Developmental Biology 318 (2008) 153–161

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Insulin-like signaling negatively regulates muscle arm extension through DAF-12 in *Caenorhabditis elegans*

Scott J. Dixon [1], Mariam Alexander [1], Kevin Ka Ming Chan, Peter John Roy *

Department of Molecular Genetics, Collaborative Program in Developmental Biology, The Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON, Canada M5S 3E1

ARTICLE INFO

Article history:
Received for publication 7 January 2008
Revised 10 March 2008
Accepted 11 March 2008
Available online 20 March 2008

Keywords:
Muscle arms
Insulin
DAF-2
DAF-4
DAF-7
DAF-12
DAF-16
FOXO
Dauer
Caenorhabditis elegans

ABSTRACT

The body wall muscles (BWMs) of nematodes are connected to motor axons by muscle membrane extensions called muscle arms. To better understand how muscle arm extension is regulated, we screened conserved receptor tyrosine kinases for muscle arm defects in *Caenorhabditis elegans*. We discovered that mutations in *daf-2*, which encodes the only insulin-like receptor tyrosine kinase, confer a *s*uper*n*umerary muscle *a*rm (Sna) phenotype. The Sna phenotype of *daf-2* mutants is suppressed by loss-of-function in the canonical downstream FOXO-family transcription factor DAF-16 in either the muscles or the intestine, demonstrating that insulin-like signaling can regulate muscle arm extension non-autonomously. Furthermore, supernumerary arm extension requires the B isoform of the down-stream DAF-12 nuclear hormone receptor, which lacks the DNA-binding domain, but retains the ligand-binding domain. *daf-2* regulates many processes in *C. elegans* including entry into dauer, which is a diapause-like state that facilitates survival of harsh environmental conditions. We found that wild-type dauers are also Sna. Unlike other changes associated with dauer, however, the Sna phenotype of dauers persists in recovered adults. Finally, disruption of a TGF-β pathway that regulates dauer formation in parallel to the insulin-like pathway also confers the Sna phenotype. We conclude that supernumerary muscle arms are a novel dauer-specific modification that may facilitate some aspect of dauer behavior.

Introduction

The neuromuscular system of *Caenorhabditis elegans* is relatively simple, having only 302 neurons and 135 muscles in the adult hermaphrodite (Sulston and Horvitz, 1977). Although many of these cells are strikingly similar to their mammalian counterparts (Sulston and Horvitz, 1977; Sulston et al., 1983; White et al., 1986), nematodes differ in at least one important respect. In mammals, motor axons project to muscle cells (Buchthal and Schmalbruch, 1980; Prin et al., 2005), but in nematodes, the body wall muscles (BWMs) extend membrane processes called muscle arms to the motor axons (Dixon and Roy, 2005; Schneider, 1866; White et al., 1986) (see Fig. 1). Muscle arms are an important feature of the neuromuscular system of nematodes as they harbor the post-synaptic elements of the neuromuscular junction (White et al., 1986).

The 95 BWMs of the *C. elegans* adult are divided into four quadrants, each of which is subdivided into a distal and a proximal row with respect to the nearest nerve cord (Fig. 1A). Muscles of the dorsal quadrant extend muscle arms to the dorsal cord, and ventral BWMs extend arms to the ventral cord. Distal BWMs typically extend between three and five muscle arms, depending on the particular identity of the muscle cell (Dixon and Roy, 2005; Hall and Hedgecock, 1991). Muscle arms develop during both embryonic and larval stages. Embryonic muscle arms are thought to arise passively through the initial attachment of myoblast membrane to the juxtaposed embryonic axons, followed by myoblast movement away from the axon, leaving a trailing membrane behind (see Dixon et al., 2005 for more details). On average, distal BWMs have between one and two embryonic muscle arms, as judged by the number of arms in newly hatched first-stage larvae (L1s) (Dixon and Roy, 2005). In contrast to the passive nature of muscle arm development during embryogenesis, post-embryonic muscle arm development is an active process that is dependent on cytoskeletal remodeling (Dixon and Roy, 2005). The number of muscle arms present in L1 hatchlings is approximately doubled sometime between the mid-L1 to L3 stages (Dixon and Roy, 2005). This doubling in number is dependent on the birth of post-embryonic motor neurons, which presumably produce a factor that elicits muscle arm extension (Dixon and Roy, 2005). This idea is consistent with the observations that muscle arm extension is dependent on UNC-104/kinesin-mediated anterograde axonal transport of synaptic vesicles (Hall and Hedgecock, 1991) and the extension of muscle arms towards misguided motor axons (Hedgecock et al.,

* Corresponding author. Fax: +1 416 978 6885.
E-mail address: peter.roy@utoronto.ca (P.J. Roy).
[1] These authors contributed equally to this work.

0012-1606/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2008.03.019

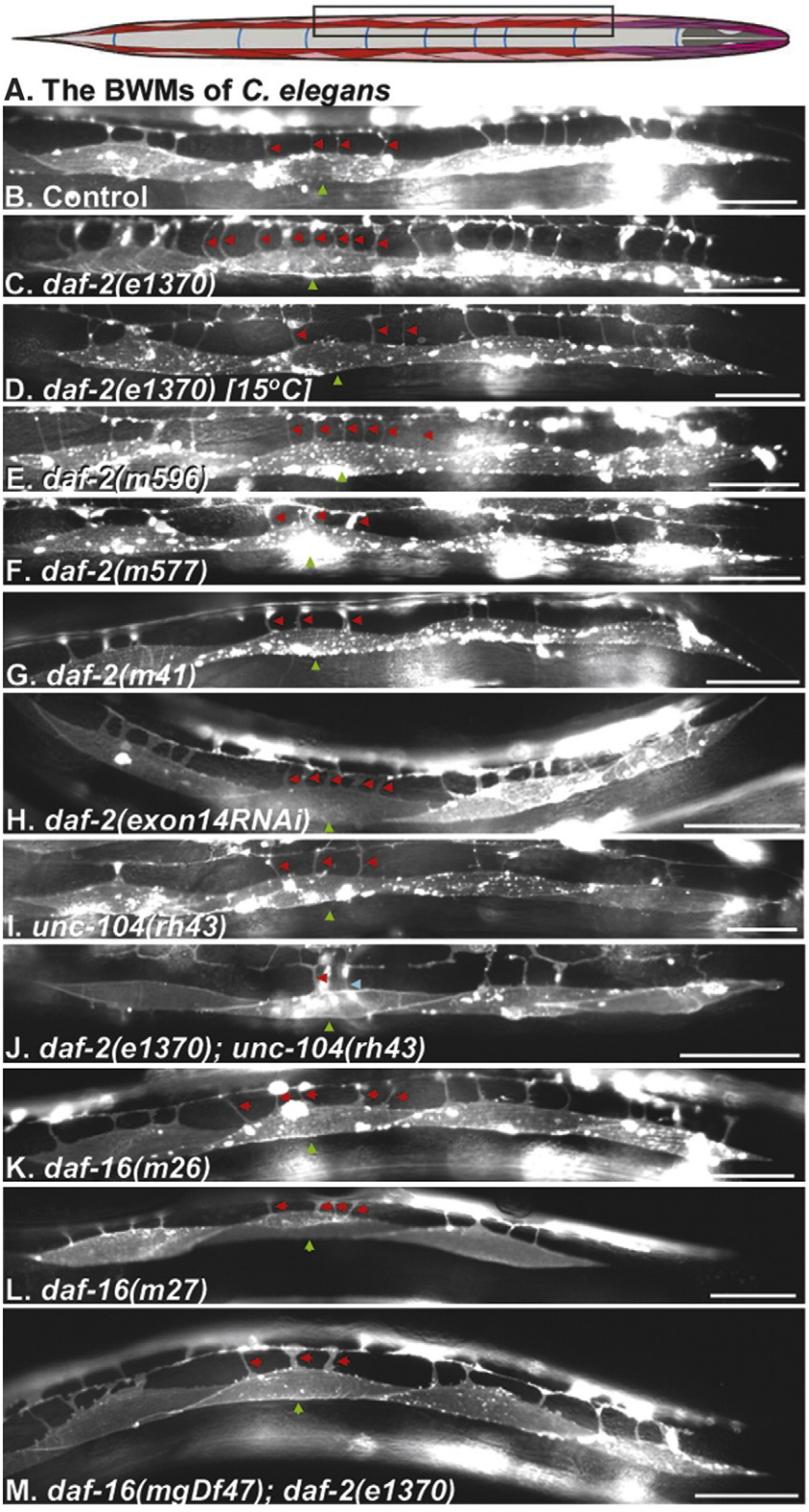

Fig. 1. Muscle arms of insulin pathway mutants. (A). A cartoon depicting the right side of the worm. The dorsal and ventral right BWM quadrants are shown along with rough position of commissural motor axons (blue). Each quadrant contains an inner and an outer row of muscles. Those muscles colored red express Mb::YFP from the *him-4* promoter. The boxed area indicates the region of the dorsal right BWM quadrant examined in all animals and presented below. (B–M) The nature of the mutation or loss of function is indicated. Muscle arms are visualized in all animals using the *trIs30* transgene, except panels K, L and M, which express MB::YFP from an extrachromosomal array *trEx* containing *him-4p::mb::yfp*. In all panels, muscles of young adults are shown and number 13 is indicated with a green arrow for reference, the muscle arms of muscle 13 are indicated with a red arrow. In panel J, a worm that has hatched internally in this animal is indicated with a blue arrow. The scale bar represents 50 uM.

1990). There is currently no marker that distinguishes larval muscle arms from embryonic ones, although null mutants that compromise muscle arm extension typically have between one and two muscle arms, which are likely embryonic in origin (Dixon et al., 2006; Dixon and Roy, 2005). In our ongoing effort to understand the development of muscle arms, we discovered that mutations in *daf-2*, which encodes

the lone insulin-like receptor tyrosine kinase in *C. elegans*, confer a *super*numerary muscle *a*rm (or Sna) phenotype.

Under non-stressed conditions, DAF-2 activates a conserved signaling cascade comprising phosphoinositide 3-kinase (PI3K), 3-Phosphoinositide-Dependent Kinase 1 (PDK-1) and AKT/Protein Kinase B (PKB)-1/2 (Morris et al., 1996; Paradis et al., 1999; Paradis and Ruvkun, 1998). AKT-1/2 phosphorylates the forkhead-family transcription factor DAF-16/FOXO, preventing it from accumulating in the nucleus, where it can regulate the expression of numerous genes that promote normal development (Cahill et al., 2001; Lin et al., 1997, 2001; Murphy et al., 2003; Ogg et al., 1997). Under persistent stress, such as overcrowding, starvation or high temperature, L1 and L2 animals down-regulate the expression of insulin-like ligands (Li et al., 2003). As a consequence, the activity of the DAF-2 signaling cascade is reduced, resulting in DAF-16 nuclear accumulation in many tissues (Henderson and Johnson, 2001; Lee et al., 2001; Libina et al., 2003; Lin et al., 2001). Nuclear DAF-16 ultimately results in reduced production of cholesterol-derived hormones by the DAF-9/cytochrome P450 synthetic pathway (Gerisch et al., 2001; Jia et al., 2002; Motola et al., 2006), which likely includes the Rieske-like oxygenase, DAF-36 (Rottiers et al., 2006). Acting in parallel to the insulin pathway, a TGF-β pathway similarly regulates the activity of DAF-9 (Liu et al., 2004; Rottiers and Antebi, 2006; Shaw et al., 2007).

In the absence of a DAF-9-derived hormone during early larval development, the nuclear hormone receptor DAF-12 suppresses genes involved in reproductive development and activates unknown genes that promote dauer formation (Antebi et al., 1998, 2000; Ludewig et al., 2004; Riddle and Albert, 1997). Dauers are an alternative L3 stage that facilitate survival of harsh environments and dispersal to new ones (Cassada and Russell, 1975; Golden and Riddle, 1984; Maupas, 1899). Dauer perdurance is promoted by decreased metabolism via a dauer-specific buccal cavity plug that prevents feeding and a dependence on anaerobic respiration (Riddle et al., 1981; Wadsworth and Riddle, 1989). In addition, a stress-resistant dauer cuticle, and increased Hsp90 and superoxide dismutase expression likely extend the life of existing cellular machinery (Cassada and Russell, 1975; Dalley and Golomb, 1992; Larsen, 1993). In contrast to dauer perdurance, less is known about the cellular changes that govern dispersal behaviors, such as the proclivity to climb and bursts of rapid movement (Cassada and Russell, 1975).

Our finding that multiple components of the insulin-like signaling pathway regulate muscle arm extension led to the discovery that wild-type dauers also extend supernumerary muscle arms. Unlike other cellular modifications elicited by the dauer program, such as the remodeling of the cuticle, pharynx and certain neurons (Riddle, 1988; Riddle and Albert, 1997), the Sna phenotype of dauers persists in recovered adults. Because the Sna phenotype is also conferred by mutations in the TGF-β pathway that regulates dauer entry, we conclude that supernumerary muscle arm extension is a novel, dauer-regulated morphological adaptation and may facilitate some aspect of dauer behavior.

Materials and methods

Strains, transgenics and induction of dauer formation

Unless otherwise indicated, nematodes were cultured at 20 °C using standard methods (Brenner, 1974). The following strains were obtained from the CGC: DR27 *daf-16(m27)* I, DR26 *daf-16(m26)* I, GR1309 *daf-16(mgDf47)* I, CF1442 *daf-16(mu86)* I; *daf-2(e1370)* III; *muEx169*, CF1514 *daf-16(mu86)* I; *daf-2(e1370)* III; *muEx211*, CF1515 *daf-16(mu86)* I; *daf-2(e1370)* III; *muEx212*, NL2099 *rrf-3(pk1426)* II, NG2615 *cam-1 (gm122)* II, CB1370 *daf-2(e1370)* III, DR1565 *daf-2(m596)* III, DR1567 *daf-2(m577)* III, DR1574 *daf-2(e1391)* III, DR63 *daf-4(m63)* III, DR62 *daf-7(m62)* III, CB1375 *daf-18(e1375)* IV, GR1310 *akt-1(mg144)* V, RB783 *scd-2(ok565)* V, JT191 *daf-28(sa191)* V, DR2281 *daf-9(m540)* X, DR20 *daf-12(m20)* X, AA82 *daf-12(rh284)* X, AA86 daf-12(rh61rh411) X. The nature of the extrachromosomal arrays *muEx169*, *muEx211* and *muEx212* has been described previously (Libina et al., 2003). The strain containing the mutation *daf-28(tm2308)* was obtained from National BioResource Project (Japan).

For most strains, muscle arm extension was examined in the background of RP247 *trIs30* I, which we have described previously (Dixon and Roy, 2005). For some strains, animals were made transgenic for an extrachromosomal array *trEx[pPRRF138.2(him-4p::mb::yfp); pPRZL44(hmr-1b::dsred2); pBluescript +KS]* using standard microinjection protocols as described previously (Dixon and Roy, 2005; Mello et al., 1991). The following plasmid concentrations were used for all microinjections: *pRRF138.2* at 10 ng μL^{-1}, pPRZL44 at 10 ng μL^{-1}, and pBluescript at 80 ng μL^{-1}. The *hmr-1b::dsred2* construct drives expression of DsRed2 in the AS, VD and DD commissural motor axons (Broadbent and Pettitt, 2002), thereby allowing us to determine if any defects in muscle arm extension are secondary to axon guidance defects (Hedgecock et al., 1990). For reference, Table 1 lists all strains used in this study. Unless otherwise noted, muscle arm numbers were counted in young adults raised at 20 °C.

Double mutant strains between *daf-2* and *unc-104* and *daf-12* mutants were constructed by crossing *trIs30*/+; *daf-2*/+ males into hermaphrodites of the second mutant. The resultant *trIs30*/+; *daf-2*/+; mutant/+ hermaphrodites were isolated and transferred to 25 °C. *daf-2* homozygous F2 progeny were isolated singly as dauers at this temperature and then transferred back to 15 °C to recover. A portion of the F3 progeny from the recovered *daf-2* homozygotes were then screened for 100% presence of the second mutation, either Unc (*unc-104*) or suppression of Daf-c at 25 °C (*daf-12*). Animals homozygous for the *trIs30* transgene were then isolated from the original F2 plates containing the confirmed double mutants. It has been reported that *daf-12(rh284)* is not Daf-d, as it forms greater than 10% dauers when cultured in large numbers on plates lacking food (Antebi et al., 1998). However, we observe that the Daf-c phenotype of *daf-2(e1370)* animals is partially suppressed in *daf-2(e1370); daf-12(rh284)* animals raised at 25 °C, allowing a portion to grow to adulthood.

RP247 *trIs30* dauer animals were isolated from plates starved for 3 weeks at 20 °C. Individual dauer animals were identified based on their distinctive morphology and hyperactive locomotion in response to prodding. These animals were then either examined directly (as dauers) or transferred to a fresh plate (10–20 per plate) with abundant food and allowed to develop to adulthood prior to examination (as recovered dauers). To cultivate animals grown under crowded conditions, we deposited 400,000 L1-staged RP247 hatchlings on a 15 cm '*E. coli*-egg yolk plate' prepared as previously described (Portman, 2006). Approximately 5% of the animals developed into dauers and we examined the muscle arms of adults that failed to enter the dauer phase.

Molecular biology and constructs

Standard laboratory protocols were used to manipulate all bacterial strains used for cloning, PCR reactions and restriction enzyme digestions (Sambrook and Russell, 2001). The plasmid pBluescript +KS is from Fermentas. *daf-2(exon9)* and *daf-2(exon14)* RNAi constructs were constructed by sub-cloning 1 kbp PCR fragments centered on the *daf-2* exon 9 and exon 14 coding regions, respectively, into the L4440 vector. The resultant constructs were then transformed into the RNAi feeding strain HT115 for use in experiments (Timmons et al., 2001). All other constructs used in this work have been described (Dixon et al., 2006; Dixon and Roy, 2005). All primer sequences and plasmids are available upon request.

RNAi

All RNAi experiments were done by feeding worms dsRNA-producing bacteria on NGM plates (Brenner, 1974) as described (Timmons and Fire, 1998) but with the following modification. Single clones of RNAi-inducing bacterial strains were grown in LB media overnight and then spotted directly onto NGM+IPTG plates without prior IPTG induction. RNAi-inducing bacteria targeting *egl-15* was obtained from the Ahringer library (Kamath et al., 2003). The negative control is the empty RNAi vector (pPD129.36) alone. For RNAi experiments L4-staged P_os were picked to plates lacking food and allowed to run for 30 min to remove any OP50 contamination. From this plate, 4 worms were picked onto each 6 cm NGM+RNAi-inducing bacterial plate for each experiment. RNAi phenotypes were scored in the F1 generation.

Temperature shift experiments, microscopy and analysis of muscle arm extension

Temperature shift experiments with *daf-2(e1370)* were performed by picking staged animals raised at either the permissive or non-permissive temperature and either maintaining them at the same temperature or transferring them to a different temperature, either 15 °C or 20 °C. Microscopy was performed as described previously (Dixon and Roy, 2005). Analysis of muscle arm extension in young adults and synchronized larvae was performed as described previously (Dixon and Roy, 2005) with one modification: Instead of repeated pair-wise comparison using the Mann–Whitney test, all counts of muscle arm number for a given genetic background were analyzed simultaneously using a one-way ANOVA and Tukey's post-test.

Results

Muscle arm extension is negatively regulated by daf-2

Receptor tyrosine kinases are required for neuromuscular development and function in *C. elegans* and other animals (Francis et al., 2005; Kummer et al., 2006). We previously examined several

Table 1
A comparison of the number of muscle arms in various mutant backgrounds

	Genotype[a]	Description	15 °C			20 °C				25 °C		
			n[b]	Mean[c]	*P* val[d]	*n*[b]	Mean[c]	*P* val[d]	*P* val[e]	*n*[b]	Mean[c]	*P* val[d]
1	*trIs30*	Young adult	15	3.6±0.1	–	30	3.5±0.1	–	–	15	3.4±0.1	–
2	*daf-2(e1370)*	Insulin RTK (Class II)	15	4.0±0.1	ns (1)	15	5.3±0.2	<0.001 (1)	–	–	–	–
3	*cam-1(gm122)*	ROR RTK	–	–	–	15	3.8±0.1^	ns (1)	–	–	–	–
4	*scd-2(ok565)*	Alk RTK	–	–	–	15	3.8±0.1	ns (1)	–	–	–	–
5	*egl-15(RNAi)*	FGFR RTK	–	–	–	15	3.2±0.1	ns (1)	–	–	–	–
6	*daf-2(m596)*	Insulin receptor (Class II)	15	4.1±0.1	<0.01 (1)	15	4.3±0.1	<0.001 (1)	–	–	–	–
7	*daf-2(e1391)*	Insulin receptor (Class II)	15	4.5±0.1	<0.001 (1)	15	4.4±0.1	<0.001 (1)	–	–	–	–
8	*daf-2(m577)*	Insulin receptor (Class I)	–	–	–	15	3.6±0.1	ns (2)	–	–	–	–
9	*daf-2(m41)*	Insulin receptor (Class I)	–	–	–	15	3.5±0.1	ns (2)	–	–	–	–
10	*rrf-3(pk1426);Ø(RNAi)*	RNAi sensitive	–	–	–	15	3.5±0.1	–	–	–	–	–
11	*rrf-3(pk1426); daf-2(exon9RNAi)*	Insulin receptor	–	–	–	15	4.3±0.1	<0.01 (10)	–	–	–	–
12	*rrf-3(pk1426); daf-2(exon14RNAi)*	Insulin receptor	–	–	–	15	4.6±0.1	<0.01 (10)	–	–	–	–
13	*Ex(p138.2,p44,pKS)*	Ex array control	–	–	–	15	3.8±0.1	–	–	–	–	–
14	*daf-28(tm2308)**	Insulin ligand	–	–	–	15	4.2±0.1	ns (13)	–	14	3.9±0.1	ns
15	*daf-28(sa191)*	Insulin ligand	–	–	–	15	3.8±0.1	ns (1)	–	–	–	–
16	*unc-104(rh43)*	Kif 3 kinesin	–	–	–	15	1.3±0.1	<0.001 (1)	–	–	–	–
17	*daf-2(e1370); unc-104(rh43)*	–	15	1.7±0.1	<0.001 (1)	15	1.7±0.1	<0.001 (2)	–	–	–	–
18	*daf-18(e1375)*	PTEN	–	–	–	15	2.6±0.1	<0.001 (1)	–	–	–	–
19	*akt-1(mg144)**	Akt S/T kinase GOF	–	–	–	15	3.2±0.1	ns (13)	–	–	–	–
20	*daf-16(m26)**	FOXO	–	–	–	15	3.7±0.1	ns (13)	–	–	–	–
21	*daf-16(m27)**	FOXO	–	–	–	15	3.5±0.1	ns (13)	–	–	–	–
22	*daf-2(e1370); daf-16(mgDf47)**	–	–	–	–	15	3.3±0.1	ns (13)	–	15	3.5±0.1	ns (13)
24	*daf-2(e1370); daf-16(mu86)**	–	–	–	–	15	3.8±0.1	ns (13)	–	–	–	–
25	*daf-9(m540)*	Cytochrome P450	–	–	–	15	4.5±0.1	<0.001 (1)	–	–	–	–
26	*daf-12(m20)*	Nuclear hormone receptor	–	–	–	15	3.4±0.1	ns (1)	–	15	3.2±0.1	ns (1)
27	*daf-2(e1370);daf-12(m20)*	–	–	–	–	15	4.7±0.1	<0.001 (1)	ns (2)	–	–	–
28	*daf-12(rh284)*	Nuclear hormone receptor	–	–	–	15	4.0±0.1	ns (1)	–	15	3.7±0.1	ns (1)
29	*daf-2(e1370); daf-12(rh284)*	–	–	–	–	15	4.0±0.1	ns (1)	<0.001 (2)	15	3.7±0.1	ns (1)
30	*daf-12(rh61rh411)**	Nuclear hormone receptor	–	–	–	15	3.7±0.1	ns (13)	–	15	3.7±0.1	ns (13)
31	*daf-2(e1370); daf-12(rh61rh411)*	–	–	–	–	15	3.3±0.1	ns (1)	<0.001 (2)	15	2.9±0.1	ns (1)
32	*trIs30*	L3-stage	–	–	–	15	3.6±0.1	ns (1)	–	–	–	–
33	*trIs30*	Dauer	–	–	–	15	4.5±0.1	<0.001 (32)	–	–	–	–
34	*trIs30*	Dauer recovered	–	–	–	15	4.5±0.1	<0.001 (1)	ns (33)	–	–	–
35	*trIs30*	5-day starvation recovered	–	–	–	15	3.3±0.1	ns (1)		–	–	–
36	*trIs30*	Crowding	–	–	–	15	3.4±0.1	ns (1)		–	–	–
37	*trIs30*	26 °C	–	–	–	–	–	–	–	15	3.3±0.1	ns (1)
38	*daf-7(m62)*	TGF-β-like ligand	–	–	–	15	4.2±0.1	<0.001 (1)	–	–	–	–
39	*daf-4(m63)*	TGF-β-like Type II Receptor	–	–	–	15	4.4±0.1	<0.001 (1)	–	–	–	–
40	*trIs30*	L1-stage	–	–	–	30	1.2±0.0	–	–	–	–	–
41	*daf-2(e1370)*	L1-stage	–	–	–	30	1.3±0.0	ns (40)	–	–	–	–

[a]All counts were done in the *trIs30* background, except those genotypes marked with an asterisk, which were done with a *him-4p::mb::yfp* extra-chromosomal array. All counts are from young adults unless otherwise noted in the 'Description' column, and raised and scored at the temperature indicated. [b]*n* values indicate the number of animals considered. For each animal, the muscle arms from each of BWMs 9, 11, 13, 15, 17 and 19 of the dorsal right quadrant were counted, except for those counts followed by a carrot (^), where only BWMs 9, 11, 13 and 15 were considered. Thus, muscle arms were counted for a minimum of 48 muscles. [c]Shown is the average number of muscle arms per BWM, followed by the standard error. [d,e]These *p*-values were derived as described in Materials and methods and are relative to the counts from the row indicated in brackets. Unless otherwise indicated, the default comparison row is 1 (*trIs30*). For those counts where only muscles 9–15 were considered, all statistical tests were done by comparing these counts to that of muscles 9–15 of the respective genotypes. ns equals not significant ($p>0.01$).

conserved RTKs for roles in muscle arm extension and showed that the EGL-15/FGF receptor is required to prevent ectopic membrane extensions from BWMs (Dixon et al., 2006). As part of these ongoing studies we asked whether insulin-like RTK signaling is involved in muscle arm extension. To test this, we examined animals with a hypomorphic, temperature-sensitive mutant allele (*e1370*) of the *daf-2* gene. Surprisingly, *daf-2(e1370)* worms raised at 20 °C have more muscle arms per BWM than *trIs30* controls (5.3 versus 3.5) ($p<0.001$) (Figs. 1B, C) (Table 1). Normal muscle arm extension is observed in mutants of three other receptor tyrosine kinases implicated in neuromuscular function or muscle development in *C. elegans*, namely *cam-1/ror*, *scd-2/alk* and *egl-15/fgfr* (Table 1) (Dixon et al., 2006; Francis et al., 2005; Gottschalk et al., 2005; Liao et al., 2004). Thus, the Sna phenotype is specific for the DAF-2 receptor tyrosine kinase. When raised at 15 °C, muscle arm extension in *daf-2(e1370)* worms does not differ significantly from controls ($p>0.01$) (Fig. 1D) (Table 1), suggesting that the phenotype is temperature sensitive in the *e1370* background.

To further investigate the relationship between *daf-2* and muscle arm development, we examined four additional *daf-2* loss-of-function alleles: *m596*, *e1391*, *m577* and *m41*. These four *daf-2* alleles fall into two different classes as defined by Gems et al. (1998). Like *e1370*, both *m596* and *e1391* are class II alleles, while *m577* and *m41* are class I alleles. Both class II and class I alleles display a high temperature constitutive dauer formation (Daf-c) phenotype, increased adult longevity and intrinsic thermotolerance (Gems et al., 1998). Class II alleles, but not class I alleles, also display other abnormalities affecting motility, gonad development and fertility (Gems et al., 1998). The nature of the differences between class I versus class II alleles at the molecular level is not clear (Gems et al., 1998). We observe that muscle arm extension in both *m596* and *e1391* animals raised at both 15 °C and 20 °C is greater than controls ($p<0.01$) (Fig. 1E) (Table 1). By contrast, muscle arm extension in the class I alleles *m577* and *m41* does not differ significantly from controls ($p>0.01$) (Table 1) (Figs. 1F, G). These results suggest that class I alleles retain a residual activity at 20 °C that is sufficient to prevent the Sna phenotype while the class II alleles do not.

Next, we treated worms with RNAi-inducing constructs targeting *daf-2* coding sequence. In the background of the RNAi-sensitive strain *rrf-3(pk1426)*, we find that both *daf-2(exon9RNAi)* and *daf-2(exon14RNAi)*-

treated animals have significantly more muscle arms than animals treated with a negative control RNAi ($p<0.01$) (Fig. 1G) (Table 1). This suggests that *daf-2(RNAi)* treatment produces an effect that mimics class II *daf-2* alleles at 20 °C. Together, these data show that muscle arm extension is negatively regulated by DAF-2 activity.

DAF-2 activity is regulated both positively and negatively by different insulin-like ligands, of which at least 38 have been predicted from the *C. elegans* genome sequence (Li et al., 2003; Pierce et al., 2001). The best understood insulin-like ligand gene, *daf-28*, is down-regulated under dauer-inducing conditions, and is therefore thought to normally prevent dauer entry by acting in a positive manner to maintain a high basal level of DAF-2 activity (Li et al., 2003). We found that muscle arm extension in both the *daf-28(tm2308)* deletion allele and the *daf-28(sa191)* gain-of-function allele is not significantly different from controls ($p>0.01$) (Table 1). Also, deletion alleles of *ins-1*, *ins-6*, *ins-18*, *ins-23*, *ins-26*, *ins-29* and *ins-38* have normal muscle arm extension, as do worms fed dsRNAs targeting *ins-5*, *ins-11*, *ins-24*, *ins-30*, *ins-31* and *ins-32* (not shown). We conclude that the insulin-like ligands examined do not by themselves regulate muscle arm extension, although redundancy between ligands cannot be excluded.

Supernumerary muscle arms are indistinguishable from wild type arms

We investigated when DAF-2 insulin-like signaling is required to regulate supernumerary muscle arm extension, and whether this period corresponds with the previously defined interval for larval muscle arm extension between mid L1 and late L3 (Dixon and Roy, 2005). First, we examined muscle arm number in newly hatched *daf-2(e1370)* L1s and found that muscle arm numbers are statistically indistinguishable from control animals ($p>0.01$) (Table 1, Fig. 2). This suggests that the additional muscle arms observed in young *daf-2* mutant adults are derived post-embryonically.

Next, we sought to narrow the developmental period during which *daf-2* is required to regulate supernumerary arm extension. To do this, we transferred staged *daf-2(e1370)* worms from the permissive temperature of 15 °C to the semi-permissive temperature of 20 °C at various times during development and then scored muscle arm extension at adulthood. We find that muscle arm extension in *daf-2(e1370)* worms hatched at 15 °C and then transferred from 15 °C to 20 °C as L3s or L4s does not differ significantly from muscle arm extension in worms raised continuously at 15 °C ($p>0.05$) (Fig. 2A). By contrast, worms transferred from 15 °C to 20 °C as L1s are Sna and statistically indistinguishable from worms raised continuously at 20 °C ($p>0.05$) (Fig. 2A). This suggests DAF-2 is required before L3 to regulate muscle arm extension. Performing the converse experiment, we find that *daf-2(e1370)* worms transferred from the semi-permissive temperature of 20 °C to the permissive temperature of 15 °C at any point after L2 (e.g. as L3s or L4s) do not differ significantly from animals raised continuously at 20 °C ($p>0.05$) (Fig. 2B). However, muscle arm extension is significantly reduced in *daf-2(e1370)* animals that are shifted from 20 °C to 15 °C either as embryos or L1s compared to animals raised continuously at 20 °C ($p<0.01$) (Fig. 2B). Thus, the restoration of DAF-2 activity at any time after L2 is unable to rescue the Sna phenotype of these worms. Together, these results suggest that *daf-2* activity is required between L1 and L3 to negatively regulate muscle arm extension. These results are consistent with insulin-like signaling regulating muscle arm extension during the period of larval muscle arm outgrowth during the mid L1 to late L2 larval stage (Dixon and Roy, 2005).

In wild-type animals, the anterograde kinesin UNC-104 is required to transport an unidentified factor(s) that is essential for larval muscle arm extension to the nerve cords (Dixon and Roy, 2005; Hall and Hedgecock, 1991). If the supernumerary muscle arms of *daf-2* mutants exhibit the same properties as the wild-type arms, *unc-104(rh43)* should be epistatic to *daf-2(e1370)* and preclude the formation of all

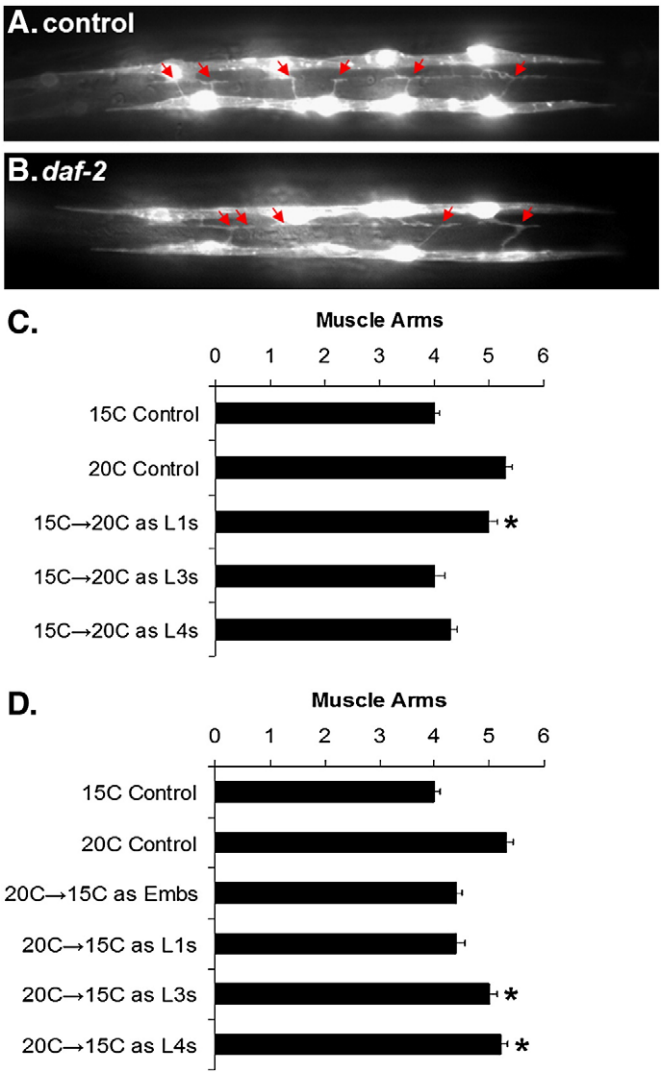

Fig. 2. An analysis of when *daf-2* is required to regulate supernumerary muscle arm extension. (A) The muscle arms of the distal BWMs of the left (above) and right (below) dorsal quadrant of an L1 hatchling. Anterior is to the right. Red arrowheads point to the muscle arms extended by the muscles of the dorsal right quadrant, which we infer to be of embryonic origin. (B) The same as in panel A, but in a *daf-2(e1370)* background. The animals depicted in panel A and B were raised at 20 °C. (C) *daf-2(e1370); trIs30* animals were raised continuously at the permissive temperature of 15 °C (15C), the less-permissive temperature of 20 °C (20C) or shifted from 15 °C to 20 °C as L1s, L3s or L4s. In panel C, a black asterisk indicates that the number of muscle arms per BWM in animals shifted as L1s is significantly greater than in animals raised continuously at 15 °C or in those transferred from 15 °C to 20 °C as L3s or L4s ($p<0.01$). Animals shifted as L1s do not differ significantly from animals raised continuously at 20 °C ($p>0.05$). (D) *daf-2(e1370); trIs30* animals were raised continuously at the permissive temperature of 15 °C, the less-permissive temperature 20 °C or shifted from 20 °C to 15 °C as embryos (Embs), L1s, L3s or L4s. In panel D, the black asterisks indicate that the number of muscle arms is significantly higher in animals transferred as L3s or L4s compared to those raised continuously at 15 °C or transferred as embryos or L1s ($p<0.01$). Animals shifted as L3s or L4s do not differ significantly from animals raised continuously at 20 °C ($p>0.05$). For both panel C and D, muscle arm counts are of dorsal right BWMs numbered 9, 11, 13, 15, 17 and 19 (Dixon and Roy, 2005) from between 6 and 15 animals per condition. Error bars represent the standard error of the mean.

but the one or two embryonic arms. To test this, we constructed the *rh43; e1370* double mutant. It is noteworthy that we found that *rh43* strongly enhances the Daf-c phenotype of *daf-2(e1370)* animals at 20 °C, with most double mutant animals arresting as dauer larvae (not shown). Similar dauer-enhancing effects have previously been reported for neuronal genes involved in synaptic vesicle release, such as *unc-31* and *unc-64* (Ailion et al., 1999), suggesting that *unc-104* is required to transport an insulin-like ligand that antagonizes

dauer formation. Regardless, analysis of muscle arm number in *rh43; e1370* animals that develop into adults demonstrates that *rh43* is epistatic to *e1370* (Table 1) (Figs. 1I, J). This result supports the conclusion that supernumerary arms extend during larval development and suggests that they are no different than wild type larval muscle arms.

daf-18 is required for normal muscle arm extension outside of the canonical insulin-like signaling pathway

Loss of *daf-2* activity increases muscle arm extension. We wondered if increased DAF-2 pathway activity has the opposite effect, namely a decrease in muscle arm extension. We therefore examined a loss-of-function allele of *daf-18*, which encodes a broadly expressed PTEN homolog that functions downstream of DAF-2 by antagonizing PIP3 production (Masse et al., 2005; Ogg and Ruvkun, 1998). Compared to controls, muscle arm extension in *daf-18(e1375)* animals is significantly reduced from 3.5 to 2.6 muscle arms per BWM ($p<0.001$) (Table 1). One interpretation of this result is that *daf-18* enhances PIP3 accumulation and downstream insulin-like pathway activity, leading to a reduction in muscle arm extension. Alternatively, *daf-18* may function outside of the canonical insulin-like pathway to regulate muscle arm extension. To distinguish between these possibilities, we examined an *akt-1(mg144)* mutant that expresses a constitutively active AKT-1 protein, thereby bypassing the requirement for PIP3 in insulin-like signaling (Paradis and Ruvkun, 1998). We find that muscle arm extension in *akt-1(mg144)* animals does not differ significantly from controls ($p>0.01$) (Table 1). We draw two conclusions from these results. First, DAF-18 is likely playing a role in muscle arm extension outside of the canonical insulin pathway. We speculate that excessive PIP3 accumulation in a *daf-18* background may interfere with the localization or function of other proteins required for muscle arm extension. A similar role has been proposed for DAF-18 in the process of HSN polarization and axon outgrowth in *C. elegans* (Adler et al., 2006), and would be consistent with the role of PTEN orthologues in other systems (Iijima and Devreotes, 2002). Alternatively, DAF-18 may be required to directly dephosphorylate a protein necessary for muscle arm extension. Our second conclusion is that hyperactivation of the insulin-like signaling pathway downstream of PIP3 has no effect on muscle arm extension.

Supernumerary arm extension is regulated by DAF-16 in muscle and intestine

AKT-1/2 can inactivate DAF-16 through serine phosphorylation and consequent cytoplasmic retention (Cahill et al., 2001). The absence of phenotype in the constitutively active *akt-1(mg144)* mutant suggests that inactivation or elimination of DAF-16 should have no effect on muscle arm development. Consistent with this prediction, we find that muscle arm extension in *daf-16(m26)* and *daf-16(m27)* loss of function mutants is not significantly different from wild-type controls ($p>0.01$) (Figs. 1K, L) (Table 1). Thus DAF-16 is dispensable for normal muscle arm development.

Down-regulation of DAF-2 results in nuclear localization of DAF-16, which regulates the expression of many genes (see Introduction). To test if *daf-16* is required for the Sna phenotype of *daf-2(e1370)* animals, we examined *daf-2(e1370)* worms in the background of the chromosomal deficiency *mgDf47*, which is null for *daf-16* (Ogg et al., 1997). *daf-2(e1370)*; *daf-16(mgDf47)* animals cultured at either 20 °C or 25 °C develop normally and muscle arm extension is not significantly different from wild-type animals ($p>0.01$) (Fig. 1M) (Table 1). These data show that the Sna phenotype of *daf-2* mutants requires DAF-16.

DAF-16 is expressed throughout the worm and is required in the nervous system to prevent dauer formation, and in the intestine and gonad to promote normal aging (Libina et al., 2003; Wolkow et al., 2000). To determine where insulin-like signaling is required to regulate arm extension, we examined muscle arm extension at 20 °C in *daf-16(mu86); daf-2(e1370)* mutants in which *daf-16* gene function is restored in the nervous system, body wall muscles or intestine using cell-specific promoters (Libina et al., 2003). *mu86* is a deletion allele that removes almost the entire coding sequence of *daf-16* and is likely null (Lin et al., 1997). Muscle arm development in two *daf-16(mu86); daf-2(e1370)* lines did not differ from wild-type animals ($p>0.01$) (Fig. 3) (Table 1), confirming that DAF-16 is required for the Sna phenotype. Similarly, muscle arm extension in two independent *daf-16(mu86); daf-2(e1370)*; *muEx169* lines, where DAF-16 is expressed throughout the nervous system, do not differ significantly from *daf-16(mu86); daf-2(e1370)* controls ($p>0.05$) (Fig. 3). By contrast, three independent *daf-16(mu86); daf-2(e1370); muEx212* lines, where DAF-16 is expressed in the BWMs, have significantly more muscle arms than the control strains ($p<0.01$) (Fig. 3). We also find that four independent *daf-16(mu86); daf-2(e1370); muEx211* lines expressing DAF-16 in the intestine exhibit significantly more muscle arms than controls ($p<0.01$). Together, our results demonstrate that the DAF-2 signal can function non-autonomously to regulate supernumerary muscle arm extension.

The DAF-12B isoform is required for supernumerary muscle arm extension

Daf-16/foxo is dispensable for normal muscle arm development, but required for supernumerary arm production in a *daf-2(e1370)* background. Nuclear entry of DAF-16 ultimately results in down-regulation of DAF-9/Cytochrome P450 activity, reducing the production of endogenous sterol-based hormones, leading to de-repression of DAF-12/NHR and induction of the dauer program (see Introduction). To determine if this branch of the dauer pathway also regulates supernumerary arm formation, we first examined hypomorphic *daf-9(m540)* animals that are defective in hormone biosynthesis (Jia et al., 2002). We

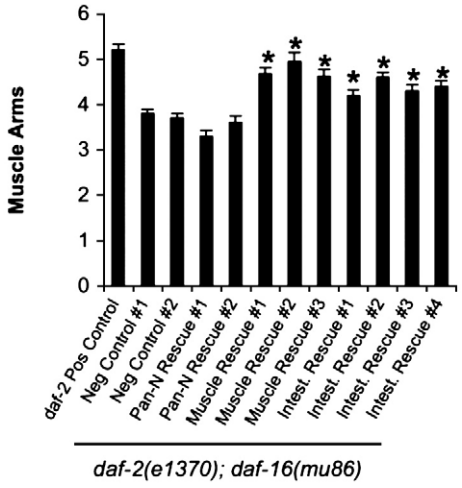

Fig. 3. Cell specific expression of DAF-16 restores the Sna phenotype in *daf-16(mu86); daf-2(e1370)* mutants. Muscle arm extension for *daf-2(e1370)* is shown for reference at the far left (*daf-2* Pos Control). Two negative control lines *daf-16(mu86); daf-2(e1370); trEx(him-4p::mb::yfp)* that do not express DAF-16 (Neg Control #1, #2) do not differ from two lines of *daf-16(mu86); daf-2(e1370); muEx169[unc-119p::GFP::daf-16; rol-6(su1006)]; trEx(him-4p::mb::yfp)* animals expressing DAF-16 pan-neuronally (Pan-N Rescue #1, #2). Three lines of *daf-16(mu86); daf-2(e1370); muEx212[myo-3p::GFP::daf-16; rol-6(su1006)]; trEx(him-4p::mb::yfp)* expressing DAF-16 in muscles (Muscle Rescue #1, #2, #3) have significantly more arms than the negative controls, as do four lines of *daf-16(mu86); daf-2(e1370); muEx211[(ges-1p::GFP::daf-16; rol-6(su1006)]; trEx(him-4p::mb::yfp)* expressing DAF-16 in the intestine (Intest. Rescue #1, #2, #3, #4) (ANOVA test with Tukey post-tests, $p<0.05$, indicated with black asterisks). The muscle arm counts are of dorsal right BWMs numbered 9, 11, 13, 15, 17 and 19 (Dixon and Roy, 2005) from between 5 and 15 animals per line. Error bars represent the standard error of the mean.

find that muscle arm extension is significantly greater in *daf-9(m540)* animals than controls ($p<0.001$), suggesting that hormone production may be required to prevent the Sna phenotype.

We next examined the role of *daf-12* in muscle arm development. Alternative splicing of the *daf-12* mRNA generates at least three different isoforms of the protein (Antebi et al., 2000). Two full length isoforms, A1 and A3, contain both a zinc-finger DNA-binding domain and a ligand-binding domain (LBD) while the shorter B isoform contains the LBD only (Antebi et al., 2000). The *m20* allele encodes a nonsense mutation predicted to truncate the full-length isoforms of the DAF-12 protein within the DNA-binding domain (Antebi et al., 2000), leaving only the B isoform intact. We find that *m20* does not alter muscle arm extension in an otherwise wild-type background ($p>0.01$) (Table 1), and does not suppress the Sna phenotype of *daf-2(e1370)* mutants ($p>0.01$) (Table 1). By contrast, both the *daf-12(rh284)* allele that disrupts the LBD of all DAF-12 isoforms, and the *daf-12* null (*rh61rh411*) (Antebi et al., 1998; Antebi et al., 2000), suppress the Sna phenotype of *daf-2(e1370)* mutants ($p<0.001$) (Table 1). These observations raise the interesting possibility that hormone production downstream of DAF-2 and DAF-9 prevents supernumerary arm formation by antagonizing the DAF-12B LBD-only isoform.

Dauers extend supernumerary muscle arms

Decreased insulin-like ligand secretion in wild-type animals results in the down-regulation of DAF-2 activity. In turn, DAF-16 enters the nucleus and promotes dauer formation (Henderson and Johnson, 2001; Lee et al., 2001; Li et al., 2003). Because insulin-like signaling regulates both dauer formation and muscle arm extension during an overlapping period of development, we investigated if wild type dauers are Sna. We find that the number of muscle arms in dauers is significantly greater than that in L3-stage-specific controls ($p<0.01$) (Figs. 4A, B) (Table 1). Next, we examined the muscle arms of adult animals recovered from dauer to determine if the Sna phenotype is reversed upon dauer exit. Surprisingly, we find that dauer-recovered animals remain Sna ($p<0.01$) (Table 1) (compare Fig. 4C to Fig. 1B). Thus, unlike other dauer-specific morphological adaptations, the Sna phenotype of wild type dauers is retained into adulthood following exit from the dauer state.

Finally, we investigated if supernumerary muscle arms are elicited specifically through the dauer program or if it is simply a consequence of stress on the animal. First, we examined animals raised at high temperature (26 °C) and found that they were not Sna (Table 1). Second, we starved synchronized L1 hatchlings for 5 days, placed them on food, and then examined the muscle arms of resulting adults and found that they were also not Sna (Table 1). Third, we grew synchronized worms in crowded conditions such that a relatively high percentage of dauers were induced, which is symptomatic of stressful conditions, and then examined the adults that failed to undergo dauer development. Adults raised in these crowded conditions were not Sna (Table 1). Finally, we examined components of a TGF-β pathway that regulates dauer-formation in parallel to the insulin-like pathway. Both pathways converge at the level of *daf-9* (Gerisch et al., 2001; Mak and Ruvkun, 2004). We found that mutations in both the TGF-β ligand, *daf-7(m62)*, and in the TGF-β type II receptor *daf-4(m63)*, conferred a Sna phenotype. Together, these results suggest that it is the dauer program, and not stress alone, that elicits supernumerary muscle arms.

Discussion

Here, we show that the dauer program elicits supernumerary muscle arm extension in *C. elegans*. Unlike other dauer-associated modifications that are reversed upon dauer exit (Riddle, 1988; Riddle and Albert, 1997), we find that the Sna phenotype of dauers persists into adulthood, suggesting that muscle arm extension is irreversible. Compromising either the insulin-like or the TGF-β pathway leads to both dauer entry and increased muscle arm number, suggesting that the Sna phenotype is not a secondary effect of modifying either pathway. Instead, the Sna phenotype is likely a direct consequence of dauer formation that is mediated by the DAF-12 nuclear hormone receptor that is downstream of the insulin-like receptor DAF-2.

We propose a model for how insulin-like signaling regulates muscle arm extension during larval development (Fig. 5). Under well-fed conditions, DAF-2 activity is sufficient to prevent DAF-16 nuclear accumulation (Henderson and Johnson, 2001; Lee et al., 2001; Li et al., 2003; Lin et al., 2001) and supernumerary arm extension (Fig. 5A). Under dauer-inducing conditions, decreased insulin-like ligand availability reduces DAF-2 activity (Fig. 5B). Consequently, DAF-16 accumulates in the nucleus of many cells (Henderson and Johnson, 2001; Lee et al., 2001; Li et al., 2003; Lin et al., 2001). We suggest that DAF-16 acts non-autonomously in both intestinal and BWM nuclei to regulate muscle arm extension based on three lines of evidence: First, intestinal *daf-16* expression can rescue the suppression of the *daf-2(e1370)* Sna phenotype by the *daf-16* null. Thus, *daf-16* does not function autonomously to regulate muscle arm extension. Second,

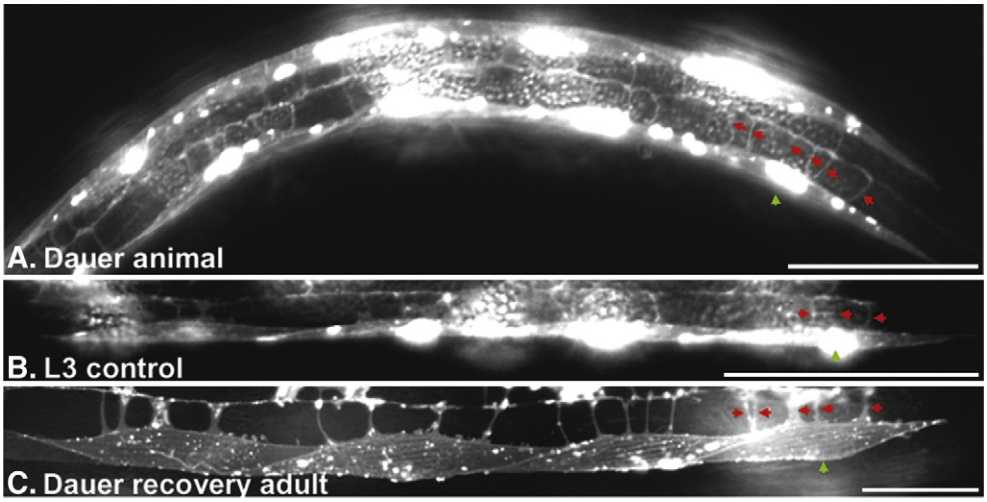

Fig. 4. Dauer animals are Sna. (A) Wild-type dauer animal. (B) Wild-type L3-staged animal. (C) An adult recovered from dauer. In all animals, muscle arms are visualized using the *trIs30* transgene. In all panels, muscle number 9 is indicated with a green arrow for reference, select muscle arm termini are indicated with a red arrow. The scale bar represents 50 μM.

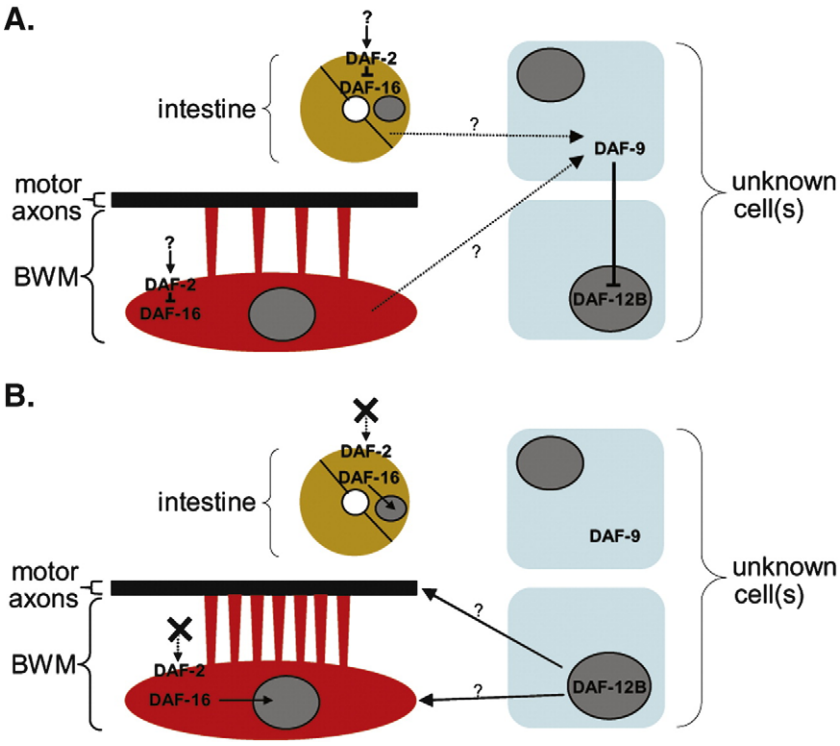

Fig. 5. A model to explain the role of the DAF-2 pathway in muscle arm development. (A) In well-fed, wild-type animals, insulin signaling is sufficient to prevent DAF-16 nuclear entry in BWM and intestine, resulting in normal development and muscle arm extension. (B). When DAF-2 receptor activity is down-regulated, DAF-16 enters the nucleus in the BWMs and/or the intestine and results in the production of a signal that somehow engages supernumerary muscle arm extension from the BWMs. The signal could be a sterol generated by DAF-9. Ultimately, the activity of DAF-12B is required to transduce decreased DAF-2 signaling into supernumerary muscle arm extension. How DAF-16 activity interfaces with DAF-9 activity is unknown. In each cartoon the nucleus is depicted as a grey circle within the cell.

because muscle-expressed *daf-16* can also rescue the suppression of the *daf-2(e1370)* Sna phenotype by the *daf-16* null, *daf-16* expression in the intestine is unnecessary. Finally, the DAF-9 cytochrome P450 enzyme is thought to act down-stream of DAF-16 in dauer development (Gerisch et al., 2001) and is required for supernumerary arm extension. However, DAF-9 is not obviously expressed in either the BWMs or intestinal cells (Gerisch et al., 2001; Jia et al., 2002; Mak and Ruvkun, 2004). We therefore surmise that DAF-16 acts in the intestine and/or the BWMs to promote supernumerary muscle arm formation by indirectly modifying the activity of DAF-9 in some other cell(s). How DAF-16 activity in one cell type regulates the activity of DAF-9 in another cell type is a known gap in our understanding of insulin-like signaling in *C. elegans* (Gerisch et al., 2001; Jia et al., 2002; Motola et al., 2006). Regardless, reduced DAF-9 activity, which is observed in *daf-2* or *daf-9* mutants or in dauer-inducing conditions, impairs the synthesis of an inhibitory ligand for DAF-12 (Antebi et al., 1998, 2000; Gerisch et al., 2001; Ludewig et al., 2004; Matyash et al., 2004; Ohkura et al., 2003; Riddle and Albert, 1997; Rottiers and Antebi, 2006). In the absence of this inhibitory ligand, DAF-12B induces supernumerary muscle arm extension (Fig. 5B). DAF-12B could do this by acting in the nervous system to stimulate increased production of a muscle arm chemoattractant, in the BWMs to hypersensitize them to existing cues, or by some other mechanism. Although alternative models are conceivable, all highlight the complex, distributed and endocrine nature of the insulin-like signaling pathway in *C. elegans*.

The identity of the insulin-like ligand that regulates supernumerary arm production is not known. Our data suggest that neither DAF-28 nor other insulin-like ligands alone regulate Sna formation. It is possible that we have simply not considered the appropriate ligand. Alternatively, DAF-28 or other INS ligands could act redundantly. A third possibility is that under standard laboratory conditions at 20 °C, ligand-independent DAF-2 activity is sufficient to prevent the Sna phenotype. A DAF-2 ligand-independent activity, termed *daf-2B*, has previously been proposed to account for the phenotypes unique to class II *daf-2* alleles (Gems et al., 1998). In our case, *daf-2B* ligand-independent activity could account for the observation that class I *daf-2* alleles (*daf-2B*$^{+}$) raised at 20 °C have normal muscle arm extension while class II alleles (*daf-2B*$^{-}$) raised at the same temperature are Sna.

Finally, the role of supernumerary muscle arms in dauer behavior is an open question. Dauer larvae are specialized for dispersion from overpopulated and food-stressed environments (Riddle and Albert, 1997). The additional muscle arms seen in dauer mutants may facilitate the process of dauer dispersion (Riddle and Albert, 1997). Addressing this issue will require the development of novel approaches to investigate worm behavior in settings that more closely mimic those found in the wild.

Acknowledgments

We thank Nicole Ricker and Guillermo Selman for technical assistance; Andrew Spence for helpful comments and use of his equipment; Mei Zhen, Ed Liao and Richard Roy for helpful comments on the manuscript; the *C. elegans* gene knockout consortium, the Mitani lab (Tokyo Women's Medical University School of Medicine, Japan), and Theresa Stiernagle and the *C. elegans* Genetic Center, which is funded by the NIH National Center for Research Resources (NCRR), for several worm strains used in this work. P.J.R. is a Canada Research Chair in Molecular Neurobiology. This work was supported by a National Cancer Institute of Canada (NCIC) operating grant funded by the Canadian Cancer Society.

References

Adler, C.E., et al., 2006. UNC-6/Netrin induces neuronal asymmetry and defines the site of axon formation. Nat. Neurosci. 9, 511–518.

Ailion, M., et al., 1999. Neurosecretory control of aging in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U. S. A. 96, 7394–7397.
Antebi, A., et al., 1998. daf-12 regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. Development 125, 1191–1205.
Antebi, A., et al., 2000. daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. Genes Dev. 14, 1512–1527.
Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. Genetics 77, 71–94.
Broadbent, I.D., Pettitt, J., 2002. The *C. elegans* hmr-1 gene can encode a neuronal classic cadherin involved in the regulation of axon fasciculation. Curr. Biol. 12, 59–63.
Buchthal, F., Schmalbruch, H., 1980. Motor unit of mammalian muscle. Physiol. Rev. 60, 90–142.
Cahill, C.M., et al., 2001. Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3-dependent and 14-3-3-independent pathways. J. Biol. Chem. 276, 13402–13410.
Cassada, R.C., Russell, R.L., 1975. The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev. Biol. 46, 326–342.
Dalley, B.K., Golomb, M., 1992. Gene expression in the *Caenorhabditis elegans* dauer larva: developmental regulation of Hsp90 and other genes. Dev. Biol. 151, 80–90.
Dixon, S.J., Roy, P.J., 2005. Muscle arm development in *Caenorhabditis elegans*. Development 132, 3079–3092.
Dixon, S.J., et al., 2006. FGF negatively regulates muscle membrane extension in *Caenorhabditis elegans*. Development 133, 1263–1275.
Francis, M.M., et al., 2005. The Ror receptor tyrosine kinase CAM-1 is required for ACR-16-mediated synaptic transmission at the *C. elegans* neuromuscular junction. Neuron 46, 581–594.
Gems, D., et al., 1998. Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. Genetics 150, 129–155.
Gerisch, B., et al., 2001. A hormonal signaling pathway influencing *C. elegans* metabolism, reproductive development, and life span. Dev. Cell 1, 841–851.
Golden, J.W., Riddle, D.L., 1984. The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. Dev. Biol. 102, 368–378.
Gottschalk, A., et al., 2005. Identification and characterization of novel nicotinic receptor-associated proteins in *Caenorhabditis elegans*. EMBO J. 24, 2566–2578.
Hall, D.H., Hedgecock, E.M., 1991. Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in *C. elegans*. Cell 65, 837–847.
Hedgecock, E.M., et al., 1990. The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. Neuron 4, 61–85.
Henderson, S.T., Johnson, T.E., 2001. daf-16 integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. Curr. Biol. 11, 1975–1980.
Iijima, M., Devreotes, P., 2002. Tumor suppressor PTEN mediates sensing of chemoattractant gradients. Cell 109, 599–610.
Jia, K., et al., 2002. DAF-9, a cytochrome P450 regulating *C. elegans* larval development and adult longevity. Development 129, 221–231.
Kamath, R.S., et al., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 421, 231–237.
Kummer, T.T., et al., 2006. Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. Curr. Opin. Neurobiol. 16, 74–82.
Larsen, P.L., 1993. Aging and resistance to oxidative damage in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U. S. A. 90, 8905–8909.
Lee, R.Y., et al., 2001. Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway. Curr. Biol. 11, 1950–1957.
Li, W., et al., 2003. daf-28 encodes a *C. elegans* insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. Genes Dev. 17, 844–858.
Liao, E.H., et al., 2004. An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. Nature 430, 345–350.
Libina, N., et al., 2003. Tissue-specific activities of *C. elegans* DAF-16 in the regulation of lifespan. Cell 115, 489–502.
Lin, K., et al., 1997. daf-16: An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. Science 278, 1319–1322.
Lin, K., et al., 2001. Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat. Genet. 28, 139–145.
Liu, T., et al., 2004. Regulation of signaling genes by TGFbeta during entry into dauer diapause in *C. elegans*. BMC Dev. Biol. 4, 11.
Ludewig, A.H., et al., 2004. A novel nuclear receptor/coregulator complex controls *C. elegans* lipid metabolism, larval development, and aging. Genes Dev. 18, 2120–2133.
Mak, H.Y., Ruvkun, G., 2004. Intercellular signaling of reproductive development by the *C. elegans* DAF-9 cytochrome P450. Development 131, 1777–1786.
Masse, I., et al., 2005. Lifespan and dauer regulation by tissue-specific activities of *Caenorhabditis elegans* DAF-18. Dev. Biol. 286, 91–101.
Matyash, V., et al., 2004. Sterol-derived hormone(s) controls entry into diapause in *Caenorhabditis elegans* by consecutive activation of DAF-12 and DAF-16. PLoS Biol. 2, e280.
Maupas, E., 1899. Arch. Zool. Exp. VII (3e série), 563–628 (as cited in Modes and Forms of Reproduction of Nematodes; Archives de Zoologie Experimentale et Generale 8: 463–624. Translated by M.A. Felix).
Mello, C.C., et al., 1991. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 10, 3959–3970.
Morris, J.Z., et al., 1996. A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. Nature 382, 536–539.
Motola, D.L., et al., 2006. Identification of ligands for DAF-12 that govern dauer formation and reproduction in *C. elegans*. Cell 124, 1209–1223.
Murphy, C.T., et al., 2003. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. Nature 424, 277–283.
Ogg, S., Ruvkun, G., 1998. The *C. elegans* PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. Mol. Cell 2, 887–893.
Ogg, S., et al., 1997. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. Nature 389, 994–999.
Ohkura, K., et al., 2003. SDF-9, a protein tyrosine phosphatase-like molecule, regulates the L3/dauer developmental decision through hormonal signaling in *C. elegans*. Development 130, 3237–3248.
Paradis, S., Ruvkun, G., 1998. *Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev. 12, 2488–2498.
Paradis, S., et al., 1999. A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in *Caenorhabditis elegans*. Genes Dev. 13, 1438–1452.
Pierce, S.B., et al., 2001. Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse *C. elegans* insulin gene family. Genes Dev. 15, 672–686.
Portman, D.S., 2006. Profiling *C. elegans* Gene Expression with DNA Microarrays. In: T. C. e. R. Community (Ed.), Wormbook. doi/10.1895/wormbook.1.104.1, http://www.wormbook.org.
Prin, F., et al., 2005. Ephrin-As play a rhombomere-specific role in trigeminal motor axon projections in the chick embryo. Dev. Biol. 279, 402–419.
Riddle, D., 1988. The Dauer Larva. In: Wood, W. (Ed.), The Nematode *Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 393–412.
Riddle, D.L., Albert, P.S., 1997. Genetic and environmental regulation of dauer larva development, *C. elegans* II. CSHLP, pp. 739–768.
Riddle, D.L., et al., 1981. Interacting genes in nematode dauer larva formation. Nature 290, 668–671.
Rottiers, V., Antebi, A., 2006. Control of *Caenorhabditis elegans* life history by nuclear receptor signal transduction. Exp. Gerontol. 41, 904–909.
Rottiers, V., et al., 2006. Hormonal control of *C. elegans* dauer formation and life span by a Rieske-like oxygenase. Dev. Cell 10, 473–482.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
Schneider, A., 1866, Monographie der Nematoden (as cited in Debell, J.T. 1965. A long look at neuromuscular junctions in nematodes. The Quarterly Review of Biology. 40. 233–251). Berline.
Shaw, W.M., et al., 2007. The *C. elegans* TGF-beta Dauer pathway regulates longevity via insulin signaling. Curr. Biol. 17, 1635–1645.
Sulston, J.E., Horvitz, H.R., 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. Dev. Biol. 56, 110–156.
Sulston, J.E., et al., 1983. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Dev. Biol. 100, 64–119.
Timmons, L., Fire, A., 1998. Specific interference by ingested dsRNA. Nature 395, 854.
Timmons, L., et al., 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene 263, 103–112.
Wadsworth, W.G., Riddle, D.L., 1989. Developmental regulation of energy metabolism in *Caenorhabditis elegans*. Dev. Biol. 132, 167–173.
White, J.G., et al., 1986. The structure of the nervous system of the nematode *C. elegans*. Philos. Trans. R. Soc. Lond. 314B, 1–340.
Wolkow, C.A., et al., 2000. Regulation of *C. elegans* life-span by insulin-like signaling in the nervous system. Science 290, 147–150.