THE FUNCTIONS OF Y-BOX BINDING PROTEINS IN *CAENORHABDITIS ELEGANS*

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ABBREVIATIONS

A site	Acceptor site
ADMA	Asymmetrical dimethylation
AF	Assembly factor
ARE	AU-rich element
APA	Alternative polyadenylation
CEY	*C. elegans* Y-box binding protein
CDS	Coding sequenc
CPE	Cytoplasmic polyadenylation element
CPEB	Cytoplasmic polyadenylation element binding protein
CSD	Cold shock domain
Csp	Cold shock protein
CTD	C-terminal domain
DTC	Distal tip cell
E site	Exit site
eEF	Eukaryotic translation elongation factor
eEF2K	eEF2 kinase
eIF	Eukaryotic translation initiation factor
IRES	Internal ribosome entry site
miRISC	miRNA-induced silencing complex
miRNA	micro RNA
mRNA	messenger RNA
mRNP	messenger ribonucleoparticle
NGD	No-go decay
NMD	Nonsense-mediated decay
NRD	Non-functional rRNA decay
NSD	Non-stop decay
P bodies	Processing bodies
P site	Peptidyl-tRNA site
PABP	Poly(A) binding protein
PIC	Preinitiation complex
PTC	Premature termination codon
PTGR	Post-transcriptional gene regulation
RBP	RNA binding protein
RNAi	RNA interference

RNA pol	RNA polymerase
RPF	Ribosome protected fragment
rRNA	Ribosomal RNA
SUnSET	Surface sensing of translation
TGR	Transcriptional gene regulation
tRNA	transfer RNA
UTR	Untranslated region
YBP	Y-box binding protein

1. SUMMARY

Members of the highly conserved family of Y-box binding proteins (YBPs) have a broad spectrum of functions in both transcriptional and post-transcriptional regulation of gene expression. However, most information comes from *in vitro* or single cell experimental systems. In addition, these proteins have not been studied in one of the major model organisms, *Caenorhabditis elegans*. Here, we provide a functional characterization of YBPs in this nematode, thereby also generally adding to the very scarce knowledge on the developmental functions of YBPs in a multicellular context. Our data suggests a conserved and essential role for *C. elegans* YBPs (CEYs) in packaging and stabilizing maternal mRNAs during oogenesis. In the soma, the absence of CEYs also affects the abundance of many messages, interestingly, with a strong bias for highly translated mRNAs. The most striking observation is the soma-specific requirement for CEY proteins to accumulate large polysomes. Surprisingly, this loss has no negative impact on translation rates and *cey* mutant animals show no apparent defects in somatic development. Finally, our data also suggests a potentially novel function for YBPs, namely, a direct involvement in ribosome biology.

2. GENERAL INTRODUCTION

2.1 The multiple layers of gene regulation

The “building plan” for any organism is encoded in its DNA, more precisely, by a large array of individual modules, so called genes. Correct temporal and spatial regulation of gene expression is essential from a developmental point of view, but also as a mean to respond to changes in environmental conditions. In eukaryotes, the DNA is located in the nucleus, which is separated by the nuclear membrane from the cytoplasm. Genes are transcribed by so called RNA polymerases (RNA pol) to yield RNA molecules. One particular type of RNA, the messenger RNA (mRNA), transcribed by RNA pol II, is exported to the cytoplasm, where it is translated into proteins by ribosomes. The regulation of gene expression can be roughly divided into three classes: Transcriptional gene regulation (TGR), post-transcriptional gene regulation (PTGR) and protein modifications that happen post-translationally. TGR includes modifications on histones and the DNA itself, which allow or prevent the binding of specific transcription factors, thereby providing a highly dynamic and complex regulatory network. However, despite the fact that TGR primarily determines if an RNA product is made or not, regulation at the post-transcriptional level plays an equally important role. This is especially apparent during early stages of embryogenesis, when transcription is silent due to major rearrangements in the genome, and protein production, therefore, relies entirely on maternal messages previously stored in the oocyte. The fate of an mRNA molecule in the cytoplasm, that is translation, degradation or storage (translation repression and stabilization), largely depends on the subset of associated RNA-binding proteins (RBPs), which often rely on the presence of specific sequences or structural properties on the bound mRNA (Figure 1). Importantly, the expression level of a gene is not simply determined by independent regulatory events happening at each step of the pre-mRNA/mRNA life cycle, but in fact is the result of an integrated network. The cytoplasmic fate of an mRNA often depends on events happening already at the transcriptional/co-transcriptional level, such as the choice of the transcription start site, alternative splicing events or alternative polyadenylation (APA) (Lutz and Moreira, 2011) (Figure 1). Such alterations allow the inclusion/exclusion of important mRNA regulatory elements. Furthermore, recent studies suggest that RNA-binding factors that influence mRNA stability in the cytoplasm can associate with their targets in a co-transcriptional manner (Haimovich et al., 2013a). More surprisingly, the vice versa also appears to be true, as components of the mRNA decay machinery have been found to bind to promoter regions (Haimovich et al., 2013b). It has recently been proposed that this

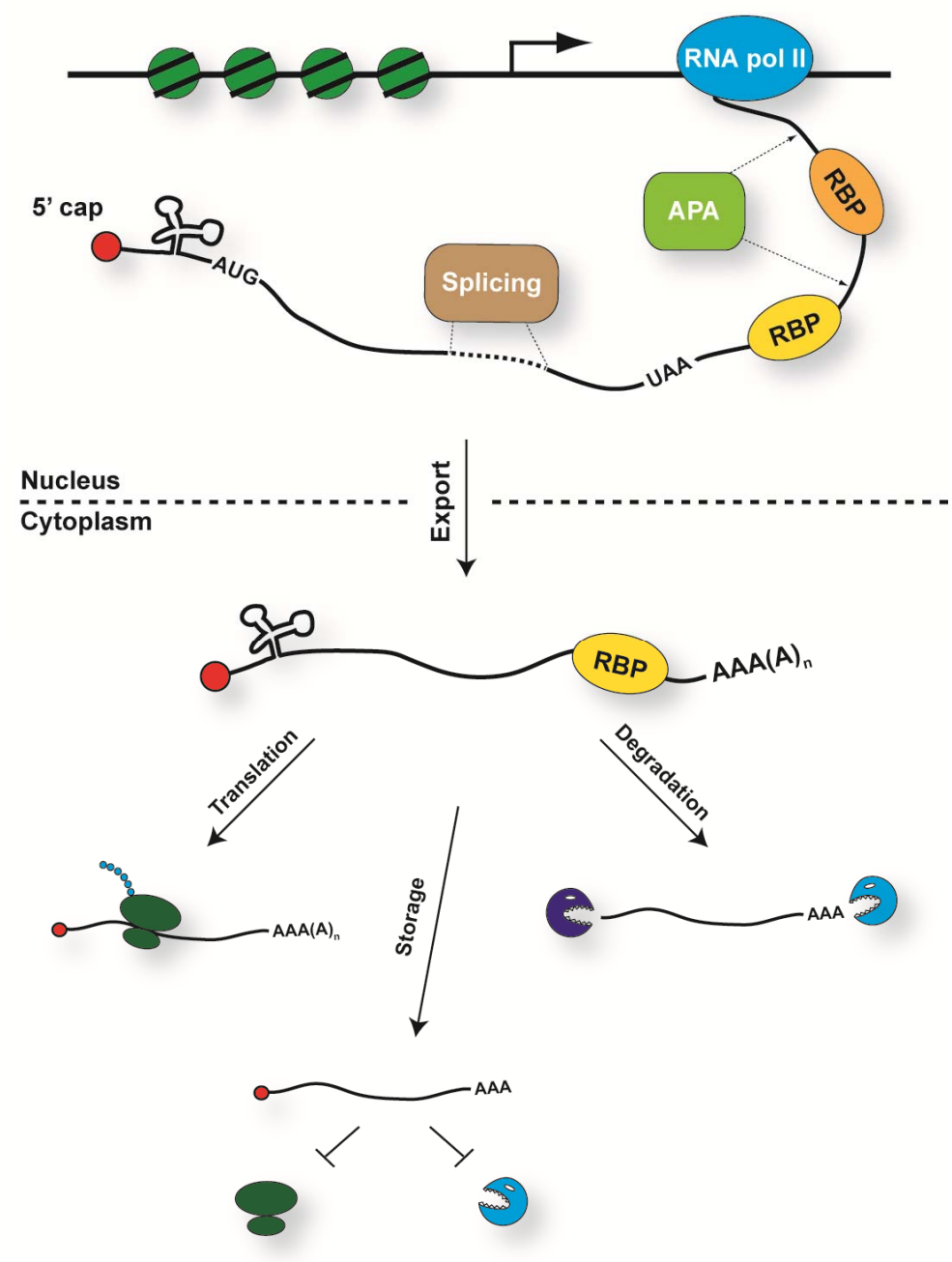

Figure 1. Post transcriptional gene regulation

The pre-mRNA is processed as soon as it emerges from the RNA pol II machinery. The added cap structure protects its 5' end and plays an important role in translation. Splicing events eliminate introns (dotted line), whereby alternative splicing can lead to the inclusion or exclusion of certain exons/introns. The length of the 3'UTR depends on the site of polyadenylation, which can vary due to APA and include or exclude certain *cis*-elements, thereby affecting PTGR. Once mature, the mRNA molecule is exported to the cytoplasm. In the cytoplasm, the composition of bound RBPs, potential secondary structures and the overall condition a cell is in will decide if an mRNA is directed for translation, degradation or storage.

coupling between transcription and mRNA decay may lie at the core of eukaryotic gene expression regulation (Haimovich et al., 2013a). Despite this striking interdependence of nuclear and cytoplasmic events in mRNA regulation, the following introduction will focus mainly on PTGR in the cytoplasm. The major reason for neglecting nuclear events, is our finding that YBPs in *C. elegans*, the functions of which I was studying during my graduate studies, appear to be present exclusively in the cytoplasm. Therefore, a direct involvement in nuclear processes is very unlikely. First, I will introduce two of the central subjects in cytoplasmic PTGR, the mature mRNA and the ribosome, before focusing more closely on the different fates an mRNA molecule can have in the cytoplasm. Then I will introduce the family of YBPs and what is known from other model organisms regarding their functions in cytoplasmic PTGR. Finally, I will introduce our model organism, *C. elegans*, with a major focus on its germline.

2.2 The mature messenger RNA molecule

The mature mRNA can be subdivided into several different parts, each of which serves specific functions and thereby contributes to the correct functionality of the molecule. As its name suggests, the cap structure is located at the 5' end of the mRNA. It is added co-transcriptionally as soon as the nascent pre-mRNA molecule emerges from the RNA exit channel of RNA pol II (Figure 1). An RNA triphosphatase removes the 5'-γ-phosphate from the first transcribed nucleotide, which is followed by the addition of a guanine monophosphate to the RNA 5'-diphosphate end (catalyzed by guanylyltransferase). The guanosine cap (GpppN) is subsequently methylated by an RNA methyltransferase to produce the 7-methylguanosine cap (m^7GpppN). In the nucleus the cap is bound by the nuclear cap binding complex, which besides stimulating pre-mRNA splicing, is also essential for mRNA export. In the cytoplasm, the cap on the one hand protects mRNAs against exonucleases, thereby promoting mRNA stability, and on the other hand plays an important role in promoting translation by serving as a docking site for the eukaryotic translation initiation factor 4E (eIF4E), which then recruits the remaining components of the eIF4F complex (see section 2.4.1) (Topisirovic et al., 2011).

The coding sequence (CDS), which consists of a number of sequential exons, represents the central element of every mRNA molecule, as it carries the codon information required for the ribosome to produce the corresponding protein. The CDS begins at the start codon (AUG) and ends at the termination codon (UAA, UAG, UGA). In pre-mRNA molecules the individual exons are separated by non-coding introns. These are removed co-transcriptionally by splicing, which is catalyzed by the large spliceosome complex (Matera

and Wang, 2014) (Figure 1). Specific exons can also be included or excluded during splicing, a process known as alternative splicing. This alters the CDS of the mature mRNA molecule and is one example of the important regulatory functions splicing can have in PTGR (Kornblihtt et al., 2013).

The cleavage and polyadenylation machinery, which is comprised of multiple factors, cleaves the nascent pre-mRNA at a specific site in the 3' untranslated region (UTR) and, with the exception of histone mRNAs, adds a poly(A) tail of approximately 250 adenosine residues (Di Giammartino et al., 2011). Interestingly, some pre-mRNAs can be cleaved at different sites in their 3'UTR, causing APA and thereby alter the length of the corresponding 3'UTR (Tian et al., 2005) (Figure 1). The poly(A) tail represents a crucial target for PTGR. In the cytoplasm, its length depends on the interplay between poly(A) polymerases and deadenylases, which lengthen and shorten the poly(A) tail, respectively. Long poly(A) tails or an increase in poly(A) tail length usually directs an mRNA towards translation, while shortening it, in most cases, marks the first step in mRNA degradation. However, short poly(A) tails can also prevent translation without triggering mRNA decay, a phenomenon observed for instance in germ cells or neurons, where messages need to be "stored" in a translationally quiescent state for a longer period of time (see section 2.6) (Weill et al., 2012). The poly(A) tail is bound and stabilized by the poly(A) binding protein (PABP), a central component in cytoplasmic PTGR (Mangus et al., 2003).

Finally, the 5'UTR and 3'UTR correspond to the stretches of nucleotides upstream of the start codon and downstream of the stop codon, respectively. The average length of 5'UTRs usually ranges between 100 and 200 nucleotides, while the length of 3'UTRs ranges from an average of 200 nucleotides in plants and fungi to around 800 nucleotides in humans and other vertebrates (Mignone et al., 2002). Stable secondary structures, internal ribosome entry sites (IRES), which allow cap-independent translation and upstream ORFs, so called uORFs, are examples of features that are present in 5'UTRs and play important roles in regulating translation initiation (Figure 1). Furthermore, regulatory elements (see section 2.5.3), which are present predominantly in 3'UTRs, often specify the composition of bound proteins and thereby influence the stability and/or translatability of an mRNA. Therefore, UTRs are primarily of regulatory importance (Mignone et al., 2002).

2.3 The ribosome

2.3.1 Ribosome biogenesis

The eukaryotic 80S ribosome consists of a small 40S and a large 60S subunit. Ribosome biogenesis is a highly complex process, which requires the coordinated activity of all three RNA polymerases and the orchestrated work of more than 200 transiently associated ribosome assembly factors (AF) (Thomson et al., 2013). The mature 40S subunit consists of the 18S ribosomal RNA (rRNA) and at least 33 ribosomal proteins, while the 60S subunit contains three rRNAs (25S, 5.8S, 5S) and a least 46 ribosomal proteins (Thomson et al., 2013). All ribosomal proteins are transcribed by RNA pol II, translated in the cytoplasm and transported back into the nucleus. The 5S rRNA is transcribed by RNA pol III and is then transported into the nucleolus (Ciganda and Williams, 2011). The 18S, 5.8S and 25S are transcribed in the nucleolus by RNA pol I as a single polycistronic transcript, which is co-transcriptionally modified at over 100 rRNA residues by more than 60 small nucleolar ribonucleoparticles (Kos and Tollervey, 2010). The emerging rRNA transcript also acquires predominantly small subunit ribosomal proteins and AFs to form the so called 90S pre-ribosomes or small subunit processome complexes (Bernstein et al., 2004, Dragon et al., 2002, Grandi et al., 2002). This complex is then cleaved into pre-40S and pre-60S particles. Whereas pre-40S particles are believed to be exported relatively rapidly to the cytoplasm (Schäfer et al., 2003), pre-60S complexes have to undergo more extensive nuclear maturation processes before being exported (Nissan et al., 2002). Once in the cytoplasm, both subunits undergo multiple maturation steps, which include the sequential release of AFs, the association of remaining ribosomal proteins and processing of rRNA, thereby rearranging the subunits allowing them to acquire their ultimate structure and functionality (Thomson et al., 2013).

2.3.2 Ribosome maturation and quality control

The energy invested into the production of ribosomes is enormous (Warner, 1999). These high costs in combination with the complexity of ribosome biogenesis call for elaborate quality control mechanisms to ensure the formation of a functional end product. One major challenge is to prevent the engagement of premature ribosomes in translation. In eukaryotes this is partially resolved by separating ribosome biogenesis in the nucleus/nucleolus from translation in the cytoplasm. However, as stated above, both subunits undergo final maturation in the cytoplasm in the presence of all components of the translation machinery.

Especially, the small subunit, which binds tRNA, mRNA, and translation factors during translation initiation (see section 2.4.1), needs to be protected at multiple sites to prevent precocious ligand binding. Indeed, a recent cryo-EM structure of a cytoplasmic 40S assembly intermediate from yeast showed that seven bound AFs cooperate to prevent every step in the translation initiation pathway (Strunk et al., 2011). The large subunit on the other hand does not interact with mRNA or tRNA on its own but binds directly to the small subunit only late during translation initiation (see section 2.4.1). Thus, blocking the interaction with the 40S subunit is sufficient to prevent 60S precursors from precociously entering translation. The protein eIF6 is one such example, as it binds to the 60S subunit interface and blocks the formation of 80S ribosomes (Gartmann et al., 2010; Klinge et al., 2011).

Preventing premature assembly of ribosomes is important, however, it is useless if the protected subunits eventually turn out to be non-functional. For this reason there have to be ways to test the integrity of 40S and 60S before releasing them into the translation pool. A recent study suggests that the 40S assembly intermediate (including the seven bound AFs) undergoes a translation-like cycle in order to mature, thereby testing multiple functions of the small subunit, such as correct 60S recruitment, interactions with translation initiation factors and proteins important for translation termination (Strunk et al., 2012). Similarly, Efl1, a protein with high homology to the eukaryotic translation elongation factor 2 (eEF2), plays an important role in releasing the eIF6-mediated block during 60S maturation. Efl1 thereby appears to mimic the function of eEF2-mediated translocation of tRNAs during translation elongation (see section 2.4.2) and therefore serves as a way to test the integrity of the large subunit (Bussiere et al., 2012; Karbstein 2013).

Finally, potential aberrant intermediate or mature subunits need to be eliminated. Non-functional mature ribosomes will stall on an mRNA molecule during translation elongation, which will ultimately cause the degradation of both mRNA and ribosomal subunits via the no-go-decay (NGD) (see section 2.5.4) and non-functional rRNA decay (NRD) pathways, respectively (Cole et al., 2009). However, due to the endonucleolytic degradation of the mRNA during this process, this should not occur on a larger scale. Therefore, quality control and degradation should happen before translation starts. The TRAMP complex currently represents the best characterized machinery for detecting and degrading ribosome assembly intermediates in the nucleus. This complex, composed of the poly(A) polymerase Trf4/5, the RBP Air1/2, and the helicase Mrt4, marks defective nuclear RNAs, including rRNA, with short poly(A) tails, and then targets them for degradation by the exosome (La Cava et al., 2005; Karbstein, 2013). However, due to the complexity of ribosome biogenesis, potential defects can occur at many steps, including cytoplasmic maturation, and it is still a mystery on how the surveillance system can cope with all of this. One idea is that quality control pathways do not primarily detect individual defects but sense potential delays in

assembly and maturation due to abnormalities, which then cause the removal of such complexes (Thomson et al., 2013).

2.3.3 Ribosome heterogeneity

Despite the high complexity of the ribosome, it was for a long time considered to be a uniform structure. However, more recent findings suggest that the ribosome is actually far more heterogeneous than previously imagined. Some ribosomal proteins are encoded by more than one gene and the expression patterns as well as the loss-of-function phenotypes of such paralogues can be distinct (Xue and Barna, 2012). Even the expression of core ribosomal proteins can differ depending on tissue and cell type, as has been shown for a developing vertebrate embryo (Kondrashov et al., 2011). Furthermore, post-translational modifications, such as acetylation, methylation, phosphorylation and ubiquitylation play an important role in regulating ribosomal proteins, whereby the phosphorylation of RPS6 represents the best studied example to date (Gressner and Wool, 1974). Ribosome-associated factors can also enhance or inhibit the translation of mRNAs. The *D. melanogaster* protein Reaper, for instance, inhibits cap-dependent translation by binding to the 40S subunit and disrupting AUG recognition by the scanning 48S complex (Colon-Ramos et al., 2006). Another example is RACK1, which among other functions has been shown to recruit the miRNA-induced silencing complex (miRISC) to the ribosome, thereby facilitating microRNA (miRNA)-induced repression (Jannot et al., 2011). Finally, heterogeneity can also be found in rRNA molecules, an extreme example of which is found in the malaria parasite *Plasmodium falciparum*, which carries two classes of rDNA genes, thereby producing two different kinds of ribosomes depending on the stage of its life cycle (Gunderson et al., 1987).

How different versions of the ribosome (differential expression of ribosomal proteins) specifically affects gene expression is still largely unknown. However, some examples regarding the function of “specialized ribosomes” in cellular and developmental processes are becoming evident. One particularly interesting finding concerns the loss of the *Rpl38* gene in mice, which causes a perturbation in tissue patterning. The resulting defects in the axial skeleton appear to stem from reduced translation of several Hox mRNAs and RPL38 has been shown to regulate 80S formation (as part of the ribosome) during translation initiation on these mRNAs. Consistently, *Rpl38* transcripts are enriched in specific regions of the embryo where these defects occur (Kondrashov et al., 2011; Xue and Barna, 2012). Another example concerns the use of a whole ribosome out of its usual context. Mitochondrial ribosomes, which are smaller than normal eukaryotic ribosomes, are found outside mitochondria in polar granules in the embryo of *D. melanogaster* (Amikura et al.,

2001). Even though the underlying mechanisms are still unknown, specifically blocking mitochondrial ribosomes results in defects in germ cell formation (Amikura et al., 2005).

These variations of ribosomal proteins/ribosomes in different tissues or at different developmental stages combined with the evidence for specific effects on gene expression, strongly implies that ribosome heterogeneity adds another layer of complexity to PTGR in the cytoplasm.

2.4 Translating mRNA

In order to produce proteins, mature mRNAs exiting the nucleus are translated by the ribosome. The process of translation represents a highly regulated process and involves many more factors than just the mRNA and the ribosome.

2.4.1 Translation initiation

Translation initiation represents the most complex and rate-limiting step in translation (Hinnebusch and Lorsch, 2012) (Figure 2). The canonical way of initiating translation on mRNAs is cap-dependent. However, cap-independent initiation via so called IRESs also exists. These specialized secondary structures are localized close to the start codon. This enables the recruitment of the translation machinery directly to the starting point of translation, thereby circumventing 5' UTR scanning by the small subunit. IRESs were initially discovered in viral RNA, but are also present in cellular mRNAs, where they might enable the translation of specific messages in conditions when cap-dependent translation is compromised (Jackson, 2013). Cap-dependent translation starts by binding of the ternary complex, which consists of initiator methionyl-tRNA and GTP-bound eIF2, to the 40S subunit to form the 43S preinitiation complex (PIC). This interaction is promoted by additional eIFs (eIF1, eIF1A, eIF5, eIF3). Binding of the 43S PIC to the mRNA close to the cap structure is facilitated among other proteins by eIF3 and the eIF4F complex, which consists of the cap-binding eIF4E, the scaffold protein eIF4G and the RNA helicase eIF4A, which is thought to provide the "landing platform" for the small subunit. eIF4G has binding sites for both eIF4E and PABP, thereby bringing the 5' UTR and 3' UTR in close proximity to form a circular messenger ribonucleoparticle (mRNP), known as the "closed-loop" structure. This stable structure is thought to give the translation machinery an advantage over competing RNA-protein and protein-protein interactions and therefore appears to play an important role to promote translation. Once bound to the mRNA, the 43S PIC scans the 5' UTR until it finds an

AUG in a suitable context, where the anticodon of the methionyl-tRNA and the AUG in the peptidyl-tRNA (P) site of the 40S subunit base-pair. This event causes the conversion of

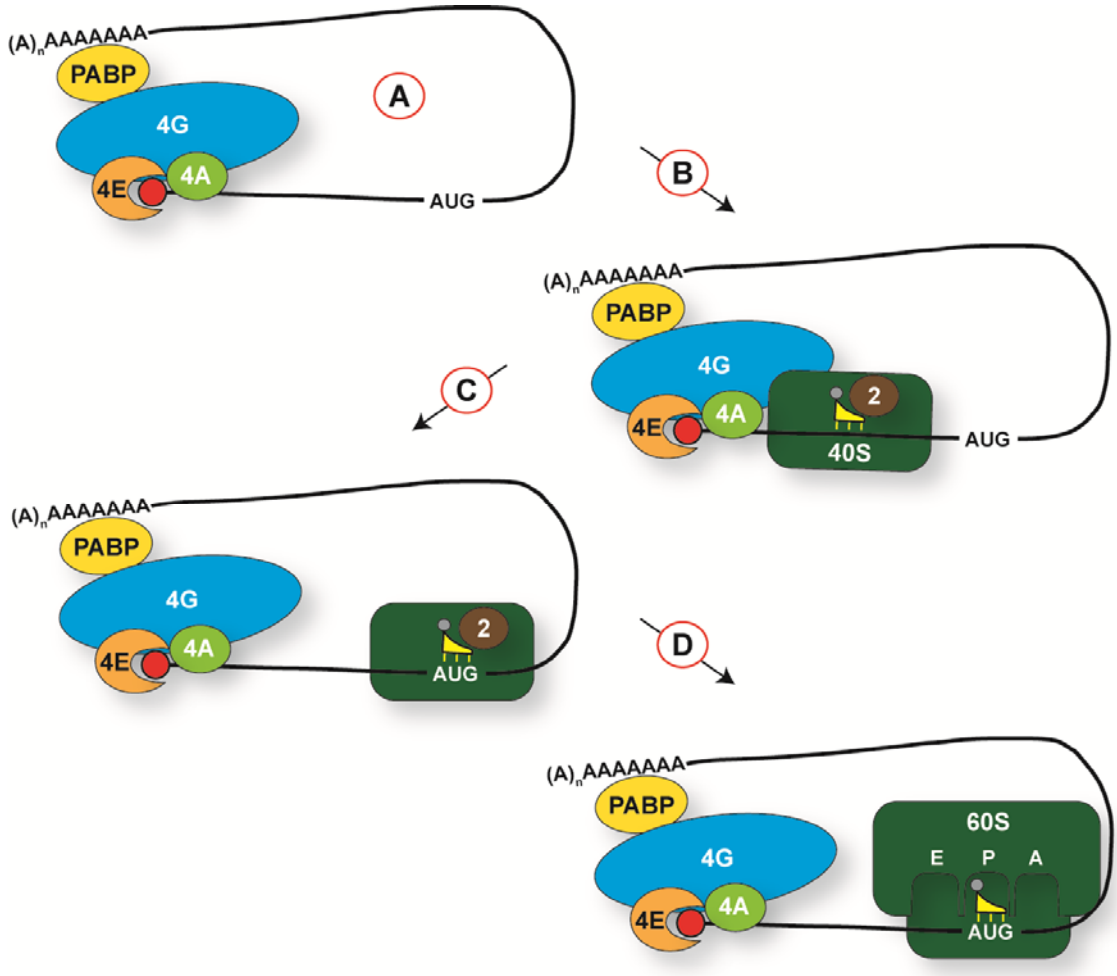

Figure 2. Translation initiation

(A) The “closed loop” is formed by the scaffold protein eIF4G, which bridges the cap-bound eIF4E and the poly(A)-bound PABP. (B) The helicase eIF4A provides a landing platform for the 43S PIC in the 5’ UTR. (C) The 43S PIC scans along the mRNA until it encounters the AUG start codon. (D) AUG recognition results in eIF2-GTP hydrolysis, the release of eIF2-GDP and joining of the 60S subunit to form the 80S ribosome ready for translation elongation.

eIF2-GTP to eIF2-GDP. After eIF2-GDP and additional eIFs have been released from the PIC, the 60S subunit joins (catalyzed by eIF5B) to form the 80S ribosome, which is then ready to enter the translation elongation step (Hinnebusch and Lorsch, 2012). One way to globally reduce or block translation initiation events, is by phosphorylating the α-subunit of eIF2, which prevents the recycling of its inactive GDP-bound form to its active GTP-bound

version (Krishnamoorthy et al., 2001). This phosphorylation event frequently occurs under stress conditions (Wek et al., 2006).

2.4.2 Translation elongation and termination

Following translation initiation, the ribosome starts translocating along the mRNA, a process known as translation elongation, thereby decoding one codon after the other to produce the corresponding polypeptide (Figure 3A-D). The elongation factor eEF1A is responsible for binding amino-acyl-tRNA and it does this in a GTP-dependent manner. Once the tRNA recognizes the codon in the A site, it triggers GTP hydrolysis and the dissociation of eEF1A, which is recycled to its active GTP-bound state by a guanine nucleotide exchange factor. The peptidyl transferase centre then positions the amino-acyl-tRNAs in the A and P site in a way that peptide bond formation can occur. Following this process, ratcheting of the ribosomal subunits locks tRNAs into so-called hybrid P/E and A/P states, with the acceptor ends of the tRNAs in the E and P sites, while the anticodon ends remain in the P and A sites. Full translocation of tRNAs to the E and P sites requires the GTPase eEF2 in complex with GTP, which stabilizes the hybrid state. GTP hydrolysis and accompanying conformational changes in eEF2 allow tRNA and mRNA movement, ultimately locking the ribosome in the posttranslocation state. At this point the A site is vacant and ready for the binding of the next amino-acyl-tRNA (Dever and Green 2012). An important modification in respect to controlling the elongation step of translation is the phosphorylation of eEF2 by the eEF2 kinase (eEF2K), which is thought to block translation by preventing eEF2 binding to the ribosome (Carlberg et al., 1990). Regulating global translation at the elongation step via eEF2 phosphorylation has been shown, for instance, to be crucial as a response to nutrient deprivation (Leprivier et al., 2013). Translation of an mRNA ends when a stop codon (UAA, UAG, UGA) enters the A site of the ribosome (Figure 3D-G). The termination step is catalyzed by eRF1 and the translational GTPase eRF3. Both proteins form a ternary eRF1:eRF3:GTP complex where eRF3 is supposed to deliver eRF1 to the ribosome. The N-terminus of eRF1 recognizes the stop codon in the A site, while upon GTP hydrolysis and eRF3 release, the middle domain of eRF1 extends into the peptidyl transferase centre where its Glycine-Glycine-Glutamine motif catalyzes peptide release. An additional ATPase, ABCE1/Rli1, has been shown to promote the release of the peptide by eRF1:eRF3 in an ATP-independent fashion. Finally, the same factor, this time in an ATP-dependent manner, plays a crucial role in the release/recycling of the 80S complex from the bound mRNA (Dever and Green 2012).

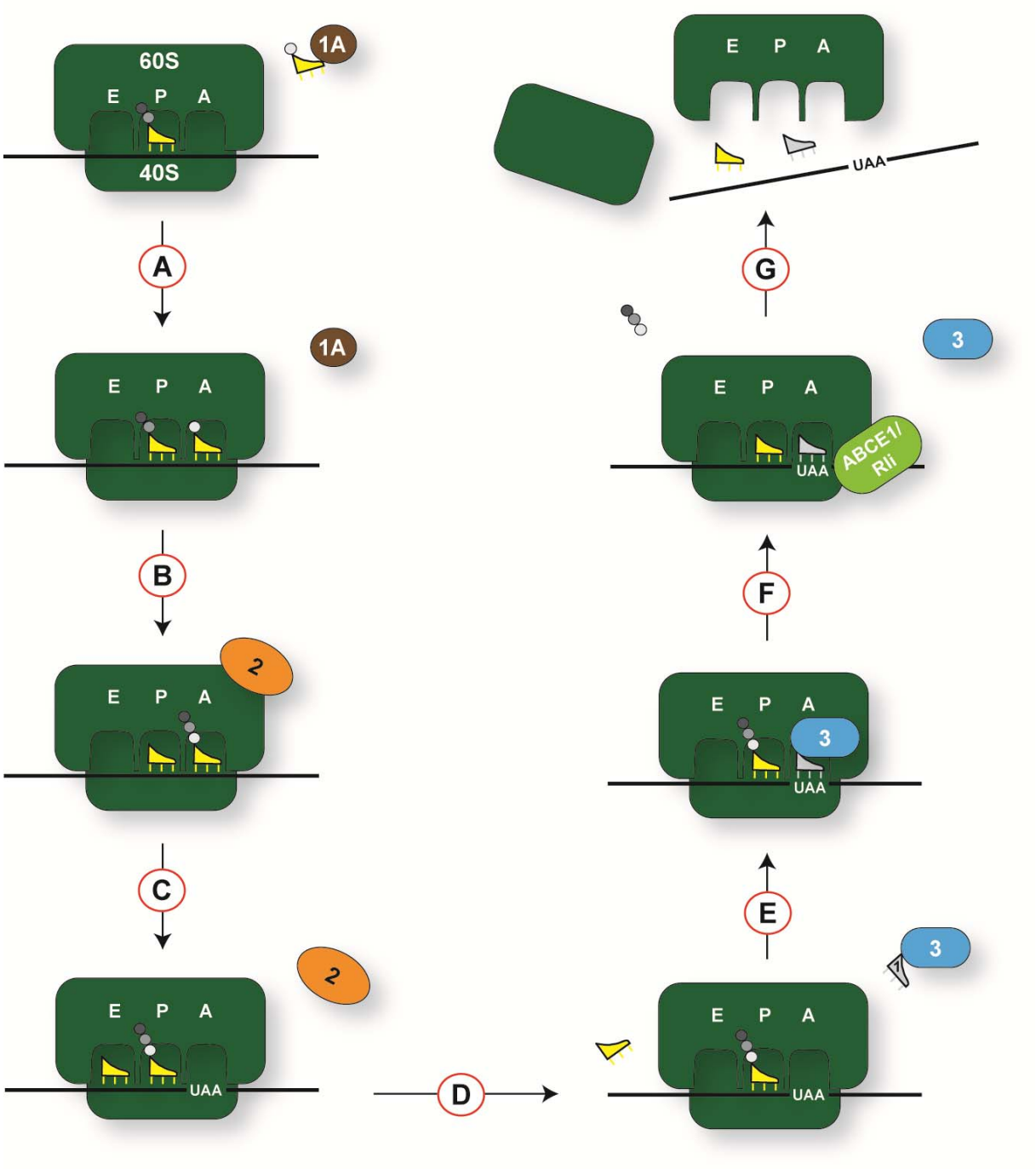

Figure 3. Translation elongation and termination

(A) The next aminoacyl-tRNA is delivered by eEF1A to the A site. Upon codon recognition eEF1A-GTP is hydrolysed and eEF1A-GDP is released. (B) Peptide bond formation takes place and eEF2 binds to the ribosome. (C) The hydrolysis of eEF2-GTP mediates ribosome translocation and eEF2-GDP is released. (D) The tRNA is released from the E site. eRF3 delivers eRF1. (E) eRF1 recognizes the stop codon present in the A site. (F) eRF3-GTP hydrolysis and the ATPase ABCE1/Rli (ATP-independent) promote peptide release. eRF3-GDP is released. (G) ABCE1/Rli (ATP-dependent) is involved in 80S complex dissociation from the mRNA.

2.5 Degrading mRNA

The relevance of mRNA translation for a cell is obvious. However, the turnover of messages as a mean to regulate what and how many proteins are produced plays an equally pivotal role in PTGR. Degradation can be roughly divided into two classes: Mechanisms that remove abnormal and potentially toxic mRNAs, which are detected by quality control pathways, and mechanisms that determine the half-life of "healthy" messages and thereby affect the abundance of a given protein. Insights into decay mechanisms and especially the interrelations between mRNA degradation and translation come predominantly from work done in yeast (Huch and Nissan, 2014). However, many factors are conserved across species, including *C. elegans*.

2.5.1 Degradation mechanisms

The 5' cap and the poly(A) tail, both of which are important for translation, also represent crucial targets of the mRNA degradation machinery (Figure 4). It should be noted that while poly(A) tail shortening is revertible, a paradigm of which is found in maternal mRNA regulation in *Xenopus* oocytes (see section 2.6.1), an mRNA that has lost its cap is irreversibly destined for degradation. This might be one reason why the decay of most mRNAs in eukaryotes starts by deadenylase-dependent shortening of the poly(A) tail. Major deadenlyases include PAN2-PAN3, the Ccr4/Pop2/Not complex and PARN. Once the poly(A) tail has been shortened, mRNAs are degraded either from 3'-to-5' by the exosome complex or more commonly from 5'-to-3' by the exoribonuclease XRN1 (Garneau et al., 2007). However, to make the 5' end accessible to XRN1, the cap structure has to be removed. The two major decapping enzymes are Dcp1 and Dcp2, whereby the latter provides the catalytic activity (Steiger et al., 2003). The decapping efficiency is often enhanced by accessory factors, such as the Lsm1-7 complex or the DEAD-box helicase Dhh1 (Tharun et al., 2000; Coller et al., 2001). A more unusual route of decay has been reported in yeast for both *RPS28B* and *EDC1* mRNAs, which recruit the decapping complex in the absence of deadenylation (Badis et al., 2004; Muhlrad et al., 2005). Finally, in addition to degradation by exonucleases, mRNA molecules can also be targeted by endonucleases to initiate turnover, whereby the 5' and 3' cleavage products are subsequently degraded by the exosome and XRN1, respectively (Schoenberg, 2011).

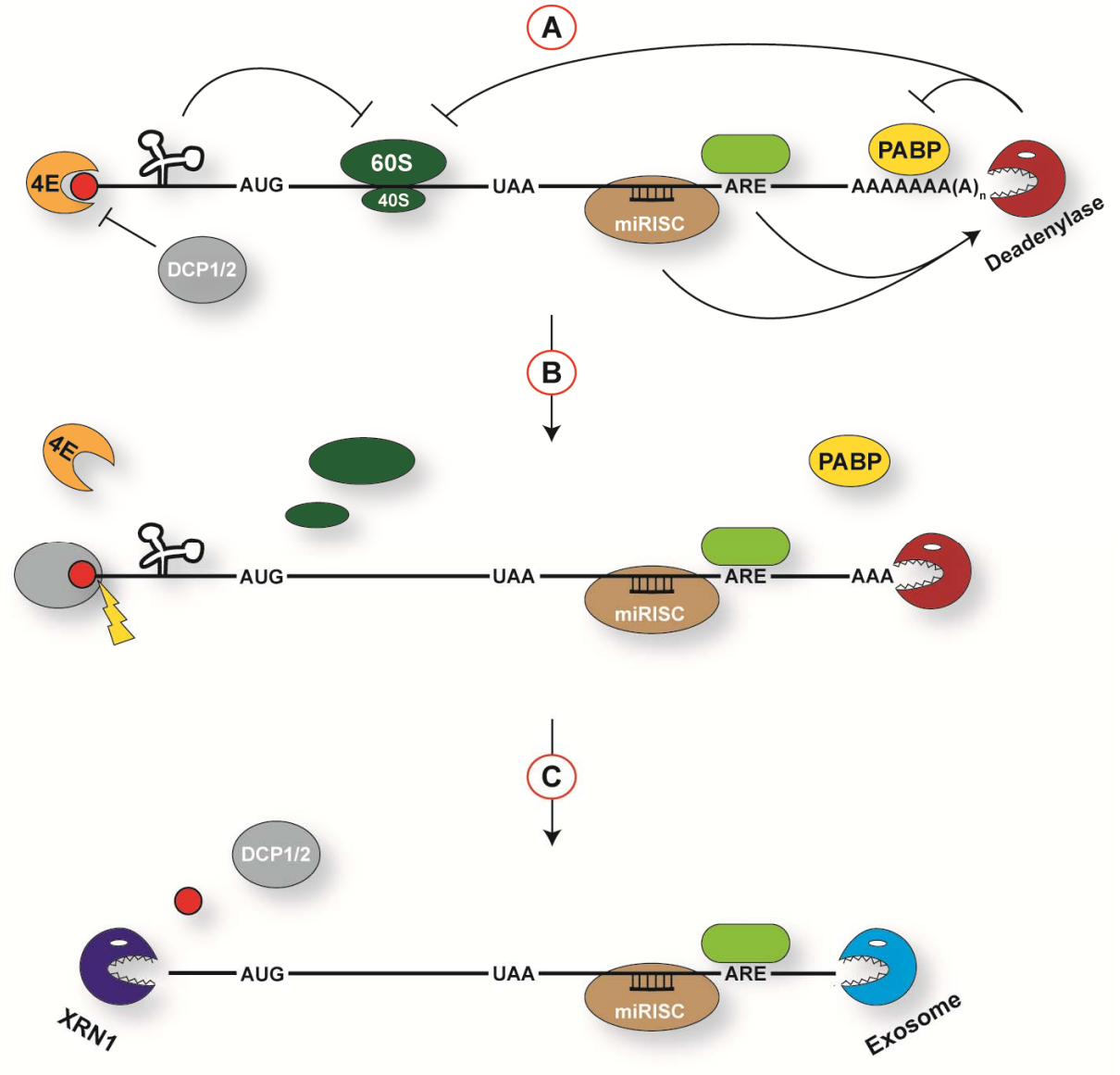

Figure 4. Translational repression can promote mRNA degradation

(A) *Cis*-acting elements repress translation either directly by blocking the ribosome (secondary structures, etc.) or more indirectly (miRNA binding sites, AREs, etc.) by recruiting deadenylases. (B) Ribosomes are removed from the mRNA. The repressed mRNA can subsequently be directed for mRNA decay by further shortening of the poly(A) tail and DCP1/2-dependent removal of the cap. (C) Subsequent degradation can take place from 5'-to-3' by XRN1 or 3'-to-5' by the exosome or by both pathways simultaneously.

2.5.2 Translation versus mRNA degradation

Cap- and poly(A) tail-binding factors, such as eIF4E and PABP, promote translation not only by driving the initiation process, but also by protecting an mRNA from the degradation machinery, such as decapping enzymes and deadenlyases, respectively (Schwartz et al.,

1999; Schwartz et al., 2000; Tucker et al., 2002). Vice versa, negatively affecting the process of translation initiation, for instance by mutating components of the cap-binding complex (Schwartz et al., 1999), appears to promote mRNA decay under normal conditions. Consistently, in addition to promoting decapping, several factors such as the DEAD box helicase Dhh1, Pat1 or Scd6, also act as translation repressors by targeting specific steps during translation initiation (Coller and Parker, 2005; Nissan et al., 2010). This suggests a strong reciprocal relationship between active translation and mRNA decay. Interestingly, a block in translation elongation using cycloheximide reduces the extent of mRNA degradation, suggesting that bound ribosomes protect messages from being turned over (Beelman et al., 1994). In the case of 5'-3' decay, these findings led to the model that translational repression precedes decapping and subsequent degradation of the message (Coller and Parker, 2005) (Figure 4). Surprisingly, however, more recent findings suggest that mRNA turnover can also occur co-translationally (Hu et al., 2009).

Translationally silenced mRNAs are not always turned over immediately. The decision to degrade or stabilize/store a repressed mRNA depends largely on the underlying mechanism (*cis*-acting elements and *trans*-acting factors) that is responsible for the block in translation. Maternal messages represent the most prominent example, as they can remain in a silent but stable state for a very long period of time (see section 2.6). Furthermore, extrinsic effects, such as rapid changes in environmental conditions and the resulting stress response generally cause repression of many mRNAs without causing immediate degradation. This allows a cell to redirect most of its resources towards stress adaptation, but also enables a rapid return to its previous state in case the stressful situation passes. Inhibition of deadenylation appears to be responsible for enhanced stability of mRNAs under stress conditions (Hilgers et al., 2006).

2.5.3 Promoting the repression/degradation of "healthy" mRNA

To promote the repression/degradation of a certain mRNA or a group of messages without affecting others, requires sophisticated ways to target transcripts in a specific manner. Specificity is achieved by elements present on the mRNA molecule itself, so called *cis*-acting elements, in combination with their interacting proteins (*trans*-acting factors). Some *cis*-acting elements are of structural origin and, if present in the 5'UTR, can slow down or even block translation initiation and thereby promote decay (Muhlrad et al., 1995). Others, found predominantly in 3'UTRs, consist of specific motifs or an enrichment of certain nucleotides (Figure 4). The AU-rich element (ARE), for instance, represents one of the most intensively studied *cis*-acting mRNA stability determinants so far. Several ARE-binding proteins have

been identified, which has shed light on how mRNA decay is promoted, namely, by recruiting components of the decay machinery directly to the target mRNA (Chen et al., 2001; Gherzi et al., 2004). Interestingly, AREs can also serve as direct docking sites for the exosome (Anderson et al., 2006). Puf proteins represent another family of *trans*-acting factors involved in promoting mRNA repression/decay. These proteins interact with UG-rich sequences in the target mRNA and recruit the Ccr4/Pop2/Not deadenylase complex (Goldstrohm et al., 2006, 2007). Furthermore, *trans*-acting factors need not necessarily be proteins. miRNAs, for example, have been predicted to control many protein-coding genes in mammals (Bushati and Cohen, 2007). miRNAs represent a class of small RNAs of around 21 nucleotides, which base-pair with their binding sites present predominantly in the 3'UTRs of mRNAs. Thereby, miRNAs act as a guide for the actual effector complex, the miRISC. Once bound to an mRNA, the miRISC complex recruits deadenylases causing translational repression and in some cases subsequent mRNA decay (Fabian et al., 2010, Fabian and Sonenberg, 2012). In summary, the combination of many *cis*-acting elements and *trans*-acting factors make sure that the gene expression program, regarding "healthy" mRNAs, is regulated correctly at the level of cytoplasmic PTGR.

2.5.4 Degradation of aberrant mRNA

Faulty mRNA molecules have to be efficiently removed from a cell to prevent the accumulation of aberrant proteins, which might have toxic effects. Defects in mature mRNAs are usually detected co-translationally by an unusual behaviour of the ribosome. Three mRNA quality control mechanisms have been described to date: Nonsense mediated decay (NMD), no-go decay (NGD), and non-stop decay (NSD) (Figure 5).

NMD is activated by precocious termination of translation at premature stop codons (PTCs). PTCs can form due to errors in transcription or splicing, genetic mutations or recombination event. Premature termination of a ribosome attracts the NMD machinery to the faulty mRNA, which is then degraded either via endonucleolytic cleavage, decapping or deadenylation (Figure 5A). How NMD factors distinguish a premature termination event from a canonical one is not fully understood. The presence of splicing-derived exon junction complexes, which are usually removed during the first round of translation, appear to play a role (Le Hir et al., 2001). Furthermore, the 3'UTR and its interacting proteins, such as PABP, stimulate translation termination. A too big distance from the 3'UTR might reduce the efficiency of this eRF1-eRF3-mediated event and then serve as an entry point for NMD

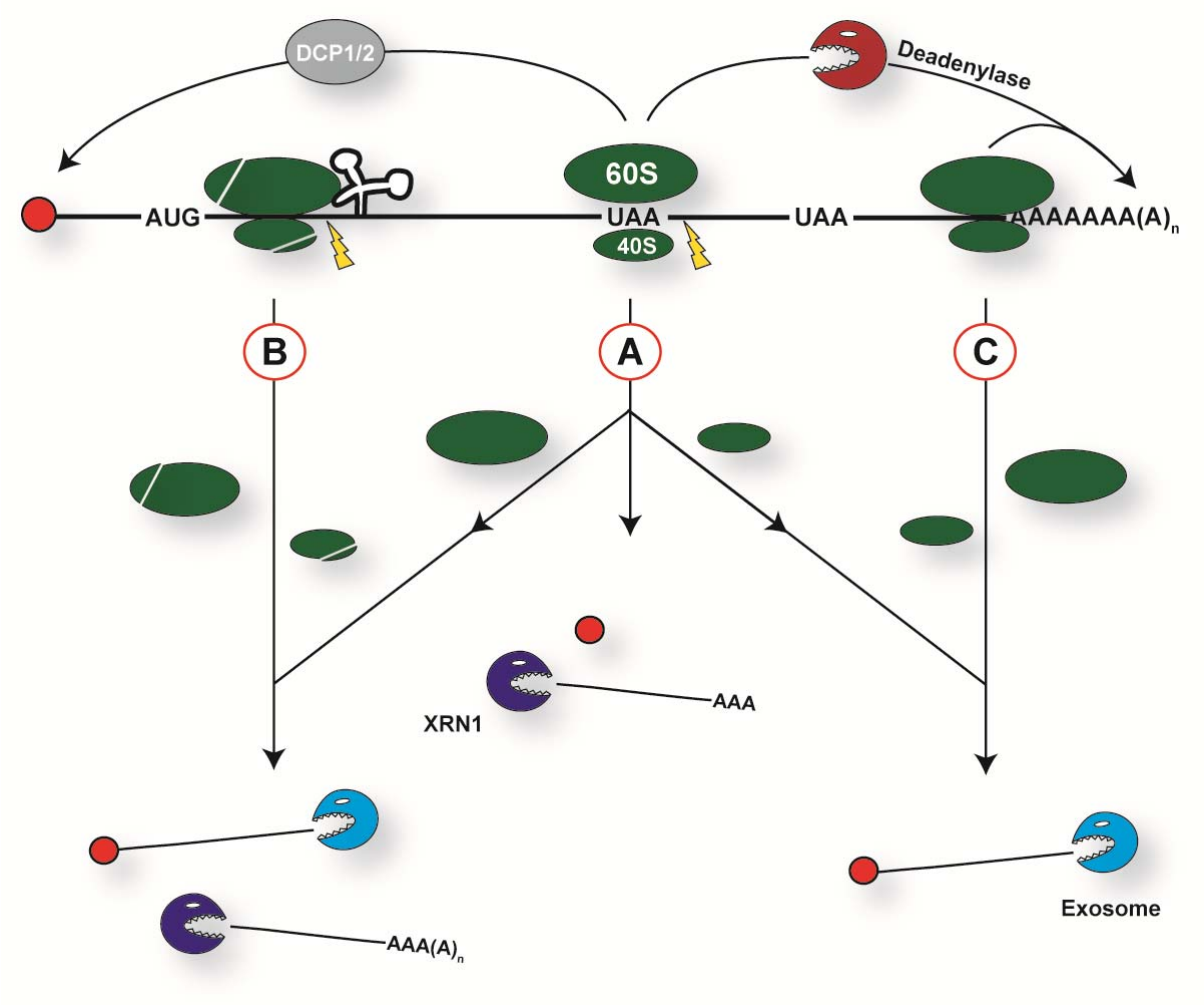

Figure 5. Degradation of aberrant mRNAs

(A) NMD is triggered by stalling of a ribosome at a premature stop codon. Subsequent mRNA turnover can take place from 5'-to-3' (XRN1), 3'-to-5' (exosome), or by an endonucleolytic cleavage event. (B) NGD is triggered by prolonged stalling of a ribosome during translation elongation, which occurs either due to alterations on the mRNA (secondary structure) or due to ribosome abnormalities. Degradation of the underlying mRNA is initiated by an endonucleolytic cleavage event. (C) NSD is triggered by ribosomes that continue translation into the 3'UTR. Subsequent mRNA turnover occurs from 3'-to-5' (exosome).

(Amrani et al., 2004). Interestingly, some endogenous mRNAs appear to be natural targets of NMD, most likely for regulatory purposes (Mendell et al., 2004; Guan et al., 2006).

The NGD machinery is attracted to defective mRNAs, on which a ribosome has stalled during translation elongation. This type of quality control pathway has so far only been reported in yeast and *Drosophila* (Doma et al. 2006; Passos et al., 2009). NGD is initiated by an endonucleolytic cleavage event followed by turnover of the two degradation intermediates (Figure 5B). The Hbs1-Dom34 complex, which highly resembles the eRF1-eRF3 translation

termination complex, is crucial for NGD. On the one hand Hbs1 and Dom34 appear to stimulate endonucleolytic cleavage of the aberrant mRNA (Doma et al., 2006; Passos et al., 2009) and on the other hand they are important to dissociate the stalled ribosome from the message to allow mRNA degradation (Tsuboi et al., 2012). The cleavage event appears to strongly enhance Hbs1-Dom34-mediated ribosome disassembly, which suggests a sequential order of the two processes (Lykke-Andersen and Bennet, 2014). The identity of the endonuclease is still unknown. In the case of NGD the dissociated ribosome is usually also degraded as the cell cannot distinguish if the block in translation elongation is due to abnormalities in the mRNA or the ribosome (Karbstein, 2013).

The third quality control pathway, NSD, deals with aberrant mRNAs that do not contain the canonical stop codon, which causes ribosome translocation into the 3'UTR (Figure 5C). Premature polyadenylation inside the coding region of an mRNA is thought to be the major cause for the loss of the termination codon. NSD is triggered when a ribosome reaches the 3' end of the message (Frischmeyer et al., 2002; van Hoof et al., 2002). The protein Ski7 appears to play a crucial role in NSD. Its C-terminal domain, which resembles eRF3, is thought to interact with the empty A-site of the ribosome, while the N-terminal part is responsible for recruiting the exosome (van Hoof et al., 2002). Furthermore, the Hbs1-Dom34 complex promotes disassembly of the ribosome. Similar to NGD, ribosome release appears to be important for exosome-mediated 3'-5' decay to occur (Tsuboi et al., 2012).

2.5.5 Processing bodies and stress granules

The remodelling of individual mRNPs, thereby exchanging the set of bound RBPs, regulates the fate of an mRNA. In addition, single mRNPs can aggregate into dynamic, microscopically visible granules, such as processing bodies (P bodies) or stress granules. Blocking global translation at the initiation stage increases the number of mRNAs in both types of granules, whereas polysome-associated messages decrease (Kedersha et al., 2005; Teixeira et al., 2005). Vice versa, preventing the release of mRNAs from polysomes by globally blocking translation elongation reduces granule formation (Teixeira et al., 2005). These observations are reminiscent of the reciprocal relationship of translation and mRNA decay. Therefore, not surprisingly, P bodies contain many core proteins involved in translational repression and mRNA degradation and are thought to represent centres for mRNA decay. However, some reports suggest that repressed mRNAs located in P bodies can also re-associate with ribosomes (Brengues et al., 2005). Stress granules share some components with P bodies, but in addition contain factors involved in translation initiation including the 40S ribosomal subunit. Therefore, translationally repressed mRNAs are thought to transiently accumulate in

stress granules, however, with an apparent capacity to re-enter active translation (Decker and Parker, 2012). Despite the extensive research performed on granule biology in the past years, the actual significance of forming P bodies and stress granules and their interrelation still remains unclear. Their disruption, for instance, appears to have little effect on processes such as decapping or translational repression (Decker et al., 2007; Buchan et al., 2008). Therefore, it has been suggested that P bodies and stress granules might act more as enhancers of certain processes, rather than having essential functions. For example, these granules might locally increase the concentration of certain, potentially limiting factors, thereby enhancing the efficiency of specific reactions, such as decapping or the assembly of translation initiation complexes. Furthermore, granules might also act as a buffering system, for instance, to keep translation efficient by sequestering mRNAs to ensure that the translation machinery can accurately cope with the number of mRNA molecules presented (Decker and Parker, 2012).

Some microscopically visible aggregates are also unique to the germline, such as P granules in *C. elegans*, sponge bodies and polar granules in *Drosophila*, or germinal granules in *Xenopus*. These germ granules contain a wide variety of essential proteins involved in maternal mRNA regulation. However, similar to P bodies and stress granules, the functional relevance of forming larger aggregates and how this might impact central aspects of maternal mRNA regulation in oocytes, such as stability, transport, or translation remains to be determined (Schisa, 2012).

2.6 Storing mRNA

Some mRNAs, when exported to the cytoplasm, are prevented from entering the translation pool without being degraded. Instead these messages associate with a specific subset of proteins, which assist in packaging the particular mRNA into a silent and stable mRNP. One of the few somatic tissues, in which prolonged repression and stabilization of mRNAs appears to be essential, is in neurons. Due to their extensive dimensions compared to many other cell types, mRNAs generated in the neuronal soma destined to be expressed at distant synapses have to be transported there in a repressed state. Furthermore, upon arrival at their destination many mRNAs only become activated upon certain neuronal stimuli. This system is regulated by a complex network of *cis*-acting elements and *trans*-acting proteins (Doyle and Kiebler, 2011). Interestingly, PTGR plays a crucial role in the plasticity of synapses and therefore also in processes such as learning and memory. Not surprisingly, abnormalities in synaptic PTGR have been implicated in several neurological diseases (Darnell and Richter, 2012). Despite the importance of translational repression and

stabilization of mRNAs in neurons, the term “mRNA storage” is primarily associated with the regulation of maternal messages in the germline. Mammalian oocytes, for instance, can remain in a quiescent state for months or even years before they are activated. During all this time the integrity of maternal mRNA has to be maintained. Data coming from many different organisms, including *C. elegans*, has shed light on many essential RBPs and underlying *cis*-acting elements involved in maternal mRNA regulation. The best mechanistic insights into how maternal mRNAs are stored, but also how these are reactivated at the appropriate time, come from studies performed in *Xenopus* and *Drosophila* (Richter and Lasko, 2011).

2.6.1 Storing maternal mRNA in the *Xenopus* oocyte

Regulation of maternal mRNAs during *Xenopus* oocyte maturation serves as a paradigm for how modulating the poly(A) tail regulates gene expression. Importantly, varying the length of the poly(A) tail as a mean to control gene expression is not restricted to germ cells but also plays an important role in neurons, for example (Weill et al., 2011). The poly(A) tail is added in the nucleus. In addition, some pre-mRNAs contain a cytoplasmic polyadenylation element (CPE) in their 3’UTR. Still inside the nucleus the CPE is bound by CPEB and exported in the presence of additional factors, one of which is Maskin (Lin et al., 2010). Once exported into the cytoplasm the complex acquires two proteins with opposing functions, the poly(A) polymerase Gld2 and the deadenylase PARN. As PARN is more active than Gld2, the poly(A) tail is kept short (Kim and Richter, 2006). However, this is not sufficient to keep the mRNA repressed, but in addition requires the action of Maskin, which binds to both eIF4E and 3’UTR-bound CPEB (Stebbins-Boaz et al., 1999). These observartions led to the proposition that mimicking the “closed loop”, thereby precluding eIF4G, is one way to block translation of maternal mRNAs, an intriguing model that indeed holds true in *Drosophila* (see below) (Figure 6). However, the “Maskin-model” itself is highly debated (Minshall et al., 2007).

Relieving translation repression represents another important step in maternal mRNA regulation. One way translation repression is thought to be relieved upon progesterone stimulation in *Xenopus* is by phosphorylating CPEB. This causes the release of PARN from the complex, and allows Gld2 to polyadenylate the mRNA (Kim and Richter, 2006) (Figure 6).

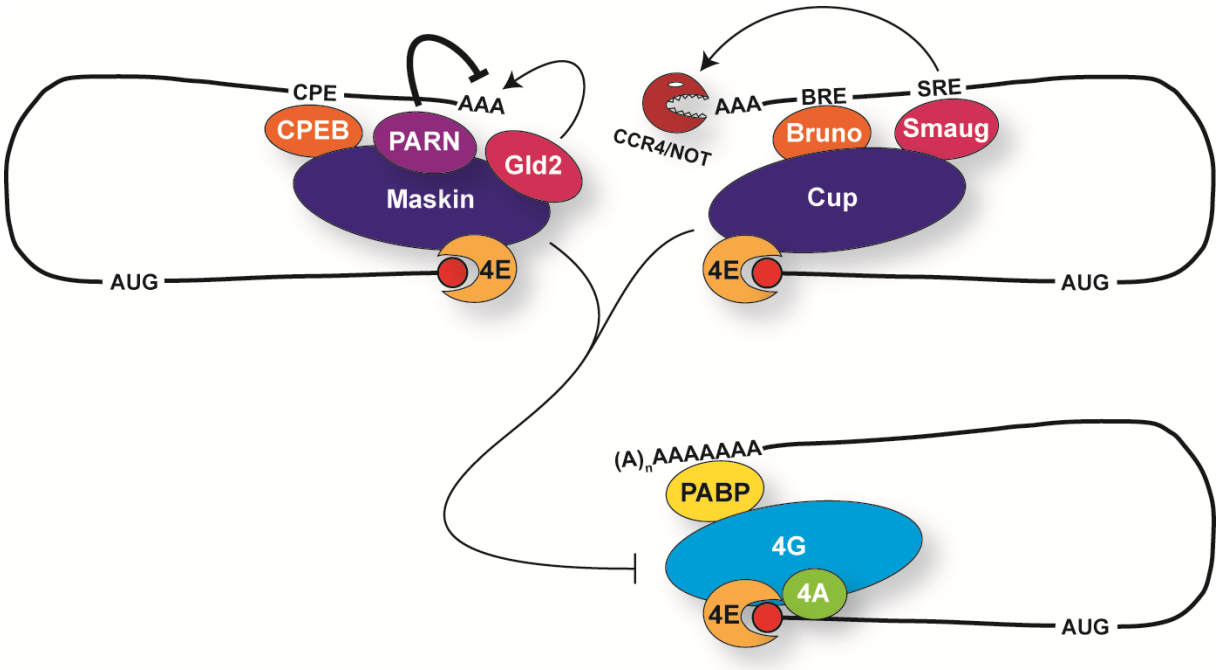

Figure 6. Mimicking the "closed loop" to prevent translation

In *Xenopus* oocytes translation is repressed by a complex including Maskin, which bridges the cap-bound eIF4E and the 3'UTR-bound CPEB, thereby mimicking the "closed loop" usually formed by eIF4E-eIF4G-PABP. The poly(A) tail is kept short by the recruitment of the deadenylase PARN and upon its release is extended by the poly(A) polymerase Gld2. In *Drosophila* oocytes translation is repressed by a complex including Cup, which bridges eIF4E and either of the two 3'UTR-bound proteins Bruno or Smaug, the latter of which has been shown to recruit the deadenylase CCR4/NOT.

2.6.2 Storing maternal mRNA in the *Drosophila* oocyte

Proper embryogenesis in *Drosophila* depends largely on the correct spacial deposition of maternal mRNAs during oogenesis and its subsequent localized translation. Not surprisingly, *Drosophila* oogenesis has also served as a convenient model to investigate the mechanisms behind localized transport of mRNA molecules (Becalska and Gavis, 2009). The translation regulation of several specific maternal mRNAs has been studied in depth. A recurring mechanism to repress translation, first suggested in *Xenopus,* as stated above, is the mimicking of the "closed loop" usually formed by eIF4E, eIF4G and PABP during normal rounds of translation (Figure 6). The translational repressor Cup, for instance, blocks *oskar* mRNA translation through binding to eIF4E and thereby competes with eIF4G (Nakamura et al., 2004). Another factor termed Bruno interacts with specific Bruno-Response Elements in the 3'UTR of the *oskar* mRNA (Kim-Ha et al., 1995). Furthermore, Cup and Bruno directly interact with one another, thereby closing the loop (Nakamura et al., 2004). The *nanos*

mRNA is regulated in a similar way. Its 3'UTR contains a Smaug-Recognition Element, which is bound by the translational repressor Smaug (Smibert et al., 1996). Smaug interacts with eIF4E-bound Cup and this interaction is essential for repression (Nelson et al., 2004). Smaug also promotes target mRNA deadenlyation by recruiting the Ccr4/Pop2/Not deadenylase complex (Zaessinger et al., 2006) (Figure 6).

Translation activation in *Drosophila* is thought to involve Orb, one of several additional factors present in *oskar* mRNPs and the *Drosophila* homologue of *Xenopus* cytoplasmic polyadenylation element binding protein (CPEB). Orb binds to the 3'UTR of *oskar* mRNA and promotes translation, potentially by promoting polyadenylation (Chang et al., 1999). This is supported by the fact that Orb interacts directly with the two poly(A) polymerases PAP and Wispy (Benoit et al., 2008). Translational activation of *nanos* mRNA has been proposed to depend on Oskar-dependent displacement of Smaug, thereby preventing Ccr4/Pop2/Not-mediated deadenylation (Zaessinger et al., 2006). Finally, the translation of *gurken* mRNA appears to be promoted by the DEAD-box helicase Vas. Vas interacts directly with eIF5B and the recruitment of this initiation factor is thought to be crucial for the activation of *gurken* mRNA (Johnstone and Lasko, 2004).

2.7 The importance of general RNA-binding proteins in PTGR

Despite the importance of *cis*-acting elements and *trans*-acting factors for the regulation of specific mRNAs, RBPs that interact with messages in a more general, often sequence-independent manner also appear to have crucial functions in PTGR. The highly conserved family of DEAD-box helicases, for instance, such as Me31B in *Drosophila* and Xp54 in *Xenopus*, are among the essential constituents found in maternal mRNPs and contribute to translational repression (Nakamura et al., 2001; Minshall et al., 2001). However, members of this family are also present in the soma and are therefore not restricted to maternal mRNA regulation. Their ATP-dependent unwinding/remodelling activity combined with their general affinity for RNA has DEAD-box helicases affect PTGR in many ways in both nucleus and cytoplasm (Weston and Sommerville; 2006; Linder and Jankowsky, 2011). Similarly, the so called Y-box binding proteins represent another class of RBPs that are frequently found among the constituents of mRNPs.

2.8 The family of Y-box binding proteins

YBPs belong to a subgroup of the superfamily of cold shock domain (CSD) containing factors. The CSD, which is around 70 amino acids in length, is highly conserved, showing more than 40% identity and more than 60% similarity in bacteria and vertebrates (Sommerville, 1999). Its name comes from bacterial cold shock proteins (Csps), which essentially consist of one CSD. These small Csps are induced upon cold stress and bind to single stranded RNA. They are believed to function as RNA chaperones to prevent unwanted, potentially toxic folding events of RNA molecules (Horn et al., 2007). The CSD acquires the so called oligosaccharide-/oligonucleotide-binding (OB) fold, which consists of five antiparallel β-strands that form a β-barrel (Kloks et al., 2002). The OB-fold can interact with diverse ligands such as nucleic acids or proteins (Arcus, 2002). The CSD appears to bind primarily to single stranded nucleic acids, whereby the two motifs, RNP1 and RNP2, located in β-strand 2 and β-strand 3, respectively, play a central role (Landsman et al., 1992; Mihailovich et al., 2010). YBPs got their name from initial observations that YB-1 interacts with the Y-box element in the promoter of major histocompatibility complex class II genes (Didier et al., 1988). However, apart from acting as transcription factors, YBPs are among the most abundant proteins in cytoplasmic mRNPs in both soma and germline (Bouvet et la., 1994; Evdokimova et al., 1995). Due to their ability to generally bind and package mRNAs, YBPs have also been termed “RNA histones” (Sommerville, 1999). Not surprisingly, YBPs have a very broad spectrum of functions, a fact that is nicely exemplified by YB-1.

2.8.1 YB-1: A paradigm for pleiotropic functionality

YB-1 represents the best-studied member of the YBP family to date. Besides the central CSD, YB-1 contains an N-terminal alanine/proline-rich domain and a C-terminal domain (CTD), which consists of positively and negatively charged clusters. While the CSD folds into a specific structure, the other two domains appear to be intrinsically disordered, albeit being important for YB-1 function. A disordered region can become ordered once the interaction with a specific substrate has been established. Therefore, a certain degree of disorder might increase the number of different targets and could explain why proteins, such as YB-1, have multiple different functions (Lyabin et al., 2014). In the nucleus, YB-1 participates in the regulation of transcription, in DNA repair, and in mRNA splicing. In the cytoplasm, YB-1 appears to be the most prominent constituent of repressed mRNPs, but is also a major component of polysomes (Minich and Ovchinnikov, 1992). At a low YB-1/mRNA ratio, resembling the situation in polysomes, individual YB-1 proteins appear to interact via both

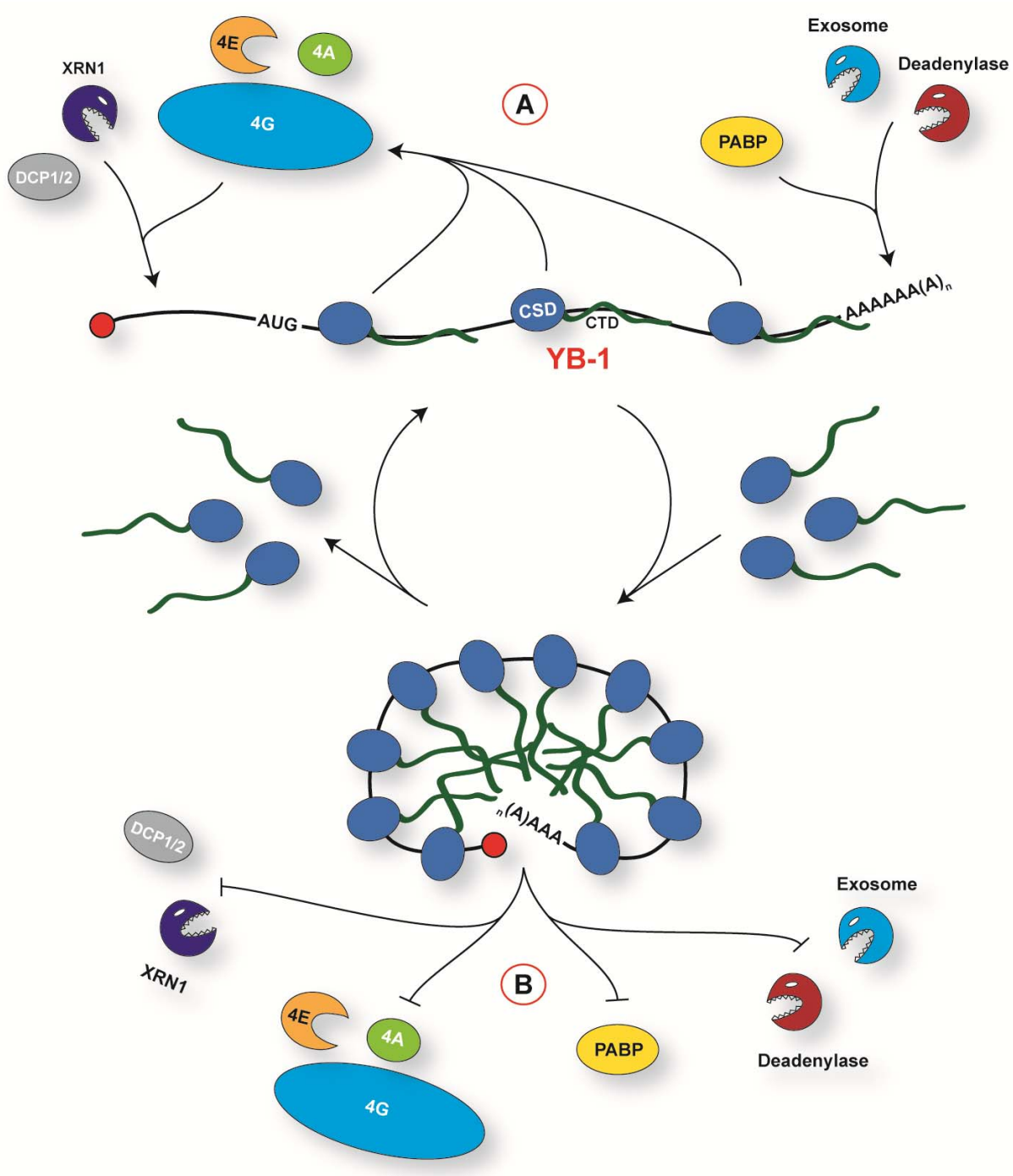

Figure 7. Cytoplasmic functions of YB-1 (Adapted from Skabkin et al., 2004)

(A) At low YB-1/mRNA concentrations both the CSD and the CTD bind to the mRNA molecule. The 5' and 3' end of the mRNA are accessible by the translation and degradation machineries. YB-1 binds along the mRNA and thereby promotes translation by preventing unwanted binding of initiation factors along the message. (B) At high YB-1/mRNA concentrations the CSD still binds to the mRNA, but the CTD is now involved in YB-1 multimerization. The ends of the mRNA are buried in the densely packed structure, thereby preventing both translation and decay.

the CSD and CTD with the RNA and promote unwinding of the transcript. In this scenario, the ends of the mRNA molecule are accessible to both the translation and decay machineries (Skabkin et al., 2004) (Figure 7). The idea of YB-1 playing an important role in active translation is supported by the observation that translation initiation is blocked in the absence of YB-1 (Evdokimova et al., 1998). One hypothesis how general mRNA binding proteins, such as YB-1, promote translation is by accumulating along an mRNA molecule, thereby allowing the translation initiation complex to bind only at the 5' end of the message (Svitkin et al., 1996). In addition to simply binding RNA, YB-1 also has the ability to melt RNA secondary structures *in vitro*, thereby acting similar to an RNA helicase (Evdokimova et al., 1995). This unwinding activity of YB-1 might allow this protein to promote translation initiation also by enhancing the efficiency of ribosomal scanning (Skabkin et al., 2001). At high YB-1/mRNA ratios, mimicking the situation of repressed mRNPs, YB-1 still appears to interact via the CSD with the RNA, however, the CTD now promotes YB-1 multimerization. This results in packaging of the mRNA molecule including its 5' and 3' ends, thereby not only preventing translation but also exonuclease-mediated degradation (Skabkin et al., 2004) (Figure 7). Indeed, it was shown that both PABP and the eIF4F complex are displaced by YB-1 at high concentrations, causing translational repression at the initiation stage and the stabilization of the underlying mRNA (Evdokimova et al., 2001; Nekrasov et al., 2003). Not surprisingly, based on this global function in translation repression, YB-1 has been found to localize to both P bodies and stress granules (Kedersha and Anderson, 2007; Yang and Bloch, 2007). Furthermore, at high YB-1/mRNA ratios YB-1 appears to promote the association of mRNA with microtubules and might therefore play an important role in the localization of repressed mRNAs (Chernov et al., 2008). YB-1 also directly interacts with actin (Ruzanov et al., 1999). Therefore, YB-1 and YBPs in general cannot be considered solely as nucleic acid binding proteins (Eliseeva et al., 2011).

The concentration-based effect of YB-1 on global translation is due to its strong affinity for single-stranded RNA in general. However, several more recent studies suggest that YB-1 can also interact with more specific sequences and thereby regulate translation and stability of individual mRNAs (Eliseeva et al., 2011; Lyabin et al., 2014). One of the best examples is the apparent autoregulatory loop, whereby YB-1 represses its own production by interacting with a specific regulatory element in its 3 'UTR (Lyabin et al., 2011).

The presence of YB-1 in both nucleus and cytoplasm, in combination with unspecific and specific recognition of its targets, not surprisingly, has this protein function in many different processes in a cell. The pleiotropic functions of YB-1 include a role in cell proliferation, where it has been shown, for instance, to repress translation of cyclins, growth factors and translation factors. Many functions described for YB-1, such as preventing apoptosis or driving differentiation, are related to cancer cells, in which YB-1 often appears to

be highly expressed. Therefore this protein serves as a good marker for, but also as a potential drug target against such malignant cells (Lasham et al., 2013). YB-1 also plays an important role in a variety of stress responses (Eliseeva et al., 2011; Lyabin et al., 2014).

Most insights into the different functions of YB-1 stem from either *in vitro* or single cell systems. Studies looking closer into potential developmental functions of YBPs in the soma are still rare. In mouse, two studies reported that the loss of YB-1 in the embryo is fatal, showing defects in neurotubule formation and prominent growth retardation (Lu et al., 2005; Uchiumi et al., 2006). Therefore, further *in vivo* studies performed in a multicellular context are required to gain more insights into how this very interesting class of proteins actually influences somatic development.

2.8.2 The role of Y-box binding proteins in maternal mRNA regulation

Most knowledge on the function of YBPs in the germline comes from studies on FRGY2 in *Xenopus*, which plays an essential role in packaging and storing maternal mRNAs in oocytes (Tafuri and Wolffe, 1993). The requirement for FRGY2 specifically for maternal mRNA regulation is underlined by the fact that the protein levels strongly decline during early embryogenesis and are no longer detectable once the pool of stored maternal mRNAs has been used up (Wolffe et al., 1992). Similar to YB-1, FRGY2 can generally interact with mRNAs, but also shows some preference for certain nucleotide sequences, whereby the CSD plays the predominant role in recognizing these (Bouvet et al., 1995). The N- and C-terminal regions, on the other hand, contribute more to the unspecific interaction with mRNA, but appear to play the predominant role for translationally repressing the bound messages (Matsumoto et al., 1996). Even though the exact mechanism by which FRGY2 acts is unclear, a steric occlusion of the translation and degradation machinery due to the tight packaging of mRNAs into dense RNPs is most likely (Matsumoto et al., 2003). Interestingly, injecting *in vitro* transcribed mRNA directly into the cytoplasm of *Xenopus* oocytes caused less potent translational repression compared to *in vivo* transcribed messages. It was proposed that this relies on the binding of RBPs, including FRGY2, in the nucleus and was one of the first observations that nuclear history matters for subsequent cytoplasmic regulation of mRNAs (Bouvet and Wolffe, 1994).

In the mouse, the deletion of the YBP termed MSY2 results in sterility, whereby both sperm and oocyte development are affected (Yu et al., 2004; Yang et al., 2005, 2006). As for FRGY2, MSY2 protein starts to decrease strongly upon fertilization and it is present only at very low levels in the two-cell stage embryo, a turnover pattern that strongly coincides with the one of maternal mRNAs (Yu et al., 2001). Consistent with a function in the regulation of

maternal messages, the stability of many mRNAs is affected in oocytes of *Msy2*$^{-/-}$ homozygotes. The fact that a mutant version of MSY2 that cannot bind to RNA is unable to restore stability (compared to the wild-type version), suggests that MSY2, at least in part, plays a direct role in global stabilization of maternal mRNAs (Medvedev et al., 2011).

Yps in *Drosophila* is part of the mRNP complex that is important for *oskar* mRNA regulation in the oocyte. It has been suggested that Yps has an antagonistic function to the putative translational activator Orb to prevent precocious expression prior to reaching its correct posterior location in the oocyte. However, the mechanism by which it functions is unclear (Mansfield et al., 2002). It is likely that Yps also plays a role in regulating additional messages, such as *bicoid* mRNA (Wilhelm et al., 2000).

In zebrafish, Ybx1 has been shown to bind to the dorsal localization element in the 3' UTR of squint (sqt) maternal mRNA and the loss of Ybx1 results in embryonic lethality due to defects in Sqt/Nodal signalling. In addition to the 3' UTR, Ybx1 also interacts with the cap-binding factor eIF4E and might thereby mimic the "closed loop" to occlude eIF4G binding and prevent translation initiation (Kumari et al., 2013).

2.9 The nematode *Caenorhabditis elegans*

This model system was introduced in the 1960s (Brenner, 1974). The adult size of only around one millimetre in length, the fact that bacteria are a sufficient food source combined with the production of a large number of progeny (~240 at 15°C, ~250 at 20°C, ~150 at 25°C), allow for easy cultivation of this organism. In addition, *C. elegans* has a short life cycle, the length of which depends on the temperature the animals are grown at (~96h at 15°C, ~50h at 20°C, ~40h at 25°C). The embryo develops inside a protective eggshell, before hatching as a so called L1-stage larva. Subsequently, an animal proceeds through three additional larval stages (L2, L3, L4), before reaching adulthood. Each stage is separated by a molt, during which a new "skin" (cuticle) is formed, which allows the animal to grow. In the case of nutrition deprivation *C. elegans* can also enter the "Dauer" state at the L1-to-L2 transition, which allows prolonged survival without any food intake. The genome of this diploid organism is comparably small (10^8 bp compared to $3x10^9$ bp in humans) and is packed into five autosomes and one sex chromosome comprising around 20'000 protein-coding genes. Importantly, many genes have homologues in mammals. A very useful feature is the transparency of its cuticle throughout development. This enables internal observations to be made directly *in vivo* and is especially powerful in combination with fluorescently-tagged proteins. *C. elegans* adults consist of 959 somatic cells, which form a series of different tissues. Due to its transparent nature, cells can be traced during development,

providing insights into how the body plan of a multicellular organism is established. The nervous system, for instance, with only 302 neurons, compared to the billions present in humans, is perfectly suited for studying basic principles of neuronal development and function (White et al., 1986).

2.9.1 The reproductive system

C. elegans occurs predominantly as a hermaphrodite (XX) and the resulting self-fertility is another feature that contributes significantly to the easy cultivation of this organism. Nevertheless, males (X0) also exist, thereby enabling crosses, a central aspect for genetics. The self-fertility of hermaphrodites is based on sperm production and storage in the spermatheca during the L4 stage, before switching to oogenesis as young adults. An adult hermaphrodite is a genuine “progeny production machinery” and the two symmetrical gonad arms that merge on a common uterus constitute a large part of its body mass (Figure 8A). Signalling coming from the distal tip cell (DTC), a somatic cell that sits at the distal end of the gonad, is responsible for maintaining a pool of mitotic stem cells (Kimble and White, 1981) (Figure 8B). However, upon moving further proximally, DTC-mediated signals weaken and germline stem cells enter the transition zone where germ cell development starts by initiating meiosis. While germ cells continue to move proximally, maternal messages and proteins accumulate in the common cytoplasm. After the bend region, individual cellularized oocytes become apparent (Figure 8B). These arrest in meiosis I and are only reactivated by signals derived from the stored sperm in the spermatheca, such as the major sperm protein (Miller et al., 2001). Subsequently, an activated oocyte is pushed through the spermatheca and fertilized, an event that on average takes place every 23 minutes (McCarter et al., 1999) (Figure 8B). The two rounds of meiosis are completed before the oocyte and sperm nuclei fuse to form the one-cell stage embryo. As in other organisms, global transcription is turned off during late stages of oogenesis and is only reactivated in the three somatic blastomeres of the four-cell stage embryo, while transcription remains silent in the germline precursor cell (Seydoux et al., 1996). Therefore, very early development depends entirely on stored maternal factors also in *C. elegans*. Two crucial RBPs for maternal mRNA regulation are GLD-1 and CGH-1.

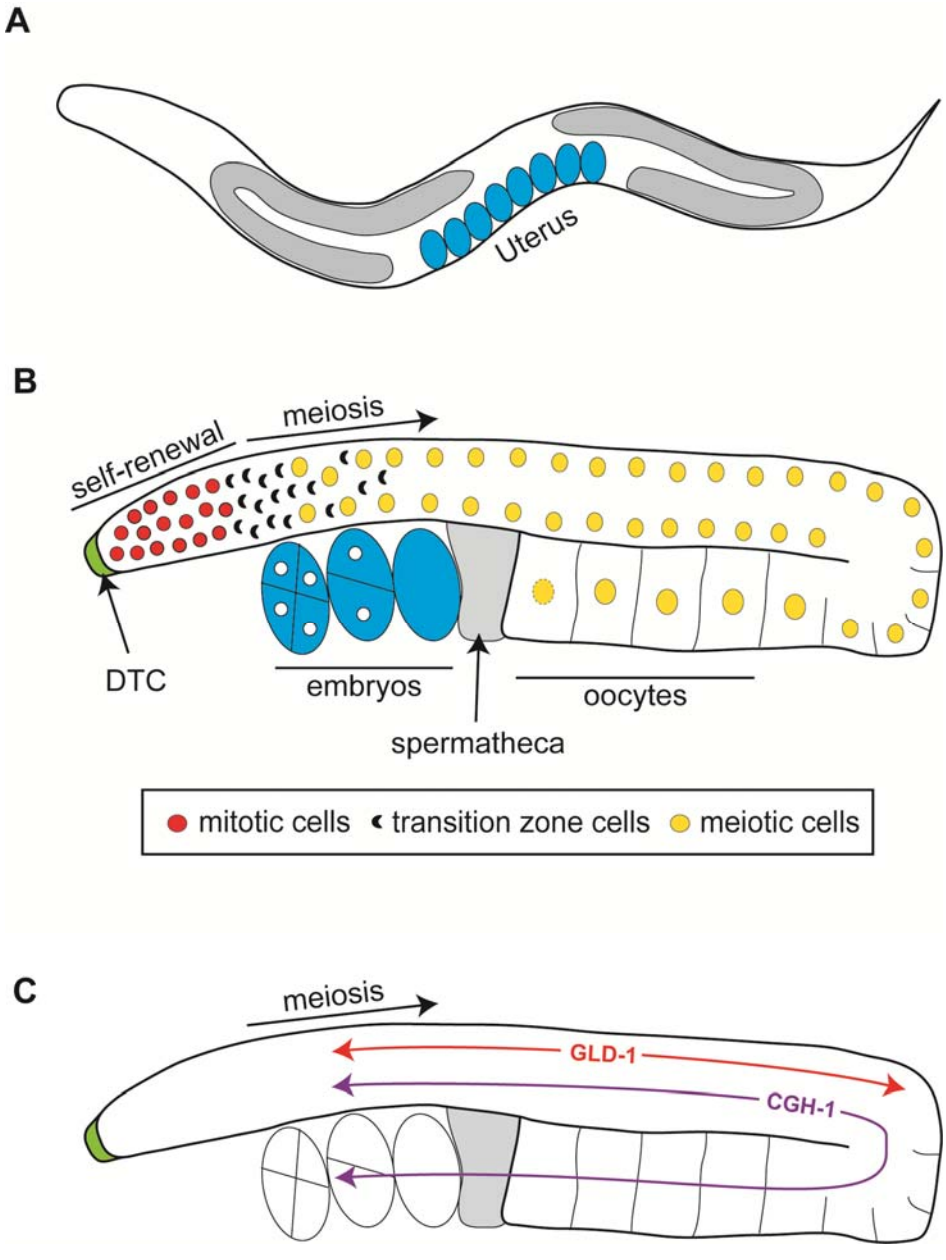

Figure 8. The *C. elegans* reproductive system

(A) *C. elegans* has two gonadal arms (in grey) that merge on a common uterus (embryos in blue). (B) A schematic gonad with embryos (in blue). The distal tip cell (DTC) promotes the self-renewal of germline stem cells in the most distal part of the gonad. More proximally, germ cells enter meiosis via a so-called transition zone and, in adults, eventually differentiate into oocytes. Ovulated oocytes become fertilized by sperm stored in the spermatheca (in grey). Embryogenesis follows. (C) Both GLD-1 and CGH-1 are expressed upon meiotic entry. GLD-1 is only present in the medial gonad, while CGH-1 is detected throughout oogenesis as well as in early embryos.

2.9.2 GLD-1 and CGH-1: Two essential RNA-binding proteins in oogenesis

GLD-1 belongs to the conserved family of Quaking-related RNA-binding proteins, a subgroup of the STAR (signal transduction and activation of RNA) family (Biedermann et al., 2010). In oogenesis, GLD-1 starts to be expressed when germ cells enter meiosis and disappears at the bend region (Jones et al., 1996) (Figure 8C). It is therefore expressed at a time point when many newly synthesised maternal messages need to be stored. Indeed, GLD-1 has been shown to have an essential function in translationally repressing maternal mRNA and a more recent study also suggested a role in stability (Lee and Schedl, 2001; Biedermann et al., 2009; Wright et al., 2011; Scheckel et al., 2012). This protein is not a general RNA binder, but specifically recognizes a heptanucleotide sequence, known as the GLD-1 binding motif, which is found predominantly in the 3'UTR of its target mRNAs (Wright et al., 2011, Daubner et al., 2014). The loss of GLD-1 results in the re-entry of meiotic cells into mitosis and the formation of a tumour. GLD-1-mediated translational repression of cyclin E mRNA during meiosis has been shown to play a very important role to prevent the re-entry into mitosis (Biedermann et al., 2009). Such tumorigenic cells can further differentiate into somatic cells, such as neuron or muscle, thereby forming a germline teratoma (Ciosk et al., 2006). This extreme phenotype underlines the role of GLD-1 as an essential constituent of many maternal mRNPs, thereby acting as a central regulator of germ cell development.

CGH-1 is a conserved DEAD-box helicase closely related, for instance, to the maternal mRNP components Xp54 in *Xenopus* or Me13B in *Drosophila*. Its homologue in yeast, Dhh1, is involved in mRNA repression and turnover and is a regular constituent of P bodies (Coller and Parker, 2005; Nissan et al., 2010). CGH-1 accumulates upon the entry of germ cells into meiosis. However, differing from GLD-1, CGH-1 is present throughout oogenesis and eventually disappears in the embryo with the exception of the germline precursor cell (Navarro et al., 2001; Boag et al., 2008) (Figure 8C). Interestingly, CGH-1 appears to play a dual role. During oogenesis it associates with other translational regulators, such as GLD-1 and YBPs, and a larger, but apparently specific subset of maternal messages to prevent their turnover (Boag et al., 2005, 2008; Scheckel et al., 2012). However, as DEAD-box helicases are thought to bind mRNA in an unspecific manner, it is perfectly possible that it also generally regulates maternal mRNAs but prefers some over others. In the somatic tissue of embryos, CGH-1 appears to be part of P body-like structures, which include decapping enzymes, potentially participating in maternal mRNA turnover (Boag et al., 2008). The phenotype of *cgh-1* mutant animals is less severe than the teratoma observed in the absence of GLD-1, and germ cells do not re-enter mitosis. This might be due to the fact that maternal mRNAs are not precociously expressed as in the *gld-1* mutant. Nevertheless, animals lacking CGH-1 are sterile. Apparent defects are an enhanced level of germ cell

apoptosis and the accumulation of aberrant oocytes (Navarro et al, 2001). Therefore, just like GLD-1, CGH-1 is an essential germline RBP.

3. SCOPE OF THIS PHD THESIS

Two previous studies in *C. elegans* had looked into CGH-1- and GLD-1-interacting proteins, respectively (Boag et al., 2005; Scheckel et al., 2012). In both cases, CEYs were detected, suggesting a conserved function of these factors in maternal mRNP regulation. Despite the fact that YBPs had been studied in many different model organisms, at the time nothing was known about these proteins in *C. elegans*. Due to the interaction with both GLD-1 and CGH-1 we knew that CEYs must be present in the germline. Furthermore, as RBPs important for oocyte development in *C. elegans*, in particular GLD-1, were of major interest in our lab, we primarily wanted to know what functions CEYs might have during oogenesis. Nevertheless, considering the scarce amount of studies published on the function and potential importance of YBPs during somatic development, we were also interested if CEYs were expressed in the soma and what their function might be. Finally, as most knowledge on YBPs had derived from single cell or *in vitro* systems, investigating the functions of YBPs in a multicellular context, such as in *C. elegans*, was bound to contribute valuable information to the field.

4. RESULTS

4.1 Functional characterization of *C. elegans* Y-box binding proteins reveals tissue-specific functions and a critical role in the formation of polysomes

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4.1.1 Abstract

The cold shock domain is one of the most highly conserved motifs between bacteria and higher eukaryotes. Y-box binding proteins represent a subfamily of cold shock domain proteins with pleiotropic functions, ranging from transcription in the nucleus to translation in the cytoplasm. These proteins have been investigated in all major model organisms except *Caenorhabditis elegans*. In this study, we set out to fill this gap and present a functional characterization of CEYs, the *C. elegans* Y-box binding proteins. We find that, similar to other organisms, CEYs are essential for proper gametogenesis. However, we also report a novel function of these proteins in the formation of large polysomes in the soma. In the absence of the somatic CEYs, polysomes are dramatically reduced with a simultaneous increase in monosomes and disomes, which, unexpectedly, has no obvious impact on animal biology. Because transcripts that are enriched in polysomes in wild-type animals tend to be less abundant in the absence of CEYs, our findings suggest that large polysomes might depend on transcript stabilization mediated by CEY proteins.

4.1.2 Introduction

The cold shock domain (CSD) is one of the most ancient and highly conserved protein domains known, sharing more than 40% identity and 60% similarity between bacteria and vertebrates [1]. This nucleic acid binding motif enables the proteins to bind to both single stranded RNA and/or DNA [2]. A small subgroup of the CSD protein superfamily includes the so-called Y-box binding proteins (YBPs). Apart from the CSD, YBPs can contain additional motifs, such as basic/aromatic or glycine-rich stretches in vertebrate and plant proteins, respectively, and RG/RGG repeats in a range of invertebrate proteins [1,3]. Even though YBPs act predominantly as nucleic acid binding proteins, they can also bind proteins, as has been shown for human YB-1 [4]. These interactions usually depend on motifs located outside the CSD. YB-1, for example, binds to actin filaments via its alanine- and proline-rich N-terminal domain [5].

Previous work from many laboratories revealed that YBPs function in different cellular processes, best represented by the intensively studied human YB-1 (reviewed in [4]). In the nucleus, for instance, this protein is involved in transcription, DNA repair, and pre-mRNA splicing, while in the cytoplasm it has an important role in mRNA regulation, which includes both mRNA stability and translation repression or activation. Another family member, FRGY-2, is expressed specifically in *Xenopus* oocytes. Its main function is to package newly synthesized maternal messages and keep them stable and translationally inactive until

needed [6,7,8]. Further examples of YBPs with important functions in the germline are MSY-2, which is important for the stability of many maternally provided mRNAs in mice [9,10], Yps, which plays a role in correct localization and expression of maternal oskar mRNA in *Drosophila* [11], and Ybx1, which regulates maternal sqt1 mRNA translation and thereby ensures correct development of the zebra fish embryo [12]. Due to their ability to bind and package mRNA, YBPs have also been referred to as “RNA histones” [1]. Just like YBPs, the so-called DEAD-box helicases appear to be common constituents of mRNA/protein granules (RNPs) and it has been suggested that these enzymes help to establish and stabilize the interaction of YBPs with single-stranded RNA [13]. A previous study identified *C. elegans* YBPs (CEYs) as interaction partners of the DEAD-box helicase CGH-1 [14], which is essential for correct oogenesis [15]. The abnormal oocytes that form in the *cgh-1* mutant appear in part to be a result of the formation of large aberrant RNP granules [16,17,18], which have been proposed to represent solid aggregates of abnormal RNPs [19].

Here, we present a comprehensive characterization of CEYs that expands our understanding of the function of these proteins in animal biology. We show that CEYs are essential for the production of viable progeny and have a conserved role in the formation of maternal mRNPs. Additionally, we present an unexpected function of these proteins in the soma. We find that, in the absence of CEYs, there is a spectacular loss of large polysomes with the concomitant increase of mono- and disomes, suggesting that CEYs are essential for the proper accumulation of multiple ribosomes on mRNAs. Surprisingly, however, this loss of large polysomes appears to have little consequences for animal development and homeostasis. The potential roles of CEYs in polysome biogenesis and in animal biology are discussed.

4.1.3 Materials and methods

Culturing animals

Animals were usually grown on 3.5-15 cm NG 2% plates seeded with OP50 bacteria. For large scale experiments, animals were grown on 15 cm peptone-rich plates seeded with OP50 bacteria. Gravid adults were then bleached and allowed to hatch on empty plates o/n. The next morning, synchronized L1s were counted and a defined number of larvae were transferred to seeded plates. Animals were then grown to young adulthood and harvested in liquid N_2. The two temperature sensitive strains, *glp-1*(*e2144*) and *glp-4*(*bn2*), were maintained and grown to large numbers at 15°C, before bleaching gravid adults and then shifting staged L1s to 25°C.

Strains

cey-1(*ok1805*), *cey-2*(*ok902*), *cey-4*(*ok858*), *glp-1*(*e2144*), *glp-4*(*bn2*), *efk-1*(*ok3608*), *ced-9*(*n1950*), and *ced-1*(*e1735*) mutants were obtained from CGC. The *cey-3*(*tm2839*) mutant was provided by the Mitani lab through the National Bio-Resource Project of the MEXT, Japan. All strains were outcrossed at least 4x before use. The AIR-2-GFP transgenic line was provided by the Colaiácovo lab [20]. Transcription activator-like effector nucleases (TALENs) [21,22] were used to delete *cey-3* in the *cey-2* mutant background to obtain the *cey-2,-3* double mutant. A common cross of the two single mutants was not attempted due to very close proximity of the two genes (<0.1 cM). We obtained an 8 bp deletion in the first exon, which created a premature stop codon soon after, making this *cey-3*(*rrr11*) mutant a functional null (confirmed by sterile phenotype of the *cey-1,-2,-3,-4* mutant).

Despite a 539 bp deletion, the *cey-1*(*ok1805*) mutant still gave rise to a severely truncated version of CEY-1. This protein contained the first part of the cold shock domain with both RNA binding motifs (RNP1 and RNP2) [2,23]. Due to unpredictable effects of such a protein, we used the Cas9/CRISPR system to obtain a functional null mutant [24]. We obtained a 5 bp deletion in the second exon that generated a premature stop codon soon after. The aberrant transcript was recognized and degraded by the NMD pathway (confirmed by semi-quantitative PCR).

Transgenic lines

Multisite Gateway (Life Technologies) was used to clone almost all transgenes. Only CEY-3-GFP was obtained from the TransgeneOme project [25]. For the expression of FLAG-tagged CEY-4, the ubiquitous *dpy-30* promoter had to be used instead of the endogenous *cey-4* promoter (also ubiquitous) due to technical problems during cloning. An operon system [26] was used to monitor expression of FLAG-tagged transgenes. Table S4 shows a list of transgenes generated for this study. Except for CEY-3-GFP, which was obtained by bombardment, all transgenic lines were obtained using MosSCI [27]. Each line was outcrossed at least 2x before use.

RNAi

Young adult hermaphrodites were injected with *cey-2,-3* dsRNA (500 ng/µl each). Injected animals were allowed to lay eggs for 10-12 h, before being transferred to new plates and grown at 25°C. *cgh-1* RNAi was performed by feeding [28].

Counting progeny numbers

For each strain, 10 L1s were picked to individual plates and grown to adulthood at the corresponding temperature (20°C, 25°C or 26°C). Adults were picked every 24 h to new plates (2-3 times). This allowed for a more accurate counting of the progeny number.

Microscopy

Images were captured with a Zeiss AxioImager Z1 microscope, equipped with an Axiocam MRm REV2 CCD camera. All images were acquired in the linear mode of the Axiovision software (Zeiss) and processed using Image J and Adobe Photoshop CS4 in an identical manner.

Live imaging

Animals were transferred into a drop of 0.04 M levamisol on an agarose pad, covered with a cover slip and immediately imaged.

Cell death assays

To measure germ cell death, animals were incubated for 3-4 h on NGM plates containing acridine orange (AO) (500 µl of 100 mM AO/plate) and viewed by fluorescent microscopy [14]. Cell corpses were counted visually using DIC optics.

Immunostaining

Immunostaining was performed as previously described [15]. The following primary antibodies were used: α-CEY-4 (Supplementary Figure S1), α-GLD-1 [29], α-CGH-1 [14], α-CAR-1 [14], α-pH3 (phospho-histone H3) (Upstate Biologicals) and α-activated MAPK-YT (Sigma). DNA was visualized using 4"- 6-Diamidino-2-phenylindole (DAPI).

RNA extraction

One millilitre of Trizol (Life Technologies) was added to each frozen *C. elegans* pellet (50-200 µl) and then ground using a mortar and pestle in the presence of liquid N_2. Extracts were centrifuged at 4°C for 10 min at 12,000 x g to get rid of remaining debris. Supernatants were transferred to new microcentrifuge tubes and 200 µl of chloroform were added, vortexed for 0.5 min, and then centrifuged at 4°C for 15 min at 12,000 x g. The aqueous phase was transferred to a new microcentrifuge tube and 500 µl of isopropanol were added, mixed well and incubated at RT for 15 min. The samples were then centrifuged at 4°C for 10 min at

12,000 x g. Subsequently, the RNA pellet was washed once with 75% ethanol, before being air dried and resuspended in nuclease-free water (Ambion). To obtain germline-specific RNA, gonads were manually dissected and RNA was purified using the Arcturus PicoPure RNA Isolation Kit (Life Technologies).

DNA and rRNA removal

DNAse treatment was performed with RNeasy Mini columns (Qiagen) using the corresponding RNAse-free DNAse set (Qiagen). The RNA cleanup protocol was followed. Removal of rRNA was performed using the Ribo-Zero^{TM} Magnetic Gold Kit (Human/Mouse/Rat, Epicentre). For qRT-PCR, RNA samples were only DNAse treated. For total RNA sequencing, RNA samples were DNAse treated and rRNA was removed. rRNA removal was checked on the Agilent Bioanalyzer using the Pico RNA chip.

qRT-PCR

Reverse transcription reactions were performed using the ImProm-II Reverse Transcription System (Promega) using random primers (Promega). For subsequent qRT-PCR reactions, one primer in each pair overlapped an exon-exon junction to avoid amplification from non mRNA molecules.

Polysome profiling

Polysome profiling and RNA extraction from sucrose fractions was performed as previously described [30]. A 15% (w/v) to 60% (w/v) sucrose gradient was used for polysome profiles shown (each profile was obtained at least twice). For ribosome profiling, a 5% (w/v) to 45% (w/v) sucrose gradient was used.

Ribosome profiling

Nematode lysates were prepared as described for polysome profiling [30], however, without adding RNAse inhibitor to the lysis buffer. RNAse I (200 U / 110 OD, Ambion) was added and the mixture was incubated at 23°C for 1 h. The remaining extract was used for total RNA extraction for subsequent total RNA sequencing. After digestion, the lysate was immediately loaded on the gradient and centrifuged. Samples were then fractionated into 24 collection tubes instead of the usual 12. This allowed cleaner isolation of monosomes. Ribosome-protected fragments (RPFs) were then isolated as described above for total RNA extraction. RNA was loaded on a Novex 15% TBE-Urea gel (Life Technologies) and a piece between 27 nt and 31 nt (oligos from Genscript) was excised from the gel and the RNA was eluted from

the gel piece o/n at RT. The library was then prepared using the TruSeq Small RNA kit (Illumina), whereby the RNA was precipitated for at least 4 h in between each of the following steps. RNA was first dephosphorylated using T4 PNK (NEB), followed by 3' adapter ligation (T4 RNA ligase 2 truncated, NEB). The 5' end was then re-phosphorylated using T4 PNK (NEB) supplied with ATP, followed by 5' adapter ligation, cDNA synthesis and PCR. The PCR product was then loaded on a Novex 6% TBE-Urea gel (Life Technologies) and the band around 150 bp (5' adapter + 30 nt RPF + 3' adapter) was excised from the gel. The DNA was then eluted from the gel piece and sent for sequencing.

RNA sequencing and data analysis

For total RNA sequencing, the samples were prepared using the ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre) and then sequenced. Both the total RNA sequencing data and the ribosome profiling data were analyzed as previously described [31].

Microarray and data analysis

Sample preparation, microarray, and subsequent data analysis were performed in the *glp-4*(*bn2*) mutant as previously described for wild type [29].

Western blot analysis and antibodies used

Depending on the pellet size, 300-600 µl extraction buffer (50 mM HEPES, 100 mM KOAc, 5 mM MgAc, 0.1% Triton X-100, 10% Glycerol (w/v), 20mM β-glycerophosphate) were added to the pellet. Protein extracts were then prepared as for RNA extraction by grinding with a mortar and pestle in the presence of liquid N_2. Animal debris were removed by a 20-min spin at 20,000 x g. Protein concentrations were measured using the Bradford assay (Biorad). The required amounts of 4x LDS sample buffer (Life Technologies) and 10x sample reducing agent (Life Technologies) were added to the samples, followed by 10 min of heating at 70°C. The samples were then loaded on a gel (NuPAGE® Novex® 4-12% Bis-Tris Protein Gels, 1.0 mm, 10 well or 17 well) and ran for 55 min at 200 V. Proteins were transferred to the membrane using the Trans-Blot® Turbo™ system (Biorad). Membranes were then washed 2 x 10 min in ddH_2O, Ponceau stained for 5-10 min, and cut if necessary. After washing for 2 x 5 min with PBS-T (Tween 1:1000), membranes were blocked with 4% milk (in PBS-T) for 1 h. The primary antibody was then added. The primary α-PAB-1 [29], α-CEY-4 (Supplementary Figure S1), α-RME-2 [32], α-ACT-1 (MAB1501, Millipore), α-EEF-2 (Cell Signaling Technology), and α-EEF-2-P (Cell Signaling Technology) antibodies were incubated o/n at 4°C. The next day, membranes were washed 3 x 5 min in 4% milk (in PBS-T) before the

secondary (HRP-coupled) antibody was applied (GE Healthcare) for 1 h at RT. The membranes were then washed 3 x 5 min in PBS-T and then developed using Pierce ECL Western Blotting Substrate (Thermo Scientific). For FLAG detection, we used the primary monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody (Sigma). This antibody is coupled to HRP. After 2 h of incubation at RT, the membrane was washed 3 x 5 min in PBS-T and then developed using Pierce ECL Western Blotting Substrate (Thermo Scientific).

Protein extraction from sucrose fractions

One hundred and fifty microlitres from each sucrose fraction were transferred to a new 1.5 ml microcentrifuge tube and filled up with washing buffer (20 mM Tris-HCl pH 7.9, 140 mM KCl, 5 mM $MgCl_2$) to 500 µl. The entire volume was then loaded onto an Amicon Ultra-0.5 Centrifugal Filter Unit (Milipore) and centrifuged at 4°C for 30 min at 14,000 x g. 7 µl 4x LDS sample buffer (Life Technologies) and 3 µl 10x sample reducing agent (Life Technologies) were added to each sample (17 µl), which was then heated at 70°C for 10 min. Subsequently, 14 µl were loaded on the gel.

Surface Sensing of Translation (SUnSET)

L1s were hatched in M9 o/n at 150 rpm. Per sample, 12,000 L1s were grown to young adulthood on NG 2% plates seeded with OP50 bacteria. Animals were washed off the NG 2% plates and washed twice in S-basal [33]. 4 ml of S-medium [33] were added to the animals and transferred to a 50 ml Erlenmeyer. An o/n culture of OP50 bacteria was 10x concentrated and the pellet was resuspended in S-medium. 750 µl bacteria solution was added to the Erlenmayer. Finally, 250 µl of puromycin stock solution (10 mg/ml) were added, resulting in a final volume of 5 ml and a final puromycin concentration of 0.5 mg/ml. Animals were grown in the presence of puromycin for 4 h at 200 rpm and then harvested. 4 h allowed detection of a puromycin signal by western blot analysis, without having an obvious effect on general translation (no abnormalities observed on polysome profiles, data not shown). 50 µg of total protein were loaded per well for western blot analysis. This allowed for a good signal at a reasonable exposure time of 5-10 min. The monoclonal α-puromycin antibody (Millipore, [34]) was used at a dilution of 1:5000 in 4% milk (in PBS-T). For GFP detection, we used monoclonal GFP antibodies (Roche). GFP (GFP-RPS-1) was used as a spike and served as an external loading control.

^{35}S-methionine incorporation assay

The assay was performed as previously described [35].

CEY-1 and CEY-4 immunoprecipitation and mass-spectrometry analysis

CEY-1 and CEY-4 associated proteins were identified by anti-FLAG IPs, using FLAG-coupled Dynabeads (Life Technologies). The FLAG IPs were performed on FLAG-CEY-1 and FLAG-CEY-4 transgenic lines, and wild type. An on-bead RNAse digestion was performed with 0.1 mg/ml RNAse A (Sigma) for 2 h at 4°C. After washing, samples were eluted using FLAG peptides (Sigma). Samples were TCA precipitated and submitted for mass-spectrometry. The protein pellets were dissolved in 0.5 M Tris pH 8.6, 6 M guanidinium hydrochloride, reduced with 16 mM TCEP for 30 min, and alkylated in 35 mM iodoacetamide for 30 min in the dark. The proteins were digested with 0.2 µg Lys-C (Wako chemicals, Osaka, Japan) for 6 h after 3x dilution in 50 mM Tris 5mM CaCl2 (pH 7.4), followed by 0.2 µg trypsin after an additional 2x dilution, overnight. The peptides were analyzed by capillary liquid chromatography tandem mass spectrometry with an EASY-nLC 1000 using the two column set up (Thermo Scientific). The peptides were loaded in buffer A onto a peptide trap (Acclaim PepMap 100, 75 µm x 3 cm, C18, 3 µm, 100 Å) at a constant pressure of 500 bar. Then they were separated, at a flow rate of 200 nl / min with a gradient of 2–44% buffer B in buffer A in 67 min (Buffer A: 0.1% formic acid in water, buffer B: 0.1% formic acid in acetonitrile) using a 75 µm x 25 cm Reprosil-PUR C18, 3 µm, 100 Å PicoFrit column mounted on a DPV ion source (New Objective). The data were acquired on an Orbitrap Velos (Thermo Scientific) using 60000 resolution for the peptide measurements in the Orbitrap and a top 20 method with CID fragmentation and fragment measurement in the LTQ, according the recommendation of the manufacturer. Mascot (Matrix Science, London, UK) searching UniProt data base version 2012_09 was used to identify the peptides. The enzyme specificity was set to trypsin allowing for up to three incomplete cleavage sites. Carbamidomethylation of cysteine (+57.0245) was set as a fixed modification, oxidation of methionine (+15.9949 Da), acetylation of protein N-termini (+42.0106 Da), dimethylation of Arginine (+28.0312 Da) and phosphorylation of Serine and Threonine (+79.9663) were set as variable modifications. Parent ion mass tolerance was set to 5 ppm and fragment ion mass tolerance to 0.6 Da. The results were validated with the program Scaffold Version 4.0 (Proteome Software, Portland, USA). Peptide identifications were accepted if they could be established at greater than 50% probability as specified by the Peptide Prophet algorithm [36]. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [37]. Post translational modification sites were further evaluated with the software ScaffoldPTM 2.1.2.1 (Proteome Software) and validated

manually. Relative quantification of the proteins was done with the program ProgenesisLC (Nonlinear Dynamics).

Phylogenetic tree

Protein sequences (fasta files) were obtained from www.uniprot.org. Sequence alignment and phylogenetic tree were constructed using Clustal Omega (http://www.ebi.ac.uk/ Tools/msa/ clustalo/). Njplot (http://pbil.univ-lyon1.fr/software/ njplot.html) was used to modify the phylogenetic tree.

Chemotaxis

Chemotaxis was tested towards different volatile chemo-attractants as described [38]. Briefly, animals were given a choice between a spot of 0.1% (vol/vol) attractant in ethanol with 20 mM sodium-azide and a counter spot with ethanol and sodium-azide. After 1 h the animals were counted and a chemotaxis index was calculated as described [38]. 50-200 synchronized young adults were used per plate and each experiment was done in triplicates and repeated three times.

Olfactory conditioning and memory

Olfactory conditioning and memory was assessed as described previously [39]. Starvation conditioning was performed using young adult animals on conditioning plates without food in the presence of 2 µl undiluted diacetyl spotted on the lid and trained for 1 h on 10 cm CTX plates (5 mM KH_2PO_4/K_2HPO_4 pH=6.0, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 2% agar). Naive and conditioned animals were given a choice between a spot of 0.1% (vol/vol) DA in ethanol with 20 mM sodium-azide and a counter spot with ethanol and sodium-azide for 1 h, and chemotaxis index was calculated as described [38]. Memory was assessed as described [40] where diacetyl conditioned animals were tested for their preference toward the attractant following a 60 min resting period in absence of food and odorant.

4.1.4 Results

CEYs are ubiquitous cytoplasmic proteins

The *C. elegans* genome encodes five CSD-containing proteins, which include the well-studied developmental regulator LIN-28 [41,42] and four previously uncharacterized proteins, CEY-1-4, which are the focus of this study. CEYs are relatively small proteins, ranging from

around 200 amino acids (CEY-1) to almost 300 amino acids (CEY-4). CEY-2 and CEY-3 are around 270 amino acids in length and are 70% identical. As their expression patterns also overlap (see below), CEY-2 and CEY-3 very likely have redundant functions. The CSD represents one of the most highly conserved protein domains [1]. However, outside the CSD, CEYs share only limited similarity with YBPs from other species (Figure 1A). In addition to the CSD, CEY-1 and CEY-4 contain a large number of RG/RGG repeats (Figure 1B), which is reminiscent of such repeats in other invertebrate YBPs [43,44,45].

To find out where the different *cey* genes are expressed, we generated GFP reporters. The results revealed that *cey-1* and *cey-4* were present in both the soma and the germline, whereas *cey-2* and *cey-3* were only expressed in the germline (Figure 1C and D). This was confirmed by qRT-PCR performed on RNA isolated from either wild-type animals or temperature-sensitive *glp-4(bn2)* mutants, which, when grown at 25°C, have no germline (Supplementary Figure S2A). In addition, the reporters revealed that *cey-2* and *cey-3* were lowly expressed in the distal-most part of the gonad that contains self-renewing undifferentiated germ cells, but became strongly expressed upon the entry of germ cells into meiosis (Figure 1C and D). Furthermore, we observed the disappearance of GFP-tagged versions of CEY-2 and CEY-3 during the oocyte-to-embryo transition, such that these proteins were no longer detected in early embryos (Figure 1E).

YBPs in other organisms have been implicated in gene regulation at both transcriptional and posttranscriptional levels. Accordingly, they are present in both the cytoplasm and the nucleus, best illustrated by the human YB-1 [4]. In contrast, immunostaining and GFP localization experiments showed that CEY proteins were only present in the cytoplasm in both soma and germline (Figure 1E and Supplementary Figure S2B). Knockdown of *xpo-1*, the homologue of yeast, vertebrate and *Drosophila* CRM1/exportin-1, which represents the major receptor for the export of proteins out of the nucleus [46], did not cause an accumulation of CEY-1 or CEY-2 in the nucleus (data not shown). This supports a predominantly cytoplasmic localization for CEYs, allowing the analysis of posttranscriptional roles of these proteins independently from potential functions in the nucleus, for example in transcription or mRNA splicing.

Both CEY-1 and CEY-4 contain multiple clusters of RG/RGG repeats. The arginine residues can serve as targets for protein arginine methyl transferases (PRMTs), which can either mono- or di-methylate arginines and thereby alter protein function [47]. Our mass-spectrometry data (data not shown) suggested that some of the RG/RGG repeats were indeed methylated and western blotting experiments confirmed this, showing that both CEY-1 and CEY-4 were asymmetrically di-methylated (ADMA) (Supplementary Figure S2C and D). The RG/RGG methylation was recently shown to depend on PRMT-1 in *C. elegans* [48]. Consistently, we found that CEY-4 no longer carried the ADMA mark in the *prmt-1*(*ok2710*)

mutant (Supplementary Figure S2D). Interestingly, CEY-4 protein levels appeared to increase upon de-methylation (Supplementary Figure S2D and E), suggesting that the methylation status of its RG/RGG repeats may regulate CEY-4 protein levels.

CEY proteins are required for multiple aspects of germline development

The loss of YBPs in the germline has a major impact on the production and viability of progeny in other organisms [9,10,12]. While *cey-1* and *cey-4* single mutants showed wild-type brood sizes, the loss of germline-specific CEY-2 or CEY-3 caused a significant reduction in progeny number (Supplementary Figure S3A). This defect was strongly exacerbated upon the loss of both CEY-2 and CEY-3 (Supplementary Figure S3B). By contrast, the loss of both CEY-1 and CEY-4 had little or no effect on brood size at either 20 or 25°C. Only at 26°C, an extreme growth temperature for *C. elegans*, was the sterility observed in *cey-1,-4* double mutants (Supplementary Figure S3B). Finally, knocking out all four *cey* genes led to sterility, whereby the severity of the phenotype was strongly temperature-dependent, with more or less normal-looking oocytes at 20°C and a double row of smaller oocytes at 25°C (Figure 2). In summary, while CEY-1 and -4 have some function, CEY-2 and -3 appear to be the major CEY proteins in the germline.

Maintenance of stem cells and transit-amplifying cells in the most distal part of the gonad, control of germ cell apoptosis, and correct timing of oocyte maturation, are key events required for the production of healthy gametes. Focusing on the two germline-specific CEYs, CEY-2 and CEY-3, we wanted to evaluate a potential requirement for them in these processes. We first examined germ cell proliferation in animals subjected to *cey-2* and *cey-3* RNAi (*cey-2,-3* RNAi for brevity). To assess cell cycle progression, we used an antibody against Ser10-phosphorylated histone H3 (pH3), which stains condensed chromosomes marking cells in the M phase of the cell cycle. Animals depleted of CEY-2 and -3 had a significantly reduced number of proliferating cells in the distal gonad (Figure 3A) and, consistently, the mitotic zone was shorter in these gonads than in control wild-type gonads (Figure 3B). The length of the so-called transition zone, where germ cells enter into the meiotic prophase, remained similar between *cey-2,-3* RNAi and control animals (Figure 3C).

Next, we examined the effect of *cey-2,-3* knockdown on germ cell apoptosis in the mutant strain *ced-9(n1950)*. In this mutant, only the physiological apoptosis pathway is active, which removes defective germ cells and ensures oocyte quality [49]. We observed a significant increase in the number of apoptotic cells in the RNAi-ed animals (Figure 3D). This could be due to increased rates of cell death or reduced rates of cell corpse clearance. To distinguish between these two alternatives, we RNAi-ed *cey-2,-3* in the cell corpse engulfment-defective mutant *ced-1(e1735)*. We observed a significant increase in the

number of germ cell corpses in the *ced-1(e1735); cey-2,-3* RNAi animals compared to mock-RNA-ied animals (Figure 3E), indicating that CEY-2 and -3 depleted animals have increased levels of germ cell apoptosis. Thus, CEY-2 and -3 are required for normal levels of germ cell proliferation and survival.

Finally, we examined the ability of oocytes to undergo normal oocyte maturation in CEY-2,-3 depleted animals. To monitor oocyte maturation, we used two phosphorylation-specific markers; one for the mitogen-activated protein kinase 1 (MPK-1) and one for Aurora B kinase (AIR-2). In wild type gonads, these markers highlight the most proximal oocytes (so-called -1 and -2 oocytes). In contrast, depleting CEY-2,-3 resulted in the appearance of these markers in more distal oocytes (Figure 3F and G), suggesting premature maturation.

CEYs are essential components of germline mRNPs

A major function of YBPs in the germline is to bind and package mRNAs into mRNPs for cytoplasmic storage [8,50]. To look at the association between CEYs and mRNAs globally, we isolated RNA following FLAG IPs performed on extracts of animals expressing FLAG-tagged versions for CEY-1, CEY-2, and CEY-4. MYC IPs performed on the same extracts served as controls. The different replicates correlated with each other very well (Supplementary Figure S4A), indicating high reproducibility. Comparing the FLAG IP data to the respective MYC control IPs revealed no striking enrichment of specific mRNAs (Supplementary Figure S4B-D). We thus conclude that CEYs do not have a clear preference for binding specific subsets of mRNAs, a result expected for proteins that either do not bind mRNA or interact with messages in an unspecific fashion. As YBPs from other model organisms often interact more generally with mRNA, we believe that this is also the case in *C. elegans*. Curiously, we found that many mRNAs were depleted in the case of all three FLAG IPs compared to MYC control IPs (Supplementary Figure S4B-D). This was most strongly apparent for the germline-specific CEY-2 (Supplementary Figure S4C). We speculated that the observed depletion of mRNAs might stem from varying expression of a given CEY protein in different tissues. To test this, we selected the mRNAs ‘depleted’ from FLAG-CEY-2 IPs and monitored their expression in the soma versus the gonad (Supplementary Figure S4E and F). As predicted, these mRNAs were predominantly expressed in the soma, a tissue in which the germline-specific CEY-2 is not present.

Two previous studies had shown that both the conserved RNA helicase CGH-1/DDX6 and the STAR-domain RNA-binding protein (RBP), GLD-1/Quaking, interact with CEYs via RNA [14,29]. In a reverse immunoprecipitation experiment, we purified FLAG-tagged CEY-1 and CEY-4 in the presence or absence of RNAse. The samples were then examined by MS analysis to obtain a global view of RNA-dependent and RNA-independent protein

interactions. We found that CGH-1 and GLD-1 co-purified with CEYs in an RNA-dependent fashion (Supplementary Table S2 and 3 and Figure S5 and 6, green squares). These data were confirmed by co-IPs performed again with or without RNAse treatment on FLAG-CEY-2 (chosen instead of FLAG-CEY-1 to include a germline-specific CEY protein) and FLAG-CEY-4, and detecting specific proteins by western blot (Figure 4A). Intriguingly, while RNAse treatment led to the loss of the interaction between CEYs and several germline RBPs, we observed that the interaction with multiple ribosomal proteins was maintained (Supplementary Table S2 and 3 and Figure S5 and 6, red dots), suggesting a potential direct link between CEYs and ribosomes.

The absence of CGH-1 causes the accumulation of aberrant RNP granules, which have been proposed to represent an aggregation of abnormal mRNPs [16,17,18,19]. Indeed, knockdown of *cgh-1* also resulted in the localization of GFP-tagged CEY-3 to abnormal granules in the cytoplasmic core of the gonad (Figure 4B). To test if CEYs play a role in RNP regulation, we stained CEY(-) gonads for both CGH-1 and CAR-1/Rap55, a conserved RBP that is usually present on CGH-1-bound mRNAs [14]. Indeed, both factors localized to aberrant RNP granules in the *cey-1; cey-2 cey-3; cey-4* (for brevity *cey-1,-2,-3,-4*) quadruple mutant (Figure 4C). Furthermore, we found that GLD-1 was also present in aberrant RNPs (Figure 4D). Thus, CEYs are important for normal RNP appearance in the *C. elegans* germline, as their homologs are in other organisms.

CEY-1 and CEY-4 are required for the formation of large polysomes in the soma

Besides germline defects, the loss of YBPs can impact somatic development. For example, YB-1 depleted mice are embryonic lethal [51], though it remains possible that this lethality may reflect a maternal function of YB-1. Initially, we observed that the *cey-1(ok1805)* mutant grew significantly slower than wild type at 20°C. However, we found that the *ok1805* mutation, thought to be a null, gave rise to a truncated protein that contained both RNA binding motifs (RNP1 and RNP2) of the CSD (data not shown) [2,23], and might therefore still bind RNA. To create a functional null, we generated a new *cey-1* mutation (*rrr12*), using the CRISPR/Cas9-system [24]. We used this mutation in subsequent studies. We found that *cey-1(rrr12)* mutants, thereafter referred to simply as *cey-1*, no longer displayed the growth delay observed in *cey-1(ok1805)* mutants. This might suggest a slight dominant-negative effect of the truncated CEY-1 protein encoded by the *cey-1(ok1805)* mutant. Also the *cey-1,-4* double mutant had no apparent developmental defects compared to wild type under normal growth conditions. Furthermore, apart from the sterile phenotype observed at 26°C, the *cey-1,-4* mutant showed no defects in its response to different stress cues, such as low or high temperature or food deprivation. The same was true for longevity (data not shown). Similarly,

neither the two single mutants (*cey-1* and *cey-4*) nor the double mutant (*cey-1,-4*) showed any significant irregularities in diacetyl chemotaxis (Supplementary Figure S7), despite the fact that CEY-1 was strongly expressed in neurons (Figure 1D).

YBPs can act as translational repressors [4,6,7]. For this reason, we monitored mRNA translation by polysome profiling. In this approach, 'ribosomal' fractions harbour ribosomes actively engaged in translation (Supplementary Figure S8A). While the depletion of the germline-specific CEY-2 and -3 caused no obvious change in the distribution of ribosomes (Figure 5A), the removal of CEY-1 and -4 resulted in a striking loss of large polysomes (multiple ribosomes associated with mRNAs) with a concomitant increase in monosomes and disomes (Figure 5B). This was very surprising, considering the absence of any obvious phenotype in the *cey-1,-4* mutant. Polysome profiles of heat-shocked animals (Supplementary Figure S8B and C), suggested that the effect observed in *cey-1,-4* mutants did not reflect a stress response. Furthermore, the *cey-1,-4* mutant responded to the heat stress as wild-type animals did, namely, by reducing translating ribosomes, consistent with our previous observation that these mutants deal with stress as well as wild-type animals.

As most cells in adult *C. elegans* are germ cells, we performed polysome profiling also on germline-less *glp-4* mutants to find out what contribution the germline has on the polysome profile. Strikingly, we found that virtually all subpolysomal (repressed or poorly translated) mRNAs were germline-specific (disappeared in the *glp-4* mutant background) (Supplementary Figure S8D and E). Thus, it seemed unlikely that the loss of polysomes in *cey* mutants was caused by germline defects. To test this directly, we crossed the *cey-1,-4* mutant into another temperature-dependent germline-less mutant, *glp-1(e2144)*. In these animals, we still observed the loss of large polysomes and, additionally, some intermediate ribosomal peaks (Figure 5C). Because CEY-4 expressed specifically in the germline and CEY-2 expressed from the *cey-1* promoter both failed to rescue the polysome defect (Figure 5D and E and Supplementary Figure 8F and G), we conclude that, in general, accumulation of large polysomes depends on the somatic CEY proteins. Interestingly, by monitoring a FLAG-tagged version of CEY-1 and the endogenous CEY-4, we found that the distribution of CEY-1 and CEY-4 between sub- and polysomal fractions from wild-type animals was not identical. While CEY-4 was present in both sub- and polysomal fractions, potentially indicating an interaction with ribosomes, CEY-1 was mainly present in the sub-monosomal fractions (Figure 5F). Consistent with non-identical distributions along the gradient, the polysome profiles of individual *cey-1* and *cey-4* single mutants did not match. The *cey-4* single mutant showed a drop in larger polysomes and an increase in mono- and disomes, similar to the *cey-1,-4* double mutant, albeit to a lesser extent (Supplementary Figure S8H). The *cey-1* single mutant, on the other hand, showed only a slight decrease of large polysomes without affecting mono- or disomes (Supplementary Figure S8I). Thus, CEY-1

and -4 may have specific functions, though the fact that the loss of CEY-1 further increases both the loss of large polysomes and the accumulation of mono- and disomes in *cey-4* single mutants, suggests that there is some degree of redundancy between CEY-1 and CEY-4.

CEY-1 and -4 appear dispensable for normal levels of protein synthesis

Considering the importance attributed to polysomes in maintaining high translation rates, we were surprised to see that the *cey-1,-4* double mutant grew similar to wild type. To assess global protein synthesis rates in these animals, we used SUrface SEnsing of Translation (SUnSET) [34]. SUnSET is based on the incorporation of puromycin into newly synthesized peptides and the subsequent detection of the incorporated puromycin by western blotting using a specific monoclonal antibody. Consistent with the normal growth of *cey-1,-4* mutants, we detected no obvious decrease in puromycin incorporation (Figure 6A), suggesting that, on the global scale, there is no major change in the translation rates in *cey-1,-4* mutants**.** To confirm this, we additionally performed the ^{35}S-Met labeling assay [35]. The results were consistent with the data obtained from the SUnSET experiment (Figure 6B). In agreement with those findings, the total amounts of protein were similar between wild type and the *cey-1,-4* mutant (Supplementary Figure S9A).

The global loss of large polysomes in *cey-1,-4* mutants suggested that there could be less ribosomes per specific mRNAs in the mutant compared to wild type. A recent study in human cells showed that a decrease in ribosome number activates the elongation machinery via a controlled feedback loop [52]. The amount of translation-inhibiting EEF-2 kinase (*eef-2k*) decreases, thereby reducing the amount of phosphorylated EEF-2, the inactive form of this essential elongation factor. This might allow fewer ribosomes to translate more efficiently, producing the same protein output as in wild type. In the *cey-1,-4* mutant, however, we found that both *efk-1* (*C. elegans* homologue of *eef-2k*) mRNA levels (data not shown) as well as the EEF-2 phosphorylation status remained unchanged compared to wild type (Supplementary Figure S9B), suggesting that the above-mentioned feedback mechanism is unlikely to compensate for the loss of polysomes.

CEY proteins are broadly required for mRNA accumulation

To obtain a more detailed view of mRNA levels and translation in *cey-1,-4* mutants, we performed ribosome profiling combined with total RNA sequencing [53]. This data may reveal relative changes of mRNA levels and translation within a given sample but cannot be used to compare absolute mRNA levels between different samples. The replicates correlated very well with one another for both the ribosome profiling experiment as well as for the total RNA sequencing (Supplementary Figure S10), indicating high reproducibility. We first created

start- and stop codon profiles of the 5' ends of all ribosome-protected fragments (RPFs), but found no apparent differences between profiles from wild type and *cey-1,-4* mutants (Supplementary Figure S11). Interestingly, we observed a gradual increase of reads along mRNA in both wild-type and mutant animals (Supplementary Figure S11), suggesting that the speed of translation elongation gradually decreases towards the end of messages in *C. elegans*. This could help, for example, in co-translational protein folding that might become more problematic with increased polypeptide length [54].

To determine the impact of CEY-1 and -4 on mRNA levels and translation, we globally compared the changes on the mRNA level to those on the RPF level in a scatter plot. We observed a group of transcripts that showed relatively small changes in mRNA abundance but displayed a pronounced reduction of RPFs (Figure 7A, red arrow). We found that the majority of the messages that were downregulated predominantly at the RPF level were germline-specific (Figure 7B and Supplementary Figure S12A and B). As germline mRNAs tend to be poorly translated, we observed that also messages normally depleted from ribosomal fractions were downregulated at the RPF level (Figure 7C and Supplementary Figure S12C). Interestingly, many of these CEY-1 and -4 regulated germline mRNAs encode factors important for the oocyte-to-embryo transition, such as RME-2, EGG-1,-2,-4,-5 and OMA-2 (Supplementary Figure S12D-F and Supplementary Table S4). Thus, reduced translation of these messages may be responsible for the fertility defects observed in *cey-1,-4* mutants at restrictive temperature (Supplementary Figure S3B). In stark contrast, we found that messages normally enriched in ribosomal fractions (translated) (Supplementary Figure S12G and H) showed, in general, a decrease in mRNA abundance in the mutants (Figure 7D). We performed a series of qRT-PCR experiments to validate these global observations. Indeed, the abundance of "ribosome-associated mRNAs" (mainly ubiquitous or soma-specific mRNAs) was in most cases strongly reduced in the absence of CEY-1 and -4 (Figure 8A), but remained constant in *cey-2,-3* mutant animals (Figure 8A). We also found that germline-specific mRNAs were more strongly affected in *cey-2,-3* than in *cey-1,-4* mutants (Figure 8B), which is consistent with a predominant role for CEY-2 and -3 in the germline. Nevertheless, the absence of all four CEY proteins further reduced mRNA levels (Figure 8B). As expected, this reduction in mRNA abundance had a major effect on protein accumulation in the germline, as exemplified by RME-2 (Figure 8C). Finally, as RPF levels of oocyte-to-embryo transition factors decreased more strongly compared to mRNAs levels (Supplementary Figure S12D-F and Supplementary Table S4), we looked for potential redistribution of these messages from mono- and polysomal fractions to submonosomal fractions in the *cey-1,-4* mutant (Supplementary Figure S13A). Indeed, we observed a redistribution of *rme-2*, but not of *oma-2* or *egg-1* (Supplementary Figure S13B). It is possible that these messages shift predominantly from heavier to lighter polysomes. However, as

mRNAs of oocyte-to-embryo transition regulators are generally present at low levels in polysomal fractions, detecting such redistributions may be difficult.

4.1.5 Discussion

The conserved requirement for CEY proteins in the germline

All four CEYs are expressed in the germline. However, whereas CEY-1 and -4 are present in the self-renewing germ cell compartment in the most distal gonad, the most likely redundant CEY-2 and CEY-3 are only weakly expressed in this gonadal region. They become strongly upregulated more proximally, i.e. upon the entry into meiosis. Our observations that the *cey-2,-3* mutant animals produce only around half of wild-type progeny, while the *cey-1,-4* double mutants have less progeny only when challenged with extreme temperature, suggest that CEY-2 and -3 are the predominant CEY proteins in the germline. We speculate that they are induced to support CEY-1 and -4 to cope with the bulk of newly synthesized maternal mRNAs, which are then transported with general cytoplasmic flow into growing oocytes [55]. Consistent with this idea, CEY-2 and -3 disappear in early embryos, coincidently with the degradation of most maternal mRNAs [56,57]. Indeed, the abundance of tested maternal mRNAs was more strongly affected in *cey-2,-3* mutant animals, compared to *cey-1,-4* double mutants, supporting a predominant role for CEY-2 and -3 during oogenesis. Essential RBPs and germline RNP components, such as GLD-1, CGH-1, and CAR-1, are also strongly upregulated when germ cells enter meiosis [14,15,58] and interact, via RNA, with CEYs [14,29], suggesting that all these proteins are present in the same RNPs during oogenesis. The observation that both the *cgh-1* mutant [15] and the *cey-1,-2,-3,-4* mutant display similar germline defects, such as reduced germ cell proliferation, enhanced apoptosis, and defective oocytes, supports a functional connection between these proteins. Even though the aberrant RNP granules observed in *cey-1,-2,-3,-4* gonads appear distinct from the solid square sheets found in the absence of *cgh-1* [16,17,18,19], the formation of abnormal aggregates is likely to impact the regulation of germline messages. Although the connection between large RNP formation and function in mRNA regulation remains unclear, we found that the abundance of maternal mRNAs was strongly affected in the absence of CEYs. We therefore believe that CEY proteins play an important role in the binding and stabilization of maternal messages during oogenesis, similar to what has been postulated for mouse and *Xenopus* YBPs [6,7,8,9,10]. This may involve the formation, maintenance and/or disassembly of functional RNP granules to guarantee correct spatial and temporal mRNA translation.

A function of CEY proteins in the accumulation of large polysomes

In contrast to the expected results in the germline, we found that the loss of CEY proteins in the soma causes a striking reduction of large polysomes, with a simultaneous increase in monosomes and disomes. Our ribosome profiling data combined with mRNA sequencing and subsequent qRT-PCR validation suggest that the abundance of messages normally enriched in the polysomes decreases. One possibility, therefore, is that CEY proteins are also required for mRNA stability in the soma, consequently permitting accumulation of more ribosomes on mRNAs and thus allowing the formation of larger polysomes. In this scenario, CEYs might promote mRNA stability via direct association with mRNAs and, by doing so, protect mRNAs from destabilizing factors such as deadenylases or de-capping enzymes. We attempted to test the stability of specific mRNAs in *cey* mutant adults by inhibiting Pol II-mediated transcription and monitoring mRNA decay. However, although several drugs inhibited Pol II transcription, they also interfered with the expression of ribosomal RNAs and, consequently, with translation, rendering these experiments inconclusive.

Interestingly, the germline-specific protein CEY-2, when expressed in the soma from the *cey-1* promoter, was not able to rescue the polysome defect observed in the *cey-1,-4* mutant. This suggests that, to some extent, CEY-1 and -4 have different functions than CEY-2 and -3, which might be related to their association with the ribosome. This idea is based on the immunoprecipitation results, which suggest that CEY-1 and CEY-4 might directly associate with ribosomal proteins. Other proteins have been shown to bind and potentially regulate ribosomes. For example, nucleolin binds ribosomal proteins via its RG/RGG motifs [59]. Whether this represents a broader role of the RG/RGG motifs in ribosomal interactions remains unknown, but it is intriguing that the human genome encodes over one thousand proteins with at least one RG/RGG repeat, in most cases with unknown molecular functions [47]. Similarly, the two somatic nematode CEY proteins also have RG/RGG repeats that can be di-methylated. A potential role of this methylation in polysome accumulation seems unlikely, as we found that polysomes were normally present in *prmt-1* mutants (our unpublished observation). However, this does not exclude the possible role of the RG/RGG repeats of CEYs in regulating some aspect of ribosome biology, which could have an impact on efficient translation and/or mRNA stability. Testing this will require mutating the RG/RGG motifs in otherwise rescuing proteins and is an interesting objective for the future research. Intriguingly, in addition to the loss of large polysomes in *cey-1,-4* mutants, we observed the accumulation of potentially partial or aberrant ribosomes. Partial ribosomal peaks have been described previously. However, these were found on the heavier side of ribosomes and were shown to represent so called “half-mers” [60,61,62]. They form due to problems in 60S binding or availability and represent a 40S subunit bound to mRNA. In contrast, the unusual

"peaks" in *cey* mutants accumulate to the lighter side of ribosomes (observed for di- and trisomes). The formation of potentially abnormal ribosomes suggests that the overall number of "healthy" ribosomes might be lower in *cey* mutant animals. However, the causal relation between the appearance of these, potentially abnormal, ribosomes and the loss of large polysomes remains to be determined.

The biological (in)significance of *C. elegans* polysomes

Our most striking finding is that the apparent loss of large polysomes in *cey-1,-4* mutants appears to have little or no negative impact on global translation rates. This stands in stark contrast with the general view that large polysomes contribute significantly to overall protein production. Puromycin release assays showed that large polysomes are indeed engaged in translation in *C. elegans*. So, in the absence of polysomes, how can protein synthesis rates remain at wild-type levels? One possible explanation is that an enhanced speed of translation elongation might compensate for the loss of polysomes. If we assume that the number of functional ribosomes is reduced in *cey-1,-4* mutant animals, maintaining wild-type EEF-2 levels means that the ratio of available elongation machinery factors per ribosome may increase, possibly providing a "passive" way to make translation elongation more efficient. However, testing this hypothesis will require developing methodology to measure the speed of elongation in *C. elegans,* which is currently not feasible*.* Independently of whether elongation speed is affected or not, our study has raised a fundamental question: what is the accumulation of multiple ribosomes on messages required for? A regulatory function appeared to us most likely. However, under several different stress conditions the *cey-1,-4* mutant performed as well as wild type did. Nevertheless, it remains possible that a potential disadvantage of the mutant might only become apparent in non-laboratory conditions, where slight defects could have a large impact on the overall fitness.

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4.1.9 Figures

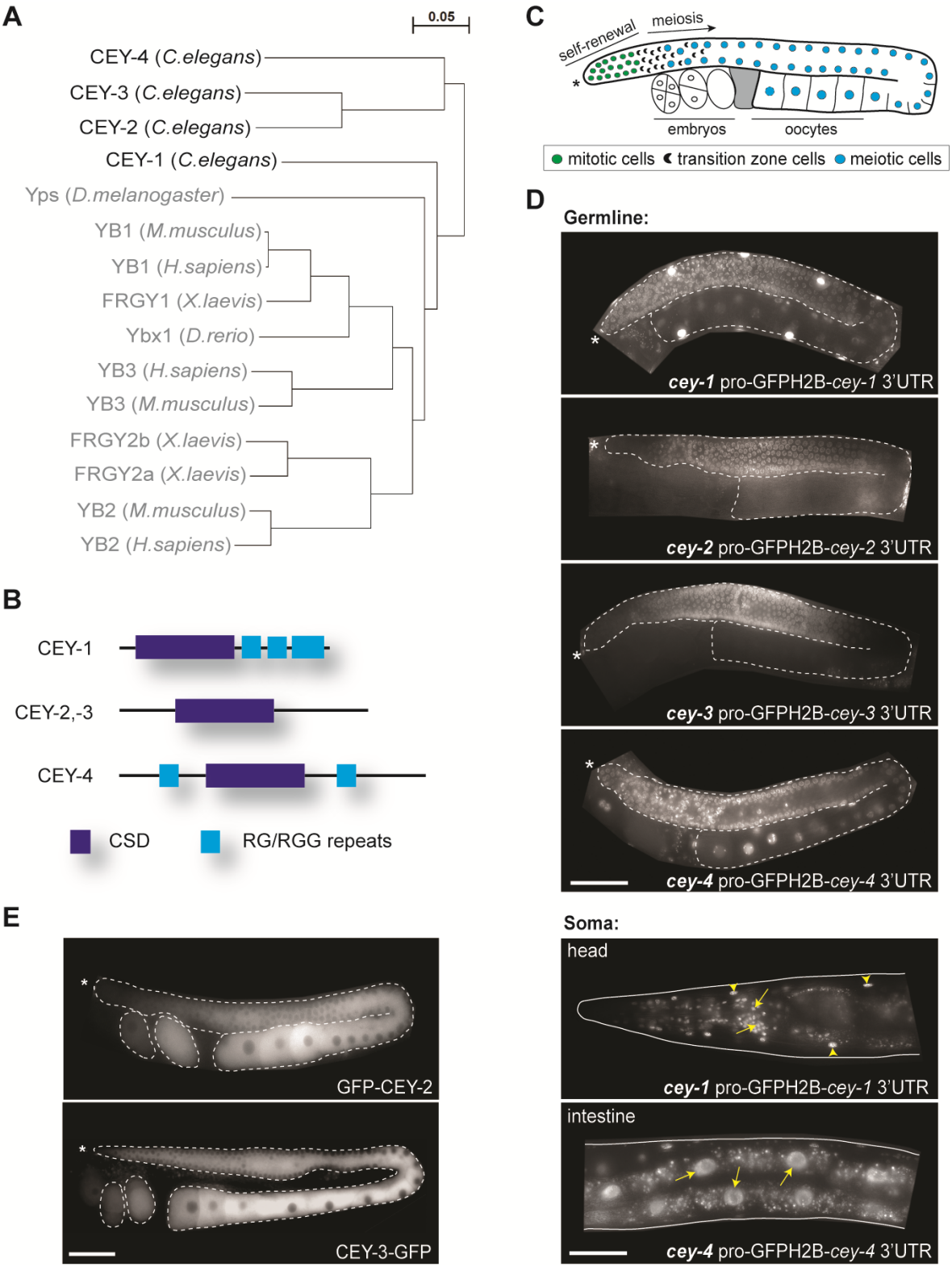

A
0.05
CEY-4 (C.elegans)
CEY-3 (C.elegans)
CEY-2 (C.elegans)
CEY-1 (C.elegans)
Yps (D.melanogaster)
YB1 (M.musculus)
YB1 (H.sapiens)
FRGY1 (X.laevis)
Ybx1 (D.rerio)
YB3 (H.sapiens)
YB3 (M.musculus)
FRGY2b (X.laevis)
FRGY2a (X.laevis)
YB2 (M.musculus)
YB2 (H.sapiens)
B
CEY-1
CEY-2,-3
CEY-4
CSD
RG/RGG repeats
C
self-renewal
meiosis
embryos
oocytes
mitotic cells
transition zone cells
meiotic cells
D
Germline:
cey-1 pro-GFPH2B-cey-1 3'UTR
cey-2 pro-GFPH2B-cey-2 3'UTR
cey-3 pro-GFPH2B-cey-3 3'UTR
cey-4 pro-GFPH2B-cey-4 3'UTR
Soma:
head
cey-1 pro-GFPH2B-cey-1 3'UTR
intestine
cey-4 pro-GFPH2B-cey-4 3'UTR
E
GFP-CEY-2
CEY-3-GFP

Figure 1. Characteristics of CEY proteins

(A) Phylogenetic tree showing a relation between CEYs and Y-box-binding proteins in other organisms, the closest being Yps in *D. melanogaster*. The distance values show the number of amino acid substitutions as a proportion of the length of the alignment. (B) Besides the CSD, CEY-1 and CEY-4 contain RG/RGG repeats, which are absent in CEY-2 and CEY-3. (C) A schematic gonad with embryos. Self-renewing germ cells are located in the most distal part of the gonad. More proximally, germ cells enter meiosis via a so-called transition zone and, in adults, eventually differentiate into oocytes. Ovulated oocytes become fertilized by sperm stored in the spermatheca (in grey). Embryogenesis follows. (D) Fluorescent micrographs from live animals expressing reporter constructs for each of the four *cey* genes. The indicated *cey* promoters and the corresponding 3'UTRs drive expression of GFP fused to histone H2B (localizing GFP to the nucleus). The gonads are outlined by dotted lines and the animals by solid lines. Asterisks indicate the distal ends of the gonads. In the germline, *cey-1* and *cey-4* begin to be expressed in the distal most region of the gonad. In contrast, *cey-2* and *cey-3* are very lowly expressed distally but become strongly upregulated more proximally, when germ cells enter meiosis. In the soma, *cey-1* and *cey-4* are expressed in all tissues, albeit at different levels. Upper panel: *cey-1* reporter is expressed in neurons and muscles (yellow arrows point to exemplary neurons and yellow arrowheads to muscles). Lower panel: *cey-4* reporter is strongly expressed in the intestinal cells (yellow arrows). Scale bars = 50 μm. (E) Fluorescent micrographs from live animals expressing GFP-tagged CEY-2 and CEY-3. Gonad and embryos are outlined by dotted lines. Consistently with the reporters shown above, both GFP-CEY-2 and CEY-3-GFP are upregulated upon the meiotic entry. The GFP signal starts to decrease in most proximal oocytes and disappears in early embryos. Scale bar = 50 μm.

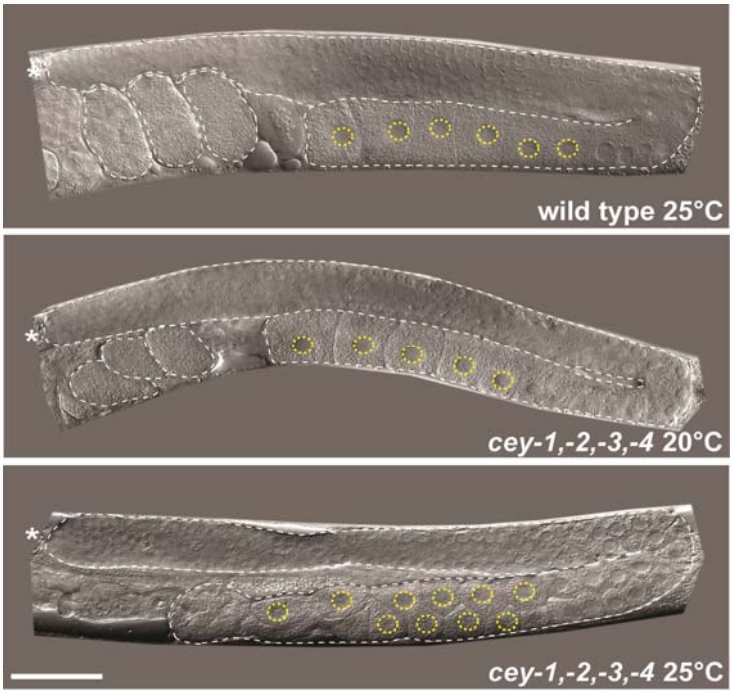


Figure 2. CEY proteins are essential for fertility

Light micrographs from live wild-type and mutant animals grown at the indicated temperatures. Gonads and embryos are outlined by white dotted lines, and oocyte nuclei by yellow dotted circles. Asterisk indicates the distal end of the gonad. Wild type looking oocytes form in the *cey-1,-2,-3,-4* quadruple mutant at 20°C, but all embryos fail to develop. A double row of smaller oocytes form in the *cey-1,-2,-3,-4* quadruple mutant at 25°C. Scale bar = 50 µm.

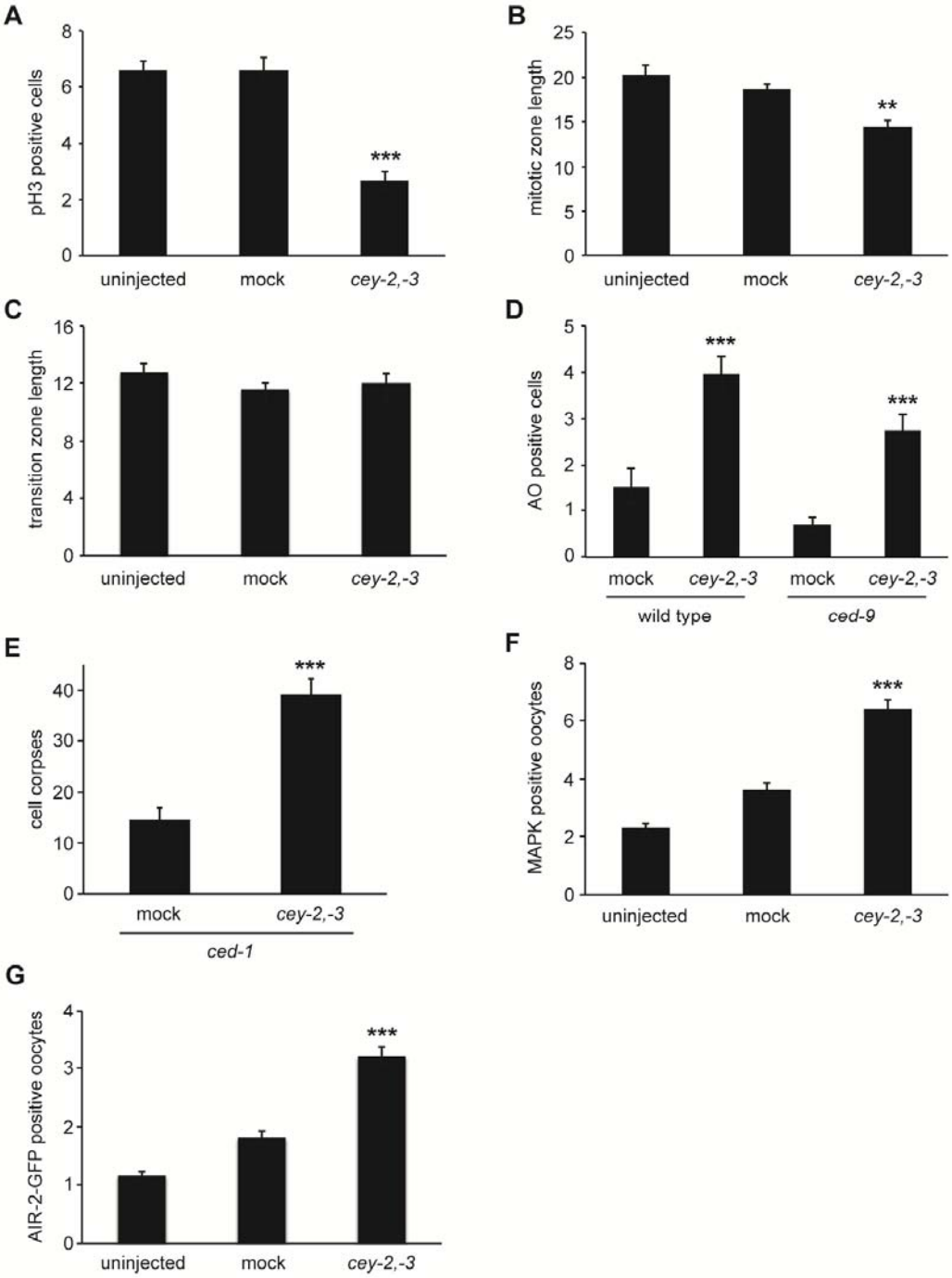

A
pH3 positive cells
8
6
4
2
0
***
uninjected
mock
cey-2,-3
B
mitotic zone length
25
20
15
10
5
0
**
uninjected
mock
cey-2,-3
C
transition zone length
16
12
8
4
0
uninjected
mock
cey-2,-3
D
AO positive cells
5
4
3
2
1
0
***
***
mock
cey-2,-3
mock
cey-2,-3
wild type
ced-9
E
cell corpses
40
30
20
10
0
***
mock
cey-2,-3
ced-1
F
MAPK positive oocytes
8
6
4
2
0
***
uninjected
mock
cey-2,-3
G
AIR-2-GFP positive oocytes
4
3
2
1
0
***
uninjected
mock
cey-2,-3

Figure 3. Germline defects in the absence of CEY proteins

Gonads were either not injected (uninjected), control injected (mock RNAi) or injected with RNAi clones targeting both *cey-2* and *cey-3* (*cey-2,-3* RNAi). (A-G) Error bars represent SEM. Asterisks denote *p-values*<0.01 by *t*-test. (A) Proliferative germ cells were stained with anti-pH3 antibody and the number of positive cells was subsequently quantified by fluorescence microscopy. (B) The proliferative zone was shorter in *cey-2,-3* RNAi gonads compared to controls, while the length of the transition zone remained constant (C). (D) Depletion of CEY-2,-3 caused an increase of acridine orange (AO) stained apoptotic cells in both wild-type and *ced-9(n1950)* animals. (E) RNAi of *cey-2,-3* in the cell corpse engulfment-defective strain, *ced-1(e1735),* resulted in a significant increase in the number of germline corpses. (F-G) RNAi of *cey-2,-3* resulted in premature appearance of activated MAPK in proximal oocytes and AIR-2-GFP on the chromosomes of oocytes in diakinesis.

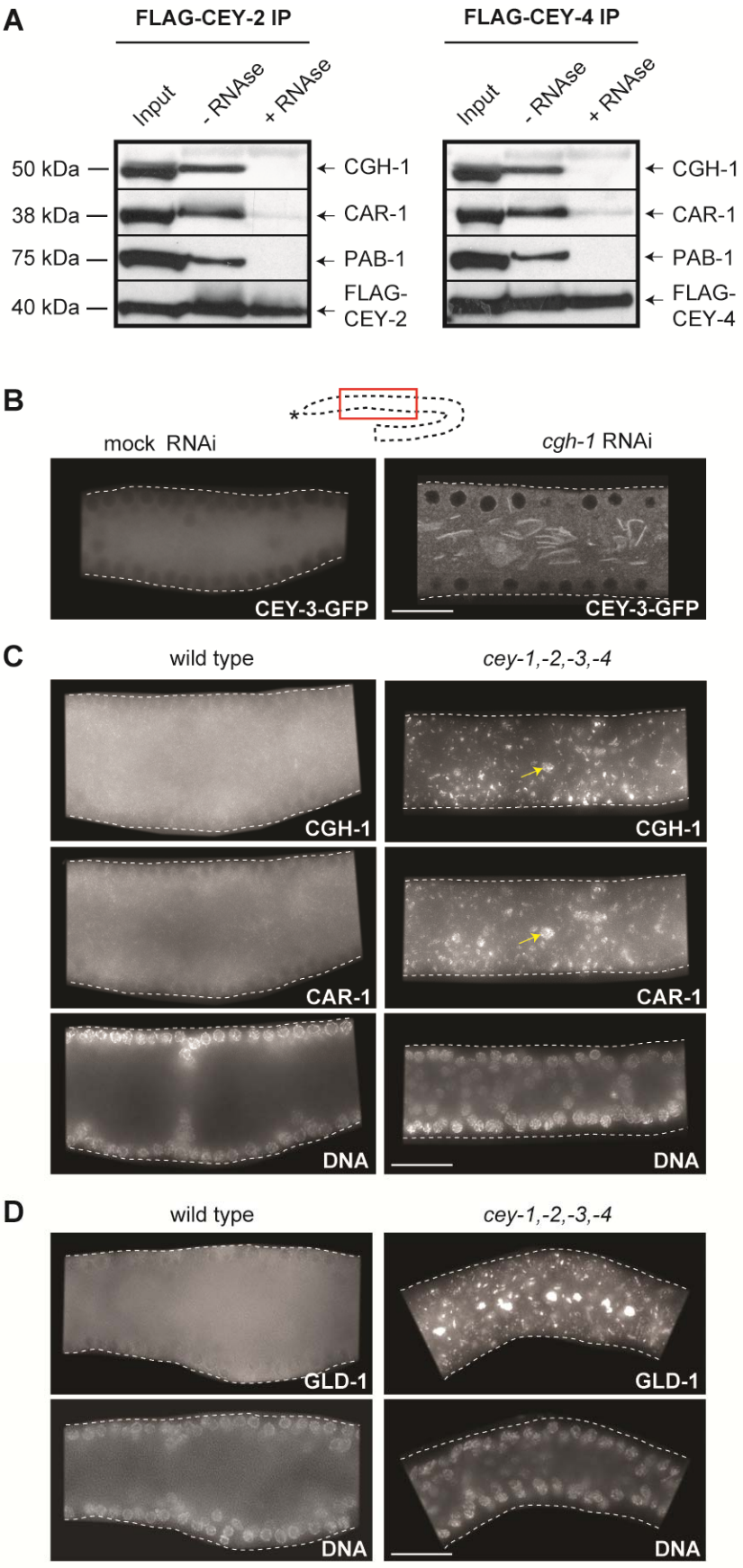

A
FLAG-CEY-2 IP
FLAG-CEY-4 IP
Input
- RNAse
+ RNAse
50 kDa
38 kDa
75 kDa
40 kDa
CGH-1
CAR-1
PAB-1
FLAG-CEY-2
FLAG-CEY-4
B
mock RNAi
cgh-1 RNAi
CEY-3-GFP
C
wild type
cey-1,-2,-3,-4
CGH-1
CAR-1
DNA
D
GLD-1

Figure 4. CEYs are required for the integrity of germline mRNPs

(A) FLAG IPs performed in the presence or absence of RNAse on extracts from transgenic animals expressing either FLAG-CEY-2 or FLAG-CEY-4. In both cases, CGH-1, CAR-1, and PAB-1 were no longer co-IPed upon RNAse treatment. (B-D) All shown images come from the medial gonadal region, boxed in red on the schematic gonad. All partial gonads are outlined by dotted lines. Asterisk indicates the distal end of the gonad. (B) Fluorescent micrographs of medial gonads from live animals expressing CEY-3-GFP. In the cytoplasm of wild-type gonads, the CEY-3-GFP was distributed evenly. Upon *cgh-1* RNAi, CEY-3-GFP localized to sheet-like structures. Scale bar = 20 μm. (C) Fluorescent micrographs of wild-type and *cey-1,-2,-3,-4* mutant gonads co-immunostained for CAR-1 and CGH-1, and additionally stained with DAPI to visualize DNA. Both proteins localized to aberrant RNP granules in *cey* mutant germlines (yellow arrow points to an exemplary RNP granule). Scale bar = 20 μm. (D) Fluorescent micrographs of wild-type and *cey-1,-2,-3,-4* mutant gonads immunostained for GLD-1 and stained with DAPI. GLD-1 also localized to aberrant granules in the absence of CEY proteins. Scale bar = 20 μm.

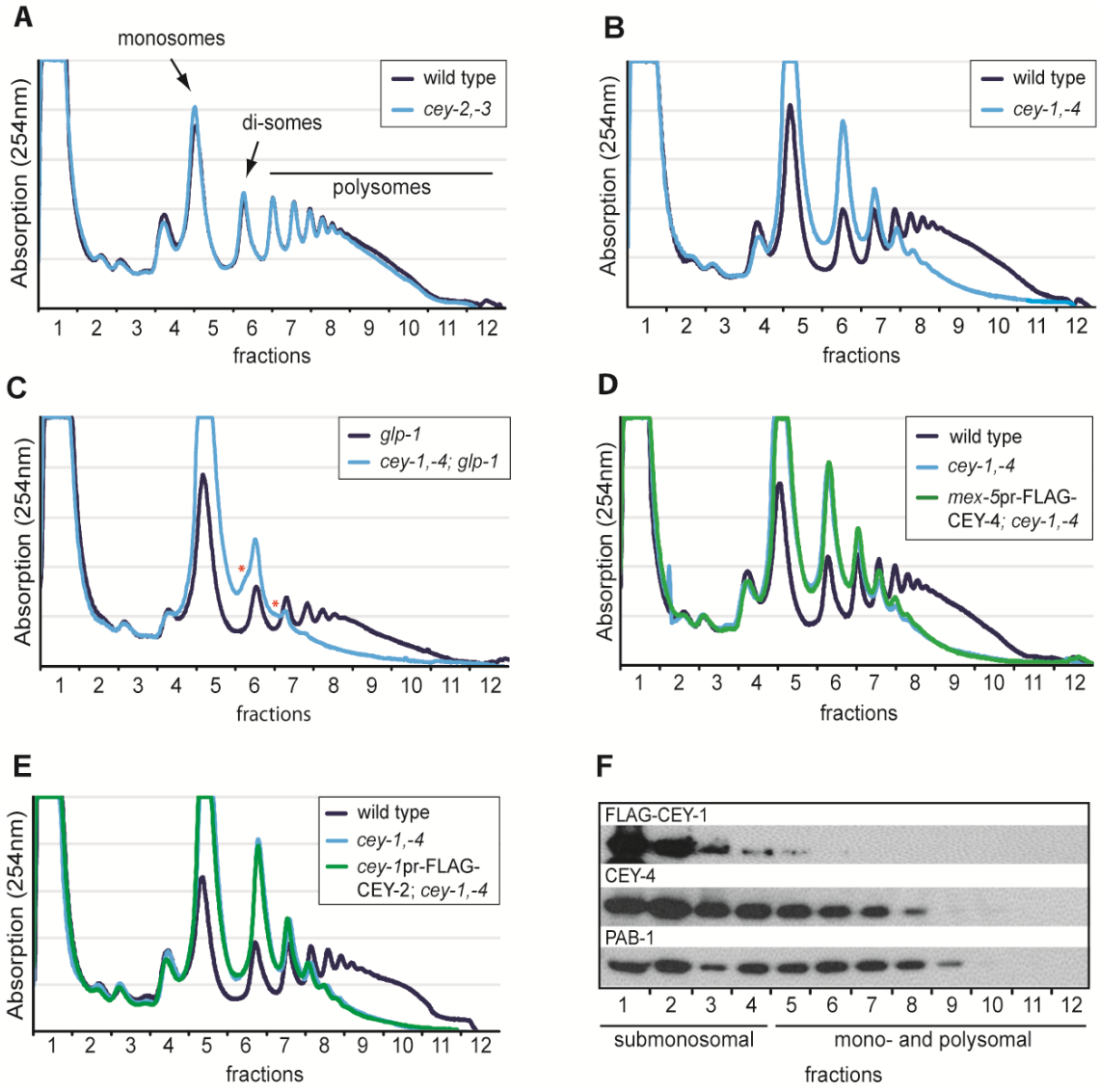


Figure 5. CEY-1 and CEY-4 are essential for the assembly of large polysomes

(A) Polysome profiles from wild type and *cey-2,-3* mutants were indistinguishable. Indicated are the positions of mono-, di-, and polysomes. (B) The depletion of CEY-1 and CEY-4 caused a strong decrease of large polysomes with a concomitant increase of mono- and disomes. (C) The loss of polysomes observed in *cey-1,-4* mutants was also observed in the germline-less *glp-1(e2144)* background. Red asterisks indicate the positions of additional small peaks present to the lighter side of normal di- and trisome peaks. (D) A FLAG-tagged CEY-4 transgene (FLAG-CEY-4) expressed specifically in the germline from the *mex-5* promoter could not restore polysomes in the *cey-1,-4* mutant. The same fusion protein when expressed ubiquitously, partially restored polysomes (see Fig. S7F). (E) Expressing a FLAG-tagged CEY-2 transgene (FLAG-CEY-2) from the *cey-1* promoter also did not restore polysomes in the *cey-1,-4* mutant. Expression of a FLAG-tagged CEY-1 transgene (FLAG-

CEY-1) from the *cey-1* promoter partially restored polysomes (see Fig. S7G). (F) Proteins were extracted from each of the 12 fractions from a polysome profiling experiment and analyzed by western blot. CEY-1 (FLAG-tagged) was mainly present in sub-polysomal fractions, while a significant part of CEY-4 was additionally found in ribosomal fractions. PAB-1 was, as expected, present in both submonosomal and ribosomal (mono- and polysomal) fractions and served here as a control.

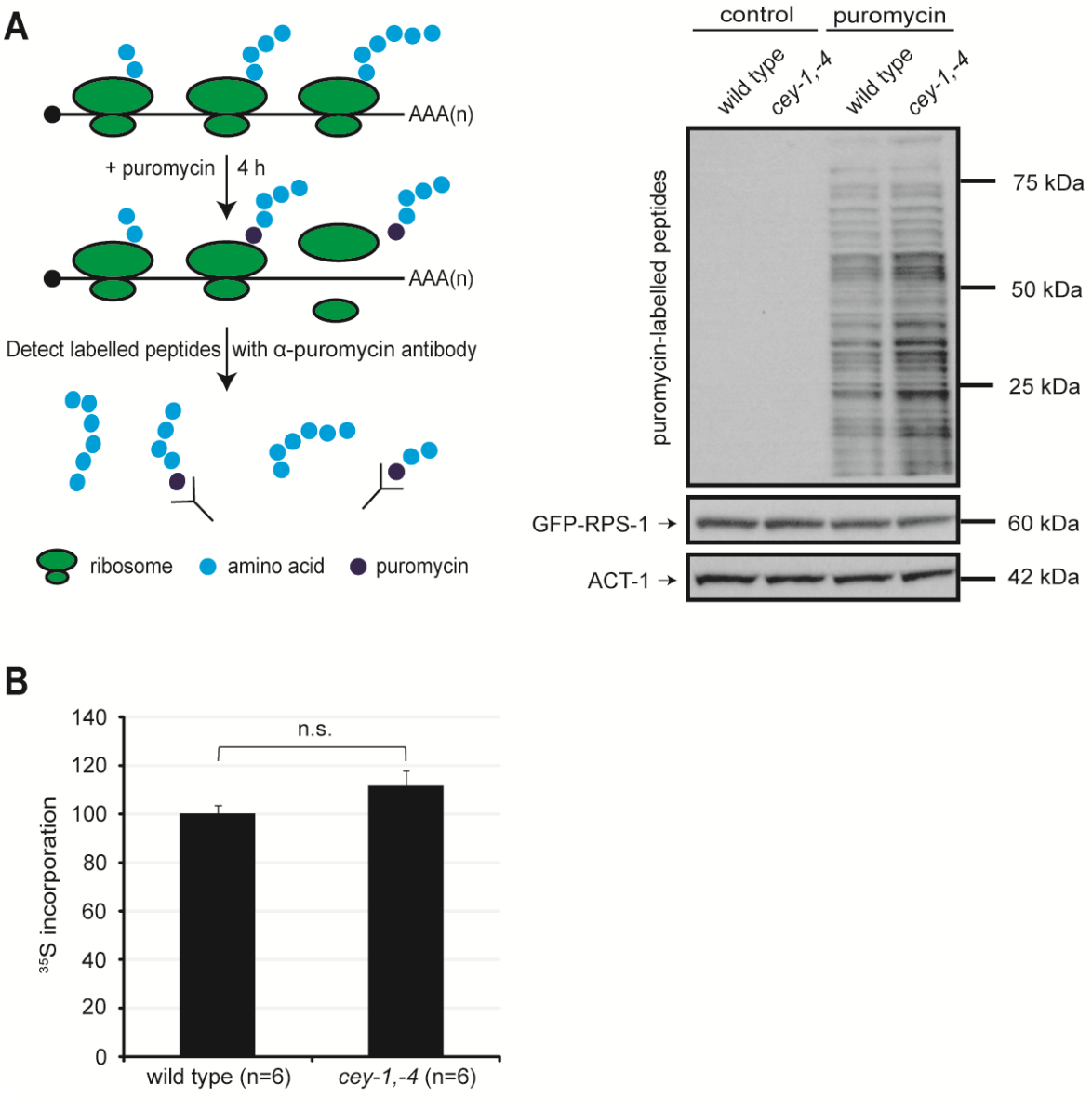


Figure 6. Global protein synthesis rates are similar between wild type and *cey-1,-4* mutants

(A) SUrface SEnsing of Translation (SUnSET) was adapted for *C. elegans*. Animals were grown with or without puromycin. A 4 h treatment was sufficient to detect puromycin incorporation into nascent proteins on a western blot, but, importantly, did not yet affect global translation (data not shown). Actin (ACT-1) was used as a loading control. Additionally, as an external loading control, extracts were spiked with an identical amount of extract from animals expressing GFP-tagged RPS-1, which were grown without puromycin. (B) Animals were grown in ^{35}S-methionine labelled OP50 bacteria for 3 h, and the amount of radioactivity incorporated into newly synthesized proteins was measured for wild type and *cey-1,-4* mutants. The wild type value was set to 100.

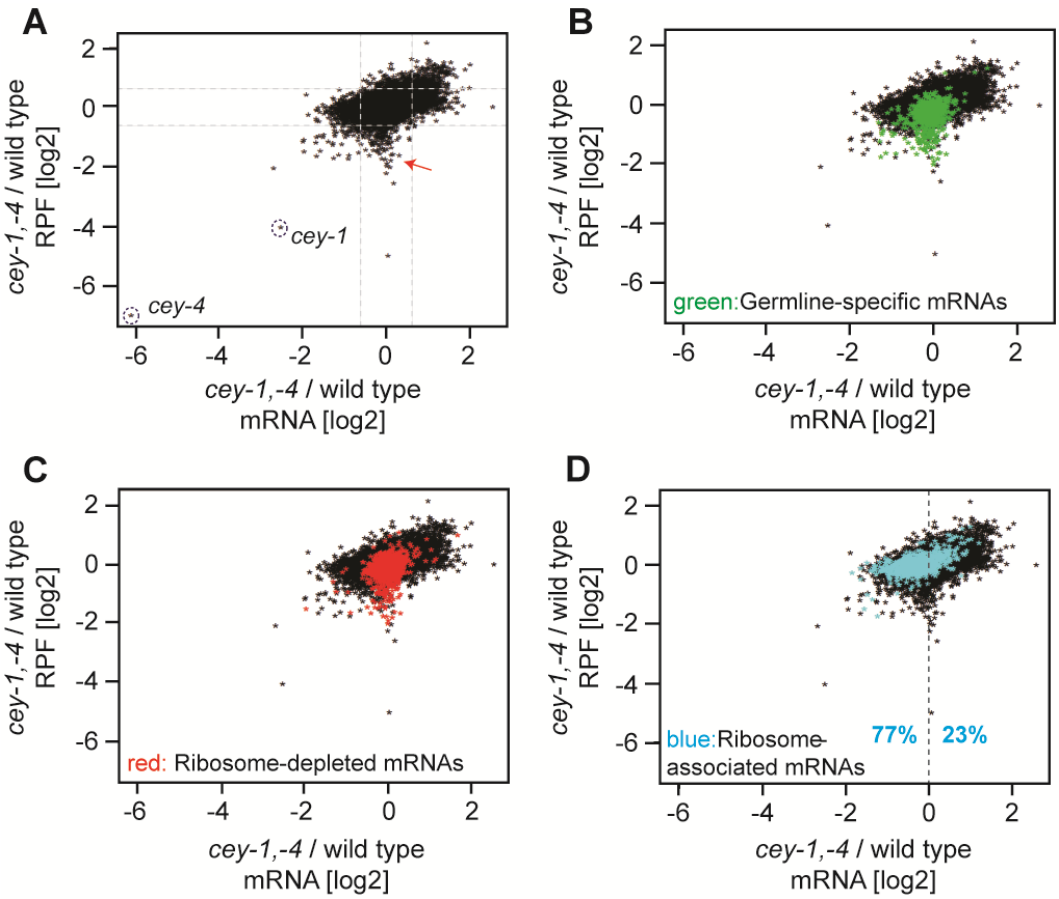


Figure 7. CEY-1 and CEY-4 regulate mRNA translation and abundance

Both ribosome profiling and total RNA sequencing were performed in duplicates. The mean values were calculated and the wild-type values were subtracted from the *cey-1,-4* values. The changes in mRNA levels were then plotted against the changes in RPF levels (indicating translation). The same plot is shown in A-D. The “germline-specific mRNAs”, “ribosome-depleted mRNAs”, and “ribosome-associated mRNAs” (marked in B, C, and D, respectively) were selected as shown in Fig. S11A-C and G-H. (A) Gray dotted lines demarcate 1.5-fold changes. As expected, *cey-1* and *cey-4* reads were strongly depleted in the mutants. We found that a sub-population of transcripts (red arrow) displayed little or no change in mRNA levels but showed reduced association with ribosomes. (B) In green are marked mRNAs expressed in gonads (mostly germline mRNAs) but not in the soma (see Fig. S11A-B). (C) In red are marked mRNAs that are depleted from mono- and polysomes (i.e. are either poorly translated or repressed) in wild-type animals (see Fig. S11C). (D) In blue are marked mRNAs enriched in mono- and polysomes in wild type animals (see Fig. S11G-H). The vertical dotted line marks no change at the mRNA level in wild type and mutant. The majority of “ribosome-associated mRNAs” (77%) appear to the left of the dotted line. Therefore, compared to all

mRNAs, genes in this subset have a higher chance to be lower in abundance in the mutant (*p-value*<2.2e-16 by *t*-test).

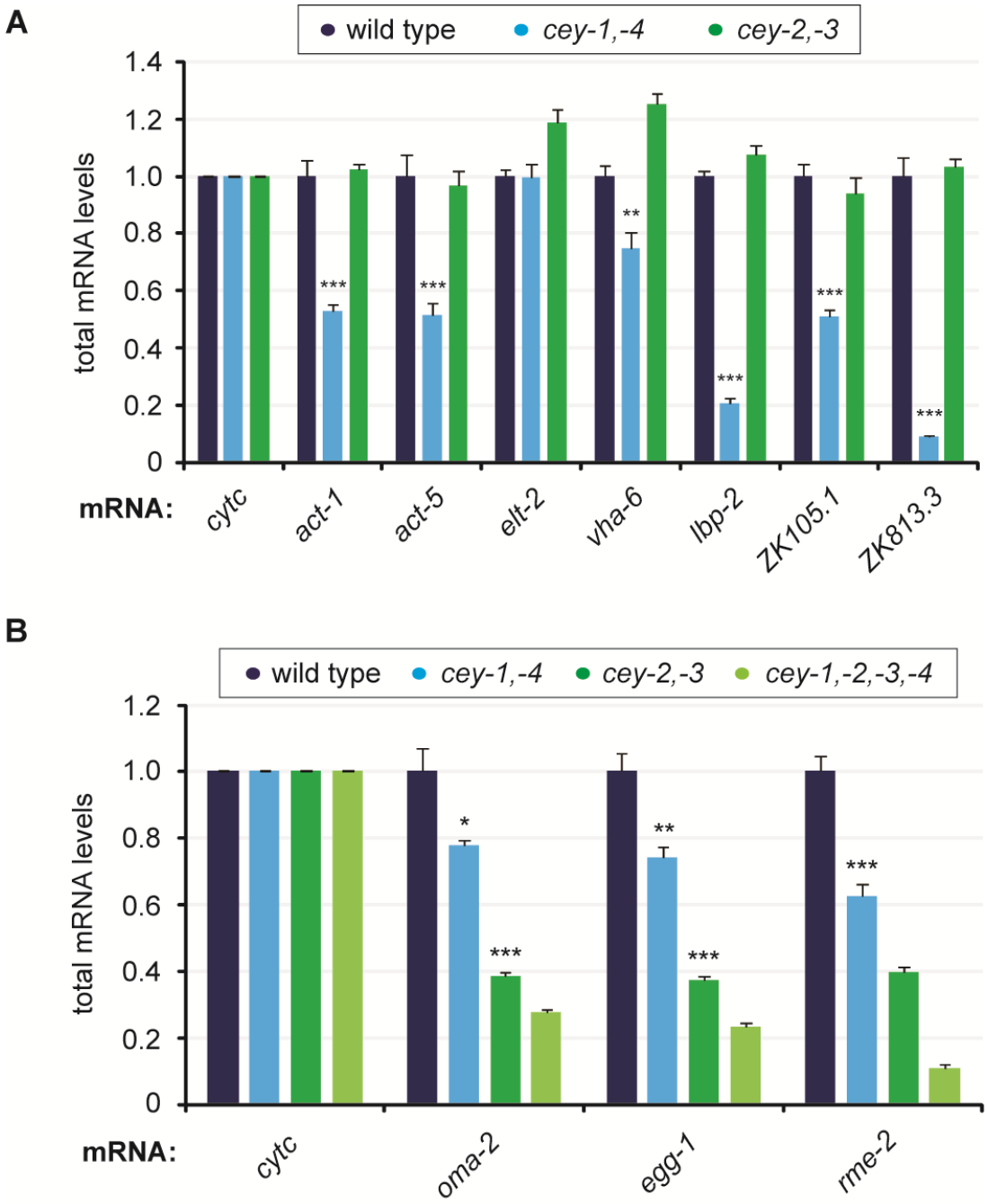
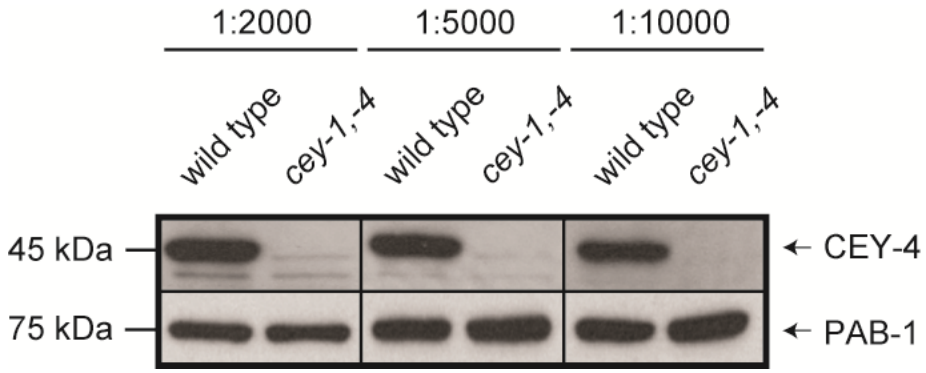


Figure 8. CEYs promote mRNA abundance in the soma and the germline

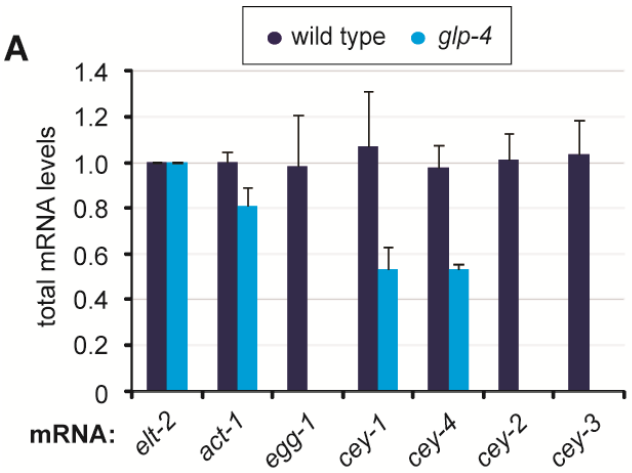
(A-B) Polysome profiling was performed for wild-type, *cey-1,-4*, *cey-2,-3*, and *cey-1,-2,-3,-4* animals. qRT-PCR analysis was performed on RNA extracted from pooled sucrose fractions 1-12 (total RNA) (see Fig. S12A). The data was normalized to a mouse mRNA, cytc (cytochrome c), to correct for any discrepancies during RNA extraction and cDNA synthesis. One asterisk denotes *p-value*<0.05 by *t*-test. Two asterisk denotes *p-value*<0.01 by *t*-test. Three asterisks denotes *p-value*<0.005 by *t*-test. Error bars represent SEM. (A) The abundance of tested ubiquitous (expressed in germline and soma) or soma-specific mRNAs was reduced in the *cey-1,-4* mutant but not in the absence of the germline-specific CEY-2 and -3. (B) The abundance of germline-specific transcripts was more strongly affected in the *cey-2,-3* double mutant compared to *cey-1,-4* mutant animals. The mRNA levels dropped even further in the *cey-1,-2,-3,-4* quadruple mutant. (C) Changes in RME-2 protein levels mirrored the changes of mRNA levels (B), showing the strongest decrease in the *cey-1,-2,-3,-4* mutant

4.1.10 Supplementary figures and tables



Supplementary Figure S1. Specificity of the CEY-4 antibody

The CEY-4 antibody (obtained from sdix, affinity purified, rabbit, polyclonal) was tested at different dilutions (1:2000, 1:5000, 1:10000) on either wild-type or *cey-1,-4* protein extract. The antibody is specific as the signal disappears in the absence of CEY-4.


A
wild type
glp-4
total mRNA levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
mRNA:
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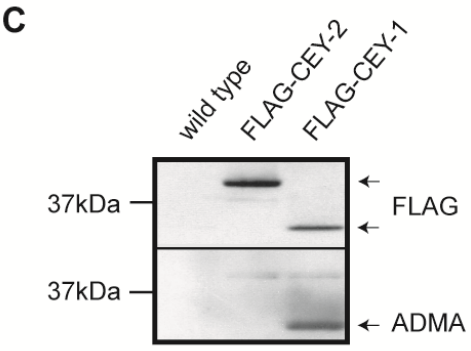
act-1
egg-1
cey-1
cey-4
cey-2
cey-3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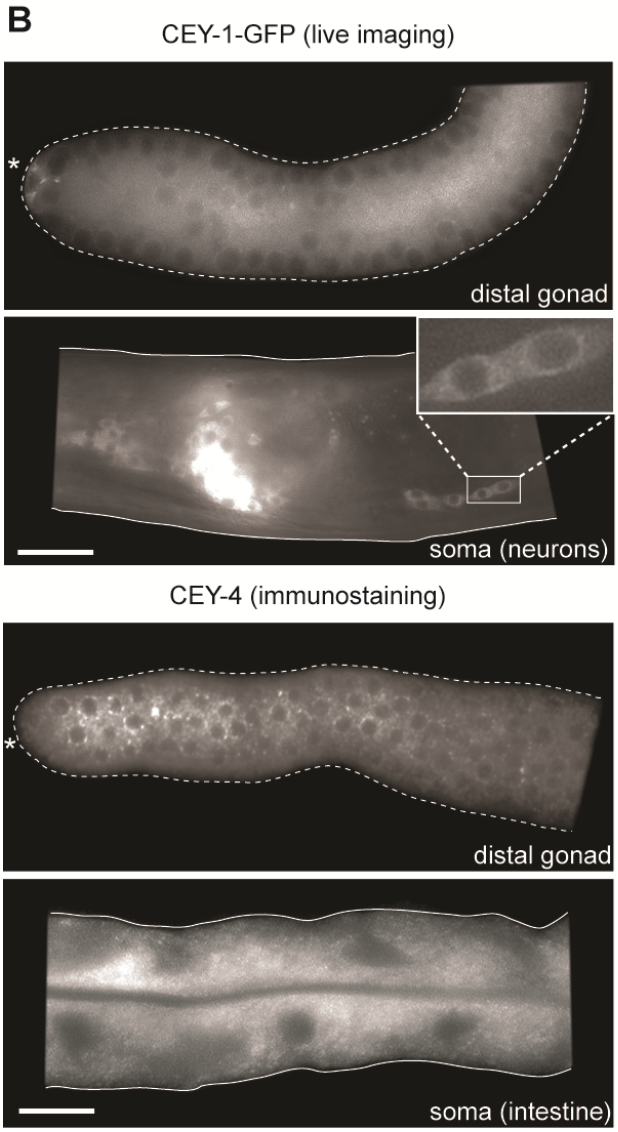
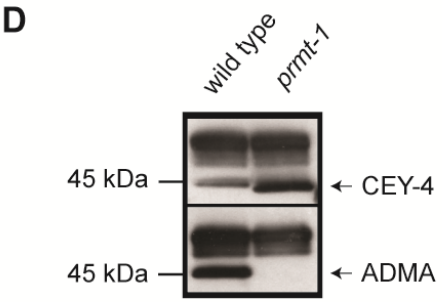
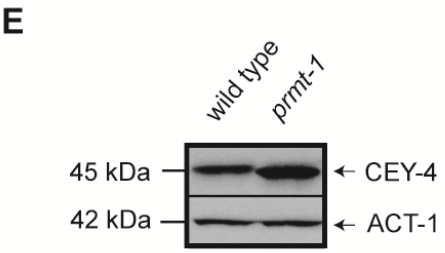
FLAG-CEY-1
37kDa
37kDa
FLAG
ADMA

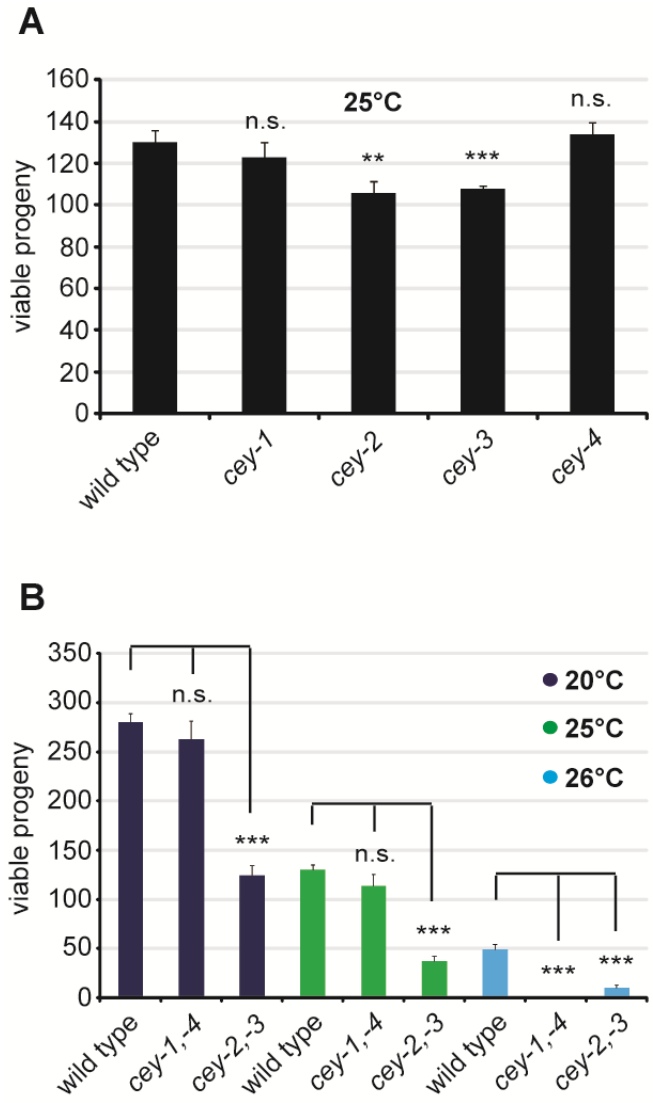

B
CEY-1-GFP (live imaging)
distal gonad
soma (neurons)
CEY-4 (immunostaining)
distal gonad
soma (intestine)


D
wild type
prmt-1
45 kDa
45 kDa
CEY-4
ADMA


E
wild type
prmt-1
45 kDa
42 kDa
CEY-4
ACT-1

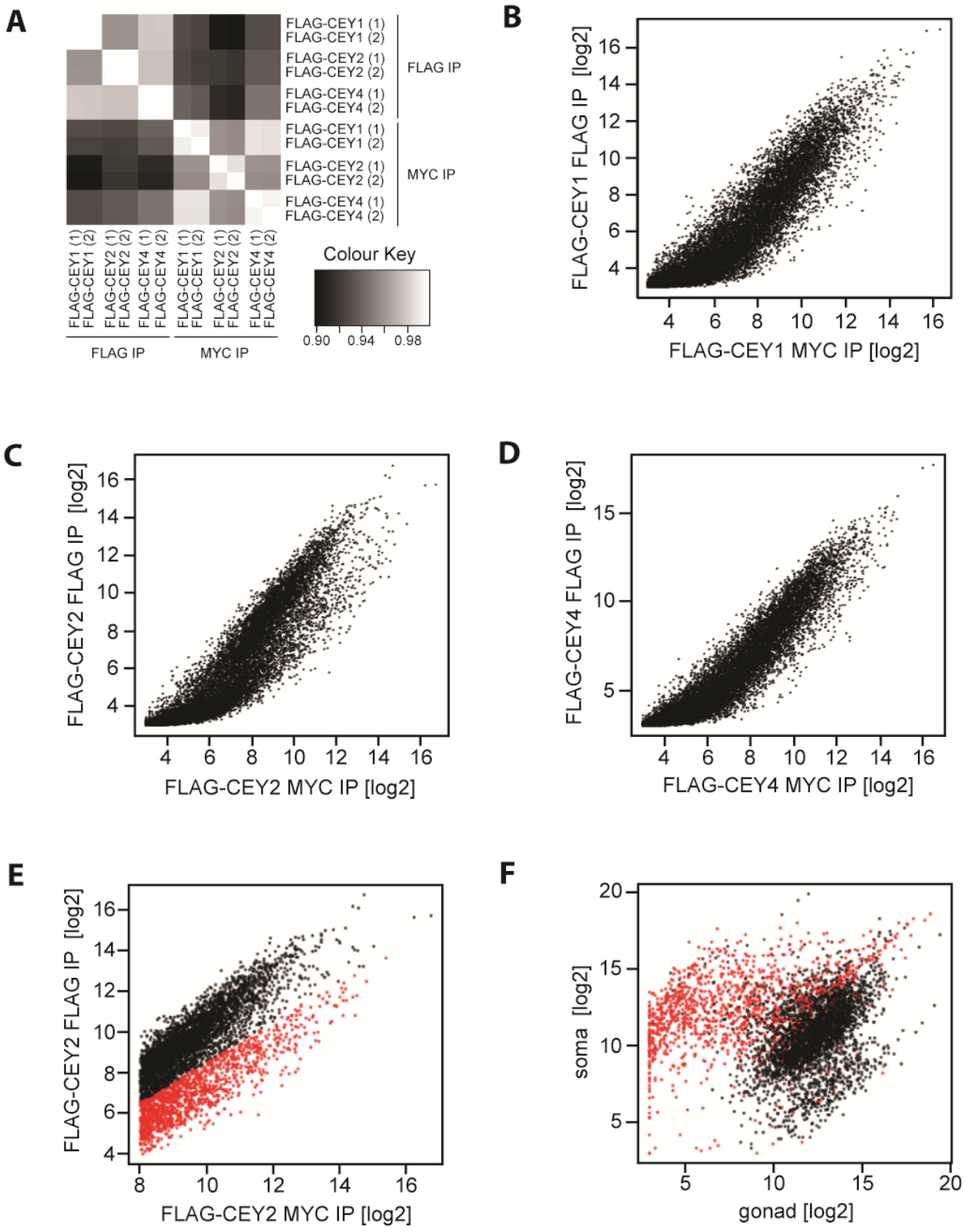
Supplementary Figure S2. Characteristics of CEY proteins

(A) qRT-PCR analysis on wild type and germline-less *glp-4(bn2)* mutants showed that *cey-2* and *cey-3* are only expressed in the germline. Controls include soma-specific *elt-2*, ubiquitously expressed *act-1* and germline-specific *egg-1*. Error bars represent SEM. (B) Fluorescent micrographs of GFP-CEY-1 (live animals) and of CEY-4 immunostainings. The gonad and somatic structures are outlined by a dotted and solid line, respectively. Asterisk indicates the distal end of the gonad. CEY-1 and CEY-4 proteins are cytoplasmic in both the soma (intestine and neurons) and the germline. Scale bar = 20 µm. (C-D) IPs performed on CEY-1 (FLAG-CEY-1), CEY-2 (FLAG-CEY-2), and CEY-4 (endogenous), revealed that the RG/RGG repeats in CEY-1 and CEY-4 are asymmetrically di-methylated (ADMA). (C) CEY-2 has no RG/RGG repeats and consistently showed no ADMA signal. (D) The ADMA mark depends on *prmt-1*. In addition, the amount of CEY-4 protein increased upon the loss of ADMA. This was confirmed by western blot analysis of total protein (E).



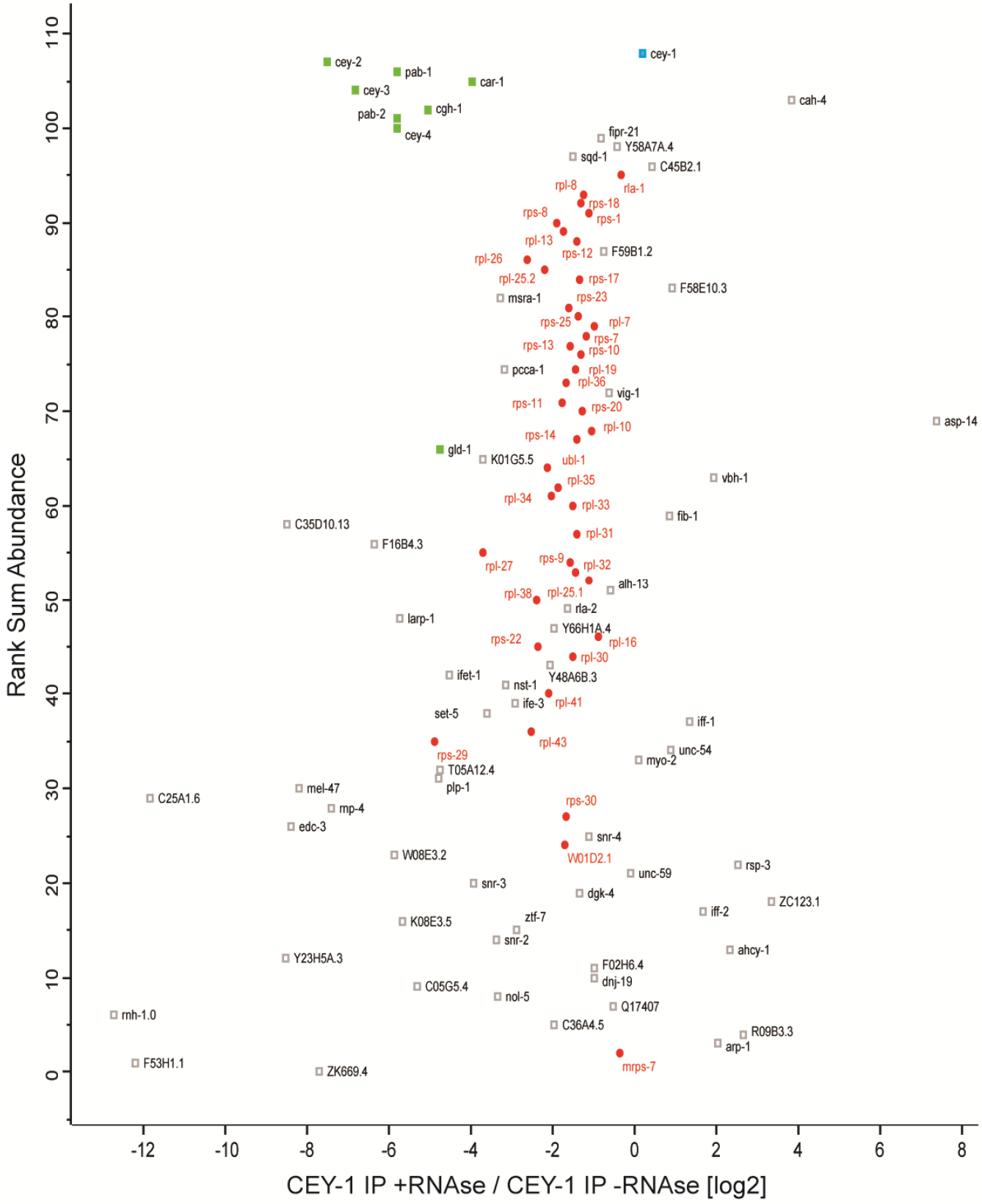
Supplementary Figure S3. CEY-2 and CEY-3 have a predominant role in producing progeny

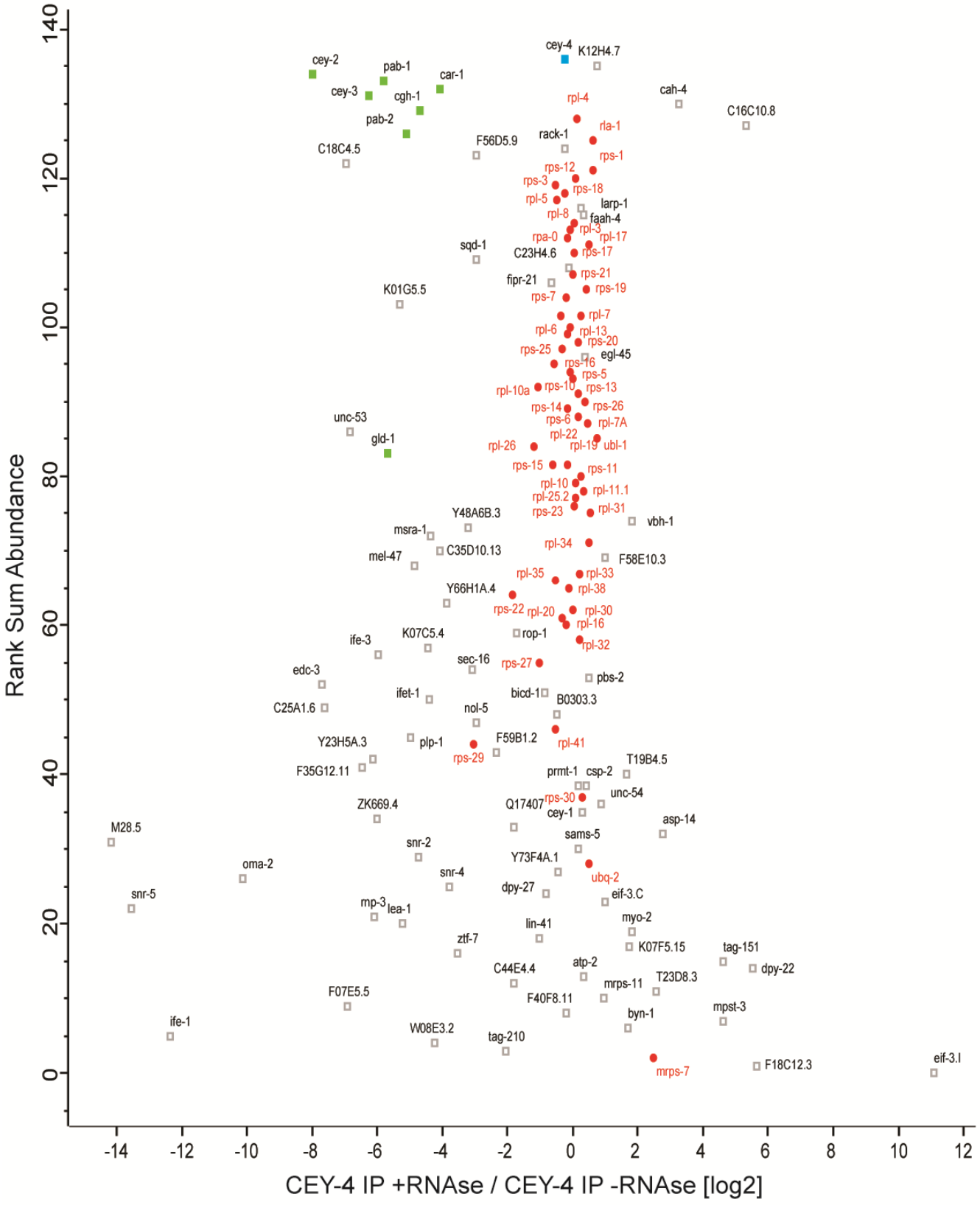
The number of progeny per animal (n = 8-10) was counted at the indicated temperature. Error bars represent SEM. Two asterisks denotes *p-value*<0.01 by *t*-test. Three asterisks denotes *p-value*<0.005 by *t*-test. (A) The loss of either *cey-2* or *cey-3* caused a significant reduction of viable progeny at 25°C. (B) Consistently, knocking out both *cey-2* and *cey-3* simultaneously caused a strong decrease in progeny number at all three tested temperatures. The *cey-1,-4* double mutant only started showing severe fertility defects at 26°C.


A
FLAG-CEY1 (1)
FLAG-CEY1 (2)
FLAG-CEY2 (1)
FLAG-CEY2 (2)
FLAG-CEY4 (1)
FLAG-CEY4 (2)
FLAG IP
MYC IP
Colour Key
0.90 0.94 0.98
B
FLAG-CEY1 FLAG IP [log2]
FLAG-CEY1 MYC IP [log2]
C
FLAG-CEY2 FLAG IP [log2]
FLAG-CEY2 MYC IP [log2]
D
FLAG-CEY4 FLAG IP [log2]
FLAG-CEY4 MYC IP [log2]
E
FLAG-CEY2 FLAG IP [log2]
FLAG-CEY2 MYC IP [log2]
F
soma [log2]
gonad [log2]

Supplementary Figure S4. Global analysis of mRNAs bound by CEY proteins

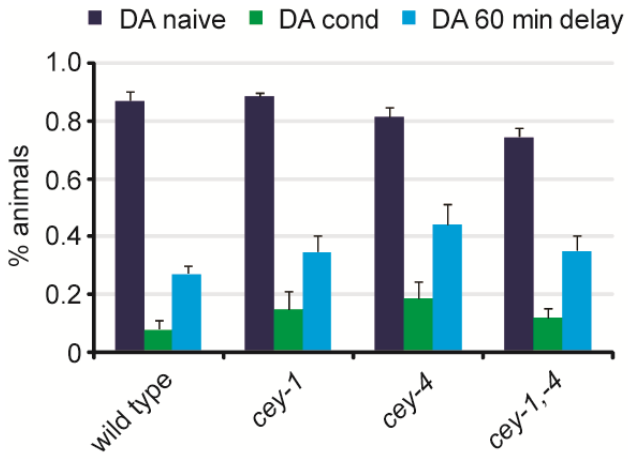
(A) Pairwise correlation heatmaps for FLAG IPs performed on FLAG-CEY-1, -2, and -4 and their respective MYC IP controls performed in duplicates. (B-D) Scatter plots comparing MYC IPs and FLAG IPs for CEY-1 (B), CEY-2 (C) and CEY-4 (D). (E) Scatter plot for the highly expressed genes (>8, $\log_2$ scale), highlighting in red those that were depleted by more than 2.25 fold in the FLAG IPs performed on FLAG-CEY-2 compared to the respective MYC control IPs. (F) Scatter plot comparing expression in the gonad to expression in the soma (*glp-4(bn2)*) for the same genes as in (E) highlighting the identical subset of genes as in (E).


Rank Sum Abundance
CEY-1 IP +RNAse / CEY-1 IP -RNAse [log2]
cey-1
cey-2
pab-1
car-1
cey-3
cgh-1
pab-2
cey-4
cah-4
fipr-21
Y58A7A.4
sqd-1
C45B2.1
rpl-8
rla-1
rps-18
rps-8
rps-1
rpl-13
rps-12
F59B1.2
rpl-26
rpl-25.2
rps-17
F58E10.3
msra-1
rps-23
rps-25
rpl-7
rps-7
rps-13
rps-10
pcca-1
rpl-19
rpl-36
vig-1
rps-11
rps-20
asp-14
rpl-10
rps-14
gld-1
K01G5.5
ubl-1
vbh-1
rpl-35
rpl-34
rpl-33
fib-1
C35D10.13
rpl-31
F16B4.3
rps-9
rpl-32
rpl-27
alh-13
rpl-38
rpl-25.1
rla-2
larp-1
Y66H1A.4
rps-22
rpl-16
rpl-30
Y48A6B.3
ifet-1
nst-1
ife-3
rpl-41
set-5
iff-1
rpl-43
unc-54
rps-29
myo-2
T05A12.4
plp-1
C25A1.6
mel-47
rps-30
mp-4
edc-3
snr-4
W01D2.1
W08E3.2
unc-59
rsp-3
snr-3
dgk-4
ZC123.1
iff-2
K08E3.5
ztf-7
snr-2
ahcy-1
Y23H5A.3
F02H6.4
dnj-19
C05G5.4
nol-5
Q17407
rnh-1.0
C36A4.5
R09B3.3
arp-1
F53H1.1
mrps-7
ZK669.4
0
10
20
30
40
50
60
70
80
90
100
110
-12
-10
-8
-6
-4
-2
0
2
4
6
8


Rank Sum Abundance
CEY-4 IP +RNAse / CEY-4 IP -RNAse [log2]
cey-4
K12H4.7
cey-2
pab-1
car-1
cey-3
cgh-1
pab-2
C18C4.5
F56D5.9
rack-1
rla-1
rpl-4
cah-4
C16C10.8
rps-1
rps-12
rps-3
rps-18
rpl-5
larp-1
rpl-8
faah-4
rpl-3
rpa-0
rpl-17
sqd-1
C23H4.6
rps-17
fipr-21
rps-21
K01G5.5
rps-19
rps-7
rpl-7
rpl-6
rpl-13
rps-20
rps-25
egl-45
rps-16
rps-5
rpl-10a
rps-10
rps-13
rps-14
rps-26
unc-53
rps-6
rpl-7A
rpl-22
gld-1
rpl-26
rpl-19
ubl-1
rps-15
rps-11
rpl-10
rpl-11.1
rpl-25.2
Y48A6B.3
rps-23
rpl-31
vbh-1
msra-1
rpl-34
C35D10.13
mel-47
F58E10.3
rpl-35
rpl-33
Y66H1A.4
rpl-38
rps-22
rpl-20
rpl-30
rpl-16
ife-3
K07C5.4
rop-1
rpl-32
sec-16
rps-27
edc-3
pbs-2
ifet-1
bicd-1
B0303.3
C25A1.6
nol-5
Y23H5A.3
plp-1
F59B1.2
rpl-41
rps-29
T19B4.5
F35G12.11
prmt-1
csp-2
unc-54
rps-30
ZK669.4
Q17407
cey-1
asp-14
M28.5
sams-5
snr-2
Y73F4A.1
oma-2
snr-4
ubq-2
dpy-27
snr-5
eif-3.C
mp-3
lea-1
myo-2
lin-41
ztf-7
K07F5.15
tag-151
C44E4.4
atp-2
dpy-22
T23D8.3
mrps-11
F07E5.5
F40F8.11
mpst-3
byn-1
ife-1
W08E3.2
tag-210
mrps-7
F18C12.3
eif-3.I

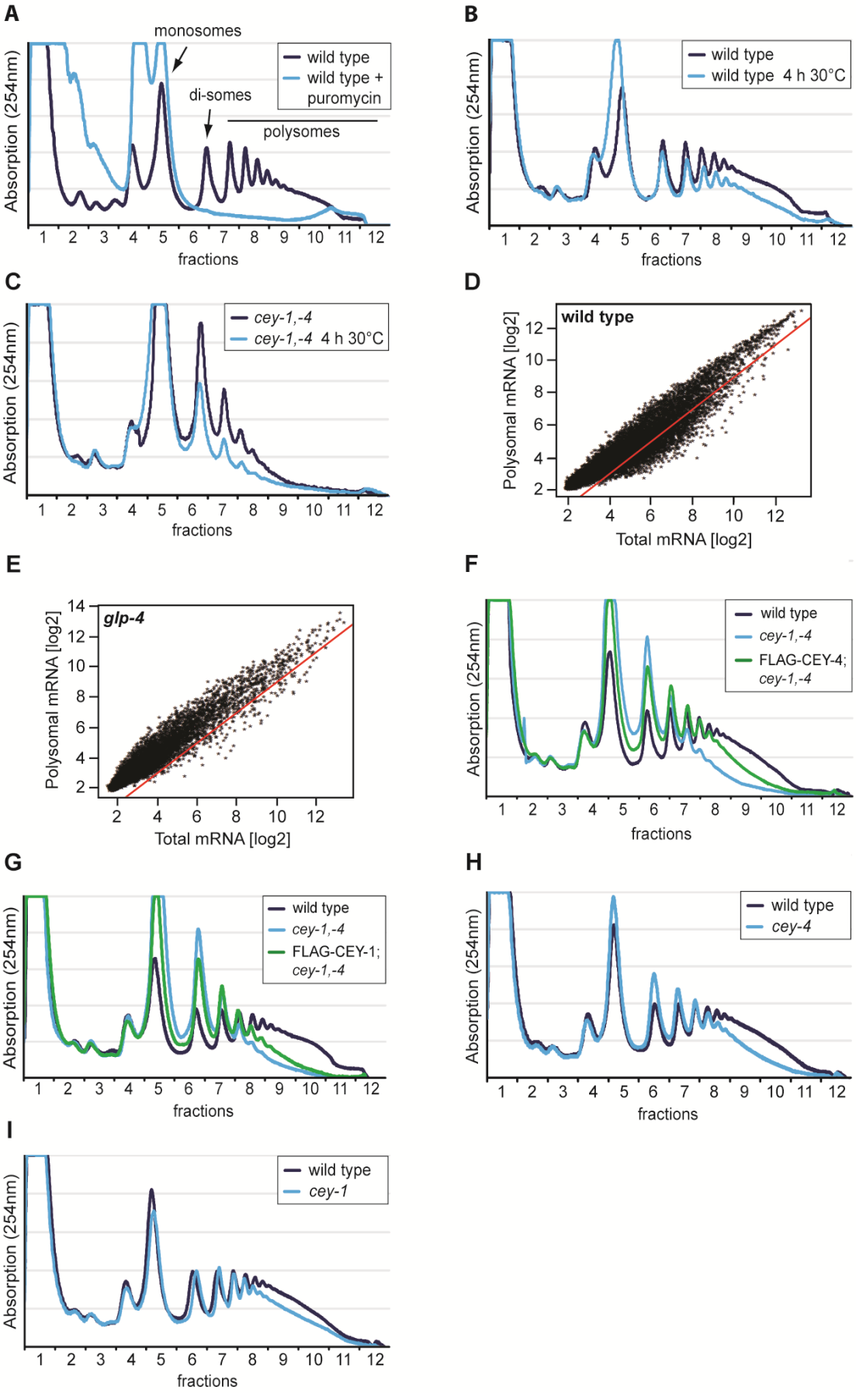
Supplementary Figures S5 and S6. Mass-spectrometry data on the interaction partners of CEY-1 and CEY-4 in the presence or absence of RNA

The $\log_2$ ratio of the protein abundances from the RNase treated CEY-1 or CEY-4 IP to the non-treated CEY-1 or CEY-4 IP is plotted on the X-axis. The proteins are ranked according to their abundance on the Y-axis. The data points are labeled with the gene names. Proteins quantified with two or more peptides are shown. Ribosomal proteins are marked in red circles. A group of proteins either shown (see Fig. 2A) or suspected to undergo RNA-dependent interactions with CEY-1 and CEY-4 are marked with green squares. The protein used for the IP (either CEY-1 or CEY-4) is marked with a blue square.



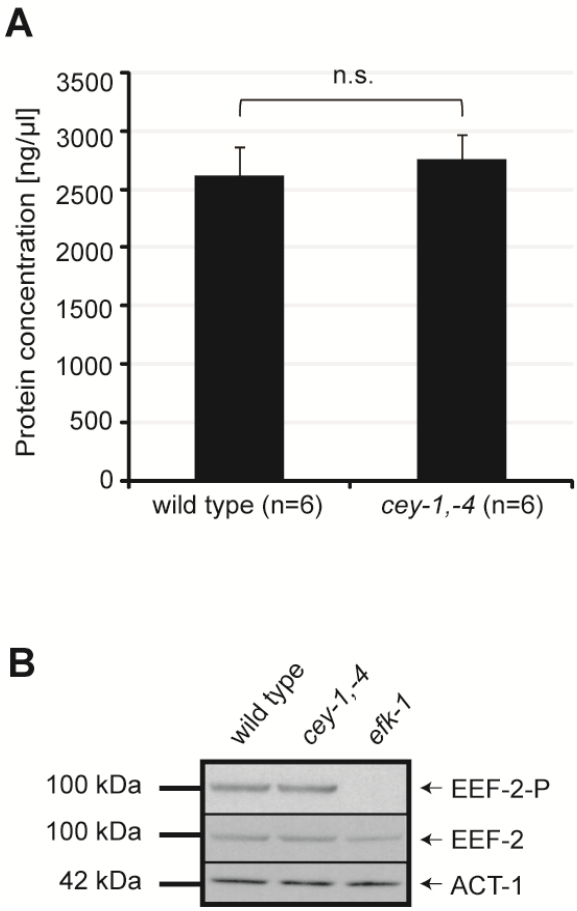
Supplementary Figure S7. Neurological functions do not appear to be compromised in the *cey-1,-4* mutant

Neither the two single mutants (*cey-1* and *cey-4*) nor the double mutant (*cey-1,-4*) showed deficiencies in chemotaxis (DA naive), learning (DA cond) or memory (DA 60 min delay). “cond”: conditioned; “DA”: diacetyl.


A
monosomes
di-somes
polysomes
wild type
wild type + puromycin
Absorption (254nm)
fractions
B
wild type
wild type 4 h 30°C
Absorption (254nm)
fractions
C
cey-1,-4
cey-1,-4 4 h 30°C
Absorption (254nm)
fractions
D
wild type
Polysomal mRNA [log2]
Total mRNA [log2]
E
glp-4
Polysomal mRNA [log2]
Total mRNA [log2]
F
wild type
cey-1,-4
FLAG-CEY-4; cey-1,-4
Absorption (254nm)
fractions
G
wild type
cey-1,-4
FLAG-CEY-1; cey-1,-4
Absorption (254nm)
fractions
H
wild type
cey-4
Absorption (254nm)
fractions
I
wild type
cey-1
Absorption (254nm)
fractions

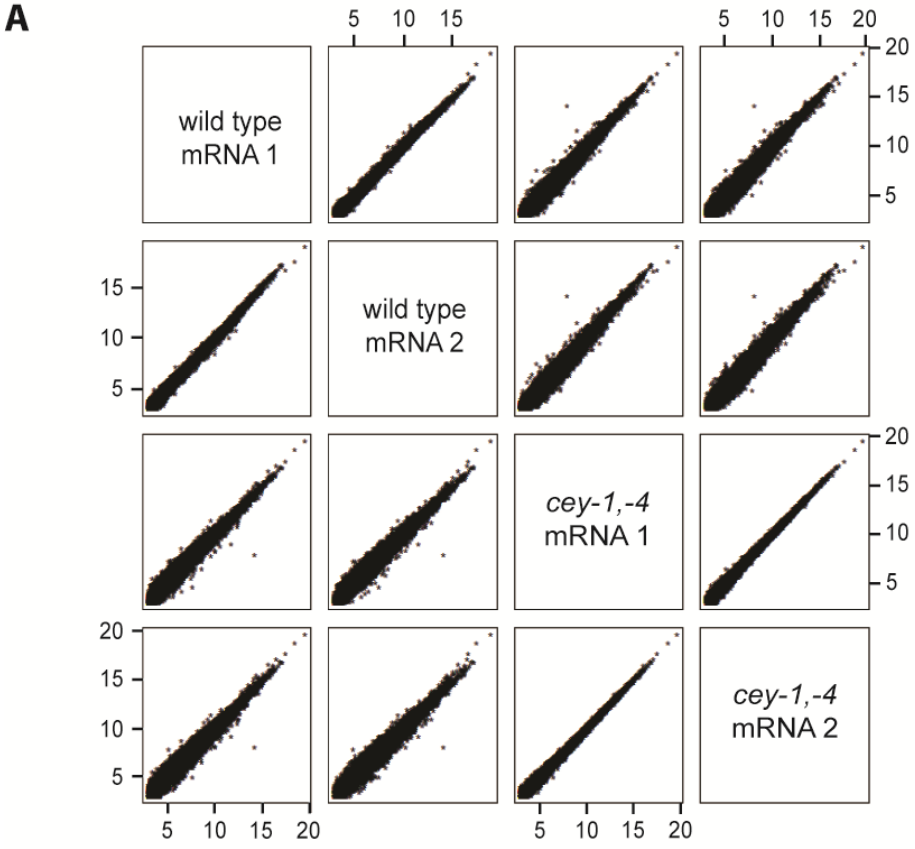
Supplementary Figure S8. Additional polysome profiling data

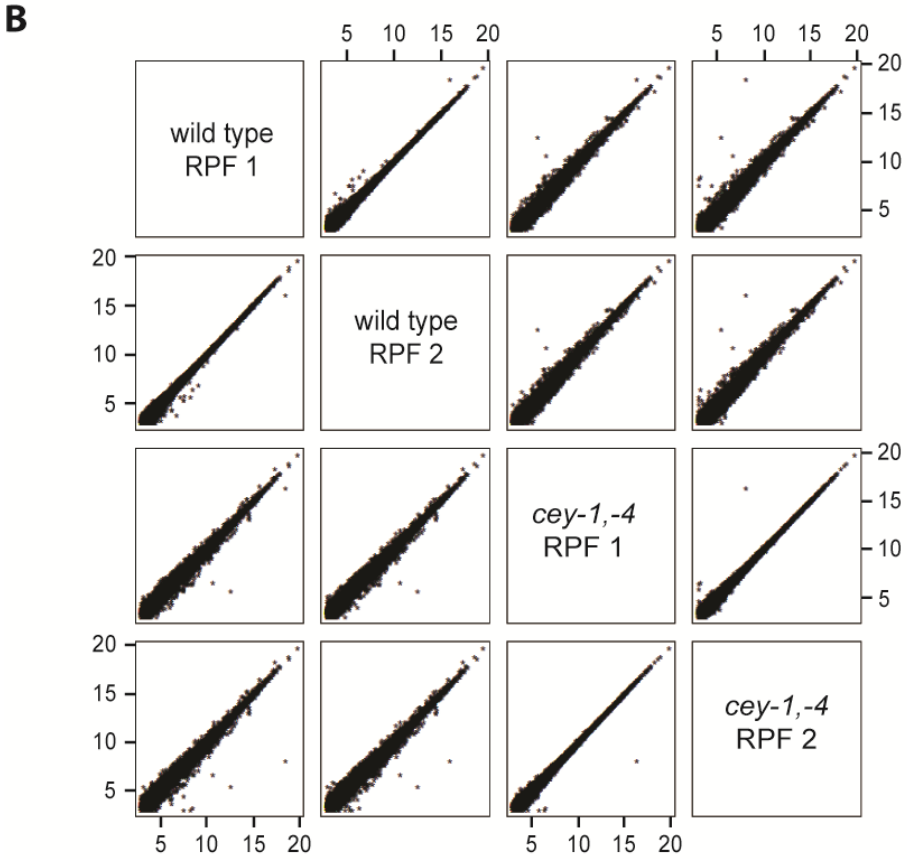
(A) Extract was treated with puromycin prior to loading on sucrose gradients. In the presence of puromycin, the majority of polysomes were lost, suggesting that they correspond to actively translating/elongating ribosomes. Indicated are the positions of mono- and disomes (two ribosomes), and of polysomes. (B-C) Wild-type and *cey-1,-4* mutant animals were heat-shocked for 4 h at 30°C and compared to control animals. (B) Polysome profiles for wild type showed a decrease of polysomes as a response to heat stress. (C) Similar to wild type, *cey-1,-4* mutants responded to heat shock by a further reduction of translating ribosomes. (D-E) Polysome profiling was performed on wild type (previously published in [29]) and germline-less (*glp-4(bn2)*) mutants. RNA was isolated either from sucrose fractions containing monosomes and polysomes (termed polysomal mRNA) or from all sucrose fractions (termed total mRNA). The global abundance of transcripts was measured by microarray analysis. Polysomal mRNAs were then plotted against total mRNAs. The red diagonal demarcates transcripts that were more than two-fold depleted from polysomal fractions (to the right of the line). Most mRNAs found below the two-fold cutoff in wild type disappeared upon removal of the germline (E). (F-G) FLAG-tagged CEY-1 and CEY-4 were partially able to restore polysomes in the *cey-1,-4* mutant. (H) The polysome profile of the *cey-4* single mutant showed a decrease of large polysomes and an increase of mono-, di-, and trisomes. (I) Loss of CEY-1 caused a slight decrease of large polysomes but no increase of mono-, di-, or trisomes.



Supplementary Figure S9. Total protein levels and EEF-2 phosphorylation status remain unchanged in the *cey-1,-4* mutant

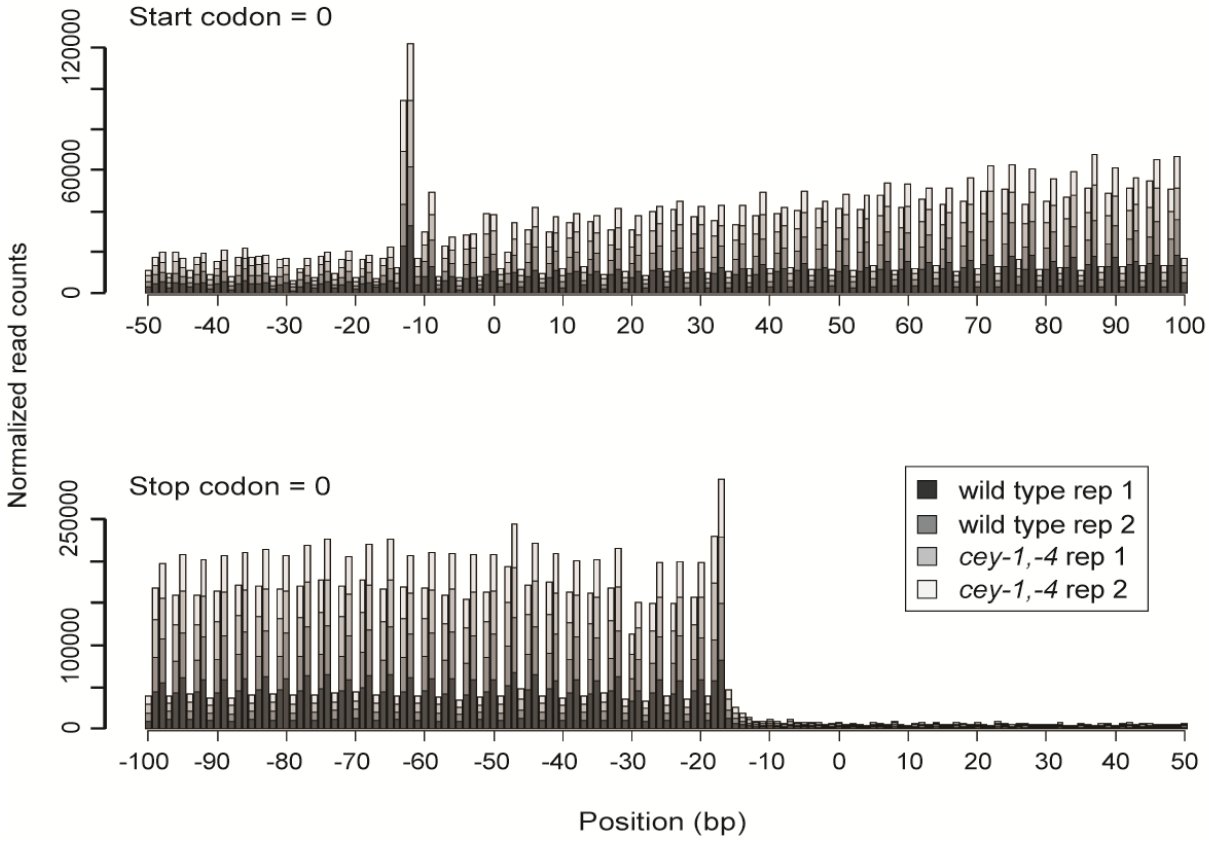
(A) Protein was extracted from an equal amount of wild-type and *cey-1,-4* mutant animals. The total volume (not shown) and concentration of mutant extract were not affected in the *cey-1,-4* mutant. (B) Western blot analysis of EEF-2 and its phosphorylated (inactive) form (EEF-2-P) in wild-type, *cey-1,-4* mutant, and *efk-1* (EEF-2 kinase homologue in *C. elegans*) mutant animals. Both EEF-2 and EEF-2-P levels remained unchanged in wild type and *cey-1,-4* mutant. As expected, the EEF-2-P signal was lost in *efk-1* mutant animals. ACT-1 was used as a loading control.


A
wild type mRNA 1
wild type mRNA 2
cey-1,-4 mRNA 1
cey-1,-4 mRNA 2
5 10 15 20


B
wild type RPF 1
wild type RPF 2
cey-1,-4 RPF 1
cey-1,-4 RPF 2
5 10 15 20

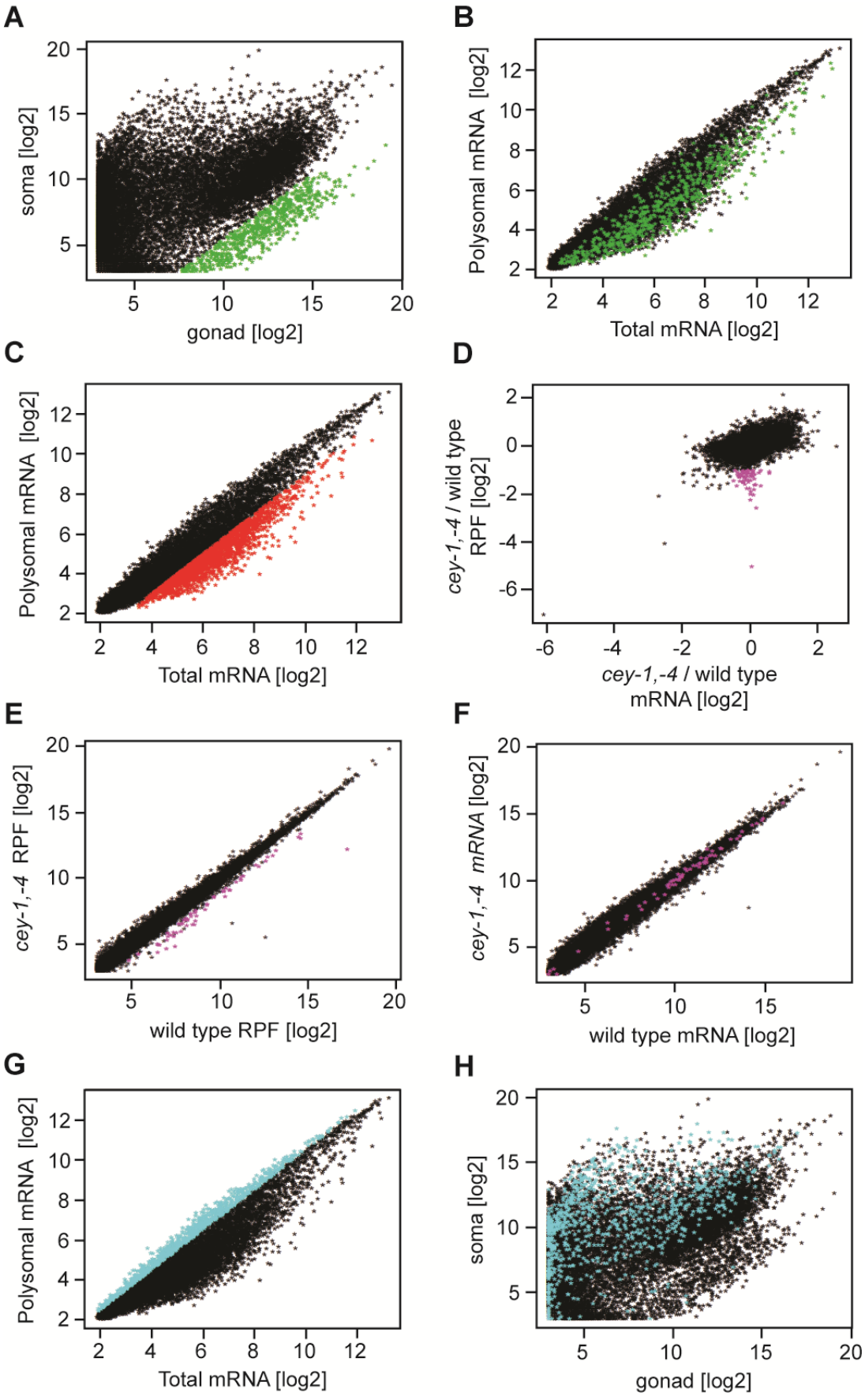
Supplementary Figure S10. RNA sequencing and ribosome profiling data

Pairwise correlation plots shown for the mRNA sequencing data (A) and the ribosome profiling data (B). Axes are in $\log_2$ scale. “1”: first replicate, “2”: second replicate. “RPF”: ribosome protected fragment. In all cases the replicates correlated very well.



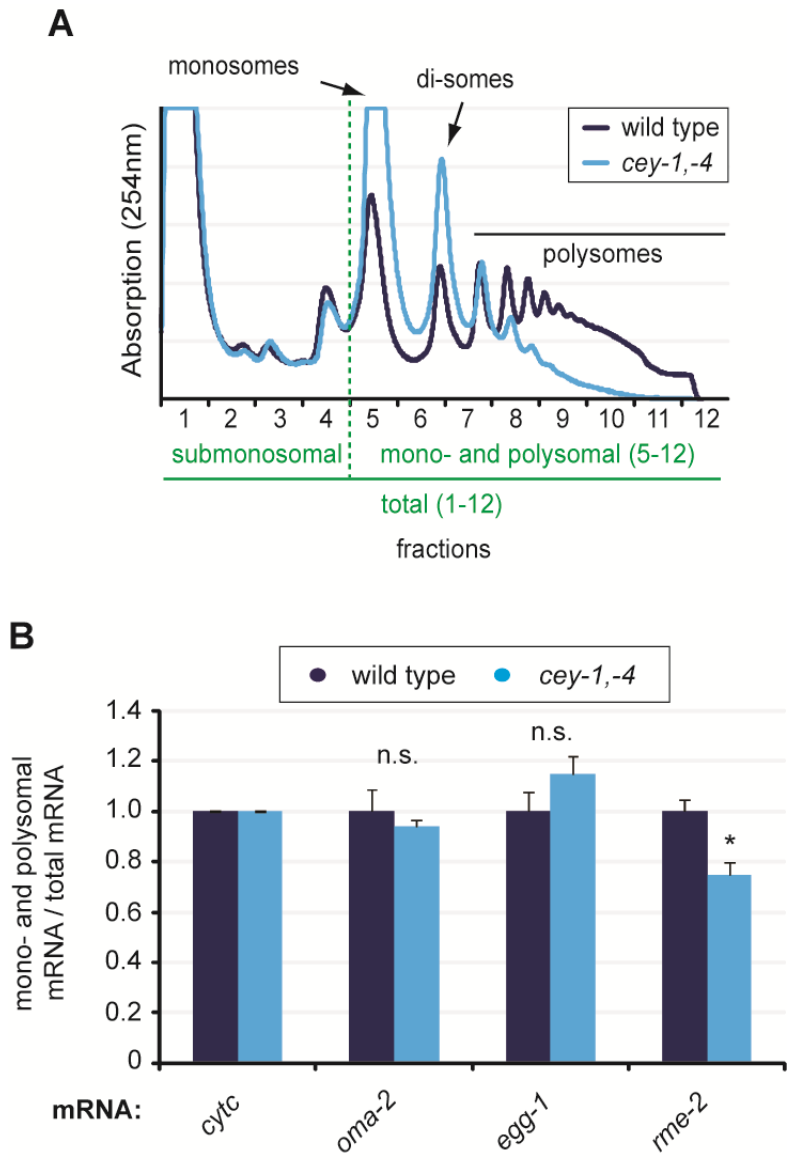
Supplementary Figure S11. RPFs mapped to the transciptome show no difference in wild type and *cey-1,-4* mutant

Ribosome profiling was performed in duplicates for both wild type and *cey-1,-4*. The positions of the 5’ ends of the reads for ribosome protected fragments (RPFs) were used for counting. Only genes with an annotated 3’UTR or 5’UTR of at least 50 bp were used. To reduce the impact of highly expressed transcripts, we normalized the coverage of each transcript by its expression (coverageNorm=coverage/ (expression+8) *avgExpression). The pseudocount of 8 was used to reduce the impact of transcripts with very low expression levels. avgExpression denotes the average transcript expression and was used as a global constant to scale back the counts. Three nucleotide periodicity and depletion of RPFs from 5’UTRs and 3’UTRs supports that they originate from mRNAs undergoing translation. “rep”: replicate.


A
soma [log2]
gonad [log2]
B
Polysomal mRNA [log2]
Total mRNA [log2]
C
Polysomal mRNA [log2]
Total mRNA [log2]
D
cey-1,-4 / wild type RPF [log2]
cey-1,-4 / wild type mRNA [log2]
E
cey-1,-4 RPF [log2]
wild type RPF [log2]
F
cey-1,-4 mRNA [log2]
wild type mRNA [log2]
G
Polysomal mRNA [log2]
Total mRNA [log2]
H
soma [log2]
gonad [log2]

Supplementary Figure S12. Selecting specific subsets of transcripts

The three different subsets of mRNAs ("germline-specific", "ribosome-depleted", "ribosome-enriched") marked in Fig. 7 were selected as follows. (A) The "germline-specific" mRNA subset was selected by plotting gene expression data derived from germline-less *glp-4(bn2)* animals (soma) against those derived from dissected gonads (gonad). mRNAs were arbitrarily selected as being "germline-specific" if they were more than 22-fold enriched in gonads (green). (B) Consistent with the finding that most ribosome-depleted mRNAs were found in the germline (Fig. S7D-E), germline-specific genes showed a bias towards ribosome-depletion (green). (C) The "ribosome-depleted" mRNA subset was selected by plotting polysomal mRNA versus total mRNA (described in Fig. S7D-E). The chosen cutoff represents transcripts depleted at least two-fold from polysomal fractions (red). (D) In magenta are marked mRNAs that show similar mRNA abundance between wild-type and *cey-1,-4* mutant animals, but are more than two-fold reduced in ribosomal association in the mutants. This group of mRNAs encodes many regulators of oocyte-to-embryo transition; see Table S3. These changes were real, as marked genes were expressed at both the mRNA and RPF level in wild type and *cey-1,-4* mutant animals shown by wild-type mRNA vs *cey-1,-4* mRNA or wild-type RPF vs *cey-1,-4* RPF plots (E-F). (G) The "ribosome-enriched" mRNA subset was selected by plotting polysomal mRNA versus total mRNA (described in Fig. S7D-E). The chosen cutoff represents transcripts enriched at least 1.4-fold in polysomal fractions (blue). As expected this subset was absent from germline-expressed transcripts (H).



Supplementary Figure S13. The distribution of mRNAs in submonosomal versus mono- and polysomal fractions

(A) Fractions 1-12 from a polysome profiling experiment were pooled and RNA was extracted (total mRNA). Fractions 5-12 were pooled and RNA was extracted (mono- and polysomal mRNA). (B) qRT-PCR analysis for germline-specific mRNAs. The values for mono- and polysomal mRNA were divided by the values of total mRNA. *rme-2* mRNA levels appeared to drop in mono- and polysomal fractions in *cey-1,-4* mutants compared to wild type. This was not the case for *oma-2* and *egg-1* mRNAs. Asterisk denotes *p-value*<0.05 by *t*-test. Error bars represent SEM.

Strain number	Figure	Strain name
#928	S1B	rrrSi120 [*cey-1*pro::*cey-1*ORF::GFP::FLAG::*cey-1* 3'-UTR; *unc-119*(+)] IV
#1143	1E	rrrSi245 [*cey-2*pro::GFP::*cey-2*ORF::*cey-2* 3'-UTR; *unc-119*(+)] I
Boag lab	1E	*cey-3* pro::*cey-3*ORF::GFP::FLAG(3x)::*cey-3* 3'UTR
#1099	6A	rrrSi235 [*rps-1* pro::GFP::*rps-1*ORF::*rps-1* 3'-UTR; unc-119(+)] I
#1087	1D	rrrSi228 [*cey-1*pro::PEST:GFPH2B::*cey-1* 3'-UTR; *unc-119*(+)] I
#1090	1D	rrrSi231 [*cey-2*pro::PEST:GFPH2B::*cey-2* 3'-UTR; *unc-119*(+)] I
#1091	1D	rrrSi232 [*cey-3*pro::PEST:GFPH2B::*cey-3* 3'-UTR; *unc-119*(+)] I
#1088	1D	rrrSi229 [*cey-4*pro::PEST:GFPH2B::*cey-4* 3'-UTR; *unc-119*(+)] I
#1092		rrrSi233 [*cey-2*pro::FLAG(1x)::*cey-2*ORF::*cey-2* 3'-UTR::operon linker::PEST:GFPH2B::tbb-2 3'UTR; unc-119(+)] I
#1308		rrrSi300 [*cey-1*pro::FLAG(1x)::*cey-1*ORF::*cey-1* 3'-UTR::operon linker::PEST:GFPH2B::tbb-2 3'UTR; *unc-119*(+)] I
#1335		rrrSi307 [*dpy-30*pro::FLAG(1x)::*cey-4*ORF::*cey-4* 3'-UTR::operon linker::PEST:GFPH2B::tbb-2 3'UTR; *unc-119*(+)] I **(*)**
#1502		rrrSi325 [*mex-5*pro::FLAG(1x)::*cey-4*ORF::*cey-4* 3'-UTR::operon linker::PEST:GFPH2B::tbb-2 3'UTR; *unc-119*(+)] I
#1537		rrrSi332 [*cey-1*pro::FLAG(1x)::*cey-2*ORF::*cey-1* 3'-UTR::operon linker::PEST:GFPH2B::tbb-2 3'UTR; *unc-119*(+)] I
#1069		*cey-2(ok902)*; *cey-3(rrr11)*
#1352		*cey-1(rrr12)*

Supplementary Table S1. Summary of acquired transgenic strains used in this study

(*) the ubiquitous *dpy-30* promoter had to be used instead of the *cey-4* promoter (also ubiquitous) due to cloning problems.

Accession	Peptides	Score	Description	Summed peptide ion abundance		
				CEY-1 + RNAse	CEY-1 - RNAse	wild type ctrl
Ribosomal proteins						
RS3A_CAEEL	17	838.34	RPS-1	1.20E+05	2.59E+05	3.04E+04
RL8_CAEEL	15	813.31	RPL-8	1.23E+05	2.88E+05	4.82E+04
RS8_CAEEL	13	733.52	RPS-8	7.67E+04	2.85E+05	5.49E+04
RL13_CAEEL	13	649.94	RPL-13	8.15E+04	2.73E+05	3.70E+04
O18240_CAEEL	13	630.39	RPS-18	1.17E+05	2.88E+05	4.88E+04
RS17_CAEEL	12	607.13	RPS-17	7.80E+04	1.99E+05	2.64E+04
RL10_CAEEL	11	550.09	RPL-10	4.70E+04	9.67E+04	1.30E+04
RS12_CAEEL	8	523.37	RPS-12	9.41E+04	2.48E+05	3.57E+04
RL19_CAEEL	12	513.95	RPL-19	5.21E+04	1.41E+05	1.31E+04
RS7_CAEEL	11	509.24	RPS-7	6.62E+04	1.49E+05	2.15E+04
RS13_CAEEL	12	455.13	RPS-13	5.12E+04	1.53E+05	1.91E+04
RLA1_CAEEL	7	453.79	RLA-1	2.47E+05	3.09E+05	5.92E+04
Q20206_CAEEL	11	415.23	RPS-11	3.77E+04	1.27E+05	2.20E+04
Q8WQA8_CAEEL	8	398.64	RPS-20	4.68E+04	1.13E+05	2.24E+04
RS14_CAEEL	6	396.76	RPS-14	3.74E+04	9.79E+04	1340.98
RL7_CAEEL	11	376.49	RPL-7	7.31E+04	1.45E+05	2.81E+04
R23A2_CAEEL	9	374.41	RPL-25.2	4.98E+04	2.29E+05	1.34E+04
O01869_CAEEL	6	346.71	RPS-10	5.65E+04	1.40E+05	2.24E+04
RS9_CAEEL	11	340.91	RPS-9	2.50E+04	7.45E+04	1.41E+04
RL31_CAEEL	7	308.05	RPL-31	3.04E+04	8.03E+04	6732.43
RL26_CAEEL	7	301.68	RPL-26	4.12E+04	2.53E+05	2.64E+04
RL35A_CAEEL	8	281.34	RPL-33	3.00E+04	8.50E+04	1.49E+04
RL13A_CAEEL	8	280.75	RPL-16	2.61E+04	4.84E+04	3064.33
RL37A_CAEEL	6	271.68	RPL-43	7263.06	4.17E+04	7036.81
RS25_CAEEL	6	268.22	RPS-25	5.61E+04	1.68E+05	3.26E+04
RL27_CAEEL	5	268.16	RPL-27	7457.44	9.60E+04	1.03E+04
RS23_CAEEL	5	260.62	RPS-23	6.16E+04	1.59E+05	1.23E+04
RL36_CAEEL	5	255.67	RPL-36	4.18E+04	1.33E+05	8340.94
Q22716_CAEEL	5	245.06	RPL-32	2.60E+04	7.04E+04	1954.95
A3QMC5_CAEEL	5	228.86	RPL-34	2.29E+04	9.23E+04	1.33E+04
Q9U1X9_CAEEL	5	220.07	RLA-2	1.92E+04	5.98E+04	7131.92
O62388_CAEEL	7	219.11	W01D2.1	6413.02	2.06E+04	2303.11
P90983_CAEEL	5	213.51	RPS-29	1602.78	4.72E+04	1180.4
RL35_CAEEL	5	207.9	RPL-35	2.51E+04	9.16E+04	4887.81
Q9XWS4_CAEEL	4	202.89	RPL_30	1.91E+04	5.39E+04	7841.98
RS27A_CAEEL	2	201.41	UBL-1	2.27E+04	9.91E+04	9931.76
R23A1_CAEEL	4	196.18	RPL-25.1	2.80E+04	6.02E+04	1.17E+04
O17218_CAEEL	3	138.96	RPS-22	1.22E+04	6.18E+04	1.78E+04
RL38_CAEEL	2	122.93	RPL-38	1.37E+04	7.19E+04	2481.09

RL44_CAEEL	5	120.84	RPL-41	1.17E+04	5.02E+04	2626.83
Q18231_CAEEL	3	89.82	RPS-30	7607.51	2.40E+04	1854.05
RT07_CAEEL	2	82.33	MRPS-7	2122.53	2717.21	0

Other proteins						
Q9U302_CAEEL	52	3214	PAB-1	9.89E+04	5.51E+06	1.20E+05
Q9XW17_CAEEL	29	2144.94	CAR-1	3.22E+05	5.04E+06	3.34E+05
CGH1_CAEEL	35	2093.3	CGH-1	5.17E+04	1.70E+06	3.89E+04
P91306_CAEEL	19	1832.24	CEY-2	5.95E+04	1.07E+07	6.19E+04
P91398_CAEEL	20	1673.54	CEY-3	3.39E+04	3.82E+06	4.56E+04
Q19579_CAEEL	22	1461.93	PAB-2	2.24E+04	1.24E+06	2.89E+04
Q8WQD8_CAEEL	18	1411.73	CAH-4	2.31E+06	1.60E+05	1.41E+04
O62213_CAEEL	16	1392.65	CEY-1	3.30E+07	2.87E+07	4.98E+04
G5EDV3_CAEEL	17	1292.14	CEY-4	2.11E+04	1.17E+06	4811.64
O02089_CAEEL	9	879.68	MSRA-1	2.29E+04	2.19E+05	1579.89
Q9XUW5_CAEEL	22	865.95	F58E10.3	1.69E+05	8.96E+04	3.13E+04
PCCA_CAEEL	15	709.09	PCCA-1	1.91E+04	1.74E+05	1.68E+05
Q8MXR6_CAEEL	12	661.53	SQD-1	1.95E+05	5.51E+05	7.49E+04
DKC1_CAEEL	13	613.23	K01G5.5	9173.24	1.20E+05	7523.09
D5MCN2_CAEEL	12	604.26	LARP-1	1414.81	7.56E+04	1.04E+04
H2KYR1_CAEEL	11	586.51	VIG-1	6.67E+04	1.02E+05	1.58E+04
Q94271_CAEEL	9	465.16	ASP-14	1.57E+05	931.43	3146.47
GLD1_CAEEL	11	445.29	GLD-1	4736.04	1.27E+05	5716.97
Q18490_CAEEL	7	434.72	C35D10.13	316.5	1.13E+05	545.02
MYO4_CAEEL	10	430.99	UNC-54	3.11E+04	1.68E+04	3434.74
Q20277_CAEEL	6	390.14	FIPR-21	4.04E+05	7.02E+05	5.72E+04
FBRL_CAEEL	8	369.18	FIB-1	7.37E+04	4.07E+04	4825.66
G1K0V8_CAEEL	3	296.64	IFF-1	3.69E+04	1.45E+04	6942.51
GAR1_CAEEL	4	289.54	Y66H1A.4	1.52E+04	5.94E+04	1866.14
Q94230_CAEEL	8	282.77	PLP-1	1342.86	3.67E+04	185.68
Q9N3F4_CAEEL	6	276.73	VBH-1	9.51E+04	2.50E+04	1.13E+04
MEL47_CAEEL	6	256.85	MEL-47	128.22	3.73E+04	1420.83
IF4E3_CAEEL	5	229.5	IFE-3	6612.3	5.00E+04	1056.53
O61880_CAEEL	4	219.68	F59B1.2	1.26E+05	2.09E+05	5580.79
Q21740_CAEEL	4	219.51	EDC-3	82.18	2.79E+04	184.29
Q95YC6_CAEEL	5	216.17	C45B2.1	3.86E+05	2.83E+05	2.00E+04
NHP2_CAEEL	4	207.47	Y48A6B.3	1.30E+04	5.40E+04	1.05E+04
Q21832_CAEEL	2	151.7	RNP-4	200.74	3.39E+04	1077.31
Q20898_CAEEL	5	150.73	IFET-1	2603.01	6.01E+04	3548.16
NOP10_CAEEL	3	148.73	C25A1.6	8.86	3.61E+04	3950.43
O76616_CAEEL	3	142.69	Y23H5A.3	30.06	1.13E+04	615.11
Q20448_CAEEL	3	139.2	ZTF-7	1852.2	1.37E+04	584.22
SMD2_CAEEL	2	138.95	SNR-4	8598.32	1.86E+04	1824.47

G5EDE4_CAEEL	3	126.75	W08E3.2	413.86	2.40E+04	0
Q17407_CAEEL	2	108.42	T27E9.1a	2855.68	4050.76	0
O45012_CAEEL	2	108.41	NOL-5	639.06	6466.18	244.7
M1ZJ32_CAEEL	2	103.33	SNR-3	1067.21	1.64E+04	581.59
ODB2_CAEEL	2	92.64	ZK669.4	10.66	2430.89	163.99
SAHH_CAEEL	2	91.74	AHCY-1	9525.78	1899.79	0.08
IF5A2_CAEEL	4	85.56	IFF-2	1.24E+04	3871.42	1494.24
RSMB_CAEEL	3	84.84	SNR-2	1192.22	1.23E+04	3267.49
MYO2_CAEEL	3	82.74	MYO-2	2.44E+04	2.28E+04	559.63
O45713_CAEEL	2	79.72	R09B3.3	5241.94	828.89	437.66
Q18494_CAEEL	2	79.52	C36A4.5	1378.99	5327.85	161.96
H2KZC9_CAEEL	3	75.99	DGK-4	4798.14	1.20E+04	1168.87
Q86MP4_CAEEL	3	74.7	RNH-1.0	0	6763.26	4008.3
Q9XUS4_CAEEL	2	70.17	K08E3.5	306.36	1.56E+04	4177.95
SUCA_CAEEL	3	63.79	C05G5.4	207.24	8295.48	3955.17
RSP3_CAEEL	2	61.86	RSP-3	1.87E+04	3199.36	1.82E+04
Q9NA98_CAEEL	2	59.58	ARP-1	4717.06	1146.44	0
O16303_CAEEL	2	59.39	DNJ-19	2935.68	5718.93	0
G5EEE4_CAEEL	4	59.06	SET-5	4022.79	4.87E+04	3110.35
ALH13_CAEEL	2	55.37	ALH-13	3.46E+04	5.13E+04	558.67
GNL3_CAEEL	3	54.89	NST-1	6309.97	5.56E+04	776.05
Q9U334_CAEEL	2	54.52	UNC-59	1.01E+04	1.08E+04	602.23
O44633_CAEEL	2	53.12	F16B4.3	1272.41	1.03E+05	2315.26
Q966A5_CAEEL	3	53.07	Y58A7A.4	3.68E+05	4.90E+05	3.90E+04
H2L0J8_CAEEL	2	52.62	T05A12.4	1522.94	4.07E+04	457.29
Q965K2_CAEEL	2	51.94	F53H1.1	0	4677.86	573.44
O45021_CAEEL	2	51.1	ZC123.1	1.51E+04	1487.48	874.26
O62131_CAEEL	2	50.82	F02H6.4	3231.94	6391.15	530.64

Accession	**Peptides**	**Score**	**Description**	**Summed peptide ion abundance**		
				CEY-4 + RNAse	**CEY-4 - RNAse**	**wild type ctrl**
Ribosomal proteins						
RL4_CAEEL	33	1963.59	RPL-4	7.23E+05	6.54E+05	1.42E+05
RS3_CAEEL	22	1282.38	RPS-3	2.73E+05	3.92E+05	5.86E+04
RL5_CAEEL	19	1151.56	RPL-5	2.53E+05	3.52E+05	5.84E+04
RS3A_CAEEL	23	1123.67	RPS-1	5.86E+05	3.78E+05	3.20E+04
RL3_CAEEL	22	1068.35	RPL-3	2.65E+05	2.78E+05	5.29E+04
RL8_CAEEL	17	1040.61	RPL-8	2.82E+05	2.74E+05	3.68E+04
RLA0_CAEEL	18	1001.35	RPA-0	2.53E+05	2.86E+05	3.74E+04
RS6_CAEEL	16	1001.23	RPS-6	1.52E+05	1.35E+05	2.62E+04

RS15_CAEEL	13	799.1	RPS-15	9.50E+04	1.47E+05	1.20E+04
RS7_CAEEL	13	757.65	RPS-7	2.01E+05	2.28E+05	2.80E+04
RL10_CAEEL	14	723.34	RPL-10	1.17E+05	1.10E+05	2.22E+04
RS16_CAEEL	12	691.76	RPS-16	1.54E+05	1.91E+05	1.58E+04
RS12_CAEEL	11	684.41	RPS-12	3.74E+05	3.48E+05	4.36E+04
RS17_CAEEL	14	683.69	RPS-17	2.50E+05	2.39E+05	2.28E+04
O18240_CAEEL	14	680.29	RPS-18	2.88E+05	3.38E+05	3.52E+04
RL6_CAEEL	13	667.74	RPL-6	1.76E+05	2.26E+05	3.47E+04
RL13_CAEEL	13	647.35	RPL-13	1.94E+05	2.07E+05	3.94E+04
RL7A_CAEEL	17	630.19	RPL-7A	1.66E+05	1.28E+05	3.00E+04
RS5_CAEEL	11	620.68	RPS-5	1.36E+05	2.04E+05	3.63E+04
RL19_CAEEL	13	599.47	RPL-19	1.15E+05	1.27E+05	2.40E+04
RS13_CAEEL	13	596.3	RPS-13	1.64E+05	1.65E+05	2.31E+04
Q8WQA8_CAEEL	12	589.45	RPS-20	1.84E+05	2.03E+05	2.34E+04
RL7_CAEEL	13	584.19	RPL-7	2.19E+05	1.83E+05	3.72E+04
RS21_CAEEL	8	580.42	RPS-21	2.28E+05	2.24E+05	3.36E+04
RLA1_CAEEL	6	576.37	RLA-1	7.18E+05	4.68E+05	1.07E+05
RL17_CAEEL	13	561.55	RPL-17	2.97E+05	2.13E+05	4.04E+04
RL10A_CAEEL	9	554.88	RPL-10A	1.06E+05	2.20E+05	3.15E+04
Q20206_CAEEL	11	553.9	RPS-11	1.30E+05	1.08E+05	1.43E+04
RS19_CAEEL	11	485	RPS_19	2.50E+05	1.86E+05	4.15E+04
RS14_CAEEL	8	446.12	RPS-14	1.38E+05	1.54E+05	2.97E+04
O01869_CAEEL	7	433.66	RPS-10	1.63E+05	1.70E+05	2.03E+04
RL35A_CAEEL	10	422.26	RPL-33	8.33E+04	7.28E+04	1.23E+04
RS25_CAEEL	7	401.66	RPS-25	1.89E+05	1.66E+05	2.86E+04
Q22716_CAEEL	8	380.18	RPL-32	6.06E+04	5.17E+04	4155.61
RS26_CAEEL	5	375.47	RPS-26	1.61E+05	1.44E+05	1.11E+04
RL11_CAEEL	9	370.94	RPL-11.1	1.25E+05	9.83E+04	1.78E+04
RL18A_CAEEL	9	352.1	RPL-20	5.37E+04	6.69E+04	9293.01
O17218_CAEEL	4	346.78	RPS-22	2.88E+04	1.03E+05	1.66E+04
R23A2_CAEEL	8	335.65	RPL-25.2	1.08E+05	1.02E+05	1.65E+04
RS23_CAEEL	6	325.55	RPS-23	1.06E+05	1.02E+05	1.82E+04
RL26_CAEEL	8	319.79	RPL-26	8.25E+04	1.87E+05	1.65E+04
RL13A_CAEEL	8	318.86	RPL-16	5.48E+04	6.21E+04	3284.68
RL31_CAEEL	6	318.16	RPL-31	1.21E+05	8.33E+04	5781.92
RL22_CAEEL	6	299.9	RPL-22	1.65E+05	1.20E+05	2.49E+04
Q9XWS4_CAEEL	5	274.5	RPL-30	6.43E+04	6.42E+04	8360.82
A3QMC5_CAEEL	6	227.14	RPL-34	9.79E+04	6.83E+04	1.67E+04
RS27A_CAEEL	3	210.36	UBL-1	1.70E+05	1.01E+05	1.40E+04
P90983_CAEEL	5	198.9	RPS-29	4765.88	3.90E+04	769.58
RS27_CAEEL	4	185.97	RPS-27	2.89E+04	5.80E+04	5917.36
RL35_CAEEL	6	181.78	RPL-35	6.36E+04	9.18E+04	1.49E+04
RL38_CAEEL	3	145.77	RPL-38	6.35E+04	6.91E+04	6824.36

RL44_CAEEL	4	121.15	RPL-41	1.81E+04	2.65E+04	1011.38
Q18231_CAEEL	3	95.73	RPS-30	1.62E+04	1.33E+04	2170.1
Q23155_CAEEL	2	95.71	MRPS-11	5823.49	2972.62	748.17
RT07_CAEEL	2	89.8	MRPS-7	3593.36	648.88	0
RL40_CAEEL	2	61.66	UBQ-2	9051.73	6458.58	1125.02
Others proteins						
G5EDV3_CAEEL	47	3801.7	CEY-4	3.58E+07	4.18E+07	1.55E+05
Q9U302_CAEEL	54	3454.79	PAB-1	1.11E+05	6.23E+06	1.26E+05
Q9XW17_CAEEL	31	2169.46	CAR-1	2.89E+05	4.93E+06	3.34E+05
P91306_CAEEL	20	2053	CEY-2	5.44E+04	1.39E+07	6.75E+04
YM67_CAEEL	22	2003.15	K12H4.7	9.14E+06	5.46E+06	4.32E+04
CGH1_CAEEL	32	1885.77	CGH-1	5.62E+04	1.47E+06	2.69E+04
P91398_CAEEL	19	1581.58	CEY-3	6.31E+04	4.83E+06	5.45E+04
Q19579_CAEEL	25	1409.15	PAB-2	3.37E+04	1.17E+06	4.09E+04
GBLP_CAEEL	18	1215.6	RACk-1	5.25E+05	6.15E+05	8.48E+04
DKC1_CAEEL	18	1166.38	K01G5.5	1.01E+04	3.98E+05	1.65E+04
Q8WQD8_CAEEL	14	1127.91	CAH-4	1.39E+06	1.47E+05	4.19E+04
Q9XUW5_CAEEL	19	801.05	F58E10.3	1.07E+05	5.42E+04	1.83E+04
GLD1_CAEEL	16	759.08	GLD-1	5076.07	2.63E+05	1.39E+04
O02089_CAEEL	10	692.22	MSRA-1	7828.31	1.59E+05	1503.79
Q8MXR6_CAEEL	11	686.58	SQD-1	5.43E+04	4.23E+05	6.52E+04
MEL47_CAEEL	13	682.93	MEL-47	5319.37	1.54E+05	1.10E+04
YZVL_CAEEL	12	654.91	K07C5.4	4981.51	1.07E+05	1.69E+04
EIF3A_CAEEL	16	597.83	EGL-45	1.94E+05	1.50E+05	2.62E+04
RO60_CAEEL	14	597.17	ROP-1	2.60E+04	8.68E+04	6251.13
Q21740_CAEEL	8	490.46	EDC-3	312.47	6.49E+04	350.6
O45012_CAEEL	8	451.02	NOL-5	5137.3	4.04E+04	1213.8
Q18490_CAEEL	9	442.7	C35D10.13	9017.96	1.54E+05	3161.09
Q20277_CAEEL	5	403.3	FIPR-21	1.72E+05	2.67E+05	1.75E+04
Q9N3F4_CAEEL	9	392.76	VBH-1	1.46E+05	4.16E+04	1.32E+04
IF4E3_CAEEL	6	346.68	IFE-3	1567.62	9.94E+04	2008.83
O76616_CAEEL	6	312.85	Y23H5A.3	458.44	4.03E+04	817.87
Q20057_CAEEL	3	285.74	F35G12.11	576.43	4.10E+04	283.56
NHP2_CAEEL	6	283.82	Y48A6B.3	1.73E+04	1.61E+05	9827.66
GAR1_CAEEL	6	283.6	Y66H1A.4	8246.91	1.21E+05	2782.61
D5MCN2_CAEEL	7	227.35	LARP-1	3.10E+05	2.63E+05	1755.47
Q94230_CAEEL	7	224.34	PLP-1	1373.52	4.28E+04	290.26
ODB2_CAEEL	5	222.96	ZK669.4	421.92	2.75E+04	1428.7
NOP10_CAEEL	4	218.77	C25A1.6	249.96	5.00E+04	0.12
Q17407_CAEEL	4	211.87	T27E9.1a	5597.62	1.98E+04	3719.14
Q20898_CAEEL	7	211.01	IFET-1	2549.01	5.39E+04	559.81
MYO4_CAEEL	5	205.9	UNC-54	1.84E+04	1.00E+04	332.87

P91453_CAEEL	8	193.38	T19B4.5	3.08E+04	9779.6	3813.85
O62213_CAEEL	4	178.16	CEY-1	1.56E+04	1.27E+04	774.44
Q94271_CAEEL	4	170.35	ASP-14	1.72E+04	2499.48	1842.37
Q9U2X0_CAEEL	4	167.41	PRMT-1	1.56E+04	1.40E+04	140.09
EIF3C_CAEEL	3	167.39	EIF-3	8618.62	4373.58	1718.24
TSR1_CAEEL	4	160.14	TAG-151	9835.96	399.61	154.95
I7J4C6_CAEEL	4	152.99	K07F5.15	8336.33	2502.25	992.21
P91223_CAEEL	3	149.57	F07E5.5	71.44	8678.71	0
YQ58_CAEEL	3	141.79	C16C10.8	1.20E+06	3.03E+04	4318.42
LIN41_CAEEL	2	130.82	LIN-41	3601.14	7263.32	523.37
O01806_CAEEL	3	128.94	C44E4.4	2117.33	7419.46	177.42
DMON2_CAEEL	2	121.8	Y73F4A.1	6439.86	8689.51	0
B6VQ85_CAEEL	6	114.84	C23H4.6	2.17E+05	2.38E+05	2.90E+04
SMD2_CAEEL	2	111.63	SNR-4	1007.89	1.41E+04	487.59
NH2L1_CAEEL	2	108.17	M28.5	0	1.86E+04	161.08
BYN1_CAEEL	2	107.96	BYN-1	5677.28	1743.01	106.13
Q23359_CAEEL	3	102.88	OMA-2	12.55	1.51E+04	830.35
Q20448_CAEEL	2	101.38	ZTF-7	829.13	9745.79	124.48
H2FLK6_CAEEL	4	101.05	LEA-1	289.87	1.09E+04	6933.22
G5EFM7_CAEEL	2	98.29	F18C12.3	3610.85	71.6	2584.59
ATPB_CAEEL	2	96.38	ATP-2	5403.34	4261.5	1041.76
Q21323_CAEEL	3	96.05	RNP-3	163.73	1.11E+04	1373
OLA1_CAEEL	2	92.99	TAG-210	946.79	3906.58	461.39
H9G340_CAEEL	2	92.38	MPST-3	7714.84	311.34	243.52
G5EDE4_CAEEL	2	84.82	W08E3.2	254.45	4813.82	62.44
Q0G828_CAEEL	2	81.4	F40F8.11	3836.36	4445.36	5.13
LTV1_CAEEL	3	81.15	T23D8.3	7582.97	1272.74	524.1
O61880_CAEEL	2	79	F59B1.2	7031.69	3.60E+04	2237.45
METK5_CAEEL	3	76.3	SAMS-5	9658.74	8660.12	1779.37
RUXF_CAEEL	2	74.9	SNR-5	0	1.20E+04	872.01
Q20878_CAEEL	5	74.68	F56D5.9	1.28E+05	9.83E+05	9.72E+04
D1MN85_CAEEL	2	73.25	IFE-1	0	5277.7	131.21
RSMB_CAEEL	2	68.76	SNR-2	591.14	1.58E+04	1248.53
MED12_CAEEL	3	67.18	DPY-22	9956.49	214.47	4643.34
M1ZK05_CAEEL	4	66.44	SEC-16	7713.54	6.52E+04	8831.31
MYO2_CAEEL	2	64.03	MYO-2	8590.38	2446.91	0
G5EBX1_CAEEL	4	60.18	UNC-53	2393.6	2.72E+05	1.65E+04
EIF3I_CAEEL	2	57.36	EIF-3.I	2198.92	0	695.81
Q94174_CAEEL	3	57.15	BICD-1	2.11E+04	3.82E+04	3070.6
Q8I4C5_CAEEL	2	54.15	FAAH-4	3.13E+05	2.48E+05	1873.46
O62102_CAEEL	2	53.34	PBS-2	3.86E+04	2.76E+04	679.6
G5ECY5_CAEEL	2	52.08	CSP-2	1.70E+04	1.26E+04	592.77
DPY27_CAEEL	3	51.58	DPY-27	4915.97	8606.28	1197.02

YKA3_CAEEL	3	50.1	B0303.3	2.00E+04	2.78E+04	600.33
H2KYV8_CAEEL	3	50.02	C18C4.5	8691.7	1.08E+06	1045.22

Supplementary Tables S2 and S3. Mass-spectrometry data

List of interacting proteins identified by mass spec following FLAG IPs performed on either FLAG-tagged CEY-1 or CEY-4. IPs were performed with and without RNAse treatment. Proteins were divided into “ribosomal proteins” and “others”. “peptides”: number of unique peptides used for the quantification; “score”: sum of the peptide ion scores from MASCOT; “summed peptide ion abundance”: relative protein quantification was done with the program Progenesis LC-MS. All peptide ion abundances for a protein were summed up and listed in the table.

Wormbase ID	Name	*cey-1,-4* / wild type (mRNA)	*cey-1,-4* / wild type (RPF)
WBGene00000386	cdc-25.1	0.78	0.43
WBGene00000465	cpg-1	0.87	0.35
WBGene00000866	cyb-2.1	1.12	0.33
WBGene00000867	cyb-2.2	1.24	0.32
WBGene00000870	cyd-1	1.06	0.48
WBGene00000913	daf-18	0.96	0.36
WBGene00001372	exo-3	0.95	0.5
WBGene00001569	gei-12	0.93	0.3
WBGene00001606	gln-5	0.96	0.37
WBGene00001647	gna-2	0.93	0.48
WBGene00003184	mei-2	1.06	0.43
WBGene00003229	mex-3	1.10	0.45
WBGene00003865	oma-2	0.76	0.32
WBGene00004027	pie-1	1.06	0.49
WBGene00004086	pph-4.2	1.01	0.44
WBGene00004217	ptr-2	0.97	0.48
WBGene00004239	puf-3	0.95	0.5
WBGene00004352	rgs-9	0.90	0.33
WBGene00004374	rme-2	0.84	0.49
WBGene00004819	skr-13	0.73	0.5
WBGene00004984	spn-4	1.01	0.25
WBGene00006619	try-1	0.79	0.45
WBGene00007643	C17E4.3	1.01	0.27
WBGene00008218	nasp-2	0.92	0.48
WBGene00008219	C50B6.3	1.20	0.46
WBGene00009035	F22B3.4	0.84	0.42
WBGene00010351	cbd-1	0.88	0.41
WBGene00010353	H02I12.5	0.98	0.29
WBGene00010492	meg-1	1.08	0.29
WBGene00010493	meg-2	1.01	0.37
WBGene00010621	K07A12.2	0.87	0.46
WBGene00010674	K08E7.6	1.03	0.03
WBGene00010939	M163.7	1.00	0.21
WBGene00011320	T01C3.3	0.95	0.42
WBGene00011352	rskn-1	0.92	0.44
WBGene00011501	rmd-1	0.80	0.41
WBGene00011986	T24D1.3	0.96	0.46
WBGene00012077	T27A8.5	1.07	0.25
WBGene00012220	W03C9.2	0.89	0.41
WBGene00012328	W07G1.1	1.13	0.17
WBGene00013380	Y62E10A.14	0.81	0.36
WBGene00013862	wdr-5.3	1.24	0.5
WBGene00014117	clec-91	0.87	0.49
WBGene00015083	egg-1	0.81	0.41
WBGene00015102	cpg-2	0.82	0.44

WBGene00016263	C30F12.4	1.03	0.45
WBGene00016440	C35D10.2	1.48	0.46
WBGene00016485	C36C9.1	0.71	0.37
WBGene00017548	F18A1.7	0.94	0.38
WBGene00017843	F26G5.1	0.76	0.47
WBGene00017986	F32D1.7	1.09	0.33
WBGene00019095	F59A7.8	1.09	0.39
WBGene00019606	clec-88	0.86	0.36
WBGene00019811	egg-2	0.91	0.47
WBGene00020035	egg-5	1.05	0.42
WBGene00020652	egg-4	1.01	0.42
WBGene00020910	W01A11.2	1.04	0.49
WBGene00021035	W05F2.3	0.79	0.42
WBGene00021056	W06B4.1	0.85	0.44
WBGene00021206	Y17G9B.9	0.90	0.38
WBGene00021891	Y54G2A.27	1.48	0.48

Supplementary Table S4. Transcripts down-regulated predominantly at the RPF level in the *cey-1,-4* mutant encode many oocyte-to-embryo transition regulators

Shown are the expression changes at the mRNA level and the level of translation (RPFs) for the subset of genes marked in magenta in Fig. S11D. Genes (*rme*-2, *oma-2*, *egg-1*) validated by qRT-PCR are highlighted in yellow.

4.2 RG/RGG repeats regulate the abundance of CEY proteins

4.2.1 Introduction

Post-translational modifications are present on many proteins and the methylation of histones or the phosphorylation of other proteins at specific amino acids are well known examples of how such covalent attachments can affect a protein's functionality. Similarly, the arginines found in RG/RGG repeats can be methylated by PRMTs, which is apparently the case for both CEY-1 and CEY-4 (see section 4.1.4). Furthermore, RG/RGG repeats, in general, have been shown to play important roles in PTGR (Thandapani et al., 2013). The fact that both CEY-2 and CEY-3 are devoid of RG/RGG repeats combined with the observation that CEY-2, when expressed from a *cey-1* promoter, is unable to restore large polysomes, prompted us to hypothesize that these motifs in CEY-1 and CEY-4 including their methylation status might be connected to the observed shift on polysome profiles in the *cey-1,-4* mutant.

4.2.2 Materials and Methods

See section 4.1.3.

4.2.3 Results and discussion

We previously could show that the methyltransferase PRMT-1 is responsible for the asymmetrical di-methylation of RG/RGG repeats in both CEY-1 and CEY-4 (see section 4.1.4). To find out if these methyl marks are connected to the loss of large polysomes in the absence of CEY-1 and -4, we compared polysome profiles of wild-type and *cey-1,-4* mutant animals with the one in the *prmt-1* mutant background (Figure 9A). The results did not support this hypothesis. However, it should be noted that PRMT-1 has several targets, among which is also the stress response gene *daf-16* (Takahashi et al., 2011). Furthermore, the *prmt-1* mutant takes more than ten hours longer than wild type to reach adulthood at 20°C. This suggests a negative effect on global translation, which might cover up potential similarities to the polysome profile of *cey-1,-4* mutants. Therefore, to look at a potential function of these repeats in a more specific way, we obtained a transgenic line expressing a

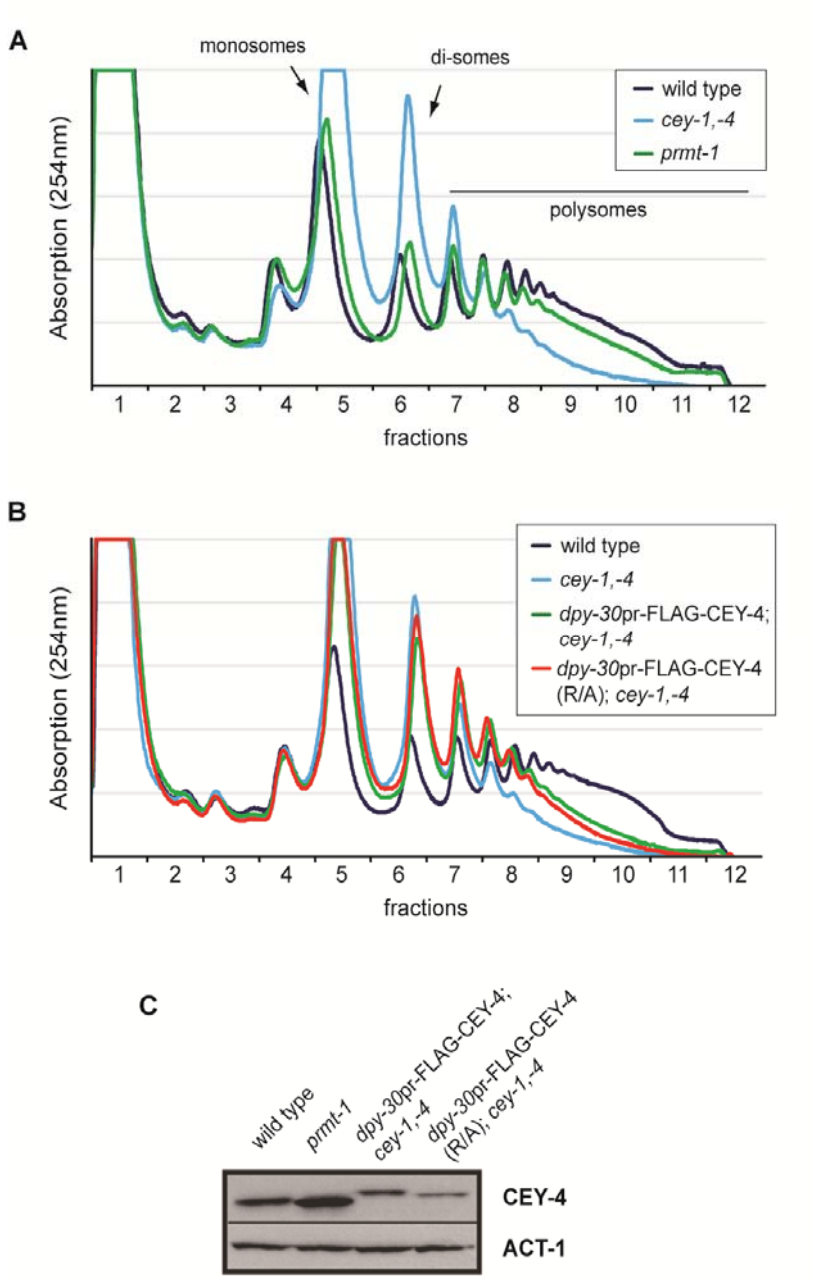


Figure 9. RG/RGG repeats affect the protein abundance

(A) The polysome profile from *prmt-1* mutants was much more similar, albeit not identical, to the one from wild-type animals compared to the strong shift observed in *cey-1,-4* mutants. Indicated are the positions of mono-, di-, and polysomes. (B) Mutating the RG/RGG repeats to AG/AGG did not abolish the partial rescue of the polysome profile shift observed for the wild-type version of the CEY-4 transgene. (C) The amount of CEY-4 protein increased in the *prmt-1* mutant, while mutating the RG/RGG repeats to AG/AGG had a negative effect on CEY-4 transgene levels.

version of CEY-4, in which all RG/RGG repeats, apart from those present in the CSD, had been mutated to AG/AGG. The GFP signal coming from the operon construct was the same for the wild-type and mutant CEY-4 transgenes, indicating very similar expression levels.

When crossed into the *cey-1,-4* mutant background, the mutant version of CEY-4 was able to rescue the profile shift almost to the extent the wild type CEY-4 transgene could (Figure 9B). Therefore, it appears that the RG/RGG repeats, at least in the case of CEY-4, are not essential for the assembly of large polysomes in the soma. We had previously found that CEY-4 protein levels increase upon preventing asymmetric di-methylation in the *prmt-1* mutant (see section 4.1.4). Interestingly, mutating the RG/RGG repeats had the opposite effect (Figure 9C), suggesting that these motifs influence protein stability/abundance. One hypothesis here is that, similar to other YBPs, basic amino acids found outside the CSD might participate in mRNA binding (Matsumoto et al., 1996). Therefore, modifying or mutating these RG/RGG repeats might positively or negatively affect the affinity for mRNA and the strength of RNA binding might correlate with the stability of CEY-4.

5. GENERAL DISCUSSION AND OUTLOOK

5.1 Functions of CEY proteins in the germline

The apparent general association of CEY proteins with mRNA combined with the significant reduction of maternal mRNA levels and the sterile phenotype observed in the *cey* quadruple mutant support a conserved and essential role for YBPs in maternal mRNA regulation in *C. elegans*, whereby the two germline-specific CEY-2 and -3 play a predominant role. Consistently, their expression pattern strongly resembles the one of maternal messages, showing a strong decrease during early stages of embryogenesis similar to what has been reported for FRGY2 and MSY2 (Wolffe et al., 1992; Yu et al., 2001). I propose that CEYs associate with maternal messages in a predominantly unspecific manner and, as proposed for YB-1 at high concentrations (Skabkin et al., 2004), play a central role to densely pack and maintain mRNPs, thereby stabilizing the bound mRNA (Figure 10A and B)). This would fit well to the general idea of YBPs functioning as "RNA histones" (Sommerville, 1999). Specificity is most likely provided by other maternal mRNP components, such as GLD-1 for example. Based on my model, the large aberrant granules that appear in the absence of CEY proteins form due to the accumulation and aggregation of faulty mRNPs. This hypothesis might be further supported by the observation that CEY-2 tagged with GFP shows an even distribution in a wild-type background, but localizes to abnormal granules when the endogenous CEY-2 and -3 are removed (Appendix 1). Even though highly speculative, this might be due to an increase in the amount of mRNA-bound GPF-CEY-2, whereby the enhanced number of GFP molecules starts to sterically interfere with the compact packaging of the underlying mRNA. If this interpretation is correct, this data might also suggest that indeed multiple CEY molecules bind to a single mRNA molecule simultaneously (Figure 10).

In addition to protecting maternal mRNA from degradation, YBPs, such as FRGY2 in *Xenopus* oocytes, play an important role to translationally repress bound messages (Matsumoto et al., 1996). A global analysis of ribosome association using ribosome profiling either on the *cey* quadruple mutant or the *cey-2,-3* double mutant compared to wild type should clarify if CEYs also affect the translation status of maternal mRNAs.

Besides the importance for proper formation and maintenance of mRNPs, maternal mRNAs also need to be reactivated in a temporally and spatially correct manner. An interesting question to be addressed in the future is if and how CEYs might be involved in translational reactivation. Despite the fact that most CEY-2 is found in submonosomal fractions, some of it is also present on polysomes (Appendix 2). Therefore, CEY proteins

might remain bound to and protect an mRNA until being stripped of the message by a ribosome during the first round of translation (Figure 10C). CEYs might also enhance the efficiency of maternal mRNA reactivation by preventing unspecific binding of translation initiation factors along the message, as has been proposed for YB-1 (Svitkin et al., 1996) (Figure 10C). What role might protein modifications play? The phosphorylation of FRGY2, for instance, has been suggested to modulate the binding to RNA (Kick et al., 1987; Murray et al., 1991). Indeed, in addition to the methylation of RG/RGG repeats, our MS data identified phosphorylation sites for both CEY-1 and CEY-4 (data not shown) and it would be interesting

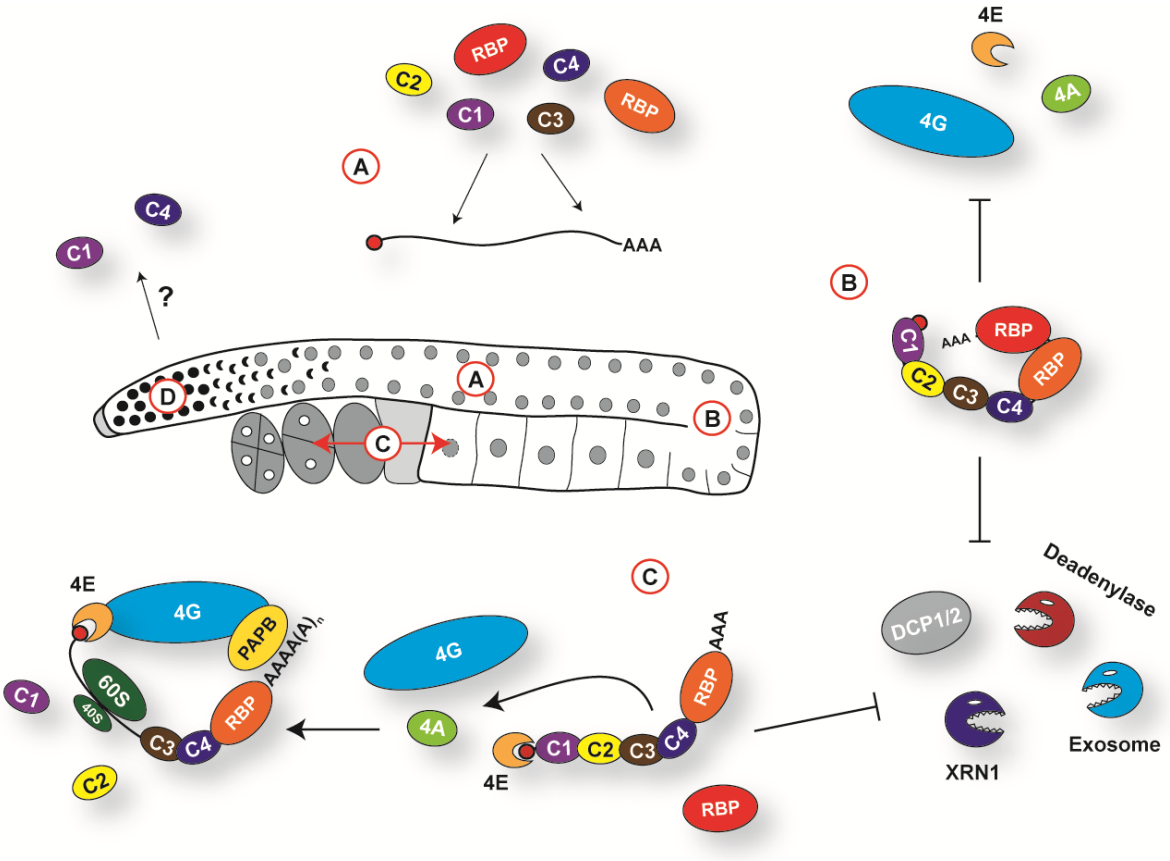


Figure 10. Model on how CEYs might participate in maternal mRNA regulation

(A) CEYs and additional RBPs, such as GLD-1 or CGH-1, bind to maternal mRNAs produced upon meiotic entry of germ cells. (B) The densely packed mRNA is protected from the decay and translation machinery while it moves proximally. (C) Many maternal messages are released from their translational repression during the oocyte-to-embryo transition. CEYs might promote translation by preventing unspecific binding of initiation factors along the message and by blocking the access of decay factors. CEYs are then stripped of the mRNA by the first elongating ribosome. (D) Only CEY-1 and CEY-4 are expressed in self-renewing germline stem cells. Their functions in this gonadal region are unknown.

to find out if such sites also exist in the two germline-specific proteins. Finally, considering the importance of poly(A) tail regulation, especially in *Xenopus* oocytes (Richter and Lasko, 2011), one could also compare the length of poly(A) tails of some candidate maternal mRNAs in the *cey* mutant with those in wild type to figure out if this might be an issue in the absence of CEY proteins.

What might the role of CEY-1 and CEY-4 in the very distal stem cell niche be (Figure 10D)? These are mitotic cells and it is possible that CEY-1 and CEY-4 have similar functions in these cells as they have in the soma, where I could not make out any deleterious phenotypic abnormalities in their absence (see below).

5.2 Functions of CEY proteins in the soma

Besides its sterile phenotype, we found another apparent abnormality in the *cey* quadruple mutant, namely, a significant loss of larger polysomes and a simultaneous increase in mono-, di-, and trisomes. This effect turned out to be specifically due to the absence of CEY-1 and CEY-4. Curiously, the inability to assemble large polysomes has no negative effect on the overall translation rate in the *cey-1,-4* mutant. Therefore, our data strongly questions the importance of accumulating multiple ribosomes on mRNAs and it would be interesting to know what consequences the loss of larger polysomes has in other model organisms. The observed profile shift also appears to be specific to the soma. Therefore, larger polysomes are found predominantly in this tissue, which suggests that, at least in part, translation is regulated by different means in the germline versus the soma and that translation in the latter becomes “germline-like” in the absence of CEY-1 and CEY-4. How might these two proteins regulate the accumulation of polysomes in the soma? The pleiotropic functionality of YB-1 is thought to derive predominantly from its intrinsic disorder (Lyabin et al., 2014). Considering the degree of disorder found in both CEY-1 and CEY-4, mainly due to their RG/RGG repeats, the strong shift on polysome profiles, might be the final readout of various alterations affecting mRNA translation and/or degradation. Furthermore, interrelations and feedback mechanisms in between translation and degradation make it difficult to distinguish direct from indirect effects (Huch and Nissan, 2014). Indeed, highly translated mRNAs apparently have a higher chance to be destabilized in *cey-1,-4* mutants. We previously speculated that a reduction in the half-life/stability of such messages could prevent the accumulation of a higher number of ribosomes. While CEY-4 is present in submonosomal and polysomal fractions and thereby might contribute to the stability of mRNAs also during active translation, CEY-1 is found almost exclusively in submonosomal

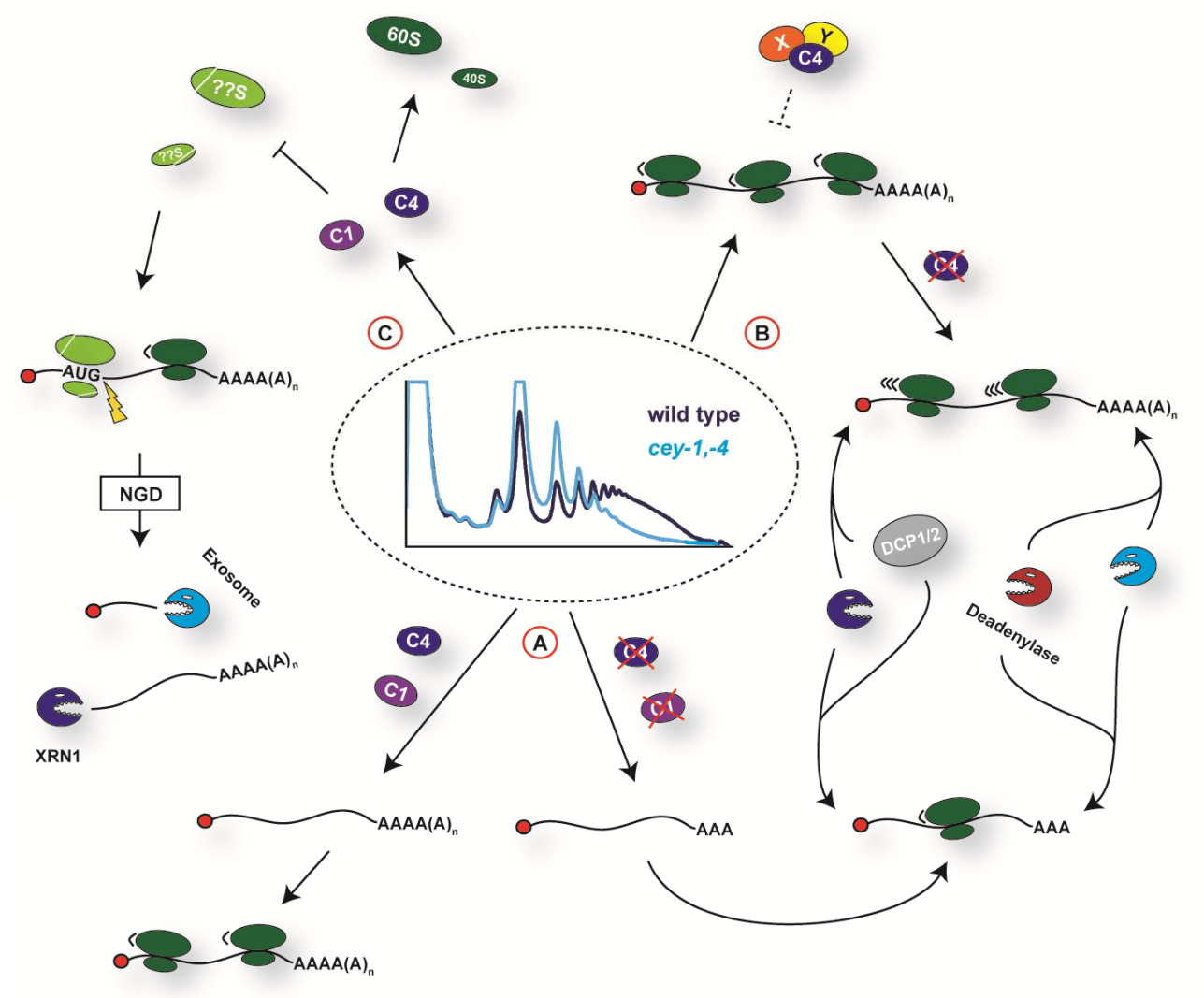


Figure 11. Model on how CEY-1 and CEY-4 could promote polysome assembly in the soma. (A) CEY-1 and CEY-4 promote correct poly(A) tail length prior to translation to guarantee normal mRNA half-lives. This allows multiple rounds of translation and the accumulation of larger polysomes. In the absence of CEY-1 and CEY-4 mRNAs with shorter poly(A) tails enter translation, which allows for only a few ribosomes to initiate translation before the mRNA is targeted for degradation. This prevents the build up of larger polysomes. (B) CEY-4, as part of a soma-specific complex, actively slows down elongating ribosomes, thereby causing the accumulation of larger polysomes. In its absence, the speed of translation elongation is enhanced, which reduces the number of ribosomes present on mRNAs. Furthermore, as a result of the enhanced elongation speed mRNAs might also be turned over more rapidly. (C) CEY-1 and CEY-4 prevent the formation of aberrant ribosomes. In their absence, an increased occurrence of faulty ribosomes enters the translation pool and arrests at the start codon, thereby triggering NGD and subsequent mRNA decay.

fractions, suggesting that it can only directly affect the properties of an mRNA prior to translation. One characteristic of almost every mRNA molecule with a strong influence on its "lifespan" is the poly(A) tail, the shortening of which in many cases represents the first step in mRNA degradation. One way, therefore, in which CEYs might influence the half-life of a message also prior to translation is by regulating its poly(A) tail length, a fact that might be especially important for highly translated mRNAs (Figure 11A). This could be tested by comparing the poly(A) tails of some candidate genes in the *cey-1,-4* mutant and wild type. Increased half-lives of mRNAs in the soma compared to those in the germline might be one reason for the soma-specific accumulation of larger polysomes. In addition, it is also possible that CEY-1 and CEY-4 promote translation initiation rates and thereby drive the accumulation of larger polysomes. Again, in the case of CEY-1, this could only include the first round of translation due to its absence from polysomes.

Despite these above mentioned intruiging hypotheses, the fact that the protein synthesis rate does not drop in *cey-1,-4* mutants, had us conclude that the profile shift must, at least in part, include an alteration that does not negatively and may even positively affect the translational output. The only hypothesis here that fits to the shift observed on the polysome profile is an increase in the speed of translocating ribosomes. Actually, a more pronounced regulation at the translation elongation step in the soma might be another explanation as to why larger polysomes accumulate there but not in the germline, where translation is predominantly regulated at the initiation stage in the form of stored mRNPs. We speculated that due to a potential drop in the number of mature ribosomes (see below), the ratio of elongation factors per ribosome might increase in the mutant, thereby passively enhancing the efficiency of elongation. However, considering the presence of CEY-4 on polysomes, one could also propose a more direct function. This protein, potentially as part of a soma-specific complex, might be important to slow down elongating ribosomes, thereby playing a central role in controlling the translational output (Figure 11B). Consistently, the SUnSET results suggest that the protein synthesis rate is even higher in the *cey-1,-4* mutant compared to wild type, which might indeed be due to enhanced elongation rates. Furthermore, the finding that slowing down translation at the elongation step can stabilize mRNAs (Beelman et al., 1994), might suggest, vice versa, that the mRNA stability issue in the *cey-1,-4* mutant is a direct consequence of enhanced ribosome translocation speed. The fact that only CEY-4 is present on polysomes, means that SUnSET and qRT-PCR experiments (to look at the abundance of several highly translated transcripts) will have to be performed in the *cey-4* single mutant background to reveal if the above mentioned hypothesis holds true or not. An encouraging fact is that the polysome profile shift observed in the *cey-4* single mutant does resemble the one in *cey-1,-4* mutant animals, albeit being less severe. Unfortunately, as mentioned previously, a methodology to measure translation

elongation rates in *C. elegans* is still missing and *C. elegans* cell culture has not yet been established.

The potential direct link to ribosome biology adds significantly to the complexity regarding the functions of CEY-1 and CEY-4. We cannot rule out that our MS results, which suggest a predominantly RNA-independent interaction of both proteins with numerous ribosomal factors, might be artefacts due to the high abundance of both CEY proteins and ribosomal constituents. However, the abnormal ribosomal peaks observed in a soma-only background suggest that there might indeed be some ribosome-related function of CEY-1 and CEY-4. I can only speculate as to what these peaks might be, but based on their location to the lighter side of genuine ribosomal peaks, they appear to represent “smaller” variants of the ribosome. Are these defective or different versions of the ribosome? The fact that CEYs are not present in the nucleus suggests that they could only function during cytoplasmic maturation/quality control processes and that in their absence the overall number of mature ribosomes decreases and/or the chance for non-functional/aberrant ribosomes entering the translation pool increases. Such ribosomes would then most likely fail to enter the elongation step, thereby activating the NGD pathway, which ultimately results in an endonucleolytic cleavage of the underlying message (Figure 11C). This could be another reason for the reduced mRNA half-lives, especially of those that accumulate many ribosomes, thereby contributing to the apparent drop in the abundance of highly translated transcripts. Our ribosome profiling data did not reveal any apparent increase of RPFs at the start codon in *cey-1,-4* mutant animals compared to wild type, a characteristic that would be expected due to prolonged occupation of aberrant ribosomes at the translation start site. However, such ribosomes might not be stable enough during the RNAse digestion step of ribosome profiling and therefore might not yield any RPFs. Another possibility is that these additional peaks represent an alternate, but functional version of the ribosome. The growing amount of literature on the heterogeneity of ribosomal proteins and ribosomes and its importance for the development of specific tissues or as a response to changes in the environment, makes this hypothesis not so unlikely (Xue and Barna, 2012). The idea here would be that CEYs usually suppress the synthesis of this variant, which is only produced under certain conditions. Either way, this direct interaction of CEY-1 and CEY-4 with ribosomal proteins will first have to be accurately validated by co-IP experiments performed in the absence of RNA, before addressing any further questions.

Despite the fact that the strong shift is only observed when both *cey-1* and *cey-4* are deleted simultaneously, the polysome profiles of the two single mutants do not match. In addition, our MS data revealed that while CEY-1 IPs readily pulled down CEY-4 (RNA-dependent interaction), the vice versa was not as apparent, suggesting that CEY-4 is also frequently found in mRNPs, possibly polysomes, in the absence of CEY-1. Even though

these discrepancies might derive from the fact that CEY-4 has additional functions on polysomes, it is also possible that the functions of the two proteins in submonosomal fractions are actually not fully redundant. Therefore, one might want to consider studying CEY-1 and CEY-4 individually. In any case, the primary task for the future will be to find conditions, in which these mutant animals show an apparent, if possible drastic somatic phenotype. If this could then also be linked to the loss of polysomes, we would know what the accumulation of multiple ribosomes on mRNAs is good for. As suggested previously, it is possible that a multitude of existing slight defects only have a negative impact under non-laboratory conditions. The potential misregulations in ribosome biogenesis and translation in the *cey-1,-4* mutant, two major energy consuming processes in the cell, might weigh strongly in nature, especially under scarce food conditions, giving these mutant animals a major disadvantage compared to wild type.

6. ACKNOWLEDGEMENTS

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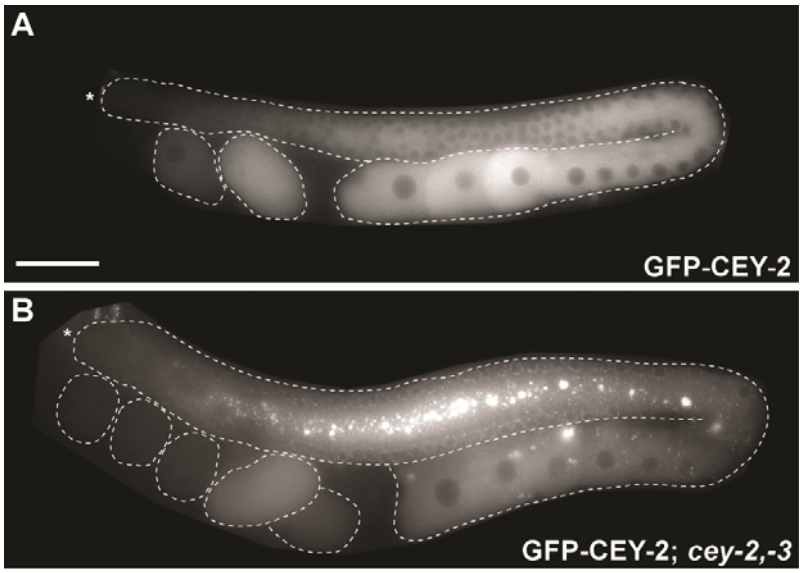
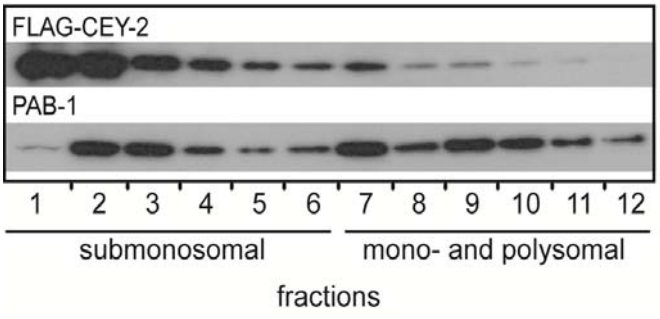
8. APPENDICES



Appendix 1. GFP-CEY-2 localizes to aberrant granules in the *cey-2,-3* mutant

Fluorescent micrographs from live animals expressing an N-terminally GFP-tagged CEY-2 transgene from the *cey-2* promoter. The gonads and embryos are outlined by dotted lines. Asterisks indicate the distal ends of the gonads. In a wild-type background the GFP signal is evenly distributed in the gonadal cytoplasm. In the *cey-2,-3* double mutant GFP-CEY-2 localizes to large aberrant granules.



Appendix 2. CEY-2 protein is also present in polysomal fractions

Proteins were extracted from each of the 12 fractions from a polysome profiling experiment and analyzed by western blot. The majority of CEY-2 (FLAG-tagged) was found in sub-monosomal fractions, however, some protein was also present in ribosomal (mono- and polysomal) fractions. PAB-1 was, as expected, present in both submonosomal and ribosomal fractions and served as a control.