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

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Accepted 12 June 2013
Journal of Cell Science 126, 4274–4285
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doi: 10.1242/jcs.132936

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

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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,

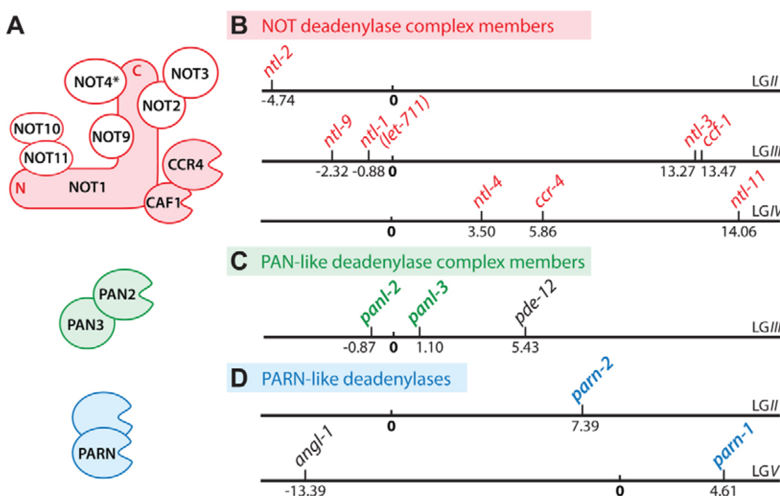

Fig. 1. Genomic locations of predicted deadenylases in *C. elegans*. (**A**) Graphic of the human Ccr4–Not complex (red), Pan2–Pan3 complex (green) and PARN (blue). NOT4 is a non-constitutive member of the complex (*). With the exception of NOT10, all other components are represented in the *C. elegans* 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.

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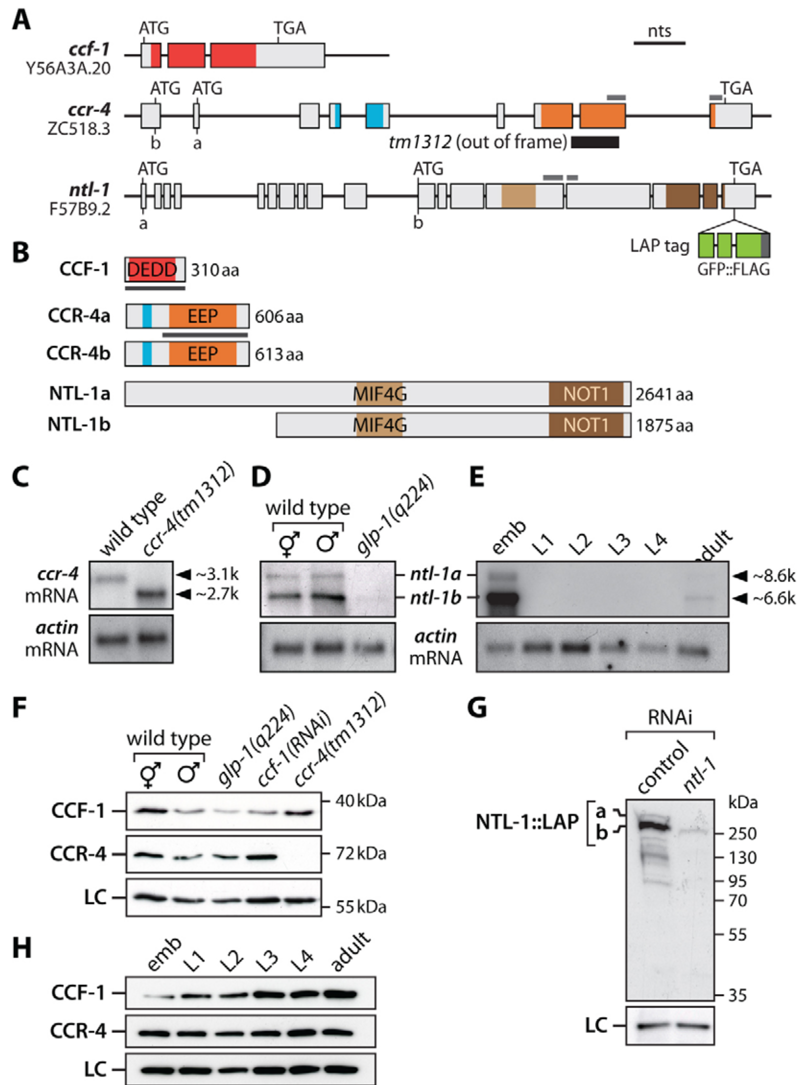

Fig. 2. Genomic locus, mutants and expression products of *ccf-1*, *ccr-4* and *ntl-1*. (**A**) Gene structure of *ccf-1*, *ccr-4* and *ntl-1*. Gray parts of the boxes represent exons. Colors indicate protein domains. Deleted regions in mutants and their effect on the coding potential are indicated below 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**) Developmental 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

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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, suggesting a likely common role in female germline development.

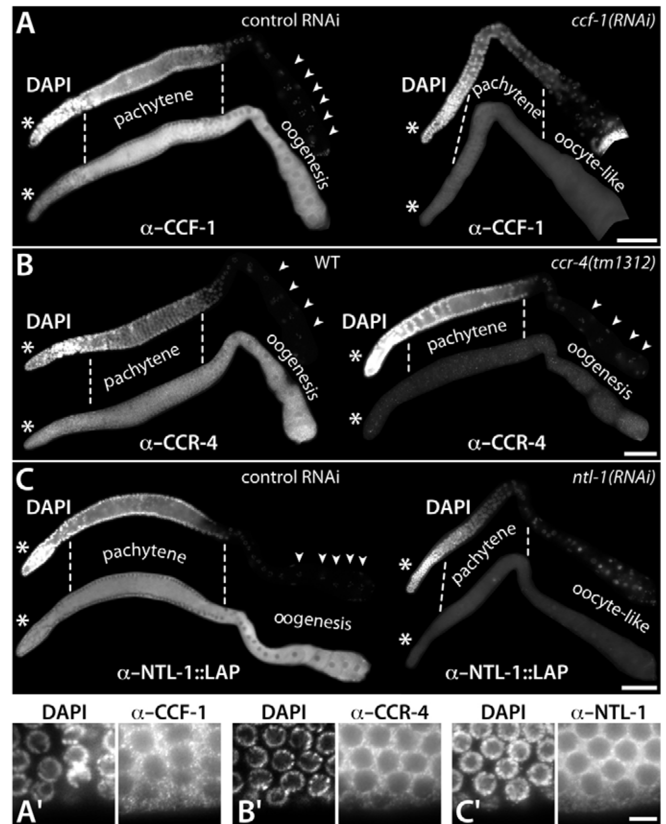

Fig. 3. Germline expression of CCF-1, CCR-4 and NTL-1. Epifluorescent images of extruded hermaphrodite gonads stained with DAPI and primary antibodies against endogenous (A) CCF-1, (B) CCR-4, and (C) anti-FLAG antibodies against LAP-tagged NTL-1 protein (NTL-1::LAP). Anti-GLH-2 staining was used as a 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.

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

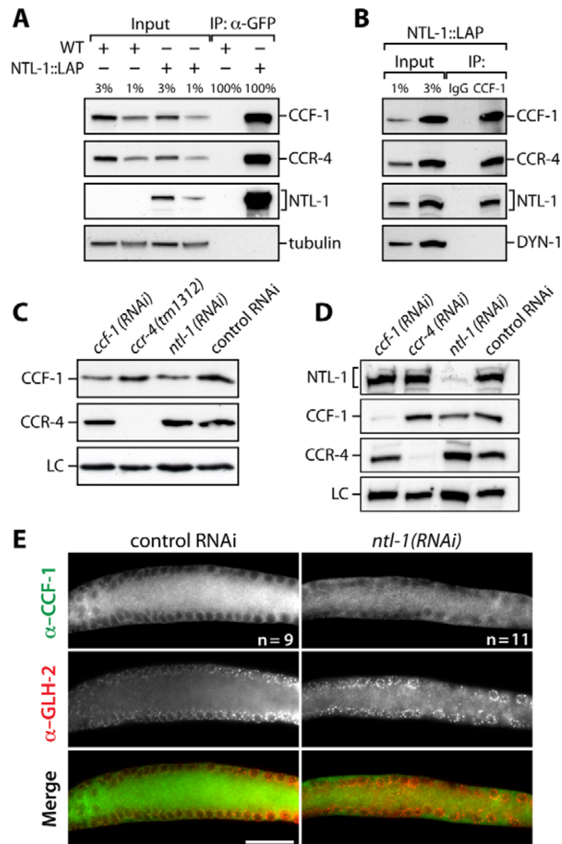

Fig. 4. CCF-1, CCR-4 and NTL-1 form a stable Ccr4–Not core complex in *C. elegans*. (**A**,**B**) Co-immunoprecipitation experiments of CCF-1, CCR-4 and NTL-1::LAP. Whole-worm extracts were prepared from mixed stage wild-type and transgenic NTL-1::LAP expressing animals. Immunoprecipitations were performed 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°C (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).

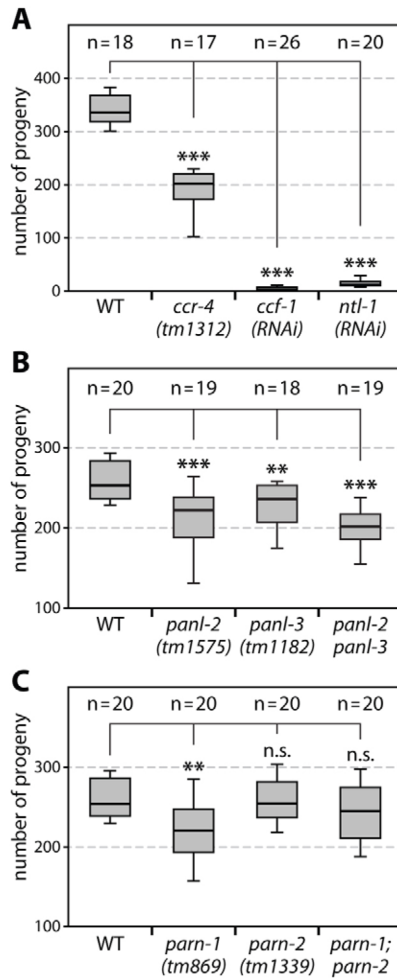

Fig. 5. Deadenylase mutants display a reduced fecundity. The fertility of parental hermaphrodites (n) was analyzed by counting hatched F1 larvae (progeny). (**A**) At 20℃, core Ccr4–Not complex components are required for fertility. An RNAi knockdown of *ccf-1* or *ntl-1* induces sterility. The brood size of *ccr-4(tm1312)* mutants is also significantly reduced. (**B**) At 25℃, Pan2–Pan3 complex components display a significant reduction in their brood sizes. (**C**) At 25℃, 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. $^{**}P \geq 0.01$, $^{***}P \geq 0.001$.

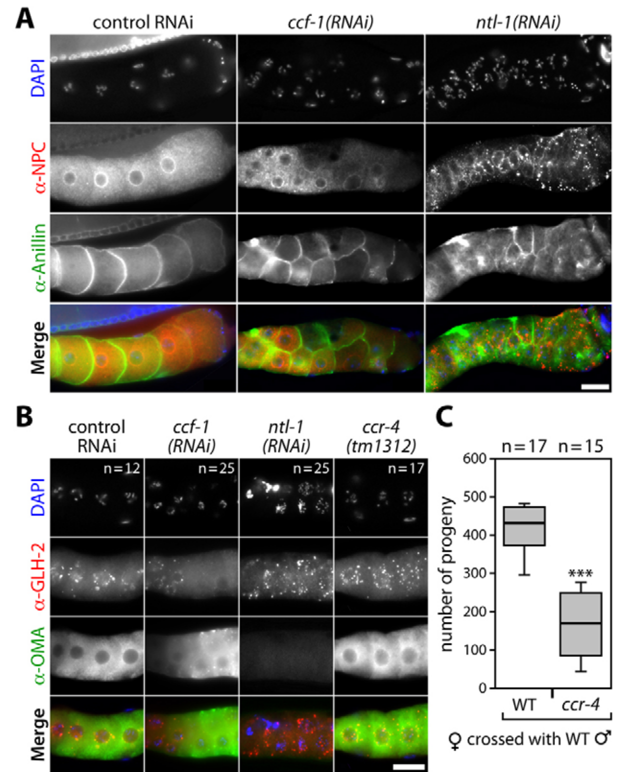

Fig. 6. CCF-1 and NTL-1 are essential for germline organization and oogenesis. (**A**,**B**) Extruded gonads of given genotypes were stained for either (A) nuclear pore complexes (NPC) and the cell cortex (anillin), or (B) P granules (GLH-2) or an oocyte-enriched maturation marker (OMA). Meiotic stages 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

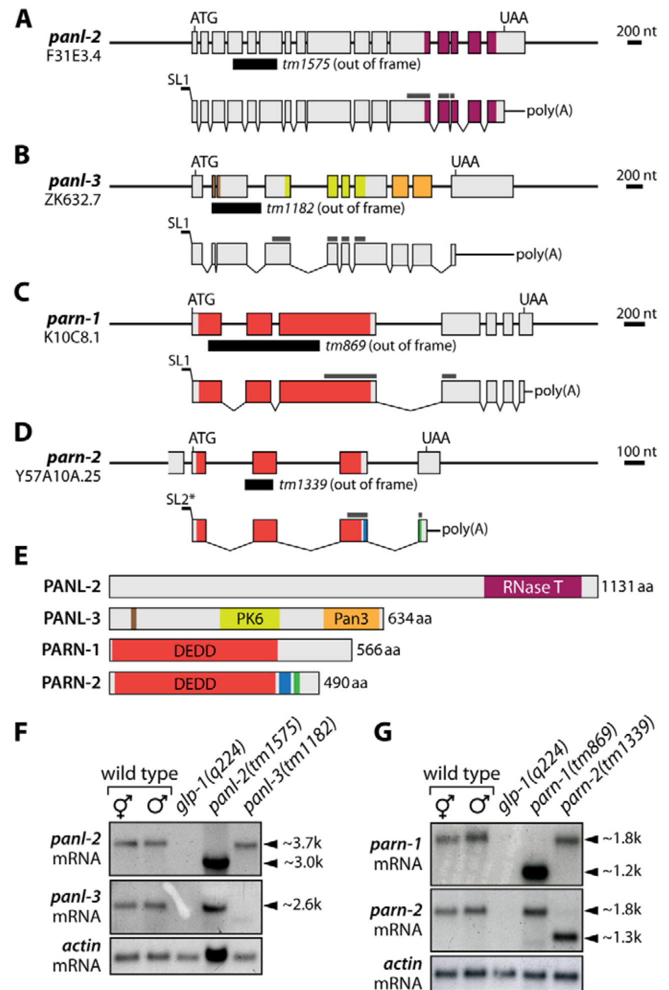

Fig. 7. Genomic locus, mutants and expression products of *panl-2*, *panl-3*, *parn-1* and *parn-2*. (**A–D**) Gene and mRNA structures are shown of (A) *panl-2*, (B) *panl-3*, (C) *parn-1* and (D) *parn-2*. Colors indicate protein domains, as given in E. Deleted regions in mutants and their effect on 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,

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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*.

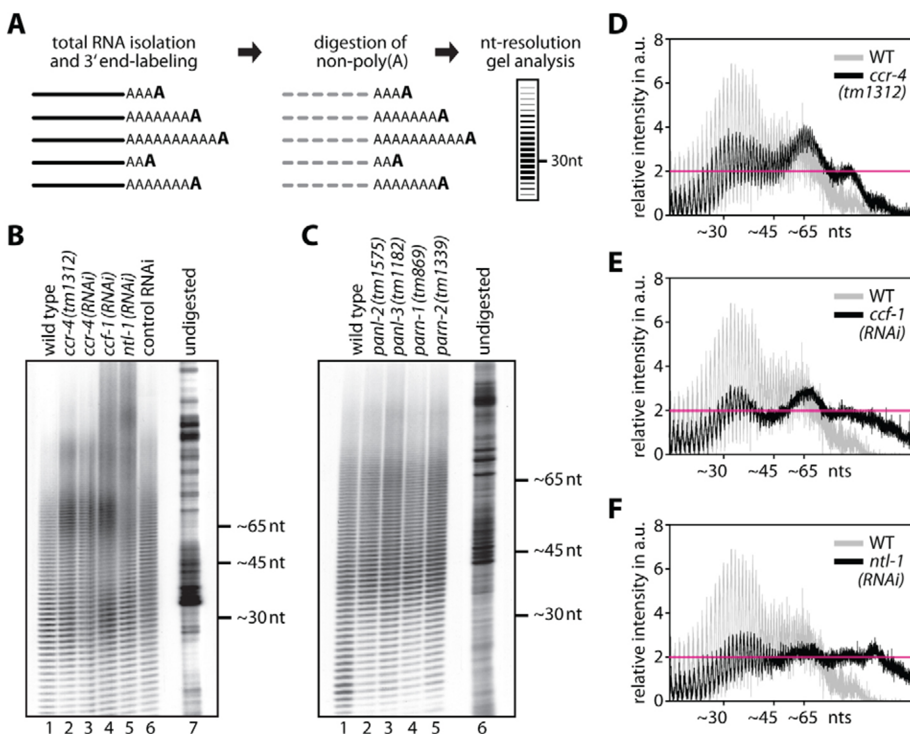

Fig. 8. Bulk poly(A) tail length measurements of total RNA. (**A**) Scheme of the experimental procedure. Total RNA was isolated from adult animals, labeled with radioactive cordycepin, digested by RNases A and T1, and size-separated on a sequencing gel. Undigested RNA of wild-type animals was loaded 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.

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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

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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℃ 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 el 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.

Acknowledgements

We are indebted to Walter Keller (University of Basel) for providing us with an expression clone of bPAP; Elmar Wahle, Claudia Temme (University of Halle), and Stephanie Hilz for introducing us to measuring bulk poly(A) tails. We are grateful to the MPI-CBG antibody and protein expression facilities for technical assistance; the MPI-CBG bioinformatic facility for help with clarifying the evolutionary protein relationships; Karen Bennett (University of Missouri-Columbia), David Drechsel and Antony Hyman (MPI-CBG) for sharing reagents; Yuji Kohara (NIG, Mishima) for providing us with cDNA clones; and the CGC (funded by the NIH Center for Research Resources) and the Japanese Knockout Consortium (led by Shoei Mitani) for providing strains. We also thank Agata Rybarska, Thomas Preiss and Dirk Ostareck for critical reading of the manuscript.

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.

Funding

C.E. is an MPI-CBG investigator. This work was supported by the Max Planck Society and funds from the Deutsche Forschungsgemeinschaft [grant number EC369/2-1] to C.E.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.132936/-/DC1

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