sized with sodium pentobarbital. A silver clip (0.2 mm in diameter) was placed on the left renal artery in the preparation of the renal hypertensive rats. In the preparation of the DOCA-salt hypertensive rats, the left kidney was removed and a DOCA pellet (50 mg) was implanted subcutaneously. The DOCA rats were then fed an 8% salt diet. Rats from both groups were used after 8 weeks in the experiments, together with male, 17–22-week-old spontaneous hypertensive rats. The average systolic pressure in these groups of hypertensive rats ranged from 209 to 237 mm Hg, and no significant difference was found between groups. Eight-week-old male Wistar rats were used as controls. Their average systolic pressure was 139 mm Hg. Y-27632 was administered orally. The systolic blood pressure was measured by the tail cuff method at 1, 3, 5, 7 and 24 h. The rats were prewarmed to 40°C for 10 min before each measurement. No toxicity was found in rats treated with 30 mg kg⁻¹ of Y-27632 administered per os once per day for 10 days.

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Letters to nature

The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans

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In mammals, insulin signalling regulates glucose transport together with the expression and activity of various metabolic enzymes. In the nematode Caenorhabditis elegans, a related pathway regulates metabolism, development and longevity. Wild-type animals enter the developmentally arrested dauer stage in response to high levels of a secreted pheromone, accumulating large amounts of fat in their intestines and hypodermis. Mutations in DAF-2 (a homologue of the mammalian insulin receptor) and AGE-1 (a homologue of the catalytic subunit of mammalian phosphatidylinositol 3-OH kinase) arrest development at the dauer stage. Moreover, animals bearing weak or temperature-sensitive mutations in daf-2 and age-1 can develop reproductively, but nevertheless show increased energy storage and longevity. Here we show that null mutations in daf-2 suppress the effects of mutations in daf-2 or age-1; lack of daf-2 bypasses the need for this insulin receptor-like signalling pathway. The principal role of DAF-2/AGE-1 signalling is thus to antagonize DAF-16, which is widely expressed and encodes three members of the Fork head family of transcription factors. The DAF-2 pathway acts synergistically with the pathway activated by a nematode TGF-β-type signal, DAF-7, suggesting that DAF-16 cooperates with nematode SMAD proteins in regulating the transcription of key metabolic and developmental control genes. The probable human orthologues of DAF-16, FKHR and AFX, may also act downstream of insulin signalling and cooperate with TGF-β effectors in mediating metabolic regulation. These genes may be dysregulated in diabetes.

The metabolic, longevity, and developmental defects caused by daf-2 and age-1 mutations are suppressed by daf-16 mutations (Fig. 1 and Table 1). Wild-type animals do not arrest at the dauer stage in low pheromone, whereas temperature-sensitive mutations in daf-2 cause 100% arrest at the dauer larval stage (Table 1). daf-2 mutant animals also shift metabolism towards fat accumulation and show large drops of fat in the intestine and hypodermis of dauer larvae, as measured by Sudan black staining (Fig. 1a). However, animals bearing both a daf-2 mutation and the daf-16(mgDf47) or daf-16(mgDf50) null mutations (see below) do not arrest at the dauer stage or store large amounts of fat, developing instead into reproductive adults (Table 1 and Fig. 1b). Therefore, daf-16 is a major target of DAF-2/AGE-1 signalling. A parallel DAF-7 TGF-β pathway also regulates C. elegans metabolism and development. daf-16 gene activity is not necessary for the metabolic shift towards energy storage or dauer arrest induced by lack of DAF-7 TGF-β signalling (Fig. 1c). For example, like daf-7(e1372) mutant animals, daf-16(mgDf50); daf-7(e1372) mutant animals arrest at the dauer stage (172/174 animals arrest) and accumulate fat. The arrest, however, is not complete. The pharynx is not contracted (2/100 arrested animals have a constricted pharynx) and the intestine is less refractile than normal dauers, as has been reported using the daf-16(m26) allele (see below). Thus
placement of *daf-16* in the insulin receptor-like genetic pathway and not the DAF-7 TGF-β-like pathway is confirmed with a null *daf-16* allele. In support of the parallel pathway model, the *daf-3(mgD90)* null mutation, which potently suppresses *daf-7(e1372)* (ref. 8), does not suppress *daf-2(e1370)* (Table 1 and Fig. 1d).

*daf-16* and *daf-3* do not represent the only output of pheromone signalling. *daf-16(mgD50)* animals arrest at the dauer stage in dauer pheromone, although the arrested animals are partial dauers, with large pharynxes and low refractivity (Table 1; see below). *daf-3(mgD90)* single mutants form apparently normal dauers in the presence of pheromone (Table 1). *daf-16*, *daf-3* double-null mutant animals arrest as partial dauers in dauer pheromone, like the *daf-16* single mutant (Table 1). Thus, *daf-16* gene activity is necessary for some aspects of dauer arrest, and other pathways besides those coupled to *daf-3* and *daf-16*, for example the pathways defined by *daf-11* or *daf-12* (ref. 3), may also regulate arrest at the dauer stage in pheromone. *daf-16* and *daf-3* have no obvious function during reproductive development. Reproductively growing animals bearing null mutations in either *daf-16* or *daf-3*, or mutations in both genes do not dramatically alter fat accumulation in larvae or adult animals compared to wild type (data not shown).

We genetically mapped *daf-16* to a 300-kilobase cosmid and YAC contig (Fig. 2a). Multiple *daf-16* mutations induced with γ rays or ethylmethane sulphonate (EMS) were isolated as suppressors of the dauer constitutive phenotype induced by the *daf-2(e1370)* mutant. DNAs isolated from these *daf-16* alleles were surveyed for deletions or rearrangement breakpoints using cosmids in this contig (Fig. 2a). Deletion and point mutations associated with *daf-16* alleles (see below) were localized within cosmid R1318H to a gene that encodes a member of the Fork head family of transcription factors. The *daf-16(mgD50)* null allele (see below) was rescued by injection of cosmid R1318H into the germ line of a *daf-16(mgD50)/daf-2(e1370)* double mutant and showing that transgenic progeny arrest at the dauer stage. A neighbouring cosmid did not rescue this *daf-16* mutant allele.

Analysis of *daf-16* CDNAS revealed three alternatively spliced forms, designated *daf-16a1*, *daf-16a2* and *daf-16b* (Fig. 2a). *daf-16a* and *daf-16b* have distinct, but highly related, Fork head type DNA-binding domains (73% identical (87/119 amino acids); Fig. 2c). The amino-terminal half of the DNA-binding domain is encoded by exons 3 and 4 for *DAF-16a* and exon 5 for *DAF-16b*. The C-terminal half of each DNA-binding domain is encoded by exons 6 and 7 which are common to both transcripts (Fig. 2). Two similar isoforms of *DAF-16a*, *DAF-16a1* and *DAF-16a2* are encoded by the same 11 exons, except that exon 3 is alternatively spliced, resulting in the addition of two amino acids to *DAF-16a1* outside the DNA-binding domain (Fig. 2a, b).

The *DAF-16a* and *DAF-16b* isoforms are probably generated from distinct promoters, suggesting that they could be expressed in and mediate DAF-2 signalling in distinct tissues or cell types (see below). These isoforms have distinct Fork head DNA-binding domains that may interact with distinct partners (for example, *DAF-3*, *DAF-8* or *DAF-14*; see below) or may bind to distinct downstream promoters. Significantly, the probable DNA-binding specificity determinant of Fork head proteins in helix 3 (ref. 3) is common to the *DAF-16* isoforms. Thus, the expression and differential splicing of the two distinct DAF-16 Fork head DNA-binding domains is expected to be functionally important but is not obviously implicated in DNA-binding specificity.

The most closely related proteins in GenBank to *DAF-16* are human FKHR and AFX (Fig. 2c, d). Within the Fork head DNA-binding domain, *DAF-16a* is 65% identical (78/120) to FKHR and 62% identical (74/120) to AFX. *DAF-16b* is 50% identical (60/120) to FKHR and 47% identical (56/120) to AFX. Within this region, FKHR and AFX are 81% identical (95/117). In addition, there is a region at the amino-terminal end of each protein that is 55% identical (10/18) between *DAF-16a* and FKHR and AFX. This region is not conserved between other Fork head-related proteins.

**Table 1.** Effect of *daf-16* null mutations on dauer formation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>L4 and Dauer adults</th>
<th>Dead eggs</th>
<th>Other*</th>
<th>N†</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 Bristol (wild type)</td>
<td>98.6</td>
<td>0</td>
<td>0.3</td>
<td>1.1</td>
</tr>
<tr>
<td><em>daf-2(e1370)</em></td>
<td>0</td>
<td>95.2</td>
<td>2.8</td>
<td>2.0</td>
</tr>
<tr>
<td><em>daf-16(mgD90);daf-2(e1370)</em></td>
<td>79.6</td>
<td>0</td>
<td>18.1</td>
<td>2.3</td>
</tr>
<tr>
<td><em>daf-16(mgD47);daf-2(e1370)</em></td>
<td>84.1</td>
<td>0</td>
<td>10.2</td>
<td>5.7</td>
</tr>
<tr>
<td><em>daf-16(mgD50);daf-2(e1370)</em></td>
<td>95.1</td>
<td>0</td>
<td>3.5</td>
<td>1.4</td>
</tr>
<tr>
<td><em>daf-16(mgD41);daf-2(e1370)</em></td>
<td>89.6</td>
<td>0.4</td>
<td>9.2</td>
<td>0.8</td>
</tr>
<tr>
<td><em>daf-3(mgD90)</em></td>
<td>98.6</td>
<td>0</td>
<td>ND</td>
<td>1.4</td>
</tr>
<tr>
<td><em>daf-2(e1370);daf-3(mgD90)</em></td>
<td>0</td>
<td>97</td>
<td>ND</td>
<td>3.0</td>
</tr>
<tr>
<td>Pheromone + wild type</td>
<td>64.2</td>
<td>35.8</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Pheromone + <em>daf-16(mgD50)</em></td>
<td>73.9</td>
<td>23.8</td>
<td>ND</td>
<td>2.2</td>
</tr>
<tr>
<td>Pheromone + <em>daf-3(mgD90)</em></td>
<td>61.0</td>
<td>37.7</td>
<td>ND</td>
<td>1.3</td>
</tr>
<tr>
<td>Pheromone + <em>daf-16(mgD80);daf-3(mgD90)</em></td>
<td>56.1</td>
<td>41.8</td>
<td>ND</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* Under these conditions the dauers were not fully formed. These larvae were similar to dauer in having a shrunken cuticle, but the pharynx pumped and was not shrunken, the intestine was not dark and sterile and indistinct.

† Total number of animals scored.

*Other* includes animals that could not be classified as dauer or non-dauer because the animal was young, had grossly aberrant morphology, or was dead.

**Figure 1.** Metabolic control by *daf-16* and *daf-3*. Fat accumulation assayed by Sudan black in dauer larval stage and the comparable reproductive larval stage 3 and 4 in animals grown at 25°C. a, *daf-2(e1370)*. b, Suppression of fat accumulation in *daf-16(mgD50);daf-2(e1370) animals. c, Suppression of *daf-7(e1372)* fat storage and arrest by *daf-3(mgD90)*. d, *daf-16(mgD50)* mutant does not suppress the fat accumulation or arrest of a *daf-7(e1372)* mutant.
nor between DAF-16b and FKHR or AFX. DAF-16a is only 36% identical to Freac-4 and DAF-16b is only 33% identical to Freac-3 (Fig. 2d), which are the next most closely related proteins in the GenBank or dbEST databases. DAF-16a and DAF-16b are similarly distinct from the closest C. elegans homologues (Fig. 2d). Phylogenetic tree analysis of the Fork head domain of DAF-16 and its closest relatives show that FKHR, AFX and DAF-16 constitute a distinct class (Fig. 2d).

The high degree of sequence similarity suggests that FKHR and AFX may correspond to the human orthologues of DAF-16 and constitute major transcriptional outputs of insulin or IGF-1 receptors in mammals. FKHR and AFX, however, were identified as oncogene breakpoints and not, like DAF-16, as metabolic transcriptional regulators. The oncogenic translocations fuse a disrupted FKHR or AFX Fork head DNA-binding domain and intact carboxy-terminal activation domain to other DNA-binding domains\cite{10,11}. The PAX-3/FKHR fusion protein is a more potent transcriptional activator than PAX-3 (ref. 12). Although FKHR and AFX activities are not yet known to be regulated by insulin or IGF, growth of rhabdomyosarcomas is suppressed by declines in IGF-1 receptor signalling\cite{13,14} and these tumours accumulate large amounts of glycogen\cite{15}. The HNF3 Fork head transcription factor has been implicated in insulin regulation of metabolic gene transcription\cite{16}. Because distantly related Fork head proteins bind to similar sites\cite{17}, HNF3-binding cis-acting sites from insulin-responsive metabolic control genes may actually be regulated by the FKHR or AFX protein in mammals. Non-null mutations in FKHR or AFX that decouple these transcription factors from the insulin signalling cascade may underlie type II diabetes in some pedigrees.

Identification of \textit{daf}-16 null alleles establishes that the most

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**Figure 2** \textit{daf}-16 encodes a Fork head/HNF3-related transcription factor. \textbf{a}, Top, genetic and physical map of the \textit{daf}-16 region, bottom, exon/intron structure of \textit{daf}-16. Coding regions are filled boxes, non-coding regions are open boxes, and introns are lines. The Fork head regions are indicated in grey. \textbf{b}, Molecular identity of six mutations identified in \textit{daf}-16. The genomic region of \textit{daf}-16 is shown. Boxes indicate exons, and areas in grey indicate regions homologous to the Fork head domain. Two point mutants (\textit{mg54} and \textit{mg53}) are shown simply as arrows pointing to the location of the lesion (see also \textbf{c}). Two point mutants (\textit{mg26} and \textit{mg87}) affect splice sites and are shown below. The extent of the \textit{mgDM47} and \textit{mgD50} deletions is indicated. The area between the vertical lines is deleted, and areas of uncertainty are indicated by dotted lines. \textbf{c}, DNA-binding domains of DAF-16a, DAF-16b and human FKHR (U02310). AFX (X93996) and HNF3a were aligned using Pileup (GGC). Amino acids that are identical in at least three of the proteins are highlighted. Horizontal arrow indicates the beginning of exon 6 which is shared between DAF-16a and DAF-16b. Point mutations within the DNA-binding domains of DAF-16 are indicated; \textit{daf}-16(\textit{mg54}) is specific for DAF-16a, whereas \textit{daf}-16(\textit{mg53}) is common for both DAF-16a and DAF-16b. Vertical arrow indicates the fusion point for the PAX-3/FKHR and HTRX1/AFX1 fusion proteins that are caused by chromosomal translocations found in human tumours. \textbf{d}, Pileup (GGC) was used to align the DNA binding domains of the listed proteins. Human proteins are indicated in bold text and \textit{C. elegans} proteins are indicated by plain text. C47G2.2 is a gene-finder-predicted ORF on cosmid C47G2. The accession numbers for the \textit{daf}-16 sequences are AF020342 \textit{daf}-16a1; AF020343 \textit{daf}-16a2 and AF020344 \textit{daf}-16b.

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**Table 2** Synergy of insulin receptor-like and TGF-\(\beta\) receptor-like signalling

<table>
<thead>
<tr>
<th>Genotype</th>
<th>15°C (n)</th>
<th>18°C (n)</th>
<th>20°C (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{daf}-2(e1370)</td>
<td>0 (611)</td>
<td>5 (487)</td>
<td>5 (506)</td>
</tr>
<tr>
<td>\textit{daf}-1(m40)</td>
<td>0 (434)</td>
<td>0 (732)</td>
<td>1 (909)</td>
</tr>
<tr>
<td>\textit{daf}-1(m40); \textit{daf}-2(e1370)</td>
<td>17 (652)</td>
<td>99 (861)</td>
<td>100 (718)</td>
</tr>
</tbody>
</table>

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**Figure 3** Expression of a \textit{daf}-16::GFP fusion gene in an L1 animal.
Importantdaf-16functionisindauerarrestandmetabolichereregulation. daf-16(mgDf47) and daf-16(mgDf50) wereisolatedafter γ-raymutagenesis and are molecularnull alleles that deleterelaxeddomains or nearly all of the daf-16 coding region (Fig. 2b). Asalready described, both of these daf-16nullalleles have the sametypes: viable and complete suppression of the daf-2 dauerconstitutivelongevity and energy storage phenotypes.

Molecular analysis of other daf-16 mutant alleles revealed that the twomain DAF-16 isoforms are not redundant. daf-16(mg54) is anamber stop mutation within the DNA-binding domain of DAF-16 but ispredicted not to affect DAF-16b (Fig. 2b, c). daf-16(mg54) suppresssthedauer constitutivelongevity phenotype caused by daf-2 muta-
tions (Table 1). Two other daf-16 alleles, m26 and m87, are also
specific to the daf-16a isoform (Fig. 2c). daf-16(m26) incompletelysuppresses daf-2(e1370) (ref. 7). The identification of daf-16a-specificmutations indicates that daf-16b activity is not sufficient toinduce dauer arrest in a daf-2 mutant. No mutation that is
specific for the daf-16b Fork head region was detected in a collectionof 11 daf-16 alleles sequenced, so it is not known if this isoform is
necessary for daf-2 and age-1 induced dauer arrest. The onlypoint mutation that affects both daf-16 isoforms, daf-16(mg53), changesa conservedserine in the Fork head DNA-binding domain to leucine
(Fig. 2b, c).

Arrest ata dauer larval stage affectsthe morphology andmetabolism of many C. elegans tissues1. DAF-16 might act directlyin these target tissues or indirectly in neuronal or other signalling
cells that in turn regulate the development and metabolism of target
tissues. daf-16expression was addressed using a daf-16a::greenfluorescent protein (GFP) fusion gene. Transgenic daf-16a::GFP
animals show strong GFP fluorescence in most cells including
ectoderm, muscles, intestine and neurons (Fig. 3). The broad
expression pattern of daf-16a::GFP is also seen in late embryos,
larvae and dauer larvae. No expression was seen in the pharynxeven though the morphology of the pharynx is altered in dauer larvae,
and daf-16 gene activity is essential for this pharyngeal change in
pheromone (Table 1) or daf-7-induced dauers2. It is possible that
daf-16b is expressed in the pharynx and serves this function, that the
daf-16a::GFP fusion gene does not bear those regulatory sequences,
or that daf-16 acts non-autonomously to affect the pharynx.

The expression of daf-16ain the many target tissues that are
metabolically and developmentallyshifted in dauers is consistentwith its action in target tissues to regulate dauer arrest, metabolism
and longevity. In addition to the metabolic changes that occur in
dauer larvae, some tissues are remodelled or developmentally
arrested. The hypodermis shrinks and stores fat. The intestinallumen is shrunk and becomes refractile. The collagen composition
of the cuticle is altered by changes in the expression of collagen
genes3. daf-16 is expressed in all of these tissues, suggesting that
these phenotypic changes, including the metabolic shifts, may be
induced by DAF-16-responsive genes.

daf-2 and age-1 mutations also cause an increase in lifespan4,5,18.

These effects are suppressed by daf-16 mutations4,5,18. Thus DAF-16
might directly regulate the genes necessary for increased longevity.
For example, dauer larvae express higher superoxide dismutase
levels, which might protect them from oxygen radicals19. This gene
can be directly regulated by DAF-16. The suppression of the
longevityincreases of daf-2 and age-1 mutants by daf-16 mutations
suggests that other outputs of the DAF-2/AGE-1 signalling pathway,
for example the regulation of glucose transport or metabolic
enzyme activities, are not relevant to lifespan or dauer arrest in
the absence of DAF-16 transcriptional regulation.

In extensive genetic screens for daf mutants, the only suppressors
of daf-2 that have been identified are daf-16 (many alleles)6,20 and
daf-18 (one allele)20. Therefore, daf-16 is likely to represent the
principal negatively regulated output of this insulin receptor-like
signalling pathway. The lack of other daf-2 suppressors argues
towards the existence of another entire ligand/receptor signalling
pathway between daf-2 and daf-16 and suggests that daf-16 acts in
the same cell as daf-2.

The finding that DAF-16 encodes proteins highly related to the
Fork head class of transcription factors suggests a molecular model
for the negative regulation of DAF-16 by upstream DAF-2/AGE-1
signalling (Fig. 4). DAF-2 insulin receptor-like engagement by an
unidentified ligand activates the AGE-1 phosphatidylinositol–3
OH kinase (PI(3)K). In mammals, PI(3)K signalling activates protein kinase cascade, including the AKT/PKB kinase21 that
regulates metabolic enzyme activities as well as glucose transport22.
We suggest that the homologous DAF-2/AGE-1 regulated kinase
cascade couples to the DAF-16 transcription factor (as well as to
metabolic enzymes and transporters). We suggest that in the
absence of DAF-2/AGE-1 signalling inputs, DAF-16 acts as a
repressor of metabolic genes that mediate energy usage (Fig. 4b).
The repression of these genes causes a metabolic shift to energy
storage that can be relieved by loss of daf-16 gene activity. When the
DAF-2/AGE-1 signalling cascade is activated under reproductive
growth conditions, we suggest that a kinase cascade leads to the
modification of the transcriptional activity of DAF-16 (Fig. 4a).
This signalling may either cause DAF-16 to be an activator (Fig. 4a)
or inactivate DAF-16 repressor function to now allow the expression
of metabolic genes necessary for reproductive development.

There is precedent for the model that DAF-16 transcriptional
activity is modulated by upstream tyrosine kinase signals: in C.
elegans vulval cell lineages, the activity of the LET-23 EGF receptor
kinase is coupled to the activity of the LIN-31 Fork head transcrip-
tion factor (P. Tan and S. Kim, personal communication). And
similar to the suppression of daf-2 mutant phenotypes by daf-16
null mutations, lin-31 mutations bypass the normal requirement
of upstream LET-23 EGF-like receptor signalling for the execution
of particular vulval cell lineages23.

In addition to the DAF-2/DAF-16 pathway, the activity of the
parallel DAF-7 TGF-β pathway also regulates C. elegans metabo-
lism. We find genetic synergy between the DAF-2 and DAF-7
signalling pathways by comparing the phenotype of a daf-2; daf-1 double mutant to the phenotype of each single mutant (Table 2). This synergy, together with the similar phenotypes of mutants in the two pathways, implies that the pathways cooperate to control reproductive versus dauer development and metabolism. DAF-7 TGF-β is expressed by the exposed sensory neuron ASI under conditions that induce reproductive development but not in dauer pheromone.\(^1\) Mutations that decrease or eliminate DAF-7 activity or the activities of the type I receptor DAF-1 (ref. 23) and type II receptor DAF-4 (ref. 26), or probable downstream SMAD proteins DAF-8 (A. Estvez and D. L. Riddle, personal communication), and DAF-14 (T. Inoue and J. Thomas, personal communication) cause arrest at the dauer stage and a metabolic shift to fat storage (Fig. 2). The dauer constitutive and fat storage phenotypes caused by mutations in these upstream TGF-β signalling genes are suppressed by null mutations in daf-3, which also encodes a SMAD protein\(^1\) (Fig. 2). Thus daf-3 acts in this TGF-β pathway analogously to daf-16 in the insulin-like pathway: daf-3 gene activity is negatively regulated by upstream TGF-β signalling.

Similar convergence between tyrosine kinase receptor-mediated and TGF-β-related signalling pathways has been noted in early vertebrate development.\(^22\) Precedent from the regulation of Xenopus early development by activin/Smad2 signalling suggests a molecular model for convergent insulin receptor tyrosine kinase and TGF-β receptor regulation of development and metabolism: the Fork head protein FAST1 directly interacts with Smad2 cooperatively to bind to an activin-response element on a gene that is transcriptionally regulated by activin.\(^26\) The synergy between mutations in the daf-2 insulin receptor-like and daf-1 TGF-β receptor suggests that insulin-like transducing DAF-16 may interact with the TGF-β signal transducing SMAD proteins DAF-3, DAF-8 and/or DAF-14 on the promoters of genes that regulate metabolism and reproductive versus dauer development (Fig. 4).

We suggest that under low-pheromone conditions, when both DAF-2 ligand and DAF-7 endocrine signals are produced, DAF-16 activity is modified by an upstream DAF-2/AGE-1 kinase cascade, and DAF-8 and DAF-14 are phosphorylated by upstream DAF-7/DAF-1/DAF-4 receptor kinase signalling to induce a heteromeric Fork head/SMAD transcription factor complex (Fig. 4a). The complex activates expression of genes that mediate reproductive growth and metabolism. Under dauer-inducing conditions, we suggest that a low level of DAF-2 ligand does not activate phosphorylation of DAF-16 by the DAF-2/AGE-1 signalling cascade, and a low level of DAF-7 TGF-β does not activate phosphorylation of DAF-8 and DAF-14 by the DAF-1/DAF-4 receptor kinases. In these dauer-inducing conditions, we suggest that DAF-16 and DAF-3 form heteromers that repress the expression of reproductive development and metabolic genes.

Because daf-3 (ref. 8), daf-4 (ref. 8) and daf-16 GFP fusion genes are widely expressed, we suggest that the integration of DAF-2 insulin receptor-like and DAF-7 TGF-β signalling takes place in the target tissues where the metabolic shifts are noted (Figs 1 and 4). The genes regulated by the Fork head/SMAD transcriptional regulatory complex may correspond to the C. elegans homologues of mammalian genes, such as those that encode PEPCK and the Glut4 glucose transporter, which are transcriptionally regulated by insulin.\(^25\) However, the molecular model does not depend on the signal integration taking place in target tissues. The signals may converge only in key regulatory cells, which in turn generate signals that regulate target tissue metabolism and development. In such a case, the downstream genes whose transcription is regulated by DAF-16 and DAF-3, DAF-8 and DAF-14 would not be target-tissue metabolic-control genes analogous to those regulated by insulin, but rather genes that encode other endocrine hormones.

It may be significant to the development of treatments for diabetes that C. elegans carrying mutations in daf-16 can grow reproductively even if they also carry daf-2 mutations that disable insulin-receptor-like metabolic-control signals (Table 1). Thus the genetics yield the surprising result that these animals can live normally without insulin-receptor-like signalling if they are also mutant in daf-16. Some of the human metabolic defects that result from declines in insulin signalling in both type I and type II diabetes may be caused by unregulated activity of the human DAF-16 orthologues FKHR and AFX, in turn to cause changes in the expression of metabolic control enzymes. Inhibition of FKHR or AFX activities, for example by pharmaceutical agents that inhibit DNA binding or association with transcriptional partners, may ameliorate the metabolic defects caused by declines in insulin signalling. The more acute insulin responses, for example in glucose uptake and in metabolic enzyme activities, would not be directly affected by these transcriptional regulators.

The molecular model for convergent insulin-like and TGF-β signalling suggests that these pathways meet on the promoters of metabolic-control genes. Given such a mechanistic connection between the C. elegans insulin-like and TGF-β transcriptional outputs, this molecular complex may be conserved across phylogeny. We propose that, as in C. elegans, both insulin and a DAF-7-like neuroendocrine signal may be necessary for metabolic control in humans. Accordingly, the failure of target tissues to respond to insulin signals in type II diabetic patients could be due to defects in either the insulin or TGF-β signalling pathways. Genetic predisposition to type II diabetes has been noted. Declines in signalling from the human orthologues of DAF-7 (or in any of the signalling molecules downstream of DAF-7), could underlie the lack of response to insulin in type II diabetes, just as lack of DAF-7 signalling in C. elegans causes very similar metabolic defects as lack of DAF-2 signalling. Obesity is a major risk factor in type II diabetes. One attractive model for the connection between obesity and insulin signalling defects is that metabolic signals analogous to the dauer pheromone, which may be a fatty acid\(^16\), or hormonal signals from fat cells may downregulate production of human DAF-7.

Methods
daf-16 molecular genetics. DNA segments from the gap in the cosmid coverage of the daf-16 genetic region were identified by probing a fosmid library with the end of cosmid T22A3, which defined the left endpoint of the gap that contains daf-16. One end of each fosmid so identified was identical to nucleotide sequence of two overlapping cosmids (C43H8 and B0511) near the end of a previously unlocalized 300 kb contig. Cosmids or PCR products from genomic sequence distributed along the length of this contig were used to detect RFLPs between C. elegans strains Bristol N2 and Bergerac RC301 (mgP45 on cosmid C39H11, mgP46 on cosmid F28D9, mgP49 on cosmid C35E7, and mgP50 on cosmid C43H8). These RFLP loci were mapped relative to the lin-11 daf-16 unc-75 genetic interval. Thirty-three Daf non-Unc recombinants from a daf-16(m27) unc-75(e950); daf-2(e1370)/daf-16(+)/RC301 unc-75(+)/RC301 strain and four Daf non-Lin recombinants from the strain lin-11(n566) daf-16(m27); daf-2(e1370)/lin-11(+)/RC301 daf-16(+)/RC301 were recovered. In the Daf non-Unc class, 13/33 recombinant chromosomes carried the Bergerac RC301 of RFLP mgP46, 2/30 recombinant chromosomes carried the Bergerac RC301 allele of mgP49, and 0/11 and 0/2 carry the Bergerac RC301 allele of RFLP mgP45 and mgP50. In the Daf non-Lin class, 3/4 of the recombinant chromosomes carried the Bergerac RC301 allele of RFLP mgP45 and 0/4 of the recombinant chromosomes carried the Bergerac RC301 allele of mgP49, daf-16(mgD50); daf-2(e1370) animals were injected with 100 ng µl\(^{-1}\) FR48 and 5 ng µl\(^{-1}\) of either cosmid RB3 or RB205. The extent of the deficiencies associated with daf-16(mgD47) and daf-16(mgD50) was further characterized by PCR and Southern blotting. To characterize point mutations, genomic DNA from daf-16(mg53), daf-16(mg54), daf-16(m26), daf-16(mg87) and other daf-16 mutant alleles was PCR-amplified and directly sequenced.

Assaying dauer formation. Characterization of daf-16 null and daf-3 null suppression of daf-2: synchronized egg broods were grown at 25 °C and scored ~48 h later. Numbers represent totals from 7 or 8 trials for each genotype performed on two different days. Assays for dauer formation on pheromone
were done as described\textsuperscript{31}. Genetic synergy between daf-1 and daf-2: synchronized egg broods were scored at 48 h and rescored at 72 h for 25°C trials, at about 66 and 90 h for both 18°C and 20°C trials, or at 95 and 120 h for 15°C trials. Numbers in Table 1 represent the summary of five trials of each genotype in two experiments performed on different days.

**Expression of daf-16.** A daf-16aGFP fusion was constructed in the following manner. The daf-16a promoter region containing ~6.9 kb of genomic DNA upstream of the daf-16 5′ UTR and including the 5′ UTR and most of exon 1 from daf-16 was PCR-amplified using primers containing the appropriate restriction sites. This genomic fragment was digested with Smal and BamHI and ligated to pHt95.75 (gift from A. Fire) digested with Smal and BamHI. The daf-16aGFP plasmid was injected at 50 ng μl\textsuperscript{-1}, together with prRF4 at 100 ng μl\textsuperscript{-1}.

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**Structure of the cyclin-dependent kinase inhibitor p19\textsuperscript{Ink4d}**

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In cancer, the biochemical pathways that are dominated by the two tumour-suppressor proteins, p53 and Rb, are the most frequently disrupted. Cyclin-dependent kinases phosphorylate Rb to control its activity and they are, in turn, specifically inhibited by the Ink4 family of cyclin-dependent kinase inhibitors (CDKIs) which cause arrest at the G1 phase of the cell cycle. Mutations in Rb, cyclin D1, its catalytic subunit CdK4, and the CDKI p16\textsuperscript{Ink4a} which alter the protein or its level of expression, are all strongly implicated in cancer. This suggests that the Rb ‘pathway’ is of particular importance\textsuperscript{1}. Here we report the structure of the p19\textsuperscript{Ink4d} protein, determined by NMR spectroscopy\textsuperscript{2,4}. The structure indicates that most mutations to the p16\textsuperscript{Ink4a} gene, which result in loss of function, are due to incorrectly folded and/or insoluble proteins\textsuperscript{2}. We propose a model for the interaction of Ink4 proteins with D-type cyclin-Cdk4/6 complexes that might provide a basis for the design of therapeutics against cancer.

The sequences of the Ink4 family of CDKIs are highly conserved (Fig. 1): there are at present four known family members, p15\textsuperscript{Ink4b}, p16\textsuperscript{Ink4a}, p18\textsuperscript{Ink4c} and p19\textsuperscript{Ink4d}. In contrast to the p21\textsuperscript{Cip1}/p27\textsuperscript{Kip1} family of CDKIs, which inhibit a broad range of CDKs, the Ink4 family is specific for CdK4 and CdK6 (refs 4, 6). The Ink4 proteins, which consist of four or more ankyrin repeats\textsuperscript{4}, are expressed in distinct tissue-specific patterns, suggesting that although they have essentially indistinguishable biochemical properties\textsuperscript{4}, they are not strictly redundant\textsuperscript{1}. p15\textsuperscript{Ink4b} is induced in human keratinocytes in response to the transforming growth factor TGF-β (ref. 8). Consistent with its role as a potent inhibitor of Rb phosphorylation, p16\textsuperscript{Ink4a} is altered in familial melanomas\textsuperscript{4}, and Ink4a\textsuperscript{-/-}\textsuperscript{12} mice spontaneously develop tumours\textsuperscript{13}. In addition, methylation of the 5′ CpG island is associated with transcriptional silencing of p16\textsuperscript{Ink4a} in human cancers\textsuperscript{11}. Both p18\textsuperscript{Ink4c} and p19\textsuperscript{Ink4d} are periodically expressed in proliferating macrophages (maximally during the S-phase of the cell cycle), suggesting that they might limit cyclin-D-dependent kinase activity once cells exit the G1 phase\textsuperscript{4}.

Intact mouse p19\textsuperscript{Ink4d} protein was purified from Escherichia coli following coexpression with the chaperone proteins GroEL and GroES\textsuperscript{22}. The recombinant p19\textsuperscript{Ink4d} protein was able to inhibit cyclin D1/Cdk4 at similar concentrations to p16\textsuperscript{Ink4a} (ref. 13, and data not shown). We found that p16\textsuperscript{Ink4a} and p19\textsuperscript{Ink4d} were equally defective in binding an R24C mutant of Cdk4 (ref. 14, and data not shown). These results are consistent with others suggesting that the two proteins have indistinguishable biochemical activities\textsuperscript{4}. However, p19\textsuperscript{Ink4d} aggregated less and gave better NMR spectra than p16\textsuperscript{Ink4a} at the high concentrations required for structural studies. H\textsuperscript{15}C and H\textsuperscript{15}N chemical shifts of p19\textsuperscript{Ink4d} were assigned after recording three- and four-dimensional (3D, 4D) double and triple resonance NMR spectra of H\textsuperscript{15}N- and H\textsuperscript{15}C/H\textsuperscript{15}N-labelled proteins. The elements of secondary structure (Fig. 1) were identified from the Cα and Cβ