Differential Metabolic Sensitivity of Insulin-like-response- and mTORC1-Dependent Overgrowth in *Drosophila* Fat Cells

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ABSTRACT

The glycolytic/lipogenic axis promotes the synthesis of energetic molecules and building blocks necessary to support cell growth, although the absolute requirement of this metabolic axis must be deeply investigated. Here, we used *Drosophila* genetics and focus on the mTOR signaling network that controls cell growth and homeostasis. mTOR is present in two distinct complexes, mTORC1 and mTORC2. The former directly responds to amino acids and energetic levels, whereas the latter is required to sustain the signaling response downstream of insulin-like-peptide (Ilp) stimulation. Either signaling branch can be independently modulated in most *Drosophila* tissues. We confirm this independency in the fat tissue. We show that ubiquitous over-activation of mTORC1 or Ilp signaling affects carbohydrate and lipid metabolism, supporting the use of *Drosophila* as a powerful model to study the link between growth and metabolism. We show that cell-autonomous restriction of glycolysis or lipogenesis in fat cells impedes overgrowth dependent on Ilp- but not mTORC1-signaling. Additionally, ubiquitous deficiency of lipogenesis (*FASN* mutants) results in a drop in mTORC1 but not Ilp signaling, whereas, at the cell-autonomous level, lipogenesis deficiency affects none of these signals in fat cells. These findings thus, reveal differential metabolic sensitivity of mTORC1- and Ilp-dependent overgrowth. Furthermore, they suggest that local metabolic defects may elicit compensatory pathways between neighboring cells, whereas enzyme knockdown in the whole organism results in animal death. Importantly, our study weakens the use of single inhibitors to fight mTOR-related diseases and strengthens the use of drug combination and selective tissue-targeting.

45 **INTRODUCTION**

46 Growth of a multicellular organism is coordinated by signaling pathways that adjust
47 intracellular processes to environmental changes. These signaling pathways include the
48 mTOR (mechanistic Target Of Rapamycin) regulatory network that integrates the
49 growth factor response as well as the nutritional and energetic status (LAPLANTE AND
50 SABATINI 2012; HOWELL *et al.* 2013; LAMMING AND SABATINI 2013; SHIMOBAYASHI AND HALL
51 2014; CARON *et al.* 2015; SAXTON AND SABATINI 2017; MOSSMANN *et al.* 2018). Activation
52 of this network promotes basal cellular functions, thereby providing building blocks to
53 sustain cellular growth. However, despite a plethora of studies on the mTORC signaling
54 network, the requirement of basal metabolism—glycolytic/lipogenic axis— for cell
55 growth has not been systematically investigated. The *Drosophila* model provides a
56 powerful genetic system to address these issues (UGUR *et al.* 2016), since both the
57 intermediates of this signaling network and the basal metabolic pathways are conserved
58 in the fruit fly (MONTAGNE *et al.* 2001; HAY AND SONENBERG 2004; PADMANABHA AND
59 BAKER 2014; ANTIKAINEN *et al.* 2017; WANGLER *et al.* 2017; LEHMANN 2018).

60 The mTOR protein kinase is present in two distinct complexes, mTORC1 and mTORC2
61 that comprise raptor and rictor, respectively (KIM *et al.* 2002; SARBASSOV *et al.* 2005).
62 Regulation of mTORC1 activity by ATP and amino acids depends on a multi-step
63 process that results in the recruitment of an mTORC1 homodimer at the lysosomal
64 membrane in the vicinity of the small GTPase Rheb (Ras homologue enriched in brain)
65 (GOBERDHAN *et al.* 2009; MA AND BLENIS 2009; DIBBLE AND MANNING 2013; GROENEWOUD
66 AND ZWARTKRUIS 2013; MONTAGNE 2016). Rheb stimulates mTORC1 activity (YANG *et al.*
67 2017), which in turn regulates several downstream targets. S6Kinase1 (S6K1) is one
68 such kinase, sequentially activated through the phosphorylation of its T389 and T229
69 residues by mTORC1 and by PDK1 (Phosphoinositide-dependent protein kinase 1),

70 respectively (MONTAGNE AND THOMAS 2004; MAGNUSON *et al.* 2012). Further, Rheb
71 activation of mTORC1 is repressed by the tumor suppressor TSC (Tuberous sclerosis
72 complex) that comprises subunits TSC1 and TSC2 (RADIMERSKI *et al.* 2002a; GARAMI *et*
73 *al.* 2003; INOKI *et al.* 2003a; DIBBLE *et al.* 2012). The integrity of mTORC2 is required to
74 sustain the downstream insulin-signaling response (SARBASSOV *et al.* 2005). Binding of
75 insulin or related peptides (Ilps) to their cognate receptors results in recruitment of class
76 I PI3K (Phosphoinositide 3-kinase) to the membrane. PI3K phosphorylates inositol lipids
77 producing phosphatidylinositol-3,4,5-triphosphate (PIP3) (ENGELMAN *et al.* 2006;
78 HAEUSLER *et al.* 2018), while the tumor suppressor PTEN acts as a lipid phosphatase to
79 counteract this process (CULLY *et al.* 2006; GOBERDHAN *et al.* 2009). PIP3 constitutes a
80 membrane docking site for the protein kinase Akt whose activity requires the
81 subsequent phosphorylation of its S473 and T308 residues by mTORC2 and PDK1,
82 respectively (LIEN *et al.* 2017).

83 Constitutive activation of mTORC1 in MEFs (Mouse embryonic fibroblasts) has been
84 shown to stimulate a metabolic network, including glycolysis, the pentose phosphate
85 pathway and the biosynthesis of fatty acid (FA) and cholesterol (DUVEL *et al.* 2010).
86 Most of the genes encoding glycolytic enzymes are over-expressed in these cells as are
87 those encoding LDH (lactate dehydrogenase) and Pdk1 (Pyruvate dehydrogenase
88 kinase 1; an inhibitor of mitochondrial pyruvate processing). This suggests that
89 mTORC1-activated MEFs potentiate anaerobic glycolysis and repress the tricarboxylic
90 acid (TCA) cycle. Conversely, adipose-specific knockout of raptor to impede mTORC1
91 formation, results in enhanced uncoupling of mitochondrial activity (POLAK *et al.* 2008).
92 The increased lipogenesis observed in mTORC1 stimulated cells depends on a
93 downstream transcriptional regulatory axis involving the cofactor Lipin 1 along with a
94 SREBP (Sterol responsive element binding-protein) family member, which activates

95 genes encoding lipogenic enzymes (DUVEL *et al.* 2010; PETERSON *et al.* 2011).
96 Congruently, another study revealed that TSC2 mutant cells become addicted to
97 glucose as a result of mTORC1 hyper-activity (INOKI *et al.* 2003b). In addition, inhibition
98 of mTORC1 activity revealed that these TSC2 mutant cells become also dependent on
99 glutamine catabolism (CHOO *et al.* 2010); mTORC1 potentiates this catabolism to feed
100 TCA anaplerosis, through 1) a S6K/eIF4B/Myc axis that increases glutaminase protein
101 levels (CSIBI *et al.* 2014) and 2) the repression of SIRT4, a mitochondrial sirtuin that
102 inhibits glutamine dehydrogenase (CSIBI *et al.* 2013). Besides mTORC1 mediated
103 regulation, Ilp-signaling also impinges on basal metabolism. Intracellular activation of
104 Akt increases ATP levels (HAHN-WINDGASSEN *et al.* 2005; ROBEY AND HAY 2009) through
105 the stimulation of GLUT4-mediated glucose uptake (JALDIN-FINCATI *et al.* 2017) and the
106 enhancement of the expression and activity of glycolytic enzymes (GOTTLOB *et al.* 2001;
107 HOUDDANE *et al.* 2017). Akt also dampens glucose production by suppressing PEPCK
108 (gluconeogenesis), glucose-6-phosphatase (glycogenolyse) and the glycogen synthesis
109 repressor GSK3 (NAKAE *et al.* 2001; MCMANUS *et al.* 2005). However, in contrast to
110 mTORC1, Akt also promotes mitochondrial metabolism and oxidative phosphorylations
111 (GOTTLOB *et al.* 2001; MAJEWSKI *et al.* 2004). Conversely, hepatic knockout of the
112 mTORC2 specific-subunit rictor results in constitutive gluconeogenesis and impaired
113 glycolysis and lipogenesis (HAGIWARA *et al.* 2012; YUAN *et al.* 2012). Taken together,
114 these studies strongly emphasize the role of mTOR in metabolic-related diseases and in
115 adjusting metabolism to the nutritional and energetic status (MOSSMANN *et al.* 2018).

116 In the present study, we investigated the requirement of the glycolytic/lipogenic axis for
117 the cellular growth induced by hyper-activation of mTORC1 signaling and Ilp response
118 in *Drosophila*. As previously demonstrated, mTORC1 and Ilp signaling reside on
119 independent branches in most *Drosophila* tissues (RADIMERSKI *et al.* 2002a; RADIMERSKI

120 *et al.* 2002b; DONG AND PAN 2004; MONTAGNE *et al.* 2010; PALLARES-CARTES *et al.* 2012).
121 Here, we confirmed this independency in the *Drosophila* fat body (FB), the organ that
122 fulfils hepatic and adipose functions to control body homeostasis (PADMANABHA AND
123 BAKER 2014; ANTIKAINEN *et al.* 2017; LEHMANN 2018). We show that ubiquitous over-
124 activation of mTOR or Ilp signaling provokes an apparent enhancement of metabolite
125 consumption. Furthermore, our study reveals that metabolic restriction at the organismal
126 level has dramatic consequences on animal survival, but minor effect at the cell-
127 autonomous level, suggesting that within an organism, alternative pathways may
128 operate to compensate local metabolic defects. Nonetheless, at the cell-autonomous
129 level, metabolic restriction can partially restrain overgrowth dependent on hyper-
130 activation of Ilp- but not mTORC1-signaling, indicating that the potential compensatory
131 metabolic pathways do not fully operate in the context of Ilp-signaling stimulation.

132

133 **MATERIAL & METHODS**

134 **Genetics and fly handling**

135 Fly strains: *P[w[+mC]=tubP-GAL80]LL10,P[ry[+t7.2]=neoFRT]40A*, *daughterless(da)-*
136 *gal4*, *tub-gal80^{ts}*, *UAS-Dcr-2* (Bloomington Stock Center); *FASN$^{1-2}$* (GARRIDO *et al.*
137 2015); *mTOR$^{\Delta P}$* (ZHANG *et al.* 2000); *mTOR^{2L1}* and *PTEN* (OLDHAM *et al.* 2000);
138 *EP(UAS)-Rheb* (STOCKER *et al.* 2003);); inducible interfering RNA (*UAS-RNAi*) lines to
139 *PTEN* (NIG 5671R-2), *FASN1* (VDRC 29349), *PFK1* (VDRC 3017), *PK* (VDRC 49533)
140 *PDH* (VDRC 40410), *LDH* (VDRC 31192) (DIETZL *et al.* 2007). The *Minute* stock used
141 was previously referred to as *FRT40/P(arm-LacZ w^{+})* (BOHNI *et al.* 1999) but exhibit
142 both developmental delay and short and slender bristles, typically reported as *Minute*
143 phenotype (MORATA AND RIPOLL 1975). To generate MARCM clones in the *Minute*

144 background, these flies were recombined with the *P[w[+mC]=tubP-*
145 *GAL80]LL10,P[ry[+t7.2]=neoFRT]40A* chromosome.

146 The standard media used in this study contained agar (1g), polenta (6g) and yeast (4g)
147 for 100ml. Lipid- (beySD) and sugar-complemented media were prepared as previously
148 described (GARRIDO *et al.* 2015).

149 To select *FASN*$^{1-2}$ mutant larvae, we used a GFP-labelled CyO balancer chromosome.
150 Flies were let to lay eggs on grape juice plates for less than 24 hrs. Then, some beySD
151 media was put in the middle of the plates; larvae that do not express GFP were
152 collected the next day and transferred to fresh tubes. Prepupae were collected once a
153 day to evaluate developmental delay and to measure body weight.

154

155 **Molecular biology and Biochemistry**

156 To test RNAi-knockdown efficacy to the glycolytic enzymes (Figure S2), *UAS-Dcr-2;da-*
157 *gal4,tub-gal80*^{ts} virgin females were mated with UAS-RNAi males. Flies were let to lay
158 eggs overnight and tubes were kept at 19°C for two days. Tubes were then transferred
159 at 29°C and two days later, larvae of roughly the same size were collected. Reverse
160 transcription and quantitative PCR were performed as previously described (PARVY *et*
161 *al.* 2012).

162 Protein extracts for western-blotting were prepared as previously described (MONTAGNE
163 *et al.* 2010). Antibody used in for western-blotting have been previously described
164 (MONTAGNE *et al.* 2010) or commercially provided for Akt (Cell signaling 4054).

165 For metabolic measurements, parental flies were let to lay eggs in tubes for less than 24
166 hrs at 25°C. Tubes were then transferred at 29°C to strengthen the gal4/UAS effect,
167 and using a *UAS-Dcr-2* to strengthen the RNAi effect. Larvae were either maintained in

168 the same tubes or selected prior to L2/L3 transition and transferred on 20%-SSD.
169 Collection of prepupae and metabolic measurements were performed as previously
170 described (GARRIDO *et al.* 2015).

171

172 **Clonal analysis**

173 All the clones were generated using the MARCM strategy (LEE AND LUO 2001). Parental
174 flies were let to lay eggs at 25°C for seven hrs. Tubes were then heat shocked for 65
175 minutes in a water bath at 38°C so that recombination happens while FB precursor cells
176 are in dividing process. FB from feeding larvae at the end of the L3 stage where
177 dissected, fixed, membranes were labelled with phalloidin and nuclei with DAPI, and FB
178 were mounted as previously described (GARRIDO *et al.* 2015). Image acquisitions were
179 obtained using a Leica SP8 confocal laser-scanning microscope. For immuno staining
180 the phospho-S6 antibody has been previously described (ROMERO-POZUELO *et al.*
181 2017) and the phospho-Akt commercially provided (Cell signaling 4054). The cell size
182 calculation have been performed as previously described (GARRIDO *et al.* 2015) and
183 correspond to a set of experiments that spanned a two-year period. It represent too
184 many replicates, so that it was not possible to make them at the same time. Therefore,
185 for the graphs of cell size measurement (Figure 1M, 5M and 7M), values are reused
186 when they correspond to the same genotype and conditions. This allows a direct
187 comparison between the experiments.

188

189 **Statistical analysis**

190 Statistical analyses were performed with R version 3.4.4, scripts are available on
191 request. Significance for the statistical tests was coded in the following way based on

192 the p-values: ***: 0 < p < 0.001; **: 0.001 < p < 0.01; *: 0.01 < p < 0.05. P-values were
193 corrected for multiple testing by a Holm-Bonferroni method (HOLM 1079). Clone sizes
194 were analyzed with a mixed-effect linear model on the logarithm of cell area,
195 considering the treatment (Genotype and Sucrose conditions) as a fixed effect and
196 Series/Larva as random effects (Figures 1, 5, and 7, Table S1). The reported effects
197 (and the corresponding P-values) were obtained from the difference between the (log)
198 area of marked clonal cells and that of control surrounding cells from the same
199 treatment, by setting the appropriate contrast with the "multcomp" package (HOTHORN *et*
200 *al.* 2008), according to the pattern: EA,B = log(MA) – log(WA) – [log(MB) – log(WB)],
201 where EA,B is the difference between treatments (genotype and sucrose levels) A and
202 B, MA and MB standing for the area of marked cells, and WA, WB for the area of control
203 cells in those treatments. This is equivalent to testing whether marked/control cell area
204 ratios differ between treatments. PS6+ clone frequencies were treated as binomial
205 measurements in a mixed-effect generalized linear model "lme4" package (BATES *et al.*
206 2015), featuring Genotype as a fixed effect, and Series/Larva as random effects. Both
207 datasets of pupal weights were analyzed independently with linear models including
208 Sex, Genotype, and Sucrose level effects and all their interaction terms (Figure 3A-B
209 and Table S3 for PTEN knockdown and Rheb overexpression; Figure 6B and Table S4
210 for *FASN*$^{1-2}$ mutants). TAG, Protein, Glycogen, and Threalose concentrations were also
211 analyzed with linear models involving Genotype, Sucrose level, and their interactions as
212 fixed effects (Figure 3 and Table S3).

213

214 **Data and reagent availability statement**

215 Fly stocks are available upon request. Supplementary materials include Figures S1-S2,
216 Tables S1-S4 and supdata/script files available on the GSA figshare portal.

217

218 **RESULTS**

219 **mTORC1 and Ilp signaling independency in the fat body**

220 Activating either the mTORC1 or the Ilp signaling branch can be performed by
221 overexpressing Rheb or depleting PTEN, respectively. To investigate this independency
222 in the FB, we generated somatic clones either over-expressing Rheb (*Rheb*$^{+}$) (STOCKER
223 *et al.* 2003) or homozygote for a *PTEN* mutation (*PTEN*$^{-/-}$) (OLDHAM *et al.* 2000). The
224 precursors of FB cells divide in the embryo; during larval life, the differentiated cells do
225 not divide but endoreplicate their DNA content to reach a giant size (EDGAR AND ORR-
226 WEAVER 2001). Therefore, to precisely evaluate the effect on cell growth, somatic
227 recombination events were induced during embryogenesis at the stage of proliferation
228 of the FB cell precursors and the resulting MARCM clones were analyzed in the FB of
229 late feeding L3 larvae, prior to the wandering stage that precedes metamorphosis entry.
230 Both *PTEN*$^{-/-}$ and *Rheb*$^{+}$ clonal cells were bigger than the surrounding control cells and
231 this cell size effect was dramatically increased in *PTEN*$^{-/-}$;*Rheb*$^{+}$ combined clones
232 (Figure 1A-D and 1M). We next analyzed this growth increase in the context of the
233 previously described *mTOR*^{2L1} and *mTOR*$^{\Delta P}$ mutations. However, we could not find
234 mutant clones in the FB. Consistent with previous studies reporting that mTOR is
235 critically required for cell growth of endoreplicative tissues (OLDHAM *et al.* 2000; ZHANG
236 *et al.* 2000), we reasoned that these clonal cells were likely eliminated by cell
237 competition (MORATA AND RIPOLL 1975). Thus, we generated somatic clones in a *Minute*
238 background to slow down the growth of the surrounding control cells. In these
239 conditions, *mTOR* mutant clones could indeed be recovered. Both *mTOR*^{2L1} and
240 *mTOR*$^{\Delta P}$ mutant cells exhibited a dramatic size reduction (Figure 1G and 1J) and this
241 phenotype was dominant in *Rheb*$^{+}$ combined clonal cells (compare Figure 1E to 1H and

242 1K). In contrast, *mTOR*$^{\Delta P}$ but not *mTOR*^{2L1} exhibited a clear dominant phenotype over
243 the *PTEN*$^{-/-}$ mutation; the size of *mTOR*$^{\Delta P}$,*PTEN*$^{-/-}$ clonal cells was dramatically reduced,
244 whereas *mTOR*^{2L1},*PTEN*$^{-/-}$ clonal cells were giant (compare Figure 1F to 1I and 1L).
245 These findings indicate that the *mTOR*^{2L1} mutation affects mTORC1 but not ilp
246 signaling, whereas *mTOR*$^{\Delta P}$ affects both signaling branches.

247 Next, we used phospho-specific antibodies in immunostaining assays to analyze the
248 phosphorylation of Akt (P-Akt) and of the dS6K target, ribosomal protein rpS6 (P-S6). In
249 *PTEN*$^{-/-}$ clonal cells, we observed an increase in the P-Akt intracellular signal (Figure
250 2A). Importantly the P-Akt intracellular signal was absent in *mTOR*$^{\Delta P}$ cells (Figure 2B)
251 but not affected in *mTOR*^{2L1} cells (Figure 2C). Staining with the rpS6 phospho-specific
252 antibody revealed a patchy signal, with only a subset of cells expressing the P-S6 signal
253 in the FB (Figure 2E-J), a pattern previously described in the wing imaginal disc
254 (ROMERO-POZUELO *et al.* 2017). Therefore, to evaluate mTORC1 activity, we measured
255 the ratio of P-S6 positive cells among the population of GFP^{+} clonal cells. For control
256 clones, only labeled by GFP, about half of them were P-S6 positive (Figure 2E and 2K),
257 whereas most of the *mTOR*^{2L1} and *mTOR*$^{\Delta P}$ clones were P-S6 negative (Figure 2F, 2G
258 and 2K). Importantly, almost all the *Rheb*$^{+}$ cells were P-S6 positive (Figure 2H and 2K),
259 whereas the ratio of P-S6 positive cells was slightly but not significantly increased in the
260 *PTEN*$^{-/-}$ cell population (Figure 2I and 2K). Taken together, these findings confirm that
261 mTORC1 and Ilp signaling operate independently in FB cells and reveal that the
262 *mTOR*^{2L1} mutation affects only mTORC1, whereas the *mTOR*$^{\Delta P}$ mutation affects both
263 signaling branches.

264

265 **Activating mTORC1 or Ilp signaling impacts basal metabolism**

266 A number of studies support the notion that the mTOR signaling network controls
267 metabolism to sustain cellular growth. To evaluate how mTORC1 and Ilp affect basal
268 metabolism in *Drosophila*, we analyzed various metabolites in whole animals that
269 express the ubiquitous *da-gal4* driver to direct Rheb overexpression (*Rheb*$^{++}$) or PTEN
270 knockdown by RNA interference (*PTEN-RNAi*). Larvae were fed either a standard or a
271 20%-sucrose supplemented diet (20%-SSD) and 0-5h prepupae were collected, as this
272 is a convenient phase to stage the animals after the feeding period. When fed a
273 standard diet, a high rate of lethality was observed for *Rheb*$^{++}$ and *PTEN-RNAi* larvae,
274 although a sufficient number of prepupae could be collected for metabolic analysis. In
275 contrast, none of the *Rheb*$^{++}$ and *PTEN-RNAi* larvae reached the prepupal stage when
276 fed a 20%-SSD. Nonetheless, when *Rheb*$^{++}$ and *PTEN-RNAi* larvae were fed a
277 standard diet during early larval life and transferred onto a 20%-SSD at the L2/L3
278 molting transition, we could recover a few prepupae for metabolic measurements. For
279 both males and females fed a standard diet, the body weight of *Rheb*$^{++}$ and *PTEN-RNAi*
280 prepupae was roughly similar to that of controls (Figure 3A and 3B). Conversely,
281 providing a 20%-SSD resulted in a drop of the prepupal weight of control animals that
282 was significantly compensated in *Rheb*$^{++}$ and *PTEN-RNAi* prepupae (Figure 3A and
283 3B).

284 Next, we measured the total amounts of protein, triacylglycerol (TAG), glycogen and
285 trehalose—the most abundant circulating sugar in *Drosophila*. Although variations in
286 protein levels were observed, none of them were statistically significant (Figure 3C).
287 TAG levels in control prepupae were not affected by sucrose supplementation and did
288 not vary in *PTEN-RNAi*, but were significantly decreased in *Rheb*$^{++}$ animals (Figure 3D).
289 Feeding larvae a 20%-SSD since the L2/L3 molting transition resulted in a marked in
290 increase in glycogen and trehalose levels in control prepupae (Figure 3E-F). In *Rheb*$^{++}$

291 and, in lower extent, in *PTEN-RNAi* prepupae, glycogen levels were significantly lower
292 than those measured in controls (Figure 3E). Finally, trehalose levels were strongly
293 decreased in both *Rheb*$^{++}$ and *PTEN-RNAi* prepupae fed either a standard or a 20%-
294 SSD as compared to the control (Figure 3F). Taken together, these findings suggest
295 that a ubiquitous increased activity of either mTORC1 or Ilp signaling provokes an
296 apparent increase in metabolite consumption. This metabolic rate is correlated with a
297 relative increase in body weight for larvae fed a 20%-SSD, but not for those fed a
298 standard diet. We previously observed that increasing dietary sucrose induced a
299 reduction in food intake (GARRIDO *et al.* 2015) that may account for the body weight
300 reduction of control animals. Potentially, food intake could be less affected in *Rheb*$^{++}$
301 and *PTEN-RNAi* animals, thereby leading to a compensatory effect on body weight.
302 Measuring food intake in *Rheb*$^{++}$ or *PTEN-RNAi* larvae was not applicable since most of
303 them die during larval stage and thus, terminate feeding earlier. In sum, our data
304 indicates that basal metabolism is altered in the few *Rheb*$^{++}$ or *PTEN-RNAi* larvae that
305 survive and further suggests that in most cases stronger metabolic disruption
306 happened, resulting in lethal homeostatic defects.

307

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

309 Since manipulating mTOR resulted in a decrease in the levels of TAG and glycogen
310 stores and of circulating trehalose (Figure 3), we asked whether the basal energetic
311 metabolism affected mTORC1- and/or Ilp-signaling. First, we ubiquitously expressed
312 interfering RNA against phosphofructokinase1 (*PFK1-RNAi*), pyruvate kinase (*PK-*
313 *RNAi*) pyruvate dehydrogenase (*PDH-RNAi*) and lactate dehydrogenase (*LDH-RNAi*).
314 PFK1 catalyzes the third glycolytic reaction to form fructose 1,6-bisphophate; PK
315 catalyzes the final glycolytic reaction to form pyruvate; PDH directs the mitochondrial

316 fate of pyruvate, whereas LDH directs its anaerobic fate (Figure 4A). When directed with
317 the ubiquitous *da-gal4* driver, *PK-RNAi* provoked early larval lethality, *PFK1-RNAi* and
318 *PDH-RNAi* provoked larval lethality at L2 or L3 stages, whereas *LDH-RNAi* induced a
319 semi-lethal phenotype at larval or pupal stages (Figure 4B).

320 Second, we monitored the phosphorylation of the *Drosophila* S6Kinase (dS6K) and Akt
321 as read-out of the activity of mTORC1- and Ilp-signaling respectively. To circumvent the
322 early lethality, the *da-gal4* driver was combined with a ubiquitous thermo-sensitive form
323 of the Gal4 inhibitor, Gal80^{ts} (*tub-gal80^{ts}*) that blocks Gal4 activity at 21°C but not at
324 29°C, thereby allowing RNAi expression after temperature shift. Each RNAi was
325 ubiquitously induced at early L1 stage and protein extracts were prepared two days later
326 using late L2 larvae. At this stage the larvae were still viable, although those expressing
327 PK-RNAi did not undergo L2/L3 transition and eventually died (Figure 4B). Western-
328 blotting using these L2 protein extracts revealed that RNAi-knockdown of PFK1, LDH or
329 PDH did not affect Akt or dS6K phosphorylation (Figure 4C). In contrast, PK knockdown
330 strongly decreased dS6K phosphorylation and to a lower extent Akt phosphorylation
331 (Figure 4C). These results indicate that mTORC1 signaling may be affected when
332 knocking down PK, but not when knocking down any other enzyme directly linked to
333 glycolysis. Nonetheless, the lethal phenotype of PK-RNAi larvae occurring at the late L2
334 stage (Figure 4B) might weaken the larvae, inducing a subsequent effect on mTOR
335 signaling.

336 To evaluate the requirement of glycolysis for adult survival, RNAi-knockdown was
337 induced by temperature shift to 29°C in newly emerged flies and lethality was counted
338 every second day. In both males and females, PK and PFK1 knockdown provoked
339 lethality between 10 to 14 days after temperature shift (Figure 4D). Knockdown of PDH
340 and LDH also induced adult lethality, although not as soon as PK and PFK1 knockdown

341 (Figure 4D). As a comparison, to evaluate the consequence of disrupting fatty acid
342 synthesis, we knocked-down FASN (Fatty Acid Synthase, Figure 4A) in adults; about a
343 quarter of *FASN-RNAi* flies died between 10 to 14 days, while the others survived
344 nearly as well as control flies (Figure 4D). Taken together, these data indicate that
345 glycolysis is essential for both larval development and adult survival. However, prior to
346 the appearance of the deleterious phenotype, glycolysis knockdown is unlikely to
347 impinge on mTOR signaling.

348

349 **Cell-autonomous requirement of glycolysis for Ilp- but not mTORC1-dependent**
350 **overgrowth**

351 To investigate the requirement of glycolysis to sustain cell-autonomous overgrowth
352 dependent on Ilp- and mTORC1-signaling, *PFK1-RNAi*, *PK-RNAi*, *PDH-RNAi* and *LDH-*
353 *RNAi* were induced in *PTEN*$^{-/-}$ or *Rheb*$^{+}$ clones. Except a moderate effect of *PK-RNAi*,
354 clones expressing interfering RNA against these metabolic enzymes did not significantly
355 affect the growth of FB cells (Figure 5A-D and 5M). In combined clones, none of the
356 RNAi affected the growth of *Rheb*$^{+}$ clones (Figure 5E-H and 5M). In contrast, the size of
357 *PTEN*$^{-/-}$ clones was significantly decreased when co-expressing RNAi against any of
358 these metabolic enzymes (Figure 5I-M). These findings indicate that both aerobic and
359 anaerobic glycolysis are required to sustain cell-autonomous overgrowth dependent on
360 Ilp signaling. In contrast, reducing glycolysis does not counteract cell-autonomous
361 overgrowth dependent on mTORC1 signaling, suggesting the existence of
362 compensatory pathways.

363

364 **Linking Lipogenesis to mTORC1- and Ilp-signaling**

365 Since glycolysis and FA synthesis are tightly connected metabolic pathways (GARRIDO
366 *et al.* 2015), we investigated whether lipogenesis affects Ilp or mTORC1 signaling. FA
367 synthesis is catalyzed by FASN (Figure 4A). The *Drosophila* genome encodes three
368 *FASN* genes, *FASN1* is ubiquitously expressed but not *FASN2* or *FASN3* (PARVY *et al.*
369 2012; CHUNG *et al.* 2014; WICKER-THOMAS *et al.* 2015). The deletion of the *FASN1* and
370 *FASN2* tandem ($FASN^{\Delta 24\text{-}23}$ deletion, hereafter called $FASN^{1\text{-}2}$) results in a lethal
371 phenotype that can be rescued by feeding larvae a lipid-complemented diet (beySD)
372 (GARRIDO *et al.* 2015; WICKER-THOMAS *et al.* 2015). We observed that beySD-rescued
373 $FASN^{1\text{-}2}$ mutant larvae exhibited a delay in development, as measured by the duration
374 of larval development to metamorphosis entry (Figure 6A). Further, when beySD-
375 rescued $FASN^{1\text{-}2}$ mutant larvae were transferred at the L2/L3 larval transition onto a
376 10% sucrose-supplemented-beySD, only a few of them completed the third larval stage
377 and, after an extreme developmental delay, entered metamorphosis (Figure 6A). Delay
378 in development can be due to a default in ecdysone production that results in giant
379 pupae (PARVY *et al.* 2014) or to impaired mTOR signaling that results in reduced body
380 growth (MONTAGNE *et al.* 1999; OLDHAM *et al.* 2000). Measurements of prepupal weight
381 revealed that $FASN^{1\text{-}2}$ mutant prepupae exhibited a severe reduction in body weight,
382 whether or not they were supplemented with sucrose (Figure 6B), suggesting a default
383 in mTOR signaling. Therefore, we analyzed the phosphorylation of the *Drosophila*
384 S6Kinase (dS6K) and Akt in protein extracts of late feeding L3 larvae. Western-blotting
385 revealed that the dS6K protein resolved in several bands in $FASN^{1\text{-}2}$ extracts, whereas
386 Akt protein was unchanged (Figure 6C). These results suggest that dS6K but not Akt
387 might be degraded in the $FASN^{1\text{-}2}$ mutant background. In addition, dS6K
388 phosphorylation decreased in $FASN^{1\text{-}2}$ extracts and became barely detectable when
389 $FASN^{1\text{-}2}$ larvae were fed a sucrose-supplemented-beySD (Figure 6C). Conversely, the

390 phosphorylation of Akt was unaffected in larvae fed a beySD, although it was slightly
391 decreased in larvae fed a sucrose-supplemented-beySD (Figure 6C). This finding
392 contrasts with our previous observation showing that FB explants of *FASN*$^{1-2}$ mutant
393 larvae were hypersensitive to insulin (GARRIDO *et al.* 2015). However, *FASN*$^{1-2}$ mutants
394 also exhibited a decrease in food intake (GARRIDO *et al.* 2015), which might induce a
395 systemic suppression of dS6K phosphorylation, while FB explant were cultured in
396 nutrient media supplemented with insulin. Therefore, to determine whether *FASN*
397 mutation affects mTOR signaling at the cell-autonomous level, we analyzed P-S6 and
398 P-Akt in *FASN*$^{1-2}$ mutant clones in the FB. As for control clones, about half of the
399 *FASN*$^{1-2}$ clonal cells were P-S6 positive (Figure 2J and 2K). Furthermore, no effect on
400 P-Akt was observed in *FASN*$^{1-2}$ clonal cells (Figure 2D). In summary, these findings
401 reveal that disrupting FA synthesis does not significantly affect mTORC1 and Ilp
402 signaling at the cell-autonomous level, although it seems to impinge on mTORC1
403 signaling when inhibited in the whole animal whether directly or indirectly.

404

405 **Cell-autonomous requirement of FA synthesis for Ilp- but not mTORC1-dependent**
406 **overgrowth**

407 To determine, whether lipogenesis is required at the cell-autonomous level to sustain
408 mTORC1 and/or Ilp dependent growth, we analyzed *FASN*$^{1-2}$ clones while enhancing
409 either of the mTOR signaling branch in FB cells. We previously reported (GARRIDO *et al.*
410 2015) that *FASN*$^{1-2}$ clonal cells in the FB were slightly reduced in size and that this
411 effect was dramatically increased in larvae fed a 20%-SSD (Figure S1 and Figure 7M).
412 Therefore, we generated *PTEN*$^{-/-}$ and *Rheb*$^{+}$ clones combined or not with the *FASN*$^{1-2}$
413 mutation and analyzed them in the FB of larvae fed either a standard diet or a 20%-
414 SSD. As compared to the standard diet, feeding larvae a 20%-SSD had no effect on the

415 size of *Rheb*$^{+}$ clonal cells, but significantly reduced the size of *PTEN*$^{-/-}$ and of *PTEN*$^{-/-}$
416 ;*Rheb*$^{+}$ clonal cells (Figure 7A-F and 7M). Further, when combined with the *FASN*$^{1-2}$
417 mutation, *PTEN*$^{-/-}$ but not *Rheb*$^{+}$ clones were significantly reduced in size (Figure 7G-H
418 and 7M). The *FASN*$^{1-2}$ mutation also provoked a severe size reduction of *PTEN*$^{-/-}$,*Rheb*$^{+}$
419 clones (Figure 7I and 7M). Moreover, as compared to the standard diet, feeding larvae
420 a 20%-SSD induced a significant size reduction of *FASN*$^{1-2}$;*Rheb*$^{+}$, *FASN*$^{1-2}$,*PTEN*$^{-/-}$ and
421 *FASN*$^{1-2}$,*PTEN*$^{-/-}$;*Rheb*$^{+}$ clonal cells (Figure 7J-L and 7M). Of note, except for the
422 *FASN*$^{1-2}$;*Rheb*$^{+}$ clonal cells in larvae fed a 20%-SSD that exhibited a size roughly
423 identical to that of the surrounding control cells (Figure 7J), the cell size was always
424 bigger than the controls (Figure 7M). These findings indicate that, in larvae fed a
425 standard diet, FA synthesis is at least in part required to sustain over-growth induced by
426 Ilp, but not mTORC1. They also reveal that additional dietary sucrose is rather
427 detrimental for the growth of cells either deficient for FA synthesis or over-active for Ilp
428 signaling, suggesting that these cells have a restricted homeostatic ability to adjust to
429 an unbalanced diet, whereas mTORC1 activated cells at least in part maintain this
430 ability.

431

432 **DISCUSSION**

433 In this study, we used the powerful *Drosophila* genetics to investigate the functional
434 links between the glycolytic/lipogenic axis and mTORC1- or Ilp-dependent growth. In
435 agreement with previous studies (RADIMERSKI *et al.* 2002a; RADIMERSKI *et al.* 2002b;
436 DONG AND PAN 2004; MONTAGNE *et al.* 2010; PALLARES-CARTES *et al.* 2012), we show
437 that mTORC1 and Ilp signaling work independently in the *Drosophila* FB. Further, we
438 provide evidence that the previously described *mTOR*^{2L1} mutation that likely results in a
439 kinase-inactive protein (OLDHAM *et al.* 2000) affects mTORC1 but not Ilp signaling.

440 Congruently, a study on a *Drosophila rictor* mutant reported that the mTORC2 complex
441 was not required to sustain Akt-dependent growth, but rather to play as a rheostat for
442 this signaling branch (HIETAKANGAS AND COHEN 2007). Although this study suggests that
443 mTOR is dispensable for Akt activity, we show that Akt activity and Ilp-dependent
444 overgrowth are suppressed in *mTOR*$^{\Delta P}$ mutant indicating that the mTOR protein is
445 required for these processes.

446 On one hand, to mimic the effect that might be induced by drug treatment with a
447 systemic inhibitor, we dampened the glycolytic/lipogenic axis or enhanced mTORC1 or
448 Ilp signaling in the entire organism. On the other hand, to monitor the cell growth
449 process that spans the entire developmental program at the cell-autonomous level, we
450 analyzed clonal FB cells in mosaic animals. Intriguingly, our study reveals apparent
451 contradictory effects between perturbations at the whole body and cell-autonomous
452 levels. At the organismal level, knockdown of glycolytic enzymes or deficiency of FASN
453 result in animal lethality. However, *FASN*$^{1-2}$ mutant animals supplemented with dietary
454 lipids can survive but exhibit a dramatic overall growth suppression. This growth defect
455 might result from a decrease in mTORC1 activity that is strongly reduced in *FASN*$^{1-2}$
456 mutant animals, suggesting that mTORC1 but not Ilp signaling relies on lipogenesis. In
457 contrast, at the cell autonomous level, the mutation of *FASN*$^{1-2}$ restrains Ilp but not
458 mTORC1 dependent overgrowth in FB cells. These apparent contradictory findings,
459 suggest that the growth defect and the reduction of mTORC1 activity in *FASN*$^{1-2}$
460 mutants are not due to the addition of cell-autonomous effects but rather to a systemic
461 regulation. Potentially, FASN default might affect the activity of a specific tissue, as for
462 instance, the neurosecretory cells that synthesize and secrete Ilps, which promote
463 systemic body growth (RULIFSON *et al.* 2002). Alternatively, considering that mTORC1
464 directly responds to nutrients (DIBBLE AND MANNING 2013; GROENEWOUD AND

465 ZWARTKRUIS 2013; MONTAGNE 2016), the drop of mTORC1 activity may be a
466 consequence of feeding, since we previously reported a decrease in nutrient uptake in
467 *FASN*$^{1-2}$ mutant animals (GARRIDO *et al.* 2015). Consistently, a previous study on the
468 transcription factor Mondo —the *Drosophila* homologue of mondoA and ChREBP that
469 regulate the glycolytic/lipogenic axis in response to dietary sugar (MATTILA *et al.* 2015;
470 RICHARDS *et al.* 2017)— suggests the existence of a FASN-dependent effect in the FB
471 on the control of food intake (SASSU *et al.* 2012). FB-knockdown of *mondo* results in the
472 lack of sucrose-induced expression of *FASN1* and in a decrease in food intake. This
473 study suggests that the FASN default perturbs body homeostasis and indirectly affects
474 the neuronal control of feeding behavior. However, it does not exclude that a lipogenic
475 defect in neuronal cells may also directly impinge on feeding behavior. Finally, the drop
476 of mTORC1 activity observed in *FASN*$^{1-2}$ mutants may be a consequence of malonyl-
477 CoA accumulation, since mTOR malonylation has been reported to inhibit mTORC1-
478 but not Ilp/mTORC2-dependent activity (BRUNING *et al.* 2018). Malonylation of mTOR
479 may also account for the size reduction of *FASN*$^{1-2}$ mutant cells over-expressing Rheb
480 in animals fed a 20%-SSD, consistent with the increased expression of lipogenic
481 enzymes induced by dietary sucrose (GARRIDO *et al.* 2015). Thus, mTOR malonylation
482 and the subsequent decrease in mTORC1 activity might occur only when interfering
483 with a context of high demand for lipogenesis, an issue that should be investigated in
484 the future.

485 Our study reveals that over-activation of mTORC1 and to a lesser extent of Ilp signaling,
486 results in a decrease in glycogen and TAG stores and in circulating trehalose,
487 suggesting that activation of either signaling branch enhances metabolite consumption
488 to sustain cell growth. It is therefore surprising that activation of neither mTORC1 nor Ilp
489 signaling induces an increase in body weight. Nonetheless, overall body growth

490 depends on an intricate regulatory network that integrates cell-autonomous effects and
491 humoral messages. For instance, previous studies reported that activation of Ilp
492 signaling within the ring gland, results in a systemic decrease in body growth (CALDWELL
493 *et al.* 2005; COLOMBANI *et al.* 2005; MIRTH 2005). Therefore, ubiquitous activation of
494 mTORC1 or Ilp signaling is likely to promote the growth of most cells but might
495 concurrently perturb endocrine signals dampening overall growth. Of note, we observed
496 that larvae fed a 20%-SSD result in pupae with reduced body weight, an effect that is
497 partially suppressed when either mTORC1 or Ilp signaling is over-activated. The fact
498 that the overall body weight of these animals is maintained within a range likely
499 compatible with organismal survival contrasts with the observed high rate of lethality.
500 The decrease in stores and circulating sugars suggests that in these animals each cell
501 tends to increase its basal metabolism evoking an egoist behavior that might perturb the
502 equilibrium between cell-autonomous and systemic regulation. Thus, in a stressful
503 situation, as when animals are fed a 20%-SSD, the need of a tight adjustment to an
504 unbalanced diet may enhance the distortion between cell-autonomous effects and
505 systemic regulation, resulting in an increased rate of lethality.

506 A plethora of studies in mammalian cells indicate that mTOR activation directs
507 metabolism towards glucose consumption, storage and anabolism (GOTTLOB *et al.*
508 2001; INOKI *et al.* 2003b; HAHN-WINDGASSEN *et al.* 2005; DUVEL *et al.* 2010; PETERSON *et*
509 *al.* 2011; HOUDDANE *et al.* 2017; JALDIN-FINCATI *et al.* 2017; WIPPERMAN *et al.* 2019). Our
510 study rather suggests that in the *Drosophila* larvae, mTOR promotes metabolite
511 consumption through glycolysis but not storage. However, at the cell-autonomous level,
512 we observe that inhibition of lipogenesis or glycolysis restrains neither larval FB cell
513 growth nor overgrowth induced by mTORC1 stimulation in these cells. These findings
514 counteract the idea that mTORC1 potentiates a glycolytic/lipogenic axis (DUVEL *et al.*

515 2010) to sustain cell growth. To overcome the lack of glycolytic products and of
516 membrane lipids, these cells may benefit of a transfer from neighboring cells and might
517 favor alternative metabolic pathways, including glutamine catabolism to feed TCA
518 anaplerosis, which has been shown to be a crucial pathway in mTORC1-stimulated
519 mammalian cells (CHOO *et al.* 2010; CSIBI *et al.* 2013; CSIBI *et al.* 2014). Nonetheless,
520 such compensatory processes do not fully operate to sustain Ilp-dependent overgrowth.
521 In these cells, the mutation of PTEN potentially impedes the ability to modulate this
522 signaling branch. Therefore, it is tempting to speculate that the modulation of Ilp
523 signaling at least in part contributes to the regulation of these compensatory processes.

524 As a coordinator of growth and metabolism, mTOR plays a central role in tumor
525 development (DOWLING *et al.* 2010; HARACHI *et al.* 2018; MOSSMANN *et al.* 2018; TIAN *et*
526 *al.* 2019). PTEN, the tumor suppressor that counteracts PI3K activity downstream of the
527 Ilp receptor, is deficient in several human cancers (CULLY *et al.* 2006). Mutation of TSC1
528 or TSC2, which results in mTORC1 hyper-activation, is associated with benign tumors
529 but also with brain, kidney and lung destructive diseases (HENSKE *et al.* 2016). To
530 investigate the role of mTOR regarding tumor development, a recent study reported the
531 generation of liver-specific double knockout mice for TSC1 and PTEN (GURI *et al.*
532 2017). These mice develop hepatic steatosis that eventually progresses to
533 hepatocellular carcinoma. Both processes are suppressed in mice fed the mTORC1/2
534 inhibitors INK128, but not the mTORC1 inhibitor rapamycin, supporting an Ilp/mTORC2
535 specific effect. The combination of inhibitors against mTOR and metabolism is currently
536 under clinical investigation to fight cancers (MOSSMANN *et al.* 2018). Importantly, our
537 study reveals that ubiquitous inhibition of basal metabolism produces dramatic effects
538 during development, while at the cell-autonomous level, it only moderates growth
539 induced by over-activation of Ilp/mTORC2 signaling. Therefore, the use of drug therapy

540 to fight cancer must be taken with caution, in particular if organismal development is not
541 complete and most efforts should be made to selectively target sick tissues.

542

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552

553 **AUTHOR CONTRIBUTIONS**

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

556

557 **FIGURE LEGENDS**

558 **Figure 1: mTORC1- and Ilp-dependent growth in FB cells.** (**A-L**) MARCM clones
559 labeled by GFP (green) in the FB of L3 larvae. Nuclei were labeled with DAPI (silver)
560 and membranes with phalloidin (red). Control (A), *Rheb*$^{+}$ (B), *PTEN*$^{-/-}$ (C) and *PTEN*$^{-/-}$
561 ;*Rheb*$^{+}$ (D) clones were generated in a wild type background. *Rheb*$^{+}$ (E), *PTEN*$^{-/-}$ (F),
562 *mTOR*^{2L1} (G) *mTOR*^{2L1}, *Rheb*$^{+}$ (H) *mTOR*^{2L1},*PTEN*$^{-/-}$ (I), *mTOR*$^{\Delta P}$ (J), *mTOR*$^{\Delta P}$,*Rheb*$^{+}$

563 (K) and *mTOR*$^{\Delta P}$,*PTEN*$^{-/-}$ (L) clones were generated in a *Minute* (*M*) background. Scale
564 bars: 50µm. (**M**) Relative size of control (Co), *Rheb*$^{+}$, *PTEN*$^{-/-}$, and *PTEN*$^{-/-}$;*Rheb*$^{+}$ clonal
565 cells generated in a wild type background.

566

567 **Figure 2: mTORC1 and Ilp signaling activity in FB cells.** (**A-J**) MARCM clones
568 labeled by GFP (green) in the FB of L3 larvae. Clones were generated in a wild type
569 (A,D,E,H,I,J) or a *Minute* (B,C,F,G) background and nuclei were labeled with DAPI
570 (silver). FB tissues with *PTEN*$^{-/-}$ (A), *mTOR*$^{\Delta P}$ (B), *mTOR*^{2L1} (C) and *FASN*$^{1-2}$ (D) clones
571 were stained with a phospho-AKT antibody. FB tissues with control (E), *mTOR*^{2L1} (F),
572 *mTOR*$^{\Delta P}$ (G) *Rheb*$^{+}$ (H), *PTEN*$^{-/-}$ (I) and *FASN*$^{1-2}$ (J) clones were stained with a
573 phospho-S6 antibody. Scale bars: 50µm. (**K**) Percentage of P-S6 positive clones with
574 respect to the total number of MARCM clones for control, *FASN*$^{1-2}$, *PTEN*$^{-/-}$, *Rheb*$^{+}$,
575 *mTOR*^{2L1} and *mTOR*$^{\Delta P}$ genotypes.

576

577 **Figure 3: Enhanced mTORC1 or Ilp signaling affects larval metabolism.** (**A-B**)
578 Body weight of female (A) and male (B) prepupae formed from larvae fed either a
579 standard (0%) or a 20%-SSD (20%) as from the L2/L3 transition. (**C-F**) Measurement of
580 total protein (C), TAG (D), glycogen (E) and trehalose (F) levels in prepupae fed either a
581 standard or a 20%-SSD. Prepupae used in these measurements were the F1 progeny
582 from *da-gal4* virgin females mated to either control (Co), *EP(UAS)-Rheb* (*Rheb*$^{++}$) or
583 *UAS-PTEN-RNAi* (*PTEN-Ri*) males.

584

585 **Figure 4: Glycolysis knockdown in whole organisms.** (**A**) Scheme of basal
586 metabolism. Glucose and trehalose enter glycolysis as glucose-6P, whereas fructose

587 follows a distinct pathway to triose-P. Enzymes investigated in the present study are
588 marked in red. (**B**) Phenotype of ubiquitous RNAi knockdown of PFK1, PK, LDH and
589 PDH. Flies were left to lay eggs overnight either at 29°C (column 0h) or at 19°C and
590 transferred to 29°C the day after (column 24h); then development proceeded at 29°C
591 (i.e. the temperature that inactivates Gal80). (**C**) Western-blot analysis of total (top) or
592 phosphorylated (mid) dS6K (left) or Akt (right) proteins; tubulin (bottom) was used as a
593 loading control. Protein extracts were prepared with late L2 control larvae (Co) or L2
594 larvae expressing RNAi against the indicated metabolic enzymes. (**E-F**) Survival at
595 29°C of male (top) and female (bottom) control flies or flies expressing RNAi against the
596 indicated metabolic enzymes as from adult eclosion.

597

598 **Figure 5: Cell-autonomous requirement of glycolysis for Ilp- but not mTORC1-**
599 **dependent overgrowth.** (**A-G**) MARCM clones labeled by GFP (green) in the FB of L3
600 larvae. Nuclei were labeled with DAPI (silver) and membranes with phalloidin (red).
601 Genotypes of MARCM clones are: *PFK1-RNAi* (A), *PK-RNAi* (B), *LDH-RNAi* (C), *PDH-*
602 *RNAi* (D), *Rheb*$^{+}$,*PFK1-RNAi* (E), *Rheb*$^{+}$,*PK-RNAi* (F), *Rheb*$^{+}$,*LDH-RNAi* (G),
603 *Rheb*$^{+}$,*PDH-RNAi* (H), *PTEN*$^{-/-}$;*PFK1-RNAi* (I), *PTEN*$^{-/-}$;*PK-RNAi* (J), *PTEN*$^{-/-}$;*PDH-RNAi*
604 (K) and *PTEN*$^{-/-}$;*PDH-RNAi* (L). Scale bars: 50µm. (**M**) Relative size of clonal cells
605 corresponding to the clones shown in A-L, and in Figure 1A for control (Co).

606

607 **Figure 6: *FASN*$^{1-2}$ mutation affects developmental growth and mTORC1 signaling.**
608 (**A**) Developmental duration from egg laying to metamorphosis onset of *w*^{1118} control
609 (Co) and *FASN*$^{1-2}$ (FASN) larvae fed either a beySD (0%) or a 10% sucrose-
610 supplemented-beySD as from the L2/L3 transition (10%); n: total number of larvae
611 collected for each condition. (**B**) Prepupal weight of females (left) and males (right) as

612 listed in 6A; the numbers of weighted prepupae are indicated above each sample. (**C**)
613 Western-blot analysis of (from top to bottom) total dS6K, phosphorylated dS6K, total
614 Akt, phosphorylated Akt and total tubulin as a loading control. Protein extracts were
615 prepared from feeding L3 larvae prior to the wandering stage as listed in 6A. For each
616 condition, at least 30 larvae were used to prepare protein extracts.

617

618 **Figure 7: Cell-autonomous requirement of FASN activity for Ilp- but not mTORC1-**
619 **dependent overgrowth.** (**A-L**) MARCM clones labeled by GFP (green) in the FB of L3
620 larvae fed either a standard (A-C, G-I) or a 20%-SSD (D-F, J-L). Nuclei were labeled
621 with DAPI (silver) and membranes with phalloidin (red). Genotypes of MARCM clones
622 are: *Rheb*$^{+}$ (A,D), *PTEN*$^{-/-}$ (B,E) *PTEN*$^{-/-}$,*Rheb*$^{+}$ (C,F), *FASN*$^{1-2}$;*Rheb*$^{+}$ (G,J) *FASN*$^{1-}$
623 ^{2},*PTEN*$^{-/-}$ (H,K) and the *FASN*$^{1-2}$,*PTEN*$^{-/-}$;*Rheb*$^{+}$ (I,L). Scale bars: 50µm. (**M**) Relative
624 size of clonal cells corresponding to the clones shown in A-L and in Figure S1 for
625 *FASN*$^{1-2}$ and Figure 1A for control (Co).

626

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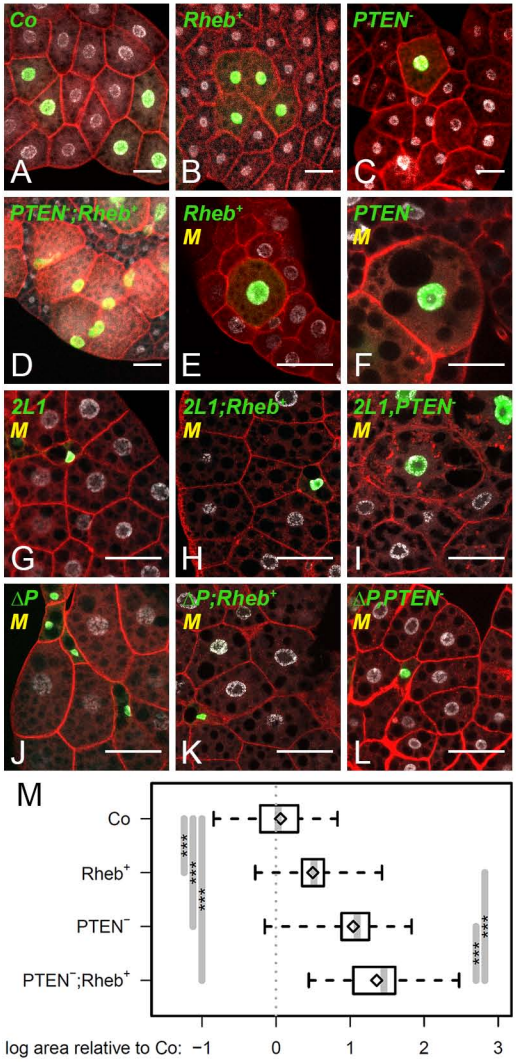
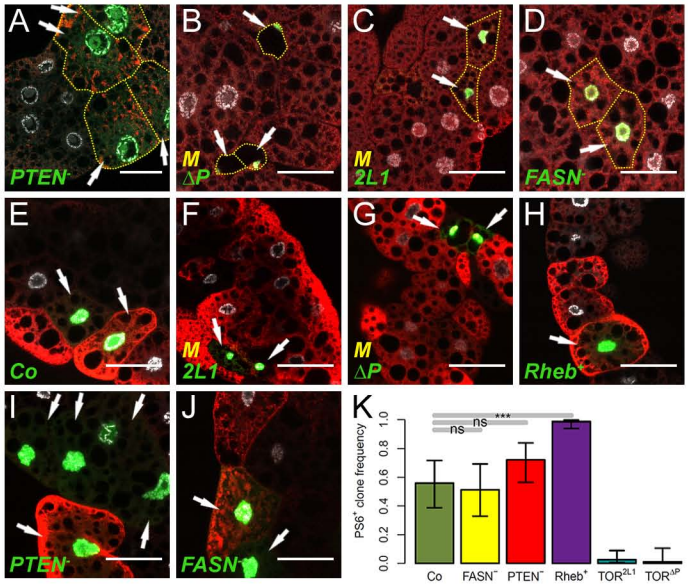
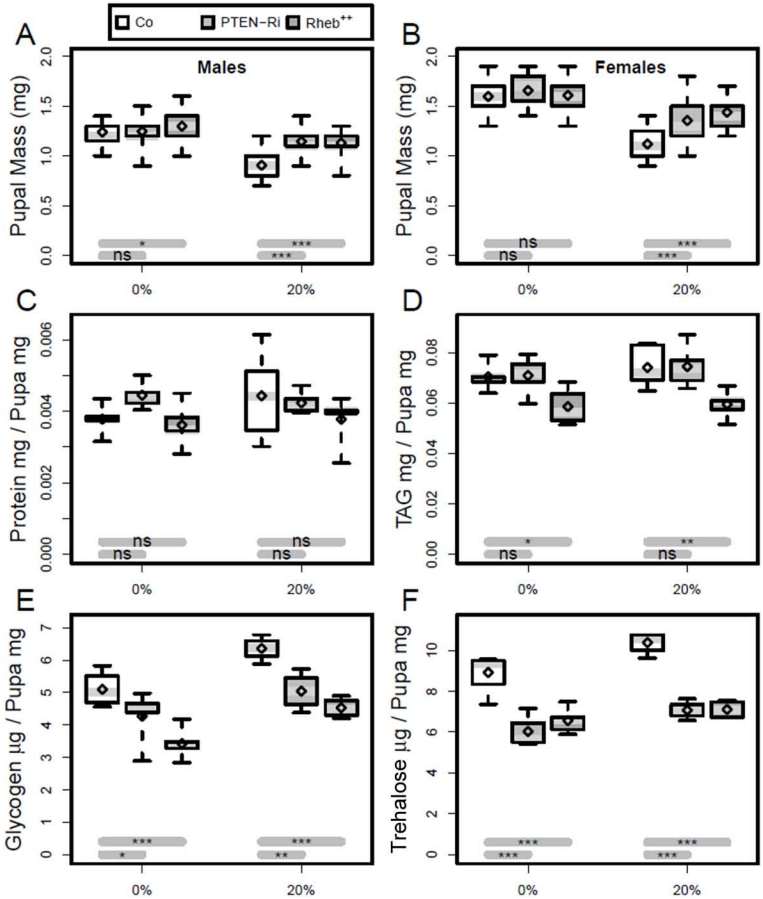

Co
A
Rheb+
B
PTEN-
C
PTEN-;Rheb+
D
Rheb+
M
E
PTEN-
M
F
2L1
M
G
2L1;Rheb+
M
H
2L1,PTEN-
M
I
ΔP
M
J
ΔP;Rheb+
M
K
ΔP,PTEN-
M
L
M
Co
Rheb+
PTEN−
PTEN−;Rheb+
***
***
***
***
***
log area relative to Co: −1
0
1
2
3


A
PTEN⁻
B
M
ΔP
C
M
2L1
D
FASN⁻
E
Co
F
M
2L1
G
M
ΔP
H
Rheb⁺
I
PTEN⁻
J
FASN⁻
K
PS6⁺ clone frequency
0.0
0.2
0.4
0.6
0.8
1.0
ns
ns
***
Co
FASN⁻
PTEN⁻
Rheb⁺
TOR2L1
TORΔP


Co
PTEN−Ri
Rheb++
A
Males
Pupal Mass (mg)
0.0
0.5
1.0
1.5
2.0
ns
*
***
***
0%
20%
B
Females
Pupal Mass (mg)
ns
ns
***
***
C
Protein mg / Pupa mg
0.000
0.002
0.004
0.006
ns
ns
ns
ns
D
TAG mg / Pupa mg
0.00
0.02
0.04
0.06
0.08
ns
*
ns
**
E
Glycogen µg / Pupa mg
0
1
2
3
4
5
6
7
*
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**
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F
Trehalose µg / Pupa mg
0
2
4
6
8
10
***
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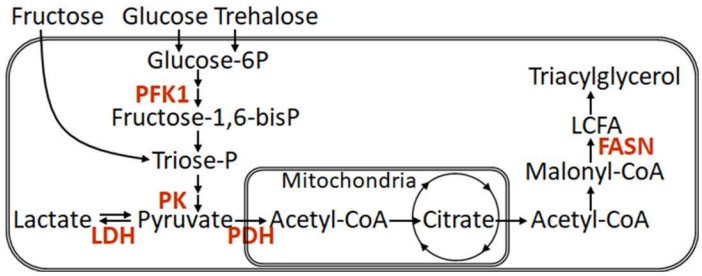
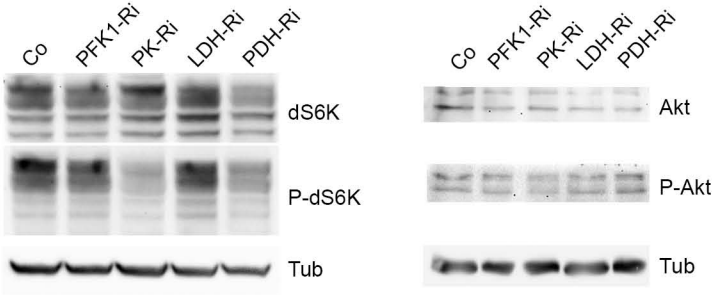
A



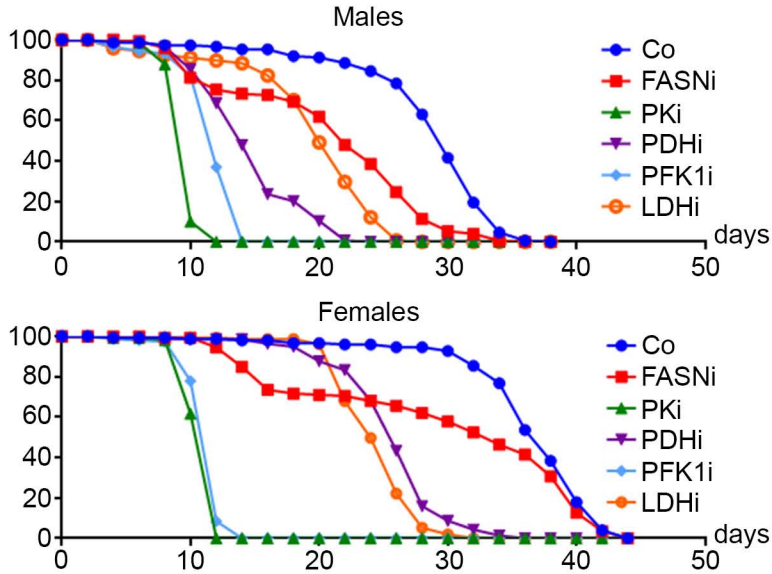
B

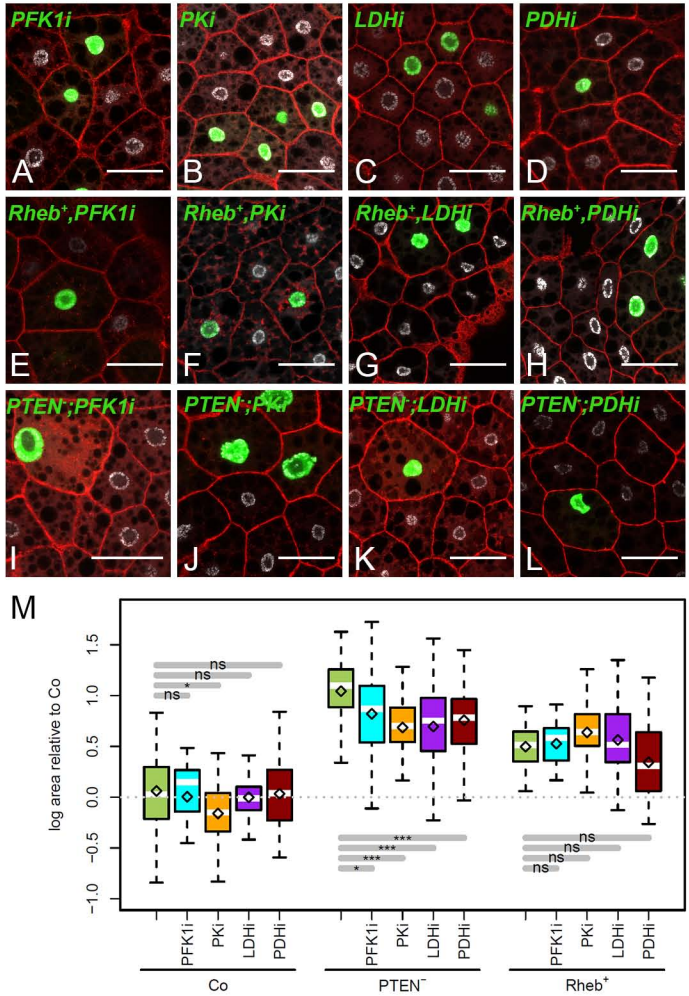
19°C>>29°C	0h	24h
PFK1-Ri	† L1/L2	† late L2
PK-Ri	† L2/L3	† L3/pp
LDH-Ri	† L2/L3	† L3/pp
PDH-Ri	½† L3/pp	½† L3/pp

C



D

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PKi
LDHi
PDHi
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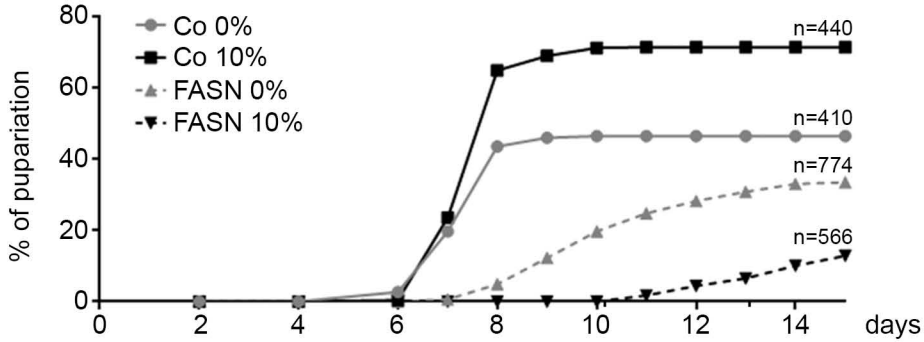
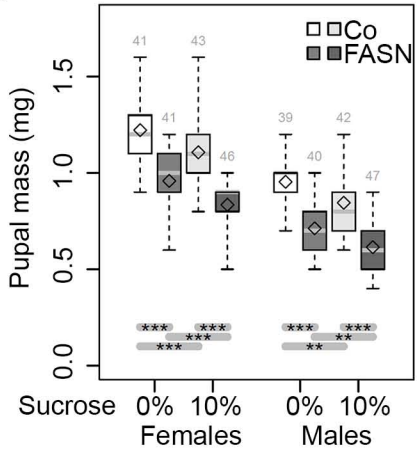
B
C
D
Rheb+,PFK1i
Rheb+,PKi
Rheb+,LDHi
Rheb+,PDHi
E
F
G
H
PTEN-;PFK1i
PTEN-;PKi
PTEN-;LDHi
PTEN-;PDHi
I
J
K
L
M
log area relative to Co
1.5
1.0
0.5
0.0
−0.5
−1.0
ns
ns
*
ns
***
***
***
*
ns
ns
ns
ns
PFK1i
PKi
LDHi
PDHi
Co
PTEN-
Rheb+

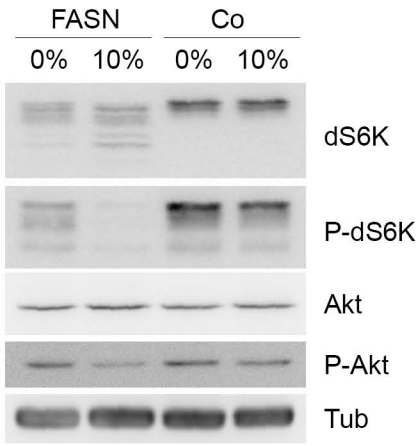
A

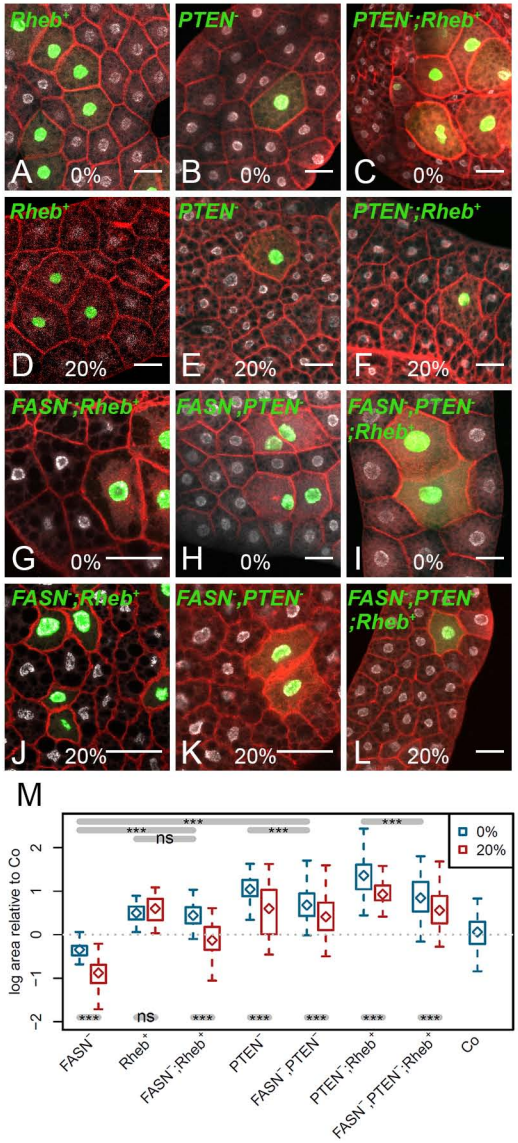

Co 0%
Co 10%
FASN 0%
FASN 10%
% of pupariation
80
60
40
20
0
0
2
4
6
8
10
12
14
days
n=440
n=410
n=774
n=566

B


Co
FASN
Pupal mass (mg)
0.0
0.5
1.0
1.5
41
41
43
46
39
40
42
47
***
***
***
***
***
**
**
Sucrose
0%
10%
0%
10%
Females
Males

C


FASN
Co
0%
10%
0%
10%
dS6K
P-dS6K
Akt
P-Akt
Tub


Rheb+
PTEN-
PTEN-;Rheb+
A 0%
B 0%
C 0%
Rheb+
PTEN-
PTEN-;Rheb+
D 20%
E 20%
F 20%
FASN-;Rheb+
FASN-;PTEN-
FASN-;PTEN-;Rheb+
G 0%
H 0%
I 0%
FASN-;Rheb+
FASN-;PTEN-
FASN-;PTEN-;Rheb+
J 20%
K 20%
L 20%
M
log area relative to Co
0%
20%
ns
***
FASN-
Rheb+
FASN-;Rheb+
PTEN-
FASN-;PTEN-
PTEN-;Rheb+
FASN-;PTEN-;Rheb+
Co