DAF-5 is a Ski oncoprotein homolog that functions in a neuronal TGFβ pathway to regulate *C. elegans* dauer development

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Summary

An unconventional TGFβ superfamily pathway plays a crucial role in the decision between dauer diapause and reproductive growth. We have studied the *daf-5* gene, which, along with the *daf-3* Smad gene, is antagonized by upstream receptors and receptor-regulated Smads. We show that DAF-5 is a novel member of the Sno/Ski superfamily that binds to DAF-3 Smad, suggesting that DAF-5, like Sno/Ski, is a regulator of transcription in a TGFβ superfamily signaling pathway. However, we present evidence that DAF-5 is an unconventional Sno/Ski protein, because DAF-5 acts as a co-factor, rather than an antagonist, of a Smad protein. We show that expressing DAF-5 in the nervous system rescues a *daf-5* mutant, whereas muscle or hypodermal expression does not. Previous work suggested that DAF-5 and DAF-3 function in pharyngeal muscle to regulate gene expression, but our analysis of regulation of a pharynx specific promoter suggests otherwise. We present a model in which DAF-5 and DAF-3 control the production or release of a hormone from the nervous system by either regulating the expression of biosynthetic genes or by altering the connectivity or the differentiated state of neurons.

Supplemental data available online

Key words: Dauer, Cyclin dependent kinase inhibitor, Dachshund box, Nematode

Introduction

Like many organisms, free-living nematodes can alter development in response to environmental conditions. When food is scarce, population density is high and temperature is high, animals arrest development after the second molt as third larval stage (L3) dauers (Golden and Riddle, 1984a; Golden and Riddle, 1984b). Dauers are resistant to environmental insults and do not age (Riddle and Albert, 1997), which allows survival and dispersal from environments with poor resources. If conditions improve, the dauer molts and resumes reproductive growth.

A transforming growth factor β superfamily (TGFβ) pathway is required for a normal dauer decision, and is thought to act as a step in a neuroendocrine pathway that couples external cues to dauer development (Riddle and Albert, 1997; Patterson and Padgett, 2000). Food availability and population density are sensed as chemosensory cues. These inputs, along with temperature, are sensed during early larval stages (Golden and Riddle, 1984b). High food and low pheromone stimulate transcription of *daf-7*, the gene for the ligand in the TGFβ pathway (Ren et al., 1996; Schackwitz et al., 1996). DAF-7 binds to the receptors DAF-1 and DAF-4, which probably function in neurons (Inoue and Thomas, 2000; Gunther et al., 2000). DAF-8 and DAF-14 are Smads that appear to be the direct targets of the receptors in the dauer TGFβ pathway (A. O. Z. Estevez, PhD thesis, University of Missouri, 1997) (Riddle and Albert, 1997; Inoue and Thomas, 2000). These Smads antagonize the function of another Smad called DAF-3 (Patterson et al., 1997). The *daf-5* gene has similar genetic properties to *daf-3* (Thomas et al., 1993), and so may be acting as a co-factor to DAF-3.

Little is known about events controlled by the receptors and Smads in this pathway, or the mechanism by which the pathway controls the dauer decision. Genetic analysis places the TGFβ pathway upstream of *daf-12* (a gene encoding a nuclear hormone receptor) and *daf-9* (a gene encoding a putative biosynthetic enzyme for a hormone that regulates DAF-12) (Thomas et al., 1993; Antebi et al., 1998; Gerisch et al., 2001; Jia et al., 2002). The *daf-9* gene appears to be expressed in the XXX cell, which is little studied, but has neuronal properties and is located in a head ganglion (Ohkura et al., 2003). These facts, as well as the expression of DAF-7 in neurons and the suggested function of DAF-4 in neurons (Inoue and Thomas, 2000), suggest a model in which DAF-3 and DAF-5 function in the nervous system. However, some evidence can be interpreted as suggesting a function for DAF-3 and DAF-5 outside the nervous system. For example, a small sequence element derived from a pharynx specific promoter binds DAF-3 in vitro, and mediates *daf-3*-dependent repression of a reporter gene in the pharynx (Thatcher et al., 1999).

The dauer TGFβ pathway is unconventional in that upstream components of the pathway directly or indirectly antagonize DAF-3, a Smad protein (Patterson et al., 1997). In other pathways, Smad transcription factors are activated, not

repressed, by the upstream components of the pathway. Anti-Smads antagonize receptor or Smad function (Shi and Massague, 2003), but DAF-3 is antagonized by receptor signaling, and functions in the absence of receptor signaling to regulate genes that control dauer formation (Patterson et al., 1997).

Genetic analysis is consistent with DAF-5 acting as a co-factor of the DAF-3 Smad. The most extensively studied roles of TGFβ superfamily pathways are in the control of cell fate and in control of the cell cycle. The neuroendocrine role of the dauer pathway is very different. The dauer TGFβ pathway has evolved a unique mode of signaling, in which the receptors and receptor-activated Smads antagonize another Smad protein, DAF-3. Studies that reveal mechanisms of *daf-5* function will help us understand how signaling pathways evolve new functions.

We show that DAF-5 is a diverged homolog of human Sno and Ski, which antagonize TGFβ signaling in cell culture by binding Smads, preventing their interaction with each other and with co-activators, and by recruiting co-repressors (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999; Xu et al., 2000; Frederick and Wang, 2002). DAF-5, Sno and Ski share a domain we call the SDS box; this domain in Ski mediates its interaction with Smad4. Yeast two-hybrid experiments demonstrate that DAF-5 interacts with DAF-3, and deletion studies are consistent with this interaction being mediated by the same domains as in the vertebrate Ski/Smad interaction. This functional similarity, combined with our phylogenetic analysis of DAF-5/Sno/Ski homologs suggests that, despite a highly divergent sequence, DAF-5 is an ortholog of Sno and Ski. DAF-5 is the first example of a Sno/Ski with a genetically defined function in a TGFβ pathway. We identified a mutation hotspot in the conserved Dachbox domain. These mutants are the first direct evidence for a function for the Dachbox in TGFβ signaling. Our analysis of regulation of a pharynx-specific promoter does not support a previously hypothesized role for *daf-3* and *daf-5* in the pharynx. Furthermore, we show that *daf-5* is expressed and functions in the nervous system. Therefore, we propose a model in which *daf-3* and *daf-5* have evolved novel functions that allow them to act in a neurosecretory pathway to control *C. elegans* dauer developmental arrest.

Materials and methods

Phenotypic assays

Details of strains used can be found in supplemental data. For egg-laying assays, worms were well fed and grown to L4 or young adult at 15°C, and were transferred to continue development and lay eggs at 25°C. Animals were scored 6 or 26 hours after the first egg was laid; for all genotypes, the data were similar for scoring at any time in this interval. The total number of eggs and the stage of the four oldest eggs inside of a worm were scored with DIC optics. For dauer formation, synchronized worms were incubated at 25-25.8°C for 3-4 days, and scored for dauer formation. High temperature Daf-c assays were very sensitive to temperature change; therefore, two printing thermometers were used to record temperature every 2 hours. Reported temperatures are an average of all readings. For scoring *rnr-1* and *cki-1* expression, animals were grown at 15°C. Gravid adults were transferred to fresh plates and allowed to lay eggs for 2-3 hours at room temperature. The progeny were incubated at 23°C. Larvae were staged by scoring the lethargus at the end of L2, and by body size and gonad size.

Mapping and transgenic strains

DNA polymorphism and mapping data were submitted to Wormbase (http://www.wormbase.org). Clones were injected as previously described (Mello et al., 1991) with pRF4 used as transformation marker.

Orthology and paralogy

We use the terms ortholog and paralog as defined in (Fitch, 1970) and (Jensen, 2001). Simply put, orthologs are duplicates of a character (e.g. a gene sequence) that arise from speciation events, whereas paralogs arise from a duplication within a single genome. Orthology and paralogy are typically hypotheses that are created based on sequence comparisons and other data. These definitions make no suggestion of conserved function.

Yeast-two-hybrid assays

Yeast two hybrid assays were performed as described (Walhout and Vidal, 2001). His/3-AT growth assay was scored on scale 0-3, and β-gal activity assay was scored on scale 0-5. All protein-coding sequences of interest were fused to the activation domain and the DNA-binding domain, and all assays were performed with both AD/DB combinations. We assigned '+' scores based on the total of His/3-AT and β-gal scores from the two combinations: ±, 1-3; +, 4-7; ++, 8 or 9; +++, 10-13; ++++, 14-16.

Results

DAF-5 is required for dauer formation and egg laying

We wished to evaluate the requirement for *daf-5* in inducing dauer formation when TGFβ pathway genes are mutant. Therefore, we carried out an epistasis study of *daf-5* using two alleles that are the most likely nulls, based on gene structure (see below). Smads and receptors in this pathway have redundant functions, and some single mutants do not completely disable the pathway (Gunther et al., 2000; Inoue and Thomas, 2000). In addition, the dauer TGFβ pathway is partially redundant with a pathway that controls body size (Krishna et al., 1999; Morita et al., 1999). Therefore, we tested for the ability of *daf-5* mutants to suppress when multiple pathway components are mutated. *daf-8(sa343)* and *daf-14(m77)* have early stop codons, and each gives the strongest known Daf-c (dauer formation constitutive) phenotype for the respective genes (A. O. Z. Estevez, PhD thesis, University of Missouri, 1997) (Inoue and Thomas, 2000). At 25°C, we see complete suppression of the Daf-c phenotype of *daf-8; daf-14* double mutants and *daf-8; daf-14; sma-2* triple mutants (Table 1). Therefore, at this temperature, *daf-5* is essential for dauer formation induced by TGFβ pathway mutants. At slightly higher temperatures, suppression is incomplete, and continues to diminish as the temperature is raised from 25.4°C to 25.8°C. Our results are consistent with previous results (Thomas et al., 1993; Ailion and Thomas, 2000), but we show that failure to completely suppress *daf-c* mutants at temperatures higher than 25°C is not caused by incomplete loss of *daf-5* function (previous studies used alleles of *daf-5* that may not be null).

Animals mutant for *daf-8* or *daf-14* Smad genes also have an Egl-d (egg laying defective) phenotype. These animals have a structurally normal reproductive system, but lay eggs less frequently than wild type, and accumulate eggs inside. This

Table 1. Dauer larva formation of *daf-c; daf-5* mutants

Genotype	Dauer larva formation*			
	25.0°C†	25.4°C§	25.6°C§	25.8°C§
daf-8(sa233); daf-14(m77)	95±3	89±9	100±0	100±0
daf-8(sa343)	99±0	97±3	100±0	100±0
daf-14(m77)	90±5	100±0	100±0	100±0
daf-8(sa343); daf-5(sa244)	0±0	16±6	25±4	40±10
daf-8(sa343); daf-5(mg89)	0±0	11±6	19±9	27±3
daf-8(sa343); daf-5(sa310)	1±1	3±1	24±3	42±11
daf-14(m77);daf-5(sa244)	0±0	8±2	62±14	42±5
daf-14(m77); daf-5(mg89)	0±0	7±3	33±6	12±8
daf-8(sa343); daf-5(sa244);daf-14(m77)	0±0	3±2	44±5	72±0
daf-8(sa343); daf-5(mg89);daf-14(m77)	0±0	1±1	21±7	50±1
daf-5(sa244)	0±0	0±0	0±0	1±0
daf-5(mg89)	0±0	0±0	2±0	21±4
daf-5(sa310)	0±0	1±1	3±3	33±5
daf-8(sa343); daf-5(sa244); sma-2(e502); daf-14(m77)	0±0	4±4	ND¶	25±1
daf-8(sa343); daf-5(mg89); sma-2(e502); daf-14(m77)	0±0	ND¶	ND¶	37±7
N2 (Wild type)	0±0	ND¶	1±1	9±3

*Percentages are average±s.e.m. from two or three plates.
†Result at 25.0°C came from three experiments.
§Result at 25.4°C, 25.6°C and 25.8°C came from single experiments with two or three plates for each genotype in each experiment.
¶Not determined.

Table 2. Eggs retained in uterus of *daf-c; daf-5* mutants

Genotype	Number of eggs*	*n*†
daf-8(sa343)	34±2	43
daf-14(m77)	27±2	33
daf-8(sa233); daf-14(m77)	31±1	84
daf-5(mg89)	11±1	42
daf-5(sa244)	9±1	41
daf-5(sa310)	10±1	44
daf-8(sa343); daf-5(mg89)	13±1	43
daf-8(sa343); daf-5(sa244)	16±1	41
daf-8(sa343); daf-5(sa310)	15±1	41
daf-5(mg89); daf-14(m77)	18±1	37
daf-5(sa244); daf-14(m77)	17±1	41
daf-8(sa343); daf-5(mg89); daf-14(m77)	24±1	46
daf-8(sa343); daf-5(sa244); daf-14(m77)	17±1	39
N2 (wild type)	17±1	41

*Number of eggs±s.e.m. is shown. These results are from a single experiment, and two other experiments gave similar results.
†Total number of animals.

phenotype is suppressed by *daf-5* and *daf-3* mutations (Trent et al., 1983; Thomas et al., 1993). *daf-8*, *daf-14* and *daf-8; daf-14* mutants contained more eggs (Table 2) and older eggs (see Table S1 and Fig. S1 at http://dev.biologists.org/supplemental), than wild type. Introducing *daf-5* mutants into *daf-8* or *daf-14* mutants or *daf-8; daf-14* double mutants fully suppressed the Egl-d phenotype, except in the case of *daf-8; daf-5(mg89); daf-14*, which was intermediate between wild type and the Daf-c mutants. Interestingly, *daf-5* single mutants have fewer eggs in the uterus than the other genotypes, and have younger eggs inside (for example, 50% of the oldest eggs in *daf-5(sa310)* have fewer than eight cells, versus 15% in wild type). This Egl-c (egg-laying constitutive) phenotype has not been previously reported for *daf-5* mutants.

Cloning of *daf-5*

We identified the *daf-5* coding region using DNA polymorphism mapping, and transgenic rescue of mutants. Our three factor mapping (data submitted to WormBase) placed *daf-5* in a ~120 kb interval (Fig. 1A). Cosmid rescue of *daf-5* mutants suggested that the *daf-5* gene was contained on the cosmid W01G7. Only one predicted gene was within the interval to which *daf-5* was mapped (Fig. 1A). We sequenced three cDNAs provided by Y. Kohara. The longest clone, *yk130g8*, is identical to the structure inferred from a concatenation of EST sequences shown by WormBase (http://www.wormbase.org) and GenBank (NM_064540).

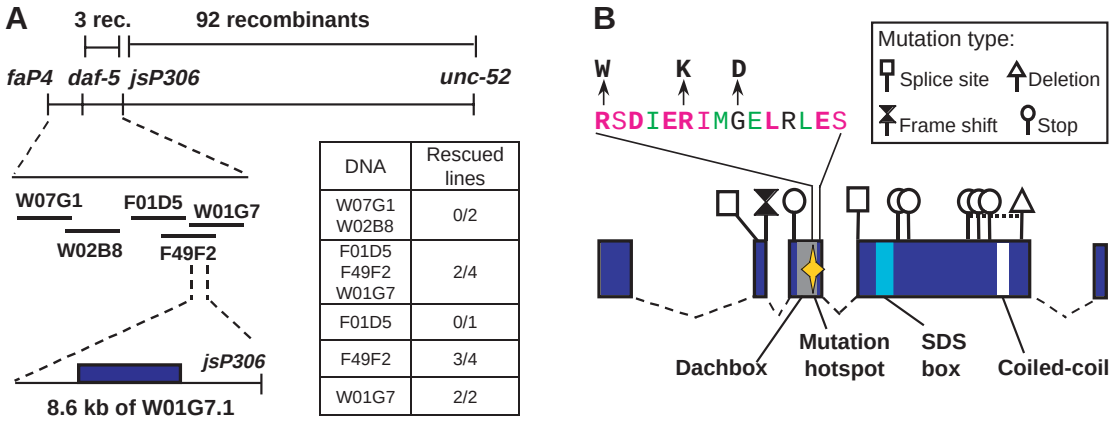
DAF-5 is similar to Ski

A blast of the predicted DAF-5 protein sequence against databases at NCBI reveals similarity to the oncoproteins Ski (for Sloan Kettering Virus) and Sno (for Ski-related novel sequence). A careful examination of many Sno/Ski homologs demonstrates that the homology of DAF-5 is significant, and we suggest that DAF-5 is the *C. elegans* ortholog of Sno/Ski. We show two domains from the Sno/Ski/Dachshund superfamily. Fig. 1C shows the SDS box (for Sno, Daf-5 and Ski); in human Ski, the SDS box and about 20 amino acids on either side constitute the minimal region required for binding to Smad4. Fig. 1D shows the Dachbox-N, a domain shared by the Sno/Ski family and Dachshund, which is a transcription regulator conserved throughout bilateria. DAF-5 has a predicted coiled-coil at the C terminus; Sno and Ski also have a coiled coil, but have a pattern of charged residues not shared by DAF-5.

This alignment is very informative regarding the relationship of DAF-5 to other members of the family. Sno and Ski are found in humans and all major groups of vertebrates, but in insects have only one ortholog of these two proteins (see Materials and methods for definitions of ortholog and paralog). Sno and Ski are more similar to each other than either is to the single *Drosophila* or mosquito ortholog; therefore Sno and Ski are probably paralogs that were duplicated after the divergence of the protostome and deuterostome lineages. We have named the insect genes *Snowski* (*Snk*) to reflect the orthology to both *Sno* and *Ski*.

A new family of proteins closely related to the Snowski group is shown in Fig. 1C,D. Humans have two genes in this group. We have named these genes *Skate* (for *Ski-related gene*) and *Icy* (for *Ski sequence family*). *Drosophila* and mosquito each have a gene that is much more similar to human *Icy* and *Skate* than to *Drosophila Snk*. We have named the single *Drosophila* and mosquito genes *iceskate* (*isk*) to reflect their orthology to both *Icy* and *Skate*.

We suggest that DAF-5 is an ortholog of Snowski or Iceskate. First, DAF-5 clearly has an SDS box, which is not found in any other protein in *C. elegans* or *C. briggsae*. This SDS box is more similar to the Snowski group than to the Iceskate group, including amino acids that are important for the ability of Ski to bind Smad proteins. Second, DAF-5 binds the DAF-3 Smad (see below). This binding is mediated by the SDS box in Ski, and may thus be a conserved function of the SDS box. Third, the rate of divergence in the Snowski/Iceskate family is so high that relatively modest sequence conservation is not surprising. This rapid change can be seen when examining the SDS box of *B. malayi* and potato cyst nematode Snowski. These two nematode proteins are more different from



DNA	Rescued lines
W07G1 W02B8	0/2
F01D5 F49F2 W01G7	2/4
F01D5	0/1
F49F2	3/4
W01G7	2/2

C

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Human Icy                CIKCGYCSMYFSP-NKFIFHSHR-TPDAKYTQPDAANFNSWRRHLKL
Human Skate              CIKCSYCNMYFSP-NKFIFHSHR-TPDAKYTQPDAANFNSWRRHLKL
Dros Iceskate            CIKCSYCGMFFSP-NKFIFHSHRITTNDRYVQPDAANFNSWRRHMSL
Mosq Iceskate            CIKCAYCSLFFSP-NKFIFHSHRLSPHDKYVQPDAANFNSWRRHLKL

Human cSKI               CIQCLDCRLMYPP-HKFVVHSHKAL--ENRTCHWGFDSANWRAYILL
Human SnoN               CIQCLECCGMFAP-QTFVMHSHRSP--DKRTCHWGFESAKWHCYLHV
Sea squirt Ski           CVSCLDCRISFSP-ARFVSHSHRGL--EKHTCHWGFDPENWRNYLLV

Dros Snowski             CIKCLECDGWFSP-QKFVGHVHRKF--ENHTCHWGFDSRNWHDYLHV
Mosq Snowski             CIECAECRGLFSP-QKFVCHQHEPQ--EIRTCHWGFNSSNWRSYIHV

Pot Nem Snowski          CFECRTCYRMFKP-EDFCRHTHQPTS-DKNICFWGFDASNWAYYIRV
Lymph Nem Snowski        QIECTECKLLFTG-EKFVSHSHSMQQ-IQRICHWGFDSSNWRHYLHL
Dog Nem Snowski          CTICIRCCRAVLK--RFSHXSHXLPSTEK--CYWEHISLLWNCTFER

C. briggsae DAF-5        SIKCRECMKMFTP-ADFILHHHYPRR-NETLEHVGLNSAKWTELITV
C. elegans DAF-5         CIECQHCEGKFTP-TDFIMHHHYPIK-PSGFVHTGCNSFQWIRLIEV
                            %  %            *% %  *    ********       **
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D

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Human Icy                GVPIVSLVIDGQERLCLAQISNTLLKNYS--YNEIHNRRVALGITCVQC-TPVQLEILRRAGA-MPISS--RRCGMITKREAERL
Human Skate              GIPIVSLVIDGQERLCLAQISNTLLKNFS--YNEIHNRRVALGITCVQC-TPVQLEILRRAGA-MPISS--RRCGMITKREAERL
Dros. Iceskate           GVQIVSLHIEGQERLCLAQISNTLLKQFS--YNEIHNRRVALGITCVQC-TPVQLEILRRAGA-MPVSS--RRCGMITRREAERL
Mosq Iceskate            GQPIISLFVENQERLCLAQISSTLLKDFS--YNEIHNRRVALGITCIQC-TPVQLEILRRAGA-MPASS--RRCGMITRREAERL

Human cSKI               GETISCFVVGGEKRLCLPQILNSVLRDFS--LQQINAVCDELHIYCSRC-TADQLEILKVMGI-LPFSA--PSCGLITKTDAERL
Human SnoN               GESISCFQVGGEKRLCLPQVLNSVLREFT--LQQINTVCDELYIYCSRC-TSDQLHILKVLGI-LPFNA--PSCGLITLTDAQRL
Sea squirt Ski           GERISCFIVGGEKRLCLPQILTSVLREFS--ISEINRTCAELHIYCSRC-SPDQLEILKVLGI-LPFNT--TQCGLITKTDAERL

Dros Snowski             GKTIGCFSVGGEMRLCLPQFLNNVLNDFS--LEQINRIFDELGIYCSQC-THDQLVEFKAAKI-LPSDV--KASGLITRTDAERL
Mosq Snowski             GKRIGCFLLGGETRLCLPQIFNNILMDFS--VEQINRSIQELMIYLYNC-TDQQLAEFKRANI-IPDTA--KSCGLITRTNAERL

Soy Nem Snowski          GKALGCFIIGGECRLSFPQMRALCLGEIP--TQKIDESMKLLAIVNALA-TEDQLTVLKRAGL-AQPSL--SKCELITKTNAERL
Root Nem Snowski         GQLLSCFVIGGECRLCFPQMKALCLGDIP--TYKIDEMIKNL----------------------------------------
Pot Nem Snowski          NKLLGCFVIGGECRLCFPQVRALCLGEIS--TQQIDEAMQFLAIANALA-SEEQLIELKRAGL-AHPSL--SKCELITKTNAERL

C. briggsae DAF-5        GRKIPALIINGEAYMPVELLQQILNESIDKKSESLQAFMAAFNIPTRYA-TRTQYTVILRKCV-ECRKLGSTSLRLISRSNMERI
C. elegans DAF-5         GVRVPALSIDGEPHLPIEILDDMLTKKDKKDQMSFQNLLRYKNVYIRMA-SPSQFRAVMEKSK-ECENLNITSLSLMSRSDIERI

Human Dach               GAKVASFTVEGCELICLPQAFDLFLKHLVGGLHTVYTKLKRLEITPVVC-NVEQVRILRGLGA-IQPGV--NRCKLISRKDFETL
Sea squirt Dach          GSKVASFSLGCDEYICLPQAFDLFLKHLVGGLHTVYTKLKRLSIAPVVC-NVEQVRILRGLGA-IQPGV--NRCKLITRKDFEIL
Dros Dach                GQKVAAFIISNETMLCLPQAFELFLKHLVGGLHTVYTKLKRLDIVPLVC-NVEQVRILRGLGA-IQPGV--NRCKLLCCKDFDIL
C. elegans Dach          GHNVAAFDINGKEMICLPQVYEVFLKNMVGGLHTVYTKLKRLYIHPMVC-NVEQVRALRSLGA-IQPGV--NRCKLLKTSDFEKL
flatworm                 LQIIQTLVFPVFVVPHIYGFHYEFLRPFR--LVQVHSICSELYLHEILSRVHKHFRNICLMLRRIPGSN--PKCQVRFQTNQHKT
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each other than insect Snowski is from Human Sno and Ski. The DAF-5 gene is even more rapidly diverging in the *Caenorhabditis* genus. *C. briggsae* and *C. elegans* proteins average more than 70% amino acid identity. The DAF-5 sequence is only 40% identical overall between *C. briggsae* and *C. elegans*. In fact, in the Dachbox and SDS box, the difference between *C. elegans* and *C. briggsae* DAF-5 is greater than the difference between insect and human Snowski.

A mutation cluster in the Dachbox domain

Sequencing of *daf-5* mutants identified a mutation hotspot. We identified mutations in 15 *daf-5* alleles (Fig. 1B; see Table S2 at http://dev.biologists.org/supplemental). All five missense mutants were found in a 16 amino acid stretch of the 627 amino acid protein. This hotspot is in the region of the Dachbox where DAF-5 is most similar to Snowski, Iceskate and Dachshund. Three of the mutants affect two very strongly conserved residues (two of the mutants are identical but independently isolated). One mutant has an in frame deletion of this region, and the final mutant is in a glycine that is unique to DAF-5. This region is critically important for DAF-5 function; the *sa310* mutation (E162K) has a phenotype as severe as putative

Fig. 1. DAF-5 encodes a *C. elegans* homolog of the Sno and Ski oncoproteins. (A) Identification of *daf-5*-coding region. The genetic map near *daf-5* and rescued transgenic lines isolated are shown. (B) DAF-5 gene structure. At the C terminus of DAF-5, a stop codon and a deletion mutant are connected by a broken line to indicate that these two mutations were identified in a single allele. A mutation hotspot is shown; an additional mutant has an in-frame deletion of the 15 amino acids shown. Colors of amino acids in hotspot represent sequence conservation as in Fig. 1C,D. See Table S2 at http://dev.biologists.org/supplemental for complete details on mutant alleles. (C,D) Alignment of SDS and Dach boxes. Consensus at each position was defined as any set of identical and similar amino acids that were found in more than one subgroup (subgroups are indicated by spaces between the rows of sequence), and in more than three proteins in total. Bold magenta residues are identical in the primary consensus (the consensus with the most matches) and plain, magenta are similar. Green residues are the secondary consensus. The % symbols below the SDS box show conserved zinc chelating residues, and the asterisks indicate amino acids that contact Smad4 in the crystal structure of a Ski/Smad4 complex. The sequence for Dog Nem. is from an EST, and has two stop codons in frame (indicated by X). These may result from sequencing errors, or the cDNA may have come from a pseudogene. Accession Numbers and abbreviations: Dros., *D. melanogaster* (Iceskate: NP_651946; Snowski: NP_609166; Dachshund: NP_723972); Mosq., mosquito (*A. gambiae*; Iceskate–XP_317739; Snowski–XP_317545); Soy. Nem., Soybean Cyst nematode (*Heterodera glycines*; CA939358); Root nem., Root Knot nematode (*Meloidogyne chitwoodi*; CB931358); Pot. Nem., Potato Cyst nematode (*Globodera rostochiensis*; AY389814); Lymph Nem, *Brugia malayi* (AY389813); Dog Nem, dog hookworm (*Ancylostoma caninum*; AW735310); *C. briggsa*e DAF-5 (found in Wormbase as CBG20832); *C. elegans* (DAF-5–NM_064540, NP_496941; Dachshund–NP_497266); Human (Dachshund–NP_542937; cSki–NP_003027; SnoN–NP_005405; Icy–XP_292349; Skate– XP_064560); sea squirt (*Ciona intestinalis*; Ski–BK001616; Dachshund–AABS01000073).

	DAF-3	DAF-3.1	DAF-3.2	Empty Vector
DAF-5 (Full-length)	+ +	+/-	—	—
DAF-5.A	+ + +	+/-	+ + + +	+/-
DAF-5.B	—	+/-	—	—
DAF-5.C	—	+/-	—	—
Empty Vector	—	+/-	—	—

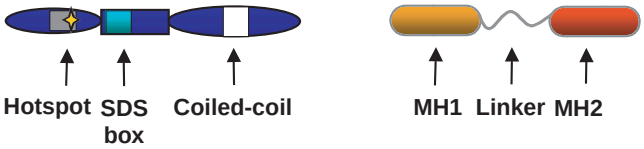

Fig. 2. Binding of DAF-3 to DAF-5 in yeast two-hybrid assays. Interactions were scored by transcriptional activation of a His reporter and a β-gal reporter. The strength of activation was assigned a score in each assay, and the symbols reflect the sum of the scores for all assays (see Materials and methods).

null alleles (Table 1). In vitro analysis showed that an insertion of four amino acids next to residue 168, which is homologous to the residue that is mutated in *daf-5(sa310)*, eliminates the transforming and myogenic activity of vSki (Zheng et al., 1997). Thus, the Dachbox is required for a gain-of-function phenotype of vSki, but how this function relates to wild-type function of cSki or to TGFβ signaling has not been experimentally determined. A point mutation in the Dachbox disrupts the interaction of Ski with NCoR (Ueki and Hayman, 2003). However, this mutation affected Vitamin D receptor-dependent gene expression, but not TGFβ-dependent gene expression. These in vitro experiments suggest possible functions for the Dachbox, but our *daf-5* mutants are the first missense mutants identified in any *Sno/Ski* gene, and thus the first in vivo evidence for the wild-type function of a particular domain of Sno/Ski.

DAF-5 binds to Smad DAF-3

A yeast two-hybrid screen for proteins that interact with DAF-3 identified the predicted protein W01G7.1 (M. Tewari, P. J. Hu, G. B. Ruvkun and M. Vidal, personal communication), which we show is DAF-5. We used yeast two-hybrid assays to identify regions of DAF-3 and DAF-5 required for interaction. We find that the DAF-3 MH2 domain strongly interacts with a DAF-5 fragment that is truncated after the SDS domain (Fig. 2, see Table S3 at http://dev.biologists.org/supplemental). Thus, as in vertebrates, the region downstream of the SDS box is dispensable for binding to Smads and the Smad domain that binds Sno/Ski is the MH2 domain (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999; Frederick and Wang, 2002; Wu et al., 2002). These interaction results suggest that DAF-3 and DAF-5 function as part of a transcriptional regulatory complex.

DAF-5 is found in nuclei of neurons, pharynx and some other tissues

We made reporter gene GFP fusions to identify the cells in which *daf-5* might function. Our full-length construct rescues a *daf-5* mutant as efficiently as a wild-type genomic clone (Table 4). We saw relatively strong expression in ganglia in the head and tail and in the anterior pharynx (Fig. 3). Cell ablation and other experiments have shown that several cells in the nervous system (ASI, ADF, ASG, ASJ and XXX) are required for normal regulation of dauer formation (Bargmann and Horvitz, 1991; Schackwitz et al., 1996; Jia et al., 2002; Gerisch et al., 2001). We examined these cells for DAF-5::GFP expression. GFP was not seen in ASI, ASJ, ADF or ASG (0 of 86 animals). Fluorescence in the anterior ganglion, where XXX is found, is at least an order of magnitude less than in the bright cells of the ganglia posterior to the nerve ring. The weakness of fluorescence has precluded identification of specific cells, but we sometimes see weak fluorescence in the ventral, anterior part of the ganglion, where XXX resides (seven out of 53 animals). A small number of animals show weak expression in

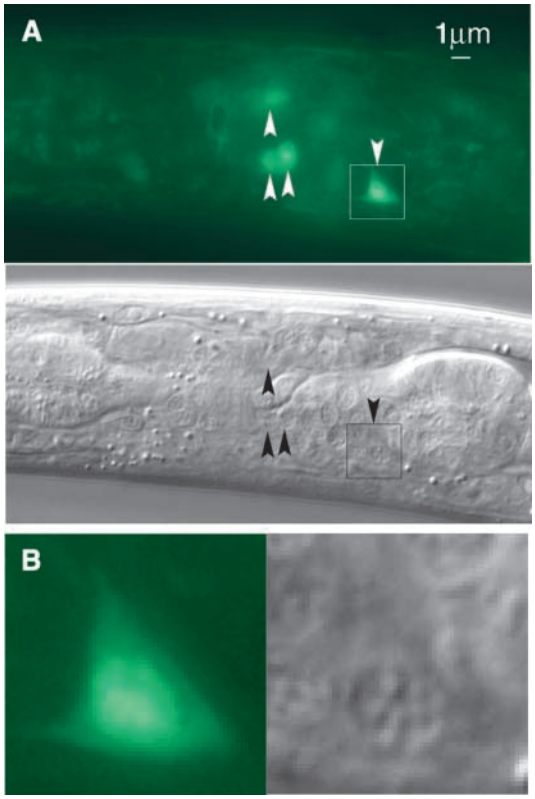

Fig. 3. *daf-5* is expressed in the nervous system and preferentially localized to nuclei. Expression of functional *daf-5::GFP.* (A) Functional *daf-5::GFP* is predominantly expressed in head ganglia. Several neurons in the head ganglia are indicated by arrowheads. (B) DAF-5 is localized to the nucleus. This panel is an enlargement of the area boxed in A. A triangle shaped neuron was glowing with DAF-5::GFP mostly found in its oval nucleus.

the hypodermis, muscles, intestine and distal tip cells (see Table S4 at http://dev.biologists.org/supplemental). We also constructed a transcriptional GFP fusion, which consists of 6.5 kb upstream of the first ATG and also the first 57 codons in the first exon. The non-rescuing GFP construct is more strongly expressed, and shows more consistent expression in the hypodermis, muscles, intestine and distal tip cells; still, its expression is strongest in the head and tail ganglia (see Table S4 at http://dev.biologists.org/supplemental). DAF-5::GFP from the rescuing construct mostly localizes to nuclei (Fig. 3, Table 3), which is consistent with the idea that DAF-5 is a transcription factor and functions in the nucleus. We examined the intensity of fluorescence and nuclear localization of DAF-5::GFP from the rescuing construct in wild-type and in TGFβ pathway mutants, including dauers, and saw no obvious differences.

daf-5 functions in nervous system

We used tissue-specific expression of *daf-5* to identify cells in which it functions. We expressed *daf-5cDNA::GFP* with various tissue-specific promoters (Aamodt et al., 1991; Okkema et al., 1993; Hsu et al., 1995; Maduro and Pilgrim, 1995; Gilleard et al., 1997; Ogura et al., 1997); these constructs had GFP inserted at the same site as a genomic construct that rescued a *daf-5* mutant (Table 4). Rescue was assayed in *daf-7; daf-5* double mutants. Rescued animals would be expected to have the Daf-c phenotype of a *daf-7* mutant. *pF25B3.3* strongly expressed *daf-5::GFP* exclusively in nervous system and rescued *daf-5* mutants as well as two positive controls. Similarly, *punc-14*, which expressed *daf-5::GFP* in nervous system at high level in addition to some non-neuronal expression, also showed strong rescue. Weak ubiquitous expression of *daf-5::GFP* from the *pdpy-30* promoter gave partial rescue. *unc-119::daf-5::GFP* was weakly expressed in the nervous system but did not rescue, perhaps owing to the low level of expression. Strong expression of *daf-5::GFP* from the muscle promoter *pmyo-3* did not rescue at all. Initial tests of two strains of *pdpy-7::daf-5::GFP* gave very weak expression and no rescue. Therefore, we isolated additional lines that had strong hypodermal expression of *daf-5::GFP*, and these did not rescue either. For unknown reasons, the expression of *pges-1::daf-5::GFP* was undetectable. Overall, our results show neuronal expression of *daf-5* is sufficient to rescue *daf-5* dauer formation defect, while muscle or hypodermal expression is not.

Table 3. Nuclear versus cytoplasmic localization of DAF-5::GFP

	Percentage of animals*					
	Mostly cytoplasmic	Cytoplasmic> nuclear	Even	Nuclear> cytoplasmic	Mostly nuclear	*n*†
L1	0%	0%	41%	49%	10%	126
L2	0%	4%	14%	65%	17%	94

*Individual neurons were examined, and fluorescence was scored by eye in each cell. Twenty-six L1 larvae and 22 L2 larvae were scored.
†Total number of cells examined.

DAF-5 is required for cell cycle arrest

In vertebrate cells, Smad proteins control the cell cycle by regulating transcription of cyclin kinase inhibitor genes (Moustakas and Kardassis, 1998). The division of hypodermal seam cells is arrested in dauers because CKI-1, a cyclin kinase inhibitor, is transcriptionally upregulated, and this upregulation is inhibited by wild-type *daf-7* (Hong et al., 1998), suggesting that *daf-5* may directly or indirectly upregulate *cki-1*. We tested whether cell cycle arrest in dauers requires *daf-5*. The reporter, *rnr-1::GFP*, drives the expression of GFP in the seam cells as they enter S phase and through the division at the beginning of L3. *daf-7* (*e1372*) mutants do not express *rnr-1::GFP* at the corresponding time, which reflects seam cell cycle arrest in dauers (Hong et al., 1998). We observed that 47% of N2 show *rnr-1::GFP* expression (Fig. 4A, Table 5), whereas none of the *daf-1*(*sa184*)-induced dauers does. Thus, this *daf-1* mutation causes a complete arrest of the cell cycle. Interestingly, in *daf*-5(*sa310*)*; daf-1(sa184)* double mutants, the percentage of animals with green seam cells is 31%, which is similar to wild type (Fig. 4B; Table 5). The reason that we did not see *rnr-1* expression in some N2 or *daf-5; daf-7* double mutants even after careful synchronization was probably due to the transient expression of the *rnr-1* gene and variation in the timing of cell cycle among individual animals. We conclude that a *daf-5* mutation suppresses the cell cycle arrest caused by *daf-1*.

We wanted to test whether *daf-5* controls seam cell cycle arrest via the cyclin kinase inhibitor, *cki-1*. In wild type, after

Table 4. Rescue of a *daf-5* mutant by tissue specific expression of *daf-5*

	Daf-c assay		Expression of GFP construct								
			Number of animals expressing GFP*								
Promoter fused to *daf-5::GFP*†	% Dauer§	*n*¶	Head neuron	Tail neuron	Ventral cord	Pharynx	Hypodermis	Muscle	Intestine	*n*¶	Intensity
*daf-5***	98	177	79	51	6	62	9	3	6	79	+††
F25B3.3	94	1101	37	37	9	-	-	-	-	37	+
unc-14	96	682	34	34	21	-	29	16	-	34	+§§
dpy-30	23	643	17	13	-	16	16	-	16	17	+/–
unc-119	2	495	21	13	-	-	-	-	5	21	+/–
dpy-7¶¶ 1st test	0	684	-	-	-	-	5	-	-	5[c]	+/–
dpy-7¶¶ 2nd test	0	986	-	-	-	-	16	-	-	16	+
ges-1	1	711	-	-	-	-	-	-	-	70***	–
myo-3	2	221	-	-	-	-	-	40	-	40	+
Negative control†††	0	208	NA§§§	NA§§§	NA§§§.	NA§§§	NA§§§	NA§§§	NA§§§	NA§§§	NA§§§

*The number of animals with expression in each tissue is indicated, with the total number of animals scored shown. '+' indicates strong expression, at least as bright as neurons of animals with the *daf-5* genomic construct, '+/–' indicates weak expression, and '–' indicates no expression.

†Promoters from the indicated genes were fused to a *daf-5* cDNA with *GFP* inserted close to the N terminus.

§Transgenic *daf-5(e1386);daf-7(e1372)* animals were scored for rescue of *daf-5*; because of *daf-7(e1372)*, rescue causes a dauer constitutive phenotype. The percentage shown is (number of transgenic dauers)/(total number of transgenic animals), with transgenic animals identified by Rol phenotype.

¶Number of animals scored.

**This construct is *daf-5* genomic DNA with *GFP* inserted into the coding region.

††Head and tail neurons and pharynx fluorescence intensity was scored as '+'; ventral cord, pharynx, hypodermis, muscle and intestine fluorescence intensity was scored as '+/–'.

§§Neuron fluorescence intensity was scored as '+'; hypodermis and muscle fluorescence intensity was scored as '+/–'.

¶¶Tests with two *dpy-7* arrays ('1st test') gave virtually no GFP fluorescence. We screened additional strains to identify strongly fluorescent strains and retested ('2nd test').

***Number of animals examined; at least 20% of them have transgenic markers.

†††Negative control is a strain with the same transgenic marker.

§§§Not applicable.

cell division is complete, *cki-1::GFP* expression is seen, reflecting the role of *cki-1* in restricting seam cells to one and only one division (Hong et al., 1998). In dauer larvae, *cki-1* is expressed during the period where seam cells would divide in wild type; as a result, the seam cells do not divide. In *daf-1(sa184)* induced dauers, 100% of the animals with the *cki-1::GFP* reporter had a continuous line of green seam cells on both sides (Fig. 4C, Table 5), confirming the regulation of *cki-1* by TGFβ signaling (Hong et al., 1998). Eighty-one percent of wild-type animals had either no green seam cells (53%) or a few faintly green seam cells (28%) expressing GFP. Similar to the N2 animals, 66% of *daf-1; daf-5* double mutants had either no green seam cells (37%) (Fig. 4D, top animal, Table 5) or a few faintly green seam cells (29%). Nineteen percent of N2 and 34% of *daf-1; daf-5* double mutants have many green cells (Fig. 4D bottom animal, Table 5). Expression in a subset of animals is expected because *cki-1* is transiently expressed at the beginning of each larval stage to limit seam cells to a single division (Hong et al., 1998). Therefore, *daf-5* mediates cell cycle arrest in dauers by controlling *cki-1* expression. However, unlike in vertebrate TGFβ signaling, this control is likely to be indirect, because *daf-5* expression in the nervous system is sufficient to cause dauer arrest.

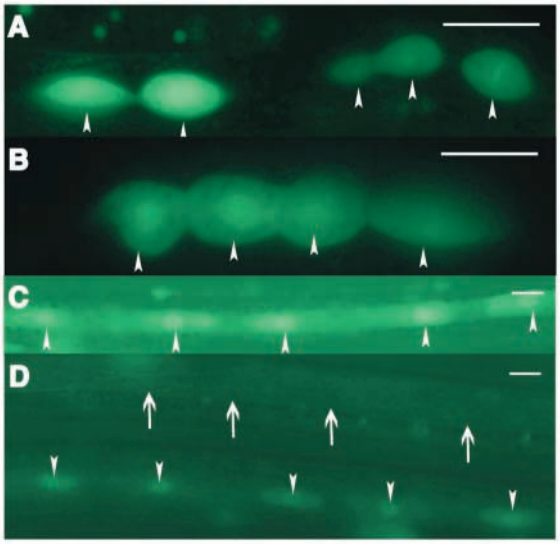

Fig. 4. *daf-5* controls cell cycle arrest in the seam cells of dauer larvae by regulating expression of a cyclin kinase inhibitor. (A,B) *rnr-1::GFP* expression in N2 and *daf-5; daf-1* double mutants. A corresponding Nomarski image can be found in Fig. S2 at http://dev.biologists.org/supplemental. White arrowheads show seam cells that express *rnr-1::GFP* in N2 and in *daf-5; daf-1*, respectively, at early L3 stage. (C,D) *cki-1::GFP* expression in *daf-1* and *daf-5; daf-1* mutants. A corresponding Nomarski image can be found in Fig. S2 at http://dev.biologists.org/supplemental. (C) *cki-1* is expressed along the seam cells in all *daf-1* dauer larvae with an average of 16 green cells. White arrowheads (E) show seam cell nuclei. (D) *cki-1* is expressed in a subset of *daf-5; daf-1* mutants in early L3. Two *daf-5; daf-1* animals are shown. The top animal is representative of the 37% of animals of this genotype that had no GFP in the seam at all. The bottom animal is representative of the 34% that expressed GFP faintly in many seam cells (between five and ten). White arrows point to the seam cells that do not express *cki-1::GFP* in the nuclei. Scale bars: 1 μm.

Regulation of *myo-2*

Previous work is consistent with DAF-5 and DAF-3 having a

Table 5. Regulation of cell cycle by *daf-5*

Genotype	Animals with green seam cells	Animals with no green seam cells	*n**
N2; rnr-1::GFP	47%	53%	32
daf-1; rnr-1::GFP	0%	100%	28
daf-1; daf-5; rnr-1::GFP	31%	69%	91

	Animals with many green seam cells	Animals with more than four green cells in midbody	Animals with no green seam cells	*n**
N2;cki-1::gfp	19%	28%	53%	32
daf-1;cki-1::gfp	100%	0%	0%	43
daf-1;daf-5;cki-1::gfp	34%	29%	37%	38

*Number of animals tested.

Table 6. Expression of a full-length *myo-2::GFP* construct in the pharynx

	L2/L2d		L3/dauer		Adult	
Genotype	Fluorescence*	*n*†	Fluorescence*	*n*†	Fluorescence*	*n*†
daf-7(e1372); daf-3(e1376)	0.9±0.4	16	1.4±0.7	20	2.5±1.3	21
daf-7(e1372)	1.3±0.5	12	0.5±0.2	10	4.0±2.0	11
daf-7(e1372); daf-5(e1386)	0.9±0.6	10	1.2±0.5	10	3.0±1.3	21
N2 (wild type)	1.1±0.6	20	1.7±0.7	10	4.5±2.8	29

*Total fluorescence of the pharynx L2, L3, and adults (or L2d, dauer and adult for *daf-7*) with a transgene fusion of a *myo-2* promoter (including 1.6 kb upstream of the translation start) to GFP was measured by photographing and quantitating fluorescence. We measured total fluorescence in the pharynx, in arbitrary units. The results shown are from a single experiment, and three additional experiments gave similar results.

†Number of animals tested.

function in the adult pharynx, but we found that expression of DAF-5 solely in the nervous system can cause dauer arrest. Therefore, we re-examined the role of DAF-3 and DAF-5 in adult pharynx. DAF-3 was shown to bind to a 5 bp sequence within a 32 base pair regulatory element isolated from the *C. elegans myo-2* promoter (the 'C subelement'). This regulatory element drives pharynx-specific gene expression when placed upstream of a minimal promoter (Thatcher et al., 1999). Expression from this construct in adults is strongly repressed in mutants of *daf-7* and other Daf-c mutants in the TGFβ pathway, but a mutation of *daf-3* or *daf-5* relieves this repression. However, all of this regulation was observed with the C subelement removed from its normal context and concatamerized upstream of a minimal promoter, and we wished to see if this regulation occurs within the normal context of the full *myo-2* promoter.

We used a GFP reporter fused to a full-length *myo-2* promoter (with 1.6 kb upstream of the translation start) to examine regulation by genes in the TGFβ pathway. If the full-length promoter is regulated similarly to the C subelement reporter, we expected that expression would be repressed in *daf-7* mutant adults relative to wild-type adults, and that mutations in *daf-5* or *daf-3* would alleviate that repression. However, expression in adults was indistinguishable in wild type and in *daf-7* mutants (Table 6). Therefore, the regulation of the full-length promoter is unlike the regulation of the C subelement reporter. Similarly, in wild-type L2 stage larvae and *daf-7* larvae in the corresponding L2d stage, expression was indistinguishable between N2 and *daf-7*, and expression in the *daf-7;daf-5* and *daf-7;daf-3* double mutants was modestly reduced. Finally, we see that expression in the *daf-7* dauer is less than in wild type or the *daf-7;daf-3* and *daf-7;daf-5* double mutants. The C subelement reporter also shows a reduction in expression in dauers, comparable with what we see. However, unlike in adults, the reduction of expression of the C subelement reporter in dauers is not dependent on *daf-3* (Thatcher et al., 1999), and is therefore mediated by some other pathway. In summary, we see no evidence that the full-length *myo-2* promoter is regulated by *daf-3* or *daf-5*.

Discussion

We show that DAF-5 is homologous to the Sno/Ski family of transcriptional co-repressors, and we argue that DAF-5 is likely to be the *C. elegans* ortholog of human Sno/Ski and *Drosophila* Snowski. In vertebrates, Sno and Ski antagonize TGFβ signaling by binding to Smads, but our analysis clearly demonstrates that DAF-5 is not an antagonist of the DAF-3 Smad. We show that DAF-5 is localized to the nucleus, and that it binds DAF-3, which strongly suggests that DAF-5 is a co-factor of DAF-3, and that DAF-5 assists DAF-3 in regulating gene expression. Our findings are novel in that we have identified a wild-type function for a Ski/Sno protein in a TGFβ pathway in vivo.

Analysis of DAF-5 identifies a role for a Snowski family protein in TGFβ signaling in vivo

Recent work in cell culture has showed that Sno and Ski function in vitro as key factors in TGFβ regulation of gene expression and cell division (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999; Wang et al., 2000; Xu et al., 2000; Frederick and Wang, 2002; Miyazono et al., 2003; Ueki and Hayman, 2003). These studies suggest numerous possible functions for Sno and Ski in TGFβ signaling, but understanding the specific contexts in which Sno and Ski function in vivo will require further work. Some studies of Sno/Ski function in vivo have used gain-of-function phenotypes in *Xenopus* development (Wang et al., 2000) or in cancer (Frederick and Wang, 2002; Miyazono et al., 2003). These studies have identified mechanisms of Sno/Ski action and suggest possible roles for the proteins in vivo, but are accompanied by a caveat. Overexpression and gain-of-function mutants might cause the genes to act in an event that is not part of the normal function of the gene. Mouse *Ski* and *Sno* mutants have interesting phenotypes, including skeletal and muscular developmental defects and cancer susceptibility (Berk et al., 1997; Shinagawa et al., 2000; Shinagawa et al., 2001). These mutants will be useful in dissecting the wild-type functions of Sno and Ski in TGFβ signaling; however, the phenotypes of these mutants are complex, and Sno and Ski function in multiple signaling pathways (Dahl et al., 1998; Nomura et al., 1999), so tying a specific phenotype to the effect of Sno or Ski on TGFβ signaling will require careful analysis. Whereas Sno and Ski function in many pathways, *daf-5* mutants exclusively affect phenotypes controlled by the dauer TGFβ pathway;

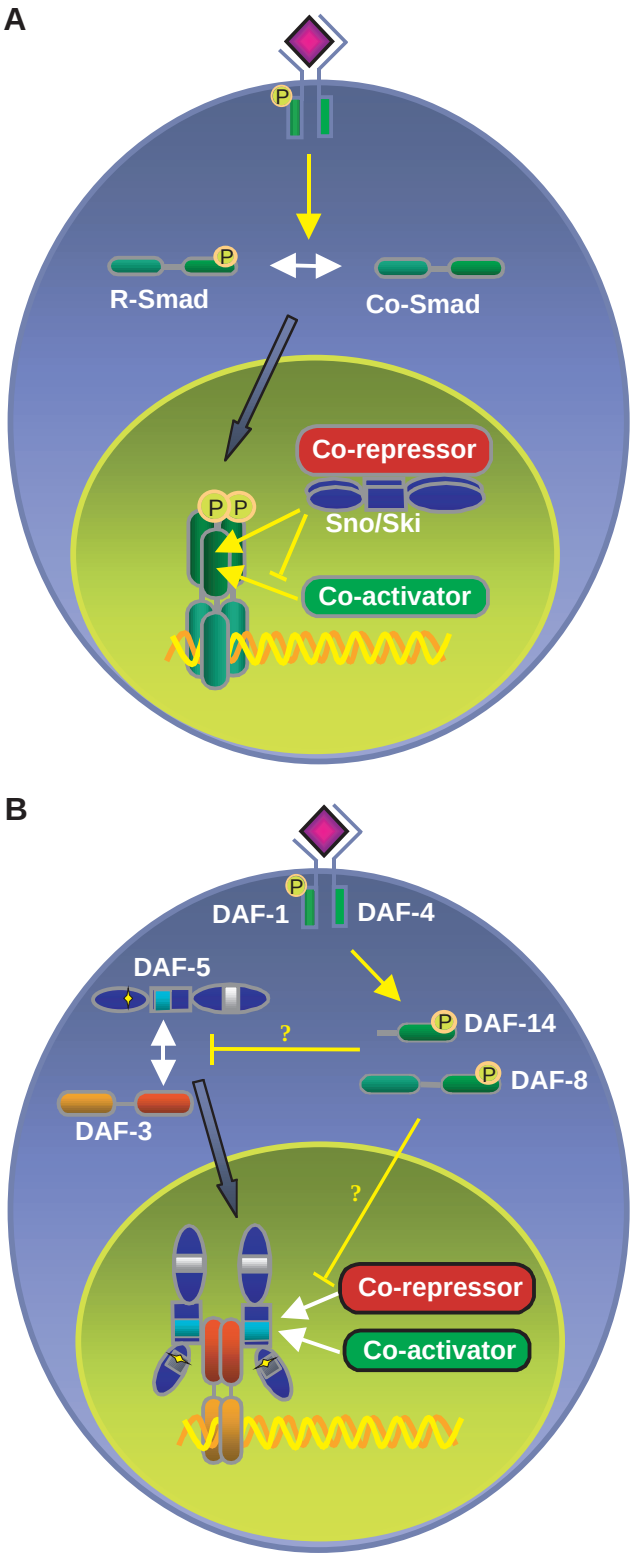

Fig. 5. Model for Sno/Ski and DAF-5 function. (A) Antagonism by Sno and Ski in vertebrates. (B) Function of DAF-5 in *C. elegans*. See Discussion for explanation of models.

this fact makes the assignment of function much more straightforward in *C. elegans*.

DAF-5 and the Snowski/Iceskate gene families

From analysis of the Snowski family, we identified several interesting new genes and relationships among them. First, we show that *Sno* and *Ski* are likely to be paralogs that arose after the divergence of protostomes and deuterostomes, perhaps as late as the divergence of urochordates and cephalochordates (because sea squirt has only one ortholog). Second, we identify the *Iceskate* family: an uncharacterized, highly conserved *Snowski*-related gene family. Genes in the *Iceskate* family have a striking difference from *Snowski*. Iceskate shows virtually no similarity to a set of amino acids in the SDS box of Ski that provide critical hydrogen bonds and van der Waals contacts in the Ski/Smad4 structure (Wu et al., 2002). To our knowledge, Iceskate proteins have not been studied experimentally; the functional relationship of Iceskate to Sno/Ski is an interesting issue for future research. Third, we find that Snowski is changing rapidly in nematode evolution. In *Caenorhabditis*, we see that *daf-5* is changing rapidly, much more rapidly than the typical *C. elegans* gene, which may explain the relatively low primary sequence conservation between DAF-5 and other Snowski proteins.

Despite the rapid evolution of DAF-5, it binds DAF-3, a Smad. We find that the region downstream of the SDS box is entirely dispensable for DAF-5 binding to Smads, and the MH2 domain of DAF-3 is sufficient for interaction with DAF-5. The region of DAF-5 homologous to the region of Ski that contacts Smad4 is shown in Fig. 1C. Sno, Ski, DAF-5 and all other members of this family have a conserved zinc finger in the SDS domain. This zinc finger is an important structural component of Ski, and presumably all members of this family, because it is absolutely conserved. Only a subset of the residues of Ski that contact Smad4 are conserved in DAF-5. The binding of DAF-5 to DAF-3 may be somewhat different from the binding of Ski to Smad4, because DAF-3 is also greatly diverged from Smad4.

Evolution of the dauer TGFβ pathway

The rapid change in *daf-5* in the *Caenorhabditis* genus prompts the question of to what extent have *daf-5* and other members of the TGFβ pathway evolved new functions? The model for Sno/Ski function in vertebrate TGFβ signaling is shown in Fig. 5A. Briefly, activation of TGFβ receptors causes phosphorylation of Smads. This activation allows the Smads to bind Sno/Ski proteins and cause their degradation. With Sno/Ski gone, the Smads are free to regulate gene expression. However, *Sno* and *Ski* transcription is activated by Smads, leading to accumulation of Sno/Ski protein after receptor activation. Sno and Ski bind to Smads and recruit a variety of co-repressors, which prevent Smads from activating gene expression.

Two major functional changes have occurred within the dauer TGFβ pathway (Fig. 5B). First, DAF-3 is unique among Smads in that its function is antagonized by receptor signaling, and that it acts in the absence of the receptors and R-Smads. DAF-3 might recruit factors that in other systems are recruited by R-Smads, or the pathway may have evolved to use other factors. Second, DAF-5 is the only known example of a Snowski protein acting in a TGFβ pathway as a co-factor rather than an antagonist. In principle, Sno or Ski could act as co-factors rather than antagonists on promoters that are negatively

regulated by TGFβ, but this function has not yet been observed. One function that DAF-5 probably does not retain is a function in a variety of signal transduction pathways. Ski acts as a co-repressor for Mad, multiple nuclear hormone receptors, Rb, and other transcriptional regulators (Nomura et al., 1999; Dahl et al., 1998); *Sno* or *Ski* mutant mice have severe developmental defects, consistent with their role in multiple pathways (Berk et al., 1997; Shinagawa et al., 2001). *daf-5* mutants, however, have much more modest defects, and *daf-5* has no known phenotypes that are not shared by *daf-3*, suggesting that DAF-5 acts only as a co-factor to DAF-3.

DAF-5 acts in the nervous system

The question of where the TGFβ pathway is acting is important, and evidence is not conclusive. Many of the genes in the pathway have broad patterns of expression, including many non-neuronal cells (Patterson et al., 1997; Gunther et al., 2000; Inoue and Thomas, 2000). We found that expression from a neuron-specific promoter efficiently rescued the *daf-5* mutant, and that expression in muscle or hypodermis did not rescue. This result is consistent with *daf-5* acting in neurons to regulate a hormonal cue for dauer.

Experiments to directly address the site of action of DAF-4 have been reported (Inoue and Thomas, 2000), and suggest that this gene also has a neuronal focus of action. In these experiments, a *daf-4* cDNA was fused to several tissue-specific reporters. A promoter expected to give neuronal and intestinal expression fused to a *daf-4* cDNA rescued a *daf-4* mutant, but putative intestine-specific, muscle-specific and other promoters did not. However, the authors could not monitor the expression of the constructs directly, and they were appropriately cautious in interpreting their results. Our results suggest that unexpected expression is not just a formal possibility. Rather, misexpression of 'tissue specific' reporter constructs may be common. Several of our constructs gave unexpected expression patterns (Table 4). The *unc-14* promoter has been reported to be neuron-specific, but we saw expression elsewhere. The *unc-119*, *dpy-7*, *dpy-30* and *ges-1* promoters gave unexpectedly weak or undetectable expression, although in the case of *dpy-7*, we were able to correct the problem by isolating transgenic strains with stronger expression.

Two reports suggest functions for *daf-3* and *daf-5* outside the nervous system, which is inconsistent with our conclusion. One report suggested that *daf-3* and *daf-5* function in the pharynx to directly regulate gene expression in adults (Thatcher et al., 1999). This work used a reporter with a small element derived from the *myo-2* reporter (the 'C subelement'). However, our examination of the full-length *myo-2* promoter indicates that *daf-3* and *daf-5* do not regulate *myo-2* gene expression in adults. In dauers, the full-length promoter does show regulation similar to that of a reporter containing only the C subelement, but regulation of the C subelement reporter in dauers is *daf-3*-independent. This *daf-3*-independent downregulation may be caused by a general reduction in expression of housekeeping genes that is seen in dauers (T. Liu and G.I.P., unpublished). We suggest that *daf-3* and *daf-5* do not actually regulate the expression of *myo-2* in a normal context. *daf-3* and *daf-5* may function in the nervous system to regulate a secondary signal that in turn regulates the reporter with the C subelement.

Cell division arrest in hypodermis and other non-neuronal cells of dauers is dependent on a cyclin kinase inhibitor, *cki-1* (Hong et al., 1998). This gene is similar to a gene that is directly regulated by Smads in vertebrates (Moustakas and Kardassis, 1998). In *C. elegans*, this gene is repressed in a *daf-7* mutant, and we found that this regulation was *daf-5* dependent, which suggests that *daf-3* and *daf-5* could act outside the nervous system to directly regulate this gene. However, our demonstration that *daf-5* expression in the nervous system is sufficient for dauer arrest suggests that regulation of *cki-1* is indirect.

Biological role of TGFβ signaling in *C. elegans*

We observed a phenotype of *daf-5* that has not been previously reported; *daf-5* mutants are Egl-c, meaning that compared with wild type, *daf-5* mutants have fewer eggs retained in the uterus and lay eggs with younger embryos. This phenotype suggests that the *daf-5* mutants have hyperactive egg-laying behavior. We were concerned that a low rate of egg production in the spermatheca might cause this phenotype, but we found that the rate of egg production was not correlated with the Egl phenotype. We found that all of the mutant genotypes in our experiment produce eggs at a rate of 1/2 to 2/3 that of wild type (data not shown), but have dramatically different Egl phenotypes. For example, the *daf-5* single mutants make eggs at the same rate as the *daf-8; daf-5; daf-14* triple mutants, which are not Egl-c. This new result suggests that *daf-5* functions to restrain egg laying even when the TGFβ pathway is active. The role of *daf-5* in egg laying is not TGFβ pathway independent, as *daf-c; daf-5* double mutants are not significantly Egl-c.

In addition to dauer and egg laying, the dauer TGFβ pathway also regulates social feeding behavior and fat accumulation (Trent et al., 1983; Thomas et al., 1993). In addition, *daf-3* and *daf-5*, but not the other Smads or receptors, have a role in chemotaxis to both olfactory and gustatory cues (Daniels et al., 2000). All of these behaviors and developmental events are also affected by chemosensory input. Thus, the role of the TGFβ pathway can be said to be a general role in coupling chemosensory events to developmental and behavioral output. How might this pathway be coupled to these events? One possibility is that all of these events are affected, directly or indirectly, by a hormonal cue or cues, and that the TGFβ pathway regulates the expression of genes needed for production of the hormone(s). Orientation to a chemical gradient is quick, occurring within minutes. This is perhaps too brief an interval for the hormone to be acting during the chemotactic process, but a hormone might cause structural changes in the nervous system that alter chemotactic behavior.

A second interesting possibility is suggested by recent work that has identified new functions for TGFβ superfamily signaling in the nervous system. Retrograde signals between *Drosophila* neurons and their postsynaptic partner cells can affect the nature of the synaptic connection (Aberle et al., 2002; Marques et al., 2002) as well as the neurotransmitters produced by the presynaptic cells (Allan et al., 2003), and genetic analysis suggests that these retrograde signals are mediated by Gbb, a TGFβ superfamily ligand, and Wit, a TGFβ superfamily receptor. Thus, the dauer TGFβ pathway might act to affect the synaptic connections or other properties of neurons to alter signaling in the nervous system. This model is not mutually exclusive with the hormone regulation model above, as

changes in connectivity or neurotransmitter production might affect synaptic signaling to a neurosecretory cell that makes hormone.

Gene regulation by the Ski superfamily

Study of the Ski and Sno proteins has provided an opportunity to learn how a co-repressor can play a variety of roles in different regulatory events. Perhaps the next important task will be to tie the biochemical and cell biological mechanisms identified in cell culture systems to important functions of Sno and Ski in vivo. Cancer biology is one field that may provide this connection, as the role of Ski and Sno in cancerous cells suggests testable hypotheses about the role of these proteins in the normal context. Sno and Ski function in multiple pathways, which makes understanding the role of the proteins in any one pathway difficult. In *C. elegans*, DAF-5 appears to function predominantly or exclusively in a single TGFβ pathway. This simplicity has allowed us the unique discovery of an unambiguous connection of a Sno/Ski family member to a specific regulatory event in vivo, and this discovery provides a genetic system in which to pursue further understanding of Sno/Ski function.

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