100). After 2
172 hrs incubation at 65°C, 700 µl of Na Acetate (0.3 M) and 130 µl of Acetic acid (1.7 M)
173 were added and tubes were centrifuged for 10 mn at 12000 rpm. For each sample,
174 three sets of 20 µl of extract were incubated overnight at 37°C, two of them contained 1
175 U/ml of either Trehalase or Amyloglucosidase (SIGMA). Next, all samples mixed with
176 500 µl of glucose oxidase (Thermo Electron, GLUCOSE (GDO-POD)) were incubated
177 20 mn at 37°C; OD was measured at 510 nm. The net concentration of trehalose and
178 glycogen was obtained by subtracting the concentration values of the extracts without
179 enzyme.

180

181 **Clonal analysis**

182 MARCM clones (LEE AND LUO 2001) were generated from 6-7hr egg collections heat-
183 shocked for 65 mn at 38°C. FB of late L3 feeding larvae were dissected, fixed and
184 labelled with Phalloidin (membranes) and DAPI (nuclei) as described (GARRIDO *et al.*
185 2015). Images were acquired on a Leica SP8 confocal laser-scanning microscope. Cell
186 size calculation has been performed as described (GARRIDO *et al.* 2015) from at least
187 eight larvae per genotype, containing a few MARCM clones surrounded by control cells.
188 Because of an extremely low ratio of larvae that fully develop and harbour mitotic
189 clones, the mean values were calculated from several experiments that could not be
190 performed at the same time, but over a long period. Therefore, for the graphs of cell
191 size measurement (Figures S2A, S2C, 5M and 7M), values are reused when they
192 correspond to the same genotype and conditions, allowing direct comparison between
193 experiments.

194

195 **Data and reagent availability statement**

196 Fly stocks and reagents are available upon request. Supplementary materials include
197 Figures S1-S5, statistical analysis (methods and Tables S1-S7) and supdata/script files.

198

199 **RESULTS**

200 **TORC1 and Ilp signaling independency in the fat body**

201 To investigate the relationship between TORC1- and Ilp-dependent overgrowth in the
202 FB, we generated somatic clones either over-expressing Rheb (*Rheb⁺*) (STOCKER *et al.*

203 2003) or *PTEN* homozygous mutants (*PTEN*^{-/-}) (OLDHAM *et al.* 2000). The precursors of
204 FB cells divide in the embryo; during larval life, the differentiated cells do not divide but
205 endoreplicate their DNA content to reach a giant size (EDGAR AND ORR-WEAVER 2001).
206 Therefore, to precisely evaluate the effect on cell growth, somatic recombinations were
207 induced during embryogenesis at the stage of proliferation of the FB cell precursors and
208 the resulting MARCM clones were analyzed in the FB of late feeding L3 larvae, prior to
209 the wandering stage that precedes metamorphosis entry. In accordance with previous
210 studies manipulating *Iip* and TOR signaling in FB cells (BRITTON *et al.* 2002; SCOTT *et al.*
211 2004), both *PTEN*^{-/-} and *Rheb*⁺ clonal cells were bigger than the surrounding control
212 cells and this cell size effect was dramatically increased in *PTEN*^{-/-};*Rheb*⁺ combined
213 clones (Figure 1A-D and Figure S2A). We next analyzed this growth increase in the
214 context of previously reported *Tor* mutations, the *Tor*^{AP} deletion (ZHANG *et al.* 2006), and
215 the *Tor*^{2L1} and *Tor*^{2L19} alleles that result in a kinase inactive and in a short truncated
216 protein, respectively (OLDHAM *et al.* 2000). Given that we could not find mutant clones,
217 which were likely eliminated by cell competition, we generated somatic clones in a
218 *Minute* background to slow down the growth of the surrounding control cells. In these
219 conditions, any of the *Tor* mutant cells exhibited a dramatic size reduction (Figure 1G,
220 1J, 1M and Figure S2C) and this phenotype was dominant in *Rheb*⁺ combined clonal
221 cells (Figure S2C and compare Figure 1E to 1H, 1K and 1N). In contrast, *Tor*^{AP} and
222 *Tor*^{2L19} but not *Tor*^{2L1} exhibited a clear dominant phenotype over the *PTEN*^{-/-} mutation;
223 the size of *Tor*^{AP},*PTEN*^{-/-} and *Tor*^{2L19},*PTEN*^{-/-} clonal cells was dramatically reduced
224 (Figure S2C and compare Figure 1F to 1L and 1O), whereas *Tor*^{2L1},*PTEN*^{-/-} clonal cells
225 were bigger than the surrounding cells (Figure 1M) and tend to be relatively bigger than
226 control clonal cells (Figure S2B and S2C). These findings indicate that the *Tor*^{2L1}

227 mutation restrains TORC1- but not IIP-dependent overgrowth, whereas *Tor^{AP}* and
228 *Tor^{2L19}* affects both.

229 Next, we used phospho-specific antibodies in immunostaining assays to analyze the
230 phosphorylation of Akt (P-Akt) and of the dS6K target, ribosomal protein rpS6 (P-S6).
231 The P-Akt intracellular signal was increased in *PTEN^{-/-}* clones, absent in *Tor^{AP}* and
232 *Tor^{2L19}* clones, but not affected in *Tor^{2L1}* clones (Figure 2A-D). Staining with the rpS6
233 phospho-specific antibody revealed a patchy signal, with only a subset of cells
234 expressing the P-S6 signal in the FB (Figure 2E-H), a pattern previously described in
235 the wing imaginal disc (ROMERO-POZUELO *et al.* 2017). Therefore, to evaluate TORC1
236 activity, we measured the ratio of P-S6 positive cells among the population of GFP⁺
237 clonal cells. About half of the control clones were P-S6 positive (Figure 2E and 2M),
238 whereas most of the *Tor^{2L1}*, *Tor^{2L19}* and *Tor^{AP}* clones were P-S6 negative (Figure 2F-H
239 and 2M). Importantly, almost all the *Rheb⁺* clones were P-S6 positive (Figure 2I and
240 2M), whereas the ratio of P-S6 positive cells was slightly but not significantly increased
241 in the *PTEN^{-/-}* clones (Figure 2J and 2M). Taken together, these findings reveal that the
242 *Tor^{2L1}* mutation affects only TORC1, whereas the *Tor^{AP}* and *Tor^{2L19}* mutations affect
243 both signaling branches and support the notion that IIP- and TORC1-dependent
244 overgrowth can be independently modulated in FB cells.

245

246 **Activating TORC1 or IIP signaling impacts basal metabolism**

247 To evaluate how TORC1 and IIP affect basal metabolism in *Drosophila*, we analyzed
248 various metabolites in whole animals that express the ubiquitous *da-gal4* driver to direct
249 Rheb overexpression (*Rheb⁺⁺*) or PTEN knockdown (*PTEN-RNAi*). Larvae were fed
250 either a standard or a 20%-sucrose supplemented diet (20%-SSD) and 0-5h prepupae
251 were collected, as this is a convenient phase to stage the animals after the feeding

252 period. When fed a standard diet, a high rate of lethality was observed for *Rheb⁺⁺* and
253 *PTEN-RNAi* larvae, although a sufficient number of prepupae could be recovered.
254 Given that none of the *Rheb⁺⁺* and *PTEN-RNAi* larvae fed a 20%-SSD reached the
255 prepupal stage, larvae were first fed a standard diet and transferred onto a 20%-SSD at
256 the L2/L3 molting transition. In this way, we could recover a few prepupae for metabolic
257 measurements. For both males and females fed a standard diet, the body weight of
258 *Rheb⁺⁺* and *PTEN-RNAi* prepupae was roughly similar to that of controls (Figure 3A and
259 3B). Conversely, providing a 20%-SSD resulted in a drop of the prepupal weight of
260 control animals that was significantly compensated in *Rheb⁺⁺* and *PTEN-RNAi*
261 prepupae (Figure 3A and 3B).

262 Next, we measured the total amounts of protein, triacylglycerol (TAG), glycogen and
263 trehalose—the most abundant circulating sugar in *Drosophila*. Although protein levels
264 did not vary in *PTEN-RNAi* and *Rheb⁺⁺* animals fed a standard diet, a mild but
265 significant increase was observed in *PTEN-RNAi* animals fed a 20%-SSD (Figure 3C).
266 TAG levels were strongly increased in animals fed a 20%-SSD (Figure 3D). When fed a
267 standard diet, TAG levels did not vary in *PTEN-RNAi* and *Rheb⁺⁺* animals, whereas,
268 when fed a 20%-SSD, they were strongly increased in *PTEN-RNAi* animals (Figure 3D).
269 Feeding larvae a 20%-SSD resulted in a moderate increase in glycogen and trehalose
270 levels in control prepupae (Figure 3E-F). In *Rheb⁺⁺* and, in lower extent, in *PTEN-RNAi*
271 prepupae, glycogen levels were significantly lower than those measured in controls
272 (Figure 3E). Finally, trehalose levels were strongly decreased in both *Rheb⁺⁺* and
273 *PTEN-RNAi* prepupae fed either a standard or a 20%-SSD as compared to control
274 (Figure 3F). Taken together, these findings suggest that a ubiquitous increased activity
275 of either TORC1 or Iip signaling provokes an apparent increase in sugar consumption.
276 This metabolic rate is correlated with a relative increase in body weight for larvae fed a

277 20%-SSD, but not for those fed a standard diet. We previously observed that increasing
278 dietary sucrose induced a reduction in food intake (GARRIDO *et al.* 2015) that may
279 account for the body weight reduction of control animals. Potentially, food intake could
280 be less affected in *Rheb⁺⁺* and *PTEN-RNAi* animals, thereby leading to a compensatory
281 effect on body weight. Measuring food intake in *Rheb⁺⁺* or *PTEN-RNAi* larvae was not
282 applicable since most of them die during larval stage and thus, terminate feeding earlier.
283 In sum, our data indicates that basal metabolism is altered in the few *Rheb⁺⁺* or *PTEN-*
284 *RNAi* larvae that survive and further suggests that in most cases stronger metabolic
285 disruption may happen, resulting in lethal homeostatic defects.

286

287 **Knocking-down glycolysis at the whole body level**

288 To determine whether the basal energy metabolism affected TORC1- and/or IIP-
289 signaling, we ubiquitously expressed RNAs against phosphofructokinase1 (*PFK1-*
290 *RNAi*), pyruvate kinase (*PK-RNAi*) pyruvate dehydrogenase (*PDH-RNAi*) and lactate
291 dehydrogenase (*LDH-RNAi*). PFK1 catalyzes the third glycolytic reaction to form
292 fructose 1,6-bisphosphate; PK catalyzes the final glycolytic reaction to form pyruvate;
293 PDH directs the mitochondrial fate of pyruvate, whereas LDH directs its anaerobic fate
294 (Figure 4A). When directed with the ubiquitous *da-gal4* driver, *PK-RNAi* provoked early
295 larval lethality, *PFK1-RNAi* and *PDH-RNAi* provoked larval lethality at L2 or L3 stages,
296 whereas *LDH-RNAi* induced a semi-lethal phenotype at larval or pupal stages (Figure
297 4B).

298 Next, we monitored the phosphorylation of the *Drosophila* S6Kinase (dS6K), Akt and
299 ERK. To circumvent the early lethality, the activity of the *da-gal4* driver was blocked
300 until early L1 stage using the thermo-sensitive Gal4 inhibitor, Gal80^{ts}, then, RNAi
301 expression was induced by a temperature shift at 29°C; protein extracts were prepared

302 two days later using late L2 larvae. At this stage the larvae were still viable, although
303 those expressing PK-RNAi did not undergo L2/L3 transition and eventually died (Figure
304 4B). Western-blotting revealed that RNAi-knockdown of PFK1 or LDH did not affect
305 phosphorylation of dS6K, Akt or ERK (Figure 4C and Figure S3A-F). In contrast,
306 knockdown of PK or PDH decreased dS6K phosphorylation, whereas PK- but not PDH-
307 knockdown mildly decreased Akt phosphorylation (Figure 4C and S3A-D).
308 Unexpectedly, knockdown of PK and LDH, and in lower extent knockdown of PFK and
309 PDH, increased the amounts of total ERK protein with minor effects on its
310 phosphorylation rate (Figure 4C and Figure S3E-F). These results indicate that
311 ubiquitous knockdown of aerobic glycolysis specifically affects TORC1 signaling.

312 To evaluate the requirement of glycolysis for adult survival, RNAi-knockdown was
313 induced by temperature shift to 29°C in newly emerged flies and lethality was counted
314 every second day. In both males and females, PK and PFK1 knockdown provoked
315 lethality between 10 to 14 days after temperature shift (Figure 4D). Knockdown of PDH
316 and LDH also induced adult lethality, although not as soon as PK and PFK1 knockdown
317 (Figure 4D). As a comparison, to evaluate the consequence of disrupting FA synthesis,
318 we knocked-down FASN1 (Figure 4A) in adults; about a quarter of *FASN1-RNAi* flies
319 died between 10 to 14 days, while the others survived nearly as well as control flies
320 (*FASN1i* in Figure 4D). Taken together, these data indicate that glycolysis is essential
321 for both larval development and adult survival.

322

323 **Cell-autonomous requirement of glycolysis for *Ilp*- but not TORC1-dependent** 324 **overgrowth**

325 To investigate the requirement of glycolysis to sustain cell-autonomous overgrowth
326 dependent on *Ilp*- and TORC1-signaling, *PFK1-RNAi*, *PK-RNAi*, *PDH-RNAi* and *LDH-*

327 RNAi were induced in *PTEN*^{-/-} or *Rheb*⁺ clones. Except a moderate effect of *PK-RNAi*,
328 clones expressing RNAi against these metabolic enzymes did not significantly affect the
329 growth of FB cells (Figure 5A-D and 5M). In combined clones, none of the RNAi
330 affected the growth of *Rheb*⁺ clones (Figure 5E-H and 5M). In contrast, the size of
331 *PTEN*^{-/-} clones was significantly decreased when co-expressing RNAi against any of
332 these metabolic enzymes (Figure 5I-M). These findings indicate that both aerobic and
333 anaerobic glycolysis are required to sustain cell-autonomous overgrowth dependent on
334 Iip signaling. In contrast, reducing glycolysis does not counteract cell-autonomous
335 overgrowth dependent on TORC1 signaling.

336

337 **Linking FA synthesis to TORC1- and Iip-signaling**

338 Since glycolysis and FA synthesis are tightly connected metabolic pathways (GARRIDO
339 *et al.* 2015), we investigated whether the deficiency of the latter affects Iip or TORC1
340 signaling. The *Drosophila* genome encodes three *FASN* genes, *FASN1* is ubiquitously
341 expressed but not *FASN2* or *FASN3* (PARVY *et al.* 2012; CHUNG *et al.* 2014; WICKER-
342 THOMAS *et al.* 2015). The deletion of the *FASN1* and *FASN2* tandem (*FASN*^{A24-23}
343 deletion, hereafter called *FASN*¹⁻²) results in a lethal phenotype that can be rescued by
344 feeding larvae a lipid-complemented diet (beySD) (GARRIDO *et al.* 2015; WICKER-
345 THOMAS *et al.* 2015). However, beySD-rescued *FASN*¹⁻² mutant larvae exhibited a
346 developmental delay to enter metamorphosis (Figure 6A). Further, when beySD-
347 rescued *FASN*¹⁻² mutant larvae were transferred at the L2/L3 larval transition onto a
348 10% sucrose-supplemented-beySD, only a few of them completed the third larval stage
349 and, after an extreme developmental delay, entered metamorphosis (Figure 6A). Delay
350 in development can be due to a default in ecdysone production that results in giant
351 pupae (PARVY *et al.* 2014) or to impaired TOR signaling that results in reduced body

352 growth (MONTAGNE *et al.* 1999; OLDHAM *et al.* 2000). Measurements of prepupal weight
353 revealed that *FASN*¹⁻² mutant prepupae exhibited a severe reduction in body weight and
354 body size, whether or not they were supplemented with sucrose (Figure 6B and Figure
355 S4), suggesting a defect in TOR signaling. Therefore, we analyzed the phosphorylation
356 of the *Drosophila* S6Kinase (dS6K) and Akt in protein extracts of late feeding L3 larvae.
357 Western-blotting revealed that the dS6K protein resolved in several bands in *FASN*¹⁻²
358 extracts, whereas Akt protein was unchanged (Figure 6C and Figure S3G and S3I).
359 These results suggest that dS6K but not Akt might be degraded in the *FASN*¹⁻² mutant
360 background. In addition, dS6K phosphorylation decreased in *FASN*¹⁻² extracts and
361 became barely detectable when *FASN*¹⁻² larvae were fed a sucrose-supplemented-
362 beySD (Figure 6C and Figure S3H). Conversely, the phosphorylation of Akt was
363 unaffected in larvae fed a beySD, although it was slightly decreased in larvae fed a
364 sucrose-supplemented-beySD (Figure 6C and Figure S3J). This finding contrasts with
365 our previous observation showing that FB explants of *FASN*¹⁻² mutant larvae were
366 hypersensitive to insulin (GARRIDO *et al.* 2015). However, *FASN*¹⁻² mutants also
367 exhibited a decrease in food intake (GARRIDO *et al.* 2015), which might induce a
368 systemic suppression of dS6K phosphorylation, while FB explant were cultured in
369 nutrient media supplemented with insulin. Therefore, to determine whether *FASN*
370 mutation affects TOR signaling at the cell-autonomous level, we analyzed P-S6 and P-
371 Akt in *FASN*¹⁻² mutant clones in the FB. As for control clones, about half of the *FASN*¹⁻²
372 clonal cells were P-S6 positive (Figure 2L and 2M). Furthermore, no effect on P-Akt was
373 observed in *FASN*¹⁻² clonal cells (Figure 2K). In summary, these findings reveal that
374 disrupting FA synthesis does not significantly affect TORC1 and Ilp signaling at the cell-
375 autonomous level, although it seems to impinge on TORC1 signaling when inhibited in
376 the whole animal whether directly or indirectly.

377

378 **Cell-autonomous requirement of FA synthesis for Ilp- but not TORC1-dependent**
379 **overgrowth**

380 To determine, whether FA synthesis is required at the cell-autonomous level to sustain
381 TORC1 and/or Ilp dependent growth, we analyzed *FASN*¹⁻² clones while enhancing
382 either TORC1 or Ilp signaling in FB cells. We previously reported (GARRIDO *et al.* 2015)
383 that *FASN*¹⁻² clonal cells in the FB were slightly reduced in size and that this effect was
384 dramatically increased in larvae fed a 20%-SSD (Figure S5A-B and Figure 7M).
385 Therefore, we generated *PTEN*^{-/-} and *Rheb*⁺ clones combined or not with the *FASN*¹⁻²
386 mutation and analyzed them in the FB of larvae fed either a standard diet or a 20%-
387 SSD. As compared to the standard diet, feeding larvae a 20%-SSD had no effect on the
388 size of *Rheb*⁺ clonal cells, but significantly reduced the size of *PTEN*^{-/-} and of *PTEN*^{-/-}
389 ;*Rheb*⁺ clonal cells (Figure 7A-F and 7M). Further, when combined with the *FASN*¹⁻²
390 mutation, *PTEN*^{-/-} but not *Rheb*⁺ clones were significantly reduced in size (Figure 7G-H
391 and 7M). The *FASN*¹⁻² mutation also provoked a severe size reduction of *PTEN*^{-/-},*Rheb*⁺
392 clones (Figure 7I and 7M). Moreover, as compared to the standard diet, feeding larvae
393 a 20%-SSD induced a significant size reduction of *FASN*¹⁻²;*Rheb*⁺, *FASN*¹⁻²,*PTEN*^{-/-} and
394 *FASN*¹⁻²,*PTEN*^{-/-};*Rheb*⁺ clonal cells (Figure 7J-L and 7M). Of note, except for the
395 *FASN*¹⁻²;*Rheb*⁺ clonal cells in larvae fed a 20%-SSD that exhibited a size roughly
396 identical to that of the surrounding control cells (Figure 7J), the cell size was always
397 bigger than the controls (Figure 7M). These findings indicate that, in larvae fed a
398 standard diet, FA synthesis is at least in part required to sustain over-growth induced by
399 Ilp, but not TORC1. Nonetheless, the metabolic dependency of Ilp-induced overgrowth
400 might be a consequence of the stronger phenotype of *PTEN*^{-/-} clones, which could be
401 more sensitive to metabolic restriction than the one of *Rheb*⁺ clones. To challenge such

402 an unspecific effect, we made use of *PTEN-RNAi* that diminish but do not abolish its
403 expression. In this setting, the size of *PTEN-RNAi* clonal cells was significantly higher
404 than the one of control clones, but not as much as the one of *Rheb*⁺ clonal cells (Figure
405 7M and Figure S5C). Importantly the size of *PTEN-RNAi* clonal cells was significantly
406 diminished when combine to the *FASN* deficiency (Figure 7M and Figure S5D), which
407 excludes a strength-phenotype dependent effect. In conclusion, overgrown cells
408 generated by *Ilp* over-activation have limited homeostatic abilities to adjust to metabolic
409 restriction, whereas TORC1 activated cells at least in part maintain these abilities.

410

411 **DISCUSSION**

412 In this study, we have investigated the functional links between the glycolysis/FA
413 synthesis axis and TORC1- or *Ilp*-dependent growth in *Drosophila*. Consistent with
414 previous studies (RADIMERSKI *et al.* 2002a; RADIMERSKI *et al.* 2002b; DONG AND PAN
415 2004; MONTAGNE *et al.* 2010; PALLARES-CARTES *et al.* 2012), we show that TORC1- and
416 *Ilp*-dependent overgrowth can be independently modulated in the *Drosophila* FB.
417 TORC1, once recruited at the lysosomal membrane, is activated by *Rheb* to
418 phosphorylate downstream targets (MONTAGNE 2016). TORC2, which comprises *rictor*,
419 resides in the *Ilp* signaling cascade to phosphorylate the S505 residue in the
420 hydrophobic motif (HM) of *Akt*. However, a study on a *Drosophila rictor* mutant reported
421 that S505 phosphorylation by TORC2 was not required to sustain *Akt*-dependent growth
422 and proposed that TORC2 plays as a rheostat for this process (HIETAKANGAS AND COHEN
423 2007). Here, we provide evidence that the previously described *Tor*^{2L1} mutation, which
424 likely results in a kinase-inactive protein (OLDHAM *et al.* 2000), affects TORC1 but not
425 *Ilp*-dependent overgrowth, whereas both overgrowth processes are suppressed by the
426 *Tor*^{ΔP} or *Tor*^{2L19} mutations that likely represent null mutants. Surprisingly, we observe

427 that the S505 residue in the Akt HM is still phosphorylated in *Tor^{2L1}*, but not *Tor^{AP}* and
428 *Tor^{2L19}* mutant clones. Taken together, these findings reveal that the TOR protein, but
429 not its kinase activity, is required for Akt HM phosphorylation and for Iip-dependent
430 overgrowth through a molecular mechanism that remains to be elucidated.

431 Intriguingly, our study reveals apparent contradictory effects between perturbations at
432 the whole body and cell-autonomous levels. At the organismal level, knockdowns of
433 glycolytic enzymes to mimic drug treatment with a systemic inhibitor result in animal
434 death. Knockdown of PDH suppress dS6K but not Akt phosphorylation suggesting that
435 aerobic glycolysis is crucial for TORC1 signaling. These observations contrast with
436 mammalian studies showing that mTORC1 stimulation promotes anaerobic glycolysis
437 (DUVEL *et al.* 2010), whereas Akt activation promotes aerobic glycolysis (GOTTLOB *et al.*
438 2001). Furthermore, at the cell-autonomous level, repression of either aerobic or
439 anaerobic glycolysis partly restrains Iip- but not TORC1-dependent overgrowth of FB
440 cells. Taken together, these findings confirm the existence of tight connections between
441 TOR signaling and basal metabolism, although the cell-autonomous effects suggest that
442 this connection integrates interactions operating between neighboring cells or organs. In
443 addition, the growth defect of beySD-rescued *FASN* null mutants might result from the
444 decrease in TORC1 activity, suggesting that TORC1 but not Iip signaling relies on FA
445 synthesis. However, at the cell autonomous level, the deficiency of *FASN* restrains Iip
446 but not TORC1 dependent overgrowth in FB cells. These apparent contradictory
447 findings, suggest that the growth defect and the reduction of TORC1 activity in *FASN*
448 mutants are not due to the addition of cell-autonomous effects but potentially to a
449 systemic regulation, as for instance, Iip secretion by neurosecretory cells (RULIFSON *et*
450 *al.* 2002). Alternatively, considering that TORC1 directly responds to nutrients (OLDHAM
451 *et al.* 2000), the drop of TORC1 activity may be a consequence of feeding, consistent

452 with the decrease in food uptake previously reported in *FASN*¹⁻² mutants (GARRIDO *et al.*
453 2015). Congruently, a study on the transcription factor Mondo, which stimulates
454 glycolysis and FA synthesis in response to dietary sugar (MATTILA *et al.* 2015;
455 RICHARDS *et al.* 2017), suggests the existence of a FASN-dependent control of food
456 intake (SASSU *et al.* 2012). Finally, the drop of TORC1 activity observed in *FASN*¹⁻²
457 mutants may be a consequence of malonyl-CoA accumulation, since mTOR
458 malonylation has been reported to inhibit mTORC1- but not Iip/mTORC2-dependent
459 activity (BRUNING *et al.* 2018). Whether TOR malonylation and the subsequent decrease
460 in TORC1 activity may also occur in *Drosophila* should be investigated in the future.

461 Our study reveals that over-activation of mTORC1 and of Iip signaling, results in a
462 decrease in glycogen stores and in circulating trehalose, suggesting that activation of
463 either signaling branch enhances glucose consumption to sustain cell growth. However,
464 the activation of neither mTORC1 nor Iip signaling induces an increase in body weight.
465 Ubiquitous activation of TORC1 or Iip signaling is likely to promote the growth of most
466 cells, but might concurrently perturb endocrine signals dampening overall growth. For
467 instance, previous studies reported that activation of Iip signaling within the ecdysone-
468 secreting gland, results in a systemic decrease in body growth (CALDWELL *et al.* 2005;
469 COLOMBANI *et al.* 2005; MIRTH 2005). Of note, we observed that larvae fed a 20%-SSD
470 result in pupae with reduced body weight, an effect that is partially suppressed when
471 either TORC1 or Iip signaling is over-activated. The fact that the overall body weight of
472 these animals is maintained within a range likely compatible with organismal survival
473 contrasts with the observed high rate of lethality. The decrease in glycogen and
474 trehalose suggests that in these animals each cell tends to increase its basal
475 metabolism evoking an egoist behavior that might perturb the equilibrium between cell-
476 autonomous and systemic regulation. Thus, in a stressful situation, as when animals are

477 fed a 20%-SSD, the need of a tight adjustment to an unbalanced diet might enhance the
478 distortion between cell-autonomous effects and systemic regulation, resulting in animal
479 death.

480 A plethora of studies in mammalian cells indicate that mTOR activation directs
481 metabolism towards glucose consumption, storage and anabolism (GOTTLOB *et al.*
482 2001; INOKI *et al.* 2003b; HAHN-WINDGASSEN *et al.* 2005; DUVEL *et al.* 2010; PETERSON *et*
483 *al.* 2011; HOUDANE *et al.* 2017; JALDIN-FINCATI *et al.* 2017; WIPPERMAN *et al.* 2019). Our
484 study suggests that in the *Drosophila* larvae, TOR promotes glucose consumption, but
485 an increase in TAG levels is observed only for larvae fed a 20%-SSD when Ilp signaling
486 is ubiquitously over-activated. However, at the cell-autonomous level, we observe that
487 inhibition of glycolysis or FA synthesis restrains neither larval FB cell growth nor
488 overgrowth induced by TORC1 stimulation in these cells. These findings counteract the
489 idea that mTORC1 potentiates a glycolysis/FA synthesis axis (DUVEL *et al.* 2010) to
490 sustain cell growth. To overcome the lack of glycolytic products and of membrane lipids,
491 these cells may benefit of a transfer from neighboring cells and/or might favor
492 alternative metabolic pathways, including glutamine catabolism to feed TCA
493 anaplerosis, which has been shown to be a crucial pathway in mTORC1-stimulated
494 mammalian cells (CHOO *et al.* 2010; CSIBI *et al.* 2013; CSIBI *et al.* 2014). Nonetheless,
495 such compensatory processes do not fully operate to sustain Ilp-dependent overgrowth,
496 potentially because these cells might be more sensitive to homeostasis perturbations or
497 because the modulation of Ilp signaling would be required for these compensatory
498 processes.

499 As a coordinator of growth and metabolism, mTOR plays a central role in tumor
500 development (DOWLING *et al.* 2010; HARACHI *et al.* 2018; MOSSMANN *et al.* 2018; TIAN *et*
501 *al.* 2019). PTEN, the tumor suppressor that counteracts PI3K activity downstream of the

502 Iip receptor, is deficient in several human cancers (CULLY *et al.* 2006). Mutation of TSC1
503 or TSC2, which results in mTORC1 hyper-activation, is associated with benign tumors
504 but also with brain, kidney and lung destructive diseases (HENSKE *et al.* 2016). To
505 investigate the role of mTOR regarding tumor development, a recent study reported the
506 generation of liver-specific double knockout mice for TSC1 and PTEN (GURI *et al.*
507 2017). These mice develop hepatic steatosis that eventually progresses to
508 hepatocellular carcinoma. Both processes are suppressed in mice fed the mTORC1/2
509 inhibitors INK128, but not the mTORC1 inhibitor rapamycin, supporting an Iip/mTORC2
510 specific effect. The combination of inhibitors against mTOR and metabolism is currently
511 under clinical investigation to fight cancers (MOSSMANN *et al.* 2018). Importantly, our
512 study reveals that ubiquitous inhibition of basal metabolism produces dramatic effects
513 during development, while at the cell-autonomous level, it only moderates growth
514 induced by over-activation of Iip/TORC2 signaling. Therefore, the use of drug therapy to
515 fight cancer must be taken with caution, in particular if organismal development is not
516 complete and most efforts should be made to selectively target sick tissues.

517

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527

528 **AUTHOR CONTRIBUTIONS**

529 JM designed the experiments; MD, DG, MP, TR and JM performed the experiments;
530 MD, DG, ALR and JM analyzed the results; and JM wrote the manuscript.

531

532 **FIGURE LEGENDS**

533 **Figure 1: TORC1- and Iip-dependent growth in FB cells.** MARCM clones labeled by
534 GFP (green) in the FB of L3 larvae. Nuclei were labeled with DAPI (silver) and
535 membranes with phalloidin (red). Control (A), *Rheb*⁺ (B), *PTEN*^{-/-} (C) and *PTEN*^{-/-};*Rheb*⁺
536 (D) clones were generated in a wild type background. *Rheb*⁺ (E), *PTEN*^{-/-} (F), *Tor*^{2L1} (G)
537 *Tor*^{2L1}, *Rheb*⁺ (H) *Tor*^{2L1},*PTEN*^{-/-} (I), *Tor*^{AP} (J), *Tor*^{AP},*Rheb*⁺ (K), *Tor*^{AP},*PTEN*^{-/-} (L) *Tor*^{2L19}
538 (M) *Tor*^{2L19}, *Rheb*⁺ (N) and *Tor*^{2L19},*PTEN*^{-/-} (O), clones were generated in a *Minute* (M)
539 background. Scale bars: 50µm.

540

541 **Figure 2: TORC1 and Iip signaling activity in FB cells. (A-J)** MARCM clones labeled
542 by GFP (green) in the FB of L3 larvae. Clones were generated in a wild type
543 (A,E,I,J,K,L) or a *Minute* (B,C,D,F,G,H) background and nuclei were labeled with DAPI
544 (silver). FB tissues with *PTEN*^{-/-} (A), *Tor*^{2L1} (B), *Tor*^{AP} (C), *Tor*^{2L19} (D) and *FASN*¹⁻² (K)
545 clones were stained with a phospho-AKT antibody. FB tissues with control (E), *Tor*^{2L1}
546 (F), *Tor*^{AP} (G), *Tor*^{2L19} (H) *Rheb*⁺ (I), *PTEN*^{-/-} (J) and *FASN*¹⁻² (L) clones were stained
547 with a phospho-S6 antibody. Scale bars: 50µm. **(M)** Percentage of P-S6 positive clones
548 with respect to the total number of MARCM clones for control, *FASN*¹⁻², *PTEN*^{-/-}, *Rheb*⁺,
549 *Tor*^{2L1}, *Tor*^{AP} and *Tor*^{2L19} genotypes.

550

551 **Figure 3: Enhanced TORC1 or Ilp signaling affects larval metabolism. (A-B)** Body
552 weight of female (A) and male (B) prepupae formed from larvae fed either a standard
553 (0%) or a 20%-SSD (20%) as from the L2/L3 transition. **(C-F)** Measurement of total
554 protein (C), TAG (D), glycogen (E) and trehalose (F) levels in prepupae fed either a
555 standard or a 20%-SSD. Prepupae used in these measurements were the F1 progeny
556 from *da-gal4;UAS-Dcr-2* females mated to either control (Co), *EP(UAS)-Rheb* (*Rheb⁺⁺*)
557 or *UAS-PTEN-RNAi* (*PTEN-Ri*) males.

558
559 **Figure 4: Glycolysis knockdown in whole organisms. (A)** Scheme of basal
560 metabolism. Glucose and trehalose enter glycolysis as glucose-6P, whereas fructose
561 follows a distinct pathway to triose-P. Enzymes investigated in the present study are
562 marked in red. **(B)** Phenotype of ubiquitous RNAi knockdown of PFK1, PK, LDH and
563 PDH. Flies were left to lay eggs overnight either at 29°C (column 0h) or at 19°C and
564 transferred to 29°C the day after (column 24h); then development proceeded at 29°C
565 (i.e. the temperature that inactivates Gal80). **(C)** Western-blot analysis of total (top) or
566 phosphorylated (mid) dS6K (left) or Akt (right) proteins; tubulin (bottom) was used as a
567 loading control. Protein extracts were prepared with late L2 larvae either control (Co), or
568 expressing RNAi against the indicated metabolic enzymes. **(D)** Survival at 29°C of male
569 (top) and female (bottom) control flies or flies expressing RNAi against the indicated
570 metabolic enzymes as from adult eclosion.

571
572 **Figure 5: Cell-autonomous requirement of glycolysis for Ilp- but not TORC1-**
573 **dependent overgrowth. (A-G)** MARCM clones labeled by GFP (green) in the FB of L3
574 larvae. Nuclei were labeled with DAPI (silver) and membranes with phalloidin (red).
575 Genotypes of MARCM clones are: *PFK1-RNAi* (A), *PK-RNAi* (B), *LDH-RNAi* (C), *PDH-*

576 *RNAi* (D), *Rheb*⁺,*PFK1-RNAi* (E), *Rheb*⁺,*PK-RNAi* (F), *Rheb*⁺,*LDH-RNAi* (G),
577 *Rheb*⁺,*PDH-RNAi* (H), *PTEN*^{-/-};*PFK1-RNAi* (I), *PTEN*^{-/-};*PK-RNAi* (J), *PTEN*^{-/-};*PDH-RNAi*
578 (K) and *PTEN*^{-/-};*PDH-RNAi* (L). Scale bars: 50µm. (M) Relative size of clonal cells
579 corresponding to the clones shown in A-L, and in Figure 1A for control (Co).

580

581 **Figure 6: *FASN*¹⁻² mutation affects developmental growth and TORC1 signaling.**

582 (A) Developmental duration from egg laying to metamorphosis onset of *w*¹¹¹⁸ control
583 (Co) and *FASN*¹⁻² (*FASN*) larvae fed either a beySD (0%) or a 10% sucrose-
584 supplemented-beySD as from the L2/L3 transition (10%); n: total number of larvae
585 collected for each condition. Each condition and genotype significantly differ from each
586 other, either by final frequency or dynamic of pupariation (Table S6). (B) Prepupal
587 weight of females (left) and males (right) as listed in 6A; the numbers of weighted
588 prepupae are indicated above each sample. (C) Western-blot analysis of (from top to
589 bottom) total dS6K, phosphorylated dS6K, total Akt, phosphorylated Akt and total
590 tubulin as a loading control. Protein extracts were prepared from feeding L3 larvae prior
591 to the wandering stage as listed in 6A. For each condition, at least 30 larvae were used
592 to prepare protein extracts.

593

594 **Figure 7: Cell-autonomous requirement of *FASN* activity for *Iip*- but not TORC1-**

595 **dependent overgrowth. (A-L)** MARCM clones labeled by GFP (green) in the FB of L3
596 larvae fed either a standard (A-C, G-I) or a 20%-SSD (D-F, J-L). Nuclei were labeled
597 with DAPI (silver) and membranes with phalloidin (red). Genotypes of MARCM clones
598 are: *Rheb*⁺ (A,D), *PTEN*^{-/-} (B,E) *PTEN*^{-/-},*Rheb*⁺ (C,F), *FASN*¹⁻²;*Rheb*⁺ (G,J) *FASN*¹⁻
599 ²,*PTEN*^{-/-} (H,K) and the *FASN*¹⁻²,*PTEN*^{-/-};*Rheb*⁺ (I,L). Scale bars: 50µm. (M) Relative

600 size of clonal cells corresponding to the clones shown in A-L and in Figure S5A-D for
601 *FASN*¹⁻² and *PTEN-RNAi* clones and in Figure 1A for control (Co).

602

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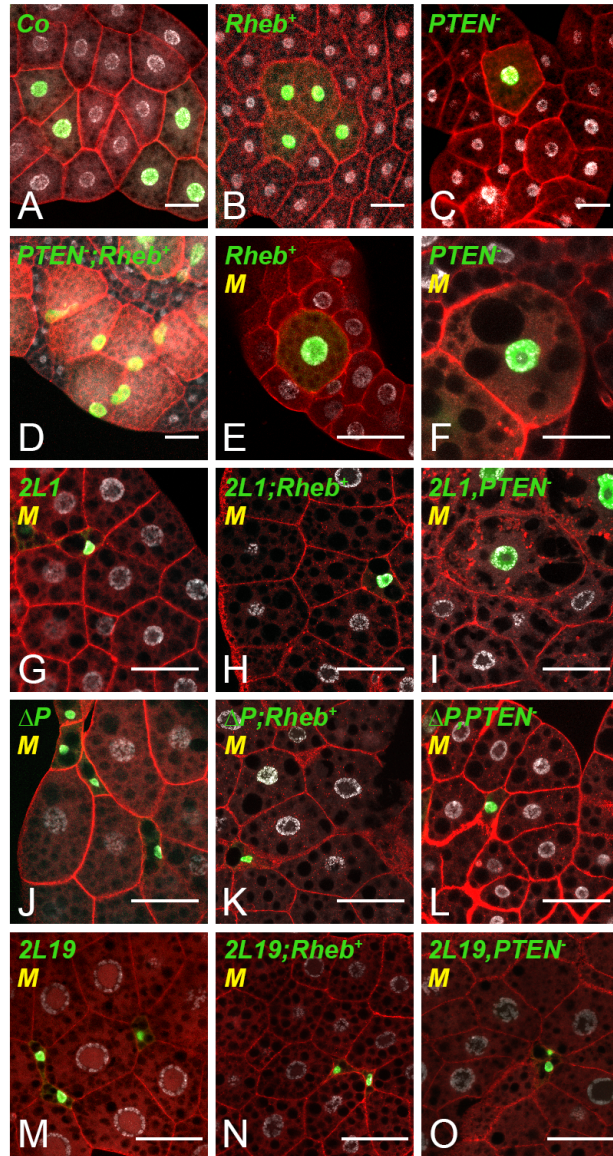
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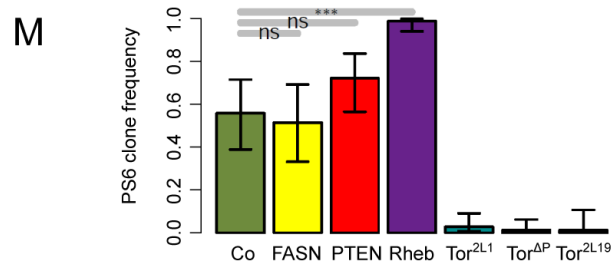
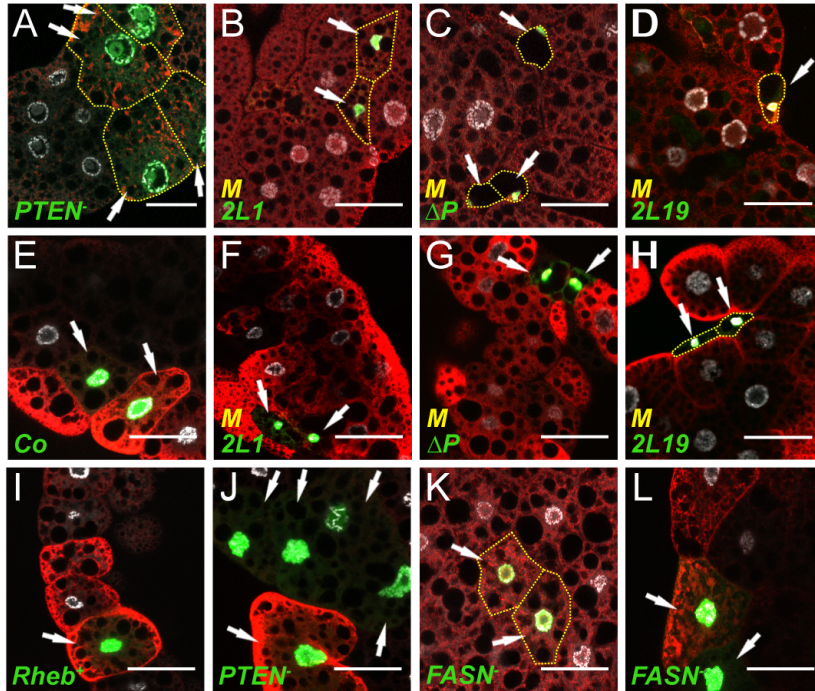
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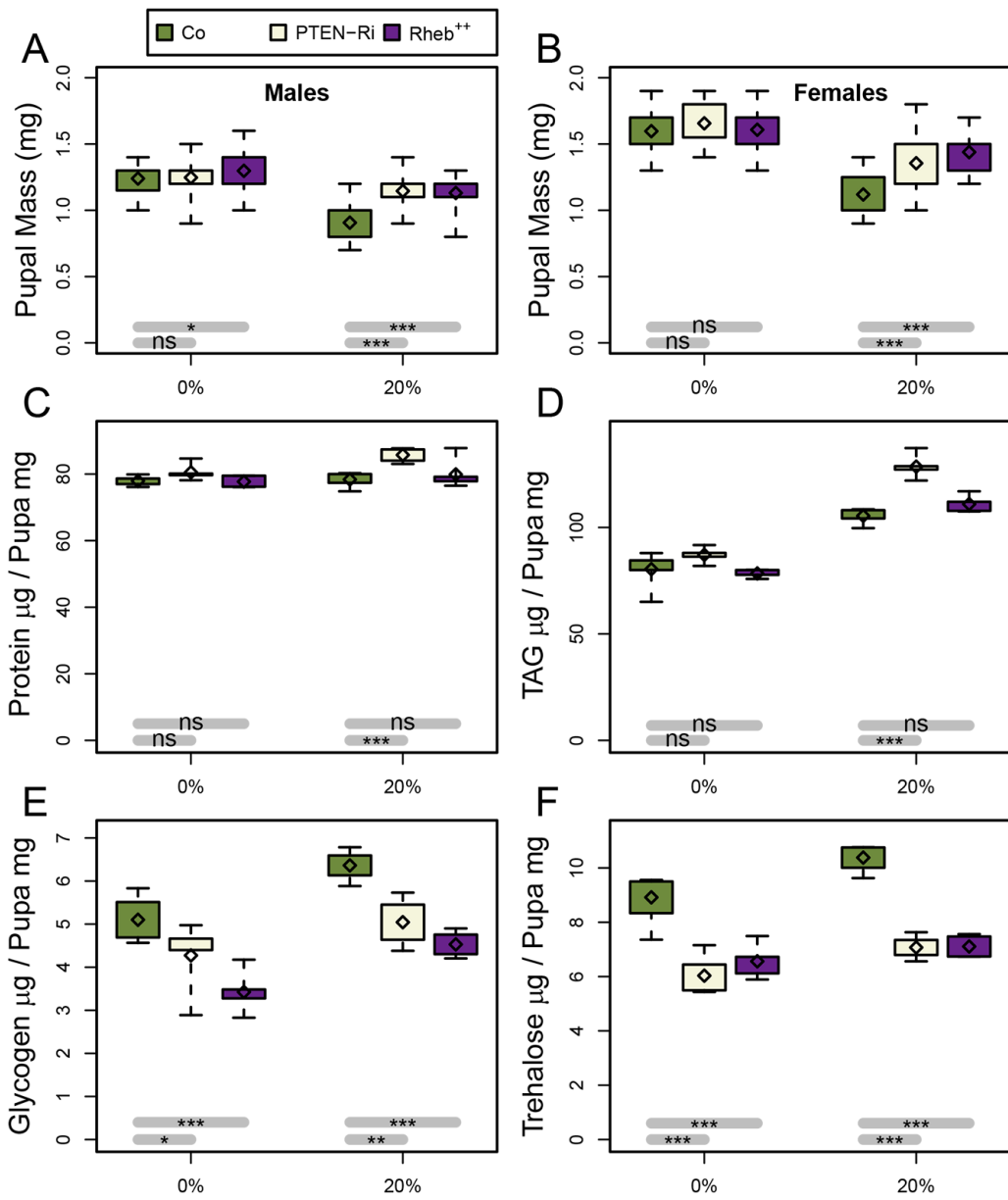
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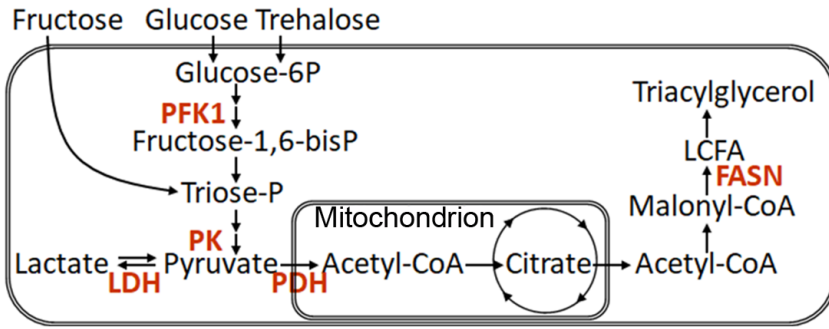
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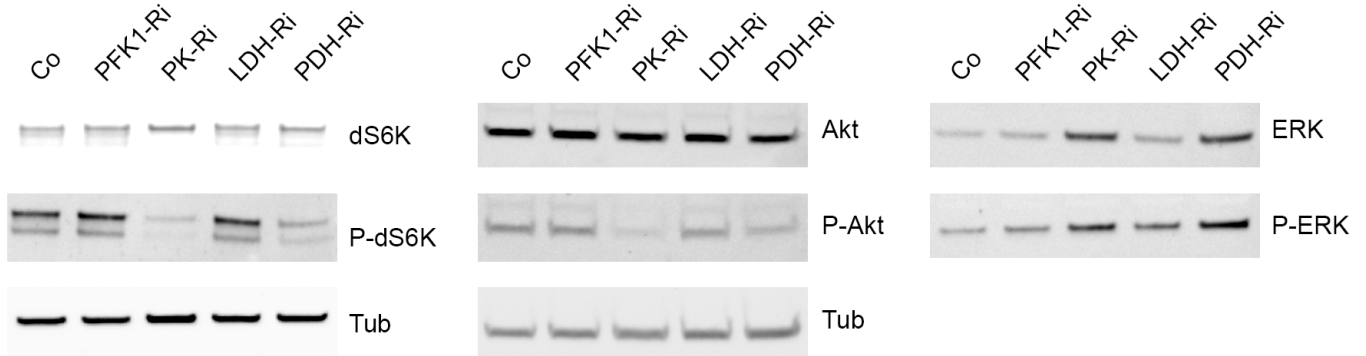
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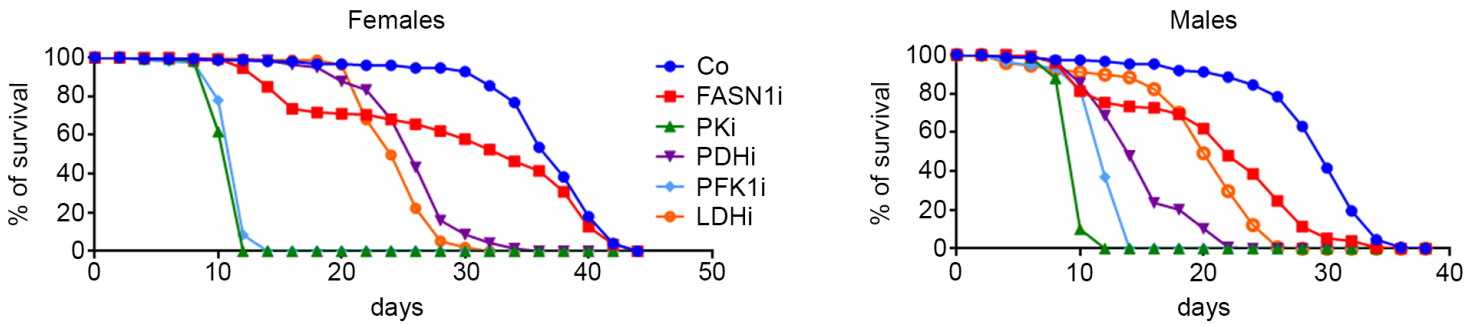
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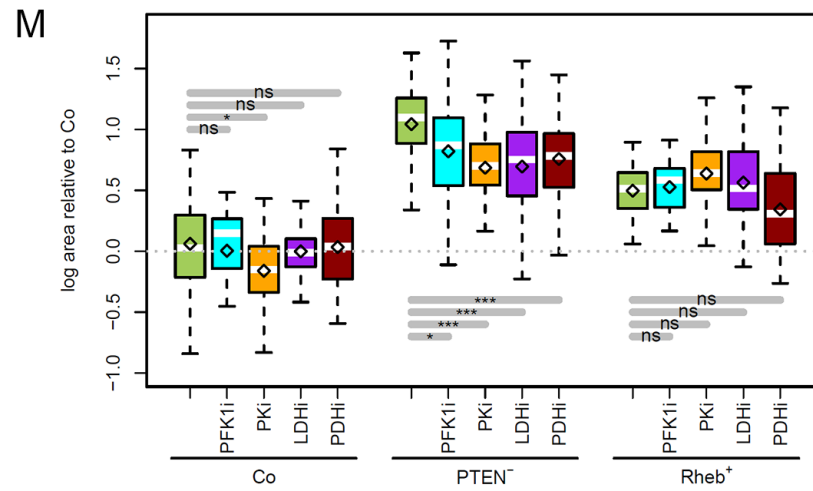
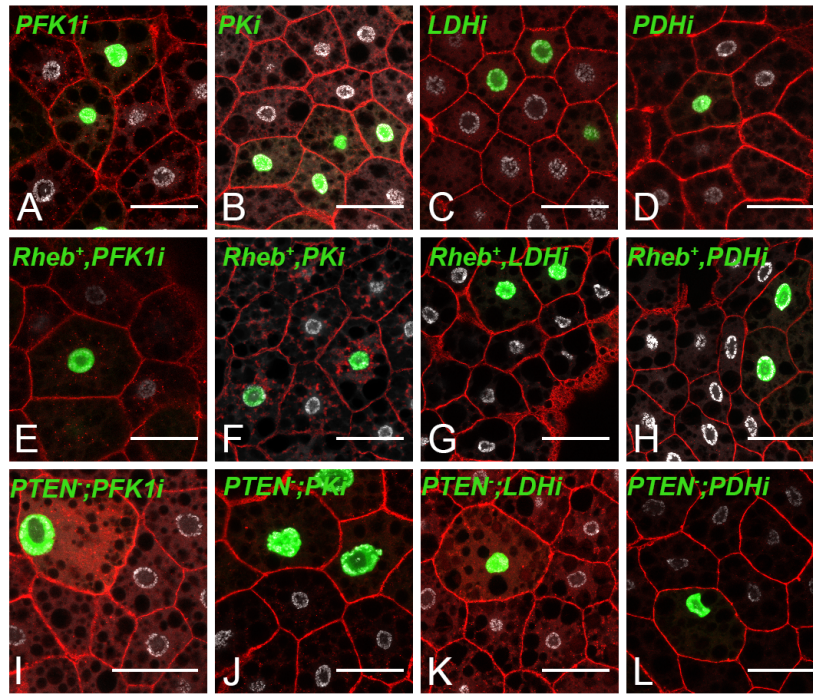
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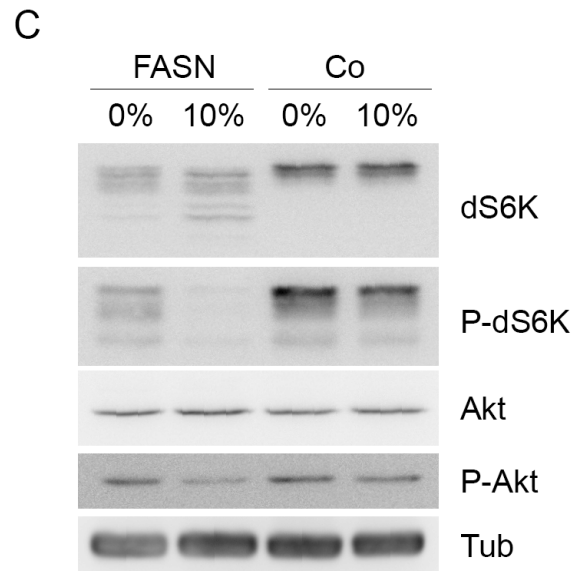
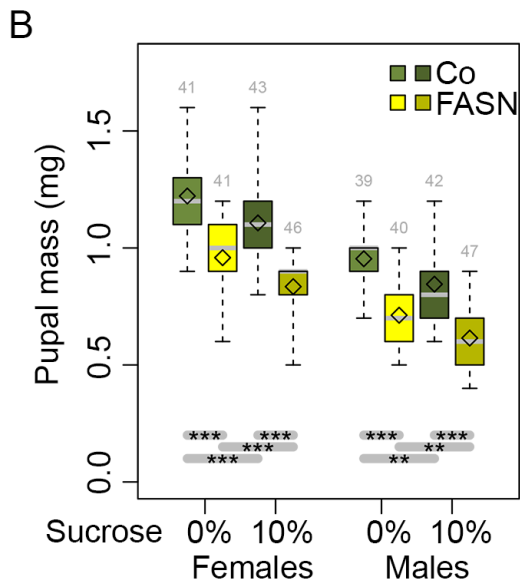
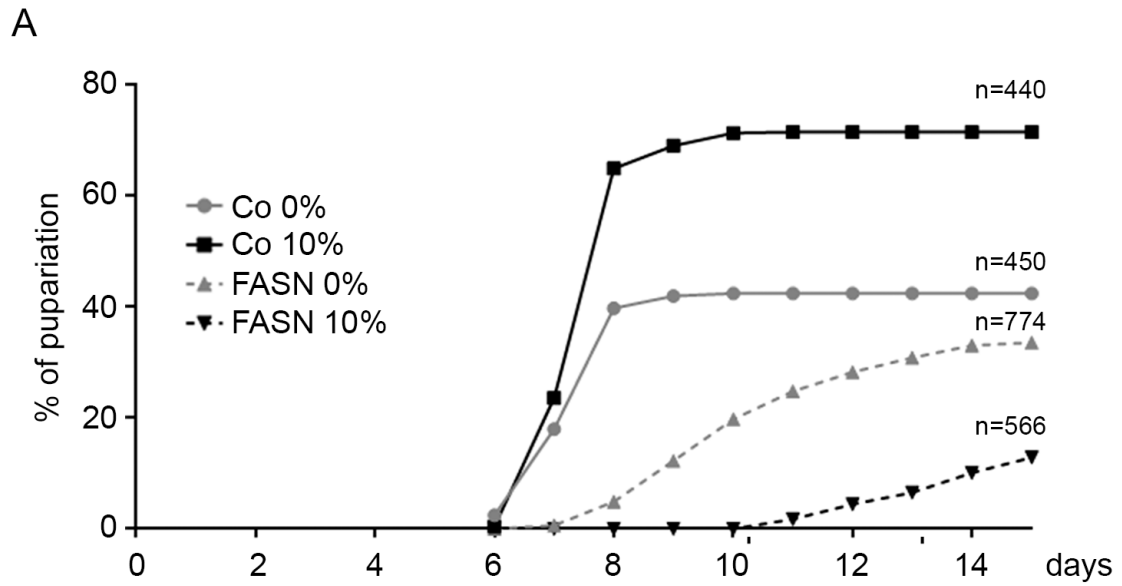
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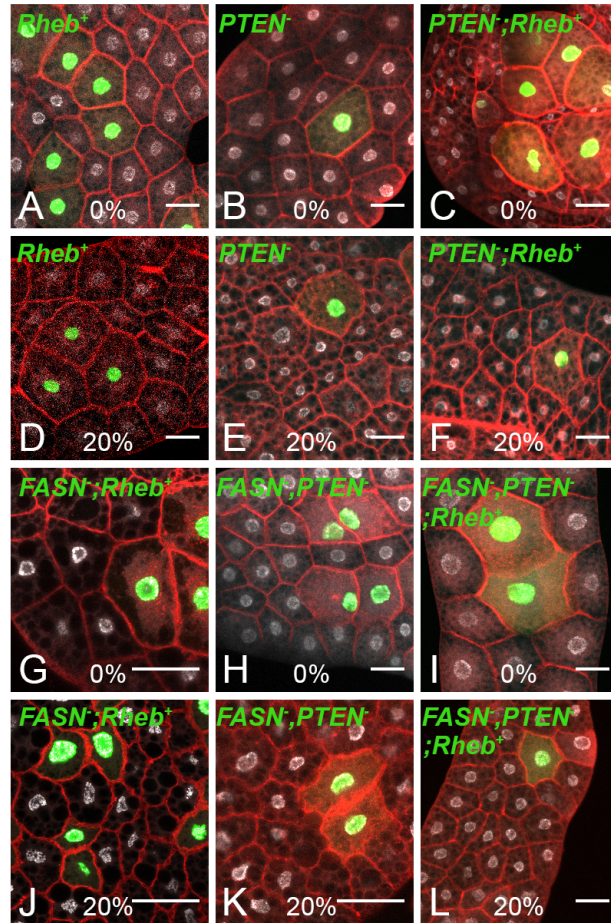


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