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A regulatory cytoplasmic poly(A) polymerase in Caenorhabditis elegans
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Messenger RNA regulation is a critical mode of controlling gene expression. Regulation of mRNA stability and translation is linked to controls of poly(A) tail length. Poly(A) lengthening can stabilize and translationally activate mRNAs, whereas poly(A) removal can trigger degradation and translational repression. Germline granules (for example, polar granules in flies, P granules in worms) are ribonucleoprotein particles implicated in translational control. Here we report that the Caenorhabditis elegans gene gld-2, a regulator of mitosis/meiosis decision and other germline events, encodes the catalytic moiety of a cytoplasmic poly(A) polymerase (PAP) that is associated with P granules in early embryos. Importantly, the GLD-2 protein sequence has diverged substantially from that of conventional eukaryotic PAPs, and lacks a recognizable RRM (RNA recog-
in the germ line and is developmentally regulated.

Database searches revealed that GLD-2 protein belongs to the DNA polymerase β superfamily of nucleotidyltransferases (NT) (Fig. 2a; refs 6, 7). Specifically, GLD-2 is a group 2 NT member, including DNA polymerase σ of Saccharomyces cerevisiae (also known as pol k and Trf4p) and eukaryotic PAPs (Fig. 2a). GLD-2 architecture and sequence is divergent from that of canonical PAPs (Fig. 2b, d), but similar to a different cluster of NT family members (Fig. 2e). GLD-2 contains three critical carboxylate side chains essential for catalytic activity (Fig. 2c, red) present in all DNA polymerase β superfamily members; furthermore, GLD-2 possesses putative ATP-interacting residues (Fig. 2c, green; Fig. 2d, green). Classical PAPs have a catalytic region (Fig. 2c, gold), a ‘central’ domain (Fig. 2c, blue), and an RRM-like region (Fig. 2c, violet)8,9. By sequence comparison, GLD-2 harbours catalytic and central domains (Fig. 2b, d, colour-coded overlines), but is highly diverged from classical eukaryotic PAPs, including C. elegans PAP-1 (C. Lütjens and M.W., unpublished results) (Fig. 2d). Classical PAPs show extensive amino-acid conservation among themselves, but limited conservation with GLD-2 (Fig. 2d black and grey boxes). Outside its catalytic and central domains, GLD-2 shares little similarity to canonical PAPs; in particular, GLD-2 has no boxes). Outside its catalytic and central domains, GLD-2 shares

![Figure 2: GLD-2 belongs to the polymerase β nucleotidyltransferase superfamily.](Image)

Figure 2: GLD-2 belongs to the polymerase β nucleotidyltransferase superfamily. a, The polymerase β superfamily (adapted from ref. 7). Small colour-coded circles, families; large grey circle, group 2 families. CCA, CCA-adding enzymes; 2’–5’A, 2’–5’ oligoA synthetases; TRF, Trf4p-like proteins; other acronyms as in ref. 7. b–d, Colour coding based on crystal structures of bovine and yeast PAPs8,9. Gold, catalytic domain; blue, central domain; violet, RRM domain. GLD-2 and PAP domains compared. Drosophila (Dm), human (Hs) and yeast (Ss). d. GLD-2 domains identified by Pfam search17; aa, amino acid; Bovine PAP 3D structure, with key residues shown in stick form (adapted from ref. 8). Created by Rasmol based on PDB file 1F5A (for bovine PAP). e, Amino-acid sequence alignment of GLD-2 and PAP core regions based on clustalW output20 and polymerase β superfamily analyses20. Mutants designated below: Red, catalytic residues; green, required for ATP binding. e, Unrooted tree of GLD-2 and its homologues, created with PHYLP programs20, based on ClustalW alignment using parsimony. Species are: Ce, C. elegans; Dm, Drosophila; Hs, human; Mm, mouse; Os, rice; Sp, S. pombe. ATP, Arabidopsis. Only homologues with E-values less than 1 x 10–15 in the first PSI blast were used; tree was built using the catalytic and central domain sequences as in d (GLD-2–2 amino acids: 528–914 and corresponding sequences of its homologues). Cid1 and GLD-2 (shown red) both function in cell cycle control; Cid13 (shown red) is involved in the replication stress response22; functions of others are unknown.
were screened and 30 gld-3 cDNAs (T07F8.3) found; using GLD-3 as bait, 1,500,000 transformants were screened and 94 gld-2 cDNAs recovered. To identify the region of GLD-2 critical for GLD-3 binding, GLD-2 variants were assayed for GLD-3 interaction. A GLD-2 fragment comprising both catalytic and central domains was essential (amino acids 544–924) (Fig. 4a). A GLD-2-E875K mutant, designed after gld-2(h292), interacted poorly with GLD-3 (Fig. 3a, E875K and Δ7). Indeed, β-galactosidase activity was reduced 7- to 16-fold by GLD-2(h292)-E875K (Fig. 4a, compare for example Δ2 to Δ7), but GLD-2 levels were equivalent (Fig. 4b). Importantly, GLD-2-E875K was present at normal levels in *C. elegans* (Fig. 3a, lane 6), even though it disrupts gld-2 function. We conclude that GLD-2 binds specifically to GLD-3, and that GLD-2-E875K is defective in GLD-3 binding. Therefore, the GLD-2/GLD-3 interaction appears to be important for development.

Given its sequence similarity to nucleotidyltransferases and its cytoplasmic location, we considered that GLD-2 might be a cytoplasmic PAP, even though its architecture and sequence diverged substantially from classical PAPs. To test this idea, we initially assayed incorporation of radiolabelled ATP into an RNA substrate. Specifically, GLD-2 was translated in vitro, either on its own or together with GLD-3. The *in vitro* translation mixture was incubated with 32P-ATP and an unlabelled poly(A) substrate, and incorporation of label into acid-insoluble material was measured (see Methods). GLD-2 on its own had low activity, whereas GLD-3 had none; however, GLD-2 and GLD-3 together gave a robust response (Fig. 4c). We also measured incorporation in three control reactions (no protein and two GLD-2 mutants together with GLD-3). GLD-2-D608A was designed to abolish the catalytic site (Fig. 2c) and GLD-2-E875K was used to disrupt GLD-3 binding (Fig. 4a). The control reactions yielded no measurable 32P-ATP incorporation (Fig. 4c). From these experiments, we argue that GLD-2 is in fact a nucleotidyltransferase and that both its predicted active site and GLD-3 binding region are essential for enzymatic activity.

We next analysed the products of the GLD-2/GLD-3 nucleotidyltransferase activity by electrophoresis and autoradiography (Fig. 4d). To this end, reactions were done as described above, except that C3A10 (see Methods) was used as substrate. Two exposures of the same autoradiogram are shown (Fig. 4d). As a marker, C3A10 was 3’ end-labelled with cordycepin triphosphate ([α-32P] 3’ dATP) (C3A10*dA; Fig. 4d left, lane 1). GLD-2 by itself exhibited modest incorporation from ATP into bands that extended the substrate by only one or a few nucleotides (Fig. 4d left, lane 2). In contrast, GLD-2 plus GLD-3 stimulated incorporation, resulting in more product with a ‘ladder’ of poly(A) extending the substrate more than 30 adenosines (Fig. 4d, lane 4). The ladder mimics the activity of bovine nuclear PAP (bPAP), but is less efficient (Fig. 4d, compare lanes 4 and 7). This difference may reflect the fact that bovine PAP acts as a monomer, whereas GLD-2 PAP activity is dependent on the interaction of two dilute proteins. Furthermore, although abundant products had only two or three nucleotides added (asterisks in Fig. 4d, lane 4), more-minor products had as many as 70 additional nucleotides. We conclude that GLD-2/GLD-3 can catalyse the addition of a poly(A) tail to an RNA substrate.

Four controls support the conclusion that GLD-2 is a PAP. First, GLD-2 PAP activity was abolished by a site-directed mutation in the inferred active site (D608A) (Fig. 4d, lane 5). Importantly, GLD-2-D608A level is equivalent to that of wild-type GLD-2 in the same assay (Fig. 4d SDS–polyacrylamide gel electrophoresis, SDS–PAGE, compare lanes 4 and 5). Thus, the GLD-2 putative active site is required for AMP addition *in vitro*. Second, GLD-2 PAP activity was abolished by the E875K mutation (Fig. 4d, lane 6), which disrupts GLD-2/GLD-3 binding (Fig. 4a). The GLD-2-E875K level was equivalent to wild-type GLD-2 (Fig. 4d, compare lanes 4 and 6). Third, GLD-2-dependent incorporation is substrate dependent and requires ATP (not shown). Thus, replacement of ATP with GTP, CTP or UTP did not yield incorporation onto the substrate. Finally, products produced by GLD-2 plus GLD-3 were selectively retained on oligo(dT) cellulose, suggesting they were polyadenylated (not shown).

The GLD-2/GLD-3 enzyme represents a new type of poly(A) polymerase (Fig. 5). Canonical PAPs, which include nuclear and cytoplasmic enzymes, are all closely related; they are monomeric and possess three key domains (Fig. 5, left)9,9. By contrast, GLD-2 appears to function as a heterodimer (Fig. 5, right). GLD-2 harbours the catalytic and central domains; GLD-3 has five consecutive K homology (KH)-related motifs which may, at least in

**Figure 3** The GLD-2 protein. Polyclonal anti-GLD-2 antibodies were affinity purified. a, Western blot of proteins from wild-type embryos (E), larvae (L1–L4), and adults (A) (lanes 1–5), and adults of genotype *gld-2(h292)* (lane 6), *gld-2(q497)*/gld-2(+)(lane 7), and *gld-2(q497)*/gld-2(q497) (lane 8). b, GLD-2 protein is in germline cytoplasm. Extruded WT adult hermaphrodite germ line; GLD-2 is abundant in pachytene region and oocytes. Magnified view shows lack of GLD-2 in nuclei (arrowheads) and presence of GLD-2 in granular form (arrows). A control *gld-2(q497)* extruded germ line showed no anti-GLD-2 staining (not shown). c, GLD-2 protein is associated with P granules in early embryos. Embryos stained with antibody to P granule marker, PGL-1, to GLD-2, and to nuclear pore antigen. Top, late P0 embryo, GLD-2 co-localizes with P granules; second panel down, 28-cell embryo, P4, white arrowhead; third panel, ~100-cell embryo, germline precursor cells, Z2 and Z3, arrows; bottom, magnified view of P2 blastomere to show PGL-1 and GLD-2 co-localization (arrows). d, e, Transgenic strain AZ212. Left, Nomarski image; right, nuclei visualized by histone::GFP maker. Both control and *gld-2(RNAi)* embryos are of approximately same age. d, Mock injected control. e, *gld-2(RNAi)*.
GLD-2 and GLD-3 are likely to function together during nematode development. First, GLD-2 and GLD-3 have similar, albeit not identical, functions in germline development and embryogenesis (refs 4, 5, and this work). Second, both are cytoplasmic and associated with P granules (ref. 5, this work), large complexes of RNA and protein that are critical for germline development30. GLD-2 and GLD-3 may polyadenylate mRNAs associated with P granules (for example, nos-2; ref. 16) or may be stored there for segregation to germline blastomeres. GLD-2 may be targeted to specific mRNAs by GLD-3, which is a Bic-C family KH protein5. Other KH proteins (EMRP, NOVA, hnRNPK) bind RNAs through sequence-specific interactions47-50. GLD-2 may also be targeted to specific mRNAs indirectly via the interaction of GLD-3 with FBF5. FBF is a sequence-specific RNA-binding protein and member of the PUF family47. PUF proteins appear to repress mRNAs by promoting poly(A) removal51. GLD-3 antagonizes FBF5, and works with GLD-2 to promote poly(A) addition (this work). Therefore, GLD-2/GLD-3 may switch FBF from a repressive to an activating mode.

Regulatory cytoplasmic PAPs of the GLD-2/GLD-3 class may be common. Within the large superfamily of DNA polymerase β-like nucleotidyltransferases, several are closely related to GLD-2 (Fig. 2e). To date, most have no assigned function, but Schizosaccharomyces pombe Cid13 and Cid1 appear to be rcPAPs22,31. The similarity between GLD-2 and Cid1 is particularly striking, as both are involved in cell cycle control. GLD-2 promotes entry into meiosis at the expense of mitosis5, and Cid1 inhibits mitosis25. We suggest that GLD-2 and Cid1 may in fact be components of an ancient regulatory circuit controlling the cell cycle, and that other GLD-2 relatives may similarly be regulatory cytoplasmic PAPs.

**Methods**

**Molecular cloning of gld-2**

Three-factor mapping places gld-2 0.05 map unit to the right of bli-4. Cosmids in this region were injected into strain JK1716 [map 4e57] glD-2(4947) [dp3-5e61] unc-13(e51) or strain JK1732 [map 4e57] glD-2[k892] [dp3-5e61] unc-13(e51). Cosmid ZC108 gave >4% germline rescue.

**Transcript analyses**

Northern blots were performed as described44. Templates for making RNA probes (gld-2 5′, middle, 3′, eft-3) were made by polymerase chain reactions (PCRs) from plK830, plK831, plK832 and pBluescript eft-3 (gift from P. Anderson). To determine the gld-2 3′ end, semi-nested PCR was performed using λAE1, C elegans mixed-stage oligo(dT) primed complementary DNA library (gift from A. Ponti). One PCR product was confirmed and sequenced. A stretch of 22 As was found at the end of the 3′ untranslated region (UTR). To determine the gld-2 5′ ends, reverse transcription (RT) were performed using SuperScript II Reverse Transcriptase (Gibco BRL) and poly(A)+ RNA from either wild-type mixed-stage worms or glp-1(q242) mutants raised at 25°C, which have no germ line. The resultant cDNA’s were then used as templates for semi-nested PCR with SL1 (a trans-spliced leader in C. elegans) as the constant 5′ primer. All PCR products were cloned into pStBlue-1 and sequenced. The 4.7-kb mRNA is SL1 trans-spliced, comprises 19 exons including an 86-nucleotide 5′ UTR and 1,105-nucleotide 3′ UTR.

**Antibody production, western blot and immunocytochemistry**

Polyclonal antibodies were generated from rabbits using a keyhole limpet haemocyanin (KLH)-conjugated peptide corresponding to GLD-2 amino acids 108–127 (Genemed Synthesis) or from rats using a GST–GLD-2 fusion protein carrying amino acids 1-330 of GlD-2. Rabbit anti-PGL-1 antibody was a gift from S. Strome. Monoclonal antibody 414, the anti-nuclear pore monoclonal, was purchased from BABCO. Western blots were performed using the GLD-2 peptide antibody as described44. Immunocytochemistry followed published procedures44 using the GST–GLD-2 fusion protein antibody, which was specific for GLD-2 as demonstrated on glD-2(q497) extruded germ lines and glD-2(RNAi) embryos.
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Poly(A) polymerase assay

Proteins were in vitro translated using the TNT coupled transcription–translation system (Promega), and assayed using buffer conditions essentially as described\(^1\). For scintillation counting, poly(A) (Roche) was used as substrate. For gel assays, we used RNA oligo, C\(_{450}\) A\(_ {500} \) (Dharmacon) a 45-nucleotide and supplemental 1 mM MgCl\(_2\). Products were analysed on 12% sequencing gels.

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