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Minireview

Shrinkage control: regulation of insulin-mediated growth by FOXO transcription factors

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Abstract

The insulin signaling pathway regulates organismal growth in response to nutrient conditions by controlling a range of metabolic and biosynthetic processes. Recent studies in *Drosophila* have shown how transcriptional responses to reduced insulin and nutrient levels can act to inhibit growth.

In the transition to multicellularity during evolution individual cells gave up autonomous control over whether to grow and divide, live or die. These processes are regulated instead by a variety of intercellular signals and the network of signal-transduction pathways they activate. Thus, proliferation of a population of cells can be regulated in concert in response to triggers that reflect the needs of the whole organism, such as patterning cues, developmental stage, and environmental conditions. Over the past several years, studies in mammalian cell culture and model organisms such as *Drosophila* have identified as a dedicated regulator of cell growth and proliferation in response to nutrition the signaling pathway from insulin at the cell surface to phosphatidylinositol (PI) 3-kinase and the protein kinase Akt (also called protein kinase B, PKB) inside the cell [1]. Mutations in this pathway result in profound changes in cell, organ and organism size, and its activation is a critical step in a number of types of cancer. Intensive efforts have therefore been directed towards gaining a molecular understanding of the mechanisms by which insulin signaling promotes growth. Three recent studies [2-4], including a paper by Jünger *et al.* in this issue of *Journal of Biology* [2], have now addressed the role played by gene expression in mediating insulin-controlled growth in *Drosophila*.

Signaling responses to insulin

The proximal steps downstream of insulin binding are well understood [5] (Figure 1). In response to ligand binding, the insulin receptor phosphorylates insulin receptor substrate (IRS) proteins (encoded by the *chico* gene in *Drosophila*), which act as docking sites for the class I PI 3-kinase. Activated PI 3-kinase increases the levels of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3) at the cell membrane; the accumulation of PIP_3 is opposed by the phosphatase activity of a negative regulator of insulin signaling, the tumor suppressor PTEN. An important downstream effector of PIP_3 is the serine threonine protein kinase Akt/PKB. In response to PI 3-kinase activation, interaction between PIP_3 and the pleckstrin homology domain of Akt causes recruitment of Akt to the cell membrane, where it is further activated by one or more additional kinases. Akt appears to be the major critical target of PIP_3 signaling in *Drosophila*, as mutations in Akt that block its ability to bind PIP_3 can restore viability to animals with high levels of PIP_3 caused by mutations in PTEN [6].

Two signaling branches downstream of Akt have been identified (Figure 1). One branch of this pathway leads to activation of the target of rapamycin (TOR) and p70 S6 kinases,

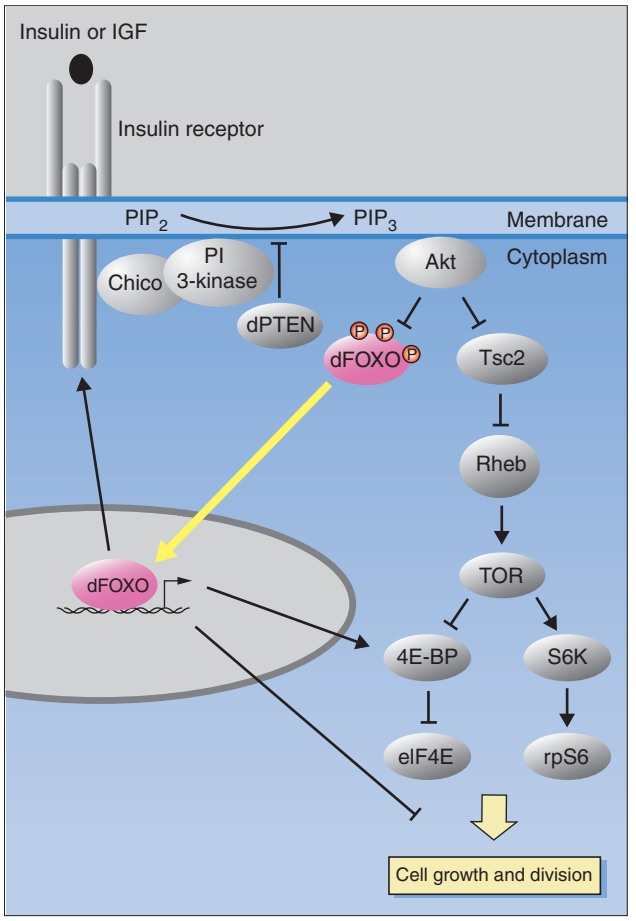

Figure 1
The dFOXO protein mediates a transcriptional response to insulin signaling. Under conditions of abundant nutrients, dFOXO is retained in an inactive state in the cytoplasm due to phosphorylation by Akt. When insulin levels fall, dFOXO is dephosphorylated and translocated into the nucleus, where it stimulates transcription of 4E-BP and presumably other negative regulators of growth. In addition, active dFOXO increases expression of the insulin receptor gene [4], which may result in increased insulin sensitivity under low insulin conditions.

which promote cell growth through a number of effects including stimulation of ribosome biogenesis [7]. The direct target of Akt in this case appears to be the product of the *tuberous sclerosis complex 2* gene [8], TSC2, which was recently found to function as a negative regulator of the small GTPase Rheb, an upstream activator of TOR [9]. Akt phosphorylates and inactivates TSC2, thereby allowing increased activity of Rheb, TOR, and S6 kinase.

A second pathway downstream of Akt was initially identified through genetic studies in *Caenorhabditis elegans*. Insulin signaling mediates responses to nutrient levels in *C. elegans* by regulating the formation of a developmentally arrested juvenile form known as the dauer, which can survive starvation conditions for an extended period [10]. Loss-of-function mutations in insulin signaling components mimic starvation, leading to inappropriate dauer formation. A number of years ago, Daf16 was identified as a negative regulator of this insulin-dependent response in worms [11]. Mutations in *daf16* can completely suppress the dauer induction caused by reduced insulin signaling. Daf16 was found to encode a transcriptional regulator of the Forkhead-box type O (FOXO) class of Forkhead-related factors, thus indicating that control of gene expression is a major output of insulin signaling in worms. Subsequent studies in cultured mammalian cells extended these results, showing that FOXO factors are negatively regulated by the insulin/PI 3-kinase/Akt pathway. In response to increased insulin levels, activated Akt phosphorylates FOXO on multiple sites, resulting in its nuclear exclusion [12]. Upon reduced insulin signaling, FOXO becomes dephosphorylated and accumulates in the nucleus, where it acts to regulate the transcription of a number of target genes.

Growth control by FOXO factors

Could FOXO-regulated transcription play a role in growth regulation by the insulin/PI 3-kinase pathway? Several lines of evidence point to such a role. First, overexpression of any of the three mammalian FOXO homologs, FOXO1, FOXO3a or FOXO4, leads to growth arrest in a variety of cell types [12]. Increased levels of insulin can suppress the growth arrest caused by overexpression of wild-type FOXO, but not of FOXO mutants lacking Akt phosphorylation sites. Second, FOXO factors regulate expression of a number of regulators of cell proliferation including $p27^{kip1}$, cyclin D, and the Retinoblastoma-related protein p107. Induction of $p27^{kip1}$, an inhibitor of cyclin-dependent kinases, appears to be a critical step in cell-cycle arrest by FOXO. The transcription of $p27^{kip1}$ is directly induced by FOXO factors in response to low insulin levels, and cells lacking the *kip1* gene are highly resistant to growth inhibition by expression of FOXO or inactivation of PI 3-kinase [13]. In addition, transcription of cyclin D is negatively regulated by FOXO, and forced expression of cyclin D can partially bypass FOXO-induced arrest [14]. Finally, a number of chromosomal translocations involving FOXO members are associated with neoplasias. For example, a t(1;13)(p36q14) translocation found in rhabdomyosarcomas results in fusion of a portion of *FOXO1* with the *PAX7* gene [15].

A potential limitation to the conclusions from these studies is that most were performed in cultured, transformed cells using non-physiological levels of transgene expression. Thus, the relevance of FOXO factors and their potential targets in growth mediated by insulin and PI 3-kinase *in vivo*

remains unclear. Indeed, genetic studies have suggested that downregulation of TSC2 and subsequent activation of the TOR/S6 kinase pathway may be the central function of insulin signaling in regulating cell growth [16].

As now described by Puig *et al.* [4], Jünger *et al.* [2] and Kramer *et al.* [3], addressing this question in *Drosophila* allows analysis of both overexpressed and endogenous *FOXO* in a variety of *in vivo* conditions. The fly genome encodes a single FOXO ortholog, dFOXO, whose sequence includes three Akt phosphorylation consensus sites similar to those found in mammalian FOXOs and nematode Daf16. As in these proteins, phosphorylation of dFOXO is stimulated by Akt activation in response to insulin, and this results in turn in its cytoplasmic localization and transcriptional inactivation [4]. Each of the three studies [2-4] demonstrates that overexpression of dFOXO or mammalian FOXO proteins in developing *Drosophila* tissues results in a significant reduction in growth. Importantly, more severe phenotypes are obtained by expression of FOXO proteins lacking their Akt phosphorylation sites, or by coexpression of wild type dFOXO with an inhibitory version of PI 3-kinase. The degree of growth suppression by dFOXO also increases in response to nutrient deprivation [2], which has been shown to reduce the levels of insulin-like protein expression. Together these results provide *in vivo* support for the idea that FOXO proteins are negative regulators of growth in response to conditions of low insulin signaling.

Although these experiments were conducted *in vivo*, the results suffer the usual caveats of studies based on overexpression. Indeed, it was found that the growth inhibition caused by dFOXO expression is due in part to induction of necrotic cell death [2], a phenotype not observed upon complete loss of insulin/PI 3-kinase signaling. This suggests that the overexpression phenotypes may not reflect normal FOXO function. To directly test the physiological requirement for dFOXO in regulating growth, Jünger *et al.* [2] generated loss-of-function mutations in the *dFOXO* gene. The predicted phenotype of disrupting a negative growth regulator is unrestrained growth, as observed in *PTEN* and *TSC* mutants. Surprisingly, this was not the case in the *dFOXO* mutants: flies lacking dFOXO were found to grow to a normal size [2]. Thus, despite its ability to potently inhibit growth when overexpressed, dFOXO is apparently not required for growth suppression under normal developmental conditions. In contrast, a genetic requirement for *dFOXO* was observed when insulin-signaling levels were experimentally lowered. Loss of FOXO significantly suppressed the reduced growth phenotype of mutations in the insulin receptor, *chico*, PI 3-kinase and Akt genes [2]. Thus, under normal conditions, insulin/PI 3-kinase signaling appears to be sufficient to maintain dFOXO in a phosphorylated state, rendering it inactive, cytoplasmic, and therefore largely irrelevant. When insulin signaling is reduced, however, dFOXO is required to provide full growth inhibition.

Like most models however, the current one has difficulty incorporating a few experimental observations. Although most parts of the fly grew normally in the *dFOXO* mutant, the wings were found to be reduced in size, an unexpected result for a growth-suppressor mutation. In addition, *dFOXO* mutants suppressed the overgrowth phenotype caused by mutations in *PTEN*, a negative regulator of insulin signaling. These results suggest that in some situations dFOXO may play a positive role in regulating growth. Recent studies have found that transient downregulation of Akt signaling and activation of FOXO3a is required for mitotic progression in NIH 3T3 cells [17]. This finding may partly explain why dFOXO mutants do not have an overgrowth phenotype - they fail to go through sufficient mitoses - and may also account for previous observations that constitutive expression of PI 3-kinase in the *Drosophila* wing can increase the rate of cell growth but not cell division [18].

Insulin signaling regulates growth by controlling both cell size and cell number, and mutations in different components of this pathway in *Drosophila* have been shown to cause distinct effects on these parameters. For example, the small flies resulting from mutations in the *chico*/IRS1 gene are comprised of both smaller and fewer cells [19], whereas loss of *dS6K* function causes a reduction in cell size without affecting cell number [20]. Where does dFOXO fit into this scheme? In general, most of the results in the recent studies [2-4] suggest that dFOXO exerts its effects largely through changes in cell number: dFOXO mutants were found to suppress the reduction in cell number but not cell size caused by *chico* mutations [2]. Furthermore, Puig *et al.* [4] found that the small eyes and wings resulting from *dFOXO* overexpression were comprised of fewer cells of normal size [4]. Thus, changes in cell size and cell number are genetically separable outcomes of insulin signaling, and dFOXO represents the first identified insulin signaling component that regulates primarily cell number.

These distinctions become somewhat blurred, however, when one considers the actual cellular processes that control the final number and size of cells in an organism, namely cell growth, cell division, and cell death. In the case of *dFOXO* overexpression, for example, the reduction in cell number but not cell size implies that rates of cell growth and division are decreased in a balanced fashion, thus maintaining normal cell size (Figure 2). In *chico* mutants, on the other hand, this balance must be slightly disrupted, with the rate of cell growth being reduced to a greater extent than that of cell division, resulting in both fewer and

smaller cells. Thus, seemingly qualitative differences amongst insulin-signaling components in their effects on final cell size and number may reflect rather modest or even trivial differences during development, such as the developmental stage at which a gene product becomes limiting. Indeed, in contrast to the conclusions of Puig *et al.* [4], Kramer *et al.* [3] found that overexpression of *dFOXO* caused reductions in both cell size and number; this discrepancy is likely to be due in part to differences in timing of overexpression, with Kramer *et al.* expressing *dFOXO* later in development, in primarily post-mitotic cells, thereby preventing a balanced reduction of growth and division. Thus, classifications of insulin signaling components on the basis of their effect on cell number and cell size probably represent somewhat artificial distinctions that do not reflect critical differences in their cellular functions.

What are the transcriptional targets that contribute to growth regulation by insulin signaling? The results of genome-wide expression analyses suggest that the number of FOXO-regulated genes is likely to be rather large. Puig *et al.* [4] identified 277 genes that were upregulated in cultured *Drosophila* cells expressing constitutively active *dFOXO*. Jünger *et al.* [2] took a complementary approach, identifying genes whose expression decreased in response to insulin. In addition, the expression profiles of *Drosophila* larvae subjected to nutrient deprivation *in vivo* have been assayed [21]. One target gene identified in each of these studies is d4E-BP, a negative regulator of translation that acts by binding and inhibiting the translation-initiation factor eIF4E. The 4E-BPs are well-established targets of phosphorylation by the TOR-dependent pathway, which disrupts the association between 4E-BP and eIF4E; the current results therefore indicate that both the expression and activity of d4E-BP are negatively regulated by insulin signaling (Figure 1). Interestingly, loss-of-function mutations in *d4E-BP* appear to have no effect on growth in an otherwise wild-type background, but they were found to suppress the reduction in growth caused by reduced insulin signaling, in a manner remarkably similar to that of *dFOXO* mutants [2]. In addition, Puig *et al.* [4] also identified the insulin receptor gene as being transcriptionally activated by dFOXO, suggesting a negative feedback loop that may serve to buffer the effects of alterations in insulin levels.

Together, these new studies in *Drosophila* significantly broaden our understanding of the multiple layers of insulin-mediated growth regulation. Control of gene expression by FOXO factors in response to insulin allows integration of transcriptional activities with other growth-related processes regulated by insulin, such as protein synthesis, carbohydrate metabolism and survival. A challenge for the future is to explore how these processes interact, and to determine what role transcription plays in their regulation. For example, by coordinating the expression of genes that induce growth arrest with genes required to survive quiescence, FOXO factors may provide a comprehensive response to conditions of low insulin or nutrient levels [22]. In addition, it will be important to understand how differences in cell type and developmental context can influence the transcriptional and physiological response to FOXO activity, regulating cell growth and proliferation in some cases and differentiation in others. Identification of the physiologically relevant target genes in these processes should provide further insights into the important process of insulin signaling.

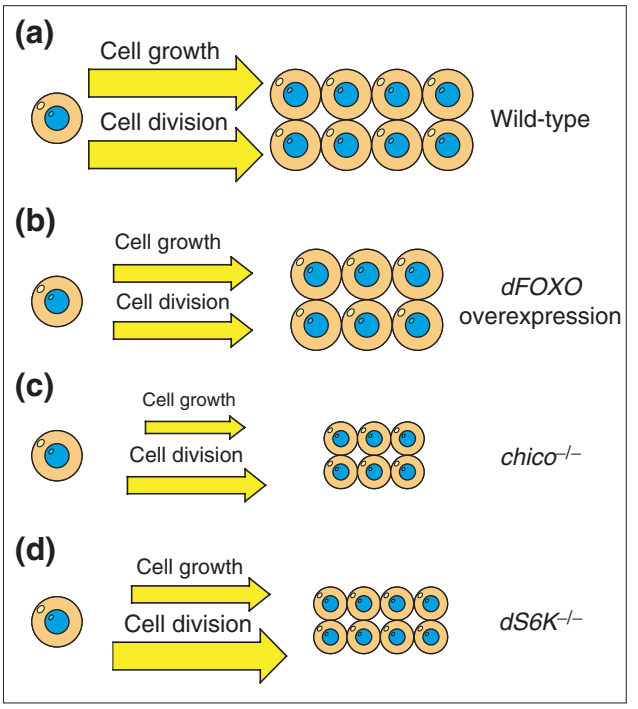

Figure 2
Insulin signaling controls cell size and number through changes in rates of cell growth and division. **(a)** Because cell growth and division rates are closely matched in wild-type cells, cell size is kept at a steady state. **(b)** By reducing cell growth and division rates in parallel, overexpression of *dFOXO* causes a reduction in cell number but maintains normal cell size. **(c)** Mutations in *chico*/IRS1 result in a reduction in both cell number and size, indicating that the rate of cell growth is decreased to a greater extent than the rate of cell division. **(d)** In *dS6K* mutants, cell size is reduced but cell number is normal, suggesting a decrease in the rate of cell growth but not cell division.

References

1. Kozma SC, Thomas G: **Regulation of cell size in growth, development and human disease: PI3K, PKB and S6K.** *Bioessays* 2002, **24:**65-71.

2. Jünger MA, Rintelen F, Stocker H, Wasserman JD, Vegh M, Radimerski T, Greenberg ME, Hafen E: **The *Drosophila* Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling.** *J Biol* 2003, **2:**20.
3. Kramer JM, Davidge JT, Lockyer JM, Staveley BE: **Expression of *Drosophila* FOXO regulates growth and can phenocopy starvation.** *BMC Dev Biol* 2003, **3:**5.
4. Puig O, Marr MT, Ruhf ML, Tjian R: **Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway.** *Genes Dev* 2003, **17:**2006-2020.
5. Cantley LC: **The phosphoinositide 3-kinase pathway.** *Science* 2002, **296:**1655-1657.
6. Stocker H, Andjelkovic M, Oldham S, Laffargue M, Wymann MP, Hemmings BA, Hafen E: **Living with lethal PIP3 levels: viability of flies lacking PTEN restored by a PH domain mutation in Akt/PKB.** *Science* 2002, **295:**2088-2091.
7. Neufeld TP: **Body building: regulation of shape and size by PI3K/TOR signaling during *Drosophila* development.** *Mech Dev,* in press.
8. Marygold SJ, Leevers SJ: **Growth signaling: TSC takes its place.** *Curr Biol* 2002, **12:**R785-R787.
9. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D: **Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins.** *Nat Cell Biol* 2003, **5:**578-581.
10. Nelson DW, Padgett RW: **Insulin worms its way into the spotlight.** *Genes Dev* 2003, **17:**813-818.
11. Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G: **The Forkhead transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*.** *Nature* 1997, **389:**994-999.
12. Burgering BM, Medema RH: **Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty.** *J Leukoc Biol* 2003, **73:**689-701.
13. Medema RH, Kops GJ, Bos JL, Burgering BM: **AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1.** *Nature* 2000, **404:**782-787.
14. Ramaswamy S, Nakamura N, Sansal I, Bergeron L, Sellers WR: **A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR.** *Cancer Cell* 2002, **2:**81-91.
15. Barr FG: **Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma.** *Oncogene* 2001, **20:**5736-5746.
16. Potter CJ, Pedraza LG, Xu T: **Akt regulates growth by directly phosphorylating Tsc2.** *Nat Cell Biol* 2002, **4:**658-665.
17. Alvarez B, Martinez AC, Burgering BM, Carrera AC: **Forkhead transcription factors contribute to execution of the mitotic programme in mammals.** *Nature* 2001, **413:**744-747.
18. Weinkove D, Neufeld TP, Twardzik T, Waterfield MD, Leevers SJ: **Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I(A) phosphoinositide 3-kinase and its adaptor.** *Curr Biol* 1999, **9:**1019-1029.
19. Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, Beckingham K, Hafen E: **Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4.** *Cell* 1999, **97:**865-875.
20. Montagne J, Stewart MJ, Stocker H, Hafen E, Kozma SC, Thomas G: ***Drosophila* S6 kinase: a regulator of cell size.** *Science* 1999, **285:**2126-2129.
21. Zinke I, Schutz CS, Katzenberger JD, Bauer M, Pankratz MJ: **Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response.** *EMBO J* 2002, **21:**6162-6173.
22. Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM: **Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress.** *Nature* 2002, **419:**316-321.