

Research news

Controlling how many cells make a fly

Pete Moore

Published: 21 August 2003

Journal of Biology 2003, **2**:16

The electronic version of this article is the complete one and can be found online at <http://jbiol.com/content/2/3/16>

© 2003 BioMed Central Ltd

Studies in *Drosophila* have revealed the Forkhead-family transcription factor FOXO to be a crucial mediator of the branch of the insulin-signaling pathway that controls cell number.

In our earliest biology lessons we learnt that all living organisms grow, and that growth requires an increase in both cell number and cell size. But how is this controlled? **Insulin** and

insulin-like growth factors (IGFs; see the 'Background' box) play a critical role, and they are also implicated in medical conditions such as cancer and diabetes. So understanding their

mechanism of action at the molecular level will have important consequences not only for our knowledge of biology, but for pathology as well.

Working at the University of Zürich, Switzerland, Ernst Hafen heads a team that is looking at the control of growth. "You can think of our work in terms of a triangle," he explains. "At the three corners are *Homo sapiens*, *Caenorhabditis elegans* and *Drosophila melanogaster*, and at the center of the triangle is the **insulin-signaling pathway**." Hafen's team has learnt important lessons about the pathway from each species, and their new findings, published in this issue of *Journal of Biology* [1], add significant evidence in support of the idea that the key functions of the pathway have been powerfully conserved through evolution. The new results also serve to tie together controls of cell size and cell number with how organisms respond to oxidative stress and nutrient availability (see 'The bottom line' box for a summary of their work).

Insulin and IGF in mammals

"We know most of the biochemistry of the system from mammalian cell-culture experiments and knockout mice," explains Martin Jünger, a PhD

The bottom line

- The homologous transcription factors FOXO and DAF-16 are known to lie on the insulin-signaling pathway, but it was unclear precisely how this pathway regulates cell size, cell number, and development in different organisms.
- In *Drosophila*, FOXO mutants have no growth phenotype, but are more sensitive than wild-type flies to oxidative stress.
- Mutations in *chico*, an upstream component of the insulin-signaling pathway, reduce both cell size and cell number; an additional FOXO mutation rescues the reduction in cell number, indicating that wild-type FOXO negatively regulates this aspect of growth. But a FOXO-*chico* double mutant still has its cell size reduced.
- Cell size is regulated by the S6 kinase branch of the insulin-signaling pathway, while FOXO regulates cell number, in part by up-regulating a protein involved in the regulation of translation.
- The insulin-signaling pathway is highly conserved in mammals, *C. elegans* and *Drosophila*, and may have evolved in the ancestor of metazoans to allow regulation of growth and development in response to stress and nutrient availability.

Background

- Both **insulin**, first identified for its role in energy metabolism, and **insulin-like growth factors** (IGFs) signal through the **insulin receptor**, a transmembrane protein kinase that initiates a signaling cascade that includes transcriptional regulation by **FOXO**, a member of the **Forkhead** family of **transcription factors**.
- The **insulin-signaling pathway** has roles in growth and development in many animal species, and is implicated in the control of lifespan, initially from studies of the genes controlling the formation of the developmentally arrested, stress-resistant **dauer** form in *C. elegans*.
- **Protein kinase B** (PKB, also known as AKT) phosphorylates FOXO and turns off its transcriptional activity. PKB also regulates growth through a pathway independent of FOXO but including the **S6 kinase**.

student in Hafen's lab. Decades of experiments have shown that insulin regulates energy metabolism, and more recent results show that it plays a key role in embryonic [2] and post-embryonic [3] growth, as well as the determination of lifespan [4].

Studies in mammalian cells have also shown that insulin negatively regulates FOXO (**Forkhead** box, subclass O) **transcription factors**, which in turn arrest the cell cycle and, in some types of cell, induce cell death. FOXO transcription factors therefore have a negative influence on growth, and their function is turned off by the insulin effector **protein kinase B** (PKB, which is also known as AKT [5]).

The worm and its dauer stage

The link between insulin and FOXO proteins initially came from experiments in *C. elegans*, where insulin signals to the FOXO equivalent, DAF-16 (see Table 1 for the names of corresponding proteins in the different species discussed in this article). In worms, the effect of modulating the insulin-signaling pathway is quite unique: rather than affecting size, it induces a change in the nematode's developmental program. Adverse conditions, such as starvation, decrease

signaling activity within the pathway, which in turn drives the worms into the developmentally arrested 'dauer stage' (DAF denotes 'dauer formation'). Dauer larvae alter their metabolism, stockpile fat and can survive in this state for at least four to eight times longer than the normal two-week lifespan of *C. elegans*.

The evidence that dauer formation is dependent on the transcription factor DAF-16 comes from genetic experiments showing that if the insulin-signaling pathway is mutated, *C. elegans* enters the dauer stage. But in a double mutant in which DAF-16 is also disabled, the worms develop as normal. The clear implication is that in normal animals the insulin pathway has its effects on dauer formation via negative regulation of DAF-16. "But the

link to growth [in worms] is not clear," says Hafen. "Because this strange worm is built by a precisely fixed number of cells, there is no relation between body size and insulin signaling." This apparent difference in action threw into question the idea that the insulin pathway has a conserved role in worms and mammals.

Drosophila and growth

Into this arena of confusion comes *Drosophila*. The clearest indication of the way that insulin signaling affects this species comes from the so-called *chico* mutant. Wild-type Chico protein functions in the insulin-signaling pathway, and flies lacking it are small with delayed development. In many ways this is similar to the situation in mammals, where mutations in the insulin/IGF pathway affect growth and body size. The flies have fewer cells, and the cells they do have are smaller in size. "This [growth] reduction is something that was never seen in *C. elegans*," says Hafen. "So, before our recent work, the best concept was that the initial pathway was the same in all species, but the readout was different," leading to growth in mammals but preventing dauer formation in *C. elegans*.

Sorting out size and number

The insulin-signaling pathway is normally triggered by insulin binding to the **insulin receptor**, which then phosphorylates Chico, an intracellular adapter protein (see Figure 1). Chico then recruits the phosphatidylinositol (PI) 3-kinase, which in turn phosphorylates

Table 1

Terms for equivalent proteins in different species

	Human	<i>C. elegans</i>	<i>Drosophila</i>
Forkhead transcription factors	Three different hFOXO proteins	DAF-16	dFOXO
Insulin effector kinases, containing pleckstrin homology (PH) domains	PDK1 and PKB/AKT 1-3	PDK1, Akt-1 and Akt-2	dPDK1 and dPKB/dAktfs

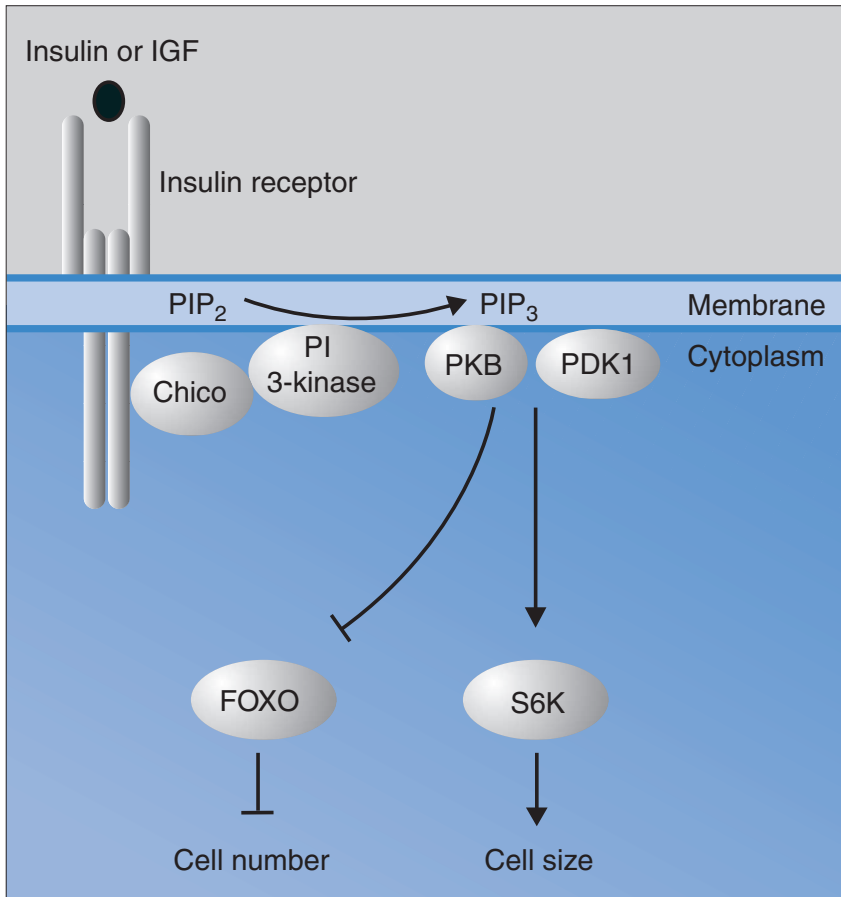


Figure 1
The key molecules of the insulin-signaling pathway, as discussed in the text.

the membrane-bound phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP₂) to phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). Hafen explains that the next key event is that PIP₃ causes kinases like PDK1 and PKB, which contain pleckstrin-homology (PH) domains, to be translocated from the cytoplasm to the membrane.

Now, Jünger, Hafen and colleagues have looked at what happens in *Drosophila* downstream of PKB [1] (see the 'Behind the scenes' box for more discussion of the background to the work). From work in mammalian cells, they knew that PKB phosphorylates transcription factors of the FOXO family, causing them to leave the nucleus and become trapped in the

cytoplasm where they cannot stimulate the initiation of transcription of target genes. "In *C. elegans*, we know that this [part of the pathway] influences development, not size, so the question for us was if size was mediated through DAF-16 in flies."

One part of the answer to this question - dealing with the size of each cell - came from a paper previously published in *Science* [6]. This showed that cell size is controlled in *Drosophila* by the S6 kinase (dS6K), an enzyme that apparently acts downstream of dPDK1 and dPKB and is named for its effects on ribosomal protein S6. Mutating *dS6K* produces small flies that have the same number of cells as in the wild type but whose

cells are small. The answer to the cell number question came from the paper by Jünger *et al.* [1], which initially set out to characterize the fly DAF-16 homolog and to assess both whether and how it fitted into the fly insulin-signaling pathway and also its growth-modulating capabilities.

When the Zürich team produced *dFOXO* mutants they were initially surprised. The flies were viable and normal-sized; there was no apparent phenotype, other than that the flies were more susceptible to oxidative stress than were their wild-type cousins. Jünger and colleagues had anticipated that removing the presumed negative influence would cause the flies to grow bigger. At first, they questioned whether they really had mutated *dFOXO*, but the genetic and molecular evidence was compelling.

As a next step, Jünger started to test the mutants in a genetic background in which other aspects of the insulin pathway were compromised. In this case, a normal fly would produce fewer, smaller cells. But take *dFOXO* away and the flies have small cells, but almost the normal number. "The reduced cell number [in insulin-pathway mutants] is rescued by the absence of the transcription factor, because [wild-type] *dFOXO* has a negative influence," he explains.

Jünger went on to show that *dFOXO* operates in part by up-regulating the gene for a binding protein called d4E-BP. With larger quantities of this binding protein produced, the translation-initiation factor eIF4E is effectively removed from the translation machinery, in turn inhibiting the initiation of protein synthesis. This shows that insulin operates not only by regulating pre-existing 4E-BP protein via phosphorylation [7], but also by influencing the intracellular abundance of 4E-BP at the gene expression level.

"We have shown that d4E-BP is a relevant target [of the pathway]," says Jünger, "but we absolutely don't postulate that it is the only one.

Behind the scenes

Journal of Biology asked Martin Jünger about how and why he set out to study *dFOXO* and its role in regulating growth.

What prompted the work?

Team members in the lab had a long-running interest in growth regulation and had performed extensive genetic screens for growth-affecting mutations. They had found many components of the insulin-signaling cascade, but did not find FOXO. As FOXO is such an established target in mammals and worms, it was an obvious issue to address.

My involvement started with my PhD thesis. I got my degree in biochemistry in Berlin and became interested in signal transduction during my diploma work. I moved to Ernst's lab for the beginning of my thesis to combine signal transduction and genetics.

How long did it take to do the experiments, and what was the team's reaction to the results?

"In total it took about three years, although when I started in December 2000, several months work had already been invested by Michael Greenberg's team at Harvard. [When we saw the results] we were surprised and excited, mainly because of FOXO's double role, the absence of a growth phenotype and the effect within the mutant context - it was a very interesting project.

What are the next steps?

We will certainly follow up on some of the results, for example the oxidative stress issue and the control of cell proliferation. More extensive expression-profiling studies should help to clarify the molecular mechanisms underlying these effects. The rather small microarray experiment in our *dFOXO* paper was something of a sidetrack.

Personally, I will invest much of my time in studying the insulin pathway in cultured cells in more detail at the transcriptome and proteome level. We have a couple of exciting collaborations going on.

It's more like a 'proof-of-principle' experiment, showing that we can find physiologically relevant targets in our rather artificial cell culture system, where we stimulate *Drosophila* cultured cells with bovine insulin! But recent microarray studies (by Puig *et al.* [8] and Ramaswamy *et al.* [9]) suggest that FOXO proteins work by modulating the transcription of large sets of target genes."

The picture that emerges for *Drosophila* is that the insulin signaling pathway forks at PKB, with an S6K element controlling cell size, and a

FOXO element taking charge of cell number (see Figure 1).

Related studies

At the same time as the Jünger *et al.* paper [1] was published, two other groups were publishing findings that support the same idea. Robert Tjian and colleagues at the University of California, Berkeley, presented biochemical evidence that when insulin is applied, *dFOXO* is phosphorylated by dPKB, leading to it being retained in the cytoplasm and therefore not being capable of initiating transcription [8].

His group reports that "targeted expression of *dFOXO* in fly tissues regulates organ size by specifying cell number with no effect on cell size". Moreover, they also found and validated *d4E-BP* as a target gene. This nicely complements the findings of Jünger *et al.* [1].

On top of this, Tjian's group had another striking result. "We found that FOXO also regulates expression of the insulin receptor," says Tjian. "This means that in the absence of insulin, FOXO is produced. This not only limits growth, but it also up-regulates sensitivity for insulin. The system is now primed to look for lower concentrations of insulin."

A third study, by Jamie Kramer and colleagues at the Memorial University of Newfoundland, Canada, presents a slightly different picture. Kramer *et al.* [10] agree with Jünger *et al.* and Tjian's group that *dFOXO* is the fly homolog of *DAF-16* and *hFOXO* (see Table 1). But, in a key difference, Kramer *et al.* found that overexpression of *dFOXO* leads to reductions in both cell size and cell number. "We have seen this effect in both the eye and the wing of *Drosophila*," says Kramer. He believes that this difference between his results and those of the other groups is most likely to arrive from his use of overexpression analysis whereas Jünger used loss-of-function techniques.

"A general problem," agrees Jünger, "is that overexpression studies are prone to artefacts, because overexpressed proteins often start doing things which under normal, physiological protein concentrations they do not." Tjian agrees; "If I got results from overexpression experiments that differ from loss-of-function work I would be inclined to trust the loss-of-function study," he says. At the same time, Tjian points out that his team's findings also came from overexpression studies. He is now keen to study the exact differences in method between his own and Kramer's work to see if this sheds light on the differences.

Completing the triangle

For Hafen, the new data complete the triangle. "In the worm, fly and human, FOXO is [a] negative [regulator of growth]," he says. "Now the pictures do not look different at all. What we see is a great underlying evolutionary conservation of this pathway." In Hafen's view, this pathway governs one of the most fundamental controls that the ancestors of multicellular organisms had to evolve. "Wild flies are not like our laboratory flies, fed on delicious food day in, day out. In nature animals often have too little food, so they have to evolve mechanisms to deal with the issue. They can't just run their metabolism at maximal speed, irrespective of whether there is food around or not; they have to find ways to adjust their metabolic rate and their speed of development according to the availability of nutrients."

He postulates that his group didn't see the full effect of the *dFOXO* mutants because the flies were growing in unnatural conditions: because the flies are fed the whole time, the insulin pathway is constantly activated. A constantly starving wild fly with a *dFOXO* mutation might have an impaired ability to limit its rate of growth to suit the nutrient availability.

Hafen likens the situation to driving a car when you know that the tank is running out of fuel. "You don't go at hundred and forty kilometers an hour, you reduce speed to reduce fuel consumption," he comments. "This is what animals had to learn to do during evolution - and they do it at least in part via the insulin-IGF pathway. The main goal of this pathway is to adjust growth rates, or the developmental program in the case of *C. elegans*, with respect to availability of food, and the mechanism is conserved right down to the level of the DAF-16 transcription factor."

Tjian is also excited by the findings. "We are starting to get a better idea of how transcription factors affect organ size and how they are used to decide when to stop putting new cells into organs," he says. And understanding the

role that FOXO plays in morphogenesis has far-reaching implications in both the laboratory and medical practice.

References

1. Jünger MA, Rintelen F, Stocker H, Wasserman JD, Végh 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.
2. Takahashi Y, Kadowaki H, Momomura K, Fukushima Y, Orban T, Okai T, Taketani Y, Akanuma Y, Yazaki Y, Kadowaki T: **A homozygous kinase-defective mutation in the insulin receptor gene in a patient with leprechaunism.** *Diabetologia* 1997, **40**:412-420.
3. Baker J, Liu JP, Robertson EJ, Efstratiadis A: **Role of insulin-like growth factors in embryonic and postnatal growth.** *Cell* 1993, **75**:73-82.
4. Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloën A, Even PC, Cervera P, Le Bouc Y: **IGF-I receptor regulates lifespan and resistance to oxidative stress in mice.** *Nature* 2003, **421**:182-187.
5. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME: **Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.** *Cell* 1999, **96**:857-868.
6. Montagne J, Stewart MJ, Stocker H, Hafer E, Kozma SC, Thomas G: ***Drosophila* S6 kinase: a regulator of cell size.** *Science* 1999, **285**:2126-2129.
7. Miron M, Verdu J, Lachance PE, Birnbaum MJ, Lasko PF, Sonenberg N: **The translational inhibitor 4E-BP is an effector of PI(3)K/Akt signalling and cell growth in *Drosophila*.** *Nat Cell Biol* 2001, **3**:596-601.
8. 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.
9. 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.
10. Kramer JM, Davidge JT, Lockyer JM, Staveley BE: **Expression of *Drosophila* FOXO regulates growth and can phenocopy starvation.** *BMC Dev Biol* 2003, **3**:5.

Pete Moore is a science writer based in Surrey, UK.
E-mail: moorep@mja-uk.org