MCB
Journals.ASM.org

LIN-35/Rb Causes Starvation-Induced Germ Cell Apoptosis via CED-9/Bcl2 Downregulation in *Caenorhabditis elegans*

L. I. Láscarez-Lagunas,[a] C. G. Silva-García,[a] T. D. Dinkova,[b] R. E. Navarro[a]

Departamento de Biología Celular y Desarrollo, Instituto de Fisiología Celular,[a] and Departamento de Bioquímica, Facultad de Química,[b] Universidad Nacional Autónoma de México, Mexico City, Mexico

Apoptosis is an important mechanism for maintaining germ line health. In *Caenorhabditis elegans*, germ cell apoptosis occurs under normal conditions to sustain gonad homeostasis and oocyte quality. Under stress, germ cell apoptosis can be triggered via different pathways, including the following: (i) the CEP-1/p53 pathway, which induces germ cell apoptosis when animals are exposed to DNA damage; (ii) the mitogen-activated protein kinase kinase (MAPKK) pathway, which triggers germ cell apoptosis when animals are exposed to heat shock, oxidative stress, or osmotic stress; and (iii) an unknown mechanism that triggers germ cell apoptosis during starvation. Here, we address how starvation induces germ cell apoptosis. Using polysomal profiling, we found that starvation for 6 h reduces the translationally active ribosomes, which differentially affect the mRNAs of the core apoptotic machinery and some of its regulators. During starvation, *lin-35*/Rb mRNA increases its expression, resulting in the accumulation of this protein. As a consequence, LIN-35 downregulates the expression of the antiapoptotic gene *ced-9/Bcl-2*. We observed that the reduced translation of *ced-9/Bcl-2* mRNA during food deprivation together with its downregulation drastically affects its protein accumulation. We propose that CED-9/Bcl-2 downregulation via LIN-35/Rb triggers germ cell apoptosis in *C. elegans* in response to starvation.

Many germ cells (known in many organisms as nurse cells) are needed to produce a single oocyte. Once they have served their purpose, nurse cells are eliminated by apoptosis. An unsolved question in the germ cell field is how apoptosis is triggered to produce and sometimes protect germ cells. Adult *Caenorhabditis elegans* nematodes that encounter food deprivation enter a diapause stage that allows them to survive for a few months and put off fertility (1, 2). Later, when animals are subjected to better conditions, they exit adult diapause and restore fertility. Germ cell apoptosis is a key element to protect gonads from starvation, as exhibited by mutants of the caspase CED-3 that are unable to restore fertility after adult diapause (1). Previously, we found that starvation triggers germ cell apoptosis (3), and in this study we proposed a model to explain how this response is regulated in *C. elegans*.

In *C. elegans*, apoptosis occurs in somatic tissues during embryonic and postembryonic development (developmental apoptosis) (4, 5) and in the adult hermaphrodite gonad (physiological germ cell apoptosis) (6). In this nematode, apoptosis is executed via a conserved pathway that consists of the proteins CED-3 (a caspase), CED-4 (APAF1-like adaptor protein), and CED-9 (BCL2 ortholog) (reviewed in references 7 and 8). Developmental apoptosis is initiated when the protein EGL-1 (BH3-only) disrupts the CED-9/CED-4 complex, thereby triggering CED-3 activation.

The mechanism that induces physiological germ cell apoptosis is independent of EGL-1 (6) and is partially induced by the ortholog of the human retinoblastoma gene (Rb) *lin-35*, which downregulates *ced-9* (9). In contrast, the *C. elegans* DP ortholog, *dpl-1*, induces physiological germ cell apoptosis via *ced-4* upregulation (9). Genotoxic agents activate germ cell apoptosis via EGL-1, CEP-1 (the p53 ortholog), and CED-13 (a BH3-only protein) (10–12). Furthermore, several conditions, including oxidative, osmotic, heat shock, and starvation stresses, can induce germ cell apoptosis via an EGL-1- and CEP-1-independent mechanism (3). Oxidative, osmotic, and heat shock stresses induce apoptosis via the mitogen-activated protein kinase kinase (MAPKK) pathway (3). However, starvation induces germ cell apoptosis via a previously unknown mechanism.

Here, we examined how starvation affects the core apoptotic machinery genes and some of their regulators to induce apoptosis in the *C. elegans* germ line. We found that during starvation, *ced-9* expression is considerably downregulated, while *lin-35* and *dpl-1* are upregulated. The translation levels of several mRNAs, including some that encode the core apoptotic machinery, like *ced-9*, as interpreted from their distribution along polyribosomal profiles, are reduced. Unexpectedly, *lin-35* mRNA is translated efficiently upon fasting, resulting in a considerable increase in its protein accumulation.

The results of the current study suggest a model during starvation in which a considerable increase in LIN-35 expression accounts for *ced-9* mRNA downregulation, which, combined with the less efficient translation of its mRNA, dramatically reduced the CED-9 protein levels, subsequently inducing germ cell apoptosis.

(This research was conducted by L. I. Láscarez-Lagunas in partial fulfillment of the requirements for a Ph.D. degree in biochemical science from the Universidad Nacional Autónoma de México. Mexico City, Mexico.)

Received 21 November 2013 **Returned for modification** 15 December 2013
Accepted 16 April 2014
Published ahead of print 21 April 2014
Address correspondence to R. E. Navarro, rnavarro@ifc.unam.mx.

doi:10.1128/MCB.01532-13

MATERIALS AND METHODS

General methods and strains. *C. elegans* strains were cultured at 20°C as previously described (13). The wild-type (wt) strain *C. elegans* var. Bristol (N2) was used in this study. The alleles used were as follows: *lin-35*(*n745*) (14), *dpl-1*(*n3643*) (15), *ced-4*(*n1162*) (16), and *efl-1*(*seI*) (17). All of the strains were obtained from the *Caenorhabditis* Genetics Center (CGC).

Stress conditions and quantification of germ cell apoptosis. For the starvation experiments, 1-day-old animals were transferred onto nematode growth medium (NGM) plates with or without bacteria and incubated for 6 h at 20°C. The animals were anesthetized using 40 mM sodium azide and mounted onto slides using 2% agarose pads; the cell corpses were then visualized using Nomarski [for *ced-1*(RNAi) experiments] or fluorescence microscopy (for *ced-1*::*gfp* strains).

Statistical analyses were performed using Prism (GraphPad Software). All *t* tests were two-tailed unpaired *t* tests (Mann-Whitney).

RNAi experiments. RNA interference (RNAi) experiments were performed by feeding as previously described (18). Constructs for the *ced-1*, *ced-3*, *lin-35*, *dpl-1*, and *efl-2* genes were obtained from the *C. elegans* RNAi, version 1.1, feeding library (Open Biosystems). The *ced-9* gene was cloned using the oligonucleotides 5′-GGAATTCCCGATATTCGAGAAG AAGCACG-3′ and 5′-GGGGTACCCCTTACTTCAAGCTGAACATCA TC-3′. The product was digested with EcoRI and KpnI and cloned into a feeding vector (PD129.36), and the resulting plasmid was transformed into *Escherichia coli* strain HT115 (DE3). RNAi experiments for *ced-1*, *ced-3*, *lin-35*, *dpl-1*, and *efl-2* were performed at 20°C, and RNAi experiments for *ced-9* were performed at 25°C. Empty plasmid was used as a control, as suggested by Conte and Mello (19).

Quantitative expression analyses. Total RNA was isolated from approximately 100 gonads dissected from 1-day-old adult animal hermaphrodites using TRIzol (Invitrogen) and purified via chloroform extraction and isopropanol precipitation. Then, 100 ng of total RNA was treated with DNase I (New England BioLabs) and reverse transcribed into cDNA using Im-Prom II reverse transcriptase (Promega) and oligo(dT) primers. Quantitative reverse transcription-PCR (qRT-PCR) was performed with primers designed specifically for each gene using the Primer Express, version 2.0, program (Applied Biosystems). Amplification and detection were performed using an ABI Prism 7000 detection system (Applied Biosystems) with the Power SYBR green PCR master mix (Applied Biosystems). γ-Tubulin (*tbg-1*) was used as an external control. The test gene threshold cycle (C_T) values were normalized to *tbg-1*. The C_T values and relative expression levels were derived using the comparative C_T method (20). The primers used in this study were as follows: for *tbg-1*, 5′-CCTG TTGTCGATCCAAATGA-3′ and 5′-AACCCGAGAAGCAGTTGAAA-3′; for *ced-3*, 5′-CGGAGTTCCTGCATTTCTTC-3′ and 5′-CGGAGTTC CTGCATTTCTTC-3′; for *ced-4*, 5′-AATTCTCGAGCAGCGTCTTC-3′ and 5′-ATGAACGACGGAATTTTTGG-3′; for *ced-9*, 5′-AAACGGAAT GGAATGGTTTG-3′ and 5′-AATTTTCCGCGTGCTTCTT-3′; for *lin-35* 5′-ACTGGAATTCCGTCCACTTG-3′ and 5′-TCCGCTCATCAATACT TCCA-3′; for *dpl-1*, 5′-AGCCACATCAAGTGCAACAG-3′ and 5′-ACC ACCTGGAGCAACAATTC-3′; for *efl-1* 5′-GGCAAAAGCGACGAATCT AC-3′ and 5′-CCGCCTTTCCACTGTATCAT-3′; and for *efl-2* 5′-AGCA ATCACTTGGGCTCATT-3′ and 5′-ACGCGTCGTTTCGAGATATT-3′.

Polysomal profiles. Polysomes and polysomal RNA were obtained from N2 1-day-old animals. Control animals or animals starved for 6 h were grown at 20°C on egg plates, collected in M9 medium, and crushed in liquid N_2. Liquid N_2-frozen pellets equivalent to 0.5 g of animals were homogenized in four volumes of lysis buffer (300 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM EGTA, 200 mg of heparin/ml, 400 U of RNasin/ml, 2.5 mM phenylmethylsulfonyl fluoride, and 0.2 mg of cycloheximide/ml). The lysate was centrifuged at 27,000 × *g* at 4°C for 15 min, and 0.5-ml aliquots of the supernatant were loaded onto an 11-ml 15 to 60% sucrose gradient in gradient buffer (140 mM NaCl, 25 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.2 mg/ml cycloheximide) and centrifuged in a Beckman SW41Ti rotor at 38,000 rpm at 4°C for 2 h. The gradients were then fractionated with continuous monitoring at an absorbance of 260 nm, and 1-ml fractions were collected.

RNA isolation from sucrose gradients and RT-PCR. For RNA isolation from the sucrose gradients, each fraction was treated with 25 μl of a 10% solution of sodium dodecyl sulfate (SDS) and 1 μl of 10 mg/ml proteinase K at 37°C for 30 min, followed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and chloroform-isoamyl alcohol (24:1). The samples were precipitated with an equal volume of isopropanol and LiCl (1.5 M final concentration). For each sample, 2 μg of RNA was treated with DNase I (New England BioLabs) and then subjected to reverse transcription using oligo(dT) primers and Im-Prom II reverse transcriptase (Promega). Equal amounts of cDNA were used as templates for gene-specific PCR with the appropriate primers. The primers used were the following: for *act-5*, 5′-CGCCTCCTCCTCCTCCCTC-3′ and 5′-GGTAG GTGGTCTCGTGGATTCC-3′; for *glh-4*, 5′-GGAATTCTTGCTTTCTT GATACCATTC-3′ and 5′-GGGGTACCCTAAACAGCACAGTTTG-3′; for *ced-3*, 5′-GAGCAAGATCTCGTTCTCGATCG-3′ and 5′-GTCGAAA ACACGGCTTATGGTTG-3′; for *ced-4*, 5′-GCCGATGCCTGTTGGAGA AAAA-3′ and 5′-CCTATTTCAAGAAGACGCTGCTCG-3′; for *ced-9*, 5′-GGAATTCCCGATATTCGAGAAGAAGCACG-3′ and 5′-GGGTACCC CTTACTTCAAGCTGAACATCATC-3′; for *lin-35*, 5′-CGGAATTCCGT GATAATTAAACTCGTACTC-3′ and 5′-GAAGATCTTCTCGTTTTTG TGGAC-3′; for *dpl-1*, 5′-TCTATGAACCTCTCACAAGCCCAA-3′ and 5′-AGACAACTCTCTGTTGCACTTGATG-3′; for *efl-1*, 5′-GGCACACA AGTTCGGCTCAGTGA-3′ and 5′-CATCATCATTGATCGTCCGCCG-3′; for *efl-2*, 5′-GTCAAATCTCACGCGTTGCTCTT-3′ and 5′-GTTTCC AAATCCTCGGTATCAATT-3′.

Relative nonpolysomal (NP) RNA or polysomal (P) RNA content was calculated as NP RNA/(NP RNA + P RNA) or P RNA/(NP RNA + P RNA), respectively, and was determined from three independent biological samples (21).

Western blot analysis. For the Western blot analysis, 100 hermaphrodites from the indicated backgrounds were grown at 20°C or 25°C and collected and washed in phosphate-buffered saline (PBS). For protein extraction, the samples were boiled for 5 min and centrifuged at 13,000 rpm for 1 min, and the resulting supernatant was loaded onto 10% or 8% polyacrylamide gels for CED-3, CED-4, CED-9, or LIN-35 protein detection. The gels were transferred onto an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) according to standard procedures. The blots were initially incubated using commercial antibodies for CED-3 (1:500) (Cc-20, sc-9192; Santa Cruz Biotechnology), CED-4 (1:750) (Cn-21, sc-9193; Santa Cruz Biotechnology), CED-9 (1:500) (ce-280, sc-33737; Santa Cruz Biotechnology), or LIN-35 (1:500) (cN-18, sc-9273; Santa Cruz Biotechnology). The secondary antibody was horseradish peroxidase-conjugated (HRP)-conjugated donkey anti-goat antibody (1:10,000) (Santa Cruz Biotechnology) for CED-3, CED-4, and LIN-35 and HRP-conjugated goat anti-rabbit antibody (1:1,000) (Pierce) for CED-9. Bound antibody was detected using a Super Signal West Pico Chemiluminescent Substrate kit (Pierce). Anti-CED-3 antibody detected several bands in the Western blot analyses. Although all of the bands were reduced after *ced-3* silencing, only one band was absent, which corresponded to the predicted molecular mass of CED-3 (56.6 kDa) (see Fig. 3A). Silencing of *ced-3* was also evaluated by RT-PCR, which showed a 70% decrease in its mRNA (see Fig. 5C). Using anti-CED-4 antibody, only one band was consistent with the predicted molecular mass of CED-4 (62.9 kDa). This band was absent in protein extracts from *ced-4*(*n1162*) mutant animals (see Fig. 3B). Using anti-CED-9 antibody, we detected several bands that were completely absent in the homogenates obtained from *ced-9*(RNAi) animals (see Fig. 3C). However, only one was consistent with the predicted molecular mass of CED-9 (32 kDa), suggesting that the smaller bands were either modifications of CED-9 or its degradation products. Silencing of *ced-9* was also evaluated by RT-PCR, and we observed an 80% decrease in its mRNA (see Fig. 5C). Anti-LIN-35 antibody detected several bands in our experiments; however, only the 110.9-kDa band was absent in the *lin-35*(*n745*) mutant protein extracts (see

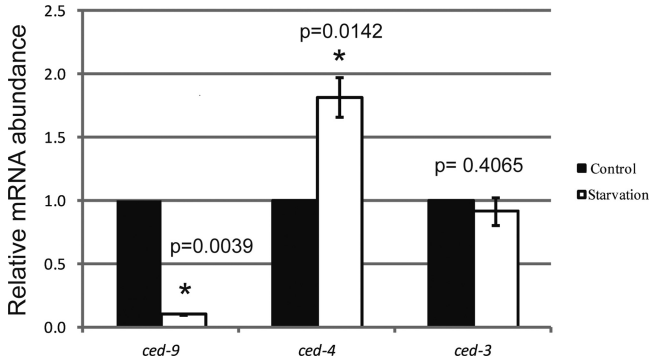

FIG 1 *ced-9* mRNA is downregulated during starvation. qRT-PCR analysis of the indicated genes from the gonads of well-fed (control) or 6-h-starved 1-day-old wild-type animals. The graph shows the average relative abundances of each mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. The *P* values were determined using a paired and nonparametric test (Wilcoxon). Significance is indicated by an asterisk ($P < 0.05$).

Fig. 4C). A monoclonal antibody raised in mouse against α-tubulin (T9026; Sigma) was used as a loading control. The ImageJ software program was used to quantify the bands.

Bioinformatics analysis of differentially translated mRNAs. Sequences of the 3′ untranslated region (UTR) were obtained from the UTRome.org database (22). The free folding energy was calculated using DINAMelt (23). The open reading frames (ORFs) and transcript lengths were obtained from WormBase, version WS236.

RESULTS

***ced-9* expression is downregulated during starvation.** The exposure of 1-day-old wild-type (wt) adult animals to 6 h of bacterial deprivation triggers germ cell apoptosis via an unknown mechanism. The gonads of 6-h-starved animals appear healthy despite their increased germ cell apoptosis (3). To investigate the mechanisms that trigger starvation-induced apoptosis in the *C. elegans* gonad, we studied the expression of genes that encode the core apoptotic machinery under starvation conditions. Extracted RNA from the extruded gonads of well-fed or 6-h-starved 1-day-old wt animals was used for quantitative real-time reverse transcription-PCR (qRT-PCR) for *ced-9*, *ced-4*, and *ced-3*. We found that after 6 h of starvation, *ced-9* mRNA expression was considerably reduced (10-fold), *ced-4* mRNA was on average higher (1.8-fold), and *ced-3* mRNA was not affected (Fig. 1). These data show that during starvation, the adjustments in the expression of genes encoding core apoptotic machinery corresponded with the initiation of apoptosis.

Starvation slows translation. The mRNA translation rate is commonly affected during stress (24, 25), but it has not been well studied in *C. elegans*. Thus, we examined the translational status during starvation using polysomal profiling in 0.5 g of well-fed or 6-h-starved 1-day-old wt adult animals (see Materials and Methods). The polysomal profiles were divided into three regions according to the absorbance at 260 nm (mainly ribosomal RNAs 18S and 28S): translationally inactive, which included mostly untranslated mRNAs present in the mRNPs (TI; fractions 1 to 4); monosomes (M, fraction 5); and translationally active (TA; polysomes; fractions 6 to 11) (Fig. 2A and B). TA fractions were further subdivided into light polysomes (LP; fractions 6 to 8) and heavy polysomes (HP; fractions 9 to 11) (Fig. 2A and B). Polysomal profiling showed that under conditions of starvation, there was an overall reduction in the number of polysomes bound to mRNAs and a slight increase in the monosomal fraction, suggesting that starvation reduces overall translation (Fig. 2A and B).

To determine how translation is affected under starvation, we analyzed nonpolysomal (NP) and polysomal (P) RNA levels as previously reported (21). The nonpolysomal RNA included mRNP complexes of <80S (40S and 60S ribosomal subunits) and 80S monosomes (fractions 1 to 5), and the polysomal RNA included mRNAs with two or more ribosomes (fractions 6 to 11). The relative proportion of RNA in NP and P complexes was determined after the precipitation of RNA from the mentioned fractions. The P RNA content in starved animals (0.26 ± 0.02) was significantly lower ($P < 0.0001$) than that determined for animals grown under control conditions (0.63 ± 0.01). Furthermore, the NP content of starved animals (0.74 ± 0.02) was significantly higher ($P < 0.0001$) than that of control animals (0.37 ± 0.01), which is consistent with reduced translation during starvation (Table 1).

To test whether the reduced translation during starvation affected housekeeping genes, we analyzed the polysomal distribution of *act-5* mRNA, which encodes actin (26). Under control conditions, 16% of the *act-5* mRNA was present in the TI and M fractions (fractions 2 to 5), while 84% was enriched in the polysomal fractions (fractions 6 to 11). During starvation, we detected an enrichment of *act-5* mRNA (32%) in TI and M fractions (fractions 2 to 5), while 60% remained associated with polysomal fractions 6 to 11 (Fig. 2C). Although *act-5* mRNA was more associated with TI fractions during starvation, its presence in the TA fractions persisted, although at a lower level, indicating that the reduced translation induced by starvation affected this housekeeping gene.

Sucrose density gradients of extruded gonad extracts are difficult to use because *C. elegans* are very small (1 mm in length), and a fair number of animals are required for polysomal profiling. To overcome this technical difficulty, we determined whether a decrease in translation could occur in the gonad by examining a germ line-specific mRNA, *glh-4* (27). In well-fed animals, most of the *glh-4* mRNA (73%) was present in the TA fractions (fractions 7 to 11) (Fig. 2D). However, when the animals were starved, only 28% of the *glh-4* mRNA remained associated with these fractions (Fig. 2D). Furthermore, we observed a shift in *glh-4* mRNA to a lighter polysomal fraction (fraction 6; 17%), the M fraction (fraction 5; 15%), and the TI fractions (fractions 2 to 4; 40%) during starvation (Fig. 2D). These results demonstrate that the decrease in the translation during starvation occurs not only in somatic tissues but also in the germ line.

Next, we examined the polysomal distribution of *ced-9*, *ced-4*, and *ced-3* mRNAs. Under control conditions, 74% of the *ced-9* mRNA was present in TA fractions (fractions 7 to 11) (Fig. 2E). *ced-9* mRNA was not abundant during starvation (Fig. 1); however, the small amount that was present was severely affected, and only 5% of this transcript remained associated with fractions 7 to 11. Most of its mRNA was enriched in lighter polysomes (fraction 6; 72%), the M fraction (fraction 5; 7%), and the TI fractions (fraction 2 to 4; 16%) (Fig. 2E). These data show that the observed decrease in *ced-9* mRNA translation was consistent with the reduction in the overall translation during starvation.

Regarding the translation of *ced-4* mRNA, we found that under control conditions, 73% of this mRNA was present in TA fractions

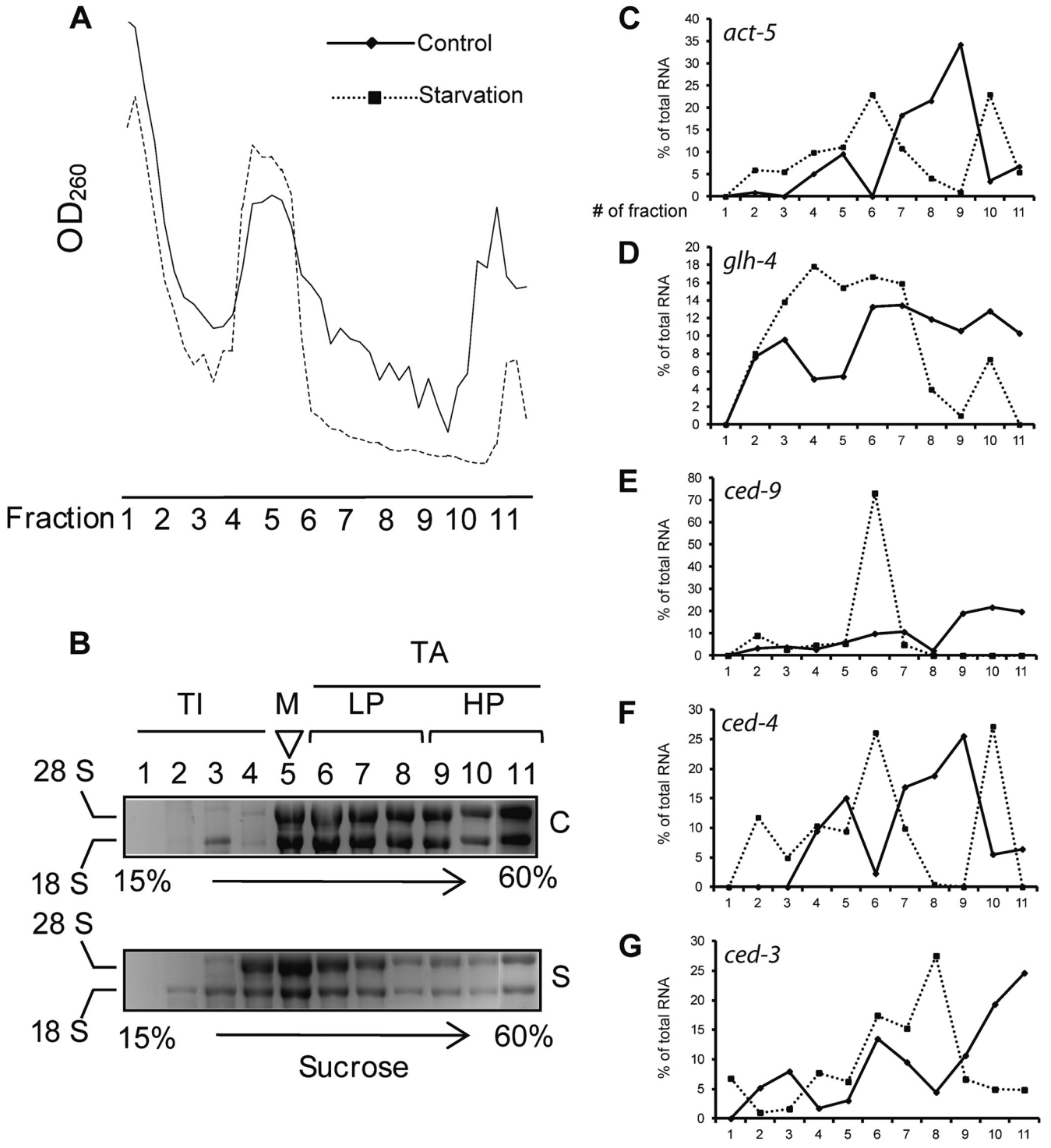

FIG 2 Starvation induces a decrease in translational rate in *C. elegans*. (A) A profile from the polysomal fractionation of 15 to 60% sucrose gradients of the extracts obtained from well-fed (control) or 6-h-starved 1-day-old wild-type animals as recorded at A_{260}. The profiles were divided into three principal regions: fractions 1 to 4, translationally inactive mRNPs (TI); fraction 5, monosomes (M); and fractions 6 to 11, translationally active (TA). Fractions 6 to 11 were in turn divided into fractions 6 to 8, which consisted of light polysomes (LP), and fractions 9 to 11, which consisted of heavy polysomes (HP). (B) rRNA fraction analyses. RNA from control (C) and starved (S) animals was extracted for each fraction and subjected to electrophoresis on 1% agarose gels. The arrows indicate the sucrose density gradient. Fraction 11 was loaded in a different gel under the same conditions because the lack of space. (C to G) Polysomal distribution of the indicated mRNAs. RNA was extracted from each fraction, and cDNA was synthesized to perform PCR on the indicated gene. The relative mRNA levels were calculated from RT-PCR using densitometry. The graphs show the percentage of mRNA present in each fraction from the total amount of mRNA (sum of all fractions). OD_{260}, optical density at 260 nm.

TABLE 1 Relative RNA content in NP and P complexes

Condition	Relative RNA content[a]	
	NP fraction	P fraction
Control	0.37 ± 0.01	0.63 ± 0.01
Starvation	0.74 ± 0.02	0.26 ± 0.02
P value	<0.0001	<0.0001

[a] The relative RNA content (total = 1) represents the average ± standard deviation from three independent biological samples. Relative nonpolysomal RNA (NP) or polysomal RNA (P) contents were calculated as NP RNA/(NP RNA + P RNA) and P RNA/(NP RNA+P RNA), respectively. The *P* value from each column was determined using an unpaired *t* test (Mann-Whitney).

(fractions 7 to 11). In contrast, only 38% of this mRNA remained associated with the same fractions during starvation (Fig. 2F). Furthermore, *ced-4* mRNA was enriched in lighter polysomes (fraction 6; 26%), monosomes (fraction 5; 9%), and TI fractions during starvation (fractions 2 to 4; 27%) (Fig. 2F). We conclude that the *ced-4* mRNA was also affected by the decreased translation rate observed during starvation.

We observed that 55% of *ced-3* mRNA was associated with HP fractions (fractions 9 to 11) under control conditions. In contrast, during starvation it was mostly associated with lighter polysomal fractions (fractions 6 to 8; 60%) (Fig. 2G), indicating that although starvation impaired *ced-3* mRNA translation, this impairment was less extreme than the mRNA shifting observed for *ced-9* and, to a lesser extent, *ced-4*.

Thus far, we have shown that starvation induces a reduction in the translation of *C. elegans*, which includes mRNAs that encode the housekeeping gene *act-5*, the germ line-specific gene *glh-4*, and apoptosis machinery genes *ced-9* and *ced-4*. However, *ced-3* mRNA continues to be translated under starvation.

CED-9 protein is undetectable during starvation. We investigated whether changes that occurred during starvation at the mRNA expression and translational levels reflected the accumulation of CED-3, CED-4, and CED-9 proteins using Western blot analyses. Extracts from 100 well-fed or 6-h-starved hermaphrodites of the indicated genetic backgrounds were used for Western blot analyses. The specificity of each antibody was tested using mutant or RNAi animals (see Materials and Methods).

Under starvation conditions, CED-3 did not show a change in its protein levels based on its procaspase molecular mass (56.6 kDa) (28, 29) (Fig. 3A). This result was consistent with our earlier observations of the presence of *ced-3* mRNA in polysomal fractions and no changes in its mRNA expression (Fig. 1 and 2G). In the case of CED-4, we observed that its protein level did not seem to change during starvation (Fig. 3B). We believe that the reduced *ced-4* mRNA translation efficiency during starvation (Fig. 2F) was most likely compensated for by the higher level of mRNA accumulation or increased mRNA stability (Fig. 1). Another possible explanation is that the CED-4 protein is stable and maintains its levels during starvation.

In contrast, CED-9 protein was dramatically reduced as a consequence of starvation (Fig. 3C). This result was consistent with the decrease in *ced-9* mRNA expression (Fig. 1) and translational rate (Fig. 2E) under starvation. We concluded that although CED-3 and CED-4 proteins do not show significant changes in their expression levels during starvation, the accumulation of the antiapoptotic protein CED-9 dramatically decreases under this condition. We suggest that the decreased expression of CED-9 under starvation could trigger apoptosis.

LIN-35 promotes starvation-induced apoptosis via negative regulation of *ced-9* expression. We decided to study how previously demonstrated *ced-9* negative regulation in response to starvation is achieved. In *C. elegans*, LIN-35 promotes physiological germ cell apoptosis by repressing *ced-9* expression (9). Thus, we studied *lin-35* expression during starvation in extruded gonads. Using qRT-PCR, we found that *lin-35* mRNA expression was considerably increased (2.6-fold) in the gonads of starved animals (Fig. 4A).

Using polysomal profiles, we observed that under normal conditions, most *lin-35* mRNA was recruited to the TA fractions (fractions 6 to 11; 72%). Compared with other mRNAs (*act-5*, *glh-4*, and *ced-9*) (Fig. 2), it is noticeable that a substantial amount of this mRNA remained associated with polysomes under starvation conditions (fractions 6 to 11; 63%) (Fig. 4B). This indicates that *lin-35* continues to be efficiently translated during starvation despite the overall reduction in translation. In addition, while LIN-35 was not detected by Western blotting in protein extracts from control animals, it was observed under starvation conditions (Fig. 4C). The combination of these results implies that during starvation, increased LIN-35 accumulation is most likely achieved by increased mRNA expression or accumulation (Fig. 4A) and persistent translation (Fig. 4B).

To test whether LIN-35 accumulation was necessary to achieve the reduced expression of *ced-9* during starvation, we analyzed the abundance of *ced-9* mRNA in extruded gonad extracts from control and 6-h-starved *lin-35*(*n745*) animals using qRT-PCR. *lin-35*(*n745*) mutant animals carry a nonsense mutation that presumably eliminates LIN-35 activity (14). In the experiment shown in Fig. 1, we demonstrated that *ced-9* expression was 10-fold lower in starved wt animals. However, *ced-9* expression did not decrease

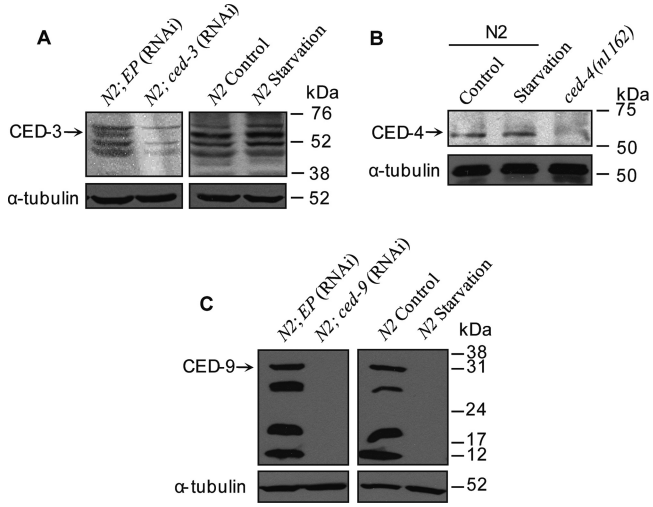

FIG 3 CED-9 protein is dramatically reduced during starvation. Western blot analyses were performed on the whole-animal protein extracts obtained from the indicated genetic backgrounds subjected to control or 6-h starvation conditions. The blots were probed using an anti-CED-3 antibody (A), anti-CED-4 antibody (B), or anti-CED-9 antibody (C). The arrows indicate the CED-3 (56.6 kDa), CED-4 (62.9 kDa), and CED-9 (32 kDa) bands, which correspond to their predicted molecular masses. For each blot, a rabbit antitubulin antibody was used as the loading control. The blots are representative of at least three independent experiments. EP, empty plasmid.

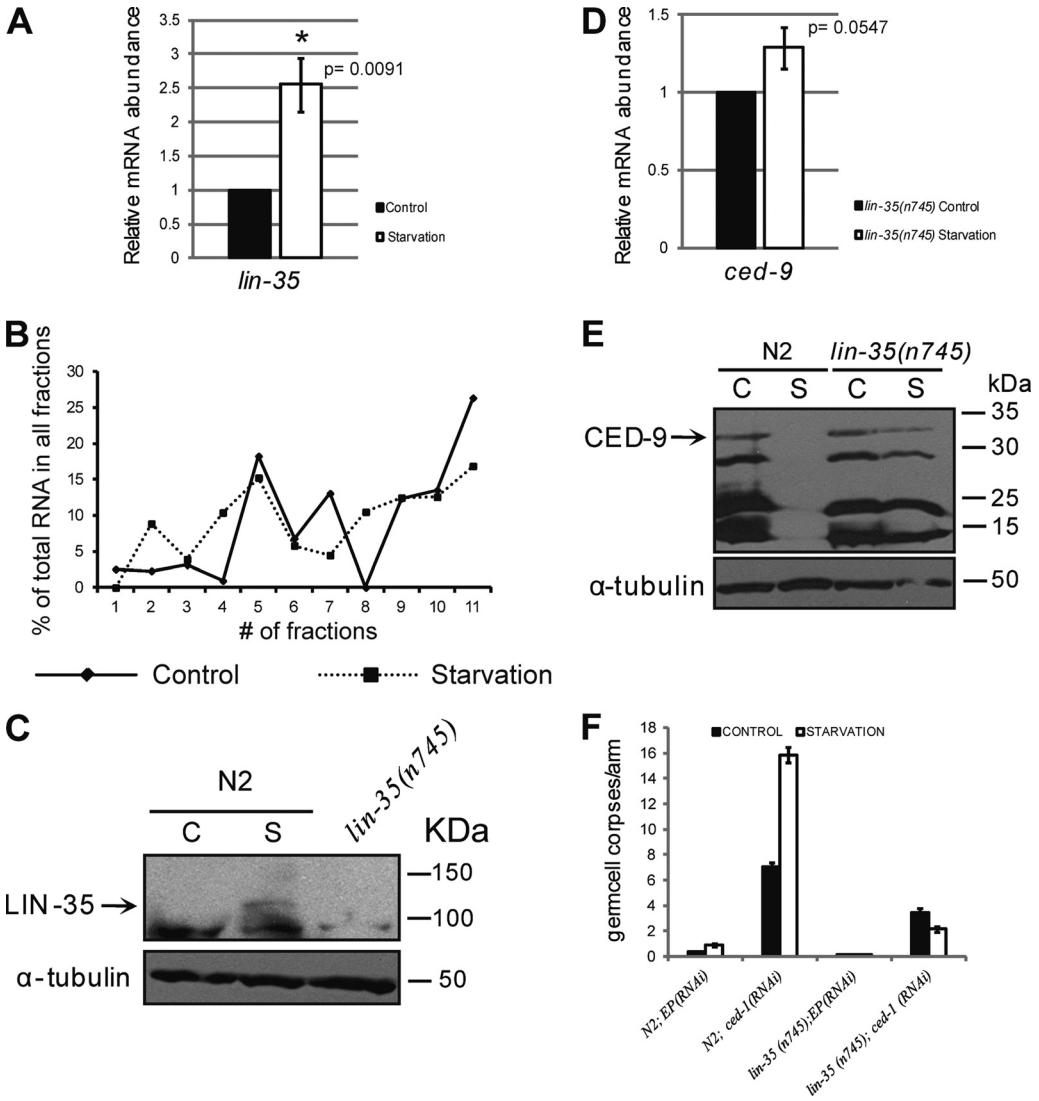

FIG 4 LIN-35 promotes starvation-induced apoptosis by avoiding general translational arrest and downregulating *ced-9* expression. (A) The gonads of 1-day-old well-fed (control) or 6-h-starved wild-type animals were extruded and processed for RNA extraction, followed by cDNA synthesis and qRT-PCR analysis. The graph shows the average relative abundance of *lin-35* mRNA from three independent experiments, each performed in triplicate. The error bars represent standard errors. (B) Polysomal distribution of *lin-35* mRNA using whole-animal extracts from control (C) and 6-h-starved (S) animals, as described in the legend to Fig. 2. (C) Western blot analysis of whole-animal protein extracts from a population of 100 1-day-old wt and *lin-35*(*n745*) animals under control or 6-h starvation conditions. The blot was probed with an anti-LIN-35 antibody. The arrow indicates the predicted molecular mass of LIN-35 (110.9 kDa). A rabbit antitubulin antibody was used as the loading control. The blot is representative of three experiments. (D) qRT-PCR analysis using the cDNA of isolated gonads from *lin-35*(*n745*) animals under normal growth and starvation conditions. The graph shows the average relative abundance of *ced-9* mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. The *P* values were determined using a paired and nonparametric test (Wilcoxon). Significance is indicated by an asterisk ($P < 0.05$). (E) Western blotting using whole-protein extracts prepared from a population of 100 *lin-35*(*n745*) animals subjected to control (C) or starvation (S) conditions. The blot was probed using an antibody generated against full-length *C. elegans* CED-9. A rabbit antitubulin antibody was used as the loading control. The blot is representative of three independent experiments. (F) One-day-old wild-type and *lin-35*(*n745*) animals treated with either an empty plasmid (EP) or *ced-1*(RNAi) were transferred to NGM plates with or without bacteria and incubated at 20°C for 6 h before being mounted and observed under a microscope to detect cell corpses. The graph represents germ cell corpses per gonad arm as a measurement of apoptosis under control and starvation conditions in the indicated genetic backgrounds. The values represent two independent experiments, and the bars show the standard error.

in starved *lin-35*(*n745*) mutant animals (1.3-fold) (Fig. 4D). These results suggest that *ced-9* downregulation in fasting animals depends on LIN-35. In agreement with this hypothesis, CED-9 protein was still detected in the whole-protein extracts from *lin-35*(*n745*) mutant animals under starvation (Fig. 4E) but not in starved wt animals (Fig. 3C and 4E). Therefore, we conclude that LIN-35 accumulation under starvation is required for *ced-9* downregulation.

Next, we tested whether *lin-35* was required to induce germ cell apoptosis upon starvation. To detect cell corpses, we used differential interference contrast optics (DIC) microscopy of *ced-1*(RNAi) animals because germ cells and particularly those produced during starvation-induced apoptosis are not easily spotted in a wild-type background (3). *ced-1* is a gene required for the efficient engulfment of apoptotic germ cells. Therefore, its depletion or deletion results in a dramatic increase in the number of

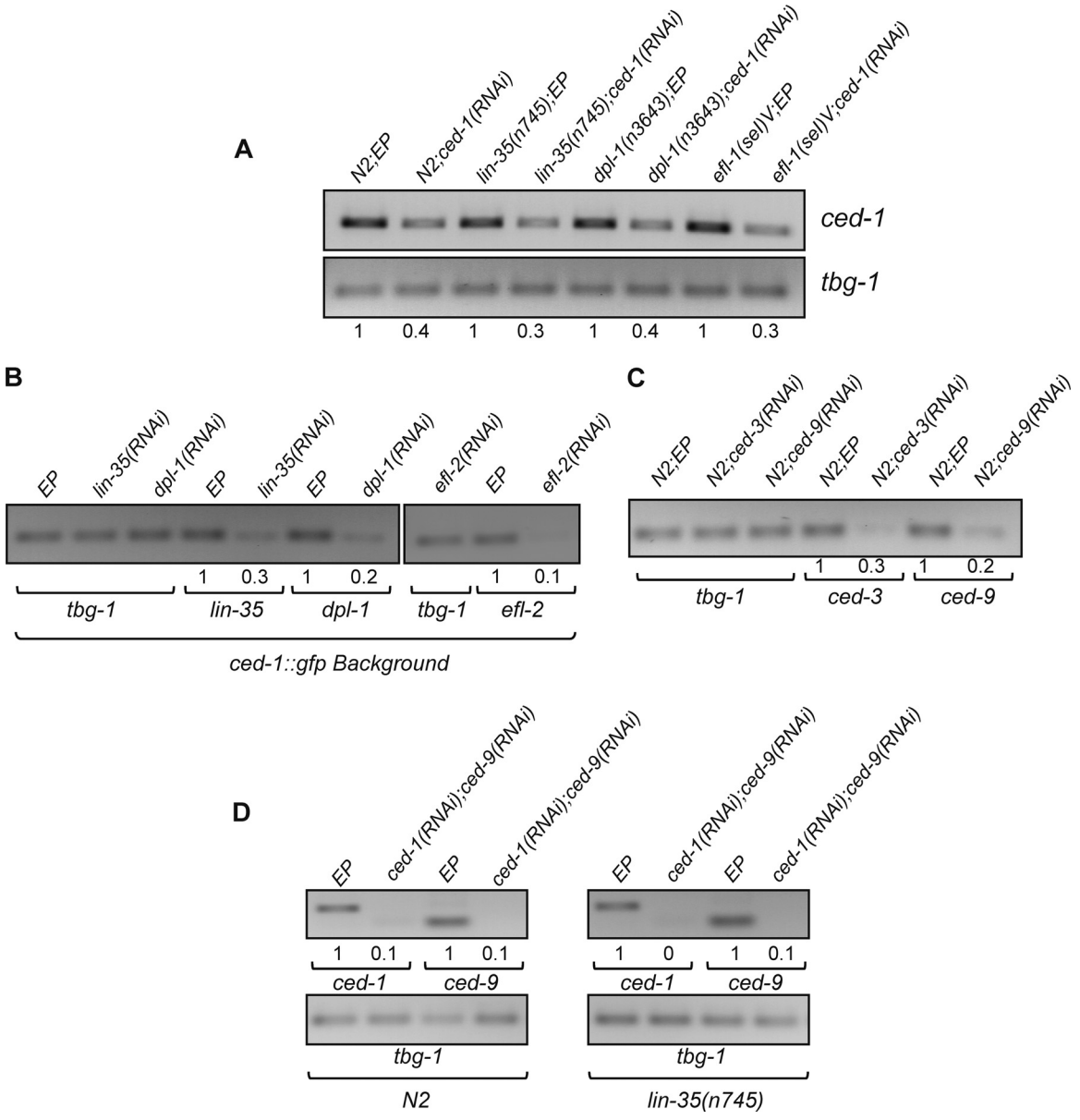

FIG 5 Efficiency of RNAi in the indicated genes and genetic backgrounds. To evaluate gene-silencing efficiency, 1-day-old animals with the indicated genetic backgrounds were subjected to control (EP) or RNAi treatment for the corresponding genes and processed for RNA extraction, followed by cDNA synthesis and PCR analysis. The relative mRNA levels were calculated from RT-PCR using densitometry and are indicated at the bottom of each gel. The gene *tbg-1* was used as a control. (A) The efficiency of *ced-1* silencing was evaluated in N2, *lin-35*(*n745*), *dpl-1*(*n3643*), and *efl-1*(*seI*)*V* backgrounds. (B) The efficiency of *lin-35*, *dpl-1*, and *efl-2* silencing was evaluated in a *ced-1*::*gfp* background. (C) The efficiency of *ced-3* and *ced-9* silencing was evaluated in a wild-type background (N2). (D) The efficiency of *ced-1* and *ced-9* silencing was evaluated in N2 and *lin-35*(*n745*) backgrounds.

apoptotic germ cells that can be easily detected (30). As a control for RNAi, we used an empty plasmid (EP) as previously suggested (19). First, we confirmed that *ced-1* silencing was efficient by RT-PCR in both wt and *lin-35*(*n745*) backgrounds (Fig. 5A). Germ cell corpse averages in *ced-1*(RNAi) animals under normal growth conditions were similar to those observed in 1-day-old *ced-1*(*e1735*) animals (3) (Fig. 4F and Table 2). N2 animals treated with EP or *ced-1*(RNAi) showed an increase in the average number of corpses under starvation conditions (2.5- and 2.2-fold, respectively) (Fig. 4F and Table 2). However, *lin-35*(*n745*); EP and *lin-35*(*n745*); *ced-1*(RNAi) animals did not exhibit increased germ cell apoptosis under the same conditions (Fig. 4F and Table 2). These results demonstrated that *lin-35* is required to induce apoptosis under starvation.

To confirm our results, we used the MD701 strain, which is commonly used to detect germ cell engulfment because it carries a construct ($P_{lim\text{-}7}$ *ced-1*::*gfp*) that is expressed in the sheath cells (12). RNAi against *lin-35* in MD701 decreased its mRNA level by 70% (Fig. 5B). We quantified the number of corpses in the gonad of 1-day-old *ced-1*::*gfp* animals treated with EP or *lin-35*(RNAi) in animals that were well fed or starved for 6 h. Unlike *ced-1*::*gfp* control animals, *ced-1*::*gfp*; *lin-35*(RNAi) animals did not increase

TABLE 2 Starvation-induced germ cell death in different genetic backgrounds

Experimental group and treatment[a]	Avg no. of corpses/gonad ± SE[f]		
	Control	Starvation	*P*[g]
lin-35(*n745*) animals[b]			
N2; EP(RNAi)*	0.36 ± 0.05 (103)	0.90 ± 0.11 (83)	0.0002
N2; *ced-1*(RNAi)	7.07 ± 0.31 (109)	15.83 ± 0.61 (108)	<0.0001
lin-35(*n745*); EP(RNAi)	0.13 ± 0.06 (38)	0.13 ± 0.05 (45)	0.9876
lin-35(*n745*); *ced-1*(RNAi)	3.42 ± 0.34 (45)	2.14 ± 0.26 (51)	0.0057
ced-1::*GFP*; *EP*(RNAi)*	6.11 ± 0.21 (135)	13.27 ± 0.36 (133)	<0.0001
ced-1::*GFP*; *lin-35*(RNAi)	3.98 ± 0.20 (41)	3.15 ± 0.17 (40)	0.0002
ced-9(RNAi) animals[c]			
N2; *ced-9*(RNAi)*	3.1 ± 0.18 (21)	3.9 ± 0.21 (21)	0.2102
N2; EP/*ced-1*(RNAi)	5.65 ± 0.23 (40)	12.66 ± 0.34 (34)	<0.0001
N2; *ced-1*/*ced-9*(RNAi)	11.05 ± 0.35 (41)	12.74 ± 0.32 (42)	0.0002
lin-35(*n745*); EP/*ced-1*(RNAi)	3.15 ± 0.15 (40)	2.19 ± 0.16 (31)	0.0013
lin-35(*n745*); *ced-1*/*ced-9*(RNAi)	10.22 ± 0.43 (44)	12 ± 0.48 (44)	0.0048
dpl-1 animals[d]			
dpl-1(*n3643*); EP(RNAi)	0.35 ± 0.08 (37)	0.22 ± 0.06 (37)	0.1962
dpl-1(*n3643*); *ced-1*(RNAi)	3.69 ± 0.27 (42)	3.39 ± 0.21 (49)	0.4304
ced-1::*GFP*; *dpl-1*(RNAi)*	5.76 ± 0.25 (29)	5.33 ± 0.26 (30)	0.2116
efl-1(*seI*)*V* and *efl-2*(RNAi) animals[e]			
efl-1(*seI*)*V*; EP(RNAi)	0.14 ± 0.06 (36)	0.33 ± 0.11 (36)	0.1962
efl-1(*seI*)*V*; *ced-1*(RNAi)	8.47 ± 0.33 (38)	19.9 ± 0.36 (40)	<0.0001
ced-1::*GFP*; *efl-2*(RNAi)*	4.98 ± 0.24 (63)	11.44 ± 0.43 (63)	<0.0001

[a] One-day-old adult animals with different genetic backgrounds were subjected to starvation and observed under Nomarski or fluorescence microscopy to detect corpses (see details in Materials and Methods). EP, empty plasmid. *, controls.
[b] *lin-35*(*n745*) animals did not respond to starvation-induced germ cell apoptosis.
[c] *ced-9*(RNAi) animals did not show evidence of starvation-induced germ cell apoptosis.
[d] DPL-1 is required to induce apoptosis under starvation.
[e] *efl-1*(*seI*)*V* and *efl-2*(RNAi) animals did respond to starvation-induced apoptosis.
[f] The number of observed gonads (one per animal) is shown in parentheses. These values represent at least two independent experiments.
[g] The *P* values were determined using an unpaired *t* test (Mann-Whitney); a *P* value of < 0.0001 was considered significant.

germ cell apoptosis under starvation conditions (Table 2). These data confirm that LIN-35 is required for starvation-induced apoptosis. We should mention that when *lin-35*(*n745*); *ced-1*(RNAi) and *ced-1*::*gfp*; *lin-35*(RNAi) animals were subjected to starvation, we observed a slight decrease in germ cell apoptosis, and we think that this could be due to the *ced-9* upregulation during starvation in a *lin-35*(*n745*) background (Fig. 4D).

Our data so far show that under starvation, LIN-35 is upregulated, its accumulation downregulates *ced-9* expression, and this effect triggers apoptosis. We have two scenarios to test our hypothesis. In the first scenario, one might think that *ced-9* silencing should have germ cell apoptosis comparable to that of the wild type under starvation. Unfortunately, we did not observe that N2 *ced-9*(RNAi) well-fed animals (3.1) have germ cell corpse numbers similar to those of wt starved worms (0.9). We think this is because starvation-induced germ cell apoptosis is not easily detected in a wt background, but it is easily observed in cell corpse-defective mutants or when a germ cell corpse marker like strain MD731 is used (3). Hence, when we used a *ced-1* background, we observed similar germ cell corpse numbers between starved control animals [N2; EP/*ced-1*(RNAi); 12.66 germ cell corpses] and well-fed N2; *ced-1*/*ced-9*(RNAi) worms (11.05 germ cell corpses), which supports our hypothesis.

In a second scenario one might think that to restore starvation-induced germ cell apoptosis in an *lin-35*(*n745*) mutant background, it would be sufficient to silence *ced-9* expression. However, because *ced-9* is required to protect cells from death (31), its absence or silencing triggers apoptosis under any condition. Indeed, when we silenced *ced-9* in wt or *lin-35*(*n745*) animals (90% knockdown efficiency) (Fig. 5D), an increase in physiological germ cell apoptosis was observed under normal growth conditions (8.6-and 3.2-fold, respectively) (Table 2). However, germ cell apoptosis did not increase further after the N2; *ced-1*/*ced-9*(RNAi) animals were exposed to starvation (0.25-fold) (Table 2). In the case of *lin-35*(*n745*); *ced-1*/*ced-9*(RNAi) animals, we observed a slightly and not statistically significant increase in apoptosis (0.17-fold); however, this increase is not comparable to the normal induction of apoptosis under starvation (Table 2). Therefore, *ced-9* silencing triggers germ cell apoptosis independently of the growth conditions or background tested, and under these conditions, starvation-induced apoptosis could not be triggered.

***ced-4* upregulation during starvation is regulated via *dpl-1* and is important for inducing germ cell apoptosis under these conditions.** Earlier, we showed that *ced-4* expression was higher during starvation (Fig. 1). In *C. elegans*, the DP ortholog DPL-1 positively regulates *ced-4*, thereby affecting germ cell apoptosis (9). Thus, we studied *dpl-1* expression during starvation. Using qRT-PCR, we observed that *dpl-1* expression increased considerably (3.6-fold) in the gonads of starved animals (Fig. 6A). Next, we determined the translational level of *dpl-1* mRNA and found that

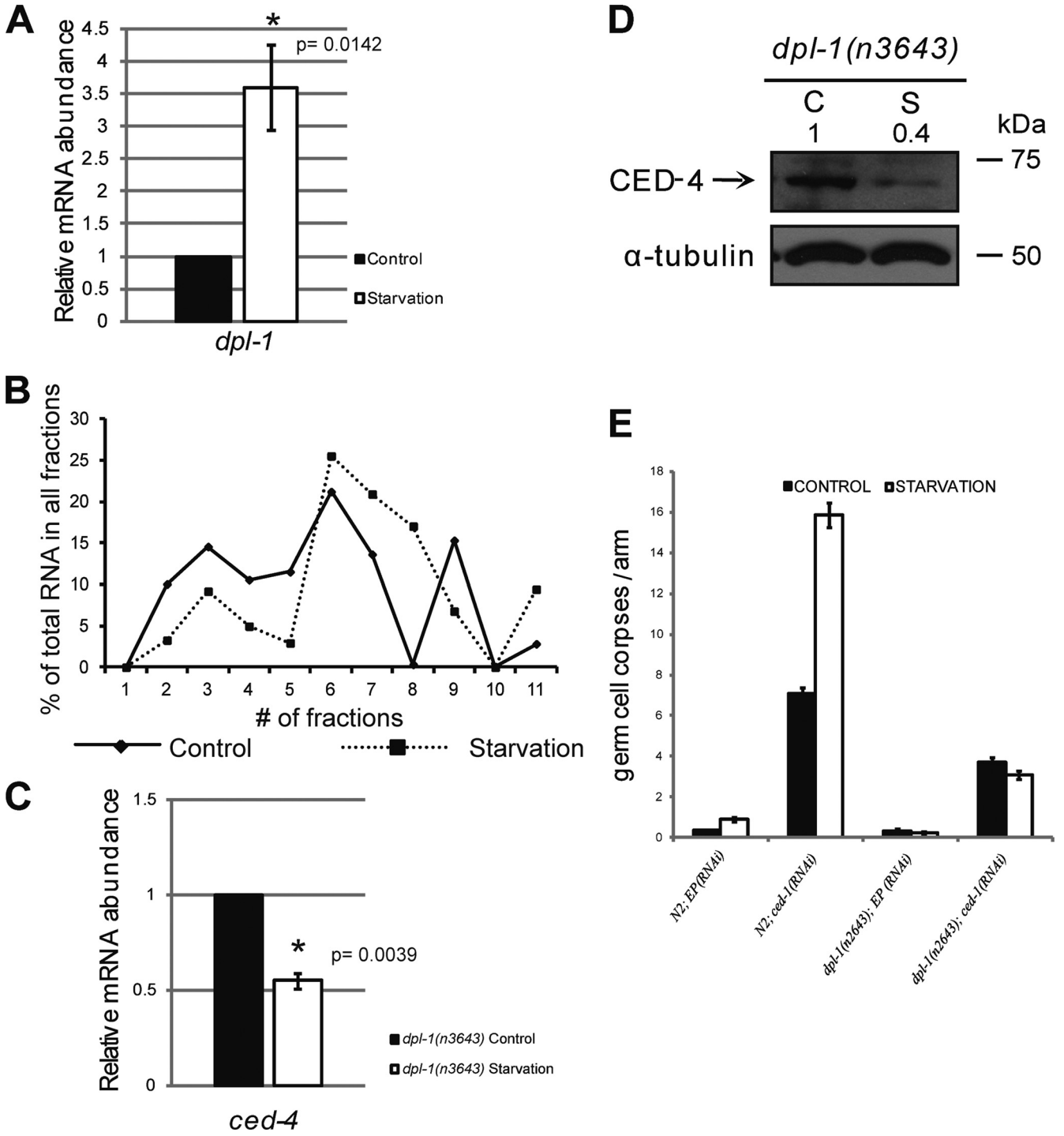

FIG 6 *dpl-1* is required for starvation-induced apoptosis. (A) The gonads of 1-day-old well-fed (control) or 6-h-starved wild-type animals were extruded and processed for RNA extraction, followed by cDNA synthesis and qRT-PCR analysis. The graph shows the average relative abundance of *dpl-1* mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. The *P* values were determined using a paired and nonparametric test (Wilcoxon). Significance is indicated by an asterisk ($P < 0.05$). (B) Polysomal distribution of *dpl-1* mRNA using whole-animal extracts from control (C) or 6-h-starved (S) animals, as described in the legend to Fig. 2. (C) qRT-PCR analysis using cDNA obtained from the isolated gonads of *dpl-1*(*n3643*) animals under normal growth or starvation conditions. The graph shows the average relative abundance of *ced-4* mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. The *P* values were determined using a paired and nonparametric test (Wilcoxon). Significance is indicated by an asterisk ($P < 0.05$). (D) Western blotting using whole-protein extracts prepared from a population of 100 *dpl-1*(*n3643*) animals subjected to control (C) or starvation (S) conditions. The blot was probed using an antibody generated against *C. elegans* CED-4 protein. A rabbit antitubulin antibody was used as the loading control. The blot is representative of three independent experiments. (E) One-day-old wild-type and *dpl-1*(*n3643*) animals treated with an empty plasmid (EP) or *ced-1*(RNAi) were transferred onto NGM plates with or without bacteria and incubated at 20°C for 6 h before being mounted and observed under the microscope to detect cell corpses. The graph represents the germ cell corpses per gonad arm as a measurement of apoptosis under control and starvation conditions in the indicated genetic backgrounds. The values represent two independent experiments, and the bars show the standard error.

under control conditions, 53% of the mRNA was present in TA fractions (fractions 6 to 11), whereas during starvation, 80% of this mRNA was associated with the same fractions (Fig. 6B). We conclude that during starvation, the expression of *dpl-1* is higher, and its mRNA continues to be efficiently translated. Unfortunately, we were unable to measure DPL-1 at the protein level due to the lack of available DPL-1 antibodies.

To determine whether DPL-1 induces *ced-4* expression during starvation, we analyzed *ced-4* mRNA abundance in wt and *dpl-1*(*n3643*) mutant animals using qRT-PCR. In contrast to the wt animals (Fig. 1), *ced-4* expression did not increase during starvation in the *dpl-1*(*n3643*) animals (Fig. 6C). In fact, its expression significantly decreased, suggesting that DPL-1 expression is important for *ced-4* upregulation during starvation.

To further confirm those results, we analyzed CED-4 protein in extracts from *dpl-1*(*n3643*) animals under normal and starvation conditions using Western blot analyses. As previously described, the levels of CED-4 appeared not to be affected under starvation conditions (Fig. 3B), probably due to its higher expression and poorly affected translational efficiency (Fig. 1A and 2F). However, in *dpl-1*(*n3643*) animals, CED-4 protein accumulation decreased by 60% under starvation conditions (Fig. 6D), indicating that CED-4 accumulation is partially dependent upon DPL-1.

We hypothesized that if DPL-1 expression is necessary to maintain CED-4 protein levels, then *dpl-1* should be required to induce apoptosis under starvation conditions. To address this requirement, we examined starvation-induced apoptosis in wt and *dpl-1*(*n3643*) animals in a *ced-1*(RNAi) background. *ced-1* RNAi decreased its mRNA level by 60% in wt and *dpl-1*(*n3643*) animals (Fig. 5A). We found that N2; EP(RNAi) and N2; *ced-1*(RNAi) animals showed starvation-induced apoptosis (Table 2 and Fig. 6E). However, control *dpl-1*(*n3643*) and *dpl-1*(*n3643*); *ced-1*(RNAi) animals did not show apoptosis in response to starvation (Table 2 and Fig. 6E).

To confirm our results, we quantified the number of corpses in 1-day-old *ced-1*::*gfp* animals treated with EP or *dpl-1*(RNAi) under control and starvation conditions. The *dpl-1*(RNAi) silencing efficiency was ~80% in the MD701 strain (Fig. 5B). Control *ced-1*::*gfp* animals increased germ cell apoptosis during starvation, in contrast to *ced-1*::*gfp*; *dpl-1*(RNAi) animals, which were unable to respond (Table 2). These results show that DPL-1 is necessary to induce starvation-induced apoptosis.

We next examined the expression of the E2F-like proteins EFL-1 and EFL-2 because they have been suggested to be positive regulators of *ced-4* expression during physiological apoptosis (9). *efl-1* is expressed in the soma and germ line, while *efl-2* is expressed only in somatic tissues (32). Using qRT-PCR, we observed a decrease in *efl-1* expression (0.6-fold) in the gonads of starved animals (Fig. 7A), while the *efl-2* expression increased considerably (2-fold) during starvation in whole-animal cDNA samples (Fig. 8A).

By polysomal profiling, we found that under control conditions, 66% of the *efl-1* mRNA was present in TA fractions (fractions 6 to 11) (Fig. 7B), whereas under starvation, only 48% remained associated with these fractions (Fig. 7B). Furthermore, we observed an enrichment of *efl-1* mRNA in the TI fractions (fractions 2 to 4) (25% control versus 45% starvation). Similarly, we observed that 60% of *efl-2* mRNA was associated with the TA fractions (fractions 6 to 11) under control conditions, while 48% remained associated with the same fractions during starvation (Fig. 8B). Furthermore, we observed a slight enrichment of the *efl-2* mRNA in fractions 2 to 4 (28% control versus 39% starvation). These results suggest that the translation of *efl-1* and *efl-2* mRNAs was diminished during starvation although not as severely as that of the *act-5*, *glh-4*, or *ced-9* mRNA.

To determine whether *efl-1* and *efl-2* played a role in the increased expression of *ced-4* during starvation, we analyzed the *ced-4* mRNA abundance in *efl-1*(*seI*)*V* mutant and *efl-2*(RNAi) animals. *efl-2* silencing decreased its mRNA level by ~90% (Fig. 8C). Similar to what was observed in the wt (Fig. 1), *ced-4* expression was increased in the gonads of starved *efl-1*(*seI*)*V* animals according to qRT-PCR (1.8-fold versus 1.7-fold) (Fig. 7C). We also observed an increase in *ced-4* mRNA in 6-h-starved N2; EP(RNAi) and *efl-2*(RNAi) animals (1.8-fold) (Fig. 8D). These results show that contrary to the transcriptional regulation model during somatic and physiological apoptosis (9, 33), neither *efl-1* nor *efl-2* mediated *ced-4* upregulation during starvation. Furthermore, similar to the wt, the amounts of CED-4 protein were similar in *efl-1*(*seI*)*V* and *efl-2*(RNAi) animals under control and starvation conditions (Fig. 7D and 8E). Finally, we tested whether *efl-1* and *efl-2* were required to induce apoptosis under starvation. To quantify apoptosis, we silenced *ced-1* on an *efl-1*(*seI*)*V* background, while for *efl-2* we used the MD701 strain. We found that the *ced-1*(RNAi) on an *efl-1*(*seI*)*V* mutant background knocked down *ced-1* as efficiently as in the wt (Fig. 5A). *efl-2* silencing in strain MD701 was also efficient (Fig. 5B). We found that under starvation conditions, *efl-1*(*seI*)*V*; *ced-1*(RNAi) animals showed increased germ cell apoptosis (2.3-fold) (Table 2 and Fig. 7D). However, we did not observed a statistically significant difference between well-fed and starved animals when we used an *efl-1*(*seI*)*V*; EP(RNAi) background because, as we said previously, starvation-induced apoptosis is not easily detected if a germ cell apoptosis tool is not used (3). *ced-1*::*gfp* animals treated with EP or *efl-2*(RNAi) showed similar apoptotic induction levels (2.1- versus 2.3-fold) in germ cell apoptosis when the animals were starved (Table 2 and Fig. 8D). These results demonstrate that EFL-1 and EFL-2 are not required for starvation-induced germ cell apoptosis.

DPL-1, EFL-1, and EFL-2 do not affect CED-9 regulation during starvation. So far, we have observed that *ced-9* and *ced-4* expression levels are regulated during starvation (Fig. 1). According to our results, such regulation is given by *lin-35*/Rb and *dpl-1*/DP (Fig. 4D and E and 6C and D). However, we wanted to determine whether this regulation changed depending on the mutant backgrounds. Therefore, we analyzed the expression of *ced-9* and *ced-4* in all the backgrounds used in this work. Similar to the wt, *ced-9* expression decreased in *dpl-1*(*n3643*), *efl-1*(*seI*)*V*, and *efl-2*(RNAi) animals during starvation (Fig. 9A). Furthermore, Western blot analyses revealed that the CED-9 protein decreased dramatically in these backgrounds (Fig. 9D). These results preclude the participation of any of these proteins in *ced-9* downregulation during starvation.

We analyzed the *ced-4* mRNA abundance in *lin-35*(*n745*) mutants and found that, similarly to control animals, during starvation *ced-4* expression was increased in the gonads of *lin-35*(*n745*) animals (Fig. 9B). Supporting our findings, the amount of CED-4 was similar in *lin-35*(*n745*) mutant animals under starvation (Fig. 9E). We concluded that the *ced-4* expression levels are not affected by *lin-35*/Rb during starvation.

The expression levels of *ced-3* were not affected by starvation in wt (Fig. 1 and 3A) or *efl-1*(*seI*)*V* and *efl-2*(RNAi) animals

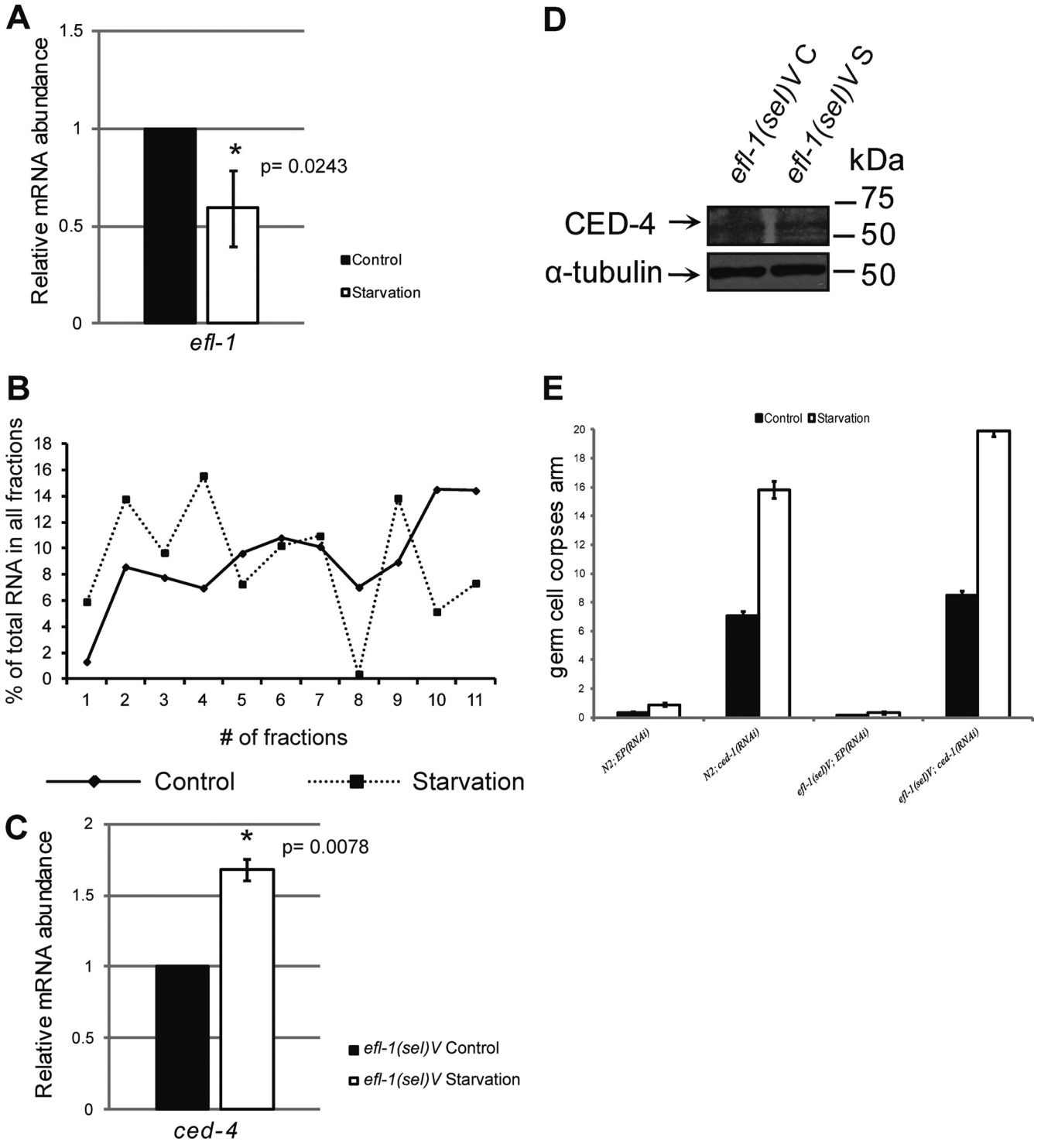

FIG 7 *efl-1* is not required for starvation-induced apoptosis. (A) The gonads of 1-day-old well-fed (control) or 6-h-starved wild-type animals were extruded and processed for RNA extraction, followed by cDNA synthesis and qRT-PCR analysis. The graph shows the average relative abundances of *efl-1* mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. The *P* values were determined using a paired and nonparametric test (Wilcoxon). Significance is indicated by an asterisk ($P < 0.05$). (B) Polysomal distribution of *efl-1* mRNA using extracts from whole control (C) and 6-h-starved (S) animals, as described in the legend to Fig. 2. (C) qRT-PCR analysis using the cDNA of isolated gonads from *efl-1*(*sel*)*V* animals under normal growth and starvation conditions. The graph shows the average relative abundance of *ced-4* mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. The *P* values were determined using a paired and nonparametric test (Wilcoxon). Significance is indicated by an asterisk ($P < 0.05$). (D) Western blotting using whole-protein extracts prepared from a population of 100 *efl-1*(*sel*)*V* animals subjected to control (C) or starvation (S) conditions. The blot was probed using an antibody generated against *C. elegans* CED-4 protein. A rabbit antitubulin antibody was used as the loading control. The blot is representative of three independent experiments. (E) One-day-old wild-type and *efl-1*(*sel*)*V* animals treated with an empty plasmid (EP) or *ced-1*(RNAi) were transferred onto NGM plates with or without bacteria and incubated at 20°C for 6 h before being mounted and observed under the microscope to detect cell corpses. The graph represents germ cell corpses per gonad arm as a measurement of apoptosis under control and starvation conditions in the indicated genetic backgrounds. The values represent two independent experiments, and the bars show the standard error.

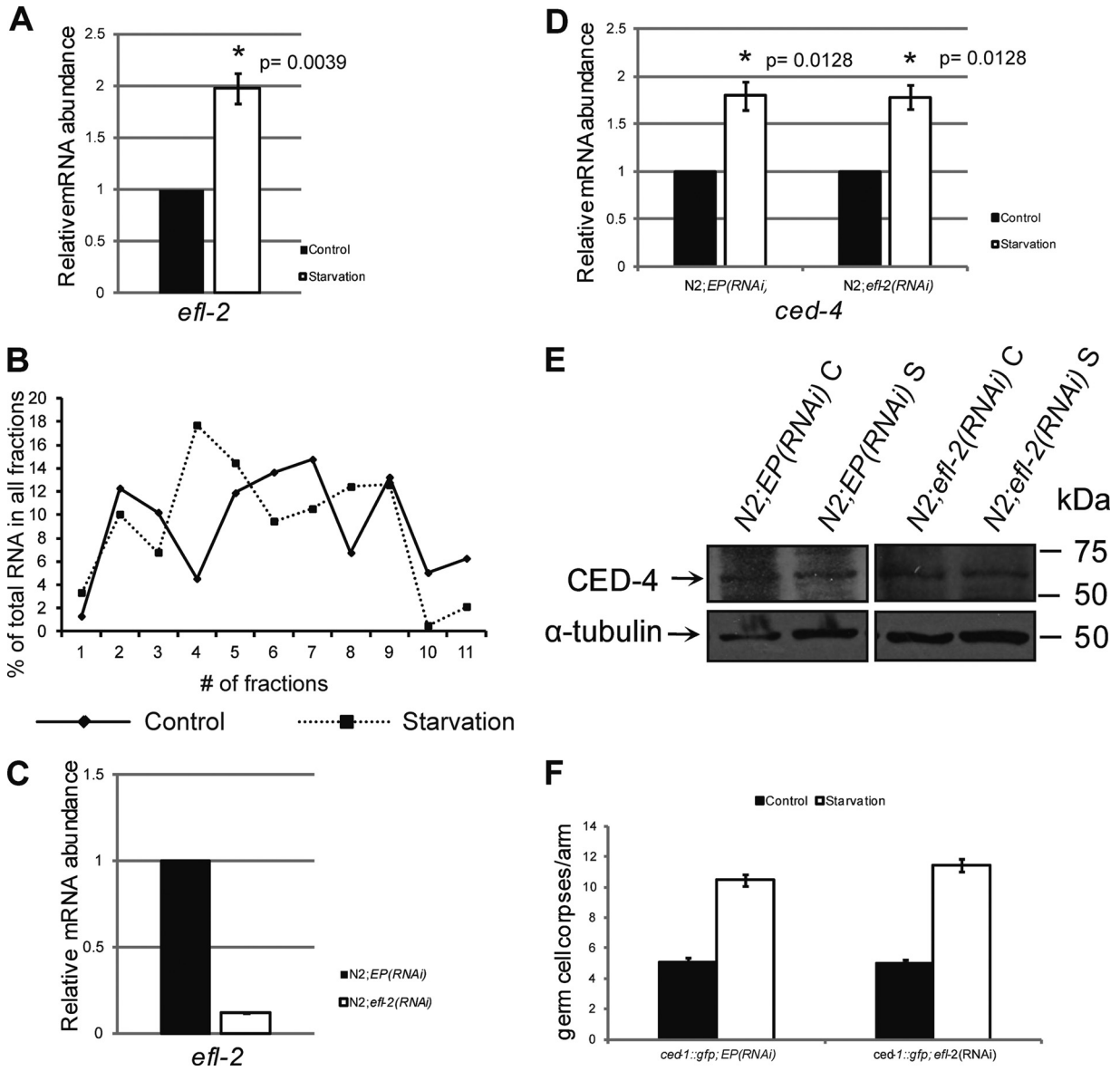

FIG 8 *efl-2* is not required for starvation-induced apoptosis. (A) One-day-old well-fed (control) or 6-h-starved wild-type animals were processed for RNA extraction, followed by cDNA synthesis and qRT-PCR analysis. The graph shows the average relative abundance of *efl-2* mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. The *P* values were determined using a paired and nonparametric test (Wilcoxon). Significance is indicated by an asterisk ($P < 0.05$). (B) Polysomal distribution of *efl-2* mRNA using extracts from whole control (C) and 6-h-starved (S) animals, as described in the legend of Fig. 2. (C) Efficiency of *efl-2* silencing by RNAi. RNAi was performed by feeding in wild-type animals using an empty plasmid (EP) as the control. We extracted total RNA, synthesized cDNA, and performed qRT-PCR for *efl-2* mRNA. The graph shows the average relative abundance of *efl-2* mRNA. (D) qRT-PCR analysis using cDNA obtained from isolated gonads of animals treated with an empty plasmid (EP) or *efl-2*(RNAi) under normal growth and starvation conditions. The graph shows the average relative abundance of *ced-4* mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. (E) Western blotting using whole-protein extracts prepared from a population of 100 EP(RNAi) or *efl-2*(RNAi) animals subjected to control (C) or starvation (S) conditions. The blot was probed using an antibody generated against *C. elegans* CED-4 protein. A rabbit antitubulin antibody was used as the loading control. The blot is representative of three independent experiments. (F) One-day-old *ced-1*::*gfp* animals treated with EP(RNAi) or *efl-2*(RNAi) were transferred onto NGM plates with or without bacteria and incubated at 20°C for 6 h before being mounted and observed under the microscope to detect cell corpses. The graph represents the germ cell corpses per gonad arm as a measurement of apoptosis under control and starvation conditions in the indicated genetic backgrounds. The values represent two independent experiments, and the bars show the standard error.

(Fig. 9C). However, in *lin-35*(*n745*) starved animals, *ced-3* mRNA levels were increased (1.64-fold) (Fig. 9C), whereas in *dpl-1*(*n3643*) animals *ced-3* mRNA decreased its expression (0.55-fold) during starvation (Fig. 9C). These changes in *ced-3* expression were not observed in CED-3 protein accumulation as analyzed by Western blotting (Fig. 9F). Our results suggest that the control of *ced-3* expression does not appear to be a determinant of starvation-induced apoptosis.

DISCUSSION

In mammals, serum deprivation induces the activation of p38 and Jun N-terminal protein kinase (JNK) mitogen-activated protein

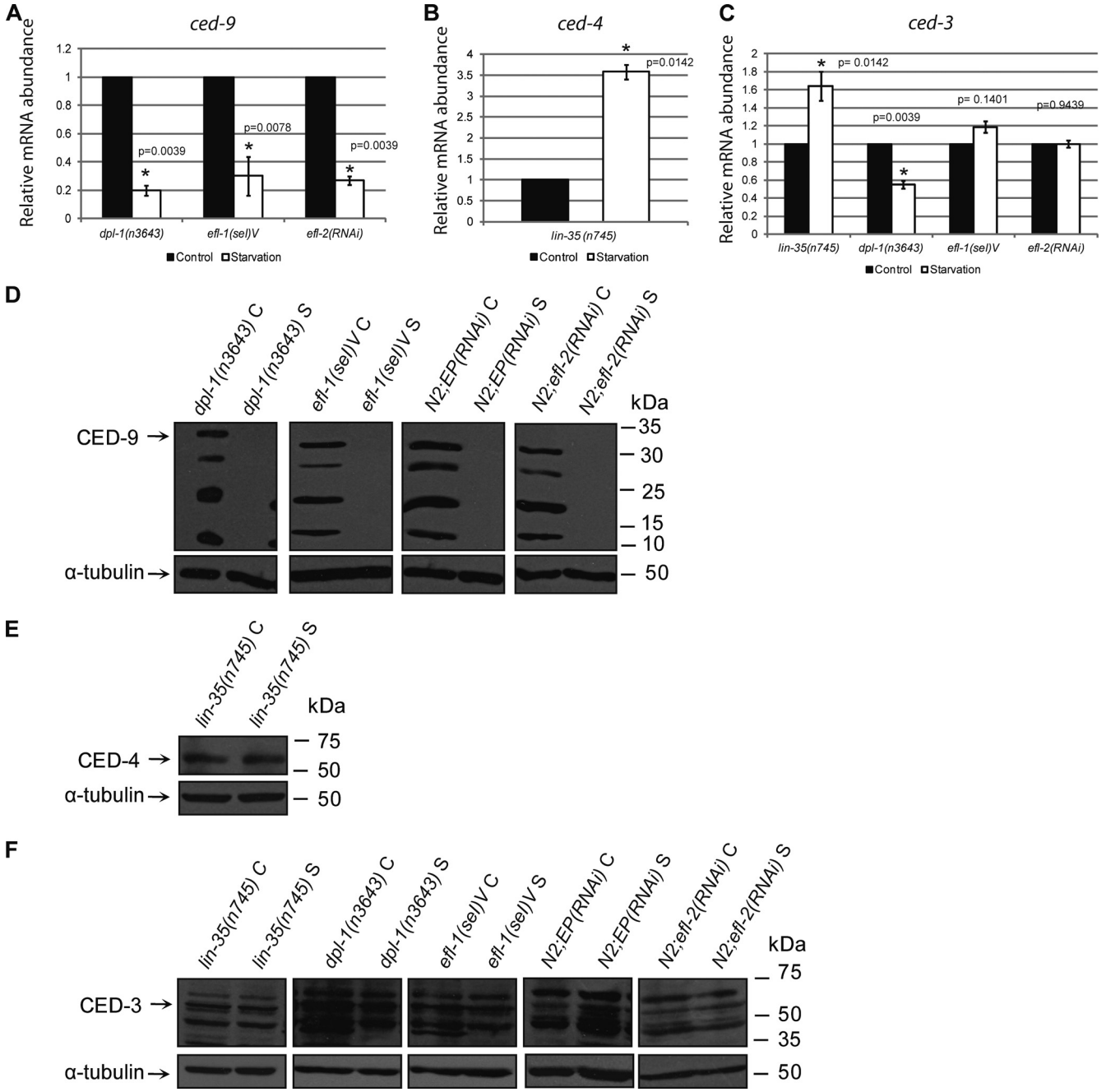

FIG 9 (A to C) qRT-PCR analysis of the indicated genes from the gonads of well-fed (control) or 6-h-starved 1-day-old animals of the indicated backgrounds. The graph shows the average relative abundances of each mRNA from three independent experiments, each performed in triplicate. The error bars represent the standard errors. The values were determined using a paired and nonparametric test (Wilcoxon). Significance is indicated by an asterisk ($P < 0.05$). (D to F) Western blot analyses were performed on the whole-animal protein extracts obtained from the indicated genetic backgrounds under control or 6-h starvation conditions. The blots were probed using an anti-CED-9 antibody (D), anti-CED-4 antibody (E), or anti-CED-3 antibody (F). The arrows indicate the CED-9 (32 kDa), CED-4 (62.9 kDa), and CED-3 (56.6 kDa) bands, which correspond to their predicted molecular masses. For each blot, a rabbit antitubulin antibody was used as the loading control. The blots represent at least two independent experiments. EP, empty plasmid.

kinases to promote cell apoptosis (34, 35). However, starvation-induced germ cell apoptosis does not require the activation of any of these pathways in *C. elegans* (3). In this study, we addressed how starvation triggers germ cell apoptosis. We found that exposing the animals to starvation induced the expression of genes such as *ced-4*, *lin-35*, and *dpl-1*, while *ced-9* expression was downregulated. Upon starvation, we observed a decrease in overall translation that severely affected the translation of *ced-9* and, to a lesser extent, *ced-4*. Under the same conditions, the association of some mRNAs with polysomes, including *lin-35*, *dpl-1*, and *ced-3*, indicated that these transcripts remain efficiently translated in spite of the overall translation reduction. Collectively, these events re-

sulted in the accumulation of LIN-35 after starvation and, consequently, the repression in *ced-9* expression. Thus, we propose that a precisely regulated reduction of CED-9 triggered germ cell apoptosis under starvation conditions.

***dpl-1* upregulation during starvation prevents CED-4 depletion during starvation.** Here, we showed that *ced-4* mRNA was upregulated as a consequence of starvation (Fig. 1). In contrast, the *ced-4* mRNA translation efficiency was diminished under the same conditions (Fig. 2D). As a result of these different responses, the overall accumulation of CED-4 appeared unaffected under starvation (Fig. 3B), which is consistent with the fact that CED-4 is required to induce apoptosis in the nematode (36).

We observed that *dpl-1* expression was increased and that its mRNA continued to be efficiently translated during starvation (Fig. 6A and B). Furthermore, under starvation conditions, *ced-4* expression was decreased in *dpl-1*(*n3643*) animals, which were unable to induce apoptosis (Fig. 6D and E). DPL-1 activates *ced-4* expression under normal growth conditions (9); thus, we propose that DPL-1 is required for starvation-induced apoptosis due to its role in *ced-4* expression upregulation under these conditions. However, the mechanisms by which the expression of *dpl-1* is regulated in the *C. elegans* gonad during starvation remain to be determined.

In mammals, DP associates with an "activating" member of the E2F family consisting of sequence-specific DNA-binding proteins (E2F1, E2F2, and E2F3) (37, 38). E2F1 regulates the transcription of APAF-1 and other proapoptotic genes (39, 40). In *C. elegans*, *dpl-1* can interact with *efl-1* to regulate some cellular processes, such as vulval development (15), the cell cycle (41), and somatic cell death (33). However, in this study, we found that although *efl-1* and *efl-2* suffer transcriptional and translational changes under starvation, they do not participate with DPL-1 in the enhancement of *ced-4* expression and initiation of germ cell apoptosis under starvation (Fig. 7 and 8).

ced-4 expression levels are slightly reduced in the mutant *lin-35*(*n745*) under control conditions (9). However, we found that the change observed in the *ced-4* expression levels during starvation in wt animals (Fig. 1 and 3) was not affected in *lin-35*(*n745*) mutant animals (Fig. 9B and E). These results suggest that *ced-4* expression levels are not regulated for *lin-35* during starvation.

The regulation of CED-3 expression does not seem to contribute to triggering apoptosis during starvation. We observed *ced-3* upregulation in *lin-35*(*n745*) animals and *ced-3* downregulation in *dpl-1*(*n3643*) animals during starvation; however, the amount of CED-3 protein did not change in either background.

Accumulation of LIN-35 during starvation affects *ced-9* expression and induces germ cell apoptosis. The expression of the antiapoptotic gene *ced-9* was significantly decreased at the mRNA and translational levels during starvation (Fig. 1 and 2E). Both events caused a significant decrease in the accumulation of CED-9 protein (Fig. 3C). Under different stress conditions, such as treatment with sodium arsenite or etoposide, Bcl-2 mRNA translation persists due to an internal ribosome entry site (IRES) element (42). Although the polysomal distribution of Bcl-2 mRNA has not been studied in mammals during starvation, prolonged serum deprivation significantly decreases the amount of its protein in cultured cells (43, 44).

Transcriptional initiation of the human Bcl-2 gene occurs via two promoters (P1 and P2) (45). The regulation of these two promoters is complex and dependent upon both the tissue type and the developmental stage (46, 47). Although little is known regarding the regulation of *ced-9* expression in *C. elegans*, microarray-based expression analyses have revealed that this gene is subject to transcriptional repression by LIN-35 (an *Rb* homolog, also known as *Rb1*) (48). Indeed, the downregulation of *ced-9* expression by LIN-35 partially regulates physiological germ cell apoptosis in *C. elegans* (9). Here, we showed that during starvation, *lin-35* expression is upregulated, and its mRNA is refractory to the observed translational inhibition, resulting in the accumulation of its protein (Fig. 4A to C). Furthermore, we demonstrated that LIN-35 accumulation during starvation was necessary for both CED-9 downregulation and germ cell apoptosis induction (Fig. 4D to F). Additionally, CED-9 downregulation during starvation did not require the DPL-1, EFL-1, and EFL-2 proteins that regulate *ced-4* under control conditions (Fig. 9A and D).

How *lin-35* expression is upregulated during starvation and how its mRNA evades the decrease in translation under starvation remains to be elucidated. It is worth highlighting that for *lin-35* mRNA, there is a correlation between the mRNA increase and the protein increase, which should be mediated by an efficient translation. The same is not observed for *ced-4*, in which the mRNA increase is not reflected by a change in protein level, probably due to its impaired translation under starvation. On the other hand, another possibility is that *lin-35* mRNA itself is a target of direct regulation, which will be discussed below.

The contribution of *lin-35*/Rb in starvation-induced apoptosis seems to be exclusively directed for *ced-9* regulation because the expression of CED-4 and CED-3 proteins were not affected in the mutant background *lin-35*(*n745*) during starvation (Fig. 9E and F).

During starvation, the translational efficiency of some mRNAs is reduced. Translational regulation allows organisms to have an immediate and rapid response to changes under physiological conditions. In this study, we showed that starvation decreased translation in *C. elegans* (Fig. 2). In other organisms, such as *Saccharomyces cerevisiae* and *Drosophila melanogaster*, there is an overall reduction in translation initiation due to glucose depletion and the availability of yeast extract in the medium, respectively (49, 50). A reduction of translation initiation decreases the number of ribosomes reinitiating translation on the same mRNA molecule, resulting in a reduced efficiency of translation. In a polysomal profile, such behavior could be observed by shifting of the mRNA from heavy polysomes to lighter polysomes and monosomes (51).

In mammals, different types of stresses trigger a sudden translational arrest, resulting in rapid polysome disassembly (52, 53). One of the most effective translation-inhibitory pathways during stress is mediated by the phosphorylation of eukaryotic initiation factor 2a (eIF2a) (24). The phosphorylation of eIF2a converts the eIF2-GTP-tRNA^{Met} ternary complex into a competitive inhibitor of the GDP/GTP exchange factor eIF2B (54). Although orthologs of eIF2a kinases exist in *C. elegans*, a similar mechanism used to inhibit general translation has not yet been described.

The mechanism that reduces the translation rate in *C. elegans* as a consequence of starvation remains to be elucidated. However, it is likely that the regulation of key translation initiation factor levels serves as a mechanism to modulate the rate of protein synthesis in response to environmental cues. In accordance with this hypothesis, previous studies have shown that IFG-1 (eIF4G) is downregulated during the stress-resistant/growth-arrested dauer state (55) and after exposing the adult animals to starvation for 2

TABLE 3 Characteristics of mRNAs that show differential translation upon starvation

mRNA group and type	3′ UTR length (nt)[a]	3′ UTR fractional GC content	3′ UTR ΔG (kcal/mol)	ORF length (nt)	Transcript length (unspliced + UTR [nt])
Not translationally repressed					
lin-35	309	0.26	−41.8	1,512	7,929
dpl-1	352	0.30	−49	2,886	2,654
ced-3	945	0.34	−165.3	1,797	5,797
Avg ± SEM	535.33 ± 205.21	0.30 ± 0.02	−85.36 ± 40.02	2,065 ± 418.66	5,460 ± 1,532
Translationally repressed					
act-5	296	0.29	−62.4	1,128	1,728
glh-4	297	0.27	−47.5	3,471	5,214
ced-9	459	0.38	−84	843	2,358
ced-4	252	0.27	−17.8	1,873	2,856
efl-1	362	0.28	−41.2	1,029	7,092
efl-2	404	0.44	−134.7	1,239	5,121
Avg ± SEM	345 ± 38.80	0.32 ± 0.03	−64.6 ± 20.42	1,597.16 ± 491.28	4,061.5 ± 1,035.25

[a] nt, nucleotide.

days (56). Furthermore, it appears that IFG-1 plays a decisive role in coordinating apoptotic events because it is a substrate for the caspase CED-3 and might promote a switch from cap-dependent to cap-independent translation (57). Coupled to this role, the translational control of gene expression by specific translation initiation factors, such as eIF4E family members, may provide an important mode of mRNA regulation in *C. elegans* (26, 58). In addition, the loss of IFE-2 (an eIF4E family member) enhances the protection against oxidative stress and extends the life span of *C. elegans* (59). Despite the generally decreased translation under stress conditions or apoptosis, it is common to detect mRNAs that are refractory to translation inhibition or even ones that exhibit stimulated translation (21, 24, 57, 60).

In eukaryotes, both untranslated regions (UTRs) and open reading frames (ORFs) possess valuable information that affects the capacity of mRNA to serve as a protein synthesis template. Regulatory features of the 5′ UTR that can have positive effects on protein production include the IRES elements (61). Although a number of cases highlighting the importance of IRES-mediated translation during stress have been previously described in yeast, *Drosophila*, and mammals (62–64), there has been only one example of a functional IRES element in *C. elegans*: *hsp-3* mRNA (65). Alternative initiation complexes may provide the means to control mRNA translation mechanisms to change cell fate in *C. elegans* (60). For example, an isoform shift between the eiF4G isoforms, IFG-1 (cap dependent), and IFG-2 (cap independent) may alter the balance of translation initiation mechanisms to favor the synthesis of proapoptotic factors in which no IRES element has been observed (60).

Although one-third of the mRNAs in *C. elegans* are not *trans* spliced (66), *trans* splicing is a prevalent mechanism (67) and generates stereotypic 5′ end sequences that replace the entire gene-encoded 5′ UTR in the most extreme cases (68). This mechanism almost always excludes the gene-specific 5′ UTR translational control sequences and may potentially explain why the 3′ UTR-mediated translational control mechanisms are more prevalent in *C. elegans* (69). When translation is inhibited, for example, during the depletion of eIF4G, genes with longer ORFs or mRNA lengths are more efficiently translated (56). However, how the ORF lengths mediate translation under stress conditions remains unclear.

We analyzed the mRNA lengths of the genes examined in this study to determine whether they showed any differences. According to their distribution in the polysomal profiles, we classified the genes into two groups: translationally repressed (*act-5*, *glh-4*, *ced-9*, *ced-4*, *efl-1*, and *efl-2*) and translationally active (*lin-35*, *dpl-1*, and *ced-3*). After examination of their 3′ UTRs, we found that the average 3′ UTR length for translationally active mRNAs was 1.6-fold higher than that of genes that were translationally repressed (Table 3). An examination of their ORFs also revealed a tendency for longer ORF lengths for genes that did not exhibit repressed mRNA translation during starvation (Table 3). An analysis of the GC content showed no significant differences between the two groups; however, the folding energy was increased in the translationally repressed mRNAs (Table 3). Unfortunately, we could not compare the 5′ UTR sequences because among the group of translationally repressed mRNAs, only one mRNA demonstrated a non-splice-leader 5′ UTR. Thus, we concluded that the intrinsic properties of mRNAs, such as their 3′ UTR and ORF lengths, might contribute to their translational control during starvation.

Mechanistic model of starvation-induced apoptosis. Physiological germ line apoptosis occurs during oogenesis and in the absence of stress. It is accomplished by the core apoptotic machinery and is induced via an unknown pathway that is independent of EGL-1 and CEP-1 (Fig. 10A). The *C. elegans* ortholog of the retinoblastoma susceptibility protein Rb (LIN-35) partially induces physiological germ cell apoptosis by downregulating *ced-9* expression (Fig. 10A) (9). In addition, subunits of the E2F transcription factor (DPL-1, EFL-1, and EFL-2) seem to promote physiological germ cell apoptosis by increasing the expression of *ced-4* (9) (Fig. 10A). Heat shock, osmotic, and oxidative stresses require the participation of the MAPKK pathway to trigger apoptosis (3). How the MAPKK pathway induces the apoptosis machinery is unknown (Fig. 10B). DNA damage induces germ cell apoptosis through CEP-1 and EGL-1 (Fig. 10C) (10–12). Furthermore, LIN-35, DPL-1, and EFL-2 regulate DNA damage-induced apoptosis through the control of the expression of unknown target genes that are downstream of *ced-9* and *ced-4* (Fig. 10C) (9). The transcriptional regulation of *ced-9*, *ced-4*, and *ced-3* does not appear to determine DNA damage-induced apoptosis (9) (Fig. 10C).

Here, we show that starvation decreases translation, differen-

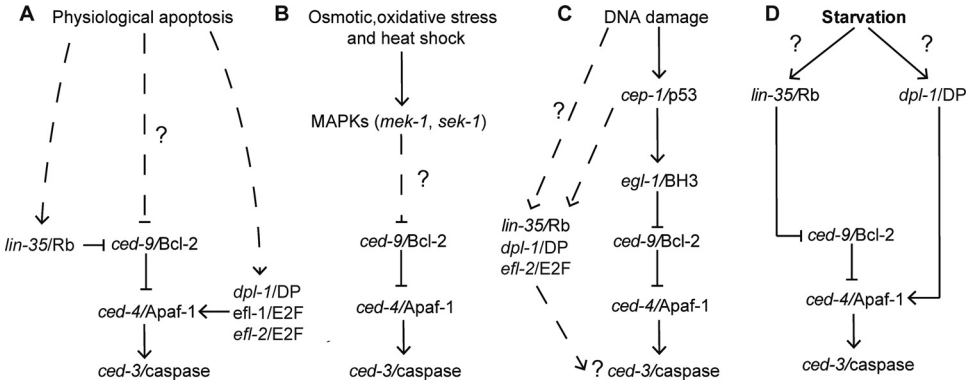

FIG 10 Proposed model for starvation-induced apoptosis. The core apoptosis machinery consists of the antiapoptotic protein CED-9, which inhibits the caspase activator CED-4 and the caspase CED-3. (A) Physiological apoptosis is triggered by an unknown pathway that partially requires *lin-35*/Rb. (B) Apoptosis induced by heat shock and osmotic and oxidative stress is activated by the MAPKK pathway. (C) The genetic pathway for DNA damage-induced germ cell apoptosis requires *cep-1/p53* and *egl-1/BH3*. *lin-35*, *dpl-1*, and *efl-2* function downstream of or in parallel to *cep-1* to cause DNA damage-induced germ cell apoptosis by controlling the expression of unknown target genes. (D) The genetic pathway for starvation-induced germ cell apoptosis involves the genes *lin-35* and *dpl-1*, which block the function of the antiapoptotic gene *ced-9* and enhance the function of the proapoptotic gene *ced-4*, respectively. Please refer to the text for further details. Solid lines represent confirmed genetic interactions. Dashed lines indicated no confirmed genetic interactions. The mammalian homolog for each protein is indicated after the slash. Question marks indicate paths which remain to be elucidated.

tially affecting the core apoptotic machinery genes and some of their regulators. We propose a model in which, LIN-35 is upregulated under starvation conditions to affect the expression of *ced-9* (Fig. 10D). *ced-9* mRNA downregulation, together with the decrease in translation, results in the depletion of CED-9 protein, thereby freeing CED-4 to interact with caspase CED-3 to induce apoptosis. Starvation also induces *dpl-1* upregulation, which in turn promotes *ced-4* accumulation (Fig. 10D). Thus, the balance between the upregulation and translational repression of *ced-4* mRNA results in constant protein levels during starvation, which activates CED-3 to induce apoptosis. During starvation, the transcription of *ced-3* does not change, and its translation is only slightly affected, resulting in the maintenance of the CED-3 protein level, which is required for the induction of apoptosis. Finally, we propose that the combination of LIN-35 and DPL-1 functions to ensure the appropriate expression levels of CED-9 and CED-4 during starvation, which is necessary to initiate starvation-induced apoptosis (Fig. 10D).

Starvation-induced apoptosis shares some regulators with physiological and DNA damage-induced apoptosis. However, there are important differences that suggest that these regulators do not act in the same way. A similarity between physiological and starvation-induced apoptosis is that DPL-1 is important for *ced-4*/Apaf-1 expression. There are two main differences between these pathways: (i) *lin-35*/Rb is completely required to induce apoptosis under starvation while its role in physiological apoptosis is only partial, which might be due to LIN-35 upregulation during starvation; (ii) EFL-1 and EFL-2 do not play a role in *ced-4* expression during starvation-induced apoptosis in contrast to physiological apoptosis.

lin-35/Rb and *dpl-1*/DP are required for DNA damage- and starvation-induced apoptosis while *efl-2*/E2F is required only in DNA damage-induced apoptosis. An important difference between starvation-induced and DNA damage-induced apoptosis is that in the latter *lin-35*/Rb and *dpl-1*/DP targets are still unknown and act downstream of *ced-9* and *ced-4*. Intriguing questions that remain to be solved are how starvation slows translation, how *lin-35* and *dpl-1* mRNAs are upregulated during fasting, and how some mRNAs, particularly *lin-35* and *dpl-1* mRNAs, continue their translation during starvation.

ACKNOWLEDGMENTS

This work was supported by grants from PAPIIT-UNAM (IN225509 and IN207412) and CONACYT-MEXICO (50896-Q and 103856-Q). L.I.L.-L. received a Ph.D. fellowship from CONACYT-México and PAPIIT-UNAM (IN207412). All strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

We thank L. Silvia Salinas, Ana Valeria Martínez Silva, and Carlos Alberto Contreras Paredes for technical assistance. We are grateful to Jorge Ramírez, Lorena Chávez-González, Simón Guzmán-León, and José Luis Santillán-Torres at the Unidad de Microarreglos, Universidad Nacional Autónoma de México, for technical support.

REFERENCES

1. **Angelo G, Van Gilst MR.** 2009. Starvation protects germline stem cells and extends reproductive longevity in *C. elegans*. Science **326:**954–958. http://dx.doi.org/10.1126/science.1178343.
2. **Seidel HS, Kimble J.** 2011. The oogenic germline starvation response in *C. elegans*. PLoS One **6:**e28074. http://dx.doi.org/10.1371/journal.pone.0028074.
3. **Salinas LS, Maldonado E, Navarro RE.** 2006. Stress-induced germ cell apoptosis by a p53 independent pathway in *Caenorhabditis elegans*. Cell Death Differ. **13:**2129–2139. http://dx.doi.org/10.1038/sj.cdd.4401976.
4. **Sulston JE, Horvitz HR.** 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. Dev. Biol. **56:**110–156. http://dx.doi.org/10.1016/0012-1606(77)90158-0.
5. **Sulston JE, Schierenberg E, White G, Thomson JN.** 1983. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Dev. Biol. **100:**64–119. http://dx.doi.org/10.1016/0012-1606(83)90201-4.
6. **Gumienny TL, Lambie E, Hartwieg E, Horvitz HR, Hengartner MO.** 1999. Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. Development **126:**1011–1022.
7. **Horvitz HR.** 2003. Worms, life, and death (Nobel lecture). ChemBioChem **4:**697–711. http://dx.doi.org/10.1002/cbic.200300614.
8. **Lettre G, Hengartner MO.** 2006. Developmental apoptosis in *C. elegans*: a complex CEDnario. Nat. Rev. Mol. Cell Biol. **7:**97–108. http://dx.doi.org/10.1038/nrm1836.
9. **Schertel C, Conradt B.** 2007. *C. elegans* orthologs of components of the RB tumor suppressor complex have distinct pro-apoptotic functions. Development **134:**3691–3701. http://dx.doi.org/10.1242/dev.004606.

10. **Derry WB, Putzke AP, Rothman JH.** 2001. *Caenorhabditis elegans* p53: role in apoptosis, meiosis, and stress resistance. Science **294:**591–595. http://dx.doi.org/10.1126/science.1065486.
11. **Schumacher B, Hofmann K, Boulton S, Gartner A.** 2001. The *C. elegans* homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis. Curr. Biol. **11:**1722–1727. http://dx.doi.org/10.1016/S0960-9822(01)00534-6.
12. **Schumacher B, Schertel C, Wittenburg N, Tuck S, Mitani S, Gartner A, Conradt B, Shaham S.** 2005. *C. elegans ced-13* can promote apoptosis and is induced in response to DNA damage. Cell Death Differ. **12:**153–161. http://dx.doi.org/10.1038/sj.cdd.4401539.
13. **Brenner S.** 1974. The genetics of *Caenorhabditis elegans*. Genetics **77:**71–94.
14. **Lu X, Horvitz HR.** 1998. *lin-35* and *lin-53*, Two genes that antagonize a *C. elegans* Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. Cell **95:**981–991. http://dx.doi.org/10.1016/S0092-8674(00)81722-5.
15. **Ceol CJ, Horvitz RH.** 2001. *dpl-1* DP and *efl-1* E2F act with *lin-35* Rb to antagonize Ras signaling in *C. elegans* vulval development. Mol. Cell **7:**461–473. http://dx.doi.org/10.1016/S1097-2765(01)00194-0.
16. **Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR.** 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β-converting enzyme. Cell **75:**641–652. http://dx.doi.org/10.1016/0092-8674(93)90485-9.
17. **Petrella LN, Wang W, Spike CA, Rechtsteiner A, Reinke V, Strome S.** 2011. synMuv B proteins antagonize germline fate in the intestine and ensure *C. elegans* survival. Development **138:**1069–1079. http://dx.doi.org/10.1242/dev.059501.
18. **Timmons L, Court DL, Fire A.** 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene **263:**103–112. http://dx.doi.org/10.1016/S0378-1119(00)00579-5.
19. **Conte D, Mello CC.** 2003. RNA interference in *Caenorhabditis Elegans*. Curr. Protoc. Mol. Biol. Chapter **26:**Unit 26.3. http://dx.doi.org/10.1002/0471142727.mb2603s62.
20. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expr. data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:**402–408. http://dx.doi.org/10.1006/meth.2001.1262.
21. **Kawaguchi R, Girke T, Bray EA, Bailey-Serres J.** 2004. Differential mRNA translation contributes to gene regulation under non-stress and dehydration stress conditions in *Arabidopsis thaliana*. Plant J. **38:**823–839. http://dx.doi.org/10.1111/j.1365-313X.2004.02090.x.
22. **Mangone M, MacMenamin P, Zegar C, Piano F, Gunsalus KC.** 2008. UTRome.org: a platform for 3′ UTR biology in *C. elegans*. Nucleic Acids Res. **36:**D57–D62. http://dx.doi.org/10.1093/nar/gkm946.
23. **Markham NR, Zuker M.** 2005. DINAMelt web server for nucleic acid melting prediction. Nucleic Acids Res. **33:**W577–W581. http://dx.doi.org/10.1093/nar/gki591.
24. **Holcik M, Sonenberg N.** 2005. Translational control in stress and apoptosis. Nat. Rev. **6:**318–327. http://dx.doi.org/10.1038/nrm1618.
25. **Kaeberlein M, Kennedy BK.** 2008. Protein translation. Aging Cell **7:**777–782. http://dx.doi.org/10.1111/j.1474-9726.2008.00439.x.
26. **Dinkova TD, Keiper BD, Korneeva NL, Aamodt EJ, Rhoads RE.** 2005. Translation of a small subset of *Caenorhabditis elegans* mRNAs is dependent on a specific eukaryotic translation initiation factor 4E isoform. Mol. Cell. Biol. **25:**100–113. http://dx.doi.org/10.1128/MCB.25.1.100-113.2005.
27. **Kuznicki KA, Smith PA, Leung-Chiu WMA, Estevez AO, Scott HC, Bennett KL.** 2000. Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1; these two P granule components are critical for fertility in *C. elegans*. Development **127:**2907–2916.
28. **Xue D, Shaham S, Horvitz HR.** 1996. The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. Genes Dev. **10:**1073–1083. http://dx.doi.org/10.1101/gad.10.9.1073.
29. **Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR.** 1993. The *C. elegans* cell death Gene *ced-3* encodes a protein similar to mammalian interleukin-1 p-converting enzyme. Cell **75:**641–652. http://dx.doi.org/10.1016/0092-8674(93)90485-9.
30. **Zhou Z, Hartwieg E, Horvitz HR.** 2001. CED-1 is a transmembrane receptor that mediates cell corpse engulfment in *C. elegans*. Cell **104:**43–56. http://dx.doi.org/10.1016/S0092-8674(01)00190-8.
31. **Hengartner MO, Ellis RE, Horvitz HR.** 1992. *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. Nature **356:**494–499. http://dx.doi.org/10.1038/356494a0.
32. **Reece-Hoyes JS, Shingles J, Dupuy D, Grove CA, Walhout AJ, Vidal M, Hope IA.** 2007. Insight into transcription factor gene duplication from *Caenorhabditis elegans* promoterome-driven expression patterns. BMC Genomics **8:**27. http://dx.doi.org/10.1186/1471-2164-8-27.
33. **Reddien PW, Andersen EC, Huang MC, Horvitz HR.** 2007. DPL-1 DP, LIN-35 Rb and EFL-1 E2F act with the MCD-1 zinc-finger protein to promote programmed cell death in *Caenorhabditis elegans*. Genetics **175:**1719–1733. http://dx.doi.org/10.1534/genetics.106.068148.
34. **Harris C, Maroney AC, Johnson EMJ.** 2002. Identification of JNK-dependent and -independent components of cerebellar granule neuron apoptosis. J. Neurochem. **83:**992–1001. http://dx.doi.org/10.1046/j.1471-4159.2002.01219.x.
35. **Lu C, Shi Y, Wang Z, Song Z, Zhu M, Cai Q, Chen T.** 2008. Serum starvation induces H2AX phosphorylation to regulate apoptosis via p38 MAPK pathway. FEBS Lett. **582:**2703–2708. http://dx.doi.org/10.1016/j.febslet.2008.06.051.
36. **Ellis HM, Horvitz HR.** 1986. Genetic control of programmed cell death in the nematode *C. elegans*. Cell **44:**817–829. http://dx.doi.org/10.1016/0092-8674(86)90004-8.
37. **Girling R, Partridge JF, Bandara LR, Burden N, Totty NF, Hsuan JJ, La Thangue NB.** 1993. A new component of the transcription factor DRTF1/E2F. Nature **362:**83–87. http://dx.doi.org/10.1038/362083a0.
38. **Helin K, Wu C-L, Fattaey AR, Lees JA, Dynlacht BD, Ngwu C, Harlow E.** 1993. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. Genes Dev. **7:**1850–1861. http://dx.doi.org/10.1101/gad.7.10.1850.
39. **Attwooll C, Denchi EL, Helin K.** 2004. The E2F family: specific functions and overlapping interests. EMBO J. **23:**4709–4716. http://dx.doi.org/10.1038/sj.emboj.7600481.
40. **Dimova DK, Dyson NJ.** 2005. The E2F transcriptional network: old acquaintances with new faces. Oncogene **24:**2810–2826. http://dx.doi.org/10.1038/sj.onc.1208612.
41. **Boxem M, van den Heuvel S.** 2002. *C. elegans* class B synthetic multivulva genes act in G_1 regulation. Curr. Biol. **12:**906–911. http://dx.doi.org/10.1016/S0960-9822(02)00844-8.
42. **Sherrill KW, Byrd MP, Van Eden ME, Lloyd RE.** 2004. BCL-2 translation is mediated via internal ribosome entry during cell stress. J. Biol. Chem. **279:**29066–29074. http://dx.doi.org/10.1074/jbc.M402727200.
43. **Bursch W, Karwana A, Mayer M, Dornetshubera J, Fröhweina U, Schulte-Hermanna R, Fazi B, Di Sanob F, Pireddab L, Piacentinib M, Petrovski G, Fésüsc L, Gernera C.** 2008. Cell death and autophagy: cytokines, drugs, and nutritional factors. Toxicology **254:**147–157. http://dx.doi.org/10.1016/j.tox.2008.07.048.
44. **Liu S-Y, Chen C-L, Yang T-T, Huang W-C, Hsieh C-Y, Shen W-J, Tsai T-T, Shieh C-C, Lin C-F.** 2012. Albumin prevents reactive oxygen species-induced mitochondrial damage, autophagy, and apoptosis during serum starvation. Apoptosis **17:**1156–1169. http://dx.doi.org/10.1007/s10495-012-0758-6.
45. **Seto M, Jaeger U, Hockett RD, Graninger W, Bennett S, Goldman P, Korsmeyer SJ.** 1988. Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. EMBO J. **7:**123–131.
46. **Smith MD, Ensor EA, Coffin RS, Boxer LM, Latchman DS.** 1998. Bcl-2 transcription from the proximal P2 promoter is activated in neuronal cells by the Brn-3a POU family transcription factor. J. Biol. Chem. **273:**16715–16722. http://dx.doi.org/10.1074/jbc.273.27.16715.
47. **Young RL, Korsmeyer SJ.** 1993. A negative regulatory element in the bcl-2 5′-untranslated region inhibits expression from an upstream promoter. Mol. Cell. Biol. **13:**3686–3697.
48. **Chi W, Reinke V.** 2006. Promotion of oogenesis and embryogenesis in the *C. elegans* gonad by EFL-1/DPL-1 (E2F) does not require LIN-35 (pRB). Development **133:**3147–3157. http://dx.doi.org/10.1242/dev.02490.
49. **Ashe MP, De Long SK, Sachs AB.** 2000. Glucose depletion rapidly inhibits translation initiation in yeast. Mol. Biol. Cell **11:**833–848. http://dx.doi.org/10.1091/mbc.11.3.833.
50. **Zid BM, Rogers AN, Katewa SD, Vargas MA, Kolipinski MC, Lu TA, Benzer S, Kapahi P.** 2009. 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in *Drosophila*. Cell **139:**149–160. http://dx.doi.org/10.1016/j.cell.2009.07.034.
51. **Davies E, Stankovicb B, Vianc A, Wood A.** 2012. Where has all the

message gone? Plant Sci. **185:**23–32. http://dx.doi.org/10.1016/j.plantsci.2011.08.001.
52. **Anderson P, Kedersha N.** 2002. Stressful initiations. J. Cell Sci. **115:**3227–3234.
53. **Spriggs KA, Bushell M, Willis AE.** 2010. Translational regulation of gene expression during conditions of cell stress. Mol. Cell **40:**228–237. http://dx.doi.org/10.1016/j.molcel.2010.09.028.
54. **Krishnamoorthy T, Pavitt GD, Zhang F, Dever TE, Hinnebusch AG.** 2001. Tight binding of the phosphorylated a subunit of initiation factor 2 (eIF2a) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. Mol. Cell. Biol. **21:**5018–5030. http://dx.doi.org/10.1128/MCB.21.15.5018-5030.2001.
55. **Pan KZ, Palter JE, Rogers AN, Olsen A, Chen D, Lithgow GJ, Kapahi P.** 2007. Inhibition of mRNA translation extends lifespan in *Caenorhabditis elegans*. Aging Cell **6:**111–119. http://dx.doi.org/10.1111/j.1474-9726.2006.00266.x.
56. **Rogers AN, Chen D, McColl G, Czerwieniec G, Felkey K, Gibson BW, Hubbard A, Melov S, Lithgow GJ, Kapahi P.** 2011. Life span extension via eIF4G inhibition is mediated by posttranscriptional remodeling of stress response gene expression in *C. elegans*. Cell Metab. **14:**55–66. http://dx.doi.org/10.1016/j.cmet.2011.05.010.
57. **Contreras V, Friday AJ, Morrison JK, Hao E, Keiper BD.** 2011. Cap-independent translation promotes *C. elegans* germ cell apoptosis through Apaf-1/CED-4 in a caspase-dependent mechanism. PLoS One **6:**e24444. http://dx.doi.org/10.1371/journal.pone.0024444.
58. **Song A, Labella S, Korneeva NL, Keiper BD, Aamodt EJ, Zetka M, Rhoads RE.** 2010. A *C. elegans* eIF4E-family member upregulates translation at elevated temperatures of mRNAs encoding MSH-5 and other meiotic crossover proteins. J. Cell Sci. **123:**2228–2237. http://dx.doi.org/10.1242/jcs.063107.
59. **Syntichaki P, Troulinaki K, Tavernarakis N.** 2007. eIF4E function in somatic cells modulates ageing in *Caenorhabditis elegans*. Nature **445:**922–926. http://dx.doi.org/10.1038/nature05603.
60. **Contreras V, Richardson M, Hao E, Keiper B.** 2008. Depletion of the cap-associated isoform of translation factor eIF4G induces germline apoptosis in *C. elegans*. Cell Death Differ. **15:**1232–1242. http://dx.doi.org/10.1038/cdd.2008.46.
61. **Jackson R.** 2005. Alternative mechanisms of initiating translation of mammalian mRNAs. Biochem. Soc. Trans. **33:**1231–1241. http://dx.doi.org/10.1042/BST20051231.
62. **Hellen CUT, Sarnow P.** 2001. Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev. **15:**1593–1612. http://dx.doi.org/10.1101/gad.891101.
63. **Spriggs KA, Stoneley M, Bushell M, Willis AE.** 2008. Re-programming of translation following cell stress allows IRES mediated translation to predominate. Biol. Cell **100:**27–38. http://dx.doi.org/10.1042/BC20070098.
64. **Stoneley M, Willis AE.** 2004. Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. Oncogene **23:**3200–3207. http://dx.doi.org/10.1038/sj.onc.1207551.
65. **Li D, Wang M.** 2012. Construction of a bicistronic vector for the co-expression of two genes in *Caenorhabditis elegans* using a newly identified IRES. Biotechniques **52:**173–176. http://dx.doi.org/10.2144/000113821.
66. **Blumenthal T, Steward K.** 1997. RNA processing and gene structure, p 117–145. *In* Riddle DL, Blumenthal T, Meyer BJ, Priess JR (ed), *C. elegans* II. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
67. **Bektesh SL, Hirsh DI.** 1988. *C. elegans* mRNAs acquire a spliced leader through a trans-splicing mechanism. Nucleic Acids Res. **16:**5692. http://dx.doi.org/10.1093/nar/16.12.5692.
68. **Eckmann CR, Kraemer B, Wickens M, Kimble J.** 2002. GLD-3, a bicaudal-C homolog that inhibits FBF to control germline sex determination in *C. elegans*. Dev. Cell **3:**697–710. http://dx.doi.org/10.1016/S1534-5807(02)00322-2.
69. **Merritt C, Rasoloson D, Ko D, Seydoux G.** 2008. 3′ UTRs are the primary regulators of gene expression in the *C. elegans* germline. Curr. Biol. **18:**1476–1482. http://dx.doi.org/10.1016/j.cub.2008.08.013.