Spargel/dPGC-1 Is a New Downstream Effector in the Insulin–TOR Signaling Pathway in *Drosophila*

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ABSTRACT Insulin and target of rapamycin (TOR) signaling pathways converge to maintain growth so a proportionate body form is attained. Insufficiency in either insulin or TOR results in developmental growth defects due to low ATP level. Spargel is the *Drosophila* homolog of PGC-1, which is an omnipotent transcriptional coactivator in mammals. Like its mammalian counterpart, Spargel/dPGC-1 is recognized for its role in energy metabolism through mitochondrial biogenesis. An earlier study demonstrated that Spargel/dPGC-1 is involved in the insulin–TOR signaling, but a comprehensive analysis is needed to understand exactly which step of this pathway Spargel/PGC-1 is essential. Using genetic epistasis analysis, we demonstrated that a Spargel gain of function can overcome the TOR and S6K mediated cell size and cell growth defects in a cell autonomous manner. Moreover, the tissue-restricted phenotypes of *TOR* and *S6K* mutants are rescued by Spargel overexpression. We have further elucidated that Spargel gain of function sets back the mitochondrial numbers in growth-limited *TOR* mutant cell clones, which suggests a possible mechanism for Spargel action on cells and tissue to attain normal size. Finally, excess Spargel can ameliorate the negative effect of FoxO overexpression only to a limited extent, which suggests that Spargel does not share all of the FoxO functions and consequently cannot significantly rescue the FoxO phenotypes. Together, our observation established that Spargel/dPGC-1 is indeed a terminal effector in the insulin–TOR pathway operating below TOR, S6K, Tsc, and FoxO. This led us to conclude that Spargel should be incorporated as a new member of this growth-signaling pathway.

TO attain proper cellular growth, availability of nutrients is imperative, because the nutrient supply fuels energy metabolism. Thus lack of nourishment cause limited growth of the organism due to reduced energy metabolism (Hietakangas and Cohen 2009; DePalma *et al.* 2012). Sensing and transport of growth signals happen in two distinct pathways: at the cellular level, the TOR pathway governs growth (Saltiel and Kahn 2001; Grewal 2009), whereas insulin signaling is responsible for subsequent adjustment of the cellular metabolism causing growth at a more systemic level. Ultimate convergence of these two signaling pathways leads to balanced growth. Therefore each member of the insulin–TOR pathway has recognized influence on cell size and cell growth as demonstrated either in cell clones or at the whole organism level. Severe growth defect during pre-natal development such as intrauterine growth restriction

and low birth weight have been linked to paucity of insulin–Tor signaling (Murakami *et al.* 2004; Gannage-Yared *et al.* 2012) and ATP production (Selak *et al.* 2003). Like in most animals, the insulin–TOR signaling pathway in *Drosophila* is dedicated to the control of growth and metabolism (Figure 1A). With its enriched genetic and genomic resources, flies have contributed significantly toward the understanding of nutritional physiology and cell growth control (Hietakangas and Cohen 2009).

Peroxisome proliferator-activating receptor gamma coactivator 1 (PGC-1) is a key transcriptional coactivator in mammals, which is involved in energy homeostasis (Lin *et al.* 2005), gluconeogenesis, fatty acid oxidation, regulation of thermal tolerance, and a more recent report suggests that PGC-1 is capable of responding to environmental cues, such as nutrients (Bhalla *et al.* 2011). The single *Drosophila* PGC-1 homolog is designated as Spargel (Tiefenbock *et al.* 2010), which shares significant homology with PGC-1 at the RNA recognition motif (RRM) and serine–arginine repeat (RS) (Gershman *et al.* 2007). Previous observation claimed that Spargel functions in the insulin signaling pathway because reduced Spargel expression abrogates the cellular

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Figure 1 Cellular localization of Spargel follows PGC-1. (A) Schematic representation of the insulin-TOR signaling pathway: insulin and TOR signaling pathway acts together to control growth in *Drosophila* and mammals. The insulin-like growth factors/insulin turns on this pathway, depending on the availability of glucose in the system. Details of insulin signaling have been reviewed elsewhere (Bier 2005; Grewal 2009). TOR plays a central role in regulating cell growth and control of mitochondrial energy. Each member of this pathway can control cell growth in a cell autonomous manner (Hennig *et al.* 2006). (B) GFP-Spargel fusion protein is localized exclusively inside the nucleus where it is not homogenously distributed; instead Spargel appears in the form of punctate structures. (C and D) Pre-mRNA splicing factor SC-35 (red), which is localized exclusively inside the nucleus in the form of nuclear speckles, colocalized with Spargel (merge) in many of these punctae. Bar, 40 μ m. (E) At higher magnification (Bar, 20 μ m) Spargel (green) and SC-35 (red) merge appears as yellow signals. As shown here inside a polytene nucleus, Spargel and SC35 are quite intimately associated, indicating a conserved role of PGC-1 and Spargel in the splicing complex. (F) Spargel carries a 16-amino-acid nuclear localization signal (NLS) as predicted by Predict-

overgrowth resulting from overexpression (OE) of insulin receptor (InR) (Tiefenbock *et al.* 2010) because Spargel acts downstream to InR. In mammalian hepatocytes, PGC-1 is known to act in the TOR pathway (Cunningham *et al.* 2007; Lustig *et al.* 2011), although how PGC-1 is related to growth remains undefined. An important first question remains: Where in the insulin-TOR signaling pathway is Spargel action required? We pursue this question with the help of genetic epistasis analysis.

Insulin-TOR signaling is central to cell growth and cell size determination, hence the loss of function of some of its members and gain of function of the others, all influencing the cell size as demonstrated either at the whole organism level or in cell clones (Hietakangas and Cohen 2009). Taking advantage of their effect on cell size, we tested the action of *spargel* hypomorph and/or Spargel gain of function on TOR, S6K, Tsc, and FoxO-induced cell growth defects. If Spargel functions downstream of any of these then Spargel overexpression should rescue or at least amend TOR, S6K, Tsc, and FoxO mutants' effect on cell size, whereas, a non-rescue will mean Spargel action is required upstream.

Materials and Methods

Stocks

UAS-*srl*⁺, Tsc2RNAi-, and FoxO-null flies were obtained from the Christian Frei, Morris Birnbaum, and Linda Partridge laboratories respectively. Act-GAL4, ap-GAL4, MS1096-GAL4, EP-*srl*⁺, UAS-TOR^{TE}, UAS-S6k^{STDETE}, UAS-S6k^{KQ}, Act-CD2>GAL4, hsp-FLP: UAS-GFP, UAS-FoxO, GMR-GAL4, and UAS-*srl* RNAi fly stocks were obtained from the Bloomington *Drosophila* Stock Center. Fly cultures were maintained in standard media at 23 $\pm$ 1° temperature. Transgenic expression of GFP-Spargel fusion protein was achieved by cloning the *spargel* cDNA into UASP-Gateway vector with N-terminal GFP.

Somatic clones and immunocytochemistry

Clones were generated in fat body cells by adopting the FLP-out technique (Zhang *et al.* 2006). Individual UAS lines were crossed with hsp-FLP UAS-GFP; Act > CD2 > GAL4 flies, so clones were formed ubiquitously in the F₁ progenies. Fat body tissue obtained from the F₁ larvae were fixed in 4% paraformaldehyde/1 $\times$ PBS/0.2% Triton-X 100. For phalloidin staining, fat bodies were permeabilized with 0.3% TX-100 in 1 $\times$ PBS for 10 min and incubated for 2 hr in 1 μ M rhodamine-tagged phalloidin at room temperature. Fat bodies were washed, mounted in Vectashield with DAPI

Protein and NLS Mapper software. (G) Cells expressing *spargel* RNAi (two GFP positive cells) appeared much smaller in size, suggesting that like all other insulin family members Spargel functions in cell growth in a cell autonomous manner. (H) Quantification of cell size confirmed that both the cytoplasmic area (CA) and nuclear area (NA) were reduced in *spargel* RNAi cell clones as compared to the control (non-GFP cells). Bar, 40 μ m. N, number of cell clones counted >25. ***P < 0.001.

(Vector Lab), and visualized under confocal microscope (Nikon).

Antibody

Phospho-4EBP (1:200) and Phospho-S6K (1:200) antibodies were obtained from Cell Signaling Technology (Miron *et al.* 2003). Antiactin (1:5000) antibody was obtained from Abcam.

Direct mitochondria visualization

Fat bodies were fixed with 4% paraformaldehyde and 20 mM formic acid. ATP5A (1: 250) (MitoSciences) antibody was used to detect mitochondria (Cox and Spradling 2009).

Microscopy

Visualization and analysis of the clones were done using a Nikon (EZ-C1) confocal microscope. Measurement of clone size and mitochondrial fluorescence intensity quantitation was done with Nikon NIS-Element software.

RT-PCR

RNA extraction was done using Qiagen RNeasy spin kit (catalog no.74104). Thirty-five flies were homogenized in 250 μ l of buffer RLT with β -mercaptoethanol. Homogenate was centrifuged at full speed for 10 min at 4°. The supernatant was added to the g-DNA spin column and centrifuged for 1 min at 11,000 rpm. The flow through was mixed with equal volume of 70% ethanol and added to the RNeasy spin column. After centrifugation, 700 μ l of buffer RW1 was added to the column and centrifuged for 1 min at 11,000 rpm. 500 μ l of buffer RPE was added, centrifuged, and the step was repeated. The columns were added in a new tube and centrifuged for 1 min at full speed. The columns were transferred to a new tube, 35 μ l of RNase-free water was added, and centrifuged at 11,000 rpm for 1 min. Collected RNA was quantified and 2 μ g of RNA was used to make the cDNA.

Western blot

Total protein was isolated by homogenization of ~30 flies in a prechilled extraction buffer (10 mM DTT, 4% glycerol, and 0.15 M Tris-Cl pH 7.5). Homogenates were spun down at 14,000 rpm for 10 min in 4°. Supernatant was collected and protein concentration was adjusted using the Bio-Rad quick start Bradford assay. Protein samples were denatured by boiling and were then electrophoresed (2 μ g/ μ l) on an SDS-PAGE (14% denaturing and 4% stacking gel) at 120 V for 1 hr at room temperature. Samples were run simultaneously alongside a protein standard (BioRad Precision Plus 250, 10 kDa). Following electrophoresis, proteins were transferred to Millipore's Immobilon-PSQ (0.2 m pore size) PVDF membrane for 60 min in cold (4°) at 120 V. The PVDF membrane was then blocked with 5% fat-free milk dissolved in 1 $\times$ TTBS (100 mM Tris-Cl pH 7.5, 0.9% NaCl and 0.1% v/v Tween 20) overnight in 4°. The membrane was washed twice (5 min each) with 1 $\times$ TTBS and probed with primary

antibody (1 μ l Ab in 5 ml TTBS containing 1% fat-free milk) for 1 hr. Excess primary antibody was washed off by rinsing three times (5 min each) with 1 $\times$ TTBS. The membrane was then incubated in secondary antibody (1 μ l Ab in 5 ml TTBS containing 1% fat-free skimmed milk) for 1 hr, and then rinsed three times with 1 $\times$ TTBS. Lastly for detection, ~4 ml of ECL was added (Amersham Biosciences) per blot. To test the phosphorylation status of S6k and 4EBP, anti-S6k antibody (1:500) (Cell Signaling) and anti-4EBP (1:500) (Cell Signaling) antibodies were used.

Statistics and software

Measured cellular and nuclear areas were compared using Student's *t*-test. Significance of pupal and adult rescues was determined using the chi-square test. Nuclear localization signal (NLS) in Spargel was predicted using two independent software programs, PredictProtein (<http://www.predictprotein.org/>) and NLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) under high stringency. The possibility of Spargel localization in the nucleus was checked with two different software programs, NucPred (<http://www.sbc.su.se/~maccallr/nucpred/>) and ESLPred (<http://www.imtech.res.in/raghava/eslpred/>).

Results and Discussion

Subcellular localization of Spargel and PGC-1 is comparable

Significant structural homologies exist between Spargel and PGC-1 (Gershman *et al.* 2007). We noticed with interest that these two molecules share a comparable subcellular localization, which may have some greater functional significance. Ubiquitous expression of a GFP-tagged Spargel protein with *Act-GAL4* driver displayed that Spargel is localized exclusively within the nucleus (Figure 1B), the same as PGC-1, which is largely located in the nucleus (Monsalve *et al.* 2000). Interestingly, Spargel localization in the nucleus is predictable with NucPred and ESLPred software (Bhasin and Raghava 2004; Brameier *et al.* 2007) with almost 93–94% accuracy. Nuclear localization of Spargel was further confirmed with a specific nuclear protein SC35, a pre-mRNA splicing factor (Fu and Maniatis 1992) (Figure 1C). In mammalian cells, PGC-1 has been shown to be associated with the splicing complexes because PGC-1 and SC35 are colocalized to the same punctate structures (Monsalve *et al.* 2000). To test whether Spargel maintains a similar characteristic, we found that Spargel and SC35 are also colocalized to the same speckles almost perfectly (Figure 1D). Under higher magnification, salivary gland polytene nuclei displayed more intimate association between SC35 and Spargel (Figure 1E, yellow signals), which help us to predict that both Spargel and PGC-1 are conserved constituents of the splicing complex. We believe that such discrete localization of Spargel into the nucleus is possibly resulting from an embedded 16-amino-acid-long Nuclear Localization signal

Figure 2 Spargel is downstream of Tor. (A) Spargel overexpression (OE) was achieved by activating an *EP(gy2)* insertion in the 5'-UTR of *spargel* (CG9809) with a *Act-GAL4* driver. (B and C) RT-PCR analysis of *spargel* OE with *EP(gy2)srl*⁺ and *UAS-srl*⁺ insertions with *Act-GAL4* driver confirmed that the *EP-srl*⁺ element overexpresses Spargel at the same level as the *UAS-srl*⁺. About twofold more *spargel* mRNA expression was achieved from both EP and UAS lines. Since *EP-srl*⁺ is located on the third chromosome, and *UAS-srl*⁺ is located on the second, the former insertion was chosen since it makes the genetic manipulations easier. $^{**}P < 0.01$; $^{***}P < 0.001$. (D) Cells expressing *TOR(DN)* (two GFP-positive cells) mutation in the fat body tissue appeared much smaller in size with tiny nuclei (blue). (E) Spargel OE helps to recover the reduced cell size of *TOR(DN)* cell clones (GFP positive), which now appears normal in size. (F) Comparison of the nuclear area (NA) and cytoplasmic area (CA) of *TOR(DN)* cell clones before and after Spargel OE confirmed that a significant increase in cell size occurs following Spargel OE ($N > 25$). Bar, 40 μ m. CA, cytoplasmic area normalized with control; NA, nuclear area normalized with control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (G) Expression of *TOR(DN)* in the wings with *MS-1094-GAL4* causes shortened and misshaped wings due to cell growth defect. (H) Following Spargel OE, the wings of *TOR(DN)* attained their normal shape and size ($N > 25$).

(NLS) (amino acids 734–750) as predicted by PredictProtein and NLS Mapper software (Rost and Liu 2003; Kosugi *et al.* 2009) (Figure 1F). Contrary to our observation, an earlier study claimed that a HA-tagged Spargel protein is localized

in the cytoplasmic compartment, which gets transported inside the nucleus following activation by insulin receptor (Tiefenbock *et al.* 2010). Based on colocalization data and other characteristics, nuclear localization of Spargel appears

Figure 3 Reduced Spargel expression suppresses the *Tsc2* RNAi-mediated overgrowth. (A) Normal eye. (B) Activation of *Tsc2*-RNAi in the eye with *GMR-GAL4* causes cellular overgrowth so the eyes appear much larger in size. (C) The overgrowth effect of *Tsc-2* reduction is mended when *spargel* expression is reduced with *spargel RNAi* so the eyes turned normal ($N > 25$). (D) *Tsc2*-RNAi effect and cosuppression of *Tsc-2*; *spargel* was normalized with control eye. Bar for eye, 100 px. (E) *Tsc2* RNAi expression in cell clones (GFP positive) causes over growth of those cells (compare with non-GFP cells). (F and G) Activation of *spargel RNAi* in *Tsc2*-RNAi cell clones ameliorates the overgrowth phenotype. Bar for cell clones, 40 μ m. * $P < 0.05$, *** $P < 0.001$.

to be more logical; however, future studies should be able to resolve this issue further.

Spargel acts downstream of TOR and S6kinase: TOR is at the core of cell and tissue growth as TOR can influence cell growth in a cell autonomous manner (Wullschlegel *et al.* 2006; Zhang *et al.* 2006). Absence of the TOR nutrient sensor causes smaller cell size and fully penetrant larval lethality (Zhang *et al.* 2000; Hennig *et al.* 2006). We wanted to test the interaction between TOR and Spargel on cell growth because many of TOR's diverse effects on cellular physiology overlap with Spargel (Zhang *et al.* 2000; Schieke *et al.* 2006). For example, clones of cells with reduced Spargel expression appear smaller in size (Figure 1, G and H) and *spargel* hypomorphic adults have smaller body size (Supporting Information, Figure S1E). This led us to conclude that Spargel can control cell growth in a cell autonomous manner similar to TOR.

Cell clones expressing TOR-dominant negative [*TOR* (*DN*)] mutation (Hennig and Neufeld 2002) (also known as TOR toxic effector domain, *TOR^{TED}*) appeared much smaller in size due to reduced cell growth (Figure 2D). *TOR^{TED}* expresses the 754-amino-acid central domain of TOR that acts in a dominant negative fashion because it is thought to sequester signaling factors. Overexpression of Spargel protein (Figure 2, A–C) in the *TOR*(*DN*) cells helps ~80% of the small-sized *TOR*(*DN*) cell to attain normal size (Figure 2, E and F). This led us to wonder whether Spargel is a downstream mediator of TOR-induced cell growth. So, we attempted to restore the tissue-restricted phenotypes of *TOR*(*DN*) with Spargel. When expressed in the wings with wing-specific *MS-1096-GAL4* driver, *TOR*(*DN*) causes a wing defect (Figure 2G) due to severe restriction of cell growth during wing development (Hennig and Neufeld 2002). Co-

overexpression of Spargel and *TOR*(*DN*) with the same GAL4 driver suppresses the wing phenotype and results in a completely normal wing shape (Figure 2H). As a final attempt, we wanted to rescue the lethal effect of *TOR*(*DN*) with excess Spargel. *TOR*(*DN*) is early lethal (Hennig and Neufeld 2002) so no pupae are recovered in this mutant (Table S1) (Hennig and Neufeld 2002). Ubiquitous overexpression of Spargel with the help of *Act-GAL4* driver in the *TOR*(*DN*) embryos resulted in 94% pupal formation of which 10–12% actually eclosed as adults, which is unprecedented (Table S1). Spargel-rescued *TOR*(*DN*) adults still appeared smaller in body size (Figure S1D) and they survived for a brief length of time. These data imply that Spargel can only take over certain functions of TOR, such as cell growth control, as documented. Yet TOR also controls a large array of cellular processes (DePalma *et al.* 2012; Laplante and Sabatini 2012) and given that the rescue was not complete, we interpret the result to indicate that Spargel does not function in all the effects mediated by TOR. This interpretation is further supported by the fact that Spargel overexpression does not influence the phosphorylation status of 4EBP (Figure S2), meaning TOR regulates 4EBP phosphorylation in a Spargel-independent manner. We therefore conclude that TOR's action on cell size and cell growth is mediated through Spargel and thus Spargel is an important downstream effector of TOR for cell growth signaling.

The above conclusion will be best supported if *spargel*-RNAi can block the TOR overexpression phenotype. Since TOR overexpression is lethal, we used the tuberous sclerosis complex (*Tsc*), which works upstream of TOR and negatively regulates TOR function (DePalma *et al.* 2012). Thus inactivation of *Tsc2* through RNAi (also known as *gigas*-RNAi) in the eye tissue causes an eye enlargement effect (Figure 3,

Figure 4 Spargel acts downstream to S6Kinase. (A) S6K gain of function in the fat body cells (GFP positive) cause cellular overgrowth. (B and C) Following *spargel RNAi*, the S6K overexpressing cells attained regular size ($N > 25$). (D) Cells carrying the *S6k(DN)* mutation negatively affects cell growth so they appeared much smaller in size. (E and F) Effect of *S6k(DN)* on cell size can be completely (100%) rescued with excess Spargel as evident from immunohistochemistry and comparison of NA and CA measurement ($N > 25$). Bar, 40 μ m. CA, cytoplasmic area normalized with control; NA, nuclear area normalized with control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (G) Expression of *S6k(DN)* on the dorsal surface of the wing with *apterous-GAL4* causes the wings to bend upward. (H) Near complete rescue of the *S6k(DN)* wing defects was possible when Spargel is overexpressed in the same wings ($N > 25$).

A and B), the same as *Tsc2* mutants (Potter *et al.* 2001). When Spargel levels are reduced (with *spargel RNAi*) in conjunction with *Tsc-2 RNAi*, this double mutant has the same phenotype as that of *spargel-RNAi* because the excess cell growth defect of *Tsc-2* was abrogated, causing the eyes to attain their normal shape (Figure 3, C and D). Similarly, the overgrowth phenotype of the *Tsc2 RNAi* cell clones (Figure 3E) in the fat body tissue are suppressed by *spargel RNAi* (Figure 3, F and G) with ~80% efficiency. These data suggest that Spargel action is necessary for *Tsc-2* to impose its effect on cell growth and most importantly provide further support that loss of Spargel can counteract the high TOR activity that is already known to be induced by the loss of *Tsc*.

S6 kinase (S6K), is primarily involved in cellular protein synthesis (Ruvinsky and Meyuhas 2006) and it is the immediate downstream effector of TOR (Figure 1A). Overexpression of S6K causes cellular overgrowth and such overgrowth phenotype can be normalized by down-regulating Spargel expression in these cells (Figure 4, A and B). On the other hand, due to reduced protein synthesis, *S6K(DN)* (also known as *S6K^{KQ}*; lysine (K₁₀₉) is replaced by glutamine generates kinase dead S6K protein) cells appear much smaller in size (Barcelo and Stewart 2002) (Figure 4D) and for the same reason *S6k* mutants have growth defects with smaller body size and high frequency of larval lethality (Montagne *et al.* 1999). Clonally expressed excess Spargel allowed

100% of the *S6k(DN)* cells in the clones to attain normal cell size (Figure 4E). *S6k(DN)* expression on the dorsal surface of the wing with an *apterous-GAL4* driver causes the wings to bend upwards due to cell size reduction on the dorsal wing surface (Figure 4G). Overexpression of Spargel with the same *apterous-GAL4* helps this bent wing phenotype to return to its normal shape and size (Figure 4H), suggesting that Spargel acts downstream, hence its overexpression is rescuing the S6K phenotype. Phosphorylation of S6K by TOR-kinase activates the S6K pathway leading to downstream protein synthesis. We therefore investigated whether Spargel overexpression changes the phosphorylation status of S6K. We did not see a change in S6K phosphorylation (Figure S2), which further supports our interpretation that Spargel acts downstream of S6K in the insulin-TOR pathway. To summarize, in the insulin-TOR pathway Spargel is a downstream effector of TOR and S6K to inflict its effect on cell size regulation.

Excess Spargel subdues FoxO overexpression defect: Fork head box-O (FoxO) transcription factor is a negative effector in the insulin-signaling pathway (Junger *et al.* 2003; Puig *et al.* 2003). When nutrients are in short supply, FoxO slows down insulin signaling by activating 4EBP (4E binding protein), which acts as a global suppressor of translation (Puig *et al.* 2003; DePalma *et al.* 2012; Laplante and Sabatini 2012). This action of FoxO minimizes the situation of

Figure 5 Spargel and FoxO interaction. (A) Overexpression of FoxO severely affects cell growth (arrows). It is possible that the FoxO OE cells will die before even reaching the adult stage. (B) Following Spargel OE, some of these tiny cells (~11%) attained increased cellular and nuclear areas as quantitated in C. (D) Normal eye. (E) FoxO OE in the eye with *GMR-GAL4* driver shows a significant reduction in ommatidial numbers. (F) FoxO OE effect on ommatidial numbers can be ameliorated by Spargel OE ($N > 50$). (G) Severe wing phenotype resulted when FoxO is overexpressed with *apterous-GAL4* driver. (H) Excess Spargel partially fixes the wing defect in H. (I and J) RT-PCR analysis of *spargel* mRNA expression in *FoxO* null background revealed that Spargel is not regulated by FoxO. Bar for cell clones, 25 μ m; bar for eye, 100 px. CA, cytoplasmic area normalized with control; NA, nuclear area normalized with control. *** $p < 0.001$; NS, not significant.

cellular energy expenditure under stress. Although FoxO has been claimed to be a negative regulator of Spargel transcription activity in *Drosophila* S2 cells (Gershman *et al.* 2007), we noticed no changes in Spargel transcription activity in the *FoxO*-null situation (Figure 5, I and J). To obtain further insight on FoxO–Spargel interaction, we co-overexpressed them in the same cell. FoxO overexpression in larval fat body causes the cells to be unhealthy with very tiny nuclei and little to no cytoplasmic area, when compared to the neighboring control cells outside the clone (Figure 5A). When Spargel is produced in excess, we found ~11% of FoxO-OE cell clones attain their normal size (Figure 5, A–C). Similarly, excess Spargel is capable of slightly improving the FoxO-mediated phenotypic severities in the wing and the eye tissue (Figure 5, D–H). While excess Spargel ameliorates the severity of FoxO overexpression in a tissue-limited fashion, the lethal effect of ubiquitous FoxO overexpression cannot be rescued with Spargel (Table S2). As a transcription factor, FoxO regulates multiple targets, which in turn influences various cellular processes in vital tissues (Accili and Arden 2004; Greer and Brunet 2005). Our experiments show that levels of Spargel overexpression that were able to rescue the TOR and S6K effects in the fat body are not capable of producing a similar high percentage of rescue of the FoxO overexpression phenotype. Therefore we suggest that Spargel does not share all of the FoxO functions and consequently cannot significantly rescue the FoxO phenotypes, or it is possible that Spargel function is not enough

to take over any of these functions, hence only a limited rescue happened.

Spargel is involved in growth signaling through mitochondrial biogenesis: Based on the above observations, we propose an extension of the insulin–TOR signaling pathway in which Spargel is incorporated as a new terminal effector in the cell growth signaling pathway (Figure 6E). Although reduced expressions of many insulin–TOR signaling partners negatively influence cell growth and Spargel does the same, Spargel gain of function is not lethal, nor does it cause any cellular or organismal overgrowth (Figure S1, A–C) (Rera *et al.* 2011). We now have reasons to believe that Spargel controls the energy flow in this system as mitochondrial energy production is upheld during Spargel overexpression with increased ATP generation (Figure 6A). This observation follows some earlier claims where Spargel overexpression was related to higher activity of electron transport complexes (Rera *et al.* 2011). Conversely, reduced Spargel level negatively impacted the electron transport chain enzymes and ATP synthesis (Tiefenbock *et al.* 2010). How is this excess ATP utilized during Spargel overexpression? One clue came from the muscle-specific overexpression of Spargel, which prolongs the vertical climbing behavior in flies (Tinkerhess *et al.* 2012), possibly due to excess ATP production.

When cells need to grow, TOR is activated through phosphorylation either by activated insulin signals or by amino acids. An activated TOR maintains nutrient and

Figure 6 Modified insulin-TOR pathway including Spargel. (A) Increased ATP production resulted from ubiquitous overexpression of Spargel, (***) $P < 0.001$, NS, not significant). (B) Immunohistochemical analysis with mitochondria-specific ATP-5A antibody revealed that the small-sized *TOR(DN)* cell clones (yellow border) carry fewer mitochondria (white speckles). Inset shows that *TOR(DN)* cells were identified as GFP-positive cells. Large fat droplets appeared as black holes are free of mitochondria. (C) Following Spargel OE, *TOR(DN)* cell clones (three GFP-positive cells in the inset marked with yellow border) attain normal size and their mitochondrial numbers appear to have increased significantly. (B' and C') Enlarged view of the portions of the cell clones showing much fewer mitochondria in *TOR(DN)* cells; however Spargel OE in these cells has vastly improved the mitochondrial numbers in C'. (D) Quantification of mean fluorescence intensity confirmed three times more mitochondria are present in the *TOR(DN)* cells rescued with Spargel ($N > 25$). (***) $P < 0.001$. (E) Based on our results we propose a modified insulin-TOR pathway including Spargel as a terminal effector downstream of Tsc-2, TOR, S6K, and FoxO. Since Spargel is a terminal effector, we anticipate that all participants of the insulin-signaling pathway impose their effect on cell size and cell growth through Spargel because Spargel regulates mitochondrial energy metabolism.

energy balance by promoting transcription, protein synthesis, and mitochondrial function (Schieke *et al.* 2006). So, to consider the possibility that Spargel action is tied to energy supply, we checked the mitochondrial content during Spargel overexpression. Indeed, in *TOR(DN)* cell clones, the number of mitochondria were far fewer (Figure 6, B, B', and D) compared to the *TOR(DN)* cell clones overexpressing Spargel (Figure 6, C, C', and D). This observation supports our model that Spargel regulates mitochondrial energetics in the growth-signaling pathway (Figure 6E). Being a transcriptional coactivator, Spargel controls an array of genes involved in mitochondrial function, glucose, fat, and protein metabolism (Tiefenbock *et al.* 2010). In addition to their function in protein production and energy generation, possible involvement of these other factors in cell growth process cannot be ruled out. Finally, based on colocalization of Spargel with splicing complexes suggest that Spargel/dPGC-1 may be involved with processing of a number of cellular mRNAs (Monsalve *et al.* 2000). Some of those mRNA's might be important for cell growth as well, although mitochondrial

energetics is possibly the centerpiece in this process. Thus, Spargel serves as a final checkpoint in the insulin-TOR pathway for regulation of cell size and cell growth; however, the mechanism of Spargel action remains to be elucidated.

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GENETICS

Supporting Information

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Spargel/dPGC-1 Is a New Downstream Effector in the Insulin–TOR Signaling Pathway in *Drosophila*

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Figure S1 Spargel OE has no influence on body size. (A) Spargel OE doesn't influence the body size. (B and C) Spargel OE cell clones (GFP positive) shows no cellular overgrowth phenotype (N > 25) (D) Adult rescuers expressing Tor(DN) with Spargel OE can live up to the adulthood. Although these flies appeared smaller in body size and has a very short life span. Scale bar = 40um. NS = Not Significant. (E) Though Spargel gain of function does not seem to affect systemic growth, *spargel* hypomorph (*srl¹*) and pervasive expression of *spargel* RNAi show growth retardation phenotype.

Figure S2 Spargel over expression does not influence phosphorylation of S6k and 4EBP. S6k and 4EBP are phosphorylated by TOR kinase. Spargel OE causes no change in the phosphorylation status of either S6K or 4EBP. This observation agrees well with the genetic analysis since Spargel is downstream to TOR, so it doesn't influence the Tor kinase activity.

File S1

Supporting Methods

GFP tagged Spargel: *spargel* cDNA was cloned in *UASpN* terminal GFP tag gateway vector (DGRC) and injected into fly lines (Best Gene). Flies were crossed with *Lsp-GAL4* and 3rd instar larvae were dissected, fixed with 4% paraformaldehyde in 1X PBS; washed with 0.3% PBX and mounted for visualization under confocal microscope.

Table S1 Spargel OE improve the effects of TOR (DN) during development

Genotypes	# of pupae formed	# of adults eclosed
Tor and Spargel interaction:		
<i>Act-Gal4 > UAS-Tor (DN)</i>	0	0
<i>Act-Gal4 > UAS-Tor (DN);EP-srl+</i>	71*** (94.6%)	9** (12%)
<i>UAS-Tor (DN); EP-srl+/ TM6, Tb (Control)</i>	75	75 (100%)

Chi-square test determined the significance of the spargel-mediated rescue. ** P < 0.01, *** P < 0.001. DN =

Dominant Negative, OE = Overexpression. Parentheses show percentage of pupae and adults.

Table S2 Spargel OE fails to rescue the FoxO OE induced lethality.

Genotypes	# of pupae formed	# of adults eclosed
FoxO and Spargel interaction		
<i>Act-GAL4>FoxO</i>	0	0
<i>Act-GAL4>FoxO; EP-srl+</i>	0 ^{NS}	0 ^{NS}
<i>UAS-FoxO; EP-srl+ (Control)</i>	190	190

FoxO over expression is developmental lethal. Spargel OE can't rescue this lethality.

NS = Not Significant. OE = Over Expression.