Activated FOXO-mediated insulin resistance is blocked by reduction of TOR activity

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Summary

Reducing insulin/IGF signaling allows for organismal survival during periods of inhospitable conditions by regulating the diapause state, whereby the organism stockpiles lipids, reduces fertility, increases stress resistance, and has an increased lifespan. The Target of Rapamycin (TOR) responds to changes in growth factors, amino acids, oxygen tension, and energy status; however, it is unclear how TOR contributes to physiological homeostasis and disease conditions. Here, we show that reducing the function of *Drosophila* TOR results in decreased lipid stores and glucose levels. Importantly, this reduction of dTOR activity blocks the insulin resistance and metabolic syndrome phenotypes associated with increased activity of the insulin responsive transcription factor, dFOXO. Reduction in dTOR function also protects against age-dependent decline in heart function and increases longevity. Thus, the regulation of dTOR activity may be an ancient "systems biological" means of regulating metabolism and senescence, that has important evolutionary, physiological, and clinical implications.

Introduction

The major cause of metabolic syndrome (defined as a cluster of metabolic abnormalities such as elevated glucose and lipid levels, related to a state of insulin resistance) and diabetes in humans is reduction of insulin signaling, but the underlying pathways and mechanisms are not completely understood. Likewise, caloric excess can lead to nutrient toxicity and desensitization of insulin signaling. Thus, dysregulation of energy homeostasis can lead to metabolic disturbances and predisposition to a variety of endocrine diseases including diabetes, cardiovascular disease, and cancer (Biddinger and Kahn, 2006; Kahn et al., 2005; Lee and White, 2004).

One major system that regulates energy homeostasis in higher metazoa is the insulin/IGF pathway. The functionally conserved components of the insulin/IGF pathway like insulin, the insulin receptor (InR), insulin receptor substrate (IRS), phosphoinositide 3-kinase (PI3K), protein kinase B (PKB, a.k.a. Akt) and the forkhead transcription factor FOXO have been shown to be involved in glucose and lipid homeostasis (Accili and Arden, 2004; Barthel et al., 2005; Biddinger and Kahn, 2006; Kahn et al., 2005; Lee and White, 2004) as well as growth and aging (Burgering and Kops, 2002; Finch and Ruvkun, 2001; Greer and Brunet, 2005; Kenyon, 2005; Tran et al., 2003). Loss of insulin signaling in the periphery and in pancreatic β cells can lead to hyperglycemia and diabetes (Biddinger and Kahn, 2006; Lee and White, 2004). For example, disruption of the *InsR* gene in the pancreatic β cells reduces islet size and insulin secretion (Kulkarni et al., 1999). *IRS1* knockout mice are hyperglycemic, but their pancreatic β cells hypertrophy to compensate for increased peripheral insulin resistance (Araki et al., 1994; Burks and White, 2001). In contrast, *IRS2* knockout mice are diabetic because their pancreatic β cells are absent due to increased cell death (Burks and White, 2001). Additionally, systemic loss of insulin signaling in metazoans leads to elevated lipids as seen in the *Daf-2* mutant worms, *Chico/IRS* mutant flies, and *IRS2* ablated mice (Böhni et al., 1999; Burks et al., 2000; Kenyon et al., 1993; Kimura et al., 1997).

Many of these insulin/IGF-mediated metabolic effects depend on the winged helix transcription factor, FOXO. FOXO was first identified in the worm, *C. elegans* as *Daf-16*, a mutation that can suppress the increased lipid levels and longevity caused by loss of *Daf-2*, the worm *InR* ortholog (Lin et al., 1997; Ogg et al., 1997). There is a single evolutionarily conserved *Drosophila FOXO* ortholog (Junger et al., 2003; Kramer et al., 2003; Puig et al., 2003) and three mammalian *FOXO* genes (*FOXO1*, *FOXO3a*, and *FOXO4*). FOXO1 controls glucose homeostasis in both peripheral tissues and pancreatic β cells (Accili and Arden, 2004). For example, expression of a constitutively activated FOXO1 (resistant to insulin/IGF-mediated inactivation) in liver and pancreatic β cells causes hepatic insulin resistance and loss of pancreatic β cells via increased apoptosis, whereas reduction of FOXO1 function can reverse the loss of pancreatic β cells and hyperglycemia seen in the *IRS2* ablated mice (Kitamura et al., 2002; Nakae et al., 2002). Thus, FOXO is a critical mediator of insulin signaling in both insulin sending and receiving tissues.

The Tuberous Sclerosis Complex (TSC1-2)/Target of Rapamycin (TOR) pathway responds to changes in insulin/IGF levels, amino acid levels, energy charge, lipid status, mitochondrial metabolites, and oxygen tension by adjusting cell growth (Abraham, 2002; Fingar and Blenis, 2004; Kim and Sabatini, 2004; Kozma and Thomas, 2002; Long et al., 2004; Oldham and Hafen, 2003; Wullschleger et al., 2006). In addition to its

well-defined role in controlling cell growth, the TSC1-2/TOR pathway may also potentially be a critical regulator of glucose and lipid homeostasis as TSC1-2/TOR signaling functionally interacts with the insulin/IGF pathway. A role for TOR signaling in glucose and lipid homeostasis in mammalian systems is demonstrated by the *S6K1* knockout mice. These mice are hyperglycemic caused by diminished insulin secretion due to reduced pancreatic β cell mass (Pende et al., 2000; Um et al., 2004). This result is in keeping with studies that show that rapamycin treatment leads to decreased levels of translation, growth, and survival of pancreatic β cells (Bell et al., 2003; Kwon et al., 2004; McDaniel et al., 2002). However, the *mS6K1* mutant mice have low lipid levels because of adipocytes that have increased fatty acid β-oxidation. Additionally, the *mS6K1* mutant mice show enhanced glucose uptake upon exogenous insulin addition due to insulin hypersensitivity in peripheral tissues via loss of a negative feedback loop on IRS (Um et al., 2004). Thus, TOR signaling via S6K can modulate insulin sensitivity by altering Ser307 and Ser636/639 phosphorylation and IRS protein levels (Carlson et al., 2004; Haruta et al., 2000; Patti et al., 1998; Tremblay et al., 2005; Tzatsos and Kandror, 2006; Ueno et al., 2005; Um et al., 2004).

There are additional levels where TSC1-2/TOR signaling may positively and negatively regulate insulin signaling. There are data that suggest that the IRS Ser302 site is required for signaling to TOR and S6K (Giraud et al., 2004). Thus, ser/thr phosphorylation of the IRS proteins may mediate both positive and negative signals for energy homeostasis. Furthermore, Akt/PKB activity may also be directly regulated by the nutrient-sensitive TOR pathway. Although the insulin/IGF pathway can signal to the TSC1-2/TOR pathway, recent evidence suggests that TOR may directly control Akt/PKB function because Akt/PKB activation depends on TORC2 complex-specific TOR Ser473 phosphorylation of Akt/PKB (Sarbassov et al., 2005). Thus, these studies suggest that dysregulation of TSC1-2/TOR signaling may contribute to the pathological progression of metabolic syndrome and diabetes, yet the direct role and function of TOR is unclear in this context.

Another functionally conserved energy homeostatic pathway is the AMPK pathway. This pathway responds to altered energy states caused by cellular stresses like mitochondrial dysfunction, anti-diabetic drugs, and exercise (Carling, 2004; Hardie, 2005; Kahn et al., 2005). Activation of the energy sensing AMPK pathway by activated AMPK as well as metformin or AICAR treatment results in decreased lipogenesis and gluconeogenesis via both central and peripheral effects (Carling, 2004; Hardie, 2005; Kahn et al., 2005). Activated AMPK can phosphorylate TSC2, which inhibits TOR signaling, while loss of AMPK activity causes an increase in TOR signaling (Bolster et al., 2002; Dubbelhuis and Meijer, 2002; Inoki et al., 2003; Kimura et al., 2003; Shaw et al., 2004). However, the requirement of TOR function for the AMPK energy response is not known. These effects may also be mediated by targets including glycogen synthase, hormone-sensitive lipase, acetyl-CoA carboxylase-2, HMG-CoA reductase, p300, and p53 (Carling, 2004; Hardie, 2005; Kahn et al., 2005); the different roles of these proteins in the AMPK-mediated low energy response are not well known. Furthermore, activation of AMPK leads to IRS Ser-789 phosphorylation and enhancement of insulin signaling, which suggests that the AMPK response can act separately from the TOR pathway to enhance insulin signaling (Jakobsen et al., 2001). Clearly, there is a great need to understand the regulation of TSC1-2/TOR signaling as it relates to the maintenance of energy homeostasis because TOR function is implicated in both insulin/IGF and AMPK signaling.

Although TOR occupies a central node that governs catabolic or anabolic responses to different nutritional and energy states, the resultant metabolic effects of altering TOR function in a metazoan are incompletely and poorly understood. This study examines in detail the function of *Drosophila TOR* in terms of energy homeostasis and senescent responses. We show that reduction of dTOR function results in decreased glucose and lipid levels with concomitant increase of DILP2 from the insulin producing cells. We also show that a reduction of dTOR function can block activated dFOXO-mediated insulin resistance and metabolic syndrome phenotypes. Taken together, these data indicate that dTOR function is required for the maintenance of energy homeostasis and organismal senescence. The additional ramifications of this study are that reduction of TOR function may have clinical utility for treating metabolic syndrome and insulin resistance.

Results

Discovery and analysis of a novel dTOR allele

Complete loss of dTOR function results in early larval lethality (Oldham et al., 2000; Zhang et al., 2000). In order to analyze the effects of a reduction of dTOR function on processes like energy homeostasis and senescence, we utilized a new hypomorphic mutation in *dTOR* within the kinase domain (Figure 1A) (Oldham et al., 2000). Flies transheterozygous for this *dTOR* allele ($dTOR^{2L7}$) and a *dTOR* P-element insertion ($dTOR^{l(2)k17004}$, hereafter called $dTOR^{7/P}$ mutant) are viable and 20% smaller compared to control (data not shown). We first examined how dTOR effectors are altered in the $dTOR^{7/P}$ mutant. Unexpectedly, we observed no d4EBP protein in the $dTOR^{7/P}$ mutant and only mildly decreased phospho-dS6K compared to the control (Figure 1B). This effect may be due to translational or post-translational effects, as we observe d4EBP mRNA in the $dTOR^{7/P}$ mutant (data not shown).

Reduction of dTOR function decreases lipid levels

Depending on the type of environmental perturbation, lipid levels may be increased for diapause (as seen in InR pathway mutants) or hibernation, whereas during times of energy reduction caused by dieting or exercising (which leads to AMPK activation), lipids are mobilized preferentially over protein stores for fuel because of their high energy content (Cahill and Veech, 2003; Saltiel and Kahn, 2001). We first determined how metabolic stores are altered by examining the lipid levels in the $dTOR^{7/P}$ mutant. We see that reduction of dTOR function by the $dTOR^{7/P}$ mutant causes a decrease in lipid levels in the fat body. Nile Red staining of the $dTOR^{7/P}$ mutant revealed substantially decreased lipid levels in the fat body compared to control (Figures 1C and 1D). The remaining lipids were confined to small vesicles within the cytoplasm of the fatbody cells.

These decreased lipid levels in the $dTOR^{7/P}$ mutant may be due to an increased utilization of lipids from pre-existing lipid stores and/or the prevention of lipid storage. This decrease in lipid levels correlated with an increase in the mRNA levels of a lipase (termed Brummer in *Drosophila*) involved in regulating lipid levels in the fatbody (Grönke et al., 2005), which suggests that

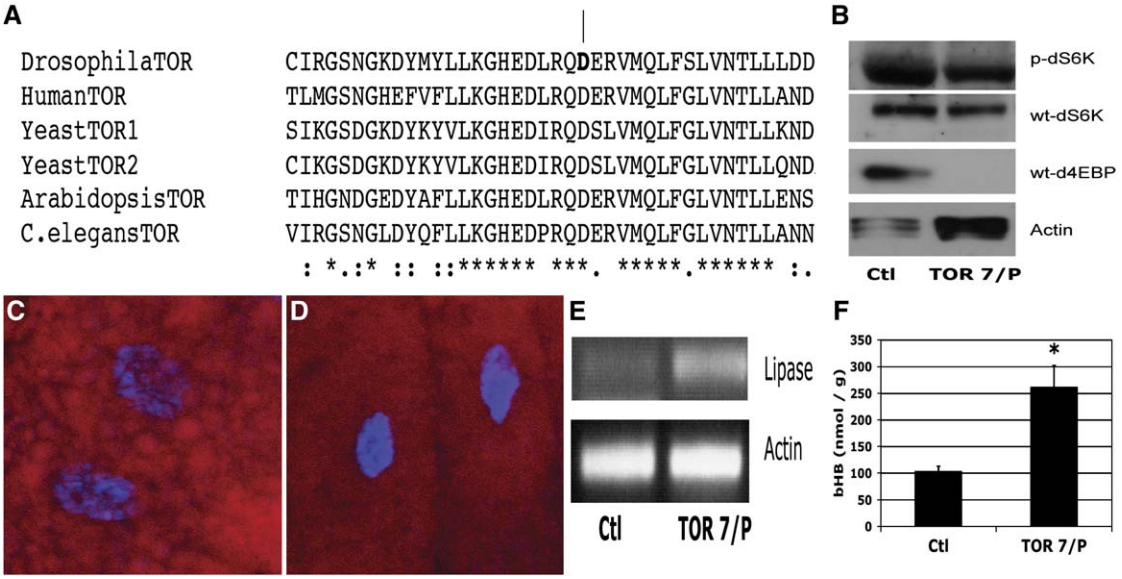

Figure 1. *Drosophila* TOR regulates lipid metabolism

A) Sequence alignment of the *dTOR*^{7} allele. The mutated residue is D2116G and is completely conserved in TOR orthologs (indicated by the line). This residue lies in the N-terminal region of the kinase domain and shows reduced kinase activity (unpublished data).

B) Slightly reduced phospho-dS6K (T398) and no wild-type d4EBP levels in the *dTOR*$^{7/P}$ mutant flies compared to control as determined by Western blotting. Actin levels are on the bottom.

C) Nile Red staining of control third instar larvae fatbody.

D) Nile Red staining of the *dTOR*$^{7/P}$ mutant larvae fatbody. DNA stained with DAPI.

E) Reduction of *dTOR* function results in increased lipase mRNA levels.

F) Reduction of *dTOR* function results in elevated ketone bodies (p = .0028, unpaired, two-tailed t test). Experiment has been performed two times independently.

reduction of dTOR function results in lipid breakdown due to increased lipase activity (Figure 1E). Consistent with the elevated lipase mRNA levels, we also observed a 27% decrease in triglyceride levels in the *dTOR*$^{7/P}$ mutant that depended on the lipase gene as a null mutant for *brummer* reversed the triglyceride decrease (data not shown). To further determine the basis of the lipid breakdown, we measured ketone bodies (β-hydroxybutyrate), whose levels also serve as an indicator of lipid utilization. The data show that there is a 155% increase in β-hydroxybutyrate levels in the *dTOR*$^{7/P}$ mutant flies, which is consistent with a conversion of lipids to ketone bodies (Figure 1F). Thus, reduction of dTOR function causes the breakdown of lipid stores and conversion into ketone bodies.

Inhibition of dTOR activity lowers glucose levels

Changes in lipid metabolism are linked with alterations in insulin signaling (Saltiel and Kahn, 2001). There are 7 insulin-like peptides in *Drosophila* (DILPs), which are structurally and functionally related to insulin and IGF (Brogiolo et al., 2001). The bilateral set of seven medial neurosecretory insulin producing cells (IPCs) in the fly brain hemispheres express DILP2 (Ikeya et al., 2002; Rulifson et al., 2002). Loss of the IPCs increase blood glucose levels and can be rescued by exogenous DILP2 expression, which shows that loss of insulin/IGF production and signaling is causally linked to altered glucose homeostasis in *Drosophila* (Broughton et al., 2005; Ikeya et al., 2002; Rulifson et al., 2002). As DILP2 is a key regulator for glucose levels, we first determined whether DILP2 protein levels are changed in the neurosecretory IPCs in the *dTOR*$^{7/P}$ mutant. We see that the *dTOR*$^{7/P}$ mutant results in an increase in DILP2 protein levels in both the cell body and axonal projections of the IPCs (Figures 2A and

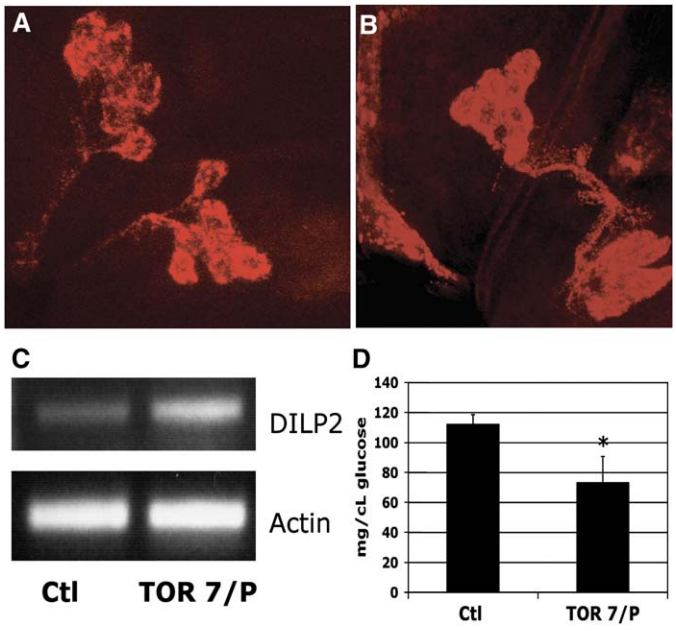

Figure 2. *Drosophila* TOR regulates DILP2 levels and glucose homeostasis

A) DILP2 shows expression in the cytoplasm of the NSC cell body and into the axonal processes in the wild-type.

B) The *dTOR*$^{7/P}$ mutant has increased DILP2 staining in both the cell body and axons. Confocal Z-series taken at 60×.

C) DILP2 mRNA levels in the *dTOR*$^{7/P}$ mutant. DILP2 (top) mRNA levels are increased in the *dTOR*$^{7/P}$ mutant flies. Actin loading control is on the bottom.

D) The *dTOR*$^{7/P}$ mutant glucose levels are significantly decreased (p = .0086, unpaired, two-tailed t test) compared to control. Experiment has been performed three times independently.

2B). To determine if the increased DILP2 protein levels due to reducing dTOR function also occurs at the mRNA level, we performed RT-PCR on the *dTOR*$^{7/P}$ mutant and looked for changes in DILP2 mRNA levels. Remarkably, we saw that the *dTOR*$^{7/P}$ mutant had increased DILP2 mRNA levels relative to the control (Figure 2C). Thus, reducing dTOR function can increase the levels of DILP2. As altered DILP2 levels lead to changes in glucose homeostasis, we next examined the *dTOR*$^{7/P}$ mutant for possible effects on glucose homeostasis. Remarkably, the *dTOR*$^{7/P}$ mutant shows a 39% decrease in blood glucose levels (Figure 2D). Thus, a reduction of dTOR function can lead to lowered glucose levels.

Lowering dTOR function blocks activated FOXO-mediated insulin resistance

The insulin/IGF pathway is a major integrator of metabolic and stress signals that depend on the function of FOXO (Matsumoto and Accili, 2005). Constitutively activated FOXO can increase the lifespan of worms and flies if expressed in appropriate spatiotemporal patterns (Giannakou et al., 2004; Hwangbo et al., 2004; Libina et al., 2003; Wolkow et al., 2000). However, constitutive FOXO activation in the liver and pancreatic β cells causes hyperglycemia in mammals (Nakae et al., 2002). Thus, activated FOXO represents the most distal step known for inducing insulin resistance and metabolic syndrome phenotypes.

Because decreased TOR signaling can enhance insulin signaling upstream of FOXO via reduced IRS ser/thr phosphorylation, we used a constitutively active dFOXO (dFOXO-TM, in which the three Akt/PKB phosphorylation sites have been inactivated) to ask whether reducing dTOR function can act downstream of dFOXO function (Hwangbo et al., 2004; Junger et al., 2003; Kramer et al., 2003; Puig et al., 2003). We first analyzed systemic dFOXO expression for changes in lipid and glucose levels. We see that expression of activated dFOXO results in increased lipid levels as assessed by Nile Red staining and measurement of triglyceride levels (Figures 3A–3C. Compare to Figures 1A and 1B). These elevated lipid levels correspond with increased dFAS mRNA levels in this background (Figure 3D). We next asked whether the *dTOR*$^{7/P}$ mutant is able to alter this activated dFOXO metabolic phenotype. We see that reduction of dTOR function can block both the lipid and glucose increase mediated by constitutively activated dFOXO (Figure 3C and data not shown). We also see that dFAS mRNA levels are decreased in the activated dFOXO-*dTOR*$^{7/P}$ mutant background (Figure 3D). We see that dFOXO is still present in the nucleus so the *dTOR*$^{7/P}$ mutant is not altering the localization of dFOXO (data not shown). As activated dFOXO expression in the head fatbody decreases DILP2 mRNA levels (Hwangbo et al., 2004), we next determined the effect of expressing activated dFOXO specifically in the IPCs. We see that activated dFOXO-mediated insulin resistance within the IPCs leads to low DILP2 mRNA levels and high glucose levels (Figures 3E and 3F). Remarkably, the low DILP2 mRNA levels and high glucose levels caused by constitutively activated dFOXO expression in the insulin producing cells are blocked by the *dTOR*$^{7/P}$ mutant (Figures 3E and 3F). Thus, in addition to upstream effects on insulin pathway components, reducing dTOR function can block dFOXO-mediated relevant metabolic targets to reverse the increased glucose and lipid phenotypes (Figure 4).

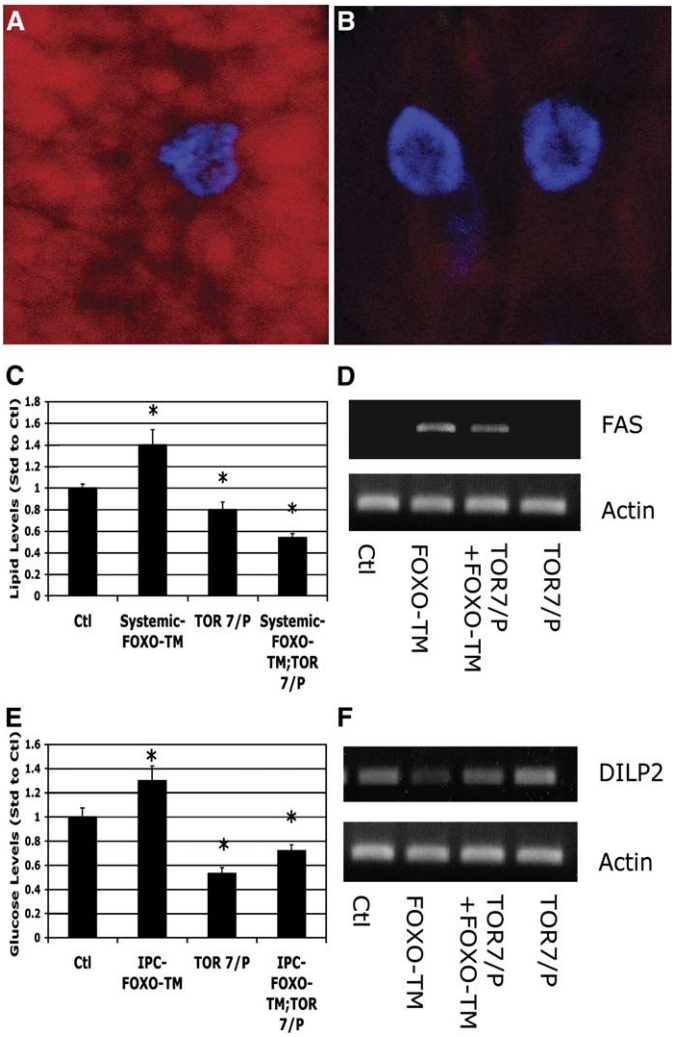

Figure 3. Reducing TOR activity blocks activated FOXO-mediated insulin resistance and metabolic syndrome phenotypes

A) Nile Red staining of *arm-Gal4; UAS-dFOXO-TM* third instar larvae fatbody.

B) Nile Red staining of the *arm-Gal4, dTOR*$^{7/P}$*; UAS-dFOXO-TM* third instar larvae fatbody.

C) The triglyceride assay shows that the *arm-Gal4; UAS-dFOXO-TM* line has increased lipid levels, while the *arm-Gal4,dTOR*$^{7/P}$*; UAS-dFOXO-TM* mutant combination has lipid levels similar to the *dTOR*$^{7/P}$ single mutant.

D) Reduction of dTOR function reverses the dFOXO-TM-mediated increase in dFAS mRNA levels.

E) The glucose assay shows that the *DILP2-Gal4; UAS-dFOXO-TM* line has increased glucose levels, while the *DILP2-Gal4,dTOR*$^{7/P}$*; UAS-dFOXO-TM* mutant combination has glucose levels similar to the *dTOR*$^{7/P}$ single mutant. Fold change in triglyceride or glucose levels where 1 equals wild-type triglyceride or glucose levels, >1 equals increased triglyceride or glucose levels, and <1 equals reduced triglyceride or glucose levels. All lines are significantly different ($p < .05$, unpaired, two-tailed t test) relative to the control.

F) Decreasing dTOR activity overcomes dFOXO-TM-mediated inhibition of DILP2 mRNA levels.

All experiments have been performed twice independently.

Dampening dTOR signaling enhances lifespan and heart performance

As TOR integrates growth factor signaling, amino acids levels, energy charge, lipid status, mitochondrial metabolites, and oxygen tension, TOR responds to many stimuli that can potentially affect both longevity and organ senescence (Kenyon, 2005; Partridge and Gems, 2002; Sharp and Bartke, 2005; Tatar et al.,

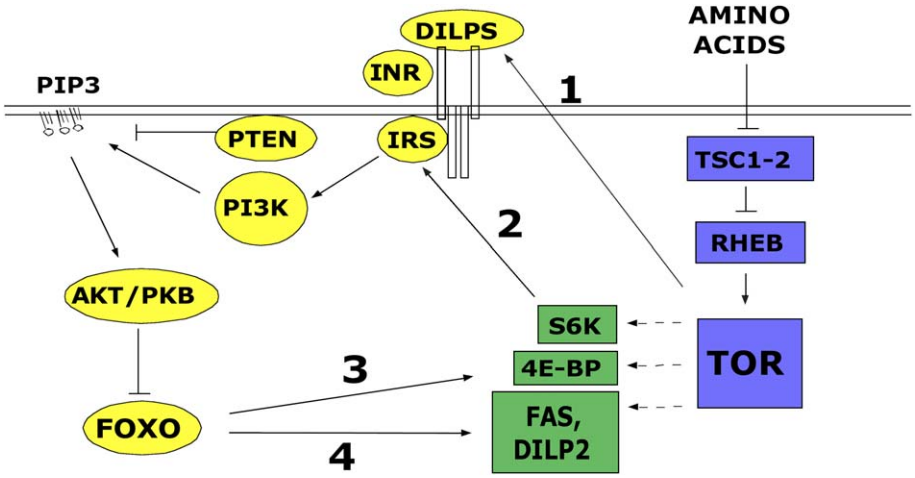

Figure 4. Model of functional interaction of TOR signaling with the insulin/IGF pathway

The numbered arrows indicate potential levels of functional interaction and are neither meant to represent whether the interaction is positive or negative nor any hierarchal dominance. TORC2 is not included.

2003). Indeed, studies in *Drosophila* using overexpression of dTOR pathway inhibitors suggest that TOR signaling may be contributing to aging (Kapahi et al., 2004). Furthermore, direct inhibition of TOR in yeast and the worm shows that loss of TOR function results in increased lifespan along with increased stress resistance (Kaeberlein et al., 2005; Powers et al., 2006; Vellai et al., 2003). We asked whether the $dTOR^{7/P}$ mutant could alter aging at the organ and organismal level, by first using an assay that measures the progressive age-dependent decline in heart function (Wessells et al., 2004). We found that the $dTOR^{7/P}$ mutant had a low heart failure rate that was observed in young flies as well as old flies (Figure 5A). Remarkably, the heart failure rate of old $dTOR^{7/P}$ mutant flies was similar to the heart failure rate of the young flies. Furthermore, in keeping with the decrease in heart failure, the median lifespan of the $dTOR^{7/P}$ mutant was increased by 20% under normal feeding conditions (Figure 5B). Thus, the age-related protection of heart function correlates well with the increased lifespan in the $dTOR^{7/P}$ mutant.

As increased longevity is frequently associated with increased stress resistance (Kenyon, 2005), we tested whether the $dTOR^{7/P}$ mutant can resist environmental stresses. We first tested the ability of the $dTOR^{7/P}$ mutant to withstand water-only starvation. Remarkably, the $dTOR^{7/P}$ mutant had no difference in the ability to withstand water-only starvation (Figure 5C). We next tested $dTOR^{7/P}$ for its ability to resist ROS production induced by paraquat treatment. Again, $dTOR^{7/P}$ behaved essentially as wild-type when subjected to oxidative stress (Figure 5D). Thus, reduction of dTOR function does not provide resistance against acute stresses. We also observed that the $dTOR^{7/P}$ mutants are fertile, have wild-type levels of ATP, and retain flight ability (data not shown). Despite the strong association between increased longevity and acute stress resistance, these results suggest that a partial reduction in dTOR function may preferentially regulate organ senescence and longevity versus acute stress responses.

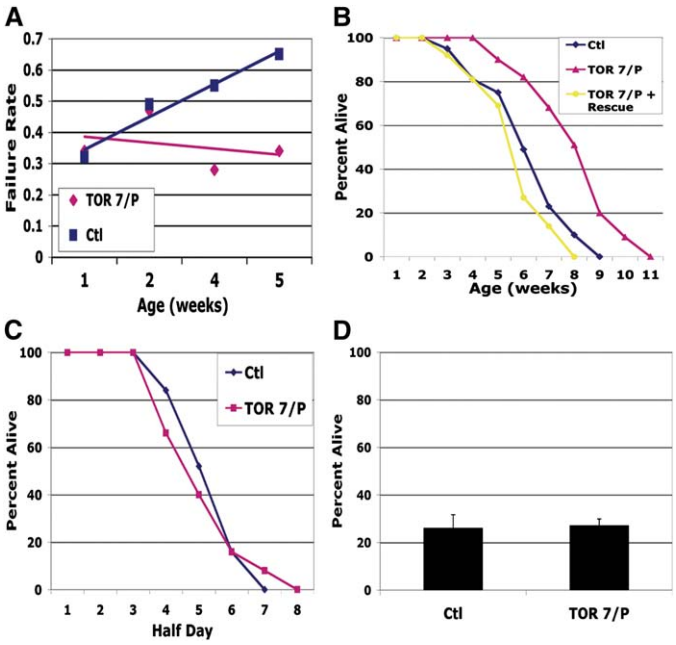

Figure 5. *Drosophila* TOR regulates aging and organ senescent responses

A) dTOR regulates age-related changes in cardiac performance. Failure rate of wild-type flies (*yw*) changes significantly with age (age-by-genotype, $\chi^2 = 59.2$, $p < .0001$). The cardiac failure rate of the $dTOR^{7/P}$ mutant exhibited a significantly decreased rate of change with age compared to *yw* (age-by-genotype, $\chi^2 = 7.49$, $p = .0519$). Numbers tested are as follows: *yw* 1 week = 100, 2 week = 47, 4 week = 79, 5 week = 92. $dTOR^{7/P}$ mutant 1 week = 245, 2 week = 231, 4 week = 177, 5 week = 200.

B) The $dTOR^{7/P}$ mutant flies exhibited a significantly extended lifespan as compared to the *yw* background ($\chi^2 = 12.42$, $p = .0004$) and to the $dTOR^{7/P}$ mutant flies which also carried a genomic rescue construct for *dTOR* ($\chi^2 = 10.56$, $p = .0012$). The $dTOR^{7/P}$ mutant flies with a *dTOR* rescue construct no longer showed a significant difference from the *yw* background ($\chi^2 = .15$, $p = .7001$). Numbers tested were as follows: *yw* = 59, *yw* + *dTOR* rescue construct = 59, $dTOR^{7/P}$ mutant = 88.

C) Reduction in *dTOR* activity has no effect on starvation resistance. The $dTOR^{7/P}$ mutant does not affect resistance to starvation conditions compared to the background *yw* genotype (genotype effect, $\chi^2 = 1.23$, $p = .2676$). Representative results from one of two replicate experiments are shown. Numbers tested for this replicate are: *yw* = 31, $dTOR^{7/P}$ mutant = 38.

D) Reduction in *dTOR* activity has no effect on resistance to oxidative stress. The $dTOR^{7/P}$ mutant had no significant effect on survival compared to the background *yw* stock (unpaired, two-tailed t test, $p = .477$). Representative results from one of two replicate experiments are shown. Numbers tested for this replicate are: *yw* = 28, $dTOR^{7/P}$ mutant = 16.

Discussion

TOR controls multiple aspects of lipid metabolism

In contrast to the elevated lipid levels caused by reduction of systemic insulin signaling (Böhni et al., 1999; Burks et al., 2000; Kenyon et al., 1993; Kimura et al., 1997), the $dTOR^{7/P}$

mutant does not show increased lipid levels. Instead, the *dTOR*$^{7/P}$ mutant shows decreased lipid levels of the fat body that depend on the function of a lipase involved in lipid metabolism (Grönke et al., 2005). We also observed elevated ketone bodies in the hypoglycemic *dTOR*$^{7/P}$ mutant, which is indicative of the increased utilization of lipids. Studies in mammalian cardiac tissue have shown that ketone bodies provide their high energy electrons directly to complex I, the NADH dehydrogenase multienzyme complex, of the mitochondrial electron transport chain (Cahill and Veech, 2003). Thus, the altered lipid levels show that dTOR has a critical role in determining the fate of fats.

It has also been shown that d4EBP is involved in lipid metabolism because the increased lipid levels caused by rapamycin treatment are blocked by a *d4EBP* mutant (Teleman et al., 2005a). Furthermore, loss of the *melted* mutant has lower lipid levels, due to lowered triglyceride production (Teleman et al., 2005b). This effect is due to increased d4EBP protein levels via dFOXO activation in the fatbody. However, there is no change in glucose levels. In this respect, the *melted* mutant resembles the *FIRKO* mouse because it shows decreased triglyceride levels without a change in glucose levels (Biddinger and Kahn, 2006). We see that the *dTOR*$^{7/P}$ mutant has a different lipid phenotype than the one caused by rapamycin treatment, which suggests that rapamycin alters dTOR function in a different manner than the *dTOR*$^{7/P}$ mutant. Additionally, we see that a novel hypomorphic *dTOR* FAT domain allele in combination with the *dTOR*^{P} allele also shows low glucose and lipid levels (data not shown), which suggests that partial reduction of dTOR activity represents a unique phenotypic class of dTOR metabolic effects versus an allele specific phenotype. Although rapamycin affects TORC1 directly, TORC2 may be altered indirectly via TOR depletion and blocking of TORC2 assembly (Sarbassov et al., 2005, 2006). Thus, it is not currently clear if the effects of rapamycin are due to inhibiting TORC1 and/or TORC2. It is known that rapamycin can impair pancreatic β cell function because it causes decreased growth and survival (Bell et al., 2003; Kwon et al., 2004; McDaniel et al., 2002). Thus, rapamycin treatment can lead to elevated glucose and lipid levels, possibly as a result of systemic insulin loss (Morrisett et al., 2003).

TOR is required for glucose homeostasis

We see that DILP2 levels are increased in the IPCs of the *dTOR*$^{7/P}$ mutant and that the *dTOR*$^{7/P}$ mutant has lowered glucose levels. Thus, reduction of dTOR function can lead to increased DILP2 levels and a reduction of glucose levels. Recent studies with the *Drosophila miRNA-278* mutant also showed elevated DILP levels, yet displayed fatbody-mediated insulin resistance as shown by elevated d4EBP and glucose levels (Teleman and Cohen, 2006). We believe that the *dTOR*$^{7/P}$ mutant represents an insulin-sensitized state because the *dTOR*$^{7/P}$ mutant shows decreased levels of the insulin resistance marker d4EBP, the *dTOR*$^{7/P}$ mutant shows decreased glucose levels, and, as discussed below, the *dTOR*$^{7/P}$ mutant blocks activated dFOXO-mediated insulin resistance phenotypes. Thus, the *dTOR* mutant phenotype resembles a whole-animal 'insulin-sensitized' state that can function below the level of constitutive dFOXO activity.

TOR signaling integrates with the insulin pathway at multiple levels

We see that overexpression of activated dFOXO in peripheral and IPC tissues results in elevated glucose and lipid levels. Although dTOR signaling can alter insulin signaling upstream of dFOXO, reducing dTOR function is able to reverse these effects. Thus, our results show that reduction of dTOR activity can block the activated dFOXO-mediated insulin resistance and metabolic syndrome phenotypes (Figure 4). These results suggest that strategies to dampen, reduce, or block TOR signaling may be able to overcome insulin resistance (i.e., hyperglycemia and hypertriglyceridemia) below the level of increased FOXO activity in mammalian systems.

Although FOXO has >100 potential targets that might contribute to the metabolic phenotype (Lee et al., 2003; McElwee et al., 2003; Murphy et al., 2003), we have identified dFAS and DILP2 as candidate mediators of the dTOR effect on the dFOXO metabolic phenotypes. The effect on dFAS is interesting because it is upregulated by dFOXO overexpression and in an *IRS*/*chico* mutant (data not shown) and may be an important determinant of the lipid levels. It has also been shown that activation of daf-16/FOXO can decrease the mRNA levels of a worm insulin gene, ins-7 (Murphy et al., 2003). This result is consistent with our results showing that DILP2 mRNA levels are decreased and reducing dTOR activity can reverse this dFOXO-mediated reduction of DILP2. These results might have parallels with a role for FOXO and TOR in the regulation of insulin levels in mammals.

We also see a selective and unexpected regulation of dTOR effectors: loss of d4EBP protein and a mild effect on dS6K Ser389 phosphorylation. It has been recently shown that the *d4EBP* gene is a target of dFOXO in *Drosophila* (Bernal and Kimbrell, 2000; Junger et al., 2003; Miron et al., 2001; Tettweiler et al., 2005) and thus may represent one of the dTOR targets responsible for contributing to the dFOXO-mediated metabolic phenotypes. It has also been shown that *daf-15*/*Raptor* is a target of *daf-16*/*FOXO* in *C. elegans* and may also contribute to the dTOR metabolic and senescent phenotypes (Jia et al., 2004). Raptor may also account for the selective difference in the regulation of d4EBP and dS6K function by TOR because Raptor binds to both S6K and 4EBP and loss of 4EBP may allow for more S6K binding to Raptor for TOR-mediated phosphorylation (Hara et al., 2002). Thus, these results suggest that reduction of dTOR function may have selective effects on translation.

TOR may control aging and organ senescence as a caloric restriction mediator

Reduction of dTOR function does not provide resistance against acute stresses or cause sterility. This result is in contrast to the yeast *TOR1* mutant, which shows elevated stress resistance (Powers et al., 2006), and the *d4EBP* mutant, which shows stress and starvation sensitivity (Tettweiler et al., 2005). Nevertheless, the *dTOR*$^{7/P}$ mutant has an increased lifespan. This result is in keeping with the yeast, worm and fly studies that show that loss of TOR signaling can increase lifespan, as a major mediator of caloric restriction (Kaeberlein et al., 2005; Kapahi et al., 2004; Powers et al., 2006; Vellai et al., 2003). Thus, alterations of TOR signaling contribute to the regulation of lifespan.

We also see that reduction of dTOR activity prevents age-dependent functional decline of heart performance. It is not currently clear how dTOR is regulating these organ and organismal responses, but the altered lipid metabolism may underlie these changes. For example, changes in lipid metabolism can both autonomously and non-autonomously affect heart function (Belke et al., 2002; Chiu et al., 2005; Park et al., 2005). Thus,

reduction of dTOR function may reallocate energy stores preferentially for the control of 'long-term' responses such as lifespan and organ maintenance. Importantly, there are many potential links between changes in energy homeostasis with alterations in aging and organ senescence (Curtis et al., 2005; Kenyon, 2005; Wessells et al., 2004). Channelling diverse stimuli like amino acids, growth factors, oxygen tension, and energy charge into the TOR pathway may be an economic method to mobilize fuel stores like lipids to counteract these fluctuations.

Drosophila is a model system to study metabolic syndrome and diabetes

The conservation of basic mechanisms between *Drosophila* and mammals is well established. It has been shown that disruption of insulin signaling in non-mammalian systems like *Drosophila* results in altered glucose and lipid levels (Broughton et al., 2005; Rulifson et al., 2002). We found that reducing dTOR function can reverse activated dFOXO-mediated insulin resistance phenotypes induced in both insulin producing and insulin receiving tissues, and thus this study provides the first direct evidence that reducing TOR function may have a clinical benefit to counter insulin resistance, metabolic syndrome, and/or diabetes. Furthermore, altering TOR signaling may underlie the benefits of various diet and nutritional regimens. These results demonstrate the utility of using the powerful genetics of this system to unravel the complex pathways involved in maintaining glucose and lipid homeostasis. In unraveling the complex genetic network of TOR and InR signaling, although far from completion, the *Drosophila* model has been indispensable in finding critical components and uncovering functionally important genetic interactions between these two pathways (Oldham and Hafen, 2003). Thus, the basic mechanisms controlling glucose and lipid homeostasis, including mechanisms by which the TSC1-2/TOR pathway influences insulin signaling as well as the influence of TSC1-2/TOR signaling on peripheral tissue and IPC physiology, are also functionally conserved.

Conclusion

We have described a new use for reducing dTOR activity to block insulin resistance, metabolic syndrome, and diabetic-like phenotypes downstream of activated dFOXO, underlining the utility of the *Drosophila* model to identify and analyze components and compounds that block insulin resistance and metabolic syndrome phenotypes as well as pathological aspects of aging and organ senescence.

Experimental procedures

Fly genetics

Standard genetic analyses were performed as described (Oldham et al., 2000). A detailed description of the new *dTOR* allele will be described elsewhere (unpublished data).

Immunohistochemistry, Western, and RT-PCR analyses

We used the DILP2 antibody to measure DILP2 protein levels by performing immunohistochemistry as described (Rulifson et al., 2002) and wt-d4EBP, phospho-4EBP, wt-dS6K, and phospho-dS6K antibodies to perform Western blotting as described (Miron et al., 2001; Oldham et al., 2000). We isolated total RNA (Qiagen) and constructed cDNA libraries (Stratagene) from the *yw* control, dFOXO-TM, the $dTOR^{7/P}$ mutant, and combinations. We then performed semiquantitative RT-PCR using primers that spanned the DILP2, dFAS, or brummer intron and used actin5C as an internal loading control (primer sequences and cycle conditions available upon request).

Metabolic assays

Lipid and glucose

We isolated the fat body from third instar larvae, fixed them with formaldehyde, and stained them with 0.5 mg/ml (final) Nile Red. The fat body was washed with 1× PBS and then mounted in Vectashield for fluorescence microscopy (Zeiss, Axiophot). Triglyceride and glucose levels were detected using a glucose oxidase assay (Pointe) and lipid assay (Thermo) as described (Rulifson et al., 2002; Teleman et al., 2005a) with exception that we extracted the lipids with a 1:1 mixture of chloroform/methanol (vol:vol).

Ketone bodies

The β-hydroxybutyrate (bHB) ketone body was measured as follows: we used 20 mg of tissue weighed on a balance cooled with cups of liquid N_2 around the balance pan. The tissue was weighed into a 1.5 or 2.0 ml screw cap Eppendorf centrifuge tube containing glass beads (1 mm diameter) that was cooled in liquid N_2. To the tissue, 4 volumes of ice-cold 0.55 M perchloric acid were added and homogenized immediately for 30 s using a Minibeadbeater (Biospec Products). The samples were then neutralized by adding 3 M KHCO3. After neutralizing, the tubes were placed in ice 30 - 60 min and spin and transferred the supernatant to a screw cap cent tube for analysis. For the fluorometric assay the wells contained: 50 mM 2-Amino-2-methylpropanol (pH 9.9), 5 mM NAD, 2 mM EDTA, 0.01% BSA. Analyses were done using a TECAN Spectrafluor Plus microplate reader (Durham, NC) using a 340 nm excitation filter and a 465 nm emission filter. We used bHB dehydrogenase (Biocatalytic) and NADH production from bHB was measured.

Stress, aging, and heart senescent assays

Starvation

Survival of male flies was tested under water-only starvation conditions as described in (Oldham et al., 2000).

ROS resistance

Survival of male flies was tested during exposure to paraquat-induced oxidative stress. Flies were exposed to 10 mM Pq in 1% sucrose and survival was assessed after 48 hr.

Lifespan

Lifespan assays were performed by taking 15–20 flies per tube and turning to a fresh tube every 2–3 days. The number of viable flies was scored at these intervals.

Heart function

Cardiac failure rates in response to electrical pacing were measured according to (Wessells et al., 2004).

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