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Tissue-Specific Coupling between Insulin/IGF and TORC1 Signaling via PRAS40 in *Drosophila*

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SUMMARY

PRAS40 has recently been identified as a protein that couples insulin/IGF signaling (IIS) to TORC1 activation in cell culture; however, the physiological function of PRAS40 is not known. In this study, we investigate flies lacking *PRAS40*. Surprisingly, we find both biochemically and genetically that PRAS40 couples IIS to TORC1 activation in a tissue-specific manner, regulating TORC1 activity in ovaries but not in other tissues of the animal. PRAS40 thereby regulates fertility but not growth of the fly, allowing distinct physiological functions of TORC1 to be uncoupled. We also show that the main function of PRAS40 in vivo is to regulate TORC1 activity, and not to act as a downstream target and effector of TORC1. Finally, this work sheds some light on the question of whether TORC1 activity is coupled to IIS in vivo.

INTRODUCTION

TOR complex 1 (TORC1) and insulin/IGF signaling (IIS) are two highly conserved pathways that sense nutrient status in animals from flies to humans. IIS is responsive to hormonal cues, thereby integrating information about organismal nutrient status. In contrast TORC1 signaling, which is conserved in unicellular organisms, senses primarily cell-autonomous information such as cellular stress, energy, and nutrients (Avruch et al., 2006; Kwiatkowski and Manning, 2005; Martin and Hall, 2005; Shaw, 2009). Both IIS and TORC1 integrate this information to regulate multiple physiological processes including carbohydrate metabolism, lipid metabolism, tissue growth, fertility and lifespan in a manner that is conserved from flies to mammals (Fontana et al., 2010; Goberdhan and Wilson, 2003; Grewal, 2009; Kozma and Thomas, 2002; Nakae et al., 2001). Of note, TORC1 is one of the most powerful anabolic signals in cells, regulating cellular growth via modulation of protein and lipid biosynthesis, leading to cellular mass accumulation. As a consequence TORC1 is hyperactivated in almost all cancers (Bjornsti and Houghton, 2004; Guertin and Sabatini, 2007).

The connection between IIS and TORC1 has been a matter of debate. In human and *Drosophila* tissue culture cells, treatment with insulin leads to rapid TORC1 activation (Cai et al., 2006; Hahn et al., 2010; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002), indicating the two pathways are linked. Less clear is whether this link is functionally relevant for cells in an animal under physiological conditions. Studies in *Drosophila* have suggested this is not the case. One molecular link connecting IIS to TORC1 is the TSC1/2 complex. In cell culture, phosphorylation of Tsc2 by Akt inactivates it, thereby relieving its suppression of TORC1 (Cai et al., 2006; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). However, in *Drosophila* in vivo, removal of the Akt phosphorylation sites on Tsc1 and Tsc2 leads to no defects in TOR activation and no physiological consequences (Dong and Pan, 2004; Schleich and Teleman, 2009), suggesting that although this molecular link exists, it does not play an important functional role under physiological conditions. Although one explanation could be the presence of redundant molecular mechanisms linking IIS to TORC1, this appears not to be the case. In *Drosophila* larvae, reduction of IIS either via removal of the insulin receptor substrate chico, or via reduction of PI3K activity ($Dp110^{A}$), has no effect on activity of S6K and hence TORC1 (Oldham et al., 2000; Radimerski et al., 2002), raising the question whether TORC1 activation is at all linked to IIS in vivo under physiological conditions. Because of the central role TORC1 plays in regulating cellular growth and metabolism, a better understanding of the mechanisms regulating its activity would have implications for both normal and pathophysiological conditions such as cancer, metabolic disease, or infertility.

Recently the protein PRAS40 has been proposed to link IIS to TORC1 in cell culture. Two reports showed that PRAS40 binds the TORC1 complex thereby inhibiting its activity, and that phosphorylation of PRAS40 by Akt relieves this inhibition (Nascimento et al., 2010; Sancak et al., 2007; Vander Haar et al., 2007). Three other studies, however, identified PRAS40 as a TORC1 substrate, suggesting that the apparent inhibitory effects of PRAS40 on the canonical TORC1 substrates 4EBP and S6K may reflect competition for substrate binding (Fonseca et al., 2007; Oshiro et al., 2007; Wang et al., 2007). This would place PRAS40 downstream, rather than upstream of TORC1. Indeed, as these studies point out, PRAS40 might function concomitantly as a TORC1 substrate and a TORC1 regulator, regulating mTORC1 activity via direct inhibition of substrate binding. These studies have led to several open questions: (1) does PRAS40 regulate TORC1 activity in vivo, as it does in cell culture? (2) does PRAS40 link IIS to TOR activation in vivo? and (3) is the main function of PRAS40 to act as a TOR substrate or as a TOR regulator? These two options can be distinguished in an animal context. If the main function of PRAS40 is to regulate

TORC1 activity (i.e., it is genetically upstream of TORC1), then *PRAS40* mutant phenotypes should be rescued by reducing activity of TORC1 or of a TORC1 target other than PRAS40. If, instead, PRAS40 functions mainly as a TOR substrate downstream of TORC1, then loss of PRAS40 cannot be rescued by manipulating TORC1. To our knowledge, no animal models for *PRAS40* loss of function have yet been reported to address these questions.

One physiological function of IIS and TORC1 of particular relevance to this present study is the regulation of fertility. In *Drosophila*, insulin-like peptides (DILPs) secreted by neurosecretory cells regulate the rate of germline stem cell division in the ovary (LaFever and Drummond-Barbosa, 2005). This links metabolic status to fertility, so that rich nutrient conditions cause high DILP secretion, leading to increased egg production. If IIS is abrogated in the ovary, as in the case of *chico* or *InR* mutants, egg production is completely blocked and the animals are sterile (Böhni et al., 1999; Brogiolo et al., 2001; Drummond-Barbosa and Spradling, 2001). The defect in *chico* mutant ovaries is ovary-autonomous because transplantation of *chico* mutant ovaries into wild-type hosts, containing normal levels of DILPS, does not rescue their defects (Richard et al., 2005). At the cellular level, IIS and TORC1 regulate almost all aspects of oogenesis including the rate of proliferation of ovarian somatic and germline cells, germline stem cell maintenance, vitellogenesis, and oocyte loss (Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2009; Hsu et al., 2008; LaFever et al., 2010; Sun et al., 2010; Thomson et al., 2010). Interestingly, the roles of IIS and TORC1 in regulating fertility are highly conserved throughout evolution, regulating similar processes in *Caenorhabditis elegans* (Michaelson et al., 2010) and in mammals. As in flies, reduction of IIS via knockout of *IGF-1* or *IRS-2* causes infertility in mice (Baker et al., 1993; Burks et al., 2000). As in flies, normal TORC1 in mice prevents oocyte loss (Thomson et al., 2010) and hyperactivation of IIS or TORC1 leads to premature activation of all primordial follicles, resulting in premature follicular depletion (Reddy et al., 2010; Sun et al., 2010). In sum, IIS and TORC1 play critical roles in regulating fertility in an evolutionarily conserved manner.

We present here a *PRAS40* loss-of-function animal model. By generating *PRAS40* knockout *Drosophila*, we study the in vivo function of *PRAS40*, as well as the connection between IIS and TORC1. We show that PRAS40 does function to link IIS to TORC1 in the animal. Unexpectedly, however, we find that it does so in a tissue-specific manner, influencing TORC1 activity predominantly in the fly ovary, but not in other tissues of the animal. As a result, PRAS40 regulates development of the ovary, but not growth or proliferation of somatic tissues, thereby influencing animal fertility but not animal growth. Because PRAS40 is present in all tissues of the fly, this indicates PRAS40 is a link between IIS and TORC1 that can be switched on and off in a tissue-specific manner. Furthermore, we find that *PRAS40* knockout phenotypes can be rescued by inhibiting TORC1 or by reducing S6K gene dosage, indicating that PRAS40 functions mainly as a TORC1 inhibitor in vivo. Finally, this work sheds light on the conundrum whether the IIS and TORC1 signaling pathways are linked under normal physiological conditions, showing that they are indeed linked, but only in particular tissues.

RESULTS

CG10109 Is *Drosophila* PRAS40

A BLAST search of the *Drosophila* proteome using human PRAS40 protein sequence identifies CG10109 as the top hit ($E = 10^{-4}$). Conversely, BLASTing the human proteome with CG10109 identifies hPRAS40 as a top hit, establishing an orthology relationship between hPRAS40 and CG10109, in agreement with previous reports (Sancak et al., 2007). The CG10109 coding sequence was previously associated with a mutant phenotype called Lobe (Chern and Choi, 2002; Wang and Huang, 2009). Lobe alleles cause preferential loss of the ventral eye domain due to aberrant Notch and JAK/STAT signaling (Chern and Choi, 2002). Although the Lobe alleles were mapped to the 51A2-B1 genomic region, which also contains *CG10109*, to our knowledge they were not molecularly mapped to the *CG10109* gene. Although Lobe loss-of-function alleles such as $L^{rev6\text{-}3}$ are lethal (Chern and Choi, 2002), we found they are rescued to viability without any obvious phenotype when put in *trans* to a deficiency uncovering the *CG10109* locus, Df(2R)ED2354, suggesting Lobe does not map genetically to *CG10109*. Furthermore, we tried to rescue viability of the $L^{rev6\text{-}3}$ allele using a *UAS-CG10109* transgene but were unable to do so. Because these results raise the possibility that Lobe might not correspond to *CG10109*, we undertook a de novo analysis of *CG10109* function, which we rename here *dPRAS40*.

We first tested whether dPRAS40 has the biochemical characteristics previously described for human PRAS40—that it binds Raptor and is phosphorylated on T246 in response to insulin stimulation. A protein alignment of dPRAS40 and hPRAS40 reveals that both the Akt phosphorylation motif containing Thr246 and the Raptor binding motif (TOS) are conserved (RPRLRS and FDLED, respectively). We expressed HA-tagged dPRAS40, immunoprecipitated (IP) it from Kc cells, and tested its phosphorylation status using a ''phospho Akt substrate'' antibody recognizing the phosphorylated R-x-R-x-x-p(S/T) motif. This revealed that insulin stimulation causes a marked induction in PRAS40 phosphorylation (Figure 1A, lanes 3 and 4). This phosphorylation is abrogated if Ser558, the equivalent site to Thr246 in hPRAS40, is mutated to alanine (Figure 1A, lanes 5 and 6), indicating that the phospho-signal is specific for S558. We next tested whether dPRAS40 can bind dRaptor by co-IP in S2 cells, and this was indeed the case (Figure 1B).

We next studied the physiological consequences of *dPRAS40* overexpression. Overexpression of *PRAS40* specifically in the posterior half of the fly wing using *engrailed-GAL4* caused a marked reduction in size of the posterior compartment (Figure 1C). This reduced tissue size was associated with reduced cell size (Figure 1D) and no defects in patterning of the veins or of the wing margin. The reduced size of the posterior compartment could not be rescued by concomitant expression of the apoptosis inhibitor p35 (Figure 1E), indicating it is not due to apoptosis. In sum, the observed phenotypes of *PRAS40* overexpression are consistent with an effect on TORC1, which regulates tissue size mainly via regulation of cell size. Consistent with this, overexpression of *Rheb*, an upstream activator of TORC1, was able to partially rescue the undergrowth caused by *PRAS40* overexpression (Figure 1F). These gain-of-function effects were not specific for the wing,

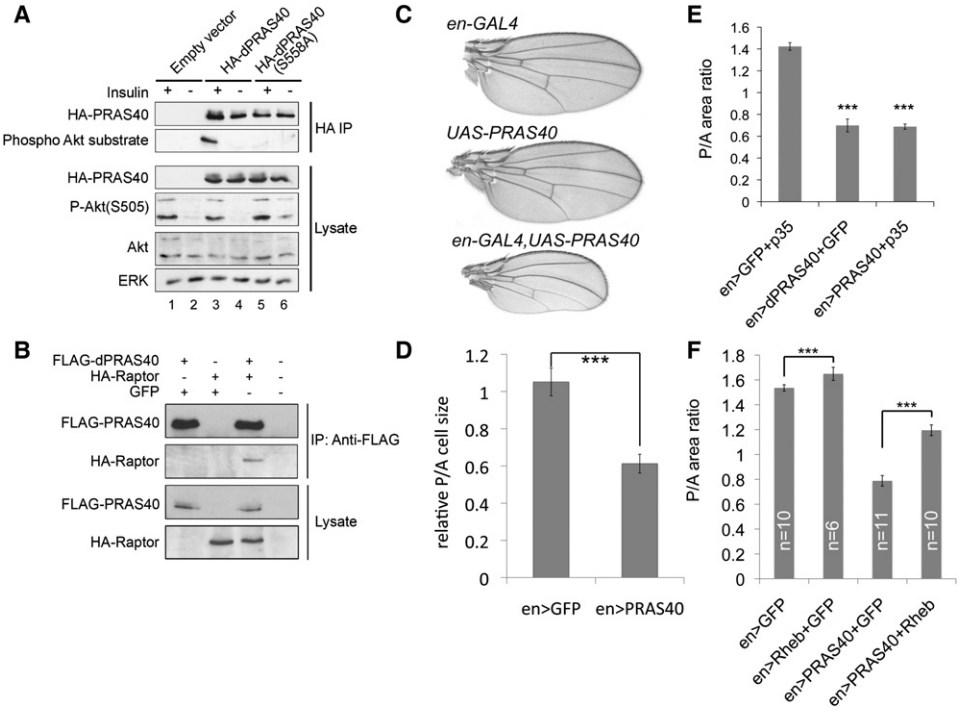

Figure 1. Overexpression of CG10109, *Drosophila* PRAS40, Causes Tissue Undergrowth

(A) *Drosophila* PRAS40 is phosphorylated on Ser558 in response to insulin. Kc cells were stimulated +/− 10 μg/mL insulin for 60 min prior to lysis. Immunoprecipitated HA-PRAS40 was then probed with phospho-Akt-substrate antibody recognizing the phospho-motif R-x-R-x-x-(S/T)*. Phosphorylation of PRAS40 increased upon insulin stimulation, but not if Ser558 was mutated to alanine.

(B) *Drosophila* PRAS40 and Raptor interact physically. FLAG-dPRAS40 and HA-dRaptor were expressed in S2 cells and anti-FLAG immunoprecipitates were probed as indicated. A GFP-expression construct was used to normalize DNA amounts in the transfection.

(C) Overexpression of *PRAS40* in the posterior compartment of the wing causes reduced tissue growth. Wings of *engrailed-GAL4*, *UAS-PRAS40* flies show a marked reduction in size of the posterior compartment, compared to the parental genotypes that do not overexpress *PRAS40*.

(D) *PRAS40* overexpression causes reduced cell size. Cell size of posterior wing cells determined by quantifying hairs per unit area in wings expressing either *GFP* or *PRAS40* in the posterior compartment with *engrailed-GAL4*. Relative size of posterior cells versus control anterior cells is indicated (n = 10).

(E) Undergrowth caused by *PRAS40* overexpression is not rescued by the apoptosis inhibitor p35. Quantification of posterior versus control anterior area of wings expressing *p35*, *PRAS40*, or both in the posterior compartment with *engrailed-GAL4*. *UAS-GFP* was used as a normalization to counteract possible GAL4 titration effects (n = 12).

(F) Overexpression of *PRAS40* and *Rheb* counteract each other phenotypically. Posterior versus anterior area for wings of indicated genotypes. Overexpression of *Rheb* partially rescues the reduced growth caused by *PRAS40* overexpression.

In all panels, ***Mann-Whitney U < 0.001, error bars denote SD.

as overexpression in the eye caused reduced eye size (see Figure S1A available online) and ubiquitous overexpression with actin-GAL4 caused reduced size of the entire animal and pupal lethality (Figure S1B).

PRAS40⁻ Flies Are Viable and Normally Sized

To study the function of endogenous PRAS40 in *Drosophila*, we generated a *CG10109* loss-of-function allele by homologous recombination-mediated gene knockout (*PRAS40^{KO}*, Figure 2A). We knocked-out 500 bp of coding sequence from the second exon, replacing it with the mini white gene, causing the remainder of the gene to be out of frame, yielding a predicted null allele. Indeed, *PRAS40⁻* animals had no detectable PRAS40 protein (Figure S2A), and their phenotypes were not exacerbated by placing the mutation in *trans* to a deficiency uncovering the locus (data not shown). We backcrossed female flies harboring the *PRAS40^{KO}* mutation to w^{1118} flies for five generations, obtaining two stocks with similar genetic backgrounds but differing by presence or absence of the *PRAS40* knockout. The resulting stocks were used for all experiments described here, and we refer to them as "*PRAS40* mutant" flies, and the *w^{1118}* stock as "controls."

Surprisingly, *PRAS40* mutant flies are viable to adulthood, fertile, and have no obvious eye patterning defects (Figure 2B). This was surprising because mutations in other known regulators of TOR such as Tsc1, Tsc2, or Rheb, are all lethal. Also contrary to our expectation, *PRAS40* knockouts are normal in size. The weight of both male and female flies is indistinguishable to those of controls (Figure 2C). Quantification of wing and cell size in *PRAS40* mutants also revealed no difference compared to controls (Figure 2D). This was unexpected from the proposed role of PRAS40 as a TOR regulator, and given that *PRAS40* overexpression has clear effects on tissue size (above). The pupation rate of *PRAS40⁻* flies also did not differ significantly from that of controls (Figure 2E), indicating that *PRAS40* mutants have a normal rate of growth. One possible explanation for

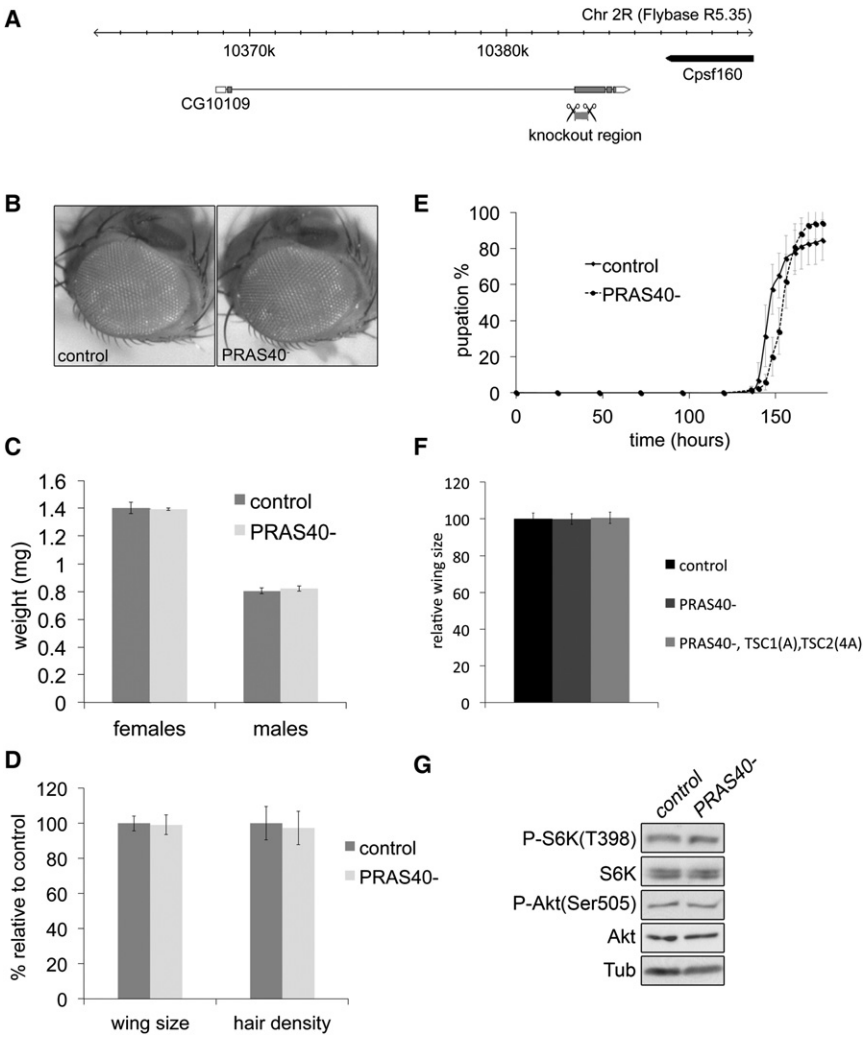

Figure 2. PRAS40^{-} Flies Are Viable and Have Normal Size

(A) Schematic representation of the *CG10109/PRAS40* locus. Knock-out region is indicated.

(B) Images of eyes from w^{1118} (control) and *PRAS40*$^{-}$ flies.

(C) *PRAS40* mutants have normal body weight. Weight per fly for control and *PRAS40*$^{-}$ males and females (n = 8, done in quadruplicate).

(D) *PRAS40* mutants have normal wing size and cell size. Total wing size of control and *PRAS40*$^{-}$ females, as well as cell size (quantified via hair density), are shown (n = 11).

(E) *PRAS40* mutants are not significantly delayed in development. Pupation curves for control and *PRAS40*$^{-}$ flies. One hundred animals per vial, 10 vials for each genotype.

(F) Flies simultaneously lacking all Akt phosphorylation sites on Tsc1 and Tsc2, as well as *PRAS40*, are normal in size. "Tsc1(A),Tsc2(4A)" indicates flies are mutant for endogenous Tsc1 and Tsc2 ($Tsc1^{29}$,gig^{192}) and contain constructs constitutively expressing mutant versions of Tsc1 and Tsc2 in which all Akt phosphorylation sites are mutated to alanine as previously described (Schleich and Teleman, 2009). Wing size relative to control wings is indicated (n = 8–2).

(G) Phosphorylation of S6K is not elevated in *PRAS40*$^{-}$ larvae. Total protein extracts from wandering third instar larvae probed with anti-phospho-S6K(Thr398), a readout for TORC1 activity.

In all panels, error bars denote SD.

the lack of a size effect in *PRAS40*$^{-}$ animals would be that PRAS40 and the Tsc1/Tsc2 complex form redundant mechanisms connecting IIS to TORC1. To test this, we generated flies in which both Tsc1 and Tsc2 were replaced with mutant versions that could not be phosphorylated by Akt (Dong and Pan, 2004; Schleich and Teleman, 2009), simultaneously harboring the PRAS40^{-} mutation ("PRAS40^{-}, Tsc1(A), Tsc2(4A)" flies). These flies were also viable and normal in size (Figure S2B), and quantification of their wing size revealed no differences compared to controls (Figure 2F). Because manipulation of TORC1 activity during *Drosophila* development causes clear and dramatic effects on final organismal size, these data suggested that removal of PRAS40 does not reduce TORC1 activity during development. To test this, we assayed phosphorylation levels of S6K on Thr398, an established readout for TORC1 activity, in third instar larvae by western blot analysis (Figure 2G). Consistent with a lack of size phenotypes in *PRAS40* knockouts, we could detect no reduction in phospho-S6K levels in the mutant larvae (Figure 2G). In sum, these data suggest PRAS40 does not regulate TORC1 activity in *Drosophila* during growth of the animal.

PRAS40 Links IIS to TORC1 in Ovaries but Not Adult Soma

We next tested whether TORC1 activity is aberrant in *PRAS40*$^{-}$ adults. Contrary to the situation in larvae, phosphorylation of the two canonical TORC1 targets 4E-BP and S6K was mildly elevated in PRAS40^{-} adults compared to controls (Figure 3A). Interestingly, the increase in TORC1 activity appeared to be of larger magnitude in females compared to males (Figure 3A, top). One difference between female and male flies is that a substantial fraction of the female body is comprised of ovaries. We therefore tested whether TORC1 activity is elevated in the ovaries of *PRAS40* knockouts. We separated ovaries from the rest of the female body and performed western blot analysis on these two fractions. Surprisingly, phospho-S6K levels were dramatically increased in ovaries of *PRAS40* mutants (Figure 3B, lanes 1 and 2), whereas there was no detectable difference in phospho-S6K levels in the remainder of the female body compared to controls (Figure 3B, lanes 5 and 6). This unexpected result indicates that the observable difference in TORC1 activity levels in adult females derives mainly from elevated TORC1 activity in ovaries. Furthermore, it indicates that endogenous PRAS40 represses TORC1 in ovaries but not in the rest of the female body.

In cell culture, PRAS40 links IIS activation to TORC1 activation (Nascimento et al., 2010; Sancak et al., 2007; Vander Haar et al., 2007). We tested whether this is also the case in vivo in the fly, in

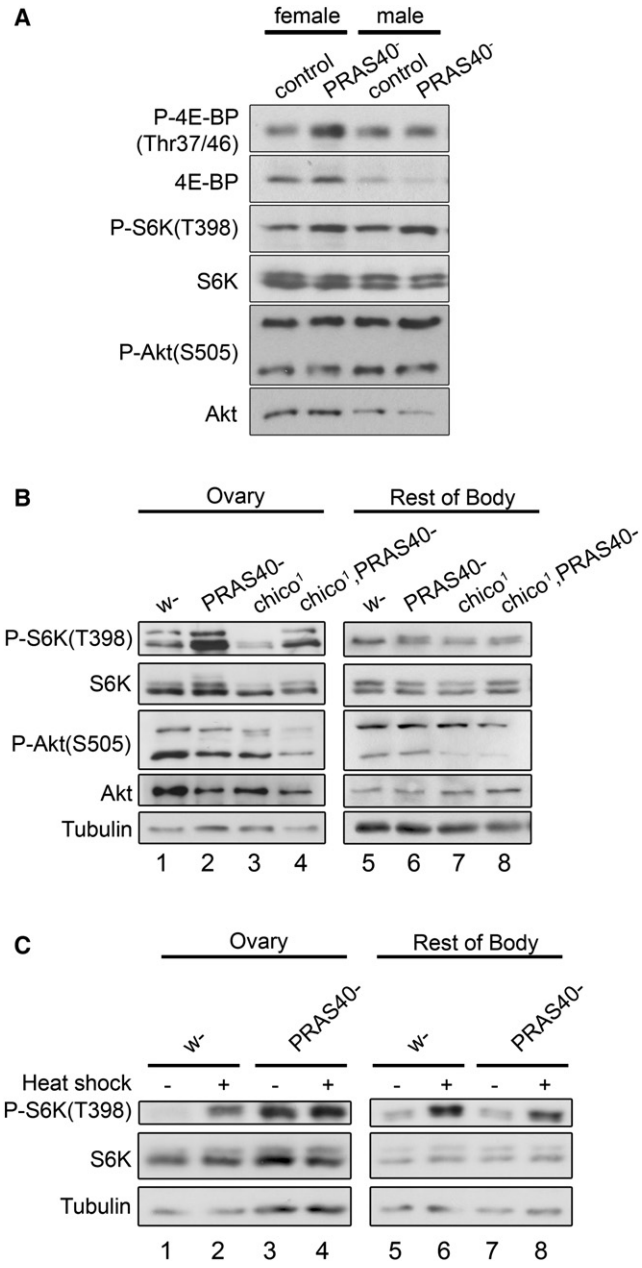

Figure 3. PRAS40 Links IIS to TORC1 in Ovaries but Not in Rest of Body

(A) TORC1 activity is mildly elevated in *PRAS40*$^{-}$ adult flies, with a more significant effect in females. Total body protein extracts from *PRAS40*$^{-}$ or control flies were probed with antibodies detecting phosphorylation of S6K on Thr398 and 4E-BP on Thr37/Thr46, two readouts for TORC1 activity.

(B) TORC1 activity is elevated specifically in ovaries of *PRAS40*$^{-}$ females, and removal of *PRAS40* rescues the reduced S6K phosphorylation of chico^{1} mutant ovaries. Female flies of indicated genotypes were dissected into "ovaries" and "rest of body" and protein extracts were probed with indicated antibodies.

(C) Increase in TORC1 activity in response to activation of PI3K is blunted in *PRAS40*$^{-}$ ovaries but not in the rest of the animal. Female flies carrying *heatshock-GAL4*, *UAS-Dp110-CAAX* were heat-shocked for 30 min at 37°C and allowed to recover for 6 hr. Ovaries were separated from the rest of the body and protein extracts were probed with indicated antibodies.

the adult ovary where PRAS40 represses TORC1. We first reduced IIS via removal of the insulin receptor substrate *chico* and monitored TORC1 activity via phosphorylation of S6K. *Chico*^{1} homozygous null animals had significantly reduced S6K phosphorylation in ovaries (Figure 3B, lane 1 versus lane 3) but not in the rest of the body (Figure 3B, lanes 5–8), the latter being in agreement with previous reports that loss of chico does not affect TORC1 activity (Oldham et al., 2000; Radimerski et al., 2002). This indicates that unlike most tissues of the fly, in the ovary IIS and TORC1 are linked. In contrast, *chico*^{1} null animals had reduced Akt phosphorylation in both ovaries and the rest of the body, as observed by others (Werz et al., 2009). We next asked whether this differential coupling between IIS and TORC1 is due to PRAS40. Indeed, removal of *PRAS40* in the *chico*^{1} mutant rescued pS6K levels back to normal in ovaries (Figure 3B, lane 1 versus lane 4). In contrast, simultaneous removal of *chico* and *PRAS40* had no effect in the rest of the female body (Figure 3B, lane 8) consistent with the phenotype of the single mutants.

In a set of complementary experiments, we increased IIS by expressing heat-shock inducible activated PI3K (*hsGAL4*, *UAS-Dp110-CAAX*), and assessed the effect on TORC1 activity in ovaries versus rest of the body (Figure 3C). In this case, ectopic activation of IIS caused increased S6K phosphorylation in the soma of both control and *PRAS40*$^{-}$ adult females (Figure 3C, lanes 5–8). This is consistent with many findings that stimulation of IIS above physiological levels can induce TORC1 activity, for instance in cell culture or in explants of larval tissues (data not shown), whereas modulation of IIS within physiological range does not (Figure 3B). In the same animals, ectopic activation of IIS also induced S6K phosphorylation in ovaries of control animals (Figure 3C, lanes 1 and 2) however the magnitude of induction was strongly blunted in *PRAS40*$^{-}$ ovaries (Figure 3C, lanes 3 and 4). In sum, both experiments, either increasing or decreasing IIS, indicate that PRAS40 is required for IIS to properly activate TORC1 in the ovaries but not in the rest of the body.

PRAS40 Rescues the Sterility of IIS Loss-of-Function Conditions and Regulates Fertility

The above-mentioned biochemical analyses suggest that *PRAS40* acts epistatically downstream of IIS to regulate TORC1 specifically in ovaries but not in the rest of the animal. If this model is correct, it predicts that *PRAS40* mutation should rescue the physiological defects caused by reduced IIS in ovaries but not in the rest of the animal. We tested this via genetic epistasis experiments. As mentioned above, *PRAS40* mutant females are fertile and normally sized compared to controls (Figure 4A). In contrast, as previously reported (Böhni et al., 1999), *chico*^{1} homozygous females have significantly reduced body size and are completely sterile (Figure 4A). Consistent with the model mentioned above, introducing the *PRAS40* mutation into the *chico*^{1} mutant background does not rescue the reduced body size of *chico* mutants (Figure 4A). The wing size of *chico*^{1}, *PRAS40*$^{-}$ females is the same as that of *chico*^{1} mutants, and ~60% that of control flies (Figure 4B). Likewise, the reduced wing cell size of *chico*^{1} mutants was not rescued by removal of *PRAS40* (Figure 4C). Strikingly, however, removal of *PRAS40* completely rescues the infertility of *chico*^{1} females.

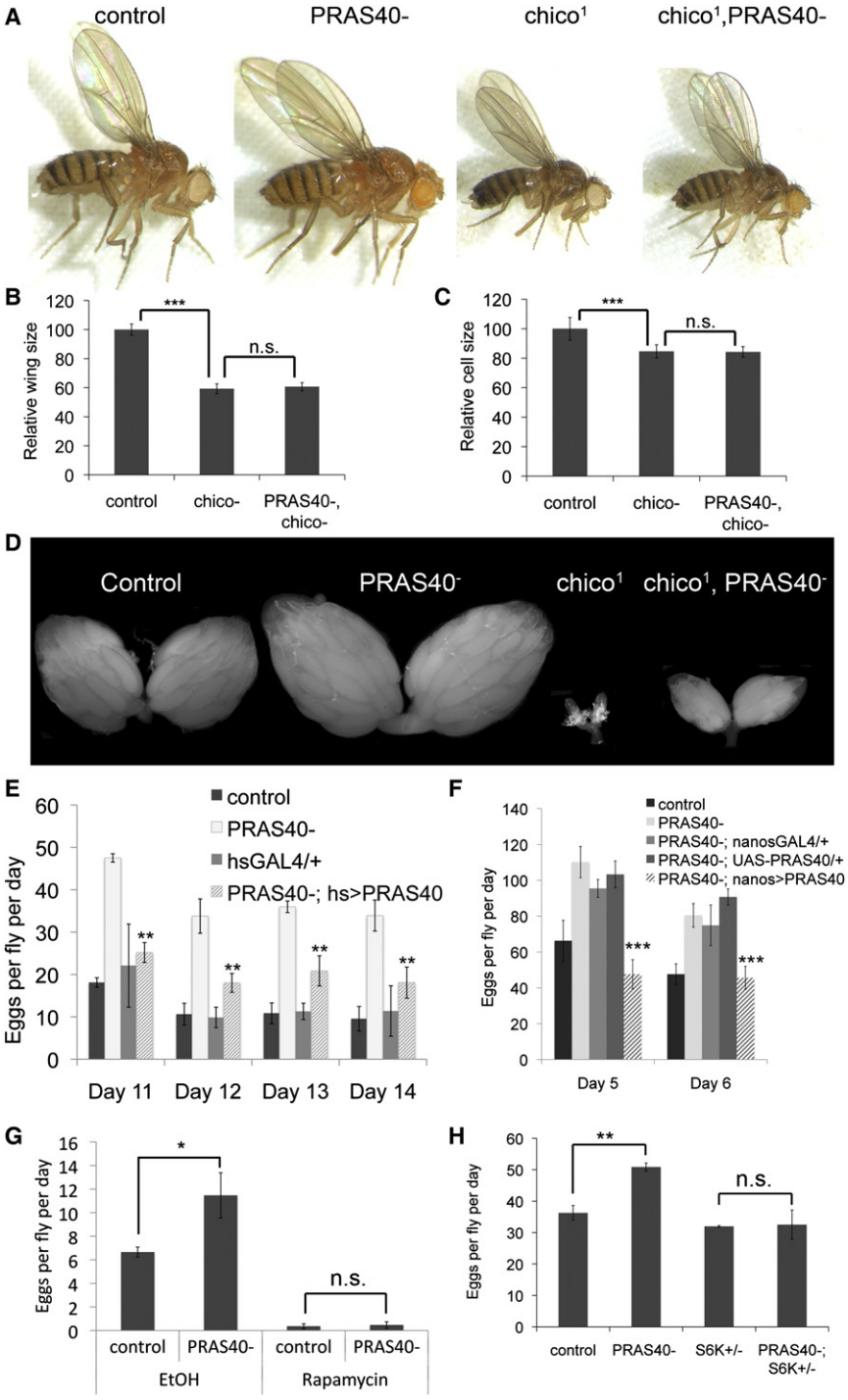

Figure 4. PRAS40 Mutation Rescues the Sterility of *chico* Mutant Flies, But Not Their Reduced Body Size

(A) Removal of *PRAS40* in the *chico*^{1} background rescues the fertility of *chico*^{1} flies, but not their reduced size. Images of female flies of indicated genotypes, grown under growth-controlled conditions. The overall body size of *chico*^{1}, *PRAS40*$^{-}$ flies is the same as that of chico^{1} flies (e.g., head size or wing size) but the abdomen has the normal "inflated" appearance of fertile females.

(B) Removal of *PRAS40* does not rescue the reduced size of *chico* mutants. Wing size of females, relative to controls, of indicated genotypes, grown under growth-controlled conditions. ***t test = 10^{-16}. n.s., not significant (t test = 0.27) (n > 10).

(C) Removal of *PRAS40* does not rescue the reduced cell size of *chico* mutants. Wing cell size, relative to controls, quantified by measuring hairs per unit area, in wings of females of indicated genotypes, grown under growth-controlled conditions. ***t test = 10^{-5}. n.s., not significant (t test = 0.85) (n > 10).

(D) Removal of *PRAS40* rescues the infertility of *chico* mutant females. Ovaries from females of indicted genotypes. *Chico*^{1} ovaries halt development prior to vitellogenesis, whereas *chico*^{1}, *PRAS40*$^{-}$ ovaries contains fully developed eggs.

(E and F) *PRAS40*$^{-}$ females lay more eggs per day than controls, and this phenotype is rescued by expressing PRAS40 ubiquitously with heatshock-GAL4 (E) or specifically in the germline with nanos-GAL4 (F). Eggs laid per fly per day in the presence of yeast is shown for indicated genotypes. Flies were aged 11–14 days (E) or 5–6 days (F) post-eclosion and the experiment done at 29°C without heat shock (E) or 25°C (F). **t test < 0.01, ***t test < 0.001 relative to PRAS40^{-} for the respective day (n > 10 flies, done in quadruplicate).

(G) The increased egg-laying rate of *PRAS40*$^{-}$ females is rescued by feeding rapamycin. Rapamycin was supplemented in food at 200 μM for 5 days in the absence of yeast. Twenty-five females per tube, done in triplicate. *t test = 0.04. n.s., not significant (t test = 0.6) (n = 25 flies, done in triplicate).

(H) The increased egg-laying rate of *PRAS40*$^{-}$ females is rescued by removing one copy of S6K. Assay done in presence of yeast. S6K+/− indicates S6K$^{07084/+}$. **t test = 0.002, n.s., = not significant (t test = 0.89) (n = 15 flies, done in triplicate).

In all panels, error bars denote SD.

The abdomens of *chico*^{1} females are "deflated" compared to controls, due to absence of fully-developed ovaries, whereas the abdomens of *chico*^{1}, *PRAS40*$^{-}$ females have the usual inflated appearance (Figure 4A). Dissection of the ovaries from these animals revealed that *chico*^{1} ovaries do not have cysts reaching the vitellogenic stage, whereas ovaries from *chico*^{1}, *PRAS40*$^{-}$ double mutants contain cysts of all stages including fully-developed eggs (Figure 4D). The total size of *chico*^{1}, *PRAS40*$^{-}$ ovaries is reduced compared to those of control females, fitting with the reduced total body and abdomen size of these animals (Figures 4A and 4D). *chico*^{1}, *PRAS40*$^{-}$ females nonetheless lay viable eggs, and *chico*^{1}, *PRAS40*$^{-}$ animals can be maintained as a viable homozygous stock (data not shown). Interestingly, the eggs laid by *chico*^{1}, *PRAS40*$^{-}$ mutant females are small (Figures S3A and S3A′). In sum, *PRAS40* acts epistatically downstream of *chico* specifically in the ovary, rescuing the

infertility of *chico* mutant animals, but not their reduced body size, in agreement with PRAS40 linking IIS to TORC1 in ovaries but not in the rest of the body. Intriguingly, mutation of *PRAS40* also rescues the infertility of $PDK1^{4/5}$ females and males, suggesting that PRAS40 may function also in the male gonad (data not shown). Indeed, a mild increase in TORC1 activity can also be observed in total extracts from adult males (Figure 3A).

Ovaries from PRAS40^{-} females appear larger than those of control females (Figure 4D). This is consistent with *PRAS40*$^{-}$ ovaries having elevated levels of phospho-S6K in comparison to controls (Figure 3B). Therefore, we asked whether PRAS40 also regulates fertility in a wild-type background where IIS is normal. Indeed, *PRAS40*$^{-}$ females lay significantly more eggs than controls, reaching up to three times as many eggs per day as controls, depending on culturing conditions and fly age (Figure 4E). This phenotype can be rescued by reintroducing *PRAS40* via a *UAS* construct driven by *heat-shock-GAL4* (Figure 4E). TORC1 regulates almost all aspects of oogenesis including the rate of proliferation of ovarian somatic and germline cells, germline stem cell maintenance, vitellogenesis, and oocyte loss (Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2009; Hsu et al., 2008; LaFever et al., 2010; Sun et al., 2010; Thomson et al., 2010). Consistent with this, we could detect PRAS40 protein in all cells of the ovary, including follicle cells, nurse cells, oocytes, and cells of the germarium (Figures S4A and S4B). PRAS40 staining is diffusely cytoplasmic with intensely-staining cytoplasmic speckles. PRAS40 levels appear strongest in the germarium and during early stages of egg development (Figures S4A and S4B). Reintroducing PRAS40 specifically in the germline using nanos-GAL4 rescues the elevated fertility phenotype of PRAS40 mutants (Figure 4F). Therefore, although PRAS40 may also be acting in the somatic ovary, germline expression of PRAS40 appears to be sufficient to rescue the phenotype. Intriguingly, testes from PRAS40 mutant males are also enlarged compared to controls (Figure S4C), raising the possibility that PRAS40 also regulates male fertility.

One open question regarding PRAS40 is whether its main role is to function as a regulator of TORC1 (perhaps via competition for TORC1 binding with S6K and 4E-BP) or whether its main function is to act as a downstream target of TORC1. This can be tested in vivo. If the phenotypes of *PRAS40*$^{-}$ flies result from elevated TORC1 activity, they should be rescued by reducing activity of TORC1 or a TORC1 effector such as S6K. If instead PRAS40 acts downstream of TORC1 as a TORC1 effector, then the absence of PRAS40 cannot be rescued by manipulating activity levels of TORC1 or other TORC1 targets. To address this, we first asked whether the increased fertility of *PRAS40* mutants can be rescued by feeding them rapamycin, which inhibits TORC1. Indeed, this was the case (Figure 4G). Most of the effect of TORC1 on fly fertility has been ascribed to S6K (LaFever et al., 2010; Montagne et al., 1999). Therefore, as a more stringent test, we asked whether the increased fertility of *PRAS40* mutants can be rescued by reducing *S6K* gene dosage. Indeed, removing one copy of *S6K* brought the egg laying of *PRAS40* mutants back down to control levels (Figure 4H). This indicates the increased fertility of *PRAS40* knockouts is mainly due to elevated S6K activity, suggesting that the main physiological function of PRAS40 is to act as a TORC1 inhibitor.

Tissue-Specific Function of PRAS40

Both the biochemical data and the genetic data presented above suggest that PRAS40 functions in a tissue-specific manner, regulating TORC1 activity in ovaries but not in other tissues of the adult or in larvae. One possible explanation is that *PRAS40* is expressed specifically in ovaries. We performed quantitative RT-PCR to detect *PRAS40* expression and found that it is expressed at all developmental stages of the animal (Figure 5A). In third instar larvae, *PRAS40* is expressed in all tissues that we assayed, with relative enrichment in the intestine (Figure 5B). In adults, *PRAS40* was expressed in ovaries, in the female gut, and in the remainder of the female body, as well as in testis, with relative enrichment in the ovary (Figure 5C). Likewise, PRAS40 protein could be detected in all assayed tissues and developmental stages (Figures S5A and S5B). Therefore, *PRAS40* is not expressed only in adult ovaries. PRAS40 is thought to regulate S6K phosphorylation via competition for substrate binding to TORC1. Another possible explanation for why PRAS40 functions in a tissue-specific manner might be that this competition mechanism functions in ovaries but not in other tissues of the animal. However, we find no evidence for substrate competition in the ovary because removal of another TORC1 target, 4E-BP, does not lead to elevated levels of S6K phosphorylation on Thr398 (Figure 5D). Having excluded these two possibilities, one remaining possibility is that the activity of PRAS40 is regulated in a tissue-specific manner, for instance by posttranslational modification. If PRAS40 were not functional in tissues other than the ovary, this would explain why removing it has no consequences. To test this possibility, we performed isoelectric focusing (IEF) of proteins from various tissues of the animal, followed by western blotting to detect endogenous PRAS40. On an IEF gel, PRAS40 forms multiple bands of varying pI (Figure 5E), in agreement with it being phosphorylated on multiple sites (Nascimento et al., 2010; Wang et al., 2008). Interestingly, PRAS40 was significantly more shifted toward isoforms with higher pI in extracts from ovaries compared to extracts from larvae or adult males (lacking ovaries) (Figure 5E), indicating that the state of posttranslational modification of PRAS40 is different in ovaries compared to other tissues. We favor the possibility that PRAS40 is maintained in an inactive state in tissues other than ovaries. This would explain why reduction of IIS via removal of chico causes reduced TORC1 activity in ovaries but not in other tissues (Figure 3B). Consistent with this, the isoelectric focusing of PRAS40 in ovaries changed in response to treatment with wortmannin (Figure S5C) whereas the isoelectric focusing of PRAS40 in larval tissues was comparatively wortmannin unresponsive (Figure S5C). These observations suggest that tissue-specific factors may be regulating the ability of PRAS40 to respond to insulin, however further work will be necessary to decipher these mechanisms.

DISCUSSION

The question whether IIS is coupled to TORC1 in vivo has been an issue of debate, because activation of TORC1 in response to IIS can be observed in some systems (Cai et al., 2006;

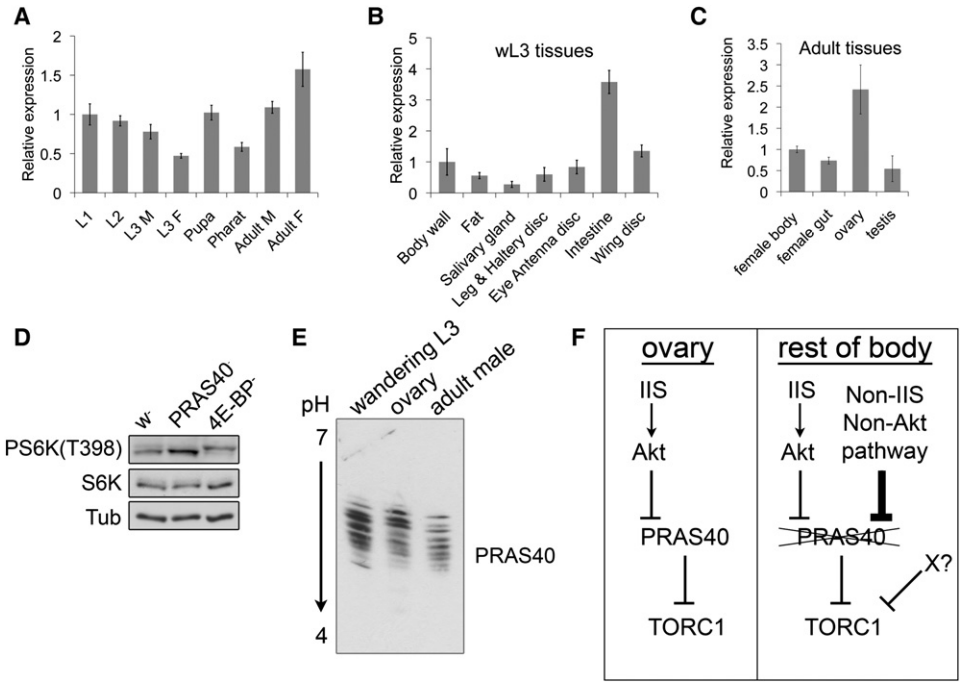

Figure 5. PRAS40 Is Differentially Posttranslationally Modified in Ovaries versus Other Tissues

(A–C) *PRAS40* expression is not restricted to ovaries. Expression of *PRAS40* determined by quantitative RT-PCR relative to *rp49* in various tissues and developmental stages of the fly, as indicated.

(D) Removal of 4E-BP does not lead to increased phosphorylation of S6K on Thr398 in ovaries. Protein extracts from ovaries of w^{1118} (w^-), $PRAS40^-$, or $Thor^2$ ($4E\text{-}BP^-$) mutant flies were probed with indicated antibodies.

(E) PRAS40 is differentially posttranslationally modified in ovaries compared to other tissues. Lysates from wandering third instar larvae (wandering L3), ovaries or whole adult males were subjected to isoelectric focusing on a pH4–7 gradient, followed by immunoblotting to detect endogenous PRAS40. PRAS40 in ovaries is more concentrated in isoforms with higher pI compared to other tissues.

(F) Schematic diagram of TORC1 regulation by PRAS40. In the ovary, PRAS40 inhibits TORC1, and this inhibition is relieved by IIS via Akt. In other tissues, PRAS40 is present but unable to inhibit TORC1 both under high and low IIS conditions, indicating that PRAS40 is held inactive by an IIS/Akt independent mechanism. This leads to uncoupling of TORC1 from IIS in the rest of the body. Additionally, other factors may be inhibiting TORC1 in rest of body ("X").

In all panels, error bars denote SD.

Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002), but not in others (Dong and Pan, 2004; Hall et al., 2007; Radimerski et al., 2002). Quite surprisingly, our work suggests that the answer to whether IIS activates TORC1 in vivo is neither "yes" nor "no," but rather that it depends in a tissue-specific manner. Both biochemically and genetically we find that PRAS40 and IIS do not affect TORC1 activity in most tissues during growth of the fly. Removal of *PRAS40* does not cause elevated TORC1 activity in larvae (Figure 2G) and, in agreement with previous reports (Oldham et al., 2000; Radimerski et al., 2002), removal of *chico* does not lead to reduced TORC1 activity in the adult body (Figure 3B) or in larvae (data not shown). Removal of *PRAS40* does not cause any size abnormalities in the fly, which is a very sensitive readout for TORC1 activity during development (Figures 2C and 2D). We were surprised to find, however, that in ovaries both IIS and PRAS40 do affect TORC1 activity. TORC1 activity drops in ovaries of $chico^-$ animals, and increases in ovaries of $PRAS40^-$ animals (Figure 3B). Furthermore, in ovaries, PRAS40 links IIS to TORC1 in that removal of both *chico* and *PRAS40* leads to renormalized TORC1 activity. These biochemical data are reflected by genetic epistasis data. Chico mutant flies are completely infertile, laying no eggs, and this phenotype is rescued by removal of *PRAS40* (Figure 4D). These data indicate that under normal physiological conditions, IIS activates TORC1 in a tissue-specific manner.

Does PRAS40 also link IIS to TORC1 in the male germline? The fact that mutation of *PRAS40* rescues the infertility of $PDK1^{4/5}$ mutant males (data not shown), and that *PRAS40* mutant testes are larger than control testes (Figure S4C) suggests that it does. $PRAS40^-$, $chico^-$ mutant testes also appear mildly increased in size compared to $chico^-$ mutant testes (Figure S4C), however, the result is not as clear cut as with ovaries, because *chico* mutant females are completely sterile whereas *chico* mutant males have only mildly reduced fertility. Further work will be required to look at this carefully.

All these data, indicating an ovary-specific link between IIS and TORC1 result from manipulations within physiological range. In contrast, overexpression of PRAS40 does cause reduced tissue growth (Figures 1C and 1D; Figure S1) as well as reduced TORC1 activity (data not shown), indicating that PRAS40 can inhibit TORC1 in most tissues when overexpressed. Furthermore, in contrast to the tissue-specific link between IIS and TORC1 under normal physiological conditions, we also observe that hyper-stimulation of IIS above physiological range does activate TORC1 in most tissues, for instance in tissue explants treated with insulin (data not shown), or in animals

overexpressing activated PI3K (Dp110-CAAX) (Figure 3C). This mechanism might be relevant for pathophysiological conditions with elevated IIS, such as in cancer cells. This may occur via elevated ATP production in the cell, inhibiting AMPK (Hahn et al., 2010), because we observe this activation also in tissues simultaneously lacking PRAS40 and all Akt phosphorylation sites on Tsc1 and Tsc2 (data not shown).

One open question is whether the main function of PRAS40 is to regulate TORC1 activity or whether it functions mainly as a downstream target and effector of TORC1. Our data suggest the former is the case. If PRAS40 had effector functions downstream of TORC1, these functions would not be rescued by additional removal of other TORC1 substrates such as S6K. Instead, we find that the elevated fertility of *PRAS40* mutants is rescued by removal of one copy of S6K (Figure 4H), suggesting that the phenotype we find in *PRAS40* mutants is due to elevated S6K activity.

Why does PRAS40 regulate TORC1 activity in ovaries but not in other tissues of the animal? PRAS40 is expressed in all tissues that we tested (Figures 5A–5C; Figure S4B). Therefore, the fact that removal of PRAS40 from larval tissues, for instance, has no effect on TORC1 activity must mean that larval PRAS40 protein is inactive (Figure 5F). We present data suggesting that the state of phosphorylation of PRAS40 may be different in larval tissues compared to ovaries (Figure 5E), providing a possible explanation for this inactivation. To date, a number of phosphorylations on PRAS40 have been reported, all of which are inhibitory in terms of TORC1 binding. These include phosphorylations by Akt (Sancak et al., 2007), TORC1 itself (Fonseca et al., 2007; Oshiro et al., 2007; Wang et al., 2007, 2008), PIM1 (Zhang et al., 2009), and PKA (Blancquaert et al., 2010). Intriguingly, this correlates with the observation that PRAS40 is highly phosphorylated in many cancers and that PRAS40 phosphorylation correlates with bad prognosis (Huang and Porter, 2005; Johnson et al., 2009; Madhunapantula et al., 2007; McBride et al., 2010). We favor the possibility that PRAS40 phosphorylation on an inhibitory site could be regulated by a kinase that is absent in ovaries, or a phosphatase that is enriched in ovaries compared to other tissues. Future studies will shed light on this issue.

TORC1 has multiple physiological roles in various tissues. In *Drosophila*, TORC1 in the growing larva regulates both growth and metabolism of the animal whereas in the adult fly, it regulates mainly metabolic parameters. TORC1 in ovaries regulates fertility of the animal (LaFever et al., 2010; Sun et al., 2010; Zhang et al., 2006), whereas in the nervous system it regulates dendritic tiling (Hong and Luo, 2009). Therefore, unless TORC1 activity can be differentially regulated in various tissues, all these physiological functions would have to be controlled in a correlated fashion. Tissue-specific differential regulation of PRAS40 presents a mechanism that allows TORC1 activity to be uncoupled in a tissue-specific manner.

EXPERIMENTAL PROCEDURES

Constructs and Fly Strains

PRAS40^{-} flies were generated by cloning 4 kb of upstream and downstream genomic flanking sequences into the NotI and XhoI sites of pRK1 (Huang et al., 2008), respectively. Knockout flies were generated as described in Huang et al. (2008). Epitope-tagged PRAS40 and raptor were generated by introducing N-terminal HA or FLAG tags via PCR, and were then cloned into pMT containing a copper-inducible metallothionein promoter. Sequences of all oligos used for cloning are provided in the Supplemental Experimental Procedures.

Antibodies

Anti-4EBP was described (Teleman et al., 2005); anti-CG10109 was a kind gift from Kwang-Wook Choi (Chern and Choi, 2002). All other antibodies are described in the Supplemental Experimental Procedures.

Quantitative RT-PCR

RNA was extracted using Trizol (Invitrogen), and reverse-transcribed with SuperScript III (Invitrogen). Q-PCR was performed using Maxima SYBR Green/ROX (Fermentas), normalized to rp49. Oligos provided in the Supplemental Experimental Procedures .

Isoelectric Focusing

Isoelectric focusing was performed using Servalyte ampholytes (Serva Electrophoresis) in 1-D slab format as described in Unit 10.2 of Current Protocols in Protein Science (Ploegh, 1995). Culturing of explants is described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2011.10.029.

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REFERENCES

Avruch, J., Hara, K., Lin, Y., Liu, M., Long, X., Ortiz-Vega, S., and Yonezawa, K. (2006). Insulin and amino-acid regulation of mTOR signaling and kinase activity through the Rheb GTPase. Oncogene *25*, 6361–6372.

Baker, J., Liu, J.P., Robertson, E.J., and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. Cell *75*, 73–82.

Bjornsti, M.A., and Houghton, P.J. (2004). The TOR pathway: a target for cancer therapy. Nat. Rev. Cancer *4*, 335–348.

Blancquaert, S., Wang, L., Paternot, S., Coulonval, K., Dumont, J.E., Harris, T.E., and Roger, P.P. (2010). cAMP-dependent activation of mammalian target of rapamycin (mTOR) in thyroid cells. Implication in mitogenesis and activation of CDK4. Mol. Endocrinol. *24*, 1453–1468.

Böhni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell *97*, 865–875.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr. Biol. *11*, 213–221.

Burks, D.J., Font de Mora, J., Schubert, M., Withers, D.J., Myers, M.G., Towery, H.H., Altamuro, S.L., Flint, C.L., and White, M.F. (2000). IRS-2 pathways integrate female reproduction and energy homeostasis. Nature *407*, 377–382.

Cai, S.L., Tee, A.R., Short, J.D., Bergeron, J.M., Kim, J., Shen, J., Guo, R., Johnson, C.L., Kiguchi, K., and Walker, C.L. (2006). Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J. Cell Biol. *173*, 279–289.

Chern, J.J., and Choi, K.W. (2002). Lobe mediates Notch signaling to control domain-specific growth in the Drosophila eye disc. Development *129*, 4005–4013.

Dong, J., and Pan, D. (2004). Tsc2 is not a critical target of Akt during normal Drosophila development. Genes Dev. *18*, 2479–2484.

Drummond-Barbosa, D., and Spradling, A.C. (2001). Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. Dev. Biol. *231*, 265–278.

Fonseca, B.D., Smith, E.M., Lee, V.H., MacKintosh, C., and Proud, C.G. (2007). PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signaling downstream of this complex. J. Biol. Chem. *282*, 24514–24524.

Fontana, L., Partridge, L., and Longo, V.D. (2010). Extending healthy life span—from yeast to humans. Science *328*, 321–326.

Goberdhan, D.C., and Wilson, C. (2003). The functions of insulin signaling: size isn't everything, even in Drosophila. Differentiation *71*, 375–397.

Grewal, S.S. (2009). Insulin/TOR signaling in growth and homeostasis: a view from the fly world. Int. J. Biochem. Cell Biol. *41*, 1006–1010.

Guertin, D.A., and Sabatini, D.M. (2007). Defining the role of mTOR in cancer. Cancer Cell *12*, 9–22.

Hahn, K., Miranda, M., Francis, V.A., Vendrell, J., Zorzano, A., and Teleman, A.A. (2010). PP2A regulatory subunit PP2A-B′ counteracts S6K phosphorylation. Cell Metab. *11*, 438–444.

Hall, D.J., Grewal, S.S., de la Cruz, A.F., and Edgar, B.A. (2007). Rheb-TOR signaling promotes protein synthesis, but not glucose or amino acid import, in Drosophila. BMC Biol. *5*, 10.

Hong, W., and Luo, L. (2009). Dendritic tiling through TOR signalling. EMBO J. *28*, 3783–3784.

Hsu, H.J., and Drummond-Barbosa, D. (2009). Insulin levels control female germline stem cell maintenance via the niche in Drosophila. Proc. Natl. Acad. Sci. USA *106*, 1117–1121.

Hsu, H.J., LaFever, L., and Drummond-Barbosa, D. (2008). Diet controls normal and tumorous germline stem cells via insulin-dependent and -independent mechanisms in Drosophila. Dev. Biol. *313*, 700–712.

Huang, B., and Porter, G. (2005). Expression of proline-rich Akt-substrate PRAS40 in cell survival pathway and carcinogenesis. Acta Pharmacol. Sin. *26*, 1253–1258.

Huang, J., Zhou, W., Watson, A.M., Jan, Y.N., and Hong, Y. (2008). Efficient ends-out gene targeting in Drosophila. Genetics *180*, 703–707.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. *4*, 648–657.

Johnson, M.D., O'Connell, M., Vito, F., and Bakos, R.S. (2009). Increased STAT-3 and synchronous activation of Raf-1-MEK-1-MAPK, and phosphatidylinositol 3-Kinase-Akt-mTOR pathways in atypical and anaplastic meningiomas. J. Neurooncol. *92*, 129–136.

Kozma, S.C., and Thomas, G. (2002). Regulation of cell size in growth, development and human disease: PI3K, PKB and S6K. Bioessays *24*, 65–71.

Kwiatkowski, D.J., and Manning, B.D. (2005). Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. Hum. Mol. Genet. *14 Spec No. 2*, R251–R258.

LaFever, L., and Drummond-Barbosa, D. (2005). Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. Science *309*, 1071–1073.

LaFever, L., Feoktistov, A., Hsu, H.J., and Drummond-Barbosa, D. (2010). Specific roles of Target of rapamycin in the control of stem cells and their progeny in the Drosophila ovary. Development *137*, 2117–2126.

Madhunapantula, S.V., Sharma, A., and Robertson, G.P. (2007). PRAS40 deregulates apoptosis in malignant melanoma. Cancer Res. *67*, 3626–3636.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol. Cell *10*, 151–162.

Martin, D.E., and Hall, M.N. (2005). The expanding TOR signaling network. Curr. Opin. Cell Biol. *17*, 158–166.

McBride, S.M., Perez, D.A., Polley, M.Y., Vandenberg, S.R., Smith, J.S., Zheng, S., Lamborn, K.R., Wiencke, J.K., Chang, S.M., Prados, M.D., et al. (2010). Activation of PI3K/mTOR pathway occurs in most adult low-grade gliomas and predicts patient survival. J. Neurooncol. *97*, 33–40.

Michaelson, D., Korta, D.Z., Capua, Y., and Hubbard, E.J. (2010). Insulin signaling promotes germline proliferation in C. elegans. Development *137*, 671–680.

Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., and Thomas, G. (1999). Drosophila S6 kinase: a regulator of cell size. Science *285*, 2126–2129.

Nakae, J., Kido, Y., and Accili, D. (2001). Distinct and overlapping functions of insulin and IGF-I receptors. Endocr. Rev. *22*, 818–835.

Nascimento, E.B., Snel, M., Guigas, B., van der Zon, G.C., Kriek, J., Maassen, J.A., Jazet, I.M., Diamant, M., and Ouwens, D.M. (2010). Phosphorylation of PRAS40 on Thr246 by PKB/AKT facilitates efficient phosphorylation of Ser183 by mTORC1. Cell. Signal. *22*, 961–967.

Oldham, S., Montagne, J., Radimerski, T., Thomas, G., and Hafen, E. (2000). Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. Genes Dev. *14*, 2689–2694.

Oshiro, N., Takahashi, R., Yoshino, K., Tanimura, K., Nakashima, A., Eguchi, S., Miyamoto, T., Hara, K., Takehana, K., Avruch, J., et al. (2007). The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mammalian target of rapamycin complex 1. J. Biol. Chem. *282*, 20329–20339.

Ploegh, H.L. (1995). One-Dimensional Isoelectric Focusing of Proteins in Slab Gels (Hoboken, NJ: John Wiley & Sons).

Potter, C.J., Pedraza, L.G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. Nat. Cell Biol. *4*, 658–665.

Radimerski, T., Montagne, J., Rintelen, F., Stocker, H., van der Kaay, J., Downes, C.P., Hafen, E., and Thomas, G. (2002). dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1. Nat. Cell Biol. *4*, 251–255.

Reddy, P., Zheng, W., and Liu, K. (2010). Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. Trends Endocrinol. Metab. *21*, 96–103.

Richard, D.S., Rybczynski, R., Wilson, T.G., Wang, Y., Wayne, M.L., Zhou, Y., Partridge, L., and Harshman, L.G. (2005). Insulin signaling is necessary for vitellogenesis in Drosophila melanogaster independent of the roles of juvenile hormone and ecdysteroids: female sterility of the chico1 insulin signaling mutation is autonomous to the ovary. J. Insect Physiol. *51*, 455–464.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol. Cell *25*, 903–915.

Schleich, S., and Teleman, A.A. (2009). Akt phosphorylates both Tsc1 and Tsc2 in Drosophila, but neither phosphorylation is required for normal animal growth. PLoS ONE *4*, e6305.

Shaw, R.J. (2009). LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. Acta Physiol. (Oxf.) *196*, 65–80.

Sun, P., Quan, Z., Zhang, B., Wu, T., and Xi, R. (2010). TSC1/2 tumour suppressor complex maintains Drosophila germline stem cells by preventing differentiation. Development *137*, 2461–2469.

Teleman, A.A., Chen, Y.W., and Cohen, S.M. (2005). 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. Genes Dev. *19*, 1844–1848.

Thomson, T.C., Fitzpatrick, K.E., and Johnson, J. (2010). Intrinsic and extrinsic mechanisms of oocyte loss. Mol. Hum. Reprod. *16*, 916–927.

Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J., and Kim, D.H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat. Cell Biol. *9*, 316–323.

Wang, Y.H., and Huang, M.L. (2009). Reduction of Lobe leads to TORC1 hypoactivation that induces ectopic Jak/STAT signaling to impair Drosophila eye development. Mech. Dev. *126*, 781–790.

Wang, L., Harris, T.E., Roth, R.A., and Lawrence, J.C., Jr. (2007). PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. J. Biol. Chem. *282*, 20036–20044.

Wang, L., Harris, T.E., and Lawrence, J.C., Jr. (2008). Regulation of proline-rich Akt substrate of 40 kDa (PRAS40) function by mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation. J. Biol. Chem. *283*, 15619–15627.

Werz, C., Köhler, K., Hafen, E., and Stocker, H. (2009). The Drosophila SH2B family adaptor Lnk acts in parallel to chico in the insulin signaling pathway. PLoS Genet. *5*, e1000596.

Zhang, Y., Billington, C.J., Jr., Pan, D., and Neufeld, T.P. (2006). Drosophila target of rapamycin kinase functions as a multimer. Genetics *172*, 355–362.

Zhang, F., Beharry, Z.M., Harris, T.E., Lilly, M.B., Smith, C.D., Mahajan, S., and Kraft, A.S. (2009). PIM1 protein kinase regulates PRAS40 phosphorylation and mTOR activity in FDCP1 cells. Cancer Biol. Ther. *8*, 846–853.