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A novel thioredoxin-like protein encoded by the *C. elegans dpy-11* gene is required for body and sensory organ morphogenesis

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SUMMARY

Sensory ray morphogenesis in *C. elegans* requires active cellular interaction regulated by multiple genetic activities. We report here the cloning of one of these genes, *dpy-11*, which encodes a membrane-associated thioredoxin-like protein. The DPY-11 protein is made exclusively in the hypodermis and resides in the cytoplasmic compartment. Whereas the TRX domain of DPY-11 displays a catalytic activity in vitro, mapping of lesions in different mutant alleles and functional analysis of deletion transgenes reveal that both this enzymatic activity and transmembrane topology are essential for determining body shape and ray morphology. Based on the abnormal features in both the expressing and non-expressing ray cells, we propose that the DPY-11 is required in the hypodermis for modification of its substrates. In turn, ray cell interaction and the whole morphogenetic process can be modulated by these substrate molecules.

Key words: *C. elegans*, Ray and body morphogenesis, Ram, *dpy-11*

INTRODUCTION

Morphogenesis is an important developmental process that eventually determines the form and shape of an organ or body structure. This process usually involves multiple regulated steps with cell shape changes, cell movement and cell-cell communication as the major events. Provision of a competent extracellular environment and proper display of cell surface proteins are both essential to ensure that normal cell migration and cellular communication occur.

Molecules in the extracellular matrix are known to be critical for establishing the morphology of *C. elegans* (Kramer, 1994). Collagens encoded by a number of *dpy* loci, when mutated, give a dumpy phenotype (von Mende et al., 1988; Levy et al., 1993). These molecules that are essential for establishing the shape and integrity of animal body are often extensively modified posttranslationally (Norman and Moerman, 2000; Friedman et al., 2000). Therefore, molecular modification of the extracellular matrix (ECM) can be used to modulate and facilitate both cellular differentiation and morphogenetic processes (Vitale et al., 1997). In addition to the matrix proteins, ligand molecules and cell surface receptors are also targets for modification. For example, proteolytic modification is essential for the synthesis of a biologically active ligand in the Hedgehog signaling pathway that controls segment polarity (Wodarz and Nusse, 1998; McMahon, 2000). Glycosylation of Notch by Fringe can regulate the downstream signaling activity in *Drosophila* (Bruckner et al., 2000; Fortini, 2000; Moloney et al., 2000). Covalent addition of polysialic acid to NCAM or integrin molecules can alter cellular adhesion properties and tissue differentiation (Hoffman and Edelman et al., 1984; Pretzlaff et al., 2000). In *C. elegans*, the *dpy-18* gene encoding a prolyl-4-hydroxylase has been shown to hydroxylate its target substrate for protein disulfide isomerase (PDI) modification and maintenance of the body shape (Friedman et al., 2000; Hill et al., 2000; Winter and Page, 2000). Failure to modify the substrate molecules can also result in incorrect protein folding or assembly leading to retention of the precursor molecules intracellularly and possibly cellular swelling (Wallis et al., 1990; Walmsley et al., 1999). In addition, many proteins and signaling molecules involved in cell-cell communication are cysteine-rich and can form disulfide bonds, e.g., Frizzled and its related proteins, fibronectin, collagen and cell adhesion molecules (CAMs) (Wodarz and Nusse, 1998; Balzar et al., 2001; Ichii et al., 2001). Intra- and intermolecular disulfide bond formation in these proteins can determine their molecular folding as well as biological activity.

Thioredoxin is an essential cellular redox cofactor for a variety of biochemical reactions and cellular functions. Most thioredoxins are soluble proteins of about a hundred amino acids. It facilitates the thioredoxin reductase-mediated electrons flow from NADPH to FAD, acts as a hydrogen donor for the ribonucleotide reductase in the process of deoxyribonucleotide synthesis and also functions as ‘protein disulfide oxido-reductase’ to reduce the disulfide bonds in proteins like insulin and NFκB transcription factor (Holmgren, 1985; Qin et al., 1995). With such diverse functional roles, thioredoxin gene-knockout animals exhibit embryonic lethality, although the primary cause of this phenotype was not identified (Matsui et al., 1996). Thus, while the three-dimensional structure of thioredoxin has been resolved, the

developmental function of thioredoxin-like molecule remains a mystery (Eklund et al., 1991; Holmgren, 1995; Martin, 1995). In the past decade, two N-terminal membrane associated thioredoxin-like molecules were isolated from the soybean plant and the symbiotic bacteria inhabiting its root nodules (Loferer et al., 1993; Shi and Bhattacharyya, 1996). Their protein structure has led to the hypothesis that these thioredoxin variants may have an additional function of facilitating communication between the host and its symbionts. More recently, a human thioredoxin-like molecule bearing a transmembrane domain was reported to be the product of a TFGβ inducible gene (Matsuo et al., 2001). Although it resides predominantly on the endoplasmic reticulum and displays an apoptosis-suppressing effect in HEK293 cells treated with low doses of brefeldin, its functional role in normal developmental process was not addressed.

We report here that the *C. elegans dpy-11* gene encodes yet another variant of the thioredoxin-like proteins with both a signal peptide and a transmembrane domain. While *dpy-11* is distinctly different from the related gene found in human, we show that its expression in hypodermal cells is essential for establishing normal body shape in larval and adult nematodes. The function of its gene product in the context of body and sensory ray morphogenesis will be discussed.

MATERIALS AND METHODS

Worm cultures

Worm strains were cultured and maintained as described by Brenner (Brenner, 1974) at 20°C unless stated otherwise. All *dpy-11* alleles were either obtained from the *Caenorhabditis* Genetics Center, Minnesota, or kindly provided by the Hodgkin laboratory and the Baillie laboratory.

Phenotypic analysis of *dpy-11* alleles

All *dpy-11* mutants were characterized by their respective body and male tail phenotypes under Nomarski microscopy. Except the reference allele *e224* that was coupled with *him-5(e1490)V*, all alleles were in *him-8(e1489)IV* background. The average body length was obtained by measuring 30 males with a micrometer. The ray phenotypes were recorded using a Ram Index defined by the degree of sensory ray swelling, with 0 indicating wild type (Fig. 1G) and 5 representing the most severe phenotype (Fig. 1L). Thirty males of each genotype were scored and the value was averaged.

Mapping the *dpy-11* and lesions in mutant alleles

dpy-11 was mapped between *gad-1* and *egl-46*. Cosmids spanning this region were injected individually into *dpy-11*(*e224*) animals with *dpy-13::gfp*, a reporter driven by the *dpy-13* promoter, at a concentration of 100-150 ng/μl (Stinchcomb et al., 1985; Mello et al., 1991). A positive rescuing cosmid W06G7 was identified. An *Acc*65I-*Sph*I rescuing fragment from W06G7 had been identified and subcloned into pSP72 as p72WAS. Further deletion derivatives of p72WAS were generated by restriction enzyme cleavage and self-ligation.

The genomic DNA of 15 *dpy-11* alleles was extracted as described previously (Sulston and Hodgkin, 1988). About 400-600 ng of genomic DNA was used for PCR amplification with Taq polymerase (Gibco) and *dpy-11* gene-specific primers. The product DNA fragment was sequenced with the BigDye Terminal Sequencing Kit (Perkin Elmer) using the same primer sets.

RNA interference of *dpy-11* gene and characterization of cellular defects

RNAi experiments were performed as described by Fire et al. (Fire et al., 1998). Sense and antisense RNA were synthesized from the EST cDNA clone, yk109h7, with T3 and T7 polymerase respectively (Promega in vitro Transcription Kit). F_1 progeny from injected individuals were examined under Nomarski microscopy.

gfp reporter driven by one of the different cell-type specific promoters, *dpy-11* or *dpy-13* (hypodermal cell, this study), *ram-5* (structural cell) (Yu et al., 2000) and *sek-1* (neuronal cell) (Yu et al., 2000) were introduced into *dpy-11* mutant animals. The transformed animals were examined by fluorescence microscopy. RNAi was also performed in these marker strains to reveal the severe cellular defects.

Engineering of reporter, expression and deletion constructs

A *Hin*cII-*Bsm*I 1.6 kb fragment carrying the *dpy-11* promoter region from p72WAS.3 was linked to the worm cDNA yk109h7 and human cDNA DKFZp564E1962 to generate p*d11*cDNA and p*d11*hcDNA respectively. For ectopic expression of *dpy-11*, a *Sph*I site was first introduced in front of the start codon in *dpy-11* cDNA by site-directed mutagenesis with primer KC293:5′-CCAGCATGCTGCTCCG-3′ (Barik, 1993). A 3 kb *Sac*I/*Sph*I fragment carrying the *ram-5* promoter was cloned in front of this modified *dpy-11* cDNA to generate p*r5*cDNA (Yu et al., 2000).

A 2.7 kb 5′ flanking region of *dpy-11* was subcloned into pPD95.70 to construct a *gfp* reporter transgene with a nucleus localization signal (NLS), p*d11*GFPN. A non-nucleus localized version of reporter, p*d11*GFP-N, was generated by deleting the NLS from p*d11*GFPN. These constructs were injected with markers pRF4 (*rol-6*^{d}) into wild-type animals or with p*d11*cDNA into *dpy-11* mutants for expression pattern examination.

The DPY-11 localization and rescue efficiency were examined by transforming *dpy-11* animals with the testing constructs. The wild-type pattern was first revealed with the full length fusion transgene, p*d11*D11GFP, which had the *gfp* fragment from pPD95.69 ligated to a *Hin*dIII site introduced at the stop codon of *dpy-11* cDNA by site-directed mutagenesis with KC 296: 5′-CGAAGAAGACCAAGCTTTAAATTCTATGCAACTTCC-3′ and KC297: 5′-GGAAGTTGCATAGAATTTAAAGCTTGGTCTTCTTCG-3′. The plasmid encoding a tagged and C terminus-truncated version of DPY-11, p*d11*ΔCTGFP, was made by deleting an internal *Ava*II/*Hin*dIII fragment in p*d11*D11GFP followed by self-ligation. A 3 kb *ram-5* promoter fragment was inserted into p*d11*D11GFP to replace the *dpy-11* promoter to generate p*r5*D11GFP for examining the subcellular localization of DPY-11 in a different cell type. A *Hin*cII site was introduced in front of the CT-domain in the *dpy-11* cDNA by site-directed mutagenesis with primers KC273: 5′-CGTTATCATCATCGTCGACCAAGTATTCCC-3′ and KC274: 5′-GGGAATACTTGGTCGACGATGATGATAACG-3′. Subsequently, the *Hin*cII cut CT fragment was cloned into the 3′ end of the GFP gene in pD119.16 with *Hin*cII-*Sma*I. This GFP with CT domain at the 3′ end was used to replace the GFP in p*d11*ΔCTGFP and was designated p*d11*AGFPC.

Starting with the functional *dpy-11* genomic cDNA fusion fragment, p*d11*cDNA, p*d11*ΔSP was generated with the signal peptide (aa 2-34) removed by *Bsm*I and *Bgl*II digestion followed by self-ligation. p*d11*ΔS was made to remove the spacer fragment from aa 135-177 by joining the *Sma*I site (introduced at aa134/135 by mutagenesis with a primer KC299: 5′-GTACCCGGGAACTGGGTCGATCAC-3′) and an *Ehe*I site (5′ to the transmembrane domain generated with primers KC271: 5′-GGGCCAGCTACGGCGCCCTCTTCGCCGGAG-3′ and KC272: 5′-CTCCGGCGAAGAGGGCGCCGTAGCTGGCCC-3′). A *Hin*cII site was introduced at position aa 198/199 with primers KC273 and KC274 (mentioned above). The fragment with the TM domain from aa178-198 was removed by *Ehe*I and *Hin*cII digestion to generate p*d11*ΔTM. p*d11*ΔCT was engineered by introducing a frameshift mutation at the *Ava*II site (aa 205), which resulted in truncation of the C-terminal tail. To replace the transmembrane domain of DPY-11, a *Bsp*EI (blunted)/*Apa*I fragment containing a *pat-3* TM *gfp* fusion from

pPD122.39 was used to substitute the DPY-11 TM at the *Ehe*I to *Apa*I of the cDNA to generate p*d11*TMGFP.

For protein expression, the thioredoxin-like domain of DPY-11 (*Ppu*MI to *Xho*I from cDNA, yk109h7) was subcloned into *Ppu*MI and *Xho*I site of pPD95.75. After a *Sma*I site was introduced after the TRX domain by site-directed mutagenesis with primer KC299 as described above, the *Sma*I fragment encompassing aa 19-133 was subcloned into the pGEX-2T vector to generate pG2TD11T (wild type). A G76E mutant version of the thioredoxin-like domain with glycine 76 changed to glutamic acid was engineered by mutagenesis with primer KC369 (5′-GGAATCAAGGTTGAAGAAGTCGATG-3′) and KC370 (5′-CATCGACTTCTTCAACCTTGATTCC-3′) and was designated pG2TG76E.

Protein production and purification

A 2 l culture of BL21(DE3) transformed with pG2TD11T was grown to log phase followed by induction with 1 mM IPTG at 30°C for 4-5 hours. The culture was lysed in PBS by French Press (SLM Instruments), sonicated for 15 minutes on ice and passed through a glutathione sepharose 4B column (Amersham Pharmacia Biotech, cat. no.17-0756-01). The GST-tagged TRX-like domain was eluted with 20 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) and quantified by the Bradford assay. After overnight dialysis in PBS, the protein was either temporarily stored in 4°C or frozen at –70°C. The G76E mutant protein and GST control protein were expressed using the respective expression plasmids. The purified protein samples of wild type (pG2TD11T), G76E mutant (pG2TG76E) and GST (pGEX2T) were separated using 12% SDS-PAGE.

Insulin reduction assay

Insulin stock solution (10 mg/ml; Sigma, cat. no. I5500) was prepared according to the method of Holmgren (Holmgren, 1979). The *E. coli* recombinant thioredoxin (Sigma cat. no. T0910) and GST were used as positive and negative control respectively. 70 μl of insulin stock solution was added to the cuvettes with 630 μl assay solutions containing 1 mM dithiothreitol (DTT) and 200 μl enzyme at different concentrations (0 μM, 1 μM, 2 μM and 3 μM) to start the reaction. The final reaction mixture contained 1× PBS, 2 mM EDTA, 1 mg/ml insulin and 1 mM DTT. The absorbency was measured in a Beckman 650 spectrophotometer at wavelength of 650 nm at 25°C. The data was recorded at 5 minutes intervals for a period of 30 minutes.

RESULT

Characterization of *dpy-11* mutant phenotypes

dpy-11 mutant was first isolated based on its dumpy body phenotype observed in all larval and adult stages (Brenner, 1974). Its swollen ray (Ram, *ra*y *m*orphology abnormal) phenotype as noted by Baird and Emmons (Baird and Emmons, 1990) indicates that *dpy-11* is also required for sensory organ morphogenesis. We examined the available *dpy-11* alleles on these two diagnostic features, and the results are summarized in Table 1. The Ram Index (RI) is a measure of the severity of swollen rays: mutants score the highest of 5 (Fig. 1L) and wild-type animals score 0 (Fig. 1G,H). The *dpy-11* reference allele *e224* together with *e390*, *e455* and *e733* had mildly swollen rays with a RI of about 2 (Fig. 1I). These mutants were weakly dumpy with the body length ranging from 0.60 mm to 0.66 mm (Fig. 1D). Strong Dpy and Ram phenotypes were found in *e33*, *e207*, *e504*, *e752*, *e794*, *e1180* and *s261* alleles (Fig. 1B,J). Their body length ranged from 0.38 mm to 0.46 mm, and their respective RI from 3.5 to 4.8 (Fig. 1J). In general, there was a good correlation of the severity of dumpiness and ray swelling.

Table 1. The phenotypes of *dpy-11* mutant alleles

Allele	Mutation (amino acid changes)	Male tail Ram Index (0-5)	Male body length (mm)*
him-8(e1489)	–	0.0	0.88±0.04
e33	W173*Opal*	4.3	0.41±0.03
e207	R4*Opal*	4.5	0.46±0.03
e504	W34*Amber*	4.6	0.38±0.03
e752	W50*Amber*	4.7	0.38±0.04
e794	P20†	4.8	0.41±0.02
e1180	P20†	4.7	0.45±0.06
s261	H100‡	3.5	0.42±0.02
e224	G76E	1.9	0.63±0.02
e390	G76E	1.9	0.66±0.04
e455	G76E	2.3	0.60±0.04
e733	G76E	2.1	0.64±0.03
e395	G187E	0.0	0.65±0.04
e431	G187E	0.1	0.65±0.03
s10	G191R	0.5	0.43±0.05
s360	Δ195-197aa	0.0	0.49±0.02
e207/e224	–	2.7	0.48±0.03

*Thirty worms were measured and the value averaged for each allele. The Ram Index of the most severe ray phenotype is 5 and that of the wild type is 0.
†Frame-shift beyond the twentieth amino acid.
‡Mutation at the 5′ donor site of intron 2.

However, there were exceptions. *s10* and *s360* mutants had a strong Dpy phenotype (0.43 mm to 0.49 mm) but exhibited almost wild-type rays (Fig. 1C). *e395* and *e431* alleles had weak Dpy body (from 0.63 mm to 0.65 mm) with normal rays (Fig. 1H). When these four alleles were tested for temperature sensitivity, only *s10* mutants presented a swollen ray tip phenotype with greater than 90% penetrance at 25°C. The other three mutants had wild-type male tails, independent of the testing temperature.

When heterozygous animals of these 15 alleles were examined, they all showed wild-type body length and rays. This observation suggests that these mutations are loss-of-function mutations. In addition, double heterozygous worms of *e207* (putative null) and *e224* alleles (weak dumpy) had a strong Dpy phenotype with intermediate Ram rays of RI of 2.7 (Table 1). Hence, the body morphology, in comparison with ray development, appears to be more sensitive to the reduction of *dpy-11* gene activity.

Cellular defects of *dpy-11* mutant sensory ray

To characterize the sensory ray abnormality in *dpy-11* mutants, cell-specific marker *gfp* transgenes driven by hypodermal, structural and neuronal cell-specific promoters were introduced into animals carrying the *e224* allele. The hypodermis and structural cell processes of the mutant animals had abnormal swelling (Fig. 2D,F) in contrast to those of the wild-type worms (Fig. 2C,E). The neuronal processes in this reference allele appeared less abnormal although the processes were rough and irregular in shape with occasional nodule-like swelling. These neuronal nodules were obvious in RNAi-treated males (Fig. 2H, arrowhead), which also exhibited more severe swelling of hypodermis and structural cell processes (data not shown).

Definition of the *dpy-11* gene locus

dpy-11 was genetically mapped between *egl-46* and *gad-1*. Cosmids spanning this region were injected into *e224* mutants

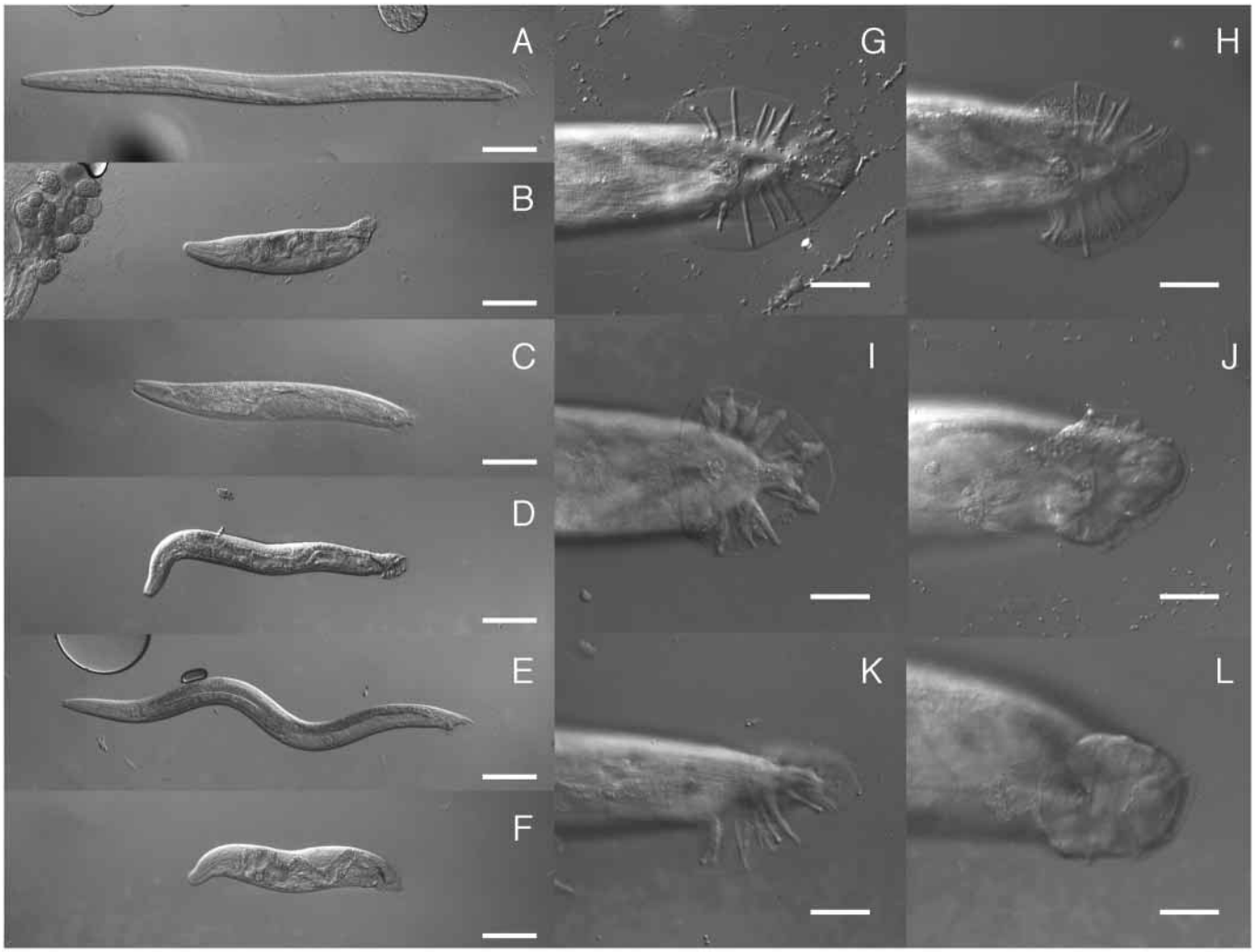

Fig. 1. Body and tail phenotype of (A,G) wild-type worms, and mutants carrying various *dpy-11* alleles: (B) *e504*, (C) *s360*, (D,I) *e224*, (H) *e431*, (J) *s261*. (E,K) Mutant worm rescued by p72WCP and (F,L) wild-type worm with *dpy-11* RNAi. Scale bar: 100 μm (A-F); 20 μm (G-L).

to test for rescue activity. Positive rescuing cosmids, W06G7 and F46E10, were identified (Fig. 3A). The physical location of *dpy-11* was simultaneously confirmed with another overlapping cosmid T04F11 (J. Pak and A. Spence, personal communication). Serial deletion constructs derived from W06G7 were made to localize the rescuing activity (Fig. 3A). The p72WCP fragment contained the F46E10.9 gene and a truncated F46E10.8 ORF. Exhibition of wild-type *dpy-11* activity by this fragment suggests that F46E10.9 is *dpy-11*. The identity was subsequently confirmed by two approaches: (1) a transgene with a F46E10.9-derived cDNA, yk109h7, linked to the F46E10.9 5′ flanking sequence rescued the mutant phenotype efficiently; (2) RNAi with this cDNA phenocopied the *dpy-11* Ram and Dpy phenotypes in wild-type animals (Fig. 1F,L). This RNAi result confirms again that both phenotypes seen in all our mutants are due to loss-of-function of *dpy-11*.

dpy-11 is exclusively expressed in the hypodermis

The spatial and temporal expression patterns of *dpy-11* were examined with a reporter assay. The green fluorescent protein gene (*gfp*) under the control of a 2.7 kb *dpy-11* 5′ flanking sequence, which could rescue mutants when coupled to the cDNA, was used to generate transgenic worms in both wild-type and mutant backgrounds. The reporter constructs, with or without the nuclear localization signal (NLS), revealed a similar pattern of signals independent of the *rol-6*^{d} or p*d11*cDNA markers used for transgenesis. The expression profile is therefore reflective of the authentic *dpy-11* transcriptional activity and related to the body and ray morphogenesis (Fig. 4A,B). Fluorescence was detected in the hypodermal cells as early as the comma stage (data not shown) and throughout all the larval stages in the body hypodermis (Fig. 4A,B) with the exclusion of seam cells (Fig. 4B, arrowheads). At the late L3 to L4 stages when ray cell differentiation took place, no *dpy-11* promoter driven GFP signal was detected in the ray precursor (Rn) cells. Neither was there any signal detected in cells of the Rn.a branch differentiating into future neuronal derivatives. GFP signal was observed only in the Rn.p cells after they were born, all of which eventually fused to form the tail seam or fused with the body hypodermal syncytium (data not shown).

To examine the subcellular localization of the DPY-11 protein, the fusion transgene encoding full-length DPY-11 with

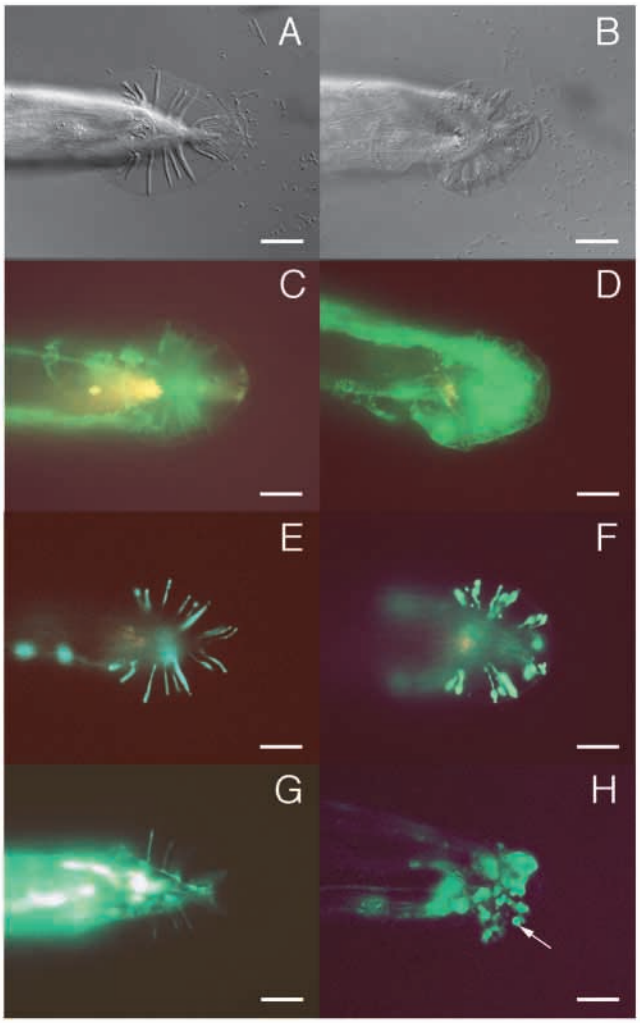

Fig. 2. Cellular features of wild type (A,C,E,G) and *dpy-11* (*e224)* (B,D,F) or *dpy-11*(RNAi) (H) male tails as visualized by (A,B) Nomarski optics; (C-H) cell-type specific markers to identify: hypodermal cells (C,D; marked by p*d11*GFP-N), structural cell (E,F; marked by *ram-5::gfp*) and neuronal processes (G and H; marked by *sek-1::gfp*). Scale bars: 20 μm.

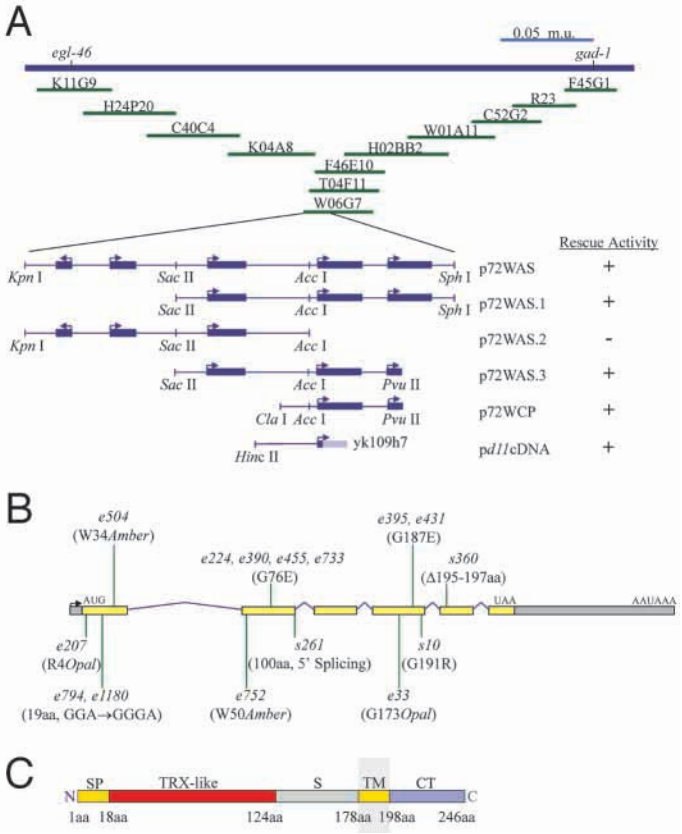

Fig. 3. The *dpy-11* locus and the predicted protein structure. (A) Cosmids and their deletion derivatives used for defining the *dpy-11* locus. (B) Genomic organization of *dpy-11* and the lesions in mutant alleles. (C) Predicted structure of the DPY-11 protein. The GenBank accession number of the *dpy-11* gene is AF250045.

a GFP tagged between the transmembrane domain and the C terminus was constructed (p*d11*AGFPC, Fig. 8, construct 10). This fusion transgene was almost as active as a wild-type transgene in complementation rescue assay. The fusion products presented a heterogeneous signal with a membranous organization in the expressing hypodermis (Fig. 4C,D). A similar pattern was noted when a C terminus-tagged GFP fusion DPY-11 (p*d11*ΔCTGFP, Fig. 8, construct 8) was examined (Fig. 4E,F). The pattern is distinctly different from that of cytosolic GFP (Fig. 4B). This distribution pattern suggests that the DPY-11 protein is associated with subcellular membranous organelles. In addition, in some dying transgenic embryos where cells started to disintegrate, membrane-associated GFP signal could be observed (Fig. 4G,H, arrowheads) while non-dying cells displayed the same heterogeneous profile (Fig. 4G,H, arrows). These results suggest that DPY-11 molecules are residing on a membranous structure, possibly endoplasmic reticulum or Golgi apparatus.

Structural organization of DPY-11

DPY-11 has 246 amino acids as predicted from the ORF present in the cDNA. Based on the secondary structure and sequence analyses, it has a putative signal peptide at the N-terminus (SP), a thioredoxin-like domain (TRX), a 50 amino acid spacer (S), a putative transmembrane domain (TM) and a 46 amino acid C-terminal region (CT) (Fig. 3C) (Schultz et al., 2000). The thioredoxin-like domain shared 48-53% similarity with thioredoxin molecules from bacteria and eukaryotes (Fig. 5A). DPY-11 also had a potential ER retention signal sequence, KKTK, at its C terminus (Hong, 1996).

Full protein sequence alignment has identified two proteins, from human and *Drosophila* respectively, sharing structural similarity to DPY-11 (Fig. 5B). These DPY-11-like proteins have an N-terminal signal peptide, a thioredoxin-like (TRX) domain, a spacer sequence, a transmembrane domain and a C terminus of different lengths. In their respective TRX domains, they all had a putative catalytic site with CPAC sequences, in contrast to the conserved CGPC sequence found in most thioredoxins. Such conservation suggests that these DPY-11-like proteins may indeed be functionally related. However, when a construct with the human *dpy-11*-like cDNA driven by the worm *dpy-11* promoter was introduced into *dpy-11* mutants, no rescue activity was observed, possibly because of the divergence in their sequences and thus biological specificity (Fig. 8, construct 2).

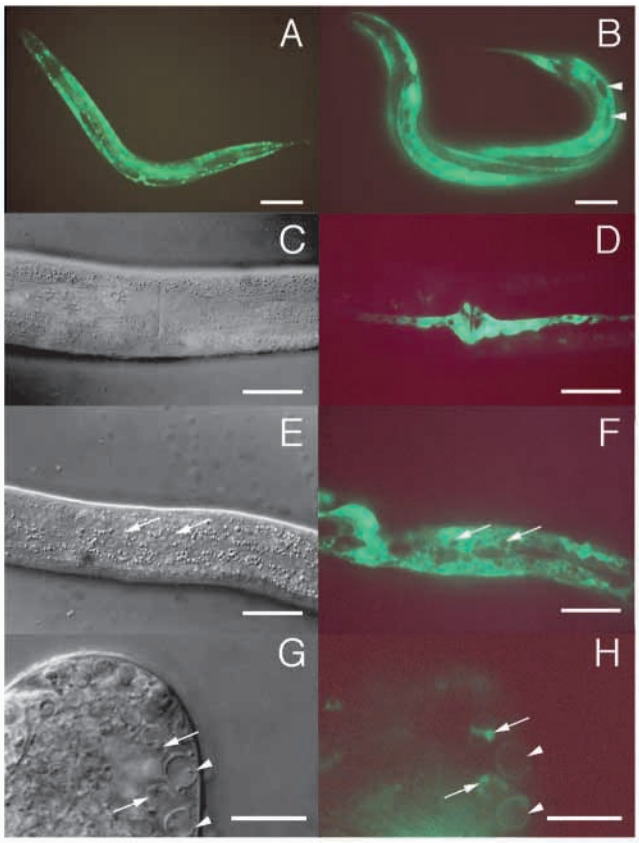

Fig. 4. Expression patterns of *dpy-11* and subcellular localization of its product. (C,E,G) Nomarski images of D,F,H, respectively. (A) *dpy-11* promoter-driven *gfp* reporter with a nuclear localization signal in L3 larva. (B) Cytosolic *gfp* reporter in L4 larva showed no seam cell expression (arrowhead). (C,D) The DPY-11 tagged with GFP at the C-terminus (p*d11*ΔCTGFP) presented a heterogeneous cytosolic expression pattern in the hypodermis around the vulva. (E,F) The fusion transgene encoding full-length DPY-11 with a GFP tagged between the transmembrane domain and the C-terminus (p*d11*AGFPC) presented a heterogeneous cytosolic expression pattern in the body hypodermis. (G,H) In gastrulating embryos, cytosolic expression (p*d11*ΔCTGFP) was detected in unfused hypodermal cells (arrows) with membrane association detected in some dying cells (arrowheads). Scale bars: 100 μm (A,B); 10 μm (C-H).

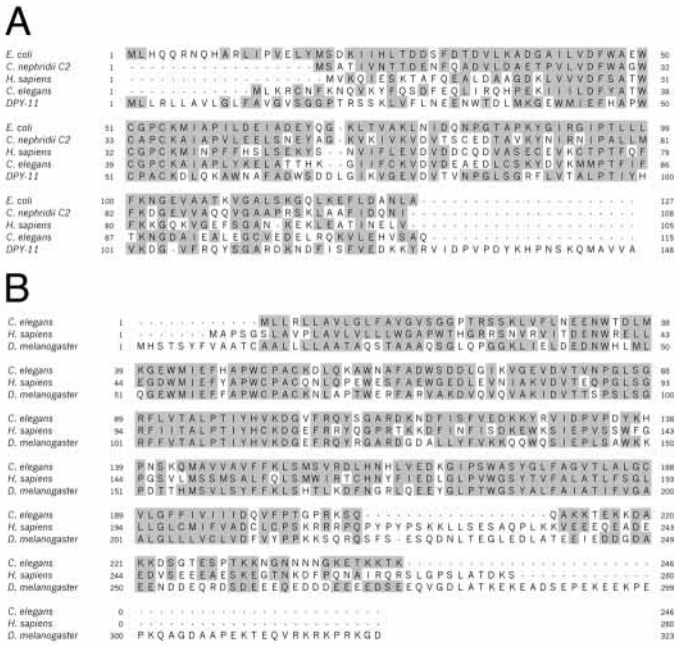

Fig. 5. Amino acid sequence alignment of DPY-11. (A) Thioredoxin domains in different organisms: *C. elegans* (Q09433), *C. nephridii* (X14630), *E. coli* (K02845), *H. sapiens* (J04026). (B) DPY-11 homologs in human (AL080080) and fruitfly (AAF47072). The numbers in brackets are GenBank accession numbers.

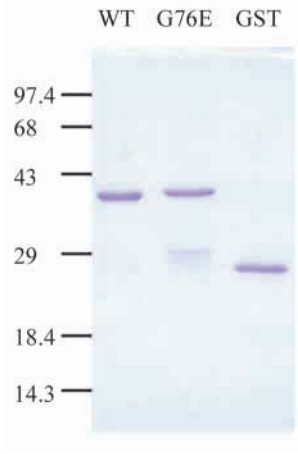

Fig. 6. Expression and purification of the GST fusion construct. WT represents the GST fusion product of the TRX-domain from amino acids 19-133 of DPY-11. G76E is a similar fusion protein with the glycine at position 76 changed to glutamic acid. GST is the expression of an empty pGEX-2T GST vector, which produced only the GST tag.

DPY-11 TRX domain has reducing activity

To verify that the TRX-like domain indeed has catalytic activity, we over-produced, in bacteria, a truncated DPY-11 fragment with aa 19-133 encompassing the TRX domain to test for its reducing activity. When the wild-type and the G76E mutant fusion proteins were produced and purified, a 40 kDa band were detected with SDS-PAGE in WT and G76E lanes respectively (Fig. 6). A band of about 26 kDa, possibly representing the GST protein, was also noted in GST lane. This result is consistent with the predicted size of 14 kDa for the recombinant truncated DPY-11 fragment. After these proteins were purified, thrombin treatment was performed to release the DPY-11 TRX domain. However, insoluble precipitates with no enzymatic activity were obtained (data not shown), so that GST fusion proteins were used subsequently for all the enzymatic assays.

The insulin reduction assay monitors the break down of the disulfide bond between insulin chains A and B in the presence of DTT (Holmgren, 1985). In such an assay, wild-type fusion protein displayed higher reducing activity than the G76E mutant version and the two negative controls (Fig. 7A). By 30 minutes into the reaction, this wild-type TRX domain displayed an activity comparable to the level of the *E. coli* thioredoxin control. However, it is interesting to note that while thioredoxin could reduce the insulin soon after the reaction was started, GST-DPY11 exhibited a 10 minutes delay.

When 20 minutes was chosen as the reaction time for examining the concentration effect of these recombinant enzymes, the wild-type fusion protein again showed higher activity than the mutant version and negative control in all concentrations tested (Fig. 7B). The wild-type TRX-like domain was less active than the positive control, but clearly

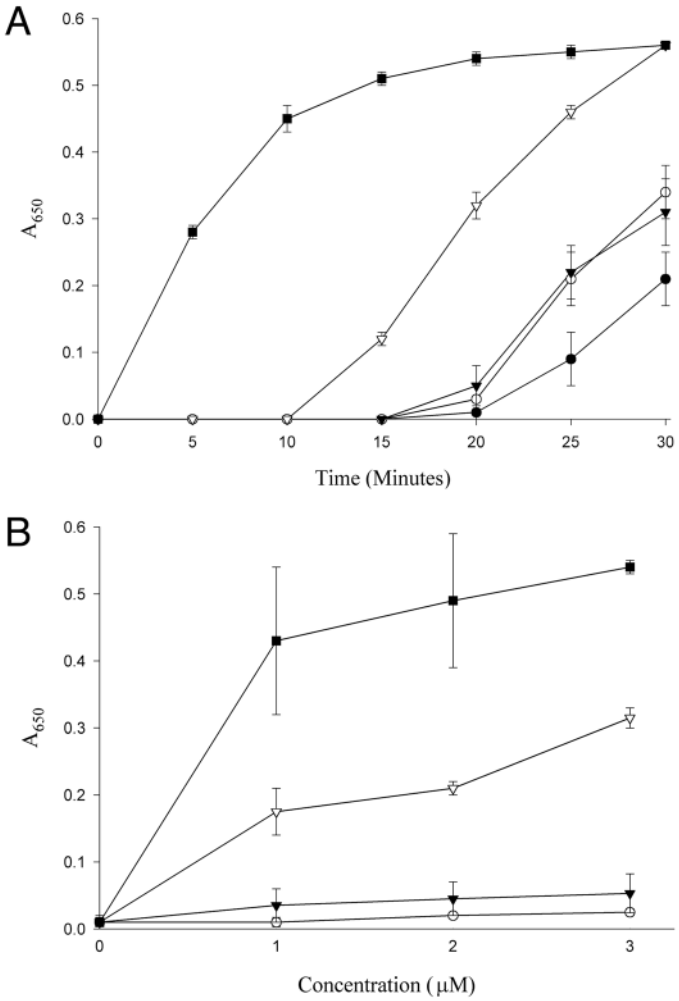

Fig. 7. Insulin reduction assay of GST-DPY-11 protein. (A) A time course of the enzymatic activity assayed at a fixed concentration (3 μM) of the tested proteins. (B) A comparison of the catalytic activities of the fusion protein and thioredoxin at different concentrations and a fixed reaction time of 20 minutes. Black squares represent the *E. coli* recombinant thioredoxin; white triangles represent the GST fusion protein of wild-type TRX domain; black triangles represent the mutant fusion protein with glycine 76 mutated to glutamic acid; white circles represent the GST alone and black circles, the buffer alone.

displayed a reducing activity. Hence, the intrinsic property of this TRX-like domain is probably relevant to the biological function of DPY-11 in body and ray morphogenesis.

The DPY-11 function within the hypodermal cell is dependent on the signal peptide, TM domain and its C terminal tail

DPY-11 protein has a unique domain organization. Its enzymatic property may not account for the full spectrum of its biological activity. Deletion constructs were made and tested for the structural requirement of the domains of this molecule (Fig. 8). Product from the p*d11*TMGFP transgene (construct 12) had the authentic TM and C terminus eliminated and could present a fusion product with only the DPY-11 N-terminal region on the hypodermal cell surface (data not shown). This molecule exhibited no mutant rescue activity at all. The same was true when the TM domain alone was deleted (construct 6). Similarly, deletion of either the signal peptide or the spacer also completely abolished the mutant rescue activity (construct 4 and 5). These results argue that the topology and the membrane anchorage of the protein appear to be important for its biological function.

In contrast, deletion of 41 amino acids of the C-terminal tail, as in the product encoded by p*d11*ΔCT or p*d11*ΔCTGFP (construct 7 and 8), did not abolish the rescue activity completely. About 20% of its wild-type activity was retained. The results show that these 41 amino acids at the C terminus are critically required though it is not essential for DPY-11 function. When the complete CT region was included in the transgene, such as in p*d11*D11GFP (construct 9), rescue efficiency was much improved. Placing the CT domain on the very C terminus, as in p*d11*AGFPC (construct 10), could increase the rescue efficiency to above 90%, i.e., only a mild compromise of the wild-type transgene activity.

As the expression pattern of *dpy-11* was confined to the hypodermal tissue and the topology of the encoded product appeared critical, we tested if the expression of *dpy-11* in other ray cells could provide the same biological activity possibly mediated through protein modification outside the expressing cell. When the *dpy-11* cDNA was driven by the *ram-5* promoter in the structural cell of *dpy-11* mutants, no reversion to the wild-type phenotype was observed (Fig. 8, constructs 3 and 11). We therefore hypothesize that DPY-11 modifies its substrate molecules within the hypodermis. The *dpy-11*

Fig. 8. A schematic representation of different mutant constructs made for the detection of rescue activity and subcellular localization. The percentage of transformed mutant animals reverted to wild type is indicated and the number of animals scored is in brackets. Localization of the tagged mutant DPY-11 proteins within the expressing cells is shown on the right column. The GFP part is not to scale.

activity present in the structural cell using the *ram-5* promoter was mis-expressed either in tissue or temporal specificity, or even both, so that wild-type ray morphogenesis process could not be restored.

Mapping of *dpy-11* mutants

With a number of mutant alleles of the *dpy-11* gene available, the mapping of their lesions could provide further support to the deduction from our deletion analysis. The mapping results are shown in Table 1 and Fig. 3B. Nonsense mutations were detected in *e207*, *e504* and *e752* alleles on the 5′ end of the predicted TRX domain. These mutations led to the synthesis of truncated proteins with the entire TRX domain and the sequence beyond missing. They represent putative null mutations. The *s261* allele had a mutation at the 5′ donor site of intron 2. With the intron retained, a stop codon in intron 2 would be used, resulting in a truncated protein. The *e33* allele had a stop codon present on the N-terminal side of the transmembrane domain resulting in a mutant protein with no membrane anchorage. This allele has a strong Dpy and Ram phenotype just like that of the null mutation suggesting that although the truncated protein synthesized had a complete TRX domain, it presumably had no biological activity. Alleles *e794* and *e1180* were generated by ICR191 acridine treatment known to introduce guanine into a poly-guanine sequence (T. Barnes and S. Hekimi, personal communication). The insertion of extra nucleotides to the poly-guanine track right after the predicted signal peptide region resulted in a frame shift upstream of the TRX domain and thus gave the same null phenotype.

A second group of alleles, *e224, e390, e455* and *e733*, had mutations all mapped at the same position converting a glycine residue in the TRX domain to glutamic acid (G76E). The conversion of a non-charged residue to a charged one in this domain probably impaired the catalytic function, as demonstrated in the in vitro enzymatic assay, and therefore lead to a partial loss-of-function phenotype with mild Dpy body and Ram rays.

Mutant animals of *e395, e431, s10* and *s360* alleles had Dpy bodies with wild-type rays. They had the lesions clustered within the predicted transmembrane region. The *s360* allele, resulting from formaldehyde mutagenesis, had nine nucleotides deleted, which led to the shortening of the encoded transmembrane domain. The *s10* allele had glycine 191 changed to a positively charged arginine. Both *s10* and *s360* had a strong Dpy phenotype. The *e395* and *e431* alleles had identical mutations changing glycine 187 to glutamic acid, and mutant animals were mildly Dpy. These four mutant alleles might have weakened the membrane association of the mutant product to a different extent such that they all had a Dpy but not a Ram phenotype. The results suggest that aberrations in the TM domain do not have the major impact on DPY-11 function in the rays that they do on body morphogenesis.

DISCUSSION

DPY-11 is a modifying enzyme

We present in this study the cloning of *dpy-11* gene, which encodes a novel thioredoxin-like molecule. This putative DPY-11 protein is much larger than most thioredoxins (246 aa versus 105-127 aa) and has several distinct molecular features. The DPY-11 TRX domain is highly similar to thioredoxins of prokaryotic and eukaryotic origins, although the CPGC catalytic site consensus was substituted by a CPAC sequence. Nevertheless, the non-consensus CAPC active site has been found in an active thioredoxin, C-2 in *Corynebacterium nephridii* (Fig. 5A) (McFarlan et al., 1989). In this study, we demonstrated biochemically that a bacterially expressed truncated DPY-11 TRX domain had a reducing activity comparable to that of a true thioredoxin from *E. coli*. A similar result was also reported for the TRX domain of the human DPY-11-like protein with the same CPAC sequence (Matsuo et al., 2001). Because four *dpy-11* mutant alleles with a reduced gene activity, *e224, e390, e455* and *e733*, had their lesions mapped within this TRX domain and could reduce the catalytic activity of this domain, as illustrated in the enzymatic assay, the catalytic property of this domain apparently represents a key function of DPY-11 protein.

It is interesting to note in our *in vitro* enzyme assay a lag phase in the first ten minutes. A similar 10 minutes lag was also observed when human DPY-11-like product was tested (Matsuo et al., 2001). The recombinant protein displayed a sigmoid curve of catalytic activity over time. It appears that DPY-11 requires an initial activation phase before enzymatic activity could be detected. Although no precedence of such a feature has been reported for thioredoxins, the existence of such property in DPY-11 may be due to the difference of substrate specificity between this DPY-11 TRX domain and other thioredoxin. Alternatively, it may be caused by the GST tag present in the fusion recombinant proteins used in this study. This possibility could not be ascertained at this point as removal of the GST tag reduced the protein solubility tremendously and prevented us from conducting the enzymatic assay.

DPY-11 modifies substrates within the hypodermal tissue

Our deletion analysis reveals that all the structural domains appear to be essential in making a functional DPY-11 protein. The signal peptide together with the single TM domain contribute to its topology. The prediction is that DPY-11 may have the TRX domain located inside endoplasmic reticulum, acting within the hypodermis to modify its substrates, which will be displayed on the cell surface or secreted to the extracellular matrix. As an alternative, DPY-11 protein may also reside on the hypodermis surface to modify substrates deposited by the same hypodermis or by its neighboring cells into the matrix. Failure to rescue the Dpy and Ram phenotypes by displaying the catalytic domain on the hyp cell surface with p*d11*TMGFP transgene, or expressing full-length DPY-11 protein in the structural cell with p*r5*cDNA are consistent with the former prediction, i.e., DPY-11 is acting within the hypodermis. Should the latter prediction be correct, these two transgenes could have provided the necessary modified function needed in the ECM within a ray, and reverted the mutant phenotypes into wild type.

Furthermore, active full-length DPY-11 protein with a GFP tagged near the C terminus was consistently detected in the cytoplasmic compartment, with a heterogeneous membranous distribution. Thus, DPY-11 may be associated with membranous organelles. This notion is supported by two

additional observations: (1) myc-tagged human DPY-11-like protein was found associated with ER in mammalian cells (Matsuo et al., 2001). (2) DPY-11 has a putative ER retention signal at the C terminus (Hong, 1996). Although apparent change of the cytosolic appearance was not observed in fusion DPY-11-GFP proteins without this KKTK sequence (p*d11*ΔCTGFP) or in fusion protein with the KKTK sequence masked by GFP (p*d11*D11GFP), these fusion proteins had a much lower mutant rescue efficiency. Obviously, the C-terminal tail is not dispensable. Whether it contributes solely to the protein localization or is also involved, in association with other cellular factors, in regulatory function remains to be determined. However, the retention of DPY-11 in the cytoplasm is definitely governed by motif(s) residing in the TM region and this C-terminal tail, since recombinant protein without these two regions, encoded by p*d11*TMGFP, was displayed on the cell surface instead.

The results from the deletion study also illustrate the importance of DPY-11 membrane association in both body and ray morphogenesis. Mutant protein lacking its TM domain had no mutant rescue activity at all. The *e33* allele with the TM domain and sequence beyond eliminated, also presented a null phenotype. Both results argue that a secreted DPY-11 enzyme is non-functional, such that its substrate is unlikely modified in situ at the extracellular matrix, but more likely is modified within the hypodermis and is deposited extracellularly afterwards. More convincingly, mutations that would hamper the membrane anchorage ability of the mutant proteins, either by amino acids deletion (*s360*) or by introducing a charged amino acid into the hydrophobic TM domain (*e395*, *e431* and *s10)*, could reduce the DPY-11 activity and resulted in Dpy animals. Although they had normal ray morphology at 20°C, the *s10* animals at 25°C, exhibiting a swollen ray tip phenotype at high penetrance, suggest that this allele has reduced *dpy-11* activity (Baird and Emmons, 1990; Yu and Chow, 2000). The lack of mutant phenotype at 20°C may reflect the presence of a low but sufficient level of membrane-associated DPY-11 to sustain the less demanding ray morphogenesis. After all, membrane anchorage of DPY-11 is still important for both ray and body morphogenesis.

DPY-11 may modulate cellular interaction through its matrix substrates

Based on the gene expression pattern, the catalytic property, the localization of DPY-11 within the expressing cell and the impact of its mutations on the differentiation of all three ray cell types, we propose that DPY-11 protein is made in the hypodermal syncytium to modify its substrate molecules. These substrates, which may be signaling molecules themselves, will be deposited in the ECM to facilitate interaction between the ray cells. A large number of cuticular collagen molecules, such as those encoded by *dpy-2*, *dpy-7*, *dpy-10* and *dpy-13* genes, are made, modified upon maturation and secreted by the hypodermal tissues (Gilleard et al., 1997; Johnstone, 2000). Mutations of these genes produce a body phenotype very similar to that of *dpy-11* mutants. They may represent good candidate substrates of DPY-11.

In addition, mutations of some of these collagen genes, e.g., *dpy-2* and *dpy-10*, can suppress mutant phenotypes of *glp-1* and *mup-1* (Maine and Kimble, 1989; Goh and Bogaert, 1991). While *glp-1* encodes a transmembane molecule required for communication between the distal germ nuclei and the somatic gonad, *mup-1* is needed for body wall muscle attachment to the hypodermis possibly through cellular communication (Kimble and White, 1981; Austin and Kimble, 1987; Goh and Bogaert, 1991). Since both *glp-1* and *mup-1* are shown to genetically interact with these collagen genes, it has been postulated that there are common mechanisms shared between the developmental events guided by these two genes (Nishiwaki and Miwa, 1998). The ability of *dpy-11* to suppress *mup-1* suggests that *dpy-11* may indeed modulate the developmental or signaling process through modifying the collagen molecules in the body (Nishiwaki and Miwa, 1998). Moreover, male tail specific collagen molecules required for normal morphogenesis of sensory rays do exist (Baird and Emmons, 1990) (R. Y. Yu and K. L. Chow, unpublished data). They are deposited in the extracellular matrix around the ray cells during the tail retraction period (R. Y. Yu and K. L. Chow, unpublished data). It is highly probable that the matrix environment within a ray would be altered in the absence of *dpy-11* modifying activity, which subsequently results in impaired cellular communication and abnormal ray morphogenesis.

In summary, *C. elegans dpy-11* gene encodes a thioredoxin-like protein with a transmembrane domain. Although it shares conserved structural organization with other *dpy-11*-like molecules, the biological and developmental relevance of such related molecules has not been determined (Matsuo et al., 2001). In this present study, we showed that the membrane association and catalytic function as well as its residence within the hypodermis are important for body and ray morphogenesis. Hence, the study of *dpy-11* regulation and future identification of its substrates would offer a platform for further analysis of the disulfide oxidoreductase system in matrix function and are instrumental to our investigation on regulated cellular communication during sensory ray morphogenesis.

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