orsai, the Drosophila homolog of human ETFRF1, links lipid catabolism to growth control

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Abstract

Background: Lipid homeostasis is an evolutionarily conserved process that is crucial for energy production, storage and consumption. Drosophila larvae feed continuously to achieve the roughly 200-fold increase in size and accumulate sufficient reserves to provide all energy and nutrients necessary for the development of the adult fly. The mechanisms controlling this metabolic program are poorly understood.

Results: Herein we identified a highly conserved gene, orsai (osi), as a key player in lipid metabolism in Drosophila. Lack of osi function in the larval fat body, the regulatory hub of lipid homeostasis, reduces lipid reserves and energy output, evidenced by decreased ATP production and increased ROS levels. Metabolic defects due to reduced Orsai (Osi) in time trigger defective food-seeking behavior and lethality. Further, we demonstrate that downregulation of Lipase 3, a fat body-specific lipase involved in lipid catabolism in response to starvation, rescues the reduced lipid droplet size associated with defective orsai. Finally, we show that osi-related phenotypes are rescued through the expression of its human ortholog ETFRF1/LYRm5, known to modulate the entry of β-oxidation products into the electron transport chain; moreover, knocking down electron transport flavoproteins EtfQ0 and walrus/ETFA rescues osi-related phenotypes, further supporting this mode of action.

Conclusions: These findings suggest that Osi may act in concert with the ETF complex to coordinate lipid homeostasis in the fat body in response to stage-specific demands, supporting cellular functions that in turn result in an adaptive behavioral response.

Keywords: CG6115, LYR, ETFRF1, Fat body, Lipid metabolism, Lipid droplets, Drosophila melanogaster

Background

A balanced interplay between different metabolic pathways is key to cellular homeostasis and ultimately to the survival of the organism. A fundamental aspect of this balance is the coordination between carbohydrate and lipid metabolism [1].

Abnormal lipid metabolism could result either from the inability to properly metabolize lipids when they are needed to sustain cell homeostasis (i.e., as a result of a deficient enzymatic function [2, 3]) or could result from an overuse of lipid reserves even in the presence
of more readily available energy sources [4]. In humans, lipid metabolism disorders span a broad spectrum of conditions, from hypercholesterolemia and hypertriglyceridemia [5] to those where the cell is incapable of breaking down lipids such as Tay-Sachs and Gaucher diseases; in fact, there are up to 22 fatty acid oxidation disorders caused either by disruption of mitochondrial β-oxidation or the transport of fatty acids through the carnitine transporter [6, 7]. Genetic conditions that lead to a deficient regulation of β-oxidation may cause hypotonia, myopathies, neuropathies, organ failure, and even developmental delay, intolerance to fasting, and death [8].

*Drosophila* has organ systems that perform essentially the same metabolic functions as their vertebrate counterparts [9]. For example, there are oxidative and glycolytic muscles. In addition, the fat body, which stores excess lipids in the form of triglycerides, combines functions of the liver and the white adipose tissue. Lipids are stored as lipid droplets that can be mobilized in times of need using lipases that are orthologous to those found in mammals [9, 10].

During development, the *Drosophila* embryo undergoes a metabolic switch from oxidative phosphorylation to aerobic glycolysis, where the synthesis of amino acids and nucleotides is promoted [11]. A characteristic of this carbohydrate-dependent developmental stage is the uncoupling of β-oxidation to promote the synthesis of lipids that will be used as building blocks to support membrane homeostasis and cell growth [12]. This transition allows larvae to generate sufficient biomass to support the nearly 200-fold increase in size associated with larval development [13, 14]. Such energy reserves are mainly stored in lipid droplets within the fat body and under normal conditions would not be used until the onset of metamorphosis.

The inability to exploit an energy resource due to metabolic dysfunction not only correlates with an energy-depleted state. It may also preclude the use of specific nutrients which causes aberrant behaviors such as hyperphagia [15], a change of diet choice [16], or early wandering [17], and even the inability to live on certain diets or survive starvation [18–20].

In the present study, we investigated the function of a novel *Drosophila* gene involved in lipid metabolism that we named *orsai* (*osi*). The lack of *osi* in larvae causes an overactive lipid catabolism, which impacts cellular dynamics and feeding behavior, ultimately leading to death in early stages of development. We show that Orsai has a critical role in the regulation of β-oxidation and provide evidence that it is an ortholog of human ETFRF1/LYRm5. Delving into Orsai’s function sheds light not only on the control of lipid metabolism and its relationship with growth promotion but also on the link between metabolism and behavior.

**Results**

**Identification of a novel mutant displaying stunted growth and developmental arrest**

A number of years ago, we carried out a genetic screen to identify genes associated with cellular homeostasis [21]. One of the identified insertions (P[UAS]100B), as homozygotes, resulted in small and translucent larvae, which apparently die as first instar (L1). To better understand the impact of this mutation, the size of carefully staged larvae grown on standard agar plates (3% sucrose and a yeast patch mixed with food colorant) was examined at 24, 48, and 72 h after egg laying (AEL, Fig. 1). Three-day-old homozygous P[UAS]100B mutants barely increased their initial size, while heterozygous ones appeared to catch up after an initial delay (Fig. 1A). In fact, homozygous P[UAS]100B larvae did not molt and died around 72–80h AEL still exhibiting mouth hooks with the morphology of a wild-type L1 (Fig. 1B–E). These results strongly suggest that the mutation causes developmental arrest followed by premature death.

A closer inspection at the fair/translucent P[UAS]100B homozygotes suggested abnormal feeding behavior. To begin to dissect the origin of this phenotype, we characterized homozygous mutant larvae in more detail. Food intake was assessed by monitoring the presence of colored food inside the gut (Fig. 1F). While most control animals (90%) had blue-colored gut within the hour that the experiment lasted, about 50% of P[UAS]100B mutants showed a clear one. Notwithstanding, mutant larvae were able to ingest food (Fig. 1F), suggesting that no gross structural defects in feeding structures is responsible for the altered behavior.

Wild-type larvae feed continuously and tend to remain inside or near appetitive food. This characteristic behavior is easily quantified by assessing the number of larvae present in a yeast patch placed at the center of an agar plate. In this context, P[UAS]100B homozygotes showed a weak preference to stay in the food during the first 48h AEL, shifting to no preference whatsoever at later time points (Fig. 1G, H). While only a small amount (<20%) of control larvae were found outside the food, over 50% of homozygous mutants did so around 24h AEL. To explore whether abnormal feeding derived from the inability to sense the food source, we performed a two-choice olfactory assay [22] that indicated that P[UAS]100B mutants were equally capable of responding to an attractive cue present in the food as control larvae (Fig. 1I), ruling out the possibility that a generally defective sensory response could be the origin of the abnormal food-seeking behavior.
Considering the tendency of the mutant to be outside of the expected place at any given time, we named this mutant *orsai* (*osi*, Argentinian street language for the football term “offside”).

The P(UAS)^100B insertion reduces the levels of endogenous CG6115

Plasmid rescue analysis [23] revealed that P(UAS)^100B is inserted 218 base pairs upstream of the transcriptional
start site of CG6115 (osi), and it is also upstream of the start site of tweek (Fig. 2A), but it does not physically interrupt any splice variant so far described for either gene. The P element is located in reverse orientation with regard to transcription at the CG6115. osi encodes a protein of 85 amino acids containing a Complex I Lyr motif (LYRm) according to Flybase (http://flybase.org/reports/FBgn0040985.html). The highly conserved tripeptide motif “LYR” is LYK in Drosophila; downstream, another highly conserved residue (a phenylalanine) is also present in Osi [24]. No additional motifs were identified. Sequence analysis retrieved putative orthologs in metazoans (Fig. 2B). Remarkably, Osi displays 50% identity and 76% similarity to the human LYRm5, whose molecular function has been examined and it has now been renamed Electron Transfer Flavoprotein Regulatory Factor 1 (ETFRF1 [25], Fig. 2C). In Drosophila, no functional characterization of CI_LYRm-containing proteins has been reported. Sequence analysis identified 7 entries in the fly genome according to Uniprot (CG6115, CG7712, CG42372, CG13191, CG34229, CG42372, CG3717). To explore the conservation within the Complex I_LYR family, Drosophila and human sequences were retrieved. The resulting set included 11 reviewed human as well as the 7 fly proteins (Fig. 2D). LYRm domain-containing proteins are characterized by the Lyr/K motif. However, it is worth mentioning that their similarity extends beyond those 3 amino acids, showing two blocks of conserved residues. This is remarkable, particularly taking into account the divergence of this group of proteins (with an average of 25% identity). There is a striking conservation of four hydrophobic and one positive residue nearby the LYR/K motif, along with an absolutely conserved phenylalanine as well as one hydrophobic and two positively charged amino acids within the second block. This analysis suggests that a functional Lyr/K domain likely contains those additional residues.

Quantitative real-time RT-PCR (qPCR) analysis indicated that, while heterozygous P[UAS]<sup>100B</sup> did not affect osi mRNA levels, the homozygous one reduced them to about one third of the controls (Fig. 2E), without affecting tweek (Fig. 2F), which is located immediately downstream of the insertion (Fig. 2A), suggesting that reduced osi mRNA levels are responsible for the phenotype of the homozygous mutant larvae. We characterized a new insertion in CG6115 that we renamed osi<sup>G4Δ</sup>). It contains a Gal4 gene trap that landed in the first and only coding intron; this cassette was designed so that it truncates the encoded protein [26]. qPCR analysis indicated that the insertion reduces osi levels to 50% compared to wild-type controls (Fig. 3A). As homozygotes, most osi<sup>G4Δ</sup> individuals died throughout development (only 2/50 individuals emerged as adults, Fig. 3B), development was delayed although not arrested, confirming that reduced osi levels compromise survival. A proportion of osi<sup>100B</sup>/osi<sup>G4Δ</sup> individuals, on the other hand, reached adulthood.

**Reduced osi levels correlate with an arrest of larval development**

Genome-wide RNA profiling revealed that osi transcript levels are normally moderate to high in every tissue starting at early development, becoming very high at around 12-h-old L3 instar larvae [27, 28]. The temporal correlation between the surge in osi expression in controls and the onset of lethality in the mutant (72h AEL), prompted us to assess osi’s relevance during development by an independent method. Thus, we resorted to the expression of osi<sup>RNAi</sup>, a RNAi line with no predicted OFF-target effects, in the context of dicer 2 co-expression to increase RNAi efficacy. We first measured steady state levels of osi mRNA by qPCR in total RNA extracts from controls (actG4+−) and larvae expressing the RNAi driven by the actin-Gal4 driver (from now onwards referred to as actG4>dcr2;osi<sup>RNAi</sup>). Ubiquitous osi knockdown (actG4>dcr2;osi<sup>RNAi</sup>) resulted in over 80% reduction in overall osi mRNA levels (Fig. 3C).

Newly hatched larvae were placed in agar plates and were imaged every day to monitor progression of development.
Fig. 2 (See legend on previous page.)
larval development for as long as experimental larvae were alive. The ubiquitous expression of osiRNAi phenocopied the original mutant; actG4 > osiRNAi larvae displayed a similar feeding behavior and failed to grow and progress beyond the L1 stage. RNAi-mediated osi down-regulation triggered complete lethality around 96h AEL (Fig. 3D).

**orsai is highly expressed in the larval fat body**

Publicly available data predicts that osi is expressed in most tissues. Within the larvae, levels are particularly elevated in the fat body (up to three times, according to FlyAtlas [27]). To independently confirm osi expression in the larval fat body, we resorted to osiGal4 to drive expression of a membrane associated mCherry. As shown in Fig. 4A, osiGal4 is expressed throughout the fat body. Interestingly, expression of a tagged Osi version suggests this protein localizes in the proximity of the nucleus in control fat body cells (Fig. 4B).

**Partial loss of osi function reduces cell size**

The observation that Osi was localized in fat body cells prompted us to examine the consequences of osi loss-of-function in this tissue. To test the possibility that depleted Osi could be associated with reduced cellular size, we dissected the fat body of controls, heterozygous and homozygous mutants at 24, 48, and 72h AEL. Figure 4C–G shows that while homozygous mutant cells were similar in size to matched controls at 24h AEL, their size was significantly reduced at 48h. These differences were even more pronounced at 72h AEL.

We further explored this possibility through expression of osiRNAi in fat body cells by means of the pplG4 driver, whose expression at this stage was first confirmed (Fig. 4H). Not surprisingly given the phenotypes observed in the mutants, Osi downregulation triggered a reduced cellular size (Fig. 4I–K). These results suggest that impaired osi function leads to a defective control of cell and/or organ size.

**Mosaic analysis uncovers a cell autonomous role for Osi**

Depletion of Osi arrests larval development. To dissect Osi’s role in the context of a viable organism, we generated mosaic animals in which somatic cell clones expressed osiRNAi along with a GFP reporter using the site-specific recombination flp/FRT system [29]. This method generates cell clones expressing Gal4 (and thus, GFP along with osiRNAi) once a heat shock triggers recombination between cis-acting sites. We applied this method to obtain clones in the fat body (Fig. 5). Twenty-four-hour AEL larvae were exposed to a very brief heat shock, and their fat body was dissected at 24-h intervals. The frequency of clones is dependent on the duration of the heat shock and larval age. Figure 5A illustrates the results obtained. Initially, no clear differences in cell size or morphology could be observed between control
Fig. 4 oso downregulation is associated with a smaller cell size. A Osi is expressed in the fat body. mCherry expression driven by osoGal4 shows strong consistent signal in the fat body. B Confocal images of a fat body of larvae expressing Osi-FLAG under actG4, stained for Flag (in red). Scale bar represents 50 μm (A) and 100 μm (B). C–G oso mutants develop cellular defects in time. C, D Representative confocal images of fat body of control and mutant animals at 72h stained with DAPI (nucleus) and phalloidin (cell outline). Scale bar represents 20 μm. E–G Quantification of cellular area of larval fat bodies of 24h (E), 48h (F), and 72h (G) AEL individuals of control, heterozygous, and homozygous mutants. Five or six fat bodies were photographed per genotype on each time point. Dots in each graph represent individual cell sizes. H GFP directed by pplG4 confirms fat body expression. Bars represent 20 μm. I–K: Phalloidin and DAPI stained fat bodies from 72h AEL control (I) and larvae expressing osoRNAi under pplG4 (J). Bars represent 10 μm. K Larvae expressing osoRNAi show reduced cell size. Quantitation of cell size of fat body cells from 72h AEL control and larvae expressing osoRNAi under pplG4. Three fat bodies were photographed per genotype. Dots in each graph represent individual cell sizes. All graphs describe the mean ± SEM. In E, F, and G, a Kruskal-Wallis with Dunn's multiple comparisons test was performed. In K, a Mann-Whitney test was performed. All panels: the total number of observations is indicated under each data set. A Shapiro-Wilk test was used for normality assessment (see Additional file 2). Different letters indicate significant differences, p<0.05; treatments sharing any letter are not statistically different. The total number of samples analyzed is detailed below the corresponding dataset. All datasets and statistical analysis on which the conclusions are based are included in Additional file 2.
(GFP−) and osiRNAi (GFP+) expressing cells (Fig. 5A, 24 and 48h). However, 72h after the heat pulse, GFP+ cells were smaller in size and also displayed an abnormal morphology, which became more dramatic later on (96h), when GFP+ clones were barely detectable, prior to their complete disappearance. Thus, mosaic analysis confirmed prior observations suggesting that Osi is relevant for the control of cell size and revealed that Osi acts in a cell-autonomous manner.

**Human LYRM5/ETF regulatory factor 1 (ETFRF1) rescues Osi loss of function**

As shown in Fig. 2, Osi shares 50% identity with human ETFRF1/LYRm5. In vitro, ETFRF1/LYRm5 was found to inhibit ETF by promoting the removal of flavin from the ETF holoenzyme, thus potentially regulating the rate of β-oxidation [25]. To investigate whether the human protein could counteract the cell-autonomous phenotypes associated with loss of Osi function, codon-optimized ETFRF1/LYRm5 was expressed in the context of osiRNAi in the same experimental setting already described (Fig. 5A). Figure 5B–G shows that, while clonal expression of osiRNAi results in a statistically significant reduction of cell size, co-expression of human ETFRF1/LYRm5 rescues this defect to a large extent, indistinguishable from the one achieved through the expression of a RNAi-resistant osi (osiSM), supporting the notion that ETFRF1/LYRm5 is the human ortholog of Osi.

As already mentioned, larvae with ubiquitous osiRNAi expression die at first instar, judged by the morphol-
Fig. 5 (See legend on previous page.)
functional analysis with an additional fat body-specific driver (cgGal4, Fig. 7E). Consistent with a key role for \(osi\) in the fat body as opposed to the gut, \(cg\text{-}Gal4>osi\text{-}RNAi\) triggered early lethality (Fig. 7G). Taken together, these data demonstrate that the aberrant behavior associated with Osi depletion is a consequence of the alteration of a metabolic program within the fat body that only in time affects food-seeking behavior.

**osi mutants have impaired cellular metabolism**

Reduced \(osi\) levels elicited developmental arrest at a stage of active growth, coincident with times of increased energy demand. This observation coupled with the fact that \(osi\) dysfunction leads to a reduced cell size in a tissue that has key metabolic functions, prompted us to consider that lethality could be linked to a defective cellular metabolism. Mitochondrial extracts were prepared from 72h AEL homozygote \(osi^{100B}\) larvae along with controls. Fresh extracts were then assayed for protein content and ATP production employing a quantitative bioluminescence kit. As shown in Fig. 8A, mutant extracts generated ATP levels three times lower than those of the controls, confirming that reduced Osi correlates with an impaired ATP production. We next estimated oxygen consumption rate (OCR) through a "Mitostress" protocol in control and mutant larvae, which relies on the sequential addition of inhibitors of the respiratory chain to assess the function of specific complexes. To improve access of the inhibitors, an open larval preparation where all the organs are exposed was selected. However, no changes in OCR became detectable in control preparations, with the exception of the addition of Rotenone/Antimycin A that resulted in the expected reduction in OCR, in line with previous reports describing similar body
Fig. 7  A key role for *osi* in the fat body. **A** *osi* downregulation in the nervous system using elavG4 or nSybG4 does not affect viability. *N*=3. **B–D** *osi* downregulation in the gut does not affect viability. NP1G4 is expressed in the gut of 72h AEL larvae. **B, C** show GFP driven by NP1G4 confirming its expression in the gut (**B**) but not in the fat body (**C**). *N*=3. **E–M** cgG4 recapitulates *osi*-related phenotypes. **E** cgG4 directed expression of GFP to the fat body. **F** qPCR shows *osi* levels are reduced about 50%. *N*=4; each replica included 20 larvae. **G** Survival indicates the percentage of living individuals. *N*=2, one representative experiment is shown. **H** Lip3 downregulation in the fat body accounts for partial survival. Adult eclosion of the indicated genotypes was quantified. The graph shows the proportion of animals obtained relative to the expected (“Methods”). *N*=1. **I** LYRm5 expression in the fat body partially rescues the lethality associated to reduced *osi* levels. Adult eclosion was quantified. *N*=2. **J** Downregulation of *osi* and EtfQ0 in the cgG4 pattern progress through development, molt and pupate; even though no viable adult eclosed. A representative experiment (*N*=2) is included. **K** wsl (EtfA) downregulation in the fat body fully rescues survival with reduced *osi* levels. *N*=3. **L, M** *osi* downregulation in the fat affects lipid droplet content. **L** Reactive area of lipid droplets stained with BODIPY was quantified. Six to eight individuals were analyzed per genotype. Each dot represents the mean reactive area per animal. **M** representative images. All panels: different letters indicate significant differences, p<0.05; treatments sharing any letter are not statistically different. The total number of animals analyzed is indicated in **D, H, I, and K**. A Shapiro-Wilk test was used for normality assessment. A T-test or one-way ANOVA with Bonferroni's multiple comparisons was performed (see Additional file 2). The total number of samples analyzed is described below each dataset. All datasets and statistical analysis on which the conclusions are based are included in Additional file 2.
Although these results should be taken cautiously, OCR was reduced in homozygous osi100B mutants (Fig. 8B), indicative of a reduced respiratory capacity. Next, we measured the extracellular acidification rate (ECAR) under a “Glycostress” assay; these experiments showed an overall defective glycolytic capacity in osi mutants as well as an altered response to glucose intake at 72h AEL. Data was normalized to the number of larvae. Six independent samples were examined. Dihydroethidium (DHE) was employed to detect ROS by fluorescence microscopy in 24h AEL larvae. Animals from 2 different plates were examined. The total number of fat bodies analyzed is indicated. A two-tailed t-test was performed. E Sod2 expression in osi-depleted animals using actG4. Three independent samples were taken into account. A one-way ANOVA with Bonferroni’s multiple comparisons was performed. The total number of animals per condition is indicated. F Addition of the antioxidant NAC to the food did not improve survival of osi-depleted animals. The experiment was repeated twice. All panels: different letters indicate significant differences, $p<0.05$; treatments displaying the same letter (alone or in combination) are not statistically different. A Shapiro-Wilk test was used for normality assessment (see Additional file 2). The total number of samples analyzed is described below the corresponding panel. All datasets and statistical analysis on which the conclusions are based are included in Additional file 2.

This impaired metabolic function is often associated with the accumulation of charged cytotoxic species that affect membrane stability and could induce the formation of superoxide anions, thus contributing to the generation of cellular reactive oxygen species (ROS) [32]. To investigate this possibility, a chemical probe called dihydroethidium (DHE) was employed to detect superoxide radicals by fluorescence microscopy in intact larval tissue [33, 34]. Interestingly, homozygous osi100B larvae showed a 2-fold increase in overall superoxide radical levels as reported by this probe (Fig. 8D); such increase in ROS levels could per se exceed the capacity of intrinsic antioxidant systems and therefore lead to oxidative stress and cell damage.
investigate this possibility, we resorted to the expression of superoxide dismutase (SOD). SOD catalyzes the conversion of superoxide anion radicals to hydrogen peroxide which is in turn converted to molecular oxygen and water by catalase. Overexpression of SOD and catalases retards accumulation of oxidative damage associated with aging [35, 36], as well as prevents some of the deleterious effects in fly models of disease [37]. To explore the possibility that increased superoxide levels could contribute to the characteristic lethality associated with depleted Osi, we expressed sod2 in the context of the ubiquitous expression of osiRNAi (actG4>osiRNAi, Sod2). Only a marginal rescue of lethality was observed; very few individuals progressed through development into adult stages, albeit not in the expected proportion, suggesting that increased superoxide levels have a minor contribution to osi’s phenotype (Fig. 8E). To further explore the relevance of free radicals to the mutant phenotype, animals were grown in N-acetylcysteine supplemented food (NAC, a potent antioxidant); under these conditions, the survival rate was similar to the one observed upon Sod2 overexpression (Fig. 8F).

Overall, this data suggests that depleted Osi function unbalances energy metabolism.

Deregulated lipid catabolism underlies cell-autonomous and systemic phenotypes

To gain more insight into the consequences of reduced Osi, we measured Lipase 3 (Lip3) mRNA levels, a key enzyme in lipid catabolism, which is increased upon starvation [17, 38]. In effect, Lip3 levels were upregulated in osi100B/100B mutants as well as in actG4>osiRNAi, while Pepek1 levels, encoding an enzyme that regulates carbohydrate catabolism [39], were not modified at 72h AEL (Fig. 9A–D). We then tested the relevance of Lip3 upregulation to osi-related phenotypes through tissue-specific RNAi downregulation (Figs. 7H and 9F–H). Noteworthy, Lip3RNAi expression in the context of osiRNAi partially rescued lethality, suggesting that an increased lipid catabolism is clearly linked to the developmental arrest (Fig. 9F). If this was the case, then impaired osi function could correlate with a reduction of lipid reserves. To shed light on this possibility, we stained fat bodies with BODIPY, a fluorescent dye for the assessment of cellular neutral lipid content [40]. As predicted, lipid content was dramatically decreased in tissue-specific knockdowns at 72 and 96h AEL (pplG4>osiRNAi, Fig. 9G–I and S2L–M). We reasoned that if reduced Osi function increases the rate of lipid catabolism through excessive Lip3 activity, Lip3 knockdown in the context of reduced Osi levels could ameliorate the lipid storage phenotype. Interestingly, Fig. 9 shows that to be the case, whereby concomitant downregulation of the two proteins rescued lipid droplet (LD) content to background levels (Fig. 9G–I). Although we cannot rule out the contribution of fasting to increased Lip3 levels, we favor the interpretation of a more direct link since reducing Lip3 levels in the context of osi knockout associates with increased survival which would not be the expected outcome when impairing a fasting response in the context of reduced feeding.

Additionally, we tested if lipid content was decreased in osi100B/100B mutants (Fig. 10). Despite no significant reduction in total area was detectable (Fig. 10B, D, F), we did find a clear change in the individual size of LDs. As shown in Fig. 10G–I, the distribution of lipid droplet size shifted towards a higher proportion of smaller ones that were increasingly represented over time in the mutants. In addition, at the end of the experiment, mutant lipid droplets exhibited an aberrant morphology of these otherwise round shaped organelles, underscoring an increased use of lipid reserves (Fig. 10J–L). The apparent subtler phenotype in osi100B/100B mutants might simply reflect that those observations were performed at an earlier timepoint during larval development. Nevertheless, considering that Lip3 downregulation per se partially compensates for loss of Osi function (Fig. 9G–I), we propose that the overuse of lipid reserves is a key component of the phenotype observed.

The striking change in LD size and shape when Osi function is compromised prompted us to examine lipid content in more detail. Among neutral storage lipids, triacylglycerol (TAGs) are the major constituents of LDs and most abundant fatty acid source for energy production as well as signaling lipid biosynthesis [41]. We performed mass spectrometry-based lipidomics in control and osi mutant animals at 48 and 72h AEL. The total TAG content of osi100B larvae at 72h AEL was significantly reduced compared to controls, but this tendency was already present at the earlier timepoint (Fig. 11A). Interestingly, this decrease was not the result of a general reduction in every species; in fact, a clear reduction was observed in the TAG species composed of common long-chain fatty acids (LCFAs), particularly with total carbon numbers C40, C38, and C48, while lower molecular weight TAG species composed of shorter LCFAs and medium chain FAs (total carbon numbers C40, C38, and C36) were significantly increased. This differential change of the TAG profile was already evident at 48h AEL and become more pronounced at 72h AEL (Fig. 11B). Currently, we cannot judge whether these differences arise from osi-dependent developmental changes of TAG biosynthesis or breakdown. However, given that at this time in development, TAGs are not commonly used as the source of energy but as building blocks for membrane production and maintenance as well as a source of signaling molecules, this finding opens the possibility that...
Fig. 9 Preventing excessive fat body lipid catabolism rescues the behavioral effect triggered by osi downregulation. A–D Quantitation of Lip3 and pepck mRNA levels in mutants (A, B) or larvae with ubiquitous RNAi expression (C, D). Data shows a clear increase in Lip3 with no effect on pepck levels. The total number of replicas/genotype is included; each dataset consisted of 20 larvae pooled together. A Shapiro-Wilk test was used for normality assessment; if normality was confirmed, a one-way ANOVA with Bonferroni’s multiple comparisons test was performed. For nonparametric assessment, Kruskal-Wallis with Dunn’s multiple comparisons test were performed (see Additional file 2). E qPCR analysis on RNA extracted from whole larvae shows efficient reduction of Lip3 levels when Lip3RNAi is expressed under pplG4 at 96h AEL. The total number of replicas/genotype is included; each dataset consisted of 20 larvae pooled together. A two-tailed t-test was performed. F Lip3 downregulation partially rescues osiRNAi lethality. Six independent experiments were performed; the total number of animals assessed are indicated. G–I Confocal images showing fat body cells stained with BODIPY (golden lipid droplets) for control, osiRNAi, Lip3RNAi, and Lip3RNAi, osiRNAi expressing animals under pplG4 from 72 and 96h AEL. Bars represent 20 μm (I). The percentage of area covered by lipid droplets or “reactive area” is quantitated in G for 72h and H for 96h AEL. Each dot represents the mean area of each fat body analyzed. The total number of fat bodies per condition is indicated. All panels: Shapiro-Wilk test was used for normality assessment. Graphs display mean ± SEM. Different letters indicate significant differences, p<0.05; treatments sharing any letter are not statistically different. The total number of samples analyzed is described below the corresponding panel. All datasets and statistical analysis on which the conclusions are drawn are included in Additional file 2.
changes in particular TAG groups may contribute to the complex array of phenotypes associated with reduced Osi levels [12].

Epistasis analysis reveals a role for Osi at the center of lipid metabolism

ETFRF1/LYRm5 has been shown to negatively regulate the electron transfer flavoprotein complex (ETF) in vitro [25], suggesting that Osi provides a modulatory step in the response to the metabolic state of the cell. The ETF complex (including Walrus/EtfA and a yet to be defined EtfB subunit) is in charge of shuttling the electrons generated during mitochondrial fatty acid and amino acid catabolism by means of the electron transfer flavoprotein ubiquinone oxidoreductase (EtfQ0) [42].

To inquiry whether arrested development could derive from a deranged β-oxidation, we explored if reducing Etf-Q0 or ETFB could restore such balance. Thus, we combined expression of Etf-Q0RNAI and osiRNAI and monitored progression throughout development. Despite no viable adults emerged, reducing Etf-Q0 levels in the context of osi knockdown rescued the developmental arrest up to puparium formation (Figs. 7 and 12A). We next carried a similar epistasis analysis with walrus (wal). Remarkably, the combined expression of walRNAI and osiRNAI resulted in a completely viable progeny, under-scoring that Osi takes part in this crucial regulatory step (Figs. 7K and 12B).

Taken together, these results support the claim that Osi is the fly’s functional homolog of ETFRF1/LYRm5 and they reveal a new player in the regulation of lipid metabolism. On a greater note, Osi directly associates a cell-autonomous biochemical process with the overall growth of the cell and, in consequence, the organism, highlighting a potential communication between the fat body and the brain that results in a plastic food-seeking behavior.

Discussion

In the search for genes essential for cellular homeostasis, we identified Orsai, a novel Complex I_LYR domain-containing protein. A severe loss of function allele or ubiquitous knockdown triggers developmental arrest at the L1 stage, followed by larval lethality. Depleted Orsai function is associated with exacerbated lipid catabolism, which in turn leads to the depletion of energy reserves which might cause the developmental arrest. Furthermore, mosaic analysis indicated that Orsai affects cell size in a cell-autonomous manner. Interestingly, in certain biological contexts (i.e., osi downregulation in the fat body), this essential function is fully rescued through the expression of human ETFRF1/LYRm5, strongly suggesting that ETFRF1/LYRm5 (Q6IPR1) is the human ortholog of CG6115/Osi (Q9VJG4).

How does Osi contribute to cellular metabolism?

Figure 12 describes our working model regarding the contribution of Osi to the regulation of basal metabolism and the coordination of cell growth. During larval development, there is an increased demand for the activity of enzymes involved in glycolysis that promote carbohydrate metabolism, while β-oxidation of fatty acids is attenuated [12]. If Osi acts similarly as it has been shown in vitro for ETFRF1/LYRm5, it would inhibit ETF activity and block β-oxidation, favoring the accumulation of fatty acids required at later stages of development (i.e., during metamorphosis [43]). On the contrary, in the context of a severe Osi knockdown, an elevated ETF activity would be anticipated, resulting in an increase of β-oxidation. Interestingly, this scenario does not correlate with an increased electron flow to the ETC, as supported by the observation that osi mutants exhibit a reduced response to inhibitors of the respiratory chain (Fig. 8B). Instead, these electrons could input the reverse electron transfer (RET, see below), resulting in increased ROS levels (Fig. 8D) as well as an imbalance of intermediate metabolites (i.e., LCFAs) that could potentially affect membrane integrity and compromise cellular viability [44], as shown by the reduction of high molecular weight TAGs (Fig. 11B). Particularly, changes in the composition of the LD membrane might contribute to changes in their size distribution in osi mutants (Fig. 10G–I), which in turn could modulate the LD access to lipases, thereby...
increasing TAG degradation and fatty acid availability [45]; in line with this, downregulation of Lip3 counteracts the active loss of energy reserves triggered by osi depletion (Fig. 9G–I), underscoring that phenotypes might be in part triggered by excessive lipid catabolism. In addition, increased ROS production as a result of a non-regulated ETF complex could lead to an increased lipid peroxidation, thereby affecting membrane fluidity, dynamics, transport eventually leading to defects in membrane initiated signaling [46].
**A possible link to canonical pathways that regulate cell growth**

Deregulated ETF/EtfQ0 activity results in higher free radical levels due to the exacerbated electron flow, as well as an increased fatty acid catabolism due to the inability to regulate this modulatory step (Fig. 8). In the context of an enhanced electron flow, the reduced state of coenzyme Q (CoQH2) would be increased, overwhelming its oxidative capacity, in turn promoting a retrograde flux of electrons (RET) from CoQH2 to Complex I (CI) [47]. As a consequence of this reversed electron transport, CI generates superoxide radicals and increases ROS production, as detected in osi-depleted larvae (Fig. 8). In this regard, the subtle rescue of the lethality by Sod2 is in agreement with earlier reports stating that Sod2 overexpression protects only weakly against ROS generated in CI, probably due to topological restrictions that limit proper access to these complexes [47, 48]. An additional consequence of RET is a decrease in NADH/FADH reducing agents that could, in turn, slow down glycolysis and the tricarboxylic acid cycle; in fact, osi mutants show reduced glycolysis (Fig. 8C). In addition, the unrestricted use of fatty acids depletes energy reserves, signaling nutritional stress, which could in time inhibit the classical cell growth regulatory pathway AKT/TOR and promote the degradation of more fatty acids in a futile attempt to maintain ATP levels [49–53]. Low ATP levels coupled to the generation of reactive oxygen species impact on the regulation of the TOR pathway further preventing cell growth in osi mutants [54].

**How are systemic effects generated?**

Food-seeking behavior is precisely regulated by the integration of the internal energy status and external sensory signals. Upon starvation, larvae initiate a wandering-like behavior, the most prominent behavioral phenotype of osi mutants, probably in search for more nutritious food sources [55] (Fig. 1). Reducing Osi levels exclusively in the fat body is sufficient to trigger this behavior, in favor of a metabolic origin as opposed to one resulting from impaired Osi function in the brain. This behavioral response can then be reversed by EYTFR1/Lyrm5 expression in the fat body (Fig. 6B–E), further supporting its connection to lipid metabolism. In addition, osi-depleted animals exhibit increased Lip3 levels (Fig. 9A, C), a marker of starvation [38], which could be the origin of the orsai (out of patch) phenotype.

Alteration of metabolic homeostasis correlates with changes in behavioral programs that in time lead to an adaptive feeding response [9, 56–59]. Previous studies have reported a similarly aberrant food-seeking behavior as the one displayed by orsai mutants, where altering fat body homeostasis eventually leads to the sudden and early cessation of feeding behavior [17, 55]. Even though there are many instances where signaling from the CNS leads to changes in peripheral tissues [9, 56, 60], and that communication between the fat body and the CNS has clearly been established [61, 62], no specific pathway has been associated to lipid metabolism. Thus, our results reveal a novel communication from the fat body to the brain and posit this model as an ideal one to reveal peripheral signals that modify behaviors.

**Conclusion**

The data presented herein show that osi is a central player in lipid and energy metabolism and establish osi mutants as a suitable genetic model for further studies of conserved functions of the LYRm family of metabolic regulators.

**Methods**

**Fly stocks and maintenance**

Flies were grown and maintained in vials containing standard cornmeal medium at 25°C under 12:12 h light: dark cycles. The stocks used were w1118 (genetic control for transgenic flies), act-Gal4, heat shock (hs)-Flp, elav-Gal4 (8765), pumpless-Gal4 (ppl-Gal4), UAS-sod2 (24494), UAS-Lip3RNAi (65025), osiGAL4 (83190), UAS-CD8mCherry (27391), UAS-GFP (9431), UAS-Dcr2 (24650), UAS-watRNAi (34915), and UAS-EtfQ0RNAi (56664) which were obtained from the Bloomington Stock Center; UAS-osiRNAi (construct ID 29711) was obtained from VDRC; and nspy-Gal4, cgGal4, and NP1Gal4 were donated by I. Miguel-Aliaga (Imperial College, UK), M. Katz (School of Medicine, UBA, Argentina), and A. Garelli (INIBIBB, Argentina), respectively. Unless otherwise indicated in the figure legend, a
UAS-dicer2 transgene was included in all RNAi experiments to bolster the effect of the UAS-osiRNAi. The osi^{100B/100B} mutation was backcrossed to w^{1118} for 5 generations. More details about stocks and antibodies used are included in Additional file 1: Supplementary Table 1.

**Sequence analysis**

Protein sequence for CG6115 (Osi) of *Drosophila melanogaster* was obtained from the GeneBank database (NP_652578.1). Homology with different model organisms was tested by the Basic Local Alignment Search
Tool (BLAST-p, NIH, https://blast.ncbi.nlm.nih.gov). Subsequently, each selected homolog was aligned using the ClustalX 2.1 alignment tool in order to identify the degree of similarity between the different organisms. Drosophila and human sequences of the complex I LYR family (as defined by Uniprot) were retrieved. We retained for the analysis only reviewed human sequences as well as all Drosophila melanogaster entries. The resulting set has 11 human and 7 fly proteins. In fact, fly sequences were originally 8, but the unreviewed entry A0A0F6QCW0_DROME is identical to the reviewed one BCN92_DROME. All the proteins belong to the Pfam family PF05347 except for SDHF3_HUMAN and SDHF3_DROM of the PF01233 family. Both families are related and are part of the Complex I_LYR-like superfamily.

**Generation of transgenic lines**

A Nt and Ct FLAG-tagged version of Osi (with a 6xGly linker between Osi and the Flag) and an untagged version of OSI were cloned into pUAStattB and targeted to the 86F8 recombination site on the 3rd chromosome. Additionally, a human ETRF5/ LYRm5 and anosi sequence with changes in codon frequency in order
to prevent silencing through the RNAi machinery (Osi silent mutations- osiSM) were cloned into pUAStattB and targeted to the 28E7 recombination site on the 2nd chromosome.

For the generation of the pUAST-osi, pUAST-osi(flag) and pUAST-(flag)osi constructs, primers flanking the gene were used to amplify the desired sequence from larval cDNA and cloned in a pCR Blunt II-TOPO vector (450245, Thermo Fisher Scientific, USA) to be later subcloned in a pUAStattB vector (Catalog number 1419, Drosophila Genomics Resource Center, USA) by EcoR1 digestion sites. For the UAS-osi construct, the forward primer contained a Drosophila Kozak sequence before the ATG (Fw1), while the reverse primer contained a stop codon at the end (Fw2: 5′-GCCACCATGTCACAGCTGGCTGAAAG-3′; Rv1: 5′-TCAGTCATTGTAACLATAGCCTGCTG-3′). In the UAS-osi(flag) construct, we used the same forward primer while the reverse was divided in two primers used in two consecutive PCR reactions with the insertion of a 6xGly linker (Rv2) followed by the FLAG (Rv3) sequence (Fw1: 5′-GCCACCATGTCACAGCTGGCTGAAAG-3′; Rv2: 5′-GTAATCTCAATCCCTCCCCGTACTTGATACTCGGTGTCGCTGC-3′; Rv3: 5′-TCACTTTACATCATCTGCTTGTAAATCTCCACCCCCCGCTCCCCCC-3′). Similarly, the UAS-(flag)osi construct was designed to carry a 6xGly linker (Fw2)) and a FLAG following the kozak sequence (Fw3) in the forward primer, while the reverse (Rv3) one included a stop codon (Fw2: 5′-TAAGGGGGGAGCCGGGGGTTGGATCACAGCTGCGCTGAAAG-3′; Fw3: 5′-GCCACCATGTCACAGCTGGCTGAAAG-3′; Rv3: 5′-TCACTTTACATCATCTGCTTGTAAATCTCCACCCCCCGCTCCCCCC-3′).

The Drosophila codon-optimized version of human ETFR1/LYRm5 and the osiSM constructions were obtained from GeneScript (USA) and cloned separately in the pUAStattB vector by the EcoR1 and Xba1 digestion sites. A comparison of the original and codon-optimized sequences is detailed in Additional file 1: Fig. S1 in. Injections and selection of transgenic individuals were carried out by Best Gene (USA).

**Growth and survival curves**

For growth assessment, ten larvae per genotype aged 24h AEL were placed in standard agar plates (3% sucrose). Every 24h, larvae still alive were imaged to assess growth by measuring body size (see below) and transferred to a new plate. Experiments lasted as long as any mutant or RNAi-expressing individuals could be scored as alive. Survival rates were examined taking 50 larvae per genotype aged 24h AEL. Larvae that did not display a colored intestine were quantified as “clear gut” larvae.

**Larval morphology assessment**

To compare body size between larvae of different genotypes or experimental conditions, we used an Olympus DP71 camera attached to an Olympus BX10 stereo-scope to take pictures of the larvae at 24-h intervals starting with larvae aged 24h AEL. ImageJ was used to measure the area of the image occupied by the larva’s entire body. At the end of the experiment, the sclerotized mouth parts (“hooks”) were dissected and photographed for larval staging (employing an Olympus BX60 microscope).

**Colored food assay**

Young (7-day old) female flies were allowed to lay eggs for 4h. Twenty-five larvae of the corresponding age of each genotype were transferred to separate agar chambers with a patch of yeast paste (food) mixed with commercial blue food coloring. One hour later, the numbers of larvae inside or outside the colored food patch were recorded. Individual larvae were also photographed as described above. Larvae that did not display a colored intestine were quantified as “clear gut” larvae.

**Two-choice olfactory assay**

An olfactory attractive response was measured as described previously [22]. Groups of fifty 24h AEL larvae were placed in the center of an agar plate, where filter papers of 5 mm of diameter, soaked with an attractant (propionic acid) or a neutral compound (water), were placed on opposite sides of the dish. Larvae were photographed right after they were placed on the plate (initial time) and 5 min later (final time). We calculated the Response Index, defined as:

\[
RI = \frac{Na - Nn}{Na + Nn}
\]

where RI = response index, Na = number of larvae under 30 mm from the attractive stimulus, and Nn = number of larvae under 30 mm from the neutral stimulus. Positive RI represents attraction, while negative RI represents avoidance and RI near zero represents indifference. Each experimental group was tested 3 times.

**Mitochondrial extracts and functional assessments**

Mitochondrial extracts were obtained from 5 mg of either control (w1118) or homozygous osi100B 72h AEL larvae using the Mitochondria Isolation Kit for Tissue (89801, Thermo Fisher Scientific, USA) and the Dounce Homogenization for Hard Tissue protocol with trypsin.
pre-treatment. We modified the protocol and used only half of the volume of the different solutions needed. For protein quantification, we used the mitochondria lysis protocol as the manufacturer instructed, followed by the Pierce BCA Protein Assay (23227, Thermo Fisher Scientific, USA). Measurements were made with a microplate reader. From these samples, mitochondrial ATP production was measured with a commercial kit (Adenosine 5′triphosphate (ATO) bioluminescent assay kit; FLAAA-1KT, Sigma-Aldrich, USA) according to the manufacturer’s instructions. The experiment was performed 3 times independently.

Superoxide levels were assessed according to Owusu-Ansah and Banerjee as modified by Lim et al. [34, 63]. In the final step, glycerol was used instead of the ProLong Gold antifade reagent. Two independent assays were performed.

**Quantitative real-time reverse transcriptase (RT) PCR (qPCR)**

Twenty to thirty larvae (96 and 72h AEL respectively) were collected per replicate and homogenized with an Omni bead ruptor IV (USA) in 500 μl Trizol reagent (15596026, Thermo Fisher Scientific, USA) according to the manufacturer’s instructions in order to obtain total RNA. Next, synthesis of cDNA was performed from 1 μg of total RNA with the SuperScript III Reverse Transcriptase kit (18080093, Thermo Fisher Scientific, USA) according to the manufacturer’s instructions employing oligo(dt) and gene-specific primers for *tweek*, Lip3, Pepck1. qPCR was performed on a Stratagene Mx3000P (Agilent Technologies) using FastStart Universal SYBR Green Master (04913914001, Roche, USA) in a 10 μl reaction. The PCR reaction consisted of 40 cycles of a 15-s denaturation step at 95°C, a 15-s hybridization step at 60°C, and a 30-s extension step at 72°C. A minimum of three independently collected biological replicates were used in each experiment and the data was expressed as the ratio of each specific gene over *Rpl29*. The following primers were used: *rpl29* (fw 5′ GAACAAGAGCCCA TCCTCA3′; rev 5′ AGTACAGTGCTGTGCTTG3′); *Lip3* (fw 5′ TTCTTTCCGATGGGTGCTCAT3′; rev 5′ AACGTCATACATGCGATCTCGTT3′); *Pepck1* (fw 5′ AAGAAGGCTACATCAGCCGCCGCT3′; rev 5′ TCC CGCGAATCCATCTCCGCT3′; rev 5′ TGCTCTCTCTCAGTGGGCTCAT3′; rev 5′ TCTATGGTGTTGAAACCCCG3′; rev 5′ AACAACATCTCCGCTGACACTG3′; rev 5′ ATCCCTGCTCGTCTGTGGTT CAT3′).

**Dietary supplementation of NAC**

Antioxidant dietary supplementation was achieved by addition of 0.8 mg/ml N-acetyl-cysteine (NAC, Sigma-Aldrich). Twenty to thirty 24h AEL larvae were selected and placed in vials with normal food supplemented with NAC according to [64]. Survival was assessed every 24h. Three replicates of each genotype per experiment were carried out; two independent experiments were performed.

**Immunohistochemistry**

Larvae were dissected in dissection buffer (70mM Na,HPO₄, 30mM Na,HPO₄, 0.15 M NaCl, 0.3% Triton X-100, pH 7.4, -PT-) and fixed in 4% formaldehyde pH 7.4 in 100 mM PBS 1× for 45 min at RT. Larvae were rinsed three times in PT for 5 min. Non-specific binding sites were blocked by incubating the tissues for 30 min in 10% goat serum in PT. The tissues were incubated in the corresponding primary antisera at 4°C overnight. Primary antibodies were as follows: chicken anti-RFP (1/500 Rockland, USA), chicken anti-GFP (1/500 Aves, USA), and monoclonal anti Flag (1/500 Thermo Scientific, USA). Samples were washed 3 times in PT 0.3% for 5 min and incubated with the corresponding secondary antibodies (Cy2, Cy3- anti chicken, Cy5- anti mouse, Jackson ImmunoResearch) for 2h at RT. Secondary antibody incubations were stopped by replacing the solution with PT 3 times for 15 min each. For visualization of lipid droplets, 24, 48, and 72h AEL larvae were dissected as previously described and fixed in 4% formaldehyde pH 7.4 in 100 mM PBS 1× for 60 min at RT. Tissues were then incubated in BODIPY (1:500 D3922, Thermo Fisher Scientific) for 20 min at RT. Samples were then washed 3 times in PT 0.3% for 5 min and mounted.

If needed, samples were incubated with DAPI 1× and/or rhodamine-coupled Phalloidin 1× for 30 min and then rinsed 3 times for 5 min in PT. Finally, samples were mounted in 60% glycerol in PT. Images were taken with a Zeiss confocal microscope LSM 710.

**Mosaic analysis in the fat body**

The “Flip-out” technique (reviewed in [65]) was employed to investigate the effect of Osi loss of function in cell size (in the genotype hs-flip; act>STP>G4;UAS-gfp/UAS-osirNA1). A 5-min 37°C heat pulse in a water bath was used to activate the heat-shock flipase (hs-flip) in 24h AEL larvae, which in turn removed the stop cassette from an *act*-G4 driver, inducing the expression of green fluorescence marker (GFP) along with the construct of interest in a subset of cells. Fat bodies from 72h post heat-shock larvae were dissected and stained for GFP (as detailed above) and phalloidin red (1:100, R415, Sigma-Aldrich, USA) to mark cellular boundaries.

Both control (GFP−) and experimental (GFP+) cells, expressing the construct of interest, were measured using ImageJ. Measurements were normalized to the average...
cell size of control cells (inactive). We used R-studio to plot frequency distribution graphs for each genotype using function

\[
\text{hist(data$Genotype-Activation\ state, xlim=range(0:2), ylim=range(0:4), prob=TRUE)}
\]

We dissected 3 independent larvae of each genotype. Images were taken with a Zeiss LSM 710 confocal microscope. We quantified cellular area using ImageJ.

**Quantitative analysis of lipid droplets**

To assess lipid droplets size and shape 24, 48, and 72h AEL, larval fat bodies were dissected, fixed, and stained with BODIPY. Images were taken with a Zeiss LSM 880 confocal microscope. Each image was assigned a random numeric code in order to quantify the different aspects of each image in blind. To standardize the measurements among the different groups, we randomly selected a representative squared area (50μm) per image, to which all measurements were then normalized. The area span by individual lipid droplet, their roundness and percentage of reactive area were measured with ImageJ as previously described [66, 67]. Briefly, LD were delimited by free hand with the selection tool and then the area and roundness were assessed; to define the percentage of reactive area the threshold tool was employed to generate a mask representing the total area of the fat body occupied by LDs. GraphPad Prism 9.0 was used to plot and quantify the data and to construct the LD size distribution histograms.

**Respiratory assays**

The Agilent Seahorse XFp metabolic analyzer was set to a working temperature of 25°C. An Agilent Seahorse XFp cartridge (Agilent, 103721-100) was hydrated with 200 μl of calibrant solution (Agilent, 100840-000) overnight at 25°C. The next day, 20 larvae (72h AEL) for each well were dissected in phosphate buffered solution (PBS) and added to the bottom of an Agilent 8-well cell plate (Agilent, 103721-100), centered in the middle between the three raised spheres, with the help of a drop of Vetbond (3M) tissue adhesive to keep them in place. Each well was then filled with 50 μl of AHL for the mitostress and 50 μl of Agilent Seahorse assay media for the gluco-stress with the corresponding supplements required for the specific assays. Then, 150 μl of assay media was added to each well, resulting in a total of 200 μl final in each well. Cell plate was then placed on the tray of the XFp analyzer. The instrument was used as is for typical cell assays with all cycle procedures consisting of 3 min mixing, 0 min waiting, and 3 min measuring.

**Mitostress assay**

Analysis of mitochondrial respiration was conducted in Agilent Seahorse XF AHLL medium (adult hemolymph like saline containing 108 mM NaCl, 5 mM KCl, 2 mM CaCl2, 8.2 mM MgCl2, 4 mM NaHCO3, 1 mM NaH2PO4, 5 mM trehalose, 10 mM sucrose, 5 mM HEPES, pH 7.5, [68]). Basal larvae OCR was measured for 3 cycles prior to oligomycin injections. Twenty-five microliters of 100 μM oligomycin was added to injection port A, resulting in a final concentration of 10 μM of oligomycin/ well. Twenty-five microliters of 70 μM Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) was added to port B and injected after the 6th cycle, resulting in a final concentration of 7 μM FCCP. Twenty-five microliters of 100 μM Rotenone/Antimycin A were added to port C and injected after the 9th cycle, resulting in a final concentration of 10 μM Rotenone/Antimycin A.

**Glycostress assay**

Analysis of larval glycolytic function, by directly measuring the extracellular acidification rate (ECAR), was conducted in base medium (Agilent, 103334-100 base medium) supplemented with 1 mM glutamine. Samples were starved for 60 min prior to testing. Twenty-five microliters of 50 mM glucose was added to port A and injected at the 6th cycle, resulting in a final concentration of 5 mM glucose. Twenty-five microliters of 100 μM oligomycin was added to port B and injected at the 11th cycle, resulting in a final concentration of 10 μM oligomycin. Twenty-five microliters of 1 M 2- deoxyglutарате (2-DG) was added to port C and injected at the 23rd cycle, resulting in 100 mM 2-DG.

**Lipidome analysis**

All solvents were at least HPLC grade. Water, 2-propanol, and phosphoric acid were purchased from Roth (Karlsruhe, GER), methanol from J.T.Baker (Austin, TX, USA), formic acid, TAG 45:0 and TAG 51:0 from Sigma (Vienna, AUT), and ammonium acetate from Merck (Darmstadt, GER). Glass beads (0.45–0.50 mm) were from SiLi (Warmensteinach, GER).

**Sample preparation**

Approximately 50 μl of glass beads and 400 μl of isopropanol were added to 50 frozen larvae in the Eppendorf tubes. The larvae were disrupted by shaking the tubes at 4°C in a MM 40 homogenizer (Retsch, Haan, GER) for 20 min at 30 Hz. Lipid extraction was done according to the method described by [69]. In brief: The isopropanol was removed in a speed vac and 700 μl MTBE/methanol (10/3, v/v) containing TAG 45:0 and TAG 51:0 internal standards. After shaking at 4°C for 60 min with a
Thermomixer (Eppendorf), 400 μl water was added and shaking continued for another 15 min. After centrifugation (15 min, 13,000 rpm), 50 μl of the organic upper phase was transferred to an autosampler vial, dried under a stream of nitrogen and re-dissolved in 400 μl isopropanol/methanol/water (30/15/5, v/v/v) for LC-MS analysis. The remaining organic upper phase (approximately 350 μl) was collected, dried under nitrogen and stored at −80°C.

Protein analysis
The remaining aqueous phase was dried in a speed vac. Then, 400 μl of 0.1 N NaOH were added, and after shaking with the Retsch Mill (10 min at 30 Hz), the samples were incubated for 6 h at 60°C. Twenty microliters was used for protein analysis with the Pierce™ BCA protein assay kit (Thermo Fisher, Vienna, AUT).

Lipid analysis
Chromatographic separation was performed using a 1290-UHPLC system (Agilent, Waldbronn, GER) equipped with a BEH-C18-column, 2.1 × 150 mm, 1.7 μm (Waters, Manchester, UK). The autosampler compartment was set to 8°C and 1 μl sample was injected. A binary gradient was applied. Solvent A was water, solvent B was 2-propanol. Both solvents contained phosphoric acid (8 μM), ammonium acetate (10 mM), and formic acid (0.1 vol%). The linear gradient started at 50% solvent B at a constant flow rate of 0.15 ml/min and increased to 100% solvent B within 22 min. In the following, 2.5 min solvent B percentage was kept at 100%. The column was re-equilibrated for 5 min, resulting in a total HPLC run time of 30 min. The column compartment was kept at 50°C. A 4670 triple quadrupole mass spectrometer (Agilent) equipped with an ESI source was used for analysis. The following source parameters were used: source temperature 300°C, sheath gas (N2) temperature 400°C. The capillary voltage was 3.5 kV in positive ionization mode. The following source parameters were used: source temperature 300°C, sheath gas (N2) temperature 400°C. The capillary voltage was 3.5 kV in positive ionization mode. A 4670 triple quadrupole mass spectrometer (Agilent) equipped with an ESI source was used for analysis. The following source parameters were used: source temperature 300°C, sheath gas (N2) temperature 400°C. The capillary voltage was 3.5 kV in positive ionization mode.

Dynamic MRM scans (For a detailed MRM list see Additional file 1: Supplementary Table S2). List of fly stocks, antibodies and fluorescent dyes used throughout this study. Figure S1. Sequence comparison of osi100B and ETFRF1/LYRm5. Alignment of the original ETFRF1 and osi mRNA sequences, compared to the version optimized for expression in Drosophila. Table S2. List of dynamic MRM transitions. It includes the list of dynamic MRM transitions.

Additional file 2 It includes all datasets and statistical analysis on which the conclusions are based. Figure 1. P(UAS) insertional mutant exhibits abnormal food-seeking behavior. Figure 2. P(UAS)100B is inserted in CG6115 and encodes a Complex I LVR domain containing protein. Figure 3. A reduction in osi levels affects viability. Figure 4. osi down-regulation is associated with a smaller cell size. Figure 5. ETFRF1/LYRm5 rescues cellular and viability defects associated with osi knockdown. Figure 6 Expression of human ETFRF1/LYRm5 rescues lethality associated with osi downregulation in the fat body. Figure 7. A key role for osi in the fat body. Figure 8. osi loss of function produces a metabolic defect not rescued by antioxidants. Figure 9. Preventing excessive fat body lipid catabolism rescues the behavioral effect triggered by osi downregulation. Figure 10. osi mutants have a progressive shift in LD size. Figure 11. Reduced osi levels alters the storage lipid content and composition. Figure 12. A model for Osi function.

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Authors' contributions
MFA, JIR, GB, GMVC, NG, MLM, SW, LAAV, GNR, RPK, CMB, RC, CR, and MFC designed, acquired, and interpreted the data. MFA, JIR, and MFC drafted the manuscript. GNK, RPK, RC, CR, and MFC substantively revised the manuscript. All authors read and approved the final manuscript.

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in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files. Any other data can be requested from the corresponding author.

Declarations

Ethics declarations and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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