

FOXO-regulated transcription restricts overgrowth of *Tsc* mutant organs

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FOXO is thought to function as a repressor of growth that is, in turn, inhibited by insulin signaling. However, inactivating mutations in *Drosophila melanogaster* FOXO result in viable flies of normal size, which raises a question over the involvement of FOXO in growth regulation. Previously, a growth-suppressive role for FOXO under conditions of increased target of rapamycin (TOR) pathway activity was described. Here, we further

characterize this phenomenon. We show that *tuberous sclerosis complex 1* mutations cause increased FOXO levels, resulting in elevated expression of FOXO-regulated genes, some of which are known to antagonize growth-promoting pathways. Analogous transcriptional changes are observed in mammalian cells, which implies that FOXO attenuates TOR-driven growth in diverse species.

Introduction

The target of rapamycin (TOR) and insulin signaling pathways control cell growth, proliferation, and metabolism throughout organism development and adult homeostasis. The TOR pathway is an ancient signaling network conserved from yeast to humans that responds to environmental stimuli, such as nutrient and oxygen availability, as well as growth factor signaling (for reviews see Oldham and Hafen, 2003; Wullschleger et al., 2006). The insulin pathway evolved in metazoans to enable dynamic control of cell growth, proliferation, and metabolism in a systemic fashion. Multiple points of crosstalk exist between the insulin and TOR pathways, which ensure optimal activity of each pathway and hence allow adjustment to dynamic environmental and dietary conditions. For example, in response to hyperactivation of the TOR pathway by conditions such as high levels of amino acids, glucose, or free fatty acids or, genetically, by mutation of *tuberous sclerosis complex (Tsc)1* or *Tsc2*, insulin signaling is suppressed (for review see Manning, 2004). Altered activity of multiple components of the insulin and TOR pathways, such as

phosphoinositide 3-kinase, Akt, PTEN, TSC1, and TSC2 contribute to an array of human cancers (for reviews see Luo et al., 2003; Inoki et al., 2005).

Akt is an important insulin pathway protein that promotes cell growth, proliferation, and survival by phosphorylating multiple effector proteins, including the TOR pathway inhibitor TSC2 and FOXO family transcription factors (for reviews see Manning, 2004; Greer and Brunet, 2005; Wullschleger et al., 2006). FOXO transcription factors have well-defined roles in insulin-dependent control of longevity and metabolism as well as stress resistance (for reviews see Greer and Brunet, 2005; Kenyon, 2005). These proteins have also been implicated in insulin-independent control of tissue growth but their role in this process is more controversial. FOXO proteins have been shown to restrict tumor formation in mice (Paik et al., 2007). Surprisingly, *Drosophila melanogaster* mutants for the gene encoding the sole FOXO family transcription factor develop normally and are of normal size, which brings into question the role of FOXO in the developmental regulation of growth under normal circumstances (Junger et al., 2003).

FOXO has been shown to attenuate growth of *D. melanogaster* tissues with elevated TOR pathway activity (Junger et al., 2003). Here, we further characterize this phenomenon by showing that *Tsc1* mutations cause increased FOXO levels, resulting in elevated expression of FOXO-regulated genes, some of which

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Abbreviations used in this paper: chrb, charybdis; FRE, FOXO recognition element; FRT, flipase recognition target; GMR, glass multiple reporter; GOF, gain of function; HIF-1, hypoxia-inducible factor-1; hsp, heat shock protein; LOF, loss of function; MEF, mouse embryonic fibroblast; QPCR, quantitative real-time PCR; scy, scylla; tgo, tango; TOR, target of rapamycin; TSC, tuberous sclerosis complex.

The online version of this paper contains supplemental material.

Supplemental Material can be found at:
<http://jcb.rupress.org/content/suppl/2008/02/25/jcb.200710100.DC1.html>

are known to antagonize growth-promoting pathways. In addition we show that this FOXO-dependent transcriptional response is conserved in mammals.

Results and discussion

Genes encoding growth inhibitors are elevated in *Tsc1* tissue

To investigate mechanisms by which the TOR pathway controls tissue growth, we analyzed transcriptional profiles of tissue lacking *Tsc1*, which leads to hyperactivation of the TOR pathway and excessive growth (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). Eye-antennal imaginal discs from third instar *D. melanogaster* larvae were generated that were composed almost entirely of tissue derived from one of two different genotypes: *Tsc1* or wild-type isogenic control. Three biologically independent first strand cDNA samples from each genotype were hybridized to Affymetrix microarray chips. Expression levels of 157 genes were elevated 1.5-fold or more, whereas 211 genes were repressed 1.5-fold or more ($P < 0.05$) when compared with control tissue. These genes have been implicated in diverse cellular functions including metabolism, membrane transport, stress response, cell growth, and cell structure (Figs. 1 and S1 and Tables S1 and S2, available at <http://www.jcb.org/cgi/content/full/jcb.200710100/DC1>).

Observed transcriptional changes were validated for several genes using *D. melanogaster* gene-enhancer trap lines (Fig. 1, B–E), and quantitative real-time PCR (QPCR; Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200710100/DC1>). The UAS–Gal4 system was used to activate the TOR pathway in a specific tissue domain by driving expression of *Rheb* under the control of the *glass multiple reporter* (*GMR*) promoter. Induction of *astray* (*aay*) and *4E-BP* (both of which were found to be elevated in *Tsc1* tissue by microarray analysis) were observed in the *GMR* expression domain (posterior to the morphogenetic furrow) when *Rheb* was misexpressed but were not induced when the negative control *Gal4* gene was misexpressed (Fig. 1, B–E). QPCR was also used to confirm expression changes observed in *Tsc1* tissue for *charybdis* (*chr*), *scylla* (*scy*), *phosphoenolpyruvate carboxy kinase*, *4E-BP*, and *aay* (Fig. S2).

Intriguingly, several gene products whose expression was elevated in *Tsc1* tissue have been implicated in tissue growth controlled by the insulin and TOR pathways, including 4E-BP, Chrb, and Scy (Miron et al., 2001; Reiling and Hafen, 2004). 4E-BP is a repressor of cap-dependent translation. Upon phosphorylation by TOR, 4E-BP dissociates from eIF4e, allowing assembly of the initiation complex at the mRNA cap structure, ribosome recruitment, and subsequent translation (for review see Wullschleger et al., 2006). Scy and Chrb, and their mammalian orthologues REDD1 and REDD2, inhibit insulin and TOR signaling in response to hypoxia and energy stress and restrict growth during *D. melanogaster* development (Brugarolas et al., 2004; Reiling and Hafen, 2004; Sofer et al., 2005). Our finding that inhibitors of growth are highly expressed in *Tsc1* tissue led us to hypothesize that such genes are transcriptionally induced as part of a feedback loop that restricts tissue growth under conditions of excessive TOR activity. Feedback loops are an

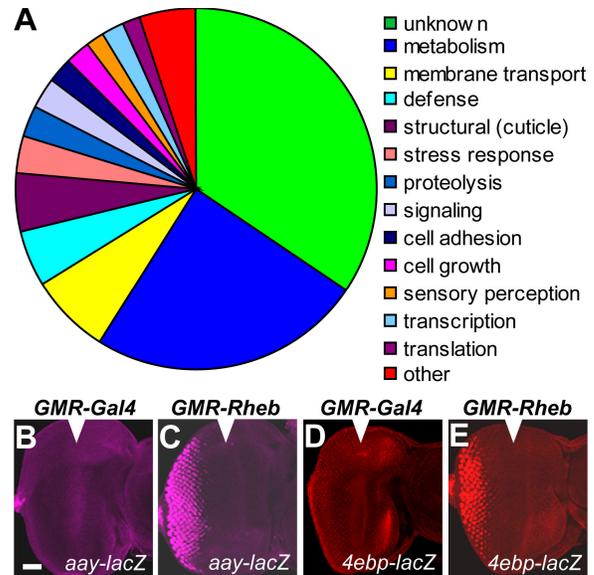


Figure 1. Genes elevated in developing *Tsc1* eye-antennal tissue. (A) Transcriptional changes observed in *Tsc1* eye-antennal imaginal discs grouped by proposed biological function. Included are genes whose expression was elevated at least 1.5-fold ($P < 0.05$) in *Tsc1* tissue compared with the wild type. (B–E) Third instar larval eye imaginal discs; anterior is shown on the right. Arrowheads indicate the morphogenetic furrow. Activity of *aay* (B and C) and *4e-bp* (D and E) enhancer trap lines in tissue misexpressing *Gal4* (B and D) or *Rheb* (C and E) under control of the *GMR* promoter. Bar, 50 μ M.

important activity-modulating feature of many signaling pathways, including the TOR and insulin pathways (for review see Manning, 2004).

Tsc1 loss-of-function (LOF) and FOXO gain-of-function (GOF) transcription profiles are overlapping

To examine the mechanism whereby transcription of growth inhibitors is induced in response to TOR hyperactivation, we sought to determine which transcription factors were responsible for their expression. One obvious candidate was FOXO, a member of the forkhead transcription factor family, which has a well-established role as an effector of insulin signaling (for review see Greer and Brunet, 2005). If FOXO has a role in inducing expression of negative regulators of growth in *Tsc* tissue, then expression of some of those genes should be elevated under conditions of increased FOXO activity. To investigate this hypothesis, we compared the expression profiles of *Tsc1* LOF tissue and *D. melanogaster* S2 cells expressing FOXOA3, a mutant version of FOXO that is insensitive to phosphorylation-dependent inhibition by Akt (*FOXO* GOF; Puig et al., 2003; Gershman et al., 2007). This analysis revealed that 25 genes were up-regulated 1.5-fold or greater in both *Tsc1* LOF and *FOXO* GOF expression profiles, which represents a highly significant degree of overlap ($P = 4.9 \times 10^{-009}$) as determined by calculation of the hypergeometric distribution. This highly statistically significant P value strongly suggests that there is a functional overlap between these two datasets that cannot be explained by random variation. Genes that were elevated at least 1.5-fold ($P < 0.05$) in both *Tsc1* LOF and *FOXO* GOF cells and that have

Gene	Tsc LOF	Foxo GOF	Biological Process
pepck	10.5	2.2	gluconeogenesis
aay	9.3	26.1	axon guidance
Prx2450-2	6.2	8.5	oxygen metabolism
scylla	5.4	5.6	growth inhibition
Hsp67Bc	3.3	3.3	stress response
ImpL2	3	4.6	cell adhesion
Esp	2.8	5.1	metabolism/transport
Hsp22	2.6	13.5	stress response
Hsp23	2.6	4.9	stress response
4e-bp	1.8	25	growth inhibition
men	1.8	6.8	citric acid cycle
Adk3	1.6	5.1	metabolism
Ald	1.6	2.4	glycolysis

Figure 2. **Common transcriptional changes in *Tsc1* tissue and cells misexpressing FOXO.** Genes elevated at least 1.5-fold ($P < 0.05$) in both *Tsc1* eye-antennal disc tissue and S2 cells misexpressing activated FOXO. Genes with a proposed biological function are listed as well as their expression level as determined by microarray analysis in each experimental condition.

been assigned to certain cellular functions are shown in Fig. 2. Interestingly, two genes previously implicated in tissue growth regulated by the insulin and TOR pathways *4E-BP* and *scy* were elevated in both microarray experiments, whereas the *chrB* growth-inhibiting gene was not (Fig. 2). Thus, a subset of genes elevated in *Tsc1* tissue appears to respond to FOXO activity and was investigated further.

FOXO activates transcription of genes whose expression is elevated in *Tsc1* tissue

4E-BP is a well-characterized FOXO target gene (Junger et al., 2003; Puig et al., 2003). To determine whether FOXO could directly activate transcription of genes that were elevated in *Tsc1* tissue other than *4E-BP*, we focused on *scy* and the phosphoserine phosphatase *aay* (one of the most highly elevated transcripts in each microarray experiment). *scy* and *aay* both possess consensus FOXO recognition elements (FREs) in their promoters comparable to those found in *dInR* and *4E-BP* promoters (Furuyama et al., 2000; Puig et al., 2003). Therefore, we examined whether these genes are bona fide FOXO targets by measuring their expression in *D. melanogaster* S2 cells misexpressing FOXOA3 in the presence of insulin (Puig et al., 2003). *aay* and *scy* mRNAs were up-regulated 19.4- and 4.3-fold, respectively, relative to a control gene, *actin*, as determined by QPCR (Fig. 3 B). Next, we used luciferase reporter assays in S2 cells to determine whether the *aay* promoter region containing putative FREs was sensitive to FOXO activity. As shown in Fig. 3 C, luciferase activity dependent on the *aay* promoter was strongly induced by FOXOA3 relative to a negative control. In addition, using in vitro band shift assays, we demonstrated that FOXO directly bound to the *aay* promoter (Fig. 3 D), which indicates that FOXO likely activates expression of *aay* by directly binding to the FRE. Surprisingly, in parallel luciferase reporter assays, we could not demonstrate activation of the *scy* promoter by FOXO, despite the fact that we observed strong binding of FOXO to the putative *scy* FRE using in vitro band-shift assays (Fig. 3 E). A possible

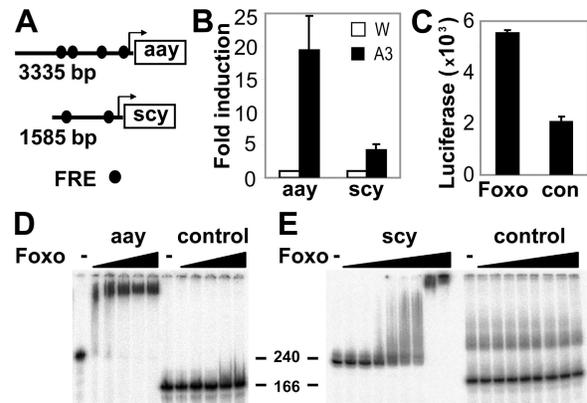


Figure 3. **FOXO can directly induce expression of genes elevated in *Tsc1* tissue.** (A) Schematic representation of FREs in *aay* and *scy* promoters. (B) QPCR analysis of *aay* and *scy* mRNA in S2 cells transfected with wild-type FOXO (W) or FOXO GOF (A3) in the presence of insulin ($n = 3$). (C) Luciferase assay ($n = 4$) measuring transcriptional activity of the *aay* promoter in S2 cells expressing either vector alone (con) or FOXOA3 (FOXO). (D and E) Band shift assays examining the ability of increasing amounts of FOXO to complex with the *aay* promoter (D), *scy* promoter (E), or negative control DNA fragments. DNA markers are indicated in bp. Error bars in B and C represent standard deviation.

explanation is that our *scy*-promoter construct lacked the minimal promoter elements required for transcription of *luciferase*.

FOXO protein is elevated and active in *Tsc1* tissue

TOR pathway hyperactivation caused by *Tsc* deficiency has been shown to strongly repress activity of Akt (Radimerski et al., 2002). FOXO is normally inactivated by Akt-dependent phosphorylation, which restricts nuclear entry of FOXO and leads to its ubiquitin-dependent destruction (for review see Greer and Brunet, 2005). Therefore, in response to TOR pathway hyperactivation, we predicted that reduced Akt activity would cause FOXO protein to accumulate. To examine this hypothesis, we analyzed expression of FOXO protein in mosaic *Tsc1* imaginal discs. We found that FOXO protein was markedly increased in *Tsc1* clones when compared with neighboring wild-type tissue (Fig. 4, A and B). In addition, FOXO protein appeared to be mostly nuclear in *Tsc1* tissue and cytoplasmic in wild-type tissue (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200710100/DC1>). Consistent with this observation, nuclear localization of the mouse FOXO orthologue FOXO1 was observed in endothelial cells of *Tsc2* mutant hemangiomas, whereas FOXO1 was mostly cytoplasmic in normal cells (Manning et al., 2005). FOXO mRNA levels were unchanged in *Tsc1* tissue as determined by microarray analysis, which suggests that changes in translation or stability of FOXO protein account for its accumulation in *Tsc1* tissue. The presence of increased FOXO protein in the nuclei of *Tsc1* cells is consistent with our hypothesis that FOXO is responsible for increased expression of some of the growth inhibitors that are up-regulated in *Tsc1* cells.

To determine whether FOXO was necessary for transcriptional induction of genes that were elevated in *Tsc1* tissue, we used QPCR analysis to measure *4E-BP*, *aay*, and *scy* expression in *Tsc1* and *Tsc1-FOXO* double mutant eye-antennal imaginal discs. Consistent with our microarray analysis, we observed increased

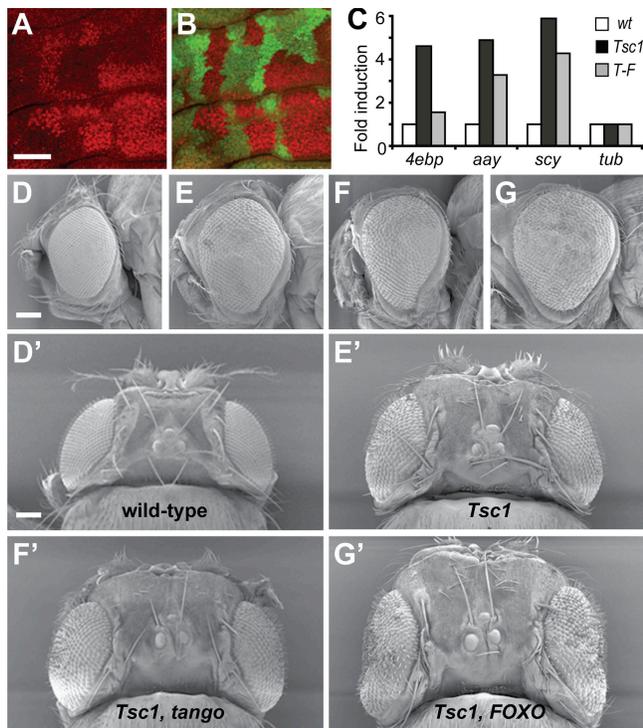


Figure 4. FOXO is elevated in *Tsc1* tissue and inhibits growth of *Tsc1* organs. (A and B) Confocal microscope images of a *Tsc1* mosaic wing imaginal disc. FOXO expression (red in A and in the merged image shown in B) was elevated in *Tsc1* clones when compared with wild-type tissue, which expresses GFP (green in B). (C) QPCR analysis of *4ebp*, *aay*, and *scy* mRNAs normalized to a *tubulin* (*tub*) control mRNA in eye-antennal imaginal disc tissue of several genotypes: wild-type (*wt*), *Tsc1*, or *Tsc1-FOXO* (*T-F*). (D–G) Scanning electron micrographs of adult eyes of genotypes: wild type (D and D'), *Tsc1* (E and E'), *Tsc1-tgo* (F and F'), and *Tsc1-FOXO* (G and G'). Data are represented as a mean expression level derived from between three and nine replicate experiments performed on pooled biological samples collected from at least 20 animals of each genotype. However, *n* was still equal to 1. Therefore, data were averaged to provide mean expression data but did not allow measurement of error values such as standard deviation or standard error of the mean. Bars: (A and B) 50 μ M; (D–G) 100 μ M.

expression of *4E-BP*, *aay*, and *scy* in *Tsc1* tissue (Fig. 4 C). In *Tsc1-FOXO* tissue, however, *4E-BP* was expressed at approximately equivalent amounts as in wild-type tissue, whereas *aay* and *scy* expression was only partially reduced (Fig. 4 C). This demonstrates that elevated expression of *4E-BP* in *Tsc1* tissue is dependent on the FOXO transcription factor and provides evidence that FOXO activity increases when the TOR pathway is hyperactivated. Expression of *aay* and *scy* appear to be partially dependent on FOXO but are likely stimulated by additional transcription factors in *Tsc1* tissue.

FOXO but not hypoxia-inducible factor-1 (HIF-1) inhibits growth in *Tsc1*-deficient tissues

Next, we sought to determine whether FOXO was required to limit growth of tissues with increased TOR pathway activity. In addition, we analyzed a potential role for another transcription factor, HIF-1, for retardation of TOR-driven growth. HIF-1 is a dual-subunit transcription factor consisting of α and β subunits that functions in response to insulin/TOR signaling

(Treins et al., 2002; Dekanty et al., 2005) and drives transcription of the growth-inhibiting genes *scy* and *chrh* (Reiling and Hafen, 2004), both of which we found to be elevated in *Tsc1* tissue.

D. melanogaster possesses several HIF-1 α subunits and a sole HIF-1 β subunit, *tango* (*tgo*), which partners with each HIF-1 α subunit. If FOXO and/or HIF-1 are required to induce expression of genes that limit tissue growth when the TOR pathway is hyperactivated, one might predict that *Tsc1-FOXO* and/or *Tsc1-tgo* double mutant tissue would possess a greater capacity to grow than *Tsc1* tissue alone. To test this hypothesis, we examined the size of *D. melanogaster* eyes comprised almost entirely of the following genotypes: control, *tgo*, *FOXO*, *Tsc1*, *Tsc1-tgo*, and *Tsc1-FOXO*. Mutant eyes were created by driving mitotic recombination of chromosomes bearing flipase recognition target (FRT) sites and the appropriate gene mutations, specifically in developing *D. melanogaster* eye-antennal imaginal discs. Eyes lacking either *tgo* or *FOXO* were approximately the same size as control eyes (not depicted), whereas *Tsc1* eyes were considerably larger (Fig. 4, D and E). *Tsc1-tgo* double mutant eyes did not exhibit a further increase in size, which suggests that HIF-1 is not required to inhibit tissue growth in response to *Tsc1* loss (Fig. 4 F). In contrast, *Tsc1-FOXO* double mutant eyes were substantially larger than *Tsc1* eyes, which is consistent with a previous study (Fig. 4 G; Junger et al., 2003). This finding is particularly significant in light of the finding that eyes lacking *FOXO* were indistinguishable in size from wild-type eyes (unpublished data; Junger et al., 2003). Thus, it appears that *FOXO* is normally dispensable for control of eye size, but when growth control is altered by virtue of increased TOR activity, *FOXO* partially offsets the increased tissue growth. These findings are consistent with our observations that FOXO protein accumulates in *Tsc1* tissue and that transcriptional profiles of *FOXO* GOF and *Tsc1* LOF cells overlap significantly.

Transcriptional changes in cells with increased TOR activity are conserved in eukaryotes

Because individual components of the insulin and TOR pathways are highly conserved among eukaryotes, important regulatory mechanisms that control tissue growth via these pathways are also likely to be conserved. To investigate this idea, we analyzed transcriptional control of mouse orthologues of genes that were elevated in *D. melanogaster* *Tsc1* tissue. Initially, we performed Northern blotting analysis on *Tsc2* primary mouse embryonic fibroblasts (MEFs; derived on a *p53* background to overcome premature senescence induced by *Tsc2* loss; Zhang et al., 2003). It is reasonable to predict that transcriptional changes that occur because of loss of either *Tsc1* or *Tsc2* should be very similar because TSC1 and TSC2 function together in an obligate fashion, and mutation of either gene leads to almost indistinguishable phenotypes (Tapon et al., 2001). We found that several gene expression changes observed in *D. melanogaster* *Tsc1* tissue were conserved in *Tsc2* MEFs (Fig. 5 A). The homologues of *aay*, *heat shock protein* (*hsp*) 23, *scy*, and *chrh* (*PSPH*, *hsp* 27, *REDD1*, and *REDD2*, respectively) were all significantly up-regulated in *Tsc2* MEFs when compared with control MEFs and expression of the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) control.

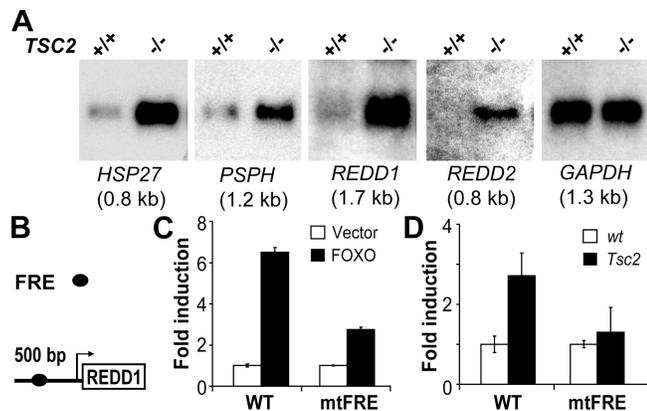


Figure 5. TOR pathway-driven transcriptional changes are conserved in eukaryotes. (A) Northern analysis of the *hsp27*, *PSPH*, *REDD1*, *REDD2*, and *GAPDH* genes in primary *Tsc2* ($-/-$) or wild-type littermate control ($+/+$) MEFs. (B) Schematic representation of the FRE in the *REDD1* promoter. (C) Luciferase assay ($n = 4$) measuring transcriptional activity of either wild-type (WT) or FRE mutant (mtFRE) *REDD1* promoters in primary MEFs expressing either vector alone (Vector) or FOXO GOF (FOXO). (D) Transcriptional activity of the *REDD1* wild type or FRE mutant promoter in wild-type (wt) or *Tsc2* MEFs ($n = 6$). Error bars represent standard deviation.

To demonstrate that these expression changes were a specific consequence of *Tsc2* loss, we reconstituted *Tsc2* expression in *Tsc2* null cells, which substantially suppressed mammalian TOR activity and expression of these genes (unpublished data). Interestingly, expression of *phosphoenolpyruvate carboxy kinase* and *4E-BP1/2* was not altered between wild-type and *Tsc2* cells (unpublished data), which might reflect tissue- or species-specific differences in the transcriptome of *D. melanogaster* epithelial cells and MEFs.

To determine whether the mode of transcription of these genes was also conserved in mammals, we analyzed expression of the *scy* homologue *REDD1*. Like *scy*, mammalian *REDD1* orthologues possess a putative consensus FRE within their proximal promoters (Furuyama et al., 2000). Cotransfection of a version of FOXO that is insensitive to phosphorylation-dependent inhibition by Akt (TM-FKHRL-1) induced robust activation of a mouse *REDD1* reporter construct in primary MEFs (Fig. 5 C). To determine whether induction was mediated through the identified FRE, we created a mutant reporter lacking this sequence. Deletion of the *REDD1* FRE consistently reduced FOXO-mediated induction of the *REDD1* promoter (Fig. 5 C). Finally, to directly assess whether FOXO-dependent transcription was activated in mammalian cells lacking *Tsc2*, we examined activity of the *REDD1* promoter reporter or the corresponding mutant FRE reporter in wild-type and *Tsc2* MEFs. As predicted, the wild-type *REDD1* promoter exhibited robust activation in *Tsc2* cells compared with wild-type cells, and this activation was substantially reduced by deletion of the FRE (Fig. 5 D). Together, these findings provide evidence that transcriptional changes resulting from *Tsc1/Tsc2* deficiency are conserved in diverse species.

Here, we report identification of an evolutionary conserved transcriptional program important for restricting tissue overgrowth driven by excessive activation of the TOR pathway. The FOXO transcription factor plays a key role in this transcriptional response, likely by stimulating expression of several growth inhibitory genes.

Thus, although the requirement for FOXO in restricting growth under normal development conditions appears dispensable, this is no longer the case under conditions of excessive TOR activation. These findings have important implications for cancer syndromes that arise because of inappropriate TOR pathway activation, such as the human hamartomatous syndrome, tuberous sclerosis. TOR-dependent feedback inhibition is thought to contribute to the benign nature of *Tsc1* and *Tsc2* tumors (Ma et al., 2005; Manning et al., 2005). Conceivably, inactivating mutations in FOXO family transcription factors and/or FOXO target genes that possess growth-inhibiting properties could promote further growth in normally benign *Tsc1* and *Tsc2* tumors.

Materials and methods

D. melanogaster stocks

The following stocks were used: *w*; *FRT82B*, *w*; *FRT82B Tsc1^{Q87X}* (Tapon et al., 2001), *w*; *FRT82B FOXO²⁵* (provided by E. Hafen, Institute of Molecular Systems Biology, Zürich, Switzerland; Junger et al., 2003), *w*; *FRT82B Tsc1^{Q87X}, FOXO²⁵* (generated by meiotic recombination), *w*; *FRT82B tgo⁵* (provided by S. Crews, University of North Carolina, Chapel Hill, NC; Emmons et al., 1999), *w*; *FRT82B Tsc1^{Q87X}, tgo⁵* (generated by meiotic recombination), *y w eyFlp*; *FRT82B P[W+] l[3]cl-R3*, *y w hsFlp*; *FRT82B P[W + ubi-GFP]*, *y w*; *P[lacW]aay*, *y w*; *P[lacW]Thor*, *UAS-Rheb* (provided by P. Patel, University of California, Los Angeles, Los Angeles, CA; Patel et al., 2003), and *GMR-Gal4*.

Microarray hybridization and analysis

Male flies of genotype *w*; *FRT82B* or *w*; *FRT82B Tsc1^{Q87X}* were crossed to *y w eyFlp*; *FRT82B P[W+] l[3]cl-R3*. Larval progeny from these crosses bore eye discs comprised almost entirely of one genotype (either wild-type or *Tsc1*). Eye-antennal discs from third instar larvae of each genotype were dissected (200 eye discs per sample in three independent samples) and total RNA was prepared using TRIZOL (Invitrogen). First strand cDNA was generated and hybridized to GeneChip *Drosophila* genome arrays (Affymetrix) by the Massachusetts General Hospital Cancer Center microarray core. Data were analyzed using Microarray Suite 5.0 (Affymetrix). Nine pairwise comparisons were performed between wild-type and *Tsc1* samples to calculate mean fold changes. Excel (Microsoft) was used to perform a *t* test to calculate significant changes ($P < 0.05$) in gene expression levels. FOXO GOF microarray experiments have been described previously (Puig et al., 2003; Gershman et al., 2007). Overlap between *Tsc1* LOF and FOXO GOF microarrays was performed using Excel (Microsoft). Measurement of significance of overlap between microarrays was determined using hypergeometric distribution calculation.

Immunohistochemistry and microscopy

Antibodies used were anti-FOXO (Puig et al., 2003), anti- β -galactosidase (Sigma-Aldrich), and anti-mouse and anti-rabbit Alexa fluor secondary antibodies (Invitrogen). The nuclear dye used was Topro-3 (Invitrogen). Confocal microscopy was performed on a confocal microscope (SP2) using software (both from Leica). Images were captured using a 20 \times NA 0.5 lens or a 40 \times NA 1.25 oil immersion lens (both from Leica) at room temperature. Scanning electron microscopy was performed according to standard protocols using a field emission scanning electron microscope (XL30 FEG; Philips; Bennett and Harvey, 2006). Any brightness or contrast adjustments were performed using Photoshop (Adobe).

Northern analysis

Total RNA was prepared from primary litter-matched wild-type or *TSC2* MEFs (provided by D. Kwiatkowski, Brigham and Women's Hospital, Boston, MA) using RNA STAT-60 (Tel-Test Inc.). 15 μ g RNA per lane was loaded and probed with the indicated ³²P-labeled cDNA probes as described previously (Sofer et al., 2005). Probes were isolated by RT-PCR from embryonic day 9.5 mouse embryo RNA.

QPCR

RNA was extracted from third instar larval eye imaginal discs or S2 cells using TRIZOL, and first strand cDNA was produced with a Superscript II kit (Invitrogen). QPCR reactions were analyzed on a sequence detection system (ABI Prism 7000; Applied Biosystems) using SYBR green reagents.

Relative levels of mRNA were compared by the comparative C_T method using *tubulin* or *actin* primers to normalize total mRNA input.

Luciferase assays

aay promoter assays were performed using a 3.3-kb *aay* promoter fragment directly upstream of the transcription start site. S2 cells were transfected with an *aay*-reporter construct and either FOXO3 or an empty vector control, and harvested 24 h later. *REDD1* promoter assays used a 0.6-kb fragment containing the proximal *REDD1* promoter and first exon (Ellisen et al., 2002). FRE mutant *REDD1* promoters were generated by PCR. Reporter constructs or luciferase controls were transfected with TM-FKHRL1 (provided by M. Greenberg, Children's Hospital, Boston, MA) into primary MEFs and luciferase activity was measured at 36 h. Luciferase assays were analyzed using the Dual Luciferase Reporter Assay system (Promega).

Band-shift assays

Band-shift assays were performed as described previously (Puig et al., 2003) using a 241-bp *aay* promoter fragment starting 1,213 bp upstream of the predicted transcription start site and a 236-bp *scy* promoter fragment starting 391 bp upstream of the predicted transcription start site.

Online supplemental material

Figs. S1–S3 provide data on expression changes observed between wild-type and *Tsc1* *D. melanogaster* larval eye-antennal imaginal discs. Fig. S1 is an accompanying figure to Fig. 1A and represents a gene ontology analysis of genes whose expression was decreased at least 1.5-fold ($P < 0.05$) in *Tsc1* tissue compared with wild-type tissue as determined by microarray analysis. Fig. S2 shows selected genes whose expression changes between *Tsc1* and wild-type tissue were confirmed by QPCR. Fig. S3 shows high-magnification images of FOXO subcellular localization in *Tsc1* mosaic larval imaginal discs. Tables S1 and S2 list all genes whose expression was increased or decreased at least 1.5-fold ($P < 0.05$) in *Tsc1* compared with wild-type tissue as determined by microarray analysis. Online supplemental materials is available at <http://www.jcb.org/cgi/content/full/jcb.200710100/DC1>.

We thank S. Crews, M. Greenberg, E. Hafen, D. Kwiatkowski, P. Patel, N. Tapon, and the Bloomington Stock Centre for *Drosophila* stocks and reagents, and T. Shioda and the MGH Cancer Center for microarray hybridizations.

K.F. Harvey is a Leukemia and lymphoma Society Special Fellow (grant 3324-06) and the recipient of a Career Development Award from the International Human Frontier Science Program Organization. Support was provided by the National Health and Medical Research Council (grant 400335 to K. Harvey), a National Institutes of Health grant (GM61672 to I.K. Hariharan), the Novo Nordisk Foundation, the Sigrid Juselius Foundation, the Finnish Diabetes Association (all to O. Puig), and an American Cancer Society grant (DDC-105895 to L.W. Ellisen).

Submitted: 16 October 2007

Accepted: 24 January 2008

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