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Broad Requirement for the Mediator Subunit RGR-1 for Transcription in the *Caenorhabditis elegans* Embryo*

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The Mediator-related transcription co-factors integrate positive and negative inputs and recruit and activate the RNA polymerase II complex. To understand the role of Mediator during transcription, it is important to identify Mediator subunits that are essential for its functions. In the yeast Mediator, the conserved component Rgr1 is associated with multiple subunits that are required for specific activation or repression events. Yeast *rgr1* is essential for viability, for certain repression mechanisms, and for activation of heat shock genes, but it is not known whether *rgr1* is generally important for transcription. Here we have performed the first analysis of *rgr-1* function in a metazoan. We found that in the developing *Caenorhabditis elegans* embryo *rgr-1* is broadly required for transcription and for phosphorylation of both Ser-2 and Ser-5 of the RNA polymerase II C-terminal domain repeat. We conclude that RGR-1 fulfills a critical Mediator function that is broadly essential for metazoan mRNA transcription and that RGR-1 may be required at an early recruitment or initiation step.

Eukaryotic mRNA transcription depends upon a set of general transcription factors (TFIIA,[1] -B, -D, -E, -F, and -H) and upon co-activator complexes that communicate with gene-specific activators and have enzymatic activities that include chromatin modification (1, 2). The Mediator co-activator enhances basal transcription *in vitro* and is required along with the general transcription factor TFIID for transcription in response to activators (1, 3–9). It is well documented that interactions between Mediator and activators facilitate transcription, possibly by recruiting RNA Polymerase II (Pol II) to promoters (5–7). Mediator also has acetyltransferase activity and is able to be recruited independently of Pol II however (10–12), suggesting that Mediator also acts through additional mechanisms. Accordingly, Mediator increases the efficiency with which the Pol II large subunit C-terminal domain (CTD) is phosphorylated during transcription (3), a process that couples transcription initiation to elongation and RNA processing (2).

Most individual Mediator subunits are not conserved between yeast and metazoans, possibly because they interact with divergent activators and repressors, and Mediator complexes that have different subunit compositions have been identified in mammals (5–7). The structural organization of yeast and mammalian Mediator is remarkably similar however, suggesting considerable conservation of function (13). Mediator changes conformation upon binding to Pol II and is induced to adopt distinct conformations by different activators (13, 14), suggesting that conformational effects may integrate positive and repressive stimuli to regulate Mediator or Pol II activity. Multiple Mediator components have been implicated in specific activation or repression events, and even various conserved Mediator subunits seem to have specialized functions. For example, in the *Caenorhabditis elegans* embryo the conserved Mediator components MED-6, MED-7, and MED-8 do not appear to be required for expression of many genes or for formation of some differentiated cell types (15). To elucidate the role of Mediator during transcription, it is important to identify Mediator components that might have broadly essential and possibly conserved functions.

The Rgr1 subunit is conserved in yeast and mammalian Mediator complexes (see Fig. 1*A*) (5–7). Within the yeast Mediator, Rgr1 is required for attachment of the Sin4·Gal11 subcomplex, which has been implicated in positive or negative regulation of specific genes (16–18). In addition, Rgr1 is present within a larger Mediator subcomplex that has multiple other components that appear to have activator-specific functions (19, 20). Yeast Rgr1 associates with certain repressors and is required for repression of particular genes (21, 22). Null *rgr1* mutants are inviable however, and *rgr1* is required for activation of metal-induced genes (23), consistent with Rgr1 additionally having positive roles. These observations raise the question of whether Rgr1 performs a specific set of activation and repression functions or is a broadly essential factor with a critical mechanistic role in yeast and metazoans as might be predicted by its conservation.

We have used RNA interference (RNAi) to investigate requirements for *C. elegans rgr-1* for transcription in the developing embryo. Because early *C. elegans* development is initiated by maternal gene products, *C. elegans* embryonic cells survive until approximately the 100-cell stage in the absence of new mRNA transcription, making it possible to manipulate expression of even generally essential transcription factors in these living cells (24, 25). We found that in *rgr-1(RNAi)* embryos, phosphorylation of both Ser-5 and Ser-2 of the CTD repeat is undetectable, development arrests with a phenotype that is consistent with a broad inhibition of transcription, and expression of multiple reporter genes that are representative of various gene classes is dramatically reduced. We conclude that RGR-1 may be more broadly required for transcription than any Mediator subunit studied so far and that the RGR-1 Mediator subcomplex plays a generally critical role early during

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[1] The abbreviations used are: TF, transcription factor; Pol II, RNA polymerase II; CTD, C-terminal domain; RNAi, RNA interference; GFP, green fluorescent protein; CDK, cyclin-dependent kinase; RT, reverse transcription; E2 cells, E cell daughters.

the transcription cycle prior to phosphorylation of the Pol II CTD.

EXPERIMENTAL PROCEDURES

C. elegans and Bioinformatics—*C. elegans* strains were maintained as described previously (25). The wild type strain was N2. Green fluorescent protein (GFP) reporter strains were provided to us as cited previously (25). The *C. elegans rgr-1* ortholog was identified by searching WORMpep and genomic databases (Sanger Centre) with human and *Saccharomyces cerevisiae* protein sequences. Alignments were produced by Megalign (DNAStar).

Immunostaining and Fluorescence Analysis—A rabbit antiserum was raised against the RGR-1 peptide PSVGQSYHHPLHHQQYPPQ with an amino-terminal Cys added and then affinity-purified (Pierce). For RGR-1 and α-Pol II staining, freeze-cracked embryos were fixed in 2% paraformaldehyde and then in methanol and washed with PBT (1× phosphate buffered saline, 1% Triton X-100, 1% bovine serum albumin) prior to staining with affinity-purified RGR-1 antiserum. RGR-1 antibody staining was competed by the immunogenic but not heterologous peptides (not shown). Staining was performed with the P-CTD (α-PSer5) (26) and H5 (α-PSer2) (Babco) antibodies as in Ref. 27. For GFP analysis, embryos were transferred to 2% agarose pads. Images were captured with a Zeiss AxioSKOP2 microscope and AxioCam digital camera, and GFP or antibody staining intensities were compared over a range of exposure times. Pixel intensities were standardized using Adobe Photoshop 5.0. Confocal images were obtained with a Bio-Rad Radiance 2000 MP multiphoton microscope and processed using Confocal Assistant 4.02 and Photoshop 5.5.

RNAi Analysis—An *rgr-1* cDNA (yk331b4) was obtained from Yuji Kohara (National Institute of Genetics, Mishima, Japan). Sense and antisense RNA was synthesized using the Ribomax kit (Promega) and then annealed and injected at 1.0 μg/μl into young adults (containing two to eight embryos). Embryos were collected for analysis at least 19 h later when uniform populations of terminally arrested *rgr-1(RNAi)* embryos were being produced. For heat shock, these *hsp-16.2*::*gfp* embryos were incubated at 37 °C for 1 min in 10 μl of M9, and then fluorescence was examined an hour later. *rgr-1* RNA was assayed in total embryonic RNA by RT-PCR using the Superscript™ One Step™ system (Invitrogen) as described in Ref. 28. *rgr-1* oligonucleotides used for RT-PCR were CCTTGAAGCTGCTGAACCAAGATTAGAAGTG and CCTCCAACTGAATGAACTGAGCTCGGTCTCC.

RESULTS

rgr-1 Is Essential during Early Embryonic Development—The *C. elegans* genome encodes a single well conserved ortholog of yeast *rgr1* and its human counterpart DRIP150/TRAP170 (Fig. 1*A*). *C. elegans rgr-1* re-identified these human and yeast genes as its closest relatives in the GenBank™ databases (not shown). To determine whether the RGR-1 protein is present in the early *C. elegans* embryo, we examined its expression by antibody staining. Using conditions optimized for early embryos, RGR-1 was readily apparent in embryonic nuclei beginning at the two-cell stage (Fig. 1*B* and not shown). RGR-1 was also detectable in adult germ line nuclei (not shown), suggesting that it is maternally expressed.

When *rgr-1* expression was inhibited in the early embryo by RNAi, RGR-1 antibody staining was reduced to background (Fig. 1*B*), and the *rgr-1* mRNA was not detected by RT-PCR (not shown), suggesting that the *rgr-1* mRNA and protein were significantly depleted. When the Pol II large subunit gene *ama-1* or other broadly essential mRNA transcription factors are inhibited by RNAi, *C. elegans* embryos arrest development after forming ~100 cells that lack any signs of differentiation (Fig. 1*C*) (24, 25, 28). A similar early developmental arrest occurred in *rgr-1(RNAi)* embryos, consistent with a broad transcription block (Fig. 1*C*).

To investigate whether this phenotype involved a general defect in maternal mRNA stores, we monitored early cell division patterns and performed parallel experiments in a transgenic strain that expresses a fusion of the maternally derived germ line protein PIE-1 to GFP. PIE-1::GFP recapitulates the endogenous PIE-1 localization pattern, which depends upon at least 20 maternal genes (29). As in *ama-1(RNAi)* embryos, in *rgr-1(RNAi)* embryos maternal PIE-1::GFP expression and localization patterns appeared normal at every stage (Fig. 1*D*

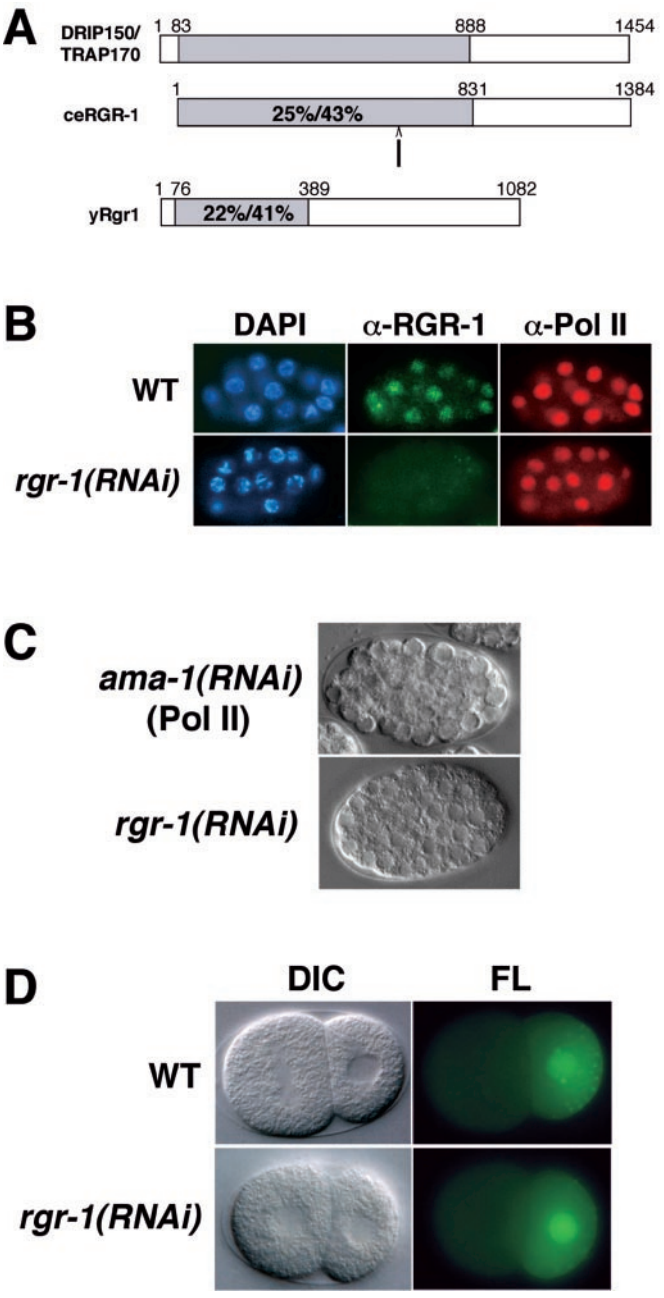

FIG. 1. **Phenotypic analysis of *rgr-1(RNAi)* embryos.** *A*, the predicted *C. elegans* (*ce*) RGR-1 protein. For *C. elegans* RGR-1 and *S. cerevisiae* (*y*) Rgr1, identity/similarity to their human ortholog DRIP150/TRAP170 within the conserved region (*gray*) is indicated. Expressed cDNA sequences predict the existence of two *C. elegans* RGR-1 isoforms, one of which contains a six-amino acid insertion that is derived from alternative splicing and insertion of an internal exon, which is shown below (*black bar*). *B*, expression of RGR-1 protein and the Pol II large subunit (AMA-1). Wild type and *rgr-1(RNAi)* embryos were stained simultaneously with RGR-1 and α-Pol II antisera (POL 3/3) (33) and with 4′,6′-diamidino-2-phenylindole hydrochloride (*DAPI*) to visualize DNA. POL 3/3 staining is abolished in *ama-1(RNAi)* embryos (25). *C*, terminal developmental arrest phenotypes. *rgr-1(RNAi)* embryos are compared with *ama-1(RNAi)* embryos. *D*, intact maternal gene functions in *rgr-1(RNAi)* embryos. Wild type (*WT*) and RNAi embryos produced by *pie-1*::*gfp* mothers were examined by differential interference (*DIC*) and fluorescence (*FL*) microscopy. *rgr-1(RNAi)* embryos were not distinguishable from wild type with respect to any aspect of PIE-1::GFP germ line and subcellular localization, including the presence of PIE-1 in germ line P granules. Late two-cell embryos are shown.

and not shown). Early cell division timing and cleavage planes were also generally normal in *rgr-1(RNAi)* embryos except for the cell cycle period of the two E cell daughters (E2 cells), which give rise to the endoderm. When early embryonic mRNA transcription is broadly inhibited, as in *ama-1(RNAi)* embryos, the E2 cell cycle length is shortened from 45 to ~22 min (24, 25). The E2 cells similarly divided after 22 min in *rgr-1(RNAi)* embryos (not shown). Together our findings suggest that depletion of embryonic RGR-1 does not detectably affect maternal mRNA stores but may broadly impair new mRNA transcription.

Early Embryonic Pol II CTD Phosphorylation and Transcription Require rgr-1—To investigate how mRNA transcription was affected in *rgr-1(RNAi)* embryos, we first analyzed phosphorylation of the Pol II large subunit CTD, which consists of repeats based on the consensus YSPTSPS (42 copies in *C. elegans*). Pol II is recruited to promoters in an unphosphorylated form, and then during transcription the CTD repeat is initially phosphorylated on Ser-5 by the TFIIH kinase (26, 30). During transcription elongation the distribution of CTD phosphorylation shifts to Ser-2 (30, 31), which in metazoans is phosphorylated by the kinase CDK-9 (28, 32). CTD Ser-5 and Ser-2 phosphorylation can be specifically detected in embryonic nuclei by staining with the P-CTD and H5 antibodies, respectively (25–27), which we refer to as α-PSer5 and α-PSer2 for clarity (Fig. 2*A*).

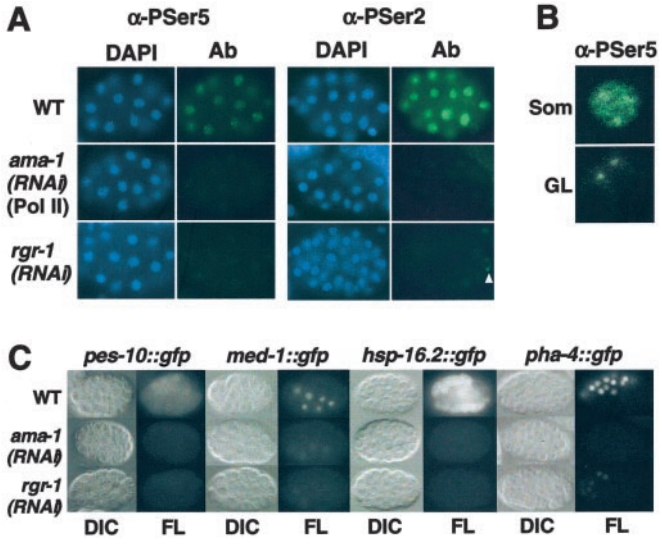

FIG. 2. **RGR-1 is required broadly for early embryonic transcription.** *A*, phosphorylation of Pol II CTD Ser-2 and Ser-5 is undetectable in *rgr-1(RNAi)* embryos. Representative wild type (*WT*) or RNAi embryos that have not reached terminal developmental arrest are shown in *rows* as indicated. These embryos were stained with α-PSer5 (*left panel*) or α-PSer2 (*right panel*) antibodies (*Ab*) and with 4′,6′-diamidino-2-phenylindole hydrochloride (*DAPI*) to visualize DNA. Throughout development of *rgr-1(RNAi)* embryos, these phosphoepitopes were not detectable above the background levels seen in *ama-1(RNAi)* embryos. In the α-PSer5-stained wild type embryo, the transcriptionally silent germ line nucleus is not located within the focal plane shown. Some previously described cross-reactive α-PSer2 staining that is independent of the Pol II epitope (28) was visible in germ line P granules (*arrowhead*) and mitotic cells (not shown). *B*, confocal microscopy of α-PSer5 staining in individual representative wild type somatic (*Som*) and germ line (*GL*) nuclei. *C*, *rgr-1* is required for expression of early embryonic genes. Wild type (*WT*) and RNAi embryos (designated in *rows*) that were derived from transgenic GFP reporter strains were analyzed by differential interference (*DIC*) and fluorescence (*FL*) microscopy (as indicated *below* the *columns*). These embryos are representative of the entire population analyzed in each of multiple independent experiments in which >35 embryos were scored. In *rgr-1(RNAi)* embryos, reduced *pha-4*::*gfp* expression was apparent in a subset of the cells in which *pha-4*::*gfp* was expressed in wild type. Reporter strains are described in Ref. 25.

In early vertebrate embryos, CTD phosphorylation that occurs independently of transcription is detectable by Western blotting (33–35), but in the *C. elegans* embryo the presence of both α-PSer5 and α-PSer2 staining appears to depend upon transcription. Staining with the α-PSer5 and α-PSer2 antibodies is not detected in embryonic nuclei until the three-to-four-cell stage when new mRNA transcription begins (27). At later stages, the patterns and intensity of this staining appear to parallel transcription activity in embryonic cells, and both types of staining are reduced to background levels when the essential initiation factor TFIIB (*ttb-1*) is depleted by RNAi (25, 27). In contrast, when the elongation factor CDK-9 is depleted by RNAi, Ser-5 phosphorylation levels appear normal, but Ser-2 phosphorylation is undetectable (28).

In *rgr-1(RNAi)* embryos, levels of the Pol II large subunit AMA-1 appeared to be unaffected (Fig. 1*B*), but phosphorylation of both Ser-5 and Ser-2 of the CTD was dramatically reduced. Ser-5 phosphorylation is detectable as a bright punctate α-PSer5 staining pattern in transcriptionally active wild type somatic nuclei (Fig. 2*A*) but is confined to two foci in the transcriptionally silent early germ line precursor (Fig. 2*B*). In contrast, in *rgr-1(RNAi)* embryos α-PSer5 staining was reduced to background levels in all cells at each embryonic stage, and the two characteristic staining foci were absent from germ line cells (Fig. 2*A* and not shown). Specific α-PSer2 staining is normally apparent in all wild type somatic cells and absent in the early germ line (27) but was not detectable in any cells in *rgr-1(RNAi)* embryos (Fig. 2*A* and not shown). These dramatic defects in phosphorylation of both CTD serines suggest that depletion of RGR-1 may broadly impair transcription and may interfere with an early step during the transcription process.

To investigate the importance of RGR-1 for expression of individual genes, we performed RNAi experiments in *C. elegans* strains that carry reporter transgenes in which regulatory regions are intact and coding regions are fused to GFP. These reporters are expressed in the early embryo in parallel to the corresponding endogenous genes. We first examined expression of *med-1* and *pes-10*, each of which is expressed as transcription initiates (Fig. 2*C*). In *rgr-1(RNAi)* embryos, PES-10::GFP and MED-1::GFP expression was reduced to very low background levels that were characteristic of *ama-1(RNAi)*

TABLE I
rgr-1 required for early embryonic gene expression

Reporter strains were scored as + when GFP was expressed at wild type levels in all embryos and as − when GFP was undetectable or present at comparable trace levels in *ama-1(RNAi)* and *rgr-1(RNAi)* embryos. −/+ indicates that some low level GFP expression above the *ama-1(RNAi)* background was detected in *rgr-1(RNAi)* embryos as in some cells in the *pha-4*::*gfp* strain (Fig. 2*C*). In each experiment, more than 35 embryos were analyzed from multiple different injected P0 animals. Reporter genes are described in Ref. 25. *let-858* is referred to as "conserved eukaryotic" because it is common to yeast and metazoans. Other genes analyzed are metazoan-specific. WT, wild type, MAP, mitogen-activated protein.

	ELT-5::GFP (ectoderm)	CKI-2::GFP (cell cycle)	SUR-5::GFP (MAP kinase pathway)	END-1::GFP (endoderm)	LET-858::GFP (conserved eukaryotic)
WT	+	+	+	+	+
ama-1(RNAi)	−	−	−	−	−
rgr-1(RNAi)	−/+	−	−	−	−/+

embryos (Fig. 2*C*). *rgr-1* was similarly required to express the heat shock gene *hsp-16.2* (Fig. 2*C*), the endodermal specification gene *end-1*, and the broadly expressed genes *sur-5* and *cki-2* (Table I). In *rgr-1(RNAi)* embryos, some genes were expressed at levels that were significantly reduced but above background. This last group of genes included the developmental specification genes *pha-4* and *elt-5* and the widely expressed gene *let-858* (Fig. 2*C* and Table I). The finding that *rgr-1* is required for appropriate expression of this diverse group of genes supports the model that RGR-1 is broadly important for transcription in the early *C. elegans* embryo.

DISCUSSION

Previous analyses implicated yeast *rgr1* in repression of particular genes and in activation of metal-induced genes (21–23), but it had not been determined in any organism whether Rgr1 performs a generally essential Mediator function. Here we have obtained multiple lines of evidence that *rgr-1* is critical for most transcription in the developing *C. elegans* embryo. Similarly to *ama-1(RNAi)* embryos, *rgr-1(RNAi)* embryos arrested development without apparent differentiation and lacked detectable Pol II CTD phosphorylation (Figs. 1*C* and 2*A*). In addition, it is striking that in *rgr-1(RNAi)* embryos each of the wide variety of embryonic genes tested was expressed at significantly reduced levels (Fig. 2*C* and Table I). The finding that some embryonic genes were expressed detectably in *rgr-1(RNAi)* embryos (Fig. 2*C* and Table I) may suggest some specialization in RGR-1 function but could derive from an incomplete RNAi effect. Despite this caveat, our findings strongly indicate that RGR-1 is broadly required for transcription and is therefore likely to be essential for most Mediator functions.

An intriguing aspect of our findings is that phosphorylation of both Ser-2 and Ser-5 of the CTD was reduced to undetectable levels in *rgr-1(RNAi)* embryos (Fig. 2*A*). A similar reduction in levels of both CTD phosphoserines occurred when the initiation factor TFIIB was inhibited by RNAi (25). In contrast, RNAi depletion of TAF-4, the homolog of the human TFIID component $TAF_{II}130$ (36), broadly inhibited Ser-2 phosphorylation but was associated with each somatic cell containing two bright and distinct α-PSer5 foci (25). These foci appeared similar to those characteristic of the transcriptionally silent germ line (Fig. 2*B*) and may have derived from aborted or stalled transcription events. Significantly, although this Ser-5 phosphorylation occurred, *taf-4(RNAi)* embryos were characterized by a more complete inhibition of reporter gene expression than *rgr-1(RNAi)* embryos (25). Our findings suggest that lack of RGR-1 may inhibit most transcription before Ser-5 phosphorylation occurs and at an earlier step than transcription is blocked in the absence of TAF-4. These conclusions are consistent with the model that Mediator is generally required at an early step during Pol II recruitment or transcription initiation.

The broad requirement for *rgr-1* that we have observed is particularly striking because the metazoan Mediator components studied to date each appear to be essential for activation of a limited subgroup of genes (5–7, 9, 15). In yeast, even the broadly required Mediator subunit Srb4 appears to be dispensable for heat- and metal-induced genes in contrast to yeast and *C. elegans rgr-1* (Fig. 2*C*) (17). Our model that RGR-1 is broadly critical for transcription provides a paradigm for explaining why some yeast *rgr1* mutations abrogate the effects of certain transcriptional repressors (21, 22) because it is consistent with the view that these repressors may block transcription by interfering with a critical positive aspect of Rgr1 function. It is possible that RGR-1 is critical for Mediator function because it is important for Mediator structural integrity. RGR-1 may be required for the presence of multiple Mediator subunits that have more specialized activities, a possibility that is consistent with Mediator structures (13, 18), or it may contribute to stability of the entire Mediator complex. Alternatively, RGR-1 might be required for an essential conformational effect, enzymatic activity, or interaction at the promoter. In either case, our findings indicate that further analysis of RGR-1 functions will elucidate fundamental principles of how Mediator complexes contribute to transcription.

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