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Regulation of Neurogenesis and Epidermal Growth Factor Receptor Signaling by the Insulin Receptor/Target of Rapamycin Pathway in Drosophila

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ABSTRACT

Determining how growth and differentiation are coordinated is key to understanding normal development, as well as disease states such as cancer, where that control is lost. We have previously shown that growth and neuronal differentiation are coordinated by the insulin receptor/target of rapamycin (TOR) kinase (InR/TOR) pathway. Here we show that the control of growth and differentiation diverge downstream of TOR. TOR regulates growth by controlling the activity of S6 kinase (S6K) and eIF4E. Loss of *s6k* delays differentiation, and is epistatic to the loss of *tsc2*, indicating that S6K acts downstream or in parallel to TOR in differentiation as in growth. However, loss of *eIF4E* inhibits growth but does not affect the timing of differentiation. We also show, for the first time in Drosophila, that there is crosstalk between the InR/TOR pathway and epidermal growth factor receptor (EGFR) signaling. InR/TOR signaling regulates the expression of several EGFR pathway components including *pointedP2* (*pntP2*). In addition, reduction of EGFR signaling levels phenocopies inhibition of the InR/TOR pathway in the regulation of differentiation. Together these data suggest that InR/TOR signaling regulates the timing of differentiation through modulation of EGFR target genes in developing photoreceptors.

A fundamental challenge during the development of any complex organism is the coordination of proliferation and differentiation. The rate of proliferation is not constant during development (Neufeld *et al.* 1998) and depends on the developmental stage as well as hormonal and nutritional cues (Britton *et al.* 2002). Coordinating growth and differentiation is a particular challenge in complex tissues, such as the nervous system. Neurogenesis is preceded by a period of proliferation, which generates a pool of precursor cells. Selected cells from this pool exit the cell cycle and initiate a complex program of gene expression that will result in the formation of the mature neuron.

The Drosophila retina is a highly tractable model for studying the relationship between growth and neuronal differentiation (Wolff and Ready 1993). Photoreceptor (PR) differentiation in Drosophila is initiated at the beginning of the third larval instar when a physical indentation, known as the morphogenetic furrow (MF), develops at the posterior of the eye imaginal disc. Over a period of ~48 hr the MF sweeps anteriorly leading to the formation of PR preclusters. The MF is initiated by the morphogen Hedgehog (Hh) and is propagated anteriorly through a combination of Hh and Decapentapalegic (Dpp) signaling (Voas and Rebay 2004). Posterior to the MF, PRs are specified sequentially through reiterative use of the Notch and EGFR pathways (Brennan and Moses 2000; Voas and Rebay 2004).

As in other neurogenic contexts, neuronal differentiation in the Drosophila eye is a temporally restricted process. Patterning of the mature cluster of eight PRs is highly stereotyped with each row forming about every 2 hr (Figure 1A) (Wolff and Ready 1993). The mechanism underlying the temporal control of PR differentiation has proven elusive. Several models have been proposed including control by receptor-mediated cell–cell interactions and intrinsic or extrinsic cellular clocks (Freeman 1997; Brennan and Moses 2000; Voas and Rebay 2004). We found that the conserved InR/TOR pathway plays a key role in controlling the timing of neuronal differentiation in Drosophila (Bateman and McNeill 2004). Using mutants in various components of the InR/TOR pathway, we showed that activation of this pathway causes precocious differentiation of neurons. Conversely, inhibition of InR/TOR signaling significantly delays neurogenesis. How the InR/TOR pathway regulates neuronal differentiation is unclear.

Temporal control of neuronal differentiation is a property of the entire InR/TOR pathway. Ligand binding to the InR causes recruitment and phosphorylation of the insulin receptor substrate (IRS) and subsequent activation of PI3K, which catalyzes the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the membrane (Leevers and Hafen 2004).

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PDK1 and PKB/AKT, both PH domain-containing kinases, become membrane localized by their interaction with PIP3 where PKB/AKT can be fully activated. InR signaling controls growth and proliferation through the inhibition of the GTPase activating protein (GAP) TSC2 (Gao and Pan 2001; Potter *et al.* 2001; Tapon *et al.* 2001; Cai *et al.* 2006). TSC2 inhibits the activity of the small GTPase Rheb, which activates TOR (Long *et al.* 2005). TOR is a phosphatidylinositol kinase-related kinase that is part of a complex (TORC1) that controls growth through the regulation of ribosome biogenesis and translation via S6K and eIF4E, respectively (Inoki and Guan 2006; Wullschleger *et al.* 2006). TOR is also a component of the TORC2 complex. TORC2 is insensitive to rapamycin and has recently been shown to phosphorylate AKT at Ser473 (Sarbassov *et al.* 2005; Guertin *et al.* 2006b). TOR has other functions including the regulation of microautophagy and fat metabolism (Rusten *et al.* 2004; Scott *et al.* 2004). In addition, inhibition of TOR by treatment with rapamycin elicits a transcriptional response involving several hundred genes (Peng *et al.* 2002; Guertin *et al.* 2006a). Recently a negative feedback loop in which S6K regulates IRS, both transcriptionally and by phosphorylation, has been shown to exist in both Drosophila (Radimerski *et al.* 2002) and mammalian systems (Harrington *et al.* 2004; Shah *et al.* 2004; Um *et al.* 2004).

What is the mechanism by which InR signaling controls the timing of neuronal differentiation? In mammalian systems activation of insulin/IGF receptor tyrosine kinases causes activation of both PI3K and Ras/mitogen-activated protein kinase (MAPK) pathways (Baltensperger *et al.* 1993; Skolnik *et al.* 1993; Downward 2003). Ligand binding to the InR results in tyrosine phosphorylation of IRS proteins and/or Shc which, through the adaptor protein Grb2, results in recruitment to the membrane of SOS for the activation of Ras (Baltensperger *et al.* 1993; Skolnik *et al.* 1993). However, flies expressing a version of the Drosophila IRS *chico,* in which the putative Drk (the Drosophila ortholog of Grb2) binding site had been mutated, are able to fully rescue the growth defects of *chico* flies (Oldham *et al.* 2002). Therefore it is currently unclear whether the InR activates MAPK signaling in Drosophila (Bateman and McNeill 2006).

In the current study we find that differentiation is temporally regulated by TOR and S6K, but not by 4EBP or eIF4E, thus providing the first branch in the differentiation pathway downstream of InR signaling in the eye. We also show that activation of the InR/TOR pathway regulates the expression, at the transcriptional level, of the EGFR pathway components Argos, rhomboid (rho), and pointedP2 (pntP2). Moreover, reducing the level of EGFR signaling, by using a *pntP2* hypomorphic allele, causes a cell-type-specific delay in differentiation, which is identical to that in mutants that inhibit the InR/TOR pathway. Finally we show that the EGFR and InR/TOR pathways genetically interact in controlling the timing of PR differentiation.

MATERIALS AND METHODS

To generate loss-of-function clones, 48- to 72-hr-old larvae were heat-shocked for 1–2 hr at 37°. Overexpression clones were generated using the "flp-out" technique (Neufeld *et al.* 1998), where 48- to 60-hr-old larvae were heat-shocked for 2.5 hr at 37°. Third instar eye discs were fixed in PBSA/4% formaldehyde (EMS Scientific) for 45 min, washed in PBSA/0.1% TritonX100 (Sigma, St. Louis) and incubated overnight with primary antibody. Primary antibodies were used as follows: mouse and rabbit anti-GFP (Molecular Probes, Eugene, OR; 1:1000), rabbit anti-Bar (a gift from K. Saigo; 1:200), mouse anti-Prospero (DHSB; 1:10), guinea pig anti-Senseless (a gift from H. Bellen; 1:1000), mouse anti-β-galactosidase (Roche, Indianapolis; 1:1000), rabbit anti-Spalt (a gift from R. Barrio; 1:500), mouse anti-Rough (DSHB; 1:100), mouse anti-Cut (DSHB; 1:20), and mouse anti-Argos (DSHB; 1:100). Secondary antibodies were from Jackson Laboratories (West Grove, PA). After staining, discs were mounted in Vectastain (Vector Laboratories, Burlingame, CA) and analyzed with a Zeiss confocal microscope or a Zeiss Apotome.

To quantify *eIF4E* mutant growth rates the mutant clone area relative to the twin-spot area was quantified using ImageJ and in three independent clones for each genotype.

The following stocks were kindly provided to us: The *pten* flies were from Sally Leevers and *tsc1* flies from Nic Tapon. The *s6k, tsc2* stock was from D. J. Pan. The *Rheb* stocks were from Ernst Hafen. The UAS-4EBP stock was from Nahum Sonenberg. *pnt* stocks were from Christian Klämbt. The rho^{X81} stock was from Matthew Freeman. *eIF4E* (11720), aos^{W11} (2513), and *TOR* (7014) mutants were from The Bloomington Stock Center. Genotypes for generating clones were as follows:

tsc1, *Rheb* mutant clones: y, *w, hs-flp*; FRT82, $dRheb^{2D1}$, $tsc1^{2G3}$/FRT82B, Ubi-GFP.
tsc2 mutant clones: y, *w, hs-flp; gig*^{56}, FRT80/FRT80, Ubi-GFP.
tsc2 mutant clones with pntP2-LacZ: y, *w, hs-flp; gig*^{56}, FRT80, pnt^{1277}/FRT80, Ubi-GFP.
s6k mutant clones: *y, w, hs-flp; s6k*^{l1}, FRT80B/FRT80, P{LacW}RpL14, eGFP.
s6k, tsc2 mutant clones: y, *w, hs-flp; gig*^{192}, *s6k*^{l1}, FRT80/FRT80, Ubi-GFP.
eIF4E mutant clones: y, *w, hs-flp; eIF4E* ^{07238}, FRT80/FRT80, arm-LacZ or y, *w, hs-flp; eIF4E*$^{715/13}$, FRT80/FRT80, arm-LacZ.
eIF4E, tsc2 mutant clones: y, *w, hs-flp; eIF4E* ^{07238}, *gig*^{56}, FRT80/FRT80, P{LacW}RpL14, eGFP.
4EBP overexpression clones: y, *w, hs-flp;* UAS-4EBP/act>y>Gal4, UAS-GFP.
tsc1 mutant clones: y, *w, hs-flp; tsc1*^{Q87X}, FRT82B/FRT82B, Ubi-GFP.
Rheb mutant clones: *hs-flp;* $Rheb^{2D1}$, FRT82/82FRT, Ubi-GFP, M[95A], Rps63.
pten mutant clones: y, *w, hs-flp; pten*^{1}, FRT40/FRT40, Ubi-GFP.
pten mutant clones with aos-LacZ: y, *w, hs-flp; pten*^{1}, FRT40/FRT40, Ubi-GFP; aos^{W11}/+.
Rheb mutant clones with aos-LacZ: *hs-flp;* aos^{W11}, $Rheb^{2D1}$, FRT82/82FRT, Ubi-GFP, M[95A], Rps63.
pten mutant clones with rho-LacZ: y, *w, hs-flp; pten*^{1}, FRT40/FRT40, Ubi-GFP; rho^{X81}/+.
pten mutant clones with pntP2-LacZ: y, *w, hs-flp; pten*^{1}, FRT40/FRT40, Ubi-GFP; pnt^{1277}/+.
TOR mutant clones: *y, w, hs-flp;* $TOR^{\Delta P}$, FRT40A/FRT40, Ubi-GFP; pnt^{1277}/+.

pntP2 hypomorph clones: *y, w, hs-flp;* FRT82, pnt^{1230}/FRT8, Ubi-GFP.
Rheb, pntP2 mutant clones: *hs-flp;* pnt^{1230}, $Rheb^{2D1}$, FRT82/82FRT, Ubi-GFP, M[95A], Rps63.
UAS-Dp110, *pntP2* clones: hs-flp, UAS-GFP; UAS-Dp110; tub-Gal80, FRT82, pnt^{1230}/FRT82, tub-Gal80.
UAS-pntP2 clones: hs-flp; act>y>Gal4, UASGFP; UAS-pntP2.
$EGFR^{ACT}$ clones: hs-flp; act>y>Gal4, UASGFP; UAS-$EGFR^{ACT}$.

RESULTS

The InR controls differentiation through a pathway including TOR and S6K, but not 4EBP/eIF4E: We have shown previously that *tsc1* loss-of-function (LOF) clones cause precocious differentiation of PRs in the developing eye (Bateman and McNeill 2004). TSC1, together with TSC2, functions as a GAP for the small GTPase Rheb. We found that loss of *Rheb* causes a strong delay in differentiation suggesting that TSC1/2 acts upstream of Rheb in controlling differentiation as it does in growth (Saucedo *et al.* 2003; Zhang *et al.* 2003). However, TSC1 has targets other than Rheb and can activate RhoGTPase and inhibit Rac1 through interaction with the ERM family of actin binding proteins (Lamb *et al.* 2000; Astrinidis *et al.* 2002; Goncharova *et al.* 2004). Therefore we asked whether TSC1 is able to affect differentiation independently of Rheb. To do this we generated *Rheb, tsc1* double-mutant clones and observed the differentiation phenotype by staining with anti-Prospero (Xu *et al.* 2000). If Rheb is absolutely required for regulation of differentiation by TSC1 then *Rheb, tsc1* double-mutant clones should have a similar phenotype to *Rheb* clones. Alternatively, if the TSC1/2 complex is able to regulate differentiation independent of Rheb, then the delayed differentiation phenotype caused by loss of Rheb should be abrogated in *Rheb, tsc1* clones. *Rheb, tsc1* double-mutant clones show a strong delay in differentiation (Figure 1B), similar to that seen in *Rheb* clones (Figure 2, C and D). This result suggests that the primary target of TSC1/2 in controlling the timing of neuronal differentiation is Rheb.

The TSC1/2 complex and Rheb regulate TOR (Leevers and Hafen 2004). TOR is part of the TORC1 complex, controls growth by phosphorylation of S6K and 4EBP, which in turn affect translation and ribosome biogenesis by regulating RpS6 and eIF4E, respectively (Inoki and Guan 2006; Wullschleger *et al.* 2006). We asked whether S6K and 4EBP are also able to control neuronal differentiation. *s6k* LOF clones do cause a slight delay in differentiation (Figure 1C), which is much weaker than the delay seen in *Rheb* or *TOR* LOF clones (Figure 2, C and D; (Bateman and McNeill 2004). To determine whether S6K mediates the precocious differentiation phenotype seen in *tsc2* clones (Figure 1D) we generated *s6k, tsc2* double-mutant clones. These clones have a wild-type differentiation phenotype (Figure 1E), indicating that S6K acts either downstream or in parallel to TSC2 in controlling differentiation.

TOR also controls growth via the translation initiation factor eIF4E and its inhibitory binding partner 4EBP. Homozygous *eIF4E* Drosophila arrest growth during larval development (Lachance *et al.* 2002). Lachance *et al.* (2002) however did not determine whether *eIF4E* mutant cells have a growth defect. To assess this we made LOF clones of cells using either weak ($eIF4E^{07238}$) or strong ($eIF4E^{715/13}$) *eIF4E* alleles. Clones made using $eIF4E^{07238}$ had a mild but significant growth defect (mean clone size = 67% ± 1% size of twin spot, $n = 3$; supplemental Figure 1), while clones made using $eIF4E^{715/13}$ had a severe growth defect (Figure 1F, compare clone to twin-spot size; mean clone size = 8.7% ± 2% size of twin spot, $n = 3$). Control clones made using a wild-type FRT chromosome were a similar size to the twin spot (mean clone size = 98% ± 1% size of twin spot, $n = 3$) as expected. Surprisingly, neither $eIF4E^{07238}$ (supplemental Figure 1) nor $eIF4E^{715/13}$ LOF clones have any effect on differentiation in posterior (Figure 1F) or anterior clones close to the MF (Figure 1G). Also, $eIF4E^{07238}$, *tsc2* mutant clones have a similarly strong precocious differentiation phenotype to *tsc2* clones (supplemental Figure 1), further suggesting that *eIF4E* is not required for InR/TOR-dependent control of PR differentiation. We also analyzed the differentiation phenotype of the eIF4E inhibitory binding partner 4EBP. In accordance with our results with *eIF4E*, overexpression of *4EBP* also has no effect on differentiation (Figure 1H). In addition, we do not observe any differentiation phenotype in clones of wild-type cells generated in a background heterozygous for a ribosomal subunit dominant mutation (a *Minute* mutant; data not shown), confirming that alteration of the overall translation rate does not affect differentiation. Taken together these data suggest that the control of the timing of neuronal differentiation is regulated by S6K and is independent of 4EBP/eIF4E, while growth is controlled by both these factors.

InR/TOR signaling controls the timing of the differentiation of a subset of photoreceptors: Each ommatidium in the Drosophila eye consists of eight photoreceptor (PR) neurons and 12 accessory cells. We have shown that the InR/TOR pathway controls the timing of differentiation of PRs 1, 6, and 7 and cone cells, but does not affect PR 8 (Bateman and McNeill 2004). The differentiation of PR 8 is followed by the sequential differentiation of PRs 2/5, then PRs 3/4, and finally PRs 1, 6, and 7 (Figure 1A). To determine whether the differentiation of PRs 2–5 is also regulated by the InR/TOR pathway we used antibodies against the transcription factors Rough (Kimmel *et al.* 1990) and Spalt (Barrio *et al.* 1999) to analyze the differentiation of PRs 2/5 and 3/4, respectively. If InR/TOR signaling does regulate the differentiation of PRs 2–5 we would expect activation of the pathway by loss of *tsc1* to cause

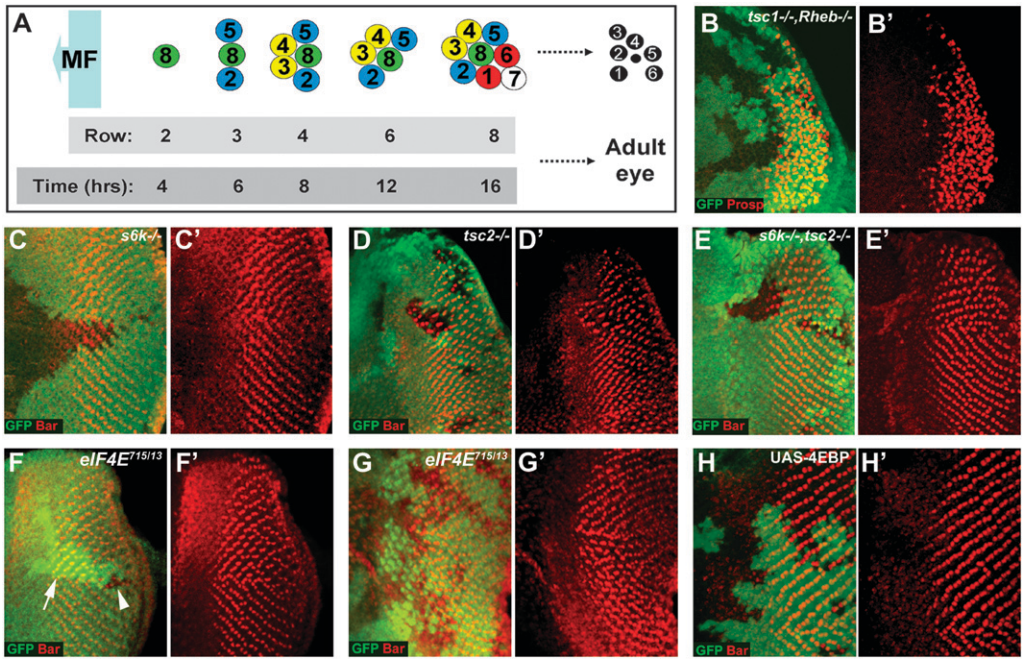

FIGURE 1.—InR and TOR signaling act through S6K, but not eIF4E to control the timing of neuronal differentiation. (A) Schematic showing the spatiotemporal nature of PR differentiation in the Drosophila eye imaginal disc. MF, morphogenetic furrow. (B and B′) $tsc1^{2G3}$, $Rheb^{2D1}$ double-mutant clones have an identical delay in differentiation (stained for Prospero expression, shown in red) to $Rheb^{2D1}$ clones (Figure 2C). (C and C′) Loss of *S6k* causes a slight delay in the differentiation of PR 7 and cone cells (stained for Bar expression, shown in red). (D and D′) *tsc2* (gig^{56}) clones cause precocious differentiation of PRs 1 and 6 (stained for Bar expression, shown in red). (E and E′) The precocious differentiation phenotype of *tsc2* cells is suppressed in *tsc2* (gig^{192}), $s6k^{11}$ clones (Bar staining in red). (F and G) $eIF4E^{715/13}$ LOF clones inhibit growth resulting in small clones, compare clone (arrowhead) to twin spot (arrow) size in F, but do not affect differentiation in posterior clones generated using hs-flp (F and F′) or clones close to the MF, generated using ey-flp (G and G′), (Bar staining in red). (H and H′) overexpression of 4EBP (shown by the presence of GFP staining) does not have any affect on differentiation of PRs 1 and 6 (stained for Bar expression, shown in red). LOF clones in B–G are marked by the loss of GFP (shown in green). Anterior is to the left in all panels.

precocious differentiation of these PRs. Both Rough and Spalt staining appeared normal within *tsc1* clones (Figure 2B and data not shown), suggesting that the InR/TOR pathway does not affect the timing of differentiation of PRs 2/5 or PRs 3/4.

We were concerned that since PRs 2/5 and 3/4 differentiate close to the morphogenetic furrow (rows 3 and 4, respectively, Figure 1A), that it might be difficult to resolve cells which are precociously differentiating. To overcome this issue we made *Rheb* LOF clones to determine whether there is any delay in the differentiation of PRs 2–5 when InR/TOR signaling is inhibited. Differentiation of PRs 1, 6, and 7 and cone cells is strongly delayed in *Rheb* clones (Figure 2, C and D and (BATEMAN and MCNEILL 2004), however, both Rough (PRs 2 and 5) and Spalt (PRs 3 and 4) staining is unaffected in these clones (Figure 2, E and F). Therefore temporal control of differentiation by the InR/TOR pathway in the developing eye is stage/cell type specific: the late differentiating PRs 1, 6, and 7 and cone cells are dependent on the InR/TOR pathway, while the early differentiating PRs 2–5 and 8 are independent of InR/TOR signaling.

Transcription of Argos, a reporter of EGFR signaling activity, is regulated by the InR/TOR pathway: The stage/cell-type-specific nature of the temporal control of differentiation suggests that the InR/TOR pathway achieves this regulation through a novel mechanism. To investigate this we asked whether any of the pathways known to be important for PR differentiation are affected by changes in InR/TOR signaling. Since the passage of the MF is unaffected by the InR/TOR pathway it seemed unlikely that Dpp, Hh, or Wingless signaling were being affected. Next we asked whether EGFR signaling is regulated by the InR/TOR pathway in the developing eye. We had previously analyzed EGFR signaling activity in two ways. First we stained with an antibody against dual phosphorylated MAPK (dpERK), which gives a direct readout of EGFR signaling levels (GABAY *et al.* 1997). Second we analyzed the level of the E26 transformation-specific sequence (ETS) protein Yan, whose accumulation in the nucleus is dependent on its phosporylation state and hence the level of EGFR activity (TOOTLE *et al.* 2003; SONG *et al.* 2005). Neither dpERK nor Yan staining are affected by activation of InR/TOR signaling (BATEMAN and MCNEILL 2004). However, we had not tested whether EGFR signaling is being affected downstream or in parallel to MAPK and Yan.

To test whether there is any overall activation of EGFR signaling by the InR/TOR pathway we looked at the expression of Argos. Argos is a secreted protein that functions as an inhibitory ligand of the EGFR (FREEMAN *et al.* 1992b). *argos* expression is induced by EGFR activation in differentiating cells and is thought to result in a feedback loop that inhibits the differentiation of surrounding cells (GOLEMBO *et al.* 1996). As a

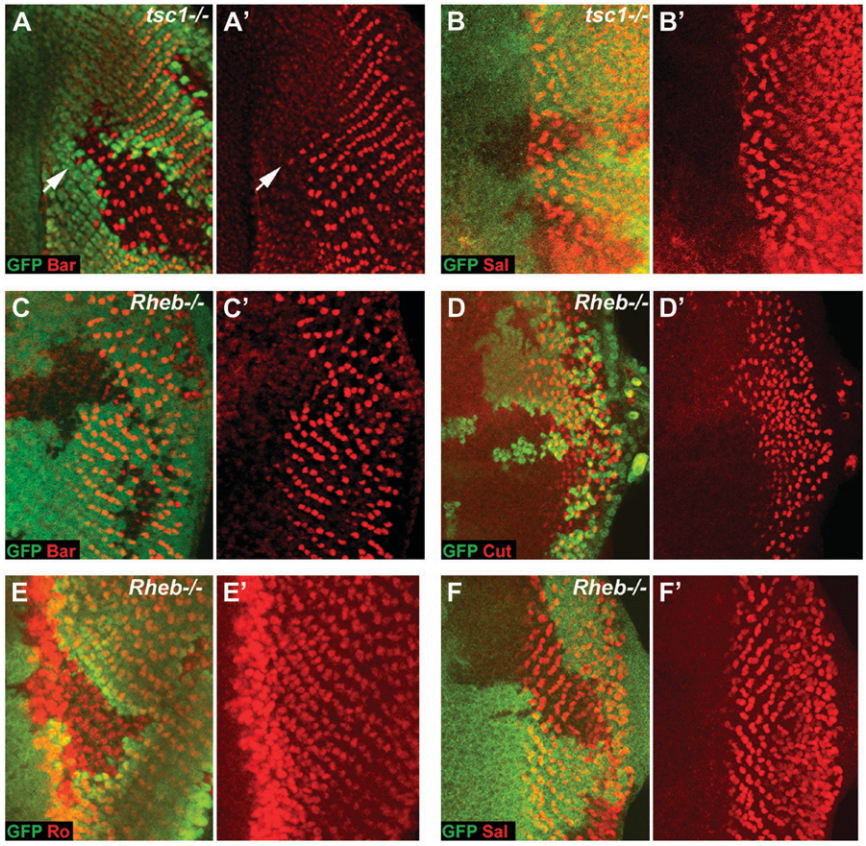

FIGURE 2.—InR/TOR signaling controls the differentiation of specific cell types in the developing eye. (A and A′) Cells mutant for *tsc1* (*tsc1*^{Q87X}) show precocious differentiation of PRs 1 and 6 (stained for Bar expression, shown in red) ahead of the wild-type differentiation front; arrow indicates an example of a precociously differentiated PR. (B and B′) Differentiation of PRs 3/4 (stained for Spalt expression, shown in red) is unaffected in *tsc1*^{Q87X} clones. (C and D) Differentiation of PRs 1 and 6 (stained for Bar expression, shown in red in C and C′) and cone cells (stained with Cut, shown in red in D and D′) is strongly delayed in *Rheb*^{2D1} clones. (E and F) PR 2/5 (stained for Rough expression, shown in red in E and E′) and PR 3/4 (stained for Spalt expression, shown in red in F and F′) differentiation is unaffected in *Rheb*^{2D1} clones. LOF clones in all panels are marked by the loss of GFP staining (shown in green). Anterior is to the left in all panels.

consequence of its dependence on EGFR activation Argos is strongly expressed in developing PRs as they differentiate (FREEMAN *et al.* 1992b). To analyze the expression of Argos in cells in which InR/TOR signaling is activated we stained *pten* LOF clones with an Argos monoclonal antibody. Although Argos stains poorly in imaginal discs we see a consistent increase in Argos accumulation in *pten* clones (Figure 3A).

Next we asked whether the ability of the InR/TOR pathway to modulate Argos levels is caused by changes in *argos* gene expression. This result would indicate that EGFR signaling is being affected, rather than a stabilization of Argos post-transcriptionally. To address this we used the *argos*^{W11} lacZ reporter line (FREEMAN *et al.* 1992a,b). Using *argos*^{W11} we observed a strong increase in *argos* expression in *pten* LOF clones (Figure 3B). Interestingly, in *pten* clones that cross the MF, strong precocious expression of *argos* is seen in the mutant cells (Figure 3B). To determine whether inhibition of the InR/TOR pathway can regulate *argos* expression we generated *Rheb* clones in larvae carrying the *argos*^{W11} allele. Loss of *Rheb* causes a strong decrease in *argos* expression in differentiating cells (Figure 3C). Thus both positive and negative regulators of the InR/TOR signaling pathway lead to alterations in *argos* expression.

Since Argos is also an inhibitory ligand of the EGFR (FREEMAN *et al.* 1992b), we analyzed the expression of *rhomboid-1* (*rho*) as an independent readout of EGFR activity. *rho* expression was monitored using the X81 enhancer trap line which is expressed strongly in PRs 2/5 and 8 (FREEMAN *et al.* 1992a). In accordance with the *argos* data, *rho* expression is upregulated in *pten* LOF clones (Figure 3D). These changes appear to be specific since the expression of several other cell fate genes is unaffected by changes in InR/TOR signaling (BATEMAN and MCNEILL 2004), including the Notch ligand Delta (supplemental Figure 2). In conclusion, these data suggest that there is crosstalk between InR/TOR signaling and the EGFR pathway and that this occurs downstream of MAPK.

Expression of pntP2 is regulated by InR/TOR signaling: *argos* expression is activated by the ETS transcription factor *pointed* (*pnt*). *pnt* is expressed as two alternatively spliced isoforms, P1 and P2, which share a C-terminal region that contains the ETS motif (SCHOLZ *et al.* 1993). *pntP2* is expressed specifically in the embryonic midline glial cells (KLAMBT 1993), and *argos* expression is lost in these cells in *pointed* (*pnt*) mutant embryos (SCHOLZ *et al.* 1997). Activation of the EGFR results in phosphorylation of MAPK, which enters the nucleus and phosphorylates pntP2 (BRUNNER *et al.* 1994; O'NEILL *et al.* 1994). In the eye imaginal disc *pntP2* is expressed in precursor cells posterior to the MF and in PRs 1, 6, and 7 and cone cells (BRUNNER *et al.* 1994). Since *argos* is a transcriptional target of pntP2 we wondered whether pntP2 expression might also be regulated by InR/TOR signaling. To test whether *pntP2* expression is regulated by InR/TOR signaling we used

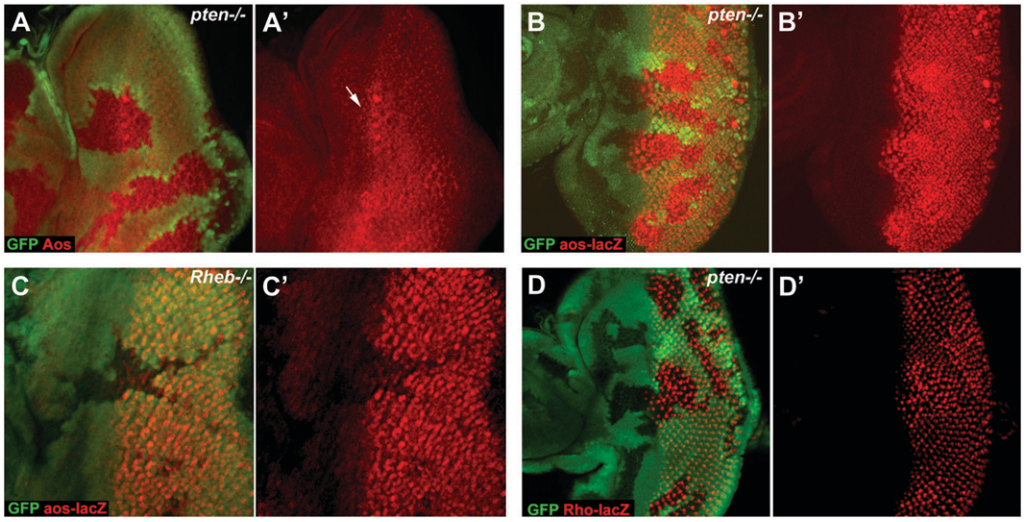

Figure 3.—*argos* and *rho* expression is regulated by InR/TOR signaling in developing neurons. (A and A′) The level of Argos protein (detected using an anti-Argos monoclonal antibody, shown in red) is increased in *pten*^{1} mutant cells (marked by loss of GFP staining). Note how Argos staining is seen ahead of the normal expression front (marked with an arrow). (B and C) *argos* expression is regulated by InR/TOR signaling at the level of transcription. Expression of β-galactosidase (stained with an anti-β-galactosidase antibody, shown in red) from the *P*{lwB}-element insertion in *argos* (*aos*^{w11}) is upregulated in *pten*^{1} clones (B and B′) and downregulated in *Rheb*^{2D1} clones (C and C′). (D and D′) *rho* expression (using the *rho*^{X81} reporter, detected by staining with a anti-β-galactosidase antibody, shown in red) is upregulated in *pten*^{1} clones. Clones are marked by loss of GFP staining and anterior is to the left in all panels.

the *pnt*^{1277} allele which contains a *P*{LacW} element within the first, noncoding exon of *pntP2* (Scholz *et al.* 1993). Using *pnt*^{1277} we observe a strong increase in *pntP2* expression in *pten* LOF clones (Figure 4A). Interestingly, the increase in *pntP2* expression differs spatiotemporally across the field of differentiating cells. *pntP2* expression is increased most strongly in cells as they differentiate, but this increase is lost once the cells become more mature. Moreover, dramatic precocious expression of *pntP2* is observed in *pten* clones that span the MF (Figure 4A). Importantly, *pntP2* expression is also upregulated in undifferentiated cells around the MF, suggesting that the increase in expression is not simply an indirect consequence of the precocious differentiation of PRs. We also observe a similar upregulation of *pntP2* expression in clones that have activated InR/TOR signaling due to loss of *tsc2* (Figure 4B). The increase in *pntP2* expression is not a result of a general increase in transcription due to increased growth, since we do not see increased expression of several other markers of PR cell fate (Bateman and McNeill 2004). To examine the effect of blocking InR/TOR signaling we examined *pntP2* expression in cells mutant for *TOR*. LOF clones of *TOR* show decreased expression of *pntP2* (Figure 4C). Therefore *pntP2* expression is sensitive to both activation and inhibition of InR/TOR signaling. To determine whether this property is specific to the eye we looked at *pntP2* expression in *pten* clones in the leg and eye discs. We did not observe any change in *pntP2* expression in these clones (supplemental Figure 3), suggesting either that InR/TOR regulation of *pntP2* is specific to the developing eye (perhaps requiring specific factors expressed close to the MF) or that the spatiotemporal nature of eye development in Drosophila makes it possible to observe changes that cannot be resolved in other imaginal discs.

Reducing EGFR signaling phenocopies loss of Rheb or TOR in developing PRs: *Argos*, *rho*, and *pntP2* expression levels are all regulated by InR/TOR signaling, suggesting crosstalk between InR/TOR and EGFR pathways. However, complete loss of EGFR or pntP2 activity (using null alleles) completely *blocks* the differentiation of all PRs except PR 8 (data not shown; (Baonza *et al.* 2001, 2002; Yang and Baker 2003), whereas inhibition of the InR/TOR pathway causes a *delay* only in the differentiation of PRs 1, 6, and 7 and cone cells (Figure 2). To reconcile these observations we wondered whether a reduction, rather than a complete loss in EGFR activity would cause the same cell-type-specific delay in differentiation as inhibition of the InR/TOR pathway.

To determine the affect of reducing EGFR signaling levels we used a hypomorphic allele of *pntP2* (*pntP2*^{1230}), which was generated by the imprecise excision of a *P* element in the first, noncoding exon of *pntP2* (Klambt 1993). We stained *pntP2*^{1230} clones with the same panel of markers that we had used to analyze the differentiation phenotype of InR/TOR pathway mutants (Figure 2). Interestingly the PR differentiation phenotype in *pntP2*^{1230} clones is identical to that in *Rheb* or *TOR* LOF clones (compare Figure 5 to Figure 2). Specifically, PR 8 (stained for Senseless expression; Figure 5A), PRs 2/5 (stained for Rough expression; Figure 5B), and PRs 3/4 (stained for Spalt expression; Figure 5C) differentiate normally in *pntP2*^{1230} clones. In contrast the differentiation of PRs 1 and 6 (stained for Bar expression; Figure 5D), PR 7 (stained for Prospero expression; Figure 5E), and cone cells (stained for Prospero expression; Figure 5E and Cut expression; Figure 5F) are strongly

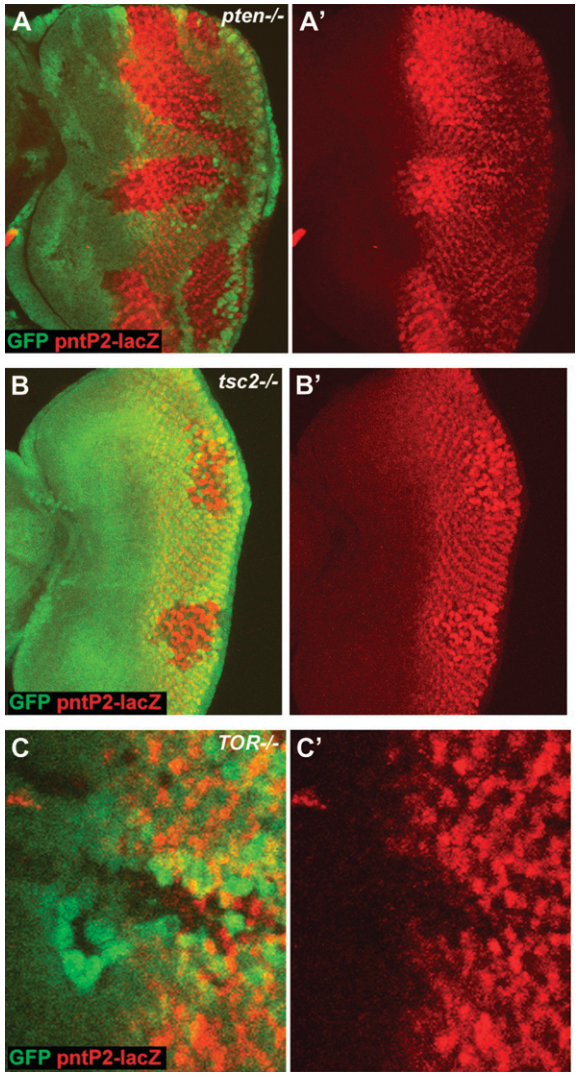

Figure 4.—pntP2 expression is regulated by InR/TOR signaling. (A and B) *pntP2* transcription, detected by staining for β-galactosidase in flies carrying a *P*{LacW} element in *pntP2* (*pnt*^{1277}), is upregulated and precocious in *pten*^{1} (A and A′) and *tsc2* (*gig*^{56}) clones (B and B′). Note that the disc shown in B is a younger disc and so *pntP2* is upregulated more posteriorly. Conversely *pntP2* transcription is downregulated in *TOR*$^{\Delta D}$ clones (C and C′). LOF clones in A–C are marked by the loss of GFP staining (shown in green). Anterior is to the left in all panels.

delayed but not completely blocked. The phenotypic similarity between PRs with reduced EGFR signaling and PRs in which InR/TOR signaling is inhibited is consistent with InR/TOR signaling modulating EGFR transcriptional outputs to control neuronal differentiation.

InR/TOR and EGFR signaling interact genetically: Since reducing EGFR pathway activity through pntP2 phenocopied inhibition of the InR/TOR pathway we wondered whether these two pathways could interact genetically. To test this we generated clones that were double mutant for *pntP2*^{1230} and *Rheb*^{2D1}. Inhibition of differentiation in these clones (Figure 6B) was much more severe than in *pntP2* (or *Rheb*) single mutant clones (Figure 6A). *pntP2*^{1230}, *Rheb*^{2D1} double-mutant clones block rather than delay the differentiation of PRs 1 and 6 (Figure 6B). Conversely, when we overexpressed Dp110 in *pnt*^{1230} clones using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo 1999), the delay in the differentiation of PRs 1 and 6 was much less severe (Figure 6D) than in *pnt*^{1230} clones alone (Figure 6C) and the precocious differentiation normally seen with Dp110 overexpression was completely suppressed, strongly suggesting that *pntP2* acts downstream of Dp110. These data demonstrate that the InR/TOR and EGFR pathways can interact genetically and are consistent with the regulation of neuronal differentiation by the InR/TOR through modulation of EGFR transcriptional output.

DISCUSSION

Tight coordination of growth and differentiation is essential for normal development. We have previously shown that InR/TOR signaling controls the timing of neuronal differentiation (Bateman and McNeill 2004) in the eye and leg in Drosophila. Here we demonstrate that the InR/TOR pathway regulates neuronal differentiation in an S6K-dependent, but 4EBP/eIF4E-independent manner. Previously we were unable to determine whether InR/TOR signaling was acting downstream or in parallel to the EGFR/MAPK pathway. Using *argos* and *rho* as reporters we have shown that the InR/TOR pathway is able to regulate EGFR/MAPK signaling downstream of MAPK. Moreover, *pntP2* expression is up- and downregulated by activation or inhibition of InR/TOR signaling, respectively, and InR/TOR and EGFR pathways interact through *pntP2*. Taken together our data suggest that temporal control of differentiation by the InR/TOR pathway is achieved by modulation of EGFR pathway transcriptional targets in differentiating PRs.

TOR is part of two multimeric complexes (TORC1 and TORC2) and is a core component of the InR pathway (Inoki and Guan 2006; Wullschleger *et al.* 2006). TORC1 activity is regulated by nutrient and energy levels (Hara *et al.* 1998; Inoki *et al.* 2003) providing a conduit for hormonal and catabolic cellular inputs. Growth is regulated by two downstream targets of TORC1: S6K and 4EBP. Our data demonstrate that upstream of TORC1, differentiation and growth are regulated by the same factors. Downstream of TORC1, differentiation and growth differ significantly in that loss of *s6k*, but not *eIF4E* (or overexpression of 4EBP) affects differentiation. eIF4E regulates 7-methyl-guanosine cap-dependent translation and is the rate-limiting factor in translation initiation (Richter and Sonenberg 2005). Our finding that eIF4E does not affect differentiation suggests that the temporal control of differentiation is not based on a translation initiation-dependent mechanism. Strikingly, we show that loss of *s6k* blocks

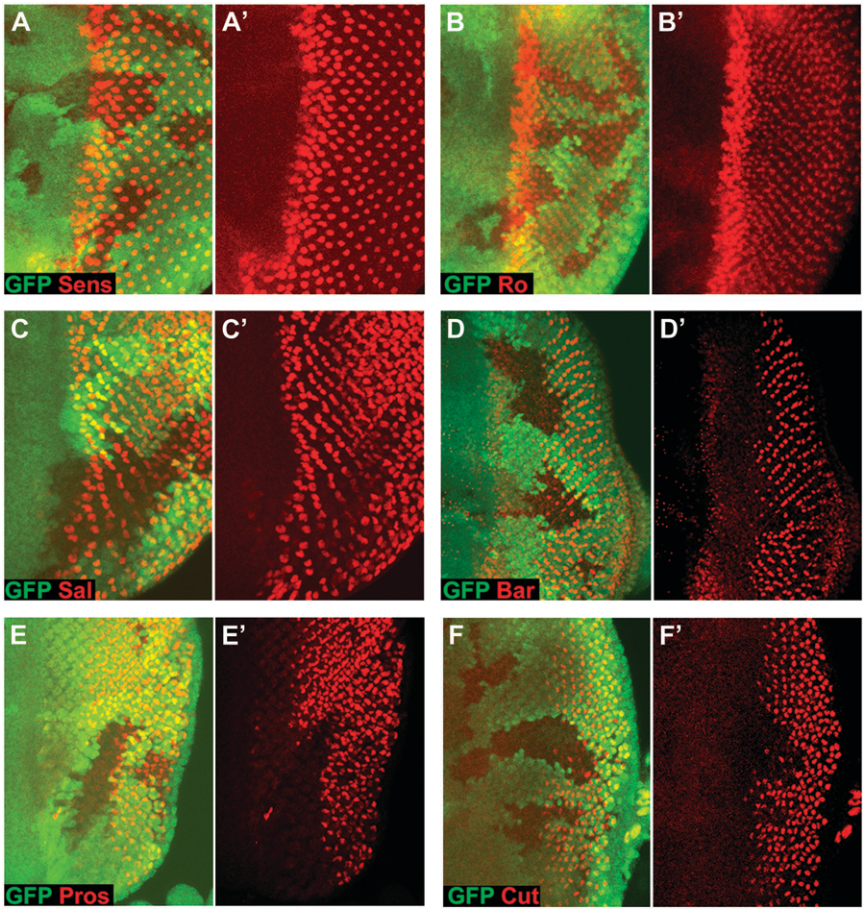

Figure 5.—Reducing EGFR signaling phenocopies the differentiation phenotype of loss of Rheb or TOR. pntP2 hypomorphic clones made using the allele $pntP2^{1230}$ show cell-type-specific delays in PR differentiation identical to those seen in LOF clones of positive effectors of InR/TOR signaling such as *Rheb* and *TOR*. (A–C) $pntP2^{1230}$ clones have no effect on the differentiation of PR 8 (stained for Senseless expression, shown in red in A and A′), PRs 2/5 (stained for Rough expression, shown in red in B and B′) or PRs 3/4 (stained for Spalt expression, shown in red in C and C′). Note that Spalt staining shows a delay toward the posterior of the disc where the antibody also stains PRs 1 and 6. In contrast $pntP2^{1230}$ clones show a strong delay in the differentiation of PRs 1 and 6 (stained for Bar expression, shown in red in D and D′), PR 7 (stained for Prospero expression, which is also expressed in cone cells, shown in red in E and E′) and cone cells (stained for Cut expression, shown in red in F and F′ and Prospero expression, shown in red in E and E′). LOF clones in all panels are marked by the loss of GFP staining (shown in green). Anterior is to the left in all panels.

the precocious differentiation induced by loss of *tsc2*. Given the relatively weak effects of loss of *s6k* this may seem surprising. However, the degree of suppression is similar to the effect of loss of *s6k* on the overgrowth phenotype caused by loss of *tsc2*, namely, *tsc2, s6k* double-mutant cells are the same size as wild-type cells (Gao *et al.* 2002). Although loss of *eIF4E* has no affect on differentiation it may act redundantly with another factor, such as *s6k*. Testing this hypothesis though is technically challenging since the Drosophila genome contains eight different *eIF4E* isoforms (Hernandez *et al.* 2005). It will be interesting in future to test whether any of these isoforms regulate differentiation or alternatively whether *eIF4E* and *s6k* act redundantly. Although further work is required to determine the precise relationship between S6K and the InR/TOR

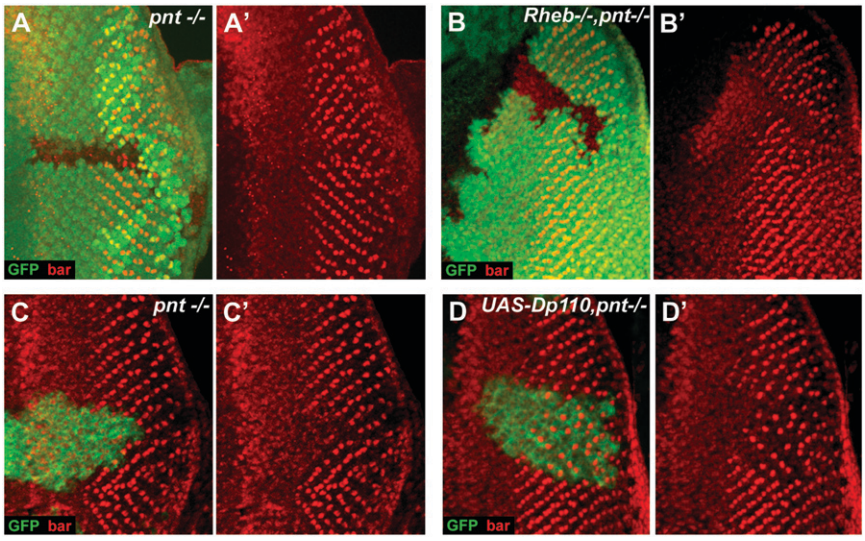

Figure 6.—InR/TOR and EGFR signaling interact genetically. (A and B) Differentiation of PRs 1 and 6 is delayed in pnt^{1230} clones (A and A′) and blocked in pnt^{1230}, $Rheb^{2D1}$ clones (B and B′). (C and D) Using the MARCM system differentiation of PRs 1 and 6 is delayed in pnt^{1230} clones (C and C′), whereas the delay is significantly weaker in pnt^{1230} clones overexpressing Dp110 (D and D′). Mutant cells are marked by the absence of GFP in A and B, but by the presence of GFP in C and D. PRs 1 and 6 are shown by Bar staining (red) in all panels.

pathway, our data point to a critical role of S6K in coordinating neuronal differentiation and growth.

As in other neuronal systems, differentiation of PRs in the Drosophila eye occurs in a stereotyped manner. The advantage of the Drosophila retina as an experimental system is that the PRs differentiate spatiotemporally. Using this feature, as well as a series of cell-type-specific antibodies, we have demonstrated that InR/TOR signaling is selective in the cell-types that it affects. The differentiation of PRs 2/5, 3/4, and 8 are unaffected by perturbations in InR/TOR signaling, whereas PRs 1, 6, and 7 and cone cells are dependent on this pathway for temporal control of differentiation. Interestingly the affected cells all differentiate after the second mitotic wave. However, we have shown that regulators of the cell cycle do not affect the temporal control of differentiation (Bateman and McNeill 2004). Why then are PRs 1, 6, and 7 and cone cells specifically affected? In cells with increased InR/TOR signaling, the expression of *argos*, *rho*, and *pntP2* is precocious and increased throughout the clone, suggesting that the upregulation of EGFR signaling occurs in all cells. However, decreasing EGFR activity using a hypomorphic *pntP2* allele specifically affects the differentiation of PRs 1, 6, and 7 and cone cells. Interestingly, *pntP2* expression in differentiated cells is also restricted to PRs 1, 6, and 7 and cone cells. These observations suggest that differentiation of PRs 1, 6, and 7 and cone cells is critically dependent on EGFR levels signaling through *pntP2*. Therefore, although activation of InR/TOR signaling causes upregulation of EGFR transcriptional targets in all cells as they differentiate, the phenotypic effect is only seen in PRs 1, 6, and 7 and cone cells since these cells are highly sensitive to EGFR activity signaling through *pntP2*. This possibility is supported by the fact that precocious differentiation caused by overexpression of Dp110 can be suppressed by the simultaneous reduction of *pntP2* levels (Figure 6). The complete suppression of the Dp110 differentiation phenotype by simultaneous reduction of *pntP2* strongly suggests that *pntP2* acts downstream of Dp110 and InR/TOR signaling in a pathway that regulates the temporal control of differentiation. It has been suggested that later differentiating PRs require higher levels of EGFR activity than their earlier differentiating neighbors. In particular, the activation of PR 7 requires both EGFR and Sevenless RTKs (Freeman 1996). In the case of InR/TOR pathway activation it may be that, through its regulation of EGFR downstream targets, the "second burst" of RTK activity is enhanced causing PRs 1, 6, and 7 and cone cells to differentiate precociously. There may also be other as yet unidentified factors through which the InR/TOR pathway controls the expression of Aos and rho in PRs 2–5 and 8.

Activation of insulin and insulin-like growth factor receptors in mammalian systems is well known to elicit a response via the Ras/MAPK pathway (Baltensperger *et al.* 1993; Skolnik *et al.* 1993; Downward 2003;). However, loss of the *InR* in the Drosophila eye does not result in a loss of PRs, a hallmark of the Ras pathway (Brogiolo *et al.* 2001), nor does mutation of the putative Drk binding site in *chico* affect the function of the Drosophila IRS (Oldham *et al.* 2002). In accordance with these data we do not observe any change in dpERK staining when the InR/TOR pathway is activated in the eye disc. Rather than a direct activation of Ras signaling by the InR, our data suggest that in the developing eye crosstalk between these pathways occurs at the level of regulation of the expression of EGFR transcriptional outputs. The most proximal component of the EGFR pathway that is regulated by InR/TOR signaling is *pntP2*. However, our data suggest that temporal control of PR differentiation requires concerted regulation of EGFR transcriptional outputs, since overexpression of *pntP2* alone is not sufficient to cause precocious differentiation, whereas overexpression of activated EGFR is sufficient (supplemental Figure 4). Interestingly, microarray analyses of Drosophila and human cells have shown that the InR/TOR pathway regulates the expression of hundreds of genes (Peng *et al.* 2002; Guertin *et al.* 2006a). The mechanism by which this transcriptional control is exerted has yet to be elucidated. It will be interesting in future to determine the extent of transcriptional crosstalk between InR/TOR and EGFR pathways in developing neurons.

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LITERATURE CITED

Astrinidis, A., T. P. Cash, D. S. Hunter, C. L. Walker, J. Chernoff *et al.*, 2002 Tuberin, the tuberous sclerosis complex 2 tumor suppressor gene product, regulates Rho activation, cell adhesion and migration. Oncogene **21:** 8470–8476.

Baltensperger, K., L. M. Kozma, A. D. Cherniack, J. K. Klarlund, A. Chawla *et al.*, 1993 Binding of the Ras activator son of sevenless to insulin receptor substrate-1 signaling complexes. Science **260:** 1950–1952.

Baonza, A., T. Casci and M. Freeman, 2001 A primary role for the epidermal growth factor receptor in ommatidial spacing in the Drosophila eye. Curr. Biol. **11:** 396–404.

Baonza, A., C. M. Murawsky, A. A. Travers and M. Freeman, 2002 Pointed and Tramtrack69 establish an EGFR-dependent transcriptional switch to regulate mitosis. Nat. Cell Biol. **4:** 976–980.

Barrio, R., J. F. de Celis, S. Bolshakov and F. C. Kafatos, 1999 Identification of regulatory regions driving the expression of the Drosophila spalt complex at different developmental stages. Dev. Biol. **215:** 33–47.

Bateman, J. M., and H. McNeill, 2004 Temporal control of differentiation by the insulin receptor/tor pathway in Drosophila. Cell **119:** 87–96.

Bateman, J. M., and H. McNeill, 2006 Insulin/IGF signalling in neurogenesis. Cell. Mol. Life Sci. **63:** 1701–1705.

Brennan, C. A., and K. Moses, 2000 Determination of Drosophila photoreceptors: timing is everything. Cell. Mol. Life Sci. **57:** 195–214.

Britton, J. S., W. K. Lockwood, L. Li, S. M. Cohen and B. A. Edgar, 2002 Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. Dev. Cell **2:** 239–249.

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

Brunner, D., K. Ducker, N. Oellers, E. Hafen, H. Scholz *et al.*, 1994 The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. Nature **370:** 386–389.

Cai, S.-L., A. R. Tee, J. D. Short, J. M. Bergeron, K. Kim *et al.*, 2006 Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J. Cell Biol. **173:** 279–289.

Downward, J., 2003 Targeting RAS signalling pathways in cancer therapy. Nat. Rev. Cancer **3:** 11–22.

Freeman, M., 1996 Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. Cell **87:** 651–660.

Freeman, M., 1997 Cell determination strategies in the Drosophila eye. Development **124:** 261–270.

Freeman, M., B. E. Kimmel and G. M. Rubin, 1992a Identifying targets of the rough homeobox gene of Drosophila: evidence that rhomboid functions in eye development. Development **116:** 335–346.

Freeman, M., C. Klambt, C. S. Goodman and G. M. Rubin, 1992b The argos gene encodes a diffusible factor that regulates cell fate decisions in the Drosophila eye. Cell **69:** 963–975.

Gabay, L., R. Seger and B. Z. Shilo, 1997 In situ activation pattern of Drosophila EGF receptor pathway during development. Science **277:** 1103–1106.

Gao, X., and D. Pan, 2001 TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev. **15:** 1383–1392.

Gao, X., Y. Zhang, P. Arrazola, O. Hino, T. Kobayashi *et al.*, 2002 Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat. Cell Biol. **4:** 699–704.

Golembo, M., R. Schweitzer, M. Freeman and B. Z. Shilo, 1996 Argos transcription is induced by the Drosophila EGF receptor pathway to form an inhibitory feedback loop. Development **122:** 223–230.

Goncharova, E., D. Goncharov, D. Noonan and V. P. Krymskaya, 2004 TSC2 modulates actin cytoskeleton and focal adhesion through TSC1-binding domain and the Rac1 GTPase. J. Cell Biol. **167:** 1171–1182.

Guertin, D. A., K. V. Guntur, G. W. Bell, C. C. Thoreen and D. M. Sabatini, 2006a Functional genomics identifies TOR-regulated genes that control growth and division. Curr. Biol. **16:** 958–970.

Guertin, D. A., D. M. Stevens, C. C. Thoreen, A. A. Burds, N. Y. Kalaany *et al.*, 2006b Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev. Cell **11:** 859–871.

Hara, K., K. Yonezawa, Q. P. Weng, M. T. Kozlowski, C. Belham *et al.*, 1998 Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J. Biol. Chem. **273:** 14484–14494.

Harrington, L. S., G. M. Findlay, A. Gray, T. Tolkacheva, S. Wigfield *et al.*, 2004 The TSC1–2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J. Cell Biol. **166:** 213–223.

Hernandez, G., M. Altmann, J. M. Sierra, H. Urlaub, R. Diez del Corral *et al.*, 2005 Functional analysis of seven genes encoding eight translation initiation factor 4E (eIF4E) isoforms in Drosophila. Mech. Dev. **122:** 529–543.

Inoki, K., and K. L. Guan, 2006 Complexity of the TOR signaling network. Trends Cell Biol. **16:** 206–212.

Inoki, K., T. Zhu and K. L. Guan, 2003 TSC2 mediates cellular energy response to control cell growth and survival. Cell **115:** 577–590.

Kimmel, B. E., U. Heberlein and G. M. Rubin, 1990 The homeo domain protein rough is expressed in a subset of cells in the developing Drosophila eye where it can specify photoreceptor cell subtype. Genes Dev. **4:** 712–727.

Klambt, C., 1993 The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development **117:** 163–176.

Lachance, P. E., M. Miron, B. Raught, N. Sonenberg and P. Lasko, 2002 Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth. Mol. Cell. Biol. **22:** 1656–1663.

Lamb, R. F., C. Roy, T. J. Diefenbach, H. V. Vinters, M. W. Johnson *et al.*, 2000 The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho. Nat. Cell Biol. **2:** 281–287.

Lee, T., and L. Luo, 1999 Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron **22:** 451–461.

Leevers, S. J., and E. Hafen, 2004 Growth regulation by insulin and TOR signalling in Drosophila, pp. 167–192 in *Cell Growth*, edited by M. N. Hall, R. Raff and G. Thomas. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Long, X., Y. Lin, S. Ortiz-Vega, K. Yonezawa and J. Avruch, 2005 Rheb binds and regulates the mTOR kinase. Curr. Biol. **15:** 702–713.

Neufeld, T. P., A. F. de la Cruz, L. A. Johnston and B. A. Edgar, 1998 Coordination of growth and cell division in the Drosophila wing. Cell **93:** 1183–1193.

O'Neill, E. M., I. Rebay, R. Tjian and G. M. Rubin, 1994 The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell **78:** 137–147.

Oldham, S., H. Stocker, M. Laffargue, F. Wittwer, M. Wymann *et al.*, 2002 The Drosophila insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. Development **129:** 4103–4109.

Peng, T., T. R. Golub and D. M. Sabatini, 2002 The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. Mol. Cell. Biol. **22:** 5575–5584.

Potter, C. J., H. Huang and T. Xu, 2001 Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell **105:** 357–368.

Radimerski, T., J. Montagne, M. Hemmings-Mieszczak and G. Thomas, 2002 Lethality of Drosophila lacking TSC tumor suppressor function rescued by reducing dS6K signaling. Genes Dev. **16:** 2627–2632.

Richter, J. D., and N. Sonenberg, 2005 Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature **433:** 477–480.

Rusten, T. E., K. Lindmo, G. Juhasz, M. Sass, P. O. Seglen *et al.*, 2004 Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. Dev. Cell **7:** 179–192.

Sarbassov, D. D., D. A. Guertin, S. M. Ali and D. M. Sabatini, 2005 Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science **307:** 1098–1101.

Saucedo, L. J., X. Gao, D. A. Chiarelli, L. Li, D. Pan *et al.*, 2003 Rheb promotes cell growth as a component of the insulin/TOR signalling network. Nat. Cell Biol. **5:** 566–571.

Scholz, H., J. Deatrick, A. Klaes and C. Klambt, 1993 Genetic dissection of pointed, a Drosophila gene encoding two ETS-related proteins. Genetics **135:** 455–468.

Scholz, H., E. Sadlowski, A. Klaes and C. Klambt, 1997 Control of midline glia development in the embryonic Drosophila CNS. Mech. Dev. **64:** 137–151.

Scott, R. C., O. Schuldiner and T. P. Neufeld, 2004 Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev. Cell **7:** 167–178.

Shah, O. J., Z. Wang and T. Hunter, 2004 Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr. Biol. **14:** 1650–1656.

Skolnik, E. Y., A. Batzer, N. Li, C. H. Lee, E. Lowenstein *et al.*, 1993 The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science **260:** 1953–1955.

Song, H., M. Nie, F. Qiao, J. U. Bowie and A. J. Courey, 2005 Antagonistic regulation of Yan nuclear export by Mae and Crm1 may increase the stringency of the Ras response. Genes Dev. **19:** 1767–1772.

Tapon, N., N. Ito, B. J. Dickson, J. E. Treisman and I. K. Hariharan, 2001 The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell **105:** 345–355.

Tootle, T. L., P. S. Lee and I. Rebay, 2003 CRM1-mediated nuclear export and regulated activity of the receptor tyrosine kinase antagonist YAN require specific interactions with MAE. Development **130:** 845–857.

Um, S. H., F. Frigerio, M. Watanabe, F. Picard, M. Joaquin *et al.*, 2004 Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature **431:** 200–205.

Voas, M. G., and I. Rebay, 2004 Signal integration during development: insights from the Drosophila eye. Dev. Dyn. **229:** 162–175.

Wolff, T., and D. Ready, 1993 Pattern formation in the Drosophila retina, pp. 1277–1326 in *The Development of Drosophila melanogaster*, edited by M. Bates and A. Martinez-Arias. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wullschleger, S., R. Loewith and M. N. Hall, 2006 TOR signaling in growth and metabolism. Cell **124:** 471–484.

Xu, C., R. C. Kauffmann, J. Zhang, S. Kladny and R. W. Carthew, 2000 Overlapping activators and repressors delimit transcriptional response to receptor tyrosine kinase signals in the Drosophila eye. Cell **103:** 87–97.

Yang, L., and N. E. Baker, 2003 Cell cycle withdrawal, progression, and cell survival regulation by EGFR and its effectors in the differentiating Drosophila eye. Dev. Cell **4:** 359–369.

Zhang, Y., X. Gao, L. J. Saucedo, B. Ru, B. A. Edgar *et al.*, 2003 Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat. Cell Biol. **5:** 578–581.

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