Synergism between altered cortical polarity and the PI3K/TOR pathway in the suppression of tumour growth

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Loss of function of *pins* (partner of inscuteable) partially disrupts neuroblast (NB) polarity and asymmetric division, results in fewer and smaller NBs and inhibits *Drosophila* larval brain growth. Food deprivation also inhibits growth. However, we find that the combination of loss of function of *pins* and dietary restriction results in loss of NB asymmetry, overproliferation of Miranda-expressing cells, brain overgrowth and increased frequency of tumour growth on allograft transplantation. The same effects are observed in well-fed *pins* larvae that are mutant for *pi3k* (phosphatidylinositol 3-kinase) or exposed to the TOR inhibitor rapamycin. Thus, pathways that are sensitive to food deprivation and dependent on PI3K and TOR are essential to suppress tumour growth in *Drosophila* larval brains with compromised *pins* function. These results highlight an unexpected crosstalk whereby the normally growth-promoting, nutrient-sensing PI3K/TOR pathway suppresses tumour formation in neural stem cells with compromised cell polarity.

Keywords: target of rapamycin; phosphatidylinositol 3-kinase; nutrient; growth; polarity; tumour suppression

EMBO reports (2012) 13, 157–162. doi:10.1038/embor.2011.230

INTRODUCTION

Asymmetric mitosis of *Drosophila* neuroblasts (NBs) generates a ganglion mother cell, which normally divides just once before differentiation, and a self-renewed NB that repeats this process until the programme of neurogenic mitoses comes to an end (Campos-Ortega, 1991; Knoblich, 2008). At the onset of asymmetric mitosis, the Partitioning-defective (PAR) complex proteins, PAR3, PAR6 and atypical protein kinase C (aPKC), accumulate on the apical cortex of the NB. The apical cortex also accumulates partner of inscuteables (PINS) and Gα_i that recruit

MUD (mushroom body defect) and associate with PAR3 through Inscuteable. Apical complexes control spindle orientation and the basal cortical localization of the adaptor proteins Partner of Numb and Miranda (MIRA), which bind to the cell fate determinants NUMB, PROS (Prospero) and BRAT (Brain Tumour), respectively (reviewed in Knoblich, 2008).

Larval brains mutant for any of several genes required for the asymmetric mitosis of NBs overgrow *in situ* and develop frankly malignant neoplasms on allograft transplantation in adult hosts (Caussinus & Gonzalez, 2005; Castellanos *et al*, 2008). Larval brains mutant for *pins* also develop malignant neoplasms on allograft (Caussinus & Gonzalez, 2005), but they actually have fewer and smaller NBs than wild-type brains and do not overgrow *in situ* (Lee *et al*, 2006). Thus, PINS seems to have a potential tumour suppressor (TS) activity, but, unlike other components of the asymmetric cell division machinery, such a TS activity is only revealed by the concurrence of other uncharacterized factors.

It has been suggested that one such factor could be the loss of the TS *lgl* (Doe, 2008), which is a rather frequent event in *Drosophila* populations both in the wild (Golubovsky *et al*, 2006) and in established laboratory strains (Roegiers *et al*, 2009). An alternative hypothesis put forward to explain why *pins* mutant larval brains can develop tumours on allograft transplantation in the abdomen of adult hosts is based on the extended time (that is, weeks, rather than days) available under such conditions (Gonzalez, 2007).

In the present study, we show that neither of these two hypotheses is correct: *lgl* loss is not a necessary event, and larval life extension is not sufficient for *pins* larval brains to overgrow. More importantly, we demonstrate that any one of food deprivation, reduced phosphatidylinositol 3-kinase (PI3K) function or inhibition of target of rapamycin (TOR) by rapamycin is sufficient to unleash the tumorigenic potential of *pins* mutant larval brains, causing overgrowth *in situ* and enhanced malignancy in allograft assays.

RESULTS AND DISCUSSION

Starvation triggers overgrowth in *pins* larval brains

We first investigated the possible contribution of *lgl* loss to *pins* tumour growth. Our results show that the development of malignant tumours from *pins* mutant larval brains does not require loss of *lgl* (supplementary Fig S1 online).

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Fig 1 | Dietary restriction promotes growth in *pins* mutant brains. (A) Examples of wild-type and *pins* (partner of inscuteable) brains from 6 days after egg laying (dAEL) larvae raised in standard fly food (SFF), 15–20 dAEL larvae subjected to ring gland inactivation (RGI) and 15–20 dAEL larvae raised under dietary restriction (DR). Scale bar, 100 μm . Genotypes in SFF and DR experiments are as follows: wild type = *UbiGFP-tub84B*, *pins* = *UbiGFP-tub84B/+*; *pins*⁸⁹/*pins*⁶². Genotypes in RGI experiments are wild type = *phm-GAL4/UAS-smt3i*, *pins* = *phm-GAL4/UAS-smt3i*; *pins*⁸⁹/*pins*⁶². (B) Quantification of optic lobe diameter (mean $\pm$ s.d.) in larval brains corresponding to the experimental conditions and genotypes described in (A). N = number of brains. P values were calculated by Student's *t*-test. (C) Change in optic lobe diameter in *pins* mutant brains relative to wild-type brains from 6 dAEL larvae raised in SFF (grey), 15–20 dAEL larvae subjected to RGI (red) and 15–20 dAEL larvae raised under DR (blue).

We then examined the possible contribution of an extended larval stage. To this end, we delayed puparium formation by either ring gland inactivation (RGI; Talamillo *et al*, 2008) or dietary restriction (DR). RGI and DR result in the extension of the larval stage to up to 20 days after egg laying (dAEL), which is about threefold the duration of the larval stage under normal conditions (6 dAEL).

Mean optic lobe diameter in wild-type larvae raised in standard fly food (SFF) for 6 dAEL (126 $\pm$ 7 μm , *n* = 20) remains unchanged in 15–20 dAEL larvae subjected to RGI (124 $\pm$ 7 μm , *n* = 18), and is reduced in 15–20 dAEL larvae raised under DR (86 $\pm$ 4 μm , *n* = 8; Fig 1A,B). In contrast, optic lobe diameter in *pins* mutant brains from larvae raised in SFF and dissected 6 dAEL (112 $\pm$ 8 μm , *n* = 20) is slightly reduced in 15–20 dAEL larvae subjected to RGI (95 $\pm$ 8 μm , *n* = 6), but enlarged in 15–20 dAEL larvae raised under DR (130 $\pm$ 21 μm , *n* = 6; Fig 1A,B). Thus, compared with the wild type, optic lobe diameter in *pins* larvae is 12% smaller (*P* < 0.001) in 6 dAEL larvae raised in SFF and 24% smaller (*P* < 0.001) in 15–20 dAEL larvae subjected to RGI, but 50% larger (*P* < 0.001) in 15–20 dAEL DR-treated larvae (Fig 1C). In fact, mean optic lobe diameter in *pins* brains raised under DR is just as large as that of control brains from larvae raised in SFF. Indeed, changes in diameter reflect much greater changes in volume. These results show that the combination of two factors such as food deprivation and loss of *pins* function, which are on their own growth inhibitory, causes overgrowth in *Drosophila* larval brains.

TOR or PI3K loss mimics starvation on *pins* brain growth

Exposure to DR conditions has several effects, including the downregulation of key signalling molecules such as PI3K and TOR (Oldham *et al*, 2000; Zhang *et al*, 2000; Britton *et al*, 2002). To determine whether the overgrowth effect that we have found DR

conditions to exert on *pins* mutant larval brains operates through PI3K and TOR, we measured optic lobe diameter in larvae that were double mutant for *pins* and *dp110*, the gene encoding *Drosophila* PI3K, and in *pins* mutant larvae treated with the TOR inhibitor rapamycin (Fig 2).

Similarly to DR, loss of TOR or PI3K activity inhibits growth in wild-type *Drosophila* tissues (Oldham *et al*, 2000; Zhang *et al*, 2000; Britton *et al*, 2002). Consistently, mean optic lobe diameters in 15–20 dAEL rapamycin-treated wild-type larvae (97 $\pm$ 4 μm , *n* = 4) and in 10 dAEL *pi3k* mutant larvae raised in SFF (101 $\pm$ 8 μm , *n* = 18) are significantly smaller than those in 6 dAEL wild-type larvae raised in SFF (126 $\pm$ 7 μm , *n* = 20; Fig 2A,B and Fig 1A,B). However, mean optic lobe diameters in 15–20 dAEL, rapamycin-treated *pins* larvae (128 $\pm$ 13 μm , *n* = 8) and in *pi3k pins* double-mutant brains from larvae raised in SFF (133 $\pm$ 19 μm , *n* = 22; Fig 2A,B) are larger than those in 6 dAEL larvae mutant for *pins* alone and raised in SFF (112 $\pm$ 8 μm , *n* = 20; Fig 1A,B). Thus, although optic lobe diameter is 12% smaller (*P* < 0.001) in *pins* than in wild-type larvae raised in SFF, it becomes 30% larger (*P* < 0.001) in larvae treated with rapamycin (Fig 2C). Similarly, optic lobe diameter is 30% larger (*P* < 0.001) in *pi3k pins* double-mutant brains than in brains that are mutant for *pins* alone (Fig 2C), a difference that is increased to 65% (*P* < 0.001) if the larval stage is extended to 15–20 dAEL by RGI (Fig 2). Taking into account the fact that volume changes are much greater than changes in diameter, and the fact that loss of PINS, PI3K or TOR function alone significantly reduces brain size, these results indicate a strong growth-promoting synergistic interaction of PI3K and TOR downregulation with *pins* loss of function. These results also indicate that inactivation of PI3K or TOR is sufficient to mimic the overgrowth effect triggered by starvation on *pins* mutant brains.

Fig 2 | Loss of TOR or PI3K mimics the effect of dietary restriction in *pins* larval brains. (A) Examples of brains from wild-type and *pins* (partner of inscuteable), 15–20 days after egg laying (dAEL) larvae raised in the presence of rapamycin; *pi3k* and *pi3k pins*, 10 dAEL larvae raised on standard fly food (SFF); and *pi3k* and *pi3k pins*, 15–20 dAEL larvae subjected to ring gland inactivation (RGI). Scale bar, 100 μm. Genotypes: wild type = UbiGFP-tub84B/+; *pins*⁸⁹/TM6B, Tb or UbiGFP-tub84B/+; *pins*⁶²/TM6B, Tb. *pins* = UbiGFP-tub84B/+; *pins*⁸⁹/*pins*⁶². *pi3k* SFF = UbiGFP-tub84B/+; *dp110*^{6M5}/*dp110*^{6V2}. *pi3k pins* SFF = UbiGFP-tub84B/+; *dp110*^{6M5}*pins*⁸⁹/*dp110*^{6V2}*pins*⁶². *pi3k* RGI = *phm*-GAL4/UAS-*smt3i*; *dp110*^{6M5}/*dp110*^{6V2}. *pi3k pins* RGI = *phm*-GAL4/UAS-*smt3i*; *dp110*^{6M5}*pins*⁸⁹/*dp110*^{6V2}*pins*⁶². (B) Quantification of optic lobe diameter (mean ± s.d.) in larval brains corresponding to the experimental conditions and genotypes described in (A). N = number of brains. P values were calculated by Student's *t*-test. (C) Change in optic lobe diameter in *pins* mutant brains relative to wild-type brains from 6 dAEL larvae raised in standard fly food (SFF; grey), rapamycin-treated *pins* brains relative to rapamycin-treated wild-type brains from 15–20 dAEL larvae (green) and *pi3k pins* double-mutant brains relative to *pins* brains both from larvae raised in SFF (light purple) and from 15–20 dAEL larvae subjected to RGI (dark purple). PI3K, phosphatidylinositol 3-kinase; TOR, target of rapamycin.

Energy stress causes asymmetry loss and overproliferation

To further investigate the effect of DR and compromised PI3K or TOR function on *pins* mutant brains, we quantified MIRANDA-expressing (MIRA+) cells per optic lobe in the central brain (Fig 3, CB, yellow dotted line). As reported, under SFF conditions, MIRA+ cells are less abundant in *pins* larvae than in wild-type controls (Fig 3A,B). However, MIRA+ cells are significantly more abundant in *pins* larvae subjected to DR (194 ± 35, *n* = 3, *P* < 0.001) or treated with rapamycin (277 ± 176, *n* = 4, *P* = 0.008). MIRA+ cells are also significantly more abundant in *pi3k pins* double-mutant larvae, both raised in SFF (585 ± 390, *n* = 6, *P* = 0.011) or subjected to RGI (752 ± 224, *n* = 3, *P* = 0.001), than in either single mutant alone. Figure 3B (black and white panels) shows that as MIRA+ cells proliferate in rapamycin-treated *pins* brains they spread over most parts of the optic lobe. The same effect was observed in *pins* brains subjected to DR or mutant for *pi3k* (not shown). In all these cases, overproliferation of MIRA+ cells takes place mostly in the dorsal side of the optic lobe (supplementary Fig S2 online).

In wild-type central brains from larvae raised in SFF, isolated MIRA+ cells (Fig 3B, red) are surrounded by clusters of much smaller nuclear-PROS+ ganglion cells (Fig 3B, green). This pattern remains unchanged on rapamycin treatment or loss of *pins* function. However, the combined effect of *pins* loss of function and rapamycin treatment results in central brains in which, particularly in the dorsal side, MIRA and PROS coexpression is the norm, PROS

is cytoplasmic and cell size differences are negligible (Fig 3B). The same effects were observed in *pins* brains exposed to DR or mutant for *pi3k* (not shown). All these traits make these overproliferative cells indistinguishable from those that have been reported in pins-derived allografted neoplasms (Causinus & Gonzalez, 2005). These results indicate that energy stress, such as experimental conditions that mimic an energy stress response, suppresses asymmetric division in *pins* mutant MIRA+ cells and transforms them from the under-grown, under-proliferative state that is characteristic of *pins* mutant larvae raised in SFF to the uncontrolled proliferative state observed in allografted pins malignant neoplasms.

Similar results have been observed in *dlkb1 pins* double mutants. Dlk1 is the *Drosophila* homologue of the human TS kinase LKB1, which is mutated in cancer-prone Peutz–Jeghers patients and is also frequently targeted for inactivation or downregulated during sporadic cancer transformation (reviewed in Jansen *et al*, 2009). In *dlkb1* mutant larval brains, there is a moderate level of abnormal asymmetric segregation of cell-fate determinants, and overproliferation is notably enhanced in *dlkb1 pins* double mutants (Bonaccorsi *et al*, 2007). In larval NBs, loss of single members of either the PAR or PINS complexes such as *baz* or *pins* has no effect on daughter cell size asymmetry and causes only transient defects in spindle orientation and basal protein localization that are often corrected late in mitosis by ‘telophase rescue’ (Peng *et al*, 2000). However, double-mutant combinations that inactivate the two complexes, such as *pins* or *Gαi* loss

Fig 3 | Overproliferation and loss of asymmetry of MIRA + cells in *pins* larval brains under energy stress. (A) Mean number of miranda (MIRA) + cells in central brains from wild-type and *pins* (partner of inscuteable) larvae raised in SFF or subjected to RGI, DR or rapamycin (rapa) treatment, and *pi3k* and *pi3k pins* raised in SFF or subjected to RGI. *P* values were calculated by Student's *t*-test. Error bars represent standard deviations. Genotypes: wild-type SFF and wild-type DR = UbiGFP-tub84B. *pins* SFF and *pins* DR = UbiGFP-tub84B/+; *pins*⁸⁹/*pins*⁶². Wild-type RGI = *phm-GAL4/UAS-smt3i*. *pins* RGI = *phm-GAL4/UAS-smt3i*; *pins*⁸⁹/*pins*⁶². Wild-type rapamycin = UbiGFP-tub84B/+; *pins*⁸⁹/TM6B, Tb or UbiGFP-tub84B/+; *pins*⁶²/TM6B, Tb. *pins* rapamycin = UbiGFP-tub84B/+; *pins*⁸⁹/*pins*⁶². *pi3k* SFF = UbiGFP-tub84B/+; *dp110*^{6M5}/*dp110*^{6V2}. *pi3k pins* SFF = UbiGFP-tub84B/+; *dp110*^{6M5}/*dp110*^{6V2}*pins*⁸⁹. *pi3k* RGI = *phm-GAL4/UAS-smt3i*; *dp110*^{6M5}/*dp110*^{6V2}. *pi3k pins* RGI = *phm-GAL4/UAS-smt3i*; *dp110*^{6M5}/*dp110*^{6V2}*pins*⁸⁹. Low-magnification views (black and white) show MIRA + cells in white. The yellow dotted line marks the central brain (CB). Scale bar, 100 µm. High-magnification views show MIRA + cells in red and PROS + cells in green. Scale bar, 20 µm. DR, dietary restriction; dAEL, days after egg laying; RGI, ring gland inactivation.

of function, in combination with mutation in *baz* or *aPKC*, remove all asymmetry traits and cause NBs to generate two equal daughters that overproliferate (Izumi *et al*, 2004; Kitajima *et al*, 2010). This might explain the *dlkb1 pins* double-mutant phenotype, because loss of *dlkb1* abrogates proper localization of the PAR complex components BAZ/PAR3, PAR6 and DaPKC (Bonaccorsi *et al*, 2007). The situation is less clear in the case of the synergistic effect that we have found food deprivation, *pi3k* loss or TOR inhibition to exert on *pins* loss-of-function conditions. Although mutants in *pi3k* result in a strong decrease in the levels of phosphatidylinositides (Orme *et al*, 2006) that are necessary to target BAZ to the membrane in embryonic NBs (Krahn *et al*, 2010), no evidence has been obtained so far demonstrating that energy stress or inhibition of PI3K or TOR compromises PAR complex function in any way, in *Drosophila* larval brains.

Starvation, TOR inhibition & PI3K loss enhance malignancy

We determined whether the overgrowth-promoting effect that energy stress, loss of PI3K and TOR inhibition have on *pins* mutant brains has an effect on the development of allografted pins malignant tumours. To this end, we implanted in the abdomen of wild-type hosts pieces of larval brain tissue corresponding to the different experimental conditions described in this work, and plotted the fraction of hosts that developed tumours as a function of time (Fig 4).

Wild-type brains from larvae raised in SFF (*n*=34) do not develop tumours in this assay (Caussinus & Gonzalez, 2005; Janic *et al*, 2010), nor do rapamycin-treated wild-type brains (*n*=22), brains mutant for *pi3k* alone (*n*=25) or *pins* brains from larvae subjected to RGI (*n*=45). Brains from 6 dAEL *pins* mutant larvae raised in SFF develop tumours in 12% (*n*=74) of implanted hosts (Fig 4, 'pins SFF'). Remarkably, this percentage increases to

Fig 4 | Starvation, inhibition of TOR and loss of PI3K function enhance malignant traits in pins-allografted neoplasms. shown are cumulative percentages of hosts that developed malignant tumours after implantation with *pins* (partner of inscuteable) brains from 6 dAEL larvae raised in standard fly food (SFF; grey), *pi3k pins* larvae raised in SFF or subjected to RGI (light and dark purple, respectively), rapamycin-treated *pins* brains (green) and 9–11 or 15–20 dAEL *pins* larvae raised under DR (dotted blue and solid blue, respectively). Brains from wild-type larvae raised in SFF, rapamycin-treated wild-type larvae, *pi3k* larvae or *pins* larvae subjected to RGI did not develop tumours (red). Genotypes: *pins* DR, SFF and rapamycin = *UbiGFP-tub84B/+*; *pins*⁸⁹/*pins*⁶². *pi3k pins* SFF = *UbiGFP-tub84B/+*; *dp110*^{6M5}*pins*⁸⁹/*dp110*^{6V2}*pins*⁶². *pi3k pins* RGI = *phm-GAL4/UAS-smt3i*; *dp110*^{6M5}*pins*⁸⁹/*dp110*^{6V2}*pins*⁶². Wild-type SFF and DR = *UbiGFP-tub84B*. *pi3k* SFF = *UbiGFP-tub84B/+*; *dp110*^{6M5}/*dp110*^{6V2}. *pi3k* RGI = *phm-GAL4/UAS-smt3i*; *dp110*^{6M5}/*dp110*^{6V2}. Wild-type rapamycin = *UbiGFP-tub84B/+*; *pins*⁸⁹/*TM6B*, *Tb* or *UbiGFP-tub84B/+*; *pins*⁶²/*TM6B*, *Tb*. *pins* RGI = *phm-GAL4/UAS-smt3i*; *pins*⁸⁹/*pins*⁶². In all cases, implanted hosts were wild type and were maintained in SFF. *P* values were calculated by log-rank test. Inset shows a control fly with no tumour (left) and a fly, 15 days after allograft of GFP-labelled larval brain tissue, in which spread GFP signal indicates tumour growth (right). DR, dietary restriction; dAEL, days after egg laying; GFP, green fluorescent protein; PI3K, phosphatidylinositol 3-kinase; RGI, ring gland inactivation; SFF, TOR, target of rapamycin.

30% ($n = 37$, $P = 0.009$) when implanted brains are 10 dAEL *pi3k pins* double mutant, to 41% ($n = 22$, $P = 0.001$) in the case of rapamycin-treated *pins* brains and to 90% ($n = 10$, $P = 0.001$) in brains that are *pi3k pins* double mutant from 15–20 dAEL larvae subjected to RGI. The percentages of implanted hosts that develop tumours on allograft transplantation of DR-treated *pins* brains from 9–11 and 15–20 dAEL larvae are 44% ($n = 9$, $P = 0.004$) and 80% ($n = 10$, $P < 0.001$), respectively. In all these cases, the higher the final percentage of implanted hosts that develop tumours, the earlier the onset of tumour growth as revealed by the shifting of the plots towards the left side of the graphic (Fig 4). Thus, whereas the first tumour takes about 23 days after allograft to appear in hosts implanted with *pins* brains from larvae raised in SFF, it takes only 11–13 days in host implanted with brains from *pins* mutant larvae raised under DR or rapamycin treatment. From these observations, we conclude that energy stress, TOR inhibition and PI3K loss strongly enhance malignant pins tumour development.

PI3K and TOR activity are necessary for sustained growth and proliferation (Colombani *et al*, 2003; Teleman, 2010), and to resume cycling activity after developmentally programmed quiescence (Chell & Brand, 2010; Sousa-Nunes *et al*, 2011). Consistently, there is abundant experimental evidence showing that in most tumour types growth is coupled to activated PI3K, and not to its loss (Engelman *et al*, 2006; Kalaany & Sabatini, 2009). Evidence showing that low-calorie intake is a protective condition against malignant transformation is also abundant (Kritchevsky, 2002). However, our results indicate that in our model system PI3K and TOR activity and a normal food supply have a function in preventing overgrowth in neural stem cells with compromised cortical polarity. Whether this conclusion applies to vertebrates

remains to be ascertained. Interestingly, some *PI3K γ ^{-/-}* mouse strains have a high incidence of invasive colorectal tumours, which is not due to *PI3K γ* loss alone, but is suspected to result from the combined effect of *PI3K γ* loss and other unknown factors (Sasaki *et al*, 2000, 2003; Barbier *et al*, 2001). Taking into account the fact that the pathways involved in the control of cell polarity and in the response to changing nutrient conditions are largely conserved across species, we propose that similar synergistic interactions might take place in vertebrates.

METHODS

Fly strains. *UAS-smt3i* on chromosome 3 (Talamillo *et al*, 2008) was used as donor to generate a *UAS-smt3i* insertion in chromosome 2 by P-element transposition. *pi3k* alleles *dp110*^{6M5} and *dp110*^{6V2} (Orme *et al*, 2006) were obtained from E Hafen and H Stoker. We used the following lines: *UbiGFP-tub84B* (Rebollo *et al*, 2004), *phm-Gal4* (Ono *et al*, 2006), *Igl⁴* (Gateff, 1978), *pins*⁸⁹ and *pins*⁶² (Yu *et al*, 2000).

Immunofluorescence. Immunofluorescence was performed as described (Gonzalez & Glover, 1993). In brief, third-instar larval brains were dissected in 0.7% NaCl, fixed for 30 min in 3.7% PFA (paraformaldehyde) in 0.7% NaCl and blocked in 10% fetal calf serum in 0.7% NaCl and 0.1% Triton X-100 for 2 h at room temperature. Antibodies were diluted in the same blocking solution. We used rat and rabbit anti-MIRA, 1:1,000 (Mollinari *et al*, 2002; Caussinus & Gonzalez, 2005), and mouse anti-PROS (MR1A obtained from DSHB), 1:250. Secondary Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 633 antibodies were obtained from Invitrogen. DNA was stained with 4,6-diamidino-2-phenylindole and brains were mounted in Vectashield (Vector Laboratories).

Images were acquired on a Leica SP2 confocal microscope and processed with Adobe Photoshop.

Dietary restriction and rapamycin treatment. Eggs were collected on plates containing SFF for 6 h, incubated for a further 24–68 h in the same plates and passed on to new plates containing 0.05% yeast and 1.5% agar (DR media). DR medium was replaced every 3 days. For rapamycin treatment, eggs were collected for a period of 24 h on SFF. After 48 h, the medium was supplemented with 150 µl of either 50 µM rapamycin, 0.7% NaCl, 0.1% dimethylsulphoxide (DMSO) in water or 0.7% NaCl and 0.1% DMSO in water. Rapamycin and DMSO were obtained from Sigma-Aldrich.

Brain-size quantification. Optic lobe size was estimated by measuring the maximum diameter from digital stereoscope images using Image J (<http://rsbweb.nih.gov/ij/>).

Allograft assay. Tissue dissection and injection were carried out as described in Caussinus & Gonzalez (2005). In brief, donor larvae were washed in distilled water, brains were dissected in 0.7% NaCl, cut into small pieces and injected into the abdomen of CO₂-anaesthetized young female adult hosts. Implanted hosts were kept at 25 °C and checked daily for tumour growth.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank M. O'Connor, R. Barrio, J. Knoblich, C. Doe, E. Hafen, H. Stoker, the Hybridoma Bank and the Bloomington and Tübingen *Drosophila* Stock Centers for reagents and fly strains; M. Marinelli for statistical advice; and members of our laboratory for discussions. Work in our laboratory was supported by grants BFU2009-07975/BMC, CENIT ONCOLOGICA-20091016, Consolider-Ingenio C3D CSD2006-23 and SGR Agaur 2009 CG041413.

Author contribution: F.R. and C.G. designed the experiments and analysed the data. F.R. conducted the experiments. C.G. wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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