The *Drosophila* PGC-1 homologue Spargel coordinates mitochondrial activity to insulin signalling

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Mitochondrial mass and activity must be adapted to tissue function, cellular growth and nutrient availability. In mammals, the related transcriptional coactivators PGC-1 α , PGC-1 β and PRC regulate multiple metabolic functions, including mitochondrial biogenesis. However, we know relatively little about their respective roles *in vivo*. Here we show that the *Drosophila* PGC-1 family homologue, Spargel, is required for the expression of multiple genes encoding mitochondrial proteins. Accordingly, *spargel* mutants showed mitochondrial respiration defects when complex II of the electron transport chain was stimulated. Spargel, however, was not limiting for mitochondrial mass, but functioned in this respect redundantly with Delg, the fly NRF-2 α /GABP α homologue. More importantly, in the larval fat body, Spargel mediated mitochondrial activity, cell growth and transcription of target genes in response to insulin signalling. In this process, Spargel functioned in parallel to the insulin-responsive transcription factor, dFoxo, and provided a negative feedback loop to fine-tune insulin signalling. Taken together, our data place Spargel at a nodal point for the integration of mitochondrial activity to tissue and organismal metabolism and growth.

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Introduction

As animals grow, nutrients are taken up, leading to an increase in cellular mass. In this process, mitochondria have critical anabolic and catabolic functions in metabolizing nutrients and in adapting cellular physiology. Therefore, one would expect coordination of mitochondrial mass and activity with growth-promoting pathways and nutrient availability, yet this remains poorly understood. To study mitochondrial biogenesis, most studies focused on individual tissues that show an enormous increase in mitochondrial mass in response to external stimuli, for example, during the

formation of the brown adipose tissue (BAT) or perinatal heart maturation. Such studies led to identification and characterization of the PGC-1 family of transcriptional coactivators, which are potent inducers of mitochondrial biogenesis: the founding member PGC-1 α (PPAR γ coactivator 1; Puigserver *et al*, 1998), as well as its homologues PGC-1 β (Kamei *et al*, 2003) and PRC (PGC-1-related coactivator; Andersson and Scarpulla, 2001). Mice lacking PGC-1 α or PGC-1 β showed reduced expression of multiple genes encoding mitochondrial proteins, yet mitochondrial mass and respiration activity were either not or only modestly reduced, depending on the tissue (Lin *et al*, 2004; Leone *et al*, 2005; Lelliott *et al*, 2006; Sonoda *et al*, 2007). These mild phenotypes could be because of redundancy. Indeed, RNAi-mediated downregulation of PGC-1 β in a PGC-1 α ^{-/-} background led to strong additive respiration defects in adipocytes (Uldry *et al*, 2006). Similarly, mice lacking both PGC-1 α and PGC-1 β showed defective mitochondrial biogenesis in the heart and the BAT (Lai *et al*, 2008). Although these studies clearly demonstrated critical functions for these proteins in mitochondria-rich tissues, a triple knockout of PGC-1 α , PGC-1 β and PRC has not been published; therefore it remains unclear whether PGC-1s are generally required for basal mitochondrial mass.

PGC-1 family members drive mitochondrial biogenesis through coactivation of nuclear transcription factors, including nuclear respiratory factor-1 and -2 (NRF-1 and NRF-2), estrogen-related receptor- α (ERR α) and YY1, to enhance the expression of genes encoding mitochondrial proteins (Puigserver and Spiegelman, 2003; Scarpulla, 2008). Accordingly, NRF-1 and ERR α are known to be functionally important for PGC-1s to stimulate mitochondrial mass (Wu *et al*, 1999; Mootha *et al*, 2004; Schreiber *et al*, 2004). Similarly, NRF-2 promoter-binding sites were required for coactivation by PGC-1 α and PRC in certain genes (Gleyzer *et al*, 2005), and PRC can coactivate NRF-2 β (Vercauteren *et al*, 2008). However, it is not known whether NRF-2 is required for PGC-1's effect on mitochondrial function, or whether NRF-2 is controlled through other factors.

In flies and mammals, insulin signalling and TOR (target of rapamycin) function are known to link cellular growth and metabolism to nutrition (Grewal, 2008; Polak and Hall, 2009). Recent data showed that mammalian TOR (mTOR) and mitochondrial oxidative capacity are tightly linked (Schieke *et al*, 2006), and mTOR inhibition reduced the association of PGC-1 α with the transcription factor YY1, leading to lower expression of several genes encoding mitochondrial proteins (Cunningham *et al*, 2007). These data demonstrate that mTOR is a crucial regulator of mitochondrial function in mammals. In flies, nutrient starvation and subsequent inhibition of insulin signalling led to reduced expression of multiple genes encoding mitochondrial proteins (Zinke *et al*, 2002; Gershman *et al*, 2007; Teleman *et al*, 2008). However, a direct

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role of TOR has not been addressed in these studies. Moreover, the majority of genes encoding mitochondrial proteins were not responsive to TOR inhibition in *Drosophila* cells (Guertin *et al*, 2006). Therefore, mechanisms in addition to TOR must exist to adapt mitochondrial mass and function to nutrient availability.

We investigated the role of the *Drosophila melanogaster* PGC-1 homologue Spargel/CG9809 in the control of mitochondrial biogenesis and activity. The *Drosophila* genome encodes a single PGC-1 homologue (Gershman *et al*, 2007), thus providing a system in which PGC-1 function can be analysed without interfering redundancy. We focused on the larval fat body, the functional equivalent of the mammalian adipose tissue and liver (Baker and Thummel, 2007; Leopold and Perrimon, 2007). In this tissue, many genes encoding mitochondrial proteins were expressed in a nutrient-sensitive manner (Teleman *et al*, 2008; Baltzer *et al*, 2009). The larval fat body is, therefore, ideal to study how mitochondrial mass and activity are coordinated with cellular metabolism and nutrient supply. Here, we show that Spargel is required for proper expression of most genes encoding mitochondrial proteins. When complex II of the electron transport chain is stimulated, *spargel* mutants show respiration defects. Remarkably, Spargel is not required for basal mitochondrial mass, but becomes limiting in the absence of Delg, the fly NRF-2 α homologue. Moreover, from these and previous experiments (Baltzer *et al*, 2009), Spargel and Delg were shown to function in two different pathways, each regulating mitochondrial in response to nutrients. Finally, we addressed the question how insulin signalling affected mitochondria, and found that Spargel is required for the stimulation of mitochondrial respiration, and to a large extent for the transcriptional control mediated by insulin signalling, including that of genes encoding mitochondrial proteins. Moreover, insulin signalling induces Spargel gene expression and protein levels, and Spargel mediates a negative feedback loop on insulin signalling. Our data demonstrate a critical role for Spargel in the coordination of mitochondria with nutrients, and thus in cellular metabolism.

Results

The *Drosophila* PGC-1 homologue Spargel is required for growth

The *D. melanogaster* genome encodes a single PGC-1 homologue, CG9809 (Gershman *et al*, 2007). Sequence alignments have shown that the N-terminal acidic domain that mediates transcriptional coactivation for mammalian PGC-1s, and the C-terminal arginine-serine-rich and RNA recognition domains are highly conserved in the fly protein (Gershman *et al*, 2007), yet its cellular function has not been addressed. To test whether CG9809 is a functional PGC-1 homologue in flies, we analysed mutants that have a P-element insertion (KG08646) in the 5' UTR (Figure 1A). In comparison with controls (precise excision of the P-element), homozygous mutant larvae had a strong reduction in CG9809 mRNA levels (Figure 1B), adults were viable, and females were sterile. Both males and females had a 25% reduction in wet weight (Figure 1C and Supplementary Figure S1F), which correlated with reduced protein, lipid, glycogen and trehalose levels per animal (Supplementary Figure S1G). When normalized to body weight, reduced lipid and glycogen levels were observed in adult males,

demonstrating metabolic defects (Supplementary Figure S1H). When external structures that are derived from imaginal discs were analysed, such as wings or legs, we did not observe large size defects in CG9809 mutants (Supplementary Figure S1A–E). These animals have, therefore, a lean phenotype, prompting us to term CG9809 'Spargel', German for 'asparagus', and the KG08646 allele as *srl*¹. To test the specificity of this allele, we used a second P-element insertion (d04518, termed *srl*²), which showed the same phenotypes as *srl*¹ (Figure 1B and C), and created transgenic flies carrying a genomic rescue construct (*Srl*^{GR}; Figure 1A), which suppressed all mutant phenotypes (Figure 1D and data not shown). As another gene, CG31525, is located within the first intron of *Spargel*, we also created a fly line expressing a *Spargel* cDNA under the control of the UAS promoter. When driven using heat-shock Gal4, UAS-*Srl* also suppressed the mutant phenotypes (Figure 1E and data not shown), demonstrating that loss of Spargel function was responsible for the observed phenotypes. Finally, a trans-heterozygous combination of *srl*¹ with Df(3R)ED5046, a deficiency that deletes the Spargel locus, led to a further reduction in *Spargel* transcript levels (Figure 1B), yet it did not lead to a further decrease in adult weight compared with *srl*¹ homozygous mutant animals (Figure 1C). Taken together, *srl*¹ is a strong hypomorphic allele, showing a ~75% reduction in mRNA levels.

***Drosophila* Spargel is required for proper expression of multiple genes encoding mitochondrial proteins**

To address a likely Spargel function as a transcriptional regulator, we performed genome-wide microarray analysis using dissected fat bodies. Genes encoding mitochondrial proteins are highly expressed during the larval feeding period but are repressed at the end of the last (third) instar, as the animals stop feeding and prepare for pupation (White *et al*, 1999; Arbeitman *et al*, 2002). Whereas control larvae enter the third instar 72 h after egg deposition (AED) and pupate 120 h AED, *spargel* mutants enter with a 12–14-h delay, and pupate with a 24-h delay (144 h AED; see Supplementary Figure S2). To avoid artefacts due to different developmental stages, mid-third instar larvae were taken for all experiments (96 h AED for control, and 120 h AED for *spargel*). As summarized in Table I, GO annotation showed that genes involved in several mitochondrial functions, in particular oxidative phosphorylation (OXPHOS; mostly electron transport complexes I, II and V), were expressed at reduced levels in the *spargel* mutant. In total, 44% of all nuclear genes encoding mitochondrial proteins were >1.5-fold downregulated in the *spargel* mutant fat body. In addition, few non-mitochondrial functions were downregulated, including translation, gene expression and RNA biology, suggesting that Spargel functions as a transcriptional regulator. As mammalian PGC-1 proteins are best characterized in respect to mitochondrial biogenesis, we focused on Spargel's role in respect to mitochondria.

First, we verified our microarray data by quantifying mRNA levels of selected genes using qRT-PCR on dissected larval fat bodies: *mtACPI*, (mitochondrial acyl carrier protein of electron transport complex I), *Scs-fp* (succinyl CoA synthetase flavoprotein subunit of complex II), *RFeSP* (Rieske iron-sulfur protein of complex III), *CoVa* (subunit Va of complex IV), *Bellwether* (*Blw*; ATP synthase alpha subunit of complex V), *Isocitrate dehydrogenase* (*Idh*; tricarboxylic acid (TCA)

Figure 1 Mutants of *Spargel* (CG9809), the *Drosophila* homologue to mammalian PGC-1 transcriptional coactivators, show growth defects. **(A)** Representation of the *Spargel* genomic locus. Black boxes represent coding regions of the respective genes (CG9809, CG31525 or *elF3-S10*). Grey boxes indicate 5' and 3' untranslated regions of *Spargel*. The P-element insertion sites of *srl*¹ and *srl*² loss-of-function alleles are indicated as white triangles. The genomic rescue construct (*Srl*^{GR}; 8700 bp) is represented as a hatched bar. **(B)** *Spargel* transcript levels determined by qRT-PCR. mRNA was isolated from whole, mid-third instar larvae: +/+ : 4dAED; *srl*^{1/1}, *srl*^{2/2} and *srl*¹/Df(3R)5046: 5dAED. Expression was normalized to *Actin5C* (CG4027) and +/+ was set to 1. **(C)** Wet weight from adult males, genotypes as indicated. **(D)** Complete rescue of the reduced weight phenotype of adult *srl*^{1/1} mutants by the genomic rescue construct, *Srl*^{GR}, or with (E) hs-Gal4 driven UAS-*Spargel* (UAS-*Srl*) with one 1.5-h heat shock (37°C) per day. For **(E)**, no rescue is seen in the absence of heat shocks (data not shown). **(C-E)** For all weight measurements, adult males were taken 2 days after eclosion. Number of flies per genotype ≥10.

cycle), and *Cyt-c-p* (encoding cytochrome *c*) were all expressed at significantly lower levels in *spargel* mutants, but not *Glutamate dehydrogenase* (*GDH*; amino-acid metabolism; Figure 2A). As explained above, we allowed *spargel* mutant larvae to grow for additional 24 h before dissection compared with controls. To test whether this shift in developmental timing would influence our results, we isolated fat body-specific mRNA from *spargel* mutants at the same timing as controls (96 h AED). Again *RFeSP* and *Idh* levels were strongly reduced compared with control (data not shown), demonstrating that the effects seen were not due to differential timing.

Spargel and the NRF-2 α homologue Delg share many putative target genes

Mammalian PGC-1 proteins drive gene expression by coactivating multiple transcription factors, including NRF-2 (Puigserver and Spiegelman, 2003; Scarpulla, 2008). *Drosophila* Delg is the structural and functional homologue to mammalian NRF-2 α , and multiple genes encoding mitochondrial proteins are expressed at lower levels in *delg* mutants (Baltzer *et al*, 2009). We therefore used *Drosophila* Spargel and Delg as a system to study the functional interaction of PGC-1 proteins with NRF-2. First, we compared our fat body-specific microarray data of *spargel* with *delg* single mutants (Baltzer *et al*, 2009) in more detail (Figure 2B and Supplementary Table S1). When focusing on genes encoding mitochondrial proteins that are downregulated in either the *spargel* or the *delg* mutant, about half (88 genes) overlapped, including many OXPHOS and TCA cycle genes. In contrast, 46 genes were downregulated in the *spargel*, but not in the *delg* mutant. These genes function in electron transport (complex I), DNA and RNA metabolism and mitochondrial

protein synthesis and targeting. In addition, 27 genes were affected in a Delg-specific manner, including genes required for amino-acid and fatty-acid metabolism (Figure 2B and Supplementary Table S1). This demonstrates that Spargel and Delg share many putative target genes, but also affect transcription independently of each other.

To study the Spargel-Delg interaction in more detail, we dissected mid-third instar larval fat bodies from *delg* single and *spargel-delg* double mutants, and measured transcript levels of the putative Spargel target genes using qRT-PCR. All genes tested above were also expressed in a Delg-dependent manner. Importantly, these genes did not show a further decrease in the *spargel-delg* double mutant when compared with single mutants (Figure 2A). We conclude that Spargel and Delg have a common role in the expression of many genes encoding mitochondrial proteins, possibly through Spargel-mediated coactivation of Delg. At the same time, either factor is required for expression levels of a subset of these genes independently of the other.

Spargel and Delg function in parallel pathways in respect to mitochondrial mass

To test whether the reduced expression of genes encoding mitochondrial proteins would result in reduced mitochondrial abundance, we used MitoTracker, a mitochondrial-specific dye. In the larval fat body of control animals, mitochondrial staining was abundant throughout the cytoplasm. In *spargel* mutants, we detected no reduction in staining (Figure 3A and B). On the contrary, *delg* mutants showed a strong reduction in mitochondrial staining, in which residual mitochondria are concentrated around the nucleus (Baltzer *et al*, 2009). When quantified, *delg* mutants had a 24% decrease in MitoTracker staining (Figure 3B).

Table I GO term enrichment for genes that were significantly up- or downregulated (>1.5 -fold) in the *srl^{1/1}* mutant fat body compared with wild-type

GO ID	P-value	Term
<i>GO Biological Process, $>1.5 \times$ down in <i>srl^{1/1}</i> mutant fat body ($P<0.05$)</i>		
<i>Mitochondrial biogenesis and function</i>		
GO:0022900	3.45E-25	Electron (e ⁻) transport
GO:0006119	8.14E-24	Oxidative phosphorylation
GO:0042773	4.67E-23	ATP synthesis coupled e ⁻ transport
GO:0006091	6.81E-22	Generation of precursor metabolites and energy
GO:0006120	2.18E-14	Mitochondrial e ⁻ transport, NADH to ubiquinone
GO:0006839	1.80E-03	Mitochondrial transport
GO:0007005	1.57E-02	Mitochondrial organization
GO:0006123	2.03E-02	Mitochondrial e ⁻ transport, cytochrome <i>c</i> to oxygen
<i>Transcription and translation</i>		
GO:0006412	5.83E-49	Translation
GO:0010467	2.91E-33	Gene expression
GO:0019538	5.82E-15	Protein metabolic process
GO:0042254	1.52E-30	Ribosomal biogenesis and assembly
GO:0016072	1.27E-18	rRNA metabolic process
GO:0006457	6.45E-03	Protein folding
GO:0055086	1.04E-04	Nucleobase, nucleoside and nucleotide metabolic process
<i>Cell cycle</i>		
GO:0051231	1.15E-18	Spindle elongation
GO:0007052	4.69E-12	Mitotic spindle organization and biogenesis
GO:0000226	3.93E-06	Microtubule cytoskeleton organization/biogenesis
GO:0000278	3.98E-05	Mitotic cell cycle
<i>Cellular metabolic processes</i>		
GO:0008152	4.31E-36	Metabolic process
GO:0044249	3.04E-18	Cellular biosynthetic process
GO:0009059	2.08E-17	Macromolecule biosynthetic process
GO:0044267	5.42E-15	Cellular protein metabolic process
GO:0006996	2.67E-06	Organelle organization and biogenesis
<i>GO Biological Process, $>1.5 \times$ up in <i>srl^{1/1}</i> mutant fat body ($P<0.05$)</i>		
<i>Developmental process</i>		
GO:0007594	5.55E-03	Puparial adhesion
GO:0007591	4.60E-02	Molting cycle, chitin-based cuticle
<i>Lipid metabolism</i>		
GO:0016042	5.74E-05	Lipid catabolic process
GO:0006635	9.40E-04	Fatty acid β -oxidation
GO:0044242	1.44E-04	Cellular lipid catabolic process
<i>Others</i>		
GO:0006810	1.03E-03	Transport
GO:0051179	3.82E-03	Localization

GO terms that are significantly enriched (P -value <0.05) are shown.

Importantly, *spargel-delg* double mutants had a more severe phenotype compared with the *delg* single mutant, in which only few mitochondria are stained per cell. On average, these cells had a 56% reduction in MitoTracker staining compared with control or *spargel* single mutant cells (Figure 3B). To complement the MitoTracker stainings, we used NAO that specifically labels the mitochondrial phospholipid cardiolipin. Again we observed reduced mitochondrial abundance in the *delg*, but not the *spargel* mutant, and a more severe phenotype in the double mutant (Supplementary Figure S3A). To assess mitochondrial morphology, we used electron microscopy. Single mutants of Spargel or Delg did not show obvious morphological defects. In contrast, *spargel-delg* double mutants were rounded in shape, and inner-mitochondrial cristae were either missing or strongly reduced in size (Figure 3C and Supplementary Figure S3B). These data demonstrate that Spargel is not required for mitochondrial morphology and abundance under normal conditions, but

becomes limiting in the absence of Delg. In addition, we observed enlarged lipid droplets in *spargel* mutant fat body cells. This phenotype has been described previously in studies on mobilization of lipid stores in response to nutrient starvation (Zhang *et al*, 2000; Colombani *et al*, 2003). Accordingly, GO annotations of microarray data demonstrated that genes involved in fatty-acid oxidation were increased in *spargel* mutants (Table I). Yet, larval lipid content was not altered (data not shown), thus further experiments are required to address a direct role for Spargel in this process.

Respiration defects in *spargel* mutant fat bodies

Mitochondria are best known to generate energy from nutrients through oxidative phosphorylation. During this process, NADH and FADH₂, which are derived from the mitochondrial TCA cycle and fatty-acid oxidation, stimulate the electron transport chain at complex I (NADH dehydrogenase) and II (succinate dehydrogenase), respectively.

Figure 2 Spargel and the NRF-2 α homologue Delg share many putative target genes. (A) Larval fat bodies were dissected and mRNA levels of the genes indicated were determined using qRT-PCR (at least three biological replicates). Mid-third instar larvae were taken: 4d AED for +/+, 5d AED for *spargel*, 6d AED for *delg* and 8d AED for *spargel-delg* double mutant. Transcript levels were normalized to *gammaTubulin23C* (*gTub*, CG3157). In all cases, *delg*^{-/-} refers to *delg*⁶¹³/Df(3R)ro^{80b}. Significance is indicated as compared with the control sample. (B) Comparison of microarray data from *srl*^{1/1} and *delg*^{-/-} single mutants using mRNA from fat bodies dissected from mid-L3 larvae. Overlap of all nuclear-encoded mitochondrial genes that are downregulated >1.5 $\times$. Processes that are regulated in a Spargel- or Delg-specific manner, respectively, as well as overlapping gene sets are indicated below.

Electrons are transferred through complex III (cytochrome bc₁) to complex IV (cytochrome *c* oxidase), which consumes oxygen as the final electron acceptor. This generates a proton gradient across the inner-mitochondrial membrane, which is dissipated either at complex V, producing ATP, or through uncoupling proteins. To measure oxidative phosphorylation capacity, we had previously adapted an assay using dissected and digitonin-permeabilized fat bodies (Baltzer *et al*, 2009). Strikingly, we observed severe respiration defects in *spargel* mutants when complex II was stimulated: oxygen consumption was strongly reduced after the addition of the substrate succinate (state 2), ADP stimulation (state 3) and the addition of atractyloside (an ADP/ATP transporter inhibitor; state 4). In contrast, when oxygen consumption was measured in the presence of cyanide, a potent complex IV inhibitor, we did not observe a difference between *spargel* mutants and controls, demonstrating that non-mitochondrial oxygen consumption was not affected (Figure 3D). This correlates with mammalian data that showed reduced complex II-mediated respiration in tissues from PGC-1 α - or PGC-1 β -knockout mice (Leone *et al*, 2005; Lelliott *et al*, 2006). Next, we repeated these assays using pyruvate and proline, as well as glutamate and malate as substrates, which all stimulate complex I of the electron transport chain. Surprisingly, we did not detect any differences in respiration between *spargel* mutants and control fat bodies (Figure 3D and data not shown). We conclude that complex II must be particularly affected in the *spargel* mutant. Accordingly, genes encoding three of the four complex II subunits were expressed at lower levels in *spargel* mutants (Supplementary Table S1). Nonetheless, these results are surprising given the reduced expression of other electron transport complexes. However, these data are similar to those from *delg* mutants, which showed comparable low expression levels of many OXPHOS genes, yet did not have

respiration defects when complex I was stimulated (Baltzer *et al*, 2009). As postulated before, post-transcriptional mechanisms might exist to compensate for reduced expression rates (Baltzer *et al*, 2009). Alternatively, factors that are rate-limiting for complex I activity might not be affected in *spargel* or *delg* single mutants.

Given the strong synergistic functions of Spargel and Delg in respect to mitochondrial morphology and abundance, we anticipated respiration defects in the *spargel-delg* double mutant, which are not seen in either of the single mutant. Indeed, compared with heterozygous controls, respiration was strongly defective when complex I was stimulated using pyruvate and proline (Figure 3E). A similar strong defect was also observed when complex II was stimulated (data not shown). As an additional control, we re-expressed Spargel using the genomic rescue construct in the double mutant background. This led to suppression of the phenotype (Figure 3E). Importantly, not all genes involved in electron transport required Spargel for proper expression. Of the Spargel-independent genes, some showed lower expression in the *delg* mutant (Supplementary Table S1). The synergistic respiration defects of the double mutant, compared with single mutants, can therefore be explained by a more complete downregulation of electron transport genes.

To further characterize OXPHOS activity, we quantified the mitochondrial DNA (mtDNA), which encodes several factors required for electron transport, and levels of which correlate with OXPHOS activity (Rocher *et al*, 2008). When normalized to nuclear DNA, we did not detect any change in the *spargel* single mutant, but increased levels were observed in *delg* single and *spargel-delg* double mutants (Figure 3F; Baltzer *et al*, 2009). Importantly, this did not lead to enhanced mtDNA transcription, as we detected reduced transcript levels of mitochondria-encoded COX subunit I (Figure 3G).

Figure 3 Spargel is required for mitochondrial respiration and functions in parallel to Delg. **(A)** Mitochondria-specific MitoTracker stainings of larval fat bodies, 5d AED. DAPI staining is shown in insets. Bar equals 20 μ m. **(B)** Quantitation of **(A)**. Images were quantified in blind using ImageJ, $n \geq 20$. **(C)** Electron microscopy images of larval fat bodies from mid-third instar larvae. Bar equals 400 nm. **(D)** Respiration assays using dissected, digitonin-permeabilized fat bodies. For complex I stimulation, serial addition of pyruvate and proline (state 2), ADP (state 3), atractyloside (state 4) and KCN was used. For complex II stimulation, rotenone was added to inhibit complex I, and respiration was stimulated by succinate (state 2). **(E)** Respiration assay as in **(D)**, but KCN values were subtracted (see Supplementary Figure S3C for the same chart without subtraction). For **(D)** and **(E)**, oxygen consumption was normalized to fat body protein content. Averages and s.d. values of three biological replicates are shown. **(F)** Mitochondrial DNA (mtDNA) content was quantified by qRT-PCR and normalized to nuclear DNA (nDNA) levels from dissected fat bodies (six biological replicates). **(G)** Mitochondrial-encoded COX level (subunit I) was determined by qRT-PCR from RNA isolated from dissected fat bodies (three biological replicates). For experiments in **(C–G)**, mid-third instar larvae were taken as in Figure 2. **(H)** Shown are larvae 4d AED. Bar equals 1 mm. In all cases, *delg*^{−/−} refers to *delg*⁶¹³/Df(3R)ro^{80b}. Significance is indicated as compared with the control sample.

Given the general correlation between mtDNA replication and transcription in mammalian cells, this seems surprising. However, a similar discrepancy has been observed recently on downregulation of mitochondrial transcription factor B2 (Adan *et al*, 2008). In addition to the additive transcription defect in the nucleus described above, the reduced OXPHOS capacity in the *spargel-delg* double mutant might also be caused by lower rates of mitochondrial transcription. In summary, these data demonstrate that although Spargel and Delg might function together for the expression of certain genes, these two factors function independently of each other when mitochondrial activity was assayed.

As mentioned above, microarray analysis revealed that genes involved in translation, gene expression and RNA

biology were expressed in a Spargel-sensitive manner. Similarly, genes encoding ribosomal proteins are expressed at reduced rates in *delg* mutant fat bodies (Baltzer *et al*, 2009). Spargel and Delg might, therefore, in addition to mitochondrial functions, be required for cellular growth and metabolism in a mitochondria-independent manner. To address this, we measured larval growth rates. Single mutants of *spargel* or *delg* pupated with a 1 or 2-day delay, respectively. Strikingly, *spargel-delg* double mutants grew very slowly, showing strongly reduced size at 4 days AED (Figure 3H), and pupation occurred with a 4-day delay (Supplementary Figure S2). These phenotypes are consistent with additive defects in growth-related functions such as translation and ribosome biogenesis. Alternatively, flies

mutant for the mitochondrial ribosomal protein, S15, or the mitochondrial protein translocator, Tim50, have similar growth defects (Galloni, 2003; Sugiyama *et al*, 2007). Therefore, the additive growth phenotype might be caused by the synergistic mitochondrial defects described above. In either scenario, although Spargel and Delg might function together in the expression of individual genes, this supports our conclusion that Spargel and Delg function in independent pathways.

Insulin receptor-signalling requires Spargel to mediate its effects on cellular growth

In *Drosophila*, the insulin signalling pathway is a potent driver of cellular growth and metabolism. Recent microarray studies have shown that starvation, and subsequent reduced insulin signalling activity, led to lower expression of genes encoding mitochondrial proteins (Gershman *et al*, 2007; Teleman *et al*, 2008). As the insulin signalling pathway is well conserved between flies and mammals (Grewal, 2008), *Drosophila* is a unique model organism to study a functional interaction between PGC-1 and insulin signalling *in vivo*. In flies, activation of the insulin receptor (INR) stimulates a signalling pathway that includes Chico, as well as the downstream kinases PI3K/Dp110 and PKB/Akt, and subsequent inhibition of the forkhead transcription factor dFoxo (Grewal, 2008). As multiple proteins have been shown to influence insulin signalling, this pathway is not linear, but regulates and responds to other signalling pathways. Accordingly, although dFoxo seems to be the predominant factor in controlling transcription in respect to insulin signalling, additional transcription factors must exist, as many genes were regulated in a dFoxo-independent manner on starvation (Teleman *et al*, 2008). As mentioned above, multiple genes involved in cellular growth control were expressed in a Spargel-dependent manner (Table I), prompting us to test whether Spargel might be required for insulin signalling. To address this, we overexpressed INR in random clones that were marked with GFP. In wild-type fat body cells, overexpression of INR led to an enormous overgrowth phenotype that was characterized by an increase in cellular and nuclear areas (Figure 4A). Interestingly, this was accompanied by a significant reduction in lipid droplet size as revealed by Nile red, a dye specific for lipids. This probably reflects increased demands for energy during cell growth, coupled with enhanced phospholipid usage for membrane synthesis. In contrast to the wild-type background, the effects of INR overexpression on cell growth and lipid droplets were completely abrogated in the *spargel* mutant background (Figure 4A), demonstrating a requirement for Spargel.

To quantify this effect, we measured cell size using the plasmamembrane-specific marker, phalloidin, and nuclear size using DAPI. The overgrowth phenotypes on INR expression were significantly reduced in the *spargel* mutant (Figure 4B). Next, we repeated these assays using ectopic expression of activated Dp110 (Dp110^{CAAX}) or Akt (myristylated Akt). Although the overgrowth driven by Dp110 was suppressed in the *spargel* mutant background, this was not seen for myr-Akt (Figure 4B). We therefore hypothesize that Spargel functions downstream of INR and Dp110, but independently of Akt. To further support such a model, we immunostained for endogenous dFoxo protein, which is known to be excluded from the nucleus on activation of

Figure 4 Insulin signalling requires Spargel to mediate its effect on growth. (A) UAS-INR was expressed in random clones using the hs-Flp; Tub > CD2 > UAS-Gal4, UAS-GFP system in wild-type or *spargel* mutant background. Stainings from larval fat bodies, using Nile red (red) specific for lipid and DAPI (blue) specific for DNA and GFP, which marks INR-expressing cells are shown. Bar equals 20 μ m. (B) Quantification of cell area (CA) and nuclear area (NA) of clones expressing UAS-INR, UAS-Dp110^{CAAX} or UAS-myrAkt in wild-type or *srl1/1* mutant fat bodies. Fold increase compared with the surrounding control cells (ctrl) not expressing insulin signalling components is shown. $n > 25$. (C) Weight of adult males, 2 days after eclosion. Genotypes as indicated.

insulin signalling (Puig *et al*, 2003). Indeed, nuclear dFoxo staining disappeared on ectopic expression of Dp110^{CAAX} or myr-Akt. Importantly, this effect did not depend on Spargel, as mutants showed an identical nuclear exclusion (data not shown). Importantly, overgrowth driven by myr-Akt expression in the fat body was relatively mild, which is surprising as myr-Akt has been found to be a very potent growth driver in other tissues (Stocker *et al*, 2002). The relative importance of insulin signalling components may therefore be altered in the larval fat body compared with other tissues. A similar model has been proposed for insulin signalling and TOR function in the prothoracic gland (Layalle *et al*, 2008). Future study, including detailed loss-of-function studies, will be required to test this.

Flies lacking insulin signalling activity are lethal, thus genetic characterization depends either on hypomorphic mutants, or on null alleles that retain some signalling activity. The *Drosophila* IRS-1 homologue, Chico, is the best-studied insulin signalling component. Null alleles have significant growth defects (Bohni *et al*, 1999), and residual signalling activity depends on the SH2B adaptor protein Lnk (Werz *et al*, 2009). To genetically characterize Spargel's role in insulin signalling further, we generated *spargel-chico* double mutants, and used the adult weight as a readout of signalling activity. Similarly to *spargel* mutants, *chico* mutants were

Figure 5 Spargel regulates part of the transcriptional changes mediated by insulin signalling. (A) Number of genes up- or downregulated ($>1.5 \times$) in microarray studies when INR was overexpressed in the wild-type or *spargel* mutant. Numbers in the grey circle indicate how many of these were expressed in a Spargel-dependent manner. (B) GO analysis of all insulin-responsive genes that were $>1.5 \times$ up- or downregulated. The percentage of genes that are expressed in a Spargel-dependent manner is indicated in the last column. (C) Genes encoding mitochondrial proteins analyzed as in (A). (D) qRT-PCR on RNA isolated from dissected fat bodies (three biological replicates) on selected target genes. Transcript levels were normalized to *Actin5C* (CG4027). (E) Respiration assays as in Figure 3D. Significance is indicated compared with control samples.

reduced in weight, yet to a larger extent. Importantly, we did not observe a further weight reduction in *chico spargel* double mutants (Figure 4C). These data suggest that Spargel has an integrate role in the insulin signalling pathway, however given the caveat for insulin signalling mutants described above, more work is required for direct proof.

Spargel mediates transcription in response to insulin signalling in parallel to dFoxo

Prompted by these results, we asked whether Spargel might function as a transcriptional regulator downstream of insulin signalling. To test this, we ectopically expressed INR and used microarray on dissected fat bodies to analyze expression profiles. Indirect effects were minimized by dissecting relatively quickly after INR induction (13 h after induction). When INR was expressed in a control background, 2254 genes were significantly regulated (Figure 5A and Supplementary Table S2A). GO analysis showed that mitochondrial genes were highly upregulated, and this response was largely dependent on Spargel (Figure 5B and C): 33 of 232 detected

genes (14.22%) encoding mitochondrial proteins were induced by INR expression compared to a control background. In contrast, when INR was expressed in a *spargel* mutant background, significantly fewer genes were INR-responsive: 8 of 232 detected (3.45%). Thus the majority (75.75%) of INR-induced genes required Spargel, especially genes involved in OXPHOS activity, mitochondrial ribosomal proteins and amino acid metabolism (Supplementary Table S2B). We further confirmed these findings by qRT-PCR to detect mRNA levels of *SDH-B*, encoding the electron transport complex II subunit B, *Idh* and *Cyt-c-p*. While INR required Spargel to drive the expression of *SDH-B* and *Idh*, *Cyt-c-p* did not show such dependence (Figure 5D). Importantly, the requirement on Spargel for INR to drive gene expression was not limited to mitochondria: when the whole genome was analyzed, 39.84% of INR-responsive genes required Spargel, in particular genes involved in translation, RNA metabolism and transcription (Supplementary Table S2A). We conclude that Spargel is required to a large extent for the transcriptional response mediated by insulin signalling.

Figure 6 Insulin signalling affects Spargel expression levels and protein localization. (A) An HA-tagged Spargel genomic rescue construct (HA-Srl^{GR}) was used to monitor the sub-cellular localization of the Spargel protein. Antibody staining against the HA tag (α -HA; red) shows a predominant cytoplasmic localization. (B) UAS-INR was expressed in random clones, marked by GFP as in Figure 4A. HA antibody stainings revealed an increased localization of HA-Spargel to the nucleus and the cytoplasm compared with the neighbouring wild-type cells. For (A) and (B), bar equals 20 μ m. (C) Relative mRNA levels of the *Spargel* transcript were quantified by qRT-PCR on dissected wild-type fat bodies with (+) or without (−) the whole body overexpression of UAS-INR ($\uparrow$ INR).

To test whether such dependence would affect mitochondrial activity, we repeated the respiration assays using dissected and permeabilized larval fat bodies. Whereas ectopic expression of INR was sufficient to potently stimulate respiration rates, this was significantly reduced in the *spargel* mutant background. Together with the requirement for cellular growth, these data demonstrate that Spargel is a critical downstream component for insulin signalling.

Drosophila Foxo is known to partially mediate the transcriptional control in response to insulin signalling. We therefore compared our data set to published, fat body-specific microarrays, where control or *dfoxo* mutant larvae were exposed to starvation, reflecting low insulin signalling (Teleman *et al*, 2008). Remarkably, we detected only a minimal overlap between Spargel- and dFoxo dependent genes, both for genes encoding mitochondrial proteins as well as for the whole genome (data not shown). Moreover, Spargel and dFoxo are synthetic lethal: While both single mutants were viable to adulthood, the *spargel foxo* double mutant led to larval lethality (data not shown). Spargel and dFoxo might therefore function independently of each other, each representing different output branches of the insulin signalling pathway. As mentioned above, Spargel is not required for nuclear exclusion of dFoxo upon insulin signalling activation, further supporting our model.

Increased Spargel protein levels in response to INR expression

Although PGC-1 α and PRC are mostly nuclear proteins (Puigserver *et al*, 1998; Andersson and Scarpulla, 2001), rat PGC-1 α , in response to a stimulus, is known to translocate from the cytoplasm to the nucleus (Wright *et al*, 2007). To test whether Spargel localization could be affected in response to insulin signalling, we generated transgenic flies bearing a

HA-tagged Spargel on a genomic rescue construct (HA-Srl^{GR}). Similar to the untagged genomic rescue construct, this version also suppressed the reduced weight phenotype of *spargel* mutants (data not shown). When tested by immunofluorescence in the larval fat body, the majority of HA-Spargel was found in the cytoplasm with relatively little staining in the nucleus under physiological conditions (Figure 6A). When INR was expressed in random clones, we noted a strong increase in HA-Spargel, in the cytoplasm as well as in the nucleus (Figure 6B). This was supported by our microarray study, where we detected increased *Spargel* transcript levels upon INR overexpression (Figure 6C). These data show that Spargel is induced by insulin signalling, leading to elevated Spargel protein, in particular in the nucleus. This further suggests that the effects seen on insulin signalling function are not caused indirectly by reduced growth rates in the *spargel* mutant, but due to a direct Spargel role downstream of insulin signalling.

Spargel mediates a negative feedback loop on insulin signalling

Since signalling pathways often involve negative feedback loops to dampen signalling activity, we analyzed our microarray data in more detail for genes encoding insulin signalling components. Indeed, we noted enhanced INR expression in *spargel* mutants (microarray; confirmed by qRT-PCR: 1.5 $\times$ change, *P*-value = 0.0298), suggesting a negative feedback loop. Accordingly, we observed enhanced PIP₃ levels at the plasma membrane, visualized by the tGPH reporter (Figure 7A), and an increase in Akt phosphorylation, which is known to correlate with Akt activity (Figure 7B). Taken together, Spargel is not only required for insulin signalling, but also mediates a negative feedback loop, and thereby might set a threshold for insulin signalling to control metabolism.

Figure 7 Spargel mediates a negative feedback loop on insulin signalling. (A) tGPH stainings in the larval fat body, specific for PIP₃ levels in response to PI3K activity (Britton *et al*, 2002). Bar equals 20 μ m. (B) Total Akt and phosphorylated Akt (Ser⁵⁰⁵) levels were detected by western blotting and quantified using the Odyssey Detection system. Band intensities were normalized to the respective tubulin control, and presented as the ratio of *spargel* mutant compared with wild-type. Insulin signalling was induced by the expression of UAS-INR with a fat body-specific driver (CS2-Gal4). Average and s.d. value were calculated from four independent experiments. (C) Genetic model for mitochondrial biogenesis in the *Drosophila* larval fat body. Two nutrient-sensitive pathways control transcription of nuclear genes encoding mitochondrial proteins: Cyclin D/Cdk4 through Delg (Baltzer *et al*, 2009), and insulin signalling through Spargel. Most of these genes are expressed in a Spargel- and/or Delg-dependent manner. It should be noted that we have not tested a direct biochemical interaction between Spargel and Delg. In addition to these mitochondrial functions, insulin signalling requires Spargel to drive expression of genes involved in cellular growth, for example, translation. Finally, Spargel mediates an inhibitory feedback loop on insulin signalling, thereby creating a critical threshold to control the expression of metabolic genes in response to insulin signalling. The effect on larval growth is most likely a combination of mitochondrial mass and activity, as well as non-mitochondrial functions.

Discussion

Mitochondrial mass and activity must adapt to cellular growth rates and nutrient availability, yet factors involved were poorly described. Here, we show that *Drosophila* Spargel is critical for proper expression of genes encoding mitochondrial proteins, and that it mediates a link to the nutrient-sensitive insulin signalling pathway. In the larval fat body, Spargel was required for proper mitochondrial respiration when complex II was stimulated, both under normal and insulin signalling stimulated conditions. These data support the interpretation that the control of mitochondria represents an ancestral function of the PGC-1 proteins family. Importantly, we show that Spargel is not a master regulator of mitochondrial biogenesis, but becomes limiting in this respect in the absence of Delg. Furthermore, ectopic expression of Spargel was not sufficient to drive mitochondrial abundance (data not shown), which contrasts mammalian data, where PGC-1's are potent stimulators of mitochondrial mass. Mammalian PGC-1 proteins are induced by external stimuli, including cold exposure in BAT or exercise in muscle tissues, thus adapting the cellular physiology in response to such stimuli (Puigserver and Spiegelman, 2003). To test a similar function for *Drosophila* Spargel, we exposed larvae to cold shocks, and measured respiration rates from dissected fat bodies by complex I stimulation. Although cold exposure led to reduced respiration, this response was not altered in the *spargel* mutant (data not shown). Thus this function is not conserved in flies, which correlates with our previous findings that the *Drosophila* fat body resembles the mammalian white adipose tissue, which is not involved in thermoregulation (Baltzer *et al*, 2009). Furthermore, genes involved in gluconeogenesis, β -oxidation and lipogenesis, all functions linked to mammalian PGC-1 proteins (Lin *et al*, 2005), were

not reduced in the *spargel* mutant, at least not in the larval fat body. We also did not detect altered lipid levels in *spargel* mutant larvae (data not shown), thus these functions might be vertebrate-specific. Potentially, these differences can be explained by the finding that Spargel lacks the canonical LXXLL motifs, which mediate binding to multiple transcription factors for mammalian PGC-1s (Gershman *et al*, 2007). Spargel however contains a conserved C-terminal FXXLL motif (Gershman *et al*, 2007), which could mediate transcription factor binding, yet such interactors remain elusive.

In mammalian cells, overexpression of PGC-1 α led to increased expression of NRF-2 α /GABP α (Mootha *et al*, 2004), and NRF-2 binding sites in the promoters of mitochondrial transcription factors TFB1M and TFB2M were required for coactivation by PGC-1 α and PRC (Gleyzer *et al*, 2005). Similarly, PRC was shown to coactivate NRF-2 β -dependent transcription (Vercauteren *et al*, 2008), thus it was assumed that PGC-1 proteins and NRF-2 would function in the same pathway. Very importantly, these studies focused on individual genes, but not on mitochondrial function and mass, thus it is still unclear whether NRF-2 is functionally required for PGC-1 proteins in respect to mitochondrial mass. We show that although *Drosophila* Spargel and Delg may share many putative target genes, these factors function in parallel pathways in respect to mitochondrial mass, morphology and OXPHOS activity.

The respiration defects in *spargel* single mutants can be explained by reduced expression of genes involved in oxidative phosphorylation. *Spargel* mutants however did not show a reduction in mitochondrial abundance, which appears surprising. In wild-type larvae, the fat body does not attract tracheoles for gas exchange (Jarecki *et al*, 1999), and shows physiological low oxygen levels (Lavista-Llanos *et al*, 2002), suggesting low rates of mitochondrial respiration. Indeed,

compared to other larval tissues, we detected a low inner-mitochondrial membrane potential and reduced oxygen consumption (Baltzer *et al*, 2009, and data not shown). This suggests that oxidative phosphorylation might not be a critical function of the fat body mitochondria. Rather, this tissue releases lipids and amino acids, in particular proline and glutamine, providing energy sources for other tissues (Baker and Thummel, 2007). Since proline and glutamine are synthesized from the mitochondrial TCA cycle intermediate 2-oxoglutarate, we propose that such a function is rate limiting for mitochondrial mass in the fat body. Delg but not Spargel was required for proper expression of genes involved in proline and glutamine metabolism (Baltzer *et al*, 2009), thus explaining the mitochondrial abundance defect in the *delg* mutant, and the absence of such defects in the *spargel* mutant.

Spargel and Delg are distinct in respect to upstream signalling: Spargel is functionally required for insulin signalling, which was not seen for Delg. In contrast, Delg, but not Spargel, is required for Cyclin D/Cdk4 to stimulate mitochondrial abundance (Baltzer *et al*, 2009). Thus our data are genetic evidence that Spargel and Delg represent two different pathways, each regulating mitochondria in response to nutrients (Figure 7C). Although several recent reports have demonstrated a functional link between insulin signalling and PGC-1 proteins in mammalian cells, the interaction appears to be complex, and is most likely tissue and/or context dependent: Some studies showed that PGC-1 proteins were required for insulin signalling (Vianna *et al*, 2006; Pagel-Langenickel *et al*, 2008), whereas other studies found an inhibitory function for PGC-1 α (Koo *et al*, 2004; Choi *et al*, 2008). In flies, the insulin signalling pathway is well conserved, and since Spargel is the only PGC-1 protein, *Drosophila* is an ideal organism to study a functional interaction between insulin signalling and PGC-1 proteins *in vivo*.

We show that Spargel is required for insulin signalling stimulated growth, functioning downstream of Dp110, but presumably independently of Akt, and balances signalling activity through a negative-feedback loop. Very remarkably, Spargel mediates ~40% of the transcriptional control in response to insulin signalling, emphasizing a major function in the control of cellular growth and metabolism. Moreover, our data suggest that Spargel functions independently of dFoxo, therefore representing a novel transcriptional output of insulin signalling in the larval fat body. This appears to contradict recent microarray data, which showed repressed *Spargel* mRNA levels upon expression of an activated form of dFoxo in culture *Drosophila* cells (Gershman *et al*, 2007). The discrepancy could be due to tissue-inherent differences. In our study, INR expression led to an increase in *Spargel* mRNA and protein abundance, further suggesting that Spargel has a direct role within the insulin signalling pathway. Future experiments are required to establish a molecular mechanism how Spargel function and subcellular localization is mediated by insulin signalling components. Analogous to our results, reduced PGC-1 α protein levels were observed upon knockdown of the insulin receptor InsR β in mammalian cells (Pagel-Langenickel *et al*, 2008), suggesting a conserved mechanism between flies and mammals. Thus our data are further genetic evidence for a functional interaction between PGC-1 proteins and insulin signalling. Although flies and mammals are several hundred millions apart in evolution,

Spargel and its mammalian counterparts PGC-1 α , PGC1-1 β and PRC have conserved functions in respect to mitochondria and insulin signalling. In the future, this will allow us to use *Drosophila* as a powerful system to understand regulatory circuitries that control homeostasis of cellular metabolism.

Materials and methods

Fly Stocks

*Srl*¹ (P{SUPor-P}CG9809^{KG08646}), *Df*(3R)ED5046, *Df*(3R)ro^{80b} and *hs-Gal4* were obtained from the Bloomington Stock Center (Indiana University, USA); the *srl*² line (P{XP}CG9809^{d04518}) from the Exelixis Collection (Harvard Medical School, USA). We also used the following fly lines: *delg*⁶¹³ (Schulz *et al*, 1993), *chico*^{1/2} (Bohni *et al*, 1999), UAS-INR (Brogiolo *et al*, 2001), UAS-Dp110^{CAAX} (Leevers *et al*, 1996), UAS-myrAkt/PKB (Stocker *et al*, 2002), *hs-Flp*¹²² Tubulin>CD2>UAS-Gal4 UAS-GFP (Scott *et al*, 2004), tGPH (Britton *et al*, 2002), and CS2-Gal4 (Baltzer *et al*, 2009). To generate transgenic flies, we cloned the full genomic region of *Spargel* (8.7 kb; either wild-type, *Srl*^{GR}, or with two HA-tags at the N-terminus, *HA-Srl*^{GR}) into the pCaSpeR4 vector. For the UAS-*Srl* transgene, we inserted the coding sequence of *Spargel* (isoform CG9809-RB) including 5' and 3'UTRs into pUAST. Flies were grown in standard food under non-crowding conditions, at 12/12 h light/dark cycle at 25°C. For fly weight measurements, adult males were collected two days after eclosion and shock-frozen in liquid nitrogen. Wet weight was assessed using a MX microbalance (Mettler Toledo).

Microscopy

MitoTracker was performed as described before (Frei *et al*, 2005). For Nile red, inverted larvae were fixed in 8% paraformaldehyde and stained in the dark with 0.0002% Nile Red (Molecular Probes). DAPI was used at 0.5 μ g/ml. For immunofluorescence, mouse α -HA (12CA5) and α -mouse Alexa Fluor⁵⁶⁸ (Invitrogen) were used. Images were taken on a DeltaVision Olympus K70 microscope using CoolSnap HQ camera (Photometrics). Serial Z-sections were acquired at 0.2 μ m distance and deconvoluted using Softworx software (Applied Precision). Shown are the projections of the maximal intensities from six subsequent sections. For phalloidin stainings, the AxioPlan2 Imaging microscope (Zeiss) was used, and cell- and nuclear areas were measured using Photoshop. For electron microscopy, larval fat bodies were dissected, fixed in 2% glutaraldehyde and stained using 1% OsO₄. Tissues were dehydrated using ethanol, stained with 0.5% uranyl acetate, embedded in EPON and treated with H₂O₂ after cutting. Images were acquired on a FEI Morgagni 268 microscope.

Oxygen consumption assay

Respiration was measured as described in (Baltzer *et al*, 2009).

Western blot

Insulin signalling was induced using CS2-Gal4-driven UAS-Dp110^{CAAX}, and fat bodies from 6 mid 3rd instar larvae were dissected per sample. Total Akt Antibody (#9272) and Phospho-*Drosophila* Akt (Ser505) Antibody (#4054; both from Cell Signalling) were used at 1/500, anti-Tubulin (Sigma) at 1/2000. Quantifications were done using the Odyssey Infrared Imaging System (LI-COR Biosciences) using Alexa-Fluor⁶⁸⁰ (Molecular Probes) or IRDye⁸⁰⁰ (Rockland, Gilbertsville, USA) secondary antibodies.

Quantitative real-time PCR

Mid 3rd instar fat bodies were dissected into RNA later (Sigma) and mRNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel) and QIAzol Lysis Reagent (Qiagen). cDNA was synthesized with Ready-To-Go-You-Prime-First Kit (Amersham Biosciences) using oligo(dT)15 primers (Promega). For mtDNA, DNA was extracted from larval fat bodies using the NucleoSpin Tissue Kit (Macherey-Nagel). mtDNA was detected by primers against mitochondrial encoded *COX subunit I* and normalized to nuclear encoded *Cdk4*. We used the Roche LightCycler LC480 for quantitative RT-PCR. Primer sequences are available on request. In all cases, mean values and standard deviations were calculated from at least three independent experiments.

Microarray and GO analysis

mRNA from 18–20 fat bodies was isolated as described above. UAS-INR was expressed in the whole animal using the hs-Flp; Tub>CD2>UAS-Gal4 UAS-GFP system and larval fat bodies were dissected 13 h after a 30 min heat shock at 37°C. Microarrays were performed by the Functional Genomics Center Zurich (FGCZ; <http://www.fgc.ethz.ch>) using the one-cycle Affimetrix workflow. Original data, data processing and quality control protocols can be viewed at the NCBI database (Edgar *et al*, 2002), using accession number GSE14779 and GSE14780. GO analysis was performed with the GOTermFinder Software (Boyle *et al*, 2004).

Statistical analysis

In all experiments, significance was determined using the Student's *t*-distribution. *** equals $P < 0.001$; ** equals $P < 0.01$; * equals $P < 0.05$; NS: not significant.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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