

# Expression of *ram-5* in the structural cell is required for sensory ray morphogenesis in *Caenorhabditis elegans* male tail

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**Tissue morphogenesis requires complex cellular interaction and communication. The sensory ray in the *Caenorhabditis elegans* male tail has a simple cellular make-up and a non-essential function, thus providing an ideal model for studying the mechanisms guiding morphogenesis. We present here the analysis of a novel gene, *ram-5*, mutations of which are characterized by abnormal lumpy rays in the male tail. Microscopic analysis and behavioral studies revealed that lumpy rays contain operational sensory neurons. However, abnormalities were observed in the hypodermis and structural cells as well as in appositions between these two cell types. Molecular cloning and expression studies revealed that the *ram-5* gene encodes a transmembrane protein localized in sensory ray support cells, the structural cells. Expression of *ram-5* in these cells is required for normal ray morphogenesis. *ram-5*-dependent cell–cell communication is implicated in organizing the structural cell and the hypodermis, potentially through adhesion at the structural cell–hypodermal cell border.**

**Keywords:** cellular morphogenesis/*ram-5*/sensory ray

## Introduction

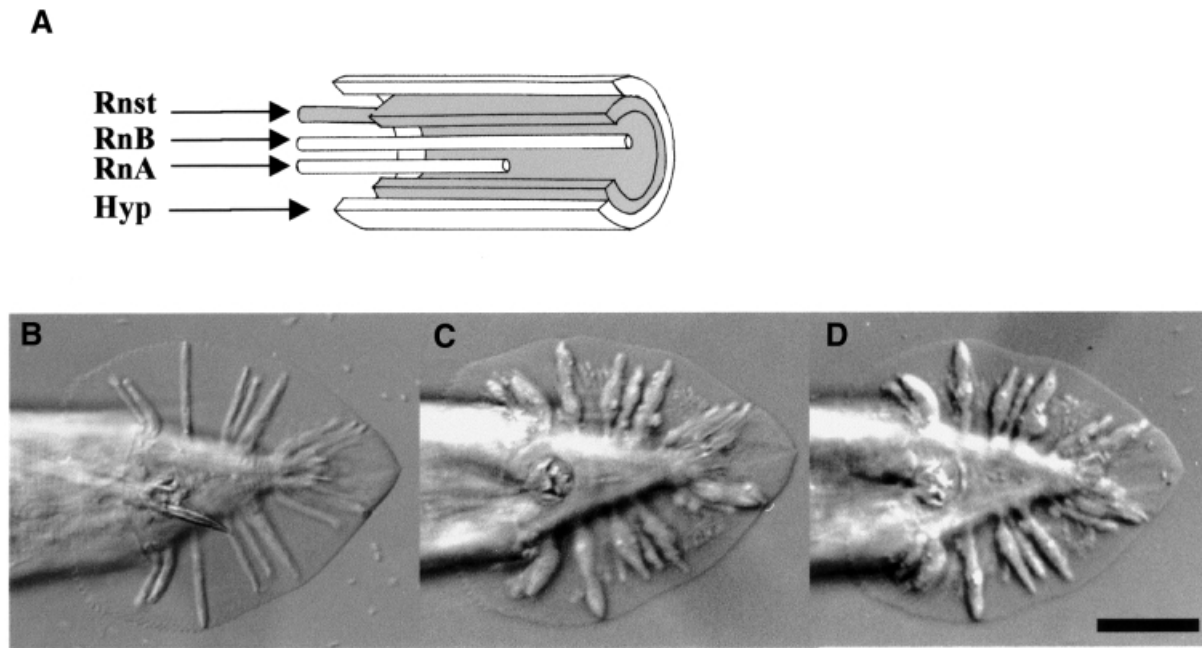
Morphogenesis guided by a tightly regulated genetic program is important in defining the functional and structural characteristics of an organism (Poodry, 1992). For instance, the morphogenesis of *Drosophila* appendages requires interactions between cellular components through secreted ligand, receptor, transcription factors and adhesion molecules for the proper propagation of morphogenetic signals (von Kalm *et al.*, 1995; Brabant *et al.*, 1998). Localized expression of proteases in the imaginal disk cells allows tissue remodeling (Appel *et al.*, 1993) while expression of adherens junction proteins and cytoskeletal molecules helps to re-establish cell–cell association and the formation of a new structural form (Geiger and Ayalon, 1992).

*Caenorhabditis elegans* has been used widely for developmental studies. Its well-documented cell lineage has facilitated the analysis of various developmental processes (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston *et al.*, 1983), including morphogenesis of

the male-specific sensory structures and the hermaphrodite vulva (Baird and Emmons, 1990; Herman *et al.*, 1999; Newman *et al.*, 1999). The sensory rays are organized bilaterally in a simple and specific pattern in the male tail and are used for active searching and locating the hermaphrodite vulva during mating (Liu and Sternberg, 1995). The ray precursor cells derive from the lateral seam cells. During the L4 stage, each precursor cell gives rise to four differentiated cells: a ray structural cell (Rnst), two ray neurons (RnA and RnB) and a hypodermal cell (hyp). Sulston and Horvitz (1977) noticed that when the Rnst cells were removed by laser microsurgery, rays failed to form. Thus, the structural cell is essential for ray formation. The hyp cells of rays 1–5 fuse together to form the tail seam (SET) and those of rays 6–9 fuse with the hyp7 compartment on the ventral surface (Sulston *et al.*, 1980). During tail retraction, the somata of Rnst, RnA and RnB of each ray migrate anteriorly, leaving behind dendritic processes with the structural cell attached to the cuticle surface. This attachment is important for ray positioning and occurs early during papilla formation (Baird *et al.*, 1991; Nguyen *et al.*, 1999). The processes of RnA and RnB eventually detach from the cuticle and are wrapped by the Rnst process near the ray tip (Figure 1A) (Chow *et al.*, 1995). Coincident with the completion of anterior migration of these cells, the hypodermis retracts dorsally and anteriorly, resulting in a thin hypodermal sheath covering the ray surface (Figure 1A) (Baird *et al.*, 1991). Although the molecular mechanisms controlling ray morphology are largely unknown, proper synthesis of adhesion molecules is believed to facilitate the cellular association and movement of ray cells during morphogenesis.

A number of genes have been shown to be necessary for male sensory ray morphogenesis (Hodgkin, 1983; Baird and Emmons, 1990; Baird *et al.*, 1991). Among them are a group of *ram* genes, mutations of which result in lumpy sensory rays (Figure 1B, C and D). It has been postulated that Ram defects are caused by the failure of ray cell assembly (Baird and Emmons, 1990). Mutations in different *ram* genes failed to complement each other, which implies interactions among the *ram* gene products. Studies of a temperature-sensitive allele of *ram-2* suggested that *ram* genes function at the final stage of ray cell morphogenesis rather than in cell fate determination steps (Baird and Emmons, 1990). In addition, tunicamycin treatment of wild-type males during the retraction period can result in the lumpy rays phenotype (Ko and Chow, 2000). In that particular study, four *ram* genes, including *ram-5*, have been suggested to act in a glycosylation-dependent pathway and interact with glycosylation-independent *ram* genes.

In this study, we explore ray morphogenesis by phenotypic characterization of *ram-5* mutants at both the



**Fig. 1.** (A) Schematic diagram showing a ray and its cellular constituents. (B–D) Morphology of wild-type and mutant male tails. Ventral view, anterior to the left with ray 1 at the most anterior position on each side of the fan. (B) *him-5*; (C) *ram-5 (bx30)*; (D) *ram-5 (bx81)*. Scale bar = 20  $\mu$ m.

molecular and cellular level. *ram-5* encodes a transmembrane protein, which is likely to be glycosylated, and interacts with other *ram* gene products to form an adhesive complex between ray cells.

## Results

### Phenotypic characterization of *ram-5* mutation

Animals with *ram-5* mutations showed observable phenotype only in males and not in hermaphrodites. In *ram-5* mutant males, all the rays are irregular (lumpy) in shape, but lie in their wild-type positions (Figure 1C and D). We used tissue-specific molecular markers and electron microscopy to examine the subcellular defect in *ram-5* mutant animals. The promoter region from *sek-1*, the nematode homolog of the mammalian SEK-1 gene, was used to drive green fluorescent protein (GFP) expression in ray neurons (*psek-1-GFP*) (K.Matsumoto, personal communication). This GFP signal in both ray neurons, RnA and RnB, was used to monitor their morphological differentiation in *ram-5* mutant males. Neuronal processes extend the full length of the rays in both mutant (Figure 2C and D) and wild type (Figure 2A and B). However, the expression of a hypodermal marker, *pmab-21-GFP* (S.S.H.Ho, G.M.K.So and K.L.Chow, in preparation), in *ram-5* mutants revealed abnormal and irregular swelling in the hypodermal tissues (Figure 2G and H) as compared with that of wild-type rays (Figure 2E and F). Consistent with our observation by tissue-specific markers, transmission electron microscope (TEM) analysis also showed abnormal morphology of the hypodermal cell with entrapped nuclei, membranous whorls and cellular swelling (Figure 3C, D and F). The neuronal cells, RnA (not shown) and RnB (Figure 3B and E), appeared essentially normal and their distal ciliated tips were enclosed in a

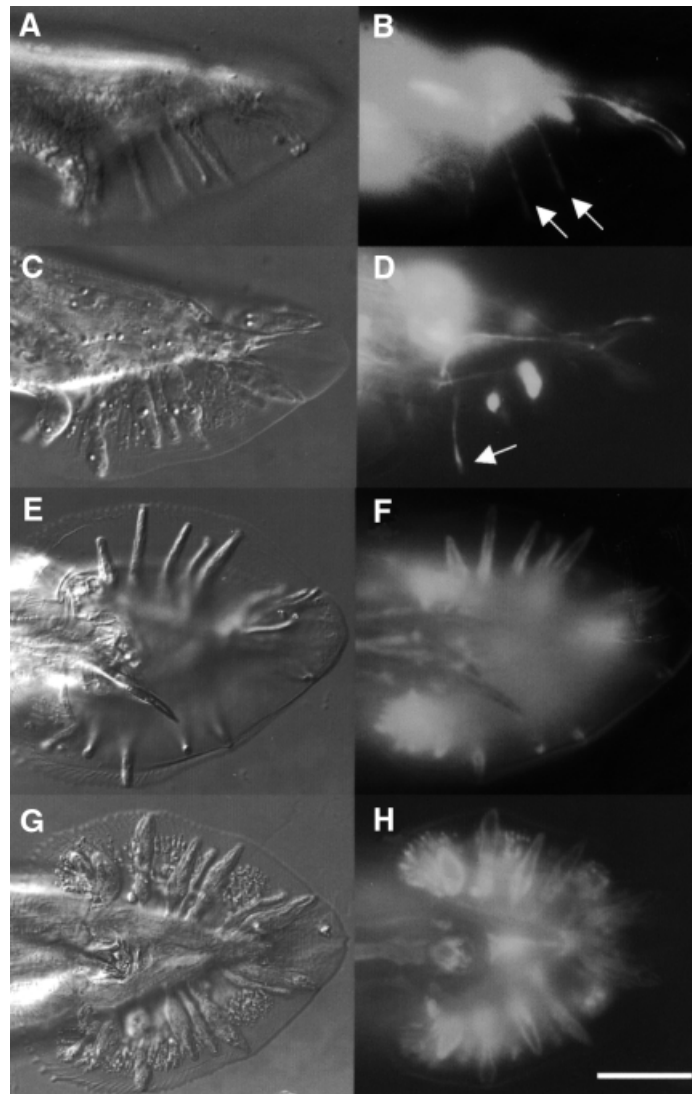
pocket of the structural cell process (not shown). In addition, acellular hypodermal debris, swollen structural cells with vacuoles and excessive membranous whorls were noted (Figure 3C and F). Hence, the lumpy ray phenotype must be due to abnormal differentiation of both structural and hypodermal cells but not gross abnormality in neurons.

*ram-5* males also displayed active mating engagement in mating tests and sired cross progeny efficiently (data not shown). While male searching behavior is dependent on the two ray neurons (Sulston *et al.*, 1980; Liu and Sternberg, 1995), our observation suggests that *ram-5* mutations have little or no effect on neuronal function of the rays.

### Cloning of the *ram-5* locus

*ram-5* was mapped on the right arm of chromosome X (Figure 4A and B). Rescue experiments with cosmids spanning this region identified three cosmids, H02I07, H01A20 and T24C2, which revert the mutant phenotype back to wild type (Figure 4D). Thus, the *ram-5* locus must fall within their overlapping region. In these experiments, a swollen ray tip was occasionally observed when rescue was incomplete. Further deletion of T24C2 defined a minimal rescue DNA fragment of 9.5 kb (Figure 4C). Since animals carrying either *bx30* or *bx81* alleles of the *ram-5* gene, obtained by ethylmethanesulfonate and diethylbutane mutagenesis, respectively, could be rescued by this fragment, the mutations have probably occurred within this fragment.

Using this 9.5 kb *ram-5* genomic DNA as a probe, Southern hybridization was performed to map both *ram-5* alleles. No hybridization signal was detected in the genomic DNA of *ram-5 (bx81)* worms, which indicates that the *ram-5* locus was deleted in this allele (Figure 5A). Consistent with this finding, the northern analysis also



**Fig. 2.** Mosaic animals showing ray neurons marked by *psek-1*-GFP in wild-type (A and B) and *ram-5* (C and D) males. The hypodermal sheath of a ray is marked by *pmab-21*-GFP in wild-type (E and F) and *ram-5* (G and H) animals. Scale bar = 20  $\mu$ m. Arrows indicate normal neuronal extension.

failed to detect a 2.3 kb or a shorter *ram-5*-specific transcript in the *bx81* allele (Figure 5B). Since this 9.5 kb fragment is sufficient to revert the mutant phenotype of *bx81* allele to wild type, we conclude that the *bx81* allele represents a null mutation of *ram-5* and demonstrates the null phenotype. No chromosomal aberration was found in the *bx30* allele. However, sequencing of the whole 9.5 kb fragment detected a nonsense mutation at Ala615 of the putative protein, changing it to an *opal* stop codon. Thus, *bx30* allele produces a truncated non-functional RAM-5 protein that lacks a critical transmembrane (TM) domain (see below).

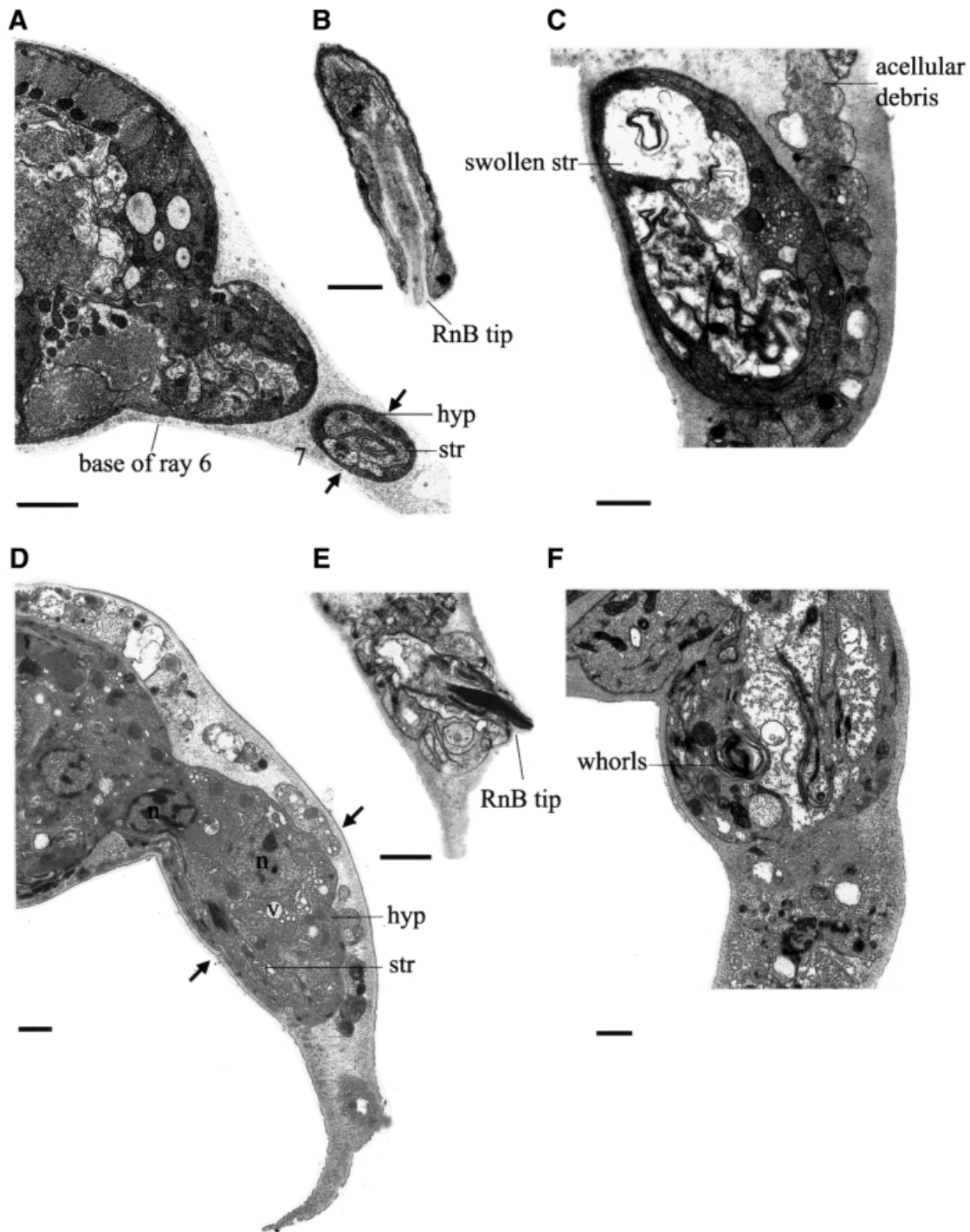
#### **Characterization of *ram-5* genomic organization and transcript**

In terms of the genomic organization, both GENSCAN and GeneFinder programs suggested that there was only one probable transcript encoded on this 9.5 kb fragment (Burge and Karlin, 1997; Solovyev and Salamov, 1997). When a series of deletion constructs were made (Figure 4C), all deletions led to a loss of the rescuing

activity except for an Afl deletion clone. Therefore, the *ram-5* gene probably spans almost the entire 9.5 kb. In addition, a *Nhe* deletion clone without the putative promoter region from nucleotides 1 to 2438 failed to rescue the mutant phenotype, while a clone with an internal deletion from nucleotides 1630 to 2779 (Afl) could. The combined results showed that the 5' promoter regulatory elements required for the rescuing activity are encompassed within the first 1630 nucleotides (Figure 4C).

To examine further the *ram-5* coding region, frame-shift experiments were performed (Figure 4C). An *SphI* site at the putative translation start site at the predicted exon 1 was eliminated from this 9.5 kb fragment by T4 DNA polymerase modification. A frame-shift mutation at the *SalI* site in the predicted exon 12 was generated by a similar approach. Both mutations caused a loss of rescuing activity (*SphI*, 0/3 lines; *SalI*, 0/4 lines), which strongly implies that both sites fall within the coding region.

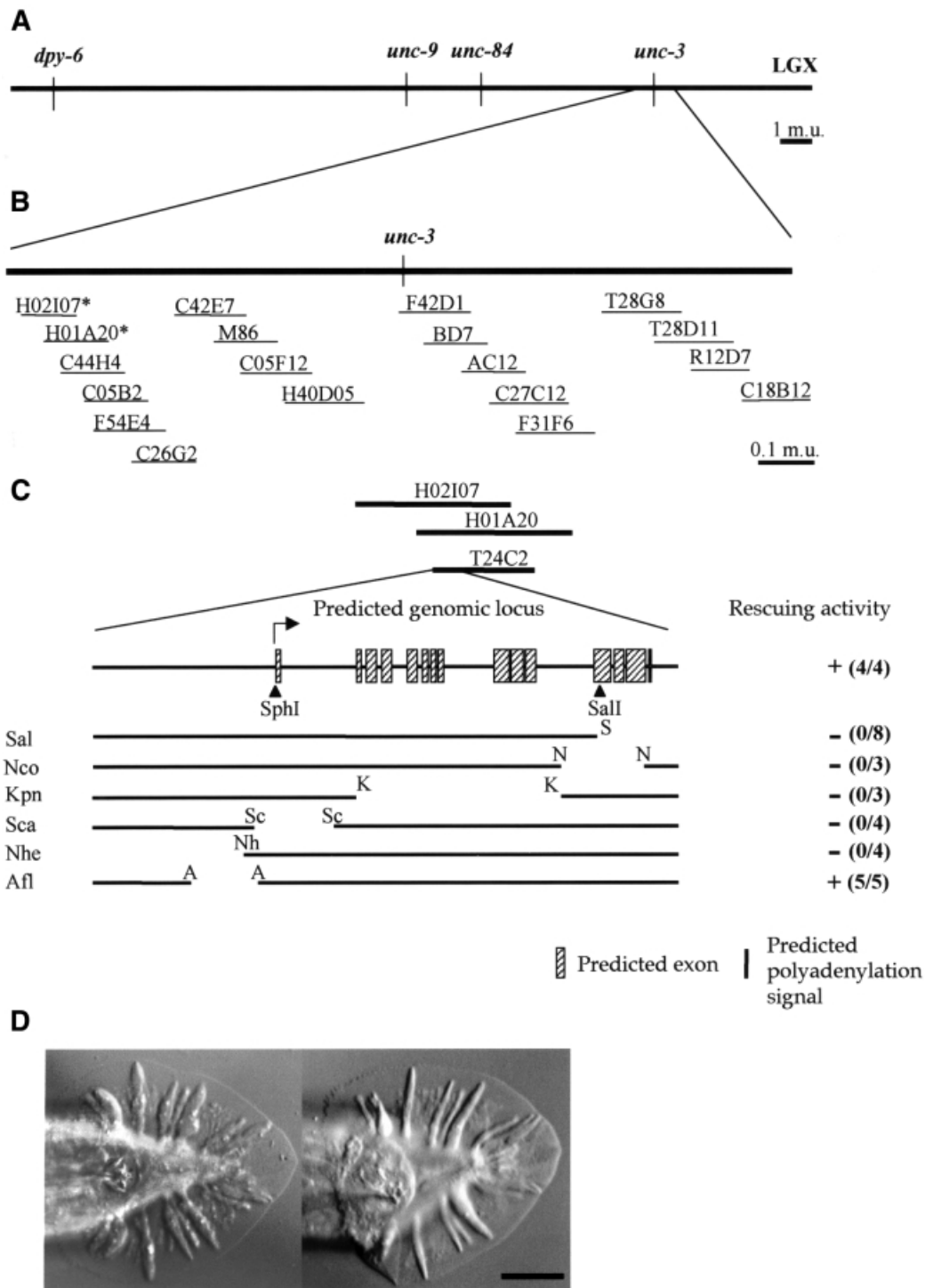
According to several prediction programs, the *ram-5* gene has 14 exons (Figure 4C). Yet, no EST clone was identified based on the putative coding sequences. Neither



**Fig. 3.** Subcellular anatomy of the rays in wild-type (A and B) and *ram-5* (C–F) animals. (A) Transverse section of *him-5* male tail showing rays 6 and 7 extending laterally to the right. Arrows indicate the girth of ray 7 at the midpoint, where a thin hypodermal (hyp) sheath wraps two neuron processes and the structural cell process (str). (B) A wild-type ray opens distally through a pore in the structural cell and cuticle to expose the tip of the RnB cilium. The ray is sheathed by a very thin layer of hyp. (C) A *ram-5* ray is swollen at its midpoint with increased hyp volume and a very swollen str cell. The border between the str cell and the hyp is increased and disordered. Irregular membranes within the str process may be a disordered version of the tight spiral of membranes that is normally present within the str process [compare with (A)]. Acellular debris fills the cuticular fan, surrounding the ray. (D) Transverse section of a *ram-5* male tail showing a fat ray at the same level as in (A). Note the increase in girth (between arrows) in comparison with wild type. The excess tissue is mostly hyp, including some vacuoles and a hyp nucleus, plus the nucleus of another cell at the base of the ray. The str cell looks normal here. (E) A *ram-5* ray opens distally to expose the tip of the RnB cilium in a normal fashion [compare with (B)], but the boundary between hyp and str cell is disordered. (F) A *ram-5* ray at midpoint is massively swollen by increased hyp volume, a very swollen str process and large whorls of membrane lying at the border, or perhaps within the hyp cytoplasm. Scale bars = 1  $\mu$ m.

did library screening yield any cDNA clone. We designed specific primers for 5' RACE, 3' RACE and RT-PCR to reconstruct a full-length *ram-5* cDNA (Figure 6B). The

cDNA obtained was ~2.3 kb in length (Figure 6A), consistent with the transcript size detected by northern analysis (Figure 5B). It has 17 exons for the coding region

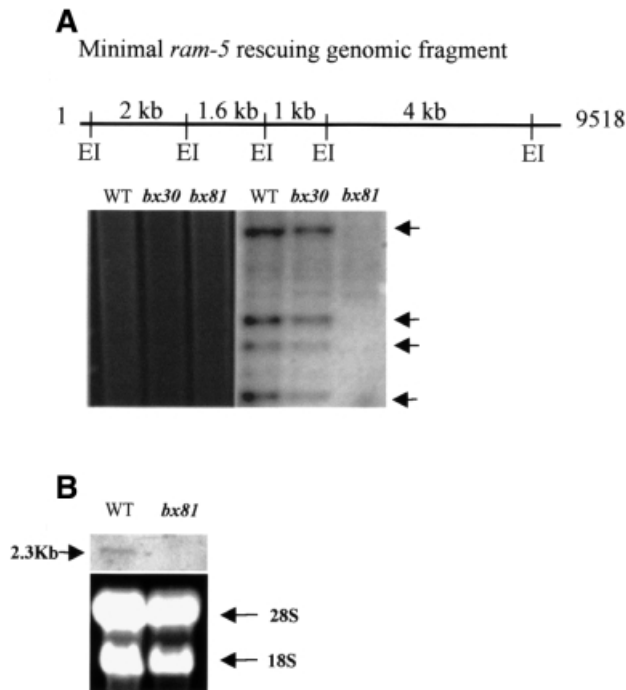


**Fig. 4.** (A) Genetic map position of the *ram-5* locus. (B) Arrangement of cosmids spanning through the mapped region. Asterisks mark the rescuing cosmids. (C) Predicted genomic organization of the *ram-5* transcript and constructs for deletion-rescue analysis in a *ram-5* mutant background. Independent transient lines were scored for the rescue activity (numbers in parentheses). Filled triangles are frameshift mutations. S, *SalI*; N, *NcoI*; K, *KpnI*; Sc, *ScaI*; Nh, *NheI*; A, *AflII*; (-), no rescue; (+), successful rescue. (D) Tail morphology of a *ram-5* mutant male (left); animal transformed with 9.5 kb rescuing DNA fragment (right). Scale bar = 20  $\mu$ m.

and is transcribed to an SL1 leader at the 5' end, thus representing a putative full-length cDNA (Figure 6B). Consensus signal sequences of splicing donor and acceptor sites at the exon-intron junctions were all confirmed in the genomic sequence.

To test the functionality of this cDNA, double-stranded RNAs corresponding to regions spanning exons 1–4 and

exons 9–12 were used for an interference study (RNAi) in wild-type animals (Fire *et al.*, 1998). Male worms with lumpy rays and swollen ray tips were generated in both experiments, suggesting that the products derived from the PCR-based experiments correspond to portions of the *ram-5* gene. When the putative full-length cDNA was put under the control of the *ram-5* promoter, including all the



**Fig. 5.** (A) Southern blot analysis of genomic DNA from wild-type and two mutant alleles of the *ram-5* gene, *bx30* and *bx81*. Four *EcoRI* fragments spanning the *ram-5* genomic region (arrows) were used as a probe to reveal the genomic aberration in the mutant animals. EI, *EcoRI*. (B) Northern blot analysis of wild-type and *bx81* allele. A single *ram-5* transcript was detected in RNA extracted from wild-type animals but not in the sample from a *bx81*-carrying strain. Ethidium bromide-stained gel showed equal loading of samples.

genomic sequence 5' to the translation start, it had full rescuing activity (*bx30*, 2/2 lines; *bx81*, 3/3 lines). Therefore, this cDNA encodes a functional RAM-5 protein.

### Sequence analysis of *ram-5*

The predicted amino acid sequence of the RAM-5 protein obtained from the functional cDNA revealed a putative signal peptide at the N-terminus of RAM-5 (Figure 6A and D) (Nielsen *et al.*, 1997). The N-terminal region shows a limited homology, 26% over 250 amino acids, to the CUT-1-like domain of cuticlin protein encoded by the *C.elegans cut-1* gene (Figure 6D). However, while the CUT-1 domain contains 12 cysteine residues with conserved spacing, the 12 cysteines in RAM-5 have a slightly different spacing. In addition, a hydrophobic  $\alpha$ -helical region between positions 636 and 657 represents a putative membrane-spanning domain of RAM-5 (Figure 6C). Because more positively charged amino acids reside in the C-terminus near the TM domain, the 'positive inside rule' predicts this protein to have a short cytoplasmic C-terminus and a large extracellular domain (Figure 6D) (Sipos and Von Heijne, 1993; Gafvelin *et al.*, 1997). This prediction is in agreement with the position of the signal peptide.

RAM-5 protein has multiple potential glycosylation sites mostly on the extracellular domain (Figure 6D). It has a weak structural resemblance to cuticlin with a large

extracellular domain and a short cytoplasmic tail (see above). Cuticlin was first identified as an insoluble non-collagenous component of nematode cuticle resistant to detergent and reducing agent treatment (Sebastiano *et al.*, 1991). Although its biological function is unknown, it is expressed specifically in dauer larva forming an ~2  $\mu$ m wide ribbon along the lateral lines beneath the alae (Sebastiano *et al.*, 1991) and may interact with the extracellular matrix in the dauer cuticle. The partial resemblance of RAM-5 to cuticlin suggests that it may interact with extracellular matrix components to dictate ray morphology.

### Expression pattern of the *ram-5* gene

To explore the expression pattern of *ram-5*, a GFP reporter was cloned into the genomic fragment at the putative translation start site at the *SphI* site (E1-GFP). Transgenic animals carrying this reporter gene were examined (Figures 7 and 8). Both hermaphrodites and males expressed GFP in a group of cells around the pharyngeal corpus (Figure 7C), which represented either the sheath or socket cells of the inner labial sensilla. Signal was also noted in the ADE socket cells (ADEso) (Figure 7C) and PDE socket cells (PDEso) (Figure 7F and G) in the midbody, and in phasid socket cells (PHso2) (Figure 7I and J) in the tail, as defined by the presence of their openings, position and morphology. The openings of ADEso and PDEso lay beneath the lateral alae (Figure 7A, B and D, E), while those of Phso2 were near the tail tip (Figure 7K). GFP expression in these sensory support cells was detected soon after their birth and was maintained into adulthood.

More importantly, a GFP signal was observed in all the ray structural cells of the male tail (Figure 8A and B), but not in the hypodermal tissue. Expression in nine pairs of structural cells began just prior to the ray retraction period, peaked during the retraction and declined upon completion of ray extension. The same pattern of expression was also noted when a genomic fragment spanning nucleotides 1–1630 was used to drive the GFP reporter. This observation, in line with the deletion results, indicates that the regulatory *cis*-elements for *ram-5* transcription activation reside within this 1.6 kb region.

### *ram-5* transcription is not dependent on expression of itself and other *ram* genes

With the E1-GFP reporter construct, we further examined the conditions for the transcriptional activation of the *ram-5* gene in the ray structural cells. In a *ram-5* null background, the transgene displayed identical spatial and temporal expression patterns to those in wild-type animals, suggesting that *ram-5* gene transcription is independent of its own expression. Similarly, when the same transgene was crossed into five different *ram* mutants, *ram-1* to *ram-4* and *mab-7*, no change of expression was noticed (data not shown). It appears that the other *ram* genes are not required for tissue-specific expression of *ram-5*. Although these *ram* mutants have similar phenotypes and interact genetically, we conclude that mutations of them do not elicit the RAM phenotype through transcriptional regulation of the *ram-5* gene.

Intriguingly, the ray structural cell morphology in *ram-5* mutants, as revealed by this reporter, is swollen and

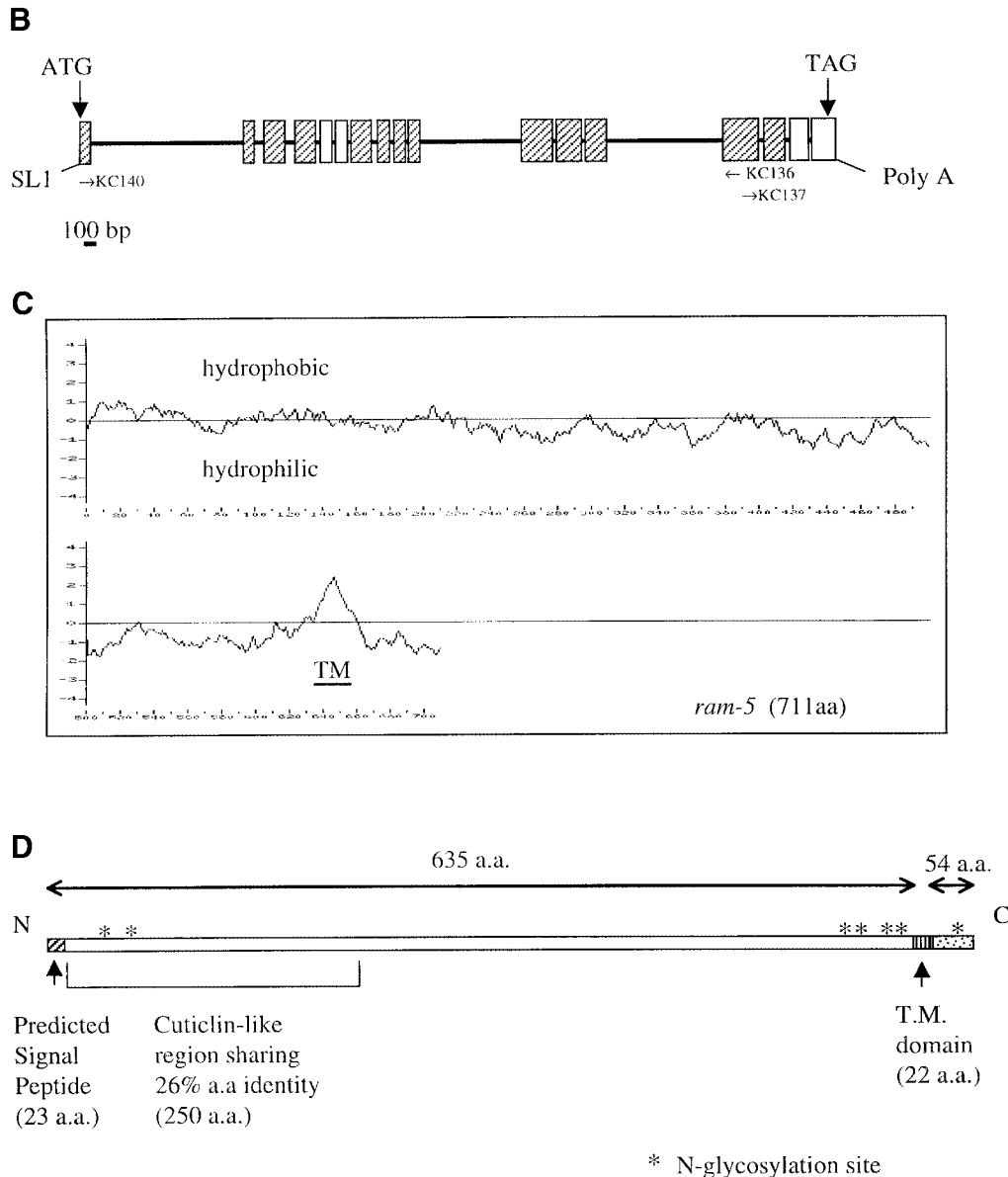
**A**

1	SL1	M Q Y S T K H V I
1	<u>GGTTTAATTACCCAAGTTTGAG</u>	<u>GATAAATATAGCATGCAATATTCACAAAACATGTCATA</u>
10	<u>G Y I F S F V V L A T S T D F D N A H V</u>	<u>GGATACATCTTCTCATTGTGCGTCTCGCCACCTCGACAGATTTTGATAATGCACATGTC</u>
61		
30	<u>M G V P Q V T C S A K L I T V S F N T N</u>	
121	<u>ATGGGGGTACCCAAGTGACATGTTCTGCAAACTTATCACAGTTAGCTTTAATACAAA</u>	
50	<u>I P F Q G R I S V F D K L F I P A C N H</u>	
181	<u>ATPCCATTTCAAGGAAGGATATCAGTATTTGACAAGTTATTCATACCAGCATGTAACCAT</u>	
70	<u>D Y S T N I Q K N A T F Q M D I L K C A</u>	
241	<u>GACTACTCACTAATATTCAAAAAATGCCACATTTCAAATGGACATTTCTGAAATGTGCC</u>	
90	<u>N P M F L K N G S R L L R A Y V E I G F</u>	
301	<u>AATCCAATGTTCTTAAAAAATGGCTCTCGATTGCTAAGAGCATATGTGGAAATTTGGGTTCT</u>	
110	<u>H P L V M T N S D R T F F I V E C L D N T</u>	
361	<u>CA'PCCGTTGCTAATGACTAATAGCGACAGAACATTTCTGGTGGAAATGCTGGATAACACA</u>	
130	<u>I M P I V N R A Q S F A D C T H L V R M</u>	
421	<u>ATAATGCCAATAGTGAATAGAGCACAAAGCTTTGCAGATTGTACACACTTGGTGAGAATG</u>	
150	<u>A S E W S S M S E F Q V G D A I V H E W</u>	
481	<u>GCTTCTGAATGGAGCAGCATGCTGAATTTCAAGTCGGCGATGCAATAGTCCATGAGTGG</u>	
170	<u>S C K L P N P A K T Q T F L T N C N A L</u>	
541	<u>AGCTGCAAGTTGCCATAATCCCGCAAAAACGCCAACCTTCCTAACAAATTCGCAACGGATTG</u>	
190	<u>S Q N G Q I I H L I D E N G C V I D S E</u>	
601	<u>TCACAAAACGGCGAGATTATTCATTTAATAGATGAAAACGGCTCGCTCATCGACTTGAA</u>	
210	<u>L M G D I V Y S D H V P K L Y A R A R I</u>	
661	<u>TTAATGGGTGACATTTGCTACAGTGACCATGTTCCGAAATTTATATGCCAGAGCTAGAATA</u>	
230	<u>F K F L T D D K Y R I E C T L E F C N N</u>	
721	<u>TTCAAGTTCTAACCGATGATAAATATCGGATAGAATGCACACTCGAGTTTTGCAATAAT</u>	
250	<u>G S P C K D R V F P P K C A F T K E E I</u>	
781	<u>GGCTCGCTTGTAAAGATCGAGTATTTCTCCGAAATCGCATTTACTAAAGAAGAAATA</u>	
270	<u>T S R S T K N Q L E Q S C M T T M P G I</u>	
841	<u>ACAAGTCGATCGACAAAATCAACTGGAACACTCCGGCATGACAACCATGCCAGGTATT</u>	
290	<u>P S S A Y D S R L K I S S A W L T I K L</u>	
901	<u>CCCAGTAGTGCCTATGATTCGAGACTAAAAATAAGTTCAGCATGGCTTACGATCAAGCTG</u>	
310	<u>N Q Y T E T K G L H P R Y H L K T F L D</u>	
961	<u>AATCAGTACCGGAAACTAAGGGATTGCATCCAAGATATCATCTGAAAACATTCCTGGAC</u>	
330	<u>P T L H E T I S P N D A D H F L M G I S</u>	
1021	<u>CCCACACTCCATGAAACAATATCCCGAATGACGGGACCACTTCTTGATGGGACTCTCC</u>	
350	<u>Y R E P I P K L S S Q S E L S H P D N N</u>	
1081	<u>TACCGGAACCAATTCCTAAATTTGTCGTCACAAAGTGAATTTGCTCACCCCGATAATAAT</u>	
370	<u>R V E G A R I L H S S A F Q P I I S P P</u>	
1141	<u>AGAGTCGAAGGAGCAGTATCCTTCATTCTTCCGCTTTTCAGCCAATCATCAGTCCACCA</u>	
390	<u>I D S H E E F I E T I T F G S N L N S E</u>	
1201	<u>ATCGACTCGCAGGAGGTTTATCGAAACAATAACCTTTGGAAGCAATTTAAATTTCCGAG</u>	
410	<u>P I V N V Q K E M H L Q K E P V H K V F</u>	
1261	<u>CCAATTTGAAACGTGCAAAAAGAGATGCATTTGCAAAAAGGAGCCCGTGCACAAAGTGT</u>	
430	<u>T H K D P L K T E K T E K F M K L V Q I</u>	
1321	<u>ACGCATAAGGACCCGCTCAAAACAGAAAAACGGAAAAATTTATGAAGCTCGTGCAGATT</u>	
450	<u>E N E D R N H G A A L E T T Q Q S R M F</u>	
1381	<u>GAGAACGAGCAGAAAACCGGTGCGGCGCTGGAACACTACTCAACAGTCAAGAATGTTCT</u>	
470	<u>T T T M P S T M I S T T T T P M T S S S</u>	
1441	<u>CAACAACAATGCCAAGTACAATGATTTCAACAACACTACTCCCATGACGCTTCATCT</u>	
490	<u>R T D K L P S E H F N H P E L D T F E K</u>	
1501	<u>AGAACCGATAAATACCTAGCGAACATTTCAATCACCCGAACTTGATACTTTTGAAAAA</u>	
510	<u>P K K S F E K S H S L V Q M T E T T A</u>	
1561	<u>CCGAAAAAGTCATTCGAAAAGAGTCACTCGCTAGTTCAAATGACTGAAACCAACTGC</u>	
530	<u>P A A N L K F Y S T T A S T K K L T P T</u>	
1621	<u>CCTGCCGCCAACCTGAAGTTCATTTCAACAACGGCGTGCACAAAAGTTGACCCCGACC</u>	
550	<u>T P Y A S A P P E S S T Q T P N S T K P</u>	
1681	<u>ACTCCGTATGCGTGGGGCCCCGGAGAGCAGCACTCAAACCTCAAACCTCGACGAAGCCC</u>	
570	<u>T S I R N V T H S V P T V K K Y D K F V</u>	
1741	<u>ACATCCATTAGAAATGTTACTTCATAGTGTGCCAACGGTTAAAAAATATGATAAATTCG</u>	
590	<u>N N N A D W R F D D K A I N D S D I V S</u>	
1801	<u>AACAATAACGGGACTGGAGATTTGACGATAAGGCAATCAATGATAGCGATATAGTGTCTG</u>	
610	<u>E K Q T S A C Y N A T I I S S Q R Q C K</u>	
1861	<u>GAGAAACAACCTTCCGCATGCTACAATGCTACAATTTTTCATCACACGGCGAGTAA</u>	
630	<u>W S G V E H L L L I W S F A S L I V W M</u>	
1921	<u>TGGTCGGGAGTAGAACATCTTTACTGATTTGGTCTTTGCGCTCGTTGATCGTTGGATG</u>	
650	<u>M L I A L F L Y R Y S S R K P Q W I G F</u>	
1981	<u>ATGCTTATAGCATTATTTCTCTACAGATACTCCAGTAGGAGCCACAATGGATTTGGTTTT</u>	
670	<u>R E Q E L R R V A Q S R V L S Q D H P W</u>	
2041	<u>CGAGAGCAAGAACTTCGGAGAGTAGCTCAATCTCGAGTGTGAGCCAAGATCATCCATGG</u>	
690	<u>L H A D A F E E R N Q S K N E I E I N H</u>	
2101	<u>CTTCACCGACGCTTTTCGAAGAGCGCAATCAAAGCAAGAATGAAATAGAGATCAATCAT</u>	
710	<u>F T *</u>	
2161	<u>TTTACTTAGAACGTTTTATATATGTAATGATTTGTTCTTTGTTTCGGAGTATGATTTG</u>	
2221	<u>ATTTGCTGATGGAAATTTTCAAAAAAGATGTTTCTATATAATAAAAATAAATAGGTTA</u>	

vacuolated at regions where the structural cell contacts the hypodermal cell ( $n = 20$ ) (Figure 8C and D). No other observable abnormality was noticed in other *ram-5*-expressing cells. In addition, structural cell bodies and processes within the animal body appear normal. This localized abnormality in the distal region of a structural cell implies that cell-specific expression of the *ram-5* gene is required for normal differentiation of the sensory ray, but apparently does not affect the structural cell fate. Using this same E1-GFP reporter, the structural cell morphology in all the other *ram* mutants was shown to have similar abnormalities to different degrees (data not shown), suggesting that the structural cell is a common cellular target for all the *ram* mutations.

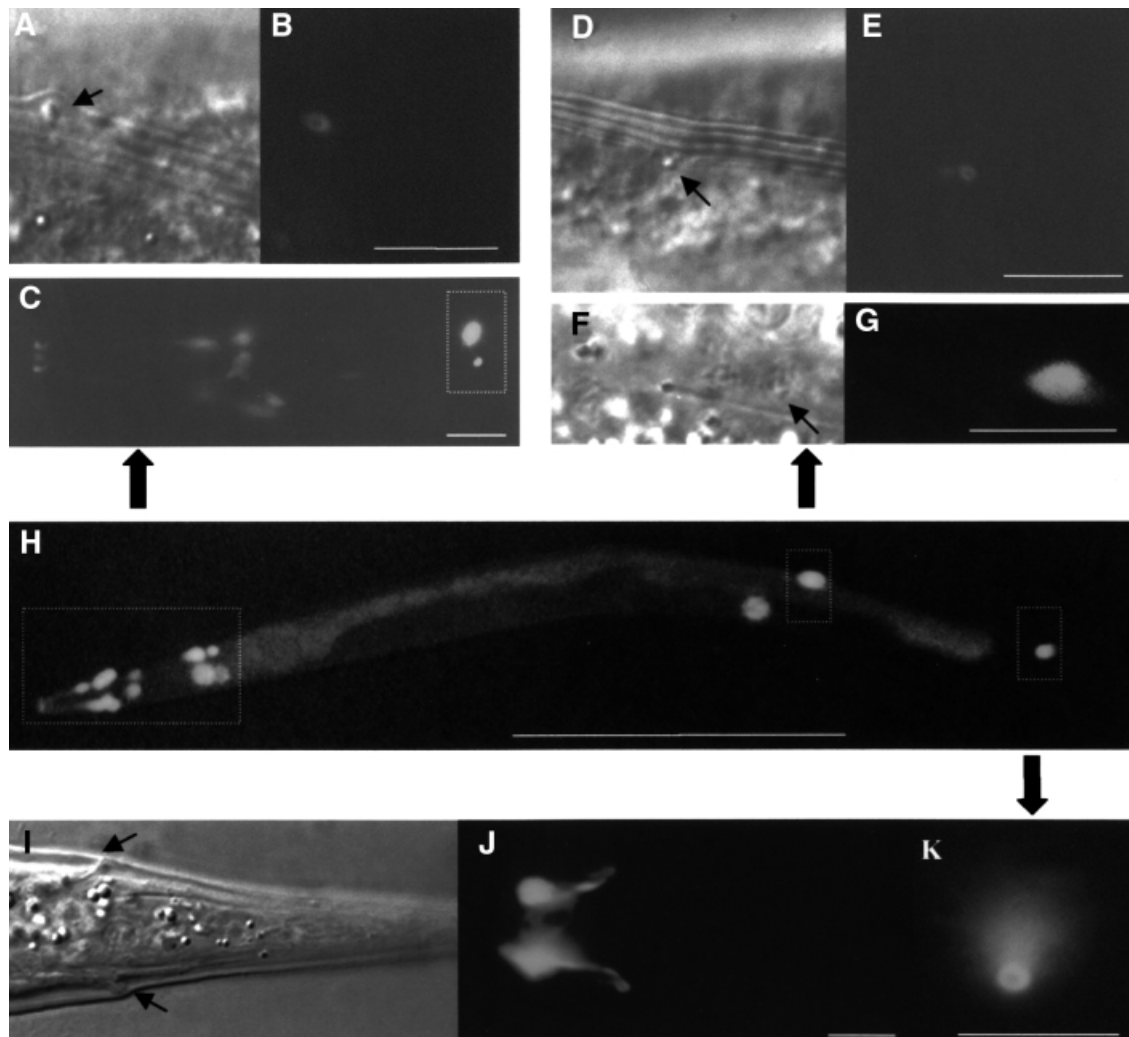
**Localized expression of RAM-5 transmembrane protein is required for structural cell differentiation**

RAM-5 protein has a TM domain. To demonstrate its membrane-bound nature, E14a-GFP was made by in-frame fusion of GFP with RAM-5 after the predicted TM domain (Figure 10). When this fusion protein was driven by the *ram-5* promoter, it behaved as a functional protein and rescued mutant ray phenotype (3/3 lines). The E14a-GFP transgene had an expression pattern identical to that of E1-GFP except that the fusion protein was detected on the structural cell surface and was highly localized near the distal pocket (Figure 9A and B). In contrast, the E1-GFP reporter presented an even distribution of GFP signal within the whole structural cell. This membrane associ-



**Fig. 6.** Cloning and sequence analysis of *ram-5* cDNA. (A) Nucleotide and predicted amino acid sequences of *ram-5* cDNA. The 5' end of cDNA contained *trans*-splice leader SL1. The predicted TM domain and stop codon are indicated by the shadowed box and by an asterisk, respectively. The underlined amino acids represents the predicted signal peptide. The 12 cysteine residues in the CUT-1-like domain are shadowed. (B) The genomic organization of the *ram-5* gene. Hatched and white boxes indicate exons that are exactly identical to or different from the prediction, respectively. The position and orientation of PCR primers are indicated as arrows. (C) Hydrophobicity plot of the predicted RAM-5 amino acid sequence with the putative TM domain marked near the C-terminus. (D) Schematic organization of RAM-5 protein.





**Fig. 7.** Common *ram-5* expression pattern in wild-type males and hermaphrodites. ADE opening is displayed as a ring on the alae (A, arrow) with the *ram-5* expression marked by fluorescence (B). (C) Labial neuron support cells around the pharyngeal corpus. The square box indicates the position of the ADE socket cell. PDE opening appears as a ring at the alae (D, arrow) with the fluorescent signal indicating *ram-5* expression (E). The PDE socket cell (F, arrow) is also marked by GFP signal (G). (H) The overall expression pattern in a hermaphrodite with the magnified region marked by squares. (I) Phasmid socket cells (PhSo2) show GFP signal at the cell bodies, processes (J) and the opening (I, arrow; K). Scale bars = 10  $\mu$ m [except (H), with scale bar = 0.5 mm].

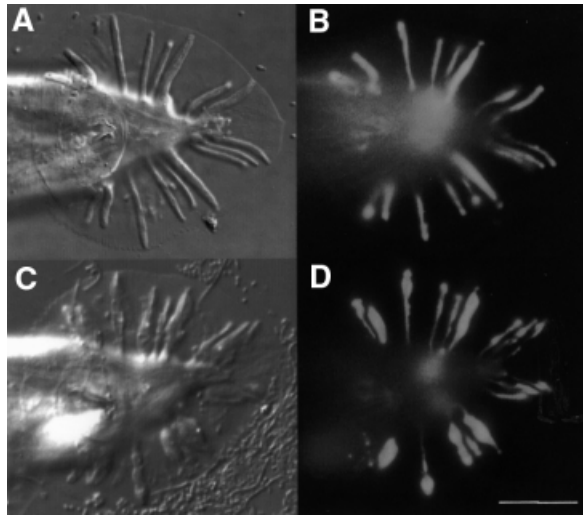
ation was further confirmed by ectopically expressing the fusion protein in the head hypodermal cells using a *mab-21* promoter (Figure 9C). The localized RAM-5–GFP fusion protein (E14a–GFP) expression near the distal portion of the ray probably reflects a property of the RAM-5 protein but not the GFP moiety alone. When the transgene TM–GFP [including at its N-terminus a hybrid signal sequence and the PAT-3 protein TM domain (Gettner *et al.*, 1995)] was expressed in the structural cells by the *ram-5* promoter, the GFP signal was evenly distributed on the membrane of the entire structural cells including their processes (Figure 10). These results argue that domain(s) encoded by the first 15 exons of the *ram-5* gene directs the subcellular localization of the protein at the ray tip.

#### **RAM-5 cytoplasmic domain is dispensable**

RAM-5 has a large N-terminal domain (635 amino acids) and only a short C-terminal tail (54 amino acids) (Figure 6D). In the E14a–GFP fusion construct, GFP

was fused to RAM-5 protein after the TM domain with the last 24 amino acids deleted. These 24 amino acids appear dispensable for the RAM-5 biological function, and the construct could rescue the mutant ray phenotype. By site-directed mutagenesis, we introduced a *Sma*I site on the C-terminal side of the TM domain so that the entire cytoplasmic domain (54 amino acids) could be replaced by GFP (RAM-5 TM GFP) (Figure 10). This fusion protein was again localized to the ray tip and had full rescuing activity (2/2 lines). Hence, we conclude that the entire cytoplasmic domain of RAM-5 is dispensable for its normal function and its subcellular localization.

Another transgene, RAM TM(–) GFP, was produced without the TM domain, so that GFP was coupled only with the extracellular domain (Figure 10). Expression of this secreted form of fusion protein was not localized within specific cells and therefore not detectable. This fusion protein had no rescuing activity (0/4 lines). However, when these transgenes were introduced into

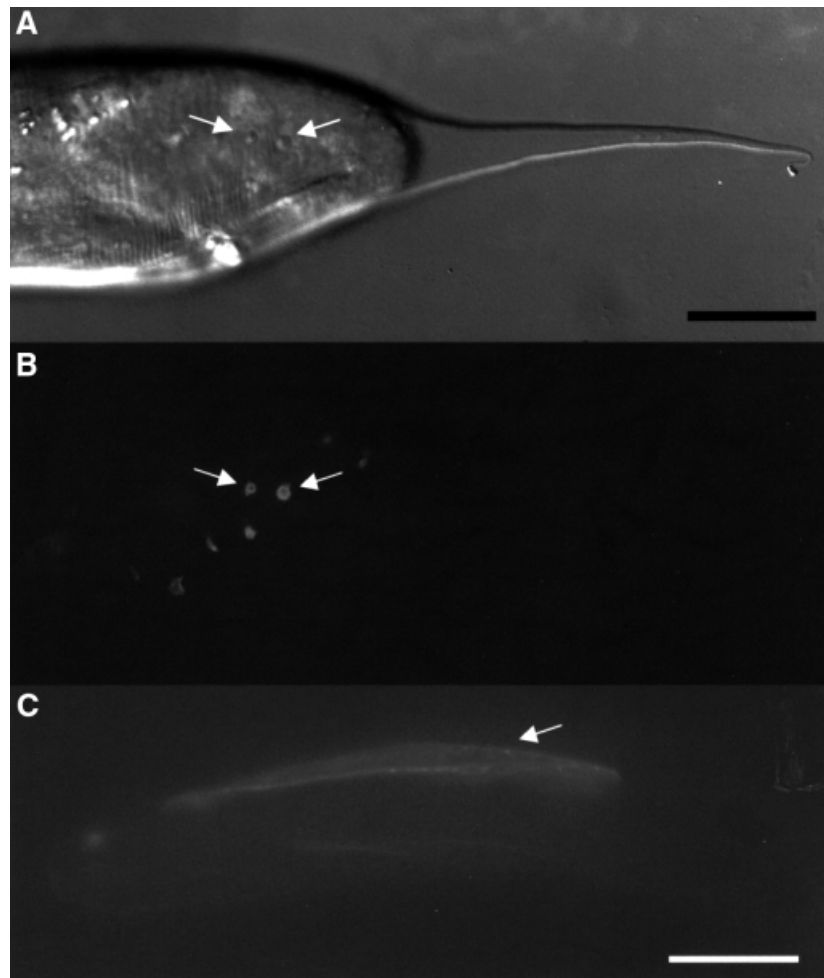


**Fig. 8.** *ram-5* (E1-GFP) expression in ray structural cells of the male tail in both wild type (A and B) and *ram-5* mutant (C and D). Expressing cells in mutant tails display abnormal swelling of the structural cells. Scale bar = 20  $\mu$ m.

wild-type animals, the secreted fusion protein could cause a lumpy ray phenotype at low penetrance while RAM-5 TM GFP or a secreted form of GFP (SEC-GFP) alone did not (Figure 10). These observations argue that the non-membrane-bound form of RAM-5 is expressed, and can interfere with normal RAM-5 function through its N-terminal extracellular domain. The lack of rescuing activity, on the other hand, highlights the importance of membrane anchorage of RAM-5 protein in normal ray morphogenesis.

**Expression of *ram-5* in structural cells can restore normal ray morphology**

*ram-5* mutants have both structural and hypodermal cell defects. To address the tissue-specific expression and requirement of *ram-5* activity, we ectopically expressed the functional RAM-5-GFP fusion, E14a-GFP, in the other ray cells. Hypodermis-specific promoters from *dpy-11* (F.C.F.Ko and K.L.Chow, in preparation) and a ray neuron-specific promoter from the *sek-1* gene were chosen to express the functional GFP-tagged RAM-5 protein in hypodermal and neuronal cells, respectively, during the



**Fig. 9.** Subcellular localization of RAM-5. (A) A lateral view of a retracting male tail with papillae (arrows) during the retraction period. (B) The same animal with E14a-GFP signal localized at the distal tip region of the ray structural cells. (C) E14a-GFP reporter driven by *mab-21* promoter in hypodermis showed a membrane-bound signal. Scale bars = 20  $\mu$ m.

		Mutant rescue activity	GFP signal	Phenotype induced in WT background
RAM-5		+	N.A.	No effect
TM-GFP		-	+(membrane )	No effect
E14a-GFP		+	+(membrane & localized in ray tips)	No effect
RAM-5 TM GFP		+	+(membrane & localized in ray tips)	No effect
RAM-5 TM(-)GFP		-	-	Lumpy rays
SEC-GFP		-	-	No effect

**Fig. 10.** Summary of mutant rescuing activity, GFP signal detection and induced phenotype in wild-type background of different versions of RAM-5 fusion protein. All the fusion constructs were driven by a *ram-5* promoter.

ray retraction period in mutant *ram-5* worms. Both constructs failed to rescue the mutant phenotype, although membrane-bound GFP signal was observed in the targeted tissues at the correct temporal windows (*pdp-11-ram-5*, 0/4 lines; *psek-1-ram-5*, 0/3 lines). Hence, RAM-5 proteins must function by anchoring on the structural cell but not other neighboring cell types.

## Discussion

### ***ram-5* product is required during the ray retraction**

*ram-5* mutant worms grow well with a normal life cycle. Embryonic lethality has not been noticed even in the null allele, *bx81*, and there is no apparent defect in hermaphrodites. Obviously, *ram-5* is not an essential gene for normal development except for establishing ray morphology in male tails. Since lumpy rays do not interfere with mating success, *ram-5* is required only for ray morphogenesis but not their sensory function. Therefore, this gene represents a true 'morphogenesis gene'.

Temporal expression of *ram-5*, as revealed by the GFP reporter E1-GFP in the structural cells, peaks at the beginning of retraction, but is absent earlier when ray cell identity or cell fate are determined. Its expression window is consistent with that defined by heat shock phenocopy and temperature-sensitive allele analysis on *ram* gene function (Baird and Emmons, 1990; Chow and Chan, 1999). Expression of *ram-5* declines substantially after the proper ray association is established. Adult male animals present little or no E14a-GFP expression near the ray tips, suggesting that *ram-5* products are quickly turned over after ray morphogenesis completes. This observation (and heat shock analysis) indicates that *ram-5* products are

required only in actively differentiating ray cells but not for maintaining the structural features of a ray once the morphology is established.

### ***Level of ram-5 activity is important for ray morphogenesis***

In a previous study, *ram-1* to *-4* mutations were shown to be semidominant or haploinsufficient. Heterozygous animals showed variably swollen ray tips, whereas homozygotes had lumpy rays (Baird and Emmons, 1990). Since *ram-5* resides on the X chromosome, haploinsufficiency or semidominance was not tested in the previous study. Our rescue and RNAi experiments offer clues that the *ram-5* expression level is also critical to its normal function. Swollen ray tips were occasionally observed in rescued animals, and were also found in dsRNA-injected animals. Two hypotheses may account for this observation: (i) mutant RAM-5 protein can act semidominantly (gene interference) or (ii) normal *ram-5* function is dependent on its expression level. Since the rescue experiment was performed in a null background (*bx81*) with a wild-type gene fragment, the swollen ray tips cannot be explained by the interference of mutant proteins. Neither could they be caused by overexpression of RAM-5, because multiple copies of rescuing transgene in wild-type animals generated no abnormality. In fact, the absence of a mutant RAM-5 protein in the *bx81* rescue experiments favors the latter hypothesis, consistent with the results from RNAi experiments where wild-type protein synthesis was reduced and no mutant protein was introduced at all. Hence, the swollen ray tips phenotype can be considered to be a result of lowering the wild-type RAM-5 protein level below a certain threshold.

### **ram-5 is expressed exclusively in the sensory support cells**

All the cells expressing *ram-5* are neuronal support cells, including the sheath and socket cells of other sensilla. Except for the rays, none of these cells was abnormal in the mutant animals as determined by molecular markers and light microscopic examination. The ADEso, PDEso, Phso2 and labial support cells in the pharyngeal region each extend fine, morphologically normal processes for their corresponding sensilla in *ram-5* mutant animals. Hence, *ram-5* expression may not be required for morphogenesis of any other sensilla except male rays. Since no sensilla in the head or midbody of *ram-5* mutants were examined by TEM, it remains possible that subtle defects went undetected in *ram-5* mutants, or functionally redundant genes do exist for the differentiation of other socket and sheath cells.

The structural cell defects in the *ram-5* mutants are distinctive. The swollen structural cell morphology seen by light microscopy and in EM thin sections is limited to the ray process and is strongest at the distal region, where it contacts with the hypodermal cell and forms a special pocket to envelop the cilia. Both these cells showed local swelling, vacuolation and membranous whorls in the mutants. The remaining portion including the cell bodies and the proximal processes within the worm body appeared normal. Consistently, the E14a-GFP fusion construct showed localized GFP signal near the structural cell pocket at the ray tip during ray extension. Based on the *ram-5* expression profile and the specific requirement of RAM-5 protein in the structural cell, we conclude that *ram-5* is needed for structural cell differentiation primarily at the structural cell-hypodermis junction during the ray retraction period. In this regard, it is noteworthy that the distal pocket does form in *ram-5* engulfing the ciliated dendrites at the tip.

The *ram-5* reporter construct (E1-GFP) also helped to uncover a similar cellular abnormality in all different *ram* mutants. The observation reveals the importance of structural cells in establishing the form and shape of sensory rays, echoing the original laser ablation results of Sulston and Horvitz (1977). This shared phenotype among many different *ram* mutants reflects an interactive mechanism for ray morphogenesis, where the Ram phenotype in other *ram* mutants did not result from the failure of transcriptional activation of the *ram-5* gene, but was more likely due to failure of regulatory and interactive events occurring at the post-transcriptional level.

### **ram-5 is required by both structural and hypodermal cells**

When cellular and functional abnormalities in *ram-5* rays were examined, both the hypodermis and the structural cell were swollen. Excess tissue, swelling, membranous whorls and vacuoles were often found in both mutant hypodermis and structural cells. Retained nuclei [of hyp, structural cell process (str) and other cells] were common in the mutant tail fan as was acellular debris, suggesting that the retraction phase of morphogenesis requires RAM-5. Disruption of the cell border between the hypodermis and structural cell was also observed. Based on the structure of RAM-5 and its localized expression, we hypothesize that RAM-5 and other *ram* gene products

participate in forming an adhesive complex at the borders between these two cells at a brief critical period of male tail retraction. We predict that specific cell surface molecules expressed on the hypodermis would bind avidly to the extracellular domain of the RAM-5 proteins. This notion is supported by the observation that normal RAM-5 function is subject to interference by a non-membrane-bound RAM-5 protein. This non-functional truncated RAM-5 at high level can act as a dominant-negative mutant molecule and competes with the wild-type RAM-5 protein for its interacting partners, impairs the cell-cell association and gives rise to a mutant phenotype.

The presence of cellular defects in both hypodermal and structural cells also indicates that RAM-5 may be required for more than an adhesion function and may facilitate a reciprocal signaling process between the expressing cell and its neighbor within a differentiating ray. Although the exact mechanism is not clear, abnormality found in both cell types in mutants implies that RAM-5 functions early in the reciprocal exchange of signals. Were *ram-5* responsible for processing a secondary (later) signal, only one of the two cell types would be affected. Interestingly, such reciprocal information exchange is evident for several other morphogenetic steps in *C.elegans* development, including early steps of male tail morphogenesis, and may act through adhesive intercellular junctions (Simske *et al.*, 1996; Nguyen *et al.*, 1999). These junctional complexes, including small adherens junctions and gap junctions, do link the structural cell pocket to the hypodermis at the distal portion of a ray (D.H.Hall, unpublished). Although the RAM-5 TM GFP fusion protein does not show the discrete punctate localization typical for either class of cell junctions, its localized expression on the distal membrane of structural cells may represent a novel cellular communication complex acting in concert with other junctional components. In addition, according to this signaling model, some of the *ram* gene products must form links to the cytoskeleton in each cell type to foster cell shape changes during retraction.

In this context, it is intriguing that the C-terminal tail of the RAM-5 protein is dispensable for its function. Should signaling events occur between the ray cells, RAM-5 may simply elicit an initiation signal to the neighboring cells through its extracellular domain alone, or it may transduce an external signal into the cytoplasm of the structural cell through an associated molecule spanning the cell membrane. In either case, RAM-5 protein must interact with other cell surface molecules, probably modulated by glycosylation of its extracellular domain. The identification of these interacting components, likely to be encoded by other *ram* genes, will help to reveal the mechanism of ray morphogenesis.

## **Materials and methods**

### **Culture of nematodes**

*Caenorhabditis elegans* strains were maintained by standard laboratory procedures (Brenner, 1974). All strains in this study carried a *him-5* (*e1490*) mutation, which gives a high incidence of male progeny (Hodgkin, 1983). Other worm strains used include: CB4088: *him-5* (*e1490*)V; EM139: *ram-1* (*bx34*)I; *him-5* (*e1490*)V; EM268: *ram-2* (*bx76*)II; *him-5* (*e1490*)V; EM97: *ram-3* (*bx32*)II; *him-5* (*e1490*)V; EM68: *ram-4* (*bx25*)IV; *him-5* (*e1490*)V; EM81: *him-5* (*e1490*)V; *ram-5*

(*bx30*)X; EM309: *him-5* (*e1490*)V; *ram-5* (*bx81*)X; and KC64: *him-5* (*e1490*)V; *mab-7* (*e1599*)X.

### Electron microscopy

Fixation, thin sectioning and TEM were performed based on standard procedures (Hall, 1995). Healthy adult *ram-5* mutant males were picked, washed in M9 buffer and fixed in 0.1 M HEPES buffer containing 2.5% glutaraldehyde, 1% formaldehyde for 90 min at room temperature. Tails were cut open in the primary fixative with a razor blade to improve tissue access. After buffer washes, samples were re-fixed in 0.1 M HEPES with 1% osmium tetroxide, 0.5% KFe(CN)<sub>6</sub> for 60 min at room temperature, washed in buffer and stained in 1% uranyl acetate, 0.1 M sodium acetate. After embedding in 3% agarose to position the tails in small groups, specimens were embedded in Scipoxy resin (Energy Beam Sciences) and heat cured. Thin sections were collected transverse to the body axis, post-stained with uranyl acetate and lead citrate, and examined in a Philips CM10 electron microscope.

### Engineering of deletion and reporter constructs

All constructs were made following standard molecular procedures described in Sambrook *et al.* (1989). *Sall*-*Bgl*II *mab-21* promoter fragments (2.3 kb) and *Hind*III-*Xba*I *sek-1* promoter fragments (2.2 kb) were subcloned into pPD95.77 to generate hypodermal and neuronal markers for ray analysis. Plasmid carrying a 9.5 kb *ram-5* genomic fragment was used as the parental vector for expression study. E1-GFP and SEC-GFP were generated by subcloning the GFP NLS<sup>-</sup> from pPD95.81 or GFP with a synthetic signal sequence from pPD95.85, respectively, downstream of the translation start at the *Sph*I site. E14a-GFP was constructed by in-frame blunt-end ligation of GFP NLS<sup>-</sup> from pPD95.77 into *ram-5* cDNA at the *Nco*I site, located in exon 14a, after T4 DNA polymerase treatment. The *ram-5* promoter in E14a-GFP was replaced with a 2.3 kb *Sall*-*Bgl*II *mab-21* promoter, 2.1 kb *Xho*I-*Bsm*I *dpy-11* promoter and 2.2 kb *Hind*III-*Xba*I *sek-1* promoter to generate *pmab-21-ram-5*, *psek-1-ram-5* and *pdp-11-ram-5*, respectively, for ectopic expression study. The TM-GFP reporter construct was prepared by cloning of TM-GFP, from pPD122.39, under the control of the *ram-5* promoter at the *Sph*I site.

Mutagenesis was performed on *ram-5* cDNA to introduce a *Sma*I site before and after the TM domain at positions 1935 and 2007 of the cDNA, respectively. Mutagenic PCR was performed with KC172 (5'-GGTCGGGAGTAGAACCCGGGATCTTTTACTGATTTGG-3')/KC173 (5'-CCAAATCAGTAAAAGATG-CCCGGGTCTACTCCCG-ACC-3') and KC174 (5'-GCATTATTCTCTACAGACCCGGGTACT-CCAGTAGGAAGCC-3')/KC175 (5'-GGCTTCTACTGGAGTACCCGGGTCTGTAGAGAAATAATGC-3') as described by Vallejo *et al.* (1995). GFP from pPD95.79 was then cloned into the cDNA at the *Sma*I site before and after the TM domain to generate the RAM-5 TM(-) GFP and RAM-5 TM GFP, respectively.

### Rescue experiment

Microinjection of DNA into gonadal syncytium of hermaphrodites was carried out as described by Mello *et al.* (1991). Tested cosmids or plasmids were co-injected with a dominant *rol-6* marker at a concentration of 100–150 ng/μl. Multiple transgenic lines were scored for each transformation experiment.

### Mutation mapping

Genomic DNA from wild-type and mutant worms was isolated as described by Sulston and Hodgkin (Wood, 1988). Five micrograms of genomic DNA were digested with *Eco*RI, run on a 0.7% agarose gel and transferred onto nitrocellulose membrane for Southern analysis as described (Sambrook *et al.*, 1989). Radiolabeled probe was prepared by random primer labeling of the 9.5 kb *ram-5* genomic fragment to detect aberration of the *bx81* allele. In the case of the *bx30* allele, nine sets of gene-specific primers were designed for amplification of exons including its splice sites from the mutant genomic DNA. At least two independent PCRs were performed for each set of primers. The amplified genomic fragments were PCR sequenced to map the point mutation.

### Extraction of total RNA, cloning of cDNA and northern analysis

Total RNA was extracted by a modified guanidinium thiocyanate-cesium chloride method (Sambrook *et al.*, 1989). RT-PCR experiments were performed using a pair of gene-specific primers, KC136 (5'-GGTCAA-CTTTTTGGTGCAGC-3') and KC140 (5'-CTTCTCATTTGTCGT-CCTCG-3'), or SL1 primer (5'-GGTTAATTACCAAGTTTGAG-3') with KC136 for amplification. A 1.6 kb cDNA fragment, which was

identical to the predicted cDNA, except that two additional exons, Ad1 and Ad2, had been inserted between the predicted exons 4 and 5 (Figure 6B). Both 5' RACE RT-PCR experiments with KC136 and an anchor primer or with KC136 and SL1 primers resulted in a 1.6 kb amplified product with SL1 sequence on the 5' end. Sequence analysis confirmed that the SL1 leader was *trans*-spliced to the 5' end of cDNA at position -11 with a 3' splice acceptor consensus (Figure 6A). 3' RACE experiment with KC137 (5'-GAAGTTCTATTCAACAACGG-3') and an anchor primer amplified a 0.7 kb fragment corresponding to the predicted 3' end with an additional intron splitting the predicted exon 14 into exons 14a and 14b (Figure 6B). Northern analysis was performed with RNA samples separated on a 1% formaldehyde-agarose gel, transferred onto nitrocellulose membrane and hybridized with a radiolabeled *ram-5* cDNA probe prepared by random labeling (Sambrook *et al.*, 1989).

### GenBank accession number

The DDBJ/EMBL/GenBank accession No. is AF218866.

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