The role of poly(A) modifying enzymes as mRNA regulators in germ cells

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Summary

RNA modifying enzymes are an important part of the gene expression cascade in eukaryotic cells. Especially, enzymes that directly modify mRNAs turn out to be potent post-transcriptional regulators. A prominent mRNA feature that is often targeted in this context is the homopolymeric adenosine stretch at its 3', also known as the mRNA poly(A) tail. The length of this structure is quite dynamic and can determine the fate of an mRNA. Short-tailed mRNAs are envisioned to be unstable and inefficiently translated, whereas long-tailed mRNAs are stable and efficiently translated. In the cytoplasm, the length of the tail is modified by two evolutionary conserved protein families known as deadenylases (DeAds), which shorten the tail, and cytoplasmic poly(A) polymerases (cytoPAP), which elongate the tail. Although, DeAds and cytoPAPs are important enzymes in RNA biology their biological roles in multi-cellular organisms were only scarcely characterized. In the work presented here, *Caenorhabditis elegans* is used as a model to investigate the roles of deadenylases and cytoplasmic poly(A) polymerases in regulating gene expression programs of developing germ cells. By combining classical genetic with biochemical and genomics approaches, I identified the cytoPAP GLD-2 and CCF-1, a DeAd of the Ccr4-Not complex as the key enzymes that are utilized for mRNA regulation in germ cells. My combined data suggests that these two factors control the level of mRNAs by modulating the length of poly(A) tails. The opposing activities of GLD-2 and CCF-1 are employed by various gene-specific RNA-binding proteins to control mRNA abundance and translation of their targets. In conclusion, this work establishes the enzymatic framework that is used for poly(A)-mediated gene expression control in germ cells and provides valuable insights into how RNA-modifying enzymes are utilized in development to control specific gene expression programs.

Zusammenfassung

RNA-modifizierende Enzyme sind ein wichtiger Teil der Genexpressionskaskade in eukaryotischen Zellen. Insbesondere Enzyme die direkt mRNAs modifizieren, haben sich als potente post-transkriptionale Regulatoren herausgestellt. Eine prominente mRNA-Struktur, auf welche in diesem Zusammenhang oft abgezielt wird, ist der homopolymerische Adenosinabschnitt am 3‘ Ende, auch bekannt als mRNA poly(A) Schwanz. Die Länge dieser Struktur ist dynamisch und kann das Schicksal einer mRNA beeinflussen. mRNAs mit kurzen Schwänzen sind instabil und werden ineffizient translatiert. Im Gegensatz dazu, sind mRNAs mit langen Schwänzen stabiler und werden effizienter translatiert. Im Zytoplasma wird die Länge eines poly(A) Schwanzes von zwei evolutionär konservierten Proteinfamilien verändert. Deadenylasen (DeAds) verkürzen und zytoplasmatische poly(A) Polymerasen (cytoPAP) verlängern diese Struktur. Obwohl DeAds und cyoPAP wichtige Enzyme in der RNA-Biologie sind, wurden deren biologische Rollen bisher nur unzureichend untersucht. In der hier präsentierten Arbeit wird der Modellorganismus *Caenorhabditis elegans* benutzt, um die Bedeutung von Deadenylasen und cytoplasmatischen poly(A) Polymerasen für die Regulierung von Genexpressionsprogrammen in sich entwickelnden Keimzellen zu studieren. Durch die Kombinierung von klassisch genetischen mit biochemischen und genomischen Herangehensweisen konnte ich die cytoPAP GLD-2 sowie CCF-1, eine DeAd aus dem Ccr4-Not-Komplex, als Schlüsselenzyme für die Regulierung von mRNAs in Keimzellen identifizieren. Meine gesammelten Daten sprechen dafür, dass diese Faktoren mRNA-Mengen kontrollieren, indem sie die Länge derer poly(A) Schwänze verändern. Dabei werden die entgegengesetzten Aktivitäten von GLD-2 und CCF-1 von spezifischen RNA-bindenden Proteinen benutzt, um gezielt die Menge sowie die Translationseffizienz von bestimmten mRNAs zu kontrollieren. Zusammenfassend stellt meine Arbeit die Bedeutung des enzymatischen Systems, welches für die poly(A)-gestützte Regulierung von Genexpressionsprogrammen in Keimzellen genutzt wird, heraus und zeigt, wie RNA-modifizierende Enzyme gezielt in der Entwicklungsbiologie eingesetzt werden können.

1. Introduction

1.1. Post-transcriptional gene expression control

The basis for all developmental processes is the ability of cells to control the spatial and temporal production of proteins. An organism needs to produce the right set of proteins at the right time in development. In general, gene expression can be controlled on the transcriptional or post-transcriptional level. Transcriptional regulation takes place in the nucleus and controls the production of mRNAs by regulating the access of RNA polymerase II to genes. Post-transcriptional regulation is much broader and can affect many different processes in a cell. This type of regulation can affect mRNA processing, splicing and export in the nucleus as well as mRNA degradation, storage and translation in the cytoplasm. The cytoplasmic events are often commonly referred to as translational regulation. Although, transcriptional and post-transcriptional regulation can be utilized in most cells some cell types such as germ cells are dominated by post-transcriptional mechanisms. Especially, translational regulation is an important aspect of germ cell development. Hence, germ cells provide valuable insides into how complex gene expression programs can be controlled primarily in the cytoplasm.

1.2. RNA-binding proteins

RNA-binding proteins (RBPs) occupy a central position in cytoplasmic regulation of gene expression. They are employed to regulate mRNAs on the global as well as on a gene-specific level. This is achieved by the ability of an RBP either to bind to a general or specific structural component of an mRNA. The typical mRNA consists of a cap structure on the 5’ end, the 5’ untranslated region (UTR), the open reading frame (ORF), the 3’ UTR, and the homopolymeric A-stretch at the 3’ end called the poly(A) tail. Proteins that bind either the cap or the poly(A) tail are considered global RNA regulators as every mRNA contains these structures (KONG AND LASKO 2012). Examples are the cap-binding protein eIF4E or the cytoplasmic poly(A) binding protein PABPC. On the cellular level, global regulators are important for stress responses (ROUX AND TOPISIROVIC 2018). For example, eIF4E activity is directly targeted

under stress conditions to regulate global protein synthesis (MUSA *et al.* 2016). RBPs that regulate just a subset of mRNAs bind to specific sequence motives which are usually present in the UTR regions. Many of these factors belong to evolutionary conserved protein families. Prominent examples that control cytoplasmic mRNAs are members of the PUF protein family (PUmilio and FBF), STAR protein family (Signal Transduction and Activation of Ribonucleic acid), Tis11 protein family or TRIM-NHL protein family (TRIpartite Motif - NCL-1/HT2A/LIN-41) (VERNET AND ARTZT 1997; BAOU *et al.* 2009; CONNACHER AND GOLDSTROHM 2020; NISHANTH AND SIMON 2020). Specific regulators fulfill important functions during the development of tissues by controlling the protein production of key developmental factors.

1.3. Translational regulation of mRNAs

Protein synthesis, also known as translation, is often the process that is directly or indirectly targeted to regulate gene expression in the cytoplasm. Translation itself is a multistep process that can be divided into three phases: initiation, elongation and termination. During the initiation phase, the two ribosomal subunits are recruited to the mRNA. During elongation, the assembled ribosome moves along the mRNA and a polypeptide chain is produced. During termination, the ribosome dissociates from the mRNA and is disassembled into its subunits. Theoretically, translational control can be exerted in any of the three phases, however the vast majority of regulatory mechanisms that have been described so far target the initiation phase (GEBAUER AND HENTZE 2004).

Translation initiation is facilitated by the canonical mRNA structures; the cap and the tail, as well as multiple proteins. More than 20 proteins called eukaryotic translation initiation factors (eIFs) are involved in mRNA recognition and ribosomal subunit recruitment (MERRICK AND PAVITT 2018). Of pivotal importance during early stages of initiation is the eIF4 complex. This multi-subunit complex consists of the scaffolding protein eIF4G, which in turn serves as a central binding hub, the cap-binding protein eIF4E, the RNA helicase eIF4A and the ribosomal interacting complex eIF3 (LAMPHEAR *et al.* 1995; MADER *et al.* 1995). Additionally, eIF4G

contains binding domains for the cytoplasmatic poly(A) binding protein (PABPC) (TARUN AND SACHS 1996). It is envisioned that eIF4G bound to eIF4E and PABPC on an mRNA leads to the formation of a closed loop conformation bringing the 5' and 3' end of an mRNA in close proximity, which in turn stimulates translation initiation (Fig.1). In general, the activity of many specific RNA-binding proteins has been proposed to interfere with the formation of the close-loop conformation of a target mRNA (GEBAUER AND HENTZE 2004).

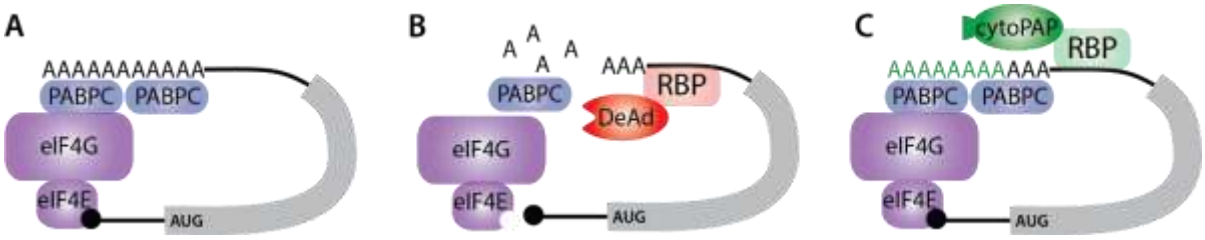

Figure 1: *Model of mRNA closed loop formation and how this process can regulate translation. (**A**) During canonical translation initiation the cap and tail structures of an mRNA are in close proximity through the interactions of eukaryotic translation initiation factors (eIF) with the poly(A) binding protein (PABPC). (**B**) Deadenylases (DeAds) shorten the poly(A) tail dispel PABPC from the mRNA and disrupt the closed loop formation. (**C**) Cytoplasmic poly(A) polymerases elongate shortened poly(A) tails reestablishing PABPC interactions and stabilizing the closed loop formation again.*

1.4. Poly(A) mediated gene expression control

Contrary to the rigid nature of the cap structure, the poly(A) tail is the most dynamic structure of an mRNA. Nuclear polyadenylation is a co-transcriptional default process that liberates the RNA from its site of transcription and assists mRNA export (SACHS AND WAHLE 1993). In the cytoplasm, the homopolymeric A-tail is removed as part of the natural mRNA decay pathway (DECKER AND PARKER 1993). However, the poly(A) tail is also a platform for regulatory translational control mechanisms that exploit its two cytoplasmic functions, enhancing mRNA stability and translatability (MATHEWS *et al.* 2007). In particular, the length of the poly(A) tail is an indicator of the mRNA's fate. A short tail makes an mRNA less attractive for translation initiation and renders it rather unstable, whereas a long tail stimulates translation initiation and stabilizes an mRNA (MUNROE AND JACOBSON 1990; DECKER AND PARKER 1993). It is commonly believed that changes in tail length directly influence ribosome recruitment by

changing the efficiency of the mRNA loop formation mediated by the eIF4 complex. Interestingly, many developmentally important RNA-binding proteins that regulate specific subsets of mRNAs have been proposed to recruit enzymes that actively change the length of the tail (GOLDSTROHM AND WICKENS 2008).

1.5. Pol(A) modifying enzymes

1.5.1. Deadenylases

Enzymes that shorten the tail of mRNAs are called deadenylases (DeAds). They represent a conserved class of exonucleases that specifically hydrolyze homopolymeric A-stretches (GOLDSTROHM AND WICKENS 2008). In terms of gene expression control DeAds have a dual role in mRNA regulation. On one hand these enzymes are regulators of global gene expression as deadenylation is part of the canonical cytoplasmic mRNA decay pathway (DECKER AND PARKER 1993). On the other hand, DeAds can be utilized to control specific subsets of mRNA by being recruited to a target via an RNA-binding protein (GOLDSTROHM AND WICKENS 2008).

The most studied deadenylases are Ccr4 (carbon catabolite repressor 4), Caf1 (Ccr4p-associated factor 1), Pan2 (PolyA nuclease 2) and PARN. All are evolutionary conserved from yeast to humans, only PARN might not be present in every organism, for example in budding yeast and flies no gene could be identified that encodes this particular DeAd (BOECK *et al.* 1996; KORNER *et al.* 1998; DAUGERON *et al.* 2001; TUCKER *et al.* 2002; GOLDSTROHM AND WICKENS 2008). In general, the strong evolutionary conservation of the different DeAds is an indicator for the crucial roles these enzymes play in RNA regulation.

Ccr4 and Caf1 are part of the multi-subunit Ccr4-Not complex. The two DeAds together with the large scaffolding protein Not1 form the central module of the complex to which a number of auxiliary factors known Not proteins can bind to (WAHLE AND WINKLER 2013). Although the components of the central module are broadly conserved, the number of additional Not proteins was increased during evolution from six in yeast to seven in mammals (BAI *et al.*

1999; BAWANKAR *et al.* 2013). Interestingly, the enzymatic balance between Ccr4 and Caf1 changed during evolution, with Ccr4 being the dominant enzyme in yeast and Caf1 in *Drosophila* (TUCKER *et al.* 2002; TEMME *et al.* 2004). In general, loss of Ccr4-Not complex components leads to a strong accumulation of polyadenylated RNAs in yeast, fly and mammalian cells (TUCKER *et al.* 2001; TEMME *et al.* 2004; YAMASHITA *et al.* 2005).

The Pan2-Pan3 complex consists of the single catalytic subunit Pan2 and its co-factor Pan3 (BOECK *et al.* 1996; BROWN *et al.* 1996). Despite the strong evolutionary conservation of this complex, loss of Pan2-Pan3 function has little effect on cytoplasmic deadenylation in yeast and fly (TUCKER *et al.* 2001; BÖNISCH *et al.* 2007). Intriguingly, PARN has a number of properties that distinguish it from the Ccr4-Not and Pan2-Pan3 complex. First, PARN acts as homodimer and binds the cap structure (DEHLIN *et al.* 2000). Second, while the two other DeAd complexes are primarily cytoplasmic, PARN can be found mainly in the nucleus (YAMASHITA *et al.* 2005; BERNDT *et al.* 2012).

In multicellular organisms Ccr4, Caf1, Pan2 and PARN are considered to be widely expressed. However, in this regard just Ccr4 and Caf1 have been analyzed in detail. Northern blot analyses in mice showed that Caf1 mRNAs are expressed in all tissues (ALBERT *et al.* 2000). In *Drosophila*, CCR4 and CAF1 proteins are detected at all developmental stages (TEMME *et al.* 2004). A similar observation was made in *C. elegans*. Here, the mRNA for the Caf1 homolog, CCF-1 is expressed during all developmental stages (MOLIN AND PUISIEUX 2005). For Pan2 and PARN only data from tissue culture systems or specific cell types provide indications into their expression pattern. Pan2 protein is detected in mouse fibroblast, monkey kidney as well as human kidney, bone and epithelia cells (YAMASHITA *et al.* 2005; ZHENG *et al.* 2008; FABIAN *et al.* 2011; BETT *et al.* 2013). PARN protein is expressed in *Xenopus* oocytes and retina cells, mouse brain tissue and myoblasts, human epithelia, blood and gastric cells (COPELAND AND WORMINGTON 2001; BAGGS AND GREEN 2003; MORAES *et al.* 2006; LEE *et al.* 2012; MARAGOZIDIS *et al.* 2012; UDAGAWA *et al.* 2012; ZHANG AND YAN 2015). Collectively, this argues that all four deadenylases, Ccr4, Caf1, Pan2 and PARN, are most likely expressed

and active in every cell of an organism. Overall, the functionality of all four enzymes has been primarily studied in a single cell context and little is known about the roles of Ccr4, Caf1, Pan2 and PARN in development.

1.5.2. Cytoplasmic poly(A) polymerases

Enzymes that elongate poly(A) tails of mRNAs in the cytoplasm are called cytoplasmic poly(A) polymerases (cytoPAPs). Contrary to nuclear PAP, these proteins belong to the group of non-canonical poly(A) polymerases that do not contain any domains that facilitate direct interactions with RNAs. Two cytoPAPs have been described so far, GLD-2 and GLD-4 (WANG *et al.* 2002; SCHMID *et al.* 2009; BURNS *et al.* 2011). Both enzymes belong to two distinct, evolutionary conserved protein families that contain no sequence homology outside their catalytic regions (MINASAKI AND ECKMANN 2012). GLD-2 proteins so far have only been identified in nematodes, flies, frogs and mammals but not yeast (WANG *et al.* 2002; KWAK *et al.* 2004; ROUHANA *et al.* 2005; BENOIT *et al.* 2008; CUI *et al.* 2008). GLD-4 belongs to the TRF4 protein family which is conserved from yeast to mammals (MINASAKI AND ECKMANN 2012). In yeast, flies and mammalian cells TRF4 homologous proteins represent the catalytic subunit of a nuclear RNA surveillance complex (SCHMIDT AND BUTLER 2013). However, the worm TRF4 ortholog GLD-4 as well as the mammalian TRF4 paralog hGLD4/PAPD5 localize predominantly to the cytoplasm (SCHMID *et al.* 2009; BURNS *et al.* 2011). This suggests that cytoPAPs might have evolved specifically in multi-cellular organisms to fulfill the demands that complex biological systems have towards RNA regulatory mechanisms.

In general, cytoPAPs by themselves seem to have weak polyadenylation activity. The best understood and most studied enzymes of this class are GLD-2 and GLD-4 from nematodes. Recombinant GLD-2 promotes poly(A) addition to a substrate only weakly, and substrate polyadenylation can be stimulated by interacting proteins such as GLD-3 or RNP-8 (WANG *et al.* 2002; KIM *et al.* 2009). Crystal structure analyses revealed that GLD-3 or RNP-8 interactions stabilize the fold of the GLD-2 catalytic domain, thereby stimulating the polyadenylation activity of the enzyme (NAKEL *et al.* 2015; NAKEL *et al.* 2016). Similar

observations were made for GLD-4, as tethering assays in Xenopus oocytes have shown that GLD-4 activity is stimulated by interactions with its co-factor GLS-1 (SCHMID *et al.* 2009). This suggests that the activity of cytoPAPs is tightly controlled via its interacting partners and that these enzymes most likely can only gain their full enzymatic potential as part of larger RNP complexes.

In development, cytoPAPs are particularly important in germ cells. Loss of GLD-2-type proteins in worms and flies leads to severe defects during spermatogenesis as well as oogenesis (KADYK AND KIMBLE 1998; CUI *et al.* 2008; SARTAIN *et al.* 2011). GLD-4 has only been characterized in worms, where the loss of the protein results in a reduction in fertility and smaller brood sizes (SCHMID *et al.* 2009). Interestingly, nematode GLD-2 and GLD-4 in germ cells counteract each other during entry into meiosis and work synergistically during the progression of meiosis (SCHMID *et al.* 2009; MILLONIGG *et al.* 2014). This nicely illustrates the multi-layered relationship these two enzymes can have during germ cell development and suggest that they promote similar as well as distinct gene expression programs. Unfortunately, little is known about how GLD-2 and GLD-4 might regulate such programs on the molecular level.

1.6. Germ cell development of the model organisms *C. elegans*

The germ line of the nematode *Caenorhabditis elegans* (*C. elegans*) has emerged as a model tissue to study RNA regulation in the context of a developmental system (NOUSCH AND ECKMANN 2013). In general, the worm has two sexes, male and hermaphrodite. Whereas males produce only sperm, hermaphrodites produce sperm at the last larval stage and switch to the production of oocytes with the onset of adulthood. The gonad in both sexes is a tube-like structure in which germ cells mature in a distal to proximal orientation. Germline stem cells divide mitotically in the most distal part of the tissue and enter meiosis further proximal. During spermatogenesis germ cells within the tissue go through all stages of meiosis and develop

into fully differentiated functional sperm cells. During oogenesis germ cells within the tissue arrest at the end of prophase I and finish meiosis after fertilization (Fig. 2).

The *C. elegans* germ line is a syncytium where many nuclei share a common cytoplasm. Despite the lack of cellular membranes to partition cells distinct protein expression patterns have to be established to guide germ cells through all the different developmental stages. Interestingly, many protein expression patterns can be recapitulated by fusing their 3' UTRs to a GFP reporter protein (MERRITT *et al.* 2008). Furthermore, it has been shown that RNA metabolism-associated factors are significantly more abundant in the germ line than in the soma and that many of them are important for germ cell development (WANG *et al.* 2009; GREEN *et al.* 2011). Collectively this argues that protein expression within this tissue is primarily controlled on the post-transcriptional level. This makes the *C. elegans* germ line the perfect tissue to study the biological roles and molecular mechanisms of RNA regulators.

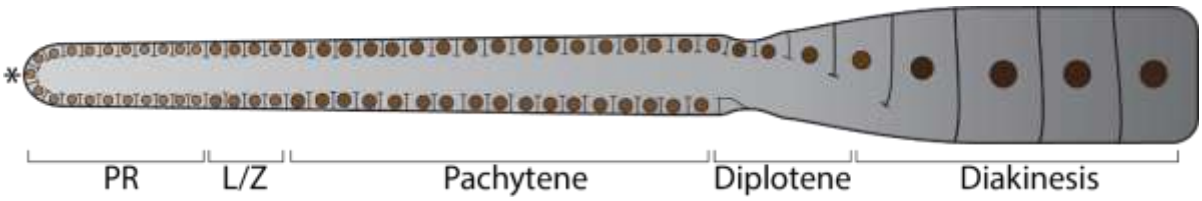

Figure 2: *Cartoon of the adult hermaphroditic germ line. Germ cells develop in a distal to proximal orientation. In the proliferative region (PR) germ cells divide mitotically. At the end of PR, cells enter and subsequently go through the indicated stages of prophase I before arresting as fully developed oocytes at the end of diakinesis. The star marks the distal end of the tissue. Sperm usually present at the proximal end of the tissue is not shown. L/Z – Leptotene/Zygotene*

2. Aims of this work

Using the germ line of the nematode *C. elegans* as a model the following questions were addressed:

(I) Which deadenylases are important for germ cell development?

(II) Which roles do cytoplasmic poly(A) polymerases have in mRNA regulation?

(III) Can cytoplasmic poly(A) polymerases have enzymatic-independent functions?

(IV) Can a tissue-specific ribosome purification method be adopted to analyze germ cell translation?

(V) How are opposing poly(A) modifying enzymes utilized during germ cell development?

3. Results

3.1. The Ccr4-Not deadenylase complex constitutes the main poly(A) removal activity in *C. elegans*

Overarching question

Which deadenylases are important for germ cell development?

Synopsis of the publication

Deadenylases have been studied quite intensively in unicellular organisms such as yeast or tissue culture systems. For example, our knowledge about the roles of DeAds in the mRNA decay pathway is based on studies using such systems (TUCKER *et al.* 2002; YAMASHITA *et al.* 2005; YI *et al.* 2018). However, little was known about DeAds in multicellular organisms in general and *C. elegans* in particular. Especially, a comparative analysis of the biological roles of the different deadenylases had never been done before in any model organism. Therefore, I used a combination of genetics and molecular methods to investigate the roles of worm homologs of the main DeAds, Ccr4, Caf1, Pan2 and PARN in *C. elegans* focusing particularly on putative roles of the different enzymes in germ cell development. I found that all deadenylases are strongly expressed in germ cells. Whereas *ccr-4*, *panl-2* (the Pan2 homolog) and *parn-1* mutants produce less offspring, the reduction of CCF-1 (the Caf1 homolog) results in complete infertile hermaphrodites. This shows that only CCF-1 is essential for germ cell development. Furthermore, I found that the two deadenylases of the Ccr4-Not complex CCR-4 and CCF-1 provide the major RNA deadenylase activity on the organismal level. In general, this work provided the basis as well as tools and reagents that allow further in-depth investigations into how deadenylases regulate gene expression in germ cells.

The Ccr4–Not deadenylase complex constitutes the main poly(A) removal activity in *C. elegans*

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Summary

Post-transcriptional regulatory mechanisms are widely used to control gene expression programs of tissue development and physiology. Controlled 3′ poly(A) tail-length changes of mRNAs provide a mechanistic basis of such regulation, affecting mRNA stability and translational competence. Deadenylases are a conserved class of enzymes that facilitate poly(A) tail removal, and their biochemical activities have been mainly studied in the context of single-cell systems. Little is known about the different deadenylases and their biological role in multicellular organisms. In this study, we identify and characterize all known deadenylases of *Caenorhabditis elegans*, and identify the germ line as tissue that depends strongly on deadenylase activity. Most deadenylases are required for hermaphrodite fertility, albeit to different degrees. Whereas *ccr-4* and *ccf-1* deadenylases promote germline function under physiological conditions, *panl-2* and *parn-1* deadenylases are only required under heat-stress conditions. We also show that the Ccr4–Not core complex in nematodes is composed of the two catalytic subunits CCR-4 and CCF-1 and the structural subunit NTL-1, which we find to regulate the stability of CCF-1. Using bulk poly(A) tail measurements with nucleotide resolution, we detect strong deadenylation defects of mRNAs at the global level only in the absence of *ccr-4*, *ccf-1* and *ntl-1*, but not of *panl-2*, *parn-1* and *parn-2*. Taken together, this study suggests that the Ccr4–Not complex is the main deadenylase complex in *C. elegans* germ cells. On the basis of this and as a result of evidence in flies, we propose that the conserved Ccr4–Not complex is an essential component in post-transcriptional regulatory networks promoting animal reproduction.

Key words: Germline development, Poly(A) metabolism, Deadenylase, Translational regulation

Introduction

Deadenylases are a conserved class of enzymes that catalyze the removal of poly(A) tails of mRNAs (Goldstrohm and Wickens, 2008). As part of cytoplasmic mRNA decay pathways, deadenylation is a regulator of global gene expression by initiating mRNA degradation (Decker and Parker, 1993). As part of translational control mechanisms, deadenylation is utilized in gene-specific expression control, leading to translational repression of mRNAs (Goldstrohm and Wickens, 2008).

Much of our knowledge about the biological roles of deadenylases was primarily inferred from studies in yeast, or tissue culture systems of flies and mammals (for recent reviews see Goldstrohm and Wickens, 2008; Parker, 2012; Wahle and Winkler, 2013). Because some deadenylases are essential, studies of their biological roles in multicellular systems have been hampered (DeBella et al., 2006; Molin and Puisieux, 2005; Neumüller et al., 2011). The deletion of a non-essential deadenylase in *Drosophila* resulted in gametogenesis defects (Morris et al., 2005). A similar observation was made for another deadenylase in mice (Nakamura et al., 2004), suggesting that deadenylases in general could have important functions in germ cell development (Nousch and Eckmann, 2013). Over recent years, the nematode model organism *C. elegans* became a paradigm for studying mRNA regulation in connection with germ cell development. The significance of poly(A)-mediated mRNA control is substantiated by the germline requirements of cytoplasmic poly(A) polymerases (Schmid et al., 2009; Wang et al., 2002). However, the roles of deadenylases remain largely unexplored in *C. elegans* or other multicellular organisms.

The best-understood eukaryotic deadenylases thus far are CCR4 (carbon catabolite repressor 4), CAF1 (Ccr4p-associated factor 1), and PAN2 (PolyA nuclease 2), which are conserved across eukaryotes from yeast to human, and PARN (PolyA-specific ribonuclease), which is not present in budding yeast and fly (Boeck et al., 1996; Daugeron et al., 2001; Goldstrohm and Wickens, 2008; Körner et al., 1998; Tucker et al., 2002). With the exception of PARN, the other three enzymes are part of larger protein complexes; CCR4 and CAF1 belong to the Ccr4–Not complex and PAN2 to the Pan2–Pan3 complex. The Ccr4–Not complex is a multi-subunit deadenylase that is composed of a core module and auxiliary factors. Three proteins build the core, the two catalytic subunits CCR4 and CAF1 (also known as Pop2) and the large scaffolding protein NOT1 (negative on TATA), which anchors the auxiliary factors to the complex. The core of the complex is broadly conserved. However, the number of CCR4 and CAF1 proteins expanded during evolution. While yeast encodes one CCR4 and CAF1 variant each, mammalian cells express two variants of either factor (Tucker et al., 2002; Yamashita et al., 2005). Additionally, the number of auxiliary factors differs among organisms. Initially, six auxiliary factors were described in *S. cerevisiae*: Not2p, Not3p, Not4p, Not5p, Caf40p (also known as Not9) and Caf130p (Liu et al., 1998). With the exception of yeast Caf130p, all are evolutionarily

conserved and orthologs have been described in flies and mammals (Denis and Chen, 2003; Temme et al., 2004). Furthermore, the complex is extended in Trypanosomes by NOT10, and in flies and mammals by NOT10 and NOT11 (Bawankar et al., 2013; Färber et al., 2013; Mauxion et al., 2012; Temme et al., 2010). The enzymatic balance between CCR4 and CAF1 changed during evolution. While CCR4 is the dominant enzyme in yeast (Tucker et al., 2002), CAF1 is the prevalent deadenylase in *Drosophila* (Temme et al., 2004). In general, loss of Ccr4–Not complex activity leads to strong cytoplasmic deadenylation defects in yeast, flies and mammals (Temme et al., 2004; Tucker et al., 2001; Yamashita et al., 2005).

The Pan2–Pan3 complex is an evolutionarily conserved heterodimer consisting of the single catalytic subunit, PAN2, and its co-factor, PAN3, in yeast and humans (Boeck et al., 1996; Brown et al., 1996; Uchida et al., 2004). However, loss of Pan2–Pan3 complex function has little effect on cytoplasmic mRNA deadenylation in yeast and fly (Bönisch et al., 2007; Tucker et al., 2001). Interestingly, the less conserved deadenylase, PARN, has a number of properties that distinguishes it from the Ccr4–Not and Pan2–Pan3 complex. First, PARN acts as a homodimer and binds the mRNA 5′ cap structure. While homodimerization of PARN is essential for RNA binding and deadenylase activity (Wu et al., 2005), its cap-binding ability stimulates its enzymatic activity only slightly (Dehlin et al., 2000). Second, while the two larger deadenylase complexes are primarily cytoplasmic, PARN is primarily localized to the nucleus (Berndt et al., 2012; Yamashita et al., 2005).

With this study, we document the importance of deadenylases for reproduction in the model organism *C. elegans*, using RNAi knockdown experiments or deletion mutants. First, we identified seven deadenylases in this nematode, including their associated protein complex members. Second, we characterized the expression of the five major deadenylase families in wild type and deadenylase mutants, investigate their biological roles in germline development and establish their global impact on bulk poly(A) tail metabolism. Third, we provide evidence that a cytoplasmic CCR-4–CCF-1–NTL-1 core deadenylase complex assembles in germ cells and is crucial for oogenesis. Intriguingly, we find that the expression of the dominant deadenylase CCF-1 relies on the co-expression of NTL-1, the presumed scaffolding subunit of the Ccr4–Not deadenylase complex.

Results

Using Blast searches with all known yeast, fly and human deadenylase factors against the *C. elegans* genome (Wormbase release WB233), we identified seven conserved deadenylases and their known protein complex subunits. They either belong to the Ccr4–Not and Pan2–Pan3 complexes or classify as orthologs of PARN (*parn-1*), Angel (*angl-1*) and 2′ phosphodiesterase (*pde-12*) deadenylase family members (Fig. 1; supplementary material Table S1). Interestingly, no ortholog for the previously described deadenylase nocturnin could be identified (Baggs and Green, 2003). We also note that by performing evolutionary distance analysis, we found the Caf1z/TOE1 deadenylase to be more similar to PARN than to Caf1/CNOT7 deadenylases (supplementary material Fig. S1); hence, we named the *C. elegans* ortholog *parn-2* (PolyA-specific ribonuclease homolog 2).

Components of the presumed Ccr4–Not complex in *C. elegans*

The *C. elegans* ortholog of Caf1 is encoded by a single genetic locus on LG *III* and is termed *ccf-1* (yeast CCR4 associated factor 1) (Fig. 1B). The production of a single transcript from that locus has previously been described (Fig. 2A) (Molin and Puisieux, 2005). The predicted protein translated from this mRNA is 310 aa in length and contains a DEDD-type nuclease domain (Fig. 2B), which is named after conserved catalytic Asp and Glu residues (Zuo and Deutscher, 2001). In order to characterize CCF-1 protein expression, specific antibodies were raised against the full-length protein. The isolated monoclonal antibody recognizes a single band at ~36 kDa in western blots (Fig. 2F). Importantly, the intensity of this band is decreased by *ccf-1(RNAi)* knockdown, which confirms the specificity of the antibody (Fig. 2F). Further analysis of hermaphrodites and males showed that CCF-1 is expressed in both sexes; a comparison of *glp-1(ts)* animals at restrictive temperature to wild-type animals showed that CCF-1 is strongly expressed in the germ line (Fig. 2F). *glp-1(ts)* hermaphrodite adults lack a germ line when grown at a restricted temperature and serve to assess the abundance of somatic versus germline expression. Moreover, CCF-1 is present at all developmental stages, albeit less abundant in embryos and L1-stage animals (Fig. 2H).

The second catalytic subunit, Ccr4, is encoded by a single genetic locus located on LG *IV* (Fig. 1B). As in budding yeast,

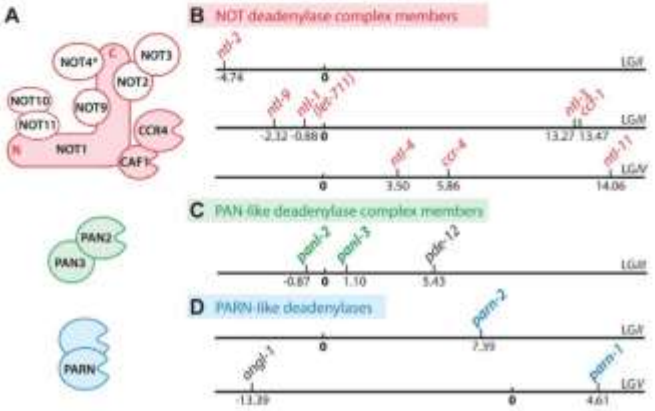

Fig. 1. Genomic locations of predicted deadenylases in *C. elegans*. (**A**) Graphic of the human Ccr4–Not complex (red), Pan2–Pan3 complex (green) and PARN (blue). NOT4 is a non-constitutive member of the complex (*). With the exception of NOT10, all other components are represented in the *C. elegans* genome. See text for an explanation of the nomenclature. (**B**) Genes encoding Ccr4–Not complex components (red). (**C**) Both subunits of the Pan2–Pan3 complex (green) map to LG *III*, including *pde-12*, which encodes the 2′ phosphodiesterase homolog. (**D**) The PARN-like enzymes *parn-1* and *parn-2* (blue). The *C. elegans* homolog of Angel, *angl-1*, is located on LG *V*. Genetic positions are not drawn to scale.

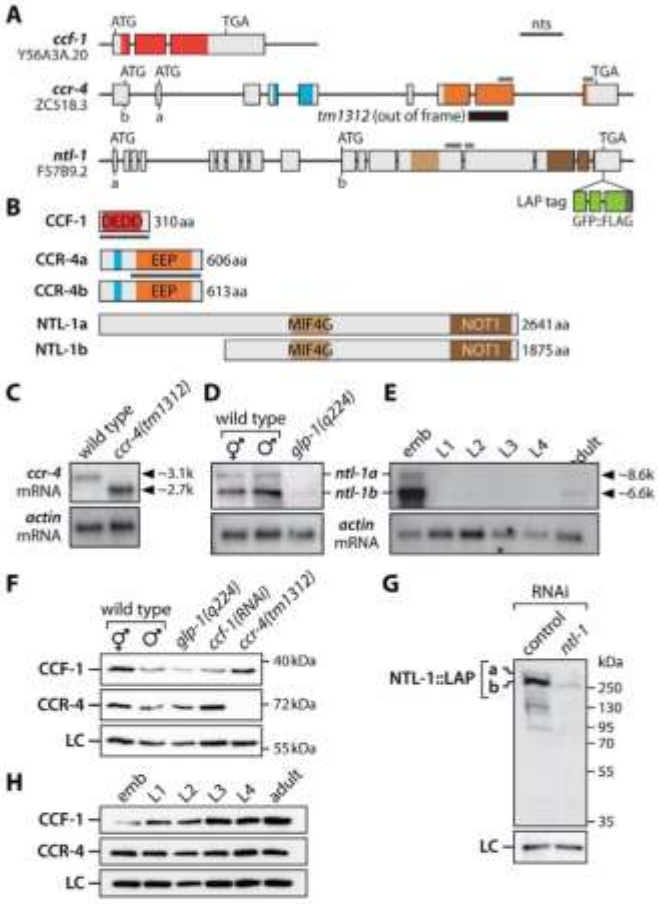

Fig. 2. Genomic locus, mutants and expression products of *ccf-1*, *ccr-4* and *ntl-1*. (**A**) Gene structure of *ccf-1*, *ccr-4* and *ntl-1*. Gray parts of the boxes represent exons. Colors indicate protein domains. Deleted regions in mutants and their effect on the coding potential are indicated below the genomic locus. Darker lines above the exons correspond to the position of the northern blot probes. Two alternative 5′ exons and corresponding start codons are present in *ccr-4* and *ntl-1*. For *ntl-1*, the insertion of the LAP tag 5′ to the stop codon of the *ntl-1*-carrying fosmid WRM0617aB06 is illustrated. Scale in nucleotides (nts): *ccf-1*=50, *ccr-4*=200, *ntl-1*=250. (**B**) The protein domain structure of Ccr4–Not core components shown in A. The different colors show the positions of known domains as annotated in PFAM: deadenylase domains DEDD (PF04857, red) and EEP (PF03372, orange). Both CCR-4 isoforms contain in addition a leucine-rich region (blue). The NOT1 (PF04054, dark brown) and MIF4G domain (PF02854, light brown) in NTL-1 were mapped by a primary sequence comparison to the human CNOT1 protein. The black lines under CCF-1 and CCR-4 indicate the corresponding antibody epitopes. (**C**) Northern blot analysis of *ccr-4* mRNAs of mixed-staged wild-type and *ccr-4(tm1312)* animals. (**D**) *ntl-1* mRNA transcript analysis of adult wild-type and *glp-1(ts)* worms. (**E**) Developenental mRNA expression analysis of poly(A)-enriched RNA of wild-type hermaphrodites. (**F**) Western blot analysis of adult worms with antibodies specific to CCF-1 and CCR-4. LC, loading control. (**G**) Western blot detection with anti-FLAG antibody. (**H**) Developmental protein expression analysis of wild-type hermaphrodite stages. Loading control is tubulin (F and H) or an unknown anti-FLAG cross-reacting background band (G).

the gene is dubbed *ccr-4* (CCR homolog 4) in *C. elegans* (Fig. 1B). According to bioinformatic predictions, multiple isoforms might be generated from that locus (WB233). Expressed sequence-tag analysis confirmed the expression of two alternative mRNAs, which are both SL1 *trans*-spliced (data not shown). The two transcripts, *ccr-4a* and *ccr-4b*, utilize a different first exon that harbors a start codon, leading to a difference of 72 nucleotides that primarily affect the 5′UTR sequence (Fig. 2A). Northern blot analysis revealed a single wild-type mRNA band, which is likely a combination of both RNA isoforms due to the limited resolution power of the agarose gel (Fig. 2C). At the amino acid level, CCR-4a and CCR-4b proteins are predicted to be 606 aa and 613 aa in size, respectively. The two proteins differ at their N-terminus; CCR4a and CCR4b possess seven and 14 unique amino acids, respectively. The remaining 599 amino acids are identical, and contain N-terminal leucine-rich repeats and a C-terminal catalytic domain, which belongs to the endonuclease-exonuclease-phosphatase superfamily (EEP) (Fig. 2B). As both proteins are highly similar to each other, and we lack tools to distinguish them, we refer to both protein isoforms as CCR-4 throughout this work. A monoclonal antibody, raised against the shared C-terminal two thirds of CCR-4 (Fig. 2B), recognized a single band at ~70 kDa in wild-type animals that is lacking in *ccr-4(tm1312)* animals, which carry a 500 nt deletion (Fig. 2A,F). Although a stable truncated out-of-frame mRNA is produced in *ccr-4(tm1312)* (Fig. 2C), no truncated protein form was detected (data not shown). CCR-4 is expressed in both sexes, present in the soma and the germline tissue, and is equally abundant throughout development (Fig. 2F,H).

The presumed scaffolding protein NOT1 is encoded by the genetic locus *let-711* on LG *III* (Fig. 1B) (DeBella et al., 2006). Since in *C. elegans* all NOT proteins are termed NOT-like proteins, we refer to *let-711* as *ntl-1* throughout the rest of this work. One transcript is predicted to be produced from the *ntl-1* locus (WB233). However, northern blot analysis showed that two distinct mRNA isoforms are generated; a prevalent ~6.6 kb and a less abundant ~8.6 kb transcript. Both mRNAs are expressed to a similar extent and ratios in wild-type hermaphrodites and males (Fig. 2D). Either transcript is substantially reduced in *glp-1(ts)* animals, suggesting that *ntl-1* is strongly expressed in the germ line (Fig. 2D). Intriguingly, both *ntl-1* transcripts are even

more abundant in embryos (Fig. 2E). Our cDNA sequence analysis confirmed the existence of two alternative transcripts, *ntl-1a* and *ntl-1b*. Both transcripts carry an SL1 sequence immediately upstream of the start codon, suggesting that they are the product of two distinct trans-splicing events (Fig. 2A). At the amino acid level, NTL-1a and NTL-1b are predicted to be 2641 aa and 1875 aa in size, respectively. While NTL-1a has a unique N-terminus of 766 aa, both isoforms are identical in the rest of the protein sequence, harboring a domain that shows similarity to a MIF4G domain, which is important for binding Caf1 in yeast and human, and a C-terminal region that is conserved among all described NOT1 proteins (Basquin et al., 2012) (Fig. 2B). As our attempts to raise antibodies against NTL-1 were unsuccessful, we decided to analyze NTL-1 protein expression using a C-terminal LAP-tagged *ntl-1* transgene (Fig. 2A). A LAP-tag is a chimera of GFP fused with three FLAG epitope tags (Cheeseman and Desai, 2005). Three transgenic lines were established and all three displayed similar expression. Using anti-FLAG antibodies, western blotting experiments detected a major band of ~260 kDa (Fig. 2G) and a minor band at ~330 kDa, which are lacking in non-transgenic worms (data not shown) and are strongly reduced in transgenic animals treated with *ntl-1(RNAi)* (Fig. 2G). The shorter NTL-1::LAP products detected by western blotting are presumably degradation products as their existence is not supported by our *ntl-1* cDNA and northern blot analysis.

Taken together, these results show that the three core members of the putative *C. elegans* Ccr4–Not complex are expressed in either sex, abundant throughout hermaphrodite development, and present in somatic and germline tissues, suggesting that the deadenylase complex might function in germ cell development.

Germline expression of *C. elegans* Ccr4–Not core complex components

The expression analysis of *ccr-4*, *ccf-1* and *ntl-1* suggests that all three proteins might be present in germ cells of adult hermaphrodites. The adult hermaphroditic germline tissue is composed of undifferentiated and differentiated germ cells that are stereotypically arranged in a linear manner with temporal and spatial resolution of each step during oogenesis. Only in its most distal part, germ cells divide mitotically. In the remaining proximal part, germ cells initiate gametogenesis and undergo the meiotic program while differentiating into oocytes. In the most proximal part, fully-grown oocytes arrest in the last stage of meiotic prophase and remain stored until ovulated.

To test if all three presumed Ccr4–Not complex components are co-expressed at any specific stage of female germ cell development, germ lines of adult hermaphrodites were extruded and subjected to indirect immunofluorescence analysis. We compared germ lines from wild type with mutant and control RNAi with gene-specific RNAi-treated animals.

While endogenous CCF-1 protein is present throughout the entire germ line, it is more abundant in germ cells that entered meiosis and progress through the pachytene stage (Fig. 3A). Endogenous CCR-4 and NTL-1::LAP are uniformly distributed throughout the entire germ line with no apparent differences in their expression (Fig. 3B,C). All three proteins are predominately localized to the cytoplasm of all germ cells (Fig. 3A′–C′). Taken together, the cytoplasmic co-expression of all three core components is consistent with the existence of a CCR-4–CCF-1–NTL-1 complex in all germ cells of adult hermaphrodites,

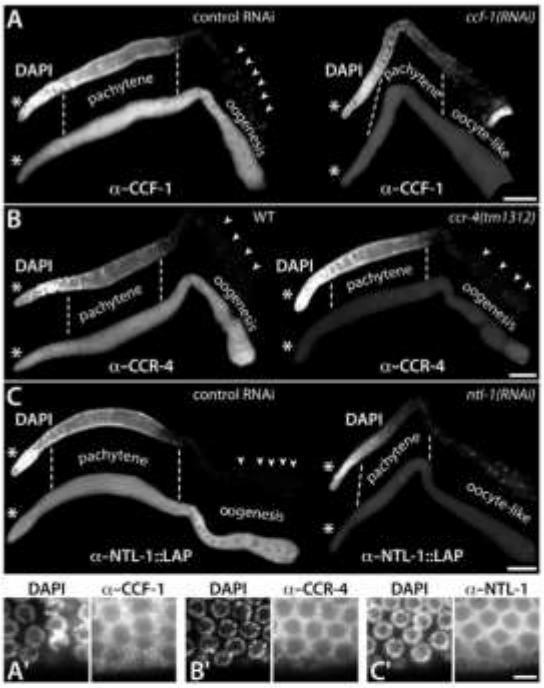

Fig. 3. Germline expression of CCF-1, CCR-4 and NTL-1. Epifluorescent images of extruded hermaphrodite gonads stained with DAPI and primary antibodies against endogenous (A) CCF-1, (B) CCR-4, and (C) anti-FLAG antibodies against LAP-tagged NTL-1 protein (NTL-1::LAP). Anti-GLH-2 staining was used as a tissue penetration control (data not shown). An asterisk marks the distal end of the gonad. Scale bars: 50 µm. (A′-C′) Magnifications of pachytene germ cells stained with DAPI (left) and the antibodies corresponding to A–C (right). Scale bar: 5 µm.

suggesting a likely common role in female germline development.

CCF-1, CCR-4 and NTL-1 form a protein complex in *C. elegans*

The Ccr4–Not complex has been biochemically characterized in yeast, fly and human (Bai et al., 1999; Lau et al., 2009; Temme et al., 2010). To assess if CCR-4, CCF-1 and NTL-1 associate with each other to form a larger protein complex, we conducted immunoprecipitation experiments. To this end, we prepared whole-worm extracts from the transgenic strain that expresses LAP-tagged NTL-1, and used it as starting material for two different immunoprecipitation experiments (Fig. 4A,B).

In the first pull-down experiment, we compared anti-GFP pellets of transgenic and non-transgenic extracts. We observed an enrichment of NTL-1::LAP with associated CCF-1 and CCR-4 from the transgenic extract only (Fig. 4A). Importantly, the interaction between the three proteins is specific as no enrichment was observed for tubulin (Fig. 4A). In the second experiment, we compared immunoprecipitations with anti-CCF-1 antibodies to non-specific IgGs. Consistent with a specific enrichment of endogenous CCF-1, endogenous CCR-4 and

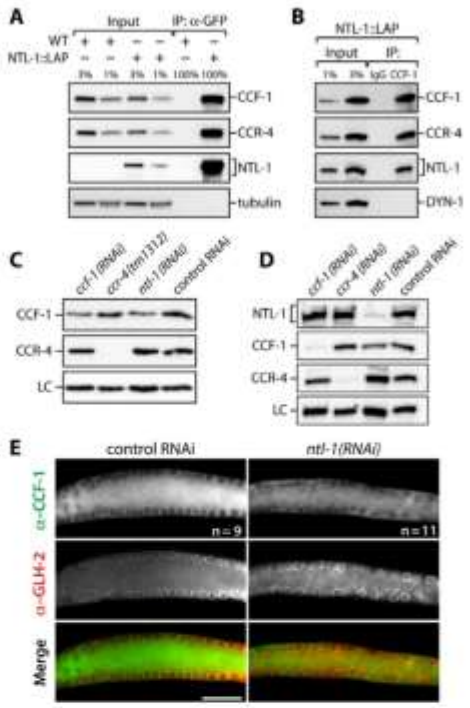

Fig. 4. CCF-1, CCR-4 and NTL-1 form a stable Ccr4–Not core complex in *C. elegans*. (**A**,**B**) Co-immunoprecipitation experiments of CCF-1, CCR-4 and NTL-1::LAP. Whole-worm extracts were prepared from mixed stage wild-type and transgenic NTL-1::LAP expressing animals. Immunoprecipitations were performed with (A) anti-GFP or (B) anti-CCF-1 antibodies and non-specific IgGs. Two independent experimental repeats each. (C-E) CCF-1 abundance is linked to NTL-1 abundance. (**C**,**D**) Western blot analysis to assess CCF-1, CCR-4 and NTL-1 protein interdependence in wild-type or NTL-1::LAP transgenic adults. Loading control (LC) is in C tubulin or in D an unknown anti-FLAG cross-reacting background band. (**E**) Extruded gonads of control or *ntl-1* RNAi-treated adults were stained for CCF-1. The anti-GLH-2 signal was used as a tissue penetration control. Distal is to the left and the region shown corresponds to late pachytene germ cells. Scale bar: 25 μm.

NTL-1::LAP co-enriched to equal levels (Fig. 4B). Importantly, the interaction between the three proteins is specific as no enrichment was observed for dynamin (Fig. 4B). We chose this control due to species incompatibility in the IP and western blot experiments as the heavy chains of the IgG protein obstructed the detection of tubulin. Taken together, the immunoprecipitation experiments suggest that a CCR-4–CCF-1–NTL-1 protein complex is formed *in vivo* in *C. elegans*. Moreover, as we observe a co-enrichment of both NTL-1 isoforms with CCF-1 precipitates, we assume that the MIF4G domain may mediate this interaction.

While testing for the specificity of our antibodies, we noticed that the protein abundance of CCF-1 depends on NTL-1 expression. A reduction of NTL-1 protein levels by RNAi caused a mild but consistent decrease of endogenous CCF-1 in wild-type or NTL-1::LAP transgenic animals (Fig. 4C,D). No changes of CCF-1 levels were detected in *ccr-4(tm1312)* or *ccr-4(RNAi)* animals (Fig. 4C,D). Contrary to CCF-1, endogenous CCR-4 levels do not change in case of *ntl-1* or *ccf-1* RNAi knockdown (Fig. 4C,D). Similarly, NTL-1 protein levels are unaltered when CCF-1 is downregulated by RNAi, or CCR-4 is absent in *ccr-4(tm1312)* (Fig. 4D). A NTL-1-dependent abundance of CCF-1 is further corroborated by comparing extruded germ lines of *ntl-1(RNAi)* with control RNAi animals stained for endogenous CCF-1 protein (Fig. 4E). Altogether, these data suggest that CCF-1, CCR-4 and NTL-1 form the enzymatic core of a Ccr4–Not deadenylase complex in *C. elegans*. Moreover, a differential protein expression dependency of one core component exists; CCF-1 abundance depends on NTL-1 presence, but not vice versa.

Ccr4–Not core components are important for fertility

To determine whether the Ccr4–Not complex is important for germline function, we asked whether its core components are necessary for general fertility, by assessing the brood size of self-fertilizing hermaphrodites. The number of living self-progeny is an indicator of germline integrity and functionality. We compared wild-type with *ccr-4(tm1312)* mutant animals and gene-specific RNAi (against *ccf-1* or *ntl-1*) with control RNAi treated animals (Fig. 5). While strong RNAi of *ccf-1* leads to a pachytene arrest of germ cells, homozygote *ccf-1* mutants show strong larval lethality (Molin and Puisieux, 2005). Homozygote *ntl-1* mutants are larval lethal, a phenotype that we could recapitulate with *ntl-1(RNAi)* (DeBella et al., 2006; Molin and Puisieux, 2005). Therefore, we used an RNAi setup that allows us to specifically address the biological relevance of CCF-1 and NTL-1 proteins in late stages of female gametogenesis (see Materials and Methods).

Wild-type or control RNAi animals produce on average ~320 viable offspring at 20℃ (Fig. 5A, data not shown). Consistent with previous data (Molin and Puisieux, 2005), animals with reduced *ccf-1* activity were either sterile with no viable offspring (7 out of 26), or produced on average ~6 viable progeny (19 out of 26) (Fig. 5A). Animals without *ccr-4* activity were fertile but produce a significantly smaller brood size, which is about one-third smaller than that of wild type (Fig. 5A). Animals with reduced *ntl-1* activity produced on average ~13 viable progeny (Fig. 5A). These results suggest that *ccf-1* and *ntl-1* are crucial for fertility, whereas *ccr-4* activity contributes less strongly to fertility. In combination, these data argue for a pivotal role of the Ccr4–Not complex in regulating reproductive capability and suggest a role in adult germ cell development.

The Ccr4–Not complex is important for germline tissue organization

Since all three core members of the Ccr4–Not complex are important for full reproductive capacity, we investigated potential underlying gametogenesis defects. Therefore, we extruded gonads from adult hermaphrodites and characterized their germline tissue organization by immunostainings (Fig. 6). We studied germ cell morphology by visualizing the actin cell cortex with antibodies against Anillin (anti-AIN-2), the nuclear envelope with antibodies against the nuclear pore complex (NPC) epitope recognized by mAb414, and the organization of the chromosomes via DAPI staining (Fig. 6A). Moreover, we analyzed oocyte-specific markers that reveal the progress of oogenesis in connection to meiotic progression (Fig. 6B).

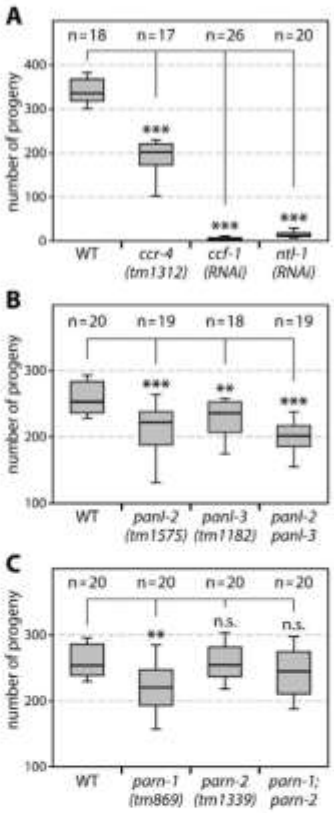

Fig. 5. Deadenylase mutants display a reduced fecundity. The fertility of parental hermaphrodites (n) was analyzed by counting hatched F1 larvae (progeny). (**A**) At 20°C, core Ccr4–Not complex components are required for fertility. An RNAi knockdown of *ccf-1* or *ntl-1* induces sterility. The brood size of *ccr-4(tm1312)* mutants is also significantly reduced. (**B**) At 25°C, Pan2–Pan3 complex components display a significant reduction in their brood sizes. (**C**) At 25°C, a reduced fertility is also present in *parn-1* but not in *parn-2* mutants. A suppression of *parn-1* sterility is observed upon additional *parn-2* removal. No detectable increase in embryonic lethality was observed for *ccr-4(tm1312)*, the *panl* mutants and *parn-1* (data not shown). Significance was calculated by two-tailed student's *t*-test. $^{**}P \geq 0.01$, $^{***}P \geq 0.001$.

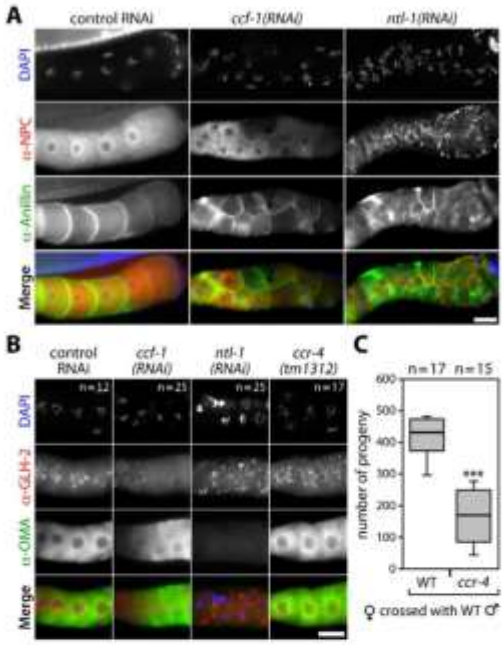

Fig. 6. CCF-1 and NTL-1 are essential for germline organization and oogenesis. (**A**,**B**) Extruded gonads of given genotypes were stained for either (A) nuclear pore complexes (NPC) and the cell cortex (anillin), or (B) P granules (GLH-2) or an oocyte-enriched maturation marker (OMA). Meiotic stages are revealed by chromosome staining (DAPI). Scale bar in (A) 20 µm, (B) 10 µm. (**C**) Brood size analysis of feminized wild-type or *ccr-4(tm1312)* animals that were crossed each with a single wild-type virgin male. Significance was calculated by two-tailed student's *t*-test. $^{***}P \geq 0.001$.

The overall germ cell organization in the distal region of the germ line is similar between wild-type, control RNAi, *ccf-1(RNAi)*, *ccr-4(tm1312)* and *ntl-1(RNAi)* animals (data not shown). However, small differences in the mitotic region and entry into meiosis were seen, affecting mostly the starting point of meiotic entry (data not shown). The most striking abnormalities were present in the proximal region of *ccf-1*- or *ntl-1*-compromized animals. In contrast to wild-type germ lines, which harbor in their proximal region oocytes that are arranged in a single-cell row, *ccf-1(RNAi)* and *ntl-1(RNAi)* germ lines possessed several cell rows of oocyte-like germ cells (Fig. 6A). While these cells still resemble diakinetic oocytes, they are much smaller in size. Moreover, Anillin is less prominently localized to the cell cortex and appears more diffusely cytosolic (Fig. 6A), indicating a defect in cellular organization. When combined, these data suggest that the meiotic program of oocytes completes prophase I, but the normal oocyte differentiation process fails upon CCF-1 or NTL-1 knockdown.

The degree of cellular disorganization was the strongest in *ntl-1(RNAi)* germ lines (Fig. 6A). The proximal oocyte-like cells are much smaller in size than in control RNAi germ lines, and many more nuclei are present in the proximal region of *ntl-1(RNAi)* germ lines, indicating that multiple cell rows are formed. While the DNA configuration resembles diakinetic nuclei, the perinuclear NPC staining is lost in the most proximal cells and is strongly granular, resembling nuclear envelope fragmentation reminiscent of maturing oocytes. Surprisingly, no obvious morphological defects were found in *ccr-4(tm11312)* germ lines (data not shown). Therefore, we conclude that differentiating oocytes are abnormal in animals compromised in *ccf-1* and *ntl-1*, but not *ccr-4* activity.

To assess if the oogenic differentiation program is affected by the absence of Ccr4–Not core components, we tested for the presence of oogenic fate markers by immunofluorescence. Extruded germ lines were stained with a pan-OMA antibody that recognizes OMA-1 and OMA-2. Both proteins are essential for oocyte maturation and gradually accumulate in the cytoplasm of growing oocytes (Detwiler et al., 2001); therefore, they indicate late stages of oogenic differentiation (Fig. 6B). While

OMA accumulation in proximal germ lines of control RNAi is essentially identical to developing wild-type oocytes, *ccf-1(RNAi)* germ lines show two defects. OMA protein expression is delayed and its subcellular localization is more granular (Fig. 6B). Conversely, the granular pattern of GLH-2 is lost in *ccf-1(RNAi)* oocytes (Fig. 6B). Interestingly, while the granular GLH-2 protein expression pattern is maintained in *ntl-1(RNAi)* oogenic cells, no OMA expression was detected (Fig. 6B). In germ lines of *ccr-4(tm1312)* animals, OMA and GLH-2 expression pattern resembled wild type (Fig. 6B). These data suggest that *ccf-1* and *ntl-1* activities are important for late oogenesis, however, the molecular pathologies are distinct upon the reduction of the two complex components. A direct involvement of the Ccr4–Not complex in OMA expression regulation remains an open question.

Although *ccr-4(tm1312)* was compromised in progeny production, no morphological defects were detected (Fig. 6B). Thus, we tested oocyte quality as a likely reason for the partial infertility. Feminized wild-type or *ccr-4(tm1312)* animals were crossed with wild-type virgin males and the resulting brood size was analyzed. Wild-type mothers ($n=17$) produce on average ~400 offspring in this assay, whereas *ccr-4(tm1312)* mothers ($n=15$) produce on average only ~170 offspring (Fig. 6C). This fertility assay indicates that a defect in *ccr-4(tm1312)* animals is associated with either oogenesis or a somatic part of the hermaphroditic gonad.

Components of the presumed Pan deadenylase complex in *C. elegans*

The Pan2–Pan3 complex consists of two proteins, Pan2 and Pan3, each encoded by a single gene on LG *III* in the *C. elegans* genome (Fig. 1A,C). The locus *panl-2* (pan-like 2) expresses a single SL1 spliced transcript (Fig. 7B), which is highly abundant in germ cells as confirmed by northern blotting (Fig. 7F). A predicted PANL-2 protein is 1131 aa in size, carrying in its C-terminus the putative catalytic domain that belongs to the RNase T exonuclease family. The *panl-2(tm1575)* deletion mutant produces a truncated *panl-2* mRNA and is expected to produce no functional protein (Fig. 7A,F).

The locus *panl-3* (pan-like 3) expresses a single SL1 spliced transcript (Fig. 7A), which is also highly abundant in germ cells (Fig. 7F). A predicted PANL-3 protein is 634 aa in size and has a putative poly(A)-binding protein-interacting motive (PAM2) in the N-terminus and is most similar to other Pan3 proteins in its C-terminus. The *panl-3(tm1182)* deletion mutant produces a truncated *panl-3* mRNA and is expected to produce no functional protein (Fig. 7B,F).

Pan-complex components affect fertility at elevated temperatures

As a putative Pan2–Pan3 complex may assemble in germ cells, we tested its impact on fertility. A brood size analysis was conducted using single and double mutant *panl-2(tm1575)* and *panl-3(tm1182)* alleles. Although both deletions are expected to be strong loss-of-function mutants, they are maintained as homozygote animals and a minor reduction of fertility was observed at 20℃ (data not shown). Therefore, we challenged the animals by raising them at elevated temperatures. At 25℃, wild-type animals produce on average ~260 offspring, whereas *panl-2(tm1575)* and *panl-3(tm1182)* single mutants have a significantly reduced number of offspring, which is similar to

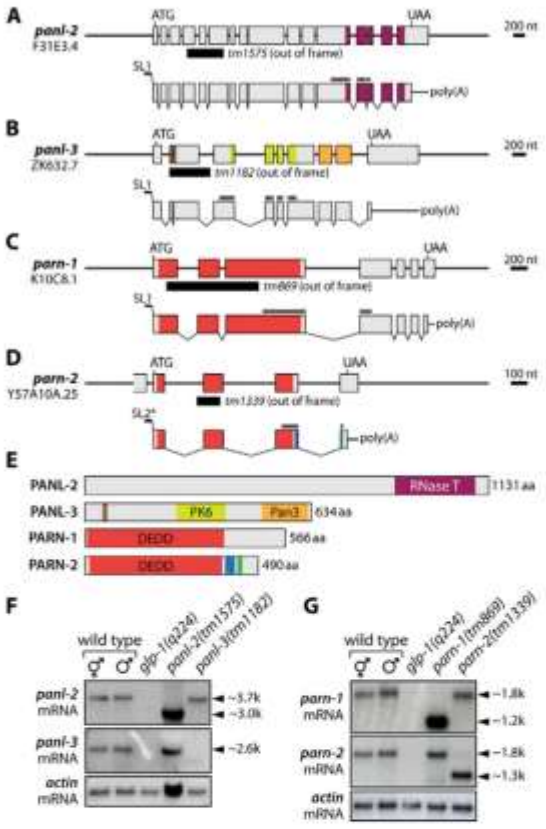

Fig. 7. Genomic locus, mutants and expression products of *panl-2*, *panl-3*, *parn-1* and *parn-2*. (**A–D**) Gene and mRNA structures are shown of (A) *panl-2*, (B) *panl-3*, (C) *parn-1* and (D) *parn-2*. Colors indicate protein domains, as given in E. Deleted regions in mutants and their effect on the coding potential are indicated below the genomic locus. Darker lines above the exons correspond to the position of the northern blot probes. (E) The protein domain structure of PANL-2, PANL-3, PARN-1 and PARN-2. Colors indicate the position of putative domains as predicted by PFAM or by protein similarity to human homologs: RNaseT exonuclease similarity (Pfam00929, violet); PABP-interacting motif PAM2 (Pfam07145, brown); catalytic domain of protein kinases (PK6, Pfam00069, yellow); conserved region in Pan3 homologs (Pan3, orange); deadenylase domain DEDD (PF04857, red). Conserved C3H zinc-finger (blue) and predicted basic nuclear localization signal (green) present in PARN-2 homologs. (F,G) Northern blot analyses of total mRNA. Transcripts encoding (F) components of the putative Pan2–Pan3 complex and (G) the two different PARN proteins are strongly expressed in the germ line.

panl-2 panl-3 double mutants (Fig. 5B). This suggests that the putative Pan2–Pan3 complex is important for robust germline function at elevated temperature.

PARN-like proteins in *C. elegans*

Two *parn*-genes, *parn-1* and *parn-2*, show similarity to the DEDD deadenylase PARN and are located on LG *V* and *II*, respectively (Fig. 1A,D). Either gene produced a single mRNA transcript, as confirmed by cDNA analysis and northern blotting,

which is highly enriched in germ cells (Fig. 7C,D,G). While PARN-1 contains only a DEDD-type catalytic domain in its N-terminus, PARN-2 contains also a C3H-zinc finger and a predicted nuclear localization signal in its C-terminus (Fig. 7E). Either deletion mutant, *parn-1(tm869)* or *parn-2(tm1339)*, produces a truncated mRNA species and is expected to produce a non-functional protein (Fig. 7C,D,G).

parn-1 but not *parn-2* activity affects fertility at elevated temperatures

As both *parn*-like genes are strongly expressed in germ cells, we tested their impact on fertility in brood size analysis. Similar to *panl*-genes (Fig. 5B), only a minor defect was observed at 20℃ for the *parn-1(tm869)* single mutant, while the *parn-2(tm1339)* single mutant was not affected (data not shown). Hence we repeated our analysis at 25℃. Again, the *parn-1(tm869)* single mutant showed a significant reduction in fertility, whereas the *parn-2(tm1339)* single mutant did not (Fig. 5C). Interestingly, an elimination of *parn-2* in combination with *parn-1* restores fertility of the *parn-1 parn-2* double mutant to wild-type levels (Fig. 5C). This suggests that the *parn-1*-associated fertility defect might be caused by the activity of *parn-2*. In summary, both *C. elegans* PARN genes, *parn-1* and *parn-2*, are expressed in the germ line and loss of *parn-1* results in reduced fertility.

The Ccr4–Not complex comprises the main deadenylase activity in *C. elegans*

Finally, we characterized the molecular function of either *C. elegans* deadenylase activity in respect to global poly(A) tail metabolism. To reveal *in vivo* enzymatic activity of the Ccr4–Not core complex, the Pan2–Pan3 complex and the two PARN-like proteins, we conducted bulk poly(A) tail measurements from wild-type, mutants or RNAi-treated adults (Fig. 8). With this method, global poly(A) tail-length changes are detected (Temme et al., 2004). To this end, total RNA was isolated from whole worms, labeled on the 3′ end and partially digested to remove non-poly(A) RNA sequences. The remaining poly(A) material is size-separated on high-resolution polyacrylamid gels (Fig. 8A).

mRNAs from wild-type or control RNAi-treated animals possess poly(A) tails from 20 to 80 nt in length, with the length distribution peaking around 30 nt (Fig. 8B, lane 1 and 6, Fig. 8D–F). This distribution is different in *ccr-4(tm1312)* mutant or *ccr-4(RNAi)* animals. Bulk poly(A) tails increase in length and an enrichment of poly(A) tails in the 60–70 nt range is observed (Fig. 8B, lane 2 and 3, Fig. 8D, and supplementary material Fig. S2A,B). Interestingly, a similar peak at 60–70 nts is observed when *ccf-1* activity is downregulated by RNAi (Fig. 8B, lane 4, Fig. 8E). Additionally, a significant population of mRNAs containing poly(A) tails longer than 70 nt appears (Fig. 8B, lane 4, Fig. 8E). In *ntl-1(RNAi)* an abundance of extra-long poly(A) tails is even more pronounced (Fig. 8B, lane 5, Fig. 8F). Together, our results suggest that all Ccr4–Not core components are important for deadenylation, albeit to different extent.

Contrary to results with the Ccr4–Not components only mild poly(A) tail-length changes were detected in *panl-2(tm1575)*, *panl-3(tm1182)*, *parn-1(tm869)* and *parn-2(tm1339)* single mutants. Neither extra-long poly(A) tails, nor the prominent 60–70 nt peak, which is detected in *ccr-4* and *ccf-1* compromised animals, are present (Fig. 8C, and supplementary material Fig. S2C–F). However, in comparison to wild type, a mild reduction of short poly(A) tails below 30 nt in length was detected (Fig. 8C, and supplementary material Fig. S2C–F). In summary, our bulk poly(A) tail measurements indicate that the Ccr4–Not complex may carry the main deadenylation activity in *C. elegans*.

Discussion

With the exception of nocturnin, we identified seven conserved deadenylases in the developmental model organism *C. elegans*.

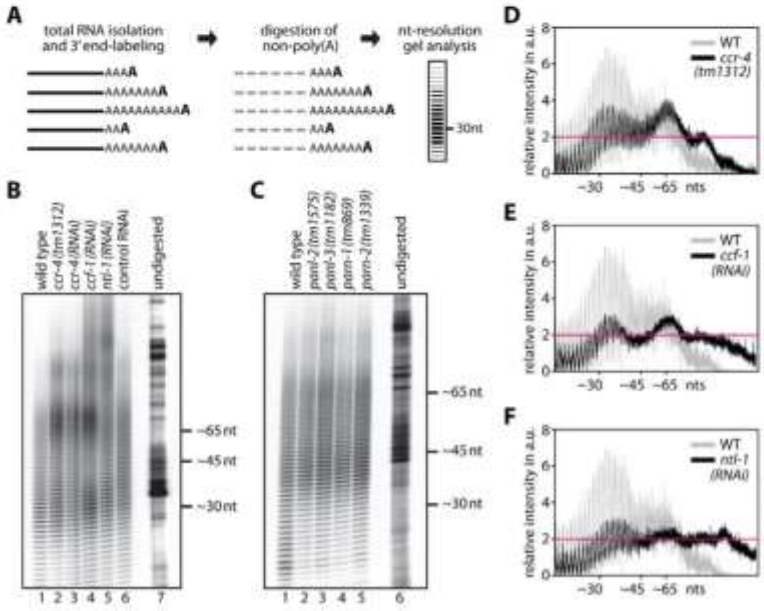

Fig. 8. Bulk poly(A) tail length measurements of total RNA. (**A**) Scheme of the experimental procedure. Total RNA was isolated from adult animals, labeled with radioactive cordycepin, digested by RNases A and T1, and size-separated on a sequencing gel. Undigested RNA of wild-type animals was loaded to control the efficiency of the digest and size markers were included. (**B**,**C**) Analysis of the length of bulk poly(A) tails of the indicated samples. Each sample was analyzed at least in three independent experiments; representative gels are shown. (B) A reduction of core Ccr4–Not complex components extends bulk poly(A) lengths. (C) Slight changes of bulk poly(A) tail lengths in Pan2–Pan3 complex component mutants and PARN mutants. (**D-F**) Line scans of wild-type (WT), *ccr-4(tm1312)*, *ccf-1(RNAi)* and *ntl-1(RNAi)* lanes of (B). The relative intensity signal of the lane is shown and given in arbitrary units (a.u.). Red line represents a visual aid.

We found that the *ccr-4*, *panl-2*, *parn-1*, and *parn-2* genes are non-essential for life and reproduction. By contrast, *ccf-1* is essential and RNAi-mediated protein knockdowns cause severe oogenesis defects in adult animals. CCF-1 protein associates with CCR-4 and NTL-1, forming a core module with enzymatic function of a presumably larger Ccr4–Not deadenylase complex that requires NTL-1 for the optimal abundance of CCF-1. Consistent with an abundant expression of all five deadenylases in the adult germline tissue, four enzymes are important for germline function, albeit to different degrees; *ccf-1* and *ccr-4* activities are required for fertility at normal temperature, whereas *panl-2* and *parn-1* are only required for fertility at elevated temperature. The prevalent biological roles of CCF-1 and CCR-4 are also apparent at the molecular level. By contrast to the other three enzymes, the two Ccr4–Not complex-associated deadenylases are required for bulk mRNA poly(A) tail shortening, suggesting that the full Ccr4–Not complex may constitute the main deadenylase for mRNA metabolism in *C. elegans*.

Components of the *C. elegans* Ccr4–Not complex and its general role in mRNA deadenylation

The Ccr4–Not complex is a multi-subunit protein assembly that is structurally organized around its largest subunit, the scaffolding component Not1 (Bai et al., 1999). We found that all but one (i.e. CNOT10) of the human Ccr4–Not complex subunits are evolutionarily conserved in *C. elegans*. Overall, the nematode Ccr4–Not complex is more similar to flies and humans than to yeast (Bawankar et al., 2013; Mauxion et al., 2012). In parallel to humans and flies (Albert et al., 2000; Temme et al., 2010), the two yeast paralogs, Not3p and Not5p, are represented by only one gene in *C. elegans*, i.e. NTL-3. Moreover, the organization of the core complex is preserved in worms; NTL-1, CCF-1 and CCR-4 are found in a stable complex with each other, whereby CCR-4 is most likely associated with NTL-1 via an interaction with CCF-1, based on the conserved leucine-rich region in CCR-4, which is the mapped interaction domain in yeast and human Ccr4 orthologs (Clark et al., 2004; Dupressoir et al., 2001). Intriguingly, RNAi-mediated reduction of NTL-1 led to a reduction of CCF-1 but not the CCR-4 subunit. This specific unidirectional dependency of CCF-1 on NTL-1 suggests that CCF-1 exists mainly as part of a NTL-1 complex, and that CCR-4 might also exist outside of the Ccr4–Not complex. Moreover, the correlated expression relationship of the CCF-1 deadenylase with the scaffolding subunit NTL-1 resembles, at least partially, the interaction dynamics of the complex observed in *Drosophila* S2 cells (Temme et al., 2010).

The deadenylase activity of the Ccr4–Not complex is provided by the two Not1-associated deadenylases, Caf1 and Ccr4 (Goldstrohm and Wickens, 2008). While the EEP domain protein Ccr4p is the major catalyst of mRNA deadenylation in yeast, the DEED domain protein CAF1 is the predominant enzyme in flies and humans (Chen et al., 2002; Mauxion et al., 2008; Sandler et al., 2011; Temme et al., 2004; Tucker et al., 2002). In *C. elegans*, we find that both enzymes, including NTL-1, have a role in global mRNA deadenylation. The defects of mRNA poly(A) tail extension in the absence of deadenylase activity are more severe for *ccf-1(RNAi)* than *ccr-4(tm1312)*. This is especially more striking when keeping in mind that feeding RNAi of *ccf-1* is incomplete, as we detect about forty percent of CCF-1 remaining in these animals. As CCF-1 abundance depends on NTL-1, the similarly strong effects of *ccf-1* and *ntl-1* RNAi knockdown on bulk mRNA poly(A) tail extension can be interpreted as a primary reduction of CCF-1 function in *ntl-1(RNAi)* animals. This functional correlation suggests that CCF-1 is the major deadenylase of the nematode Ccr4–Not complex, and that CCR-4, which does not reduce CCF-1 or NTL-1 expression, has a minor but clearly detectable role in bulk mRNA deadenylation.

The biological roles of Ccr4–Not complex in animal development

Deadenylation of mRNAs is viewed as an essential part of gene expression regulation in eukaryotes (Garneau et al., 2007). Importantly, both 5′ and 3′ decay pathways are initiated via mRNA deadenylation (Garneau et al., 2007). Besides post-transcriptional roles of the Ccr4–Not complex, transcriptional roles have been suggested that are independent of deadenylase activity and strongly linked to promoting arrested transcription elongation (Collart, 2003; Kruk et al., 2011). In this context it is interesting to note that only Not1p is an essential gene in yeast, whereas Not1 and Caf1 are essential in flies (Maillet et al., 2000; Neumüller et al., 2011), perhaps reflecting a dual role of the Ccr4–Not complex mediated by this scaffolding protein. In *C. elegans*, mutations in *ntl-1* (DeBella et al., 2006) or *ccf-1* (Molin and Puisieux, 2005) but not in *ccr-4* (this work) are lethal. This lethality is primarily due to failed embryonic development or mid-larval arrest (DeBella et al., 2006; Molin and Puisieux, 2005), suggesting a common role for *ntl-1* and *ccf-1* in early developmental processes that may reflect transcriptional and post-transcriptional roles of the Ccr4–Not complex. The overlapping biological roles of Caf1 and Not1 and their shared molecular defects at the mRNA level in nematodes and flies, suggest that deadenylation by the Ccr4–Not complex is an essential process in a multicellular organism and that Caf1 deadenylases may be the predominant enzymes involved in mRNA decay.

These combined roles appear distinct from a developmental requirement for *ccr-4*, which seems limited to female reproductive capacity. Moreover, as documented in bulk poly(A) tail measurements of an enzymatically dead *ccr-4(tm1312)* mutant, *ccr-4* deadenylase activity is important for mRNA deadenylation, which is consistent with a likely and a more exclusive post-transcriptional role of CCR-4 deadenylase. However, it remains a formal possibility that this mutant produces a truncated CCR-4 protein that is negatively influencing the activity of CCF-1, as the leucine-rich region remains intact, and the truncated mRNA is stable. We regard this as unlikely, as *ccr-4(RNAi)* is quite effectively downregulating CCR-4 protein levels and a similar extension of poly(A) tails is observed. While downregulation of CCR4 in *Drosophila* S2 cells has no consequence on bulk mRNA poly(A) tails, mutations in the *Drosophila* gene *twin*, which encodes CCR4, affect female reproductive capacity (Morris et al., 2005; Temme et al., 2004). Moreover, it appears that Ccr4 deadenylases of either species have a minor role in general mRNA deadenylation and that Ccr4 orthologs might be used for gene-specific mRNA regulation. Taken together, this picture contrasts strongly with the unicellular organism *S. cerevisiae* and suggests that a situation similar to nematodes may have been preserved in flies and humans.

An organ that is very susceptible to the loss of Ccr4–Not complex activity is the gonad. For example, *Drosophila* CCR4 is

crucial in oocytes for early stages of oogenesis (Morris et al., 2005; Zaessinger et al., 2006) and one of the two mammalian CAF-1 homologs, CNOT7, is essential in Sertoli cells to support mouse spermatogenesis (Berthet et al., 2004; Nakamura et al., 2004). In *C. elegans*, genetic elimination of *ccf-1* or *ntl-1* activity precludes an assessment of gonadal defects in the adult. However, we find that RNAi knockdown of *ccf-1* or *ntl-1* activity in hermaphrodites leads primarily to strong female gametogenesis defects, resulting in very small brood sizes or sterility. While elimination of the CCR-4 deadenylase causes a smaller brood size than wild type, we did not observe morphological oogenesis defects similar to *ccf-1(RNAi)* or *ntl-1(RNAi)* animals. By contrast, mating tests suggested that a reduction of fertility might be linked to later stages of oogenesis, such as oocyte ovulation or fertilization. Even unrecognized defects in somatic cells of the gonad may be causal. Although an involvement of *ccf-1* and *ccr-4* in post-transcriptional regulation of mRNA-specific gene expression has been suggested in *C. elegans* (Schmid et al., 2009; Suh et al., 2009; Zanetti et al., 2012), it remained unclear how prevalent Ccr4–Not complex-mediated deadenylation is at the global level. The combined biological and molecular results of this study argue for an evolutionarily conserved need of regulated mRNA poly(A) tail shortening in female germ cells that is provided broadly by CCF-1 of the Ccr4–Not complex, and probably fine-tuned by CCR-4 deadenylase activity. In parallel to a general requirement of the Ccr4–Not deadenylase complex, gene-specific deadenylation as part of translational control mechanisms may underlie observed oogenesis defects, as described in other organisms (for a recent review see Richter and Lasko, 2011).

The role of additional deadenylases in *C. elegans* reproduction

The other three deadenylases characterized in this work, *panl-2*, *parn-1* and *parn-2*, have no obvious role in the general development of *C. elegans*. Mutants of a potential Pan2–Pan3 deadenylase complex, and double mutants of both PARN enzymes, are homozygous viable with no obvious somatic phenotypes. This lack of an apparent biological need for the Pan2–Pan3 deadenylase complex or PARN deadenylases in nematodes is consistent with observation from yeast (Brown et al., 1996; Reverdatto et al., 2004), suggesting that these deadenylase enzymes may have specialized biological roles in physiology, rather than during animal development. Consistent with this idea, we observed smaller brood sizes for *panl-2* and *parn-1* mutants grown at elevated temperatures, indicating a demand for the Pan2–Pan3 deadenylase complex and PARN-1 under stress conditions.

How could PANL-2 and PARN-1 function at the molecular level to promote stress resistance? While mammalian PAN2 is mainly cytoplasmic, PARN is primarily nuclear in tissue culture cells (Berndt et al., 2012; Yamashita et al., 2005). As part of the mRNA decay pathway, Pan2 is proposed to initiate deadenylation of mRNAs, which is then followed by CCR4-mediated poly(A) shortening (Brown and Sachs, 1998; Yamashita et al., 2005). This process may not be conserved in *C. elegans* for global mRNA degradation, as we did not detect anticipated bulk mRNA poly(A) metabolism changes in Pan2–Pan3 complex mutants. However, under stress conditions the need for poly(A) tail shortening and mRNA degradation might be increased and mRNA deadenylation may have to be as efficient as possible. Therefore, *panl-2* could facilitate efficient cytoplasmic mRNA turnover at elevated temperatures together with the Ccr4–Not complex. Alternatively, bulk deadenylation via Ccr4–Not may be inhibited under certain stress conditions (Bönisch et al., 2007). At the functional level, PARN was associated with nuclear degradation of mRNAs in response to DNA damage in humans and osmotic stress in plants (Cevher et al., 2010; Nishimura et al., 2005). By analogy, *C. elegans parn-1* could be involved in nuclear deadenylation and degradation of mRNAs at elevated temperature to coordinate a cellular stress response. However, this role of PARN-1 may be antagonized by PARN-2, given the genetic interaction in regard to fertility. Future work will have to clarify the biological roles and relationship of nuclear deadenylases.

In summary, the largest and most conserved Ccr4–Not complex is also the most important deadenylase for general mRNA poly(A) tail removal in *C. elegans*. The strong correlation between mRNA deadenylation and germline developmental defects suggests that Ccr4–Not-mediated poly(A) tail shortening is an essential process for the reproduction of multicellular organisms. Other deadenylases play only minor roles for general development, if at all. Their biological importance is only apparent in environmental stress situations in *C. elegans*, indicating a functional diversification of the enzymatic class of deadenylases.

Materials and Methods

Nematode strains and transgenesis

Worms were handled according to standard procedures and grown at 20°C unless otherwise stated (Brenner, 1974). The N2 strain was used as a reference for wild type. Strains used in this study: LG *II*: *parn-2(tm1339)*, LG *III*: *glp-1(q224)*, *panl-2(tm1575)*, *panl-3 (tm1182)*; LG *IV*: *ccr-4(tm1312)*, *him-8(e1489)*; LG *V*: *parn-1(tm869)*. All *parn-x* and *panl-x* alleles were out crossed three times, *ccr-4(tm1312)* was outcrossed nine times. Based on our cDNA analysis, no functional protein is produced from any *parn-x* and *panl-x* deletion allele and they are expected to represent enzymatic null alleles. Adult germline phenotypes of homozygote animals were scored 24 hrs past L4. For additional information see supplementary material Table S2.

The NTL-1::LAP-tagged fosmid was obtained from the *C. elegans* TransgeneOme platform (Sarov et al., 2012). Transgenic animals were created by microparticle bombardment as described (Praitis et al., 2001). Three independent lines (EV465–467) were established and EV465 was used for further analysis.

For brood size analysis, L4 animals were singled and passaged to a new plate every 24 hrs until the mother stopped laying embryos. Living larvae were counted to assess brood size.

RNAi feeding constructs and procedure

The feeding construct against *ccr-4* and *fog-1* were described previously (Schmid et al., 2009). Full-length *ccf-1* or nt 5868–7584 from *ntl-1* were amplified from whole-worm cDNA and cloned into pL4440. The plasmids were transformed into HT115(DE) bacteria and induced with IPTG as described (Kamath and Ahringer, 2003). For *fog-1*, *ccr-4* and *ccf-1* RNAi-treatment, wild-type or NTL-1::LAP transgenic animals were fed from L1 stage onwards and analyzed 24 hrs past L4. For *ntl-1(RNAi)*, wild-type or NTL-1::LAP transgenic animals were placed on RNAi plates at the L4 stage and analyzed 24 hrs later.

Primary antibodies

Primary antibodies against the following proteins were used: rabbit anti-ANI-2 [(Maddox et al., 2005), a gift from Antony Hyman], goat anti-GFP (a gift from David Drechsel), chicken anti-GLH-2 [(Gruidl et al., 1996), a gift from Karen Bennett], anti-NPC (mAb414, Covance) and anti-FLAG M2 epitope (Sigma-Aldrich). The guinea pig anti-OMA (SAC38) serum was raised against the following peptides, NVNGENNEKIDEHHLC (OMA-1) and ETVPEEQQKPISHIDC (OMA-2) at Eurogentec (Belgium). Monoclonal antibodies were generated at MPI-CBG by immunizing mice with a bacterially expressed fusion-peptide that corresponded either to full-length CCF-1 (aa 1–310) or the C-terminus of CCR-4 (aa 202–606). The following clones were used in this work: anti-CCF-1 (mo2448-G25-1) and anti-CCR-4 (mo2483-B77-1).

Immunocytochemistry

Indirect immunocytochemistry of extruded and 1% PFA-fixed gonads was carried out in solution as described (Rybarska et al., 2009). Images were taken on a Zeiss

Imager M1 equipped with an Axiocam MRm (Zeiss) and processed with AxioVision (Zeiss) and Photoshop CS3 (Adobe). Secondary antibodies coupled to fluorochromes FITC, CY3 and CY5 were purchased from Jackson Laboratories (Dianova).

Western blotting and immunoprecipitations

For standard western blotting experiments, we collected individual worms by hand and boiled them in protein sample loading buffer prior to gel separation. Worm protein extracts for protein co-immunoprecipitations were made as described (Jedamzik and Eckmann, 2009), with a minor modification of the procedure; to generate liquid nitrogen-frozen worm powder we used a MR301 mill at 30 hertz (Retsch). For the immunoprecipitation procedure we coupled goat anti-GFP and mouse anti-CCF-1 antibodies to Protein G or A Dynabeads (Invitrogen), respectively. All immunoprecipitates were generated and analyzed by western blotting with ECL detection of HRPO-coupled secondary antibodies (Jackson Laboratories) as described (Jedamzik and Eckmann, 2009).

RNA isolation and northern blotting

Total RNA was isolated from whole worms by using Trizol (Invitrogen). For northern blotting, 10 μg of total RNA from wild-type hermaphrodites or *him-8(e1489)* males were treated with Terminator 5′-Phosphate-Dependent Exonuclease (Epicentre) to reduce ribosomal background. RNA transfer was performed according to standard protocols. Membrane hybridization was done using DIG Easy Hyb suspension (Roche #11796895001). For detection we used the DIG Wash and Block Buffer Set (Roche #11585762001), a 1:10,000 diluted anti-DIG antibody (Roche, #1093274) and 0.25 mM CDP-Star (Roche #1685627). RNA antisense probes were produced in an *in vitro* transcription reaction using dioxigenin-labeled (DIG) rNTPs according to the manufactures protocol (Roche #11277073910). Template sequences were generated by PCR form cDNA. Primer sequences are available upon request.

Bulk poly(A) tail-length measurements

One μg of total RNA was used to perform bulk poly(A) tail measurements following a previously described protocol (Temme et al., 2004), with the only exception that un-incorporated α^{32}P-cordycepin triphosphate (Perkin Elmer) was removed using Mini Quick Spin Columns (Roche). Each sample was analyzed from three independent biological repeats. The size markers were synthesized RNA oligos of 30 and 45 nucleotides in length and a loading dye band that corresponds to ~65 nts.

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Author contributions

M.N. and C.E. designed the experiments. M.N., N.T., D.H. and S.M. performed the experiments. M.N., D.H. and C.E. analyzed the data. M.N. and C.E. wrote the manuscript.

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Journal of Cell Science

3.2. The cytoplasmic poly(A) polymerases GLD-2 and GLD-4 promote general gene expression via distinct mechanisms

Overarching question

Which roles do cytoplasmic poly(A) polymerases have in mRNA regulation?

Synopsis of the publication

Cytoplasmic poly(A) polymerases are important enzymes for germ cell biology. For example, in *C. elegans* the two cytoPAPs GLD-2 and GLD-4 are crucial for processes such as meiotic entry and progression (KADYK AND KIMBLE 1998; WANG *et al.* 2002; SCHMID *et al.* 2009). Based on the severity of the defects that are observed in the absence of these two enzymes it has been envisioned that GLD-2 and GLD-4 are important regulators for mRNAs in germ cells. However, little was known about how these enzymes influence mRNA fates and promote gene expression in these cells. Therefore, I combined whole-genome RNA analysis with polysome profiling to explore the molecular mechanisms of GLD-2 and GLD-4 mediated RNA regulation. I investigated the impacts that a reduction of GLD-2 and GLD-4 has on abundances and translational efficiencies of germ cell mRNAs. Interestingly, the analysis showed that GLD-2 primarily affects mRNAs abundance, whereas GLD-4 affects general translation. Additionally, general RNA polyadenylation is strongly promoted by GLD-2 and only mildly by GLD-4. The overall data suggest that GLD-2- and GLD-4-type cytoPAPs influence gene expression with fundamentally different mechanisms. Furthermore, this work establishes GLD-2 as the main enzyme that is utilized for polyadenylation-mediated mRNA regulation in germ cells.

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The cytoplasmic poly(A) polymerases GLD-2 and GLD-4 promote general gene expression via distinct mechanisms

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ABSTRACT

Post-transcriptional gene regulation mechanisms decide on cellular mRNA activities. Essential gatekeepers of post-transcriptional mRNA regulation are broadly conserved mRNA-modifying enzymes, such as cytoplasmic poly(A) polymerases (cytoPAPs). Although these non-canonical nucleotidyltransferases efficiently elongate mRNA poly(A) tails in artificial tethering assays, we still know little about their global impact on poly(A) metabolism and their individual molecular roles in promoting protein production in organisms. Here, we use the animal model *Caenorhabditis elegans* to investigate the global mechanisms of two germline-enriched cytoPAPs, GLD-2 and GLD-4, by combining polysome profiling with RNA sequencing. Our analyses suggest that GLD-2 activity mediates mRNA stability of many translationally repressed mRNAs. This correlates with a general shortening of long poly(A) tails in *gld-2*-compromised animals, suggesting that most if not all targets are stabilized via robust GLD-2-mediated polyadenylation. By contrast, only mild polyadenylation defects are found in *gld-4*-compromised animals and few mRNAs change in abundance. Interestingly, we detect a reduced number of polysomes in *gld-4* mutants and GLD-4 protein co-sediments with polysomes, which together suggest that GLD-4 might stimulate or maintain translation directly. Our combined data show that distinct cytoPAPs employ different RNA-regulatory mechanisms to promote gene expression, offering new insights into translational activation of mRNAs.

INTRODUCTION

One of the unifying features of living organisms is their ability to regulate gene expression programs. Prior to protein production, many regulatory possibilities exist that operate at the transcriptional or post-transcriptional level, which are mediated by mechanisms that target DNA or RNA, respectively. The global importance of these two distinct regulatory modes appears to differ between tissues. Many somatic cell types primarily use transcriptional control mechanisms, whereas numerous examples in neurons and germ cells highlight a prevalence of post-transcriptional control mechanisms. However, the underlying functional mechanisms and their contribution to the global level of post-transcriptional gene expression regulation remain to be determined.

Messenger RNAs (mRNAs) mature in the nucleus and serve as templates for ribosome-mediated protein synthesis in the cytoplasm. However, mRNAs subjected to post-transcriptional control are withheld from entering the translational pool via targeted degradation or translational repression. Although both processes result in the down-regulation of protein levels, they are mechanistically distinct: mRNA degradation results in the final destruction of the mRNA template and is irreversible; translational repression stabilizes the target and is reversed in a process termed translational activation. Due to the flexibility of translational control, mRNA repression and activation mechanisms provide an immediate mode of gene expression regulation in dynamic biological systems.

An mRNA intrinsic feature that registers repressive and active translational control mechanisms is its poly(A) tail. Originally added in the nucleus (1), this homopolymer of adenosines at the 3′end is subject to distinct length changes influencing cytoplasmic mRNA fates. Especially in animals, poly(A) tail shortening is a key step in the mRNA decay pathway (2), establishing a clear relationship between longer poly(A) tail lengths and increased stability. Poly(A) tail length is also correlated with translational efficiency. Longer poly(A) tails enhance protein synthesis in many *in vitro* translation extracts (3). Furthermore, in developmental contexts gene expression regulation is strongly connected to poly(A) tail extension. For example, during early *Drosophila* embryogenesis or *Xenopus* oocyte maturation,

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gene-specific poly(A) shortening or elongation correlates with the protein amounts needed in the following developmental stage (4,5). This leads to the generalization that mRNAs with long poly(A) tails are better translated than short ones. However, no global correlation between poly(A) tail length and translation efficiency is observed in somatic tissue culture systems or during late stages of embryonic development (6). Only during early developmental stages, when transcriptional regulation is not present yet, a strong correlation between long poly(A) tails and high translational efficiency is detected (6). This suggests that global mechanisms of gene expression regulation, involving mRNA poly(A) tail-length changes, are best revealed in systems where post-transcriptional control is the dominant mode of gene expression.

Cytoplasmic poly(A) polymerases (cytoPAPs) represent a class of enzymes that are proposed to post-transcriptionally elongated poly(A) tails of mRNAs. Two cytoPAPs have been described so far in animals, GLD-2 and GLD-4 (7–9). Both proteins belong to two distinct, evolutionary conserved protein families of non-canonical nucleotidyltransferases that contain no sequence homology outside their enzymatic regions (10) (Figure 1A and B). Moreover, both lack predictable RNA-binding domains and, therefore, are hypothesized to rely on interactions with RNA-binding proteins to establish efficient contact with mRNA targets (7,8). For GLD-2, strong polyadenylation activity has been detected in tethering assays when probing the nematode, fly, frog and mammalian homologs (11,12). For GLD-4, only the enzyme of the nematode *Caenorhabditis elegans* was tested in such an assay; polyadenylation depended on its intact nucleotidytransferase domain and required the species-specific co-factor, GLS-1 (8). The *in vivo* function of either GLD-2 or GLD-4 as cytoplasmic poly(A) polymerases is apparent from the polyadenylation defects of specific mRNA targets in the corresponding mutants or RNAi-mediated knockdowns (9,13–19). Although it was recently suggested that GLD-2 might target numerous mRNAs in *C. elegans* and *Drosophila melanogaster* (14,18), very few mRNA targets have been reported for *C. elegans* and human GLD-4 (8,9,15,19). The global role(s) and functional mechanism(s) of either cytoPAP still remain(s) unclear.

In this work, we address the role of *C. elegans* GLD-2 and GLD-4 in global mRNA regulation. We combine polysome profiling with RNA sequencing to identify GLD-2- and GLD-4-dependent changes in mRNA abundance and translation. We find that GLD-2 primarily stabilizes mRNAs that are translationally repressed. Furthermore, GLD-2 strongly promotes bulk polyadenylation. Surprisingly, these functions of GLD-2 seem to have little impact on stimulating efficient target mRNA translation. By contrast, we find that GLD-4 promotes bulk polyadenylation only mildly and has no major role in promoting general mRNA stability. However, GLD-4 is needed for efficient polysome formation and general mRNA translation. Taken together, our data indicate that GLD-2 and GLD-4 use two distinct mechanisms to promote gene expression in germ cells.

MATERIALS AND METHODS

Strains and RNAi feeding

Worms were handled according to standard procedures and grown at 20°C unless otherwise stated (20). Mutations used in this study: LG *I*: *gld-2(q497)*, *gld-4(ef15)*, *gls-1(ef8)*, LG *III*: *glp-1(q224ts)*. With the exception of the *glp-1* mutation, all others were kept as heterozygotes over the *hT2[qIs48] I;III* balancer and homozygote F1 progeny was analyzed. Bristol N2 served as the reference wild-type strain. For all analysis, we synchronized L1 animals by starvation and harvested them after growing on feeding plates 24 h after the mid-L4 stage. The feeding constructs against *ccr-4*, *ccf-1*, *gld-4* and *fem-3* were described previously (8,21,22). The empty pL4440 vector represented control RNAi. The plasmids were transformed into HT115(DE) *Escherichia coli* cells and double-stranded RNA production was induced with IPTG according to standard methods.

Immunoblotting and antibodies

Primary antibodies against the following proteins were used: anti-GLD-2 (19), anti-GLD-4 (8), anti-α-tubulin (T5168, Sigma), rabbit anti-GLS-1 (22), anti-eIF2α (23) and anti-GST (MPI-CBG, antibody facility). Polyclonal antibodies against the two similar proteins PAB-1 and PAB-2 were generated by immunizing rabbits with a recombinant GST-tagged fusion peptide of PAB-2 (aa 517 to 692). The serum's specificity and cross-reactivity was verified by comparing *pab-1(RNAi)* or *pab-2(ok1851)* mutant extracts with that of wild-type. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch.

Sucrose gradient centrifugation

Whole worm extracts were prepared from age-matched adult worms as previously described (24). Equal amounts of total cellular protein (20 mg) were resolved through a 10 ml 17–50% sucrose gradient (25). The gradients were spun for 210 min at 30 000 rpm and 4°C in a SW40Ti rotor (Beckman Coulter). The fractionation was conducted bottom up while the absorbance profile at 260 nm was recorded. For RNA isolations, the samples were fractionated directly into three volumes of ethanol, and 150 pg of *in vitro* transcribed firefly luciferase mRNA (FFluc) was added to each fraction. No FFluc spike-in mRNA was added to the input samples. For protein analysis, 50 ng of purified GST peptide was added to each fraction as a proxy to survey sample precipitation efficiency. GST-protein purification was conducted as previously described (25).

RNA handling and bulk poly(A) tail length measurements

The ethanol/gradient mixture of input and gradient material was incubated overnight at −20°C and pelleted in a benchtop centrifuge for 20 min at 16 000 x *g* and 4°C. The pellets were TRIzol (Invitrogen) extracted following the manufacturer's protocols. The resulting RNA was either sequenced by the Dresden Genome Center on an

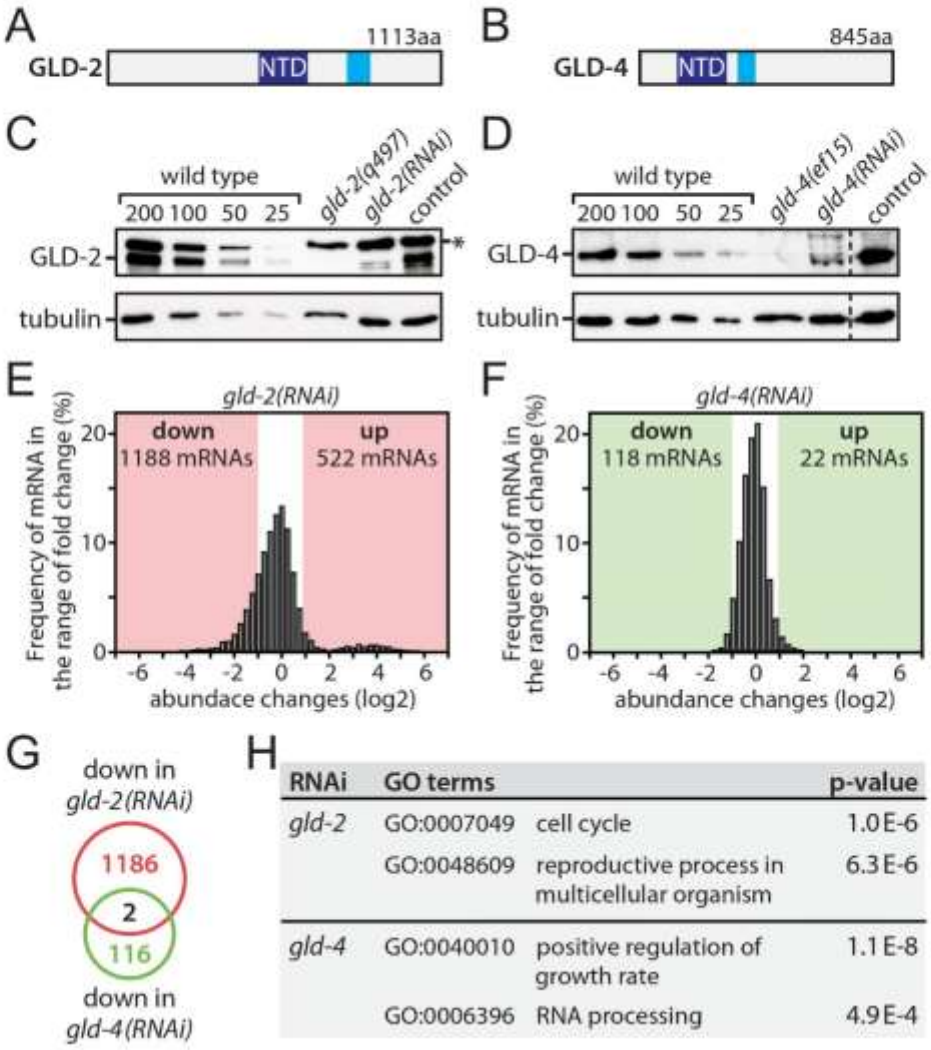

Figure 1. *gld-2* but not *gld-4* promotes mRNA abundance. (**A** and **B**) Domain structure of GLD-2 and GLD-4 proteins: dark blue–nucleotidyl transferase domain (NTD), light blue–poly(A) polymerase-associated domain. (**C** and **D**) Western blot analysis of protein levels in RNAi-treated adults. The asterisk marks a non-specific background band. Representative images are shown (n = 3). (**E** and **F**) mRNA abundance changes in RNAi-treated animals. All detectable 7649 genes are shown and the number of significant abundance changes is indicated. (**G**) The overlap of down-regulated genes between *gld-2(RNAi)* and *gld-4(RNAi)* is shown. (**H**) A GO-term analysis for *gld-2(RNAi)* and *gld-4(RNAi)* down-regulated genes was conducted and two representative categories are shown for each.

HiSeq2000 (Illumina) platform or reverse transcribed using random hexamer primers and RevertAid Premium reverse transcriptase (Fermentas), according to the manufacturer's protocols. Quantitative PCR (qPCR) was conducted on a Mx3000P qPCR system (Stratagene) using the ABsolute QPCR SYBR Green mix (Thermo) and gene-specific primers (sequences available upon request). All gradient data were normalized to the FFluc spike-in mRNA control. For bulk poly(A) tail measurements, total RNA was isolated from hand-picked adults using the TRIzol method. One microgram of total RNA was used in the 3′ end labeling assay and processed according to the published methods (26), with the only exception that un-incorporated α^{32}P-Cordycepin (Perkin Elmer) was removed using Mini Quick Spin Columns (Roche).

Library preparation and next-generation sequencing (NGS)

mRNA was isolated from 2 ug of total RNA by poly-dT25 enrichment using the NEBNext Poly(A) mRNA Magnetic Isolation Module according to the manufacturer's instructions and eluted in 15 ul 2-times first-strand cDNA synthesis buffer (NEBNext, NEB). After chemical fragmentation for 15 min at 94°C, the sample was

directly subjected to the workflow for strand-specific RNA-Seq library preparation (Ultra Directional RNA Library Prep, NEB). After ligation of custom adaptors, unused adapters were depleted by a 1-times XP-bead purification (Beckman Coulter); adaptor-oligo1: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′, adaptor-oligo2: 5′-P-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3′. Indexing was done during the following PCR enrichment (15 cycles) using custom amplification primers, carrying the index sequence indicated with 'NNNNNN'; primer1: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT, primer2: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT, primer3: CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTT. After two more 1-times XP-bead purifications, libraries were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen). For Illumina flowcell production, samples were equimolarly pooled and distributed on all lanes used for 75-bp single-read sequencing on Illumina HiSeq 2000.

Analysis of NGS data

The quality of the NGS data was analyzed using the fastqc software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). TruSeq adapter were removed using cutadapt (version 1.2.1) (27). The fastq files were mapped to the *C. elegans* genome downloaded from Ensembl (WS220) using the TopHat2 algorithm (version v2.0.10) allowing only unique mapping (28). Using the featureCount algorithm from the SubRead software package (version 1.4.0), the reads were counted from the bam files (tophat2 output) on exon-level based on the gene annotation from Ensembl, resulting in a read count for each gene (29). Using the DESeq package (R 3.0.2, DESeq version 1.14.0), the count data were normalized by the size factor to estimate the effective library size (30). After calculating the gene dispersion across all samples, the comparison of two different conditions resulted in a list of differentially expressed genes. Genes with a normalized read count smaller than 100 were ignored in the final analysis. A pre-filtering step was used to calculate the number of genes showing a high probability of being differentially regulated. In this step, genes with a probability (unadjusted *P*-value) above 0.003 were excluded from the differential expression analysis. Genes with a fold-change higher or equal to two, as well as an adjusted *P*-value of $<= 0.05$ was then defined as differentially expressed. The *P*-values are being adjusted to the multiple testing hypotheses to reduce the false discovery rate (FDR). For the analysis of the translation efficiency, we needed to correct the analysis for the bias between the non-polysomal (NP) and polysomal (P) fractions. We calculated a correction factor based on the amounts of FFluc spike-in to adjust the NP and P data for each sample.

RESULTS

GLD-2, but not GLD-4 activity stabilizes mRNAs

The animal model, *C. elegans*, is a well-established organism to study post-transcriptional gene regulation in germ cells (31). The adult animal is a self-fertile hermaphrodite and its fully developed germline tissue constitutes a large proportion of its biomass, allowing for the detection of germline-specific gene expression changes even in whole animal extracts. GLD-2 and GLD-4 are predominantly but not exclusively expressed in the germ line, and are present at almost all stages during germ cell development (7,8). To assess and compare the influence of the two cytoplasmic poly(A) polymerases on general mRNA abundance, we performed RNAi-knockdown experiments followed by RNA sequencing. The technique of RNAi feeding was used because large quantities of synchronized animals were needed for our experiments, which is difficult to obtain from *gld-2* and *gld-4* homozygote mutants.

We analyzed young adults that were fed with either control RNAi, *gld-2(RNAi)* or *gld-4(RNAi)*. A strong cytoPAP protein knockdown was observed by western blot analysis: less than ~10% of both GLD-2 (Figure 1C and Supplementary Figure S1A) and GLD-4 (Figure 1D and Supplementary Figure S1A) remained in RNAi-treated animals. In agreement with previous studies, *gld-2(RNAi)*-treated animals were sterile with somewhat less severe germline phenotypes compared to the genetic null mutant *gld-2(q497)* (data not shown) (14). The milder *gld-2(RNAi)* phenotype is considered an advantage for the expression analysis as it presumably reduces indirect effects arising from developmental changes during late oogenesis. In comparison to the strong-loss-of-function mutant *gld-4(ef15)* and consistent with previous observations (8,19), the germline defects of *gld-4(RNAi)* animals were less severe and the animals were fertile. No overt somatic defects were apparent in either cytoPAP RNAi-treated animals.

Next, we measured mRNA levels by isolating total RNA from RNAi-treated animal extracts, followed by mRNA enrichment and non-strand-specific RNA sequencing. In *gld-2(RNAi)*, reads could be mapped to 15 620 genes. In *gld-4(RNAi)*, 11 791 genes could be detected (Supplementary Data S1). Both gene lists were combined, and after removal of low expressing genes, 7649 high confidence genes were used for further analysis (Supplementary Data S1). When comparing *gld-2(RNAi)* to control RNAi, widespread RNA changes in abundance were observed: 1188 mRNAs were reduced and 522 mRNAs increased upon GLD-2 reduction (Figure 1E). By contrast, overall mRNA abundance changes in *gld-4(RNAi)* were much less frequent and less strong in amplitude: 118 mRNAs were reduced and 22 mRNA are increased (Figure 1F and Supplementary Figure S1B). The overlaps between the *gld-2(RNAi)* and *gld-4(RNAi)* expression changes were quite small (Figure 1G and Supplementary Figure S1C–E), suggesting that distinct mRNA populations are affected by the reduction of either cytoPAP protein. This view is further supported by a Gene Ontology (GO) annotation analysis of down-regulated mRNAs in *gld-2(RNAi)* and *gld-4(RNAi)* showing that different biological processes are affected (Figure 1H). A GO-term analysis of the *gld-2(RNAi)* up-regulated genes yielded as the top-scoring term pseudopodium (GO:0031143) (data not shown), pseudopod formation is the final process of spermatogenesis, suggesting that many of these genes might be increased due to indirect effects.

To focus our analysis on mRNA expression changes in the germ line, we conducted the same analysis on 2243 previously defined germline-enriched genes (32) (Supplementary Data S1). Similar to the entire dataset, germline genes show broad expression changes in *gld-2(RNAi)* with 444 mRNAs down and 283 up-regulated (Supplementary Figure S1F), and only mild changes in *gld-4(RNAi)* with 54 mRNA down and 7 up-regulated (Supplementary Figure S1G). We find that the majority of up-regulated mRNAs in the *gld-2(RNAi)* are functionally connected to spermatogenesis (237 out of 283 genes). This is consistent with *gld-2* null mutant defects (33), and the observed sterility in *gld-2(RNAi)* animals, which is partly due to spermatogenesis arrested germ cells. By contrast, wild-type germ cells complete spermatogenesis in the last larval stage before adulthood to produce fertilization-competent spermatozoa. Therefore, the increase of spermatogenic mRNAs in *gld-2(RNAi)* animals is likely due to later developmental arrests that are secondary to an initial molecular requirement of GLD-2 during earlier stages of spermatogenesis. Alternatively, indirect effects may also arise from GLD-2's function to promote a negative regulator of RNA stability, as documented for hGLD2 (9). Moreover, some down-regulated mRNAs are likely indirect due to changes in female germline development. However, we also noticed that *gld-2*-dependent mRNAs are enriched for previously identified GLD-2-associated mRNAs; of the 538 GLD-2-associated mRNAs, 272 are significantly reduced in our complete dataset (Figure 2A and Supplementary Figure S1H) (14), arguing that they likely represent direct targets of GLD-2 control. Hence, throughout the rest of this work, we will refer to them as GLD-2-stabilized mRNAs. To test whether the decrease in mRNA abundance is due to a bias in the RNA sequencing procedure, we analyzed the mRNA levels of 18 exemplary genes via quantitative RT-PCR (qRT-PCR), using random hexamers for reverse transcription. Most down-regulated mRNAs (12/14) and none of the negative controls (4/4) were significantly reduced in *gld-2*-compromised animals (Figure 2B). Taken together, these data suggest that *gld-2* but not *gld-4* activity is important to maintain or up-regulate the abundance of many mRNAs.

GLD-2 but not GLD-4 promotes strong bulk poly(A) tail elongation

The poly(A) tail status of several developmentally and post-transcriptionally controlled germ cell mRNAs depends on *gld-2* and *gld-4* activity (13–15). To assess whether these findings are also detectable on a global scale, we performed bulk poly(A) tail measurements. To this end, total RNA was isolated from different genotypes or RNAi-treated young adults, radioactively end-labeled with cordycepin, partially digested with RNases A/T1 and the remaining poly(A) sequences were analyzed on sequencing gels (21). Due to the sensitivity of this method, we also included genetic mutations of *gld-2* and *gld-4* to strengthen our analysis.

In the wild-type, bulk poly(A) tails extended on average from ~20 up to ~100nt in length (Figure 3A and B, lane 1). As previously described (21), this distribution is shifted toward longer tails in deadenylation-compromised *ccr-4(RNAi)* or *ccf-1(RNAi)* animals (Figure 3A, lane 5 and 6). The opposite effect was observed in *gld-2(q497)* mutants and *gld-2(RNAi)* animals: bulk poly(A) tails were shortened to ~20–40 nucleotides (nts), and poly(A) tails above ~40 nts were strongly reduced in abundance (Figure 3A, lane 2 and 3, and 3C). An obvious difference between *gld-2*-compromised animals to wild-type is the absence of embryos (33). To exclude that a lack of embryos may have biased our measurements, we analyzed *fem-3(RNAi)* animals that produce no embryos, due to the lack of sperm (34). However, the overall poly(A) tail profile of feminized animals was comparable to wild-type (Figure 3B, compare lanes 1 and 3, and Supplementary Figure S2C), suggesting that the observed dramatic *gld-2*-dependent poly(A) tail shortening is not a consequence of sterility.

In *gld-4(ef15)* mutants and *gld-4(RNAi)* animals, only a mild overall reduction of bulk poly(A) tails was detected (Figure 3A, lane 4, and 3D; data not shown). In comparison to wild-type, the overall *gld-4* poly(A) profile primarily differed in a reduction of lengths below ~35 nts. Compared to *gld-2*, poly(A) tails beyond ~40 nts were less severely affected. Last, as GLD-2 and GLD-4 are mainly expressed in germ cells, we assessed the contribution of the germline tissue to bulk poly(A) profiles by comparing wild-type to *glp-1(q224ts)* animals, which at the restrictive temperature do not develop a germ line (35). In germline-less *glp-1(q224ts)* young adults, only a mild reduction of bulk tails was observed compared to wild-type at the non-permissive temperature (Supplementary Figure S2A and B), affirming that our assay in general detects poly(A) changes that primarily originate from the germline tissue. Taken together, our bulk poly(A) measurements reveal that GLD-2 strongly and GLD-4 mildly promote general mRNA polyadenylation in germ cells.

GLD-2 stabilizes poorly translated mRNAs

An important aspect of translational control is the regulated access of mRNAs to ribosomes. The translational status of mRNAs can be analyzed by polysome profiling. This technique measures the distribution of mRNAs in a sucrose gradient where ribosomes are separated into active and inactive fractions, based on their density and shape (36). We performed this analysis with extracts prepared from synchronized young adults and compared the known translationally regulated *gld-1* mRNA to the presumably unregulated *rpl-25.2* mRNA. The majority of *gld-1* was present in the non-polysomal region, whereas *rpl-25.2* was mainly detected in the polysomal region of the gradient (Figure 4A). This demonstrates that mRNAs subjected to translational control can be clearly separated from unregulated mRNAs.

To analyze the translational status of mRNAs at the global level, we performed RNA-sequencing analysis of mRNAs from pooled fractions of the gradient that correspond to non-polysomal (NP) and polysomal (P) regions (Figure 4A) and mapped 15 849 genes in the control RNAi sample. The relative number of NP- to P-enriched genes is indicative of how many mRNAs are translated with low or high efficiency. In control RNAi, a comparison of the NP- to the P-dataset for all as detectable defined 7649 mRNAs shows that 537 mRNAs are at least 2-fold enriched in

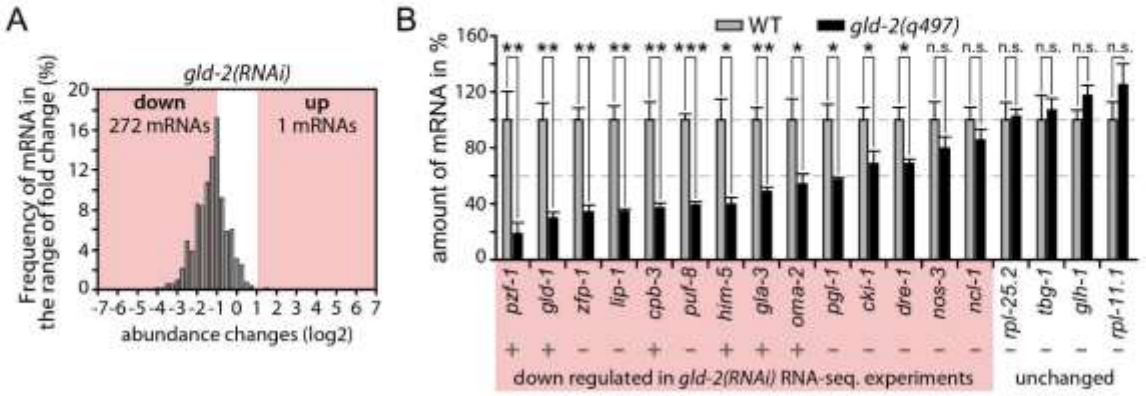

Figure 2. GLD-2-associated mRNAs are less abundant. (**A**) Abundance changes of 538 previously described GLD-2-associated mRNAs (14) in our *gld-2(RNAi)* dataset. (**B**) RT-qPCR measurements of randomly selected down-regulated and unchanged genes, comparing mRNA abundance in *gld-2(q497)* mutants to wild-type. A plus sign indicates previously proposed GLD-2-associated mRNAs (14). Shown is the mean (±SD) of three independent experiments. Significance was calculated with a Student's *t*-test: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., not significant.

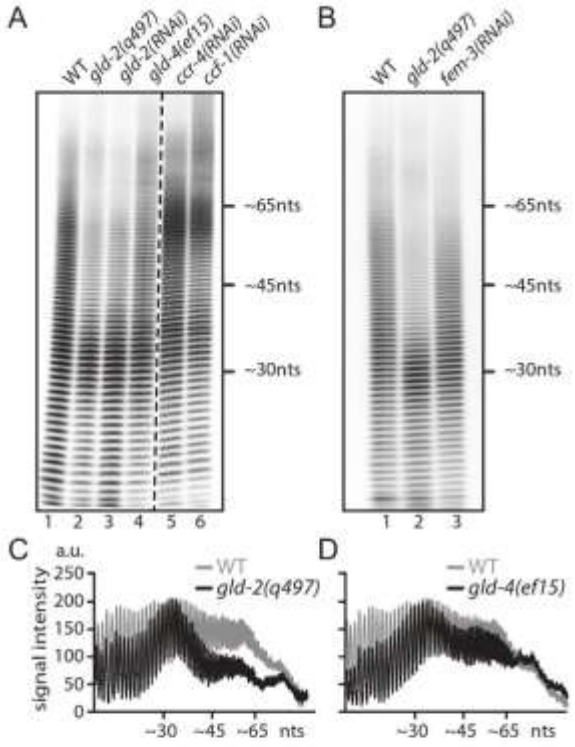

Figure 3. *gld-2* promotes bulk mRNA poly(A) tail extension. (**A** and **B**) Representative gels of at least three independent bulk poly(A) tail measurements. Equal amounts of radioactivity were loaded for each sample. WT, wild-type. (**C** and **D**) Line scans of bulk poly(A) profiles from (A).

the NP-fraction, and 2406 mRNAs in the P-fraction giving an NP/P ratio of 1:4.5 (Figure 4B and Supplementary Data S1), suggesting that the majority of all mRNAs are well translated. A different distribution is observed for germline-enriched mRNAs: 289 versus 404 mRNAs are enriched in the NP- and P-fraction, respectively, resulting in an NP/P ratio of 1:1.4 (Figure 4B and E). This indicates a shift toward non-polysomal enrichments for germline mRNAs. This trend is even more distinct for GLD-2-stabilized germline mRNAs: 133 versus 13 genes are enriched in the NP- and P-fraction, respectively, resulting in an NP/P ratio of ~10:1 (Figure 4C and E). By contrast, GLD-4-stabilized germline genes do not show this behavior: six versus six genes are enriched in the NP- and P-fraction, respectively. With an NP/P ratio of 1:1, they follow the trend of all germline genes (Figure 4D and E). This suggests that germline mRNAs are poorly translated in general. Moreover, only GLD-2 but not GLD-4 preferentially stabilizes germline mRNAs that are translated with low efficiency.

mRNAs that accumulate in the NP fraction might be targets of translational repression. Hence, we compared the overlap of less abundant germline mRNAs in *gld-2(RNAi)* and *gld-4(RNAi)* to published whole-genome interaction studies of two well-characterized translational repressors in *C. elegans* germ cells, GLD-1 and FBF-1 (37–39). We found that a large number of GLD-2-stabilized mRNAs are also putative GLD-1 and FBF-1 targets (Figure 4F). This is not the case for GLD-4-stabilized or *gld-2*-insensitive mRNAs (Figure 4F), suggesting that GLD-2 but not GLD-4 cytoPAP preferentially sustains the levels of translationally repressed mRNAs.

GLD-4 promotes general translation efficiency of mRNAs

The poly(A) tail serves to stabilize mRNAs and is implicated in the efficient recruitment of ribosomes (3). To test whether any of the two cytoPAPs has a role in promoting translation, we conducted a gradient analysis of *gld-2(RNAi)* and *gld-4(RNAi)* mRNAs and compared it to control RNAi datasets. We could map reads for 15 907 genes in the *gld-2(RNAi)* sample and 15 899 genes in the *gld-4(RNAi)* sample. We started the analysis with the 7649 genes that were classified as expressed in the input analysis. In *gld-2(RNAi)*, only minor translational changes could

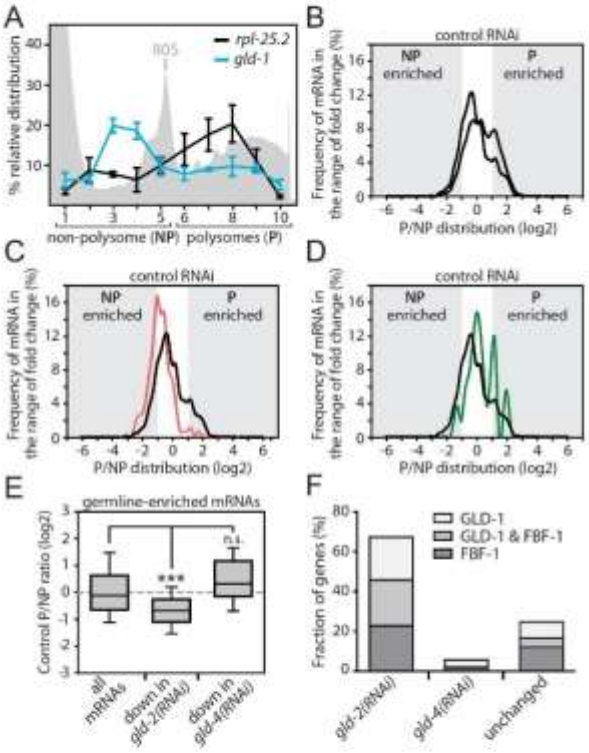

Figure 4. *gld-2* promotes abundance of poorly translated germline mRNAs. (**A**) The relative gradient distribution of *rpl-25.2* and *gld-1* mRNA in animals treated with control RNAi is analyzed by RT-qPCR. Shown is the mean (±SEM) from 10 fractions in three independent experiments. (**B**–**D**) The gradient distribution of different groups of mRNAs was analyzed between polysome (*P*) and non-polysome (*NP*) fractions for control RNAi-treated animals. Shown are (**B**) all detected genes (dotted line–7649 mRNAs), (B, C and D) germline-enriched genes (solid black line–2243 mRNAs), (**C**) germline genes that are less abundant in *gld-2(RNAi)* (solid red line–444 mRNAs) and (**D**) germline genes that are less abundant in *gld-4(RNAi)* (solid green line–54 mRNAs). (**E**) P/NP distribution of the indicated groups of germline mRNAs. Significance was calculated with a Student's *t*-test: ***$P < 0.001$; n.s., not significant. (**F**) A large percentage of GLD-2-stabilized germline mRNAs are also GLD-1 and FBF-1 targets. The percent overlap between GLD-2-regulated mRNAs and putative GLD-1 and FBF-1 target mRNAs are given. mRNAs that are not decreased in *gld-2(RNAi)* are labeled as unchanged.

be detected: 74 mRNAs were increased in the NP-fraction, and 54 mRNAs in the P-fraction (Figure 5A and C). Interestingly, in *gld-4(RNAi)* the overall translation status of mRNAs is shifted toward lighter fractions with 901 mRNAs being significantly enriched in the NP-fraction (Figure 5B and C), suggesting that many mRNAs are less well translated. Similar trends were observed in *gld-2(RNAi)* and *gld-4(RNAi)* for the 2243 defined germline-enriched genes (data not shown). A GO-term analysis of less well-translated mRNAs suggests that different groups of mRNAs are affected in *gld-2*- and *gld-4*-compromised animals (Figure 5D). Taken together, this analysis argues for a potentially broader role of GLD-4 in promoting mRNA translation than for GLD-2.

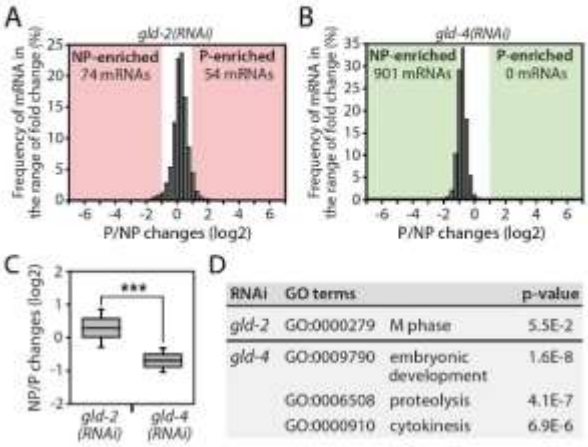

Figure 5. *gld-4* promotes general translation. (**A** and**B**) Translational efficiency changes of all 7649 detectable genes in (A) *gld-2(RNAi)* and (B) *gld-4(RNAi)*. (**C**) Statistical analysis of translational efficiency changes. (**D**) GO-term analysis of NP-enriched mRNAs in *gld-2(RNAi)* and *gld-4(RNAi)*. Student's *t*-test: ***, $P < 0.001$.

To investigate whether mRNAs might rely on a potential combined activity of both cytoPAPs, we ask how many mRNAs are less abundant in *gld-2(RNAi)* and at the same time less well translated in *gld-4(RNAi)*. We found a moderate overlap of 137 mRNAs between these two datasets (Supplementary Figure S3A). A GO-term analysis of these genes revealed an enrichment of genes connected to chromatin organization, the cell cycle and embryonic development (Supplementary Figure S3B), suggesting that a small subset of mRNAs might rely on the combined activity of GLD-2 and GLD-4 for efficient protein expression.

GLD-4 promotes polysome formation

To further corroborate a potential role for either cytoPAP in the process of translation, we used sucrose gradient centrifugation to separate initiation from post-initiation ribonucleoprotein complexes and assessed a potential co-sedimentation of GLD-2 and GLD-4 with either fraction (Figure 6). To reveal the distribution of specific proteins across the gradient, we probed for cytoplasmic poly(A)-binding protein (PABPC), translation initiation factor 2α (eIF2α), both cytoPAPs and the GLD-4-specific cofactor, GLS-1. PABPC is part of initiation and post-initiation mRNA complexes while bound to the poly(A) tail (40). In *C. elegans*, two genes encode PABPC, *pab-1* and *pab-2* (41,42). eIF2α mediates the association of the initiator tRNA with the small ribosomal subunit and serves as a marker for translation initiation complexes (43). In *C. elegans*, eIF2α is encoded by *Y37E3.10* (23).

After gradient centrifugation, proteins from individual fractions of wild-type animals were isolated (Figure 6A). To monitor the efficiency of protein precipitation, we added a purified GST peptide that also served as a loading control. Consistent with PABPC as part of initiation and post-initiation complexes, both *C. elegans* PAB proteins were abundantly detected in light and heavy fractions (Figure

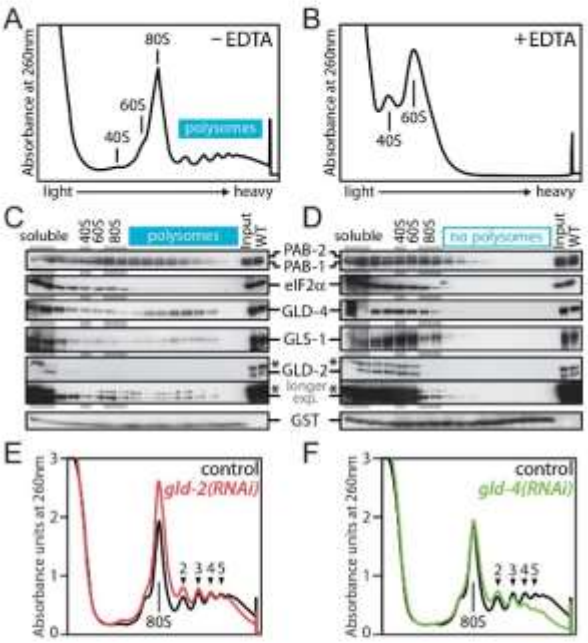

Figure 6. GLD-4 co-sediments with polysomes and promotes polysome formation. (**A** and **B**) Typical absorbance profiles of a wild-type polysome gradient (A) without or (B) with prior EDTA treatment (n = 3). The positions of major ribonucleoprotein complexes are indicated. (**C** and **D**) Western blot analysis of fractionated material from (A) and (B). Equal exposure times for each antibody in (C) and (D); for GLD-2, also a longer exposure of the same blot is shown; asterisk marks an unspecific background band. (**E** and **F**) Representative absorbance profiles from extracts of RNAi-treated animals (n > 4). The numbers indicate the position of polyribosome peaks that are clearly detected in the control RNAi sample.

6C). By contrast, eIF2α (Y37E3.10), which is expected to function only during translation initiation, is more abundant in non-polysomal than polysomal regions of the gradient (Figure 6C). The cytoPAP GLD-2 is highly abundant in non-polysomal regions and only a small fraction of the total protein is detected in polysomal regions (Figure 6C). The cytoPAP GLD-4 is also abundantly present in non-polysomal regions but, interestingly, an additional accumulation of the protein is detected in polysomal regions (Figure 6C). A similar observation is made for its cofactor GLS-1 (Figure 6C). Together this suggests that GLD-4 and GLS-1, but less so GLD-2, might associate with post-initiation complexes.

To gain further support that the polysomal migration pattern of GLD-4 and GLS-1 depends on polysome formation, we analyzed protein distribution in EDTA-treated extracts (Figure 6B). Under these conditions ribosomes are disassembled into 40S and 60S subunits, and all signals in the heavy fractions for both PABs, GLD-4 and GLS-1 disappeared (Figure 6B and D). Although EDTA treatment may also affect mRNP assemblies other than polysomes, this result is consistent with a likely association of GLD-4 and GLS-1 with post-initiation complexes.

To test whether the differential enrichment of both cytoPAPs in polysomal fractions has any functional relevance on polysome formation, we compared general ribosome distribution profiles from staged wild-type, control RNAi, *gld-2(RNAi)*, *gld-4(RNAi)*, *gld-4(ef15)* and *gls-1(ef8)* animals. No significant difference was detected by comparing *gld-2(RNAi)* with control RNAi (Figure 6E). However, in *gld-4*-compromised animals, a clear decrease of the polysomal signal was detected (Figure 6F and Supplementary Figure S4A), and this defect was also observed in *gls-1(ef8)* mutants (Supplementary Figure S4B). Taken together, these data suggest that GLD-2 has a minor role in promoting general translation. By contrast, GLD-4 may actively promote general translational efficiency, most likely together with GLS-1.

DISCUSSION

Cytoplasmic poly(A) polymerases represent an ancient class of nucleotidyltransferases that function as activators of post-transcriptional gene expression. We found that in *C. elegans*, the two cytoPAPs, GLD-2 and GLD-4, stimulate gene expression at the global level through distinct mechanisms. To a large part in germ cells, GLD-2 promotes the abundance of many translationally repressed mRNAs and facilitates bulk polyadenylation, whereas GLD-4 supports polysome formation. Our combined findings suggest that cytoPAPs globally employ diverse mechanisms to promote robust mRNA translation.

GLD-2 is a bulk mRNA poly(A) polymerase that stabilizes translationally repressed mRNAs

Across species, GLD-2 polyadenylates specific mRNAs that encode genes important for germ cell development and neuronal function (10). Initially restricted to a handful of mRNAs targets (12,13,18,44–46), the list of potential GLD-2 targets was recently expanded by two microarray studies in worms and flies (14,18). As both studies used PAT assays to measure gene-specific poly(A) tail-length changes, global changes in poly(A) lengths remained unclear. Moreover, PCR-based PAT assays notoriously underestimate the true length of poly(A) tails and exact measurements are difficult (15). Therefore, we used a method that allows bulk poly(A) measurements free of any amplification bias (26). Our analysis documented a strongly reduced bulk poly(A) tail profile in *gld-2*-compromised animals. The most prevalent change was associated with primarily germline-derived mRNAs and revealed that GLD-2 is responsible for maintaining bulk poly(A) tail lengths beyond ~35 nucleotides up to ~90–100 adenosines. This magnitude of GLD-2 activity and a similar reduction to ~40 nucleotides was also reported from other organisms. In *Drosophila*, the GLD-2 ortholog Wispy promotes tail extension of many mRNAs during late stages of oogenesis and early stages of embryogenesis (18). This argues that at least in germ cells GLD-2-type enzymes are major contributors to global cytoplasmic poly(A) metabolism.

Consistent with our bulk poly(A) measurements, our study also identifies a large set of GLD-2-stabilized germline mRNAs, and potentially expands a previous list of putative GLD-2 target mRNAs significantly. A previous study reported 538 GLD-2-associated mRNAs (14),

of which 272 mRNAs are significantly stabilized in our study, representing an overlap of ~50%. Yet, when focusing on germline-enriched RNAs (32), the number of GLD-2-associated mRNAs drops to 236 and the number of GLD-2-stabilized mRNAs among these drops further to 56, representing an overlap of ~24%. However, we found that 444 germline-enriched mRNAs are less abundant in *gld-2(RNAi)*. Extrapolating from these numbers, we assume that the number of direct GLD-2 mRNA targets in the germ line may be much larger. Several possible explanations might account for this difference. First, the previous work was based on RIP-chip analysis (14). Therefore, it is possible that the co-immunoprecipitation procedure was not comprehensive. Furthermore, it is likely that our germline filter is too stringent and potential targets are consequently excluded from our analysis. Finally, although there is a strong positive correlation between GLD-2 expression and GLD-2 target mRNA abundance in our experiments, we cannot exclude that our extended list of GLD-2-regulated mRNAs might also contain mRNAs that are indirectly changing as a consequence of developmental changes, such as late spermatogenesis and oogenesis genes. Nonetheless, we propose that our list of GLD-2-stabilized germline genes is highly enriched for direct targets, which together with the previous data represents a more complete resource for studying GLD-2-regulated mRNAs.

GLD-2-mediated mRNA regulation is important for various aspects of germ cell development. Although not the focus of this study, we noticed that genes functioning early in prophase I are significantly reduced in *gld-2(RNAi)*. So far the only known gene in this category was *gld-1* (13). We suggest that mRNAs, such as *syp-1*, *him-3*, *htp-1* or *daz-1*, might also be GLD-2 targets. On a more global scale, an mRNA stabilizing function late in prophase I was proposed for a GLD-2 sub-complex (14). By analogy, we would like to extend this thought and propose that GLD-2, most likely as part of other sub-complexes, targets also a broad range of mRNAs during early stages of prophase I. This suggests that GLD-2 impacts mRNAs at all stages of germ cell development past meiotic entry.

The regulated balance between translational repression and activation supports germline organization and function across species. GLD-2-type cytoPAPs are broadly expressed during germ cell development and likely activate gene expression (7,45). In the adult *C. elegans* germline tissue, germ cells are organised in a strict spatial and temporal manner of gametogenesis, and several translational repressors promoting germ cell development are stage specifically expressed: the PUF-protein family member FBF-1 in pre-meiotic cells, the STAR protein family member GLD-1 in early meiotic prophase and the TIS11 zinc-finger protein family member OMA-1 in late meiotic prophase (47–49). By comparison, GLD-2 protein expression is much broader: it is low in pre-meiotic cells, steadily increases during early meiotic prophase and is most abundant in late meiotic prophase (7,19). Therefore, our finding that ~77% of GLD-2-stabilized germline mRNAs are suggested targets of all three translational repressors (38,39,50) (C. Spike and D. Greenstein, personal communication) is intriguing. Given the low translational efficiency of GLD-2 targets, this observation further suggests that GLD-2 activity is primarily important for translationally repressed mRNAs. Hence, GLD-2's broad protein expression across the adult germline tissue paired with the strong mRNA target overlap of the local translational repressors indicates that GLD-2 might polyadenylate mRNAs during almost all stages of germ cell development. This argues for a central role of GLD-2-type cytoPAPs as global positive mRNA regulators that may oppose many translational repressors across species.

Beyond 3′end poly(A) extension to promote mRNA stability, the precise molecular mechanism and timing of GLD-2's enzymatic activity remains speculative. Many translational repressors are known to recruit poly(A) shortening enzymes (deadenylases) as part of their repressive function (51); prime examples are PUF proteins that associate with deadenylases from yeast to human cells (51). As deadenylation can initiate mRNA decay, GLD-2 might counteract this directed poly(A) shortening to promote mRNA stability during repression. In this scenario, a constant battle between deadenylation and polyadenylation could be envisioned, similar to the proposed antagonistic mechanism in *Xenopus* oocytes between the deadenylase PARN and xGld2 (44). Alternatively, GLD-2 activity may stabilize mRNAs after the switch from repression to activation, as short-tailed mRNAs released from repression are most likely prone to mRNA degradation. In either case, directed or globally occurring GLD-2-mediated polyadenylation is expected to protect mRNAs subject to translational control. In general, we suggest that GLD-2 represents a potent counterbalance to deadenylases employed by translational repressors.

Could GLD-2 have a role beyond mRNA stabilization? Long poly(A) tails are also important for translation efficiency (3,52). Throughout our global analysis, we find no major influence of *C. elegans* GLD-2 on the translatability of target mRNAs. However, the resolution power and the sensitivity of our analysis have to be taken into consideration here. In our genome-wide analysis, we split the gradient samples only into two fractions that allowed us to detect coarse redistributions from the polysomal to the non-polysomal regions, and vice versa. Certainly, we would have missed shifts occurring within the polysomal fractions, which would account for a more graded change of translational efficiencies, reflecting a high or lower translational initiation rate. Moreover, the majority of GLD-2 targets were low in abundance in the polysome region to begin with, arguing that the use of more sensitive techniques, such as higher resolution sucrose gradients paired with ribosome footprinting (36), might reveal a potential role of GLD-2 in stimulating mRNA translation. Hence, it remains possible that the primary effect of *gld-2* loss is translational repression combined with an enhanced secondary stimulation of mRNA degradation that overshadows positive translational regulation effects of GLD-2 function.

GLD-4 promotes general translation

The non-canonical nucleotidyltransferase GLD-4 is evolutionarily most closely related to members of the conserved TRF4 family (10). In yeast, flies and mammalian cells, TRF4 homologous proteins represent the catalytic subunits of a nuclear RNA surveillance complex that adds short

poly(A) tails of ~10 nts to its RNA substrates (53). Moreover, the mammalian TRF4 paralog, hGld4/PAPD5, localizes to the cytoplasm where it forms a complex with the RNA-binding protein CPEB, promoting polyadenylation-induced translation of the tumor suppressor p53 (9). In *C. elegans*, the TRF4 ortholog GLD-4 predominantly localizes to the cytoplasm where it forms a protein complex with the nematode-specific protein GLS-1 to promote the translation of the germ cell-specific tumor suppressor GLD-1 and the Notch receptor GLP-1 (8,19). Despite these few examples, surprisingly little is known about additional mRNA targets and the global roles of cytoplasmic GLD-4-type nucleotidyltransferase in poly(A) tail metabolism and post-transcriptional mRNA regulation.

Consistent with gene-specific poly(A) tail measurements in *gld-4* mutants (15), we found in our bulk poly(A) tail measurements that many mRNAs in *gld-4*-compromised animals have slightly shorter tails. This observation is in sharp contrast to the strong polyadenylation defects of *gld-2*-deficient animals at the bulk and gene-specific level (15,16). This suggests that GLD-4 seems to have retained the enzymatic properties of the nuclear TRF4 proteins, adding rather short adenosine stretches to its targets. A subcellular relocation of GLD-4-type nucleotidyltransferases coupled to novel interactions with additional cytoplasmic factors might therefore represent alternative ways to regulate gene expression in evolution. It will be interesting to see whether the enzymatic activity of *C. elegans* GLD-4 is similarly weak among other cytoplasmic TRF4-type nucleotidyltransferases reported from other organisms (9). Although we cannot exclude that cytoplasmic GLD-4 promotes strong polyadenylation of specific targets, our bulk poly(A) tail measurements argue that GLD-4 contributes little to overall poly(A) tail metabolism. This suggests that GLD-2 and GLD-4 have different enzymatic activities *in vivo*, promoting strong or weak poly(A) addition, respectively, which may result from potential structural differences in their catalytic domains and distinct protein interactions to additional factors.

The functional consequences of GLD-4-mediated polyadenylation are less clear. Nuclear TRF4-mediated poly(A) addition is a prerequisite for RNA substrate degradation (54). However, in GLD-4-depleted animals, we detect no major changes in mRNA abundance, arguing that tail extension via GLD-4 cytoPAP does most likely not influence mRNA degradation. Instead, our overall data indicate that GLD-4 has a potential role in promoting translation. We find that GLD-4 cytoPAP and its co-factor GLS-1 is associated with putative translating ribosomes, and the loss of either protein leads to a strong reduction in polysome formation. Although it remains to be shown whether GLD-4-mediated mRNA polyadenylation is required for polysome assembly, it is attractive to speculate that GLD-4 could promote translation re-initiation by counteracting the proposed erosion of the poly(A) tail during active translation (55). Certainly, we find it less likely that a reduction of translation factor expression may indirectly affect polysome formation efficiency, as we did not find their mRNAs strongly reduced in *gld-4(RNAi)* animals. Alternatively, GLD-4 may aid the translation of mRNAs in a poly(A) polymerase-independent manner by a yet to be identified mechanism.

Different cytoPAP mechanisms may represent a functional basis for robust and distinct gene expression control

The *C. elegans* germ line is a complex tissue that regulates its protein production primarily at the post-transcriptional level. Hence, efficient protein production is achieved via mRNA regulation. In order to satisfy the differential protein expression needs of germ cells during their developmental stages, we propose that a distinct utilization of GLD-2 and GLD-4 mechanisms would make it easy to combine or separate their activities in gene expression regulation. For example, different mRNAs could be more susceptible to one or the other mechanism. Alternatively, the combined GLD-2 and GLD-4 mechanisms might ensure that protein production is highly efficient, promoting the synthesis of large amounts of proteins in a short period of time. Such synergism is evident by the requirement of the two cytoPAPs to maintain high levels of GLD-1 protein in the germ line to ensure meiotic commitment (8). Furthermore, the overlap between *gld-2(RNAi)*-decreased and *gld-4(RNAi)*-less translated genes suggests that the combined activity of both cytoPAPs might be important for a specific set of mRNAs. In order to get a deeper understanding of the relationship between the GLD-2 and GLD-4, more work is needed to reveal their precise mechanisms. In general, we suggest that the diversification of cytoPAP mechanisms represents an additional regulatory asset to all biological systems that utilize post-transcriptional gene regulation.

ACCESSION NUMBERS

NGS data have been deposited in the GEO database (accession number: GSE58918).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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3.3. Polyadenylation is the key aspect of GLD-2 function in *C. elegans*

Overarching question

Can cytoplasmic poly(A) polymerases have enzymatic-independent functions?

Synopsis of the publication

Many enzymes fulfill important cellular functions by catalyzing biochemical reactions. Interestingly, a growing number of enzymes contribute to cellular biology also in a catalysis-independent fashion (VIVANCO *et al.* 2014; MASON *et al.* 2019; BERGE *et al.* 2020; JANISIW *et al.* 2020; SZIGETY *et al.* 2020; ZOU *et al.* 2020). Noncanonical poly(A) polymerase facilitate the addition of adenosine to the 3' end of a target RNA. Intriguingly, the enzymatic activity of yeast Trf4-type PAP in the nucleus is dispensable for its role in regulating RNAs (SAN PAOLO *et al.* 2009). This suggests that PAPs might be able to support cellular functions in an enzymatically independent manner. It had never been tested whether this is also true for any member of the GLD-2-type PAP family. The nematode *C. elegans* is the perfect system to reveal enzymatic independent functions of GLD-2. Initially discovered in the worm, the importance of *C. elegans gld-2* for the development of germ cells is well documented and characterized. Loss of the protein leads to a delayed entry into and subsequence arrest of meiosis (KADYK AND KIMBLE 1998; WANG *et al.* 2002; ECKMANN *et al.* 2004). Additionally, GLD-2 on the molecular level promotes poly(A) tail extension of mRNAs and stabilizes specific mRNAs (SUH *et al.* 2006; NOUSCH *et al.* 2017). In this work, we identified a *gld-2* allele that carries a point mutation in the catalytic domain of GLD-2. Animals that express catalytically-impaired GLD-2 present the same phenotypes on the physiological as well as the molecular level as *gld-2* alleles which do not express any GLD-2 protein. Hence, we show that GLD-2 fulfills its functions in germ cell development and RNA metabolism exclusively via its polyadenylation activity. In general, this work corroborates the importance of GLD-2-type enzymes in poly(A)-mediated RNA regulation.

Polyadenylation is the key aspect of GLD-2 function in *C. elegans*

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ABSTRACT

The role of many enzymes extends beyond their dedicated catalytic activity by fulfilling important cellular functions in a catalysis-independent fashion. In this aspect, little is known about 3′-end RNA-modifying enzymes that belong to the class of nucleotidyl transferases. Among these are noncanonical poly(A) polymerases, a group of evolutionarily conserved enzymes that are critical for gene expression regulation, by adding adenosines to the 3′-end of RNA targets. In this study, we investigate whether the functions of the cytoplasmic poly(A) polymerase (cytoPAP) GLD-2 in *C. elegans* germ cells exclusively depend on its catalytic activity. To this end, we analyzed a specific missense mutation affecting a conserved amino acid in the catalytic region of GLD-2 cytoPAP. Although this mutated protein is expressed to wild-type levels and incorporated into cytoPAP complexes, we found that it cannot elongate mRNA poly(A) tails efficiently or promote GLD-2 target mRNA abundance. Furthermore, germ cell defects in animals expressing this mutant protein strongly resemble those lacking the GLD-2 protein altogether, arguing that only the polyadenylation activity of GLD-2 is essential for gametogenesis. In summary, we propose that all known molecular and biological functions of GLD-2 depend on its enzymatic activity, demonstrating that polyadenylation is the key mechanism of GLD-2 functionality. Our findings highlight the enzymatic importance of noncanonical poly(A) polymerases and emphasize the pivotal role of poly(A) tail-centered cytoplasmic mRNA regulation in germ cell biology.

Keywords: poly(A) polymerase; poly(A) metabolism; translational regulation; germline development

INTRODUCTION

Gene expression programs are profoundly influenced by RNA-modifying enzymes. The catalytic activity of such proteins is often used to facilitate covalent RNA-structure changes, thereby providing a mechanistic basis for post-transcriptional gene regulation. Besides the obvious importance of the catalytic activity of enzymes per se, an increasing number of examples shows that enzymes also perform critical catalysis-independent functions. This is nicely exemplified in the field of signal transduction where fundamental noncatalytic roles are attributed to kinases such as scaffolding or allosteric regulation (Kung and Jura 2016). However, in the field of RNA research, it is currently unclear to which degree the molecular or biological functions assigned to many enzymes are dependent on their RNA-modifying activity.

A developmentally important class of RNA-modifying enzymes are noncanonical cytoplasmic poly(A) polymerases (cytoPAPs). The most prominent member of this class is germline development defective (GLD)-2 (Minasaki and Eckmann 2012). Originally discovered in the nematode *Caenorhabditis elegans* (Kadyk and Kimble 1998; Wang et al. 2002), numerous homologs of GLD-2 have been described in other invertebrates and vertebrates (Kwak et al. 2004; Rouhana et al. 2005; Benoit et al. 2008; Cui et al. 2008). Among the different species, GLD-2-type cytoPAPs are most commonly and predominantly expressed in germ cells (Rouhana et al. 2005), and in the case of nematodes and flies, it was found that this expression is essential for the formation of functional gametes (Kadyk and Kimble 1998; Benoit et al. 2008; Cui et al. 2008; Sartain et al. 2011). Therefore, this class of enzymes comprises important evolutionarily conserved regulators of germ cell development.

All members of the GLD-2-type cytoPAP family contain a nucleotidyl transferase domain (NTD) that is embedded in a large central domain. The crystal structure has been solved for *C. elegans* GLD-2 (Nakel et al. 2015). NTD and central domain are separated by a pronounced cleft that harbors the catalytically active site, whereby the catalytic domain itself is composed of a five-stranded β-sheet flanked by two α-helices (Nakel et al. 2015). Interestingly, the central domain, including its NTD, constitutes the main part of GLD-2 proteins

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in vertebrates (Kwak et al. 2004; Rouhana et al. 2005), whereas large protein extensions are found in nonvertebrates, such as flies and worms (Wang et al. 2002; Benoit et al. 2008; Cui et al. 2008), making nonvertebrate GLD-2 proteins prime candidates to investigate potential noncatalytic functions.

The isolated GLD-2 protein itself has only weak enzymatic activity in vitro. The purified human GLD-2 protein, although highly specific for the addition of adenosines, can only monoadenylate a wide set of RNA substrates (Chung et al. 2016). Interestingly, the activity of GLD-2 is stimulated by interacting proteins that bind to the catalytic domain (Wang et al. 2002; Kim et al. 2009). The crystal structure of worm GLD-2 in a complex with such binding partners shows that these interactions reduce the flexibility of the catalytic center, stabilize the fold of the catalytic domain, and extend its positively charged surface area for RNA interaction (Nakel et al. 2015, 2016). This suggests that GLD-2 has to be part of a protein complex in order to facilitate efficient polyadenylation.

It is envisioned that GLD-2-type cytoPAPs function as post-transcriptional activators by elongating mRNA poly(A) tails, thereby promoting mRNA stability in the cytoplasm. This idea is supported by several observations, made in different systems. In *Caenorhabditis elegans*, loss of GLD-2 protein correlates with a shortening of mRNA poly(A) tails as well as a decrease in abundance of many germline mRNAs (Kim et al. 2010; Nousch et al. 2014). Similar observations have been made in *Drosophila melanogaster*, where putative mRNA targets of the GLD-2 homolog, WISPY, are not polyadenylated in its absence, leading to inefficient protein synthesis during oocyte formation (Benoit et al. 2008; Cui et al. 2013).

The catalytic activity is not essential for the function of all noncanonical poly(A) polymerases. This is nicely illustrated by the functional requirements of Trf4-type PAPs. Most members of this protein family are involved in nuclear RNA quality control as part of the TRAMP complex, by oligoadenylating target RNAs that subsequently are degraded by the nuclear exosome (LaCava et al. 2005; Vanácová et al. 2005; Wyers et al. 2005). Loss of Trf4-type protein leads to an increase of many noncoding and nonfunctional RNAs (Kadaba et al. 2004; LaCava et al. 2005; Vanácová et al. 2005; Wyers et al. 2005; Davis and Ares 2006; Egecioglu et al. 2006). Interestingly, the overexpression of catalytically inactive Trf4 protein in yeast can restore many of these mRNAs back to wild-type levels (San Paolo et al. 2009), demonstrating that the enzymatic process of oligoadenylation is not essential for Trf4-mediated RNA degradation.

To date, all studies on *gld-2* function were made in the absence of full-length GLD-2 proteins, making it impossible to distinguish between catalysis-dependent and -independent functions. In the work presented here, we investigate the importance of the poly(A) polymerase activity GLD-2 with regard to its molecular and biological functions by studying a missense mutation that resides in the catalytic domain of *C. elegans* GLD-2.

RESULTS AND DISCUSSION

Description of *gld-2* point mutations

Several alleles of the *gld-2* gene have been reported in *C. elegans* (Kadyk and Kimble 1998). We sequenced these alleles to identify mutations that are predicted to produce full-length protein but inhibit the enzymatic activity of GLD-2. Our search settled on the allele *gld-2(q540)*, a missense mutation that results in the exchange of proline 652 to leucine (P652L), which is located within the nucleotidyltransferase domain (NTD) (Fig. 1A). According to the recently described crystal structure of *C. elegans* GLD-2 (Nakel et al. 2015), proline 652 maps to β-sheet number 4 of the catalytic domain, which is part of the cleft that harbors the active site of the enzyme (Fig. 1B). Importantly, the high degree of evolutionary conservation from worm to humans of proline 652 (Fig. 1B) suggests structural and/or functional importance.

To test whether GLD-2(P652L) protein is stably expressed in *gld-2(q540)* worms, we performed Western blot analyses, comparing the mutant protein to wild-type and two other *gld-2* alleles: *gld-2(q497)*, which is considered to be a strong loss-of-function allele producing no full-length GLD-2 protein (Fig. 1A; Wang et al. 2002), and *gld-2(h292)*,

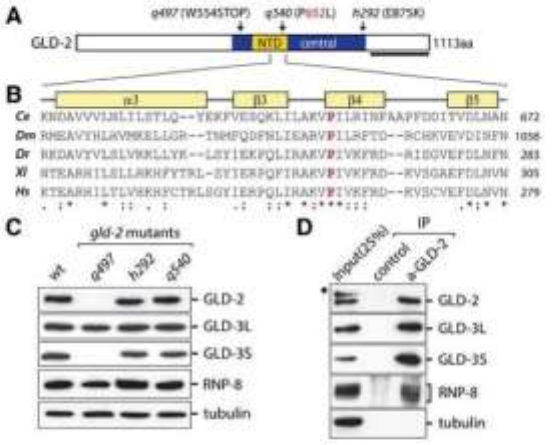

FIGURE 1. The GLD-2(P652L) protein is robustly expressed and interacts with GLD-3 and RNP-8. (*A*) Cartoon of GLD-2 protein domain structure. Position and impact of *q497*, *q540*, and *h292* mutations on GLD-2 protein are indicated. The black, thick bar indicates the region that was used to raise all antibodies against this protein. (*B*) A protein sequence alignment comprising the second half of the nucleotidyl transferase domain (NTD) from *Caenorhabditis elegans* (Ce), *Drosophila melanogaster* (Dm), *Danio renio* (Dr), *Xenopus laevis* (Xl), and *Homo sapiens* (Hs) GLD-2. On *top* of the alignment, the secondary structure elements of the *C. elegans* protein are shown (α, α-helices; β, β-sheet). The conserved proline in β-sheet number 4 is highlighted in red. (*C*) Western blot analysis of the indicated proteins in the different *gld-2* mutants. (*D*) Immunoprecipitation of GLD-2(P652L)-containing complexes from adult animal extracts, tested for a coenrichment of both known GLD-2-interacting proteins. The star indicates a background band that is detected by the mouse anti-GLD-2 antibody.

a missense mutation that replaces glutamic acid 875 with an arginine (E875R) in GLD-2 (Fig. 1A; Wang et al. 2002). Consistent with previous results, no full-length GLD-2 protein could be detected in *q497*, and an equally robust GLD-2 signal was present in wild-type as well as *gld-2(h292)* young adults (Fig. 1C; Wang et al. 2002). Importantly, a comparably strong GLD-2 signal was detected in extracts of *gld-2(q540)* animals, showing that in *q540*, full-length GLD-2(P652L) protein is present in similar amounts compared to wild-type GLD-2 or *h292* full-length GLD-2 (E875R).

To test whether germ cell-enriched GLD-2 mutant variants have an influence on the abundance of GLD-2-associated proteins, we analyzed the expression of GLD-3 and RNP-8 in the different *gld-2* mutant backgrounds. Both proteins have previously been shown to physically bind to GLD-2 (Wang et al. 2002; Eckmann et al. 2004; Kim et al. 2009; Nakel et al. 2015, 2016). In germ cells, two variants of GLD-3 are expressed via alternative splicing: GLD-3 long (GLD-3L) and GLD-3 short (GLD-3S) (Eckmann et al. 2002). In the different GLD-2 mutants, the abundance of GLD-3L is unchanged compared to wild-type (Fig. 1C). Contrary to the long isoform, GLD-3S is nearly absent in *q497* and slightly reduced in *h292*, which is also true for *q540* (Fig. 1C). RNP-8 levels are comparable among all tested genotypes (Fig. 1C). Together, this shows that only the expression level of GLD-3S, but neither GLD-3L nor RNP-8, is dependent on the presence of GLD-2. The differential requirement of both GLD-3 isoforms on GLD-2 protein presence suggests that GLD-3S might be stabilized by its integration into a GLD-2 complex.

To test whether GLD-2(P652L) protein can still establish known protein interactions in vivo, we conducted immunoprecipitation experiments from hand-picked homozygous *gld-2(q540)* adult worms. GLD-2(P652L) was efficiently enriched in pull-down experiments with specific anti-GLD-2 antibodies, whereas no GLD-2 protein was detected in pull-downs from the same extract with unspecific antibodies (Fig. 1D). Importantly, both isoforms of GLD-3 as well as RNP-8 were specifically coimmunoprecipitated with GLD-2 (P652L), whereas tubulin was not (Fig. 1D). As GLD-3 and RNP-8 occupy the same interaction surface on GLD-2, both proteins bind to GLD-2 in a mutually exclusive manner (Kim et al. 2009; Nakel et al. 2015, 2016), and our observations suggest that both proteins are able to bind to GLD-2 (P652L), presumably forming two distinct GLD-2(P652L)-containing mRNP complexes in *gld-2(q540)*. Although it cannot be excluded that the *q540* mutation disturbs the association of unknown GLD-2–interacting factors, it is unlikely that, judging from the structural position of β-sheet number 4, Proline 652 participates in intermolecular interactions (Nakel et al. 2015). Regardless, based on the robust enrichment of GLD-3 and RNP-8 with the mutated enzyme, we concluded that GLD-2(P652L) cytoPAP is incorporated into protein complexes in vivo.

The enzymatic activity of GLD-2(P652L) cytoPAP is strongly compromised in vivo

Loss of GLD-2 protein causes a decrease in mRNA poly(A)-tail lengths at the gene-specific as well as global level (Suh et al. 2006; Minasaki et al. 2014; Nousch et al. 2014). To test whether the GLD-2(P652L) enzyme polyadenylates mRNAs in vivo, we measured poly(A)-tail lengths of individual mRNAs and of bulk RNA in *gld-2(q540)* worms. Using the sPAT method (Minasaki et al. 2014), we concentrated our gene-specific analysis on two previously described GLD-2 target mRNAs: *gld-1* and *oma-2* (Suh et al. 2006; Schmid et al. 2009; Kim et al. 2010). Consistent with previous results (Suh et al. 2006; Kim et al. 2010; Minasaki et al. 2014), the average poly(A)-tail length of *gld-1* and *oma-2* mRNAs is shorter in the putative protein null allele *gld-2(q497)* than in wild-type (Fig. 2A). Interestingly, we made similar observations in *gld-2(q540)* animals, where both tested mRNAs also have shorter poly(A) tails (Fig. 2A). This suggests that *gld-1* and *oma-2* mRNAs are not efficiently polyadenylated by the GLD-2(P652L) enzyme. Still, small poly(A) tail differences are detected in *gld-2(q497)* and *gld-2(q540)* for *gld-1* mRNA, which might reflect a differential sensitivity of different mRNAs toward GLD-2 activity. To test how prevalent the polyadenylation defect is in *gld-2(q540)* animals, we conducted bulk poly(A)-tail measurements. For this assay, we isolated total RNA, labeled it with radioactive cordycepin, removed everything that is not a poly(A) stretch by RNase digestion, and visualized poly(A) distribution on a sequencing gel. In wild-type, the majority of poly(A) tails is distributed between 30 and 60 nucleotides (nt) (Fig. 2B,C). In *gld-2(q540)* animals, poly(A) tails of 40 nt and longer are significantly less abundant, and the overall poly(A) profile is similar to the one detected in the protein null allele *gld-2(q497)* (Fig. 2B, C). However, with the assays used in this study, we cannot exclude that the GLD-2(P652L) protein still possesses some residual polyadenylation activity, as indicated by the small differences between *gld-2(q497)* and *gld-2(q540)* detected in the bulk poly(A) measurements (Fig. 2C). Nonetheless, our results show that a large number of mRNAs is inefficiently polyadenylated in *gld-2(q540)* animals, indicating that GLD-2(P652L) possesses a significantly reduced poly(A)-polymerase activity and cannot efficiently promote mRNA tail extension.

The fold of the catalytic domain of GLD-2 is stabilized by interactions with GLD-3 or RNP-8 (Nakel et al. 2015, 2016), and interactions with either protein stimulate GLD-2's polyadenylase activity (Wang et al. 2002; Kim et al. 2009). As GLD-2(P652L) is still able to interact with both proteins (Fig. 1), it is unlikely that the loss of polyadenylation can be explained by an inability of the protein to interact with stimulating factors. It is more plausible that the P652L mutation has a direct effect on the catalytic efficiency of GLD-2 cytoPAP. In the structure of yeast nuclear poly(A) polymerase, PAP-1, the 3′-terminal residue of the poly(A) substrate is

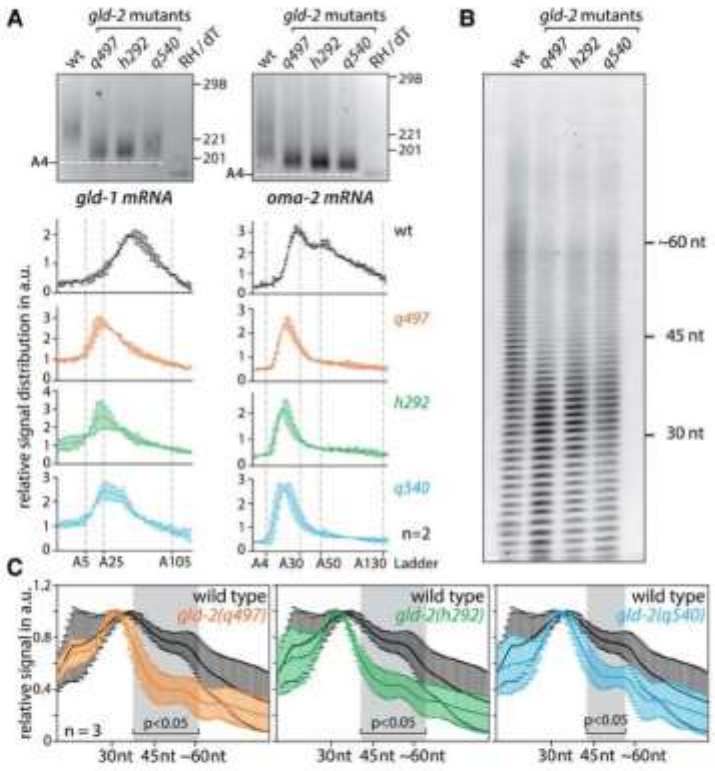

FIGURE 2. Impact of different GLD-2 mutant proteins on mRNA polyadenylation. (*A*) sPAT analysis of *gld-1* and *oma-2* mRNA in wild-type (wt) and different *gld-2* backgrounds. The lane RH/dT is a wild-type sample that was treated with oligo(dT) and RNase H prior to the sPAT assay to indicate the size of a completely deadenylated mRNA. Line scans from data generated by the sPAT assay are given below. Two independent biological repeats were analyzed. (*B*) The distribution of bulk poly(A) tails was analyzed in wild-type and the given *gld-2* backgrounds. (*C*) Line scans from bulk poly(A) measurements. Three independent biological repeats were analyzed. Regions of statistically significant differences indicated with a gray stripe and black bracket are calculated via the Student's *t*-test.

sandwiched between valine 141 and the base of the incoming ATP (Balbo and Bohm 2007). In the structure of GLD-2, this valine maps to position 651 and is thereby located one residue before the P652L mutation. With such an important nearby residue, it is plausible that the P652L mutation somehow interferes with the positioning of the substrate in the catalytic pocket and thus affects polyadenylation activity. Taken together, we therefore propose that GLD-2(P652L) is at best a catalytic null or at minimum a severely catalytically impaired enzyme.

We also included the *h292* allele in our poly(A)-tail measurements. The *h292* missense mutation has previously been described to abolish the polyadenylation activity of a GLD-2 fragment in vitro (Nakel et al. 2015). However, it was unclear whether similar negative effects would also manifest in vivo in the context of full-length GLD-2 (E875R) protein. Here, we find that both poly(A) tails of specific mRNAs (Fig. 2A) and bulk RNA poly(A)-tail profiles (Fig. 2B,C) are substantially reduced in *gld-2(h292)* animals, suggesting that also this mutation strongly affects the poly(A)-polymerase activity of GLD-2 in vivo. However, how this mutation, which is located outside of the catalytic domain, impairs GLD-2 activity is currently unclear. As part of the nucleotide recognition motif, E875 is likely to affect catalysis in a direct manner (Nakel et al. 2015). Moreover, in a yeast-two-hybrid assay, this mutation inhibits the interaction with GLD-3 (Wang et al. 2002). As the interaction with GLD-3 stabilizes the fold of the catalytic domain of GLD-2 (Nakel et al. 2015), the loss of polyadenylation in *gld-2(h292)* animals may also be due to an inability of GLD-2(E875R) to interact with GLD-3. However, a negative impact of the *h292* mutation on the GLD-2/GLD-3 interaction could not be recapitulated in vitro upon coexpression and subsequent copurification of recombinant GLD-2 and GLD-3 fragments (Nakel et al. 2015). Therefore, future studies will be needed to provide a molecular explanation on how the *h292* mutation impairs GLD-2 polyadenylation activity in vivo.

GLD-2-dependent polyadenylation is important for mRNA stability

The presence of GLD-2 protein is important for a large number of germline mRNAs, as their abundance decreases in animals where GLD-2 is down-regulated or absent (Kim et al. 2010; Nousch et al. 2014). To test whether GLD-2(P652L) stabilizes mRNAs, we measured mRNA levels of previously described GLD-2 target mRNAs in young adults by qPCR, comparing the two *gld-2* alleles, *q540* and *q497*, to wild-type. The expression of each mRNA was normalized to *rpl-11.1* levels, a germline-specific ribosomal protein and non-GLD-2 target mRNA (Maciejowski et al. 2005; Nousch et al. 2014). Consistent with previous results (Nousch et al. 2014), eight tested GLD-2 target mRNAs (*gld-1*, *zfp-3*, *lip-1*, *oma-2*, *him-5*, *cpb-3*, *gla-4*, *puf-8*) were significantly reduced in *gld-2(q497)* compared to wild-type, whereas three tested nontarget mRNAs (*pgl-1*, *glh-1*, *rpl-25.2*) remained unchanged (Fig. 3A). Interestingly, in *gld-2(q540)* animals, the abundance of all target mRNAs was comparable to *gld-2(q497)* animals (Fig. 3A). The observation that not even a partial rescue of GLD-2 targets could be detected strongly suggests that the polyadenylation-compromised GLD-2(P652L) enzyme is not able to stabilize

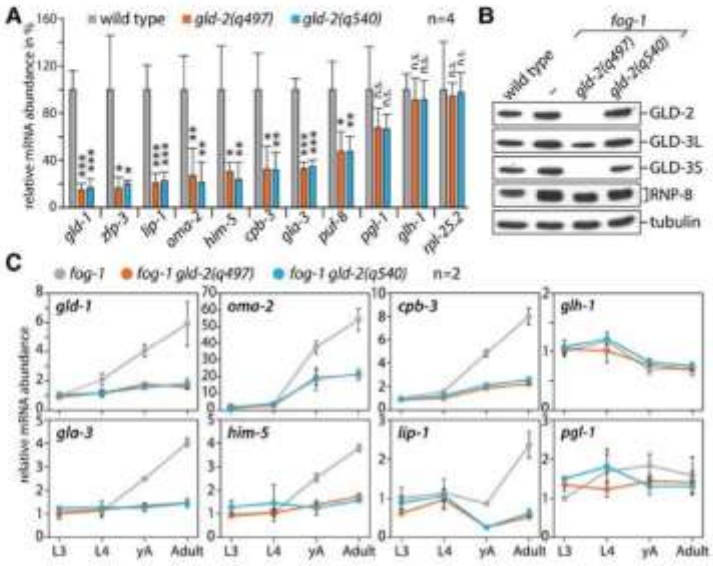

FIGURE 3. Levels of GLD-2 target mRNAs are reduced in GLD-2(P652L)-expressing animals. (*A*) Abundance measurements of the indicated mRNAs via quantitative real-time PCR. Statistically significant differences are indicated and calculated via the Student's *t*-test. (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$; n.s., not significant. (*B*) Western blot analysis of the indicated proteins in *fog-1 gld-2* double-mutant backgrounds. (*C*) mRNA abundance analysis during the development from L3 larvae to adults in given mutant backgrounds. L3, L3 larvae; L4, L4 larvae; earlyA, L4 + 12h; Adult, L4 + 24h.

mRNAs. This argues that the formation of polyadenylation-deficient cytoPAP complexes is not sufficient to promote target mRNA abundance, highlighting the importance of the enzymatic activity of GLD-2.

In order to investigate the dynamics of GLD-2-mediated mRNA regulation, we decided to measure the accumulation of GLD-2 target mRNAs during the different stages of female germline development. *C. elegans* is a hermaphrodite that produces sperm during the L3 and L4 larval stages before switching to produce oocytes for the remainder of the animal's lifetime. In order to extend the time window of our analysis, we conducted our measurements in a *fog-1 loss-of-function* (*lf*) background. Removal of *fog-1* activity feminizes the germline, and oogenesis already starts at the L3 larval stage (Barton and Kimble 1990). The absence of *fog-1* has no influence on the expression of GLD-2(P652L) or its binding partners GLD-3L and RNP-8 (Fig. 3B). Also, in this genetic background, GLD-3S remained hardly detectable in the absence of functional GLD-2 protein (Fig. 3B). We measured six GLD-2 target mRNAs (*gld-1*, *oma-2*, *gla-3*, *him-5*, *cpb-3*, and *lip-1*) and two non-GLD-2 target mRNAs (*glh-1* and *pgl-1*) at the L3 and L4 larval stage, as well as in younger (L4 + 12h) and older (L4 + 24h) adults. Each time point was normalized to the expression of *rpl-11.1*. In wild-type, most GLD-2 targets show a steady increase in expression following the L4 larval stage, with the exception of *lip-1*, which only increases during adulthood (Fig. 3C). On the contrary, these mRNAs show in both *gld-2* mutant animals, *q497* and *q540*, only a moderate increase in abundance, if any, during this time course (Fig. 3C). No such differences could be detected for *glh-1* and *pgl-1* (Fig. 3C). These data show that GLD-2 target mRNAs have similar levels and similar expression dynamics during oogenesis in the two tested *gld-2* alleles, strongly arguing that GLD-2(P652L)-containing RNP complexes cannot stabilize mRNAs during any stages of oogenesis.

gld-2(q540) recapitulates known *gld-2(0)* phenotypes in the germline

GLD-2 protein expression is essential for germ cell development. In adult *C. elegans* hermaphrodites, female germ cells gradually mature with respect to their distal-to-proximal position within the tube-like gonad: Proliferative cells are located most distally and enter meiosis further proximally, starting with leptotene and eventually arresting in diakinesis at its proximal end (Fig. 4A). GLD-2 is important for at least two aspects of germ cell development: entry into and progression through meiosis (Kadyk and Kimble 1998; Eckmann et al. 2004). As a consequence, no functional gametes are produced in the absence of GLD-2 (Kadyk and Kimble 1998).

It has previously been described that *gld-2(q540)* animals are sterile (Kadyk and Kimble 1998). However, it remained unclear whether all germ cell defects ascribed to the protein null allele *q497* are also present in *gld-2(q540)* animals. We first concentrated on *gld-2*'s function in meiotic entry in the distal part of the gonad (Fig. 4A). In the absence of GLD-2 protein, the size of the proliferative region increases, which is interpreted as a delayed entry into meiosis (Eckmann et al. 2004). We analyzed the size of the proliferative region in wild-type and *gld-2* mutants by DAPI staining. In wild-type gonads, germ cells enter meiosis around row 20 (germ cell distance measured from the distal tip) (Fig. 4B). Consistent with previous data (Eckmann et al. 2004), the size of the proliferative region is substantially increased in *gld-2(q497)* (Fig. 4B). Interestingly, the size of the proliferative region in *gld-2(q540)* is also larger than in wild-type, and no difference was detected between *q497* and *q540* (Fig. 4B), demonstrating that the *gld-2(q540)* missense mutation mimics the delayed meiotic entry phenotype of the genetic null allele *gld-2(q497)*. Thus, we conclude that the polyadenylation-compromised GLD-2(P652L) protein

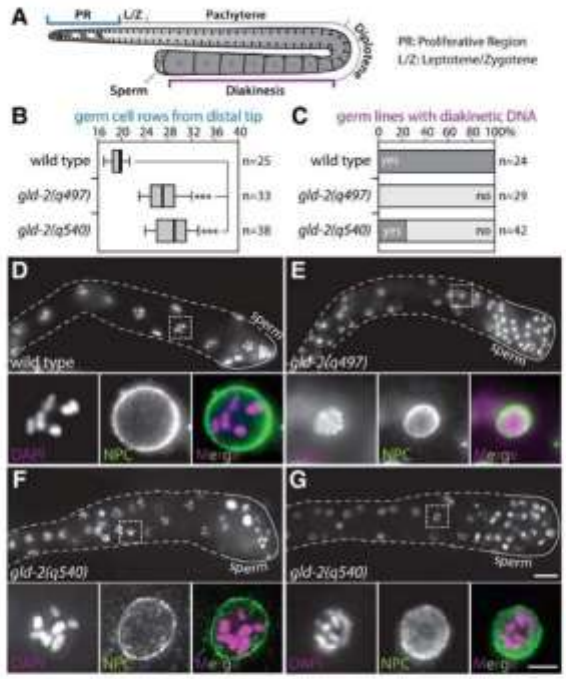

FIGURE 4. *gld-2(q540)* largely phenocopies the genetic null allele of *gld-2*. (*A*) Cartoon of the adult hermaphroditic syncytial germline tissue. Circles represent nuclei, T-structures are partial membranes. Important female germ cell stages are indicated; distal is *top left*, the position of sperm marks the most proximal end of the gonad. Color marked regions were analyzed in this work. (*B*) Analysis of the size of the proliferative region. Germ cell rows before meiotic entry were counted in the different genetic backgrounds as judged by DAPI staining. Significance was calculated by a two-tailed Student's *t*-test. (***) $P \leq 0.001$. (*C*) Percentage of germlines that positively stain for diakinetic DNA in the proximal part, assessed by DAPI staining. (*D–G*) DAPI (purple in merge) staining of the indicated genetic backgrounds. NPC (green in merge), nuclear pore complex. Scale bar: (*G*) 15 µm, (blow-up) 5 µm.

is insufficient to compensate for meiotic entry defects, and that a timely transition from mitosis to meiosis is tightly linked to GLD-2's enzymatic activity.

The second major germ cell phenotype that we analyzed by DAPI staining occurs in the proximal part of the gonad (Fig. 4A). In this part of wild-type gonads, germ cells with highly condensed chromosomes are detected (Fig. 4C,D), marking them as cells that are in the diakinetic stage of meiosis. In the absence of GLD-2 protein, as seen in *gld-2(q497)*, germ cells arrest in their meiotic program somewhere around the pachytene-diplotene stage, never reaching diakinesis (Fig. 4C,E). We made similar observations for the *q540* allele; a large fraction of gonads (~76%, $n = 42$) lacks diakinetic germ cell nuclei (Fig. 4C,G). Nonetheless, diakinetic chromosome configurations were detected in ~24% of the analyzed *gld-2(q540)* germlines (Fig. 4C,F), showing that the *q540* mutation mimics the meiotic arrest of germ cells of the *q497* mutation to a large extent, but not completely. The overall phenotypic similarities between the two analyzed *gld-2* alleles argue that polyadenylation is the most important function of GLD-2 in germ cells.

GLD-2 is broadly expressed during *C. elegans* germ cell development and forms distinct cytoPAP complexes with respect to its roles within the gonad. Based on the ubiquitous expression of GLD-3 and its phenotypic resemblance (Eckmann et al. 2002, 2004), a GLD-2/GLD-3 complex is most likely the main cytoPAP complex that regulates germ cell mRNAs in the distal part of the gonad. In combination with RNP-8, whose expression is limited to later stages of meiotic pachytene (Kim et al. 2009), at least two GLD-2 complexes are likely to operate in the proximal gonad. Interestingly, neither single mutant animals of *rnp-8* or *gld-3*, nor *rnp-8; gld-3* double mutants show the typical germ cell arrest seen in the loss of *gld-2* (Eckmann et al. 2002; Kim et al. 2009), suggesting that additional GLD-2 interactors may exist for oogenesis. In either case, our data suggest that all GLD-2 (P652L)-containing cytoPAP complexes are primarily compromised in their polyadenylation activity of target mRNAs.

Combined, our data suggest that the GLD-2 function is more diverse in later meiotic stages of germ cell development than during entry into meiosis. This may also reflect a different level of requirement or sensitivity for GLD-2-mediated polyadenylation. Although the expression of the catalytically compromised GLD-2(P652L) cytoPAP is most of the time insufficient for the progression through pachytene, germ cells occasionally develop further, suggesting that either GLD-2 (P652L) protein has some remaining polyadenylation activity that we were unable to detect in our assays, or it might have polyadenylation-independent roles. Whether such minor roles exist and are connected to mRNA regulation needs to be addressed in the future. Nonetheless, we conclude that in strong contrast to Trf4-type nucleotidyl transferases, GLD-2-type poly(A) polymerases are likely to fulfill their cellular roles almost exclusively in a polyadenylation-dependent manner.

MATERIALS AND METHODS

Nematode strains and transgenesis

Worms were handled according to standard procedures and grown at 20°C (Brenner 1974). The N2 Bristol strain was used as a reference for wild-type. Strains used in this study: Linkage group (LG) I: *gld-2 (q497)*, *gld-2(q540)*, *gld-2(h292)*, and *fog-1(q785)*. We noted that the original *gld-2(q497)* and *gld-2(q540)* strains (Kadyk and Kimble 1998) carried a background mutation in the *mut-16* locus, which is on the same LG and corresponds to the allele *mg461*. To remove this mutation, both *gld-2* stains were outcrossed with wild-type and rebalanced with hT2 [*bli-4(e937) let-?(q782) qIs48*] (I;III). Unless stated otherwise, adult germline phenotypes were scored 24 h past mid-L4 stage. For the time course experiment in Figure 3, worms were synchronized at the L1 stage via food deprivation, and developmental stages of the population were determined by DIC analysis of the developing germline of 5–10 animals.

Primary antibodies

Primary antibodies against the following proteins were used: rabbit anti-RNP-8 (Kim et al. 2009); mouse anti-NPC (Mab414, Covance), anti-tubulin (T5168, Sigma), and anti-GLD-2 (Millonigg et al. 2014); and rat anti-GLD-3 (Eckmann et al. 2002). The mouse anti-GLD-2 (Millonigg et al. 2014) recognized the C terminus of the protein. The rabbit anti-GLD-2 antibody used in the immunoprecipitation experiments was generated by immunizing New Zealand white rabbits with an epitope that covered amino acid 959–1113 of GLD-2, fused to a GST-affinity tag. The fusion protein had been expressed in BL21 bacteria and purified via a GST column to homogeneity. An analogously prepared MBP-fusion protein had been coupled to Hi-TRAP NHS columns (GE Healthcare) and used for affinity purification of the immune serum rb184.

Immunocytochemistry

Indirect immunocytochemistry of extruded and 1% PFA-fixed gonads was carried out in solution as described (Rybarska et al. 2009). Images were taken on a Zeiss Imager M1 equipped with an Axiocam MRm (Zeiss) and processed with AxioVision (Zeiss) and Photoshop CS5 (Adobe). Secondary antibodies coupled to fluorochromes FITC, CY3, and CY5 were purchased from Jackson ImmunoResearch (Dianova).

Western blotting and immunoprecipitations

For Western blotting experiments, we collected individual worms by hand and boiled them in Laemmli protein sample loading buffer prior to gel separation. Worm protein extracts for protein coimmunoprecipitations were made as previously described (Jedamzik and Eckmann 2009), with a minor modification of the procedure; to generate liquid nitrogen-frozen worm powder, we used a MR301 bead mill (Retsch) at 30 Hz. For the immunoprecipitation procedure, we coupled affinity-purified rabbit 184 anti-GLD-2 antibodies to Protein A Dynabeads (Invitrogen). All immunoprecipitates were analyzed by Western blotting with ECL detection of HRPO-coupled secondary antibodies (Jackson ImmunoResearch) as previously described (Jedamzik and Eckmann 2009).

RNA isolation and qPCR

Total RNA was isolated from hand-picked whole worms using TRIzol (Invitrogen). Total RNA (200 ng) was reverse-transcribed using random hexamer primers and ReverseAid Premium reverse transcriptase (Thermo Fisher Scientific), according to the manufacturer's protocols. Quantitative PCR (qPCR) was conducted on an iQ5 (Bio-Rad), using the ABsolute QPCR SYBR Green mix (Thermo Fisher Scientific) and gene-specific primers (sequences available upon request).

PCR-based poly(A)-tail measurement (sPAT assay)

For measuring poly(A) tails of specific mRNAs, we performed the previously described sPAT assay (Minasaki et al. 2014). In short, we isolated total mRNA from hand-picked worms, ligated an RNA anchor to the 3′ ends, performed gene-specific RT-PCR, and resolved the samples on high-resolution agarose gels. Lane quantifications were performed using Fiji (ImageJ).

Bulk poly(A)-tail length measurements

One microgram of total RNA was used to perform bulk poly(A)-tail measurements, following a previously described protocol (Temme et al. 2004), with the only exception that un-incorporated [α-^{32}P]-Codycepin (PerkinElmer) was removed using mini Quick Spin Columns (Roche). Each sample was analyzed from three independent biological repeats; size markers were synthesized RNA oligos of 30 and 45 nt in length, and a loading dye band that corresponds to approximately 65 nt. Lane quantifications were performed using Fiji (ImageJ).

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3.4 RPL-4 and RPL-9-mediated ribosome purifications facilitate the efficient analysis of gene expression in *Caenorhabditis elegans* germ cells

Overarching question

Can a tissue-specific ribosome purification method be adopted to analyze germ cell translation?

Synopsis of the publication

The cellular complexity of model organisms is a major obstacle that limits our comprehensive understanding of gene expression networks in germ cells. For the *C. elegans* germ line, several studies provided transcriptomic data in the past which come with certain limitations. Most studies extract mRNAs from whole animals and infer the identity of cell-specific transcripts by using mutants that do not generate a significant germline tissue (REINKE *et al.* 2004). This restricts the analysis towards germ cell-enriched mRNAs. Alternatively, more comprehensive results can be obtained by conducting transcriptomics from dissected germ lines (ORTIZ *et al.* 2014). However, this approach is based on a tremendous amount of manual labor to obtain reasonable quantities of isolated germline tissue for each analysis. To facilitate gene expression analyses from germ cells I adopted a ribosome-immunoprecipitation method aimed at analyzing the translatome from specific cell types, named Translating Ribosome Affinity Purification (TRAP), to the *C. elegans* germ line. The TRAP approach combines easy access to large amounts of cellular material from whole animals with the selectivity of tissue-specific dissections. It relies on the ectopic expression of a tagged ribosomal protein specifically in the tissue of choice, which is subsequently used to purify ribosome-associated mRNAs from a defined cell population. In the process, I discovered that the ribosomal protein that is used in the classical TRAP assays is not the ideal choice to purify ribosomes from germ cells. With RPL-4 and RPL-9, I established and characterized two alternative ribosomal proteins that are much better suited for this task.

INVESTIGATION

RPL-4 and RPL-9–Mediated Ribosome Purifications Facilitate the Efficient Analysis of Gene Expression in *Caenorhabditis elegans* Germ Cells

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ABSTRACT In many organisms, tissue complexity and cellular diversity create a barrier that can hinder our understanding of gene expression programs. To address this problem, methods have been developed that allow for easy isolation of translated mRNAs from genetically defined cell populations. A prominent example is the Translating Ribosome Affinity Purification method also called TRAP. Here, ribosome associated mRNAs are isolated via purification of the ribosomal protein RPL10A/uL1, which is expressed under the control of a tissue specific promoter. Originally developed to investigate gene expression in mouse neurons, it has by now been adopted to many different organisms and tissues. Interestingly, TRAP has never been used successfully to analyze mRNA translation in germ cells. Employing a combination of genetic and biochemical approaches, I assessed several ribosomal proteins for their suitability for TRAP using the *Caenorhabditis elegans* germline as a target tissue. Surprisingly, I found that RPL10A/uL1 is not the ideal ribosomal component to perform such an analysis in germ cells. Instead other proteins such as RPL4/uL4 or RPL9/eL6 are much better suited for this task. Tagged variants of these proteins are well expressed in germ cells, integrated into translating ribosomes and do not influence germ cell functions. Furthermore, germ cell-specific mRNAs are much more efficiently co-purified with RPL4/uL4 and RPL9/uL6 compared to RPL10A/uL1. This study provides a solid basis upon which future germ cell TRAP experiments can be built, and it highlights the need for rigorous testing when adopting such methods to a new biological system.

KEYWORDS
germ cell
translation
ribosomal protein
gene expression

The development of germ cells is driven by complex gene expression programs, which dictate the production of proteins to a large extent via post-transcriptional mechanisms. Two aspects make it challenging to perform a comprehensive analysis of germ cell gene expression programs in an organism. First, the tissue complexity of most multicellular organisms often limits the amounts of homogeneous biological materials that can be analyzed and methods that facilitate the enrichment for a specific cell population might introduce changes in the transcriptome (Richardson *et al.* 2015). Second, gene expression programs are usually analyzed by measuring mRNA abundances in a cell. However, especially developing female germ cells accumulate a large number of maternal mRNAs that are only translated into proteins after fertilization. Therefore, transcriptome measurements can only provide a limited understanding of the gene expression programs that drive germ cell development.

Two methods have been developed that aim at the characterization of the translatome in specific tissues named RiboTag and Translating Ribosome Affinity Purification (TRAP) (Sanz *et al.* 2009; Heiman *et al.* 2014). Both methods work with the same principle; ribosomes and their associated mRNAs are purified via a specifically tagged protein of the large subunit. The ribosomal protein (RPL) protein is expressed under the control of a tissue specific promoter. Both methods were originally designed to characterize gene expression in mouse neurons (Sanz *et al.* 2009;

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Heiman *et al.* 2014). The main difference between the two methods is the choice of the RPL protein. Whereas TRAP utilizes RPL10A/uL1, which was N-terminally fused to GFP, RiboTag uses RPL22/eL22, which was fused C-terminally to an HA-tag (Sanz *et al.* 2009; Heiman *et al.* 2014). Of the two methods only TRAP was adapted to other organisms, such as *C. elegans*, *D. melanogaster*, zebrafish and *xenopus* to investigate gene expression in a variety of tissues such as neurons, intestines and muscles (Thomas *et al.* 2012; Watson *et al.* 2012; Tryon *et al.* 2013; Gracida and Calarco 2017). However, neither RiboTag nor TRAP has been rigorously tested for their usability to analyze gene expression in germ cells.

Employing *C. elegans* as a model, the four ribosomal proteins RPL-1/uL1, RPL-4/uL4, RPL-9/uL6 and RPL-22/eL22 were tested for their TRAP-suitability in germ cells. The expression, developmental impact, integration into translating ribosomes and amounts of co-purified mRNA were analyzed from worm strains expressing the tagged ribosomal proteins. Surprisingly, this showed that the previously used RPL-1/uL1 and RPL-22/eL22 are out performed by RPL-4/uL4 and RPL-9/uL6, strongly implying that these two proteins are a much better choice for germ cell TRAP assays.

MATERIALS AND METHODS

Nematode strains and transgenesis

Worms were handled according to standard procedures and grown at 20°, if not otherwise stated (Brenner 1974). The N2 Bristol strain was used as a reference for wild type. The following strain was used in this study: LG *III: glp-1(q224)*. Transgenic strains EV850 (*efIs157[Cbr-unc-119(+) + Pmex-5::rpl-9::FLAG::tbb-2 3′UTR] II*), EV927 (*efIs173 [Cbr-unc-119(+) + Pmex-5::rpl-1::FLAG::tbb-2 3′UTR] II*) and EV928 (*efIs174[Cbr-unc-119(+) + Pmex-5::rpl-22::FLAG::tbb-2 3′UTR] II*) were generated using the Mos1-mediated single copy insertion (MosSCI) protocol (Frøkjær-Jensen *et al.* 2012). Injected constructs were assembled using the multisite Gateway cloning system (Thermo Fisher Scientific). To this end, the entire genes including introns for *rpl-1*, *rpl-9* and *rpl-22* were amplified from genomic DNA, fused with 3xFLAG-tag encoding sequences via overlap extension PCR, and inserted into the entry vector pDONR221. The assembled constructs were injected into the recipient strain EG6699 ttTi5605 II; unc-119(ed3) III; oxEx1578. EV484 (*efIs155[Cbr-unc-119(+) + Pmex-5::rpl-4::FLAG::tbb-2 3′UTR] II*) and was described previously (Nousch *et al.* 2019).

For the fertility analysis, L4 animals were singled and passaged to a new plate every 24hrs until the mother stopped laying embryos. Living larvae were counted to assess brood size.

Western blotting

For Western blotting experiments samples were prepared from hand-picked worms, age 24h past mid L4, by boiling the collected material in Laemmli protein sample loading buffer prior to gel separation on 4–12% PAGE gradient gels (Eurogentec). Gel running and blotting was done following the manufacturer's protocol. The composition of the blotting buffer was as followed: Tris base 15 mM and Glycine 192 mM. Western blots were visualized with an Odyssey Fc Imaging System (LI-COR) after incubation with IRDye secondary antibodies (LI-COR). All secondary antibodies were used at 1:10,000 dilutions in 5% Milk/PBS/0.05% Tween.

Primary antibodies

Primary antibodies against the following proteins were used at the indicated dilutions: mouse anti-FLAG M2 (Sigma-Aldrich) 1:5000, anti-tubulin (T5168, Sigma) 1:5000 and RPS5 (sc-390935, Santa cruz) 1:100; and rabbit anti-PAB-1/2 (Nousch *et al.* 2014) 1:5000. All antibodies were diluted in 5% Milk/PBS/0.05% Tween.

Extract preparation for immunoprecipitations and sucrose gradient centrifugation

Worms were synchronized at the L1 larval stage, grown on 10 cm MGM plates spotted with OP50 and harvested as young adults (24h past mid L4). For harvesting, worms were washed off the plates with M9, collected into 15 ml tubes and centrifuged for 2 min at 500xg. The worm pellet was washed twice with M9 and once with B70 buffer (HEPES pH 7.4 50 mM, KAc 70 mM, NaF 1 mM, β-glycerophosphate 20 mM, MgOAc 5 mM, Triton X-100 0.1% and glycerol 10%) using 5x the pellet volume. Finally, the pellet was resuspended in equal volume of B70, mixed carefully and droplets were made in liquid nitrogen. The resulting worm pearls were stored at -80°.

For extract preparation the frozen pearls were ground into a fine powder using a MR301 ball mill at 30 hertz (Retsch). The powder was resuspended in 300 µl of B70+Inhibitors (DTT 1 mM, PMSF 1 mM, Benzamidine 2 mM, Pepstatin A 1 µg/ml, Leupeptine 1 µg/ml, Pefabloc 0.1 µg/ml, RNAaseOUT 100 U/ml and Cycloheximide 100 µg/ml) and spun in a bench top centrifuge for 10 min at 10000xg and 4°. The clear supernatant was transferred into a clean tube and the protein concentration was measured. The extract was now ready to be used for sucrose gradient centrifugation or immunoprecipitations.

Sucrose gradient centrifugation

Equal amounts of extracts were resolved through a 10 ml 17–50% sucrose gradient. The gradients were spun for 210 min at 35000 rpm and 4° in a SW40Ti rotor (Beckman Coulter). The fractionation was conducted bottom up while the absorbance profile at 260 nm was recorded. Proteins were concentrated by TCA precipitation and all pellets were dissolved in the same amount of Laemmli protein sample loading buffer.

Immunoprecipitation of ribosomes

Cellular extracts were pre-cleared using Protein A agarose beads (Sigma-Aldrich) for 1h at 4°. Afterward, 200 µl of extract were incubated with 20 µl anti-FLAG M2 affinity agarose (Sigma-Aldrich) for 2h at 4° with gentle mixing. This was followed by washing the bead material three times with 300 fresh B70+Inhibitors. RNA was isolated from the matrix material as well as the input as described below.

RNA isolation and qPCR

RNA was isolated from extracts or bead material using Trizol (Invitrogen). 200 ng of total RNA was reverse transcribed using random hexamer primers and ReverseAid Premium reverse transcriptase (Thermo Fisher Scientific), according to the manufacturer's protocol. Quantitative PCR (qPCR) was conducted on an iQ5 (BioRad), using the ABsolute QPCR SYBR Green mix (Thermo Fisher Scientific) and gene-specific primers (sequences available upon request).

Data availability

Strains and plasmids are available up on request. Supplemental material available at figshare: https://doi.org/10.25387/g3.12901601.

RESULTS

Choice and expression of tagged ribosomal proteins

Genes encoding homologs for all ribosomal proteins of the small and large subunit are present in the *C. elegans* genome (Table S1). The

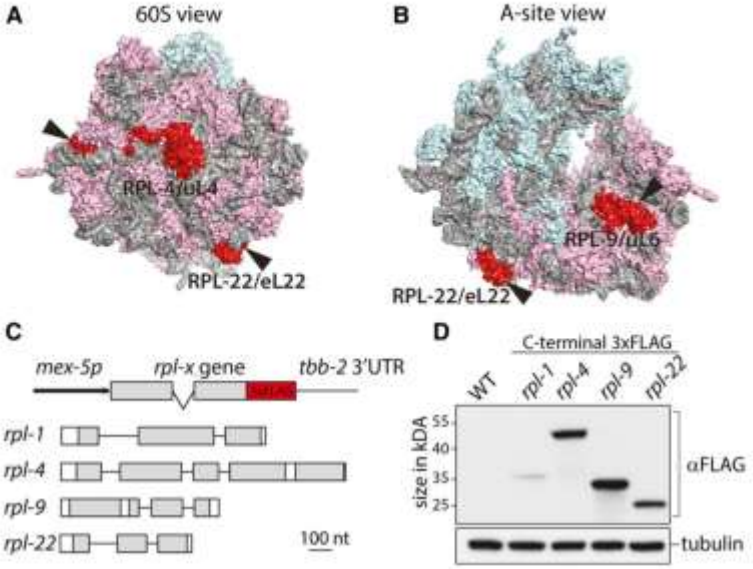

Figure 1 A differential expression is detected for tagged ribosomal proteins in germ cells. (A-B) Structure of the yeast 80S ribosome. RPL-4, RPL-9 and RPL-22 are shown in red. The arrow head indicates the position of the tag. Ribosomal RNA is shown in gray, proteins of the small subunit in light blue and proteins of the large subunit in pink. The original PyMOL file for the shown structure was generated by the Ban lab (https://bangroup.ethz.ch/research/nomenclature-of-ribosomal-proteins.html). (C) Top: Shown is the general structure of the expression constructs; Bottom: Genomic structure of *rpl-1*, *rpl-4*, *rpl-9* and *rpl-22*. Gray boxes indicate regions that define the different evolutionary conserved RPL protein regions. (D) Western blot analysis of RPL::FLAG expressing strains. Per lane 30 adult hermaphrodite were loaded.

only exception is RPL41 for which no gene could be identified. Four proteins from the large subunit were chosen to be tested for their suitability in a potential germ cell TRAP assay, as mRNAs copurified with these proteins should be part of an 80S ribosome and therefore are most likely actively translated. I investigated RPL-1/uL1 and RPL-22/eL22, because they have been used in TRAP assays in the past (Heiman *et al.* 2008; Sanz *et al.* 2009). Furthermore, because of their size and position in the ribosome, I included RPL-4/uL4 and RPL-9/uL6. In general, RPL proteins are rather small and in *C. elegans* the median size of all RPL proteins is 16 kD. RPL-4/uL4 and RPL-9/uL6 can be considered large for RPL proteins, with ~39 kD and ~22 kDA respectively. These sizes should decrease the likelihood that a small tag interferes with the functions of the two proteins. Positional information about RPL-4/uL4, RPL-9/uL6 and RPL-22/eL22 within the assembled ribosome can be inferred from the crystal structure of the 80S complex from *Saccharomyces cerevisiae* (Ben-Shem *et al.* 2011). RPL-4/uL4 is located on the solvent side of the 60S subunit (Figure 1A), RPL-9/uL6 is close to the A-site (Figure 1B) and RPL-22/eL22 is close to the interface with the 40S subunit (Figure 1A and B). The position of RPL10A/uL1, the homolog of RPL-1, has been previously mapped close to the exit channel of the mRNA (Anger *et al.* 2013). In contrast to RPLs with strong integral binding positions within rRNA (*e.g.*, uL2, eL33 or eL37) RPL10A/uL1, RPL-4/uL4, RPL-9/uL6 and RPL-22/eL22 show more peripheral attachment to rRNA and should be accessible for immune-purifications.

Expression constructs were generated for FLAG-tagged RPL-1/uL1, RPL-4/uL4, RPL-9/uL6 and RPL-22/eL22 (Figure 1C). To this end, the genetic loci of the four *rpl*-genes were C-terminally fused to 3xFLAG and cloned into plasmids, which permit germ cell specific expression. The production of mRNAs was controlled by the well characterized *mex-5* promoter (*mex-5P*) and protein production was directed by the 3'UTR of tubulin (*tbb2*), which allows translation of an mRNA during all stages of germ cell development (Merritt *et al.* 2008). The entire expression cassettes were integrated into the *C. elegans* genome as single copies using the MosSCI method (Frøkjær-Jensen *et al.* 2012). Western blot analysis of homozygous adults that carry the expression cassettes revealed strong expression of tagged RPL-4/uL4 and RPL-9/uL6, moderate expression of tagged RPL-22/eL22 and low expression for tagged RPL-1/uL1 (Figure 1D). This shows that FLAG fusion proteins can be efficiently produced for RPL-4/uL4, RPL-9/uL6 and RPL-22/eL22 but not for RPL-1/uL1 in germ cells.

Tagged ribosomal protein expression in germ cells has no influence on fertility

To test whether the expression of tagged RPL-1/uL1, RPL-4/uL4, RPL-9/uL6 or RPL-22/eL22 has an impact on germ cell development, I analyzed the brood sizes of homozygous transgenic animals grown at different temperatures. At 20°, a wildtype worm produces around 300 offspring (Figure 2A). No significant difference in brood size was detected for any of the animals expressing the tagged RPL proteins (Figure 2A). At 25°, a wildtype animal has a slightly reduced brood and produces around 250 offspring (Figure 2B). Interestingly, this number is significantly reduced to ~200 progeny for all RPL::FLAG expressing strains (Figure 2B). To test whether this might be a consequence of increased expression of the tagged RPL proteins I investigated the RPL::FLAG protein levels by Western blotting. Surprisingly, no significant difference in RPL::FLAG expression could be detected between worms grown at 20° or 25° (Figure 2C). In general, no correlation seems to exist between RPL::FLAG expression levels and fertility, suggesting that the decrease in brood size at elevated temperature might be an inherent property of the MosSCI strain that was used in this work to generate the transgenic animals. This argues that the expression of tagged RPL-1/uL1, RPL-4/uL4, RPL-9/uL6 and RPL-22/eL22 has no negative impact on germ cell functions.

Tagged RPL proteins are present in polysomes

It is important that the tagged RPLs do not influence the efficiency of general translation. Hence, I conducted a polysome analysis investigating the distribution of active and non-active ribosomes (polysomes *vs.* non-polysomes). To this end, whole animal extracts from adult wild type or transgenic worms were separated on a 17–50%

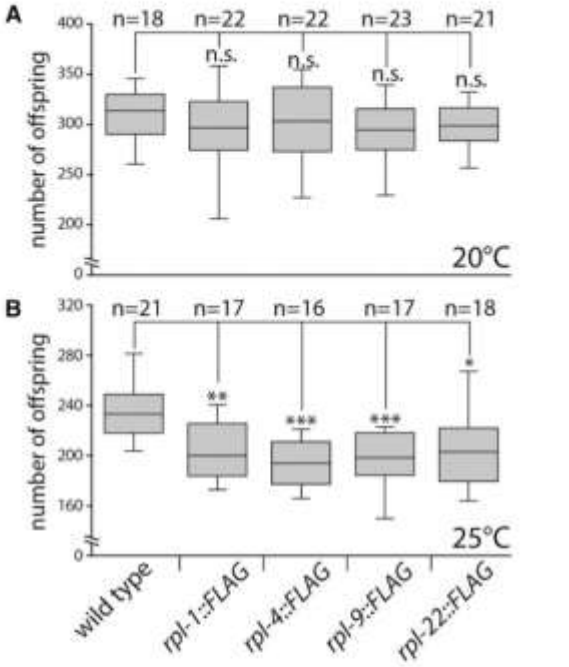

Figure 2 Expression of tagged RPLs in germ cells has no influence on fecundity. The fertility of parental hermaphrodites (n) was analyzed by counting hatched F1 larvae (progeny). (A) At 20°, no significant difference was detected in the number of median offspring generated by wild type or *rpl::flag* expressing worms. (B) At 25°, all *rpl::flag* expressing strains produce less progeny compared to wild type. (C) Western blot analysis of *rpl::flag* expressing strains grown at different temperatures. No obvious differences in expression levels are detected.

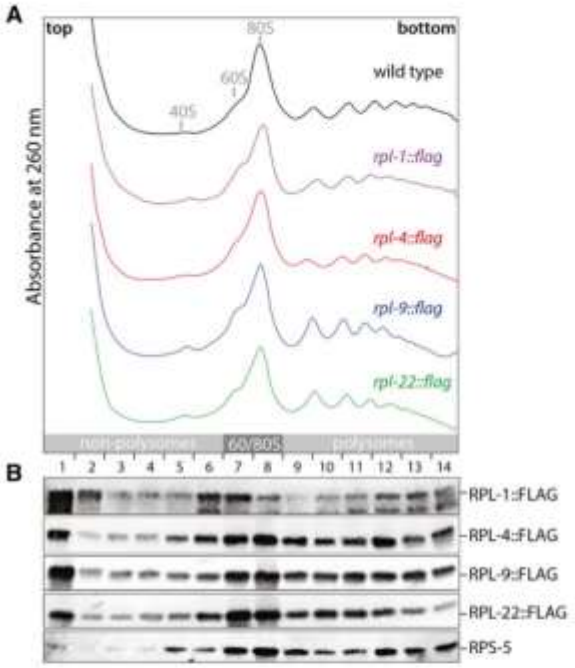

Figure 3 Differential integration of tagged ribosomal proteins into translating ribosomes. A polysome analysis was conducted comparing wild type to *rpl::flag* expressing animals. (A) Absorbance traces at 260 nm of polysomal gradients from wild type, *rpl-1::flag*, *rpl-4::flag*, *rpl-9::flag* and *rpl-22::flag* worms. Similar amounts of cellular proteins were loaded onto each gradient. The positions of the major ribosomal complexes are shown on the top. The numbers on the bottom indicate the fractions that were collected. (B) Western blot analysis of the gradient fractions. RPS-5 distribution was similar in all gradients. The image shown was generated from the *rpl-22::flag* gradient.

sucrose gradient and fractionated. During the fractionation procedure the absorbance at 260 nm was recorded in order to trace the migration pattern of ribosomes. In wild type, a prominent 80S ribosome peak was detected in the middle of the gradient dividing polysomal from non-polysomal regions (Figure 3A). Furthermore, only minor peaks corresponding to the 60S and 40S ribosomal subunits are present in this sample (Figure 3A). In the *rpl::flag* strains, the overall distribution of ribosomal complexes is highly similar to the one in wild type (Figure 3A). In adults, germ cells contribute a significant amount of biological material toward whole animal extracts. In order to judge how much RNA in lysate comes from germ cells, I measured total RNA amounts isolated from wild type and *glp-1(q224)*, a temperature sensitive strain which virtually has no germ cells at 25° (Austin and Kimble 1987). The yield of isolated RNA from *glp-1(q224)* adults grown at 25° was ~50% lower compared to wild type (Fig. S1). This suggests that half of the total RNA in an adult originates from germ cells. Therefore, severe translational defects that occur in germ cells would be detectable in a polysome profile. Only small differences are detected among the different *rpl* samples. Heavy polysomes are slightly decreased and light polysomes are increased in *rpl-9::flag* and *rpl-22::flag* compared to *rpl-1::flag* and *rpl-4::flag* (Figure 3A). This minor shift in the distribution of translating ribosomes could indicate that FLAG-tagged RPL-9 and RPL-22 either slightly decrease translation initiation rates or increase translation elongation speeds. Nonetheless, the overall absence of strong polysome abnormalities argues that the expression of RP-1::FLAG, RPL-4::FLAG, RPL-9::FLAG or RPL-22::FLAG has no major effect on the global translation efficiency of ribosomes.

Next, I investigated whether the tagged RPLs are part of active translating ribosomes. Thus, the migration behavior of the fusion proteins in the gradients was analyzed by Western Blot. RPS-5 of the small ribosomal subunit served as a marker illustrating the gradient distribution of an endogenous ribosomal component. A small amount of RPS-5 is detected in fraction one, which is the top of the gradient (Figure 3B). This is most likely corresponding to free proteins, which are not associated with larger complexes and therefore do not enter the gradient. The majority of RPS-5 is present around the 80S peak and in polysomes (Figure 3B). The RPL::FLAG

proteins accumulate stronger on top of the gradient compared to RPS-5 (Figure 3B). This might be explained by the fact that the fusion proteins have to compete with the endogenous versions of the RPLs for incorporation into the large ribosomal subunit. Regardless, all RPL::FLAG proteins in the gradient are detectable in 80S and polysomal fractions, mirroring to a large degree the distribution of RPS-5 (Figure 3B). This migration pattern suggests that all tagged RPLs can be incorporated into translating ribosomes. However, quantitative differences exist. RPL-1::FLAG is strongly present on the top and only weakly in heavy regions of the gradient. On the other hand, RPL-4::FLAG, RPL-9::FLAG and RPL-22::FLAG show robust co-sedimentation with polysomes, arguing that these three proteins are better integrated into functional ribosomes compared to RPL-1::FLAG (Figure 3B).

Analyzing the ribosome association of germline mRNAs

Next, I addressed which of the different tagged RPL proteins can be utilized to analyze mRNA translation. To this end, the different tagged RPL proteins were purified from adult whole animal extracts using anti-FLAG beads. Extracts prepared from wildtype animals, which do not express a tagged protein, were also incubated with anti-FLAG beads and served as a negative control. For better quantitative comparison, equal amounts of extracts were incubated with the same bead volume for each sample. The investigation of the bead material by Western blotting shows that all tagged ribosomal proteins could be specifically enriched with the anti-FLAG beads (Figure 4A). The purification efficiency was low for RPL-1::FLAG, medium for RPL-22::FLAG and the highest for RPL-4::FLAG and RPL-9::FLAG (Figure 4A). To test whether translation-related proteins could be co-purified, the presence of cytoplasmic poly(A) binding protein PAB-1/2 was analyzed in the immune-purifications. PAB-1/2, a factor that binds to poly(A) tails and promotes translation, was detected in RPL-4::FLAG and RPL-9::FLAG purifications (Figure 4A) (Gu *et al.* 1995). On the other hand, tubulin, a protein not associated with translation, could not be detected in any purification (Figure 4A). In summary, all tagged RPLs can be purified using the FLAG tag. However, only RPL-4 and RPL-9 allow the efficient co-purification of a translational factor, suggesting that the two RPLs are part of active ribosomes.

Finally, it was tested whether components of the ribosome can be enriched with the tagged RPL proteins. To this end, RNA was isolated from the input and RPL::FLAG purified material and analyzed for the presence of ribosomal RNA. A signal for the 18S and 26S rRNA was barely visible in the control providing the baseline for a background enrichment of rRNA (Figure 4B). In all purifications rRNA was detected above background levels with the strongest signals in RPL-4::FLAG, RPL-9::FLAG and RPL-22::FLAG (Figure 4B). This suggests that in general ribosomes can be purified with all four tagged ribosomal proteins.

To test whether germline-specific mRNAs can be co-purified with the different RPL::FLAG proteins, RNA was isolated from purified material, converted into cDNA and the amounts of specific mRNAs were measured by quantitative real-time PCR. Eight mRNAs previously described as being germline-enriched and four mRNAs that are primarily expressed in the soma were quantified by measuring their enrichment with the different RPL::FLAG proteins relative to the control purification. None of the four soma-enriched mRNAs was significantly enriched with any RPL::FLAG purification (Figure 4C). In contrast to this, significant enrichments were detected for germline enriched mRNAs (Figure 4C). Again differences existed in co-purification efficiency, with RPL-1::FLAG showing enrichment for 1/8, RPL-4::FLAG for 8/8, RPL-9::FLAG for 8/8 and RPL-22::FLAG for 7/8 measured germline mRNAs (Figure 4C). This shows that a large complement of germ cell specific mRNAs can be efficiently isolated using tagged RPL-4, RPL-9 and RPL-22. The combined immunoprecipitation results strongly argue that tagging RPL-4 or RPL-9 provide the best handle to purify active ribosome/mRNA complexes from germ cells in *C. elegans*.

DISCUSSION

The goal of this work was to identify suitable ribosomal proteins that allow an efficient analysis of mRNA translation in *C. elegans* germ cells. Surprisingly, the overall data show that of the four tested RPLs the most commonly used in the literature, RPL-1/uL1, is not a good choice for this task. The RPL-1 fusion protein is not well expressed in germ cells, does not strongly associate with ribosomes and thus allows only inefficient copurification of germ cell mRNAs. The main difference between the RPL-1/uL1 variant in my work and studies in the past is the position and nature of the tag. Whereas in the presented work a C-terminal FLAG was used, the initial study in mice employed an N-terminally GFP-tagged RPL10A/uL1 for the purification procedure (Heiman *et al.* 2008). In the original work only GFP::RPL10A/uL1 was tested and nearly all studies that adopted the TRAP assay to other organism copied this design (Thomas *et al.* 2012; Watson *et al.* 2012; Tryon *et al.* 2013; Gracida and Calarco 2017). Only in zebrafish a double-tagged RPL10A with GFP on the N- and HA on the C-terminus was used to enrich hair cell-specific mRNAs (Matern *et al.* 2018). Unfortunately, the zebrafish study did not provide any evidence to which degree the double tagged protein is truly functional, and it is entirely possible that any modification at the C-terminus affects RPL-1/10A function. Therefore, using an N-terminal FLAG tag might improve the performance of an RPL-1 fusing protein for a TRAP assay in germ cells.

The TRAP method relies on the ectopic expression of modified ribosomal proteins in specific tissues and cells. For an easy adaptation of this assay to new biological systems the perfect TRAPable ribosomal protein should show little developmental expression variation. Interestingly, many ribosomal proteins have paralogs that can be expressed in a spatial and temporal restricted manner resulting in a tissue specific composition of ribosomes (Genuth and Barna 2018). Although, no functional paralogs have been described for uL1 and eL22 in *C. elegans*, these two proteins might contribute to the diversification of ribosomes in other organisms. In *Drosophila*, uL1 proteins are encoded by two genes, RpL10Aa and RpL10Ab (Wonglapsuwan *et al.* 2011). According to modENCODE data (http://www.modencode.org/), RpL10Ab seems to be the uL1 protein variant which is expressed in most tissues, whereas RpL10Aa expression is enriched in adult testis. For eL22, paralogs with tissue-specific functions have been described in flies and zebrafish (Zhang *et al.* 2017; Mageeney and Ware 2019). Contrary to this, rpl-4/uL4 and rpl-9/uL6 are each encoded by only one gene in worms, fly, fish and mice. This lack of protein variability argues that in all organisms these two RPLs should be easily suitable for ribosome purifications from most tissues.

Surprisingly, heterogeneity of the translational machinery might occur not only on the tissue level but also has been proposed to exist within cells. Absolute abundance measurements of 15 of the 80 core ribosomal proteins in polysomes from mouse embryonic stem cells showed that six proteins are substoichiometric (Shi *et al.* 2017). One of these six proteins is RPL10A/uL1, suggesting that not every actively translating ribosome contains this protein. Additionally, the same study found that RPL10A/uL1 purifications co-enrich for specific

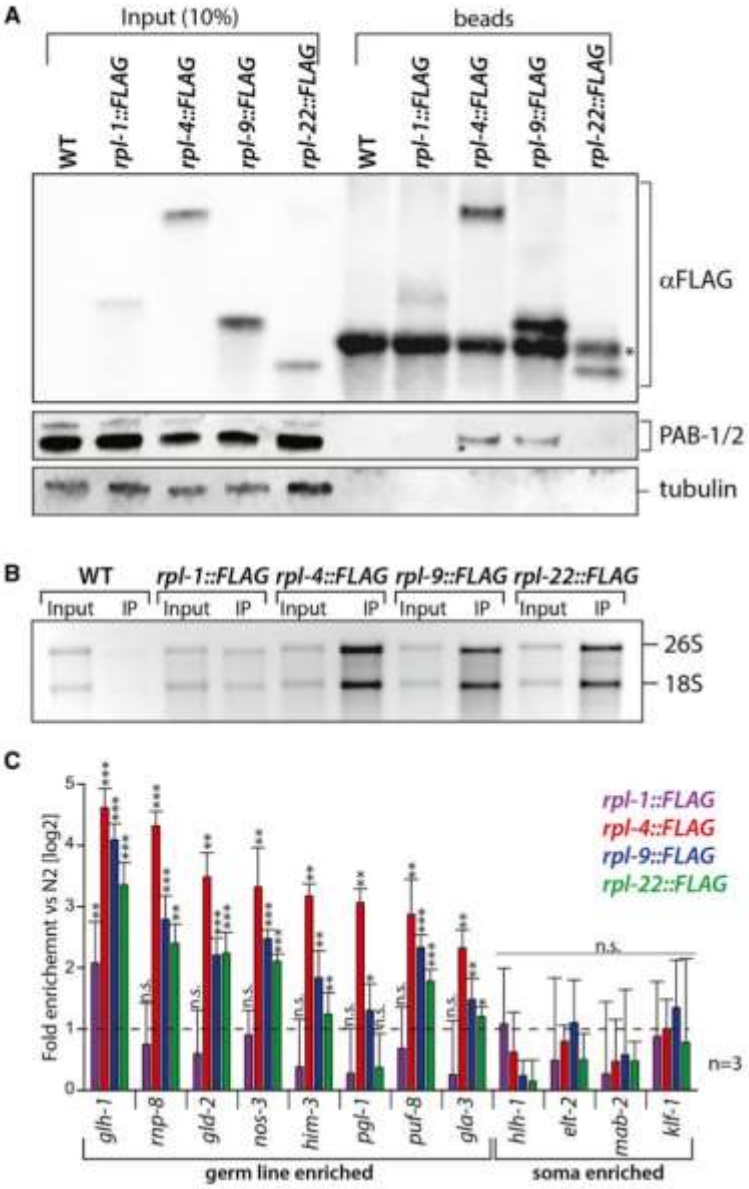

Figure 4 Translationally active mRNAs can be efficiently co-purified with RPL-4::FLAG and RPL-9::FLAG. (A) Western blot analysis of copurified RPL::FLAG proteins from adult worms. Equal amount of extract and anti-FLAG beads were used in the different immunopurifications. Input and bead material were analyzed for the indicated proteins. The asterisk in the FLAG blot markes an IgG band. (B) RNA was isolated from input and bead material and analyzed on a denaturing RNA gel stained with ethidium bromide for the presence of the 18S and 26S rRNA. Equal fractions of bead material were loaded for each immunoprecipitation. The signal in the input represents 1% of the amount of extract that was incubated with the bead material. (C) Analysis of mRNA enrichments in the different purifications using quantitative real time PCR.

mRNAs (Shi *et al.* 2017). Interestingly, the vast majority of studies that characterize tissue-specific translatomes in organisms solely rely on data generated via RPL10A/uL1 purifications (Doyle *et al.* 2008; Heiman *et al.* 2008; Thomas *et al.* 2012; Zhou *et al.* 2013; Liu *et al.* 2014; Gracida *et al.* 2017; Matern *et al.* 2018; Rodrigues *et al.* 2020). This raises the question to which degree the TRAP data generated by these studies truly reflect the global translation programs occurring in the investigated cell types. Contrary to RPL-1/uL1, the proteins RPL-4/uL4, RPL-9/uL6 and RPL-22/eL22 are most likely constitutive and, thus, a part of every ribosome in a cell (Shi *et al.* 2017). Hence, mRNAs copurified via these proteins should provide an unbiased view of translation in cells.

A growing number of studies provide evidence that ribosomal proteins play an important part in the translation of specific mRNA (Kondrashov *et al.* 2011; Zhang *et al.* 2013; Shi *et al.* 2017). Hence, the view of the ribosome has shifted away from the rigid machine that is the same in every cell toward a more dynamic macromolecular complex with specialized compositions and functions (Genuth and Barna 2018). Consequently, methods originally developed to survey global mRNA translation in cells and tissues should be reevaluated, especially if adapted to a new biological system. The ideal ribosomal protein used for TRAP assays should be a stable component of the ribosome in many biological settings. The robust performance of RPL-4/uL4 and RPL-9/uL6 in *C. elegans* germ cells combined with the evolutionary conserved nature of uL4 and uL6 homologs as core components of the ribosome on the cellular as well as the tissue level, strongly argues that these proteins might be the ideal choice for future TRAP assays in many biological systems.

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3.5 Stage-specific combinations of opposing poly(A) modifying enzymes guide gene expression during early oogenesis

Overarching question

How are opposing poly(A) modifying enzymes utilized during germ cell development?

Synopsis of the publication

By now poly(A) modifying enzymes are recognized as crucial components of the gene expression networks in germ cell development (MORRIS *et al.* 2005; SUH *et al.* 2006; BENOIT *et al.* 2008; NOUSCH *et al.* 2013). In this context, deadenylases are considered as negative and cytoPAPs as positive mRNA regulators. In my previous works, I investigated the roles of deadenylases and cytoPAPs in *C. elegans* germ cell development separately. However, the interplay between these opposing poly(A) regulators remained poorly understood. Using genetics, RNA sequencing and the TRAP assay I analyzed the developmental and molecular relationship between GLD-2 and different deadenylases in *C. elegans*. I found that Ccr4-Not is the main counterforce to GLD-2 controlling mRNA polyadenylation and abundance in germ cells. A GLD-2/ Ccr4-Not balance is vital for all steps of oogenesis where the opposing activities of these enzymes are utilized by several RNA-binding proteins to control their mRNA targets. Furthermore, this work suggests that the mechanism of GLD-2-mediated RNA regulation switches from promoting mRNA stability during early stages towards controlling translational efficiency during late stages of female germ cell development. Overall, this work identified with GLD-2 and Ccr4-Not two evolutionary conserved regulators that most likely provide the molecular framework for gene-specific poly(A) tail regulation not only in *C. elegans* but also in other biological systems that utilize the dynamic potential of the tail to control gene expression.

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Stage-specific combinations of opposing poly(A) modifying enzymes guide gene expression during early oogenesis

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ABSTRACT

RNA-modifying enzymes targeting mRNA poly(A) tails are universal regulators of post-transcriptional gene expression programs. Current data suggest that an RNA-binding protein (RBP) directed tug-of-war between tail shortening and re-elongating enzymes operates in the cytoplasm to repress or activate specific mRNA targets. While this concept is widely accepted, it was primarily described in the final meiotic stages of frog oogenesis and relies molecularly on a single class of RBPs, i.e. CPEBs, the deadenylase PARN and cytoplasmic poly(A) polymerase GLD-2. Using the spatial and temporal resolution of female gametogenesis in the nematode *C. elegans*, we determined the distinct roles of known deadenylases throughout germ cell development and discovered that the Ccr4–Not complex is the main antagonist to GLD-2-mediated mRNA regulation. We find that the Ccr4–Not/GLD-2 balance is critical for essentially all steps of oocyte production and reiteratively employed by various classes of RBPs. Interestingly, its two deadenylase subunits appear to affect mRNAs stage specifically: while a Caf1/GLD-2 antagonism regulates mRNA abundance during all stages of oocyte production, a Ccr4/GLD-2 antagonism regulates oogenesis in an mRNA abundance independent manner. Our combined data suggests that the Ccr4–Not complex represents the evolutionarily conserved molecular opponent to GLD-2 providing an antagonistic framework of gene-specific poly(A)-tail regulation.

INTRODUCTION

The ability to regulate gene expression at the mRNA level is crucial for many developmental and physiological processes. In the cytoplasm, gene expression control is achieved by the association of mRNAs with designated mRNA-binding proteins (RBPs) to repress or activate select mRNA targets, forming larger mRNA–protein (mRNP) complexes. Among sequence-specific RBPs also RNA-modifying enzymes that either shorten or elongate the length of mRNA 3′ poly(A) tails were repeatedly identified in previous biochemical purifications of mRNPs, suggesting that endured presence of these enzymes may facilitate efficient mRNA regulation (1,2). In this context, tail shortening is mediated by deadenylases (DeAds) correlating with repression of mRNAs, whereas tail elongation is mediated by cytoplasmic poly(A) polymerases (cytoPAPs) correlating with activation of mRNAs. Hence, opposing activities of DeAds and cytoPAPs might provide an antagonistic frame work for many RBPs to mechanistically regulate mRNAs in the cytoplasm.

In animal development, only one prominent example has been described of how the molecular antagonism between poly(A)-tail modifiers suppresses and reactivates mRNA activities. During frog oocyte maturation, the deadenylase PARN and cytoPAP GLD-2 form an antagonistic pair to regulate the translation of mRNAs whose protein products drive the progression through both meiotic divisions in *Xenopus laevis* (3). The PARN-GLD-2 antagonism is instructed by the sequence-specific RBP CPEB and serves as a hallmark of how a DeAd/cytoPAP rheostat may be employed in gene-specific mRNA regulation. However, whether the antagonistic PARN-GLD-2 pair has general validity and extends to other stages of germ cell biology or even to other organisms remains unclear. Currently, no studies exist that directly address other potential DeAd–cytoPAP relationships and it remains to be shown how relevant this opposing enzyme pair might be for other RBPs than CPEBs.

In contrast to PARN, which is present in most but not all multi-cellular organisms, two other prominent mRNA deadenylases have been described that are evolutionary conserved from yeast to humans: the Pan2 and the Ccr4–Not

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complex (1). While the Pan2 complex is composed of two subunits, the single enzyme Pan2 and its regulatory scaffold Pan3, the Ccr4–Not deadenylase is a multi-subunit complex that contains two enzymatic components. Both deadenylases, Caf1 (also known as Pop2) and Ccr4, are attached as a single module to the central scaffolding protein Not1 (4). The Ccr4–Not complex assumes a pivotal role in the general mRNA degradation pathway, and as such provides the major poly(A)-removing activity in all organisms tested to date (5–7). Importantly, Ccr4–Not appears to also participate in gene-specific mRNA repression in many organisms. A number of evolutionary conserved cytoplasmic RBPs, including PUF proteins or Zinc-finger-containing RBPs, utilize this complex to silence and/or degrade mRNA targets (8,9). Whereas the importance of the Ccr4–Not complex for the control of cytoplasmic gene expression regulation is undisputed, a potential antagonistic role in a likely DeAd–cytoPAP relationship and its relevance to other sequence-specific RBPs remains to be determined.

Across species, GLD-2-type poly(A) polymerases provides the major poly(A)-tail elongation activity in the cytoplasm (10,11). These cytoPAPs are terminal nucleotidyl transferases with a preference for efficient A-addition to mRNAs that already end on adenosine (12). The enzymatic activity of GLD-2 is stimulated by interacting proteins and represents the main driver of its molecular and biological functions (13–15). Consistent with its essential roles in various stages of germ cell development, GLD-2-type enzymes are strongly expressed in germ cells, however they are not uniformly abundant throughout gametogenesis (13). Although deadenylases are ubiquitously expressed in most tissues of various organisms, the Ccr4–Not complex is particularly crucial for germ cell biology; loss of its components leads to germ cell defects during female oocyte production in metazoans (5,16,17). However, whether the Ccr4–Not deadenylase complex forms an antagonistic pair with GLD-2-type cytoPAPs in tissue-specific mRNA regulation is currently not known.

In this study, we exploit the simple spatial and temporal resolution of female gametogenesis in the nematode *C. elegans* to reveal broad-scale antagonistic relationships of poly(A)-tail modifying enzymes. Unlike vertebrates, the gonad of this animal model system facilitates a refined molecular and morphological analysis of all phases of gametogenesis preceding the stage of oocyte maturation. Our work identifies, with a precision that is not possible in vertebrate animals, the Ccr4–Not complex as the major deadenylase that opposes GLD-2 cytoPAP to regulate essentially all phases of early oogenesis, reaching from germ stem cell proliferation to oocyte maturation. Interestingly, in opposition to GLD-2 clear differences exist among the two catalytic subunits of Ccr4–Not. Whereas Caf1, termed CCF-1 in worms, is primarily important for all phases of early female gametogenesis by regulating mRNA abundance, CCR-4 plays a significant role in later phases by promoting gene expression possibly in a translational rather than abundance-dependent manner. This molecular difference coincides with a likely shift of GLD-2 function from promoting mRNA stability to promoting mRNA translatability. Finally, our data suggests that several evolutionary conserved RBPs rely on the antagonistic Ccr4–Not/GLD-2 pair to regulate their target mRNAs. Our combined work reveals that the opposing forces of the deadenylase Ccr4–Not and poly(A) polymerase GLD-2 provide an antagonistic frame work to cytoplasmic gene expression regulation, which is presumably tuned by many diverse RBPs to balance mRNA abundance and translation.

MATERIALS AND METHODS

Nematode strains and transgenesis

Worms were handled according to standard procedures and grown at 20°C (18). The N2 Bristol strain was used as a reference for wild type. Other strains used in this study: Linkage group (LG) *I: gld-2(q497), rrf-1(pk1417); II: parn-2(tm1339); III: panl-2(tm1575); IV: ccr-4(tm1312); V: parn-1(tm869).* Unless stated otherwise, adult germline phenotypes were scored 24hrs past mid-L4 stage. Transgenic strains EV484 (*efIs81*[Cbr-*unc-119(+)* + *Pmex-5::rpl-4::FLAG::tbb-2 3′UTR*] II) were generated using the Mos1-mediated single copy insertion (MosSCI) protocol (19). Injected constructs were assembled using the multisite Gateway cloning system (Thermo Fisher Scientific). To this end, the entire gene of *rpl-4* was amplified from genomic DNA, fused with 2xFLAG-tag encoding sequences via overlap extension PCR, and inserted into the entry vector pDONR221. The size of the proliferative region was analysed by counting nuclei along the distal-proximal axis until a cluster of three to four nuclei with crescent shaped DNA in a circumference was detected.

RNAi feeding constructs and procedure

The feeding constructs targeting *ccf-1*, *ntl-1* and *gld-2* were described previously (5,10). For *ccf-1* RNAi treatment in wild type and *gld-2(q497)*, animals were fed from L1 onwards. The same is true for *gld-2* RNAi treatments of EV484 animals. For *ntl-1* RNAi treatment in wild-type and *gld-2(q497)*, L4 animals were fed until adulthood. For RNAi treatments of *rrf-1(pk1417)*, L4 animals were placed on RNAi plates and F1 progenies were analyzed at the adult stage.

Primary antibodies

Primary antibodies against the following proteins were used: mouse anti-FLAG M2 (Sigma-Aldrich), anti-NPC (Mab414, Covance), anti-tubulin (T5168, Sigma), DAO-5 (20) and anti-GLD-2 (21); guinea pig anti-HIM-3 (22) and anti-OMA-1/2 (5).

Immunocytochemistry

Indirect immunocytochemistry of extruded and 1% paraformaldehyde PFA-fixed gonads was carried out in solution as described (23). Images were taken on a Zeiss Imager M1 equipped with an Axiocam MRm (Zeiss) and processed with AxioVision (Zeiss) and Photoshop CS5 (Adobe). Secondary antibodies coupled to fluorochromes FITC, CY3 and CY5 were purchased from Jackson ImmunoResearch (Dianova).

Western blotting, sucrose gradients and immunoprecipitations

For Western blotting experiments, we collected individual worms by hand and boiled them in Laemmli protein sample loading buffer prior to gel separation. Specific proteins were analyzed by western blotting with ECL detection (GE Healthcare) of HRP-coupled secondary antibodies (Jackson ImmunoResearch). Worm protein extracts for sucrose gradients and ribosome immunoprecipitations were made as described (5). The sucrose gradient centrifugation and fractionation was conducted as previously described (10).

RNA isolation and qPCR

Total RNA was isolated from hand-picked whole worms or immunoprecipitated material using Trizol (Invitrogen). 200 ng of total RNA was reverse transcribed using random hexamer primers and ReverseAid Premium reverse transcriptase (Thermo Fisher Scientific), according to the manufactures protocol. Quantitative PCR (qPCR) was conducted on an iQ™5 (BioRad), using the ABsolute QPCR SYBR Green mix (Thermo Fisher Scientific) and gene-specific primers (sequences available upon request). Immunoprecipitated RNA was also visualized via denaturing agarose gel electrophoresis.

Bulk poly(A)-tail length measurements

One microgram of whole worm total RNA was used to perform bulk poly(A)-tail measurements as previously described (5). Each sample was analyzed from three independent biological replicates; size markers were synthesized RNA oligos of 30 and 45 nucleotides in length, and a loading dye band that corresponds to proximally 65nts. Lane quantifications were performed using Fiji (ImageJ).

Library preparation and next-generation sequencing (NGS)

mRNA was isolated from 1 μg DNAse-treated total RNA using the Ribo-Zero Gold Kit (human, mouse, rat) from Illumina according to the manufacturer's instructions. Final elution was done in 5 μl nuclease free water. Samples were then directly subjected to the workflow for strand-specific RNA-Seq library preparation (Ultra Directional RNA Library Prep II, NEB). For ligation, custom adaptors were used 1: (Adaptor-Oligo 5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT-3′, Adaptor-Oligo 2: 5′-P-GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC-3′). After ligation, adapters were depleted by an XP bead purification (Beckman Coulter) adding beads in a ratio of 1:1. Indexing was done during the following PCR enrichment (15 cycles) using custom amplification primers carrying the index sequence indicated with 'NNNNNN'. (Primer1: Oligo_Seq AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T, primer2: GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T, primer3: CAA GCA GAA GAC GGC ATA CGA GAT NNNNNN GTG ACT GGA GTT. After two more XP beads purifications (1:1) libraries were quantified using Qubit dsDNA HS Assay Kit (Invitrogen). Samples were equimolarly pooled and used for 75bp single read sequencing on a Nextseq 500 (Illumina).

Analysis of NGS data

First, the high quality of the data was ensured using the software fastqc (v. 0.11.5) [FastQC A Quality Control tool for High Throughput Sequence Data http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ by S. Andrews]. The fastq files were than mapped to the *Caenorhabditis elegans* genome (Ensembl genome version WBcel235) using the STAR algorithm (v. 2.5.2b) (24). Subsequently, the reads were counted per gene using the featureCounts tool from the SubRead package (v. 1.4.6-p4) (25). Reads were counted on a gene level from the bam files based on the ensemble annotations.

Using the DESeq2 package (R version 3.4.0, DESeq2 version 1.18.1) the count data was normalized by the size factor to estimate effective library sizes (26). Following a dispersion calculation across all samples, a pair-wise comparison of various conditions was done resulting in a list of differentially expressed genes for each of the compared groups. Genes with a normalized read count of higher or equal 100, a fold-change higher or equal to 1.5 and with an adjusted *P*-value lower or equal to 0.05 were defined as differentially expressed (DE).

Immunoprecipiation of ribosomes

Worms expressing rpl4::FLAG were grown on *gld-2(RNAi)* or control RNAi plates. Extract preparations and immunoprecipitations were conducted as previously described (15) using anti-FLAG M2 affinity agarose (Sigma-Aldrich). RNA was isolated from the matrix material as well as the input material using Trizol (Invitrogen). Equal amounts for each sample of isolated RNA (200 ng) were converted into cDNA and analysed via qPCR as described above. A ribosome association coefficient was calculated for each analysed mRNA (amount of ribosome-bound mRNA/ amount of input mRNA). In order to control for sample to sample variations all measurements were normalized to a negative control (*nos-3*).

RESULTS

The Ccr4–Not complex is essential for proliferation

The *Caenorhabditis elegans* adult gonad is a tube-like organ in which female germ cells are organized in a distal-to-proximal gradient of oocyte production, representing all stages of oogenesis. At its most distal end, germline stem cells divide mitotically and occupy the proliferative region together with cells preparing for meiosis. Further proximally, germ cells enter meiosis and begin to differentiate gradually while progressing through all stages of prophase I, before arresting in diakinesis at the proximal end as fully differentiated oocytes (Figure 1A). The terminal oocyte will eventually leave the germline tissue in the process of ovulation and matures to become fertilization competent.

In this nematode, homologs have been identified for all major DeAds (5). The two Ccr4–Not associated enzymes, Ccr4 and Caf1, are represented by CCR-4 and CCF-1, respectively. PANL-2 is homologous to the Pan2 deadenylase. PARN-1 and PARN-2 represent two orthologues of worm PARN. Throughout this work the function of *ccr-4*, *panl-2*,

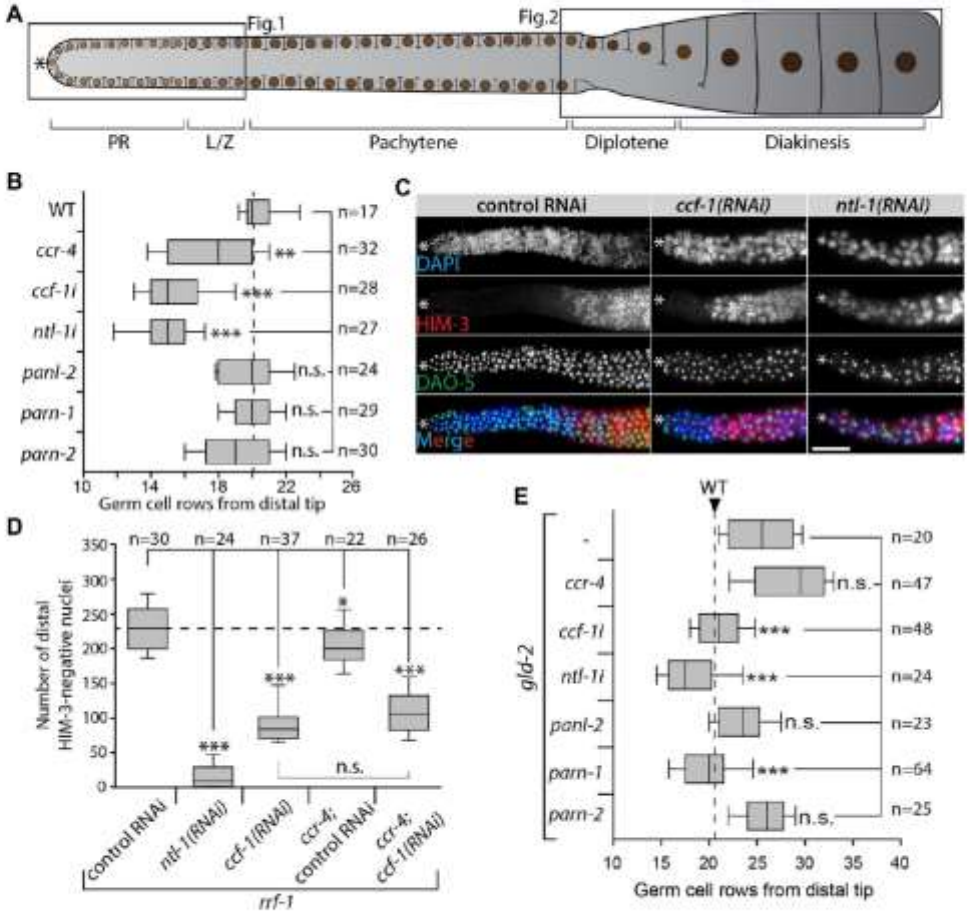

Figure 1. Ccr4–Not complex is essential for proliferation. CCF-1 and PARN-1 are opposed by GLD-2 in the regulation of meiotic entry. (**A**) Scheme of the adult female germline tissue, indicating the proliferative region (PR) and different stages of meiotic prophase I (L/Z: Leptotene and Zygotene). The asterisk indicates its distal tip; fully cellularized oocytes are located at its proximal end. Brown circles represent germ cell nuclei. The two boxed germline regions are analyzed in Figures 1 and 2. (**B**) Size of the proliferative region shown as germ cell rows until L/Z nuclei are detected in wild-type (WT), mutants, or RNAi-treated adults (indicated with an i, e.g. *ccf-1i*). Control RNAi is similar to WT and not shown for clarity. (**C**) DAPI (in blue) and antibody staining of extruded gonads of *rrf-1* adults treated with control, *ccf-1*, or *ntl-1* RNAi. HIM-3 (in red) marks nuclei that entered meiosis. Nucleolar DAO-5 (in green) serves as a penetration control; scale bar = 20μm. (**D**) Quantification of HIM-3–negative nuclei in the distal part of the germ line of indicated RNAi treatments in *rrf-1* single or *rrf-1;ccr-4* double mutants. (**E**) Size of the proliferative region in *gld-2(q497)* mutant animals that were further compromised in deadenylase functions. Data in B, D, and E was tested for statistical significance via a two-tailed Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. = not significant; *n* = number of analyzed gonads.

parn-1 and *parn-2* will be addressed using established loss-of-function alleles (5). The function of *ccf-1* or *ntl-1* (the worm Not1 homolog) will always be assessed upon conditional RNAi-mediated knockdown using feeding RNAi. The founding cytoPAP GLD-2 was initially isolated in *C. elegans*.

To test which DeAds are functionally important for germ cell proliferation and entry into meiosis, we analyzed the size of the proliferative region of extruded gonads by DAPI staining. In wild-type animals, the proliferative region is between 20 and 21 germ cell rows in size; this did not change in *pani-2*, *parn-1* and *parn-2* single mutants (Figure 1B). While the proliferative region was mildly reduced in animals lacking *ccr-4*, it was strongly reduced in *ccf-1* or *ntl-1* RNAi-treated animals (Figure 1B). This suggests that Ccr4–Not is the main DeAd complex, important for germ cell proliferation with CCF-1 being the key enzyme in the switch from proliferation to differentiation.

To test how strongly the Ccr4–Not complex is needed in proliferating germ cells, we repeated the analysis in *rrf-1* animals, which fail to mount a strong somatic RNAi response (27). This allows to conduct longer periods of RNAi feeding in order to maximize the RNAi effect in germ cells. Strikingly, such *ntl-1* or *ccf-1* depleted *rrf-1* animals contain very small gonads in which it was difficult to distinguish proliferative from meiotic germ cells by DAPI staining alone (Figure 1C). Therefore, to asses cells in meiotic prophase, we visualized the localization of marker protein HIM-3, a synap-

tonemal complex component, in immunostained extruded gonads (22). In *rrf-1* animals treated with control RNAi, we counted ~230 HIM-3-negative premeiotic cells in the distal end (Figure 1C and D). This number is slightly reduced in *rrf-1;ccr-4* and strongly reduced in *rrf-1;ccf-1(RNAi)* as well as *rrf-1;ccr-4; ccf-1(RNAi)* (Figure 1C and D). The strongest effect was detected in some *rrf-1;ntl-1(RNAi)* animals that possessed HIM-3-positive germ cells only (Figure 1C and D), arguing that all of their germ cells had entered meiosis at the expense of mitosis. Together these observations suggest that the Ccr4–Not complex is essential for maintaining germline stem cells in an undifferentiated state.

CCF-1 and PARN-1 are opposed by GLD-2 in the regulation of the switch from proliferation to differentiation

GLD-2 cytoPAP is a known positive regulator of meiotic entry and commitment (15,28,29). To test whether the proliferative defects in Ccr4–Not–compromised germ cells are a consequence of extensive GLD-2-mediated polyadenylation, we removed individual DeAds in *gld-2* mutants. In this analysis we additionally included the deadenylases *panl-2*, *parn-1* and *parn-2*. Compared to wild type, the absence of GLD-2 activity causes germ cells to delay entry into meiosis, which is characterized by a substantial size increase of the proliferative region from 20–21 germ cell rows in wild type to 27–28 in *gld-2* mutants (Figure 1E). Interestingly, an additional removal of *ccr-4*, *panl-2*, or *parn-2* did not change the size of the proliferative region (Figure 1E), arguing that none of these DeAds influence meiotic entry in a *gld-2*-dependent manner. However, the removal of *ccf-1*, *ntl-1*, or *parn-1* restored the size of the proliferative region to wild type (Figure 1E). This shows that two different DeAds, CCF-1 and PARN-1, are opposed by GLD-2 in regulating the entry into meiosis. The observation that the removal of PARN-1 in a *gld-2* mutant changes the size of the proliferative region is particularly surprising, as the loss of *parn-1* by itself had no detectable effect on germ cells in the distal germ line.

CCR-4 and CCF-1 are opposed by GLD-2 in regulating meiotic progression

To investigate the relationship between the different DeAds and GLD-2 during oocyte production, we analyzed the status of meiotic progression by DAPI staining in the proximal part of the gonad. Loss of GLD-2 activity results in a premature meiotic arrest around the pachytene/diplotene border (28). As a consequence and contrary to wild type, no nuclei with distinct bivalents corresponding to diakinetic nuclei were detected in *gld-2* mutants (Figure 2A and B). Whereas the additional removal of *parn-2* and *panl-2* did not alleviate this meiotic arrest, diakinetic nuclei were detected upon removal of *ccr-4*, or *parn-1*, or the reduction of *ccf-1* (Figure 2A and B).

To test whether compromising either Ccr4–Not or PARN-1 activity in a *gld-2* mutant would also restore the oocyte differentiation program, we analyzed the respective gonads for the presence of the two paralogous oocyte marker proteins, OMA-1 and OMA-2, jointly referred to as OMA-1/2 (30). In wild type but not in *gld-2* single mutants, OMA-1/2 expression is detected in proximally differentiating oocytes (Figure 2C). Interestingly, OMA-1/2 expression recovered by the additional removal of *ccr-4* or *ccf-1*, but not *parn-1* (Figure 2C). Overall this suggests that the entire Ccr4–Not deadenylase module is the primary opposing poly(A)-removing activity to GLD-2, guiding meiotic oocyte progression and differentiation.

The Ccr4–Not complex counteracts *gld-2*-mediated polyadenylation at the global level

GLD-2 is important for the polyadenylation of many mRNAs in the germ line (10). To test a likely influence of CCR-4, CCF-1 or PARN-1 on the polyadenylation status of mRNAs at the global level, we conducted from whole worms bulk poly(A)-tail length measurements upon their removal. Consistent with our previous findings (10), bulk poly(A) tails were significantly shorter in the absence of GLD-2 (Figure 3A and B), also illustrating that that global changes in tail length can be detected specifically form germ cells using this method. Compared to *gld-2* single mutants, no significant difference in tail length distribution was detected in *gld-2;parn-1* double mutants (Figure 3A and E), whereas longer tails were present in *gld-2;ccr-4* and *gld-2;ccf-1(RNAi)* animals (Figure 3A, C and D). This suggests that both deadenylases of the Ccr4–Not complex, CCR-4 and CCF-1, oppose GLD-2-mediated polyadenylation of target mRNAs. Contrary to this, PARN-1 has no significant global impact on the polyadenylation status of GLD-2-dependent target mRNAs, suggesting that either a small subset or no mRNAs are targeted by PARN-1.

The balance between *gld-2* and *ccf-1* regulates mRNA levels

In a previous study, we found that a large number of germline mRNAs depends on GLD-2 for efficient expression (10). To identify which DeAd is responsible for the degradation of these putative *gld-2*-target mRNAs, we assessed the abundance of mRNAs by Next-Generation Sequencing in the following genetic backgrounds: wild type, *gld-2*, *gld-2;ccr-4*, *gld-2;ccf-1(RNAi)* (henceforth called *gld-2;ccf-1*) and *gld-2;parn-1*. For each genetic background, three independent biological samples were prepared and mRNAs from hand-picked whole worms were analyzed.

First, we compared wild type to *gld-2* single mutants focusing our analysis on germline-expressed mRNAs (31). The mRNA abundance changes detected in the mutant are similar to the ones detected in our previous RNAi experiments (10), with one notable difference. Spermatogenic mRNAs are more strongly upregulated in the mutant compared to the RNAi treatment (Supplementary Figure S1A). This is consistent with the observation that an RNAi knockdown of *gld-2* has a less severe impact on germ cell development than a protein null mutant (10,15,32), paralleling its spermatogenic defect (28,32). Regardless of the extent in expression changes the correlation is high between the two data sets ($r = 0.81$; Supplementary Figure S1A), arguing that the gene expression changes are comparable between *gld-2* mutants and *gld-2(RNAi)* animals.

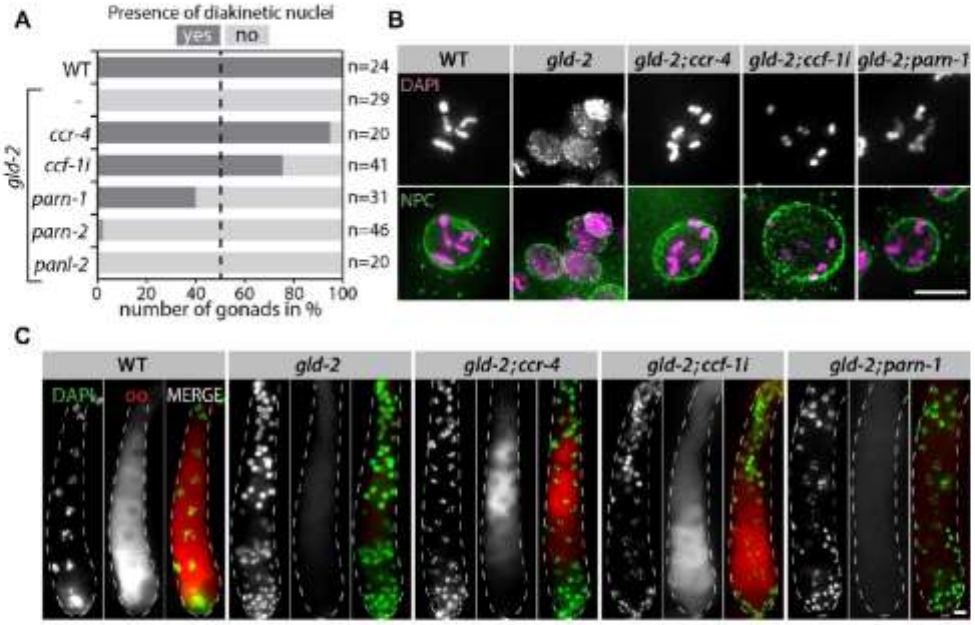

Figure 2. GLD-2 opposes CCR-4 and CCF-1 in the regulation of meiotic progression. (**A**) Diakinetic nuclei are absent from gonads lacking GLD-2 but form upon the additional removal of certain deadenylase activities. Percentage of germ lines in which diakinetic nuclei can be detected in the proximal part as judged by DAPI staining. (**B**) Examples of germ cell nuclei in the proximal part of extruded gonads from indicated genetic backgrounds. DAPI staining (purple in merge) marks DNA; NPC = Nuclear pore complex (green in merge). (**C**) Extruded gonads of indicated genetic backgrounds stained for the oocyte marker proteins OMA-1/2 (oo, red in merge); and DAPI to reveal chromatin morphologies (green in merge). Scale bars = 10 μm.

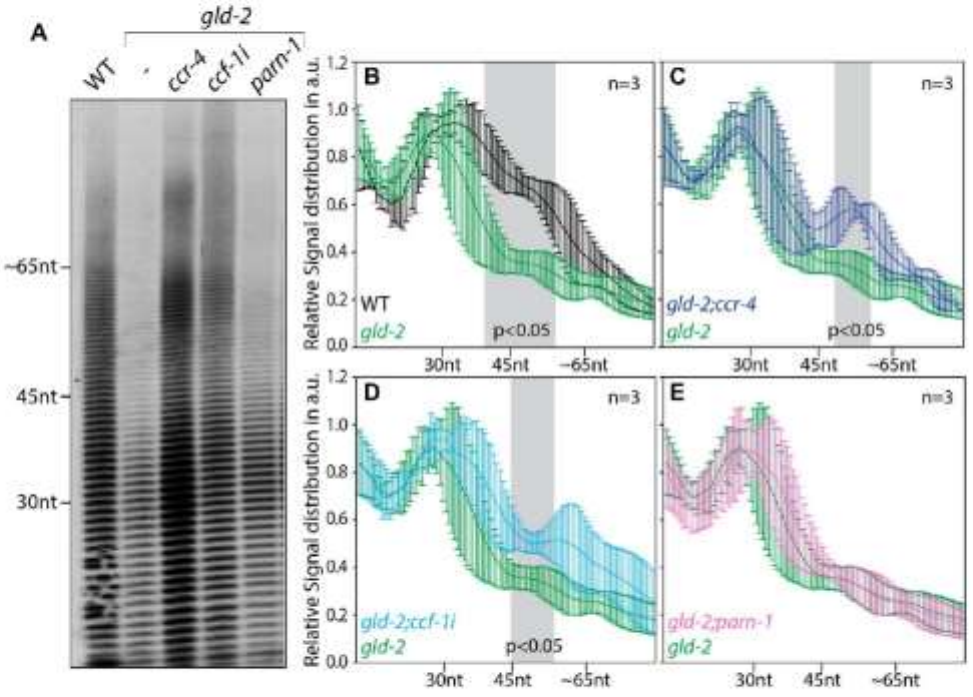

Figure 3. Reduced activities of the Ccr4–Not complex but not PARN rescue bulk polyadenylation defects in *gld-2* mutants. (**A**) The global status of poly(A)-tail lengths were analyzed in the indicated genetic backgrounds using bulk poly(A) measurements. A representative gel image is shown. (**B**–**E**) Line scans of bulk poly(A) measurements of three independent biological samples (*n*) showing the relative signal distribution in the lane in arbitrary units (a.u.). Values of statistical difference, calculated with a two-tailed Student's t-test, are highlighted in gray. nt = nucleotides.

To assess which genes are significantly changed in the *gld-2* mutant, we chose the following parameter for our analysis: minimal normalized read count ≥100; fold change ≥1.5 and *P*-adjusted < 0.05, again focusing only on germ line expressed genes. As a result, we found that 406 mRNAs are significantly down and 1056 mRNAs are significantly up-regulated (Figure 4A, Supplementary FileS1). Consistent with described phenotypic changes (28), the up-regulated mRNAs are to a large extent from spermatogenic genes, whereas down-regulated mRNAs are primarily from sex-neutral and oogenic genes (Figure 4B). To gauge the behavior of direct GLD-2 target mRNAs in our data set, we analyzed the expression changes of mRNAs that have been described to physically be associated with GLD-2 protein in an RNP complex (32). We find that the abundance of direct GLD-2 targets is significantly reduced in *gld-2* null mutants (Figure 4C). This observation suggests that the up-regulated mRNAs are most-likely indirect GLD-2 targets and their expression changes are a consequence of altered germ cell physiology. Overall these data support the previously suggested function of GLD-2 in promoting mRNA stability (10), and argues that the 406 down-regulated mRNAs are significantly enriched in direct GLD-2 targets. For this study, we define these less abundant mRNAs as GLD-2-stability targets.

To see which DeAd might be responsible for the down regulation of mRNAs in *gld-2*, we investigated the expression changes of GLD-2-stabilty targets in various *gld-2;DeAd* animals by comparing them to the *gld-2* single mutant. In comparison to the changes of all germline mRNAs, the additional removal of *ccf-1*, *ccr-4* or *parn-1* led to a statistically significant increase in the levels of GLD-2-stability targets in *gld-2* mutants (Figure 4D-F). However, quantitative differences are detected among the three analyzed DeAds: a minor increase in abundance was detected by removal of *parn-1*; a moderate increase by removal of *ccr-4;* and the largest increase upon reduction of *ccf-1* (Supplementary Figure S1B). In addition, the RNA-sequencing data were validated by analyzing the expression changes of twelve GLD-2-stability targets by RT-qPCR. These measurements showed that the mRNA levels were significantly increased for 12/12 and 5/12 mRNAs in *gld-2;ccf-1* and *gld-2;ccr-4* (Figure 4G; Supplementary Figure S1C), respectively. No significant increase was detected in *gld-2;parn-1* animals for the tested candidate mRNAs (Figure 4G; Supplementary Figure S1C). This suggests that PARN-1-mediated expression changes might be very subtle, and argues that PARN-1 most-likely plays no major role in the degradation of GLD-2-stability targets. Furthermore, this expression analysis supports the view that GLD-2 overall protects its target mRNAs from Ccr4–Not-mediated degradation, which is primary due to the activity of CCF-1.

GLD-2 and the Ccr4–Not complex regulate RNA stability during all stages of meiotic prophase I

Next, we investigated whether Ccr4–Not and GLD-2 regulate mRNAs during all stages of oocyte production. To this end, we analyzed abundance changes of mRNAs in our data set that are presumed targets of well-defined sequence-specific RBPs. We used data from representatives of four evolutionary conserved RBPs: the PUF protein FBF-1 (33), the STAR protein GLD-1 (34), the TRIM-NHL protein LIN-41 and the Tis11-type Zinc-finger protein OMA-1 (35). All of these proteins are expressed in a stage-specific manner during oocyte production (Figure 5A). FBF-1 and GLD-1 are mRNA regulators during early stages of oocyte production and are mainly expressed in proliferating or pachytene germ cells, respectively (36,37). LIN-41 and OMA-1 are mRNA regulators during late stages of oocyte production and are primarily expressed in diplotene and diakinetic germ cells (30,38). Large data sets of putative target mRNAs are available for all four RBPs that were previously co-purified with each respective RBP (33–35).

For our analysis, we focused on the top 100 FBF-1-, GLD-1-, LIN-41- or OMA-1-associated mRNAs. These target mRNAs were always compared to the behaviour of all germline genes, and only mRNAs that have previously been classified as sex-neutral or oogenic were considered in our analysis (31). We found that target mRNAs of FBF-1/PUF and GLD-1/STAR behaved similarly: they were significantly decreased in the *gld-2* single mutant, increased in *gld-2;ccf-1*, and unchanged in *gld-2;ccr-4* (Figure 5B–D). However, target mRNAs of LIN-41/TRIM-NHL and OMA-1/Tis11 behaved differently: although both were decreased in the *gld-2* single mutant and increased in *gld-2;ccf-1* (Figure 5E, F), OMA-1 targets were unchanged in *gld-2;ccr-4* as compared to *gld-2*, and a small but significant increase was detected for LIN-41 targets (Figure 5G). Taken together, this suggests that GLD-2 and CCF-1 form a functionally antagonistic pair to regulate the abundance of mRNAs during all stages of oocyte production, with a small contribution of CCR-4 during late stages. Overall, this data shows that balancing Ccr4–Not and GLD-2 activities mediate mRNA levels essentially during all different stages of early oogenesis.

GLD-2 promotes ribosome association of mRNA targets during late prophase I

Finally, we investigated whether GLD-2 cytoPAP also influences the translation of mRNAs during any stage of oocyte production. To allow tissue-specific isolation of ribosomes from multi-cellular organisms, we adopted the Translating Ribosome Affinity Purification (TRAP) method to *C. elegans* germ cell analysis (39), and expressed from a germ cell-specific promoter the FLAG-tagged RPL-4 large ribosomal subunit protein (Figure 6A). Western blot analysis of polysome gradient fractions showed that the epitope tagged ribosomal protein RPL-4::FLAG is enriched in heavy polysomal fractions (Figure 6B), indicating its functional incorporation into translating ribosomes. This was further confirmed by immunoprecipitating ribosomes from RPL::FLAG-expressing but not wild-type worm extracts using anti-FLAG antibodies. Subsequent analysis of associated RNA material revealed that both ribosomal RNAs (18S and 26S) were abundantly detected in purifications from transgenic but not in control animals (Figure 6C). As FLAG-tagged RPL-4 worms have no apparent fecundity defects [number of offspring N2: 320±47(N = 23), rpl4::FLAG: 308±61 (N = 21)], we concluded that RPL-

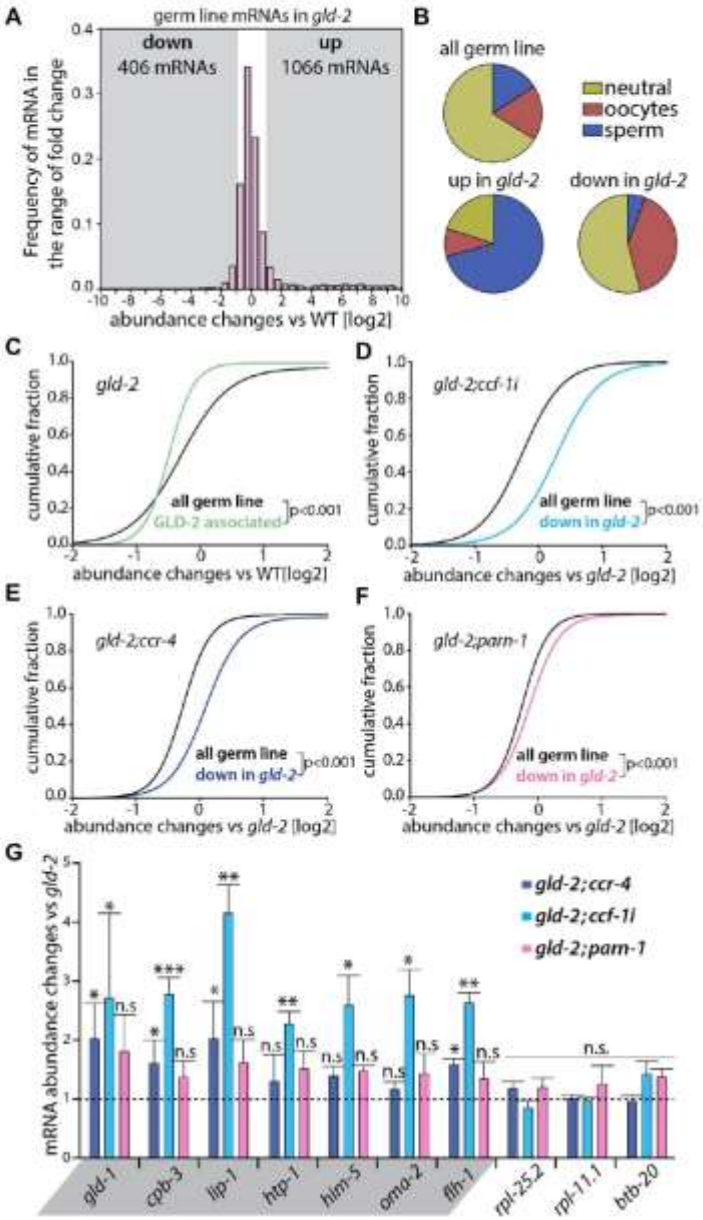

Figure 4. GLD-2-dependent mRNA targets are stabilized by the additional knockdown of CCF-1 deadenylase. (**A**) Histogram of germline mRNA abundance changes in *gld-2* animals compared to wild type. All detected 8286 germline mRNAs are shown. Gray areas mark regions of at least a twofold change. The numbers of significantly up- or down-regulated mRNAs fulfil the following parameters: read number ≥ 100, fold change ≥1.5×, *P*-adj ≤ 0.05. (**B**) Distribution of sex-neutral, sperm-, or oocyte-enriched mRNAs in the indicated data sets, according to the classification by Oritz at al. 2014. (**C**–**F**) Cumulative fractions of mRNA abundance changes comparing GLD-2-associated mRNAs to all germline mRNAs in the *gld-2* single mutant (C), and *gld-2*-down-regulated mRNAs to all germline mRNAs in *gld-2;ccf-1 (RNAi)* (D), *gld-2;ccr-4* (E), or *gld-2;parn-1* (F) animals. Given statistically significant differences were calculated with a Mann–Whitney Rank Sum Test. (**G**) Abundance measurements of putative GLD-2-dependent target mRNAs (highlighted in gray) or nontarget mRNAs. A selection of candidates is shown; for all tested mRNAs see Supplementary Figure S1C. All quantitative real-time PCR measurements ($n = 3$) were normalized to the expression values of *pab-1*. Statistically significant differences were calculated via the Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. = not significant.

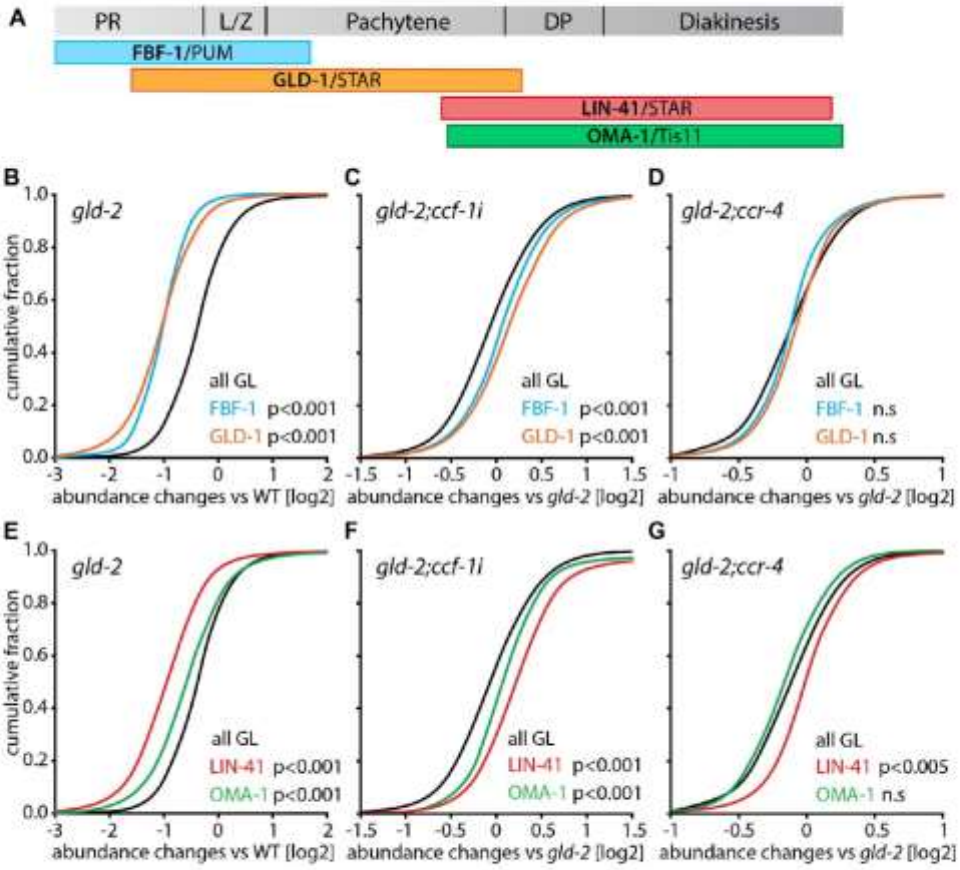

Figure 5. The Ccr4–Not complex and GLD-2 influence mRNA abundance during all meiotic stages of oocyte production. (**A**) Scheme of adult germline tissue with indicated protein expression patterns of four evolutionary conserved RNA-binding proteins that guide oogenesis. (**B**–**G**) Cumulative fractions of the abundance changes of all germline mRNAs (all GL) in the indicated genetic backgrounds and the top 100 mRNAs bound by either FBF-1/PUF, GLD-1/STAR, OMA-1/Tis11, or LIN-41/TRIM-NHL. Only mRNAs classified as sex-neutral or oocyte-enriched were considered, following the classification by Oritz *et al.* 2014. Statistically significant differences between the RBP-bound and all germline mRNAs are indicated and were calculated via a Mann–Whitney Rank Sum test.

4::FLAG expressing worms are suitable for TRAP experiments.

To test whether GLD-2 influences the translation efficiency of mRNAs during any stage of oocyte production, we examined the changes in ribosome association of FBF-1/PUF, GLD-1/STAR, LIN-41/TRIM-NHL or OMA-1/Tis11 mRNA targets upon GLD-2 knockdown by feeding RNAi. Comparative western blot analysis of control RNAi to *gld-2(RNAi)* protein extracts showed that GLD-2 protein could be efficiently reduced in RPL-4::FLAG expressing worms (Figure 6D). Following immunoprecipitations of RPL-4::FLAG from three independent RNAi treatments, co-purified mRNAs and the corresponding input materials were analyzed by quantitative real-time PCR. To account for mRNA translation changes driven by variations in general mRNA abundance, a ribosome association coefficient was calculated for each individual mRNA by normalizing the amounts of ribosome-associated mRNA to the signal from the input. Thirty candidates were chosen that represent prominent mRNA targets of the four different RBPs. While two third of the tested mRNAs (20/30) remained unchanged, one third (10/30) were significantly less associated with ribosomes in *gld-2(RNAi)* (Figure 6E and Supplementary Figure S2A). Interestingly, the majority of the translation-reduced mRNAs are targets of LIN-41 (7/9) compared to FBF-1 (7/19), GLD-1 (4/17) and OMA-1 (4/16) (Figure 6E and Supplementary Figure S2). This suggests that especially LIN-41-associated mRNAs require GLD-2 for efficient translation, further arguing that in addition to mRNA stability, GLD-2 promotes also the translation of mRNAs prior to oocyte maturation stages.

DISCUSSION

The regulation of gametogenesis in many organisms relies heavily on post-transcriptional mechanisms involving the deadenylases of the Ccr4–Not complex and the cytoPAP GLD-2. Removal of one of the two opposing activities has devastating consequences for germ cell development. We

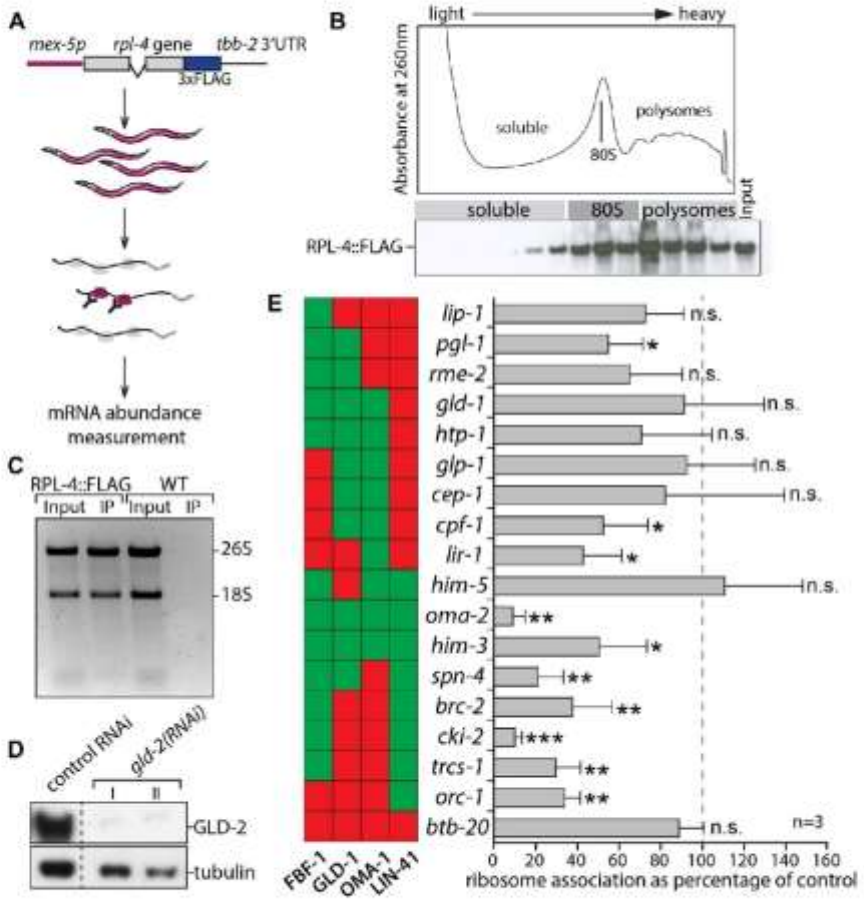

Figure 6. GLD-2 promotes the translation of LIN-41 targets. (**A**) Scheme of germ cell-specific ribosome immunopurification, for details see Materials and Methods. (**B**) Absorbance profile at 260nm of a typical polysome gradient from RPL-4::FLAG-expressing adult worms (top). Western blot of gradient fractions tested for the presence of tagged RPL-4 (bottom). The sedimentation positions of major ribonucleoprotein complexes are indicated. (**C**) A denaturing agarose gel of RNA isolated from anti-FLAG immunoprecipitation (IP) experiments from crude extracts (Input) of either RPL-4::FLAG-expressing or non-FLAG-expressing wild-type (WT) animals. Bands corresponding to the large (26S) and small (18S) ribosomal RNAs are indicated. (**D**) Western blot analysis of the indicated proteins of RPL-4::FLAG expressing animals treated with control or *gld-2* RNAi. Extracts of two out of three independent *gld-2(RNAi)* treatments (I and II) are shown for comparison. The control sample corresponds to RNAi-treatment I. Additional lanes were removed for clarity (dashed line). (E) A selection of FBF-1, GLD-1, OMA-1 or LIN-41 target mRNAs analyzed for changes in ribosome association, comparing *gld-2* to control RNAi-treated animals. The abundance of 18 mRNA was measured by RT-qPCR. Statistically significant differences are indicated and were calculated via a two-tailed Student's *t*-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s. = not significant; $n = 3$ biologically independent experiments. The color-coded columns to the left of each gene name indicates the target status of the corresponding mRNAs towards the respective RBPs (red = non-target; green = target). The complete data set of 30 mRNAs is shown in Supplementary Figure S2A.

show that the removal of both poly(A) modifying forces restores aspects of germ cell development back to wild type arguing that an intricate balance between these opposing enzymes is a key to regulate mRNAs in this tissue. Interestingly, these two opposing activities represent the major polyadenylation and deadenylation forces in female germ cells, and are likely recruited by a number of different translational repressor RBPs to stabilize and translationally regulate the oogenic mRNAs. Due to the evolutionary conservation of the poly(A) modifiers, as well as many of the RBPs investigated in this study, it is likely that Ccr4–Not and GLD-2 provide the regulatory frame work for poly(A)-mediated mRNA regulations also in other organisms.

We find that the previously characterized GLD-2 antagonist PARN plays no major role in regulating mRNAs during oocyte production. This observation is in strong contrast to the model proposed for oocyte maturation in frogs (40). The difference of the oogenic developmental stages could be one possible explanation for this finding. In general, PARN is assumed to be predominantly nuclear and by analogy to *Xenopus* oocytes is only present in the cytoplasm upon nuclear envelop breakdown during oocyte maturation (41). Hence, PARN might not be able to participate in cytoplasmic mRNA regulation during the oocyte production phase. Two PARN proteins exist in *C. elegans*, PARN-1 and PARN-2, and the subcellular localization of

either endogenous protein has not been clarified yet. Our own attempts to generate specific antibodies failed to date. While our experimental setup precluded us from detecting potential oocyte maturation functions of PARN, neither deadenylase appears to be a major factor for mRNA regulation during early female gametogenesis. In animals lacking GLD-2, only the removal of PARN-1 rescues some aspects of early oocyte development. This observation is likely indirect and might point at poly(A)-length control mechanisms in the nucleus that precede further length control in the cytoplasm. Alternatively, the rescue might be explained by other PARN-1 functions that influence germ cell development, such as piRNA biogenesis (42). Although, no data exist addressing the functionality of PARN during early female germ cell development in other organisms, a growing number of studies from tissue culture systems supports the view that PARN proteins primarily target non-coding RNAs rather than mRNAs (43–47).

The catalytic module of the Ccr4–Not complex is of central importance for animal gametogenesis. It has previously been suggested that the deadenylase CCF-1/Caf1 is important for promoting germ cell proliferation in nematodes (48). Here, we confirm this observation and extend it to other components of the Ccr4–Not complex. First, down-regulation of either the structural component NTL-1/Not1 or the catalytic component CCF-1/Caf1 elicits in all tested aspects of germ cell development similar defects, arguing that both components together comprise the active nuclease core module of the Ccr4–Not complex. Second, for regulating the transition from proliferation to differentiation the activity of CCF-1 is more important than the activity of CCR-4. Third, next to promoting germ cell proliferation, Ccr4–Not is also essential for maintaining undifferentiated germ cells. Similar observations have been made in *D. melanogaster*, where CAF1-driven CCR4-Not activity is employed to promote germline stem cell self-renewal (49). In mice, Ccr4–Not–mediated activity is required for spermatogenic stem regulation, however it is currently unknown which catalytic subunit is important for this function (50). Therefore, the evolutionary conserved Ccr4–Not complex represents the main deadenylase activity in germline stem and differentiation cells to regulate gene expression programs in metazoans.

Many RBPs that negatively regulate specific sets of target mRNAs form mRNPs that recruit the Ccr4–Not complex to facilitate translational repression (1). However, the majority of such mRNPs contain many additional RNA-associated regulators, including poly(A)-tail length modifiers. All four RBPs investigated in this work, FBF-1/PUF, GLD-1/STAR, LIN-41/TRIM-NHL, and OMA-1/Tis11 belong to distinct RBP families and were initially described to act as translational repressors (38,51–53). Although existing biochemical data on FBF-1-, LIN-41- and OMA-1-containing RNPs suggest that Ccr4–Not components and GLD-2 cytoPAP are part of each respective mRNP (35,48,54), it was unknown whether the presence of the tail modifiers has any functional relevance for the target mRNAs. Moreover no biochemical data on GLD-1/STAR-containing RNP exists currently that links their repressive function to enzymes that edit poly(A)-tail lengths. Our data predicts that all four RBPs, including GLD-1/STAR, utilize the antagonism of poly(A)-tail modifiers to regulate their mRNA targets, reminiscent of CPEB-target mRNAs during frog oocyte maturation (40). Our findings on members of four other RNA-binding protein classes suggest that a fine balance of opposing tail-length modifiers is reiteratively employed during all stages of female germ cell development to achieve differential gene expression regulation at the mRNA level. Furthermore, this concept argues that translational repressor RBPs employing deadenylases may in addition have to recruit cytoPAPs to ensure mRNA stability, either during the period of active repression or immediately after release from repression. It is conceivable that this antagonistic frame work of Ccr4–Not/GLD-2 could be a widespread phenomenon in poly(A)-mediated mRNA regulation and its use via RBPs extends beyond germ cells.

During the different stages of oocyte development, poly(A) function might shift from promoting mRNA stability towards promoting translatability. In *Drosophila*, strong GLD-2-dependent polyadenylation of mRNAs is detected in maturing oocytes (11,55,56). Furthermore, during this developmental stage the translational efficiency of mRNAs is mainly driven by its poly(A)-tail length (55,56), arguing that regulation of translation is the primary mechanisms of GLD-2-mediated gene expression control during oocyte maturation. Our current work in *C. elegans* provides evidence that GLD-2 cytoPAP promotes translation of mRNAs already prior to oocyte maturation, indicating that aspects of tail-mediated translational regulation may start to appear already during late stages of oocyte production in preparation for embryogenesis. This may reflect a change in general RNA degradation competence of female germ cells, a potentially conserved feature of animal gametogenesis.

DATA AVAILABILITY

NGS data was deposited in the GEO data base (accession number: GSE130685).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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4. Discussion

4.1 The global impact of tail regulation in germ cells

The classical views about poly(A) tail lengths influencing mRNA stability and translation were recently reexamined by RNA-sequencing studies. As a result, the general importance of tail regulation for mRNA stability could be confirmed on a genome-wide level for many different biological systems (SUBTELNY *et al.* 2014; EICHHORN *et al.* 2016; EISEN *et al.* 2020). However, this is different for translation. Especially the notion that long-tailed mRNAs are translated better seems not to be universally true. Although a coupling between mRNA tail lengths and translation efficiency is observed in early fly, frog and zebrafish embryos, it disappears after gastrulation (SUBTELNY *et al.* 2014; EICHHORN *et al.* 2016; LIM *et al.* 2016). Also, no strong influence of tail lengths on translation was observed in adult somatic tissues, tissue culture systems or yeast (SUBTELNY *et al.* 2014). This strongly implies that tail length changes have only an effect on the translation status of mRNAs in specific tissues and cell types. Unfortunately, the connection between tail lengths and translation in developing germ cells is not clear at the moment. My data shows that a global shortening of mRNA poly(A) tail correlates with the destabilization of many mRNAs and a decrease of ribosome association of some mRNAs in *C. elegans* germ cells (NOUSCH *et al.* 2014; NOUSCH *et al.* 2019). While I employed a genome-wide approach for the abundance measurements, I analyzed the impact on translation only for a small number of mRNAs yet. However, this makes it difficult to assess how broadly applicaple the influence of the tail on translation really is in germ cells. Nonetheless, taken together my data argues that tail regulation most likely promotes both, stability as well as the translation of mRNA, in developing germ cells.

4.2 Ccr4-Not a global mRNA regulator with tissue-specific importance

Deadenylation of mRNAs is generally seen as a crucial aspect of the mRNA degradation pathway in eukaryotes (MUGRIDGE *et al.* 2018). The deadenylases of the Ccr4-Not complex have been shown to provide the main poly(A) shortening activity so far only in cell culture

systems (TUCKER *et al.* 2001; YAMASHITA *et al.* 2005; TEMME *et al.* 2010; YI *et al.* 2018). I conducted bulk poly(A) measurements of total RNA isolated from whole worms and found that loss of *ccr-4*, the reduction of *ccf-1* or *nlt-1* leads to severe deadenylating defects which are not detected in other deadenylase mutants (NOUSCH *et al.* 2013). Similar observations have been previously made only for *ccr-4* in flies (TEMME *et al.* 2004). Hence, my work extends our previous knowledge about the importance of the central Ccr4-Not module at the organismal level and cements the role of the complex in mRNA deadenylation in metazoans.

Intriguingly, the loss of CAF1 or CCR4 has different effects on organismal development. In *C. elegans*, loss of the Caf1-homolog *ccf-1* is lethal (MOLIN AND PUISIEUX 2005). This is a strong contrast to the observations made in *ccr4* mutants. I found that *ccr-4* loss in worms does not affect somatic but only germ cell development, (NOUSCH *et al.* 2013; NOUSCH *et al.* 2019). This is consistent with results from other organisms. In *Drosophila*, loss of *caf1* is lethal whereas *ccr4* mutants are viable but display defects during oocyte development (MORRIS *et al.* 2005; ZAESSINGER *et al.* 2006; NEUMULLER *et al.* 2011). In mice, both DeAds are each represented by two independent genes, Ccr4 by *cnot6/6l* and Caf1 by *cnot7/8* (BIANCHIN *et al.* 2005; MORITA *et al.* 2007). Although, *cnot7* knock-out mice show several physiological abnormalities, they are viable suggesting that Cnot8 can partially compensate for the loss of Cnot7 (NAKAMURA *et al.* 2004). However, primary mouse embryonic fibroblasts undergo apoptosis when both Caf1 genes are deleted from the genome (MOSTAFA *et al.* 2020). For Ccr4, only *cnot6l* knock-out mice exist which are viable but females display reduced fertility (SHA *et al.* 2018). Mouse embryonic fibroblasts in which both Ccr4 genes are deleted remain viable (MOSTAFA *et al.* 2020). Overall, these data suggest that the two enzymatic subunits of the Ccr4-Not complex have distinct biological functions, with Ccr4 being dispensable of general but not germ cell development in metazoans.

However, when interpreting the impact of CAF1, one has to consider the structure of the Ccr4-Not complex. All characterizations of CAF1 in organisms, including my work, rely on the removal of CAF1 protein. But, CAF1 serves as a bridge between CCR4 and the scaffolding

protein NOT1 (BASQUIN *et al.* 2012; PETIT *et al.* 2012). Thus, the loss of CAF1 automatically severs the connection of CCR4 to the rest of the complex. Interestingly, my work suggests, if not part of the Ccr4-Not complex, CCR4 protein is more stable than CAF1 (NOUSCH *et al.* 2013). However, currently we do not know whether CCR4 by itself can be functional and loss of CAF1 protein most likely also leads to an activity loss of CCR4 in cells. Therefore, mutants would have to be generated in which the enzymatic activity of CAF1 is abolished without interfering with the abundances of the protein to gauge the impact of CAF1 alone on biological processes.

CAF1 and CCR4 have been investigated on the molecular level shining a light on their individual involvement in mRNA deadenylation. In mammalian cells, the strong deadenylation defects observed in the absence of CAF1 can be partially rescued by the presence of a catalytically inactive CAF1, suggesting that as part of the Ccr4-Not complex CCR4 can compensate the activity loss of CAF1 (YI *et al.* 2018). Hence, efficient mRNA tail shortening is the result of the combined activities of both enzymatic subunits. Interestingly, mechanistic differences exist between CAF1 and CCR4. An accumulation of distinct deadenylation-intermediates is detected in the presence of catalytically inactive CCR4 but not CAF1 (YI *et al.* 2018). In these CCR4 mutants, the size pattern of the poly(A) signal is consistent with the footprint of bound PABP (YI *et al.* 2018). Furthermore, *in vitro* deadenylation activity of CCR4 but not CAF1 is stimulated by the presence of PABP (YI *et al.* 2018). Based on these observations the model of Ccr4-Not-mediated deadenylation was proposed in which CAF1 trims PABPC-free A tails while CCR4 removes PABPC-bound A tails. Intriguingly, in my bulk polyA measurements from *ccr-4* mutant animals, I also detected distinct deadenylation-intermediates highly similar to the ones detected in mammalian cells (NOUSCH *et al.* 2013; NOUSCH *et al.* 2019). This suggests that also in *C. elegans* CCR-4 shortens tails in a PABP dependent manner. It is attractive to speculate that the relationship between CCR-4 and PABP might provide the basis for the specific biological roles of this enzyme.

4.3 GLD-2 a tissue-specific cytoPAP and broad mRNA regulator

In contrast to deadenylases, the cytoplasmic poly(A) polymerase GLD-2 is quite restricted in its expression pattern and thereby in its activity. The analyses of GLD-2-type proteins in worms, flies and vertebra show that this cytoPAP is only strongly produced in germ cells and neurons (WANG *et al.* 2002; ROUHANA *et al.* 2005; BENOIT *et al.* 2008; CUI *et al.* 2008). In recent years the full scale of GLD-2-mediated mRNA regulation has been revealed. In Drosophila, loss-of-function mutants for the GLD-2 homolog *wispy* display a global shortening of mRNA poly(A) tails in late oocytes (CUI *et al.* 2013; EICHHORN *et al.* 2016). This suggests that GLD-2 enzymes target large sets of mRNAs in germ cells. In my work, I could confirm this notion. I found that also in *C. elegans* loss of GLD-2 leads to a global shortening of mRNA poly(A) tail in developing germ cells (NOUSCH *et al.* 2014). Additionally, GLD-2 immunopurifications from worms coenrich a broad range of germ cell-specific mRNAs and GLD-2 activity is needed to maintain the steady-state levels of many germline mRNAs (KIM *et al.* 2010; NOUSCH *et al.* 2014; NOUSCH *et al.* 2017; NOUSCH *et al.* 2019). Interestingly, also in neurons, GLD-2 stabilizes mRNAs. Here, the depletion of GLD-2 from the mouse hippocampus leads to the decrease of hundreds of mRNAs in the tissue (MANSUR *et al.* 2020). Taken together this argues that in germ cells and neurons GLD-2 promotes the stability of not just a small subset but a broad range of mRNAs. Hence, in these tissues GLD-2-type enzymes should be considered major regulators that are heavily utilized to control most likely many gene expression programs.

4.4 Several RNA-binding proteins utilize GLD-2 and Ccr4-Not

Specific RNA-binding proteins can employ GLD-2-type enzymes and deadenylases. This concept has been proposed for the regulation of maternal mRNAs during *Xenopus* oocytes maturation. Here, the RNA-binding protein CPEB is envisioned to control mRNAs by recruiting and balancing the activities of GLD-2 and PARN (KIM AND RICHTER 2006). Although the antagonism between GLD-2 and PARN often serves as a paradigm for poly(A)-mediated gene

expression control, the generality of the GLD-2/PARN relationship is questionable. In general, GLD-2 type proteins are localized to the cytoplasm (WANG *et al.* 2002; BENOIT *et al.* 2008). PARN on the other hand seems to be primarily nuclear and becomes only cytoplasmic upon nuclear envelope break down during late stages of oocyte maturation (YAMASHITA *et al.* 2005; BERNDT *et al.* 2012). This argues that PARN/GLD-2 mediated RNA regulation is only biologically relevant during one specific stage of oocyte development. My work shows that not PARN but rather Ccr4-Not opposes GLD-2 during all stages of female germ cell development leading up to oocyte maturation (NOUSCH *et al.* 2019). Furthermore, my data suggests that the opposing activities of GLD-2 and Ccr4-Not are employed by several evolutionary conserved RNA-binding proteins, including FBF-1^{PUF}, GLD-1^{STAR}, OMA-1^{Tis11} and LIN-41$^{TRIM-NHL}$, to regulate target mRNAs. The idea that the Ccr4-Not/GLD-2 antagonism might be used by various RBPs is supported by biochemical data from other studies. FBF-1^{PUF}, OMA-1^{Tis11} and LIN-41$^{TRIM-NHL}$ have been shown to form RNPs which contain GLD-2 as well as Ccr4-Not (SUH *et al.* 2009; SPIKE *et al.* 2014; TSUKAMOTO *et al.* 2017). Overall, this suggests that the opposing activities of GLD-2 and deadenylases, in particular Ccr4-Not, could be a hallmark for RNA-binding proteins that control gene expression in the cytoplasm.

The RNA-binding protein determines how the tail modifiers affect the fate of a target mRNA. In my work I found that GLD-2 and Ccr4-Not affect the abundance of FBF-1^{PUF}, GLD-1^{STAR}, OMA-1^{Tis11} and LIN-41$^{TRIM-NHL}$ target mRNAs, indicating that the enzymes are employed by these four RBPs to regulate mRNA stability (NOUSCH *et al.* 2019). Interestingly, whereas FBF-1^{PUF}, GLD-1^{STAR}, OMA-1^{Tis11} and LIN-41$^{TRIM-NHL}$ use GLD-2 to stabilize targets, differences are detected for the Ccr4-Not-mediated destabilization mechanism. Target mRNAs of all four RBPs are increased upon loss of CCF-1, however only the targets of LIN-41$^{TRIM-NHL}$ additionally respond to the loss of CCR-4, suggesting that CCR-4 is dispensable for repressing FBF-1^{PUF}, GLD-1^{STAR} and OMA-1^{Tis11} targets. Additionally, the ribosome association of several LIN-41$^{TRIM-NHL}$ targets is decreased with the down-regulation of GLD-2, suggesting that tail-modifiers also regulate translation of LIN-41$^{TRIM-NHL}$. Although in my analysis I did not detect a

broad impact by GLD-2 of FBF-1^{PUF}, GLD-1^{STAR} and OMA-1^{Tis11} target association with ribosomes, I cannot exclude that these RBPs also control the translational status of its targets due to the small number of analyzed mRNAs. Loss of GLD-1 leads to a decrease in target abundance as well as polysome association implying that also this RBP might regulate its targets via multiple mechanisms (SCHECKEL *et al.* 2012). Therefore, genome-wide approaches are needed to assess the prominence of translation regulation that is exerted by the different RBPs. Nonetheless, my data suggests the GLD-2/Ccr4-Not balance is employed most likely by most RBPs to regulate primarily the abundance of mRNA.

4.5 Ccr4-Not and GLD-2 might also regulate non-coding RNAs

Although not the focus of my work, alternative target RNAs have been proposed for Ccr4-Not and GLD-2. In this regard, Ccr4-Not has been implicated in RNA regulation in the nucleus of germ cells. In drosophila, the depletion of Ccr4-Not complex components causes a strong derepression of telomeric retroelements resulting in mitotic defects during early embryogenesis (MORGUNOVA *et al.* 2015). It has been suggested that the Ccr4-Not complex associates with factors that are involved in piRNA-mediated silencing and that CCR4- mediates the degradation of transcribed transposable elements (KORDYUKOVA *et al.* 2020). It remains to be shown whether this role of Ccr4-Not is conserved in other organisms or if this is a fly-specific function of the complex. However, piRNAs operate exclusively in germ cells and the involvement of Ccr4-Not in piRNA-mediated transposon silencing might contribute to the general sensitivity of germ cells towards the reduction of Ccr4-Not components.

GLD-2 in mammals has been proposed to be involved in the regulation of miRNAs. Large-scale sequencing analyses in mammals and *C. elegans* revealed that many miRNAs are 3' monoadenylated (RUBY *et al.* 2006; LANDGRAF *et al.* 2007; AZUMA-MUKAI *et al.* 2008). In the liver and hippocampus of mice as well as human fibroblasts knockdown of GLD-2 leads to a loss of miRNA monoadenylations (KATOH *et al.* 2009; D'AMBROGIO *et al.* 2012; MANSUR *et al.* 2016). However, the effect of GLD-2-mediated miRNA is still debated. Whereas in the mouse

liver and human fibroblast GLD-2 seems to stabilize miRNAs, in the hippocampus loss of GLD-2 does not affect the steady-state levels of the affected miRNAs. Currently, it is unclear whether this aspect of GLD-2 functionality is relevant for the development of multi-cellular organisms in general or germ cell biology.

4.6 Enzymatic-independent functions of Ccr4-Not and GLD-2

In general, enzymes can have important biological roles independently of the biochemical reaction that they catalyze. In this respect, little is known about GLD-2 and the two enzymes of the Ccr4-Not. For the deadenylases, so far only one study indicates that the CAF1 might be functional in an enzymatically independent manner. In this study, catalytically inactive CAF-1 from *Xenopus* or humans was tethered to reporter mRNAs and able to repress the production of proteins from the target mRNA in *Xenopus* oocytes (COOKE *et al.* 2010). This observation suggests that CAF-1 could repress translation in an enzymatic independent manner. However, as the overall design of the experiment is quite artificial it remains to be tested to which degree such a mechanism also plays a significant role in the regulation of mRNAs in a physiological context.

Data from yeast indicate that the catalytic activity of Trf4-type non-canonical poly(A) polymerase might be dispensable for their functions in RNA regulation (SAN PAOLO *et al.* 2009). My findings suggest that GLD-2-type PAPs seem to promote the abundance of its target mRNAs solely via polyadenylation (NOUSCH *et al.* 2017). Unfortunately, the *C. elegans* protein is the only GLD-2-type protein that has been tested so far in this regard. However, recently the crystal structure of mammalian GLD-2 was solved which revealed significant structural differences compared to the *C. elegans* protein, showing that the mammalian protein seems to be a more stable enzyme (MA *et al.* 2020). Whereas *C. elegans* GLD-2 relies on its interaction with co-factors such as GLD-3 or RNP-8 to polyadenylate substrates efficiently, mammalian GLD-2 can robustly extent poly(A) tails by itself (NAKEL *et al.* 2015; NAKEL *et al.*

2016; MA *et al.* 2020). The evolutionary enhancement of GLD-2 enzymatic robustness might indicate that also in mammals the enzymatic activity is the key feature of this protein family.

4.7 The biological importance and roles of other deadenylases in RNA regulation

4.7.1 Pan2-Pan3 a global complex with specialized roles

Curiously, the strength of the evolutionary conservation of the Pan2-Pan3 complex is not immediately mirrored by its importance for general biology. In *C. elegans*, the removal of *panl-2* and *panl-3* individually or in parallel results in wild-type-looking animals that are fertile (NOUSCH *et al.* 2013). Lacks of obvious phenotypes are most likely also observed in flies. According to Flybase, the removal of Pan2 seems to not affect the survival or fertility of *drosophila*. This suggests that the Pan2-Pan3 complex is most likely dispensable for the global development of multi-cellular organisms. However, Pan2-Pan3 is present in the genomes from yeast to humans arguing that this complex should have biologically relevant functions (BOECK *et al.* 1996; UCHIDA *et al.* 2004; NOUSCH *et al.* 2013). Indeed, I found that in *C. elegans panl-2* or *panl-3* mutants produce significantly fewer offspring compared to wild type at elevated temperatures (NOUSCH *et al.* 2013). This suggests that Pan2-Pan3 becomes relevant for development under stress conditions. Unfortunately, up to this point *C. elegans* remains the only animal in which the biological importance of this complex was addressed and it will be interesting to see whether similar results can be obtained from other organisms. Overall, my data argues that Pan2-Pan3 might be needed for efficient deadenylation under stress conditions thereby contributing to developmental robustness in multi-cellular organisms.

How can the lack of Pan2-Pan3 biological relevance under normal cellular conditions be explained? In general, it has been proposed that on the molecular level mRNA deadenylation occurs in two steps (YAMASHITA *et al.* 2005). For efficient degradation, mRNAs with very long poly(A) tails (>150 nt) are first shortened by Pan2-Pan3 and are subsequently further deadenylated by Ccr4-Not (YAMASHITA *et al.* 2005; YI *et al.* 2018). Interestingly, RNA

sequencing studies characterizing mRNA poly(A) tails in mammalian tissue culture cells, *Drosophila* S2 cells, mouse liver, early zebrafish and *Drosophila* embryos found that the median length of an mRNA poly(A) tail in most cells is between 50 and 100 nt (CHANG *et al.* 2014; SUBTELNY *et al.* 2014; EICHHORN *et al.* 2016). Hence, a minor population of mRNAs carries tails longer than 150 nt indicating that only a small number of mRNAs is targeted by Pan2-Pan3 under normal cellular conditions.

Unfortunately, we do not know how the length of mRNA poly(A) tails change in *C. elegans* germ cells upon temperature increase. However, two observations from whole-genome studies in human cells might explain the requirement of Pan2-Pan3 under stress conditions. First, arsenic-induced stress leads to the enhanced usage of proximal alternative polyadenylation (APA) sites during mRNA production and the accelerated decay of mRNAs that have been processed at the distal APA sites (ZHENG *et al.* 2018). Second, a comparison of poly(A) tail lengths between mRNAs produced from distal or proximal APAs revealed that the longer UTRs also carry longer tails (LEGNINI *et al.* 2019). Also, in *C. elegans* a significant number of genes carry multiple alternative polyadenylation sites (JAN *et al.* 2011). Hence, PANL2-PANL3 might be required for the efficient degradation of long-tailed mRNA populations under heat stress conditions.

4.7.2 PARN a multi-task deadenylase

The biological importance of PARN proteins seems to have evolved over time. PARN in general is broadly but not universally conserved. For example, PARN is present in *S. pombe* but not in *S. cerevisiae* (REVERDATTO *et al.* 2004). Furthermore, PARN can be found in nematodes, and vertebras but not in flies (GOLDSTROHM AND WICKENS 2008). The absence of PARN especially in flies indicates that this deadenylase is not required for development in every organism. I found that *parn-1*, *parn-2* and *parn-1;parn-2* mutant animals in *C. elegans* are viable and present no obvious general developmental defects, supporting this notion (NOUSCH *et al.* 2013). However, this is different in mice where homozygote PARN knockout

animals die during early embryogenesis (BENYELLES *et al.* 2019). This shows that PARN although dispensable in worms is an essential gene in mammals.

The evolutionary increase of PARN importance might be explained by its RNA targets. PARN was initially described to regulate the deadenylation of a handful of maternal transcripts in *Xenopus* oocytes (KORNER *et al.* 1998). However, PARN seems not to be a major mRNA regulator in somatic and early germ cells. The knockdown of PARN in mouse cells results in the stabilization of only a small number of mRNAs (LEE *et al.* 2012). Also in my work, I detected only minor changes in mRNA abundances upon the loss of *parn-1* in early developing germ cells (NOUSCH *et al.* 2019). Interestingly, a genome-wide sequencing study found that PARN has not a strong influence on the length of mRNA poly(A) tails in mammalian cells, supporting the idea that the impact of PARN on mRNAs could be indirect (YI *et al.* 2018). This notion gains traction with an ever-growing number of studies that show PARN involvement in the regulation of non-coding RNAs (ncRNA), including the processing and abundance of some miRNAs, as well as the processing of Y RNAs, piRNAs, snoRNAs, rRNAs and telomerase RNAs (BERNDT *et al.* 2012; YODA *et al.* 2013; BOELE *et al.* 2014; HARWIG *et al.* 2015; MOON *et al.* 2015; TANG *et al.* 2016; ISHIKAWA *et al.* 2017; MONTELLESE *et al.* 2017; SHUKLA AND PARKER 2017; LEE *et al.* 2019; SHUKLA *et al.* 2019; NIETO *et al.* 2020). Based on these studies, a picture emerges of PARN as a major processing factor that controls the production and functions of ncRNA transcripts.

PARN-mediated regulation of ncRNAs seems to be the key to its general developmental importance. One of the biologically more impactful functions of PARN is its involvement in the maturation of the telomerase RNA component (TERC). TERC is part of the telomerase complex that catalyzes the addition of telomere DNA repeats onto the ends of linear chromosomes using the embedded TERC as the template (BLACKBURN AND COLLINS 2011). In humans, mutations in PARN are associated with Idiopathic pulmonary fibrosis, an age-related disease featuring progressive lung scarring, and dyskeratosis congenita, a life-threatening bone marrow disorder (STUART *et al.* 2015; TUMMALA *et al.* 2015). A hallmark of

both diseases is an impairment of telomere maintenance which is based on decreased levels of TERC (ROAKE AND ARTANDI 2020). In a tissue culture system, it could be shown that PARN removes a post-transcriptionally acquired oligo(A) tail from TERC that targets the RNA for degradation and the ectopic expression of PARN in PARN-deficient cells rescues TERC levels (MOON *et al.* 2015). This directly links PARN to the maintenance of TERC expression and demonstrates the requirement for PARN-mediated regulation of ncRNAs in development.

The requirements for different telomere length regulation mechanisms could explain the differential effects on the general development of *parn* mutants in worms and mammals. In most organism telomerase is essential and the developmental dynamics of telomerase loss is nicely illustrated in mice. Here, the initial loss of telomerase activity does not affect the organism due to the existence of sufficient telomeric sequences (BLASCO *et al.* 1997). However, it eventually becomes detrimental to the entire animal as a result of replication-associated telomere shortening germ cells and proliferative tissues fail to develop properly (BLASCO *et al.* 1997). Although in *C. elegans* telomerase single mutants eventually become sterile, mirroring to some degree phenotypes observed in knockout mice, the downregulation of additional factors allows for the maintenance of telomere lengths via alternative extension mechanisms rescuing the sterility phenotype (MEIER *et al.* 2006; CHENG *et al.* 2012; LACKNER *et al.* 2012). Hence, *C. elegans* is one of the few organisms that tolerates the loss of telomerase activity. Although we currently do not know whether PARN is involved in *C. elegans* telomere length regulation, the prominence of a telomerase-independent mechanism to regulate telomeres is a feasible explanation for the absence of major phenotypes in *parn* mutant worms in my work.

5. Outlook

Taken together, my work describes the developmental roles and genetic interactions of poly(A) tail modifying enzymes in developing germ cells of *C. elegans*. Although, I provided several novel and interesting findings regarding the cytoPAPs GLD-2 and GLD-4, as well the DeAds Ccr4-Not, Pan2 and PARN we still lack detailed mechanistic models that describe how such enzymes control the regulation of specific mRNAs during germ cell development. For example, the discovery that gene expression in germ cells is balanced by GLD-2 and Ccr4-Not raises the question of how these opposing activities are controlled on the molecular level. Hence, the compositions and structures of RNPs that contain specific RNA-binding proteins and the tail modifiers will have to be characterized in detail. We have to know whether RBPs directly interact with tail modifiers or if adaptor proteins are used, and if the opposing enzymatic factors are present in RNPs at all times or if two mutually exclusive RNP subpopulations exist. The analysis of immune-purified complexes from gonads using methods such as Cross-linking/mass spectrometry should help to identify interaction surfaces and provide valuable insides into the RNP architecture in germ cells (PIERSIMONI AND SINZ 2020). Detailed structural models will also be instrumental to reveal and understand how the two opposing activities of GLD-2 and Ccr4-Not are balanced and regulated in this context.

The findings that the Pan2-Pan3 complex in worms is biologically relevant at elevated temperatures argues that this complex is needed for the cellular stress response. In general, virtually nothing is known about poly(A) mediated gene expression regulation in stressed germ cells in any organism. Hence, the roles of RNA binding proteins as well as poly(A) modifiers during germ cell development should be characterized under stress conditions in *C. elegans*. This will reveal how these post-transcriptional regulators including Pan2-Pan3 are utilized and integrated into the cellular networks that control stress responses.

Finally, we need a comprehensive understanding how tail length modifications impact protein production in germ cells. As long-assumed correlation between long poly(A) tails and efficient translation seems not to be universally true; we need data that addresses this relationship in

developing germ cells. The tools that I have developed for the germ cell TRAP assay will help to fill this knowledge gap. Furthermore, by combining the TRAP assay with RNA sequencing techniques such as Tail-Seq and ribosome profiling one should be able to generate a comprehensive picture of the gene-expression landscape in early developing germ cells (INGOLIA *et al.* 2009; CHANG *et al.* 2014). By conducting such investigations not only in wild type but also in mutants of poly(A) modifiers and specific RNA-binding proteins the impact of poly(A) tail length on mRNA abundances and translation efficiencies will be revealed in this context.

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7. Erklärung

Hiermit erkläre ich, dass die vorgelegte Habilitationsschrift von mir persönlich angefertigt und ohne andere als die darin angegebenen Hilfsmittel angefertigt, sowie die wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet wurden.

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