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Stress granules form in *Brachionus manjavacas* (Rotifera) in response to a variety of stressors

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ABSTRACT

Many eukaryotes share a common response to environmental stresses. The responses include reorganization of cellular organelles and proteins. Similar stress responses between divergent species suggest that these protective mechanisms may have evolved early and been retained from the earliest eukaryotic ancestors. Many eukaryotic cells have the capacity to sequester proteins and mRNAs into transient stress granules (SGs) that protect most cellular mRNAs (Anderson and Kedersha, 2008). Our observations extend the phylogenetic range of SGs from trypanosomatids, insects, yeast and mammalian cells, where they were first described, to a species of the lophotrochozoan animal phylum Rotifera. We focus on the distribution of three proteins known to be associated with both ribosomes and SG formation: eukaryotic initiation factors eIF3B, eIF4E and T-cell-restricted intracellular antigen 1. We found that these three proteins co-localize to SGs in rotifers in response to temperature stress, osmotic stress and nutrient deprivation as has been described in other eukaryotes. We have also found that the large ribosomal subunit fails to localize to the SGs in rotifers. Furthermore, the SGs in rotifers disperse once the environmental stress is removed as demonstrated in yeast and mammalian cells. These results are consistent with SG formation in trypanosomatids, insects, yeast and mammalian cells, further supporting the presence of this protective mechanism early in the evolution of eukaryotes.

1. Introduction

Posttranscriptional control of gene expression is crucial for cell development, differentiation, immune signaling and neuronal plasticity (Richter, 2007; Anderson and Kedersha, 2008; Thomas et al., 2011). Translational repression is associated with RNA aggregation into granules, which have been identified in various eukaryotic cells (Anderson and Kedersha, 2006). Diverse types of RNA granules have been observed, including neuronal RNA granules, germ cell granules (GCGs), processing bodies (PBs) and stress granules (SGs) (Kedersha and Anderson, 2002; Navarro and Blackwell, 2005; Anderson and Kedersha, 2006; Kedersha and Anderson, 2007; Anderson and Kedersha, 2008, 2009b). These RNA granules are all functional by-products of mRNA metabolism that perform distinct functions.

Germ cell granules (GCGs) are polarized cytoplasmic aggregates. During embryogenesis, many maternal mRNAs are translationally repressed and transported to specific locations within the oocytes (Anderson and Kedersha, 2006). GCGs define sites of germ cell differentiation within organisms. Analogous GCGs have been found in multiple insect species as well as, *Xenopus laevis*, *Drosophilia melanogaster* and *Caenorhabditis elegans* (Schisa et al., 2001). The GCGs contain maternal mRNA required for germ cell specification and direct the timing of maternal mRNA translation. GCGs also contain proteins involved in translation initiation, translation control and mRNA decay (Anderson and Kedersha, 2006). Other RNA granules have also been found within GCGs (Navarro and Blackwell, 2005; Anderson and Kedersha, 2006).

PBs are cytoplasmic aggregates that contain RNA silencing or RNA decay machinery (Bashkirov et al., 1997; Ingelfinger et al., 2002; van Dijk et al., 2002; Sheth and Parker, 2003; Brengues et al., 2005; Teixeira et al., 2005; Eulalio et al., 2007; Franks and Lykke-Andersen, 2007, 2008; Buchan and Parker, 2009). PBs are constitutively expressed in cells and can be further induced upon exposure to a variety of stressors (Brengues et al., 2005; Kedersha et al., 2005; Loschi et al., 2009). PBs have been observed in yeast, plants, insects, nematodes, vertebrates and trypanosomatids (Franks and Lykke-Andersen, 2008; Buchan and Parker, 2009). PBs and SGs share some components even though they are very distinct structures.

Eukaryotic cells commonly respond to environmental stresses by altering their translational machinery and forming stress granules

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(SGs) (Kedersha and Anderson, 2002). Environmental stress prevents translational initiation, leading to the formation of unproductive and translationally silenced pre-initiation small subunits complexed with mRNAs (Anderson and Kedersha, 2002b, 2006, 2008). The pre-initiation small subunits aggregate to form microscopically visible cytoplasmic structures called SGs. Stalled small ribosomal subunits are found in the SGs along with mRNAs bound to early initiation factors (eIF4E, eIF3, eIF4A, eIFG), but not large ribosomal subunits (Table 1) (Kedersha and Anderson, 2007). SGs may also contain RNA binding proteins that regulate mRNA translation and decay such as T-cell-restricted intercellular antigen 1 (TIA-1), and other proteins involved in RNA metabolism (Kedersha and Anderson, 2007).

SGs appear only under stress conditions, and their presence correlates with translational silencing (Kedersha et al., 1999, 2000, 2002; Kimball et al., 2003; Anderson and Kedersha, 2008; Cassola, 2011). SGs are transient and may only be present for a limited period of time following exposure to stress (Anderson and Kedersha, 2008). In heat-shocked plants, SGs are believed to sequester mRNAs, transiently preventing their translation until homeostasis is restored (Nover et al., 1989).

SGs have been found in yeast, insects, trypanosomatids and mammalian cell lines (Farny et al., 2009; Thomas et al., 2011). Although they were first discovered within the cytoplasm of heat stressed cultured tomato cells (Nover et al., 1989), SGs have also been observed within mammalian cells upon environmental stress such heat, hyperosmolarity, oxidative conditions and UV irradiation (Anderson and Kedersha, 2002b; Kedersha and Anderson, 2002). It is not known how widely distributed SGs are among animals, especially invertebrates, and whether this cell protective mechanism is a general stress response or a specialized process of a few groups.

Invertebrate animals from the phylum Rotifera can sustain growth and reproduction over a wide range of environmental conditions. However, exposure to high temperature, low pH and high solute concentrations induce a generalized stress response. Brachinoid rotifers possess a full suite of heat shock genes and mobilize them in conventional ways upon exposure to high temperatures (Denekamp et al., 2009; Smith et al., 2012). Brachinoid rotifers also possess a suite of late embryo abundant (LEA) genes that assist in the osmotic adjustments during their life cycle (Denekamp et al., 2010). It is not known whether formation of SGs is part of the rotifer response to stress and whether SG formation confers resistance to future stressors.

Here, we aimed to elucidate mechanisms employed by the rotifer *Brachionus manjavacas* in response to specific, environmental challenges using fluorescence confocal microscopy and pharmacological approaches. We focus on the distribution of three proteins known to be associated with both ribosomes and SG formation: translation factors eIF3B, eIF4E and TIA-1. Our results indicate that SGs are part of the stress response repertoire of *B. manjavacas*. These observations extend the phylogenetic range of SGs to lophotrochozoan invertebrates and suggest that SG formation is a common element of the stress response of animals.

2. Materials and methods

2.1. Rotifer species and culturing

We used *B. manjavacas* (Fontaneto et al., 2007) as test animals in this study. *B. manjavacas* was originally collected from Azov Sea and previously known as *Brachionus plicatilis* (Rico-Martínez and Snell, 1997; Snell et al., 2006). *B. manjavacas* has been cultured continuously in the Snell laboratory since 1983, with periodic collection and storage of resting eggs (Stout et al., 2010). Rotifer neonates are hatched from resting eggs in 15 ppt artificial seawater (ASW, prepared from Instant Ocean salts) under constant fluorescent illumination at 25 °C in all of the following experiments.

2.2. Assessing survival of neonates during periods of environmental stress

Neonates were subjected to one of three environmental stressors within three hours of hatching to determine the effect on survivorship of the rotifers. The stressors are (1) osmotic stress, (2) heat stress or (3) nutrient deprivation. Rotifers were judged dead by immobility and lack of any ciliary or internal movements for 3 s of observation as consistent with the other studies (Snell et al., 1991; Jones et al., 2012). Twenty technical replicates were used for each of the stressors, and all survival experiments were conducted with six biological replicates.

2.3. Temperature stress

In heat stress experiments, 50 neonates in 150-μl ASW were placed in 500-μl thin-walled PCR tubes. The incubation temperature of the neonates was shifted from 22 °C to 40 °C in a PCR thermocycler for 0, 5, 10, 20, 40, 80 or 160 min. After heating, the temperature was shifted back to 22 °C for 24 h of recovery, after which the neonates were assessed for survival. In a second experiment, neonates were incubated with translation-inhibiting antibiotics to assess their ability to inhibit the formation of SGs. The rotifers were incubated in 30 μM of either cycloheximide or puromycin for 30 min prior to heat stressing the animals.

2.4. Osmotic stress

To assess survival after osmotic stress, neonates were hatched in 15 ppt and shifted to 15-ppt (control), 25-ppt, 35-ppt or 45-ppt ASW for 1 h. After the osmotic stress, the neonates were transferred back to 15-ppt ASW and were allowed to recover for 24 h and then assessed for survival.

Table 1
Selected eukaryotic initiation factors.

Initiation of translation is promoted by a number of proteins called eukaryotic initiation factors. The form a complex with the ribosome and promote scanning of mRNA. The functions of the initiation factors essential for SG formation are provided.

Multi-complexed eukaryotic initiation factors	Subunit	Function	Reference
eIF4	eIF4A	A DEAD box RNA helicase important for resolving mRNA secondary structures.	Cheng and Gallie (2006)
	eIF4B	Interacts with the 18S portion of the small ribosomal subunit and interacts non-specifically with mRNA. Acts as an anchor and is a critical cofactor for eIF4A.	Cheng and Gallie (2006)
	eIF4E	Recognizes and binds to the 5′ cap structure on mRNA.	Anderson and Kedersha (2009a)
	eIF4G	Interacts with both eIF4A and eIF4E and enhances cap binding to eIF4E.	Craig et al. (1998)
eIF3		Serves as an adaptor between eIF2, eIF4G and the small ribosomal subunit. Facilitates initiation and stabilization of the closed loop of polysomal mRNA.	Anderson and Kedersha (2009a)
eIF2	eIF2a	A GTP binding protein responsible for bringing the initiator tRNA to the P-site of the pre-initiation complex.	Rajesh et al. (2008)
	eIF2b	Catalyzes the exchange of GDP for GTP, which recycles the eIF2 complex for another round of initiation.	Rajesh et al. (2008)
	eIF2g	Required to turn off protein synthesis globally. Its phosphorylation sequesters eIF2B.	Hasenohrl et al. (2008)

2.5. Nutrient deprivation

The response of neonates to nutrient deprivation was determined by incubating the neonates without food for 24, 48, 72 and 96 h, after which survival was assessed.

2.6. Analysis of protein accumulation

2.6.1. Protein identification

We have examined three proteins normally associated with SGs in mammals: eIF3B, eIF4E and TIA-1 (Higgins et al., 1992; Thompson et al., 1994). Commercial antibodies available for these mammalian proteins are expected to cross-react with rotifer proteins, depending on sequence similarity. Rotifer protein sequences were compared to their mammalian counterparts with ClustalW (Supplementary Figs. 1–and 3) (Higgins et al., 1992; Thompson et al., 1994). Commercial antibodies raised against regions of high rotifer/mammalian sequence similarity were purchased from Santa Cruz Biotechnology (Santa Cruz, California). An eIF3B mouse monoclonal antibody contains a specific epitope mapping between amino acids 215 and 242 near the C-terminus of the eIF3B of human origin (catalog number sc-271539). A rabbit polyclonal TIA-1/TIAR antibody contains an epitope corresponding to the amino acids 21–140, mapping near the N-terminus of the TIA-1 of human origin (catalog number sc-28237). The eIF4E goat polyclonal antibody contains an epitope analogous to the C-terminus of eIF4E of human origin (catalog number sc-6968). Fluorescently tagged secondary antibodies specific for each of the primary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). A donkey anti-mouse antibody conjugated to Alexa Fluor 647 (code number 715-605-150) binds specifically to the eIF3B primary antibody. The donkey anti-rabbit antibody conjugated to Cy3 (code number 711-166-152) binds specifically to the TIA-1 primary antibody. The donkey anti-goat antibody conjugated to Alexa Fluor 488 (code number 705-545-147) binds specifically to the eIF4E primary antibody.

2.6.2. Immunohistochemistry and confocal microscopy

Fifty newly hatched female rotifers were transferred into 2-ml ASW to follow protein localization during periods of stress. The neonates were anesthetized with club soda and immediately fixed after stress exposure by adding 20% formalin directly to the rotifers with final concentration of 4%. They were incubated at room temperature for 10 min. The fixed neonates were separated from the formalin and ASW mixture by centrifugation and aspiration of the supernatant. The rotifers were then rinsed with PBS (130 mM NaCl, 10 mM NaH_2PO_4, pH 7.2) and blocked for 1 h with 5% donkey serum to reduce non-specific antibody binding. The donkey serum was removed following incubation, and the rotifers were incubated in phosphate buffer saline with Tween 20 [PBT: 130 mM NaCl, 10 mM NaH_2PO_4, pH 7.2, 0.5% (v/v) Tween 20] for 10 min. The rotifers were then incubated with the appropriate primary antibody (against eIF3B, eIF4E or TIA-1) (1:50 in PBS). After incubation for 4 h at room temperature, the primary antibody binding solution was removed and the rotifers were washed three times with the PBS solution. The rotifers were then exposed to the appropriate secondary antibody.

The rotifers were then transferred to a Nunc Lab-Tek chambered coverglass, 8-well microscope slide from Thermo Scientific (catalog number 12-565-470) for imaging. Twenty replicates were assessed for each experimental group. The rotifers were imaged using a Zeiss LSM 700-405 confocal microscope with an oil immersion objective lens magnification of 63×. An argon laser at 488 nm was used to excite Alexa Fluor 488. Emissions were collected using a 505- to 550-nm band-pass filter. A helium-neon laser was used to excite Cy3 at 543 nm, with emission collected using a band-pass 560–615 nm. Alexa Fluor 647 was excited at 633 nm and detected from 650 to 710 nm. Excitation wavelengths were 350 nm for Hoechst; emission wavelengths were 400–460 nm for Hoechst. The emission spectra of several of the fluorophores overlap. We kept the signals of the dyes separate by collecting the images from the fluorophores with overlapping spectra sequentially instead of simultaneously. Images were taken under multi-track mode. Images from fluorophores in the same track were taken simultaneously. The first track consisted of the Hoechst and Cy3 readings. The second track was collected after the first track. The second track consisted of Alexa Fluors 488 and 647. To prevent autofluorescence when imaging, the laser power and gains were set to a level to ensure that there was no

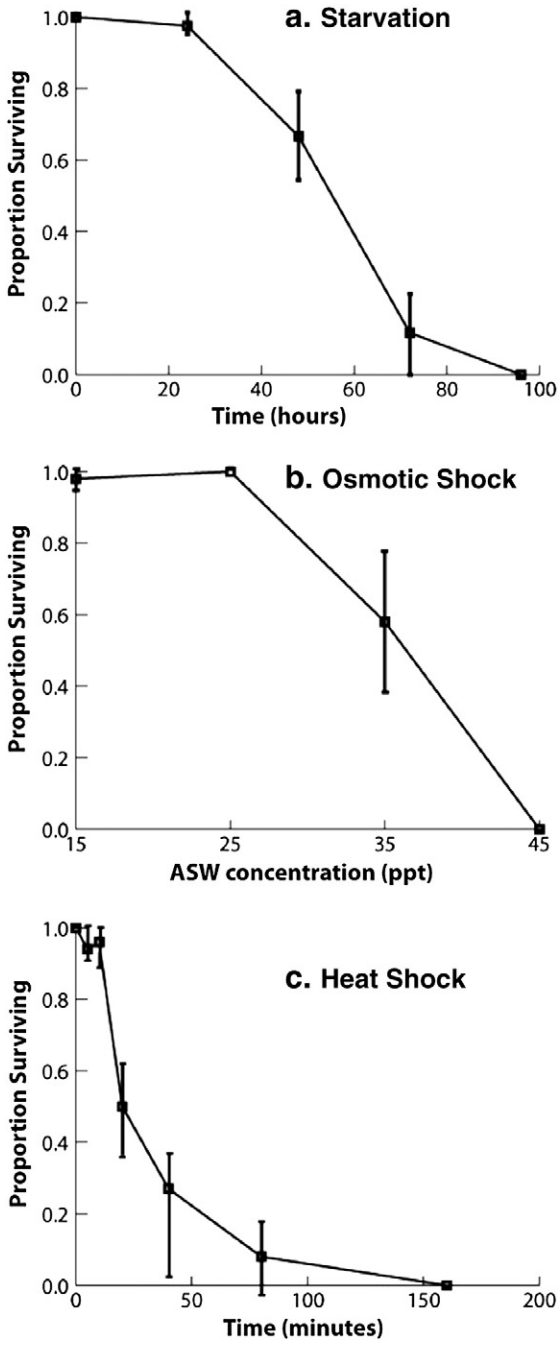
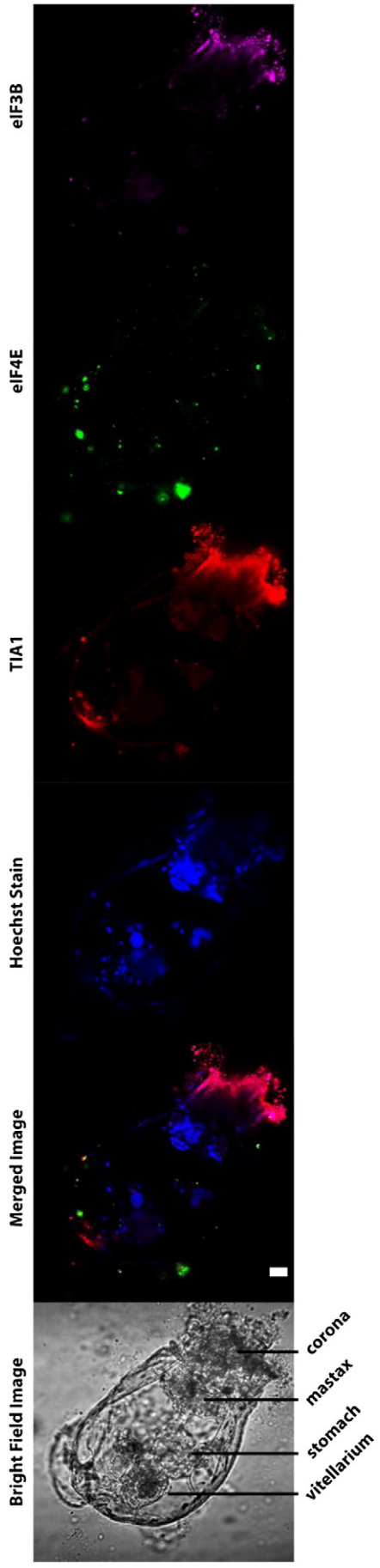


Fig. 1. *Brachionus manjavacas* neonate survival after environmental stress. (a) *B. manjavacas* neonates were starved for 24, 48, 72 or 96 h and the proportion of animals surviving at each time point was determined. (b) Neonates were exposed to 15 ppt (control), 25 ppt, 35 ppt and 45-ppt ASW for 1 h, returned to 15 ppt for 24 h, and the proportion surviving was determined. (c) Neonates were heat stressed at 40 °C for 0, 5, 10, 20, 40, 80 or 160 min, returned to 22 °C for 24 h and the proportion surviving was determined. These experiments identify three possible environmental stresses that induce stress granule (SG) formation.



fluorescence detected in our negative control samples (rotifers incubated with secondary antibodies only and not with primary antibodies).

For co-localization analysis, the voxel dimensions were set according to the Nyquist criteria (Scriven et al., 2008). Calculations were made using an online Nyquist calculator (www.svi.nl/NyquistCalculator). The *x*, *y* and *z* dimensions of the pixel sizes were calculated for 488-nm excitation wavelength as $x = 43$ nm, $y = 43$ nm and $z = 130$ nm; zoom (confocal) 3.1. The calculated *x*, *y* and *z* dimensions for the 547-nm excitation wavelength were $x = 490$ nm, $y = 490$ nm and $z = 146$ nm. The calculated *x*, *y* and *z* dimension s for the 647-nm excitation wavelength were $x = 58$ nm, $y = 58$ nm and $z = 174$ nm; zoom (confocal) = 2.4. The pinhole size was 1 Airy unit.

Fiji software was used to analyze the confocal pictures (http://fiji.sc/Fiji). The quantitative co-localization analyses were also performed with Fiji software using the Coloc_2 program. Our co-localization experiments were performed using the red and green signals. The Coloc_2 plug-in calculates multiple coefficients of co-localization, including Pearson's and Manders' coefficients. We present Manders' coefficients (R_r) in this work. R_r describes the extent of overlap between image pairs. The R_r value is between 0 and 1, with 0 being no overlap and 1 being perfect overlap of the 2 images (Manders' et al., 1992, 1993).

3. Results

3.1. Stress response of rotifer neonates when subjected to heat stress, osmotic stress and nutrient deprivation

Of the neonates, 100% were able to survive 24 h of nutrient deprivation, 70% survived 48 h without feeding, only 15% survived 72 h and none survived 96 h of nutrient deprivation (Fig. 1a). Neonates incubated in 15 and 25 ppt exhibited 100% survival after 24 h. Only about 60% of the neonates survived exposure to 35 ppt, and none of the neonates survived incubation in 45-ppt ASW (Fig. 1b). Heat-shocked neonates exhibited a drastic decline in survival after 20 min at 40 °C. Only about 10% of the neonates were able to survive 80 min at 40 °C, and no neonates were able to survive 40 °C heat stress for 160 min (Fig. 1c).

Having demonstrated the impact of our stress treatments on rotifer survival, we followed cellular responses to these treatments. We examined the intracellular distribution of three proteins associated with stress granules, eIF3B, eIF4E and TIA-1, in response to each environmental stressor. We used immunohistochemistry and confocal microscopy to follow the localization of the three proteins within the cells of stressed rotifers (Fig. 2).

Neonates were fixed immediately after nutrient deprivation at 0, 24, 48 or 72 h and probed with antibodies for localization of eIF4E (green), eIF3B (magenta) and TIA-1 (red). Nutrient deprivation led to obvious aggregation and co-localization of TIA-1, eIF4E and eIF3B into particles resembling previous descriptions of stress granules. Manders' coefficient for co-localization of the proteins was used for statistical analysis. It yielded a value of 0.349 in the control group and peaked at 0.728 at 24 h of nutrient deprivation. This was followed by a decrease in co-localization at 48 h. The Manders' coefficient dropped to 0.338 at 48 h (Fig. 3). A Manders' coefficient of 1 would indicate complete co-localization of the proteins, while a 0 indicates no localization.

For osmotic stress, neonates were transferred from 15-ppt ASW to 15-ppt (control), 25-ppt, 35-ppt or 45-ppt ASW for 1 h and changes

Fig. 2. Microscopy images from *Brachionus manjavacas* immunohistochemistry. This is a single slice of a confocal image illustrating the visualization of proteins and DNA using fluorescent tags. The neonates were probed with antibodies specific for TIA-1 (red), eIF4E (green) and eIF3B (magenta). Nuclei were stained using Hoechst (blue). The bottom panel is a bright-field microscopic image showing *B. manjavacas* neonates. This image illustrates the localization of the probed proteins and DNA within neonate tissues. The scale bar is 10 μm.

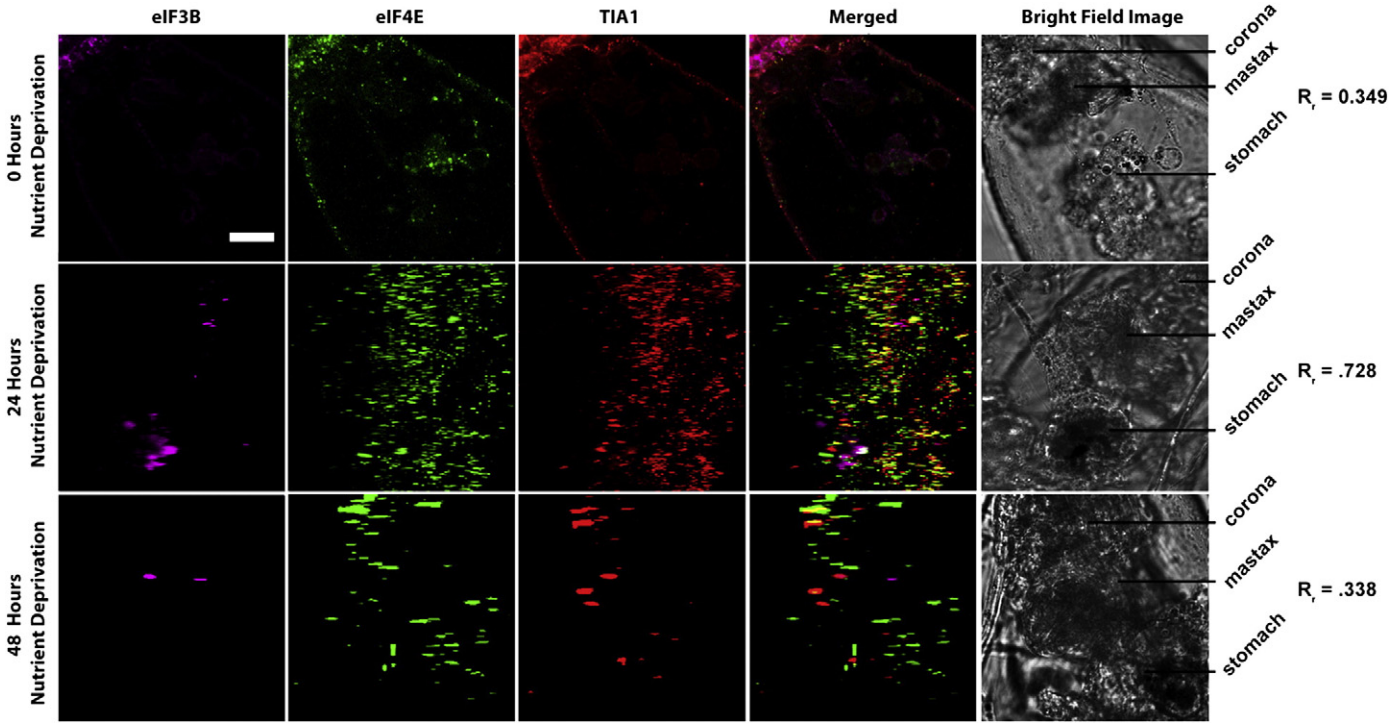


Fig. 3. eIF4E, eIF3B and TIA-1 are recruited to SGs in response to nutrient deprivation. *Brachionus manjavacas* neonates were deprived of nutrients for 0 h (control), 24 h and 48 h. They were fixed and probed with antibodies specific for eIF4E (green), eIF3B (magenta) and TIA-1 (red). The probed neonates were visualized using immunohistochemistry. The bright-field microscopy image (BF) is provided to illustrate the localization of the proteins within the organs of the rotifers. Manders' correlation coefficient (R_r) shows that the proteins increasingly co-localize with increased time period of starvation, suggesting the formation of SGs. There is also a decrease in co-localization seen with extended durations of nutrient deprivation. The scale bar is 20 μm.

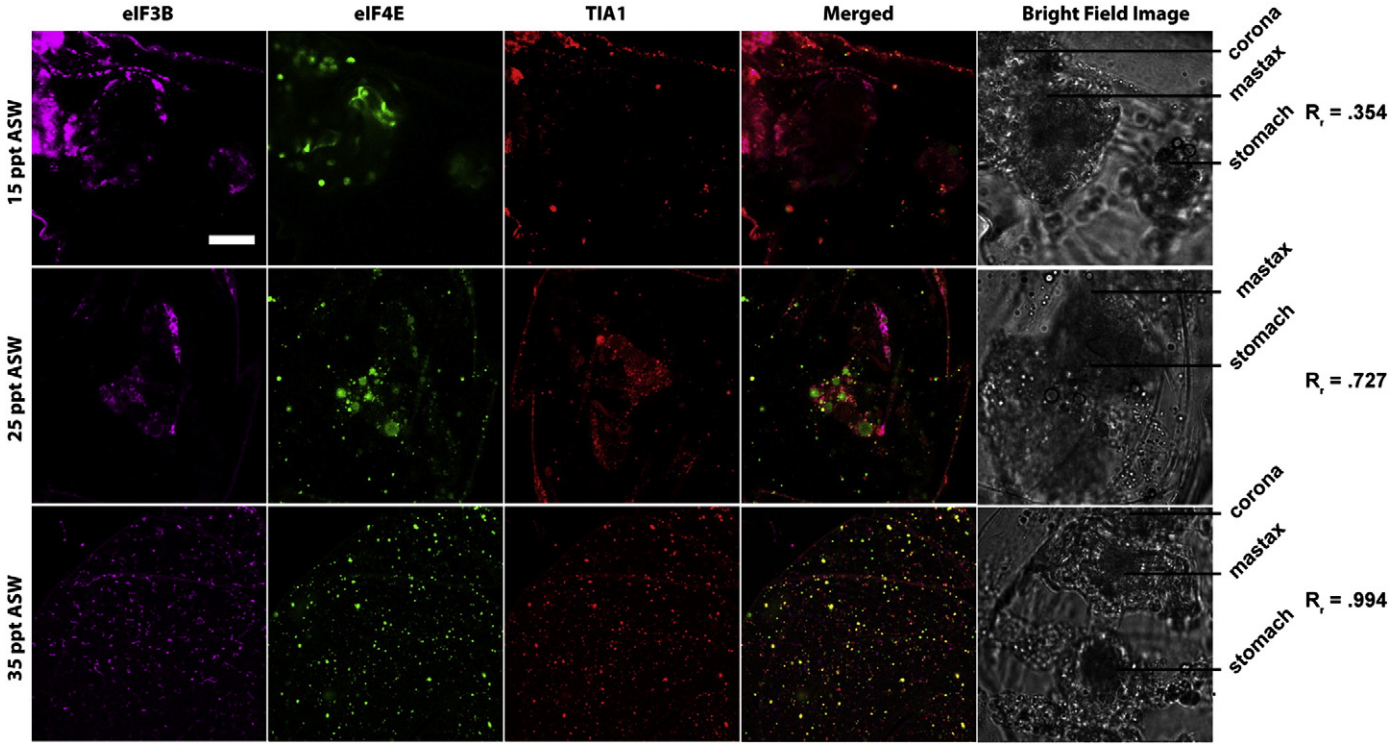


Fig. 4. eIF4E, eIF3B and TIA-1 are recruited to SGs in response to osmotic stress. *Brachionus manjavacas* neonates were incubated in 15 ppt (control), 25 ppt or 35-ppt ASW for 1 h and fixed. The neonates were probed with antibodies specific for eIF4E (green), eIF3B (magenta) and TIA-1 (red). The fluorescently tagged macromolecules were visualized using immunohistochemistry. The bright field (BF) is provided to illustrate the localization of the proteins within the organs of the rotifers. The proteins are more uniformly distributed throughout the cells in the control group (15-ppt ASW) and become co-localized with increasing osmotic shock (35-ppt ASW), indicating the formation of SGs. R_r value confirms the proteins co-localize with increased osmotic shock. The co-localization of these proteins provides further evidence for formation of SGs. The scale bar is 20 μm.

in localization of the three proteins was assessed. After treatment, the neonates were immediately fixed and probed with antibodies. Osmotic stress led to co-localization of eIF4E, TIA-1 and eIF3B within the corona, mastax, stomach and vitellarium of the rotifers. Manders' coefficient of co-localization yielded a value of 0.354 in the control (15-ppt ASW), which increased to 0.994 in the 35-ppt sample (Fig. 4).

In the third experiment, neonates were heat stressed at 40 °C for 0, 5, 10 or 20 min and immediately fixed and immunostained with antibodies. Heat stress resulted in the co-localization of TIA-1, eIF3B and eIF4E in the major organ systems of the neonates (Fig. 5). The Manders' coefficient was 0.02 in the control group (0 min heat stress) and increased to 1.00 in the rotifers subjected to 20 min of heat stress. The protein aggregation dissipated after recovery from heat stress for 3 h at 22 °C. The Manders' coefficient was 0.289 in the control group; it increased to 0.734 after 30 min of heat stress and dropped back down to 0.375 with 3 h of removal from the heat stress (Fig. 6).

3.2. The large ribosomal subunit does not co-localize to SGs

We probed the heat stressed and fixed neonates with C-puromycin conjugated to fluorescein isothiocyanate (FITC) to discern the location of the large ribosomal subunit. C-puromycin binds to the A site of the large ribosomal subunit (Luhrmann et al., 1981; Gilly and Pellegrini, 1985; Starck et al., 2004). Incubation with the fluorescently labeled C-puromycin enabled us to visualize the location of the large ribosomal subunit. The neonates were heat stressed at 40 °C for 0, 10 or 30 min. They were then anesthetized, fixed and probed with C-puromycin-FITC, eIF3B and TIA-1 antibodies. Antibodies binding to eIF4E were not used for this experiment because Alexa Fluor 488 has a very similar excitation and emission spectra as FITC. The large ribosomal subunits aggregated when subjected to heat stress; however, they did not co-localize with eIF3B and TIA-1 proteins of the small ribosomal subunit. The calculated Manders' coefficient for the co-localization of the large ribosomal subunit with eIF3B was 0.781 in the control group and decreased to 0.348 in the group subjected to heat stress (Fig. 7).

3.3. SGs respond to the presence of both puromycin and cycloheximide

Next, we tested the ability of the TIA-1, eIF3B and eIF4E proteins to aggregate in the presence of two antibiotics, cycloheximide and puromycin. Cycloheximide has been demonstrated to prevent stress granule formation in both yeast and mammalian cells, while puromycin promotes stress granule formation in both yeast and mammalian cells (Kedersha et al., 2000; Anderson and Kedersha, 2002a, 2008; Grousl et al., 2009; Cassola, 2011). These proteins aggregated in the presence of puromycin but failed to aggregate in the presence of cycloheximide. The Manders' coefficient for co-localization began at

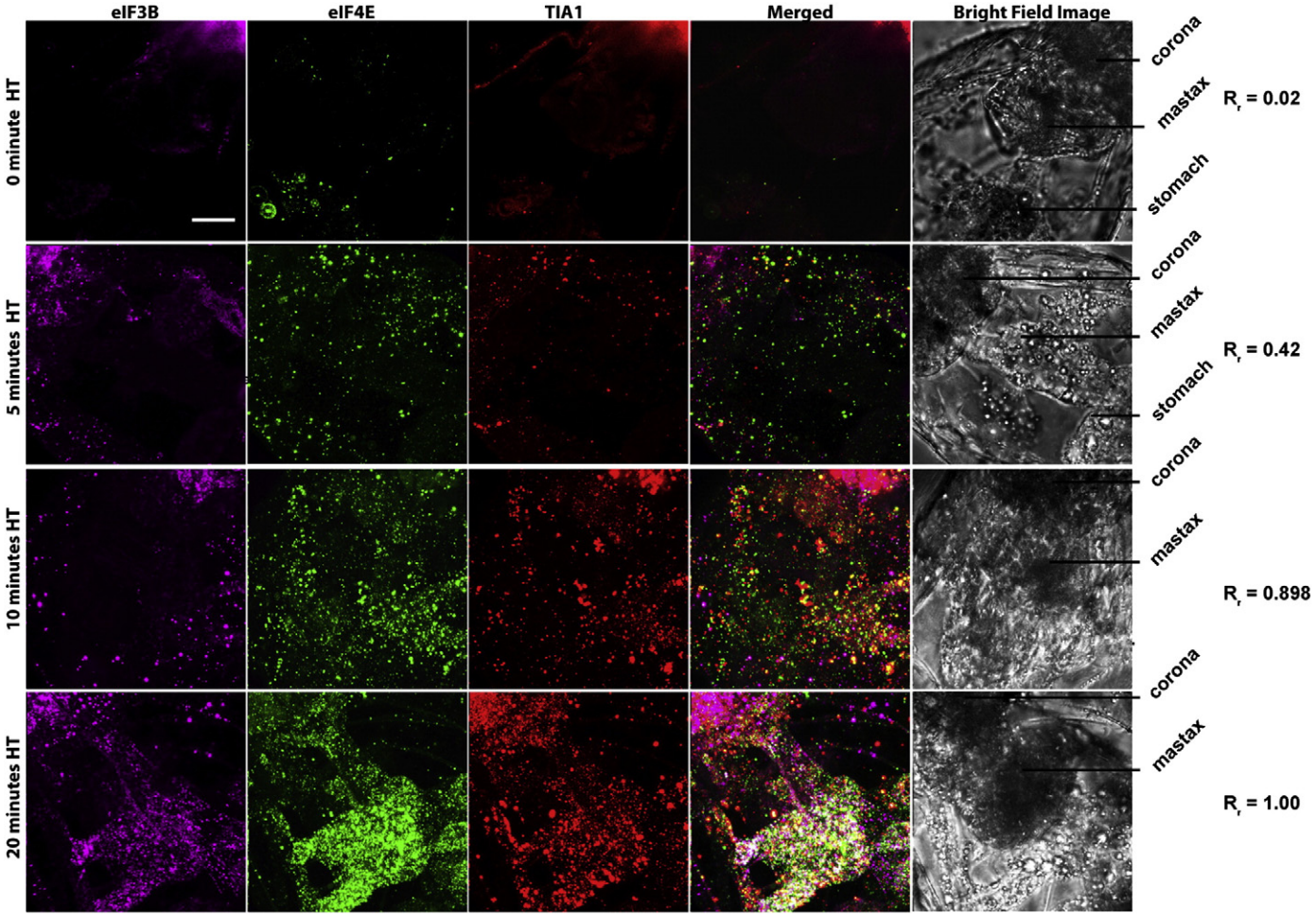


Fig. 5. eIF4E, eIF3B and TIA-1 are recruited to SGs in response to heat stress. *Brachionus manjavacas* neonates were incubated at 40 °C for 0, 5, 10 and 20 min. The organisms were immediately fixed and probed with antibodies specific for eIF4E (green), eIF3B (magenta) and TIA-1 (red). The bright field (BF) is provided to illustrate the localization of the proteins within the organs of the rotifers. The localization of the probed proteins shifts from being more uniformly distributed throughout the neonates to a significant amount of co-localization within major organs. The R_r values calculated using Manders' coefficient confirms the co-localization of the proteins. The data indicate the formation of SGs within the organs in response to heat stress. The scale bar is 20 μm.

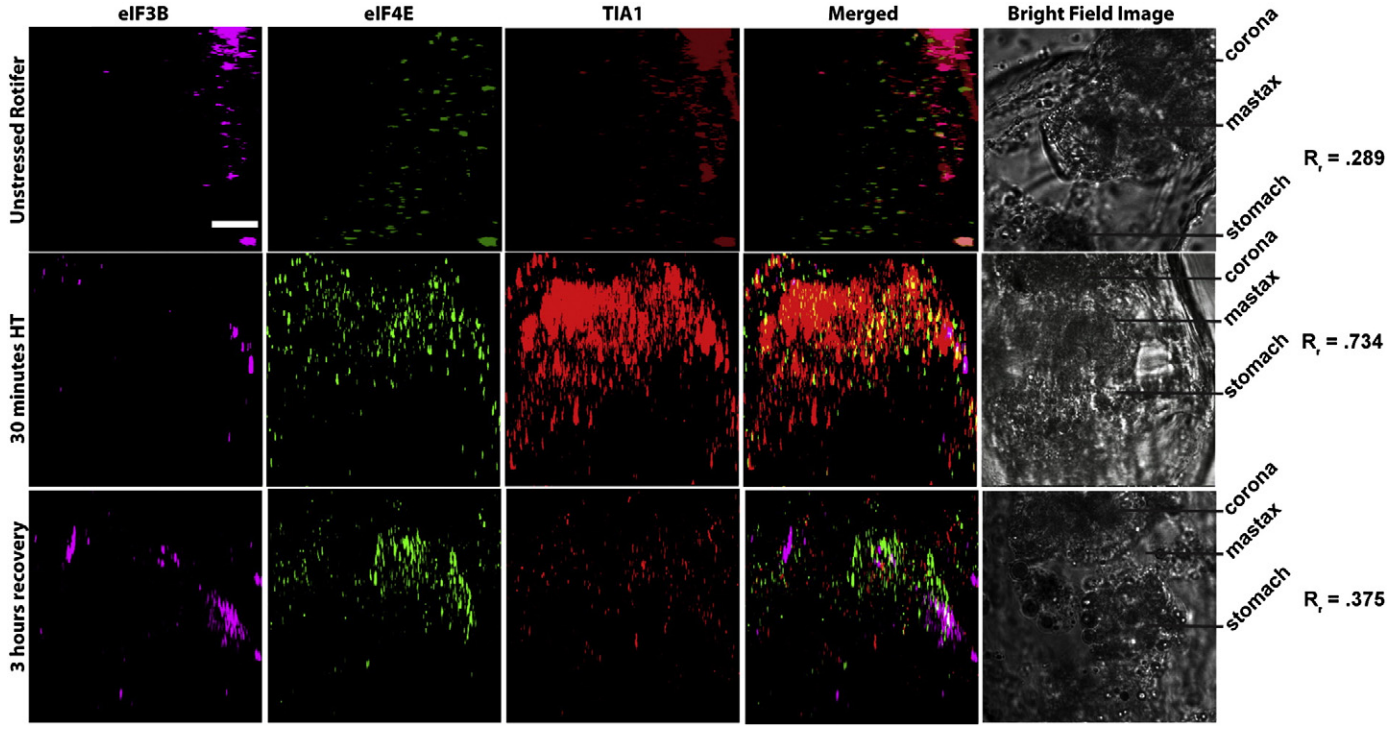


Fig. 6. The SGs disperse after removal of the organism from the environmental stress. Neonates were heated at 40 °C for 30 min then were allowed to recover for 3 h at room temperature (22 °C). Each experimental group was immediately fixed and probed with antibodies specific for eIF4E (green), eIF3B (magenta) and TIA-1 (red). The cells were visualized using confocal microscopy. The proteins co-localize at the beginning of the environmental stress and disperse after its removal. The Manders' coefficient (R_r) increases with the initial environmental stress; however, it decreases when the stress is removed. These data suggest that SG formation within neonates is a dynamic response to environmental stress. The scale bar is 20 μm.

0.358 in the control group. The Manders' coefficient increased to 0.992 in animals incubated in the presence of puromycin (Fig. 8).

4. Discussion

We have analyzed the impact of environmental stresses on *B. manjavacas* neonates. We used fluorescent confocal microscopy to demonstrate for the first time in the phylum Rotifera that mammalian-like SGs form in response to heat stress, osmotic stress and nutrient deprivation. This extension of stress granule formation to lophotrochozoan invertebrates suggests that it may be part of a generalized stress response in most animals.

Brachinoid rotifers are adapted to environments where their growth is episodically restricted by harsh environments (Denekamp et al.,

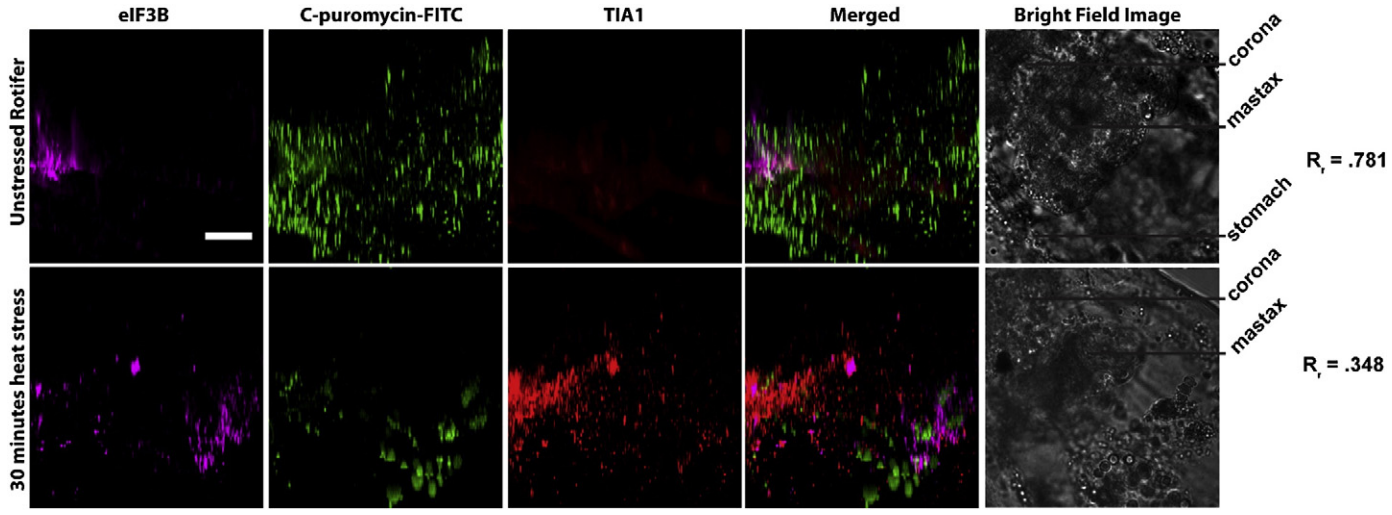


Fig. 7. The large ribosomal subunit does not localize to SGs. Neonates were heated at 40 °C for 0 and 30 min. The neonates were immediately removed from the heat stress, fixed and probed with antibodies specific for eIF3B (magenta) and TIA-1 (red). Puromycin conjugated to fluorescein isothiocyanate (FITC, green) was used to probe for the large ribosomal subunit. The cells were visualized using confocal microscopy. eIF3B and TIA-1 co-localize upon exposure to environmental stress, suggesting the formation of SGs. In contrast, the large ribosomal subunit aggregates in a separate location (green). The Manders' correlation coefficient (R_r) provides further evidence that the large ribosomal subunit does not co-localize to SGs. These data indicate that the large ribosomal subunit does not localize to stress granules. SGs found in both mammalian and yeast cells also lack the large ribosomal subunit. The constituents of SGs in *Brachionus manjavacas* neonates appear to be consistent with what has been seen in other eukaryotes. The scale bar is 20 μm.

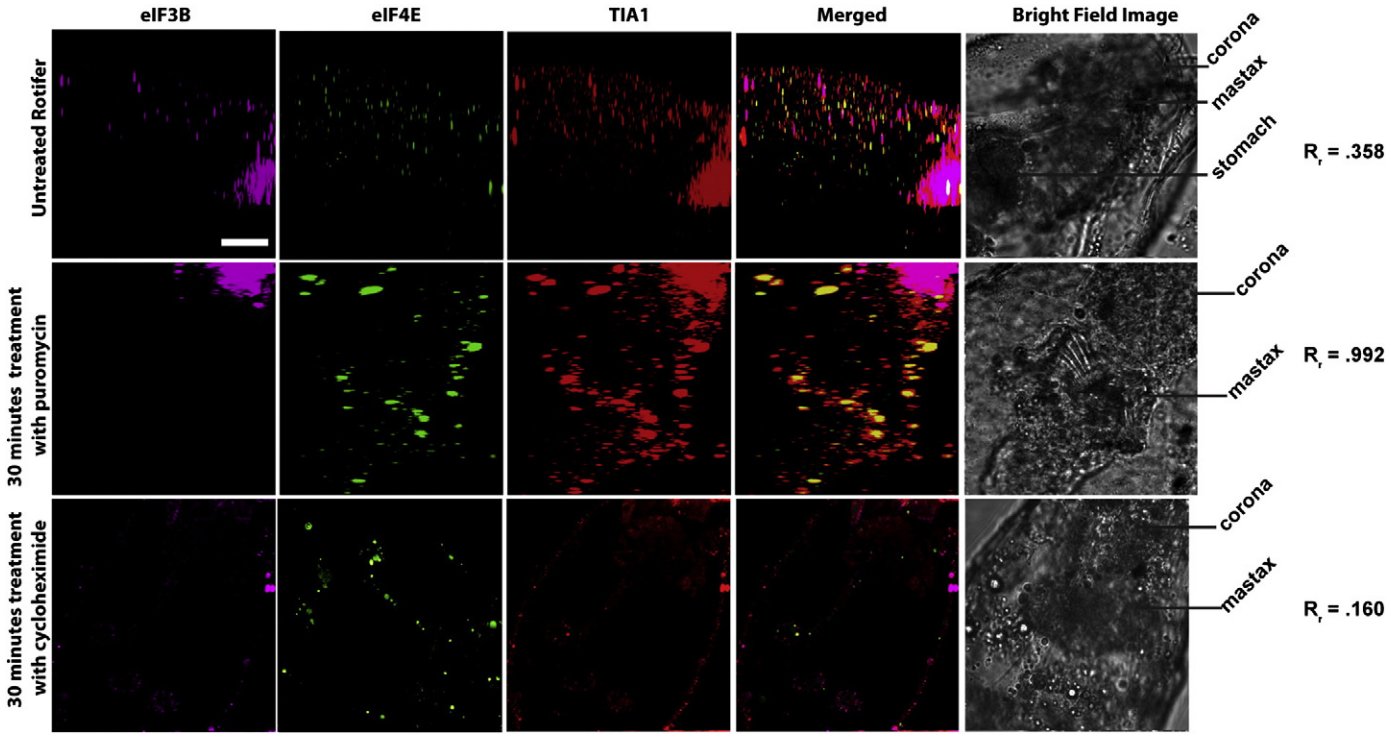


Fig. 8. eIF3B, eIF4E and TIA-1 are recruited to SGs respond to the presence of the antibiotic puromycin, not cycloheximide. *Brachionus manjavacas* neonates were incubated in 30 μM puromycin or 30 μM cycloheximide for 30 min. The neonates were probed with antibodies specific for eIF3B (magenta), eIF4E (green) and TIA-1 (red). The proteins are uniformly distributed throughout the cells in the control group (unstressed rotifers) and co-localize upon treatment with puromycin but not cycloheximide. This indicates the formation of stress granules. These results are consistent with SG formation in trypanosomatids, yeast and mammalian cells. SGs also fail to form within trypanosomatids, yeast and mammalian cells in the presence of cycloheximide. The scale bar is 20 μm.

2009). Several adaptations have been suggested to explain the capability of organisms to survive environmental stresses, particularly trehalose, sucrose, yolk proteins and LEA proteins (Crowe et al., 1998; Hoekstra et al., 2001; Boschetti et al., 2011; Hand et al., 2011; Jones et al., 2012). Although the precise function of SGs in the physiology of rotifers is yet to be understood, their formation is most probably related to survival during periods of stress. SG formation might allow for temporary suspension of energy consuming protein synthesis until the environmental stress has passed or until the rotifer moves to a more favorable environment. Interestingly, the survival of animals decreased in groups that displayed a decrease in SG formation during periods of stress. This was especially evident in rotifers with extended periods of nutrient deprivation.

Our work identifies three types of environmental factors that induce stress granule formation and inhibit translation in rotifers: nutrient deprivation, heat stress and osmotic stress. Rotifers often occupy habitats that are only temporarily suitable for growth (García-Roger et al., 2006). We found that nutrient deprivation for more than 48 h, osmotic changes where animals were transferred from 15 to more than 35 ppt and temperature stress at 40 °C for more than 1 h stressed the neonates to their limits for survival. These stressful conditions are similar to those the neonates could encounter in natural environments, and have been documented as conditions that negatively affect neonate hatching (Minkoff et al., 1983; Pérez-Legaspi and Rico-martínez, 1998; García-Roger et al., 2006; Dahms et al., 2011).

We identified TIA1-like, eIF4E-like and eIF3B-like proteins in *B. manjavacas* that bind antibodies raised against similar mammalian proteins. These proteins may play essential roles in the stress response during periods of nutrient deprivation, thermal and osmotic stress. TIA-1 is an RNA binding protein known to promote arrest of translation in environmentally stressed cells (Kedersha and Anderson, 2002; Gilks et al., 2004). The overexpression of TIA-1 induces stress granule formation even in the absence of stress (Gilks et al., 2004). Likewise, cells that lack TIA-1 exhibit impaired stress granule assembly (Kedersha et al., 1999; Gilks et al., 2004). A key conserved component of eIF3 is required for SG formation (Anderson and Kedersha, 2009a). Ohn et al. have shown that knockdown of eIF3 subunits inhibits stress granule assembly (Ohn et al., 2008).

Using immunohistochemistry, we followed the localization of TIA-1, eIF3B and eIF4E in rotifers during environmental stress. Our finding of the co-localization of these three proteins during periods of nutrient deprivation, osmotic and thermal stress are consistent with previous studies with trypanosomatids, animal and yeast models of stress granule formation (Kedersha and Anderson, 2002, 2007; Anderson and Kedersha, 2008; Kramer et al., 2008; Farny et al., 2009; Grousl et al., 2009; Cassola, 2011; Thomas et al., 2011).

Thermal stress induced the greatest extent of protein co-localization in *B. manjavacas*. Thermal stress has also been found to be a powerful inducer of stress granule formation in *Saccharomyces cerevisiae* (Grousl et al., 2009), *D. melanogaster* (Farny et al., 2009), and *Trypanosoma brucei* (Kramer et al., 2008). This pattern of SG formation is found in all animals thus far investigated, with the exception being yeast (Buchan and Parker, 2009; Grousl et al., 2009).

SGs in *B. manjavacas* neonates failed to form in the presence of cycloheximide; however, incubation in the presence of puromycin did promote stress granule formation. This result is consistent with data from trypanosomatids, yeast and mammalian cells treated with these drugs. Studies have shown that cycloheximide prevents SG formation and treatment with puromycin does not inhibit the formation of SGs in the presence during heat stress experiments (Kramer et al., 2008; Cassola, 2011). The rotifer stress granules disassembled once the heat stress was removed. This dynamic behavior is also seen in both mammalian and yeast cells suggesting a conserved evolutionary role for SGs (Anderson and Kedersha, 2002a).

While the presence of SGs in rotifers is certain, we certainly observed that the appearance, disappearance and protein make up of the observed SGs vary with the type of environmental stress as well as the duration of the environmental stress. These observations are consistent with what has been observed in the SGs of other eukaryotes, specifically both yeast and mammalian cells. Heterogeneous populations of all RNA granules have been observed (Thomas et al., 2011). Both PBs and SGs are highly dynamic. Proteins and RNA have been observed being exchange among the two. SGs and PBs can also release both transcripts and proteins in order to promote translation of messages (Anderson and Kedersha, 2006; Franks and Lykke-Andersen, 2008; Buchan and Parker, 2009). Furthermore, the composition of SGs varies during any one-stress response. Also, SGs can be subtly different depending on the nature of the stress condition (Buchan and Parker, 2009; Buchan et al., 2011).

SGs have been implicated in signaling cascades that determine survival in a variety of eukaryotes. Therefore SGs appear to be a near universal and versatile means of managing cellular stress (Nilsson and Sunnerhagen, 2011). Cells prevented from assembling SGs show a reduced level of survival during periods of environmental stress (McEwen et al., 2005; Kwon et al., 2007; Kim et al., 2012), and the assembly of SGs enhances the survival of stressed cells (Anderson and Kedersha, 2008). The identification of SGs in the Phylum Rotifera further suggests that SGs have an ancient origin, early in the evolution of animals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2013.07.009.

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