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# Biological significance of a universally conserved transcription mediator in metazoan developmental signaling pathways

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## SUMMARY

**Transcription mediators are known to be required for regulated transcription in yeast and higher eukaryotes. However, little is known about the specific roles of mediators in vivo during development. In this report, we have characterized the biological functions of the *C. elegans* gene *med-6*, which is the homolog of the yeast mediator *med-6*. We first identified a genetic mutation in the *med-6* gene by comparing genetic and physical maps and determining the molecular lesion. Next, we demonstrated that *med-6* plays an important role in metazoan development by regulating the transcription of genes in evolutionarily conserved signaling pathways. We showed that *med-6* is involved in the transcription of genes of the Ras pathway by showing that *med*-6 RNAi suppressed phenotypes associated with gain-of-function alleles of *let-23* and *let-60*, and enhanced those associated with a reduction-of-function allele of *lin*-3. We also found that *med-6* is involved in male ray development, which is partly mediated by the Wnt pathway. As MED-6 is universally conserved, including in yeast, and the mediator-related proteins that function in vulval and male ray development are metazoan specific, our results suggest the role of *med-6* as a point of convergence where signals transmitted through metazoan-specific mediator-related proteins meet. In addition, RNAi experiments in *rde-1* background showed that maternal and zygotic *med-6* activities have distinct roles in development.**

Key words: *C. elegans*, Transcriptional mediator, *med-6*, RNAi, *rde-1*, *Ras*, *Wnt*

## INTRODUCTION

Transcriptional mediators are proteins associated with the basal transcription machinery, which are required to integrate diverse gene-specific regulatory signals and to recruit basal transcription machinery to specific promoters (Malik and Roeder, 2000). The Mediator complexes were initially identified in yeast as distinct intermediary molecules that mediate signal transfer between gene-specific transcriptional activator proteins and the basal transcription machinery (Kim et al., 1994; Koleske and Young, 1994; Thompson et al., 1993). The yeast Mediator complex is composed of the Med proteins (Med1, Med2, Med4, Med6, Med8, Med9, Med10 and Med11), Gal11, Rgr1, Sin4, Hrs1, Rox3 and the Srb family of proteins (Srb2, Srb4, Srb5, Srb6 and Srb7). These Mediator components assemble into several functional modules that regulate distinct groups of genes (Lee and Kim, 1998; Myers et al., 1999).

To date, many mediator complexes have been identified in various multicellular species. These complexes include the human Srb/Med-containing co-factor complex (SMCC) (Gu et al., 1999), the negative regulator of activated transcription (NAT) complex (Sun et al., 1998), mouse and human Mediator complexes (Boyer et al., 1999; Jiang et al., 1998), and the Mediator complex in the nematode *C. elegans* (Kwon et al., 1999).

Although the biological significance of the mediators at the organismic level is largely unknown in higher eukaryotes, some experimental evidence of a physiological role for these Mediator complexes in development has come from studies in the nematode *Caenorhabditis elegans*. The *med-6*, *med-7* and *med-10* mediator genes were initially identified from a genome search for homologs of the yeast mediators (Lee et al., 1997), and we have previously reported that these mediators are required for regulated transcription of tissue- and stage-specific developmental genes, and that loss of function in any of these genes causes embryonic lethality, confirming their essential roles in development (Kwon et al., 1999). Another mediator gene characterized in the nematode was *sur-2*. *sur-2* was originally isolated as a suppressor of a *let-60 Ras* gain-of-function mutation (Singh and Han, 1995). Loss-of-function mutations in *sur*-2 alone resulted in pleiotropic, incompletely penetrant phenotypes, including a vulvaless phenotype in hermaphrodites, defects in development of the male tail, gonadal abnormalities and larval lethality. When *sur-2* was cloned, it was found to have no homology to any other gene, but later biochemical studies on human mediator complexes revealed that human SUR-2 was a component of the mediator complex. It was also shown that the adenovirus E1A protein binds to SUR-2, activating transcription in vitro (Boyer et al., 1999). A third *C. elegans* gene, *sop-1*, which encodes a transcriptional mediator-related protein, was isolated as a

suppressor of a *pal-1* mutation. *pal-1* is a gene that encodes the ortholog of the transcription factor *caudal* (Waring and Kenyon, 1991), and its gene activity is required for both early and late embryogenesis. During late embryogenesis, *pal-1* is required for specifying the fate of V6 cells. Expression of *pal-1* in V6 neuroblasts activates expression of *mab-5*, a gene encoding a homeobox protein, which in turn activates expression of *egl-5* and *lin-32*, thus defining male ray-specific properties (Costa et al., 1988; Ferreira et al., 1999; Hunter et al., 1999; Wrischnik and Kenyon, 1997). One of the *pal-1* mutations, *pal-1 (e2091)*, is a tissue-and stage-specific mutation in that it does not perturb any early embryonic development but causes defects in the fate specification of V6 cells. Mutations in the *sop-1* gene were isolated in a screen for suppressors of *pal-1(e2091)*, and the *sop-1* gene turned out to encode a homolog of TRAP230, which is a component of the human mediator-related protein complex (Zhang and Emmons, 2000).

One common feature of the two mediator-related proteins, SUR-2 and SOP-1, found in the nematode, is that these proteins do not have homologs in yeast, but are conserved in the various metazoan species studied so far, including humans, indicating that these mediator-related proteins may relay signals from metazoan-specific transcriptional regulators. As MED-6, MED-7, MED-10 and SRB-7 are conserved not only in the metazoa but also in yeast, it is conceivable that these universally conserved mediators may be the point of convergence at which diverse transcriptional signaling mediated by metazoan-specific transcription factors and mediator-related proteins converge, at least in metazoa. Based on these inferences, we became interested in resolving the issue of the relationship between the metazoan-specific mediator-related proteins and the conserved mediator components. We pursued this by studying the roles of a universally conserved mediator *med-6*, using *Caenorhabditis elegans.*

In this study, we have isolated and characterized the genetic mutation in the *med-6* gene, and have examined the in vivo functions of this mediator gene in the development of *C. elegans* by establishing biological assay systems involving the Ras and Wnt pathway, in which metazoan-specific mediator-related proteins are known to be involved. Based on our observation that the *med-6* mutant animals had a vulval defect, which is due to hypo-induction of the vulval precursor cells (VPCs), we hypothesized that *med-6* is involved in transcriptional regulation of genes involved in the Ras pathway. Accordingly we examined the effect of *med-6* RNAi on the *lin-3*, *let-23*, and *let-60* genes, which act in the Ras pathway. We also examined the effect of *med-6* RNAi on male ray development, in which pathway *sop-1*, a mediator-related gene, is involved. Another interesting finding was that RNAi of *med-6* in wild-type background caused more severe phenotypic changes than the putative null mutation of *med-6*. We examined the possibility that this phenotypic discrepancy was due to maternal rescue by employing a genetic experiment involving the *rde-1* mutation.

## MATERIALS AND METHODS

### Strains and culture

The *C. elegans* Bristol strain N2 was used as the standard wild-type strain. The alleles and strains used for cloning *med-6* were: *let-404*, BC2590 [*dpy-18(e364)/eT1 III; dpy-11(e224) let-404(s119) unc-42(e270) /eT1 V*]; *let-438*, BC2858[*dpy-18(e364)/eT1 III; unc-46(e177) let-438(s2114)/eT1 V*]; *let-468*, BC3655[*dpy-18(e364)/eT1 III; unc-46(e177) let-468(s1533)/eT1 V*]; *let-339*, BC2245[*dpy-18(e364)/eT1 III; unc-46(e177) let-339(s1444)/eT1 V*]; *let-343*, BC1919[*dpy-18(e364)/eT1 III; unc-46(e177) let-343(s1025)/eT1 V*]; *let-425*, BC3060[*dpy-18(e364)/eT1 III; unc-46(e177) let-425(s385)/eT1 V*]; and *let-332*, BC1480[*dpy-18(e364)/eT1 III; unc-46(e177) let-332(s234)/eT1 V*] (Rosenbluth et al., 1988). The alleles used for studying the mediator function in vulval development and their typical phenotypes were: *let-60(n1700) IV* (multivulva 96%), *lin-3(e1417) IV* (vulvaless, 10-30% wild type), *let-23(sa62) II*; *him-5(e1490) V* (*let-23* semi-dominant, over 95% multivulva), and *dpy-5(e61) I; syIs1* (*lin-3* over expression, over 95% multivulva). The deficiency strain we used for examination of hemizygotes of the *let-*425 mutation was of the genotype *dpy-18(e364)/eT1 III; unc-46(e177) sDf20/eT1 V.* The strains were obtained from the Caenorhabditis Genetics Center (CGC) and P. W. Sternberg (Caltech, Pasadena, CA). For male tail experiments, *pal-1(e2091) III; him-5(e1490) V* and *pal-1(e2091) III; him-5(e1490) V; sop-1(bx92) X* were used (gifts from Scott W. Emmons, Albert Einstein College of Medicine, New York, NY). The strain used for examination of *pal-1* transcription was BW1851 whose genotype is *pal-1::GFP pRF4 integrated* (a gift from L. G. Edgar and W. B. Wood, University of Colorado, Boulder, CO) For *rde-1* experiments, *unc-32(e189) III; rde-1(ne219) V* was used (Tabara et al., 1999). The culture of *C. elegans* has been previously described (Brenner, 1974).

### Identification of *let-425* as the *med-6* gene

The physical location of *med-6* was determined to be in the YAC Y57E12 (Y.-J. Kim, personal communication), on chromosome V. However, no positive cosmids were detected by Southern hybridization using med-6 cDNA as a probe. We reasoned that this was because the *med-6* gene resided in a cosmid gap that is located between the cosmid K11C4, encoding *unc-68*, and VC5, encoding *odr-2*. As the region was saturated with lethal mutations, and *sDf20* and *sDf30* delete both *unc-68* and *odr-2* while *nDf32* does not, we searched for lethal mutations mapped between the break points of the deficiencies. According to the genetic map available (http://elegans.swmed.edu), *let-404*, *let-438*, *let-468*, *let-442*, *let-339*, *let-343*, *let-425*, *let-346* and *let-332* reside in this region. We examined the phenotypes of animals containing each mutation by DIC microscopy, and decided that *let-425* and *let-332* could be candidates for the *med-6* mutation. A *let-425* mutation had been isolated as a lethal mutation with a sterile phenotype, and a *let-332* mutation isolated with embryonic lethality (Johnsen and Baillie, 1991). We amplified the genomic region containing the entire coding sequence of the *med-6* gene from either heterozygotes of *let-332* or animals homozygous for *let-425*. The primers used for amplifying the genomic region from single larvae were CM6F (5′-GGACTAGTAT-GGGACCTCCAGCAGCTGCAC-3′) and CM6R (5′-TAATTTAA-TGTTCATTTTTAGTG-3′). The amplified fragments were cloned into pGEM-T easy vector (Promega). We determined the sequences from six independent colonies with T7 sequenase 2.0 DNA sequencing kits (Amersham). To confirm the identity of the mutation, we directly determined the sequence of the PCR-amplified fragments from eight *let-425* homozygous animals, eight heterozygotes and eight N2 animals. In an effort to rescue the mutation of *let-425* with the wild-type *med-6* gene, we amplified a full-length *med-6* gene containing the upstream 2 kb region and the entire 3′UTR as well as the coding region. The PCR primers used for this purpose were CM6PF (5′-CGTCGACCACATCCTTCGCCGGAAGC-3′) and CM6PR (5′-CTCTAGACTCATAAACCACAAAGAGGAGC-3′). We microinjected the linear PCR product into wild-type animals with genomic DNA digested with *Eco*RI, which cuts the coding region of *med-6*. Heterozygous males of the genotype *dpy-18/eT1; unc-46 let-425/eT1* were mated with transgenic animals containing the *med-6*

transgene, and $F_1$ hermaphrodites that segregated Dpy animals in the next generation were individually selected. Among the siblings of the Dpy animals, rolling UNC-46 animals, whose genotype should be *unc-46 let-425; [Ex pRF4 + med-6 + fragmented genomic DNA]* were examined for their phenotypes. These UNC-46 animals were normal in vulval development and were able to lay eggs, a phenomenon never seen in the *let-425* homozygous animals. This result indicates that the linear PCR fragment containing the *med-6* gene complemented the *let-425* mutation.

### Characterization of *let-425* homozygous animals

The *let-425* mutation is maintained in the heterozygote form in the genotype *dpy-18(e364)/eT1 III; unc-46(e177) let-425(s385)/eT1 V*. In order to measure the lethality and sterility of the *let-425* homozygotes, we removed the eT1 balancer by mating *dpy-18* homozygote hermaphrodites with N2 males to produce males of the genotype *dpy-18/ +*, and the *dpy-18*/+ heterozygous males were mated with the *let-425* heterozygotes. In the next generation, Dpy virgins of the genotype *dpy-18(e364)/dpy-18(e364) III; unc-46(e177) let-425(s385)/+ V* were selected, and the phenotypes of their progeny were examined. In order to determine whether the existing allele of *let-425* is null, we mated males of the genotype *dpy-18(e364)/eT1 III; unc-46(e177) let-425(s385)/eT1 V* with hermaphrodites of the genotype *dpy-18(e364)/eT1 III; unc-46(e177) sDf20/eT1 V.* The number of non-Dpy male progeny and that of Dpy Unc male and hermaphrodite progeny were compared, and the phenotypes of the hemizygotes were examined. The expected ratio of the non-Dpy males to Dpy Unc animals (hermaphrodites and males) is 4:2, if there had been no embryonic lethality (Rosenbluth and Baillie, 1981). The observation was that the ratio of the non-Dpy males to Dpy Unc animals was close to 2.45:1 (n=913). Among the hemizygous hermaphrodites containing a single copy of the *let-425* mutation, 78% arrested at the L4 stage and the remaining 22% reach adulthood. This number is comparable with that of the homozygous *let-425* mutants. These results indicate that the *let-425* mutation is a severe reduction-of-function mutation, if not a complete null.

### RNAi and microscopy

The *med-6* dsRNA used in this study was identical to the dsRNA used in the previous study (Kwon et al., 1999). The size of the RNA used in RNAi was about 800 nucleotides, and contained the full *med-6*-coding region. The concentration of the *med-6* RNA used in the experiments was 100 μg/ml. In order to observe the effect of *med-6* RNAi on vulval development in various backgrounds, vulval invagination at the L4 stage of $F_1$ progeny laid at 6-24 hours after microinjection was observed. We confirmed the effectiveness of the RNAi by observing that the embryos laid after that time were 100% embryonic lethal. For the RNAi experiments in the *rde-1* background, *med-6* dsRNA was injected into *rde-1* mutant animals, and individual animals were mated with three N2 males. After each 12 hour period, we moved the P0 individuals to new plates and provided new males. Individual $F_1$ progeny were transferred to new plates and were examined for their phenotypes after four days of culture. The phenotype 'small brood size' was used to describe $F_1$ animals with fewer than 20 $F_2$ progeny. Thus, animals classified as normal had fewer progeny than wild-type animals in many cases. Embryonic lethality was calculated by subtracting the number of viable animals from the total number of eggs. In the *rde*-1 background experiments, we attempted to see if microinjection of dsRNA into the gonad, body cavity or intestine gave different ranges of phenotypes, but we did not see any correlation between the location of injection and severity of the resulting phenotypes. To characterize the role of *med*-6 in ray development, we likewise microinjected *med-6* dsRNA into animals of various mutant backgrounds. When using the *rde*-1 mutant background, we mated the injected animals with N2 males and examined the phenotypes of the male progeny. When using *pal-1(e2091) III; him-5(e1490) V* and *pal-1(e2091) III; him-5(e1490) V; sop-1(bx92) X* backgrounds, we examined the males from the mothers directly. To examine the change in *pal*-1 transcription by *med-6* RNAi, we microinjected *med*-6 dsRNA into the *pal-1::GFP* integration line, and observed $F_1$ embryos inside the PO animals. A Zeiss Axioplan2 microscope was used for Nomarski images, and a Zeiss AxioCam digital camera and MC200 camera (Carl Zeiss) were used for taking photographs.

## RESULTS

### MED-6 is encoded by *let-425*

In order to identify genetic mutations in the *med-6* gene, we compared the physical map to the genetic map (Fig. 1). The *med-6* gene was physically mapped to the YAC, Y57E12, by YAC grid hybridization (Y.-J. Kim, personal communication), and later by sequencing data from the genome project (C. elegans Sequencing Consortium, 1998). The *med-6* gene was located in the cosmid gap between *unc-68* and *odr-2*, and we reasoned that *med-6* should be encoded by a lethal mutation that mapped between the break points of the deficiencies, *sDf20* and *nDf32*. Among the lethal mutations that have not been cloned from this region, we found that there was a single base substitution in the *med-6* gene from the *let-425* mutant animals (Fig. 1; Materials and Methods). The mutation was a C to T transition at nucleotide 292, which we expect causes a truncated LET-425 protein with amino acids missing from the 91st residue. We confirmed the mutation by single worm PCR and sequencing of several homozygous animals containing two copies of the *let-425* mutation. We were able to rescue the *let-425* mutation with a PCR fragment containing the *med-6* gene (see Materials and Methods). In addition, the fact that the phenotypes of the *let-425* mutation showed striking similarities to the phenotypes caused by *med*-6 RNAi also supported our sequencing results. For example, the *let-425* mutant animals displayed hypo-induction of the vulval precursor cells, similar to animals affected by *med-6* RNAi (Fig. 2B,C) as well as larval arrest, a male tail defect and adult sterility. Thus, we identified *let-425* as the *med-6* gene.

### Phenotypes associated with a putative null mutation of let-425

We first determined whether the *let-425* mutation was null. As hemizygous animals containing a single copy of *let-425* mutation displayed comparable phenotypes with those of the *let-425* homozygotes (see Materials and Methods), the existing *let-425* mutation is a severe reduction-of-function mutation. The phenotypes associated with the *let-425* mutation were then examined in order to define the biological significance of the *med-6* gene in development. We examined the *let-425* homozygous animals laid by heterozygous mothers because homozygous animals are completely infertile. Two phenotypes were apparent in the *let-425* homozygous progeny; late larval arrest and adult sterility, but they exhibited no embryonic lethality. In order to check if there was any embryonic lethality associated with the mutation, we examined the progeny from heterozygous mothers with the genotype *dpy-18/dpy-18; unc-46 let-425/+*. Among the 2145 progeny laid from the heterozygous mothers, we found that 25.7% were *let-425* homozygous animals. This percentage is comparable with the expected number of homozygous *let-425* progeny if there had

not been any embryonic lethality associated with the *let-425* mutation. In another experiment, we let the animals of the genotype *dpy-18/dpy-18; unc-46 let-425/+* lay eggs and then transferred all the progeny individually to single plates and allowed them to grow. Among the 194 progeny examined, 25.8% were Dpy Unc larvae or sterile adults, while 47.9% were heterozygotes and 26.3% were *dpy-18* homozygotes, confirming that there was no embryonic lethality and that the *let-425* mutation caused 100% larval arrest or adult sterility.

Next, we examined the phenotypes of the larvae and adults of the *let-425* homozygous animals in more detail. Among the 132 animals observed under Nomarski optics, 72% were arrested in the L4 larval stage and 28% were adults with 100% sterility. The sterile adults were defective both in oogenesis and spermatogenesis: when the male progeny from RNAi-affected *him-5* hermaphrodites were mated with *unc-101* hermaphrodites, they could not produce any cross progeny (*n*=20). Additionally, in a reciprocal mating, in which RNA-affected hermaphrodites were mated with N2 males, no progeny were produced (data not shown). Because male tail defects and vulval defects, which may interfere with mating ability by themselves, are not 100% penetrant (see the following sections), enough numbers of matings should have resulted in production of cross progeny if there was no defect in spermatogenesis or oogenesis, respectively. Interestingly, the sterile adult animals showed an additional phenotype of protruding vulvae (Fig. 2F). Accordingly, we examined the *let-425* homozygous animals at the L3 molt stage, which is when the vulval precursor cells usually divide to form the vulval tissue (Sternberg and Horvitz, 1986; Sternberg and Horvitz, 1989), in order to explain the cellular defects associated with this phenotype. Out of 15 L3 molt animals, eight had less than wild-type vulval induction, in which we commonly observed induction of P6.p but not P5.p and P7.p (for example, see Fig. 2B). RNAi-affected animals displayed an identical hypo-induction phenotype (Fig. 2C). It is known that hypo-induced VPCs later form protruding vulval tissue, and in our study, we found that the animals confirmed to have hypo-induced VPCs later either arrested as L4 larvae or grew to adulthood with protruding vulvae (data not shown).

## MED-6 is involved in regulating genes involved in a Ras pathway

A possible explanation for the hypo-induction of VPCs in the *let-425* homozygous animals is that the *med-6* mutation causes transcriptional downregulation of genes involved in vulval induction. To test our hypothesis, we examined the effect of reduction of *med-6* gene function on *lin-3*, *let-23* and *let-60*, which encode an epidermal growth factor (EGF), the EGF receptor and Ras, respectively (see Table 1; Fig. 3; Aroian et al., 1990; Han and Sternberg, 1990; Hill and Sternberg, 1992). Either gain-of-function or reduction-of-function alleles of these genes were used. Gain-of-function mutations in any one of these genes result in a multivulva phenotype in which more than three VPCs are induced to make additional vulval tissue in addition to a functional vulva. On the other hand, reduction-of-function mutations in these genes cause hypo-induction of VPCs. We reasoned that reduction in *med-6* activity would result in reduction of the transcription levels of these genes, which would suppress the multivulva phenotype of the gain-of-function mutations and enhance the hypo-induction phenotype of reduction-of-function mutations. The alleles of *lin-3* that we used were *syIs1*, an allele generated by integration of multicopies of the wild-type *lin-3* gene, and *lin-3* (*e1417)*, a reduction-of-function mutation. Reduction of *med-6* activity by RNAi did not suppress the multivulva phenotype resulting from overexpression of *lin-3* driven by multicopies of this gene (Fig. 3C,D). On the contrary, the hypo-induction phenotype of

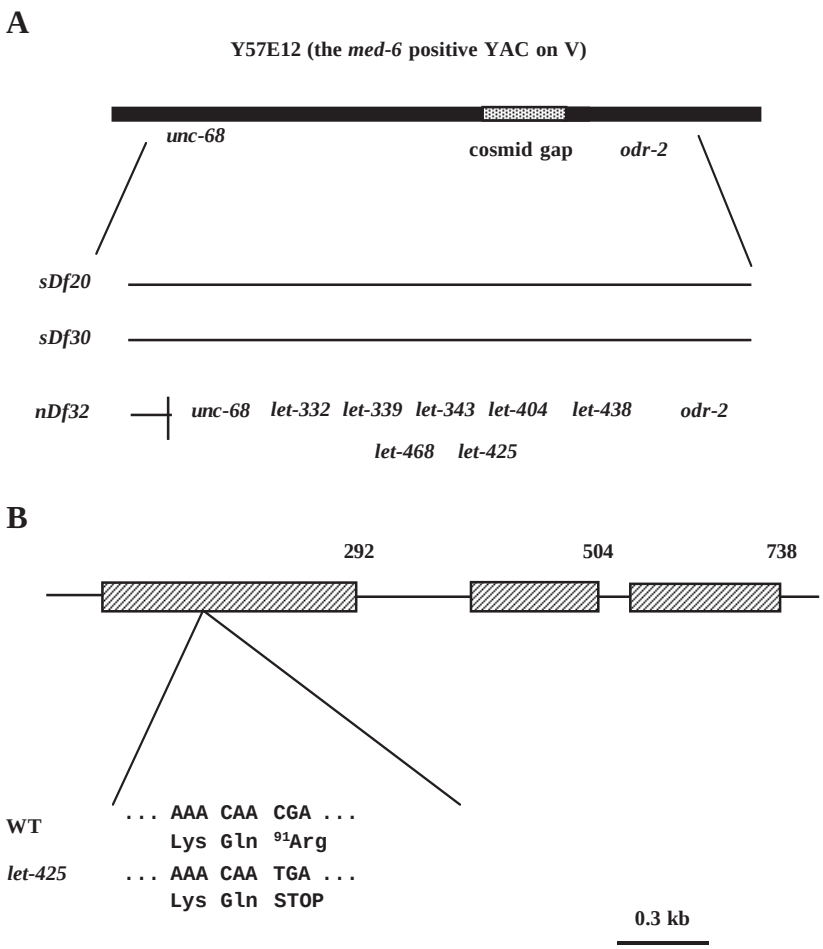


**Fig. 1.** Identification of the *med-6* mutation. (A) The physical and genetic maps of the *med-6* region. The yeast artificial chromosome (YAC) Y57E12 contained the *med-6* gene. The cosmid gap within the YAC is indicated. The genetic deficiencies, sDf20 and sDf30, delete both *unc-68* and *odr-2*, whereas the deficiency, *nDf32*, does not. Lethal mutations, *let-322*, *let-339*, *let-343*, *let-404*, *let425*, *let-438* and *let-468*, which were mapped in that region, are shown. The map is not drawn to an exact scale. (B) Genomic structure of *med-6* and the mutation identified in *let-425*. The *med-6* gene contains three exons separated by two introns. The *med-6* gene isolated from the homozygous *let-425* animals contained a point mutation that changes C to T, causing a change in the amino acid residue from Arg to a stop codon. The numbers above the genomic structure are the amino acid numbers encoded by the gene.

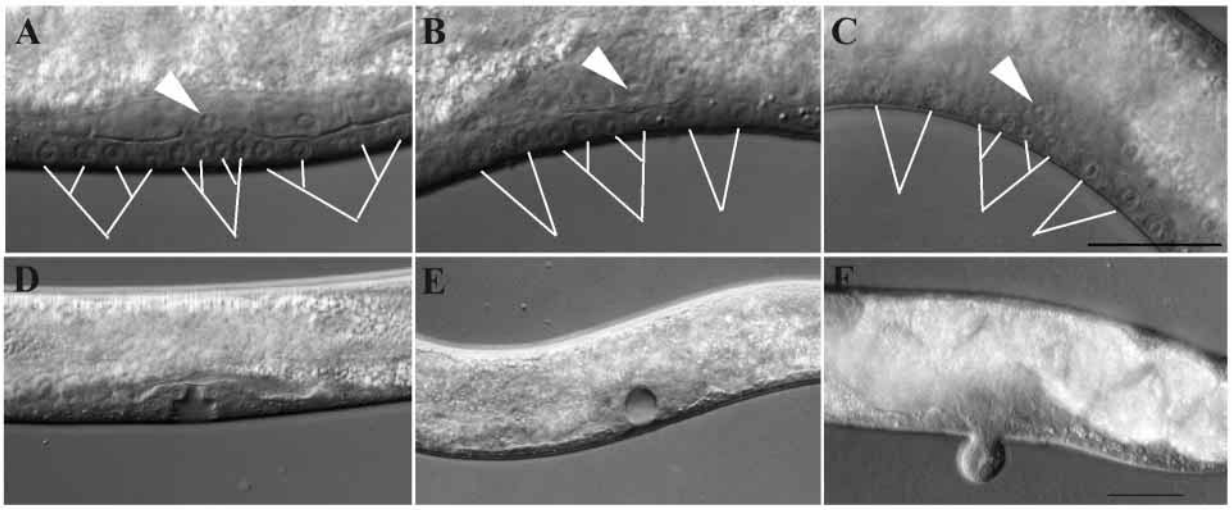


**Fig. 2.** Typical phenotypes of *let-425* homozygotes (A-C), Nomarski images of the animals at the L3 molt stage, when the induced vulval precursors cells have divided to form four progeny. (A) Wild type, (B) homozygous *let-425* mutant animal and (C) a wild-type animal affected by *med-6* RNAi. (D,E) Nomarski images of the vulval tissue at the L4 stage, when invagination of the induced vulval precursor cells occurs. (D) Wild type, (E) a homozygous *let-425* mutant animal, showing an abnormal invagination caused by less than wild-type induction of VPCs. (F) Nomarski image of a homozygous *let-425* mutant animal at the adult stage. Hypo-induced VPCs have undergone morphogenesis and formed a protruding vulva. Scale bar: 25 μm.

*lin-3 (e1417)* was significantly enhanced (Fig. 3A,B). Furthermore, gain-of-function mutations in *let-23* or *let-60* were partially suppressed to the wild-type form by reduction of *med-6* activity (Fig. 3E-H). From these results, we concluded that reduction of *med-6* activity could cause the genes involved in the Ras signaling pathway to be transcribed at lower levels.

## MED-6 is involved in regulating genes involved in ray development

A recent study has shown that a tissue-specific mediator is involved in regulating transcription of gene(s) involved in ray development (Zhang and Emmons, 2000). Transcription of *pal-1*, a gene encoding a transcription factor required for proper ray development, is regulated by two separate circuits, one of which is normally silent, owing to *sop-1* activity, which represses the transcription of *pal-1* from the 5′ upstream regulatory element. This transcriptional regulation is mediated by the Wnt pathway. We wished to examine whether *med-6* was also involved in the regulation of genes involved in ray development. We first examined the male tails of a few *let-425* homozygous animals and found that the male structures were defective (data not shown). We then used RNAi to examine the effect of *med-6* on male ray development. Because we reasoned that genes required for proper ray development should be functional at the zygotic level, we examined the male tail phenotypes associated with males produced by the mating of N2 males with *rde-1* homozygous hermaphrodites that had been microinjected with *med-6* dsRNA (see Materials and Methods; for *rde-1* experiments, see the next Result sections). We found that 17 out of 45 males observed under Nomarski optics displayed abnormal ray defects (Fig. 4B). A typical phenotype was that the V6-derived rays, 2-6, were partially or completely missing. We observed essentially identical phenotypes when we examined the tail phenotypes of the males produced from N2 hermaphrodites that had first been mated with N2 males, and then subjected to *med*-6 RNAi (data not shown). It is known that the development of the V6-derived ray structure is dependent on the expression of the caudal homolog *pal-1* in the V6 progeny. Because we did not see significant defects in ray number 1, nor in 7-9 in our experimental conditions, it is probable that *med-6* may be involved in ray development at the level of *pal-1* transcription, although we

**Table 1. RNAi in *lin-3, let-23* and *let-60* genetic backgrounds**

| | Before *med-6* RNAi | | | | |
|---|---|---|---|---|---|
| Allele examined | Normal induction (%) | Hypo-induction (%) | No induction (%) | Greater than wild-type induction (%) | *n* |
| *let-60 (n1700)* | 6 | 0 | 0 | 94 | 34 |
| *lin-3 (e1417)* | 19 | 56 | 26 | 0 | 43 |
| *let-23(sa62)* | 6 | 0 | 0 | 94 | 54 |
| *lin-3(syIs1)* | 0 | 0 | 0 | 100 | 21 |
| | After *med-6* RNAi* | | | | |
| Allele examined | Normal induction (%) | Hypo-induction (%) | No induction (%) | Greater than wild-type induction (%) | *n* |
| *let-60 (n1700)* | 35 | 0 | 0 | 65 | 37 |
| *lin-3 (e1417)* | 0 | 30 | 70 | 0 | 70 |
| *let-23(sa62)* | 23 | 0 | 0 | 77 | 43 |
| *lin-3(syIs1)* | 0 | 0 | 0 | 100 | 17 |

*In all experiments, 100 μg/ml of *med-6* dsRNA was microinjected into each strain with the specific genotypes listed and the $F_1$ progeny were collected from 6-24 hours after microinjection. We confirmed the effectiveness of the RNAi by ascertaining that almost all eggs laid after that time point were embryonic lethal.

cannot rule out the possibility that genes downstream of *pal-1* were affected. We next examined the ray phenotypes of animals of the genotype *sop-1; pal-1* in order to determine whether *med-6* is involved in the transcriptional regulation mediated by the Wnt pathway in ray development. In wild-type animals, *pal-1* is transcriptionally activated through an intronic regulatory element and the transcriptional activation by the Wnt pathway is repressed by the activity of *sop-1*. As in the *pal-1; sop-1* mutants *pal-1* is transcriptionally activated by the Wnt pathway (Zhang and Emmons, 2000), it is possible to examine if *med-6* is involved in the Wnt-mediated transcription of *pal-1* by using these mutants. If *med-6* is involved in the transcriptional activation of *pal-1* both by the original intronic regulatory element and by the Wnt pathway, then *med-6* RNAi should reduce the development of the V6-derived rays in the double mutant animals. We observed ray defects in the *pal-1; sop-1* double mutant animals that were almost identical to those caused by *med-6* RNAi in the wild-type background (Fig. 4C-F), indicating that Wnt signal-mediated transcription may also require MED-6 as its transcriptional mediator.

### Examination of maternal and zygotic functions of *med-6* using an *rde-1* mutation

Our previous RNAi result showed that RNAi using a high concentration of *med-6* double-stranded RNA in the wild-type background caused almost 100% embryonic lethality and that at a lower concentration of RNA, the animals showed both embryonic lethality and adult sterility (Kwon et al., 1999). When we examined the phenotype of the *let-425* mutation, we found no evidence of embryonic lethality (see the previous Results section). One possible explanation is that maternal *med-6* activity required for embryogenesis may still be provided by the heterozygous mother. We examined this possibility by using the *rde-1* mutation (Tabara et al., 1999). To test our hypothesis, we designed a genetic experiment in which we could selectively reduce the zygotic *med-6* gene function. Fig. 5 shows the mating schemes that we employed. As *rde-1* homozygous animals are resistant to RNAi both maternally and zygotically, and the *rde-1* phenotype is fully recessive (Tabara et al., 1999), we performed RNAi of *med-6* at a high concentration in the *rde-1* homozygous animals and mated them with N2 males. The progeny from this mating should have a zygotic RNAi effect, but not a maternal RNAi effect, because *rde-1* is now in the heterozygote form. This mating allowed for selective reduction of the zygotic *med-6* gene function (Table 2). Unlike wild-type animals, *rde-1* mutant animals were indeed 100% resistant to RNAi of *med-6*: we did not see any effect of *med-6* RNAi in the *rde-1* mutant animals. However, after mating the RNA-injected *rde-1* hermaphrodites with N2 males, the progeny showed various degrees of severity in their phenotypes. About 10% of the eggs counted ($n$=1693) were arrested at the embryonic stage, 6% grew up to adulthood and became sterile, and 28% of the eggs grew to adulthood with no obvious phenotype. About 7% of the adults had limited brood sizes. In addition, males produced in this experiment displayed ray defects and hermaphrodites showed defective vulval induction (data not shown). In control experiments, wild-type animals injected with the dsRNA, whether mated or

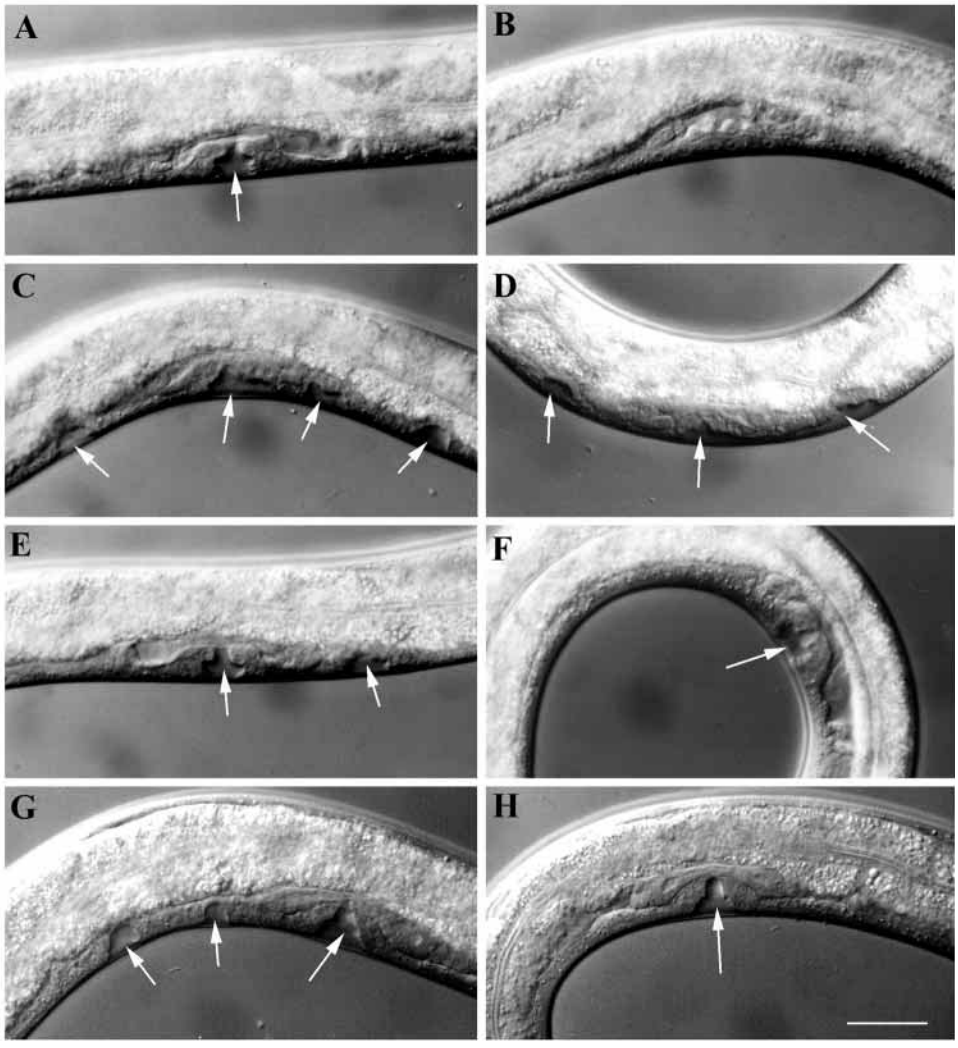


**Fig. 3.** *med-6* is involved in regulating genes in the Ras pathway: vulval phenotypes before and after *med-6* RNAi in various genetic backgrounds. The left panels show the vulval tissues at the L4 stage before *med-6* RNAi, and the right panels, after RNAi. (A,B) *lin-3 (e1417)*; (C,D) *lin-3 (syIs1)*; (E,F) *let-23(sa62)*; and (G,H), *let-60(n1700)*. This figure shows representative animals only. For example, (A) shows a wild-type vulva of an animal of the genotype *lin-3(e1417)*, whose typical phenotype is 70-90% vulvaless, with only 10-30% wild-type vulval induction. For detailed data, see Table 1. The arrows indicate vulval tissue invagination. Wild-type animals always have only one invagination. Scale bar: 25 μm.

not, produced nonviable embryos. In summary, we observed phenotypes in *rde-1* mutant RNAi that were comparable with, although less severe than, *let-425* homozygous mutant phenotypes. From these data, we propose that the maternal *med-6* is required for early embryogenesis, and that the zygotic function of *med-6* is mostly required for postembryonic development such as vulval development and ray development.

## DISCUSSION

We have previously examined transcription mediator functions by using RNAi and showed that *med-6* was required for regulated gene transcription but not for constitutive gene transcription (Kwon et al., 1999). We also showed that mediators are essential for embryonic development as RNAi of any one of the mediators caused embryonic lethality. The next goal was to characterize the biological functions of these mediators by identifying mutations in their genes. Accordingly, we identified *let-425* as the *med-6* gene by sequencing the genomic region of *med-6* in *let-425* mutant animals. We were unable to rescue the *let-425* mutation by introducing high copies of a genomic fragment that contained the full-length *med-6* gene. A possible reason for not being able to rescue the phenotype would be that the transgene, introduced by microinjection, may have interfered with endogenous mutant RNA, causing co-suppression (for example, Dernburg et al., 2000). We therefore tried low-copy transformation by co-injecting the PCR fragment and genomic DNA digested with a restriction enzyme. Using this method, we were able to rescue the *let-425* mutation, apparently by overcoming the silencing of multicopy genes (Kelly et al., 1997).

### Role of transcription mediators in development

Mediators were shown to be required for regulated gene transcription in yeast and in the nematode (Kwon et al., 1999; Lee and Kim, 1998). In yeast, *med-6* mediates transcription of about 10% of the whole genome (Holstege et al., 1998). It is therefore conceivable that *med-6* also is involved in the regulated transcription of many genes in the nematode *C. elegans*. In order to establish biological assay systems to study the role of transcription mediators in development, we examined genes acting in two signaling pathways of the nematode: the Ras and the Wnt pathways. The Ras signaling pathway is one major signaling pathway in hermaphrodite vulval development, and the Wnt pathway is involved in ray development. Because vulval and ray development should occur at the appropriate time in development at the correct place, the genes involved in those pathways should be regulated temporally and spatially. Therefore, it is conceivable that mediators may be involved in regulated transcription of the genes in these pathways. In this report, we showed that nematode *med-6* is involved in the regulation of genes associated with the Ras pathway and the Wnt pathway.

We first examined vulval development because we observed abnormal vulval morphogenesis in the *let-425* homozygous mutant animals. From the animals produced at early time points after injection of *med-6* dsRNA, we were able to show that *med-6* is indeed involved in the Ras pathway: reduction of *med-6* activity lowered the gene activity of both the gain-of-function mutant genes and a reduction-of-function mutant gene. The fact that embryos laid at later time points after RNAi were arrested at the embryonic stage indicates that the animals produced earlier might have had residual *med*-6 activity, sufficient to enable the animals to survive up to adulthood. We were unable to obtain direct evidence showing that the transcription levels

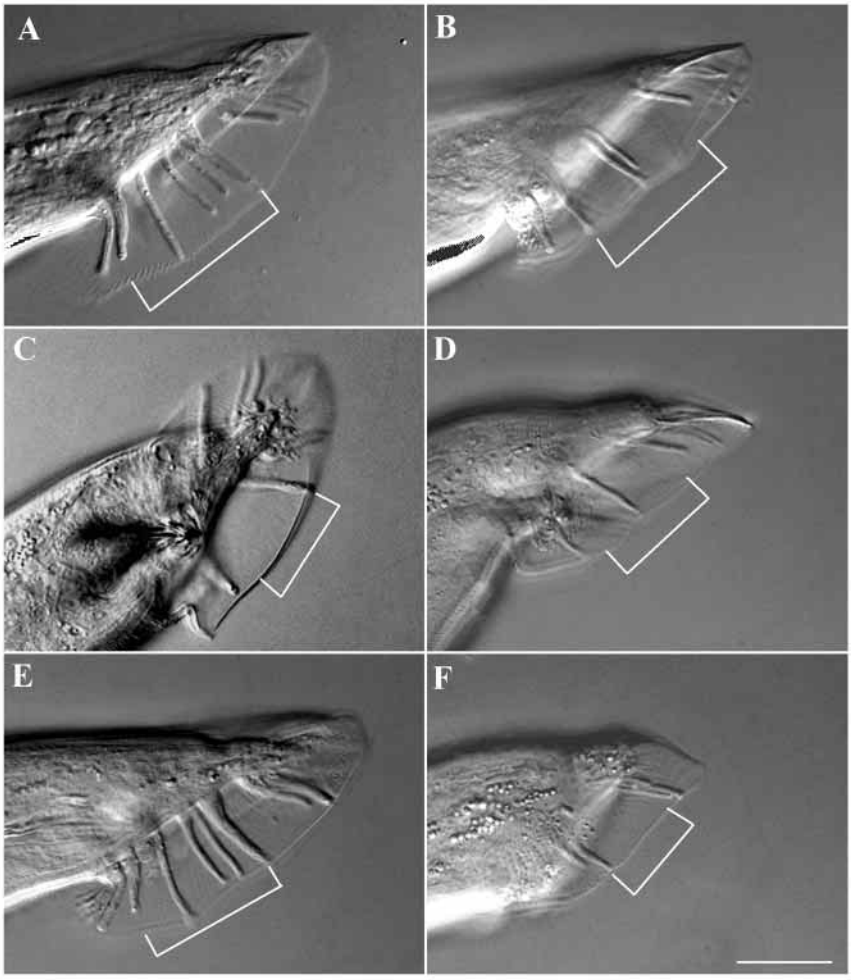


**Fig. 4.** *med-6* is involved in ray development. The locations where the V6-derived rays are supposed to be found are indicated by the lines in all figures. (A) A typical wild-type ray structure. (B) The ray phenotype caused by *med-6* RNAi in the *rde-1* background. Development of the V6-derived rays is affected by RNAi. (C) Defective V6-derived rays in a *pal-1 (e2091)* mutant animal. (D) The ray phenotype caused by *med-6* RNAi in a *pal-1(e2091)* mutant animal. (E) The restored V6-derived rays in the *pal-1; sop-1* double mutant animal. (F) The ray phenotype caused by *med-6* RNAi in the *pal-1; sop-1* double mutant background. The V6-derived rays are defective. Scale bar: 25 μm.

**Table 2. Phenotypes associated with RNAi in the *rde-1* background**

| Experiment number* | Total number of eggs on the plate | Self progeny (*n*) | Males (*n*) | Hermaphrodites | | | Embryonic lethal§ (*n*) |
|---|---|---|---|---|---|---|---|
| | | | | Sterile (*n*) | Small brood size‡ (*n*) | Normal (*n*) | |
| 1 | 146 | 0 | 59 | 7 | 8 | 40 | 32 |
| 2 | 108 | 25 | 42 | 4 | 9 | 19 | 9 |
| 3 | 161 | 0 | 66 | 1 | 0 | 65 | 29 |
| 4 | 137 | 9 | 68 | 4 | 14 | 33 | 9 |
| 5 | 105 | 14 | 42 | 27 | 7 | 6 | 9 |
| 6 | 146 | 0 | 58 | 24 | 19 | 21 | 24 |
| 7 | 156 | 0 | 69 | 9 | 16 | 41 | 21 |
| 8 | 164 | 0 | 85 | 2 | 3 | 61 | 13 |
| 9 | 119 | 15 | 47 | 0 | 3 | 47 | 7 |
| 10 | 108 | 17 | 43 | 0 | 8 | 32 | 8 |
| 11 | 141 | 0 | 63 | 16 | 18 | 37 | 7 |
| 12 | 95 | 7 | 52 | 12 | 13 | 15 | –4 |
| 13 | 107 | 0 | 52 | 0 | 0 | 54 | 1 |
| | 1693 | 87 (5%) | 746 (44%) | 106 (6%) | 118 (7%) | 471 (28%) | 165 (10%) |
| 14 | 673 | 654 (97%) | | | | | 19 (3%) |
| 15 | 934 | | | 3 (0.3%) | | | 931 (99.7%) |
| 16 | 323 | | | | | | 323 (100%) |

*In experiments 1-13, individual *rde-1* homozygote animals were microinjected with *med-6* dsRNA and then mated with several N2 males. Progeny from each P0 were treated as individual experiments. In experiment 14, five P0 *rde*-1 homozygous animals were microinjected with *med-6* dsRNA but were not mated with N2 males. In experiment 15, five N2 animals were microinjected with *med-6* dsRNA and then mated with several N2 males. In experiment 16, three N2 animals were microinjected with *med-6* dsRNA but were not mated. In all experiments, $F_1$ progeny were collected at 12-40 hours after RNAi and individual $F_1$ progeny were transferred to individual plates and subjected to phenotypic examination after 4 days of culture.

‡We defined 'small brood size' as the phenotype where hermaphrodites laid a total of fewer than 20 eggs.

§We calculated the lethality as the difference between the number of viable animals and the total number of eggs counted. In one case, the lethality was calculated as a negative value, which means that we missed some eggs while counting.

of the genes were reduced. However, from the phenotypes of the RNAi-affected animals, we could see the effects caused by the reduction of *med-6* activity. It was not surprising that reduction of *med-6* activity did not suppress the multivulva phenotype caused by multicopies of *lin-3*. We interpret this as follows: the level of *lin-3* activity is known to be crucial in determining how many VPCs are induced (Katz et al., 1995), and it is probable that reduction of *med-6* activity by RNAi was not effective enough to reduce the transcription level of *lin-3* to a point where fewer VPCs were induced. However, it is obvious that *lin-3* is directly or indirectly activated by *med-6*, because the reduction-of-function mutation of *lin-3* was enhanced by the *med-6* RNAi. We do not know at this point whether transcription of all three of the genes we examined, *lin-3*, *let-23*, and *let-60*, is directly regulated by *med-6* activity. We cannot rule out the possibility that other genes downstream of these are affected by the *med-6* RNAi, resulting in the effects we saw in the experiments. It would be necessary to establish an in vitro transcription assay system to test this directly.

It was recently reported that components of the nucleosome remodeling and histone deacetylase (NURD) complex in *C. elegans* antagonize Ras-induced vulval development by deacetylating specific target genes to repress vulval development (Solari and Ahringer, 2000). Now that it is evident that both the mediators and the chromatin remodeling complex are involved in the strict temporal and spatial regulation of sets of genes for proper development, the *C. elegans* vulval development system can serve as a model in which one can study the biological significance of the mediators and chromatin remodeling complexes.

We examined ray development because it was reported that another mediator protein was involved in repression of the Wnt pathway in ray precursor cells (Zhang and Emmons, 2000), and we subsequently found that *med-6* was also involved in regulation of the gene(s) involved in ray development. Reduction of *med-6* activity caused V6 progeny to undergo abnormal development, in that ray structures produced by these cells were missing or reduced in number. We also demonstrated that *med-6* is required for proper regulation of the genes in the Wnt signaling pathway by examining the RNAi effect in the *pal-1; sop-1* mutant animals. The data presented in this report suggest that *med-6* is involved in the development of V6-derived ray structures by regulation of *pal*-1 transcription. Consistent with this, we found that *med-6* RNAi lowered *pal-1* transcription during embryogenesis (data not shown). However, it is formally possible that *med*-6 is involved in regulated transcription of genes downstream of *pal-1*, such as *mab-5*.

The fact that SUR-2 and SOP-1 proteins do not have homologs in yeast, but are conserved only in metazoa, while MED-6 is conserved from yeast to humans, raises the possibility that MED-6 is a more general mediator of transcriptional regulatory signaling. Consistent with this, SUR-2 has been shown to have roles in the Ras pathway, but not in ray development, while SOP-1 has been shown to be involved in the Wnt pathway, but not in the Ras pathway. On the contrary, *med-6* has been shown to be involved both in the Ras

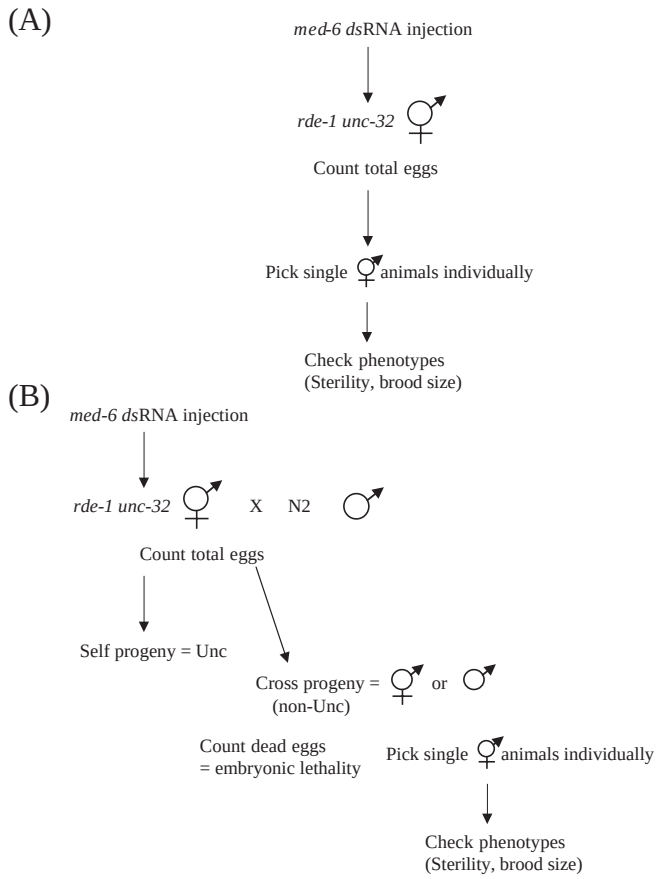


**Fig. 5.** Experimental scheme of the *rde-1* background RNAi. (A) Control experiment. Homozygous *rde-1 unc-32* animals were microinjected with *med-6* dsRNA, and the total number of eggs laid was counted. Individual progeny were transferred to individual plates, and phenotypes were examined after 4 days. (B) Maternal rescue RNAi. Homozygous *rde-1 unc-32* animals were microinjected with *med-6* dsRNA and were mated with wild-type males. The total number of eggs laid was counted. Non-Unc cross progeny were transferred to new plates individually, and checked for their sex and phenotypes. The percentages of dead embryos were determined by the differences in the numbers of eggs and viable animals.

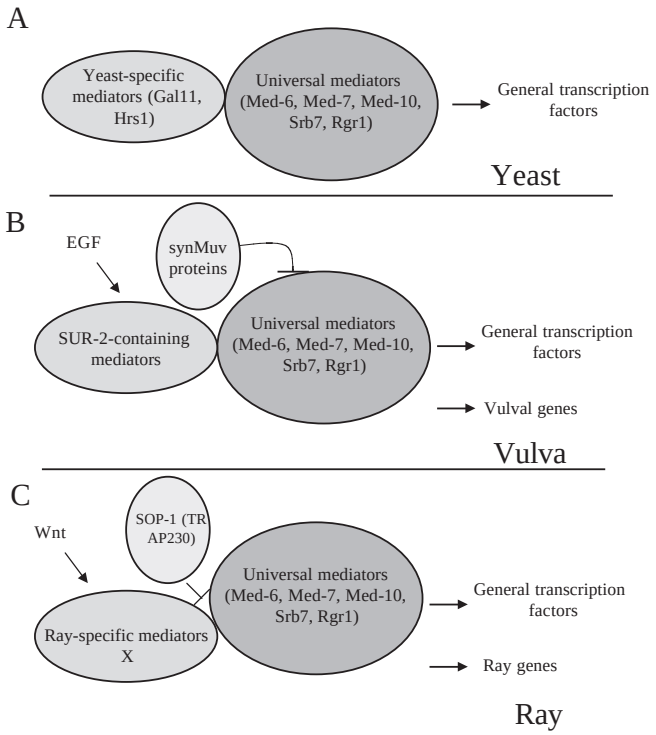


**Fig. 6.** An action model of the mediator-related proteins in yeast and *C. elegans*: the MED-6-containing mediator complex as a convergence point of transcriptional regulation. (A) In yeast, yeast-specific mediator proteins such as Gal11 and Hrs1 relay signals from transcription activators through the universally conserved mediator complex that contains MED-6 to the general transcription factors to regulate transcription. (B) In the nematode vulval development pathway, SUR-2 is a metazoan-specific mediator-related protein that interacts with outside signals (activated by EGF) and relays them through the conserved mediator complex containing MED-6 to the general transcription factors that regulate genes associated with the Ras pathway. The SynMuv proteins including the histone deacetylase complex proteins may counteract the mediator functions. (C) In the nematode ray developmental pathway, SOP-1 is a mediator-related protein that represses the Wnt-mediated transcriptional activation, which is dependent on the universally conserved mediator complex that contains MED-6.

pathway and in the Wnt pathway. Our model for the action of the mediator-related proteins in the nematode is shown in Fig. 6. We propose that the metazoan-specific mediator-related proteins interact with metazoan-specific transcription regulators to generate signals that converge at a more conserved mediator complex containing MED-6, which in turn transmits the signal(s) to the basal transcriptional machinery, leading to developmental specific gene expression.

### Using the *rde-1* mutation background as a tool to dissect maternal and zygotic gene function

The finding that the *let-425* mutant phenotypes were less severe than the effects of RNAi prompted us to examine the differences between the maternal and zygotic gene functions of *med-6* in development. If mutations are introduced in essential genes that are required both maternally and zygotically, the phenotype of homozygous mutant animals from heterozygous mothers would be masked because of maternal rescue. We were able to show by using the *rde-1* mutant background that one reason why the phenotypes of the *let-425* mutation are less severe than the effects of RNAi is due to the maternal contribution of wild-type *med-6* gene activity. As RNAi in the wild-type background does not distinguish between the reduction in maternal or zygotic functions of genes that are expressed both maternally and zygotically, we employed the *rde-1* mutant background. By using the *rde*-1 mutant background, we were able to examine the phenotypes caused by the reduction in zygotic gene expression only, leaving the maternally expressed genes intact. From the data we presented above, we could infer that the maternal contribution of *med-6* is required for normal embryogenesis and that its zygotic gene activity is mostly required for normal larval development and fertility, as well as normal ray development.

Additionally, we were able to confirm the limitations of RNAi, one of the main ones being that the effect of RNAi is not as severe as the null mutation of the gene, particularly for

genes acting late in development. The *rde-1* RNAi experiment we described above is an example of this. Sterility and larval arrest are late phenotypes compared with embryonic lethality, and quite a few progeny from the RNAi-affected *rde-1* animals were able to escape the RNAi effect, while the effect of the *let-425* genetic mutation was persistent through adulthood.

In summary, we have shown in this report that MED-6 is encoded by *let-425*, that the maternal and zygotic *let*-425 have distinct roles in development. Maternal *let-425* has been shown to be required for early embryogenesis and zygotic *let-425* has been shown to be required for late development, including hermaphrodite vulval development and male ray development. As for the action mechanism of the mediators, we suggested that MED-6, a universally conserved transcription mediator, is required for actions of metazoan-specific mediator-related proteins, such as SOP-1, for appropriate transcriptional regulation of development-specific genes. The next step will be to examine whether and how *med-6* is involved in regulated transcription of other genes in development. One way to pursue this issue on a large scale would be to perform a microarray experiment, as has been done in the yeast system.

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