

Specificity of TGF β signaling is conferred by distinct type I receptors and their associated SMAD proteins in *Caenorhabditis elegans*

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SUMMARY

In *C. elegans*, the TGF β -like type II receptor *daf-4* is required for two distinct signaling pathways. In association with the type I receptor *daf-1*, it functions in the dauer pathway. In addition, it is also required for body size determination and male tail patterning, roles which do not require *daf-1*. In an effort to determine how two different signals are transmitted through *daf-4*, we looked for other potential signaling partners for DAF-4. We have cloned and characterized a novel type I receptor and show that it is encoded by *sma-6*. Mutations in *sma-6* generate the reduced body size (Sma) and abnormal mail tail (Mab) phenotypes identical to those observed in *daf-4* and *sma-2*, *sma-3*, *sma-4* mutants (*C. elegans* Smads), indicating that they function in a common

signaling pathway. However, mutations in *sma-6*, *sma-2*, *sma-3*, or *sma-4* do not produce constitutive dauers, which demonstrates that the unique biological functions of *daf-4* are mediated by distinct type I receptors functioning in parallel pathways. We propose that the *C. elegans* model for TGF β -like signaling, in which distinct type I receptors determine specificity, may be a general mechanism of achieving specificity in other organisms. These findings distinguish between the manner in which signaling specificity is achieved in TGF β -like pathways and receptor tyrosine-kinase (RTK) pathways.

Key words: TGF β , Type I receptor, *Caenorhabditis elegans*, *sma-6*, SMAD

INTRODUCTION

One of the primary signaling pathways is the TGF β superfamily of extracellular ligands, which provides a diverse array of developmental and proliferation control cues. The dpp/BMP subfamily regulates many important developmental events, including dorsal-ventral patterning, mesoderm induction, and osteogenesis (Irish and Gelbart, 1987; Panganiban et al., 1990; Wozney et al., 1988; reviewed in Padgett et al., 1998). In *Drosophila*, *dpp*, *60A*, and *screw* encode TGF β -like ligands and are required during the embryonic and/or the pupal stages to regulate development (Padgett et al., 1987; Arora et al., 1994; Chen et al., 1998; Khalsa et al., 1998). In *C. elegans*, the *daf-7* gene, which encodes a divergent ligand, is involved in dauer formation (Ren et al., 1996), and *unc-129* is involved in axonal pathfinding (Colavita et al., 1998). In *Drosophila* and *C. elegans*, there are other TGF β -like ligands that have been identified by their respective genome sequencing projects, but their functions have not been reported.

Members of the TGF β superfamily share a conserved C-terminal region that is cleaved from a precursor polypeptide. Subsequently, homo- or heterodimerization of these domains yields functional bioactive molecules. Metalloproteases whose activity is required for the biological function of the ligands have been identified in several organisms (Childs et al., 1994;

Finelli et al., 1994; Padgett et al., 1997). Signaling is mediated by two receptor serine-threonine kinases that heterodimerize after ligand binding to transduce the signal to downstream components. Two classes of receptor kinases have been identified, the type I and II families, that are both essential to this role. The two classes of receptors are homologous, but there is greater similarity among the members within each group. A distinguishing feature of type I receptors is the existence of a glycine-serine rich stretch of residues, the GS domain, preceding the kinase region.

A model of receptor activation and signal transduction has been developed for TGF β receptors in vertebrates (Miyazono et al., 1994; Ventura et al., 1994; Wrana et al., 1994; Liu et al., 1995; Weis-Garcia and Massagué 1996). According to this model, the type II receptor kinases are constitutively phosphorylated and form a complex with a corresponding type I receptor upon ligand binding. Phosphorylation of the GS domain by the type II receptor then renders the type I kinase active. In this manner, the active receptor complex is able to transduce the signal from the ligand to downstream intracellular effectors. Further work has demonstrated that the signaling complex consists of a hetero-oligomeric or tetrameric combination of type I and type II receptors, and that type II receptors may exist freely as dimers preceding interaction with ligand (Yamashita et al., 1994).

There are two well-characterized TGF β -like pathways in *C.*

elegans, the Sma/Mab (small and male abnormal) and dauer pathways, and a recently discovered third pathway involving *unc-129* (Colavita, et al., 1998). Previously, the *daf-1* and *daf-4* genes were shown to encode TGF β -like serine-threonine kinase receptors of the type I and II families respectively, and it was demonstrated that DAF-4 is capable of binding vertebrate BMP-4 (Riddle et al., 1981; Georgi et al., 1990; Estevez et al., 1993). Mutations of *daf-4* result in small body size, male tail deformities, constitutive dauer formation, and egg-laying abnormalities. Although normal with respect to body size and male tail morphology, *daf-1* mutants share the dauer and egg-laying defects of *daf-4*. The model for receptor activation and signaling suggests that *daf-1* and *daf-4* may function together to transduce a dauer signal; however, this does not explain the additional phenotypes observed in *daf-4* animals. Investigations into other molecules that are necessary to transduce the *daf-4* signal regulating body size and male tail development led to the cloning and characterization of the *C. elegans sma-2*, *sma-3*, and *sma-4* genes, which were shown to encode Smads that function downstream of *daf-4* (Savage et al., 1996). These genes, when mutant, produce the reduced body size (Sma), and male tail abnormal (Mab) defects evident in the *daf-4* phenotypes, and were found to function non-redundantly in a common pathway (Savage et al., 1996). Collectively, *Mad*, *sma-2*, *sma-3*, and *sma-4*, and their homologs constitute the Smad family of transducers (Derynck et al., 1996).

The discovery and characterization of the Smad protein family has shown that they are integral signal transducing elements in diverse TGF β -like pathways (Sekelsky et al., 1995; Baker et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Savage et al., 1996). The Smad members identified thus far in several organisms, including *C. elegans*, *Drosophila*, and vertebrates fall into three related classes: receptor-regulated (R-Smads), common (Co-Smads), and antagonistic (Anti-Smads) (reviewed by Attisano and Wrana, 1998; Heldin et al., 1997; Massagué, 1998; Padgett et al., 1998). Phosphorylation of receptor-regulated Smads by a respective type I kinase has been demonstrated to be critical for their ability to mediate a TGF β -like signal (Macias-Silva et al., 1996; Zhang et al., 1996; Kretzchmar et al., 1997). A nuclear role for these transducers has been established by the demonstration that the phosphorylated Smads translocate to the nucleus as a heteromeric complex consisting of receptor-regulated and common Smads, and interact with transcription factors and promoter regions of target genes to regulate expression (Chen et al., 1996; Lagna et al., 1996; Kim et al., 1997; Wu et al., 1997; Feng et al., 1998; Janknecht et al., 1998). In vertebrates, FAST-1, a winged-helix *forkhead* homolog, has been observed to associate with Smad4 in signaling-responsive transcriptional regulatory complexes (Chen et al., 1996, 1997). In humans, mutations in the Smads have been implicated in tumor progression, presumably through a lack of response to growth-influencing cellular communication signals (Eppert et al., 1996; Hahn et al., 1996). The identification of the antagonistic Smads, human Smad6 (Imamura et al., 1997; Hata et al., 1998), human Smad7 (Hayashi et al., 1997; Nakao et al., 1997), and *Drosophila* DAD (Tsuneizumi et al., 1997) as competitive negative regulators of TGF β -like signaling pathways has yielded an additional mechanism by which the activity of the pathway is intracellularly regulated.

In this work, we report the identification and characterization in *C. elegans* of a novel type I receptor encoded by *sma-6*, and show that it is an essential signaling component of the Sma/Mab developmental pathway. Based on our results, the implications for TGF β signaling in general are that first, two dissimilar type I receptors may share a common type II receptor signaling partner; and second, that the corresponding developmental roles of the type I receptors can be unique through the utilization of distinct downstream transducers. Furthermore, this feature of *C. elegans* TGF β signaling contrasts with the receptor tyrosine kinase system in *Drosophila*, in which three pathways utilize the same MAPK, but achieve specificity by activating different transcription factors (Brunner et al., 1994).

MATERIALS AND METHODS

C. elegans strains and culture

Strains were manipulated and cultured using standard methods (Brenner, 1974). The strain N2 (Bristol) was used as the wild-type reference. Unless stated otherwise, all strains were maintained at 20°C.

Isolation and characterization of *sma-6* cDNAs

The degenerate primer pair 5'GCCGGAATTCCAYCGNGAYATHAARTCNAARAA3' and 5'GCCGTCTAGATCAGNAYTTCNG-GNGCCATRTA3' was used to amplify a 150 bp PCR fragment corresponding to the conserved kinase regions III and VI of TGF β /BMP receptors from oligo-dT primed reverse-transcribed total RNA extracted from N2 animals. The RT-PCR was performed according to the manufacturer's protocol (Perkin-Elmer), using 2 μ g of total RNA. The conditions for the PCR reaction were: 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute 30 seconds at 72°C. The bands of interest were purified, cloned into pBS SK+ at the *Eco*RI and *Xba*I sites, and sequenced. Inserts corresponding to *sma-6* were then excised and radiolabeled to probe a cDNA library. An N2 cDNA library in λ Zap (courtesy of R. Barstead) was screened with the 150 bp PCR fragment. Seven independent cDNA clones in pBS SK+ (Stratagene) were sequenced at the 5' and 3' termini. An approx. 2.25 kb cDNA containing the expected secretion signal and conserved intracellular kinase region was judged to be full-length, and was subsequently sequenced. Comparison with cosmid C32D5 sequence (*C. elegans* Genome Sequencing Consortium) verified the sequence.

Isolation of *sma-6* genomic fragments

The 2.25 kb fragment corresponding to the *sma-6* cDNA was radiolabeled and used to probe a *C. elegans* genomic library in EMBL3A (courtesy of Dr Philip Anderson). Phage inserts were purified and mapped through restriction analyses using the known cosmid sequence. Inserts covering the *sma-6* gene were used for generating a rescuing construct.

Transformation constructs

A genomic clone encompassing the *sma-6* region was digested with *Pst*I/*Eag*I to generate a approx. 9 kb fragment which was subcloned into the Bluescript (SK+) vector to generate pSK1. pRF4, bearing a dominant *rol-6* mutation, was used as a transformation marker (Mello et al., 1991). Stable transgenic lines were generated through selection of transformed progeny bearing the Rol phenotype, and rescue of the body size defect was assessed. pRF4 injected alone was not sufficient for rescue of the *sma-6* phenotype.

A 2 kb fragment including upstream regulatory sequences and the first four residues of SMA-6 was used to generate a translational fusion with the *lacZ* gene in the vector pPD95.03 (courtesy of A. Fire).

Transformed lines bearing a stable extrachromosomal array were selected and subjected to γ -ray irradiation followed by selection for stably integrated lines. Animals were then stained to examine the expression of *lacZ* (Fire et al., 1990; Fire, 1992).

Generation of *sma-6* alleles

sma-6(wk10) was identified in a non-complementation screen for *sma-6* mutants. *unc-4(e120)II* homozygous hermaphrodites were mutagenized with the standard EMS protocol (Brenner, 1974). Homozygous *sma-6(e1482)III* males were mated with mutagenized parental *Unc* animals, and the subsequent F₁ progeny were scored for a Sma Non-*Unc* phenotype, representing the cross progeny homozygous for a mutation in *sma-6*. *sma-6(wk7)*, *sma-6(wk8)*, and *sma-6(wk9)* were recovered in an F₂ screen for Sma animals (C. Savage and R. W. P., unpublished data) and failed to complement *sma-6*. *sma-6(wk11)* was isolated in a screen for transposition induced Sma mutants (C. Savage and R. W. P., unpublished).

Characterization of mutant alleles

All *sma-6* mutants were outcrossed at least twice. *sma-6 him-5(e1490)V* animals were generated to facilitate male tail analyses. Animals were propagated on plates and harvested with M9 buffer and transferred to tubes, followed by rounds of washing. NaN₃ was added to a concentration of 50 mM to anesthetize the animals. Approximately 100 animals were then pipetted onto 5% agar pads in batches and examined with Nomarski optics to score spicules or rays. Only complete fusions of rays were scored as defective (partial fusions include fusions of only the distal portion of the rays). *sma-6/Df* animals were generated by crossing N2 males into *sma-6* hermaphrodites and using the heterozygous F₁ males to cross into strains SP788 *unc-4(e120) mnDf96/mnC1 dpy-10(e128) unc-52(e444)II* and SP543 *mnDf30 unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444)II* to obtain F₂ *sma-6/Df* males. LT234 *sma-6(e1482) unc-4(e120)II*; *sma-4(e805)III*; *him-5(e1490)V* was generated by crossing *sma-4(e805)*; *him-5(e1490)* males into *sma-6(e1482) unc-4(e120)* animals. Wild-type F₁ hermaphrodites were then transferred to individual plates, and allowed to self-fertilize. Sma Non-*Unc* F₂ animals were isolated and transferred to individual plates. Sma *Unc* animals were isolated and transferred in the F₃ generation, and the F₄ generation was examined for the Him phenotype. Complementation tests were performed to confirm the homozygosity for *sma-6(e1482)* and *sma-4(e805)*.

Genetic interaction between dauer and Sma/Mab pathways

Mutant strains consisting of *sma-[2,3,4,6] daf-1*; *him-5* were constructed by first crossing *him-5(e1490)* males into *daf-1* hermaphrodites. Doubly heterozygous [*daf-1(m40)* or *daf-1(m213)IV*]; *him-5(e1490)V* males were then crossed into *sma-[2,3,4,6]* hermaphrodites. Non-Sma hermaphrodites, representing the cross progeny, were picked onto separate plates. The Sma progeny were then selected from these plates and allowed to self-fertilize, and the progeny were examined over two generations for the Sma Daf Him phenotypes, representing the homozygous state. These animals were transferred to individual plates, and allowed to self-fertilize for an additional generation for confirmation.

Sequencing and analyzing molecular lesions in *sma-6*

Primers corresponding to the termini of the *sma-6* gene were used to amplify a 2.5 kb product from the mutant strains CB1482, LT191, and LT78 using *Pfu* polymerase (Stratagene) to minimize the error rate. Independent clones were sequenced using an automated sequencer (Applied Biosystems). Comparison with the C32D5 cosmid sequence (*C. elegans* Sequencing Consortium) was used to identify polymorphisms. Sequence analyses were performed using the Wisconsin package (GCG Group). Genefinder (courtesy of Phil Green and Ladeana Hiller, Washington University) was used to predict open

reading frames on an Irix platform. AceDB was used for *C. elegans*-specific genetic analyses.

RESULTS

Molecular cloning of a novel type I TGF β -like receptor

The hypothesis that an unknown type I receptor participated with *daf-4* to signal through the *sma-2*, *sma-3* and *sma-4* genes argued for the existence of an additional type I receptor which, when mutant, confers the small body size and male tail abnormal phenotypes. However, no preexisting mutants had been identified that exhibited both of these specific characteristics. The conserved intracellular kinase regions of the TGF β superfamily receptors provided a strong region of homology suitable to employ an RT-PCR approach. Since this pathway was a BMP-like pathway, we chose to employ degenerate oligonucleotides corresponding to the conserved regions III and VI of *Drosophila* TKV and SAX type I kinase domains (Xie et al., 1994), RT-PCR was performed on total *C. elegans* total RNA. Several clones were sequenced and analyzed for homology with the type I receptor family. In addition to multiple instances of *daf-4* and other non-transmembrane serine-threonine kinases, a BLAST search revealed that one of the clones resided on the *C. elegans* cosmid C32D5 in LGII. We obtained full-length cDNA clones using the PCR fragment as a probe and isolated multiple 2.2 kb clones, examination of which revealed they had the structure of a TGF β -like receptor. The presence of an N-terminal signal sequence in the predicted protein indicated that the cDNA clone was full-length.

sma-6 encodes a BMP type I receptor

Cosmid C32D5 had been localized to the LGII in the central cluster by the *C. elegans* Genome Sequencing Project. In order to determine the biological role of the novel type I receptor, we sought candidate mutations for rescue. We examined the genetic region near C32D5 for genes which mutate to a Sma/Mab phenotype. Interestingly, the *sma-6* locus, which exhibits a reduced body size when mutant, maps to the C32D5 region (Fig. 1A). *sma-6* mutants, like mutants in *sma-2*, *-3* and *-4*, appear uniformly reduced in body size, both with respect to width and length (Fig. 3B). To test if the new type I receptor corresponded to *sma-6*, we used a genomic clone of the receptor for transformation rescue of the canonical *sma-6(e1482)* allele. A 9 kb *PstI/EagI* subcloned genomic fragment containing the type I receptor and flanking regions of adjacent predicted genes was sufficient to rescue the *sma-6(e1482)* allele. By sequencing the region corresponding to the cDNA in *sma-6(e1482)* mutants, we identified a molecular lesion. These results indicate that *sma-6* encodes the new receptor.

The *sma-6* gene is composed of 12 exons spanning 2.7 kb of transcribed sequence and an additional 2 kb of possible upstream regulatory sequence, as deduced from the existence of a polyadenylation sequence in an adjacent predicted transcript using Genefinder. Analysis of the *sma-6* primary structure indicated that it is homologous to other TGF β -like type I receptors, and is more similar to the majority of other type I receptors than is *daf-1* (Fig. 2A and 2B). The conserved N-terminal cysteine residues found in the extracellular

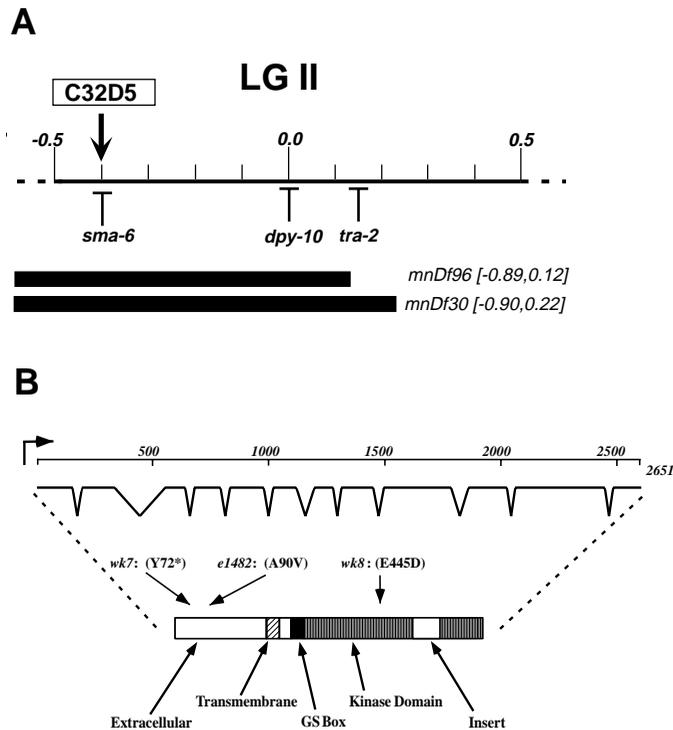


Fig. 1. Molecular cloning of *sma-6*. (A) *sma-6* genomic localization. The *sma-6* transcript was localized to cosmid C32D5, which is on LGII. A 9 kb genomic fragment encompassing the predicted gene was used to rescue *sma-6(e1482)* animals. Strains SP788 and SP543 harboring heterozygous deletions *mnDf30* and *mnDf96*, respectively, of the region containing *sma-6* were utilized for deficiency analyses. (B) Genomic organization, domain structure and identified mutations in *sma-6*. The intron/exon organization of the *sma-6* gene deduced from the cDNA is shown. The domain structure of SMA-6, including secretion, extracellular, transmembrane, GS domain, and kinase domains is similar to other type I kinases.

domains of both type I and II receptors are present in *sma-6*, as is the type I specific GS domain (Fig. 1B). SMA-6 contains a 41-amino acid insertion in the kinase domain between residues 491 and 533 which is not found in other type I receptors and which has no detectable motifs. Interestingly, an acidic amino acid modification of the TGF β type I receptor T β R-I, which renders the kinase constitutively active, 204T \rightarrow D (Weiser et al., 1995), is an inherent feature of the wild-type form of SMA-6.

Phenotypic analysis of *sma-6* alleles

The *C. elegans* male tail is a sex-specific, bilaterally symmetric copulatory structure comprising of a pair of sharp elongated features known as spicules, which are mechanically required during mating, and rays, which are chemosensory organs which also provide tactile feedback. Each of the nine pairs of rays is unique with respect to morphology and chemosensitivity (Fig. 3A). We were interested in the potential role of *sma-6* in tail morphogenesis through participation with *sma-2*, *sma-3*, *sma-4*, and *daf-4*. Previously, the tail defects in *sma-2*, *sma-3*, *sma-4*, and *daf-4* males had been characterized (Estevez et al., 1993; Savage et al., 1996). In each of these mutants, the identity of rays is transformed, leading to a ray fusion defect which can be

seen (Fig. 3A,D). The fusion defect has been characterized as a posterior to anterior transformation of identity, which results in a high fusion percentage of rays 4/5, 6/7 and 8/9 (Savage et al., 1996). Furthermore, *Sma* mutants exhibit a defect in the morphology of the spicules which results in a crumpled appearance of these structures and renders males unable to mate.

We therefore examined the tails of *sma-6(e1482)* males to look for defects similar to those observed in *daf-4* and *sma-2*, *sma-3* and *sma-4* mutants, which would indicate that these genes function together in a common signaling pathway. However, *sma-6(e1482)* males had wild-type tail morphologies. Because the nature of the molecular lesion suggested that the *sma-6(e1482)* mutation was of insufficient severity to generate tail defects, we sought to further reduce the signaling activity mediated by SMA-6. We used deficiencies in the region, *mnDf30* and *mnDf96*, which correspond to deletions encompassing the *sma-6* gene, to generate heterozygous animals that harbored both the *e1482* mutation and a deleted copy of *sma-6*. Tail defects were not observed in males heterozygous for *sma-6(e1482)* and either deficiency. The finding that *sma-6(e1482)* did not produce the ray fusions and crumpled spicule defects of the other components in the *Sma* signaling pathway invited several hypotheses, including the involvement of an unidentified third type I receptor in tail development (Table 1). However, two sources of evidence argued that *e1482* was not null and that the generation of null alleles may yield animals with abnormal tail morphologies. First, the observation that *sma-6(e1482)/Df* animals were considerably smaller than *sma-6(e1482)* alone argued that *sma-6(e1482)* was not null. Second, analysis of the molecular lesion in *sma-6(e1482)*, corresponding to a 90A \rightarrow V change in the primary structure, suggested that this mutation was likely to be relatively weak.

To examine this issue further, we generated additional alleles of *sma-6* (see Materials and Methods). Analyses of additional alleles of *sma-6* indicated that this gene is required both in determining body size and in male tail morphogenesis, consistent with the view that the canonical *sma-6(e1482)* allele was hypomorphic. Males homozygous for the *sma-6(wk7)*, *sma-6(wk8)*, or *sma-6(wk9)* alleles manifest identical tail defects to those in *daf-4*, and *sma-2*, *sma-3* and *sma-4* animals (Fig. 3B and Table 1). The percentage of fusions presented in this work differs from previous analyses of other mutants in the *Sma/Mab* pathways, such as *sma-2*, *-3*, *-4* or *dbl-1* (Savage et al., 1996; Suzuki et al., 1999) because only complete fusions of individual rays were scored as defects, and when similar criteria are applied, the percentage of fusions is similar (see Materials and Methods). An additional abnormality observed in *sma-6* mutant males that has been unreported for *daf-4* or *sma-2*, *sma-3* and *sma-4* mutants is the occasional absence of ray 5. The cause of this defect in relation to the transformation of ray identity is not currently known.

To determine if these phenotypes represent a null mutation in *sma-6*, we sequenced the *sma-6(wk7)* allele and identified the presence of a stop codon in the extracellular region at 72Y. Both the kinase domain and the transmembrane region required for function would be absent in the mutant protein, and therefore it is a molecular null. Furthermore, hemizygous *sma-6(wk7)/Df* animals did not exhibit a more severe phenotype than *sma-6(wk7)* homozygotes. In *sma-6(wk8)* animals, a nucleotide change resulting in the mutant form 445E \rightarrow D yields a phenotype that closely resembles the null, as judged through

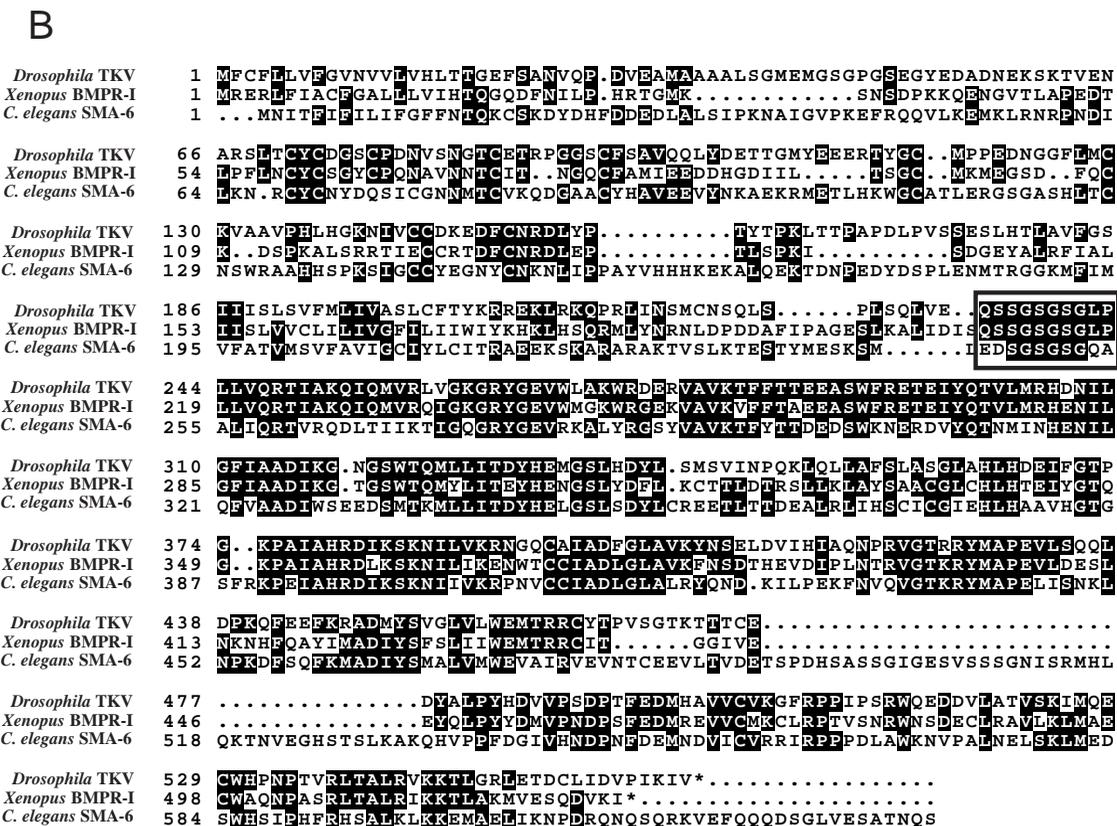
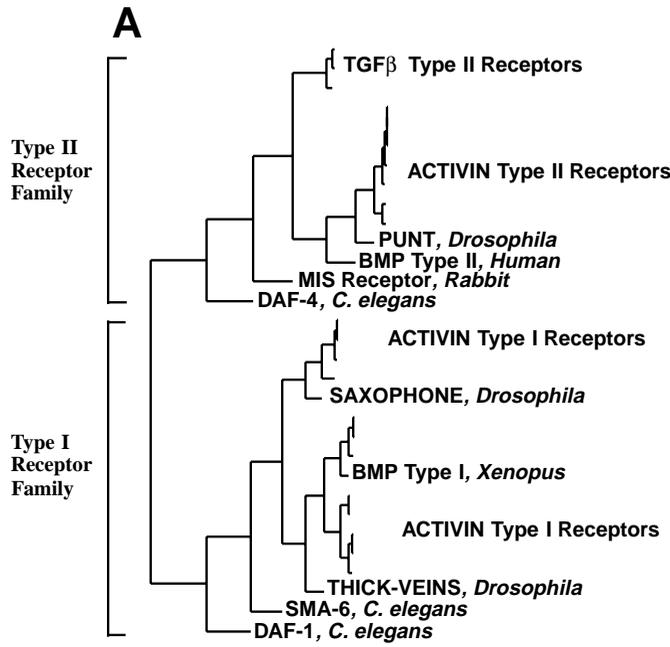


Fig. 2. *sma-6* encodes a type I receptor. (A) Receptor dendrogram. Dendrograms were produced with the Pileup Program using the Wisconsin GCG Software Package, Genetic Computer Group, Madison, WI. SMA-6 is a member of the type I class of TGFβ-like receptors, and is more conserved than its counterpart in the *C. elegans* dauer pathway, DAF-1. Also shown are the *Drosophila* type I receptors SAX and TKV. (B) Amino-acid sequence alignment of *C. elegans* SMA-6, *Drosophila* THICK VEINS, and *Xenopus laevis* BMPR-I. Several cysteine residues that contribute to the extracellular framework of the type I receptor family are conserved in SMA-6. In addition, the conserved GS domain, a hallmark of the type I family and of phosphorylation, which is necessary for the activation of the type I kinase, is present in SMA-6 (boxed). This sequence has been deposited in GenBank (AF104017).

several criteria, including comparison with the *sma-6(wk7)* body size, inspection of the male tail (Table 1), and by analysis of *sma-6(wk8)/Df* animals. Based on sequence comparison, the mutation in *sma-6(wk8)* disrupts an extremely conserved residue found in protein kinases. We examined the crystal structures of other serine-threonine and tyrosine kinase domains, and observed that the *wk8* mutation would diminish

the critical predicted hydrogen-bonding role of the wild-type residue in establishing the framework of the kinase region, primarily with residue 477R.

Differential signaling thresholds for body size and tail development

The ability to separate the *Sma* and *Mab* phenotypes in both

Table 1. Male tail developmental abnormalities in *sma-6* mutants

Genotype	Frequency of ray fusions (%)						% Abnormal spicules	<i>n</i> (half-sides)
	4	5	6	7	8	9		
<i>sma-6(e1482); him-5(e1490)</i>	0	0	0	0	0	0	0	>100
<i>sma-6(wk7); him-5(e1490)</i>	15	17	49	51	30	25	89	139
<i>sma-6(wk8); him-5(e1490)</i>	13	15	49	49	19	16	95	166
<i>sma-6(wk9); him-5(e1490)</i>	21	23	46	47	29	27	90	182
<i>sma-6(bx110); him-5(e1490)</i>	23	24	47	49	24	20	89	135
<i>sma-4(e805); him-5(e1490)</i>	0	0	0	0	0	0	0	30
<i>sma-6(e1482); sma-4(e805); him-5(e1490)</i>	6	11	5	0	25	25	50	36

sma-6(e1482) and the previously characterized *sma-4(e805)* alleles suggest that the body size regulation and male tail development functions may have distinct downstream signaling components. However, alternative hypotheses including different signaling threshold requirements for the two roles are possible. In this model, the separate biological processes each require different levels of signaling activity.

In order to distinguish between these two possibilities, quadruply homozygous *sma-6(e1482)unc-4(e120); sma-4(e805); him-5(e1490)* animals were generated and examined for the Mab phenotype. Interestingly, though neither the *sma-6(e1482)* nor the *sma-4(e805)* alleles elicit tail defects, animals homozygous for both the *sma-6(e1482)* and *sma-4(e805)* mutations exhibit ray fusions at a low penetrance (Table 1). Though crumpled spicules were also seen in this genotype, abnormalities in a single spicule were also observed (data not shown). We conclude that the activity of the *sma-6(e1482)* loss-of-function allele is sufficient for male tail development, but below the activity required for body size regulation. Furthermore, these data suggest that more signaling is needed for specifying body size than for regulating tail morphogenesis. Consistent with this model, there have been no reported mutations in *sma-2*, *sma-3*, *sma-4*, or *sma-6* that only affect tail development but not body size.

sma-6 expression is dynamic

Integrated transgenic lines bearing translational fusions of the *sma-6* gene with a *lacZ* reporter indicate that *sma-6* is expressed during embryonic, larval and adult stages (Fig. 4). During the early larval stages (L1-L2), *sma-6* is extensively expressed in the anterior pharyngeal and distal posterior regions of the animal (Fig. 4A). Soon afterwards, expression begins in the posterior intestinal nuclei and advances more anteriorly

(Fig. 4B). Expression is seen in the pairs of intestinal nuclei as they divide. Strong expression is seen in the pharyngeal muscles of the anterior and posterior bulbs of the pharynx (Fig. 4C). Once the intestinal cell divisions are complete, one can see continued expression in the intestinal cells during L2-L3 (Fig. 4D), and later disappearing in these cells. In adults, the staining pattern disappears from the midsections of the animal and persists in the pharyngeal muscles (Fig. 4E). In males, expression is seen in the tail (Fig. 4F), as predicted, but the identity of the cells is unclear. The pattern of expression in the anterior regions is strikingly similar to those reported for DAF-4/GFP constructs (Patterson et al., 1997), and resembles that of DBL-1, a candidate ligand for SMA-6 (Suzuki et al., 1999). These results are consistent with both the placement of DAF-4 and SMA-6 in a common signaling pathway, and the role of *sma-6* in male tail development.

sma-6 expression is also observed in late-stage embryos (data not shown), indicating a possible embryonic requirement that does not affect known phenotypes. Biological activity of

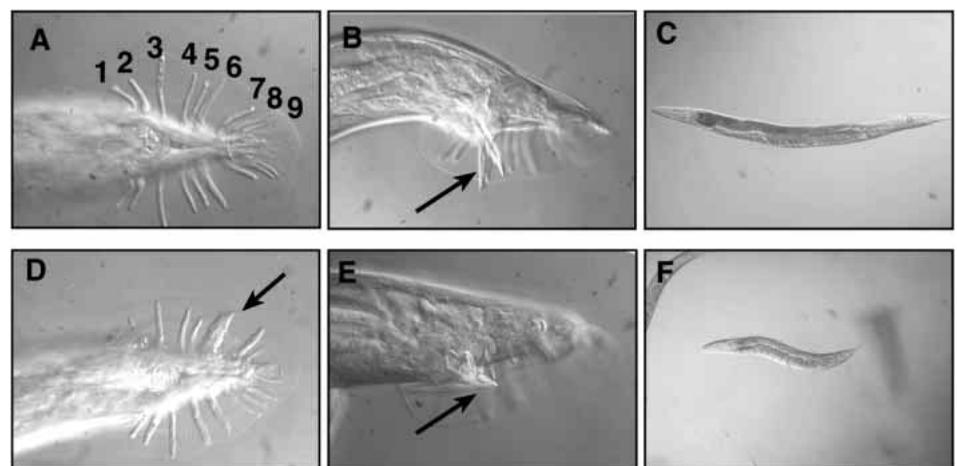


Fig. 3. *sma-6* is required for body size determination and male tail development. (A) Nomarksi micrographs of a wild-type male tail, (B) wild-type spicules, and (C) wild-type animal. The wild-type male tail is composed of laterally symmetric triplets of rays from 1 to 9, numbered anterior to posterior. (D) *sma-6* male tail, (E) crumpled spicules of *sma-6*, and (F) *sma-6* animal at same magnification as in C. The arrows in B and E point to spicules. The arrow in D points to a fusion of rays 6 and 7 in a *sma* null mutant.

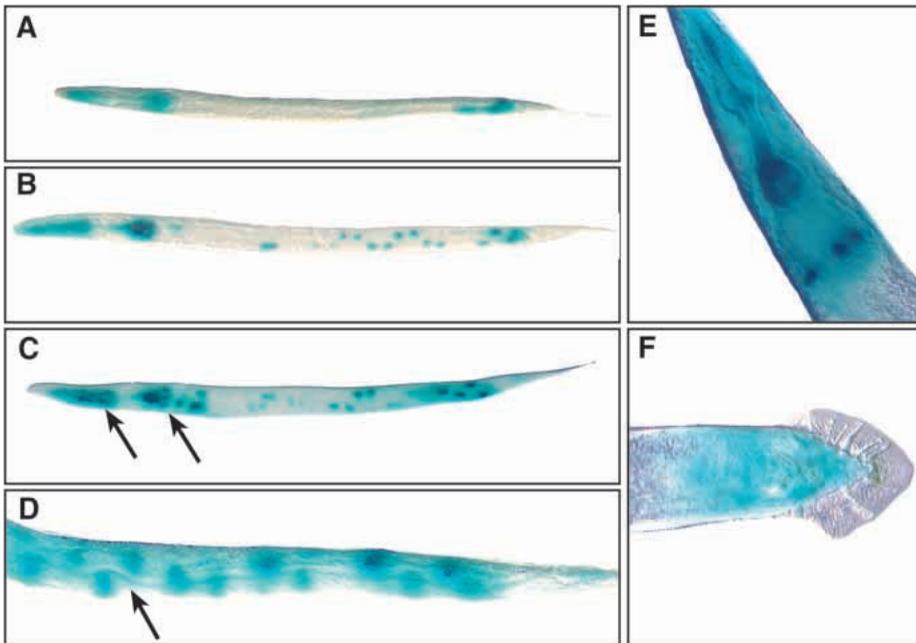


Fig. 4. Expression of *sma-6* (A-F) using *lacZ* reporter fusion assays. An integrated transgenic line bearing a translational fusion of *lacZ* and SMA-6 was used to assess *sma-6* transcriptional activity. (A) An L1-L2 animal showing staining in the pharyngeal muscles and posterior regions of the animal. (B) Soon after the expression seen in A, the expression is beginning in the posterior intestinal nuclei and extends anteriorly. (C) Expression continues in the intestinal nuclei as they are dividing and persists in the pharyngeal muscles (arrows point to the pharyngeal muscle nuclei). (D) Staining of intestinal nuclei (arrow points to the lumen of the intestine; L2-L3 animal). (E) Adult staining persists in pharyngeal muscle nuclei; (F) staining in the male tail – the resolution of this *lacZ* construct does not allow clear identification of the staining cells.

sma-6, however, would require the presence of signaling initiators and effectors, particularly the ligand, type II receptor, and downstream Smads. Our observation that the null *sma-6(wk7)* allele is not lethal implies that complete loss-of-function mutations in other components of the Sma pathway also may not produce lethality. However, we have observed that the fecundity (number of eggs) of hermaphrodites bearing mutations in *sma-6* is reduced in several independent outcrossed alleles (data not shown). Mutations in *sma-6* may contribute to low levels of embryonic or larval lethality, though a large proportion of animals reach adulthood. Therefore, further investigations are necessary to characterize the developmental role of the embryonic expression, if any.

Signaling specificity and crosstalk

The finding that two parallel signaling pathways which share a type II receptor exists in *C. elegans*, introduces the potential for mixed signaling, or ‘crosstalk’ between them. Possible interactions between *daf-1* and *sma-6* were assessed by examining the phenotype of double homozygotes. Mutations in *sma-6* were observed to enhance the dauer-constitutive (Daf-c) defects of *daf-1* mutants (Table 2). The alleles *daf-1(m213)* and *daf-1(m40)* were used to compare the effects of the doubly homozygous state. At temperatures exceeding 15°C, this difference was not observable, as the *daf-1* mutations results in a very high frequency of dauered animals (>95%). The enhancement of the Daf-c defect yielded a sensitive in vivo assay to examine crosstalk between the Sma/Mab and dauer pathways. To pursue these indications further, we investigated the genetic interactions between *sma-2*, *sma-4* and *daf-1*. Because of the large proportion of dauered animals at temperatures greater than or equal to 20° C, these data were only collected at 15° C (Table 2). All combinations of *sma-6* and *daf-1* alleles that were tested demonstrated enhancement, thereby eliminating the possibility of allele-specific genetic interactions. However, the degree of enhancement varied among the strains, as *wk7* exhibited

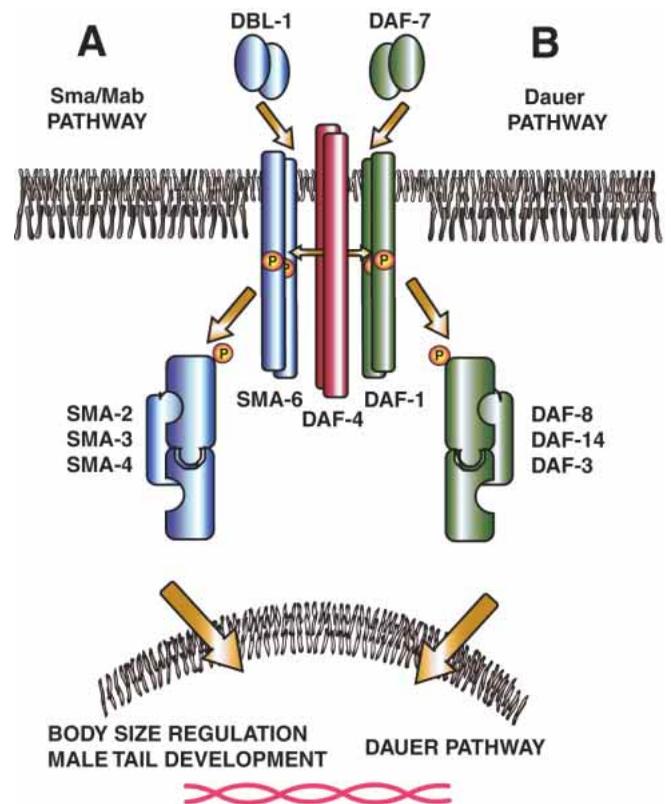


Fig. 5. A model for TGF β superfamily signaling in *C. elegans*. The type II receptor DAF-4 is shared between both developmental pathways, through interaction with the process-specific type I receptor and ligand(s). SMA-6, a type I receptor, transduces the extracellular signal to the downstream effectors SMA-2, SMA-3, and SMA-4 which regulate the expression of downstream targets. The other role of DAF-4 signaling involves the type I receptor DAF-1 and the downstream Smad mediators DAF-3, DAF-8, and DAF-14 (Georgi et al., 1990; Estevez et al., 1993; Ren et al., 1996; Patterson et al., 1997).

Table 2. Enhancement of *daf-1* mutants by mutations in the Sma pathway

Genotype	Dauer frequency (%) at:		
	15°C	20°C	25°C
<i>sma6(wk7);him-5(e1490)</i>	0 (n=261)	0 (n=238)	0 (n=295)
<i>daf-1(m213)</i>	3 (n=1266)	96 (n=597)	98 (n=437)
<i>sma-6(wk7);daf-1(m213); him-5(e1490)</i>	28 (n=406)	96 (n=317)	~100 (n=312)
<i>sma-2(e297);daf-1(m213); him-5(e1490)</i>	3.3 (n=153)	N.D.	N.D.
<i>sma-4(e729);daf-1(m213); him-5(e1490)</i>	8.7 (n=104)	N.D.	N.D.
<i>daf-1(m40)</i>	2 (n=618)	N.D.	N.D.
<i>sma-6(wk7);daf-1(m40); him-5(e1490)</i>	52 (n=266)	N.D.	N.D.
<i>sma-6(wk8);daf-1(m40); him-5(e1490)</i>	27 (n=153)	N.D.	N.D.

greater enhancement than *wk8*. *sma-4(em269)* demonstrated a moderate level of enhancement, whereas *sma-2(e297)* failed to interact with *daf-1*. These results provide evidence of possible crosstalk between the Sma and dauer pathways at low levels and may result from ancient evolutionary duplication of the signaling pathways. The fundamental developmental role of each of the two type I receptors, however, is unique because of the failure of null mutations in either *daf-1* or *sma-6* to generate overlapping mutant phenotypes.

DISCUSSION

The developmental role of *sma-6*

The results presented indicate that *sma-6* is a *dpp*/BMP-like type I receptor involved in regulating body size and male tail development in *C. elegans*. Prior to this work, the upstream components of the Sma/Mab pathway were unknown (Savage et al., 1996). Through this investigation, we demonstrate that the phenotypes resulting from mutations in *sma-6* are consistent with a model whereby DAF-4, SMA-2, SMA-3, SMA-4 and SMA-6 function in a common developmental pathway (Fig. 5). Recently, mutations in a *C. elegans* homolog of the *dpp*/BMP family of ligands have been identified that render it a candidate ligand for SMA-6 based on the similarity of phenotypes with mutations in other components of the Sma/Mab pathway (Suzuki et al., 1999). In parallel, DAF-7, DAF-1, DAF-4, DAF-3, DAF-8, and DAF-14 are involved in the regulation of the dauer pathway through the utilization of the shared type II receptor DAF-4. The genetic analysis of these genes provides in vivo evidence for how specificity is achieved in *C. elegans*. The ligands of the two pathways primarily interact with only one type I receptor, both of which function with a single common type II receptor, which in turn determines which set of Smads are utilized in downstream signaling events.

The role of TGF β signaling in determining body size of Sma mutants is unknown. Two general models can be proposed that can account for the body size differences between wild-type animals and animals mutant in the components of the Sma/Mab pathway. First, developmental cues that affect cell migration, division, or lineage specification may be rendered dysfunctional, yielding aberrant numbers or positioning of

cells in tissues which are responsive to Sma/Mab pathway signaling. Alternatively, control mechanisms involved in the regulatory phases of the cell cycle may be altered, generating correct lineages and cellular fates, but reduced cell size. The *C. elegans cul-1* gene provides an example of the latter mechanism, whereby mutations in a negative cell-cycle regulator result in simultaneous hyperplasia and reduced cell size in affected tissues, leading to developmental arrest and increased body length in mutant animals (Kipreos et al., 1996). The observation that many aspects of TGF β -like signaling are intimately involved in cell-cycle regulation and differentiation render either of these processes, or a combination of them, possible causes for the primary phenotypes of *sma-6* mutants and the Sma pathway in general. We are currently extending our studies to clarify this issue.

The early expression of *sma-6* as assayed by reporter fusion suggests a possible embryonic role for the receptor, perhaps one that has not been accounted for by the phenotypes assessed in this study. Alternatively, body size regulation involving the Sma pathway may proceed from early stages of development, or perhaps stages from before which the phenotype is evident. This notion is supported by the observations that animals defective in other components of the Sma pathway exhibit the reduced body size phenotype during late larval stages, and the difference in body size between wild-type and mutant animals continues to increase thereafter. Conversely, animals transformed with a genomic fragment corresponding to the *sma-6* gene exhibit increased body length. This result indicates that moderate hyperactivity of the Sma/Mab pathway can exceed the developmental requirement of the signal and suggests that this may be true for other components of the pathway.

There has been much progress in discerning the molecular mechanisms that contribute to male tail morphogenesis and development of the constituent structures. In *C. elegans*, HOM-C/Hox gene products play a central role in specifying ray identity. The products of *mab-5*, *egl-5*, and *mab-18* are all required to specify correct ray patterning. Mutations in these genes lead to defects in tail development, including missing and fused rays (Baird et al., 1991; Chisholm, 1991; Chow and Emmons, 1994). Aberrant regulation or dysfunction of these genes and their products in ray precursor or ancestral cells result in transformation of the respective ray identities, thereby contributing to the observed absence and fusion defects. These observations suggest there may be a connection between Hox gene function and the Sma/Mab pathway in ray patterning. In *Drosophila*, a primary regulatory target of DPP signaling is *labial*, a Hox gene that is required during midgut morphogenesis (Panganiban et al., 1990). Mutations in the components of the *dpp* pathway result in diminished expression of *labial* in response to *dpp* signal and it has recently been shown that *Drosophila* MAD binds to *labial* promoter elements (Szűts et al., 1998). It is possible the *C. elegans* Smads bind to promoter elements of the recently characterized *C. elegans labial* homolog *ceh-13* (Wittmann et al., 1997), since the expression of a *ceh-13::lacZ* reporter is highly diminished in Sma/Mab mutations (Suzuki et al., 1999).

Parallel developmental pathways

The separation of TGF β signaling in the Sma/Mab and dauer

pathways is in marked contrast with RTK pathways. For example, in *Drosophila*, the receptor tyrosine kinases encoded by *sevenless*, *torso* and the EGF receptor DER share a common downstream MAPK signaling cascade, as evident in the ability of a gain-of-function mutation in the downstream MAPK target *rolled* to activate multiple pathways (Brunner et al., 1994). Also in yeast, Ste11, a component of one of the six known MAP kinase pathways, is used in three signaling pathways that have distinct biological outputs (reviewed by Madhani and Fink, 1998). It is hypothesized that some of the specificity for these three yeast pathways may result from interactions with Ste5, which may assemble the kinases into a complex and prevent crosstalk with other MAP kinase pathways. In contrast, in the *C. elegans* TGF β pathways, specificity is achieved by the choice of the type I receptors, SMA-6 and DAF-1, which function with a distinct set of Smads, which in turn bind to specific DNA sequences on promoters. Previous studies of the two vertebrate type I BMP receptors have shown differences in biological outcomes (Zou et al., 1997), but neither these studies, nor biochemical studies, have determined the identity of the intracellular molecules that distinguish these two signals. It is possible that these vertebrate pathways function like their counterparts in *C. elegans*, but this issue will have to await genetic analysis.

What still remains to be determined in the Sma/Mab pathway is how one signal, one receptor, and one set of Smads, elicits three different developmental events – body size, spicule development and ray development. Presumably, there are additional transcription factors involved, such as a FAST-1 winged helix transcription factor (Chen et al., 1996), that are tissue specific for each of these three developmental events. It will be important to determine if other molecules exist that are required for signaling which bind to the receptors or Smads. Genetic screens are underway that may shed light on this issue. Another unanswered question regarding the Sma/Mab pathway stems from the failure to identify negatively acting Smad members that reduce the primary signaling response, such as Smad6, Smad7 and DAD. It is possible that signaling activity through SMA-6 is only required at discrete intervals during development, and this requirement would obviate the need to competitively regulate the signaling levels. Alternatively, TGF β signaling may be more rudimentary in *C. elegans*, with antagonistic Smads being a later development. However, most of the characterized genes and mechanisms involving TGF β signaling have been highly conserved in *C. elegans*.

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