The C. elegans homolog of nucleoporin Nup98 is required for the integrity and function of germline P granules

Ekaterina Voronina¹ and Geraldine Seydoux¹.².*

SUMMARY

C. elegans P granules are conserved cytoplasmic ribonucleoprotein complexes that are unique to the germline and essential for fertility. During most of germline development, P granules are perinuclear and associate with clusters of nuclear pores. In an RNAi screen against nucleoporins, we have identified a specific nucleoporin essential for P granule integrity and function. The C. elegans homolog of vertebrate Nup98 (CeNup98) is enriched in P granules and associates with the translationally repressed, P granule-enriched mRNA nos-2 (nanos homolog). Loss of CeNup98 causes P granules to disperse in the cytoplasm and to release nos-2 mRNA. Embryos depleted for CeNup98 express a nos-2 3′UTR reporter prematurely. In the mouse, Nup98 immunoprecipitates with the germ granule component MVH. Our findings suggest that, in germ cells, the function of Nup98 extends beyond transport at the nuclear pore to include mRNA regulation in the cytoplasm.

KEY WORDS: C. elegans, Nup98, P granule, Germline, Nucleoporin, Translational control

INTRODUCTION

Germ cells contain unique cytoplasmic organelles called germ granules (Saffman and Lasko, 1999). Germ granules are rich in RNA and RNA-binding proteins required for germ cell development and have been proposed to function as regulatory hubs for germ cell mRNAs (Seydoux and Braun, 2006). Germ granules share some components with another class of RNA granules called P bodies, but are in fact distinct organelles (Gallo et al., 2008; Nagamori and Sassone-Corsi, 2008; Anderson and Kedersha, 2009). Unlike P bodies, which are dispersed in the cytoplasm, germ granules are perinuclear during most of germline development. Ultrastructural studies have shown that germ granules in C. elegans (called P granules) associate with clusters of nuclear pores on the cytoplasmic face of the nuclear envelope (Pitt et al., 2000). Several translationally regulated mRNAs are enriched in P granules (Subramaniam and Seydoux, 1999; Schisa et al., 2001), raising the possibility that P granule components mark mRNAs for post-transcriptional regulation as they emerge from nuclear pores. Translational control is a common mode of gene regulation in germ cells (Merritt et al., 2008; Rangan et al., 2009), but a functional link between nuclear pores and P granules had not yet been tested.

Nuclear pore complexes (NPCs) are large macromolecular machines that regulate traffic between the nucleus and the cytoplasm. NPC architecture is conserved from yeast to human and includes ~30 proteins termed nucleoporins or Nups (Capelson and Hetzer, 2009). One-third of nucleoporins contain unstructured domains that are rich in phenylalanine and glycine (FxFG or GLFG repeats). The so-called FG-Nups localize to the cytoplasmic and nuclear faces of the NPC and also line the central channel of the pore (Elad et al., 2009). FG repeats bind directly to transport receptors (such as the mRNA export factor Nxt1) to facilitate their translocation through the pore (Carmondy and Wente, 2009). Interestingly, FG repeats are also present in the C. elegans P granule proteins GLH-1, -2 and -4 (Kuznicki et al., 2000), raising the possibility that P granules also translocate RNAs through their core (Schisa et al., 2001).

In this study, we investigate the possibility of a functional link between nuclear pores and P granules using an RNAi screen against nucleoporins. We report that the FG-Nup Nup98 associates with P granules and is essential for P granule integrity and function.

MATERIALS AND METHODS

Worm culture

All strains (see Table 1) were derived from C. elegans Bristol strain N2 and cultured using standard methods (Stiernagle, 2006). VC316+/+mtl II; npp-10(ok467)/mtl1[dpY-10(e128)] III was obtained from the C. elegans Gene Knockout Consortium (http://celeganskoconsortium.omrf.org), outcrossed six times to wild type, and rebalanced with a GFP-marked qC1 balancer to generate JH2691 npp-10(ok467)/qc1[dpY-19(e1259) gfp-1(q339) qIs26[rol-6 Plag-2::GFP]]. JH2691 was crossed to worms expressing PGL-1::GFP driven by the nmy-2 promoter (Wolke et al., 2007) resulting in JH2753. The sequence of the ok467 mutation was determined by amplifying total RNA isolated from VC316 hermaphrodites using an SL1 primer and an npp-10-specific primer and sequencing the RT-PCR product. ok467 is an in-frame deletion/insertion that removes amino acids 187-420 and mutants the junction amino acid to F (see Fig. S1 in the supplementary material).

Generation of transgenic worms

All transgenes were driven by the pie-1 promoter (maternal expression), unless otherwise indicated, and were constructed by Gateway cloning (Invitrogen) (Landy, 1989). Coding sequences and predicted 3′UTRs were amplified from N2 genomic DNA (npp-7, npp-9, npp-10). Gene structure of npp-10 was analyzed by cDNA sequencing [ends of cDNAs were verified by 5′ RACE with SL1 primer, and 3′ RACE with oligo(dT) primer], and the corrected mRNA sequence, including four additional 5′ exons, was submitted to GenBank (accession number GU174496). The GFP::mCherry construct was generated by PCR fusion. Transgenic lines were generated by microparticle bombardment (Prattis et al., 2001).

RNAi

RNAi was performed by feeding worms with bacteria expressing double-stranded RNA (Timmons and Fire, 1998). The following RNAi constructs were used: ana-1, lmn-1, mel-28, npp-1, npp-2, npp-3, npp-4, npp-5, npp-
Development 137 (9)

Table 1. Nematode strains used in the study

<table>
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<th>Strain</th>
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<th>Genotype</th>
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<tr>
<td>BN7</td>
<td>pie-1 prom::LAP::NPP-19</td>
<td>unc-119(ed3); bqls07</td>
<td>Rodenas et al., 2009</td>
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<td>JH103</td>
<td>pes-10 prom::GFPunc-54 3′UTR</td>
<td>axis3[pJH1.16, dpy-20(+)]</td>
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6, npp-7, npp-8, npp-9, npp-10, npp-12, npp-14, npp-15, npp-17, npp-19, npp-20, npp-21, npp-22 and C09C9.2 (Kamath and Ahringer, 2003); npp-10 [nt 487-2057 of the corrected ORF (see above); this study]; npp-11, npp-13, npp-16 and npp-18 (Galy et al., 2003); nup-1 (genomic fragment, nt 2206-3161 of gene model; this study); pos-1 (full-length ORF; this study); rps-6 (full-length ORF; this study); and L4440 empty vector control. For combinatorial RNAi, bacterial cultures expressing the double-stranded RNA were grown separately and mixed in equal amounts immediately prior to seeding the plates. L4 mothers were incubated on RNAi for 22 hours at 24°C before analyzing their embryos. Synchronized L1 larvae were incubated on RNAi for 22 hours at 23°C before analyzing the L2 germlines (Merritt et al., 2008).

In situ hybridization

Hybridization with the nos-2 probe was performed as described previously (Seydoux and Fire, 1994; Gallo et al., 2008).

Antibody generation and western blots

Polyclonal guinea pig anti-CeNup98 antibodies were generated against the peptides EKAPEGQELNRPAEVC (antibody 1, Ab#1) and CISA-MSKDYDGKSEELRVEDY (antibody 2, Ab#2) by Covance (highlighted in Fig. S1 in the supplemental material).

The following primary antibodies were used: guinea pig anti-CeNup98 Ab#1 at 1:800 and Ab#2 at 1:1500; mouse monoclonal anti-tubulin DM1A (Sigma-Aldrich) at 1:1000; rabbit anti-PGL-1 (Abcam) at 1:2000; mouse monoclonal anti-NPP-8 C-7 (Santa Cruz Biotechnology) at 1:1000; and mouse monoclonal anti-tyrosine kinase mAbAb414 (Covance) at 1:1000. Secondary antibodies were all HRP conjugates from Jackson ImmunoResearch: goat anti-guinea pig at 1:6500; goat anti-mouse IgG1 at 1:6000; goat anti-mouse IgG2a at 1:5000; and goat anti-rabbit at 1:8000. Blots were developed using HyGlo Quick Spray Reagent (Denville). Quantification of western signals was performed using ImageJ (NIH).

For assessing anti-CeNup98 antibody specificity, groups of 50 worms treated with different RNAi were collected into individual Eppendorf tubes, mixed with 2X NuPage LDS Sample Buffer (Invitrogen) containing 200 mM DTT, lyzed by triple freeze-thaw and heating for 10 minutes at 70°C. Lysates were separated on 7% SDS-PAGE gels (Invitrogen), and proteins were transferred to Immobilon-P membrane (Millipore). The blots were preblocked in TBS/0.06% Tween 20 with 5% milk, and probed with antibodies diluted in blocking solution. For analyzing the results of immunoprecipitations, aliquots of input and immunoprecipitation eluates were mixed with LDS sample buffer and DTT, and treated as above.

Homologous ok467 larvae were isolated for western blotting using a COPAS Biosorter (Union Biometrica). Embryos from npp-10(ok467)/qC1(dpy-19(e1259) glp-1(q339) qIs26[rol-6 Plag-2::GFP]) hermaphrodites were hatched overnight without food, plated onto NGM/E. coli OP50 plates and allowed to grow for 24 hours to reach the L2 stage. Homologous npp-10(ok467) L2 larvae were sorted as GFP-negative worms in batches of 500 using the COPAS Biosorter. In parallel, synchronized N2 L2 larvae were grown identically and collected directly from plates for western analysis.

Immunolocalization

Adult hermaphrodites were placed on poly-L-lysine-treated slides, squashed under a coverslip to release embryos, and flash-frozen on aluminum blocks chilled on dry ice. For staining with mAbAb414 and K76, the samples were fixed for 15 minutes in 100% methanol (−20°C) followed by 10 minutes in 100% acetone (−20°C). For staining of nucleoporins (anti-GFP, anti-CeNup98 Ab#1 and Ab#2, anti-CeNup96, anti-NPP-8), samples were fixed for 1 minute in 90% methanol/5 mM EDTA pH 6.0 (−20°C) followed by 1% paraformaldehyde/0.2% Triton X-100 for 10 minutes on ice. The samples were blocked for at least 30 minutes in PBS/0.1% BSA/0.1% Triton X-100 (PBT). Primary antibodies were diluted in PBT as follows: mouse anti-PGL-1 [K76, Developmental Studies Hybridoma Bank (DSHB), University of Iowa] at 1:10; mAbAbIC1D4 (DSHB) at 1:12; mAbAb414 (Covance) at 1:100; anti-CeNup98 (Ab#1) at 1:1500; anti-CeNup98 (Ab#2) at 1:400; anti-NPP-8 (Franz et al., 2005) at 1:180; and anti-CeNup96 (Franz et al., 2005) at 1:500. Rabbit anti-GFP (Invitrogen) was first preadsorbed for 1 hour against N2 acetone powder to deplete cross-reactivity to P granules, and then used at 1:150. Secondary antibodies were: Alexa Fluor 488 goat anti-rabbit at 1:300, and Alexa Fluor 488 goat anti-guinea pig at 1:240 from Invitrogen; and Cy3 goat anti-mouse IgM at 1:200, FITC goat anti-mouse IgG at 1:100, and goat anti-mouse IgG (Fcγ specific) at 1:200 from Jackson ImmunoResearch. All primary antibody incubations were overnight at 4°C; all secondary antibody incubations were for 2 hours at room temperature.

Confocal images were acquired with either a Zeiss LSM 510 laser-scanning confocal microscope and software or a Cascade QuantEM camera attached to a Zeiss Axioimager with Yokogawa spinning disk confocal scanner and
and used for RNA isolation and quantitative (q) PCR as described below.

**Immunoprecipitation**

**C. elegans**

Extracts of young adult worms expressing GFP::NPP-10° or GFP::mCherry were immunoprecipitated with anti-GFP polyclonal antibody (Clontech) conjugated to Protein A Dynabeads (Invitrogen). mRNA bound to the beads was eluted with four 25 μl washes of 100 mM glycine pH 2.5 and used for RNA isolation and quantitative (q) PCR as described below.

**Mouse**

Dissected and detunicated testes of 1.5- to 3.5-month-old C57/Bl6 mice were lyzed for 50 minutes on ice, followed by ten passes through a 25-gauge syringe. After removal of cellular debris by centrifugation, 500 μl of the cleared lysates were incubated with appropriate antibodies crosslinked to Protein A magnetic beads (NEB; 20 μg antibody/100 μl beads) for 4 hours at 4°C. We used the following commercially available antibodies: rabbit anti-MVH (Abcam) (Reynolds et al., 2007), mouse monoclonal anti-Nup98 C-7 (Santa Cruz Biotechnology), and mouse monoclonal anti-nucleoporin mAb414 (Covance) (Davis and Blobel, 1987). Immunoprecipitates were washed five times with immunoprecipitation buffer, and bound proteins were eluted by heating for 10 minutes with 1× LDS sample buffer at 70°C. Proteins were analyzed by western blotting as described above.

**qRTPCR**

Total RNA was isolated from immunoprecipitation eluates using Trizol (Invitrogen), and treated with DNase to remove DNA contamination (TURBO DNA-Free Kit; Ambion). cDNA (20 μl) was prepared from 1 μg of RNA using the iScript cDNA Synthesis Kit according to manufacturer’s instructions (Bio-Rad) using a mix of oligo(dT) and random primers. Real-time PCR reactions were performed in triplicate using 1 μl of cDNA template, iQ SYBR Green Supermix PCR Master Mix and appropriate primers in an iCycler iQ5 real-time PCR detection system (Bio-Rad) using a mix of oligo(dT) and random primers. Real-time PCR reactions were performed in triplicate using 1 μl of cDNA template, iQ SYBR Green Supermix PCR Master Mix and appropriate primers in an iCycler iQ5 real-time PCR detection system (Bio-Rad). Primers for glp-1, nos-2, rme-2 and pal-1 were designed to span exon-exon borders to prevent amplification from residual DNA. Primer binding sites for fbl-1 were separated by an intron, and gel analysis of amplification products did not detect genomic fragment amplification. For primer sequences, see Table S1 in the supplementary material. Each qPCR included a triplicate control set in which reverse transcriptase was omitted from the cDNA synthesis reaction. Average Ct values were determined by iQ5 software for each primer pair. Enrichment of the nos-2 target and several control mRNAs in the GFP::NPP-10 immunoprecipitates was calculated using ΔCt of anti-GFP and non-specific IgG immunoprecipitates, and plotted for comparison without normalization. Mean Ct in total extracts before pulldowns were as follows: nos-2, 22.28; fbl-1, 26.61; rme-2, 22.72; glp-1, 22.64; pal-1, 22.94.

**Mouse experiments**

The mouse tissues used were generated from wild-type C57BL/6 surplus male mice that were scheduled for termination as per IACUC protocol and provided to our laboratory following euthanasia. This procedure has been reviewed and approved by Johns Hopkins Animal Care and Use Committee and was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

**RESULTS**

**Identification of four nucleoporins essential for P granule integrity in C. elegans embryos**

Twenty-four NPC-associated genes (including 22 nucleoporin, or npp, genes) are annotated in WormBase version WS203. We used RNAi feeding to knock down each nucleoporin in hemaphrodites expressing the constitutive P granule component PGL-1 tagged with GFP (Kawasaki et al., 1998; Gallo et al., 2008). Four nucleoporins (npp-7, -8, -9, -10) gave a high penetrance diffuse GFP::PGL-1 phenotype in embryos (~40% broods affected), and seven others gave less penetrant phenotypes (see Table S2 in the supplementary material). All of these candidates were also recovered by Updike and Strome (Updike and Strome, 2009) in a genome-wide RNAi screen using GFP::PGL-1.

Fig. 1. A subset of C. elegans nucleoporins is required for P granule integrity. (A-H) Single-plane confocal sections of 13- to 28-cell stage embryos derived from mothers treated with the indicated RNAi, and co-stained with antibodies against nuclear pore proteins (mAb414, red) and PGL-1 (K76, green). Embryos are oriented with anterior to the left and posterior to the right in this and all subsequent figures. P granules are perinuclear in control (empty vector), npp-1(RNAi), npp-19(RNAi) and lmn-1(RNAi), but mislocalized after npp-7(RNAi), npp-8(RNAi), npp-9(RNAi) and npp-10(RNAi). (I) Comparison of the percentage of embryos expressing the zygotic transgene pes-10::GFP and those with wild-type P granules following the indicated RNAi treatments (error bars are s.e.m.). *, P<0.01 (Student’s t-test): a statistically significant reduction in the number of pes-10::GFP-positive embryos in RNAi versus control. The number of embryos scored (n) is shown below each bar.
To determine which nucleoporin affects endogenous P granules, we rescreened the 11 candidates using the anti-PGL-1 antibody K76 (Kawasaki et al., 1998). RNAi efficiency was assessed by measuring embryonic lethality. RNAi against \textit{npp-7}, -8, -9 or -10 reliably disrupted endogenous P granules (Fig. 1; see Table S2 in the supplementary material). In the P3 blastomere of affected embryos (see Fig. S2 in the supplementary material for a description of embryonic cell lineage), P granules were smaller (0.6 μm in diameter versus 1.5-2.0 μm in wild type) and more numerous (54-73 versus 14), as if fragmented. Most were dispersed in the cytoplasm, although a minority (7-29%) remained at the nuclear periphery (Fig. 1; see Fig. S2 and Table S2 in the supplementary material). We term this phenotype ‘P granule dispersal’. We observed the same phenotype in \textit{npp-10(RNAi)} embryos expressing GFP fusions to two other P granule components, GLH-1 and PGL-3 (see Fig. S3C in the supplementary material).

P granule dispersal could be a secondary consequence of a general defect in nuclear pores or could reflect a specific requirement for a subset of nucleoporins. To distinguish between these possibilities, we compared P granules and nuclear pores in embryos depleted for \textit{npp-7}, -8, -9 and -10 and embryos depleted for \textit{lmn-1}, \textit{npp-1} and \textit{npp-19}, three genes that are essential for nuclear pore organization (Liu et al., 2000; Galy et al., 2003). As predicted, nuclear pore organization was disrupted by each RNAi treatment (mAb414, Fig. 1). P granule dispersal, however, was observed only in \textit{npp-7(RNAi)}, -8(RNAi), -9(RNAi) and -10(RNAi) (Fig. 1). We also examined whether the P granule dispersal phenotype correlates with a general defect in cell function as assessed by gene expression. We used the \textit{pes-10::GFP} transgene to monitor zygotic gene expression in embryos (Wallenfang and Seydoux, 2002). \textit{npp-8(RNAi)} and \textit{npp-10(RNAi)} showed only a partially penetrant effect on \textit{pes-10::GFP} expression (Fig. 11). By contrast, \textit{npp-7(RNAi)} and \textit{npp-9(RNAi)} efficiently blocked \textit{pes-10::GFP} expression. \textit{npp-7(RNAi)} and \textit{npp-9(RNAi)} also caused defects in protein import into nuclei, not seen in \textit{npp-8(RNAi)} and \textit{npp-10(RNAi)} (see Fig. S3B in the supplementary material). Depletion of the large subunit of RNA polymerase II (AMA-1), the mRNA export factor NXF-1 and the core nucleoporin NPP-1 all blocked \textit{pes-10::GFP} expression, but these treatments had no effect on P granules (Fig. 11). These observations indicate that defects in nuclear pore organization and function do not necessarily correlate with P granule dispersal and vice versa. We conclude that \textit{npp-7}, -8, -9 and -10 represent a specialized subset of nucleoporins required for P granule integrity.

### GFP::NPP-8 and GFP::NPP-10 localize to P granules

To determine whether NPP-7, -8, -9 or -10 localize to P granules, we first used GFP fusions to examine their localization in embryos (Franz et al., 2005) (see Materials and methods). We also examined
a GFP fusion to NPP-19 as a control (Rodenas et al., 2009). As expected, all GFP::NPP fusions localized to nuclear envelopes (Fig. 2A). GFP::NPP-8 and GFP::NPP-10, in addition, were enriched in P granules (Fig. 2A). Co-localization with P granules was seen in fixed embryos (Fig. 2A), but not in live samples (data not shown). GFP fusions to NPP-7, -9 and -19 did not localize to P granules in fixed or live samples (Fig. 2A).

To determine whether P granule localization of GFP::NPP-8 and GFP::NPP-10 depends on the other nucleoporins identified in our screen, we re-examined the fusions in embryos depleted of nucleoporins by RNAi. We found that GFP::NPP-8 requires npp-9 for P granule localization, but not npp-7 or -10 (Fig. 2B). In npp-7(RNAi) and npp-10(RNAi), the P granules were dispersed but still showed enrichment for GFP::NPP-8, suggesting that localization of GFP::NPP-8 to P granules is not sufficient for P granule integrity.

By contrast, localization of GFP::NPP-10 to P granules depended on all other nucleoporins required for P granule integrity, i.e. npp-7, -8 and -9 (Fig. 2B). npp-8(RNAi) caused GFP::NPP-10 to cluster in the cytoplasm away from P granules and nuclei. In npp-7(RNAi) and npp-9(RNAi), GFP::NPP-10 could still be detected on the nuclear envelope, but was no longer enriched in P granules, suggesting that localization to the nuclear envelope is not sufficient to localize GFP::NPP-10 to P granules. We conclude that localization of GFP::NPP-10 to P granules correlates with P granule integrity under the conditions tested.

Fig. 3. Endogenous CeNup98 is enriched in P granules in embryos. (A) Confocal sections of C. elegans embryos co-stained with the indicated anti-nucleoporin antibodies (green) and anti-PGL-1 (red), with and without npp-10(RNAi). npp-10(RNAi) eliminates all anti-nucleoporin staining. (B) Western blot with anti-CeNup98 antibody 1 and with anti-tubulin antibody of extracts from wild-type worms treated with the indicated RNAi. Fifty worms were loaded per lane. Full blots are shown in Fig. S5 in the supplementary material.

CeNup98 is enriched in P granules

npp-10 encodes the C. elegans homolog of mammalian Nup98-Nup96, a gene that produces two nucleoporins (Nup98 and Nup96) from a common precursor by proteolytic cleavage (Fontoura et al., 1999). Nup98 is an FG-Nup that associates dynamically with the NPC, shuttles between the nucleoplasm and cytoplasm (Griffis et al., 2002), and has been implicated in mRNA export (Powers et al., 1997; Pritchard et al., 1999). Nup96 is a component of the Nup107-160 sub-complex, which is stably associated with the NPC (Rabut et al., 2004). If processing of NPP-10 occurs in C. elegans as in mammals, the GFP::NPP-10 fusion described above should report on the localization of the N-terminal polypeptide Nup98 (CeNup98); henceforth, we refer to this fusion as GFP::NPP-10<sub>Nup98</sub>. Western analysis of worm extracts confirmed that GFP::NPP-10<sub>Nup98</sub> migrates at a molecular weight that is consistent with a GFP::Nup98 fusion, but in some extracts the higher molecular weight uncleaved precursor was the predominant species (see Fig. S4 in the supplementary material). To examine the distribution of npp-10 products directly, we generated two antibodies against two non-overlapping CeNup98 peptides and obtained a previously characterized CeNup96 antibody (Fig. 3; see Fig. S5 in the supplementary material) (Galy et al., 2003). We found that the two CeNup98 antibodies labeled P granules, but the antibody against CeNup98 did not (Fig. 3). The CeNup98 antibodies also showed strong staining in the cytoplasm, in contrast to the CeNup96 antibody, which primarily stained nuclear envelopes (Fig. 3). Staining was eliminated by npp-10(RNAi), confirming specificity. Localization of CeNup98 to P granules was dependent on npp-7 and npp-8, as we observed for the GFP::NPP-10<sub>Nup98</sub> fusions (see Fig. S6 in the supplementary material). We conclude that CeNup98, but not CeNup96, is enriched in P granules.

P granules are mostly cytoplasmic in the P0, P1 and P2 blastomeres (Strome and Wood, 1982). We detected enrichment of GFP::NPP-10<sub>Nup98</sub> and endogenous CeNup98 in some, but not all, cytoplasmic P granules (see Fig. S7 in the supplementary material). Generally, larger P granules were more likely to show enrichment than smaller ones. We do not know whether this preference reflects the limited sensitivity of our immunostaining, or regulation. We conclude that enrichment of CeNup98 can occur even in P granules that are not in direct contact with the nuclear envelope.

P bodies are RNA granules found in both somatic and germ cells (Gallo et al., 2008). Although GFP::NPP-10<sub>Nup98</sub> and endogenous CeNup98 were abundant in the cytoplasm of somatic blastomeres, neither was enriched in P bodies. npp-10(RNAi) also had no effect on P body integrity (see Fig. S8 in the supplementary material).

An antibody against NPP-8 (Franz et al., 2005) also labeled P granules (see Fig. S9 in the supplementary material). npp-8(RNAi) eliminated the nuclear envelope staining but did not eliminate the P granule staining, indicating that the NPP-8 antibody recognizes a second epitope present in P granules. We were therefore unable to verify whether endogenous NPP-8 also associates with P granules.

CeNup98 is required for P granule integrity

The finding that CeNup98, but not CeNup96, is enriched in P granules suggests that CeNup98 is the nucleoporin required for P granule integrity. Because CeNup98 and CeNup96 are derived from a common precursor, however, RNAi against the npp-10 locus depletes both nucleoporins (see Fig. S5 in the supplementary material) (Galy et al., 2003) and thus cannot be used to distinguish between the two. To determine whether CeNup98, specifically, is required for P granule integrity, we obtained a CeNup98-specific deletion allele from the C. elegans Gene Knockout Consortium.

DEVELOPMENT

RESEARCH ARTICLE
ok467 is an in-frame deletion (see Fig. S1 in the supplementary material) which removes 234 amino acids from the CeNup98 polypeptide, including half of the FG repeats (17 out of 35) and part of the RAE1-binding GLEBS motif (Pritchard et al., 1999). Western analysis confirmed that ok467 homozygotes produce a truncated version of CeNup98 and full-length CeNup96 (Fig. 4A). ok467 homozygotes die in the L2 larval stage (data not shown). At hatching, ok467 homozygotes exhibited the wild-type perinuclear punctate PGL-1::GFP pattern (Fig. 4B), presumably owing to maternal contribution from the npp-10 locus. By the L2 stage, however, 100% of ok467 homozygotes showed only diffuse PGL-1::GFP (Fig. 4C). Immunostaining for the P granule epitope OIC1D4 confirmed that ok467 germ cells do not contain visible P granules (Fig. 4C). Identical results were obtained when wild-type larvae were treated with npp-10(RNAi) starting at the L1 stage (Fig. 4D). The effect on P granules was specific to npp-10: the GFP::PGL-1 pattern appeared wild-type in larvae depleted of other nucleoporins (Fig. 4D). We conclude that CeNup98 is essential for P granule integrity, both in embryos and in larvae.

**npp-10 is required for full translational repression of the P granule-associated mRNA nos-2**

To determine whether loss of npp-10 and P granule dispersal have any functional consequences, we examined the regulation of the P granule-associated mRNA nos-2. nos-2 is a maternal mRNA that is degraded in somatic blastomeres and maintained only in germline blastomeres (Subramaniam and Seydoux, 1999). nos-2 mRNA is distributed in the cytoplasm in the zygote, and becomes visibly enriched in P granules in the germ line P2 and P3 blastomeres (Galloy et al., 2005). nos-2 is translationally repressed throughout oogenesis and early embryogenesis and is first translated at the 28-cell stage in the germline blastomere P4. Translational regulation of nos-2 depends on its 3’UTR: a transgene containing the nos-2 3’UTR [pie-1(prom)::GFP::Histone H2B:nos-2 3’UTR] expresses the GFP reporter in P4 (28-cell stage) but not earlier (D’Agostino et al., 2006). We found that the nos-2 3’UTR reporter is activated prematurely in npp-7(RNAi), npp-8(RNAi) and npp-10(RNAi) embryos (Fig. 5A). Precocious expression was detected at the 12-cell stage in the germline blastomere P3, but not earlier.

 nos-2 regulation depends on both translational repressors (MEX-3 and SPN-4) and translational activators (POS-1), which compete for binding to the nos-2 3’UTR. POS-1 is required for translational activation of nos-2 in wild-type embryos, but not in spn-4(RNAi) or mex-3(RNAi) embryos, which activate the nos-2 transgene at the 2-cell stage (Jadhav et al., 2008). We found that premature expression of the nos-2 reporter in npp-10(RNAi) is dependent on pos-1 activity (see Fig. S10 in the supplementary material). Together with the fact that the nos-2 reporter remains repressed before the P3 stage in npp-10(RNAi), this result indicates that MEX-3- and SPN-4-mediated repression remains functional in npp-10(RNAi). We conclude that NPP-10 is required for the full extent of translational repression of nos-2, but might not be essential for all aspects of nos-2 regulation.

To examine the endogenous nos-2 mRNA directly, we used in situ hybridization combined with labeling of P granules. In wild-type embryos, 100% of P granules in the P3 blastomere were positive for nos-2 RNA (n=57). By contrast, in npp-10(RNAi), only 13% of granules were positive for nos-2 RNA (n=122) (Fig. 5B,C). The nos-2 RNA was still present in the P3 blastomere, but was found in smaller foci, most of which did not co-localize with the dispersed P granules (Fig. 5B). nos-2 mRNA was rapidly degraded in somatic blastomeres in npp-10(RNAi) as in wild type, confirming that npp-10(RNAi) does not disrupt all aspects of nos-2 mRNA regulation (Fig. 5D). We conclude that npp-10 is required for accumulation of the nos-2 mRNA in P granules.

**CeNup98 is in a complex with nos-2 RNA**

To determine whether CeNup98 associates with the nos-2 mRNA, we immunoprecipitated GFP::NPP-10(ok467) with an anti-GFP antibody (or with a control IgG) and performed real-time qPCR on
the immunoprecipitates. nos-2 mRNA was enriched an average of 465-fold in the GFP::NPP-10\textsuperscript{Nup98} immunoprecipitate compared with the IgG precipitate (Fig. 6). By comparison, control mRNAs were enriched only 2- to 10-fold. Immunoprecipitation of a GFP::mCherry fusion yielded only a 3-fold enrichment in nos-2 RNA compared with IgG. We conclude that CeNup98 associates preferentially with the nos-2 mRNA, consistent with their co-localization in P granules.

**Nup98 associates with the germ granule component MVH in mouse testes**

The chromatoid body is a mouse germ cell organelle that is related to P granules. Like P granules, the chromatoid body is rich in RNA and contains several RNA-binding proteins, including MVH (Ddx4), an RNA helicase related to Drosophila Vasa and C. elegans GLH-1 (Nagamori and Sassone-Corsi, 2008). To determine whether Nup98 is also enriched in the chromatoid body, we immunoprecipitated Nup98 and MVH from mouse testes extracts using commercially available antibodies. We detected Nup98 in MVH immunoprecipitates and MVH in Nup98 immunoprecipitates (Fig. 7). A control nucleoporin, p62 [Nup62; detected by mAb414 (Davis and Blobel, 1987)], did not co-immunoprecipitate with MVH as efficiently. The interaction between MVH and Nup98 is likely to be indirect, as we could not reproduce it by expressing MVH and Nup98 in tissue cell culture (data not shown).

**DISCUSSION**

In this study, we identify the nucleoporin CeNup98 as an essential component of P granules in *C. elegans*. Our findings suggest that Nup98 function extends beyond the nuclear pore to include mRNA regulation in the cytoplasm.

**P granule integrity requires a specific subset of nucleoporins**

In our RNAi screen of 24 NPC-associated genes, we identified four nucleoporins (npp-7, -8, -9 and -10) required for P granule integrity. Four lines of evidence indicate that the requirement for these nucleoporins is specific and not due to a general requirement for NPC function. First, RNAi depletion of other essential nucleoporins (e.g. npp-1 and npp-19) had no effect on P granules. Second, RNAi depletion of RNA polymerase II, or of the RNA export factor NXF-1, also had no effect on P granules. Third, CeNup98 is enriched in P granules and the other three nucleoporins isolated in our screen (npp-7, -8, -9 and -10) are required for this localization. Fourth, a deletion mutant that specifically disrupts CeNup98, without affecting the co-translation nucleoporin CeNup96, is sufficient to disrupt P granule integrity.

We propose that CeNup98 plays a direct role in promoting P granule integrity by interacting with P granule-associated mRNAs (see below). The role of NPP-7 and NPP-9 might be secondary to their effect on CeNup98 localization, as these proteins do not appear enriched in P granules, at least as GFP fusions. GFP::NPP-8 did localize to P granules but we were unable to verify this localization for endogenous NPP-8. Interestingly, the *Drosophila* NPP-8 homolog (Nup154) has also been reported to localize to cytoplasmic complexes when expressed as a GFP fusion in germ cells (Grimaldi et al., 2007). Consistent with NPP-8 and CeNup98 functioning together, the corresponding yeast homologs Nup157 and Nup145N have been reported to form a complex in vitro and in vivo (Lutzmann et al., 2005). GFP::NPP-8 was enriched in P granules in npp-7(RNAi) and npp-10(RNAi) zygotes suggesting that, without CeNup98, NPP-8 is not sufficient to promote P granule integrity.
The association of CeNup98 and GFP::NPP-8 with P granules is likely to be transient and dynamic because we observed co-localization only in fixed samples. Similarly, co-localization between specific mRNAs and P granules has been observed only in fixed samples, and mRNAs that are enriched in P granules are also present outside the P granules in the cytoplasm (Pitt et al., 2000; Schisa et al., 2001; Gallo et al., 2008). P granules are highly dynamic in embryos, and even core components can diffuse in and out of the granules within 30 seconds (Brangwynne et al., 2009).

The function of CeNup98 in P granules
What is the function of CeNup98 in P granules? Depletion of CeNup98 by RNAi in embryos leads to small P granules that lack nos-2 mRNA and associate inefficiently with the nuclear envelope. Live observations have revealed that P granules exhibit behaviors that are typical of liquid droplets, such as dissolution, condensation and surface wetting on nuclei (Brangwynne et al., 2009). These dynamics have suggested that P granules are held together by low-affinity interactions (Brangwynne et al., 2009). In this context, the role of CeNup98 might be to promote the ‘liquid phase’ of P granules, by increasing the number of interacting RNA-protein complexes. Our finding that perinuclear localization does not require functional NPCs is also consistent with low-specificity interactions tethering P granules to nuclei. Such interactions are likely to be favored when P granules reach a critical size. In this model, CeNup98 would not function as a direct bridge between P granules and nuclear pores, but would promote perinuclear localization indirectly by increasing P granule size. Consistent with this hypothesis, P granules are small and dispersed in embryos depleted for npp-7 and npp-9, which lack CeNup98 in P granules but maintain CeNup98 on nuclear envelopes.

Depletion of CeNup98 also leads to premature translation of the nos-2 mRNA. How does CeNup98 contribute to nos-2 repression? In Drosophila, NPP-8 (Nup154) interacts with the translation repressor Cup (Grimaldi et al., 2007), but a Cup ortholog has not yet been described in C. elegans. An alternative possibility is that

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**Fig. 6. CeNup98 is in a complex with nos-2 mRNA.** (A) nos-2 and control mRNAs were amplified by RT-PCR in GFP::NPP-10Nup98 and control GFP::mCherry immunoprecipitates. nos-2 mRNA is only detected in the GFP::NPP-10Nup98 immunoprecipitates. fbf-1 is a germline mRNA which, like nos-2, is also present in embryos. NRTC, no reverse transcriptase control. RNA was isolated from the inputs and eluates, treated with DNase and reverse transcribed. cDNA was used for qPCR with primers to nos-2 or fbf-1. Shown are products of 36 amplification cycles (linear range determined by qPCR). Input lanes are 1% of pulldown lanes. Processing of GFP::NPP-10 fusion to GFP::NPP-10Nup98 was verified by western blot (see Fig. S4, lane 2, in the supplementary material). (B) Fold enrichment of mRNAs in the anti-GFP immunoprecipitates compared with the IgG immunoprecipitates. Relative enrichment was calculated using RT-qPCR and the comparative Ct method (Pfaffl, 2001). The average of three qPCR amplifications from two independent biological replicates is reported; error bars represent s.e.m.

**Fig. 7. Mouse Nup98 interacts with the germ granule component MVH in testes.** (A) Lysates of C57/Bl6 mouse testes were immunoprecipitated using the indicated antibodies (top), followed by western blot analysis. Input lanes contain 4 or 5% of the extract used for immunoprecipitation. (B) Quantitation of results shown in A. Fold enrichment was calculated by determining the ratio of band intensities in the specific immunoprecipitations over the IgG immunoprecipitation. Plotted values are derived from the experiment shown in A. A second independent immunoprecipitation experiment (not shown) yielded similar enrichment values: 11-fold enrichment of Nup98 in the MVH immunoprecipitate and a 46-fold enrichment of MVH in the Nup98 immunoprecipitate, as compared with the IgG immunoprecipitate.
CeNup98 delays nos-2 translation simply by keeping nos-2 in large aggregates that exclude ribosomes or other translational activators. nos-2 translation is regulated by the ratio of the activator POS-1 to the repressor SPN-4, proteins that compete for binding to the nos-2 3′UTR (Jadhav et al., 2008). By maintaining nos-2 in a complex that favors SPN-4 over POS-1, CeNup98 could delay nos-2 translation. Consistent with this view, nos-2 expression in npp-10(nRNAi) embryos still depends on POS-1.

Vertebrate Nup98 interacts with the mRNA export factor RAEl (Pritchard et al., 1999; Rayala et al., 2004). RNAi depletion of the C. elegans Rael ortholog npp-17 did not affect P granules (see Table S2 in the supplemental material) (Galy et al., 2003), suggesting that CeNup98 does not function with RAEl (NPP-17) in P granules. When immunoprecipitated from worm extracts, CeNup98 is in a complex enriched for nos-2 mRNA. Presumably, CeNup98 does not bind nos-2 mRNA directly, as CeNup98 does not have a predicted RNA-binding domain. One possibility is that CeNup98 associates with proteins bound to the nos-2 mRNA when nos-2 is first synthesized during oogenesis, and is inherited with nos-2 during embryogenesis as a part of maternal complex enriched in P granules. The interaction between CeNup98 and P granules appears to be conserved in evolution because we found that mouse Nup98 immunoprecipitates with the germ granule protein MVH in testes extracts.

Nucleoporins with roles outside of the NPC

Several studies have implicated nucleoporins in activities beyond nuclear trafficking, including roles in chromosome segregation, spindle assembly and the regulation of transcription (reviewed by Lim et al., 2008; Capelson and Hetzer, 2009). These novel functions correlate with the localization of nucleoporins away from the NPC: to kinetochores, spindle microtubules and chromosomes. Our study extends this view to include a role for nucleoporins outside the nucleus: in P granules in the cytoplasm. Nup98 has been implicated in oncogenic transformation, as the FG domain of NUP98 has been found in 19 different fusion proteins associated with leukemias (Kalverda and Fornerod, 2007). In the majority of cases, the FG repeats are fused to transcription factors and the resulting oncogenic fusions have been proposed to act as aberrant transcription factors (Xu and Powers, 2009). Interestingly, oncogenic NUP98 fusions have also been observed with DDX10, a putative RNA helicase (Romana et al., 2006). The oncogenic properties of NUP98 could therefore also involve dysregulation of gene expression at the post-transcriptional level. This possibility has already been suggested for the FG-Nup, NUP214, in an oncogenic fusion with the nuclear protein DEK (Ageberg et al., 2008).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.orglookup/suppl?doi:10.1242/dev.047654/-/DC1

References


Figure S1

Protein sequence and domain structure of CeNup98

Nucleoporin Autoprotease domain predicted by homology by HHpred (http://toolkit.tuebingen.mpg.de)

Fragment deleted in npp-10(ok467) and replaced with a single F

RAE-binding motif by homology to human Nup98

CeNup98

CeNup96

SKYGLADDEPMDSPPQQALQASSPLVQIMNTSARDVNNQVQRKKVHKATDAHHQEI1 LERVPAPAALGVDVPIIRVRNVRKGLGGS GLLDSDR EESC2IGMTTE3FENSEXGHDIIIEEGQ PEKPKPLELADLEYESSRFIRNLQELKVMKANDPAHFGHHGHSAMICMGKSLID2 GIVGKGRSSHGWSTRGCLVWSAQPFRHQVLFGTIDRTSVDNENTLISMLDVNVHVESTSR KGPSQSVNSVKSLSNFTYSDYSDYSMMFAKYIDVAQQAGGDHSWVKLISALFYERR EGWSF5ERGIUGELRTAEVSKVPPDSDT6SMDGWNQLGSLG1DKAFQIAIDNQPQL ATMLQTSAVCPEATVHCFAQALQAVLWKKCETLHPIKETLKYLVMSGLSHYEWDDQGKNIH SINCLEDGLNQALQLHYWLYRAWTGLLEESYDAYQKDNAGRAASNQGLPLGLIKLACE SQHSVEVLCAEGENPNDYFLQWHWSLLYSGYTMSKTETRLHRNYSQLEASSLS KYALFVQHIDDDEERSTAVSLDDIRAFTDNDMDFISSEQFDIPSEWAADQFIASX VDDSTQFLHLAVAAKNYLEICRLFVDDIAPTAVAGHDAKLKAACMVRPFENQIPEWGA TGMYVTYCRLLNLIENDEELQDVLQVLSLTHALTISKNQLQKLQLTIGRVLFEY RADKNTLPETWKLLGHRQMFKIRDRRSSWGGIERTFIEFD
Figure S10

GFP:H2B:nos-2 3’utr

- control
- npp-10(RNAi)
- npp-10(RNAi), pos-1(RNAi)

~25-cell

~50-cell

10 μm
Figure S3

A

Vector RNAi

Defective P granules

B

Defective P granules

C

Defective P granules

GFP:GLH-1
Figure S4

1. GFP:Nup98-Nup96
2. GFP:Nup98
Figure S5

(A) CeNup98 Antibody #1

(B) CeNup98 Antibody #2

(C) Anti-Tubulin

(D) npp-10 gene locus (Nup98/96)
Figure S6

<table>
<thead>
<tr>
<th>CeNup98 localization</th>
<th>Nuclear Envelope</th>
<th>P granules</th>
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<tr>
<td>wt</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>npp-7(RNAi)</td>
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<td>-</td>
</tr>
<tr>
<td>npp-8(RNAi)</td>
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<td>-</td>
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<td>npp-19(RNAi)</td>
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</table>
Figure S7
Figure S8

A

endogenous CeNup98

GFP:NPP-10

B

vector RNAi

npp-10(RNAi)
Figure S9

anti-NPP-8 (CeNup155) staining
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<thead>
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<th>Primer sequence (5’ to 3’)</th>
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| fbf-1 | GCTCTACCGAGATTGATCGTAA
TTTGTCAACCGAAAACCTCATC |
| glp-1 | GACATACTGGTGCCCGTGCC
TCTCGTCTTCGGCAATCTTGAC |
| nos-2 | AAGGCGTGGAATATGGATGTG
GCAGTACGTCTCGGTGATT |
| pal-1 | CGTTATGGATATCAACAGTTTCAG
ACACGGACATTATCGTTCCA |
| rme-2 | GAAACGTTTTCTTCAACAAAAATCTC
TGTCCTTGTCTTTGGCTTCG |
Table S2. RNAi screen of C. elegans nucleoporins

<table>
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<tr>
<th>C. elegans gene</th>
<th>Vertebrate Nup</th>
<th>Percentage broods with abnormal GFP::PGL-1 (n)</th>
<th>Abnormal K76 staining</th>
<th>Embryonic lethality (%)</th>
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JH2107 (GFP::PGL-1) was subjected to the indicated RNAs for 22 hours by feeding and the hermaphrodites were scored for embryos with abnormal GFP::PGL-1. Percentages are the numbers of broods with one or more embryos with abnormal GFP::PGL-1. All positives and several negatives were subjected to a secondary RNAi screen using wild-type worms and endogenous PGL-1 scored by immunostaining (K76 antibody) (Strome and Wood, 1982). The efficacy of the RNAi treatments was evaluated by scoring embryonic lethality; values (including those of no lethality) were similar to those reported previously (Galy et al., 2003). Identities of vertebrate homologs are according to previous reports (Galy et al., 2003; Franz et al., 2005).

*As in Galy et al. (Galy et al., 2003).
† Defects might be secondary to general cytological abnormalities (see Fig. 1F).