Drosophila insulin-like peptide 1 (DILP1) promotes organismal growth during non-feeding stages

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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 not fully understood. Previous work showed that *dilp1*/DILP1 is transiently expressed 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. Overexpression of *dilp1* additionally increases body size of flies, but reduces stores of larval-derived energy, leading to increased feeding the first days after eclosion. No effects of *dilp1* manipulations were detected during larval development. An earlier study demonstrated interactions between *dilp1* and *dilp2* in regulation of adult lifespan. Here we monitored the effects of *dilp1, dilp2* and *dilp1/dilp2* mutations on growth and found that only the single mutants displayed lower body mass. In recently eclosed flies, survival during starvation is strongly diminished in *dilp1* mutants, but not in *dilp2* and double mutants, whereas in older flies double mutants display reduced starvation resistance. Egg to pupal viability is decreased both after overexpression of *dilp1*, and in the double mutants. In conclusion, *dilp1* promotes growth of adult tissues during the non-feeding pupal stage, likely due to reallocation of stored energy. This results in larger newly-eclosed flies with reduced stores of larval/pupal energy 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 [10,4,11]. Eight insulin-like peptides (DILP1-8), each encoded on a separate gene, have been identified in *Drosophila.* Based on sequence similarities DILP1-5 are considered related to *bona fide* insulins, DILP6 is IGF-like, whereas DILP7 and DILP8 are relaxin-like [12,10,13,14]. The genes encoding these DILPs display differential temporal and tissue-specific expression profiles, suggesting that they have different functions [12,14-17]. Specifically, DILP1, 2, 3 and 5 are mainly expressed in median neurosecretory cells located in the dorsal midline of the brain, designated insulin-producing cells (IPCs) [12,18,16,19,20]. From the IPCs the DILPs can be released into the open circulation from axon terminations in the corpora cardiaca, the anterior aorta and crop. Genetic ablation of the IPCs alters growth and metabolism, leads to increased resistance to several forms of stress and prolongs lifespan [21,18]. The functions of the individual DILPs produced by the IPCs may vary depending on the stage of the *Drosophila* life cycle. Already the temporal expression patterns hint that DILP1-3 and 5 play different roles during development; whereas DILP2 and 5 are relatively highly expressed during larval and adult stages, DILP1 and 6 are almost exclusively expressed during pupal stages under normal conditions [22,15].

DILP1 is unique among the IPC-produced peptides since it can be detected primarily during the non-feeding pupal stage and the first few days of adult life when residual larval/pupal fat body is present [15,16]. Furthermore, in female flies kept in adult reproductive diapause, where feeding is strongly reduced, *dilp1*/DILP1 expression is also high [16]. Its temporal expression profile resembles that of DILP6 although this peptide is primarily produced by the fat body, not IPCs [22,15]. Since DILP6 was shown to regulate growth of

adult tissues during pupal development [22,15], we asked whether also DILP1 plays a role in growth control. It is known that overexpression of several of the DILPs is sufficient to increase body growth through an increase in cell size and cell number, and especially DILP2 produces a substantial increase in body mass [12,23,24]. In contrast, not all single *dilp* mutants display a decreased body mass. The *dilp1*, *dilp2* and *dilp6* single mutants display decreased body weight [10,22,15], whereas the *dilp3*, *dilp4*, *dilp5* and *dilp7* single mutants retain normal body weight, indicating that some of the individual DILPs have redundant functions [10]. However, a triple mutation of *dilp2, 3, and 5* causes a drastically reduced body weight, and a *dilp1–4,5* mutation results in even smaller flies [10,25].

There is a distinction between how DILPs act in growth regulation. DILPs other than DILP1 and 6 promote growth primarily during the feeding larval stages when their expression is high [12,23]. This nutrient dependent growth is relatively well understood and is critical for production of the steroid hormone ecdysone and thereby developmental timing and induction of developmental transitions such as larval molts and pupariation [26-30]. The growth during non-feeding stages, which affects imaginal discs and therefore adult tissues, is far less studied. In this study, we investigate the role of DILP1 in growth regulation in *Drosophila*. We found that mutation of *dilp1* diminishes body mass and ectopic *dilp1* expression promotes organismal growth during the non-feeding pupal stage, similar to DILP6.

We also investigated the role of *dilp1* mutation and overexpression on early adult physiology, and found that *dilp1* manipulation affects starvation resistance in newly eclosed flies, but less so with increasing age. Testing flies that are three days or a week old shows that *dilp1/dilp2* double mutants are more sensitive to starvation than the other mutants and controls. The diminished starvation resistance in newly hatched flies after *dilp1* overexpression is probably a consequence of nutrient reallocation during the increased growth of adult tissues leading to newborn flies with low energy stores. Conditional *dilp1* overexpression in young adults results in a slightly decreased survival during starvation suggesting a need for intact DILP signaling also in adult homeostasis. Interestingly, the newly eclosed *dilp1* mutant flies are less resistant to starvation than controls and *dilp2* mutants;

the common notion is that reduced IIS increases survival during starvation [21].

Taken together, our study suggests that DILP1 promotes growth of adult tissues during the non-feeding pupal stage, and this carries over to affect the metabolism in the young adult fly. Most of the phenotypes from *dilp1* knockout and overexpression are stronger in female flies. We suggest that *dilp1*, similar to *dilp6* [15], may be a hormonal factor that ensures that a larva exposed to poor nutritional conditions will as a pupa utilize stored nutrients for growth of adult tissues, rather than keeping these stores for the first days of adult life. Our findings indicate a separate functional role of *dilp1* in the adult fly in metabolism, and lifespan.

Experimental procedures

Fly lines and husbandry

Parental flies were reared and maintained at 18°C with 12:12 Light: Dark cycle on a food recipe from Bloomington *Drosophila* Stock Center (BDSC) (http://fly-stocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm).
The experimental flies were reared and maintained at 25°C, with 12:12 Light:Dark cycle on an agar-based diet with 10% sugar and 5% dry yeast.

The following Gal4 lines were used in this study: *dilp2*-Gal4 [[18] from E. Rulifson, Stanford, CA], *Pdf*-Gal4 (obtained from BDSC, Bloomington, IN), *Ppl*-Gal4 [[31] from M.J. Pankratz, Bonn, Germany], *To*-Gal4 [[32] from B. Dauwalder, Houston, TX], Da^{GS}-Gal4 [*Daughterless gene switch*; [33] obtained from V. Monnier , Paris, France], *c929*-Gal4 [[34] from Paul H. Taghert], yw; UAS-*dilp6*, yw; UAS-*dilp2;*+ [[23] from H. Stocker, Zürich, Switzerland]. Several UAS-*dilp1* lines were produced is a previous study [35] and two of them, UAS-*dilp1* (II) and UAS-*dilp1* (III), were used here. As controls we used w^{1118} or *yw* obtained from BDSC, crossed to Gal4 and UAS lines. All flies (except yw; UAS-*dilp6*, and yw; UAS-*dilp2;*+) were backcrossed to w^{1118} for at least 6 generations.

We used a double null mutation of *dilp1/dilp2* that was previously generated by homologous recombination and verified as described by Post et al. [35]. Also single *dilp1* and *dilp2* null mutants were employed. As described

earlier [35]; these were obtained from BDSC and a residual w+ marker was Cre excised followed by chromosomal exchange to remove *yw* markers on chromosomes 2 and X.

To activate the GeneSwitch-Gal4 driven *dilp1* expression in the adult stage, *Da*^{GS}-Gal4>UAS-*dilp1* flies were raised on normal food until two days of age to allow them to mate. Thereafter the flies were transferred either to food that contained RU486 (mifepristone; Sigma, St. Louis, MO, USA) at a final concentration of 20 µM dissolved in EtOH, or food containing the same concentration of solvent. The flies were kept on this food for five days after which the experiments were performed.

Antisera and immunocytochemistry

For immunolabeling, tissues from larvae or female adults were dissected in chilled 0.1 M phosphate buffered saline (PBS). They were then fixed for 4 hours in ice-cold 4% paraformaldehyde (PFA) in PBS, and subsequently rinsed in PBS three times for 1 h. Incubation with primary antiserum was performed for 48 h at 4°C with gentle agitation. After rinse in PBS with 0.25% 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 [36] and part of the C-peptide of DILP5 [37] 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. Dircksen, Stockholm, Sweden [38], rabbit antiserum to cockroach leucokinin I (LK I) at 1:2000 [39], 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 antiserum, goat anti-rabbit Alexa 488 antiserum, and goat anti-mouse Alexa 488 antiserum (all from Invitrogen). Cy3-tagged goat anti-guinea pig antiserum (Jackson ImmunoResearch, West Grove, PA). All were used at a dilution of 1:1000.

Image analysis

Images were captured with a Zeiss LSM 780 confocal microscope (Jena, Germany) using 10×, 20× and 40× oil immersion objectives. The projection of z-stacks was 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 [40]. Thorax length was measured from the posterior tip of the scutellum to the base of the most anterior point of the humeral bristle.

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 fresh food on which they were allowed to lay eggs for four hours. Two hours after the initiation of egg laying was considered time “0”, and thereafter the number of pupae was monitored at 6 or 12-hour intervals. To investigate the viability of egg to pupae formation, one pair of 6-7 day old adult flies was allowed to lay eggs for 24 hours after which the total number of eggs was counted. Subsequently, the total number of pupae was counted and the viability of egg to pupae was determined as pupa number/egg number × 100%. The body weight (wet weight) of single adult flies was determined using a Mettler Toledo MT5 microbalance (Columbus, USA).

Starvation survival assay

Newly hatched and mated 6-7 day old adults were used for starvation resistance experiments. For newly hatched flies, we collected virgin flies every 4 hours, to be used for starvation experiments. The flies were kept in vials containing 5 ml of 0.5% aqueous agarose (A2929, Sigma-Aldrich). The

number of dead flies was counted at least every 12 hours until all the flies were died. At least 110 flies from 3 replicates were used for the analysis.

Capillary feeding (CAFE) assay

Food intake was measured using a slightly modified capillary feeding (CAFE) assay following Ja et al. [41]. In brief, female flies were placed into 1.5-ml Eppendorf micro centrifuge tubes with an inserted capillary tube (5 µl, Sigma) containing 5% sucrose, 2% yeast extract and 0.1% propionic acid. To estimate evaporation, three food-filled capillaries were inserted in identical tubes without flies. The final food intake was determined by calculating the decrease in food level minus the average decrease in the three control capillaries. Food consumption was measured daily and calculated cumulatively over four consecutive days. For this assay we used 8-10 flies in each of three biological replicates.

Metabolite quantification

Glycogen and triacyl glyceride (TAG) levels were assayed as previously described [42,43,35]. 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 protein levels were determined by BCA assay (Thermo Fisher) and metabolite levels were normalized to protein level.

Statistical analysis

All results are presented as means ± SEM. We first investigated normality of data using Shapiro-Wilk's normality test, then used one-way analysis of variance (ANOVA) or Student's t-test, followed by Tukey's multiple comparisons test. Lifespan data were subjected to survival analysis (Log rank tests with Mantel-Cox post-test) and presented as survival curves. Prism GraphPad version 6.00 (La Jolla, CA, USA) was used for generating the graphs.

Results

Mutation of *dilp1* decreases body mass

Growth in *Drosophila* is in part regulated by several of the DILPs through activation of the canonical IIS/TOR (target of rapamycin) pathway [12,11,28]. It was previously reported that decreased *dilp1* activity reduces adult body mass 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 [35]. The efficacy of these mutants was confirmed by qPCR in stage 8-9 pupae and immunolabeling in one-week-old mated female flies (Suppl Fig. 1). 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 (Suppl Fig. 1A-C). At the protein level DILP2 but not DILP3 immunofluorescence increased in *dilp1* mutants (Suppl Fig. 1D-G). These findings suggest only minor compensatory changes in other dilps/DILPs in *dilp1* mutants during the pupal stage.

We monitored the body mass (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 mass 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 mass (Fig. 1C). To determine whether decreased organismal growth was responsible for the lower body mass we

measured wing size in the female mutant flies and found no significant difference to controls (Fig. 1D). Thus, the decreased mass of the flies does not seem to reflect a decrease in organismal size.

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

In a study of *dilp6* it was shown that if third instar larvae (after reaching critical size) were put on a low protein diet they emerged as smaller adults and that this was accentuated in *dilp6* mutants, suggesting that *dilp6* is important for assuring growth of adult tissues under low protein conditions [15]. We, thus, performed a similar experiment with *dilp1* mutant larvae kept on normal food or low protein diet. Flies emerging from larvae on restricted protein indeed displayed significantly lower body mass and female *dilp1* mutants weighed less than controls under protein starvation (Fig. 1F). It was shown that *dilp6* transcript increased in larvae exposed to protein starvation [15]. Third instar larvae exposed to similar starvation did not display any change in DILP1 immunolevels in IPCs (not shown).

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

Having shown effects of the *dilp1* null mutation on adult fly mass we next explored the outcome of over-expressing *dilp1*, either in IPCs, or more broadly. For this we generated several UAS-*dilp1* lines [see [35]]. These UAS-*dilp1* lines were verified by DILP1 immunolabeling after expression with several Gal4 drivers (Suppl Fig. 2A-D) and by qPCR in stage 8-9 pupae (Suppl Fig. 3A-F). Overexpression of *dilp1* in fat body (*ppl*-Gal4 and *to*-Gal4)

and IPCs (*dilp2*-Gal4) results in a drastic upregulation of *dilp1* RNA (Suppl Fig. 3A, D), but has no effect on *dilp2* and *dilp6* expression (Suppl Fig. 3B, C, E. F), except a minor decrease in *dilp2* for *ppl*-Gal4 (Suppl Fig. 3B). 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 (Suppl Fig. 4A-E). 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 mass of female flies (Fig. 2A). Next we expressed *dilp1* in the fat body, the insect functional analog of the liver and white adipocytes in mammals [44,45]. The fat body displays nutrient sensing capacity, and is an important tissue for regulation of growth and metabolism in *Drosophila* [46,15,47-49]. It is also the tissue where DILP6 is produced and released [46,15]. 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 (Suppl. Fig. 2D). In *ppl>dilp1* flies we also found DILP1 labeling in the nephrocytes, which are highly endocytotic cells located close to the heart [50] (not shown). Possibly the immunoreactive DILP1 has accumulated from the circulation after release from the fat body.

The effect of *dilp1* overexpression in the fat body was monitored both on adult body mass and organismal size. We also measured the time to pupariation and size of pupae to determine whether *dilp1* overexpression affected larval development and growth. Using the *ppl*-Gal4 driver we did not observe any effect on the time from egg to pupa compared to controls (Fig. 2B). Pupal volume, as a measurement of larval growth, was not altered by *ppl*-Gal4>*dilp1* (Fig. 2C). As expected [15,46], over-expression of *dilp6* also had no effect on pupal size (Fig. 2C). However, as shown earlier for ubiquitously expressed *dilp2* [23], *dilp2* expression in the fat body generated a strong increase in pupal volume, suggesting growth during the larval feeding stage (Fig. 2C). Driving *dilp1* with the *c929* Gal4 line, that directs expression to several hundred *dimm*-expressing peptidergic neurons including IPCs [51], we did not observe any effect on time to pupariation or pupal volume (Fig. 2B,

C). Taken together our data suggest the ectopic *dilp1* does not affect larval growth or developmental time.

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

It was suggested that *dilp6* promotes growth of adult tissues during pupal development by utilizing nutrients stored in the larval fat body, which is carried into the pupa [15]. This may be the case also for *dilp1*, and if so, newly hatched *dilp1* overexpressing flies should have less energy stores in the form of residual larval fat body. To test this idea we monitored feeding in recently hatched *dilp1* mutant flies and controls. Indeed, flies overexpressing *dilp1* displayed increased food ingestion over the first four days after adult emergence compared to controls (Fig. 2J). Next we compared the weights of one day old and 6-7 day old flies after dilp1 overexpression with *ppl*-Gal4 and found that at both ages the female *ppl>dilp1* flies weighed more and that the older flies were heavier than the younger ones (Fig. 2K). In male flies *ppl>dilp1* also increased the body mass, but there was a loss of weight for all genotypes over the first 6-7 days of adult life (Suppl. Fig. 5A). As a comparison *dilp2>dilp1* had only minor effects on body mass of female flies, only in 6-7 d old flies there was an increase (Suppl. Fig. 5B), whereas in males a significant decrease was noted at both ages (Suppl. Fig. 5C).

Using the *to*-Gal4 fat body driver to express *dilp1* we also noted an increase in weight of recently emerged female and male flies (Suppl. Fig. 5D, E), but no change in body size except a minor increase in thorax length in females (Suppl. Fig. 5F, G). The female *to>dilp1* flies increased further in weight the first 6-7 days of adult life, but not later (Suppl. Fig. 5D), whereas

the males did not (Suppl. Fig. 5E). Furthermore, with the *to*-Gal4 driver there was no increase in pupal volume, indicating that *dilp1* does not affect larval growth (Suppl. Fig. 5H).

Ectopic expression of *dilp1* in neuroendocrine cells by means of the *c929*-Gal4 did increase body weight (Suppl. Fig. 6A), but had no effect on wing size in males or females or food intake in young flies (Suppl. Fig. 6B, C), suggesting that *dilp1* expression (or release) was not strong enough to yield major effects. Also *dilp2>dilp1* flies were tested in food intake and no effect was seen (Suppl. Fig. 6C).

Effects of *dilp1* manipulations on metabolism in newborn and older flies

To investigate whether energy is reallocated during pupal development we monitored the levels of triacylglycerids (TAG), glycogen and glucose in recently emerged and three day old *dilp* mutant and *dilp1*-overexpression female flies (Fig. 3). In newborn *dilp1* mutant flies glycogen was significantly lowered, whereas glucose and glycogen was diminished in *dilp2* mutants while in the *dilp1/dilp2* double mutants all three compounds were decreased (Fig. 3A-C). In the three-day-old flies *dilp1* and double mutants displayed reduced glycogen, whereas in *dilp1/dilp2* double mutants TAG was increased (Fig. 3D-F). Using *ppl*-Ga4 to express *dilp1* we found that the only effect was a reduction of glycogen in newborn flies; at 3 or 7 days of age no effect was noted (Fig. 3G-I). Thus, it appears that intact *dilp1* signaling is required for mobilization of glycogen stores in young flies.

dilp1 overexpression increases the size of the adult brain and neuroendocrine cells

It was previously shown that signaling through the *Drosophila* insulin receptor (dInR) can regulate growth of cell bodies of neuroendocrine cells in a cell autonomous manner, and that *dilp6* in glial cells is a candidate ligand to mediate this dInR dependent growth [52,53]. Since *dilp1* has an expression profile similar to *dilp6* and promotes growth of adult tissues in the pupal stage we asked whether *dilp1* also affects size of neuroendocrine cells that differentiate in the pupa. Thus, we overexpressed *dilp1* with the broad driver *c929*-Gal4, and monitored the cell body size of several groups of

neuroendocrine cells in the adult with specific peptide antisera. We found that the cell body size of IPCs increased in *c929>dilp1* adult flies, as shown by anti-DILP2 staining (Fig. 4A1-A3). Furthermore, the cell bodies of the adult-specific pigment dispersion factor (PDF) expressing clock neurons (l-LN$_v$s), as shown here by anti-PDF staining, were enlarged in *c929>dilp1* flies compared to the controls (Fig. 4B1-B3). Finally, 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 (Fig. 4C1-C3). However, the observed increase in cell body size may 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 (Figure 4D). The c929-Gal4 is expressed in IPCs and several other groups of neurosecretory cells that could underlie systemic release of DILP1, which affects growth. In contrast, we found that expressing *dilp1* in interneurons, such as PDF-expressing clock neurons does not induce growth of brain neurons (Suppl. Fig. 7A,B) or size of the brain (Suppl. Fig. 7C), but affected the intensity of PDF immunolabeling (Suppl. Fig. 7D). 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 (Fig. 4F), suggesting that *dilp1* overexpression has no effect on neuron growth during the larval stage. Using the *ppl*-Gal4 to drive *dilp1* in the fat body we also found an increase in the size of the PDF expressing clock neurons (Fig. 4G1-G3) and the brain (Fig. 4H) supporting the proposal that systemic DILP1 is required to promote this growth. Finally, since overexpression of *dilp6* in glial cells by *Repo*-Gal4 promotes neuronal cell body growth [53], we tested overexpression of *dilp1* in these cells, but found no significant effect on the cell-body size of PDF neurons (Suppl. Fig. 8), again indicating that to affect growth DILP1 must act systemically rather than in a paracrine fashion. Finally, there was no effect on body mass after expressing *dilp1* with the *Repo*-Gal4 (Suppl. Fig. 8C, D).

Effects of *dilp1* on adult physiology

Genetic ablation of the IPCs, which produce DILP1, 2, 3 and 5, results in enhanced starvation resistance in adult flies [21]. Thus, we asked whether the alterations of *dilp1* expression during development have effects on adult physiology such as survival during starvation or desiccation (as a proxy for effects on metabolism). We investigated the starvation resistance in newborn, three days old and one-week-old female *dilp1, dilp2* and double mutant flies. The newly eclosed *dilp1* mutant flies display strongly reduced survival during starvation and double mutants increased survival compared to control flies, whereas the stress resistance of *dilp2* mutants is similar to the controls (Fig. 5A, Table 1). In three days old virgin flies the *dilp1* and *dilp1/dilp2* mutants display reduced survival during starvation, whereas the *dilp2* mutants perform similar to the controls (Fig 4B, Table 1). In a separate study [35] it was shown that 6-7 day old female flies display a similar response to starvation: the *dilp1/dipl2* mutants exhibit the strongest reduction in survival, followed by *dilp1* mutants that also are much less stress tolerant, whereas *dilp2* mutants and control flies perform very similar (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 (Fig. 4C).

Table 1. Median lifespans of female flies exposed to starvation.

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

* Data from Post et al. [35]

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 hatched and three day old virgin flies. As seen in Fig. 5D (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 stress 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 shift in *dilp* function 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 (Suppl. Fig. 9A) where a strong decrease in *dilp1*, but not *dilp2* or *dilp6* was seen. The *dilp1*-RNAi resulted in newly eclosed flies that displayed reduced survival during starvation (Suppl. Fig. 9B), similar to *dilp1* mutant flies.

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

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

When overexpressing *dilp1* with the fat body driver *ppl*-Gal4 newly eclosed and 6-7 d old female flies become less resistant to starvation compared to parental controls (Fig. 6A, B). However, in 6-7-day-old male flies there is no difference between controls and flies with ectopic *dilp1* (Fig. 6C). Thus, in females it appears as if both knockout and over expression of *dilp1* reduces starvation resistance, maybe due to offsetting a narrow window of homeostasis. It was shown earlier that conditional knockdown of *dilp6* by RNAi during the pupal stage resulted in newborn flies with *increased* survival during starvation [15], suggesting that the effect the *dilp1* null mutation is different. After ectopic expression of *dilp1* in the fat body there was an increase in food intake (cumulative data) in one-week-old flies over four days (Fig. 6D), suggesting that metabolism is still altered in older flies.

We furthermore investigated starvation resistance in flies overexpressing dilp1 in IPCs (*dilp2>dilp1*) and found that in newborn flies overexpression reduced survival (Fig. 6E), whereas in a week old flies all genotypes displayed the same survival (Fig. 6F).

Since the effect of *dilp1* manipulations seems stronger in female flies we asked whether fecundity is affected by overexpression of *dilp1*. An earlier study showed that *dilp1* mutant flies are not deficient in number of eggs laid, or the viability of offspring (egg to pupal viability), although the *dilp1/dilp2* double mutants displayed a reduction in viability of these eggs [35]. Here, we expressed *dilp1* in fat body (*ppl*-Gal4) and neuroendocrine cells (*c929*-Gal4) and both lines resulted in flies that laid eggs that exhibited decreased viability as monitored by numbers of eggs that developed into pupae (Fig. 6G).

We next asked whether there is any physiological trigger of increased *dilp1* expression, except for diapause [16] and experimental ones such as ectopic expression of sNPF or knockdown of *dilp6, dilp2* and *dilp2,3,5* [57,16,35]. Although diminished protein diet in larvae had no effect on *dilp1* expression measured by qPCR (not shown), we found that 40 h starvation of 10 d old flies (w^{1118}) leads to a significant increase in *dilp1*, but not *dilp2* or *6* (Fig. 6H). Thus, at a time (12 d) when *dilp1* is very low under normal conditions, it is upregulated four times during starvation, further suggesting that the peptide indeed plays a role also in older adult flies.

The functional homolog of glucagon in flies, adipokinetic hormone (AKH), plays important roles both in metabolism and regulation of lifespan [58-60]. A previous paper showed that in *dilp1* mutant flies levels of AKH were not affected [35]. Here we found that *dilp1* overexpression with the *c929*-Gal4 driver induced an increase in AKH immunolabeling in one-week-old flies (Fig. 6I). Thus there appears to be an interaction between *dilp1* and AKH that may underlie some of the effects of this insulin on metabolism and stress tolerance.

To further test whether *dilp1* has direct effects on metabolism/ starvation resistance in the adult fly we over-expressed *dilp1* with the drug inducible gene-switch (GS) system [61] in one-week-old adults. We used a *daughterless* (*Da*)-Gal4^{GS} driver to conditionally express *dilp1* and fed flies RU486 to activate the Gal4 in the adult stage. Starvation resistance was significantly reduced in flies fed RU486 compared to controls that were not exposed to the drug (Fig. 7A). The *Da*-Gal4^{GS} driven expression of DILP1 was monitored by immunolabeling, and found to include median neurosecretory cells and several other neuron types in the brain, including mushroom bodies and the central body (Fig. 7B,C).

Discussion

Our study indicates a role for *dilp1* in regulation of adult tissue growth during the pupal stage, as well as roles in adult physiology, especially during the first days of adult life. The experiments herein suggest that the developmental role of *dilp1* may be to ensure nutrient reallocation in the pupa toward growth of adult tissues if the larva was exposed to restricted food sources. In the adult *dilp1* is upregulated during starvation and genetic gain and loss of function of *dilp1* signaling alters the flies' survival under starvation conditions. These novel findings combined with previous data showing high levels of *dilp1* during adult reproductive diapause [16] and its role as a pro-longevity factor during aging [35] demonstrate a wide-ranging importance of this signaling system. Not only does *dilp1* expression correlate with stages of non-feeding (or reduced feeding), these stages are also associated with lack of reproductive activity, and encompass the pupae, newly eclosed flies, and diapausing flies. Under normal conditions, diminished *dilp1*/DILP1 expression during the first

few days of the adult could relate to a metabolic transition and the onset of sexual maturation.

In *Drosophila*, the final body size is determined mainly during the larval feeding stage [23,12,11,29]. However, regulation of body size can also occur after the cessation of the feeding stage, and this process is mediated by *dilp6* acting on adult tissue growth in the pupa in an ecdysone-dependent manner [15,46]. This is likely a mechanism to ensure growth of the adult tissues if the larva is exposed to shortage of nutrition during its feeding stage. Our findings suggest that *dilp1* is another regulator of growth during the pupal stage. We show here that *dilp1* promotes organismal growth in the non-feeding pupa at the cost of stored nutrients derived from the larval stage. As a consequence large *dilp1*-overexpressing flies display increased food ingestion over the first four days as adults and an altered response to starvation. Conversely *dilp1* mutants hatched as flies with significantly smaller mass. Thus, both alterations in *dilp1* expression influence the metabolic balance in early adults as seen in reduced starvation resistance. Our study suggests that *dilp1* parallels *dilp6* [15,46] in balancing adult tissue growth and storage of resources during pupal development, and affecting adult physiology. This is interesting since *dilp6* is an IGF-like peptide that is produced in the nutrient sensing fat body [15,46], 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. As well, overexpression of *dilp1* in fat body of IPCs has no effects 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, normally *dilp6* levels are far higher than those of *dilp1* [15,46], which could balance the effects of changes in *dilp1* signaling. In adults, DILP6 is released from the fat body and is known to affect the brain IPCs to diminish DILP2 production/release and thereby extending lifespan [42]. It is not known whether DILP6 affects DILP1 release in the pupal stage.

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 is able to promote growth mainly during the non-feeding pupal stage. However, restricted protein diet during the later larval stage diminished the body mass 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 stress responses in *Drosophila* [see [10,21,62]]. Flies with ablated IPCs or genetically diminished IIS display increased resistance to several forms of stress, including starvation [21,10]. Conversely, overexpression of *dilp2* causes lethality in *Drosophila* [24]. We found that *dilp1* mutant flies displayed decreased starvation resistance. Both in newborn, 3 day and 6-7 day old flies, mutation of *dilp1* decreased survival during starvation. Curiously, overexpression of *dilp1* in the fat body also resulted in decreased survival during starvation in young and older flies. The effects on adult physiology of *dilp1* manipulations may be a consequence of the altered adult tissue growth during pupal development and associated reallocation of energy stores. However, we could show that conditional overexpression of *dilp1* in the one week old flies also reduces starvation resistance indicating action of the peptide also at this stage. Action of *dilp1* in the adult fly is also linked to reproductive diapause in females, where feeding is strongly reduced [63], and both peptide and transcript are upregulated [16]. Related to this we found here that *dilp1* mRNA is upregulated during starvation in 12 d old flies. Furthermore, it was shown that expression of *dilp1* increases lifespan in *dilp1-dilp2* double mutants, suggesting that loss of *dilp2* induces *dilp1* as a factor that promotes longevity [35]. Thus, *dilp1* activity is beneficial also during adult life, even though its expression under normal conditions is very low [16,46,15]. This pro-longevity effect of *dilp1* is in contrast to *dilp2, 3 and 5* and the mechanisms behind this effect are of great interest to unveil.

A previous study showed that in wild-type (Canton S) *Drosophila* DILP1

expression in young adults is sex-dimorphic with higher levels in females [16]. In line with this, we show here that increase in body weight the first week or adult life occurs only in female *dilp1* mutant flies, and also that starvation survival in one-week-old flies is diminished only in females. Finally, we found that *dilp1* overexpression specifically decreased starvation resistance only in female flies both in non-conditional and conditional experiments. Thus, taken together, we find that *dilp1* displays a sex-specific expression and function in young adult *Drosophila*, and the *dilp1* mutation affects body mass of newly eclosed flies mainly in females. It is tempting to speculate that the more prominent role of *dilp1* in female flies is linked to reproductive physiology and early ovary maturation, which is also reflected in the upregulation during reproductive diapause [16].

This study demonstrates that *dilp1* promotes growth during the pupal stage, and in females it regulates starvation resistance during the young adult stage, and affects fecundity. Like *dilp6*, perhaps *dilp1* acts as a signal promoted by nutrient shortage during the larval stage to ensure growth of adult tissues by reallocating nutrient stores from larval fat body. This in turn results in depleted pupal-derived nutrient stores in young adults. Thus, IPC-derived *dilp1* displays several similarities to the fat body-derived *dilp6*, including temporal expression, growth promotion, effects on adult stress resistance and lifespan. Additionally *dilp1* may play a role in regulation of nutrient allocation/metabolism during the first few days of adult life, especially in females. At this time larval fat body is still present and utilized as energy/nutrient store [54]. There is a change in the action of DILP1 between the pupal and adult stages from being a stimulator of growth (agonist of dInR) in pupae, to acting opposite to DILP2 and other DILPs in adults in regulation of lifespan and stress responses. It is not known what mechanism is behind this switch in function of DILP1 signaling, but one possibility is that DILP1 acts via different signal pathways in pupae and adults. One obvious difference between these two stages is the presence of larval fat body in the pupa and first few days of adults and its replacement by functional adult fat body in later stages. In the future it would be interesting to investigate if DILP1 act differently on larval and adult fat body and whether *dilp1* and *dilp6* interact to regulate growth and metabolism in *Drosophila*.

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Figure legends

Fig. 1. *dilp1* mutant flies display reduced body mass, but are not smaller. **A**. Expression profile of *dilp1*/DILP1 in *Drosophila*. Note that expression of transcript and peptide coincides with the non-feeding pupal stage and the first days of adult life when food intake is reduced (especially day one). It also times with the onset of the second and third ecdysone (*Ecd*) surges in the early pupa (earlier ecdysone peaks are not shown). E, embryo. **B.** Body weight of female flies 1 day and 6-7 days after adult eclosion. *dilp1* mutant flies display reduced body weight when 1 d old, but gain substantially the first week. Also dilp2 mutants weigh less, but do not gain much weight first week. The double mutants are not significantly affected compared to controls at 1 d, but after 6-7 d both *dilp2* and double mutants weigh less that controls and *dilp1* mutants. Data are presented as medians ± range, n = 25–30 flies for each genotype from three independent replicates (*p < 0.05, **p < 0.01, ***p < 0.001, two-way ANOVA followed by Tukey's test). **C.** In male flies the three mutants display weights similar to controls and controls lose weight the first week. Data are presented as medians ± range, n = 18–30 flies for each genotype from three independent replicates (**p < 0.01, two-way ANOVA followed with Tukey's test). **D.** Wing area was used as a proxy for organismal growth. The three mutants did not display altered wing size. Data are presented as medians ± range, n = 16–23 flies for each genotype from three independent replicates (One-way ANOVA followed with Tukey's test). **E**. Food intake was monitored over four days in a CAFE assay. The first day the *dilp1* mutant flies feed less than the other genotypes, whereas during the following days there is no difference between genotypes. Data are presented as medians ± S.E.M, n = 20–30 flies for each genotype from three independent replicates (***p < 0.001, one-way ANOVA followed with Tukey's test). **F.** Body weight of 7 d old flies that had been exposed to normal diet (N) or low protein diet (L) during late larval stage. The *dilp1* mutant flies displayed lower body weight than controls after low protein. Data are presented as means ± range, n = 17-29 flies for each genotype from three crosses (*p < 0.05, ***p < 0.001, unpaired Students' t-test).

Fig. 2. Overexpression of *dilp1* affects growth during pupal stage. **A.** Expression of *dilp1* in insulin-producing cells (IPCs) with *dilp2*-Gal4 driver increases body weight of 6-7 d adult flies. Data are presented as medians ± S.E.M, n = 14–23 flies for each genotype from three independent replicates (*p < 0.05, one-way ANOVA followed with Dunnett's test). **B.** Overexpression of *dilp1* in fat body (*ppl*-Gal4) or neuroendocrine cells (*c929*-Gal4) does not affect time to pupariation (larval development). Data are presented as medians ± S.E.M, n = 138-147 flies for each genotype from three independent replicates (*p < 0.05, as assessed by Log-rank (Mantel-Cox) test). **C.** Overexpression of *dilp1* using *ppl*-Gal4 or *c929*-Gal4 does not affect pupal volume (proxy for larval growth). Also *dilp6* overexpression has no effect, whereas *dilp2* expression triggers a significant increase in pupal volume. Data are presented as medians ± S.E.M, n = 15–32 flies for each genotype from three independent replicates. (***p < 0.001, one-way ANOVA followed with Tukey's test). **D** and **E**. Overexpression of *dilp1, dilp2* and *dilp6* all lead to adult flies (one week old) with increased body mass both in males and females. Data are presented as medians ± S.E.M, n = 24-30 flies for each genotype from three independent replicates. Except for ppl>dilp2, 13 flies were used (*p < 0.05, one-way ANOVA followed with Dunnett's test). **F.** Also 1 d flies weigh more than controls after *ppl>dilp1* but not after *dilp2>dilp1*. Knockdown of *dilp1* by *dilp2>dilp1*-RNAi lead to decreased body weight. Data are presented as medians ± S.E.M, n = 20-27 flies for each genotype from three independent replicates (**p < 0.01, ***p < 0.001, unpaired Students' t-test). **G.** Images of flies overexpressing *dilp1* in the fat body and controls. **H** and **I**. Overexpression of *dilp1* in fat body results in flies with increased wing area (H), and length of thorax (I) as proxies for organismal growth. Data are presented as medians ± S.E.M, (***p < 0.001, one-way ANOVA followed with Tukey's test); in H n= 17-24 flies and in H n = 9–17 flies from three independent replicates. **J.** Food intake (CAFE assay) is increased over four days (cumulative data shown) in flies overexpressing *dilp1* in fat body, but not in neuroendocrine cells (c929 Gal4). Data are presented as medians ± S.E.M, n = 15–30 flies for each genotype from three independent replicates (*p < 0.05, two-way ANOVA followed with Tukey's test). **K.** Body weight of 6-7 d

flies is increased for all genotypes compared to 1 d flies. The ppl>dilp1 flies weigh more than controls at both time points. Data are presented as medians ± range, n = 23–27 flies for each genotype from three independent replicates (*p < 0.05, ***p < 0.001, two-way ANOVA followed with Tukey's test).

Fig. 3. Contents of TAG, glycogen and glucose in female 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 stores were decreased. **D-F.** In 3 d old flies glycogen was also reduced in *dilp1* mutants and double mutants. **G-I.** Overexpression of *dilp1* in fat body (*ppl-Gal4*) only affected glycogen levels in newly hatched flies. In the above experiment 6-8 replicates per genotype with 5-6 flies in each. Data are presented as means ± S.E.M, (*p < 0.05, ***p < 0.001, compared to w^{1118} flies, as assessed by unpaired Students' t-test).

Fig. 4. The brain and neuronal cell bodies grow after *dilp1* overexpression in neuroendocrine cells. **A-C.** Using the neuroendocrine cell *Gal4* line *c929* to drive *dilp1* leads to increased size of the cell bodies of DILP2 immunolabeled insulin producing cells (A1-A3), PDF labeled clock l-LNv neurons (B1-B3) and abdominal leucokinin (LK) immunoreactive neurons, ABLK (C1-C3). **D.** The entire brain also increases in size in *c929>dilp1* flies. **E.** Expression of *dilp1* in IPCs with the *dilp2*-Gal4 line is not sufficient to obtain an increase in size of IPCs. **F.** The cell bodies of larval ABLKs are not affected by *c929>dilp1*. **G1-G3.** Expression of *dilp1* in the fat body (*ppl*-Gal4) increases the size of the l-LNv clock neurons and the entire brain (**H**). Data are presented as means ± S.E.M, n = 8–10 samples for each genotype from three independent replicates (*p < 0.05, **p < 0.01, ***p < 0.001, as assessed by unpaired Students' t-test).

Fig. 5. Effects of mutated *dilp* genes on adult responses to starvation and desiccation. **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 resistant (p<0.001). n =

109-147 flies for each genotype from three independent replicates. **B**. In three-day-old virgin female flies *dilp1-dilp2* double mutants are the least starvation resistant ($p<0.001$) followed by the *dilp1* mutants; n = 129-148 flies for each genotype from three independent replicates. **C.** In 6-7 days old male flies *dilp1-dilp2* mutants are also least resistant ($p<0.001$), followed by *dilp2* mutants ($p<0.001$), and *dilp1* mutants perform as controls; n = 125-141 flies from three independent replicates. However 6-7 d female flies perform as 3 d virgin females (see [35] and Table 1). **D.** Comparison between newly eclosed and 3 d flies exposed to starvation. Both mutants and controls survive longer as newborn flies and mutants perform worse than controls at each time point ($p<0.001$). n = 114-144 flies from three independent replicates. **E.** When exposed to desiccation female double mutants are less resistant than the other genotypes ($p<0.001$), n = 132-135 flies from three independent replicates. **F.** In males double mutants are less ($p<0.001$), and the other two mutants more resistant ($p<0.001$) to desiccation than controls, n = 134-135 flies from three independent replicates. Data are presented in survival curves and the error bars means S.E.M [***$p < 0.001$, as assessed by log-rank (Mantel–Cox) test].

Fig. 6. Over expression of dilp1 in the fat body affects starvation resistance, food intake and fecundity in adult flies. **A** and **B.** In recently eclosed (A) and 6-7 d old (B) female flies overexpression of dilp1 (with ppl-Gal4) leads to a decrease in survival during starvation n = 147-201 flies per genotype from three independent replicates. [***$p < 0.001$, as assessed by log-rank (Mantel–Cox) test]. **C.** In 6-7 d old males *dilp1* overexpression has no effect on starvation response. n = 117-128 flies from three independent replicates. **D.** In CAFE assay the *dilp1* overexpressing flies (6-7 d old females) display increased food intake over 4 days (cumulative data shown), Data are presented as medians ± S.E.M, n = 23–24 flies from three independent replicates (*$p < 0.05$, two-way ANOVA followed by Tukey's test). **E-F.** Expressing *dilp1* in IPCs with a dilp2-Gal4 driver also diminishes starvation survival in newborn flies n = 92-148 flies from three independent replicates. [***$p < 0.001$, as assessed by log-rank (Mantel–Cox) test], but not in 6-7 d flies (n = 122-132 flies from three independent replicates). **G.** The egg to

pupal viability is diminished in flies with dilp1 expressed in fat body (*ppl*-Gal4) and neuroendocrine cells (c929, using two different UAS-*dilp1*). Data are presented as means ± S.E.M, more than 276 eggs from 6 replicates were monitored (*p < 0.05, unpaired Students' t-test). **H.** *dilp1* mRNA is upregulated during starvation for 40 h in 10 d old adult w^{1118} flies (green bars), compared to 12 d old flies fed normal food (grey bars), as monitored by qPCR. No effect was seen on *dilp2* and *dilp6* levels. Data are presented as means ± S.E.M, 30 flies from 3 replicates were monitored (*p < 0.05, unpaired Students' t-test). **I.** The level of adipokinetic hormone (AKH) transcript increased after overexpression of *dilp1* by c929>*dilp1* as monitored by qPCR.

Fig. 7. Conditional overexpression of *dilp1* in adult flies decreases survival during starvation**. A.** Flies that were fed RU486 to induce the gene-switch in *Da*-Gal4^{GS}>*dilp1* display decreased survival during starvation compared to controls. n = 92-148 flies from three independent replicates (**p < 0.01, as assessed by log-rank (Mantel–Cox) test). **B.** DILP1 immunolabeling reveals neurons in the brain that express the peptide after *Da*-Gal4^{GS}>*dilp1* and RU486 feeding: e.g. median neurosecretory cells (MNC) and neurons in mushroom bodies (MB) and central complex (CX).

Supplemental material figures

S Fig. 1. Evaluation of mutant efficiency. **A**. qPCR reveals that in stage P8-9 pupae the *dilp1* and *dilp1/dilp2* mutants display *dilp1* levels that are close to zero, whereas in the *dilp6* mutant *dilp1* is upregulated and in *dilp2* mutant slightly reduced. **B.** In the *dilp2* and *dilp1/dilp2* mutants *dilp2* levels are not detectable. **C.** The *dilp6* levels are only affected in the *dilp6* mutants. Data are presented as means ± S.E.M, n = 6 replicates for each genotype with 6 pupae in each replicate. (*p < 0.05, **p < 0.01, compared with w^{1118} flies, unpaired Students' t-test). **D.** Using immunocytochemistry with antisera to DILP1-3 it can be shown that labeling of IPCs in 1-week-old flies is not detectable for anti-DILP1 in *dilp1* and double mutants and for DILP2 in *dilp2* and double mutants. **E-G**. Quantification of immunofluorescence shows that DILP1 labeling is not affected in *dilp2* mutants (E), DILP2 is increased in *dilp1*

mutants (F) and DILP3 strongly increased only in *dilp2* mutants (G). Data are presented as means ± S.E.M, n = 9-12 flies from 3 replicates. (**p < 0.01, ***p < 0.001, compared with w^{1118} flies, unpaired Students' t-test).

S Fig. 2. 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 immunofluorecsence in IPCs of one-week-old adults, using two different UAS-*dilp1*. Data are presented as means ± 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.

S Fig. 3. 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 ± S.E.M, n = 5-6 replicates per genotype with 10 pupae in each replicate. (*p < 0.05, **p < 0.01, ***p < 0.01, compared with w^{1118} flies, unpaired Students' t-test).

S Fig. 4. 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 ± S.E.M, n = 7-10 per genotype from 3 replicates. (**p < 0.01, compared with w^{1118} flies, unpaired Students' t-test). **D.** Using the broader *c929*-Gal4 to drive *dilp1* the DILP5 immunolabeling of IPCs increase. **E.** Quantification of DILP5 immunolabeling. Data are presented as means ± S.E.M, n = 9-12 from 3 replicates. (**p < 0.01, compared with w^{1118} flies, unpaired Students' t-test).

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

S Fig. 6. Effects of *dilp1* expression on weight, food intake and response to starvation. **A** The body weight increased in male flies after ectopic *dilp1* expression with *ppl*- and *c929*-Gal4, *** p<0.001, data are presented as

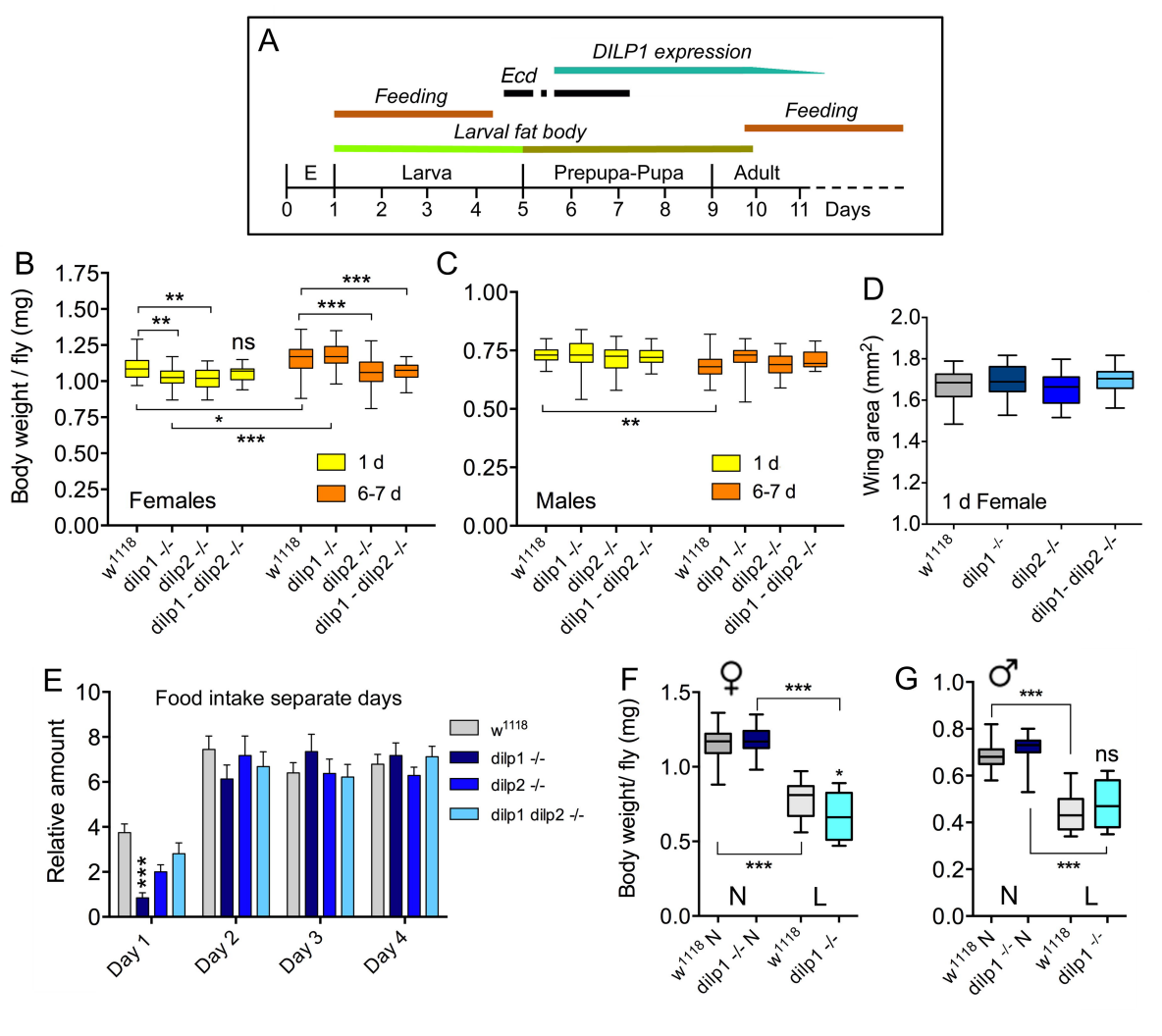
means ± S.E.M, n = 16–29 flies per genotype from three independent replicates (One-way ANOVA followed with Tukey's test). **B**. The wing area is not affected by *c929*-driven *dilp1* expression. Data are presented as means ± S.E.M, n = 15 flies from three independent replicates (One-way ANOVA followed with Tukey's test). **C.** Driving *dilp1* with *dilp2*- and *c929*-Gal4 does not affect food intake. Data are presented as means ± S.E.M, n = 24 flies from three independent replicates (two-way ANOVA followed with Tukey's test). **D** and **E**. c929-driven *dilp1* does not affect the response to starvation, n = 132-135 flies per genotype from three independent replicates, as assessed by log-rank (Mantel–Cox) test.

S Fig. 7. Ectopic expression of *dilp1* in clock neurons or larval neuroendocrine cells does not affect cell size. **A-D.** Expression of *dilp1* with the clock neuron driver *pdf*-Gal4 does not affect the size of the PDF-immunolabeled large LN_vs quantified in B. The brain size is also not affected (C). However the PDF immunolabeling is strongly increased (D). Data are presented as means ± S.E.M, n = 8 for each genotype from 3 replicates. (**p < 0.01, compared with w^{1118} flies, unpaired Students' t-test). **E.** Ectopic expression of *dilp1* with the c929-Gal4 line does not affect the size of leucokinin (LK)-immunolabeled neuronal cell bodies in the third instar larvae (quantified in F) or the size of the larval CNS (G). Data are presented as means ± S.E.M, n = 6-9 for each genotype from 3 replicates.

S Fig. 8. Ectopic expression of *dilp1* in glial cells with *repo*-Gal4 does not affect growth of neuronal cell bodies. **A.** DILP1 immunolabeling appears in cells after *Repo>dilp1*, but has no effect on the size of l-LNv clock neurons labeled with anti-PDF (quantified in **B**). Data are presented as means ± S.E.M, n = 9 for each genotype from 3 replicates. body weight of females (**C**) n = 19-25 from 3 replicates or males (**D**), n = 15-27 from 3 replicates.

S Fig. 9. Targeted *dilp1*-RNAi in IPCs reduces survival in flies exposed to starvation. **A.** The efficiency of *dilp2>dilp1*-RNAi on dilp1 levels was monitored by qPCR. A strong reduction in *dilp1* was noted, but effect was seen on levels of *dilp2* or *dilp6*. Data are presented as means ± S.E.M, n = 3 replicates per

genotype with 10 pupae in each replicate. (*p < 0.05, compared with control flies, unpaired Students' t-test). B. Flies with *dilp2>dilp1*-RNAi displayed reduced survival during starvation. n = 148-170 flies from three independent replicates. Data are presented in survival curves and the error bars means S.E.M [***p < 0.001, as assessed by log-rank (Mantel–Cox) test].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Feeding
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Prepupa-Pupa
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B
Body weight / fly (mg)
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Wing area (mm2)
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Food intake separate days
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w1118
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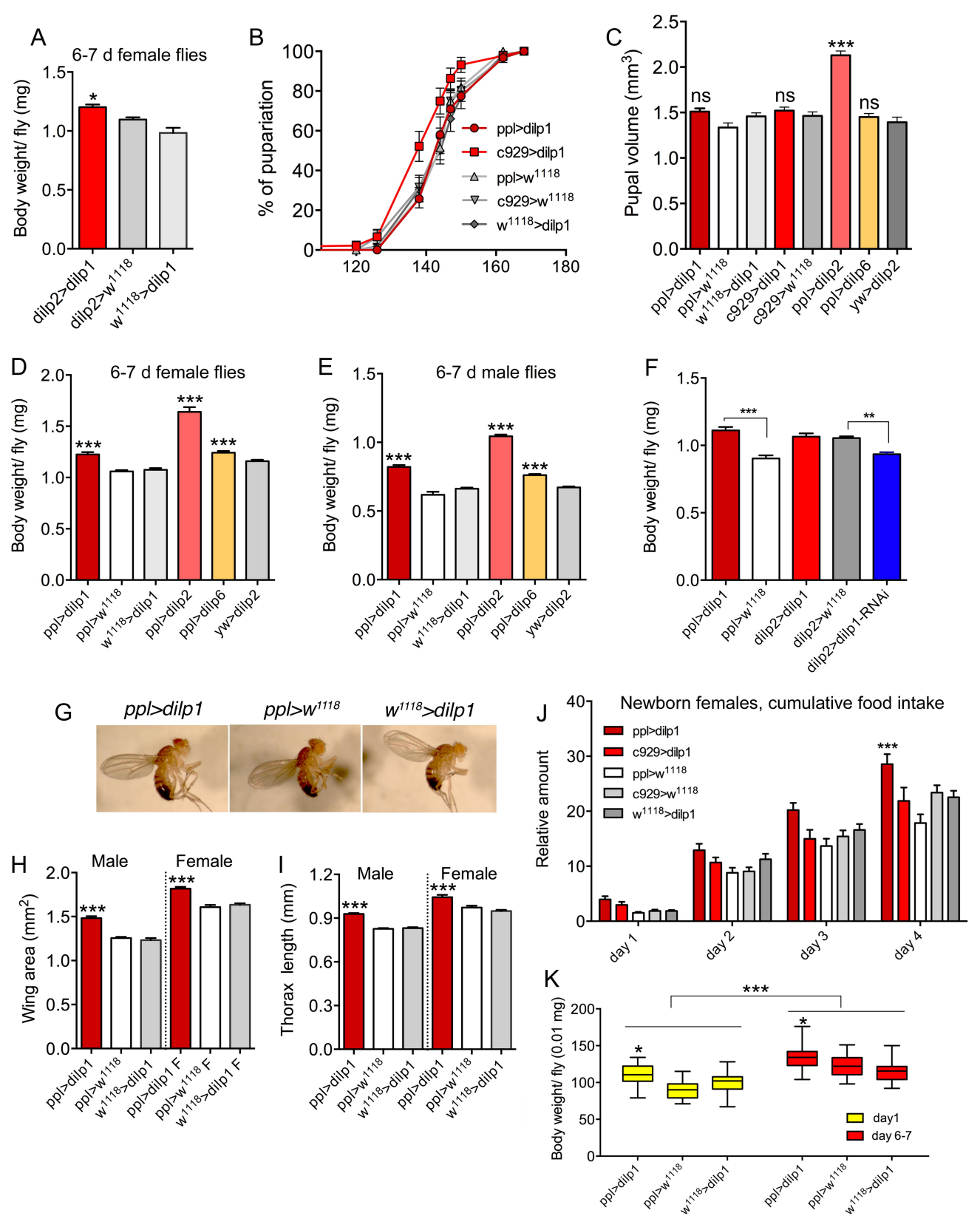
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ppl>dilp1
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H
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Female
Wing area (mm2)
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w1118>dilp1 F
I
Thorax length (mm)
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Relative amount
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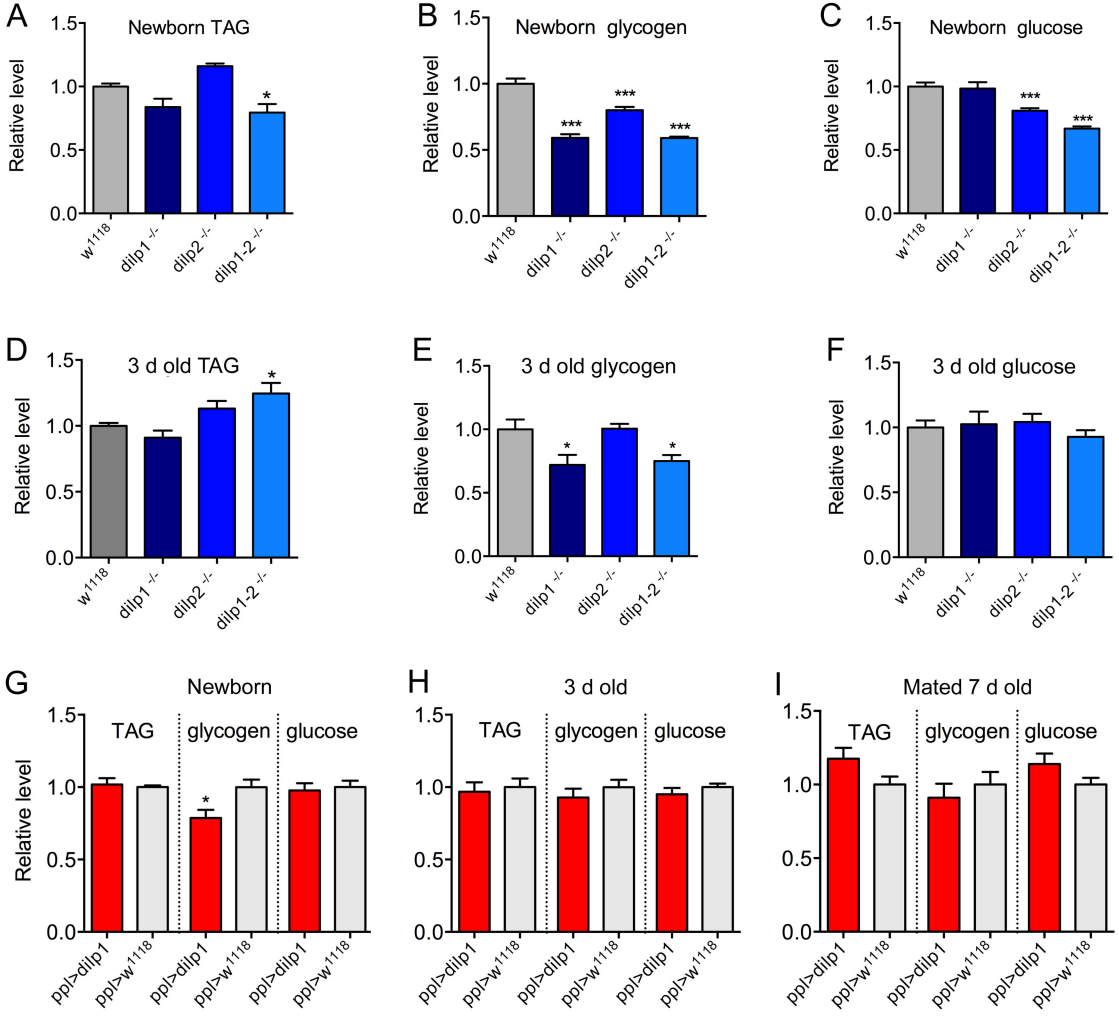
day 2
day 3
day 4
K
Body weight/ fly (0.01 mg)
day1
day 6-7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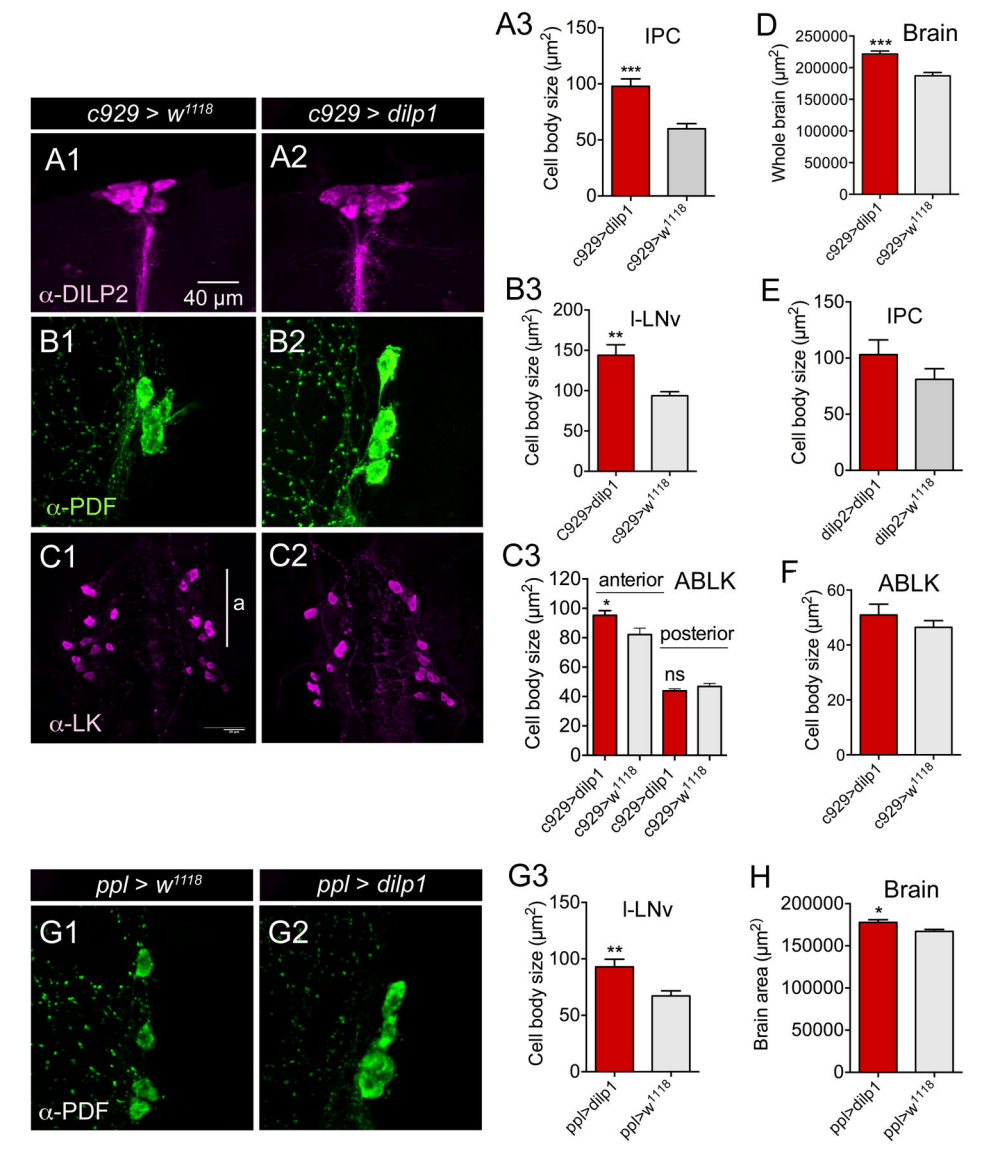
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dilp2>dilp1
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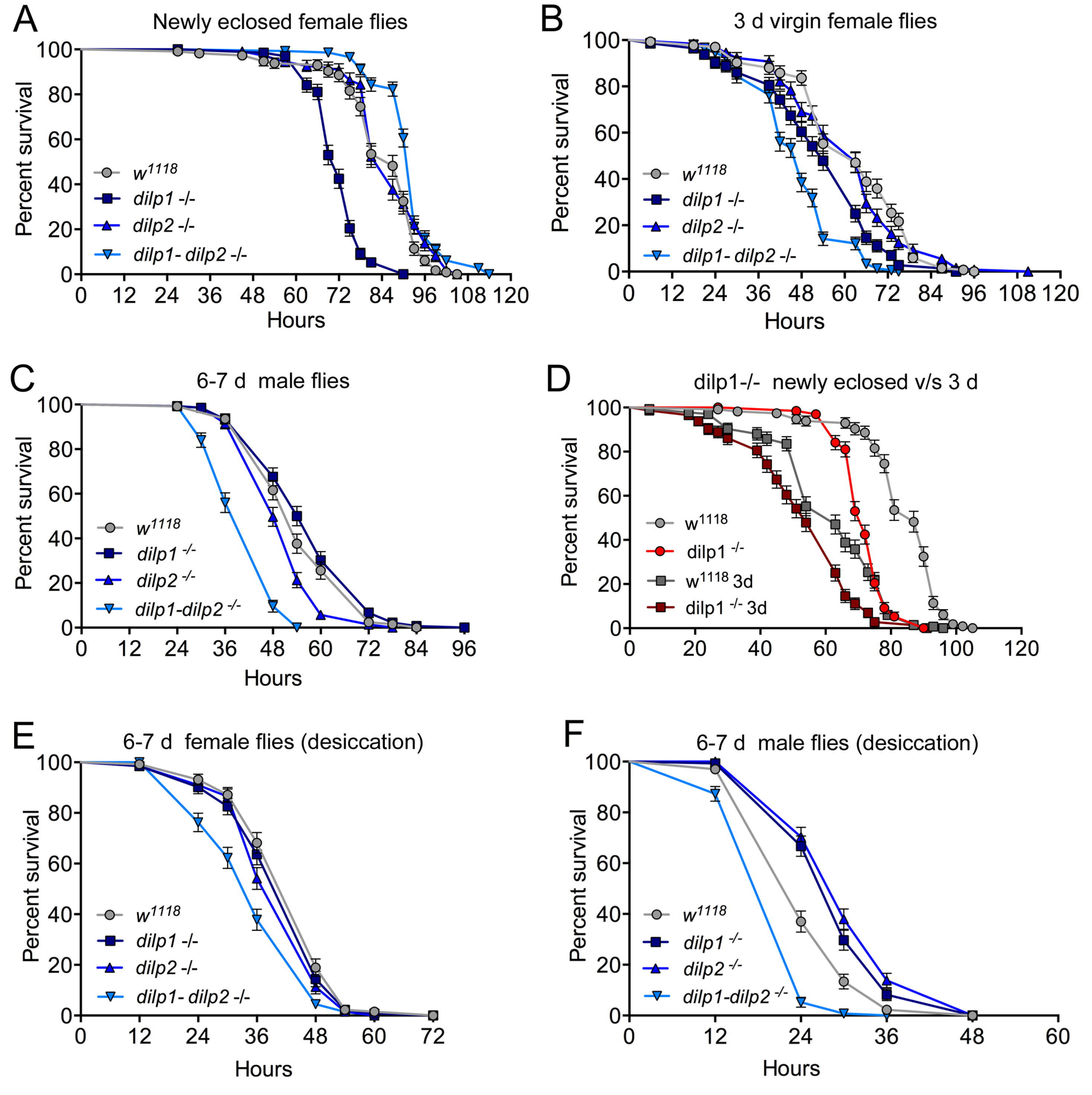
A
Newly eclosed female flies
Percent survival
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3 d virgin female flies
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dilp1 -/-
dilp2 -/-
dilp1- dilp2 -/-
C
6-7 d male flies
Percent survival
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dilp1 -/-
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dilp1-dilp2 -/-
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6-7 d female flies (desiccation)
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6-7 d male flies (desiccation)
Percent survival
Hours
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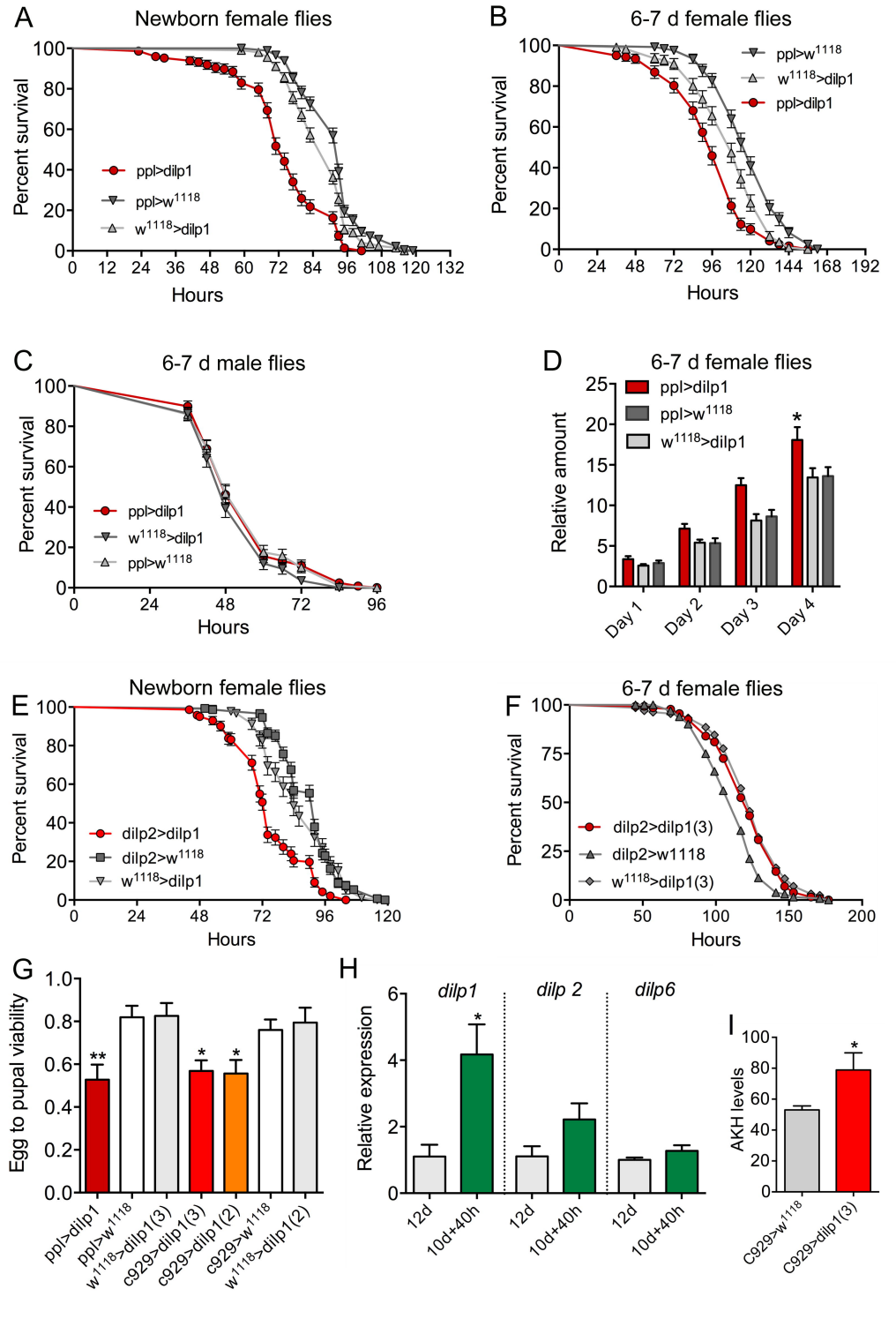
A
Newborn female flies
Percent survival
Hours
ppl>dilp1
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B
6-7 d female flies
Percent survival
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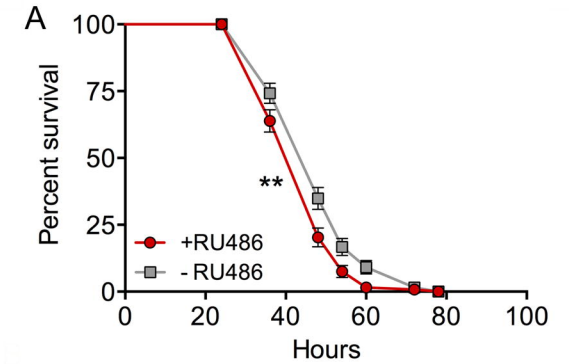
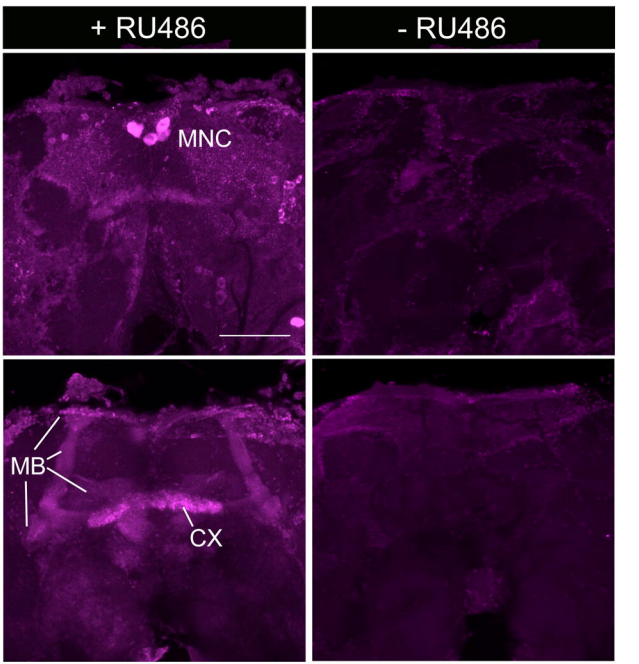
w1118>dilp1
ppl>dilp1
C
6-7 d male flies
Percent survival
Hours
ppl>dilp1
w1118>dilp1
ppl>w1118
D
6-7 d female flies
Relative amount
ppl>dilp1
ppl>w1118
w1118>dilp1
Day 1
Day 2
Day 3
Day 4
E
Newborn female flies
Percent survival
Hours
dilp2>dilp1
dilp2>w1118
w1118>dilp1
F
6-7 d female flies
Percent survival
Hours
dilp2>dilp1(3)
dilp2>w1118
w1118>dilp1(3)
G
Egg to pupal viability
ppl>dilp1
ppl>w1118
w1118>dilp1(3)
c929>dilp1(3)
c929>dilp1(2)
c929>w1118
w1118>dilp1(2)
H
Relative expression
dilp1
dilp 2
dilp6
12d
10d+40h
I
AKH levels
C929>w1118
C929>dilp1(3)


A
100
75
50
25
0
Percent survival
**
+RU486
- RU486
0
20
40
60
80
100
Hours


+ RU486
- RU486
MNC
MB
CX