Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes

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Regulation of body fat storage involves signalling between centres that regulate feeding in the brain and sites of fat storage and use in the body[1,2]. Here we describe an assay for analysing fat storage and mobilization in living *Caenorhabditis elegans*. By using RNA-mediated interference (RNAi)[3,4] to disrupt the expression of each of the 16,757 worm genes, we have systematically screened the *C. elegans* genome for genes necessary for normal fat storage. We identify 305 gene inactivations that cause reduced body fat and 112 gene inactivations that cause increased fat storage. Analysis of the fat-reducing gene inactivations in insulin, serotonin and tubby signalling mutants of *C. elegans*, which have increased body fat, identifies a core set of fat regulatory genes as well as pathway-specific fat regulators. Many of the newly identified worm fat regulatory genes have mammalian homologues, some of which are known to function in fat regulation. Other *C. elegans* fat regulatory genes that are conserved across animal phylogeny, but have not previously been implicated in fat storage, may point to ancient and universal features of fat storage regulation, and identify targets for treating obesity and its associated diseases.

We used the vital dye Nile Red (5H-benzo[α]phenoxazine-5-one, 9-diethylamino)[5] to visualize fat storage droplets in living worms (see ref. 27 and Supplementary Information for details of all Methods). Addition of Nile Red to *Escherichia coli*, the laboratory diet of *C. elegans*, resulted in uptake and incorporation of the dye into lipid droplets in intestinal cells, the principal site of worm fat storage (data not shown). The Nile Red pattern of fat staining matched the staining that resulted from feeding *C. elegans* fatty acids that had been covalently labelled with the fluorescent dye BODIPY, and was similar to the pattern of fat deposits previously observed after staining fixed animals with Sudan Black B (ref. 6 and data not shown). Nile Red staining did not affect growth rate, brood size, feeding or lifespan.

Alterations in the fat content of *C. elegans* mutants known to have modified fat storage were detectable by Nile Red staining. A neuroendocrine system comprising parallel insulin-like and transforming growth factor-β (TGF-β) signalling pathways controls whether *C. elegans* larvae grow to fast metabolizing or fat-storing adults[7]. The increased fat stores in animals with decreased *daf-2(e1370)* insulin-like signalling[6,8] were readily detectable in living animals by Nile Red staining (Fig. 1a). Consistent with epistasis analysis of the *daf* pathway, the increased fat of *daf-2* mutant animals was suppressed by mutations in the forkhead transcription factor *daf-16* (refs 9, 10) or in the *PTEN* phosphatase *daf-18* (ref. 11), but not by a mutation in the TGF-β pathway SMAD-like protein *daf-3* (ref. 7, Fig. 1a and Supplementary Fig. 1).

As in mammals, serotonin and tubby signalling pathways affect *C. elegans* body fat. Mice deficient either in *Tubby*, which encodes a conserved protein expressed in the hypothalamus, or in *HTR2C*, which encodes a serotonin receptor, are obese[12,13]. Similarly, worms with a deletion in a gene encoding a key serotonin biosynthetic enzyme, *tph-1(mg280)*[14], or in the *tubby* homologue, *tub-1(nr2004)*[15], show an increase in fat content detectable by Nile Red. *tub-1(nr2004)* mutants show roughly a twofold increase in fat content, whereas *tph-1(mg280)* mutants accumulate about 2.5-fold more fat than wild-type animals (Supplementary Fig. 1).

To correlate the differences in body fat visualized by Nile Red with actual fat content, we extracted total lipids from wild-type and mutant animals, separated them by thin layer chromatography into triacylglyceride and phospholipid components, and quantified them by gas chromatography. Consistent with the fat histochemical assessment, *daf-2(e1370)* and *tph-1(mg280)* animals had a greater total fat content than had wild-type animals (2.5-fold and 2-fold, respectively; Fig. 1b). The increased fat contents of these animals was strongly suppressed by the *daf-16(mgDf47)* null mutation. In addition, excess fat in *daf-2(e1370)* or *tph-1(mg280)* animals was largely in the form of storage triacylglycerides, suggesting that in *C. elegans*, as in mammals, triacylglycerides constitute the main form of fat storage.

C. elegans genes can be inactivated by RNAi initiated with double-stranded RNA expressed in the *E. coli* food[3,4]. A double-stranded RNAi bacterial library with 86% of the 19,000 *C. elegans* open reading frames has been constructed[4]. Analysis of this library shows that 50–90% of the genes previously identified by classical genetics can be inactivated by the clones in this library, although for genes that function in the nervous system the detection rate falls to

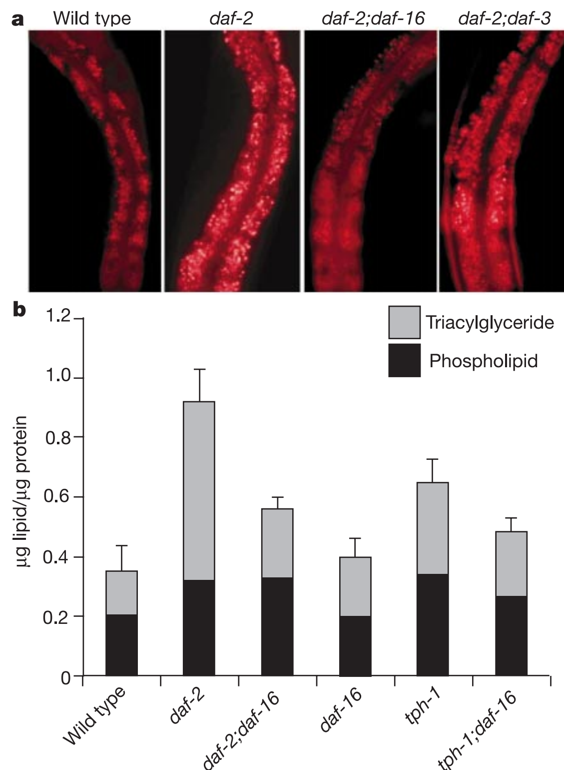

Figure 1 Changes in fat content in known mutants can be detected by Nile Red staining. **a**, Fat staining in non-starved young adult wild-type, insulin receptor mutant *daf-2(e1370)*, *daf-16(mgDf47);daf-2(e1370)* and *daf-2(e1370);daf-3(mgDf90)* animals (rhodamine filter, original magnification ×160). In each image, anterior is positioned to the bottom. **b**, Differences in Nile Red staining correspond to actual fat content measured by gas chromatography. Total lipid content, comprising triacylglycerides and phospholipids, is shown for the indicated animals. Values have been normalized to protein content extracted (refs 28, 29) from the same worms and are the means of two measurements from two independent extractions. Error bars indicate the standard deviation.

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about 12% (refs 4, 16). Using the Nile Red fat assay and the RNAi library, we screened for gene inactivations that affect fat content, fat droplet morphology and the pattern of fat droplet deposition. Of 16,757 genes tested by RNAi, 1.8% (305 genes) caused reduced fat or a distorted fat deposition pattern, and 0.7% (112 genes) resulted in animals with increased fat or an enlarged fat droplet size (Tables 1 and 2 give a partial gene list; see Supplementary Tables 1 and 2 for the full gene lists. DNA and protein sequences corresponding to each *C. elegans* GenePairs designation are available at Wormbase; www.wormbase.org). RNAi inactivation of another 261 genes caused reduced fat but was also accompanied by larval arrest, embryonic lethality or sterility (data not shown). This latter group includes known genes with essential roles in fat biosynthesis and metabolism, such as acetyl-coenzyme A (CoA) carboxylase, fatty-acid synthase and the fatty-acid desaturase *fat-7*. Although the identity of these genes endorses the approach, the decreased viability makes it difficult to disentangle the fat regulatory activity of many of these 261 genes from their roles in general cellular maintenance.

The list of gene inactivations that cause altered fat content, but do not appreciably affect viability or fertility, reflects a wide range of pathways and biochemical identities. Some of these genes are already known to have a key role in mammalian fat or lipid metabolism. For example, reduced amounts of stored fat resulted from RNAi of genes encoding worm homologues of enzymatic components of the membrane lipid biosynthetic machinery (such as choline/ethanolamine phosphotransferase, and CDP-alcohol phosphatidyltransferase), β-oxidation (Δ2, Δ4, dienoylCoA reductase, 3-hydroxyacyl-CoA dehydrogenase, long chain acyl-CoA thioesterase), fatty-acid elongation enzymes and cytosolic fatty-acid- and acyl-CoA-binding proteins. Although it is not surprising that a decrease in gene activity of fat biosynthetic enzymes would lead to a reduction in fat storage, it is less obvious why decreased gene expression of fat β-oxidation machinery would result in a similar phenotype. It is possible that inhibition of β-oxidation may produce feedback signals that alter the intake and metabolism of nutrients (see Fig. 2 for examples).

Reduced or disorganized fat deposition patterns were also caused by RNAi of several known components of sterol metabolism (such as sterol regulatory element-binding protein[17]), by RNAi of *C. elegans* homologues of genes that function in mammalian flux of glucose and glycerol energy metabolism (such as glyceraldehyde-3-phosphate dehydrogenase[18] and phosphoenolpyruvate carboxykinase[19]), and by RNAi of *C. elegans* genes whose mammalian homologues are involved in gastrointestinal digestion and uptake of food (such as the oligopeptide transporter PepT1 (ref. 20) and lipases; Fig. 2).

In addition, RNAi of specific nuclear hormone receptor genes caused a reduction or an increase in fat content. Nuclear hormone receptors are known regulators of mammalian fat and sterol metabolism[21]. Notably, increased fat was caused by RNAi of a worm homologue of hepatocyte nuclear factor 4-α (HNF4-α), mutations of which are associated with maturity onset diabetes of the young[22]. We also observed fat phenotypes produced by RNAi of several cytochrome P450 enzymes. These enzymes may be regulated by or metabolize the ligands of the nuclear hormone receptors. The predicted *C. elegans* gene, C43H6.8, a potential orthologue of the haematopoetic/neurogenic transcription factor Nhlh-2/Nscl-2 (ref. 23), is another fat regulatory transcription factor that is conserved between mammals and *C. elegans.* Knock out of this

Table 1 **RNAi clones that reduce fat in wild type and high-fat mutants**

GenePairs*	Description	WT	*daf-2*	*tph-1*	*tub-1*
Metabolic enzyme					
F52B11.2	Phosphomannomutase 2	Y	Y		
Y55F3C.c	Thioredoxin	Y	Y		
Y49A3A.1	CE phosphotransferase	Y	Y		
C06E7.3	*S*-adenosylmethionine synthase	Y	Y	Y	Y
C36A4.9	Acetyl-CoA synthetase	Y	Y	Y	Y
F08F8.2	HMG-CoA reductase	Y	Y	Y	Y
K10B3.7	GAPDH	Y	Y	Y	Y
F11E6.5	Fatty acid elongase	Y	Y	Y	Y
F13D11.1	Lysosomal acid phosphatase	Y	Y	Y	Y
K07C6.5	Cytochrome P450 2C2	Y	Y	Y	Y
K09D9.2	Cytochrome P450	Y	Y	Y	Y
Fat/lipid interacting					
R07B7.9	Brush border esterase/lipase	Y	Y		
F13D12.6	Esterase/lipase	Y	Y	Y	Y
Transcription factor					
ZK686.4	Zinc-finger C_2H_2 type	Y	Y		
F11A1.3	NHR (dihydroxyvitamin D3)	Y	Y		
T09F3.1	Zinc-finger C_2H_2 type	Y	Y		
K08A2.b	NHR (HNF-4α)	Y	Y		
F22A3.5	Pre-B-cell leukaemia factor	Y	Y		Y
Y47D3B.7	SREBP	Y	Y	Y	Y
Signal transduction					
C33H5.17	D111/G-patch domain	Y	Y		
T04D3.2	EF-hand family domain	Y	Y		
ZC302.1	Serine/threonine protein phosphatase	Y	Y		
ZC504.4	Tyrosine and serine/threonine kinase	Y	Y		
T19D2.2	Dual specificity phosphatase	Y	Y	Y	
F46G11.3	Protein kinase	Y	Y	Y	Y
M01B12.5	Tyrosine kinase	Y	Y	Y	Y
ZK675.1	Patched	Y	Y	Y	Y
Receptor					
H09F14.1	GPCR	Y	Y		
C38C10.1	Neurokinin-type receptor	Y	Y		
F07C4.1	GPCR	Y	Y	Y	Y
T07C12.5	GPCR	Y	Y	Y	Y
T14E8.3	Dopamine receptor	Y	Y	Y	Y
Y40H7A.7	SRA family chemoreceptor	Y	Y	Y	Y
Neuronal					
H27A22.1	Glutaminyl cyclase	Y	Y		
T19B4.7	DCC/axon guidance	Y	Y		

GenePairs*	Description	WT	daf-2	*tph-1*	*tub-1*
Transporter					
C37A5.1	Ion channel	Y	Y		
ZK682.2	Sugar transporter	Y	Y		
K05F1.6	OCT1 organic carrier	Y	Y		
C13D9.7	Na^+/Ca^{2+} exchanger	Y	Y		Y
C32C4.1	Potassium channel	Y	Y		Y
F15H10.4	Lysosomal transporter	Y	Y	Y	Y
K04E7.2	PepT1 symporter	Y	Y	Y	Y
C34G6.4	ABC transporter	Y	Y	Y	Y
Energy metablism					
F20D1.9	Uncoupling protein	Y	Y		
F28H6.2	Energy transfer protein	Y	Y		Y
Vesicular transport					
F11A5.3	RAB2	Y	Y		
R01H2.3	Sortilin	Y	Y	Y	Y
F53H8.1	Clathrin adaptor chain	Y	Y	Y	Y
Protein degradation					
Y65B4B10e	Ubiquitin protein ligase	Y	Y		
F49E12.4	Ubiquitin-conjugating enzyme	Y	Y	Y	
C49C3.3	Ubiquitin family	Y	Y	Y	Y
Other					
Y77E11A	Collagen	Y	Y		
C06G3.2	Kinesin motor domain	Y	Y	Y	
R04A9.4	Translation initiation	Y	Y	Y	
No function assigned					
Y24D9A.b	Unknown	Y	Y		
B0034.2	Unknown	Y	Y		
C30G4.5	Unknown	Y	Y		
T10D4.1	Unknown	Y	Y		
R08F11.2	Unknown	Y	Y		
K12D12.4	Unknown	Y	Y		
B0554.7	Unknown	Y	Y		
F46F5.10	Unknown	Y	Y		
F10A3.11	Unknown	Y	Y		
B0554.6	Unknown	Y	Y	Y	
T01D3.4	Unknown	Y	Y		Y
C18E9.5	Unknown	Y	Y		Y
K09H11.2	Unknown	Y	Y	Y	Y
ZK131.8	Unknown	Y	Y	Y	Y
F52C6.12	Unknown	Y	Y	Y	Y
F59F5.2	Unknown	Y	Y	Y	Y

Clones were tested on wild-type (WT), *daf-2(e1370)*, *tph-1(mg280)* and *tub-1(nr2004)* animals. 'Y' indicates that RNAi caused a reduced fat phenotype in the fat content of the test strain. ABC, ATP-binding cassette. NHR, nuclear hormone receptor; GPCR, G-protein-coupled receptor; DCC, deleted in colorectal cancer.

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transcription factor causes obesity in mice[23] and RNAi of C43H6.8 resulted in an increase in fat content (Fig. 2).

RNAi of several *C. elegans* genes that may function in food sensation and neuroendocrine signalling also resulted in aberrant fat content. RNAi of a likely glutamate receptor and of a protein with homology to rat hippocampal somatostatin receptor led to increased fat storage. Reduced fat was caused by RNAi of the potential orthologue of glutaminyl cyclase (required for biosynthesis of pyroglutamyl peptides), a dopamine D2-like receptor, and several chemoreceptors and olfactory receptors. The list of genes that regulate fat is unexpectedly diverse. A future challenge for genome-wide functional studies is the organization and prioritization of the identified genes. It is likely, for example, that both signal transduction components and their targets have been identified in this screen.

To organize the newly identified fat regulatory genes into signalling pathways, we tested the effect of each of the 305 gene inactivations on three *C. elegans* mutant strains with neuroendocrine signalling defects that result in increased fat: the *daf-2* insulin receptor mutant[6], the *tph-1* serotonin deficient mutant[14], and the *tub-1* tubby orthologue mutant[15]. Of the 305 RNAi clones tested, 32 (11%) caused substantial reductions in fat in wild type and in each of the three obese strains (Table 1). This group may represent the key fat regulatory genes that, when inactivated, potently and specifically affect fat storage without obvious side-effects on the general health of the animal. A third of the genes on this list correspond to metabolic enzymes and other well-characterized fat homeostasis genes. But many receptors, signal transduction molecules, transporters and vesicular transport molecules are also present on this list.

The transporters and signal transduction components may constitute central factors in the transport of fat, or in the feedback signalling from fat storage tissues to neuronal regulators of feeding and metabolism. Similarly, the vesicular sorting and protein degradation components may be the molecules that regulate the cellular trafficking of fat/metabolite transporters and receptors. These findings endorse the notion that body weight is the integration of several inputs, even in a relatively simple organism like *C. elegans*. Notably, most of these *C. elegans* fat regulatory genes have mammalian homologues.

Another set of RNAi clones showed a distinct pattern of reversing the high fat phenotype: numerous RNAi clones that potently reduced fat content in wild-type or *daf-2* animals failed to alter the increased fat content of *tph-1* and *tub-1* mutant animals (Table 1). There was a strong correlation (86%) between the effectiveness or failure of these RNAi clones to modulate fat quantities in either *tph-1* or *tub-1* mutant animals. Failure of an RNAi clone in this group to generate fat reduction in either the *tph-1* or *tub-1* mutant could not be attributed to weak or partial RNAi activity, because RNAi clones in this group were effective against *daf-2* animals, which had the most fat among the strains tested. These results indicate that the fat-increasing signals caused by the *tph-1* and *tub-1* mutations share at least one common pathway that is separable from the fat-increasing signal caused by a *daf-2* mutation. This notion is supported by the fact that both worm serotonin[14] and tubby signalling pathways act in the nervous system (unpublished observations), whereas the fat regulatory activity of the insulin like pathway is not limited to the nervous system[24,25].

By contrast, another set of RNAi clones reduced fat in wild type, *tph-1* or *tub-1* animals, but not in *daf-2* mutant animals. Notably, this list was enriched in signal transduction molecules and receptors (Supplementary Table 3). We suggest that the vesicular transporters, receptors, and signal transduction molecules on this list are candidates for transmitting fat regulatory signals from tissues affected by the *tph-1* and *tub-1* mutations. Analogous roles in neuroendocrine control of body weight regulation for mammalian homologues of these genes would be expected. Finally, 52% of the fat-reducing RNAi clones caused fat reduction only in wild type, but did not affect the greater fat content of *daf-2*, *tph-1* and *tub-1* mutants (Supplementary Table 1). These genes may have modulatory roles in the regulation of organismal fat.

The diverse array of the genes identified in our screen provides an unprecedented glimpse of the cellular machineries that regulate body fat content and deposition. To highlight the shared ancestry of *C. elegans* and mammalian fat storage regulation, we could identify *C. elegans* genes for which the mammalian homologues have already been implicated in body weight content. The concordance between the list of *C. elegans* and mammalian fat regulatory and obesity genes suggests that these animals show ancient and common pathways of body fat regulation.

Notably, over 50% of the *C. elegans* fat regulatory genes identified in our screen have mammalian homologues that have not been previously implicated in regulating fat storage. It is possible that homologues of these newly identified *C. elegans* fat regulatory genes also control mammalian body weight. Their contributions to

Table 2 **Partial list of RNAi targets that produce increased fat content**

GenePairs*	Description	GenePairs*	Description
Metabolic enzyme (7)		Signal transduction (15)	
E04F6.3	Hydroxysteroid 17-β dehydrogenase	K08F8.1	Ribosomal S6 kinase
F28F8.2	Long-chain fatty-acid CoA ligase	F56H11.6	Casein/tau-tubulin kinase
ZC513.1	Phospholipid transfer protein	C04G2.2	Serine/threonine kinase
C33A12.6	UDP-glucosyl transferase	F39B1.1	Phosphoinositide 3-kinase
VF13D12L.1	Myo-inositol-1-phosphate synthase	W08D2.1	Wnt-1 family kinase
Transcription factors (9)		W03A5.4	Guanylate kinase associated
C43H6.8	Nhlh2/Nscl-2	C24F3.2	Glucokinase-associated. phosphatase
K10C3.6	NHF-4 α subtype NHR	F46C5.6	Protein phosphatase PP2A
F33D4.1	Oestrogen-type NHR	T04C9.1	Oligophrenin-1 GTPase
C56C10.10	Aryl hydrocarbon NHR	Y11D7A.9	FGF receptor activating protein
C37F5.1	Elk-1	C18H9.7	RAPSN
R11H6.5	Interleukin enhancer factor 2	Channels/transporters (4)	
H12C20.3	C4-type steroid receptor	ZC410.4	Potassium channel
Receptors (6)		F52H2.2	Amino acid permease
F56B6.5	Somatostatin receptor-type	F14E5.1	Glucose transporter-3
C43H6.9	Glutamate receptor	Cell surface/structural (5)	
Y27F2A.g	Chemoreceptor	C34F6.3	Collagen triple helix repeat
Y46H3C_11.b	GPCR	Y38F1A.9	Contactin 6/myopalladin
Vesicular transport (3)		No function assigned (62)	
F08H9.5	Cubilin/endocytic receptor		
C04G2.4	Vesicle associated protein		

*For each RNAi clone the Research Genetics GenePairs designation is provided. Genes corresponding to the RNAi clones were grouped into functional classes. Total number of genes assigned to each functional category appear in parenthesis. A full list including the extent of increase in fat content is given in the Supplementary Information. FGF, fibroblast, growth factor. NHR, nuclear hormone receptor; GPCR, G-protein-coupled receptor; RAPSN, acetylcholine receptor-associated protein.

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mammalian body fat regulation can be studied in tissue culture or in rodent models. Given the complex interactions between fat cells and the central nervous system[1,2], however, the study of fat regulation in a physiologically intact animal, even an animal as distant from mammals as *C. elegans*, can provide insights that are not possible from studying, for example, isolated mammalian adipocytes.

Indeed, an important aspect of body weight regulation in both mammals and *C. elegans* is the profound link between feeding and body fat content in both systems. Despite the fact that *C. elegans* lacks dedicated adipocytes and sequence identifiable leptin, worm body fat is likely to be coupled to food sensation. In addition to the known impact of insulin and serotonin signalling, our results suggest the involvement of dopaminergic and glutamate signalling in regulating body fat. The G-protein-coupled receptors (GPCRs) detected in our RNAi analysis may lead to identification of neuropeptide and other neuroendocrine signalling pathways that regulate fat content and feeding behaviour. Further studies of the rate of feeding in animals with deficits in these receptors might distinguish whether the affected pathways regulate food intake behaviour or metabolism.

Many other *C. elegans* genes that regulate body fat remain to be identified. Not all genes are responsive to RNAi inactivation. Some genes, most notably neuronally expressed genes, may be only partially inactivated by RNAi. The current RNAi library covers about 86% of the known and predicted genes, and certain classes of gene may be missing or underrepresented in it. In addition, some gene inactivations probably manifest phenotypes only under specific environmental conditions or in specific sensitised genetic backgrounds.

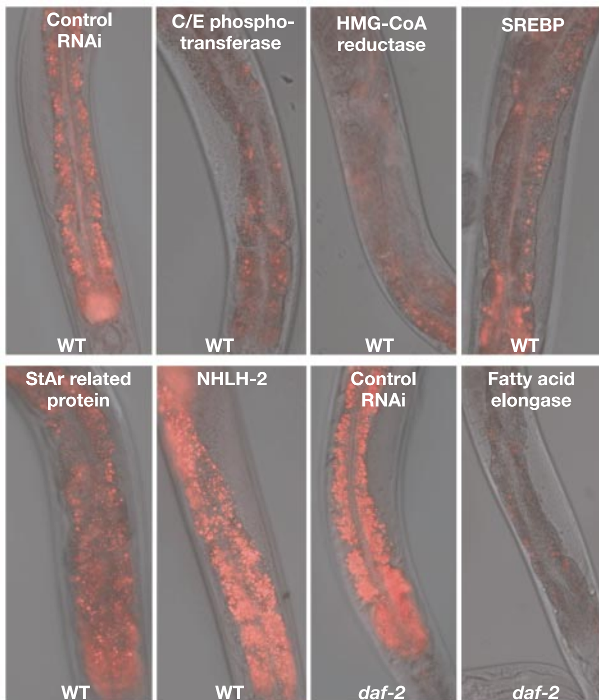

Figure 2 Examples of RNAi targets that produce alterations in fat content. Wild-type and *daf-2(e1370)* animals stained with Nile Red are shown. Reduced fat staining of wild-type animals was caused by RNAi of Y49A3A.1 (choline/ethanolamine phosphotrasferase), F08F8.2 (hydroxymethylglutaryl-CoA reductase) and Y47D3B.7 (SREBP). Distorted fat deposition pattern was caused by RNAi of K02D3.2 (steroidegenic acute regulatory related protein). Increased fat was caused by RNAi of C43H6.8 (Nhlh2 transcription factor). RNAi of F11E6.5 (fatty-acid elongase) caused reduced fat in *daf-2(1370)* animals. An L4440 vector was used for control RNAi. Overlays of Nomarski and rhodamine images are shown. In each image, anterior is positioned to the bottom.

A genome-wide RNAi screen combines the capacity of genetics to identify key components of pathways, regardless of transcriptional status, with the comprehensiveness and facility of transcriptional profiling studies. Unlike the list of genes generated from transcriptional profiling studies, a genetic function in *C. elegans* is already established for the list of genes generated by this genome-wide RNAi screen. But a useful way to prioritize our list of fat regulatory genes will be to compare it with the lists of transcriptionally regulated genes in various models of obesity.

An immediate application of the study of *C. elegans* fat regulation will be to pedigree analysis in human obesity and lipodystrophy syndromes and to quantitative trait studies in rodents. Genes discovered in *C. elegans* can point to candidate obesity or diabetes loci in the broad chromosomal regions identified in human or rodent studies[26]. Our list of *C. elegans* fat regulatory genes identifies about 150 of the 30,000 mammalian genes that might be tested for variants in obese or diabetic populations. About 20% of the *C. elegans* genes identified in this screen caused an increase in fat when inactivated, suggesting that loss-of-function mutations in their mammalian homologues could underlie obesity. But most of the fat regulatory genes that we identified caused a decrease in fat storage and thus would be expected to cause mammalian obesity only if activated by mutation. Because inactivation of some these genes diminishes fat content even in *C. elegans* mutants with defects in insulin, serotonin and tubby signalling pathways, these genes are promising candidates for developing drugs to treat obesity and its associated diseases. It is significant that many of the genes encode enzymes or proteins that are members of families with known small-molecule regulators (such as nuclear hormone receptors, potassium channels, neurotransmitter receptors and kinases), or small active sites that are good candidates for drug development. □

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Coupling of agonist binding to channel gating in the $GABA_A$ receptor

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Neurotransmitters such as acetylcholine and GABA (γ-amino-butyric acid) mediate rapid synaptic transmission by activating receptors belonging to the gene superfamily of ligand-gated ion channels (LGICs)[1]. These channels are pentameric proteins that function as signal transducers, converting chemical messages into electrical signals[2]. Neurotransmitters activate LGICs by interacting with a ligand-binding site[3–7], triggering a conformational change in the protein that results in the opening of an ion channel[8]. This process, which is known as 'gating', occurs rapidly and reversibly, but the molecular rearrangements involved are not well understood[9]. Here we show that optimal gating in the $GABA_A$ receptor, a member of the LGIC superfamily, is dependent on electrostatic interactions between the negatively charged Asp 57 and Asp 149 residues in extracellular loops 2 and 7, and the positively charged Lys 279 residue in the transmembrane 2–3 linker region of the α_1-subunit. During gating, Asp 149 and Lys 279 seem to move closer to one another, providing a potential mechanism for the coupling of ligand binding to opening of the ion channel.

Many inherited mutations in the transmembrane 2–3 linker (2–3L) region of LGICs alter gating and are associated with human diseases: defects in the nicotinic acetylcholine receptor (nAChR) give rise to forms of myasthenic syndrome[10], defects in the glycine receptor are associated with hyperekplexia[11], and defects in the $GABA_A$ receptor ($GABA_A$-R) are associated with rare forms of epilepsy[12]. The clustering of gating mutations in the 2–3L region has led to the suggestion that this loop is essential for communicating the conformational changes that result from interaction of neurotransmitter with the ligand-binding site to the transmembrane domain and its integral ion channel[9,13].

An acetylcholine-binding protein (AChBP) in the snail *Lymnaea stagnalis* has been found to have a high degree of homology with the extracellular domains of LGIC subunits, and its structure has been solved at high resolution[4]. The location of two flexible loops (loops 2 and 7) in this structure is striking. As pointed out by others[9], "in the full receptor, the signature loop is positioned to interact directly with the membrane, or possibly with the transmembrane regions (or the short sequence connecting two of them) of the receptor. The implication is that the signature loop might be involved in 'gating'.". In the LGIC subunits, the 'signature loop' (loop 7) is formed between two cysteine residues that are joined by a disulphide bond, which is an essential feature of these proteins. We also reasoned that homologous loops in the $GABA_A$-R would be positioned close to the interface between the extracellular domain and the membrane, enabling loops 2 and 7 to interact with the 2–3L region.

We aligned the amino acid sequences of the $GABA_A$-R α_1 subunit and the AChBP (see Supplementary Information) and found that loops 2 and 7 in the $GABA_A$-R α-subunits contain several highly conserved acidic residues, whereas the 2–3L region contains two highly conserved basic residues. We therefore proposed the hypothesis that electrostatic interactions between one or more residues in loops 2 and 7 and those in the 2–3L region contribute to the gating mechanism in the $GABA_A$-R. This hypothesis was tested by site-

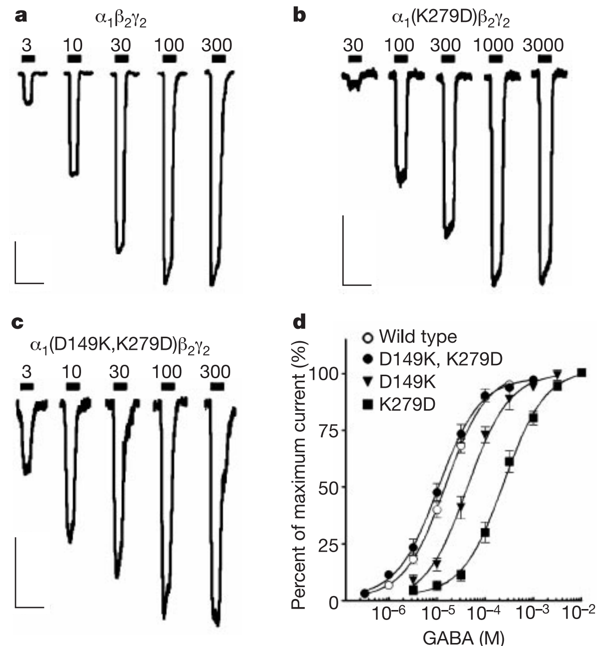

Figure 1 Charge exchange between positions 149 and 279 restores optimal GABA sensitivity. **a**–**c**, Representative recordings from HEK 293 cells transfected with $\alpha_1\beta_2\gamma_{2s}$ (**a**), α_1(K279D)$\beta_2\gamma_{2s}$ (**b**) and α_1(D149K,K279D)$\beta_2\gamma_{2s}$ (**c**) receptors. The bars above the recordings indicate the durations of GABA application, and the numbers above each bar indicate the concentration of applied GABA in micromoles. Scale bars, 200 pA and 10 s. **d**, Concentration–response curves for $\alpha_1\beta_2\gamma_{2s}$ (open circles), α_1(D149K)$\beta_2\gamma_{2s}$ (filled triangles), α_1(K279D)$\beta_2\gamma_{2s}$ (filled squares) and α_1(D149K, K279D)$\beta_2\gamma_{2s}$ (filled circles).

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