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

Ekaterina Voronina[1] and Geraldine Seydoux[1,2,*]

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 Nxf1) to facilitate their translocation through the pore (Carmody 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.

[1]Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA. [2]Howard Hughes Medical Institute, 4000 Jones Bridge Road, Chevy Chase, MD 20815-67, USA.

*Author for correspondence (gseydoux@jhmi.edu)

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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 *+/mT1 II; npp-10(ok467)/mT1[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) glp-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 mutates 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 (Praitis 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: *ama-1*, *lmn-1*, *mel-28*, *npp-1*, *npp-2*, *npp-3*, *npp-4*, *npp-5*, *npp-*

Table 1. Nematode strains used in the study

Strain	Transgene description	Genotype	Reference
BN7	*pie-1* prom::LAP::NPP-19	*unc-119(ed3); bqIs07*	Rodenas et al., 2009
JH103	*pes-10* prom::GFP*unc-54* 3′UTR	*axIs36[pJH1.16, dpy-20(+)]*	Golden et al., 2000; Wallenfang and Seydoux, 2002
JH1999	*pie-1* prom::GFP::H2B::*nos-2* 3′UTR	*unc-119(ed3); axIs1448[pCM1.01]*	D'Agostino et al., 2006
JH2017	*pie-1* prom::GFP::PGL-3::*pgl-3* 3′UTR	*unc-199(ed3); axIs1464[pCM4.10]*	Merritt et al., 2008
JH2080	*pie-1* prom::GFP::EXOS-1::*exos-1* 3′UTR	*unc-119(ed3); axIs1506[pCG136]*	Gallo et al., 2008
JH2107	*pie-1* prom::GFP::PGL-1::*pgl-1* 3′UTR	*unc-119(ed3); axIs1491[pCM4.11]*	Gallo et al., 2008
JH2184	*pie-1* prom::GFP::NPP-9::*npp-9* 3′UTR	*unc-119(ed3); axIs1595 [pEV3.01]*	This study
JH2281	*pie-1* prom::LAP::mCherry::*nos-2* 3′UTR	*unc-119(ed3); axIs1729[pEV1.04]*	This study
JH2330	*nmy-2* prom::PGL-1::GFP::*nmy-2* 3′UTR; *pie-1* prom::mCherry::PATR-1::*pie-1* 3′UTR		Gallo et al., 2008
JH2458	*pie-1* prom::LAP::NPP-10::*npp-10* 3′UTR	*unc-119(ed3); axIs1735 [pEV6.01]*	This study
JH2464	*pie*-1 prom::mCherry::H2B::*tbb-2* 3′UTR	*unc-119(ed3); axIs1779 [pCM1.119]*	This study
JH2686	*pie-1* prom::LAP::NPP-7::*npp-7* 3′UTR	*unc-119(ed3); axIs1844 [pEV6.09]*	This study
JH2688	*pie-1* prom::LAP::GLH-1::*nos-2* 3′UTR	*unc-119(ed3); axIs1927[pEV1.02]*	This study
JH2691		*npp-10(ok467)/qC1(dpy-19(e1259) glp-1(q339) qIs26[rol-6 Plag-2::GFP]) III*	This study
JH2753	*nmy-2* prom::PGL-1::GFP::*nmy-2* 3′UTR	*npp-10(ok467)/qC1(dpy-19(e1259) glp-1(q339) qIs26[rol-6 Plag-2::GFP]) III; PGL-1::GFP*	This study
VC316		*+/mT1 II; npp-10(ok467)/mT1[dpy-10(e128)] III*	*C. elegans* Gene Knockout Consortium
XA3546	*pie-1* prom::GFP::NPP-8::*pie-1* 3′UTR	*unc-119(ed3); qaIs3546*	Franz et al., 2005
	nmy-2 prom::PGL-1::GFP::*nmy-2* 3′UTR		Wolke et al., 2007

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 *C09G9.2* (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); *nxf-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-MSKYDGKSIEELRVEDY (antibody 2, Ab#2) by Covance (highlighted in Fig. S1 in the supplementary 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-MVH (Abcam) at 1:2000; mouse monoclonal anti-Nup98 C-7 (Santa Cruz Biotechnology) at 1:100; and mouse monoclonal anti-nucleoporin mAb414 (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 2× 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 eluate were mixed with LDS sample buffer and DTT, and treated as above.

Homozygous *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 NNGM/*E. coli* OP50 plates and allowed to grow for 24 hours to reach the L2 stage. Homozygous *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 aluminium blocks chilled on dry ice. For staining with mAb414 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 EGTA 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; mAbOIC1D4 (DSHB) at 1:12; mAb414 (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 IgM 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

Slidebook software (Intelligent Imaging Innovations). Image quantification was performed in Slidebook, and image processing in Adobe Photoshop CS2.

Immunoprecipitation

C. elegans

Extracts of young adult worms expressing GFP::NPP-10^{Nup98} 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.

qRT-PCR

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). 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 *fbf-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; *fbf-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 hermaphrodites 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.

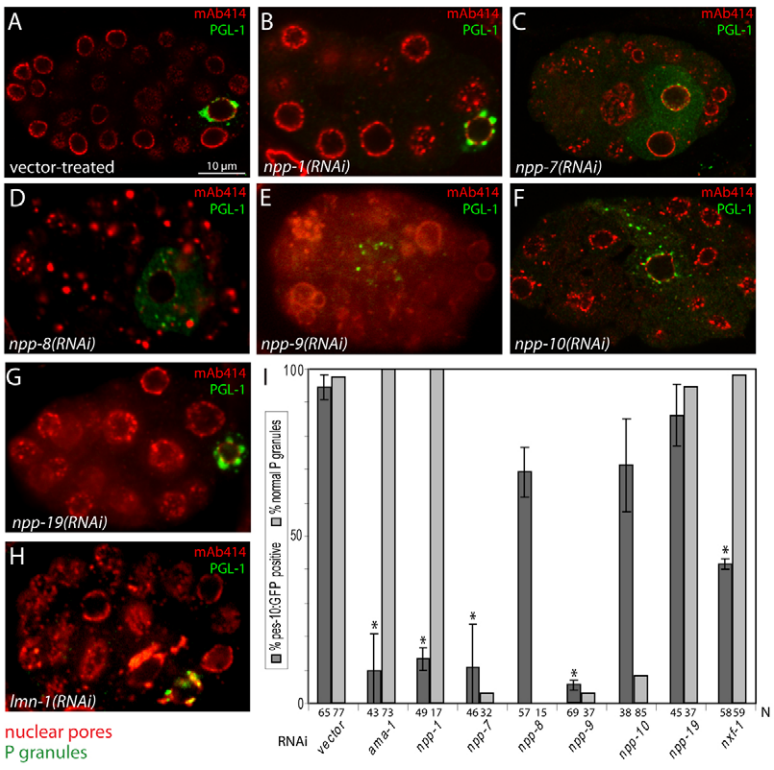


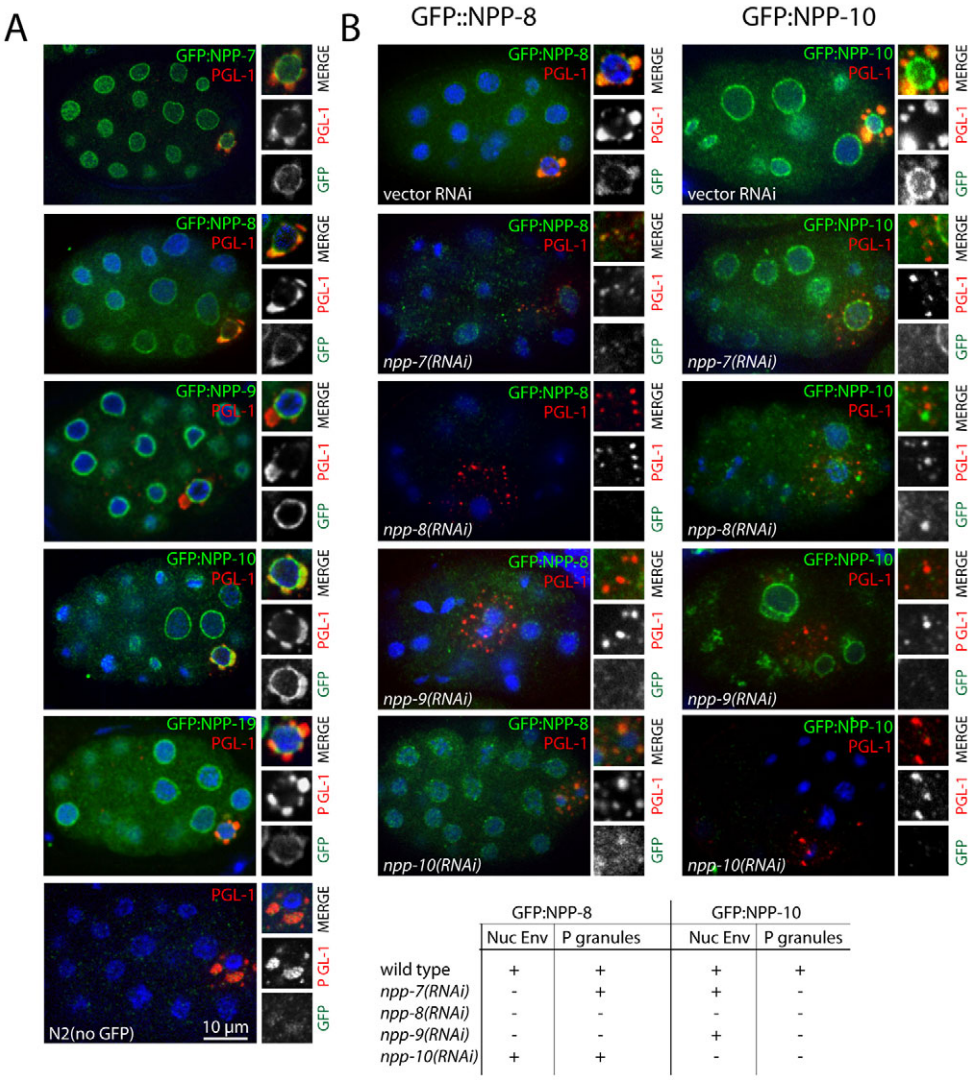
Fig. 1. A subset of *C. elegans* nucleoporins is required for P granule integrity. (**A-H**) Single-plane confocal sections of 13- to 28-cell stage embryos derived from mothers treated with the indicated RNAi, and co-stained with antibodies against nuclear pore proteins (mAb414, red) and PGL-1 (K76, 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 *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 *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 *npp-7*, *-8*, *-9* and *-10* and embryos depleted for *lmn-1*, *npp-1* and *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 *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 *pes-10*::GFP transgene to monitor zygotic gene expression in embryos (Wallenfang and Seydoux, 2002). *npp-8(RNAi)* and *npp-10(RNAi)* showed only a partially penetrant effect on *pes-10*::GFP expression (Fig. 1I). By contrast, *npp-7(RNAi)* and *npp-9(RNAi)* efficiently blocked *pes-10*::GFP expression. *npp-7(RNAi)* and *npp-9(RNAi)* also caused defects in protein import into nuclei, not seen in *npp-8(RNAi)* and *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 *pes-10*::GFP expression, but these treatments had no effect on P granules (Fig. 1I). 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 *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



	GFP:NPP-8		GFP:NPP-10	
	Nuc Env	P granules	Nuc Env	P granules
wild type	+	+	+	+
npp-7(RNAi)	-	+	+	-
npp-8(RNAi)	-	-	-	-
npp-9(RNAi)	-	-	+	-
npp-10(RNAi)	+	+	-	-

Fig. 2. GFP::NPP-10 is enriched in P granules and this localization depends on *npp-7*, *npp-8* and *npp-9*. (**A**) Single confocal plane images of 25- to 30-cell stage *C. elegans* embryos expressing the indicated GFP fusions and double stained with anti-GFP (green) and anti-PGL-1 (red) antibodies. A non-transgenic wild-type (N2) embryo is shown for comparison. (**B**) Same as A, but with the indicated RNAi treatments. Note that GFP::NPP-10 disappears from P granules under all RNAi treatments, whereas GFP::NPP-8 remains associated with dispersed P granules after *npp-7(RNAi)* and *10(RNAi)*. A summary of the results is shown at the bottom.

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.

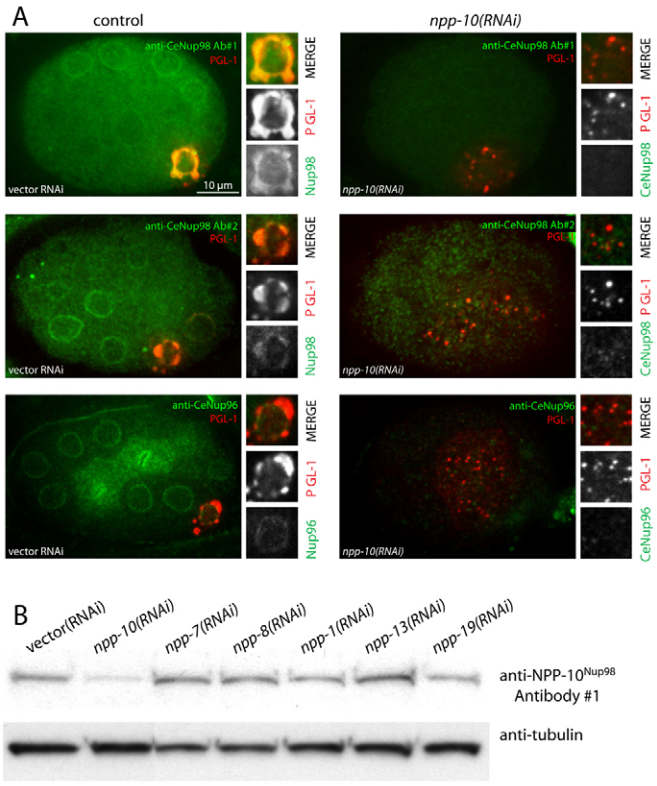


Fig. 3. Endogenous CeNup98 is enriched in P granules in embryos. (**A**) Confocal sections of *C. elegans* embryos co-stained with the indicated anti-nucleoporin antibodies (green) and anti-PGL-1 (red), with and without *npp-10(RNAi)*. *npp-10(RNAi)* eliminates all anti-nucleoporin staining. (**B**) 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^{Nup98}. Western analysis of worm extracts confirmed that GFP::NPP-10^{Nup98} 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 CeNup96 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^{Nup98} 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^{Nup98} 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^{Nup98} 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.

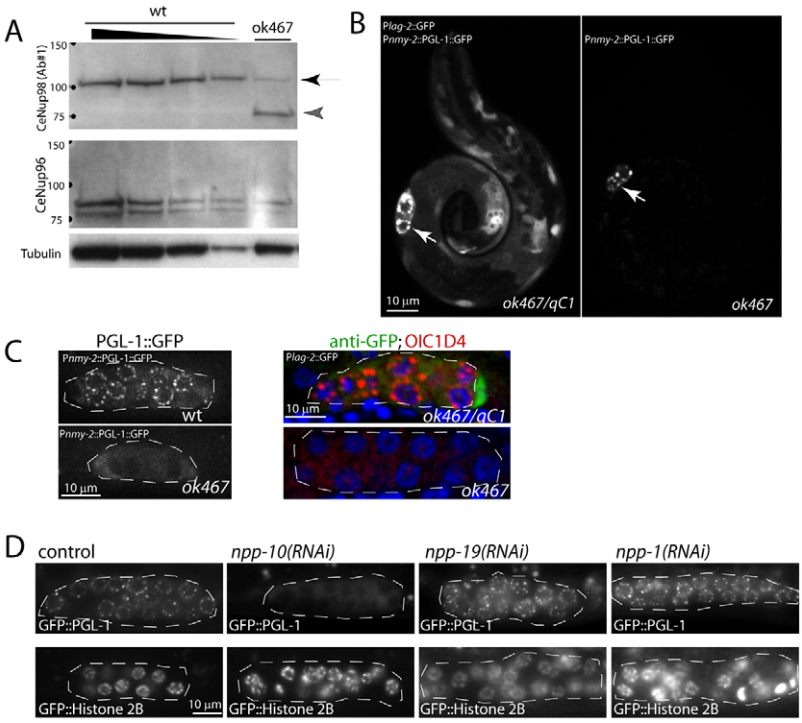


Fig. 4. CeNup98 is required for P granule integrity in larvae. (**A**) Western blot of wild-type and *ok467 C. elegans* larvae extracts probed with anti-CeNup98, anti-CeNup96 and anti-tubulin (loading control) antibodies. Black arrowhead points to full-length CeNup98 and gray arrowhead points to the CeNup98 truncation product. Low levels of full-length CeNup98 are detected in the *ok467* extract, which is likely to be maternal CeNup98. The CeNup96 antibody recognizes a doublet; both bands are eliminated by *npp-10(RNAi)* (Galy et al., 2003). (**B**) Fluorescence micrographs of an *ok467/qC1* L1 larva (expressing *lag-2*::GFP in several somatic cells) and an *ok467/ok467* L1 larva (lacking the *lag-2*::GFP-marked qC1 balancer). Both show the wild-type PGL-1::GFP pattern in the two primordial germ cells (arrow). (**C**) Fluorescence micrographs of L2 gonads of the indicated genotypes expressing PGL-1::GFP, or stained with anti-GFP antibody (green; denoting the presence of the qC1 balancer) and OIC1D4 antibody (red; P granules). (**D**) Fluorescence micrographs of live L2-stage gonads expressing GFP::PGL-1 or GFP::Histone H2B (to highlight nuclei). RNAi treatments were applied by feeding starting at the L1 stage. Note that only *npp-10(RNAi)* disrupts GFP::PGL-1.

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 distribution 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 germline P2 and P3 blastomeres (Gallo et al., 2008). *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^{Nup98} with an anti-GFP antibody (or with a control IgG) and performed real-time qPCR on

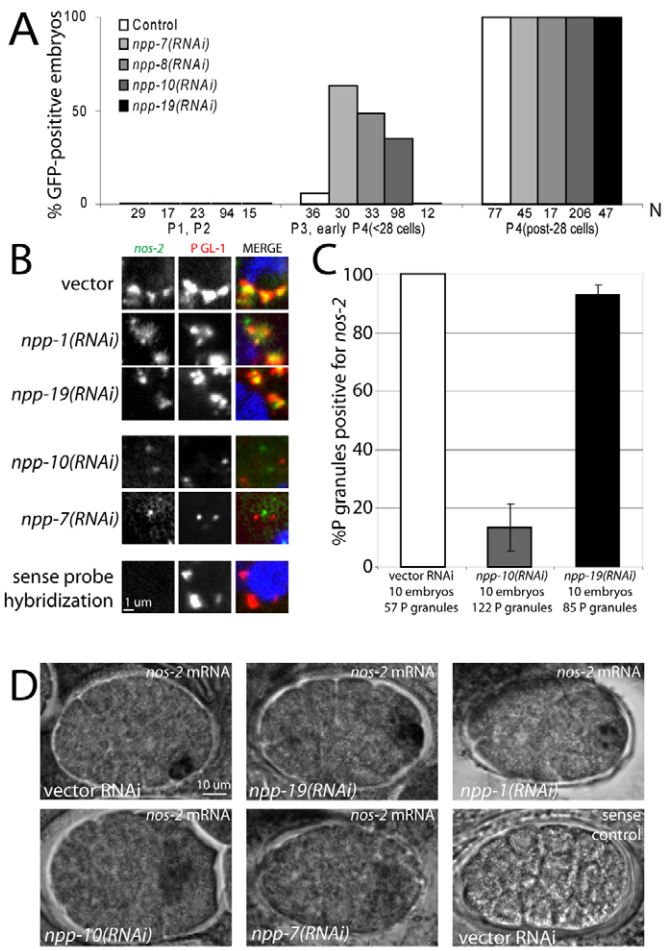


Fig. 5. The P granule-associated mRNA *nos-2* is prematurely translated and released from P granules in *npp-7(RNAi)* and *npp-10(RNAi)* embryos. (**A**) *C. elegans* JH1999 hermaphrodites (GFP::Histone H2B::*nos-2* 3′UTR) were treated with the indicated RNAi for 22 hours and their embryos co-stained for PGL-1, GFP and DNA. The percentage of embryos positive for GFP is reported for each indicated stage (*n* is shown below each bar). We observed two GFP-positive cells in 30% of 12- to 28-cell stage embryos following *npp-8(RNAi)* and a single GFP-positive cell in the remaining positive embryos at the 12- to 28-cell stage. Excess positive cells were observed in 100% of post-28-cell embryos after *npp-7(RNAi)*, *npp-8(RNAi)* and *npp-10(RNAi)*, and in ~30% of embryos after *npp-19(RNAi)*. (**B**) Confocal sections of P3 blastomeres hybridized with *nos-2* probe (green) and immunostained with anti-PGL-1 (red). Co-localization of green and red foci is lost in *npp-10(RNAi)* and *npp-7(RNAi)* embryos. (**C**) The average percentage of P granules positive for *nos-2* mRNA from ten embryos for each RNAi treatment. Bar colors match those in A. Confocal sections of P3 blastomeres co-stained as in B were scanned for P granules. Each P granule was scored as *nos-2* mRNA-positive if the pixel intensity of the green fluorescence (*nos-2* probe) overlapping the granule was above background. Error bars are s.e.m. (**D**) Wild-type embryos treated with the indicated RNAi and hybridized with *nos-2* antisense or sense probes (dark coloration indicates hybridization). Under conditions that do not disrupt P granules [vector, *npp-1(RNAi)*, *npp-19(RNAi)*], the *nos-2* signal is concentrated around the nucleus of one cell (the P blastomere). In *npp-7(RNAi)* and *npp-10(RNAi)* embryos, *nos-2* RNA is diffuse in the cytoplasm and in two cells (presumably the P blastomere and its somatic sister).

the immunoprecipitates. *nos-2* mRNA was enriched an average of 465-fold in the GFP::NPP-10^{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* and *-9*) are required for this localization. Fourth, a deletion mutant that specifically disrupts CeNup98, without affecting the co-translated 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.

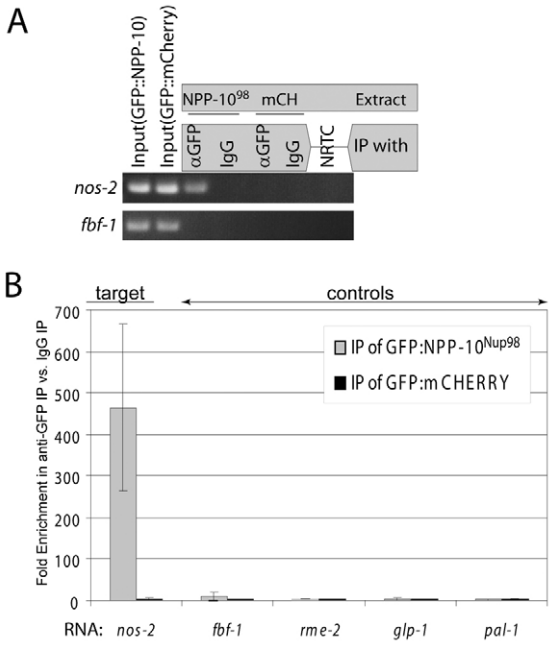


Fig. 6. CeNup98 is in a complex with *nos-2* mRNA. (**A**) *nos-2* and control mRNAs were amplified by RT-PCR in GFP::NPP-10^{Nup98} and control GFP::mCherry immunoprecipitates. *nos-2* mRNA is only detected in the GFP::NPP-10^{Nup98} immunoprecipitates. *fbf-1* is a germline mRNA which, like *nos-2*, 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-10^{Nup98} 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.

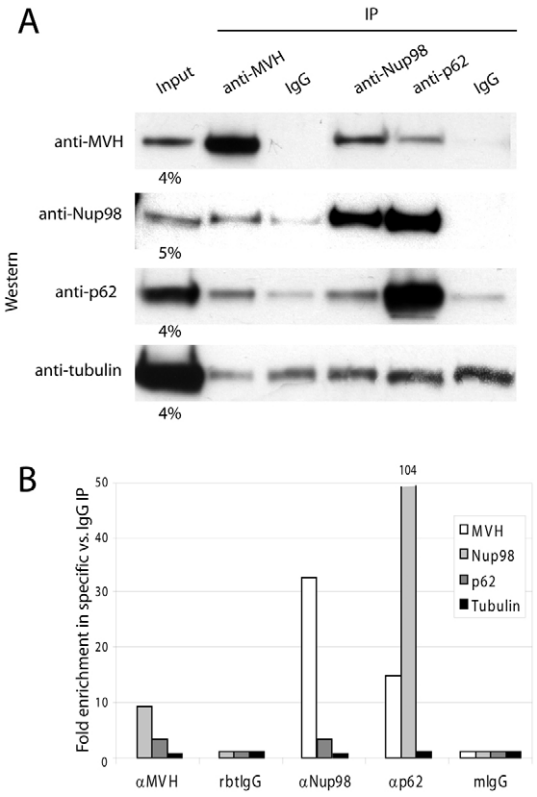


Fig. 7. Mouse Nup98 interacts with the germ granule component MVH in testes. (**A**) Lysates of C57/Bl6 mouse testes were immunoprecipitated using the indicated antibodies (top) followed by western blot analysis. Input lanes contain 4 or 5% of the extract used for immunoprecipitation. (**B**) Quantitation 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.

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

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(RNAi)* embryos still depends on POS-1.

Vertebrate Nup98 interacts with the mRNA export factor RAE1 (Pritchard et al., 1999; Rayala et al., 2004). RNAi depletion of the *C. elegans Rae1* ortholog *npp-17* did not affect P granules (see Table S2 in the supplementary material) (Galy et al., 2003), suggesting that CeNup98 does not function with RAE1 (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

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