

PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids

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Summary

Growth in normal and tumour cells is regulated by evolutionarily conserved extracellular inputs from the endocrine insulin receptor (InR) signalling pathway and by local nutrients. Both signals modulate activity of the intracellular TOR kinase, with nutrients at least partly acting through changes in intracellular amino acid levels mediated by amino acid transporters. We show that in *Drosophila*, two molecules related to mammalian proton-assisted SLC36 amino acid transporters (PATs), CG3424 and CG1139, are potent mediators of growth. These transporters genetically interact with TOR and other InR signalling components, indicating that they control growth by directly or indirectly modulating the effects of TOR signalling. A mutation in the *CG3424* gene, which we have named *pathetic* (*path*), reduces growth in the fly. In a

heterologous *Xenopus* oocyte system, PATH also activates the TOR target S6 kinase in an amino acid-dependent way. However, functional analysis reveals that PATH has an extremely low capacity and an exceptionally high affinity compared with characterised human PATs and the CG1139 transporter. PATH and potentially other PAT-related transporters must therefore control growth via a mechanism that does not require bulk transport of amino acids into the cell. As PATH is likely to be saturated in vivo, we propose that one specialised function of high-affinity PAT-related molecules is to maintain growth as local nutrient levels fluctuate during development.

Key words: Insulin signalling, *Drosophila*, Cancer, TOR, Diabetes, Nutrition, SLC36

Introduction

Cells and tissues in multicellular organisms typically grow in a coordinated fashion at a rate that can be modified by nutritional status. Recent studies in *Drosophila* have highlighted a crucial role for the insulin receptor (InR) pathway in regulating the endocrine response to nutrient levels (reviewed by Stocker and Hafen, 2000; Goberdhan and Wilson, 2003a). This evolutionarily conserved system not only affects cell, tissue and organismal growth, but also modulates multiple metabolic functions, developmental timing, fertility and longevity. Elevated InR signalling also plays a central role in tumorigenesis; mutations in *PTEN*, a negative regulator of the pathway, occur at remarkably high frequency in several different human cancers (reviewed by Goberdhan and Wilson, 2003b).

Eukaryotes possess a second and more ancient nutrition-regulated system, involving the kinase TOR (target of rapamycin) (Jacinto and Hall, 2003), which is responsive to local nutrient levels, particularly amino acids. In higher eukaryotes TOR controls downstream translational regulators, including p70-S6 kinase (S6K) and 4E-binding protein (4EBP) and is essential for normal cell-autonomous growth (Zhang et al., 2000; Oldham et al., 2000). Studies in *Drosophila* have recently linked the endocrine InR signalling system with TOR (reviewed by Goberdhan and Wilson, 2003a; Aspuria and Tamanoi, 2004). It is proposed that Akt, a key InR-regulated kinase, phosphorylates the tumour suppressor protein,

Tuberous sclerosis complex 2 (Tsc2), dissociating the Tsc1/Tsc2 complex. This activates the G protein Rheb, which positively regulates TOR.

TOR activity can be affected by changes in intracellular amino acid levels (Christie et al., 2002; Beugnet et al., 2003). Experiments in cell culture indicate that this response is modulated by amino acid transporters, but no amino acid transporter has yet been shown to regulate growth of non-endocrine cells in vivo. We have therefore genetically screened for such transporters. Although a number of different classes of mammalian amino acid transporter have been identified (see Hediger, 2004), many still have poorly characterised in vivo functions. In *Drosophila*, members of two transporter classes have been implicated in growth: *minidiscs* encodes a component of the heterodimeric family of transporters (Martin et al., 2000); and *slimfast* (*slif*) (Colombani et al., 2003) encodes a cationic amino acid transporter. These genes are required for normal growth, but they both primarily act in the fat body, an amino acid-sensitive, growth-regulatory endocrine organ with functional similarities to the mammalian liver and white adipose tissue (Britton et al., 1998).

Here, we show that two genes encoding proteins related to a third class of transporters of previously unknown biological function, the proton-assisted transporter (PAT or SLC36) family (Birmingham and Pennington, 2004), specifically modulate tissue-autonomous growth in multiple non-endocrine tissues in vivo. They also genetically interact with TOR and

other InR signalling components, indicating a direct or indirect regulatory link with this signalling system. One of these transporter genes, *pathetic* (*path*), is expressed in a wide range of tissues and is essential for both endocrine and local growth regulation. *PATH* has unusual functional properties, establishing it as the founder member of a new class of low capacity, very high-affinity PAT-related transporters, which control growth via a novel amino acid sensing mechanism. As the functions of *path* can be substituted by another *Drosophila* PAT-related transporter whose properties are much more similar to mammalian PATs, we propose that this gene family has a conserved and unique role in regulating TOR-dependent growth and the response to insulin-like molecules in development and disease.

Materials and methods

Drosophila stocks and culture

The following fly strains were used: *Rheb^{AV4}* (Patel et al., 2003), *UAS-Dp110^{D954A}*, *UAS-Dp110* (Leevers et al., 1996), *UAS-Dakt1* (Staveley et al., 1998), *UAS-Tor*, *UAS-Tor^{TEB}* (Hennig and Neufeld, 2002), *Tsc2¹⁹²*, *Tsc1²⁹* (Gao and Pan, 2001), *UAS-S6K* (Zhang et al., 2000), *UAS-slif* (Colombani et al., 2003) and GS lines (Toba et al., 1999). GS inserts near *path* (13857, 11111) and *CG1139* (10666, 7120) produce similar phenotypes (see Fig. 2). GS inserts near *CG1139* lie on the other side of a gene (*CG11815*), which is transcribed in the opposite direction; overexpression of a *UAS-CG1139* construct confirmed that growth effects were specifically mediated by *CG1139*. Other GS lines tested included 9983, 10651, 11951, 13265 and 13758. Flies were reared on standard cornmeal agar food.

Measurements and data analysis

Ommatidial sizes were calculated from stereomicroscope images (Leica Wild M3Z microscope and Axiovision software) (Radimerski et al., 2002b); the area of a 3×3 set of ommatidia in the centre of each eye was measured. Wing area data were collected using the same equipment, while wing cells were imaged using a Zeiss Axioplan 2 microscope. The number of wing cells in a rectangular 47250 μm² box next to the posterior cross vein was counted, and scaled up to give total cell number in the posterior compartment. Data are presented as mean±s.e.m. for at least six measurements per genotype and using female flies, unless otherwise stated.

Molecular analysis

Putative full-length *path-RA* (RH24992; almost all 80 available ESTs represent the RA form) and *CG1139* (LP06969) cDNAs were subcloned into transformation vector pUAST (Brand and Perrimon, 1993) using the restriction sites flanking the cDNA inserts (*EcoRI/XbaI* and *EcoRI* respectively). Transgenics were produced using standard procedures. At least four independent lines were established and tested. In situ hybridisation was based on work by Tautz and Pfeifle (Tautz and Pfeifle, 1989), using a PCR-amplified *path* template cDNA. cRNA was synthesised by in vitro transcription (mMessage mMachine, Ambion).

Functional analysis in *Xenopus* oocytes

Healthy looking *Xenopus laevis* oocytes (stage V and VI) were obtained as described (Meredith et al., 1998), and maintained at 18°C in modified Barth's medium. Transport measurements were performed at least 72 hours after microinjection of oocytes with 27.6 nM cRNA (1 μg/μl) with daily medium changes. Uptake assays with [³H]-alanine (59 Ci/mMole, Amersham Biosciences) were performed essentially as described, with five oocytes per data point (Meredith et al., 1998). Membrane potential was measured using a Dagan CA-1B amplifier. Oocytes impaled with a AgCl/3M KCl-filled glass

microelectrode (resistance approximately 0.5 MΩ) were superfused with uptake medium containing 2 mM amino acid or uptake medium alone. Intracellular amino acid concentrations were calculated based on the work of Christie et al. (Christie et al., 2002).

Western blotting followed standard procedures. Oocytes were lysed in 3 μl/oocyte lysis buffer and loaded with 1:1 (v:v) loading buffer. PVDF membranes were probed with 1:1000 (v:v) anti-phospho-p70 S6K (Thr389) antibody (Signaling Technologies, NEB) or 1:200 (v:v) anti-total S6K (Santa Cruz), followed by 1:2000 goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz) and the membrane developed using the ECL system (Amersham).

Results

PAT-related transporters regulate growth in the eye and wing

We screened for amino acid transporters that play a crucial role in TOR regulation in growing tissues. Our hypothesis was that these transporters might, like other characterised components of the InR/TOR signalling network (e.g. Leevers et al., 1996; Goberdhan et al., 1999; Goberdhan and Wilson, 2003a), affect growth when overexpressed in the eye or wing. Using the GAL4/UAS system (Brand and Perrimon, 1993), we attempted to overexpress genes predicted to encode transporters, by employing GS insertions near these genes (Toba et al., 1999) and UAS constructs.

Heterodimeric and cationic transporter genes produced little or no effect on growth when expressed in differentiating ommatidia using *GMR-GAL4* (e.g. *slif*; Fig. 1B). By contrast, overexpression of GS elements upstream of *CG3424* (subsequently renamed *path*) and *CG1139*, which encode proteins similar to the mammalian PATs (>30% identity; see Fig. S1A in the supplementary material), increased both ommatidial and overall eye size (Fig. 1C,D, respectively). When two GS insertions in *path* (Fig. 1E,L) or in *CG1139* (Fig. 1F,M) were co-expressed, the resulting eyes bulged more from the head capsule and the ommatidial array was mildly disorganised, indicating a more extreme dose-dependent overgrowth phenotype. Similar growth effects were also produced by *UAS-path* (e.g. Fig. 2K-M) and *UAS-CG1139* (Fig. 1H) transgenes, confirming that these genes caused the GS-associated phenotypes.

As previously reported (Hennig and Neufeld, 2002), expression of *UAS-Tor* under *GMR-GAL4* control produced no obvious eye phenotype (Fig. 1G). However, when co-overexpressed with either *path* (Fig. 1I) or *CG1139* (Fig. 1J), *UAS-Tor* significantly enhanced the overgrowth phenotype induced by these transporters alone (Fig. 1C,D respectively).

CG1139 and *path* also modulated growth in the developing wing, but their effects differed. Although *slif* had no significant effect with the *MS1096-GAL4* driver (Fig. 2B), which is expressed throughout the developing adult wing, but preferentially on the dorsal surface, in animals overexpressing *CG1139* GS insertions, overall wing size was increased (Fig. 2D) and excess growth on the dorsal surface caused the wing to turn down. However, the effects of *CG1139* on wing growth were not simple. Surprisingly, overexpression of the *UAS-CG1139* transgene, which has a more potent growth-stimulating effect in the eye than *CG1139* GS inserts (Fig. 1H), reduced wing growth and produced upturned wings (Fig. 2F),

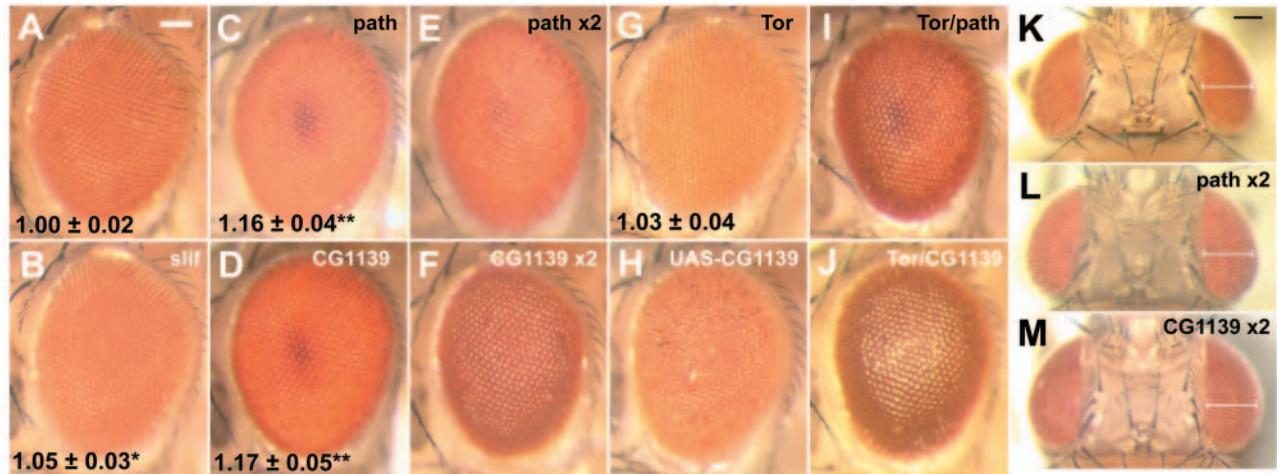


Fig. 1. Overexpression of *Drosophila* PAT-related transporters induces overgrowth in the differentiating eye. *GMR-GAL4* was used to overexpress transgenes in differentiating cells of the fly eye (B–J, L, M). Compared with controls (A, K), much greater overgrowth was induced by *path*^{GS13857} (C, E, L), *CG1139*^{GS10666} (D, F, M) and *UAS-CG1139* (H), than by *slif* (B). *Tor* had no obvious effect on growth by itself (G), but enhanced the overgrowth phenotype induced by either *path*^{GS13857} (I) or *CG1139*^{GS10666} (J). Where the ommatidial array was not disorganised, relative ommatidial size was measured (bottom of panel: mean±s.d.; **P*<0.01; ***P*<0.001). Eyes bulged from the head in more extreme cases of overgrowth (compare dorsal views in L and M with control in K; white bars are of equal length in these panels), so that only the central portion of the eye is in focus in lateral images (E, F, H–J).

suggesting that this gene might inhibit growth in some developmental contexts, if sufficiently overexpressed.

This effect was reminiscent of *UAS-Tor*, which, despite the well-documented growth-promoting functions of *Tor*, reduces growth when overexpressed in the wing (Fig. 2E) (Hennig and Neufeld, 2002). It has been proposed that TOR overexpression interferes with proper formation of a TOR-containing multiprotein complex in the wing to inhibit growth. To test whether a similar dominant-negative mechanism might explain the growth inhibitory effects of *CG1139*, we overexpressed two *CG1139* GS insertions. Although both these insertions promoted growth when expressed independently, in combination they significantly reduced growth (Fig. 2I). In addition, co-expression of a *CG1139* GS insertion with *UAS-CG1139* enhanced the growth inhibitory effect of the latter construct (Fig. 2J).

Co-overexpression of *CG1139* GS inserts with *Tor* greatly exaggerated the reduced growth phenotype produced by *Tor* alone (Fig. 2H), suggesting that these molecules might exert their dominant-negative effects on interacting complexes. Overexpression of *path* GS insertions or *UAS-path* reduced wing size in the *MS1096-GAL4* assay (e.g. Fig. 2C), suggesting that *path* also has dominant-negative effects in the wing. In addition, like *CG1139*, *path* overexpression strongly enhanced the dominant-negative effects of *Tor* (Fig. 2G).

To quantify growth changes in the wing, the transporter genes were overexpressed in the posterior wing compartment with *en-GAL4*. Effects on overall wing growth mirrored those observed with the *MS1096-GAL4* driver (Fig. 2K) and in most cases total cell number was significantly reduced (Fig. 2M). Unexpectedly, however, *path* and *CG1139* overexpression produced an increase in cell size, even when overall wing area was reduced (Fig. 2L). For each transgenic line tested, the increase was approximately inversely related to the reduction in cell number. Again, we tested whether these effects might be comparable with those of TOR. It has been reported that

TOR overexpression in wing imaginal discs reduces the size of proliferating cells, as does a dominant-negative form of TOR, TOR-TED (Hennig and Neufeld, 2002). However, they did not analyse the size of cells in the adult wing. In fact, posterior wing cells were enlarged in *en-GAL4 UAS-Tor* adult animals, despite the reduction in overall compartment size, mimicking the effects of PAT-related transporters (Fig. 2L). The effects of overexpressing *PATH*, *CG1139* and *TOR* on cell growth and proliferation therefore vary in parallel, depending on developmental context, with their growth inhibitory actions in the wing most likely being caused by dominant-negative effects during the proliferative stage of development.

path normally promotes growth in flies

To test whether PAT-related transporters normally promote growth in flies, we searched for specific mutations in either *CG1139* or *path*. We identified a transposable element insertion, *path*^{KG06640}, located within the first intron of the larger *path* transcription unit, RA (see Fig. 3A). The KG transposon contains gene silencing elements, which increase the likelihood that insertions will affect gene expression (Roseman et al., 1995). Flies homozygous for *path*^{KG06640} were markedly smaller than wild-type animals (Fig. 3B) and ~40% lighter in weight (Fig. 3C). Wing size was decreased by nearly 20% (Fig. 3D), with significant reductions in both cell number and size (*P*<0.001). Mutant eyes contained small ommatidia (compare Fig. 3E with 3F). Like many viable mutants in which InR signalling is disrupted (e.g. Böhni et al., 1999), *path*^{KG06640} homozygotes were developmentally delayed, eclosing roughly 3 days later than normal. Homozygous females also laid no eggs.

Animals transheterozygous for the *path*^{KG06640} chromosome and a large deficiency uncovering the *path* gene, *Df(3L)AC1*, were even smaller than *path*^{KG06640} homozygotes (Fig. 3B–D, G), suggesting *path*^{KG06640} is not a null allele. Transheterozygotes were also developmentally delayed and female sterile.

Transposase-mediated excision of the *path*^{KG06640} element reverted all recessive phenotypes in 90% of cases, linking these defects to the KG insertion. Expression of a *UAS-path* transgene under the control of a constitutive *GAL4* driver, *arm-GAL4*, also significantly rescued the growth, developmental delay and

female sterility mutant phenotypes (see Fig. 3C,D,H). Rescued flies were slightly smaller than controls, but this was probably caused by constitutive overexpression of *path*, as overexpressed *path* slightly reduced growth in otherwise normal flies (Fig. 3C). *CG1139*^{GS10666} also rescued *path*^{KG06640} mutant phenotypes (Fig. 3C,D). Finally, reducing the copy number of both the *Tsc1* and *Tsc2* genes, which normally antagonise TOR signalling, also partially rescued the *path* mutant phenotypes. Rescued flies were not developmentally delayed, the *path* growth phenotype was strongly suppressed (Fig. 3C,D) and about 10% of females laid a small number of eggs, some of which developed into adult flies.

path^{KG06640} homozygous larvae and *path*^{KG06640}/*Df(3L)AC1* transheterozygotes were small and took roughly 3 days longer than wild-type larvae to pupate (Fig. 3I). However, in contrast to several other developmentally delayed mutants with reduced InR signalling, imaginal discs from *path* mutant wandering third instar larvae were not reduced in size (Fig. 3J,K; Fig. 4E-H). *path* mutant discs were small when compared with similarly aged normal discs, but the extended mutant larval period allowed discs to grow to roughly normal size by pupariation. By contrast, the length of pupal development was not noticeably affected in *path* mutants, which probably explains why normal-sized adult structures are not formed. Unlike the larval endoreplicative tissues (ERTs) of viable *Rheb*, *Tor* and *slif* mutants, which are greatly reduced in size (Oldham et al., 2000; Stocker et al., 2003; Colombani et al., 2003), ERTs such as the salivary gland and the fat body were only modestly reduced in *path* mutants and fat body cells had non-starved morphology (data not shown).

Overall, we conclude that the recessive phenotypes associated with the *path*^{KG06640} mutant chromosome are caused by insertional mutation in *path* and are modified by at least one InR signalling complex: *Tsc1/Tsc2*. Based on the mutant growth phenotypes, we named the gene *pathetic* (*path*).

path is expressed in growing tissues during development and regulates both global and local growth signals

path is highly represented in the EST database. RNA in situ

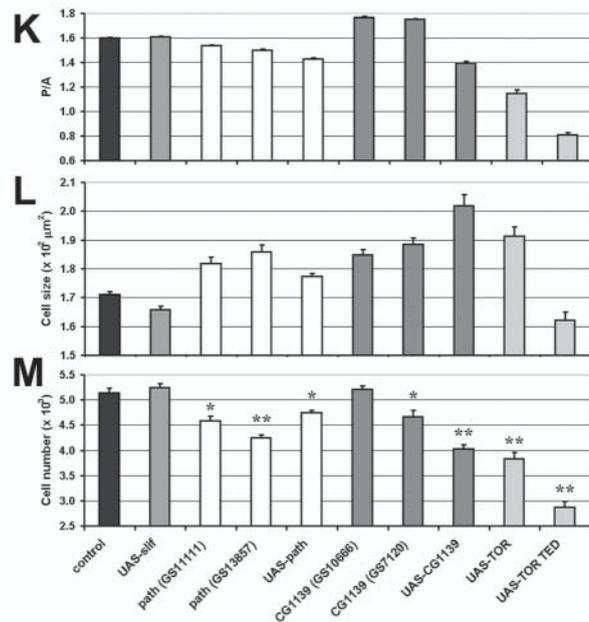
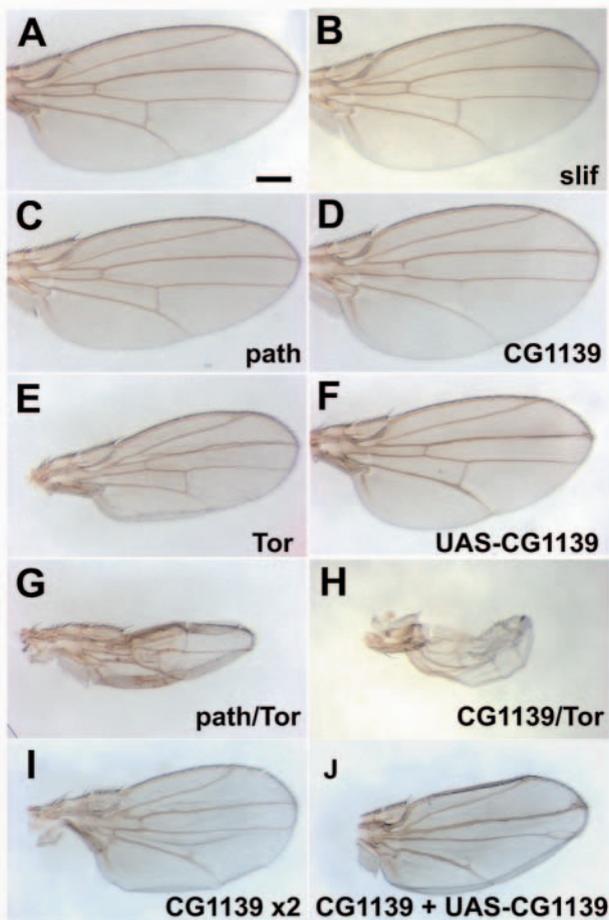


Fig. 2. PAT-related transporters phenocopy the effects of TOR on growth and cell number in the wing. (A-H) Expressing transgenes under the control of *MS1096-GAL4*, which drives transcription predominantly on the dorsal surface of the wing, resulted in an overall increase in wing size for *CG1139*^{GS10666} (D), but a reduction for *path*^{GS13857} (C), *Tor* (E) and *UAS-CG1139* (F). Overexpression of *slif* had no significant effect (B) relative to control wings (A). Both *path*^{GS13857} (G) and *CG1139*^{GS10666} (H) significantly enhanced the phenotype produced by overexpressing *Tor*. Co-expression of two *CG1139* GS insertions (*CG1139*^{GS10666} and *CG1139*^{GS120}) inhibited growth (I) and *CG1139*^{GS10666} enhanced the growth inhibitory effect of *UAS-CG1139* (J). (K-M) Histograms showing measurements of posterior compartment size (ratio of a specific wing area expressing the transgene and a non-expressing region=P/A) (K), cell size (L) and cell number (M) taken from adult female wings expressing transgenes under *en-GAL4* control. Expression of each PAT-related transporter (two GS insertions and a UAS-transgene), TOR or a dominant-negative form of TOR (TOR-TED), but not *Slif*, caused highly significant changes in wing and cell size ($P < 0.001$). Cell number was significantly reduced upon overexpression of TOR, TOR-TED and those PAT-related transporter constructs that have the greatest effects on growth (* $P < 0.01$; ** $P < 0.001$).

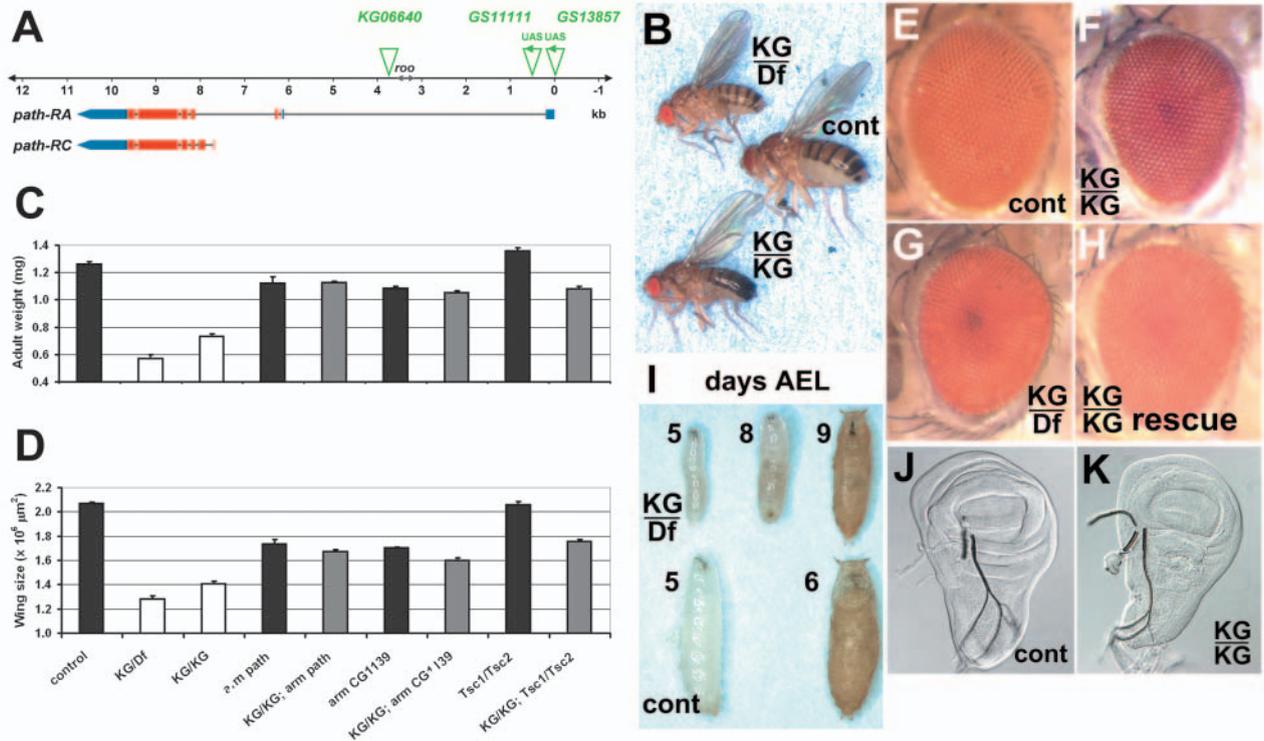


Fig. 3. *path* regulates growth in *Drosophila*. (A) Map of the *path* (CG3424) genomic region. Two predicted transcription units for *path* have been defined: RA and RC. Coding (red) and non-coding (blue) regions are indicated. *roo* (grey) is a naturally occurring transposable element found in *path*. *KG06640*, *GS13857* and *GS11111* insertions are shown in green. (B) *path*^{KG06640} homozygotes and *path*^{KG06640}/*Df(3L)AC1* transheterozygotes were reduced in size compared with wild-type controls. (C,D) Histograms show adult weights and wing sizes for different *path* mutant genotypes. Values for mutant flies were strongly reduced compared with wild type ($P < 0.001$), and were significantly rescued ($P < 0.001$; light grey bars) by both *UAS-path* and *CG1139*^{GS10666} expression under *arm-GAL4* control and by the presence of combined heterozygous mutations in *Tsc1* and *Tsc2*, which produce only a very modest size increase in otherwise normal flies. (E-H) Eyes from control (E), a *path*^{KG06640} homozygote (F), a *path*^{KG06640}/*Df(3L)AC1* transheterozygote (G) and a *path*^{KG06640} homozygote expressing *UAS-path* constitutively (H). (I) *path* mutant animals (here *path*^{KG06640}/*Df(3L)AC1* transheterozygotes) were reduced in size throughout larval and pupal development, and developmentally delayed. However, *path*^{KG06640} homozygous climbing third instar larvae had wing imaginal discs of roughly normal size (K) compared with wild type (J). Genotype of *Tsc1/Tsc2*-rescued flies in C and D is *path*^{KG06640} *Tsc2*¹⁹²/*path*^{KG06640} *Tsc1*²⁹.

hybridisation performed on embryonic and late larval stages revealed that it is expressed in most tissues. However, transcript levels were highly dynamic in embryogenesis with surges of expression in many structures, including muscle primordia, salivary glands, proventriculus (and other parts of the gut), trachea and gonads (see Fig. 4A-C). Expression was strongly reduced in homozygous *path*^{KG06640} mutants (Fig. 4D). Larval imaginal discs also expressed *path* in all or most cells. Expression was particularly strong in the pouch and hinge regions of the wing disc (Fig. 4E) and in the morphogenetic furrow of the eye disc (Fig. 4G). Transcript levels were reduced in *path*^{KG06640} homozygotes (Fig. 4F,H), but the reduction was less severe than in embryos, particularly in the eye.

The relatively modest reduction in *path* expression in mutant imaginal discs suggested that the significant growth defects associated with this mutant might not be explained by a cell-autonomous reduction in PATH activity. Indeed, when the *eyFLP/FRT* approach (Newsome et al., 2000) was used to generate eyes in which many ommatidia were derived from *path*^{KG06640} homozygous cells, no significant growth disadvantage was observed in these cells. Even when almost all the eye was mutant for *path*, because the other clonal material produced was homozygous for a cell lethal

chromosome, the resulting eyes were indistinguishable in size from normal eyes (compare Fig. 4J with 4I and 4K).

However, *path*^{KG06640} could produce tissue-autonomous effects in the eye in specific genetic backgrounds. For example, in our hands, it was difficult to produce viable animals with eyes that mainly consisted of homozygous *Tsc2* clones, using *eyFLP*-induced recombination with a cell lethal chromosome. Less than 1% of females and no males of the appropriate genotype survived through pupal stages to adulthood. When clonal material in the eye was mutant for both *Tsc2* and *path*^{KG06640} in an otherwise heterozygous animal, the lethal effects of *Tsc2* were suppressed, and about 10% of mutant animals, both males and females, eclosed. Their eyes were smaller and bulged less than *Tsc2* mutant eyes (compare Fig. 4M,N), revealing a tissue-autonomous effect of *path* on the *Tsc2* phenotype.

FOXO-induced cell death is specifically enhanced by the TOR signalling cascade and overexpression of PAT-related transporters

Our genetic data showed a role for PAT-related transporters in growth regulation and indicated that, like other amino acid transporters, this effect might involve TOR. However, they did

not reveal whether these transporters directly modulate TOR signalling or have an indirect effect via a parallel growth

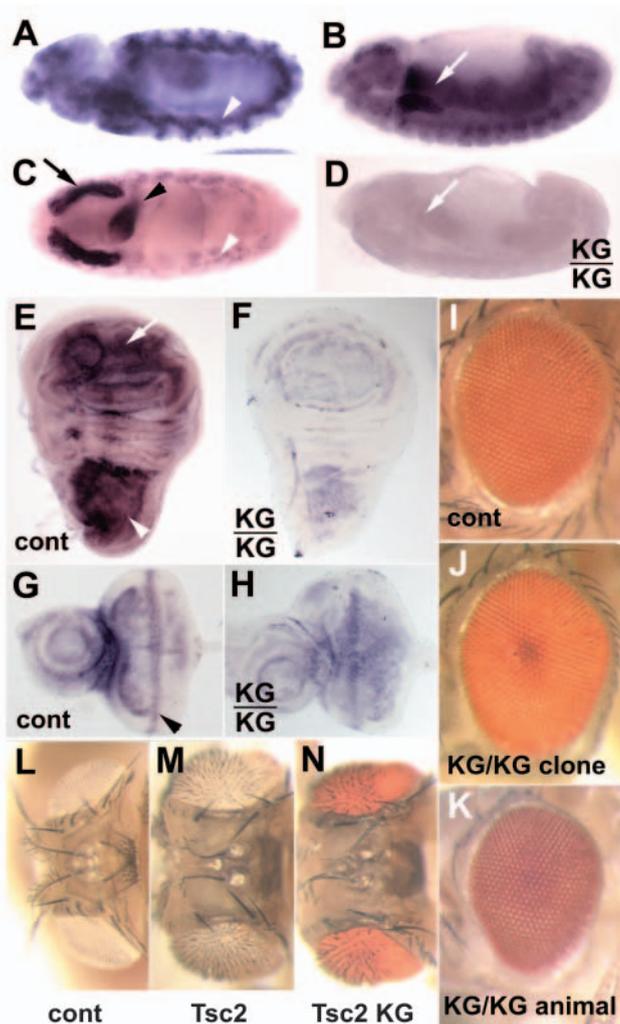


Fig. 4. *path* is broadly expressed during development and affects global and local growth signals. (A-C) In situ hybridisation with a *path* antisense probe reveals expression in almost all tissues during embryonic development, including stages 11 (A), 13 (B) and 16 (C). High transcript levels were observed in specific tissues, such as the muscle primordia (white arrowhead), midgut (white arrow), proventriculus (black arrowhead) and salivary glands (black arrow). A *path* sense probe gave no signal (data not shown). In *path*^{KG06640} homozygous embryos (D), a signal was observed only after extended staining times. (E-H) In situ hybridisation of wing (E,F) and eye antennal (G,H) imaginal discs from wild-type (E,G) and *path*^{KG06640} homozygous (F,H; overstained to detect signal) climbing third instar larvae. *path* is normally most strongly expressed in the pouch (arrow) and hinge (arrowhead) regions of the developing wing and at the morphogenetic furrow (arrowhead) in the eye. (I-K) Eyes that are almost entirely generated from homozygous *path*^{KG06640} cells via the *eyFLP/FRT* cell lethal method (J) were very similar in size to eyes of normal flies (I), and not small like those of *path*^{KG06640} homozygotes (K). (L-N) Dorsal views of eyes almost entirely generated from *Tsc2* mutant cells (M) and *Tsc2 path*^{KG06640} double mutant cells (N) were compared with normal controls (L). Fly genotypes are: *y w eyFLP/w; path*^{KG06640} *FRT80B/rps17⁴ P[w⁺] FRT80B* (J); *y w eyFLP/w; Tsc2¹⁹² FRT80B/rps17⁴ P[w⁺] FRT80B* (M); and *y w eyFLP/w; path*^{KG06640} *Tsc2¹⁹² FRT80B/rps17⁴ P[w⁺] FRT80B* (N).

regulatory pathway. To investigate this issue further, we developed an in vivo genetic assay to detect changes in TOR/S6K signalling activity in a tissue where PAT-related transporters stimulate growth. The forkhead transcription factor FOXO is a target of InR signalling in higher eukaryotes, including flies (Jünger et al., 2003; Puig et al., 2003). FOXO normally inhibits cell proliferation and stimulates apoptosis, but is inactivated when phosphorylated by InR-regulated Akt. Overexpression of a GS insertion in the first intron of *foxo*, *foxo*^{GS9928}, with *GMR-GAL4* produced distinctive ommatidial loss particularly in the posteroventral part of the eye (Fig. 5B) (Jünger et al., 2003).

This phenotype was strongly enhanced by co-expressing inhibitory molecules in the InR pathway, such as a dominant-negative form of Dp110 (Fig. 5E) (Leevers et al., 1996) and completely suppressed by activating components, such as the *Drosophila* PI3-kinase, Dp110 and Akt1 (Staveley et al., 1998) (Fig. 5C,F). By contrast, other positive growth regulators, which are believed to act independently of the InR pathway, did not produce similar suppression effects (see Fig. S2 in the supplementary material).

Radimerski et al. (Radimerski et al., 2002a; Radimerski et al., 2002b) have provided biochemical and genetic evidence that TOR signalling can negatively feed back to the InR signalling cascade, thus inhibiting Akt1 activity. We therefore reasoned that co-overexpression of TOR signalling components with *GMR-GAL4 foxo*^{GS9928} might enhance the growth inhibitory effects of FOXO. Indeed, S6K, which normally promotes modest overgrowth with *GMR-GAL4* (Fig. 3M) (Zhang et al., 2000), strongly enhanced the *GMR-GAL4 foxo*^{GS9928} reduced eye phenotype (Fig. 3N), mirroring the effects of dominant-negative Dp110 (Fig. 5E). A UAS-containing insertion in *Rheb*, *Rheb*^{AV4} (Patel et al., 2003), which by itself produces a greatly enlarged eye (Fig. 5G,J) (Patel et al., 2003), enhanced FOXO activity even more strongly in the eye, leading to loss of virtually all ommatidia in females (Fig. 5H) and pupal lethality in males with massive tissue degeneration in the head of late pupae. As expected, these phenotypes were suppressed by Akt1 overexpression (Fig. 5I,L), but not by co-expression of other UAS constructs (e.g. *UAS-GFP*, *UAS-lacZ*). Thus, growth-stimulating TOR signalling components act entirely differently to all other positive growth regulators in this assay, reducing eye size when co-expressed with FOXO.

Co-expression of *foxo* with either *CG1139* or *path* also enhanced the *foxo* reduced eye phenotype (Fig. 5P,S), an effect completely suppressed by Akt1 (Fig. 5Q,T), consistent with a role for these transporters in modulating TOR signalling. Interestingly, although TOR alone did not affect the *GMR-GAL4 foxo*^{GS9928} phenotype (Fig. 5X), it did enhance the effects of a weakly expressed *UAS-path* transgene (compare Fig. 5V with 5Y). Thus, TOR and the PAT-related transporters interact genetically in multiple developmental assays, and produce similar phenotypes when overexpressed in multiple tissues and in an in vivo assay that is specific for the TOR signalling pathway.

PATH defines a new type of PAT-related transporter with remarkably high substrate affinity and a novel signalling mechanism

To examine the transport properties of PATH and CG1139, we

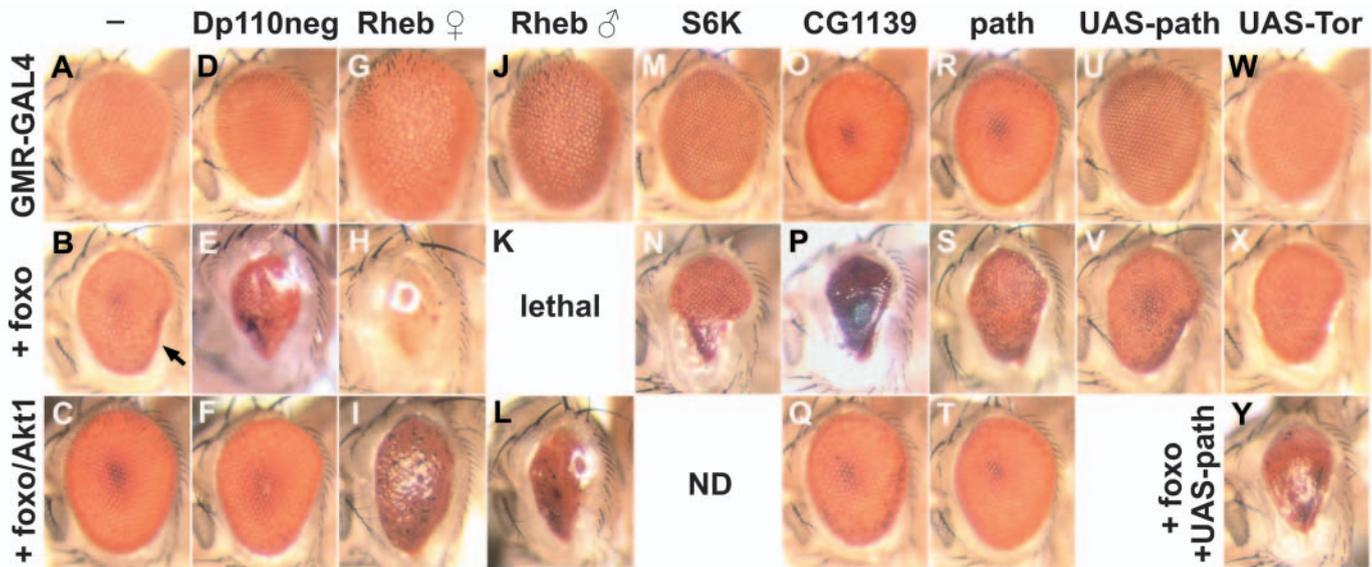


Fig. 5. PAT-related transporters and InR/TOR signalling components enhance FOXO activity by inhibiting the InR pathway. *GMR-GAL4* was used to drive expression of the following transgenes in the differentiating eye: dominant-negative *Dp110^{D954A}* (D-F), *Rheb^{AV4}* (G-L), *UAS-S6K* (M,N), *CG1139^{GS10666}* (O-Q), *path^{GS13857}* (R-T), *UAS-path* (U,V,Y) and *UAS-Tor* (W-Y). Flies overexpressing these transgenes together with *foxo^{GS9928}* (E,H,K,N,P,S,V,X,Y), or with *foxo^{GS9928}* and *UAS-Akt1* (F,I,L,Q,T) were compared with *GMR-GAL4* controls expressing the transgenes alone (D,G,J,M,O,R,U,W), no transgene (A), *foxo^{GS9928}* alone (B) or a combination of *foxo^{GS9928}* and *UAS-Akt1* (C). A small eye phenotype and posteroventral reduction (arrow in B) is produced by *GMR-GAL4 foxo^{GS9928}*. All eyes except G-I are from adult males, which produced stronger *foxo^{GS9928}* genetic interactions.

overexpressed each in *Xenopus* oocytes. This heterologous system has been extensively employed in the characterisation of many mammalian amino acid transporters, including PATs (e.g. Boll et al., 2002; Chen et al., 2003b). CG1139 behaved very similarly to mammalian PATs in radiolabelled amino acid influx (Fig. 6A,B) and inhibition (Fig. 6C,E) assays, and in electrophysiological studies (Fig. 6F,G). Like human PAT1 and PAT2, CG1139 specifically transported alanine, glycine and proline in an electrogenic and proton-stimulated fashion. Not only was influx of radiolabelled amino acids enhanced by extracellular acidification (Fig. 6A,B), but when compared with results obtained at pH 7.4, uptake of alanine, glycine and proline produced a larger plasma membrane depolarisation at pH 5.5, with reversal of membrane polarity (Fig. 6F,G). CG1139 and PAT1 have similar affinity for alanine [K_m of 1.2 ± 0.2 mM ($R^2=0.99$; Fig. 6E) versus an IC_{50} of 1.7 ± 0.23 mM for PAT1 (Chen et al., 2003a)], glycine (estimated $IC_{50}=3$ mM, 2.3 mM for PAT1; Fig. 6C) and proline (estimated $IC_{50}=8$ mM, 2.0 mM for PAT1).

By contrast, PATH behaved very differently in all these assays. Initial influx experiments using radioactive alanine, under the same conditions as for CG1139, revealed little if any change in influx at different amino acid concentrations. However, when non-radiolabelled competitor was greatly reduced in concentration, significant amino acid influx was observed (Fig. 6B). The affinity of PATH for alanine was found to be 100- to 3000-fold higher than mammalian PATs (K_m of 2.7 ± 1.0 μ M, $R^2=0.95$; Fig. 6E). In addition, the transport activity of PATH was higher at pH 7.4 than at pH 5.5 (Fig. 6B), unlike all other characterised PAT-related transporters. Inhibition studies revealed that although PATH also had a high affinity for glycine, it did not for proline (Fig. 6D).

In vivo genetic studies described above show that PATH and CG1139 have similar effects on growth and in *path* rescue experiments. However, in *Xenopus* oocytes, PATH has a transport capacity about 400 times lower than CG1139. It would thus be expected to increase overall intracellular levels of alanine by less than 0.2% during a 30 minute incubation. We tested whether despite this low transport capacity, extracellular alanine could activate the downstream target of TOR, S6K, in PATH-expressing *Xenopus* oocytes. S6K activation was detected in oocytes microinjected with leucine or alanine using an antibody that specifically crossreacts with the phosphorylated activated form of this molecule (Fig. 6H; data not shown) (Christie et al., 2002). Extracellular alanine had no effect on control oocytes, but did stimulate S6K signalling in oocytes expressing PATH (Fig. 6H). Thus, PATH can activate growth and a TOR target, S6K, in the absence of significant overall change in intracellular amino acid concentration.

Discussion

Using in vivo genetic analysis combined with transport studies in the heterologous *Xenopus* oocyte system, we have shown a novel and unexpected function for the PAT-related transporters in growth control and multiple genetic interactions with the TOR and InR signalling cascades. *CG1139*, a gene encoding a fly transporter with similar properties to mammalian PATs, modulates growth in multiple tissues. PATH, which has very different transport properties, produces surprisingly similar effects in vivo, suggesting that despite their differences, members of this family of sequence-related transporters have a conserved role to play in growth regulation (Fig. 7 and later discussion).

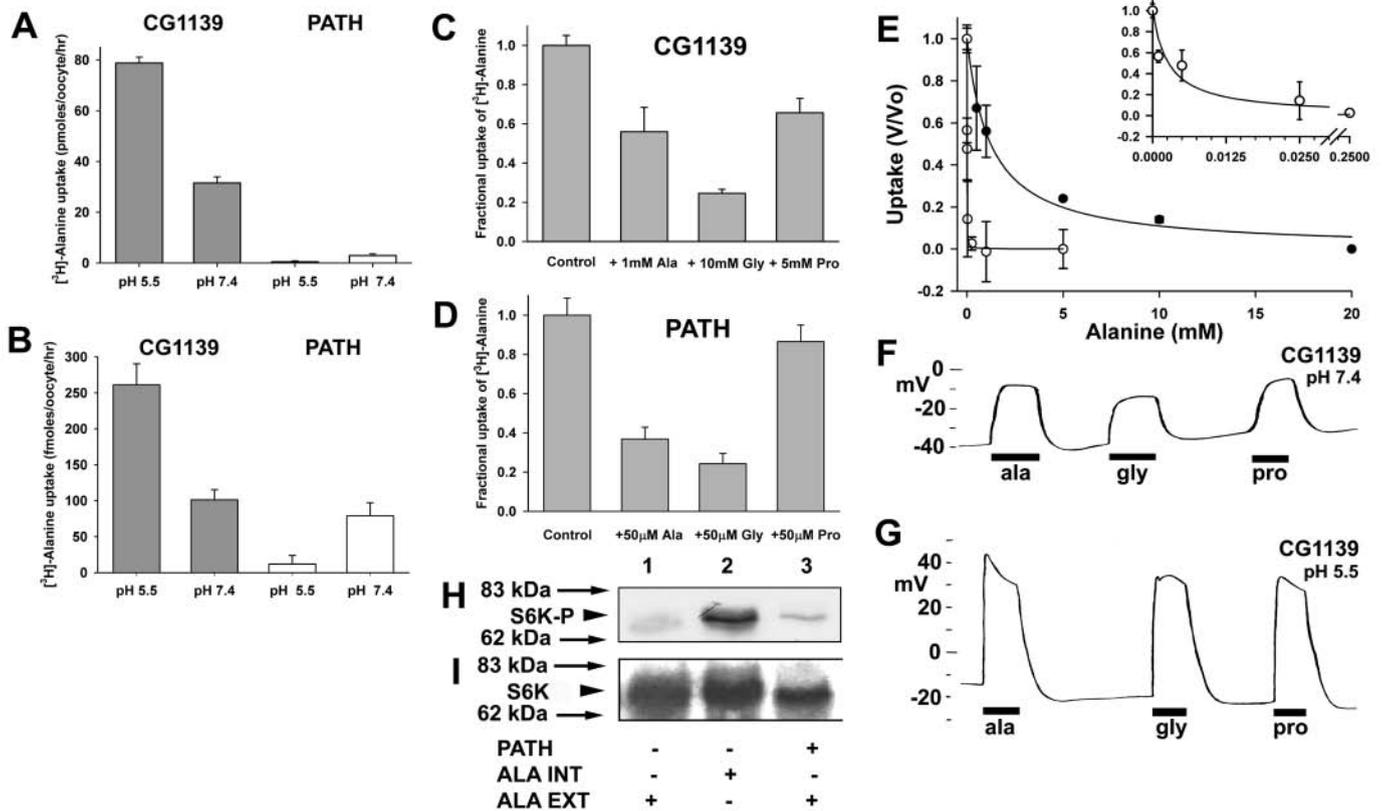


Fig. 6. PATH has very different transport properties from CG1139 and mammalian PATs. (A,B) Histograms showing pH-dependent differences ($P < 0.001$) in [^3H]-alanine uptake by *Xenopus laevis* oocytes expressing either CG1139 (grey) or PATH (white) in acidic (pH 5.5) versus neutral (pH 7.4) medium, using 50 μM (A) and 75 nM (B) extracellular alanine (both well below the K_m of CG1139). (C,D) Histograms showing inhibition of [^3H]-alanine uptake in CG1139-expressing (C; 50 μM [^3H]-alanine, pH 5.5) and PATH-expressing (D; 75 nM [^3H]-alanine, pH 7.4) oocytes by exogenous alanine, proline and glycine. All effects are statistically significant ($P < 0.03$), except that of proline on PATH. (E) Self-inhibition of alanine uptake into oocytes expressing either CG1139 (50 μM [^3H]-alanine, pH 5.5; black circles) or PATH (75 nM [^3H]-alanine, pH 7.4; white circles). Inset shows data for PATH over low concentration range (0 to 0.25 mM). (F,G) Measurements of membrane potential from oocytes expressing CG1139 in the absence or presence of exogenous alanine, glycine and proline at pH 7.4 (F) and pH 5.5 (G). None of the other common amino acids produced a response. (H,I) Western blots carrying identical oocyte extracts probed with antibodies specific for activated S6K (H) and total S6K (I; phosphorylated and non-phosphorylated S6K are not fully separated in this gel). The blots were subsequently stained with Coomassie Blue to confirm equal loading. Oocytes incubated with external alanine (10 mM, 30 min; lane 1) produced no detectable signal, while S6K was activated by microinjection of 27 nl of 10 mM alanine (lane 2). Incubation of PATH-expressing oocytes in an alanine-containing solution (10 mM, 30 min; lane 3) also activated S6K.

PAT-related transporters control growth and may specifically modulate TOR signalling

Four mammalian PAT family members have been cloned (Boll et al., 2002; Chen et al., 2003a; Chen et al., 2003b). The first identified member was christened LYAAT1, owing to its lysosomal localisation in rat brain (Sagne et al., 2001), but it is now clear that these transporters are also found at the cell surface. The human version of LYAAT1, PAT1, resides at the apical brush border membrane of intestinal cells (Chen et al., 2003a) and mouse PAT2 is predominantly localised to the endoplasmic reticulum and recycling endosomes in neurons (Rubio-Aliaga et al., 2004). The finding that PAT-related transporters modulate growth reveals an important new biological role for this class of molecules.

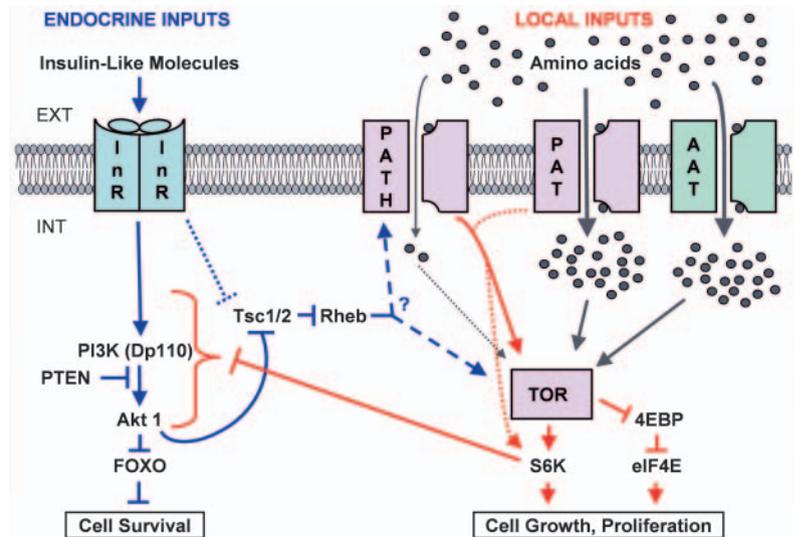
path and *CG1139* phenocopy the growth effects of *Tor* in the eye and wing, and strongly interact with *Tor* in multiple genetic assays. In addition, like positive growth regulatory components of the TOR signalling pathway, the transporters

reduce eye size in the FOXO interaction assay. Members of other classes of transporter, including the cationic transporter Slif, did not produce this range of effects. Our data are therefore consistent with a model in which both TOR (Fang et al., 2001; Wedaman et al., 2003) and the PAT-related transporters act within one or more membrane-associated molecular complexes required for TOR activation. We cannot eliminate the possibility that PATs act in a parallel growth regulatory pathway, but our data indicate that one function of this pathway would be to alter the sensitivity of the cell to InR activation (Fig. 7).

The TOR signalling pathway feeds back to negatively regulate InR signalling

Under normal conditions, the net result of TOR activation is overgrowth, but when FOXO, a direct target of Akt1 that is not part of the Akt1/Tsc/Rheb/TOR link (Fig. 7), is overexpressed in the differentiating eye, the TOR-induced reduction in Akt1

Fig. 7. Regulation of growth by PAT-related transporters. Model illustrating the role of PAT-related transporters in *Drosophila* cell growth. Although general amino acid transporters (AAT) and PATs can potentially activate TOR by increasing bulk intracellular amino acid concentrations (grey arrows), PAT-related transporters (here labelled PAT) have unique growth regulatory properties that, at least in the case of PATH, involve a local sensing mechanism (red arrows from PATH and PAT), which is modulated by InR/Tsc/Rheb. Genetic data suggest that these transporters modulate TOR signalling, but they may also affect growth via a parallel pathway that appears to affect S6K activation. The TOR signalling pathway negatively feeds back to upstream InR signalling via a mechanism that is uncharacterised in *Drosophila* (red bracket).



signalling increases cell death. Currently, the feedback mechanism involved has not been fully elucidated, although studies in mammals, where a similar phenomenon occurs, suggest that the phosphorylation state of IRS-1, an adaptor molecule for the InR, is involved (Jaeschke et al., 2002; Um et al., 2004; Harrington et al., 2005). One consequence of Akt repression by TOR signalling is to make cells more insulin resistant, a defining cellular and genetic defect in Type II diabetes. As PAT-related transporters enhance the effects of FOXO, partial cell surface inhibition of PAT activity might prove beneficial in individuals with Type II diabetes.

PATH regulates growth via local and global signalling mechanisms

Consistent with our proposal that *path* functions by modulating the response to InR/TOR signalling, the effects of reduced *path* on growth, developmental timing and female fertility broadly mirror those seen in InR signalling mutants. *path* is still transcribed, albeit at reduced levels, in *path*^{KG06640} mutant imaginal discs, and clonal analysis reveals that at least in the eye, an altered external growth signal is primarily responsible for the *path* mutant phenotype. As this phenotype is strongly suppressed by a combination of heterozygous *Tsc1* and *Tsc2* mutations, this global function of *path* probably involves modulation of InR/TOR signalling. *Tor* also affects endocrine as well as cell-autonomous growth regulation, at least in part by modulating fat body signalling (Colombani et al., 2003). However, the endocrine effects of *path* are unlikely to involve the fat body, as this organ is relatively unaffected in *path* mutants and *path* is weakly expressed in this tissue. These differences in tissue expression may explain why, unlike *Tor* mutants, ERTs and primordia of adult structures ultimately grow at a slower rate to roughly normal size in *path* larvae.

The partial suppression of a *Tsc2* clonal overgrowth phenotype in the eye by the *path*^{KG06640} mutation indicates that this allele does have tissue- and presumably cell-autonomous effects on growth in non-endocrine tissues in sensitised InR signalling backgrounds. As we have recently identified another broadly expressed PAT-related transporter with growth regulatory activity, the relatively modest effects of the *path*

mutation in imaginal discs may also be partly explained by functional redundancy in this family.

The transport properties of PATH reveal a novel mechanism for growth regulation

Analysis of the transport characteristics of CG1139 in the heterologous *Xenopus* oocyte system revealed remarkable similarities to mammalian PATs. By contrast, PATH displayed many novel properties, despite sharing similar levels of sequence identity with mammalian PATs (see Fig. S1A,B in the supplementary material). These include a mechanism inhibited by extracellular acidification, altered substrate specificity, remarkably high substrate affinity and low transport capacity. Although these experiments were not performed *in vivo*, to our knowledge there is no reported example where the functional properties of an amino acid transporter in the *Xenopus* oocyte system are not very similar to its properties *in vivo*, and it seems particularly unlikely that the substrate affinity of PATH would specifically increase by ~500-fold when synthesised in the oocyte.

Taken together with our primary finding that PATH and CG1139 are important regulators of growth *in vivo*, these data have at least three major implications. First, amino acid transporters have previously been shown to modulate TOR by changing intracellular amino acid concentrations (Christie et al., 2002; Beugnet et al., 2003), although observations in whole tissues suggest sensing of extracellular amino acid levels may be more important in regulating protein metabolism *in vivo* (Mortimore et al., 1992; Bohé et al., 2003). PATH can promote growth *in vivo* and in the heterologous oocyte system it can modestly activate S6K, despite its extremely limited transport capacity, which, during the time course of our *Xenopus* assays, is predicted to change bulk intracellular amino acid concentrations by less than 0.2%. PATH must therefore control growth via an alternative and novel mechanism, either by modulating local amino acid concentrations in a restricted compartment near the transporter, or through transport-independent amino acid sensing (Fig. 7). Based on genetic and biochemical experiments, our current hypothesis is that this mechanism involves TOR activation and is likely to be conserved for other PAT family members, given the ability of

CG1139 to largely substitute for *path* function in vivo. Interestingly, if PATs regulate growth via a transport-independent mechanism, the recent discovery that short chain fatty acids are also substrates for human PAT1 and PAT2 (Foltz et al., 2004) opens up the possibility that these molecules could act as novel growth regulators.

Second, the fact that PATH and *CG1139*, a transporter with 500-fold lower affinity, can have similar effects on growth in vivo, strongly suggests that PATH ($K_m \sim 3 \mu\text{M}$) is saturated in developing tissues. Indeed, extracellular amino acid concentrations are estimated to be in the millimolar range in flies (e.g. Pierce et al., 1999). Thus, the growth-promoting ability of PATH is effectively determined by its pattern and levels of expression and not by the extracellular amino acid concentration. Of course, it is possible that in some physiological conditions or within certain tissues, the high affinity of PATH will be crucial, but to date, rescue experiments of *path*^{KG06640} mutant animals with both *path* and *CG1139* have revealed no obvious differences between these molecules.

If PATH couples to TOR signalling, its properties ensure that all tissues that express this transporter will normally activate TOR at least at a basal level. Under these conditions, endocrine signalling by insulin-like molecules will therefore partially dominate over local nutrient signals in determining cell growth rates, consistent with previous observations (Britton et al., 2002) (Fig. 7). Interestingly, other insect genomes encode proteins with high sequence homology to PATH (XM 308237 in the mosquito *Anopheles gambiae*; XP 396451 in the Western honeybee *Apis mellifera*; see Fig. S1A,B in the supplementary material), and presumably employ similar mechanisms. Although there is no evidence at present for vertebrate sequence homologues, it is noteworthy that standard transport assays, which initially suggested PATH was an orphan transporter, have also failed to identify substrates for PAT3 and the ubiquitously expressed PAT4.

Finally, PAT-related transporters have been identified at the cell surface and in lysosomes. Subcellular shuttling of transporters has a precedent in yeast, where TOR modulates movement of at least one amino acid permease between the cell surface and the vacuole (Chen and Kaiser, 2003). Interestingly, yeast Tsc1, Tsc2 and Rheb have also been implicated in the regulation of amino acid transporters (Urano et al., 2000; van Slegtenhorst et al., 2004). However, contrary to expectations from multicellular organisms, where the Tsc complex inhibits TOR signalling, yeast Tsc upregulates and Rheb downregulates one or more amino acid permeases. Perhaps in higher eukaryotes Rheb positively regulates some transporters and negatively regulates others (Saucedo et al., 2003). Alternatively, endosomal and lysosomal targeting of PAT-related transporters upon Rheb activation could increase activity, although in this model, activation of PATH might only occur if it acts via a transport-independent mechanism, as its transport activity appears to be inhibited by acidification. Resolution of this issue will require a biochemical and cell biological analysis of the interactions between upstream InR signalling components, TOR complexes and PAT-related transporters.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/10/2365/DC1>

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