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Drosophila insulin-like peptide 1 (DILP1) promotes organismal growth and catabolic energy metabolism during the non-feeding pupal stage

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Abstract

The insulin/IGF-signaling pathway is central in control of nutrient-dependent growth during development, and in adult physiology and longevity. Eight insulin-like peptides (DILP1-8) have been identified in *Drosophila* and several of these are known to regulate growth, metabolism, reproduction, stress responses and lifespan. However, the functional role of DILP1 is far from understood. Previous work has shown that *dilp1*/DILP1 is transiently expressed mainly during the non-feeding pupal stage and the first days of adult life. Here we show that mutation of *dilp1* diminishes organismal weight during pupal development, whereas overexpression increases it, similar to *dilp6* manipulations. No growth effects of *dilp1* or *dilp6* manipulations were detected during larval development. We next show that *dilp1* and *dilp6* increase metabolic rate in the late pupa and promote lipids as the primary source of catabolic energy. This lipid mobilization in the pupa is not correlated with transcriptional changes of adipokinetic hormone. The effects of *dilp1* manipulations carry over to the adult fly. In newly eclosed flies, survival during starvation is strongly diminished in *dilp1* mutants, but not in *dilp2* and *dilp1-dilp2* double mutants, whereas in older flies only double mutants display reduced starvation resistance. In conclusion, *dilp1* and *dilp6* promote growth of adult tissues during the non-feeding pupal stage, likely by utilization of stored lipids. This results in larger newly-eclosed flies with reduced stores of pupal-derived nutrients and diminished starvation tolerance and fecundity.

Introduction

The Insulin/IGF signaling (IIS) pathway plays a central role in nutrient-dependent growth control during development, as well as in adult physiology and aging [1-5]. More specifically, in mammals insulin, IGFs and relaxins act on different types of receptors to regulate metabolism, growth and reproduction [6-9]. This class of peptide hormones has been well conserved over evolution and therefore the genetically tractable fly *Drosophila* is an attractive model system for investigating IIS mechanisms [4,10,11]. Eight insulin-like peptides (DILP1-8), each encoded on a separate gene, have been identified in *Drosophila* [10,12-14]. The genes encoding these DILPs display

75 differential temporal and tissue-specific expression profiles, suggesting that
76 they have different functions [12,14-17]. Specifically, DILP1, 2, 3 and 5 are
77 mainly expressed in median neurosecretory cells located in the dorsal midline
78 of the brain, designated insulin-producing cells (IPCs) [12,16,18-20]. The IPC
79 derived DILPs can be released into the open circulation from axon
80 terminations in the corpora cardiaca, the anterior aorta and the crop. Genetic
81 ablation of the IPCs reduces growth and alters metabolism, and results in
82 increased resistance to several forms of stress and prolongs lifespan [18,21].

83 The functions of the individual DILPs produced by the IPCs may vary
84 depending on the stage of the *Drosophila* life cycle. Already the temporal
85 expression patterns hint that DILP1-3 and 5 play different roles during
86 development. Thus, whereas DILP2 and 5 are relatively highly expressed
87 during larval and adult stages, DILP1 and 6 are almost exclusively expressed
88 during pupal stages under normal conditions [15,22].

89 DILP1 is unique among the IPC-produced peptides since it can be
90 detected primarily during the non-feeding pupal stage and the first few days of
91 adult life when residual larval/pupal fat body is present [15,16]. Furthermore,
92 in female flies kept in adult reproductive diapause, where feeding is strongly
93 reduced, *dilp1*/DILP1 expression is also high [16]. Its temporal expression
94 profile resembles that of DILP6 although this peptide is primarily produced by
95 the fat body, not IPCs [15,22]. Since DILP6 was shown to regulate growth of
96 adult tissues during pupal development [15,22], we asked whether also DILP1
97 plays a role in growth control. It is known that overexpression of several of the
98 DILPs is sufficient to increase body growth through an increase in cell size
99 and cell number, and especially DILP2 produces a substantial increase in
100 body weight [12,23,24]. In contrast, not all single *dilp* mutants display a
101 decreased body mass. The *dilp1*, *dilp2* and *dilp6* single mutants display
102 slightly decreased body weight [10,15,22], whereas the *dilp3*, *dilp4*, *dilp5* and
103 *dilp7* single mutants display normal body weight [10]. However, a triple
104 mutation of *dilp2*, 3, and 5 causes a drastically reduced body weight, and a
105 *dilp1-4,5* mutation results in even smaller flies [10,25].

106 There is a distinction between how DILPs act in growth regulation.
107 DILPs other than DILP1 and 6 promote growth primarily during the feeding
108 larval stages when their expression is high [12,23]. This nutrient dependent

109 growth is relatively well understood and is critical for production of the steroid
110 hormone ecdysone and thereby developmental timing and induction of
111 developmental transitions such as larval molts and pupariation [26-30]. The
112 growth during non-feeding stages, which affects imaginal discs and therefore
113 adult tissues, is far less studied. In this study, we investigate the role of
114 *dilp1*/DILP1 in growth regulation in *Drosophila* in comparison to *dilp6*/DILP6.
115 We found that mutation of *dilp1* diminishes body weight and ectopic *dilp1*
116 expression promotes organismal growth during the non-feeding pupal stage,
117 similar to *dilp6*. Determination of metabolic rate and respiratory quotient as
118 well as TAG levels during late pupal development provides evidence that *dilp1*
119 and *dilp6* increase the metabolic rate and that the associated increased
120 metabolic cost is fueled by increased lipid catabolism. We, however, find no
121 evidence for a role of the lipid mobilizing adipokinetic hormone (AKH) [31-33]
122 in the altered lipid catabolism in pupae.

123 We also investigated the role of *dilp1* mutation and overexpression on
124 early adult physiology. Interestingly, the newly eclosed *dilp1* mutant flies are
125 less resistant to starvation than controls and *dilp2* mutants. Thus, *dilp1* acts
126 differently from other *dilps* for which it has been shown that reduced signaling
127 increases survival during starvation [21]. The decreased starvation resistance
128 in newly hatched flies after *dilp1* overexpression may be a consequence of
129 diminishment of stored nutrients in the pupa during the increased growth of
130 adult tissues, and thus less residual pupal fat body in newborn flies. Also early
131 egg laying and fecundity are affected by *dilp1*.

132 Taken together, our data suggest that *dilp1*/DILP1 promotes growth of
133 adult tissues during the non-feeding pupal stage, and that this process mainly
134 utilizes stored lipids to fuel the increased metabolic rate. The effect of this
135 increased metabolic rate in the pupa carries over to affect the metabolism in
136 the young adult fly. We suggest that *dilp1*, similar to *dilp6* [15], ensures that if
137 a larva is exposed to poor nutritional conditions it will after pupariation utilize
138 stored nutrients for growth of adult tissues, rather than keeping these stores
139 for the first days of adult life.

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Results

Mutation of *dilp1* decreases body weight

Growth in *Drosophila* is in part regulated by several of the DILPs through activation of the canonical IIS/TOR (target of rapamycin) pathway [11,12,28]. It was previously reported that decreased *dilp1* activity reduces adult body weight in *Drosophila*, but it was not investigated at what developmental stage this occurred [10,19]. This is relevant to ask since *dilp1* displays a restricted temporal expression during the *Drosophila* life cycle (see Fig 1A). To analyze growth effects of *dilp1* and possible interactions with its tandem-encoded paralog *dilp2*, we employed recently generated *dilp1*, *dilp2* and double *dilp1-dilp2* null mutants [34]. The efficacy of these mutants was confirmed by qPCR in stage 8-9 pupae and immunolabeling in one-week-old mated female flies (S1 Fig). It can be noted that in *dilp1* mutant pupae the mRNA levels of *dilp2*, *dilp3* (not shown) and *dilp6* were not altered, but in *dilp6* mutants the *dilp1* level was upregulated (S1A-C Fig). At the protein level DILP2 but not DILP3 immunofluorescence increased in *dilp1* mutants (S1D-G Fig). These findings suggest only minor compensatory changes in other dilps/DILPs in *dilp1* mutants during the pupal stage.

We monitored the body weight (wet weight) of *dilp1*, *dilp2* and *dilp1/dilp2* double mutants. First we measured the body weight both in newborn and 6-7 day old adult mated *dilp1* mutant flies. In female flies the newly hatched *dilp1* mutants displayed a decrease in body weight compared to controls (Fig 1B). However, this difference in body weight was no longer detectable in 6-7-day-old mated flies kept under normal feeding conditions; a significant weight increase was observed (Fig 1B). Also *dilp2* mutant female flies have significantly lower body weight than controls one day after emergence, but in contrast to *dilp1* mutants they did not increase the weight over 6-7 days of feeding (Fig 1B). Interestingly the weight of *dilp1/dilp2* double mutants was not significantly affected compared to the single mutants (and control) and no weight increase was seen the first week, except in control flies (Fig 1B). Thus, there was no additive effect of the two mutations. In male flies none of the mutant flies displayed altered body weight (Fig 1C). To determine

176 whether decreased organismal growth was responsible for the lower body
177 weight we measured wing size in the female mutant flies and found no
178 significant difference to controls (Fig 1D). Thus, the decreased weight of the
179 flies does not seem to reflect a significant decrease in organismal size.

180 We next asked whether the weight gain over the first 6-7 days seen in
181 Fig 1B was caused by increased feeding. Using a capillary feeding (CAFE)
182 assay over four days, we found that during the first day of assay the *dilp1*
183 mutant flies actually fed less than the other mutants and control flies (Fig 1E).
184 The subsequent days food intake was not significantly different between the
185 genotypes. Thus, the food intake profile does not explain the weight gain over
186 the 6-7 days (Fig 1E); possibly the female *dilp1*^{-/-} flies excrete less waste or
187 spend less energy. It was shown earlier that 1 week old *dilp1* mutant flies
188 display a two-fold increased expression of *dilp6* transcript [16], that might
189 compensate for the loss of *dilp1*. However, in the midpupal stage there is no
190 significant upregulation of *dilp6* in *dilp1* mutants (S1C Fig).

191 In a study of *dilp6* it was shown that if third instar larvae (after reaching
192 critical size) were put on a low protein diet, they emerged as smaller adults
193 and that this was accentuated in *dilp6* mutants [15]. This suggests that *dilp6* is
194 important for assuring growth of adult tissues under low protein conditions.
195 We, thus, performed a similar experiment with *dilp1* mutant larvae kept on
196 normal food or low protein diet. Flies emerging from larvae on restricted
197 protein indeed displayed significantly lower body weight and female *dilp1*
198 mutants weighed less than controls under protein starvation (Fig 1F). In male
199 flies this latter effect was not seen in the mutants (Fig 1G).

200 We then asked whether mutation of both *dilp1* and *dilp6* would result in
201 a further decrease of body weight and generated a recombinant *dilp1-dilp6*
202 mutant. Using qPCR we found that these flies displayed virtually no
203 detectable *dilp1* and *dilp6* RNA (S2A Fig.). The weights of *dilp1/dilp6* mutants
204 were significantly reduced compared to controls (S2B Fig.). However, their
205 weights were not diminished more than those of the single *dilp1* and *dilp6*
206 mutants, indicating that there was no additive effect of loss of both *dilps*.

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Overexpression of *dilp1* promotes growth during the non-feeding pupal stage

Having shown effects of the *dilp1* null mutation on adult flyweight we next explored the outcome of over-expressing *dilp1*, either in IPCs, or more broadly. For this we generated several UAS-*dilp1* lines [see [34]]. These UAS-*dilp1* lines were verified by DILP1 immunolabeling after expression with several Gal4 drivers (S3A-D Fig) and by qPCR in stage 8-9 pupae (S4A-F Fig). Overexpression of *dilp1* in fat body (*ppl*-Gal4 and *to*-Gal4) and IPCs (*dilp2*-Gal4) results in a drastic upregulation of *dilp1* RNA (S4A, D Fig), but has no effect on *dilp2* and *dilp6* expression (S4B, C, E, F Fig), except a minor decrease in *dilp2* for *ppl*-Gal4 (S4B Fig). At the protein level *dilp1* overexpression resulted in minor changes in DILP2, 3 and 5 immunolevels in IPCs of one week old adult female flies (S5A-E Fig). One line, UAS-*dilp1* (III), was selected for subsequent experiments since it generated the strongest DILP1 immunolabeling.

First, we used a *dilp2*-Gal4 driver to express *dilp1* in the IPCs and detected a significant increase in body weight of female flies (Fig 2A). We then expressed *dilp1* in the fat body, the insect functional analog of the liver and white adipocytes in mammals [35-37]. The fat body displays nutrient sensing capacity, and is an important tissue for regulation of growth and metabolism in *Drosophila* [15,37-41]. It is also the tissue where DILP6 is produced and released [15,38]. To investigate the effect of ectopic *dilp1* expression in the fat body, we used the fat body-specific *pumpless* (*ppl*) and *takeout* (*to*) Gal4 drivers. The efficiency of the drivers was confirmed by DILP1 immunostaining of larval fat body of *ppl*>*dilp1* and *to*>*dilp1* flies, but not in the control flies (S3D Fig). In *ppl*>*dilp1* flies we also found DILP1 labeling in the nephrocytes (not shown), which are highly endocytotic cells located close to the heart [42]. The immunoreactive DILP1 is likely to have accumulated from the circulation after release from the fat body since the *ppl*-Gal4 is not expressed in the nephrocytes.

Before monitoring the effect of *dilp1* overexpression in the fat body on adult body weight and organismal size, we wanted to determine whether *dilp1* has an effect on larval development. We therefore measured the time to pupariation and size of pupae to determine whether *dilp1* overexpression

244 affected timing of larval development and growth during this stage. Using the
245 *ppl*-Gal4 driver we did not observe any effect on the time from egg to pupa
246 compared to controls (Fig 2B). Pupal volume, as a measurement of larval
247 growth, was not altered by *ppl*-Gal4>*dilp1* (Fig. 2C). As expected [15,38],
248 over-expression of *dilp6* also had no effect on pupal size (Fig 2C). However,
249 as shown earlier for ubiquitously expressed *dilp2* [23], *dilp2* expression in the
250 fat body generated a strong increase in pupal volume, suggesting growth
251 during the larval feeding stage (Fig 2C). Driving *dilp1* with the *c929* Gal4 line,
252 that directs expression to several hundred *dimm*-expressing peptidergic
253 neurons including IPCs [43], we did not observe any effect on time to
254 pupariation or pupal volume (Fig 2B, C). Taken together our data suggest the
255 ectopic *dilp1* does not affect larval growth or developmental time.

256 Next, we determined the body weight of mated 6-7 d old flies. Body
257 weight increased significantly in *ppl*>*dilp1* flies compared to the controls both
258 in female (Fig 2D) and male flies (Fig 2E). Here we additionally noted
259 increased weight for *ppl*>*dilp2* and *ppl*>*dilp6* flies. We also monitored the
260 weight of one day old flies and found that *ppl*>*dilp1*, but not *dilp2*>*dilp1* flies
261 displayed increased weight (Fig 2F). However, *dilp2*>*dilp1*-RNAi induced a
262 decrease in body weight (Fig 2F). Moreover, organismal size, estimated by
263 wing size (Fig 2G, H) and thorax length (Fig 2G, I), increased after ectopic
264 expression of *dilp1* in the fat body. Since we see no effect of *dilp1* expression
265 on developmental time or pupal volume, but register increased body weight
266 and size of adults, we propose that *dilp1*, like *dilp6*, promotes growth of adult
267 tissues during the pupal stage.

268 It was suggested that *dilp6* promotes growth of adult tissues during
269 pupal development by utilizing nutrients stored in the larval fat body, which is
270 carried into the pupa [15]. This may be the case also for *dilp1*, and if so, newly
271 hatched *dilp1* overexpressing flies should have less energy stores in the form
272 of residual larval fat body. To test this we monitored feeding in recently
273 hatched *dilp1* mutant flies and controls. Indeed, flies overexpressing *dilp1*
274 displayed increased food ingestion over the first four days after adult
275 emergence compared to controls (Fig 2J). Next we compared the weights of
276 one day old and 6-7 day old flies after *dilp1* overexpression with *ppl*-Gal4 and
277 found that at both ages the female *ppl*>*dilp1* flies weighed more than controls

278 and that the older flies were heavier than the younger ones (Fig 2K). In male
279 flies *ppl>dilp1* also increased the body weight, but there was a loss of weight
280 for all genotypes over the first 6-7 days of adult life (S6A Fig). As a
281 comparison *dilp2>dilp1* had only minor effects on body weight of female flies,
282 only in 6-7 d old flies there was an increase (S6B Fig), whereas in males a
283 significant increase was noted at both ages for *dilp2>dilp1*, and a loss of
284 weight over the next six days for all genotypes (S6C Fig).

285 Using the *to*-Gal4 fat body driver to express *dilp1* we also noted an
286 increase in weight of recently emerged female and male flies (S6D, E Fig), but
287 no change in body size except a minor increase in thorax length in females
288 (S6F, G Fig). The female *to>dilp1* flies increased further in weight the first 6-7
289 days of adult life, but not later (S6D Fig), whereas the males did not (S6E Fig).
290 Furthermore, with the *to*-Gal4 driver there was no increase in pupal volume,
291 supporting that *dilp1* does not affect larval growth (S6H Fig).

292 Ectopic expression of *dilp1* in neuroendocrine cells by means of the
293 *c929*-Gal4 increased adult body weight (S7A Fig), but had no effect on wing
294 size in males and females or food intake in young flies (S7B, C Fig),
295 suggesting that *dilp1* expression (and/or systemic release) was not strong
296 enough to yield major effects. Also *dilp2>dilp1* flies were tested in food intake
297 and no effect was seen (S7C Fig).

298

299 **Overexpression of *dilp1* increases the size of the adult brain and** 300 **neuroendocrine cells**

301 It was previously shown that signaling through the *Drosophila* insulin receptor
302 (dInR) can lead to an enlargement of cell bodies of neuroendocrine cells in a
303 cell autonomous manner, and that *dilp6* in glial cells is a candidate ligand to
304 mediate this dInR dependent growth [44,45]. Since *dilp1* has a temporal
305 expression profile similar to *dilp6*, and promotes growth of adult tissues in the
306 pupal stage, we asked whether *dilp1* also affects size of neuroendocrine cells
307 that differentiate in the pupa. Thus, we overexpressed *dilp1* with the broad
308 neuroendocrine cell driver *c929*-Gal4 [43,46], and monitored the cell body
309 size of several groups of neuroendocrine cells in the adult CNS with specific
310 peptide antisera. We found that the cell body size of IPCs increased in adult
311 *c929>dilp1* flies, as shown by anti-DILP2 staining (S8A1-3 Fig, Table 1).

Furthermore, the cell bodies of the adult-specific pigment-dispersion factor (PDF) expressing clock neurons (l-LN_s), as shown here by anti-PDF staining, were enlarged in *c929>dilp1* flies compared to the controls (S8B1-3 Fig, Table 1). Next, we monitored the cell-body size of leucokinin (LK) producing neurons in the abdominal ganglia (ABLKs), and found that the adult-specific anterior, but not the larval-derived posterior ABLKs, displayed increased size in *c929>dilp1* flies (S8C1-3 Fig, Table 1).

However, the observed increase in cell body size appears to be partly due to a broader growth of the adult fly tissues, since we found that also the size of the brain increased in *c929>dilp1* flies (S8D Fig, Table 1). The *c929*-Gal4 is expressed in IPCs and several other groups of peptidergic neurosecretory cells [43,46], which could be the source of systemic release of ectopic DILP1 that affects brain and cell growth. To support that systemic DILP1 is required to promote this growth we employed the *ppl*-Gal4 to drive *dilp1* in the fat body and found an increase in the size of the PDF expressing clock neurons (S8F1-3 Fig, Table 1) and the brain (S8G Fig, Table 1). In contrast, we found that expressing *dilp1* in interneurons, such as PDF-expressing clock neurons does not induce growth of brain neurons (S9A, B Fig, Table 1) or size of the brain (S9C Fig, Table 1), but affected the intensity of PDF immunolabeling (S9D Fig). Thus, paracrine release of DILP1 in the brain does not seem to affect growth of neurons. Interestingly, we found that in third instar larvae, the cell body size of ABLK neurons or the size of the CNS were not different in *c929>dilp1* larvae compared to controls (S9E-G Fig, Table 1), further supporting that *dilp1* overexpression has no effect on cell growth during the larval stage. Finally, since overexpression of *dilp6* in glial cells by *Repo*-Gal4 promotes increase in size of neuronal cell bodies [45], we tested overexpression of *dilp1* in these cells, but found no significant effect on the cell-body size of PDF neurons (S10A, B Fig, Table 1). This again indicates that to affect cell/tissue growth DILP1 must act systemically rather than in a paracrine fashion.

Table 1. Overexpression of *dilp1* in neurosecretory cells and fat body affects size of brain and certain neurons

Genotype	Neuron/tissue ¹	Size change	Significance	Figure
<i>c929>dilp1</i>	IPC	+	p<0.001	S Fig. 7A
<i>c929>dilp1</i>	I-LNv	+	p<0.01	S Fig. 7B
<i>c929>dilp1</i>	ABLK _a	+	p<0.05	S Fig. 7C
<i>c929>dilp1</i>	ABLK _p	nc	ns	S Fig. 7C
<i>c929>dilp1</i>	Whole brain	+	p<0.001	S Fig. 7D
<i>c929>dilp1</i>	ABLK larva	nc	ns	S Fig. 8E, F
<i>c929>dilp1</i>	CNS larva	nc	ns	S Fig. 8G
<i>dilp2>dilp1</i>	IPC	nc	ns	S Fig. 7E
<i>ppl>dilp1</i>	I-LNv	+	p<0.01	S Fig. 7G
<i>ppl>dilp1</i>	Whole brain	+	p<0.05	S Fig. 7H
<i>pdf>dilp1</i>	I-LNv	nc	ns	S Fig. 8A, B
<i>pdf>dilp1</i>	Whole brain	nc	ns	S Fig. 8C
<i>repo>dilp1</i>	I-LNv	nc	ns	S Fig. 9A, B

Notes

+, increased size

nc, no change

ns, not significant

¹ Neurons or tissues monitored for size

Metabolic rate and respiratory quotient in pupae of different genotypes

To investigate the role of *dilp1* in utilization of nutrients during pupal development we determined metabolic rate (MR) and respiratory quotient (RQ) in pupae of different genotypes. First we characterized the metabolic trajectory in control pupae (*w¹¹¹⁸*) by measuring cumulative MR daily throughout pupal development (Fig 3A). These data show the exponential MR curve typical for developing insects, including *D. melanogaster* [47]. To minimize handling stress, we chose to investigate only the end of pupal development in more detail and measured MR and RQ in 4-day-old pupae (that is the cumulative MR between hours 96 and 120 after pupation). For this experiment we used only *ppl*-Ga4 overexpression animals, since the mutant

365 animals displayed high mortality in the respirometry setup used here. As can
366 be seen in Fig 3B and 3C the *ppl>dilp1* and *ppl>dilp6* differed significantly
367 from the controls. The MR was higher and RQ lower in the overexpression
368 flies than in the control flies. RQ values, around 0.6 in both overexpression
369 lines, suggest pure lipid metabolism [48], and lipids are known to be a major
370 or sole fuel during metamorphosis of insects [49,50]. Our findings strongly
371 suggest that *dilp1* (and *dilp6*) affects metabolism in the pupa, maybe to
372 ensure that enough fuel is allocated for growth of adult tissues.

373

374 **TAG, carbohydrates and AKH signaling in pupae of different genotypes**

375 To determine whether it indeed are lipids that fuel growth of adult tissues in 4
376 day old pupae we determined TAG levels after over expression of *dilp1* and
377 *dilp6* in fat body (*ppl-Gal4*). Pupae of both genotypes displayed increased
378 weight (Fig. 3D) and also significantly reduced TAG levels (Fig. 3E),
379 compared to controls of the same age. The decreased glycogen levels in
380 pupae after ectopic expression of *dilp1* and *dilp6* were not significant (Fig. 3F)
381 and glucose levels were not significantly changed (S11 Fig).

382 Since AKH is known to mobilize lipids in insects, including *Drosophila*
383 [31-33,51], we determined levels of *akh* transcript in pupae with *dilp1* and
384 *dilp6* overexpression (using *ppl-Gal4*) and in *dilp* mutants, at two different time
385 points (2 and 4 d old pupae). There was no significant alteration in *Akh*
386 transcript after *dilp1* or *dilp6* overexpression; the only phenotype was a slight
387 upregulation in *dilp1* mutants in 4 d pupae (S11A-D Fig). Next we analyzed
388 levels of transcript of brummer (*bmm*), a lipase known to promote TAG
389 mobilization [52], in pupae of the same stages and found no significant
390 change in expression for any genotype (S11E-H Fig). We also measured
391 transcript of the α -glucosidase *tobi*, which regulates glycogen levels and is a
392 target of both DILPs and AKH [53], and found no effect of overexpression (not
393 shown) or loss of function of *dilp1* at either stage (S11 I, J Fig).

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395

396

Table 1). Here we tested also 6-7 day old male flies and found that they survived starvation in a manner different from females with *dilp2* and double mutants displaying diminished stress resistance whereas *dilp1* mutants survive similar to controls (S12A Fig).

435

Table 2. Median lifespans of female flies exposed to starvation.

437

Genotype	Median lifespan (calculated as % of w^{1118})		
	Newly eclosed	3 d adults	6-7 d adults*
w^{1118}	100	100	100
<i>dilp1</i> -/-	83 (p<0.001)	86 (p<0.001)	78 (p<0.001)
<i>dilp2</i> -/-	100	100	100
<i>dilp1-dilp2</i> -/-	107 (p<0.001)	76 (p<0.001)	67 (p<0.001)
<i>ppl>w^{1118}</i>	100	-	100
<i>ppl>dilp1</i>	80 (p<0.001)	-	90 (p<0.001)

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* Data from Post et al. [34]

440

As seen above, our data suggest a change in the response to loss of *dilp* function over the first week of adult life. It is known that newly hatched wild type flies are more resistant to starvation than slightly older flies [54]. Thus, we compared the survival during starvation in recently emerged and three day old virgin flies. As seen in Fig 5C (based on data in Fig 5A and B), recently hatched control flies (w^{1118}) indeed exhibit increased starvation resistance compared to controls that were tested when three days old. Also the *dilp1* mutant flies are more starvation resistant when tested as newly hatched than as older flies, and the mutants perform less well than controls at both ages (Fig 5D). However, the most drastic change within the first week is that *dilp1* mutants yield the strongest phenotype as newborn flies and then in 3d and 6-7 d old flies the *dilp1/dilp2* mutants are the ones with the lowest stress resistance. Thus, a change in the role of *dilp1* seems to occur as the fly matures during the first few days of adult life. To provide additional evidence that *dilp1* impairs starvation resistance we performed *dilp1*-RNAi using a *dilp2*-Gal4 driver. The efficiency of the *dilp2>dilp1*-RNAi was tested by qPCR (S13A Fig) where a strong decrease in *dilp1*, but not *dilp2* or *dilp6* was seen.

458 The *dilp1*-RNAi resulted in newly eclosed flies that displayed reduced survival
459 during starvation (S13B Fig), similar to *dilp1* mutant flies.

460 It is also interesting to note that the diminished starvation resistance in
461 *dilp1* and *dilp1/dilp2* mutants is opposite to the phenotype seen after IPC
462 ablation, mutation of *dilp1-4*, or diminishing IIS by other genetic interventions
463 [10,21,55,56]. Thus, in recently hatched flies *dilp1* appears to promote
464 starvation resistance rather than diminishing it. Furthermore, the decreased
465 survival during starvation in female *dilp1* mutants is the opposite of that shown
466 in *dilp6* mutants [15], indicating that *dilp1* action is different from the other
467 insulin-like peptides.

468 Next we investigated the effect of the mutations on the flies' response
469 to desiccation (dry starvation). One-week-old flies were put in empty vials and
470 survival recorded. Female *dilp1/dilp2* mutants were more sensitive to
471 desiccation than controls and the single mutants (Fig 5D). In males the double
472 mutants also displayed higher mortality during desiccation, whereas the two
473 single mutants were more resistant than controls (S12B Fig). Thus, there is a
474 sex dimorphism in how the different mutants respond to both desiccation and
475 starvation.

476 When overexpressing *dilp1* with the fat body driver *ppl*-Gal4 newly
477 eclosed and 6-7 d old female flies become less resistant to starvation
478 compared to parental controls (Fig 6A, B). However, in 6-7-day-old male flies
479 there is no difference between controls and flies with ectopic *dilp1*, using *ppl*-
480 and *c929*-Gal4 drivers (S13C-D Fig). We furthermore investigated starvation
481 resistance in flies overexpressing *dilp1* in IPCs (*dilp2>dilp1*) and in most
482 neuroendocrine cells (*c929>dilp1*) and found that in newborn flies
483 overexpression reduced survival (Fig 6C, E), whereas in a week old flies all
484 genotypes displayed the same survival (Fig 6D, F). Thus, in females it
485 appears as if both knockout and over expression of *dilp1* reduces starvation
486 resistance, maybe due to offsetting a narrow window of homeostasis. It was
487 shown earlier that conditional knockdown of *dilp6* by RNAi during the pupal
488 stage resulted in newborn flies with *increased* survival during starvation [15],
489 suggesting that the effect the *dilp1* null mutation is different.

490 After ectopic expression of *dilp1* in the fat body there was an increase
491 in food intake (cumulative data) in one-week-old flies over four days (Fig 7A),

492 suggesting that metabolism is still altered in older flies. Since the effect of
493 *dilp1* manipulations seems stronger in female flies we asked whether
494 fecundity is affected by overexpression of *dilp1*. An earlier study showed that
495 *dilp1* mutant flies are not deficient in number of eggs laid, or the viability of
496 offspring (egg to pupal viability), although the *dilp1/dilp2* double mutants
497 displayed a reduction in viability of these eggs [34]. Here, we expressed *dilp1*
498 in fat body (*ppl*-Gal4) and detected an increase in number of eggs laid over 24
499 h in 6-7 d old flies (Fig. 7B). Both *ppl*-Gal4- and *c929*-Gal4-driven *dilp1*
500 decreased the viability of eggs laid as monitored by numbers of eggs that
501 developed into pupae (Fig 7C). As a comparison we noted no difference in
502 number of eggs in 3-day-old *dilp1* mutant flies (Fig. 7D).

503 We next asked whether there is any physiological trigger of increased
504 *dilp1* expression in adult flies, except for diapause [16] and experimental ones
505 such as ectopic expression of sNPF or knockdown of *dilp6*, *dilp2* and *dilp2,3,5*
506 [16,34,57]. Although diminished protein diet in larvae had no effect on *dilp1*
507 expression measured by immunolabeling (not shown), we found that 40 h
508 starvation of 10 d old flies (*w¹¹¹⁸*) leads to a significant increase in *dilp1*, but
509 not in *dilp2* or *dilp6* (Fig 7E). Thus, at a time (12 d) when *dilp1* is very low
510 under normal conditions, it is upregulated four times during starvation, further
511 suggesting that the peptide indeed plays a role also in older adult flies.

512 The functional homolog of glucagon in flies, AKH, plays important roles
513 both in lipid mobilization, metabolism and regulation of lifespan [31,51,58,59].
514 A previous paper showed that in *dilp1* mutant flies levels of AKH were not
515 affected [34]. Here we found that *dilp1* overexpression with the *c929*-Gal4
516 driver induced an increase in AKH immunolabeling in one-week-old flies (Fig
517 7F). Thus in adult flies (in contrast to larvae) there appears to be an
518 interaction between *dilp1* and AKH that may underlie some of the effects of
519 this DILP on metabolism and stress tolerance.

520

521 Discussion

522 Our study indicates a role for *dilp1* in regulation of adult tissue growth during
523 the pupal stage, as well as a function in adult physiology, especially during the
524 first days of adult life. The experiments herein suggest that the developmental

525 role of *dilp1* may be to ensure nutrient utilization in the pupa to support growth
526 of adult tissues if the larva was exposed to restricted food sources. In the
527 adult *dilp1* is upregulated during starvation and genetic gain and loss of
528 function of *dilp1* signaling alters the flies' survival under starvation conditions.
529 These novel findings combined with previous data showing high levels of *dilp1*
530 during adult reproductive diapause [16] and its role as a pro-longevity factor
531 during aging [34] demonstrate a wide-ranging importance of this signaling
532 system. Not only does *dilp1* expression correlate with stages of non-feeding
533 (or reduced feeding), these stages are also associated with lack of
534 reproductive activity, and encompass the pupae, newly eclosed flies, and
535 diapausing flies. Under normal conditions, the diminishing *dilp1*/DILP1
536 expression during the first few days of adult life may be associated with a
537 metabolic transition (fat body remodeling; [60]) and the onset of sexual
538 maturation.

539 In *Drosophila*, the final body size is determined mainly during the larval
540 feeding stage [11,12,23,29]. However, regulation of adult body size can also
541 occur after the cessation of the feeding stage, and this process is mediated by
542 *dilp6* acting on adult tissue growth in the pupa in an ecdysone-dependent
543 manner [15,38]. This is likely a mechanism to ensure growth of the adult
544 tissues if the larva is exposed to shortage of nutrition during its feeding stage.
545 Our findings suggest that *dilp1* is another regulator of growth during the pupal
546 stage. We show here that *dilp1* promotes organismal growth in the non-
547 feeding pupa at the cost of stored nutrients derived from the larval stage. This
548 is supported by RQ-data that clearly shows a shift from mixed-energy
549 substrate energy metabolism in control flies towards almost pure lipid
550 catabolism at the end of pupal development in the *dilp1* overexpression flies
551 (also seen for *dilp6*). Furthermore, TAG (but not carbohydrate) levels in *dilp1*
552 overexpression flies were clearly decreased, which likely reflects the shift in
553 catabolic energy substrate also seen in the R/Q using respirometry. It should
554 be noted that insects predominantly use lipids as fuel during metamorphosis
555 [49,50] and *dilp1* overexpression increases lipid catabolism. As a
556 consequence large *dilp1*-overexpressing flies display increased food ingestion
557 over the first four days as adults and an altered response to starvation.
558 Conversely *dilp1* mutants hatched as flies with significantly smaller weight.

Both alterations in *dilp1* expression influence the metabolic balance in early adults as manifested in reduced starvation resistance at this stage. Our study hence suggests that *dilp1* parallels *dilp6* [15,38] in balancing adult tissue growth and storage of nutrient resources during pupal development, and thereby probably affecting adult physiology. This is interesting since *dilp6* is an IGF-like peptide that is produced in the nutrient sensing fat body [15,38], whereas the source of the insulin-like *dilp1* is the brain IPCs.

We showed earlier that young adult *dilp1* mutant flies display increased *dilp6* and vice versa [16], suggesting feedback between these two peptide hormones. This feedback appears less prominent in *dilp1* mutants during the pupal stage with no effects on *dilp2*, *dilp3* or *dilp6* levels. However, *dilp1* is slightly upregulated in *dilp6* mutant pupae. Furthermore, overexpression of *dilp1* in fat body or IPCs has no effect on pupal levels of *dilp2* and *dilp6*. Thus, at present we cannot postulate any compensatory changes in other DILPs in pupae with *dilp1* manipulations. However, under normal conditions *dilp6* levels are far higher than those of *dilp1* [38] (see also [modENCODE mRNA-Seq tissues](#) [61]), which could buffer the effects of changes in *dilp1* signaling.

Ectopic overexpression of *dilp1* in neuroendocrine cells or fat body not only increases growth of wings and thorax, but also increases the size of the brain and the cell bodies of several kinds of neuroendocrine cells in adult flies. However, there was no change in the size of neuronal cell bodies or CNS during larval development after overexpression of *dilp1*. Thus, taken together, our findings suggest that *dilp1*/DILP1 promotes growth mainly during the non-feeding pupal stage. Interestingly, restricted protein diet during the later larval stage diminished the body weight of adult flies more in *dilp1* mutants than in controls, similar to findings for *dilp6* [15]. This suggests that *dilp1* function is accessory to *dilp6* in maintaining growth of adult tissues in situations where larvae obtain insufficient protein in their diet.

DILPs and IIS are involved in modulating responses to starvation, desiccation and oxidative stress in *Drosophila* [see [10,21,62]]. Flies with ablated IPCs or genetically diminished IIS display increased resistance to several forms of stress, including starvation [10,21]. Conversely, overexpression of *dilp2* increases mortality in *Drosophila* [24]. We found that *dilp1* mutant flies displayed diminished starvation resistance. Both in newborn

593 and 3 day old flies, mutation of *dilp1* decreased survival during starvation (but
594 not in 6-7 day old ones). Curiously, overexpression of *dilp1* in the fat body
595 also resulted in decreased survival during starvation in young and older flies.
596 The effects on adult physiology of *dilp1* manipulations may be a consequence
597 of the altered adult tissue growth during pupal development and associated
598 increase in utilization of nutrient stores. Action of *dilp1* in the adult fly is also
599 linked to reproductive diapause in females, where feeding is strongly reduced
600 [63], and both peptide and transcript are upregulated [16]. Related to this we
601 found here that *dilp1* mRNA is upregulated during starvation in 12 d old flies.
602 Furthermore, it was shown that expression of *dilp1* increases lifespan in *dilp1*-
603 *dilp2* double mutants, suggesting that loss of *dilp2* induces *dilp1* as a factor
604 that promotes longevity [34]. Thus, *dilp1* activity is beneficial also during adult
605 life, even though its expression under normal conditions is very low [15,16,38].
606 This pro-longevity effect of *dilp1* is in contrast to *dilp2*, 3 and 5 and the
607 mechanisms behind this effect are of great interest to unveil.

608 A previous study showed that in wild-type (Canton S) *Drosophila* DILP1
609 expression in young adults is sex-dimorphic with higher levels in females [16].
610 In line with this, we show here that increase in body weight the first week of
611 adult life occurs only in female *dilp1* mutant flies, and also that starvation
612 survival in one-week-old flies is diminished only in females. Finally, we found
613 that *dilp1* overexpression specifically decreased starvation resistance only in
614 female flies. Thus, taken together, we found earlier that *dilp1* displays a sex-
615 specific expression [16] and here we show sex-specific function in young adult
616 *Drosophila*, and the *dilp1* mutation affects body weight of newly eclosed flies
617 mainly in females. It is tempting to speculate that the more prominent role of
618 *dilp1* in female flies is linked to metabolism associated with reproductive
619 physiology and early ovary maturation, which is also reflected in the *dilp1*
620 upregulation during reproductive diapause [16]. In fact, we show here that
621 egg-laying increased after *dilp1* overexpression, and an earlier study
622 demonstrated a decreased egg laying in *dilp1* mutant flies [16].

623 This study demonstrates that *dilp1* promotes growth of adult tissues
624 during the pupal stage, and in females it influences starvation resistance
625 during the young adult stage, and affects fecundity. Like *dilp6*, perhaps *dilp1*
626 acts as a signal promoted by nutrient shortage during the late larval stage to

627 ensure growth of adult tissues by recruiting nutrient stores from larval fat body.
 628 This in turn results in depleted pupal-derived nutrient stores in young adults.
 629 Thus, IPC-derived *dilp1* displays several similarities to the fat body-produced
 630 *dilp6*, including temporal expression pattern, growth promotion, effects on
 631 adult stress resistance and lifespan. Additionally *dilp1* may play a role in
 632 regulation of nutrient utilization/metabolism during the first few days of adult
 633 life, especially in females. At this time larval fat body is still present and
 634 utilized as energy fuel/nutrient store [54] and also contribute to egg
 635 development [64]. Curiously, there is a change in the action of DILP1 between
 636 the pupal and adult stages from being a stimulator of growth (agonist of dInR)
 637 in pupae, to acting opposite to DILP2 and other DILPs in adults in regulation
 638 of lifespan and stress responses. It is not known what mechanism is behind
 639 this switch in function of DILP1 signaling, but one possibility is that DILP1 acts
 640 via different signaling pathways downstream the dInR in pupae and adults.
 641 One obvious difference between these two stages is the presence of larval fat
 642 body in the pupa and first few days of adults and its replacement by functional
 643 adult fat body in later stages [37,54]. Also there seems to be a difference in
 644 the interactions with AKH signaling. During pupal development we did not see
 645 any effect of *dilp1* on transcripts of *Akh* or *tobi*, whereas in adult flies *Akh*
 646 expression is induced by *dilp1* [34]. This is in agreement with earlier work,
 647 which showed that AKH plays no role in development or lipid and
 648 carbohydrate metabolism in the pupa [51]. In the future it would be interesting
 649 to investigate whether DILP1 acts differently on larval/pupal and adult fat body,
 650 or act on different downstream signaling in the two stages of the life cycle,
 651 and whether *dilp1* and *dilp6* interact to regulate growth and metabolism in
 652 *Drosophila*.

653

654 Experimental procedures

655

Fly lines and husbandry

656 Parental flies were reared and maintained at 18°C with 12:12 Light:Dark cycle
 657 on food based on a recipe from Bloomington *Drosophila* Stock Center (BDSC)
 658 (http://fly-stocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm).

659 The experimental flies were reared and maintained at 25°C, with 12:12
660 Light:Dark cycle on an agar-based diet with 10% sugar and 5% dry yeast.

661 The following Gal4 lines were used in this study: *dilp2*-Gal4 [[18] from
662 E. Rulifson, Stanford, CA], *Pdf*-Gal4 (obtained from BDSC, Bloomington, IN),
663 *ppl*-Gal4 [[65] from M.J. Pankratz, Bonn, Germany], *To*-Gal4 [[66] from B.
664 Dauwalder, Houston, TX], *c929*-Gal4 [[46] from Paul H. Taghert], *yw*; UAS-
665 *dilp6*, and *yw*; UAS-*dilp2*;+ [[23] from H. Stocker, Zürich, Switzerland]. Several
666 UAS-*dilp1* lines were produced for a previous study [34] and two of them,
667 UAS-*dilp1* (II) and UAS-*dilp1* (III), were used here. UAS-*dilp1*-RNAi flies were
668 from Vienna *Drosophila* Resource Center (VDRC), Vienna, Austria. As
669 controls we used *w*¹¹¹⁸ or *yw* obtained from BDSC, crossed to Gal4 and UAS
670 lines. All flies (except *yw*; UAS-*dilp6*, and *yw*; UAS-*dilp2*;+) were backcrossed
671 to *w*¹¹¹⁸ for at least 6 generations.

672 We used a double null mutation of *dilp1/dilp2* that was previously
673 generated by homologous recombination and verified as described by Post et
674 al. [34]. Also single *dilp1* and *dilp2* null mutants were employed. We refer to
675 these three null mutants as *dilp1*, *dilp2* and *dilp1/dilp2* mutants for simplicity.
676 As described earlier [34], these were obtained from BDSC and a residual *w*+
677 marker was Cre excised followed by chromosomal exchange to remove *yw*
678 markers on chromosomes 2 and X.

679 To generate a recombinant *dilp6*;;*dilp1* double mutant, the *dilp1* and
680 *dilp6*⁶⁸ mutants [10] were used for crossing with a double balancer fly,
681 4E10D/FM7,dfd;Vno/TM3,dfd, obtained from Dr. Vasilios Tsarouhas
682 (Stockholm University). The efficiency of the *dilp6*;;*dilp1* double mutant was
683 validated by qPCR.

684

685 **Antisera and immunocytochemistry**

686 For immunolabeling, tissues from larvae or female adults were dissected in
687 chilled 0.1 M phosphate buffered saline (PBS). They were then fixed for 4
688 hours in ice-cold 4% paraformaldehyde (PFA) in PBS, and subsequently
689 rinsed in PBS three times for 1 h. Incubation with primary antiserum was
690 performed for 48 h at 4°C with gentle agitation. After rinse in PBS with 0.25%
691 Triton-X 100 (PBS-Tx) four times, the tissues were incubated with secondary

antibody for 48 h at 4°C. After a thorough wash in PBS-Tx, tissues were mounted in 80% glycerol with 0.1 M PBS.

The following primary antisera were used: Rabbit or guinea pig antiserum to part of the C-peptide of DILP1 diluted 1:10000 [16]. Rabbit antisera to A-chains of DILP2 and DILP3 [67] and part of the C-peptide of DILP5 [68] all at a dilution of 1:2000, rabbit anti-AKH (1:1000) from M.R. Brown, Athens, GA, rabbit anti-pigment-dispersing hormone (1:3000) from H. Dirksen, Stockholm, Sweden [69], rabbit antiserum to cockroach leucokinin I (LK I) at 1:2000 [70], mouse anti-green fluorescent protein (GFP) at 1:000 (RRID: AB_221568, Invitrogen, Carlsbad, CA).

The following secondary antisera were used: goat anti-rabbit Alexa 546, goat anti-rabbit Alexa 488, and goat anti-mouse Alexa 488 (all from Invitrogen). Cy3-tagged goat anti-guinea pig antiserum (Jackson ImmunoResearch, West Grove, PA). All were used at a dilution of 1:1000.

706

707 **Image analysis**

Images were captured with a Zeiss LSM 780 confocal microscope (Jena, Germany) using 10x, 20x and 40x oil immersion objectives. The projections of z-stacks were processed using Fiji (<https://imagej.nih.gov/ij/>). The cell body outlines were extracted manually and the size and staining intensity were determined using ImageJ (<https://imagej.nih.gov/ij/>). The background intensity for all samples was recorded by randomly selecting three small regions near the cell body of interest. The final intensity value of the cell bodies was determined by subtracting the background intensity.

Images of pupae, adult flies and fly wings were captured with a Leica EZ4HD light microscope (Wetzlar, Germany). The size of the adult fly body and wings were determined using Fiji. The pupal volume (v) was calculated using the equation $v = 4/3 \pi (L/2) \times (l/2)^2$, in which L = length and l = width [71]. Thorax length was measured from the posterior tip of the scutellum to the base of the most anterior point of the humeral bristle.

722

723 **Pupariation time, egg to pupae viability and adult body weight**

To determine time to pupariation, 6-7 day old adult females were crossed in the evening. The following morning, adult flies were transferred to vials with

725

726 fresh food on which they were allowed to lay eggs for four hours. Two hours
727 after the initiation of egg laying was considered time “0”, and thereafter the
728 number of pupae was monitored at 6 or 12-hour intervals. To investigate the
729 viability of egg to pupae formation, one pair of 6-7 day old adult flies was
730 allowed to lay eggs for 24 hours after which the total number of eggs was
731 counted. Subsequently, the total number of pupae was counted and the
732 viability of egg to pupae was determined as pupa number/egg number $\times$
733 100%. The body weight (wet weight) of single adult flies was determined
734 using a Mettler Toledo MT5 microbalance (Columbus, USA). The number of
735 eggs of stage 10-14 in ovaries was counted in 3-day-old flies.

736

737

Starvation survival assay

738 Newly hatched and mated 6-7 day old adults were used for starvation
739 resistance experiments. For newly hatched flies, we collected virgin flies every
740 4 hours, to be used for starvation experiments. The flies were kept in vials
741 containing 5 ml of 0.5% aqueous agarose (A2929, Sigma-Aldrich). The
742 number of dead flies was counted at least every 12 hours until all the flies
743 were dead. At least 110 flies from 3 replicates were used for the analysis.

744

745

Capillary feeding (CAFE) assay

746 Food intake was measured using a slightly modified capillary feeding (CAFE)
747 assay following Ja et al. [72]. In brief, female flies were placed into 1.5-ml
748 Eppendorf micro centrifuge tubes with an inserted capillary tube (5 μ l, Sigma)
749 containing 5% sucrose, 2% yeast extract and 0.1% propionic acid. To
750 estimate evaporation, three food-filled capillaries were inserted in identical
751 tubes without flies. The final food intake was determined by calculating the
752 decrease in food level minus the average decrease in the three control
753 capillaries. Food consumption was measured daily and calculated
754 cumulatively over four consecutive days. For this assay we used 8-10 flies in
755 each of three biological replicates.

756

757

Quantitative real-time PCR (qPCR)

758 Total RNA was extracted from whole bodies of middle or late pupal stages of
759 *Drosophila* by using Trizol-chloroform (Sigma-Aldrich). Quality and

concentration of the RNA were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific). The concentration of the RNA was adjusted to 400 ng/μl. Totally 2ug RNA was used for cDNA synthesis. The cDNA syntheses were performed by using random hexamer primer (Thermo Scientific) and RevertAid reverse transcriptase (Thermo Scientific). The cDNA products were then diluted 10 times and applied for qPCR using a StepOnePlus™ instrument (Applied Biosystem, USA) and SensiFAST SYBR Hi-ROX Kit (Bioline) followed the protocol from the manufacturer. The mRNA abundance was normalized to ribosomal protein (rp49) levels in the same samples. Relative expression values were determined by the $2^{-\Delta\Delta CT}$ method [73]. The sequences of primers used for qPCR were those used previously [16,34,74]:

dilp1 F: CGGAAACCACAACTCTGCG
 dilp1 R :CCCAGCAAGCTTTACGTTT
 dilp2 F: AGCAAGCCTTTGTCCTTCATCTC
 dilp2 R: ACACCATACTCAGCACCTCGTTG;
 dilp6 F: CCCTTGGCGATGTATTTCCCAACA
 dilp6 R: CCGACTTGCAGCACAAATCGGTTA
 akh F: GCGAAGTCCTCATTGCAGCCGT
 akh R: CCAATCCGGCGAGAAGGTCAATTGA
 tobi F: CCACCAAGCGAGACATTTACC
 tobi R: GAGCGGCGTAGTCCATCAC
 bmm F: GGT CCC TTC AGT CCC TCC TT
 bmm R: GCT TGT GAG CAT CGT CTG GT
 rp49 F: ATCGGTTACGGATCGAACAA
 rp49 R: GACAATCTCCTTGCGCTTCT

786

787 **Metabolite quantification**

Glycogen and triacyl glyceride (TAG) levels were assayed as previously described [34,75,76]. For glycogen assays, 5-6 adult female flies per sample were homogenized in PBS and quantified using the Infinity Glucose Hexokinase reagent by spectrophotometry. For TAG assays, 5-6 adult female flies per sample were homogenized in PBS + 0.05% TBS-T and quantified using the Infinity Triglycerides reagent by spectrophotometry. The fly lysate

793

794 protein levels were determined by BCA assay (Thermo Fisher) and metabolite
795 levels were normalized to protein level.

796 To measure the amount of TAG during late pupal stages, 6 replicates
797 with 4 pupae in each were collected and then homogenized in PBS + 0.05%
798 Triton-X 100 with a tissuelyser II from Qiagen. The TAG levels was
799 determined with a Liquick Cor-TG diagnostic kit (Cormay, Poland) using a
800 linear regression coefficient from a standard curve made with 2.2 µg/µl TAG
801 standard (Cormay, Poland). Absorbance of samples was measured at 550 nm
802 with a micro-plate reader (Thermo scientific). Data are expressed as
803 micrograms of TAG related to protein levels. Protein levels were determined
804 using a Bradford assay according to Diop et al. [77].

805

806 **Dynamic injection respirometry**

807 Carbon dioxide (CO₂) production and oxygen (O₂) consumption of individual
808 pupae of both sexes were measured during pupal development at 25°C to
809 assess metabolic rate (MR) as described previously [49]. Pupae were placed
810 in 1 ml syringes (i.e. respirometry chambers) that were filled with air scrubbed
811 of CO₂ with ascarite (Acros Organics, USA) that then passed through filtered
812 acidified water (pH < 4.5, checked weekly), closed with three-way luer valves,
813 and kept for roughly 24 hours at 25°C with 12:12 Light:Dark cycle. An empty
814 syringe served as control. CO₂ production was measured using a Sable
815 Systems (Las Vegas, NV, USA) differential respirometry setup. Two
816 independent lines of outdoor air scrubbed of H₂O and CO₂, using drierite (WA
817 Hammond Drierite, USA) and ascarite scrubbers respectively, were pushed at
818 a steady rate of 150 ml min⁻¹ using a SS-4 pump (Sable Systems) and two
819 separate mass flow controllers (840 Series; Sierra Instruments Inc, California,
820 USA). The syringes containing pupae were placed after the mass valve
821 controllers in the first line (sample) and 0.45 ml pushed into the airflow. The
822 push rate was recorded through a second flowmeter downstream of the
823 syringe and approximated a flow rate of 162 ml min⁻¹ downstream of the
824 syringe. The line was then scrubbed of H₂O with drierite and entered the
825 sample line of a Li-7000 CO₂ analyser (LiCor, Lincoln, NE, USA). The second
826 line (reference) proceeded the same way, mimicking the exact length of the
827 sample line (including an empty measurement chamber), entering the

reference line of the CO₂ analyser. The lines then proceeded through a second set of ascarite CO₂ scrubbers and entered an Oxzilla FC-2 O₂ analyser (Sable Systems) after which air was ejected. Preliminary measurements were performed to ensure stability of flow rate through either channel by measuring the flow rate of air ejected from the O₂ analyser. After the measurement pupae were weighed using a Mettler Toledo MT5 microbalance (Columbus, USA) and left at 25°C with 12:12 Light:Dark cycle until adult eclosion, at which point they were sexed.

Differential CO₂ and O₂ were calculated by subtracting the output of the reference line from the output of the sample line. For all measurements sampling rate was 1 Hz. In the program Expedata (version 1.9.10) the raw output was baseline corrected against the reference line value, fractioned and multiplied with flow rate to yield CO₂ and O₂ in ml min⁻¹ [78]. The values were then corrected by subtracting the readings from the empty control syringe from the sample values. MR was calculated by first integrating the fractioned CO₂ and O₂ (ml min⁻¹) values against time to yield CO₂ and O₂ in ml produced while pupae were in the syringes. Next VCO₂ and VO₂ were corrected by accounting for the fraction of air that was still left in the syringe and the time spent in the syringe using the formula (only calculation for VCO₂ is shown) $VCO_2 = (CO_2 \times (0.6 / 0.45)) / \text{hours in syringe}$ (Lighton, 2008). Then the respiratory quotient (RQ) was calculated as $RQ = VCO_2 / VO_2$. RQ values provide an estimate on what energy source is being catabolized to fuel metabolism [48]. MR (in Watts = Joules s⁻¹) was converted from VO₂ using the formula $MR = (VO_2 \times (16 + (5.164 \times RQ))) / (60 \times 60)$ (Lighton 2008) and finally divided by body weight in mg to yield MR mg⁻¹.

In the present study we monitored single identified individuals throughout pupal development, and sexed them after eclosion. For the vast majority eclosion was successful and therefore we could use the true weight of the individual for the calculation above. However, for individuals that failed to eclose properly we instead used the average weight for that sex and treatment to calculate MR.

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860
861

862 Statistical analysis

863 All results are presented as means $\pm$ SEM. We first investigated normality of
864 data using Shapiro-Wilk's normality test, then used one-way analysis of
865 variance (ANOVA) or Student's t-test, followed by Tukey's multiple
866 comparisons test. Lifespan data were subjected to survival analysis (Log rank
867 tests with Mantel-Cox post-test) and presented as survival curves.

868 For the respirometry data we used the natural logarithm of MR mg⁻¹
869 due to deviations from normality. A factorial two-way ANOVA was used with
870 MR mg⁻¹ or RQ as dependent variable, and sex and treatment as factorial
871 explanatory variables. Non-significant interactions and main effects were
872 removed from final models [79]. The respirometry data were analyzed with the
873 IBM SPSS statistics 23.0 (IBM SPSS Inc., Chicago, IL, USA) statistical
874 software package. Prism GraphPad version 6.00 (La Jolla, CA, USA) was
875 used for generating all the graphs.

876

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1092

1093 **Figure legends**

1094 **Fig. 1.** *dilp1* mutant flies display reduced body weight, but are not smaller. **A.**
1095 Expression profile of *dilp1*/DILP1 in *Drosophila*. Note that expression of
1096 transcript and peptide coincides with the non-feeding pupal stage and the first
1097 days of adult life when food intake is reduced (especially day one). It also
1098 times with the onset of the second and third ecdysone (*Ecd*) surges in the
1099 early pupa (earlier ecdysone peaks are not shown). E, embryo. **B.** Body
1100 weight of female flies 1 day and 6-7 days after adult eclosion. *dilp1* mutant
1101 flies display reduced body weight when 1 d old, but gain substantially the first
1102 week. Also *dilp2* mutants weigh less, but do not gain much weight first week.
1103 The double mutants are not significantly affected compared to controls at 1 d,
1104 but after 6-7 d both *dilp2* and double mutants weigh less than controls and
1105 *dilp1* mutants. Data are presented as medians $\pm$ range, n = 25–30 flies for
1106 each genotype from three independent replicates (*p < 0.05, **p < 0.01, ***p <
1107 0.001, two-way ANOVA followed by Tukey's test). **C.** In male flies the three
1108 mutants display weights similar to controls and controls lose weight the first

1109 week. Data are presented as medians $\pm$ range, $n = 18-30$ flies for each
1110 genotype from three independent replicates (** $p < 0.01$, two-way ANOVA
1111 followed with Tukey's test). **D.** Wing area was used as a proxy for organismal
1112 growth. The three mutants did not display altered wing size. Data are
1113 presented as medians $\pm$ range, $n = 16-23$ flies for each genotype from three
1114 independent replicates (One-way ANOVA followed with Tukey's test). **E.** Food
1115 intake was monitored over four days in a CAFE assay. The first day the *dilp1*
1116 mutant flies feed less than the other genotypes, whereas during the following
1117 days there is no difference between genotypes. Data are presented as means
1118 $\pm$ S.E.M, $n = 20-30$ flies for each genotype from three independent replicates
1119 (** $p < 0.001$, two-way ANOVA followed with Tukey's test). **F** and **G.** Body
1120 weight of 7 d old flies that had been exposed to normal diet (N) or low protein
1121 diet (L) during late larval stage. The female *dilp1* mutant flies displayed lower
1122 body weight than controls after low protein. Data are presented as medians $\pm$
1123 range, $n = 17-29$ flies for each genotype from three replicates (* $p < 0.05$, *** p
1124 < 0.001 , one-way ANOVA followed by Tukey's test).

1125 **Fig. 2.** Overexpression of *dilp1* affects growth during pupal stage. **A.**
1126 Expression of *dilp1* in insulin-producing cells (IPCs) with *dilp2*-Gal4 driver
1127 increases body weight of 6-7 d adult flies. Data are presented as means $\pm$
1128 S.E.M, $n = 14-23$ flies for each genotype from three independent replicates
1129 (* $p < 0.05$, one-way ANOVA followed by Tukey's test). **B.** Overexpression of
1130 *dilp1* in fat body (*ppl*-Gal4) or neuroendocrine cells (*c929*-Gal4) does not
1131 affect time to pupariation (larval development). Data are presented as means
1132 $\pm$ S.E.M, $n = 138-147$ flies for each genotype from three independent
1133 replicates (* $p < 0.05$, as assessed by Log-rank (Mantel-Cox) test). **C.**
1134 Overexpression of *dilp1* using *ppl*-Gal4 or *c929*-Gal4 does not affect pupal
1135 volume (proxy for larval growth). Also *dilp6* overexpression has no effect,
1136 whereas *dilp2* expression triggers a significant increase in pupal volume. Data
1137 are presented as means $\pm$ S.E.M, $n = 15-32$ flies for each genotype from
1138 three independent replicates. (** $p < 0.001$, one-way ANOVA followed with
1139 Tukey's test). **D** and **E.** Overexpression of *dilp1*, *dilp2* and *dilp6* in fat body all
1140 lead to adult flies (one week old) with increased body weight both in males
1141 and females. Data are presented as means $\pm$ S.E.M, $n = 24-30$ flies for each

1143 genotype from three independent replicates. Except for *ppl>dilp2*, 13 flies
1144 were used (**p* < 0.05, one-way ANOVA followed with Tukey's test). **F.** Also
1145 one-day-old female flies weigh more than controls after *ppl>dilp1*, but not after
1146 *dilp2>dilp1*. Knockdown of *dilp1* by *dilp2>dilp1*-RNAi lead to decreased body
1147 weight. Data are presented as means $\pm$ S.E.M, *n* = 20-27 flies for each
1148 genotype from three independent replicates (***p* < 0.01, ****p* < 0.001,
1149 unpaired Students' *t*-test). **G.** Images of flies overexpressing *dilp1* in the fat
1150 body and controls. **H** and **I.** Overexpression of *dilp1* in fat body results in flies
1151 with increased wing area (**H**), and length of thorax (**I**) as proxies for
1152 organismal growth. Data are presented as means $\pm$ S.E.M, (****p* < 0.001, one-
1153 way ANOVA followed with Tukey's test); in **H** *n* = 17-24 flies and in **I** *n* = 9-17
1154 flies from three independent replicates. **J.** Food intake (CAFE assay) is
1155 increased over four days (cumulative data shown) in flies overexpressing
1156 *dilp1* in fat body, but not in neuroendocrine cells (*c929 Gal4*). Data are
1157 presented as means $\pm$ S.E.M, *n* = 15-30 flies for each genotype from three
1158 independent replicates (**p* < 0.05, two-way ANOVA followed with Tukey's
1159 test). **K.** Body weight of 6-7 d female flies is increased for all genotypes
1160 compared to 1 d flies. The *ppl>dilp1* flies weigh more than controls at both
1161 time points. Data are presented as medians $\pm$ range, *n* = 23-27 flies for each
1162 genotype from three independent replicates (**p* < 0.05, ****p* < 0.001, two-way
1163 ANOVA followed with Tukey's test).

1164

1165 **Fig. 3.** Metabolic rate trajectories and respiratory quotients (RQ) during pupal
1166 development respond to *dilp1* and *dilp6* overexpression in the fat body. **A.**
1167 Metabolic rate in *w¹¹¹⁸* flies increased exponentially as a function of time. For
1168 the ensuing overexpression analysis we studied the period 96-120 hours after
1169 pupation. Data are presented as means $\pm$ S.E.M, *n* = 10-15 flies from three
1170 independent replicates. **B.** Metabolic rate was significantly elevated during
1171 this period in *dilp1* and *dilp6* overexpression flies (*ppl-Gal4*) when compared
1172 to *w¹¹¹⁸* flies. Data are presented as means $\pm$ S.E.M, *n* = 10-15 flies for each
1173 genotype from three independent replicates (****p* < 0.001, compared to *w¹¹¹⁸*
1174 flies, as assessed by two-way ANOVA followed with Tukey's test). Data are
1175 from both males and females as no difference was found in the ANOVA for
1176 sex. **C.** RQ, reflecting catabolic energy substrate, was significantly lower in

the overexpression flies when compared to the control flies and indicates a shift from mixed fuel catabolism (RQ = 0.7-0.8) to predominantly lipid catabolism (RQ < 0.7). Data are presented as means $\pm$ S.E.M, n = 10–15 flies for each genotype from three independent replicates (**p < 0.001, compared to *w¹¹¹⁸* flies, as assessed by one-way ANOVA followed with Tukey's test). Data are from both males and females as no difference was found in the ANOVA for sex. **D.** Four day old pupae (mixed male and female) were weighed (wet weight) before extraction and TAG determination. Overexpression of *dilp1* and *dilp6* both resulted in increased pupal weight. **E.** Levels of TAG were measured in the pupae used for weighing in D. Overexpression of each *dilp* resulted in decreased TAG levels. **F.** Glycogen levels in 4 d old pupae (no significant changes). In **D-E** 12 replicates per genotype with 4 pupae in each replicate (each data point represents 4 pupae), in F 6 replicates per genotype with 4 pupae in each replicate (*p < 0.05, **p < 0.01, one-way ANOVA followed by Tukey's test).

Fig. 4. Contents in female flies of TAG, glycogen and glucose in mutants and after ectopic *dilp1* expression. **A-C.** Contents of TAG and carbohydrates in newborn mutants and controls. Note that for *dilp1* mutants only glycogen was diminished, whereas for *dilp1-2* mutants all three compounds were decreased. 8 replicates per genotype with 5-6 flies in each replicate (*p < 0.05, ***p < 0.001, one-way ANOVA followed by Tukey's test). **D-F.** In 3 d old flies glycogen was also reduced in *dilp1* mutants and double mutants. 8 replicates per genotype with 5-6 flies in each replicate (*p < 0.05, ***p < 0.001, one-way ANOVA followed by Tukey's test). **G-I.** Overexpression of *dilp1* in fat body (*ppl-Gal4*) only affected glycogen levels in newly hatched flies. 6-8 replicates per genotype with 5-6 flies in each. Data are presented as means $\pm$ S.E.M, (*p < 0.05, ***p < 0.001, compared to *w¹¹¹⁸* flies, as assessed by unpaired Students' t-test).

Fig. 5. Effects of mutated *dilp* genes on adult responses to starvation and desiccation change in early adult life. **A.** In newly eclosed female flies *dilp1* mutant flies display reduced survival during starvation (p<0.001) compared to the other mutants and control. The double mutant is significantly more

1211 resistant ($p < 0.001$). $n = 109-147$ flies for each genotype from three
 1212 independent replicates. **B.** In three-day-old virgin female flies *dilp1-dilp2*
 1213 double mutants are the least starvation resistant ($p < 0.001$) followed by the
 1214 *dilp1* mutants; $n = 129-148$ flies for each genotype from three independent
 1215 replicates. **C.** Comparison between newly eclosed and 3 d flies exposed to
 1216 starvation. Both mutants and controls survive longer as newborn flies and
 1217 mutants perform worse than controls at each time point ($p < 0.001$). $n = 114-$
 1218 144 flies from three independent replicates. **D.** When exposed to desiccation
 1219 6-7 d old female double mutants are less resistant than the other genotypes
 1220 ($p < 0.001$), $n = 132-135$ flies from three independent replicates. Data are
 1221 presented in survival curves and the error bars show S.E.M, as assessed by
 1222 log-rank (Mantel–Cox) test].

1223

1224 **Fig. 6.** Over expression of *dilp1* in the fat body affects starvation resistance in
 1225 adult flies. **A** and **B.** In recently eclosed (A) and 6-7 d old (B) female flies
 1226 overexpression of *dilp1* (with *ppl-Gal4*) leads to a decrease in survival during
 1227 starvation $n = 147-201$ flies per genotype from three independent replicates.
 1228 [*** $p < 0.001$, as assessed by log-rank (Mantel–Cox) test]. **C** and **D.**
 1229 Expressing *dilp1* in IPCs with a *dilp2-Gal4* driver also diminishes starvation
 1230 survival in newborn flies $n = 92-148$ flies from three independent replicates.
 1231 [*** $p < 0.001$, as assessed by log-rank (Mantel–Cox) test], but not in 6-7 d
 1232 flies ($n = 122-132$ flies from three independent replicates). **E** and **F.** Using
 1233 *c929* to drive *dilp1* in newborn and 6-7 d adult flies altered starvation
 1234 resistance only in the newborn ones [*** $p < 0.001$ as assessed by log-rank
 1235 (Mantel–Cox) test, $n = 132-135$ flies per genotype from three independent
 1236 replicates.

1237

1238 **Fig. 7.** *Dilp1* overexpression affects food intake and fecundity. **A.** In CAFE
 1239 assay the *dilp1* overexpressing flies (6-7 d old females) display increased
 1240 food intake over 4 days (cumulative data shown), Data are presented as
 1241 means $\pm$ S.E.M, $n = 23-24$ flies from three independent replicates (* $p < 0.05$,
 1242 two-way ANOVA followed by Tukey's test). **B.** Number of eggs laid in 24
 1243 hours by 6-7 day old flies. We analyzed 19-29 pairs of flies from 3 replicates,
 1244 (** $p < 0.01$, one-way ANOVA followed by Tukey's test). **C.** The egg to pupal

1245 viability is diminished in flies with *dilp1* expressed in fat body (*pp1-Gal4*) and
1246 neuroendocrine cells [*c929-Gal4*, using two different UAS-*dilp1* (2 and 3)].
1247 Data are presented as means $\pm$ S.E.M, more than 276 eggs from 6 replicates
1248 were monitored (* $p < 0.05$, unpaired Students' t-test). **D.** Number of eggs in
1249 ovaries of 3 days old flies is not affected in *dilp1* mutants. 25-33 flies from 3
1250 replicates were analyzed. **E.** *dilp1* mRNA is upregulated during starvation for
1251 40 h in 10 d old adult *w¹¹¹⁸* flies, compared to 12 d old flies fed normal food,
1252 as monitored by qPCR. No effect was seen on *dilp2* and *dilp6* levels. Data are
1253 presented as means $\pm$ S.E.M, 3 replicates with 10 flies in each replicates were
1254 monitored (* $p < 0.05$, unpaired Students' t-test). **F.** The level of adipokinetic
1255 hormone (AKH) immunolabeling in 4-5 d old female flies increased after
1256 overexpression of *dilp1* by *c929>dilp1*. 7-10 flies from 3 replicates, (* $p < 0.05$,
1257 unpaired Students' t-test).

1258

1259 Supplemental material figures

1260

1261 **S1 Fig.** Evaluation of mutant efficiency. **A.** qPCR reveals that in stage P8-9
1262 pupae the *dilp1* and *dilp1/dilp2* mutants display *dilp1* levels that are close to
1263 zero, whereas in the *dilp6* mutant *dilp1* is upregulated and in *dilp2* mutant
1264 slightly reduced. **B.** In the *dilp2* and *dilp1/dilp2* mutants *dilp2* levels are not
1265 detectable. **C.** The *dilp6* levels are only affected in the *dilp6* mutants. Data are
1266 presented as means $\pm$ S.E.M, $n = 6$ replicates for each genotype with 6 pupae
1267 in each replicate. (* $p < 0.05$, compared with *w¹¹¹⁸* flies, unpaired Students' t-
1268 test). **D.** Using immunocytochemistry with antisera to DILP1-3 it can be shown
1269 that labeling of IPCs in 1-week-old flies is not detectable for anti-DILP1 in
1270 *dilp1* and double mutants and for DILP2 in *dilp2* and double mutants. DILP3 is
1271 upregulated in *dilp2* mutants. **E-G.** Quantification of immunofluorescence
1272 shows that DILP1 labeling is not affected in *dilp2* mutants (E), DILP2 is
1273 increased in *dilp1* mutants (F) and DILP3 strongly increased only in *dilp2*
1274 mutants (G). Data are presented as means $\pm$ S.E.M, $n = 9-12$ flies from 3
1275 replicates. (** $p < 0.01$, *** $p < 0.001$, compared with *w¹¹¹⁸* flies, unpaired
1276 Students' t-test).

1277

S2 Fig. Recombinant *dilp1/dilp6* mutant flies display reduced body mass. **A.** Transcripts of *dilp1* and *dilp6* in one-day-old *dilp1/dilp6* mutant flies. Data are presented as means $\pm$ S.E.M, $n = 3$ replicates for each genotype with 6 pupae in each replicate. (** $p < 0.001$, *** $p < 0.0001$, compared with w^{1118} flies, unpaired Students' t-test). **B.** Body weights are significantly reduced in single mutants and recombinant double mutants, but no additive effect of the double mutation was detected. ($n = 11-16$ flies per genotype from three replicates, One-way ANOVA followed by Tukey's test).

S3 Fig. Verification of ectopic *dilp1* expression by DILP1 immunolabeling. **A.** After *dilp2*-Gal4-driven *dilp1* expression strong DILP1 immunolabeling can be detected in IPCs of 3rd instar larvae as well as 1 and 3 week old adults, but not in controls (*dilp2*> w^{1118}). **B.** Quantification of DILP1 immunofluorescence in IPCs of one-week-old adults, using two different UAS-*dilp1* (2 and 3). Data are presented as means $\pm$ S.E.M, $n = 5-7$ flies from 3 replicates. (*** $p < 0.001$, compared with control flies, unpaired Students' t-test). **C.** Using the c929 driver DILP1 immunolabeling can be detected in numerous neuroendocrine cells in the CNS of larvae and brain of adults, but not in controls (c929> w^{1118}). **D.** Using two different fat body Gal4 drivers (*ppl* and *to*) DILP1 immunolabeling can be detected in adipocytes.

S4 Fig. Verification of ectopic *dilp1* expression by qPCR in stage P8-9 pupae. **A.** Using the fat body Gal4 drivers *ppl* and *to* a drastic increase of *dilp1* transcript was seen. **B.** The *dilp2* level was diminished after *ppl*-driven *dilp1*. **C.** No significant effect was seen on *dilp6* levels after *dilp1* expression. **D-F.** Driving *dilp1* in IPCs with *dilp2*-Gal4 drastically increases *dilp1*, but has no effect on *dilp2* or *dilp6*. Data are presented as means $\pm$ S.E.M, $n = 5-6$ replicates per genotype with 10 pupae in each replicate. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with w^{1118} flies, unpaired Students' t-test).

S5 Fig. Effects of ectopic *dilp1* expression on peptide levels of DILPs in one-week-old adults. **A.** Expressing *dilp1* in IPCs (*dilp2*>*dilp1*) increases DILP2 immunolabeling and decreases DILP3. **B** and **C.** Quantification of immunolabeling. Data are presented as means $\pm$ S.E.M, $n = 7-10$ per

1312 genotype from 3 replicates. (**p < 0.01, compared with w¹¹¹⁸ flies, unpaired
1313 Students' t-test). **D.** Using the broader *c929-Gal4* to drive *dilp1* the DILP5
1314 immunolabeling of IPCs increase. **E.** Quantification of DILP5 immunolabeling.
1315 Data are presented as means ± S.E.M, n = 9-12 from 3 replicates. (**p < 0.01,
1316 compared with w¹¹¹⁸ flies, unpaired Students' t-test).

1317

1318 **S6 Fig.** Effects of ectopic *dilp1* expression on body weight and organismal
1319 size. **A.** Expressing *dilp1* in the fat body (*ppl-Gal4*) of male flies leads to
1320 increased weight compared to controls in both young and older flies.
1321 However, in contrast to female flies, shown in Fig. 2K, there is no gain in
1322 weight over the first 5-6 days as adults, rather a decrease. Data are
1323 presented as medians ± range, n = 14–25 flies from three independent
1324 replicates (*p < 0.05, **p < 0.01, ***p < 0.001, two-way ANOVA followed with
1325 Tukey's test). **B.** Driving *dilp1* in IPCs with *dilp2-Gal4* in females increases the
1326 weight compared to controls in older flies. Data are presented as medians ±
1327 range, n = 14–23 flies from three independent replicates (*p < 0.05, **p <
1328 0.01, two-way ANOVA followed with Tukey's test). **C.** Driving *dilp1* in IPCs
1329 increases the weight of one-day-old and 6-7 day old male flies, compared to
1330 both controls. Furthermore the younger flies weigh more than the older ones
1331 for all genotypes. Data are presented as medians ± range, n = 14–24 flies per
1332 genotype from three independent replicates (**p < 0.01, ***p < 0.001, two-way
1333 ANOVA followed with Tukey's test). **D and E.** Using *to-Gal4* the body masses
1334 show the same patterns as with *ppl-Gal4* (Fig. 2K and **S5A Fig**), where body
1335 masses increase after *dilp1* over expression, and in females there is an
1336 additional weight gain over the first 5-6 days. The following days (13-14 d) no
1337 additional increase is seen. Data are presented as medians ± range, n = 9–27
1338 flies per genotype from three independent replicates (*p < 0.05, **p < 0.01,
1339 ***p < 0.001, two-way ANOVA followed with Tukey's test). **F-H.** The *dilp1*
1340 expression obtained with the *to-Gal4* does not result in a significant increase
1341 in wing area, (n = 16–22 flies per genotype from three replicates, One-way
1342 ANOVA followed with Tukey's test), whereas thorax length increased slightly
1343 (n = 19-32 flies per genotype (*p < 0.05 unpaired Students' t-test), but no
1344 effect on pupal volume (n = 29 flies per genotype from three replicates, One-
1345 way ANOVA followed with Tukey's test).

1346

1347 **S7 Fig.** Effects of *dilp1* expression on weight, wing area and food intake. **A**

1348 The body weight increased in male flies after ectopic *dilp1* expression with

1349 *c929-Gal4*, *** $p < 0.001$, data are presented as means $\pm$ S.E.M, $n = 16-29$

1350 flies per genotype from three independent replicates (One-way ANOVA

1351 followed with Tukey's test). **B.** The wing area is not affected by *c929-driven*

1352 *dilp1* expression. Data are presented as means $\pm$ S.E.M, $n = 15$ flies from

1353 three independent replicates (One-way ANOVA followed with Tukey's test). **C.**

1354 Driving *dilp1* with *dilp2-* and *c929-Gal4* does not affect food intake. Data are

1355 presented as means $\pm$ S.E.M, $n = 24$ flies from three independent replicates

1356 (two-way ANOVA followed with Tukey's test). dent replicates, as assessed by

1357 log-rank (Mantel–Cox) test.

1358

1359 **S8 Fig.** The brain and neuronal cell bodies grow after *dilp1* overexpression in

1360 neuroendocrine cells. **A-C.** Using the *c929-Gal4* line to drive *dilp1* in

1361 neuroendocrine cells leads to increased size of the cell bodies of DILP2

1362 immunolabeled insulin producing cells (A1-A3), PDF labeled I-LNV clock

1363 neurons (B1-B3) and abdominal leucokinin (LK) immunoreactive neurons,

1364 ABLK (C1-C3). **D.** The entire brain also increases in size in *c929>dilp1* flies.

1365 **E.** Expression of *dilp1* in IPCs with the *dilp2-Gal4* line is not sufficient to obtain

1366 an increase in size of IPCs. **F1-F3.** Expression of *dilp1* in the fat body (*pp1-*

1367 *Gal4*) increases the size of the I-LNV clock neurons and the entire brain (**G**).

1368 Data are presented as means $\pm$ S.E.M, $n = 8-10$ samples for each genotype

1369 from three independent replicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as

1370 assessed by unpaired Students' t-test).

1371

1372 **S9 Fig.** Ectopic expression of *dilp1* in clock neurons or larval neuroendocrine

1373 cells does not affect cell size. **A-D.** Expression of *dilp1* with the clock neuron

1374 driver *pdf-Gal4* does not affect the size of the PDF-immunolabeled large LNVs

1375 quantified in B. The brain size is also not affected (C). However the PDF

1376 immunolabeling is strongly increased (D). Data are presented as means $\pm$

1377 S.E.M, $n = 8$ for each genotype from 3 replicates. (** $p < 0.01$, compared with

1378 w^{1118} flies, unpaired Students' t-test). **E.** Ectopic expression of *dilp1* with the

1379 *c929-Gal4* line does not affect the size of leucokinin (LK)-immunolabeled

1380 neuronal cell bodies in the third instar larvae (quantified in F) or the size of the
1381 larval CNS (G). Data are presented as means $\pm$ S.E.M, n = 6-9 for each
1382 genotype from 3 replicates.

1383

1384 **S10 Fig.** Ectopic expression of *dilp1* in glial cells with *repo*-Gal4 does not
1385 affect growth of neuronal cell bodies. **A.** DILP1 immunolabeling appears in
1386 cells after *Repo>dilp1*, but has no effect on the size of I-LNv clock neurons
1387 labeled with anti-PDF (quantified in **B**). Data are presented as means $\pm$
1388 S.E.M, n = 9 for each genotype from 3 replicates.

1389

1390 **S11 Fig.** Overexpression and mutation of *dilps* have little effect on AKH
1391 signaling as determined by qPCR. **A - J.** Transcripts of AKH (*Akh*), brummer
1392 lipase (*bmm*) and the glucosidase target of brain insulin (*tobi*) were measured
1393 by qPCR in different genotypes at two stages of pupal development: two day
1394 old and 4 day old. For overexpression in fat body we used *ppl*- and *to*-Gal4
1395 drivers. We analyzed 3 replicates with 6 pupae in each replicates (*p < 0.05,
1396 one-way ANOVA followed by Tukey's test). **K.** Glucose levels were
1397 determined in 4 d pupae; 6 replicates per genotype with 4 pupae in each
1398 replicate (No significant differences; analysis by one-way ANOVA followed by
1399 Tukey's test). This panel is associated with Fig. 3D – F.

1400

1401 **S12 Fig.** Effect on starvation and desiccation in male *dilp* mutant flies. **A.** In 6-
1402 7 days old male flies *dilp1-dilp2* mutants are least resistant to starvation
1403 (p<0.001), followed by *dilp2* mutants (p<0.001), whereas *dilp1* mutants
1404 perform as controls; n = 125-141 flies from three independent replicates.
1405 However 6-7 d female flies perform as 3 d virgin females (see [34] and Table
1406 1). **B.** In males double mutants are less (p<0.001), and the other two mutants
1407 more resistant (p<0.001) to desiccation than controls, n = 134-135 flies from
1408 three independent replicates. Data are presented in survival curves and the
1409 error bars means S.E.M, as assessed by log-rank (Mantel–Cox) test].

1410

1411 **S13 Fig.** Targeted *dilp1*-RNAi in IPCs reduces survival in flies exposed to
1412 starvation. **A.** The efficiency of *dilp2>dilp1*-RNAi on *dilp1* levels was monitored
1413 by qPCR. A strong reduction in *dilp1* was noted, but no effect was seen on

1414 levels of *dilp2* or *dilp6*. Data are presented as means $\pm$ S.E.M, n = 3 replicates
 1415 per genotype with 10 pupae in each replicate. (*p < 0.05, compared with
 1416 control flies, unpaired Students' t-test). **B.** In newly eclosed female flies
 1417 *dilp2>dilp1*-RNAi resulted in reduced survival during starvation. n = 148-170
 1418 flies from three independent replicates. Data are presented in survival curves
 1419 and the error bars means S.E.M [***p < 0.001, as assessed by log-rank
 1420 (Mantel–Cox) test]. **C.** In 6-7 d old males *dilp1* overexpression in fat body (*ppl*-
 1421 Gal4) has no effect on starvation response. n = 117-128 flies from three
 1422 independent replicates. **E.** c929-driven *dilp1* does not affect the response to
 1423 starvation, n = 132-135 flies per genotype from three independent replicates.

A

6-7 d female flies

B

Eggs laid in 24 hours

C

Egg to pupal viability

D

Egg number

E

Relative expression

F

AKH levels