nature communications

Article

Nucleolar stress induces nucleolar stress body formation via the NOSR-1/NUMR-1 axis in *Caenorhabditis elegans*

Received: 25 October 2023

Accepted: 13 August 2024

Published online: 23 August 2024

Check for updates

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Environmental stimuli not only alter gene expression profiles but also induce structural changes in cells. How distinct nuclear bodies respond to cellular stress is poorly understood. Here, we identify a subnuclear organelle named the nucleolar stress body (NoSB), the formation of which is induced by the inhibition of rRNA transcription or inactivation of rRNA processing and maturation in C. elegans. NoSB does not colocalize with other previously described subnuclear organelles. We conduct forward genetic screening and identify a bZIP transcription factor, named nucleolar stress response-1 (NOSR-1), that is required for NoSB formation. The inhibition of rRNA transcription or inactivation of rRNA processing and maturation increases nosr-1 expression. By using transcriptome analysis of wild-type animals subjected to different nucleolar stress conditions and nosr-1 mutants, we identify that the SR-like protein NUMR-1 (nuclear localized metal responsive) is the target of NOSR-1. Interestingly, NUMR-1 is a component of NoSB and itself per se is required for the formation of NoSB. We conclude that the NOSR-1/NUMR-1 axis likely responds to nucleolar stress and mediates downstream stressresponsive transcription programs and subnuclear morphology alterations in C. elegans.

The nucleus contains several different dynamic bodies that are variously composed of proteins or specific RNA molecules¹. Intriguingly, nuclear bodies are not surrounded by membranes yet are retained as separate and distinct entities within the nucleoplasm, formed by a process termed liquid-liquid phase separation (LLPS)^{2,3}. It is well established that LLPS-associated proteins that contain folded domains, intrinsically disordered regions (IDRs), and low complexity (LC) regions play vital roles in the process of nucleation⁴. For instance, paraspeckles require NONO, PCPC1, and PSF^{5,6}. Cajal bodies are disrupted or disappear in the absence of COILIN, SMN, FAM118B, or WRAP53^{7,8}, PML bodies are nucleated by PML⁹, and SON and SRRM2 are essential for nuclear speckle formation¹⁰.

Nuclear body formation is generally dynamic, especially in response to a variety of stress conditions¹¹⁻¹³. For example, heat shock, UV radiation, and chemical toxicity trigger the formation of nuclear stress bodies (nSBs), which may further influence the reprogramming

¹Department of Obstetrics and Gynecology, The First Affiliated Hospital of USTC, The USTC RNA Institute, Ministry of Education Key Laboratory for Membraneless Organelles & Cellular Dynamics, Hefei National Research Center for Physical Sciences at the Microscale, Center for Advanced Interdisciplinary Science and Biomedicine of IHM, School of Life Sciences, Division of Life Sciences and Medicine, Biomedical Sciences and Health Laboratory of Anhui Province, University of Science and Technology of China, Hefei, Anhui 230027, China. ²School of Basic Medical Sciences, Anhui Medical University, Hefei, China. ³These authors contributed equally: Minjie Hong, Xiaotian Zhou. e-mail: hxy1996@ustc.edu.cn; xychen91@ustc.edu.cn; fengxuezhu@ahmu.edu.cn; sguang@ustc.edu.cn of gene expression¹⁴. Mitochondrial stress leads to altered morphology and numbers of nuclear paraspeckles¹⁵. Polycomb Group (PcG) bodies were shown to repress damaged DNA upon UV irradiation¹⁶. However, how these nuclear bodies respond to cellular stresses and their physiological roles are still poorly understood.

The nucleolus is the most prominent subnuclear structure, where the main processes of rRNA and ribosome biogenesis occur. In the mammalian nucleolus, rDNA genes are transcribed by RNA polymerase I into 47S pre-rRNA, which is subsequently processed and modified to generate 28S, 18S, and 5.8S rRNAs. Genes encoding 5S rRNA are transcribed by Pol III^{17,18}. These rRNAs are assembled with ribosomal proteins (RPs) to form small and large preribosome subunits, which are exported to the cytoplasm and eventually form the mature 40S and 60S ribosome subunits¹⁹.

Ribosome assembly factors maintain their greatest steady-state concentrations within nucleoli under normal growth conditions²⁰, and blocking ribosome biosynthesis stimulates nucleolar stress²¹. For instance, actinomycin D inhibits Pol I transcription elongation and causes nucleolar segregation²². Starvation or rapamycin treatment downregulates mTOR signaling in yeast and mammalian cells, leading to a significant nucleolar size reduction and inhibition of rRNA transcription²³. Mutations in ribosome assembly factors or ribosomal proteins (r-proteins) lead to nucleolar stress and induce a number of human disorders²⁴.

In mammals, nucleolar stress signaling pathways are classified into two types: p53-dependent and p53-independent pathways²⁰. p53 is a crucial transcription factor that responds to many cellular stresses. Nucleolar stress releases p53 from inhibition by MDM2 and activates a set of target genes^{25,26}. The mechanism of the p53-independent pathway remains poorly understood. Nucleolar stress may induce cell proliferation or cell cycle arrest via the regulation of c-Myc, E2F-1, p21 Waf1/Cip1, or p27Kip1, independent of p53²⁷⁻³⁰. Yeast and plants do not express p53 or MDM2 and, therefore, use mechanisms independent of the p53 pathway. In plant cells, the plant-specific transcription factor ANAC082 may play critical roles in the response to nucleolar stress³¹.

C. elegans expresses p53/CEP-1 but lacks MDM2. Active p53/CEP-1 is regulated at the translational level^{32,33}. Mutation in the conserved nucleolar protein NOL-6 enhances resistance to bacterial infection by activating p53/CEP-1 and its target gene, *sym-1*³⁴. The depletion of a nucleolar protein, WDR-46, activates xenobiotic detoxification genes via Nrf/SKN-1 and p53/CEP-1³⁵. Interestingly, the transcription factor FoxA/PHA-4, instead of p53/CEP-1, acts as a nucleolar stress sensor to induce the expression of lipogenic genes and lipid accumulation³⁶, suggesting additional mechanisms for the nucleolar stress response.

Previously, we reported that nucleolar stress could induce the accumulation of erroneous rRNAs that lead to the recruitment of RdRPs to synthesize antisense ribosomal siRNAs (risiRNAs), which subsequently turn on the nuclear RNAi-mediated gene silencing pathway to inhibit pre-rRNA expression^{37–39}. In addition, EXOS-10 exhibits nucleoplasmic translocation upon cold-worm shock in *C. elegans*⁴⁰. Third, abnormal accumulation of 27SA₂ rRNA intermediates upon the depletion of class I RPLs reshaped spherical nucleoli to a ring-shaped nucleolar structure with an enlarged nucleolar vacuole (NoV) in *C. elegans*⁴¹. Therefore, it is very likely that a number of cellular events may occur concurrently to respond to nucleolar stress.

Here, we identified that nucleolar stress induced the formation of a subnuclear organelle, named the nucleolar stress body (NoSB), in *C. elegans.* The inhibition of rRNA transcription by actinomycin D, inactivation of rRNA processing and maturation, or mutation in the exosome ribonuclease complex, which degrades erroneous rRNAs, results in NoSB formation. To search for factors required for the formation of nucleolar stress-induced NoSB, we conducted forward genetic screening and identified a bZIP-containing protein, nucleolar stress required-1 (NOSR-1). In the absence of NOSR-1, nucleolar stress failed to induce NoSB formation. Interestingly, NOSR-1 acts as a sensor of nucleolar stress to activate the expression of the SR-like protein NUMR-1, consequently promoting NoSB formation. Therefore, our work revealed a NOSR-1/NUMR-1-involved pathway that responds to nucleolar stress and mediates nuclear substructure alterations in *C. elegans*.

Results

Deficiency in rRNA processing and maturation induced the formation of a distinct nucleolar stress body

Our recent work showed that the depletion of class I RPL proteins and a number of 26S rRNA processing factors led to nucleolar reshaping, in which the 27SA₂ rRNA intermediate accumulated, nucleolar rings formed, and nucleolar vacuoles enlarged (Fig. 1A)⁴¹. To further investigate the nucleolar stress-induced substructure alterations in the nucleus, we generated an mCherry::DIS-3 strain and visualized the structure of the nucleus and nucleolus in hypodermal cells upon a number of nucleolar stresses. DIS-3 (also known as Rrp44 or EXOSC11) is a subunit of the 3' to 5' exoribonuclease complex, which degrades erroneous rRNAs in the nucleus and accumulates in the nucleoplasm (Fig. 1B)^{38,42}. The single-copy mCherry::DIS-3 transgene was integrated into the C. elegans' chromosome III of the strain EG8080 by MosSCI technology³⁸. The transgene was driven by its own promoter, did not affect the brood size, but rescued dis-3(ust56) dysfunction in risiRNA biogenesis, suggesting that the transgene recapitulated the function of endogenous DIS-338.

We performed feeding RNAi experiments and knocked down approximately 100 genes involved in rRNA processing and modification in mCherry::DIS-3 animals (Supplementary Data 1). Then, we visualized the hypodermal cells using Nomarski (DIC) and fluorescence microscopy. Consistent with previous work, we observed the formation of nucleolar ring structures and enlarged nucleolar vacuoles upon knocking down a number of genes involved in pre-60S ribosome maturation (Fig. 1B, C)⁴¹. The nucleolar vacuole (NoV) is an evolutionarily conserved nucleolar subcompartment, present in the nucleoli of various plants and animals⁴³⁻⁴⁶. Our recent work showed that faithful rRNA processing is essential to maintain the structure of nucleolus⁴¹. When the rRNA precursor 27SA₂ rRNA was inappropriately accumulated, the spherical nucleolus transformed into a ring-shaped nucleolus, in which a NoV appears. In the circumstance, the rRNA transcription and processing machineries were enriched in the ring, while many usual nucleoplasmic proteins accumulated in the NoV⁴¹. NoVs may have important roles in germline development or mRNA metabolism and may be involved in the transport of nucleolar substances from nucleolus to nucleoplasm and temporary storage of certain materials.

Interestingly, we noticed the appearance of an additional vacuole per cell in the nucleoplasm in hypodermal cells (Fig. 1B, C). The vacuole excludes DIS-3 and is also visible by DIC microscopy. We named this vacuole the nucleolar stress body (NoSB) (see below).

Ribosomal proteins of the small subunit (RPS) fall into two main categories according to their functions in pre-rRNA processing⁴⁷. i-RPSs are strictly required for initiating the processing of sequences flanking the 18S rRNA. p-RPS proteins are required for subsequent nuclear and cytoplasmic maturation steps. Knocking down both i-RPS and p-RPS proteins induced the formation of NoSB but not the formation of nucleolar rings (Fig. 1B, C and Supplementary Fig. 1A, C)⁴¹.

RPLs are proteins of the 60S ribosome large subunit that are involved in pre-rRNA processing, including 27SA₂, 27SA₃, 27SB prerRNAs, and 7S pre-rRNA maturation⁴⁸. *C. elegans'* genome encodes 48 large subunit (*rpl*) genes. Our recent work classified RPL proteins into two main functional categories according to whether they are required for the processing of 27SA₂ pre-rRNAs and the suppression of ringshaped nucleoli and enlarged nucleolar vacuoles (NoV)⁴¹. The Class I RPLs are required for 27SA₂ pre-rRNA processing, and the knockdown of this group significantly increased the proportion of vacuole-



Fig. 1 | **Deficiency in rRNA processing and maturation induced the formation of an uncharted nucleolar stress body. A** Schematic diagram of the formation of nucleolar rings, enlarged nucleolar vacuoles, and nucleolar stress bodies (NoSBs) in *C. elegans.* **B** DIC and fluorescence microscopy images of nuclei in hypodermal cells after knockdown of the indicated genes in the indicated L4 stage animals (scale bar, 5 µm). The nucleoplasm is marked by mCherry::DIS-3, the yellow arrows indicate

NoSB. The images were taken by the Leica THUNDER imaging System. C Quantification of NoSB and ring-shaped nucleoli after RNAi knockdown of the indicated genes. The percentage of animals containing at least three cells out of hypodermal cells that have NoSB or ring-shaped nucleoli structure was shown (20 independent animals were tested). contained nucleoli and the accumulation of 27SA₂ pre-rRNA⁴¹. However, Class II RPLs were likely involved in the processing of other prerRNA intermediates, and the knockdown of Class II RPLs did not lead to the formation of NoV⁴¹. The depletion of both classes of RPL proteins induced NoSB formation, yet only class I RPLs triggered the formation of ring-shaped nucleoli (Supplementary Fig. 1B, C).

Taken together, these results suggested that defective rRNA biogenesis and ribosome assembly could induce alterations in subnuclear structures in *C. elegans*.

Mutation of exosome ribonucleases induced NoSB formation

To investigate the mechanism of NoSB formation, we first conducted forward genetic screening to search for mutants that reveal NoSB formation. We mutagenized the mCherry::DIS-3 strain with ethyl methanesulfonate (EMS), followed by clonal screening with DIC and fluorescence microscopy (Fig. 2A). From the 2000 haploid genome, we isolated two mutants, *ust242* and *ust243*. Using whole-genome resequencing, we identified two point mutations in the coding regions of *exos-4.2 (ust243, G54R)* and *exos-10 (ust242, D368N)* (Fig. 2B). Both EXOS-4.2 and EXOS-10 are core factors of the exosome 3'-5'-exoribonuclease complex^{38,42,49}. *exos-4.2* encodes a ribonuclease (RNase) pleckstrin homology (PH)-like protein, and *exos-10* encodes a 3'-5'-exoribonuclease.

To confirm that the exosome complex suppresses NoSB formation, we generated additional alleles of *exos-4.2* and *exos-10* by CRISPR/ Cas9 technology and obtained mutants of other exosome subunits. The mutation of *exos-1, exos-4.2,* and *exos-10* resulted in NoSB formation (Fig. 2C–E). Consistently, feeding mCherry::DIS-3 animals dsRNAs targeting *exos-2, exos-3, exos-4.1, exos-5, exos-7, exos-8,* or *exos-9* induced the formation of NoSB (Supplementary Fig. 2A).

We measured the size of NoSB and nucleoli in the exosome mutants. The size of NoSB is -10% of that of nucleoli, and the exosome mutants exhibited modestly enlarged nucleoli (Fig. 2E and Supplementary Fig. 2B). Similarly, in *S. cerevisiae* and *D. melanogaster*, the depletion of ribosomal proteins also led to enlarged nucleoli⁵⁰.

LMN-1 is an ortholog of human lamin A/C and localizes to the inner side of the nuclear membrane. NoSB localized inside the nuclei, as shown by LMN-1::mCherry (Supplementary Fig. 2C). MTR-4 and NRDE-3 are two nucleoplasm-localized proteins and are involved in nuclear and nucleolar RNAi³⁸. Our previous work showed that deficient rRNA processing and maturation led to the accumulation of erroneous rRNAs and induced the production of a class of antisense ribosomal siRNA (risiRNA) which further translocated the Argonaute protein NRDE-3 from the cytoplasm to nucleoli37-39. Similarly, the disruption of RNA exosomes also resulted in the production of risiRNAs and triggered the nucleolar accumulation of NRDE-3³⁸. To test whether nucleolar stress could also induce the production of risiRNAs and enrich NRDE-3 in NoSBs, we visualized the subcellular localization of NRDE-3 and MTR-4 upon exos-9 RNAi. However, both proteins were excluded from the exos-9 depletion-induced NoSB (Supplementary Fig. 2D), suggesting that it is unlikely risiRNA could enrich in NoSB.

Therefore, we concluded that a deficient exosome ribonuclease complex could induce NoSB formation.

The inhibition of RNA polymerase I induced NoSB formation

Previous work showed that actinomycin D inhibits rRNA transcription and induces nucleolar stress^{41,51,52}. We generated an mCherry::DIS-3;FIB-1::GFP strain and visualized the structure of the nucleolus in hypodermal cells in the presence of actinomycin D. FIB-1 encodes the *C. elegans* ortholog of human fibrillarin and *Saccharomyces cerevisiae* Nop1p⁵³ and localizes to the nucleolus.

At a concentration of $5 \mu g/ml$ actinomycin D, we did not observe pronounced nuclear and nucleolar substructure changes in hypodermal cells (Fig. 2F and Supplementary Fig. 3A). However, at a higher concentration of 20 to 30 μ g/ml actinomycin D, FIB-1 likely condensed and detached from some unidentified nucleolar border. The NoSB excludes DIS-3 in the nucleoplasm, which is also visible by DIC and fluorescence microscopy. FIB-1 did not accumulate in NoSB, suggesting that the vacuole is distinct from nucleoli. The sizes of NoSB increased at a higher concentration of actinomycin D (Supplementary Fig. 3B). LMN-1::mCherry confirmed that actinomycin D-induced NoSB localized in the nucleus (Supplementary Fig. 3C). MTR-4 and NRDE-3 were also excluded from actinomycin D treatment has been shown to inhibit the formation of nucleolar ring and nucleolar vacuole⁴¹, which is oppositive to actinomycin D-induced NoSB formation.

We tested whether other kinds of environmental conditions could induce the formation of NoSB. However, growing animals at 15 or 25 °C, heat shock at 37 °C or stravation failed to induce the formation of NoSB (Supplementary Fig. 3E–G, also see Supplementary Fig. 18A, B). In addition, the ectopic expression of mcherry::DIS-3 did not affect the efficacy of NoSB formation (Supplementary Fig. 4A, B).

Taken together, these data suggested that the inhibition of RNA polymerase I by actinomycin D induced the formation of NoSBs.

NoSB is not a portion of nucleoli that blebbed off upon nucleolar stress

In mammalian cells, the factors involved in rRNA transcription and processing and ribosome assembly usually localize to the FC, DFC, and GC nucleolar subcompartments. Recent work showed that *C. elegans* nucleoli contain two phase-separated subcompartments, with FIB-1 and GARR-1 in the FC and NUCL-1 and LPD-6 in the GC region⁴³.

To test the possibility that NoSB may be a portion of the nucleolus that has blebbed off upon nucleolar stress, we generated a number of single-copy transgenes of nucleoli-localized proteins by CRISPR-Cas9 technology (Fig. 3A–C and Supplementary Fig. 5A). Among them, FIB-1 is a methyltransferase for pre-rRNA processing and modification. RBD-1 is an ortholog of human RBM19, which is required for 90S preribosome maturation. RRP-8 is a rRNA processing factor involved in N1-methyladenosine (m1A) modification of 26S rRNAs. DAO-5 is an rRNA transcription factor. T06E6.1 is an rRNA processing factor. GARR-1 is involved in snoRNA-guided rRNA pseudouridine synthesis. RPOA-1, RPOA-2, and RPAC-19 are subunits of the RNA polymerase I complex. We observed that upon *exos-9* RNAi, all these proteins were excluded from the NoSB (Fig. 3B, C, Supplementary Fig. 5A, and summarized in Supplementary Fig. 5B).

To further test that NoSB may be a reservoir for partially constructed ribosomes, we generated single-copy transgenes of ribosomal subunits by CRISPR-Cas9 technology and then examined their localization upon knocking down *exos*-9 by RNAi (Supplementary Fig. 5A, B). RPS-4 and RPS-19 are small ribosomal subunit proteins and are involved in ribosomal small subunit biogenesis. RPL-7 and RPL-14 are large ribosomal subunit proteins and are involved in ribosomal large subunit biogenesis. RPS-4 is enriched in nucleolus; RPS-19 accumulates in the nucleolus and nucleoplasm during the larval stage; RPL-7 is enriched in nucleolus, and RPL-14 is likely evenly distributed in the cell. Upon knocking down *exos*-9 by RNAi, however, RPS-4, RPS-19, RPL-7, and RPL-14 were all excluded from NoSB (Supplementary Fig. 5A, B).

Therefore, we concluded that NoSB is not a portion of the nucleolus that has blebbed off upon nucleolar stress, or the reservoir for partially constructed ribosomes.

NoSB is a distinct subnuclear organelle

To test whether NoSB is a distinct subnuclear organelle or colocalizes with other known subnuclear structures, we generated a number of fluorescence-labeled protein markers to reveal the Cajal body, paraspeckle, PcG body, and nuclear stress body (Fig. 3A)⁵⁴. The *exos*-9 depletion-induced NoSB did not colocalize with any of these known



subnuclear organelles (Fig. 3D and Supplementary Fig. 6A–D). The heat shock protein HSF-1 responds to heat stress by forming subnuclear structures termed nuclear stress granules in *C. elegans*. However, we did not find colocalization of NoSB with HSF-1::GFP (Fig. 3D).

We tested whether NoSB is a nuclear lipid droplet. Recent work reported that lipid droplets, which form at the nuclear envelope, may enter the nucleoplasm by penetrating the nuclear lamina and associated peripheral heterochromatin to form nuclear lipid drops (nLDs)^{55,56}. Actinomycin D-triggered nucleolar stress could induce excessive lipid accumulation in *C. elegans*³⁶. To test whether NoSB is a kind of nLD, we used Oil Red O to stain neutral lipids of postfixed animals with or without *exos*-9 RNAi but failed to observe lipid accumulation in NoSBs (Supplementary Fig. 6E). Therefore, NoSB is unlikely to form nuclear neutral lipid droplets.

Fig. 2 | Mutation of exosome ribonucleases induced NoSB formation.

A Schematic diagram of forward genetic screening to search for factors suppressing NoSB formation in *C. elegans* nuclei. **B** Schematic of the gene structure of exosome genes. *exos-4.2 (ust243)* and *exos-10 (ust242)* are the mutants obtained from the forward genetic screen; *exos-1 (ust57)* and *exos-10 (ok2269)* are obtained from the National Bioresource Project and the CGC; *dis-3 (ust56)*, a conserved amino acid Arg363, was mutated to cysteine^{18,40}. **C** DIC and fluorescence microscopy images of nuclei in hypodermal cells of respective L4 stage animals expressing the mCherry::DIS-3 (scale bar, 5 µm). The yellow dashed line represents the nucleus outlines, and the yellow arrows indicate NoSB (see also in Fig. 2F). **D** Quantification of NoSB in hypodermic cells in exosome mutants. Red dots indicate the percentage of hypodermis cells containing NoSB in each worm. *n* indicates the number of

An interesting observation is NUCL-1. NUCL-1 is located in the GC region and is required for nucleolar vacuole formation⁴¹. NUCL-1 encodes an evolutionarily conserved protein exhibiting extensive homology to yeast and human nucleolin and has been shown to interact with exosome proteins⁵⁷. The N-terminal domain of NUCL-1 is a long IDR containing a GAR/RGG domain that is required for subnucleolar compartmentalization⁴³ (Fig. 3E). IDRs are likely one of the driving forces of phase separation and have been identified in many proteins capable of phase separation. RRP-8 encodes an evolutionarily conserved protein exhibiting extensive homology to yeast and human RRP-8. The N-terminal domain of RRP-8 is also a long IDR (Fig. 3E). We constructed GFP- or mCherry-tagged NUCL-1, NUCL-1ARGG, RRP-8, and RRP-8AIDR transgenic animals. The depletion of RNA exosome did not translocate NUCL-1 or NUCL-1(ΔRGG) into NoSB in live worms (Supplementary Fig. 7B, C). However, further treatment with NaN₃ following actinomycin D or exos-9 RNAi could induce an accumulation of NUCL-1 or NUCL-1(Δ RGG) to NoSB (Fig. 3F and Supplementary Fig. 7A-C). Yet nucl-1 is not required for the formation of NoSB (Supplementary Fig. 7D). Double treatment of nucleolar stress followed by NaN₃ could recruit NUCL-1 to NoSB, yet the mechanism is unclear. Neither RRP-8 nor RRP-8(ΔIDR) translocated to NoSB upon nucleolar stress followed by NaN₃ treatment (Fig. 3F).

In conclusion, we speculated that NoSB is a distinct subnuclear structure induced by nucleolar stress.

Forward genetic screening identified a bZIP transcription factor NOSR-1 required for NoSB formation

To further understand the mechanism and regulation of NoSB, we performed a second forward genetic screening to search for factors that were required for NoSB formation. We chemically mutagenized mCherry::DIS-3;*exos*-10(*ust242*) animals followed by clonal screening with DIC and fluorescence microscopy and isolated mutants in which NoSB was depleted (Fig. 4A). From approximately 2000 haploid genomes, we isolated one mutant, *ust303*, in which NoSB was depleted in mCherry::DIS-3;*exos*-10(*ust242*) animals (Fig. 4B). We named the gene nucleolar stress response-1 (*nosr-1*).

We deep-sequenced the *nosr-1(ust303)* genome and identified Y17G7B.20 (Fig. 4C), in which the amino acid threonine242 was mutated to a proline (Fig. 4C). Y17G7B.20 was predicted to have a basic leucine zipper (bZIP) domain that can bind to DNA and mediate protein dimerization^{58,59}. To further confirm that *y17g7b.20* is *nosr-1*, we generated three additional deletion alleles of *y17g7b.20* by CRISPR/Cas9 technology with two single-guide RNAs (sgRNAs)⁶⁰ and examined NoSB formation in *exos-10(ust242)* animals. All three alleles, *ust307, ust308*, and *ust309*, deleted most of the gene sequence and caused a frame shift; therefore, they are likely null alleles. In the three *y17g7b.20* mutants, *exos-10(ust242)* mutation or RNAi knockdown of *exos-9* failed to induce NoSB formation (Fig. 4B, D and Supplementary Fig. 8A, B). In addition, the *nosr-1* mutation blocked actinomycin D-induced NoSB formation (Supplementary Fig. 8C). Therefore, *y17g7b.20* is *nosr-1*.

To test whether NOSR-1 is the key player in NoSB formation from all types of defects in ribosome synthesis, we observed that (1) *nosr-1* is independent animals tested. For box plots, the horizontal line represents the median value, the lower and upper quartiles represent the 25th and 75th percentile, and the whiskers show the maximum and minimum values; a two-tailed *t*-test was performed to determine statistical significance (see also in Fig. 2E). **E** The size of NoSB in exosome mutants. Blue dots indicate the area of NoSB. *n* of nucleoli from 20 independent animals were measured. **F** DIC and fluorescence microscopy images of *C. elegans* nuclei in hypodermal cells after actinomycin D treatment for 48 h. The nucleoplasm is marked by mCherry::DIS-3 (magenta), and the nucleolus is marked by FIB-1::GFP (green). Three times were repeated independently with similar results. All images were taken by the Leica THUNDER imaging System. Source data are provided as a Source Data file.

required for both *exos-9* RNAi- and actinomycin D-induced NoSB formation (Supplementary Fig. 8A–C); (2) after knocking down the components of snoRNP, pre-40S and pre-60S particles, and RPSs and RPLs, -50% hypodermal cells in each worm revealed NoSB structure which also depended on *nosr-1* (Supplementary Figs. 9A, B, 10A, B). These data suggested that NOSR-1 likely suppresses the formation of NoSB induced by all types of defects in ribosome biogenesis.

Interestingly, the depletion of *nosr-1* inhibited *rpl-14* knockdowninduced NoSB formation but did not prohibit the formation of ringshaped nucleoli (Fig. 4E, F), suggesting that the formation of NoSB and ring-shaped nucleoli involved two distinct mechanisms.

NOSR-1 promotes animal fertility and lifespan under nucleolar stress conditions

The bZIP transcription factor family is a gene family conserved from yeast to humans and has roles in a wide variety of processes⁶¹. *C. elegans* underwent a relatively recent expansion of bZIP genes, some of which do not appear to have direct homologs in mammals, likely including NOSR-1^{58,61}.

We investigated the role of NOSR-1 in *C. elegans. nosr-1* mutants revealed brood sizes similar to those of wild-type N2 animals at 20 °C but strongly reduced fertility at 25 °C (Fig. 5A, B). In addition, *nosr-1* mutation reduced the brood size of *exos-10* animals (Fig. 5C, D).

We failed to detect a significant change in the lifespan of *nosr-1* animals compared to that of N2 animals (Supplementary Fig. 11A, B). However, the *nosr-1* mutation shortened the lifespan of *exos-10* animals (Fig. 5E, F). The depletion of *nosr-1* did not significantly change the oxidative stress resistance or heat stress resistance in N2 background worms (Supplementary Fig. 11C, D).

Therefore, we concluded that *nosr-1* is required for fertility and lifespan regulation in animals under nucleolar stress conditions.

Nucleolar stress activates nosr-1 expression

To investigate the mechanism of *nosr-1* in NoSB regulation, we tested whether *nosr-1* responds to nucleolar stresses. We compared the transcriptomes of animals with or without nucleolar stress using mRNA deep-sequencing. The *nosr-1* mRNA levels were upregulated upon actinomycin D treatment, *exos-10* mutation, or RNAi knockdown of *exos-9* and *rpl-14* (Fig. 6A).

Then, we constructed a GFP-fused transcription fusion *nosr-1_P::NLS::GFP* and inserted it at the ttTi4348 site on chromosome I using CRISPR/Cas9 gene editing technology to monitor *nosr-1* expression⁶⁰. Actinomycin D treatment significantly increased the expression of *nosr-1_P::NLS::GFP* (Fig. 6B and Supplementary Fig. 12A). Knocking down *exos-9* by RNAi or mutations in exosome genes significantly increased the expression of *nosr-1_P::NLS::GFP* (Fig. 6C, D and Supplementary Fig. 12B, C). We measured the mRNA levels of endogenous *nosr-1* mRNA by quantitative real-time PCR and identified an increase in the mRNA levels of *nosr-1* (Fig. 6E, F). Similarly, knocking down rRNA biogenesis and ribosome assembly factors, the mutation of which induced NoSB formation, increased the expression of *nosr-1_P::NLS::GFP* as well (Supplementary Fig. 12D–G).

A		
Nuclear bodies	Vertebrate protein	C.elegans
Nucleolus	RBM19,RRP8	RBD-1,RRP-8
Cajal body/Nucleolus	Nopp40	DAO-5
Paraspeckle	NONO/p54nrb	NONO-1
PcG body	EZH1/2	MES-2
Nuclear stress body	HSF1	HSF-1

В

Е



F



С TagRFP::DAO-5 RNAi: exos-9 DIC RFP MERGE

5 µm



NUCL-1 Disorder tendency 0.0 200 400 600 0 (aa) GAR/RGG domain Acidic stretches AdoMet_MTases RRM domain RRP-8 1.0 Disorder tendency

NaN3					
GFP::NUCL-1		GFP::NUCL-1(△RGG)			
L4440	exos-9	L4440	exos-9		
		10-	0.8		
		۰			
RRP-8::GFP		RRP-8:(△IDR):mCherry			
L4440	exos-9	L4440	exos-9		
0.	0.	103	109)		
٠			T		
			5 μm		

Methyltransf_8

Deletion

200

300



100

similar results. E Schematic diagram of the domain structure and predicted intrinsically disordered regions of NUCL-1 and RRP-8. F DIC and fluorescence microscopy images of nuclei with the indicated transgenes after exos-9 knockdown (scale bar, 5 μm). Three times were repeated independently with similar results. All images were taken by the Leica THUNDER imaging System.

0.5

0.0



Fig. 4 | Forward genetic screening identified a bZIP transcription factor NOSR-1 required for NoSB formation. A Schematic diagram of the forward genetic screening to search for factors required for NoSB formation in *C. elegans* nuclei.
B DIC and fluorescence microscopy images of nuclei in the indicated L4 stage animals expressing the mCherry::DIS-3 (scale bar, 5 µm). The yellow dashed line represents the nucleus, and the yellow arrows indicate NoSB (see also in Fig. 4E).
C Schematic of the *nosr-1* exon structure. *nosr-1* (*ust303*) is the mutant obtained from the forward genetic screen. The deletion alleles *ust307*, *ust308*, and *ust309* were constructed by CRISPR/Cas9 technology (see Methods). D Quantification of NoSB in hypodermic cells in the indicated animals. Red dots indicate the percentage of hypodermis cells containing NoSB in each L4 stage worm. *n* indicates the number of independent animals tested. A two-tailed *t*-test was performed to determine statistical significance. For box plots, the horizontal line represents the

median value, the lower and upper quartiles represent the 25th and 75th percentile, and the whiskers show the maximum and minimum values (see also in Fig. 4F). **E** Top: Schematic diagram of the formation of nucleolar rings, enlarged nucleolar vacuoles and nucleolar stress bodies (NoSBs) in *rpl-14(RNAi)* animals. Bottom: Images of mCherry::DIS-3 transgene in *nosr-1(-);rpl-14(RNAi)* animals (scale bar, 5 µm). The green arrows indicate the nucleolar ring. **F** Quantification of nucleolar rings and NoSB in hypodermic cells in the indicated animals. Every dot indicates the percentage of hypodermis cells containing NoSB or nucleolar rings. *n* indicates the number of independent animals tested. A two-tailed *t*-test was performed to determine statistical significance. All images were taken by the Leica THUNDER imaging System. Source data are provided as a Source Data file.



Fig. 5 | **NOSR-1 is required for fertility and lifespan.** A–D Brood size of the indicated animals at 20 and 25 °C. Bleached embryos were hatched and grown at 20 or 25 °C. Then, L3 worms were transferred individually onto fresh NGM plates. *n* of progeny worms was scored. A two-tailed *t*-test was performed to determine statistical significance. **E** Survival curves of the indicated animals at 20 °C. Three

biological replicates were performed. **F** Histogram displaying the average lifespan of the indicated animals. Mean \pm SD of three independent experiments. *n*, the number of animals tested. A two-tailed *t*-test was performed to determine statistical significance. Source data are provided as a Source Data file.

Taken together, these results suggested that nucleolar stress could activate the expression of *nosr-1*.

nosr-1 may engage in an uncharted nucleolar stress response pathway

We tested whether other known stress response pathways are involved in the formation of nucleolar stress-induced NoSB. CEP-1 is an ortholog of mammalian p53 in *C. elegans* and has an essential role in nucleolar stress³⁴. SKN-1 senses nucleolar stress to activate xenobiotic detoxification genes³⁵. PHA-4 is a sensor of nucleolar stress and transactivates the expression of lipogenic genes³⁶; DAF-16 is a FOXO transcription factor involved in lifespan regulation as well as in oxidative stress and heat stress resistance^{62,63}. However, none of these genes are required for *exos-10(ust242)* mutation-induced NoSB formation (Supplementary Fig. 13A, B). Therefore, we speculated that *nosr-1* may engage in an uncharted nucleolar stress response pathway.

Transcriptome analysis identified numr-1 as the NOSR-1 target

We performed mRNA-seq to explore the NOSR-1-targeted genes involved in NoSB formation. We first compared transcriptomes of wildtype and *nosr-1*(-) mutant animals under different stress conditions (Fig. 7A and Supplementary Fig. 14A–C) and identified 11 genes that were consistently upregulated by nucleolar stress (Fig. 7B), which depend on the presence of functional NOSR-1 (Fig. 7C–E)



Fig. 6 | **Nucleolar stresses activate** *nosr-1* **expression.** A Volcano plot comparing the transcriptomes of the indicated animals. Volcano plot showing log_2 (fold change) of gene product-annotated transcript abundances in stressed vs control-animals (*x* axis) and $-log_{10}$ (*P* value) (*y* axis), calculated using an exact test in edgeR. The dashed line represents the significance threshold. The expression of *nosr-1* is highlighted. **B** Fluorescence microscopy images of *nosr-1*_p::*NLS::GFP(ustIS285)* after actinomycin D (abbreviated as Act D) treatment. **C** Fluorescence microscope images of *nosr-1*_p::*NLS::GFP(ustIS285)* in the indicated animals. **D** Fluorescence

We then tested whether nucleolar stress-induced NoSB depends on the 11 genes. We performed feeding RNAi experiments and knocked down the 11 genes in mCherry::DIS-3;*exos-10(ust242)* animals (Fig. 7F). RNAi knockdown of *numr-1* results in the prohibition of NoSB formation, suggesting that nucleolar stress-induced NoSB requires the presence of NUMR-1.

Nucleolar stress induced the accumulation of NUMR-1 in NoSB NUMR-1 (nuclear-localized metal responsive) was previously identified as an SR-like protein that promotes cadmium tolerance (Fig. 8A)⁶⁴⁻⁶⁶. microscope images of *nosr-1p::NLS::GFP* after knocking down the indicated genes by RNAi (scale bar, 20 μ m). All worms were imaged at the L4 stage and all images are representative of more than ten animals (**B**–**D**). All images were taken by the Leica THUNDER imaging System. **E**, **F** Relative mRNA levels of *nosr-1* measured by quantitative real-time PCR in the indicated animals. Data were presented as the means ± SD of three and four biological replicates, respectively. A two-tailed *t*-test was performed to determine statistical significance. Source data are provided as a Source Data file.

NUMR-1 contains an RNA-recognition motif (RRM), serine- and arginine-rich regions that are common in SR proteins, and a C-terminal tail rich in histidine and glycine, suggesting that NUMR-1 might function in RNA metabolism^{67–69}. Interestingly, the SR family of proteins is strongly associated with liquid–liquid phase separation (LLPS) and is enriched in nuclear bodies, especially in nuclear speckles⁷⁰. These features suggest that NUMR-1 might function in RNA metabolism and promote the formation of various biomolecular condensates.

To study the function of NUMR-1 in NoSB formation, we constructed a 3xFLAG-GFP-tagged NUMR-1 transgene (abbreviated



Fig. 7 | **Transcriptome analysis identified NOSR-1 targets. A** Schematic of the animal with or without nucleolar stress. **B** Venn diagrams reveal the overlapping target genes that are upregulated or downregulated under three nucleolar stress conditions. **C**–**E** Heatmap of the standardized fragments per kilobase of transcript per million mapped reads (FPKM) for the 11 coupregulated target genes in the indicated animals. The expression levels are indicated by the color bar. **F** DIC and

fluorescence microscopy images of hypodermal nuclei in *exos-10(ust242)*;mCherry::DIS-3 worms after knockdown of the indicated genes by RNAi (scale bar, 5 µm). The yellow dashed line represents the nucleus, and the yellow arrows indicate NoSB. Three times were repeated independently with similar results. Images were taken by the Leica THUNDER imaging System.

GFP::NUMR-1) using CRISPR/Cas9-directed in situ gene editing technology. In wild-type animals cultured under normal laboratory conditions, we did not observe explicit GFP::NUMR-1 expression (Fig. 8B and Supplementary Fig. 15A). Strikingly, upon nucleolar stress by the inhibition of rRNA transcription using actinomycin D or RNAi knockdown of *exos-9* or *rpl-14*, GFP::NUMR-1 expression was activated, and the protein accumulated in distinct nuclear foci under fluores-cence microscopy (Fig. 8B and Supplementary Fig. 15A). DIC



Fig. 8 | **NUMR-1 is a component of NoSB. A** Schematic of the NUMR-1 protein structure. **B** DIC and fluorescence microscopy images of hypodermal nuclei after actinomycin D treatment and RNAi knockdown of *exos-9* and *rpl-14* in the indicated animals expressing the GFP::NUMR-1 (scale bar, 5 μm). The yellow arrows indicate NoSB. The images were taken by the Leica THUNDER imaging System. **C** Quantification of NoSB in indicated tissues with indicated treatment. Every dot

indicates the percentage of cells containing NoSB in hypodermis cells (red), intestine cells (blue), germline cells (black), and oocyte cells (purple). *n*, the number of independent animals tested, see also Supplementary Fig. 16A–D. The horizontal line represents the median value, the lower and upper quartiles represent the 25th and 75th percentile, and the whiskers show the maximum and minimum values. Source data are provided as a Source Data file.

microscopy indicated that NUMR-1 foci colocalized with NoSB (Fig. 8B). In addition, RPOA-1 is the core subunit of Pol I and contributes to the polymerase activity. Feeding GFP::NUMR-1 animals with dsRNAs targeting *rpoa-1* induced the formation of NoSB (Supplementary Fig. 15B, C), which is consistent with the result that inhibiting Pol I activity by actinomycin D elicited NoSB formation. As expected, the *nosr-1* mutation inhibited GFP::NUMR-1 expression (Fig. 8B and Supplementary Fig. 15A).

We also quantified the percentage of NoSB formation in different tissues (Fig. 8C and Supplementary Fig. 16A–D). NoSB was mostly found in the hypodermal and intestinal cells, but rarely noticeable in germline and oocyte cells upon nucleolar stress.

To test whether NUMR-1 alone is sufficient to direct NoSB formation, we generated a *fib-1p::*NUMR-1::GFP::*fib-1utr* transgene to constitutively express NUMR-1 in all tissues. The ectopically expressed *fib-1p::*NUMR-1::GFP evenly diffused in the nucleoplasm without condensation into NoSB in the absence of nucleolar stress (Supplementary Fig. 17A, B). The nucleolar stress treatment via actinomycin D or *exos-9* RNAi induced the NoSB accumulation of *fib-1p::*NUMR-1::GFP, similar to the endogenous *numr-1* promoter-driven NUMR-1::GFP.



Fig. 9 | **NoSB did not reveal obvious liquid-liquid phase separation property. A** Z-stack images of the *exos-10(ust242)*; GFP::NUMR-1 worms with a step size of 0.5 μm. **B**, **C** (Top) FRAP assay of GFP::NUMR-1 transgene in indicated regions after actinomycin D treatment or in *exos-10(ust242)* mutants. (Bottom) Quantification of

FRAP data. Mean \pm SD, n = 3 independent animals. Scale bar, 2 µm. All images were taken by the Zeiss LSM980 confocal microscope. Source data are provided as a Source Data file.

Interestingly, this constitutively expressed *fib-1p:*:NUMR-1::GFP did not rescue *nosr-1* mutation and failed to direct NoSB formation upon nucleolar stress. In the *nosr-1(-); fib-1p::NUMR-1::GFP::fib-1utr* animal, we did not observe noticeable condensation of NUMR-1::GFP and formation of NoSB. We speculated that there are additional NOSR-1 dependent factors besides NUMR-1 to synergistically mediate NoSB formation, therefore the constant expression of NUMR-1 only is not sufficient to rescue all *nosr-1(-)* defects. The identification of the components, both RNAs and proteins, in NoSB will greatly help to clarify the function and regulation of NoSB.

Together, these data suggest that NUMR-1 is a downstream target of NOSR-1 and is a crucial component of NoSB. Additional factors, besides NUMR-1, could act downstream of NOSR-1 and mediate NoSB formation, as NUMR-1::GFP failed to replace NOSR-1 functionality in terms of forming NoSB.

NoSB may not obviously contain liquid-liquid phase separation (LLPS) properties

To test whether NUMR-1 can undergo phase separation, we first visualized the fine structure of NoSB by collecting Z-stack images of GFP::NUMR-1 in NoSB (Fig. 9A). GFP::NUMR-1 revealed even distribution throughout NoSB, and did not display noticeable subcompartments.

Then, we induced NoSB formation via actinomycin D treatment or *exos-10* depletion and performed fluorescence recovery after photobleaching (FRAP) assays (Fig. 9B, C). However, we failed to observe the recovery of GFP::NUMR-1 foci after photobleaching. These data

suggested that NoSB may not obviously contain liquid-liquid phase separation (LLPS) properties.

To explore the recovery dynamics of NoSB from nucleolar stress, we treated worms with actinomycin D for 48 hours, then transferred the stressed worms to normal growth media without actinomycin D, and quantified the percentage of NoSB thereafter (Supplementary Fig. 18A–C). As expected, the percentage of NoSB, as well as the expression of GFP::NUMR-1 gradually decreased upon the removal of actinomycin D (Supplementary Fig. 18B, C), suggesting that constant nucleolar stress is required for the maintenance of NoSB condensation.

Using GFP::NUMR-1 as the marker, we tested again whether other kinds of environmental conditions besides nucleolar stress could induce the formation of NoSB. However, growing animals at 15 or 25 °C, heat shock at 37 °C, or stravation failed to induce the formation of NoSB (Supplementary Fig. 19A, B, also see Supplementary Fig. 3E–G). Yet occasionally, we observed one or two small GFP::NUMR-1 foci in a few animals at normal growing conditions as well as under certain environmental stresses (Supplementary Fig. 19A, B), which were hardly detectable by DIC.

Cadmium stress-induced NUMR-1 expression and *nosr-1*-independent NoSB formation

NUMR-1 (nuclear-localized metal responsive) was previously identified as an SR-like protein that promotes cadmium tolerance^{64–66}. Cadmium was shown to increase *numr-1/-2* mRNA expression in pharyngeal and intestinal cells. Cadmium arrests larval development and decreases



Fig. 10 | **Cadmium stress-induced NoSB formation in a** *nosr-1*-independent **manner. A** DIC and fluorescence microscopy images of hypodermal and intestine nuclei after cadmium treatment in the indicated animals expressing the GFP::NUMR-1. Arrows indicate NoSB (scale bar, 5 µm). All images were taken by the Leica THUNDER imaging System. **B** Quantification of NoSB in hypodermic cells and intestine cells from (**A**). Every dot indicates the percentage of hypodermis or intestine cells containing NoSB in each worm. Red dots indicate wild-type animals,

blue dots *nosr-1(ust303)* mutants, green dots *nosr-1(ust308)* mutants. *n*, the number of animals tested. The horizontal line represents the median value, the lower and upper quartiles represent the 25th and 75th percentile, and the whiskers show the maximum and minimum values. A two-tailed *t*-test was performed to determine statistical significance. **C** A working model of NoSB formation under nucleolar stress in *C. elegans*. Source data are provided as a Source Data file.

body size, and these effects were exacerbated by *numr-1/-2* RNAi⁶⁴. We then tested whether cadmium exposure could translocate NUMR-1 to NoSB.

As expected, the treatment with cadmium increased the expression of GFP::NUMR-1 (Supplementary Fig. 20A). In addition, cadmium induced the aggregation of GFP::NUMR-1 in the nucleus in intestine cells, but not in the hypodermis (Fig. 10A, B). Interestingly, the cadmium-induced NUMR-1 expression and condensation did not dependent on *nosr-1*. In *nosr-1* mutants, cadmium could still stimulate NUMR-1 expression and aggregation (Fig. 10A, B and Supplementary Fig. 20A), suggesting a *nosr-1*-independent pathway is involved in the formation of cadmium-induced NUMR-1 condensation.

https://doi.org/10.1038/s41467-024-51693-z

Discussion

Environmental stresses not only alter gene expression but also trigger structural changes in cells. However, how distinct subcellular structures respond to stresses remains largely mysterious. Here, we showed that nucleolar stress induced the formation of a distinct subnuclear organelle, nucleolar stress body (NoSB), which is regulated by a bZIP transcription factor, NOSR-1. Further experiments identified that NUMR-1 is the critical component of NoSB, is induced by NOSR-1 and is per se required for NoSB formation (Fig. 10C), suggesting that NUMR-1 may be involved in building up the scaffold and recruiting NoSB components.

Failures in ribosome biogenesis or functions result in a condition termed nucleolar stress, which ultimately leads to disruptions in cell homeostasis. The RNA exosome is a conserved, multi-subunit ribonucleolytic complex that contributes to the 3' to 5' processing and degradation of RNAs in eukaryotic cells⁷¹. All RNA exosome components are required during the 3' processing of the 5.8S rRNA⁷². The disruption of RNA exosomes resulted in the deficiency of rRNA processing, which may ultimately lead to nucleolar stress.

In mammals, the tumor suppressor protein p53/CEP-1 is considered an essential factor in monitoring nucleolar stress and the integrity of ribosome biogenesis²⁰. Additional pathways have also been identified to respond to nucleolar stresses in the absence of functional p53^{73,74}. The ubiquitin E3 ligase MDM2 negatively regulates p53 by marking it for ubiquitin-mediated proteasomal degradation. In plant cells, nucleolar stress activates the expression of ANAC082 by releasing ribosome stalling at the upstream open reading frame (uORF)³¹. These multiple layers of regulation enable transcription factors to execute cellular responses to specific nucleolar stress conditions. Here, we found that the disruption of RNA exosomes or nucleolar stress activates the expression of NOSR-1 and identified an uncharted NOSR-1/NUMR-1-dependent pathway that links nucleolar stress with the formation of NoSB. However, how the NOSR-1/NUMR-1 axis senses perturbed ribosome biogenesis and nucleolar disorders is unclear. Further investigation is required for searching the factors that activated the NOSR-1/NUMR-1 pathway upon the disruption of RNA exosome or nucleolar stress.

NUMR-1 is upregulated by NOSR-1 and is required for NoSB formation upon nucleolar stress, suggesting that NUMR-1 functions downstream of NOSR-1. We also identified a diverse set of mRNAs that are upregulated by NOSR-1 but do not participate in NoSB formation, indicating that NOSR-1 may be involved in a variety of additional cellular processes. Further experiments, for example, chromatin immunoprecipitation followed by deep sequencing (ChIP-seq), are required to identify the direct targets of NOSR-1.

NUMR-1 encodes RRM and serine- and arginine-rich regions that are found in canonical SR proteins⁶⁴. Many SR proteins have reported functions in protein/RNA nucleation. For example, the lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) localizes to nuclear speckles where it interacts with SR proteins⁷⁵. SRSF1 and SRSF9 assemble nSBs when cells are exposed to high temperatures⁷⁶. Some SR proteins (SRSF1, SRSF2, SRSF3, SRSF7, and SRSF10) are recruited to SGs along with nontranslated mRNAs⁷⁷. Whether and how NUMR-1 uses a similar mechanism to promote NoSB formation requires further investigation.

RNAs, especially noncoding RNA (ncRNA), may act as the molecular scaffold during the formation of phase-separated subcellular organelles, provide multivalent sites to bind one or more RNA binding proteins (RBPs), and drive the occurrence of phase separation. For example, our recent work showed that 27SA₂ rRNA intermediate could promote nucleolar reshaping⁴¹. Ribosome deficiency may lead to the accumulation of erroneous RNAs and abnormal rRNA intermediate accumulation. The RRM regions in NUMR-1 implies its potential to interact with these RNA molecules and, therefore, elicit the formation of NoSB. Yet whether or how ncRNAs or specific types of ncRNAs are involved in NoSB formation is unclear. Since the Argonaute protein NRDE-3, which binds endogenous 22 G RNAs, did not accumulate in NoSB (Supplementary Fig. 2D), we guess that it is very unlikely that siRNAs or their targeted mRNAs could enrich in NoSB. In addition, the ribosomal subunit proteins also did not enrich in NoSB (Supplementary Fig. 5A, B), implying that rRNAs may not enrich in NoSB either. In the future, we will try to identify the types of RNAs in NoSB by utilizing ncRNA-seq and smFISH, and explore their roles in NoSB.

The components of NoSB are very intriguing. Till now, we found that (1) treating worms with actinomycin D or inactivation of RNA exosome did not directly translocate NUCL-1 or NUCL-1(Δ RGG) into NoSB. However, further treatment with NaN₃ after actinomycin D or inactivation of RNA exosome could induce an accumulation of NUCL-1 or NUCL-1(Δ RGG) to NoSB. (2) An SR-like protein NUMR-1 is specifically enriched in NoSB after nucleolar stress via actinomycin D, inactivation of RNA exosome, or cadmium treatment.

NoSB and GFP::NUMR-1 are rarely detectable in steady-state cells; however, their expression drastically increases in the hypodermal cells and intestinal cells upon nucleolar stress. We speculated that the reason for tissue-specificity of NoSB formation upon nucleolar stress may be: (1) NOSR-1/NUMR-1 axis mostly takes place in somatic cells, yet the pathway is not activated in germ cells upon nucleolar stress; (2) other soma-specific factors, including RNAs or proteins, may cooperate with NUMR-1 to engage in the formation of NoSBs.

The NoSB also disassembles following stress removal. Therefore, we speculated that NUMR-1 might recruit stress-induced proteins and RNAs to NoSB upon nucleolar stress. How NoSB interact with stress responsive proteins and RNAs is unknown. Further investigation to identify the protein and RNA components in NoSB is crucial to elucidate the functions of NoSB and the underlying mechanisms.

NaN₃ can quickly immobilize nematodes, which makes it an ideal tool to paralyze worms and is widely used in imaging experiments^{78,79}. Treating C. elegans with NaN₃ induces hypoxia and eventually leads to cell death⁸⁰. For all the proteins tested in the work, including markers for nucleolus and other subnuclear organelles, we only observed that NUCL-1 and NUCL-1(Δ RGG) aggregated to NoSB after the treatment of NaN₃ following the nucleolar stress. NUCL-1 is not required for NoSB formation. The reason is not clear yet. Nucleolin likely functions in rRNA transcription and processing. In C. elegans, NUCL-1 is located in the GC region and is required for nucleolar vacuole formation⁴¹. We did observe that double treatment of nucleolar stress followed by NaN₃ could recruit NUCL-1 to NoSB. The exact role of NaN3 in NUCL-1 recruitment to NoSB is unclear. We guess that NaN₃ treatment or hypoxia stress might change the biochemical properties of NUCL-1 or induce other stress reactions and import NUCL-1 into NoSB. In the future, we will focus on how and why NUCL-1 is used in NoSBs.

Membraneless organelles (MLOs) possess a continuum of dynamicity, ranging from liquid to solid, or even amyloid-like aggregates. Many MOLs possess liquid-like properties, and molecules diffuse in and out of MLOs and exchange with the surrounding environment. Some RNA granules including stress granules and processing bodies (P bodies) are highly dynamic and behave like liquid droplets. Yet other MLOs, for example, centrosomes, nuclear pores, and amyloid-like bodies, revealed less dynamic properties. NUMR-1 did not exhibit fluidlike dynamics by FRAP assay, suggesting that NoSB might be a relatively stable structure with a slow turnover in stressed cells.

NUMR-1 was previously identified as an SR-like protein that promotes cadmium tolerance⁶⁴⁻⁶⁶. Cadmium arrests larval development and decreases body size, and these effects were exacerbated by *numr*-*1/2* RNAi⁶⁴. Cadmium was shown to increase *numr-1/2* mRNA expression. Therefore, we speculated that NUMR-1/2 and NoSB may involve in protecting animals from adverse environmental conditions. The identification of the components, both RNAs and proteins, in NoSB will greatly help to clarify the function and regulation of NoSB.

Methods

Strains

Bristol strain N2 was used as the standard wild-type strain. All strains were grown at 20 °C unless otherwise specified. The strains used in this study are listed in Supplementary Data 2.

Construction of transgenic strains

For ectopic transgene expression of *nosr-1_p::NLS::GFP*, the NOSR-1 promoter and UTR were amplified from N2 genomic DNA. The EGL-3 NLS sequence was amplified from N2 genomic DNA. A GFP::3xFLAG sequence was PCR amplified from SHG326 genomic DNA. The vector fragment was PCR amplified from the plasmid pSG274. These fragments were joined together by Gibson assembly to form the repair plasmid with the ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech, Nanjing, China, Cat. No. C113-O1/O2). The transgene was integrated into *C. elegans* chromosome I via a modified counterselection (cs)-CRISPR method⁶⁰. The sequences of the primers are listed in Supplementary Data 3.

For in situ knock-in transgenes, the 3xFLAG::GFP sequence was PCR amplified from YY178 genomic DNA. The GFP::3xFLAG sequence was PCR amplified from SHG326 genomic DNA. The tagRFP coding sequence was PCR amplified from YY1446 genomic DNA. Homologous left and right arms (1.5 kb) were PCR amplified from N2 genomic DNA. The backbone was PCR amplified from the plasmid pCFJ151. All these fragments were joined together by Gibson assembly to form the repair plasmid with the ClonExpress MultiS One Step Cloning Kit. The plasmid was coinjected into N2 animals with three sgRNA expression vectors, 5 ng/ μ l pCFJ90, and the Cas9 II-expressing plasmid. The sequences of the primers are listed in Supplementary Data 4.

Construction of deletion mutants

For gene deletions, triple sgRNA-guided chromosome deletion was conducted⁶⁰. To construct sgRNA expression vectors, the 20 bp *unc-119* sgRNA guide sequence in the pU6::unc-119 sgRNA(F + E) vector was replaced with different sgRNA guide sequences. Addgene plasmid #47549 was used to express Cas9 II protein. Plasmid mixtures containing 30 ng/µl of each of the three or four sgRNA expression vectors, 50 ng/µl Cas9 II-expressing plasmid, and 5 ng/µl pCFJ90 were coinjected into N2 animals. Deletion mutants were screened by PCR amplification and confirmed by sequencing. The sgRNA sequences are listed in Supplementary Data 5.

Imaging and quantification

Images were collected on a Leica DM4 B microscope. The nucleolar area and NoSB area were quantified manually with the freehand tool using Fiji software.

Actinomycin D treatment

Actinomycin D (MedChemExpress no. HY-17559, CAS: 50-76–0) was prepared at 20 mg/ml in DMSO as a stock solution. The actinomycin D stock solution was diluted to 5 to 30 μ g/ml with concentrated OP50. NGM plates were prepared and placed at room temperature overnight before use. Synchronized L1 worms were placed onto the seeded plates and grown for 48 h before imaging.

RNAi

RNAi experiments were performed at 20 °C by placing synchronized embryos on feeding plates⁸¹. HT115 bacteria expressing the empty vector L4440 (a gift from A. Fire) were used as controls. Bacterial clones expressing dsRNAs were obtained from the Ahringer RNAi library and sequenced to verify their identity. Some bacterial clones were constructed in this work, which are listed in Supplementary Data 1. Images were collected using a Leica DM4 B microscope.

Forward genetic screening

To identify factors prohibiting the formation of NoSB, we chemically mutagenized the *mCherry::DIS-3* strain by ethyl methanesulfonate (EMS), followed by a clonal screen. The F2 progeny worms were visualized under a fluorescence microscope at the L4 stage. Two mutants that exhibited NoSB formation were isolated from two thousand haploid genomes. *exso-10 (ust242)* and *exos-4.2 (ust243)* were identified by genome resequencing.

To identify factors required for the formation of NoSB, we chemically mutagenized the *exso-10(ust242);mCherry::DIS-3* strain by ethyl methanesulfonate (EMS), followed by a clonal screen. The F2 progeny worms were visualized under a fluorescence microscope at the L4 stage. One mutant that disrupted NoSB formation was isolated from two thousand haploid genomes. *nosr-1(ust303)* was identified by genome resequencing.

Postfix Oil Red O staining and bright field imaging

Briefly, L4 stage animals were fixed in 1% paraformaldehyde/PBS for 30 min with rocking. Samples were then frozen on dry ice/ethanol and thawed with running tap water three times. Samples were washed three times with 1× PBS, dehydrated in 60% isopropanol for 2 min, and stained with 0.5 ml 60% Oil Red-O (#A6000395-50G, BBI) working solution for 30 min with rocking. Oil Red O stock solution was dissolved in isopropanol at a concentration of 0.5 g/100 ml and equilibrated for several days. The Oil Red-O working solution was prepared fresh by mixing 60% volume of stock with 40% volume of water. The mix was equilibrated for 10 min and then filtered with a 0.22- μ m syringe filter. Stained samples were then washed three times with 1× PBS, rehydrated in 1× PBS, and mounted in 1× PBS onto agarose-padded slides for imaging.

RNA isolation

Synchronized L4 worms were incubated with TRIzol (Invitrogen) reagent followed by five quick liquid nitrogen freeze-thaw cycles. RNA was precipitated by isopropanol followed by DNasel digestion (Thermo Fisher).

mRNA deep-sequencing

The Illumina-generated raw reads were first filtered to remove adapters, low-quality tags, and contaminants to obtain clean reads at Novogene. The clean reads were mapped to the reference genome of WBcel235 via HISAT2 software (version 2.1.0)⁸². Differential expression analysis was performed using custom R scripts. A twofold-change cutoff was applied when filtering for differentially expressed genes. All plots were drawn using custom R scripts.

qRT-PCR

cDNAs were generated from RNA using HiScript III RT SuperMix for qPCR (Vazyme), which includes a random primer/oligo(dT)20VN primer mix for reverse transcription. qPCR was performed using a MyIQ2 real-time PCR system (Bio-Rad) with AceQ SYBR Green Master mix (Vazyme). The primers used in qRT–PCR are listed in Supplementary Data 6.

Brood size

L4 hermaphrodites were singled onto NGM plates and transferred daily as adults until embryo production ceased, and the progeny numbers were scored.

Lifespan assay

Worm populations were synchronized by placing young adult worms on NGM plates for 4–6 h and then removing them. The hatching day was counted as day 1 for all lifespan measurements. Worms were transferred every other day to new plates to eliminate confounding progeny. Animals were scored as alive or dead every day. Worms were scored as dead if they did not respond to repeated prods with a platinum pick. Worms were censored if they crawled off the plates or died from vulval bursting and bagging. For each lifespan assay, >50 worms were used.

Hydrogen peroxide assay

Ten synchronized worms on day 1 of adulthood were transferred to each well, which contained 1 ml of worm S-basal buffer with various concentrations of H_2O_2 in a 12-well plate at 20 °C. Four hours later, 100 µl of 1 mg/ml catalase (Sigma, C9322) was added to neutralize H_2O_2 , and the mortality of worms was scored.

Heat-shock assay

Ten synchronized hermaphrodites on day 1 of adulthood were transferred to new NGM plates and incubated at 35 °C, and their mortality was checked every 1 h until there were no living worms.

Starvation assay

L4 worms were transferred to empty NGM plates without any bacterial lawn for 12 h, and then the animals were imaged.

Cadmium treatment

Synchronized L1 worms were fed with OP50 on NGM agar plates containing 0, 100, or $300\,\mu\text{M}$ of cadmium chloride for 48 h before imaging.

Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed using a Zeiss LSM980 laser scanning confocal microscope at room temperature. Worms were anesthetized with 2 mM levamisole. A region of interest was bleached with 70% laser power for 3–4 s, and the fluorescence intensities in these regions were collected every 5 s and normalized to the initial intensity before bleaching. For analysis, image intensity was measured by mean and further analyzed by GraphPad Prism 9.0 software.

Z-stack imaging experiment

Z-stack imaging experiments were performed using a Zeiss LSM980 laser scanning confocal microscope. Worms were anesthetized with 2 mM levamisole. By capturing the filtered emissions within the 495–590 nm range, with steps of 0.5 μ m, the images per section in a z-stack were acquired.

Statistics and reproducibility

For each microscopy result in Figs. 2F, 3B, D, F, 6B, 7F and Supplementary Figs. 2A, C, D, 3C–G, 5A, 6A–E, 7A–D, independent experiments were performed three times.

The mean and standard deviation of the results are presented in bar graphs with error bars. All experiments were conducted with independent *C. elegans* animals for the indicated number (N) of times. Statistical analysis was performed with the two-tailed Student's *t*-test as indicated. GraphPad Prism 9 or R scripts were used for statistical analysis.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files, and that all additional data are publicly available. The high throughput data reported in this paper have been deposited in the Genome Sequence Archive in the National Genomics Data Center (China National Center for Bioinformation/Beijing Institute

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Acknowledgements

We are grateful to the members of the Guang lab for their comments. We are grateful to the International *C. elegans* Gene Knockout Consortium and the National Bioresource Project for providing the strains. Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). This work was supported by grants from the National Key R&D Program of China (2022YFA1302700 to S.G., and 2019YFA0802600 awarded to X.F.) and the National Natural Science Foundation of China (32230016 awarded to S.G., 32270583 awarded to C. Z., 32070619 awarded to X.F., 2023M733425 awarded to X.H., and 32300438 awarded to X.H.), and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB39010600 awarded to S.G.), the Research Funds of Center for Advanced Interdisciplinary Science and Biomedicine of IHM (QYPY20230021 awarded to S.G.) and the Fundamental Research Funds for the Central Universities awarded to S.G.

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Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-024-51693-z.

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Peer review information *Nature Communications* thanks the anonymous reviewer(s) for their contribution to the peer review of this work. A peer review file is available.

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