Minimal effects of spargel (PGC-1) overexpression in a Drosophila mitochondrial disease model

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ABSTRACT
PGC-1α and its homologues have been proposed to act as master regulators of mitochondrial biogenesis in animals. Most relevant studies have been conducted in mammals, where interpretation is complicated by the fact that there are three partially redundant members of the gene family. In Drosophila, only a single PGC-1 homologue, spargel (srl), is present in the genome. Here, we analyzed the effects of srl overexpression on phenotype and on gene expression in tko25t, a recessive bang-sensitive mutant with a global defect in oxidative phosphorylation, resulting from a deficiency of mitochondrial protein synthesis. In contrast to previous reports, we found that substantial overexpression of srl throughout development had only minimal effects on the tko25t mutant phenotype. Copy number of mtDNA was unaltered and srl overexpression produced no systematic effects on a representative set of transcripts related to mitochondrial OXPHOS and other metabolic enzymes, although these were influenced by sex and genetic background. This study provides no support to the concept of Spargel as a global regulator of mitochondrial biogenesis, at least in the context of the tko25t model.

KEY WORDS: Mitochondria, Mitochondrial biogenesis, Mitochondrial disease, Transcriptional co-activator

INTRODUCTION
The PGC-1 coactivators are widely considered to be global regulators of bioenergy metabolism, specifically acting to promote mitochondrial biogenesis in many different contexts (Spiegelman, 2007). However, the fact that there are three such factors encoded in mammalian genomes (PGC-1α, PGC-1β and PPRC1, also denoted as PRC) complicates their analysis, due to the combination of tissue or physiological specialization and genetic redundancy (Finck and Kelly, 2006).

In the Drosophila genome, a single member of the PGC-1 coactivator family, spargel (srl), is present. A srl hypomorph, carrying a P-element promoter insertion, was found to have decreased weight, decreased accumulation of storage nutrients in males and female sterility (Tiefenböck et al., 2010). In the mutant larval fat body there was decreased respiratory capacity and diminished expression of genes required for mitochondrial biogenesis and activity, with evidence of co-operation with the Drosophila NRF-2α homologue Delg, and with insulin signaling.

These findings are consistent with Spargel acting as a general regulator of mitochondrial biogenesis in the fly. Many subsequent studies have been construed similarly (Mukherjee et al., 2014).

As part of a previous study of phenotypes connected with the Drosophila mutant tko25t, we found evidence consistent with such a role for Spargel in regard to mitochondrial functions (Chen et al., 2012). tko25t carries a point mutation in the gene encoding mitoribosomal protein S12 (Royden et al., 1987; Shah et al., 1997), which confers larval developmental delay, bang sensitivity, defective male courtship and impaired sound responsiveness (Toivonen et al., 2001). The mutant has an under-representation of mitoribosomal small subunit rRNA and is deficient in all four enzymes of the oxidative phosphorylation (OXPHOS) system that depend on mitochondrial DNA (mtDNA)-encoded subunits (Toivonen et al., 2001, 2003). The tko25t phenotype can be rescued by an additional genomic copy of the mutant tko locus (Kemppainen et al., 2009) and partially compensated by altered mtDNA background (Chen et al., 2012) or low-sugar diet (Kemppainen et al., 2016).

In our earlier study, flies overexpressing srl showed a modest but statistically significant alleviation of the mutant phenotype (Chen et al., 2012). When we later catalogued our strain collection, we concluded that this experiment may have used a strain carrying a genomic duplication of srl (designated srlGR, Tiefenböck et al., 2010), rather than the GAL4-dependent srl cDNA construct. In order to clarify the effects on tko25t phenotype of srl overexpression at different levels, we proceeded to combine the mutant with different srl constructs, having first profiled their effects on expression. In an initial experiment using srlGR, we were able to substantiate the earlier finding of a modest alleviation of developmental delay. However, this was not upheld in subsequent repeats of the experiment, nor by other strain combinations that overexpress srl at a higher level; nor did srl overexpression systematically modulate mtDNA copy number or the expression of genes for OXPHOS subunits, the mitochondrial nucleoid protein TFAM or other metabolic pathways. We thus find no consistent evidence to support a role for srl in boosting mitochondrial biogenesis in tko25t flies.

RESULTS
srl expression in wild-type and tko25t mutant flies
To assess the effects of srl overexpression in tko25t mutant flies and heterozygous controls, we first measured the extent of overexpression using qRT-PCR, after combining the relevant chromosomal backgrounds carrying srlGR, UAS-srl, the ubiquitously acting daGAL4 driver, the tko25t mutation and appropriate balancer chromosomes (Fig. 1). To reproduce as closely as possible the previously studied conditions, we created tko25t flies that were hemizygous for both srlGR and daGAL4, even though there should be no UAS construct present (Fig. 1A). We also analyzed the sexes separately since, in initial trials, we observed a consistently higher endogenous srl expression in females than males. Hemizygosity for the srlGR construct conferred an increase in srl RNA in both sexes,
proportionate to gene dosage (Fig. 1A). In contrast, UAS-srl driven by daGAL4 resulted in a more substantial increase in srl RNA: ∼4-fold in females and >100-fold in males (Fig. 1B). srl RNA was at lower abundance in tko25t females (though not males: Fig. 1C), and was restored to the wild-type level by srlGR (Fig. 1D). To test whether increased srl RNA due to UAS-srl expression was reflected at the protein level, we generated two antibodies against peptides of Spargel, which each detected a major band of approximate molecular weight ∼105 kDa and a minor band of ∼125 kDa (Fig. 2A), close to the predicted molecular weight of the protein (118 kDa). These bands were detected in both males and females (Fig. 2B: note that the ∼125 kDa band appears more faintly in females, but is always present at long exposure). The same two bands were detected in S2 cells induced to express V5 epitope-tagged Spargel after transient transfection (Fig. 2C,D). At higher magnification (Fig. 2Ci,ii), immunocytochemistry revealed a ‘speckled’ nuclear localization similar to that observed by Mukherjee and Duttaroy (2013) using srl-GFP, providing further validation of the (AA214) antibody. UAS-srl driven by daGAL4 led to a modest increase in detected Spargel protein, based on western blot signal compared with the GAPDH loading control (Fig. 2E,F). Note, however, that this increase (∼20–50% depending on background), was proportionately far smaller than that seen at the RNA level. The large disparity in srl RNA between males and females (Fig. 1) was not evident in the detected protein, which was actually at a higher level in males (by ∼40%).

**srl overexpression has no systematic effects on tko25t phenotype**

To clarify the effects of srl overexpression on the phenotype of tko25t we conducted a number of tests in which we varied the overexpression construct used and the genetic background. Using the srlGR construct to produce modest overexpression we recorded a small decrease in the developmental delay of tko25t flies (Fig. 3A). However, this was influenced by the presence of the daGAL4 driver, since the eclosion day of tko25t flies lacking both daGAL4 and the...
**srl**

**construct** was not significantly different from that of flies endowed with both. Furthermore, although the alleviation of developmental delay was significant in this first experiment, as inferred previously (Chen et al., 2012), it was not seen in any of the three repeats of the experiment (e.g. the one shown in Fig. 3B). There was also no significant difference in eclosion time between **tko25** flies homozygous for the **srl** construct and **tko25** controls in either sex (Fig. 3C). Furthermore, hemizygosity for the extra copy of **srl** produced no rescue of bang-sensitivity (Fig. 3D). More substantial overexpression of **srl** driven by **da** GAL4 using the UAS-**srl** construct did not alleviate developmental delay: rather there was a trend towards a slight deterioration, although this was significant only in one repeat of the experiment and in males only, as shown (Fig. 3E).

**srl expression is not altered by diet during development**

Previously, Spargel was shown to mediate insulin signaling (Mukherjee and Duttaroy, 2013), which is considered the primary system linking growth to nutritional resources. In contrast, **tko25** exhibits an apparently paradoxical growth retardation when cultured on high-sugar diet (Kemppainen et al., 2016), suggesting that insulin signaling has been abrogated or even reversed, for example, as a result of a counteracting signal arising from mitochondrial dysfunction. We therefore considered the hypothesis that **srl** was downregulated in **tko25** by a diet-dependent mechanism and that its expression and growth-promoting function might be restored in **tko25** larvae or adults cultured on minimal medium.

For this, we compared flies grown on standard high-sugar medium, containing complex dietary additives, with those grown on a minimal medium containing only agar and (10%) yeast. As previously, the low-sugar minimal medium partially accelerated the development of **tko25** flies (Fig. 4A), whilst at the same time retarding that of controls (Fig. 4A,B). However, diet-induced effects on the expression of **srl** were minimal. **srl** expression in control (wild-type Oregon R) L3 larvae of both sexes was slightly decreased in minimal medium compared with high-sugar medium (Fig. 4C,D), although this was not statistically significant in all experiments (e.g. Fig. 4C, right-hand panel). **srl** expression in **tko25** larvae (Fig. 4C, right-hand panel) was lower than in controls by approximately the same factor as in adults, but was unaffected by the different culture media, as was **srl** expression in **tko25** adults (Fig. 4D).

**Overexpression of srl has no systematic effects on genes related to core mitochondrial functions**

Despite the fact that **srl** overexpression had no impact on the **tko25** phenotype, we explored whether such overexpression nevertheless influenced the level of mtDNA or that of transcripts related to core functions of mitochondria, specifically OXPHOS subunits and the major nucleoid protein TFAM (Fig. 5). With the exception of TFAM,
all genes studied showed a similar profile of expression in the different strains tested, with higher relative expression in males, higher expression in the tkot25t background, including tkot25t heterozygotes over the FM7 balancer, attenuation of this increase by the daGAL4 driver and further slight attenuation by UAS-srl. These observations are consistent with expression levels being determined by sex and by genetic background, possibly involving effects on the RpL32 reference transcript, rather than by srl expression, which followed a different pattern (Fig. 1B). They provide no support for any enhancing effect of srl. In the case of TFAM, expression was slightly lower in males than in females, and was little affected by daGAL4 or UAS-srl (Fig. 5, top right). Note that srl overexpression was verified (Fig. 1B) in the same RNA samples.

In previous studies, the expression of genes for some of the enzymes participating in other metabolic pathways known to be influenced by PGC-1 homologues in various contexts, such as lipid catabolism, including beta-oxidation of fatty acids (Huang et al., 2017), or gluconeogenesis (Rhee et al., 2003), were found to be upregulated in tkot25t, both in larvae (Kemppainen et al., 2016) and adults (Fernández-Ayala et al., 2010). We therefore tested whether srl over-expression driven by daGAL4 was able to influence the expression of genes for such enzymes in tkot25t, despite the absence of any effect on growth rate. Once again, using the same materials as in the experiment shown in Fig. 5, we found no significant effect of srl overexpression on the transcripts of two genes for enzymes of fatty acid oxidation (yip2 and Thiolase) and two for gluconeogenesis (PCB and Pepck1), although all of them were affected by sex, by genetic background and by the interaction of these factors (two-way ANOVA, Fig. 6A).

Next, we measured relative mtDNA copy number in tkot25t and control flies, with and without srl overexpression. ANOVA revealed no significant differences between groups (Fig. 6B). Thus, srl overexpression does not appear to influence mitochondrial or metabolic functions in tkot25t in any systematic way.

**DISCUSSION**

Previous studies, where the expression of srl was downregulated either in the whole fly or in a specific tissue, suggested a global role for srl in growth regulation. Here we tested whether overexpression of srl was able to compensate the phenotype of tkot25t, a mutant with decreased...
mitochondrial biosynthesis and which grows slowly. We found that srl RNA was at decreased levels in tko25t flies (Figs 1 and 4) even when cultured in minimal medium where the growth defect is partially alleviated. As suggested in a previous study, we initially detected a small compensatory effect of srl overexpression in tko25t flies endowed with an additional copy of srl (Fig. 3A). However, further repeats (e.g. Fig. 3B) and trials with srlGR in two copies (Fig. 3C) failed to substantiate any rescue of developmental delay or bang-sensitivity (Fig. 3D). Even the much more substantial srl overexpression produced by UAS-srl (Fig. 1B) was ineffective (Fig. 3E).

Overexpression of srl had no effect on mtDNA copy number, nor on transcripts of genes connected with mitochondrial OXPHOS or other metabolic pathways. There are several potential explanations for these essentially negative results that we now consider, noting that srl overexpression was also previously found not to compensate for decreased OXPHOS capacity resulting from a mutation in the adenine nucleotide translocase (Vartiainen et al., 2014).

Translational and post-translational regulation

The first possibility is that, as suggested by the lack of congruence between RNA and protein levels, srl is translationally regulated, negating any effect of overexpression. Translational regulation is well established (see recent reviews by Zhao et al., 2019; Shi and Barna, 2015), applies to mitochondrial biogenesis (Zhang and Xu, 2016), and is prominent in early development (Winata and Korzh, 2018; Barckmann and Simonelig, 2013), playing roles in axial specification and other processes in Drosophila (Wilhelm and Smibert, 2005; Kugler and Lasko, 2009). It is also a cardinal feature of the integrated stress response (Ryoo and Vasudevan, 2017), which can be activated by mitochondrial dysfunction.

A second idea is that srl might be post-translationally regulated, which could also override effects of overexpression. Post-translational regulation is brought about by many different mechanisms (Gill, 2004; Lee et al., 2005; Johnson, 2009; Bauer et al., 2015; Narita et al., 2019; Klein et al., 2018). Many of them have been documented as affecting the PGC-1 family in mammals (reviewed by Austin and St-Pierre, 2012), which is also subject to differential splicing (Meirhaeghe et al., 2003; Martínez-Redondo et al., 2015). The two antibodies that we generated against Spargel detect the same bands on western blots, validated by epitope tagging in S2 cells. The higher molecular weight band (∼125 kDa) probably represents the predicted full-length protein of 119 kDa. The nature of the processing that generates the major (∼105 kDa) band is unknown, but can be considered a suggestive indicator of post-translational regulation of Spargel.

tko25t signaling

A third possible explanation for the finding that srl overexpression fails to modify the tko25t growth phenotype could be that the mutation might elicit a growth-inhibitory signal, overriding any effect of srl. Therefore, we should not just dismiss the conventional view that the PGC-1 coactivators are global regulators of mitochondrial biogenesis. Such a function may apply in many other physiological
contexts. Indeed, if Spargel acts in this way as a ‘master switch’, its effects may still be masked by metabolic signaling at a lower level in the hierarchy of gene regulation. Based on previous data (Kemppainen et al., 2016), a strong candidate for growth regulation in $tko^{25t}$ is ribosomal protein S6 kinase (S6K), which is influenced by multiple signaling pathways including mTOR (Magnuson et al., 2012), insulin/Akt (Manning, 2004) and AMPK (Mihaylova and Shaw, 2011). Contradicting this idea, Mukherjee and Duttaroy (2013) found that $srl$ can partially override defects in cell growth mediated by defective insulin/mTOR signaling and that mutants in S6K can be rescued by $srl$ overexpression. However, since S6K regulation in $tko^{25t}$ seems to be at the level of the protein itself, not its phosphorylation, $srl$ over-expression may be insufficient to negate it.

A different role for spargel
Spargel may also play a broader role than just promoting mitochondrial biogenesis. Although mitochondrial biogenesis is reciprocally affected by PGC-1$\alpha$ knockout (Lin et al., 2004; Leone et al., 2005) and overexpression (Lehman et al., 2000; Lin et al., 2002), the PGC-1 family also impacts thermogenesis in brown fat (Uldry et al., 2006), neuromuscular differentiation (Lin et al., 2002; Handschin et al., 2007), hepatic gluconeogenesis (Yoon et al., 2001) and oxygen radical detoxification (St-Pierre et al., 2006). As a coactivator, PGC-1 interacts with sequence-specific transcription factors which specify the genes to be regulated, but the known transcriptional targets of $srl$ are not limited to those involved in mitochondrial biogenesis (Tiefenböck et al., 2010), and it has elsewhere been implicated in various cell differentiation and cell survival programs, or in functional maintenance during aging (Tinkerhess et al., 2012; Wagner et al., 2015; Merzetti and Staveley, 2015; Diop et al., 2015; Ng et al., 2017; Staats et al., 2018). Rera et al. (2011) reported an increase in mitochondrial markers in flies globally overexpressing $srl$. However, this is also consistent with a general enhancement of muscle differentiation. Finally, we should not exclude the possibility that $srl$ could promote mitochondrial biogenesis by an effect other than on transcription, even if this would not affect the levels of mtDNA/TFAM, mitoribosomes or mitochondrial mRNAs in $tko^{25t}$ flies, nor modify the $tko^{25t}$ phenotype. However, since there is no precedent for a transcriptional coactivator influencing the levels of target proteins but not their mRNAs, this must be considered highly unlikely.

Issues in fly genetics
Our initial results using $srli^{GR}$ (Fig. 3A) were consistent with previous studies, but partial rescue of $tko^{25t}$ could not subsequently be reproduced. The reasons for the discrepancy are not clear, but we posit that both the original, apparent rescue and its non-reproducibility are most probably attributable to genetic background effects, and subject to genetic drift during stock maintenance. Unknown and therefore uncontrolled environmental variables may also have played a role. Note, in addition, that our initial finding (Fig. 3A) indicates a small negative effect of the da$\text{GAL4}$ driver. Transgenes, drivers and deleterious mutations are routinely maintained over balancer
chromosomes. Balancers are preferable to homozygosity, so as to prevent the inadvertent selection of suppressors, and are unavoidable in cases where homozygosity is lethal. However, balancers also allow new mutations to accumulate, protected from negative selection. These too potentially compromise the reproducibility of effects on new mutations to accumulate, protected from negative selection. Whilst burdensome, our study highlights the value of multiple repeat experiments to confirm quantitatively minor phenotypic variations, preferably with retesting in different backgrounds. Such measures are nevertheless much easier to implement and interpret in Drosophila, compared with mammalian models where inconsistent or strain-dependent findings abound.

Although we found no effects on phenotype, mtDNA copy number or gene expression from srl overexpression in tko256, it should be noted that all our assays were conducted on whole adult flies. Therefore, our findings largely reflect the situation in the muscle-rich thorax, where mtDNA and its transcription and translation products are at their most abundant (Calleja et al., 1993). Although we cannot exclude an srl-dependent effect in some tissue other than muscle, to detect it would require extensive dissection procedures or the use of highly tissue-specific drivers. The present results provide no basis upon which to embark on such a study.

MATERIALS AND METHODS

Drosophila strains and culture

The srl178 and UAS-srl strains (Tiefenböck et al., 2010), both supplied over a CyO balancer, were a kind gift from Christian Frei (ETH Zürich, Switzerland). The tko256 strain, originally sourced through Kevin O’Dell (University of Glasgow, UK), was backcrossed into Oregon R background (Toivonen et al., 2001) and maintained long-term in our laboratory over the FM7 balancer. The Oregon R wild-type and daGAL4 driver strains were originally obtained from Bloomington Stock Center and the tubG3 driver was the kind gift of Scott Pletcher (University of Michigan, USA). All stocks were maintained at room temperature and grown experimentally in plugged plastic vials at 25°C on a 12 h light/dark cycle in standard high-sugar medium (HS, Kemppainen et al., 2016) or, where specified in figures, in a minimal medium (MM) consisting of agar, 10% yeast and standard antimicrobial agents (0.1% nipagin and 0.5% propionic acid, Sigma-Aldrich).

Molecular cloning

Genomic DNA was extracted from adult Drosophila and used as a PCR template with chimeric gene-specific primers to amplify srl from the start codon up until, but not including, the stop codon. The chimeric primers contained EcoRI and NotI restriction sites for restriction digestion and insertion into the copper-inducible plasmid pMT-V5/HisB (Thermo Fisher Scientific), resulting in the introduction of an in-frame C-terminal V5 epitope tag. A primer deletion strategy was used on this plasmid to create an intronless version of srl tagged with V5. Both resulting plasmids were sequence-verified before use in transfections.

Developmental time and bang-sensitivity assays

Three replicate crosses were set up and tipped five times to fresh vials for egg laying, as previously described (Kemppainen et al., 2009). The mean developmental time to eclosion (at 25°C), as well as bang-sensitivity, were measured as described previously (Kemppainen et al., 2009). Unweighted means and standard deviations of eclosion day for each sex and inferred genotype were then computed for each cross, and used in statistical analyses, generally applying Student’s t-test (unpaired, two-tailed) to compare the mean eclosion day of flies of a given sex and genotype with and without the expression of a given srl overexpression construct. For bang-sensitivity,

![Fig. 6. srl overexpression in tko256 does not increase mtDNA copy number or transcript levels for other metabolic enzymes. (A) qRT-PCR measurements of RNA levels (n=4 batches of 10 flies) of the indicated genes (symbols as in flybase.org) in adult flies of the indicated genotypes and sex normalized to corresponding values for Oregon R (wild-type) females. Statistical analysis confirmed a significant effect of both sex and genotype of and interaction between these factors for all four genes (two-way ANOVA, P<0.001). However, as for the genes studied in Fig. 5, narrowly comparing expression of flies of a given sex in the presence of daGAL4, with or without UAS-srl, showed no significant differences (Student’s t-test with Bonferroni correction), despite the general trend of slight decrease in males. (B) qPCR measurements of mtDNA copy number (n=4 batches of five flies), means±s.d. normalized to Oregon R females. There were no significant differences between groups (one-way ANOVA).](image-url)
medians and quartiles of recovery time for flies of a given sex and genotype were plotted in a box-plot format.

**RNA analysis**

Total RNA was extracted from batches of ten 2-day-old flies and from L3 (wandering stage) larvae using a homogenizing pestle and trizol reagent as previously described (Kempainen et al., 2016). cDNA was synthesized using the High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to manufacturer’s instructions. Expression levels were determined by qRT-PCR using Applied Biosystems StepOnePlus™ Real-Time PCR System with Fast SYBR® Green Master Mix kit (Applied Biosystems) with, as template, 2 µl of cDNA product diluted 10-fold, in a 20 µl reaction, together with 500 nM of each gene-specific primer pair as follows (all given 5’ to 3’): Rpl32 (CG7939), TGTGCACAGGAACTTCTTGGAA and AGGCCCAAAGACTGTGAAAGAA; ND-ACP (CG9160), ACAAGATCGACCCGCAAG and ATGTCCGAGCTTAAAGCAG: ND-30 (CG12079), AAGCGGATAAGCCCTC and GCAATAAGCCCTCCAGCTC; mt:COX5A (CG14724), GGACTGGTGGTGGTAATCTGGGG and GTCCGCACACCCTTTC and TGCAGCAATGACAAAAGCGAG; mt:ND5 (CG34083), GGGTGAGATGGTTTAGGACTTG and GGATTCAA and AACTGGTCGAGCTCCAATTC; mt:Cyt-b (CG3612), GACTGGTAAGACCGCT- Blw TACATTCGGTGCTGGTGCTT; mt:CoII (CG10664), TCTTCGTGTACGATGAGCTG and GGTTGATTTCCAGGTGATCGATCCCAGCAAG and ATGTCGGCAGGTTTAAGCAG: mt:RFeSp (CG1516), AATCGGTGGCGGTCTACTC and TTGCCCACTATGTACGAG and GGTAT and CAAAGCCGGTTGATTCCAAGG; mt:16S (CG17725), TGATCCCGAACGCAC-CATC and CTCAGGAACTCCACTTGAA and AGG–TGTGCACCAGGAACTTCTTGAA and AGG –

**Protein analysis**

Batches of ten 2-day-old adult flies were crushed in 100 µl of lysis buffer (0.3% SDS in PBS plus one EDTA-free cOmplete™ Protease Inhibitor Cocktail Tablet, Roche), incubated for 15 min and centrifuged at 15,000 g_max for 10 min (all manipulations at room temperature). Supernatants were decanted and protein concentrations determined by the Bradford assay. Aliquots of 50 µg protein in SDS-PAGE sample buffer containing 0.2 M dithiothreitol were heat-denatured for 5 min at 95°C then electrophoresed on AnyKDY midi criterion gels (Bio-Rad) in ProSieve™ EX running buffer (Lonza). Transfer to Nitrocellulose membrane (Perkin-Elmer) was performed using ProSieve™ EX transfer buffer (Lonza). Membranes were blocked in 5% non-fat milk in PBS-0.5% Tween (Medicabo) for 30 min at room temperature, with gentle agitation. Primary antibody diluted in the same buffer was added and reacted at 4°C overnight. After three 10 min washes, secondary antibody was added in the same buffer containing 5% non-fat milk for a further 2 h. Membranes were washed twice for 10 min in PBS-0.5% Tween and then for a final 10 min in PBS. Primary antibodies and dilutions were as follows: Srl214AA (against peptide CFDLADFITKDFAENL) and Srl306AA (against peptide CPMQKQTPDELRYVDNVKA), custom rabbit polyclonal antibodies (21st Century Biochemicals, both 1:5000), GAPDH (Everest Biotech EB06377, goat polyclonal, 1:5000), anti-V5 (Thermo Fisher Scientific, mouse monoclonal #R60205, 1:10,000). Appropriate HRP-conjugated secondary antibodies (Vector Laboratories, 1:5000). 5 ml of Luminata™ Crescendo Western HRP substrate solution (Merck) was added for 5 min before imaging with a Bio-Rad imager.

**mtDNA copy number measurement**

Batches of five adult flies of a given sex were crushed in 500 µl DNA lysis buffer (75 mM NaCl, 50 mM EDTA, 20 mM HEPES/NaOH, pH 7.8). 5 µl of 20% SDS and 20 µl of Proteinase K (10 mg/ml, Thermo Fisher Scientific) were added to each sample and vortexed to mix. Samples were briefly centrifuged at 16,000 g for 1 min to pellet debris. Supernatants were added to each sample and vortexed to mix. Samples were briefly centrifuged at 16,000 g for 1 min to pellet debris. Supernatants were decanted and nucleic acid was precipitated with 420 µl of isopropanol with repeated inversion and overnight incubation at ~20°C. Samples were centrifuged at 16,000 g_max for 30 min at 4°C to pellet the DNA, which was washed with 500 µl of ice-cold 70% ethanol. Final pellets were left to air dry for 10 min, then resuspended in 100 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) overnight at 50°C. DNA concentration was measured by nano-drop spectrophotometry and samples were diluted to 2.5 ng/µl. Relative DNA levels of Rpl32 (single-copy nDNA) and mt:hrRNA (16S, mtDNA) were determined by qPCR using Applied Biosystems StepOnePlus™ Real-Time PCR System with Fast SYBR® Green Master Mix kit (Applied Biosystems), using as template 2 µl of DNA in a 20 µl reaction, together with gene-specific primer pairs each at 500 nM, as follows (all given 5’ to 3’): Rpl32–TGTGCACAGGAACTTCTTGGAA and AGGCCCAAAGACTGTGAAAGAA and AGGCCCAAAGACTGTGAAAGAA; mt:hrRNA–ACCTGGTCTACCGGTTTGGAA and GGGTGAGATGGTTTAGGACTTG and GGTAT and CAAAGCCGGTTGATTCCAAGG.

**Image processing**

Images were cropped and/or rotated for clarity and optimized for contrast and brightness, but without other manipulations.

**Transfections and immunochemistry**

Transfection and induction of S2 cells with V5-tagged srl constructs and subsequent staining for imaging was performed as previously (González de Cózar et al., 2019). The primary antibody used was mouse anti-V5 (Life Technologies) along with the corresponding Alexa Fluor® 488 or Alexa Fluor® 647 secondary antibodies (Abcam), with image acquisition by confocal microscopy.

**Statistical analysis**

Data were analyzed using Student’s t-test (two-tailed, with Bonferroni multiple-test comparison where indicated), one-way or two-way ANOVA, as appropriate (Microsoft Excel and GraphPad Prism). n numbers (batches of flies, representing at least 20 individual flies in total in each case, or replicate vials, representing 50–250 individual flies in total in each case) as indicated in figure legends. No exclusion criteria were applied. Note that, for statistical analysis of quantitative PCR data, ΔC_T values were used, because they are normally distributed, whereas the extrapolated fold-changes are not, having been subjected to an exponential transformation. Thus, to apply standard statistical tests such as ANOVA or Student’s t-test, the ΔC_T values must be used.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: J.G., H.T.J.; Methodology: J.G., H.T.J.; Formal analysis: J.G., H.T.J.; Investigation: J.G.; Resources: H.T.J.; Data curation: H.T.J.; Writing - original
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