Genetic control of nucleolar size: An evolutionary perspective

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ABSTRACT Exploiting a C. elegans mutant (ncl-1) exhibiting nucleolar abnormalities, we recently identified the let-7/ncl-1/br-1 genetic cascade underlying proper rRNA abundance and nucleolar size. These 3 factors, let-7 (a miRNA), NCL-1 (a member of the TRIM-NHL family), and brillarin (a nucleolar methyltransferase), are evolutionarily conserved across metazoans. In this article, we provide several lines of bioinformatic evidence showing that human and Drosophila homologues of C. elegans NCL-1, TRIM-71 and Brat, respectively, likely act as translational suppressors of brillarin. Moreover, since their 3'-UTRs contain putative target sites, they may also be under the control of the let-7 miRNA. We hypothesize that let-7, TRIM and brillarin contribute activities in concert, and constitute a conserved network controlling nucleolar size in eukaryotes. We provide an in-depth literature review of various molecular pathways, including the let-7/ncl-1/br-1 genetic cascade, implicated in the regulation of nucleolar size.

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Introduction

The most prominent structure inside the nucleus is the nucleolus, or “small nucleus,” which was first described in 1835 and later coined in 1838. By 1953, the ultrastructure of this membrane-less organelle was revealed by electron microscopy to be comprised of 3 parts: the dense fibrillar component (DFC), the fibrillar center (FC) and the granular component (GC) (for a historical review, see 1). Since 1962, the nucleolus has been known to be the site of ribosome biogenesis or the “factory of the ribosome,” as evidenced by isotope-tracing of rRNA synthesis and the absence of new rRNA synthesis in Xenopus mutant embryos lacking nucleoli.2 In the last 3 decades, the nucleolus has been shown to be the site of multiple new functions, including signal recognition particle assembly, small RNA modification, cell cycle control, and cell stress sensing.3,4 In addition, it is now known to act as an “incubator” for more than 20 different types of viruses, facilitating their replication and propagation.5,6 The emerging role of the nucleolus as a harbor of regulatory non-coding RNAs (ncRNAs) has also been illustrated by various studies. Works by Audas et al. have revealed that ncRNA transcribed from the nucleolar intergenic spacer capture and immobilize proteins containing a nucleolar detention sequence (NoDS) within the nucleolus in response to diverse stimuli.7 Moreover, recent findings show that more than 10 microRNAs (miRNAs), including miR-664, miR-24, and miR-21—as well as some target mRNAs—reside in the nucleolus, further expanding the number of known nucleolar functions.8-10 Within the last 20 y, Lamond and his colleagues have also identified ~700 nucleolar proteins by powerful mass spectrometric tools. Among them, 20% to 32% are associated with ribosomal proteins and/or ribosome biogenesis; however, 32% are novel and uncharacterized.11,12 Taken together, these data reveal
that the nucleolus is a multifunctional organelle, and that additional functions may be identified as the >200 novel resident proteins are further characterized.

The nucleolus exhibits dynamic morphological changes when cells enter mitosis. A gradual disappearance of the nucleolus is observed in prophase, while nucleolus assembly (nucleologenesis) is seen at the beginning of telophase. The molecular control of nucleolus disassembly or assembly is associated with the inactivation or activation of rRNA transcription by Pol I, respectively. In this context, the Pol I transcriptional machinery and RNP processing complex are under the control of cell cycle checkpoint components CDK-1, cyclin B kinase, and PP1 phosphatases (for a review, see 13). During nucleolus disassembly, the nucleolar proteins for pre-RNA processing exit the DFC and GC prior to the cessation of rDNA transcription. By contrast, nucleologenesis entails several distinct steps. First, rRNA transcription is initiated in the rDNA cluster domains called nucleolar organizing regions (NOR) at the same time that early and late nucleolar processing proteins accumulate in the pre-nucleolar bodies (PNBs). Next, the PNBs are targeted to the NOR, the DFC and GC are assembled, and the PNBs become indistinguishable prior to the formation of a mature nucleolus.13

How is nucleolar size controlled?

The mechanism by which the size and scale of organisms, cells, or organelles are controlled is a longstanding and fundamental question in biology. Numerous studies in different model systems have provided important pieces to this puzzle. In 1970, Miller and Gurdon demonstrated that the size of the nucleolus is smaller in Xenopus mutants carrying a haploid rDNA gene.14 Nucleolar size has also been reported to be influenced by the environment or nutrient availability. For example, the nucleolar size of liver cells is changed when rats are subjected to partial hepatectomy or fed a methionine-free diet.15 Notably, most cancer cells share the feature of enlarged nucleoli. Abnormal nucleolar size and number can be used by pathologists as a cancer marker and to classify malignancy degree.16 Since enlarged nucleoli reflect increased ribosome biogenesis and nuclear DNA synthesis, cells with a larger nucleolus should have larger volumes of the nucleus and cytoplasm. Using the fission yeast model, 2 groups independently demonstrated that nuclear size is determined by the amount of cytoplasm, following a nucleus to cytoplasm (N/C) ratio.17,18 Neumann and Nurse further extended their study to determine a nucleolus to nucleus (No/N) ratio that governs the size of the nucleolus.18 This appears to be true in the case of human sensory ganglia neurons in which the sizes of nuclei and nucleoli increase with cell size.19

For both membrane-enclosed and membrane-less organelles, being proportional in size to the overall dimensions of the cell is called “organelle size scaling.”20 Chan and Marshall proposed the existence of size-sensing control mechanisms either involving direct measurements by sensor molecules or indirect functional readouts.21 Fibrillarin, a nucleolar methyltransferase conserved from archaea to eukaryotes,22 likely serves as one such molecular sensor for regulating nucleolar size.23 Brangwynne and his colleagues introduced the notion that membrane-less nuclear bodies, including the nucleolus, behave like “liquid-phase droplets” and that “phase separation” is a general mechanism for their assembly.24 By using fibrillarin-fused green fluorescence protein (FIB-1::GFP) as a reporter, they demonstrated that nucleolar assembly is controlled by a concentration-dependent phase transition in C. elegans embryos.25 In addition, they showed that rRNA transcription is important to create a thermodynamically favorable condition for the nucleation of nucleolar components at the NOR.26 The finding that fibrillarin can methylate histone H2A at the rDNA loci,27 thereby promoting Pol I transcriptional activity, is in line with the scenario that concentration of fibrillarin plays an important role in regulation of nucleolar activity and size.

Molecules linked to the assembly of membrane-less nuclear bodies are hypothesized to contain a domain of “low sequence complexity” (LCS), such as RG, QN and YG amino acid repeats, for interaction with multiple associating proteins and/or RNAs.28 The LCS domain, a signature feature of intrinsic fibrillarins as well as in C. elegans DAO-5/Nopp140, a nucleolar protein to facilitate Pol I transcription activity and its human homolog Nolc130 (Fig. 1). Hepatitis D viral antigen (HDAg), which is constantly observed in the nucleolus upon infection, has also been shown to be an intrinsically
disordered protein. Interestingly, HDAG is perfectly co-localized with fibrillarin in HeLa cells but not in C. elegans intestinal cells, possibly reflecting distinct nucleolar constituents or other host differences between worms and humans.

In an attempt to systematically map the species-specific genetic networks that regulate nucleolar size and influence Pol I-mediated transcription, Neumuller et al. recently employed D. melanogaster and S. cerevisiae models to identify conserved or non-conserved molecular complexes. Their data indicated that the TRAMP complex (Trf4/Air2/Mtr4p polyadenylation complex) as well as other molecular complexes participating in histone acetylation; poly(A)+ mRNA export; or ER-to Golgi vesicle-mediated transport are evolutionarily conserved regulators of nucleolar size. In a genome-wide RNAi screen in Drosophila, they also found that loss of rpl23, sip3a and brat led to enlarged nucleoli whereas loss of tif1a, nopp140 and fibr reduced nucleolar size. The mechanisms employed by brat and fibrillarin to regulate size were not identified. Our recent work extended their findings by revealing that NCL-1, a C. elegans homolog of Drosophila Brat, is in fact a translational suppressor of fibrillarin, and its expression is regulated by the let-7 miRNA.

A genetic cascade of let-7/ncl-1/fib-1 regulates nucleolar size in C. elegans

There are several advantages to using C. elegans as a model for studying nucleolar size regulation. The adult worms are composed of ~1000 somatic cells with well-characterized cell types and lineages. They have a transparent body and can be easily cultured in laboratories. Furthermore, their cells display various sizes of nucleoli ranging from less than 1 to ~5–7 microns. Intriguingly, nucleoli are undetectable in the ~1 oocyte, which is adjacent to the spermatheca and blastomeres of early embryos. A previously identified ncl-1 mutant with enlarged nucleoli in nearly all cells could serve as a valuable tool for determining the mechanism of nucleolar size control as well as for identifying upstream and downstream genes involved in the process.

In our recent work, we showed that the amount of C. elegans fibrillarin (FIB-1) is correlated with...
Figure 2. *ncl-1* is an upstream translational suppressor of *fib-1* and *dao-5*. The amount of FIB-1 and DAO-5 present can be correlated with the size and function of the nucleolus. (A) Comparison of nucleolar size in embryos of various genetic backgrounds at the 4–cell stage. The nucleolus of ABp cell is highlighted and magnified in the insets. Scale bar, 20 μm. (B) Western blot analysis shows the amount of fibrillarin (FIB-1), which is correlated with the nucleolar size shown in (A). Actin serves as the loading control. (C) Quantitative representation of the nucleolar size from (A). Figures (A–C) are partially adapted and modified from Yi et al., (2015)²³ with permission of PLoS Genetics. (D) Total RNAs were extracted from embryos of wild-type (N2) and *ncl-1* mutants and fractionated by centrifugation in a sucrose gradient for polysome profile analysis. The amounts of *fib-1* and *dao-5* mRNA in each fraction were then determined by RT-qPCR and indicated by solid line (N2) or dotted line (*ncl-1*). The ribosome fractions from low to high sucrose gradients are indicated from left to right, representing a transition from monosomes to polysomes.
nucleolar size, and that fib-1 acts directly downstream of ncl-1. In wild-type embryos with no observable nucleoli, a smaller amount of FIB-1 was observed compared to ncl-1 mutant embryos, in which nucleoli were clearly detected (Fig. 2A and B). When C. elegans ncl-1 mutants were fed with dsRNA-expressing bacteria to knockdown fib-1 expression, nucleolar size in embryos decreased (Fig. 2A and C), similar to observations in a Drosophila cell culture–based RNAi screen. In addition, we demonstrated that NCL-1 cooperates with 2 RNA-binding post-transcriptional regulators, Pumilio (PUM or PUF) and NANOS, to suppress FIB-1 translation by binding to the fib-1 3’-UTR. When the PUF (PUM) binding sequences in the fib-1 3’-UTR were mutated, FIB-1::GFP expression was up-regulated approximately 3-fold.

Interestingly, absence of Nopp140/dao-5 expression in ncl-1 embryos also led to a reduced nucleolar size (Fig. 2A and C), suggesting that NCL-1 may regulate size by suppressing both fib-1 and dao-5 expression. To test whether NCL-1 is a translational suppressor of fib-1 and dao-5, we performed polysome profile analysis of wild-type and ncl-1 embryonic lysates and analyzed fractionation of fib-1 and dao-5 mRNAs. As shown in Fig. 2D, higher levels of fib-1 and dao-5 mRNA were detected in the lighter fractions for wild-type embryos, which represent monosomes and messenger ribonuclear proteins. The distribution of fib-1 or dao-5 mRNAs shifted to the heavier fractions for ncl-1 mutant embryos, strongly indicating that NCL-1 is a translational suppressor of fib-1 and dao-5 expression.

While searching for upstream factors that regulate ncl-1 expression, our bioinformatic analyses uncovered target sequences for 2 miRNAs, let-7 and mir-49, in the ncl-1 3’-UTR. We examined the expression of GFP reporters fused with ncl-1 3’-UTRs carrying wild-type or mutated let-7 binding sequences and detected a higher GFP level in animals with the latter. Fluorescence microscopic examination confirmed these GFP reporter expression patterns in seam cells and the vulva, which are known to express higher levels of let-7. Further confirmation of the possibility that ncl-1 is regulated by let-7 was obtained by assessing nucleolar size in vulva cells of a temperature-sensitive hypomorphic let-7(n2853) mutant. Compared to animals grown at the permissive temperature (15°C), we observed a 25% decrease in nucleolar size at the non-permissive temperature (25°C). No discernible difference in nucleolar size was detected when a let-7; ncl-1 double mutant was used for the assay (Fig. 3A and B).

Figure 3. let-7 regulates nucleolar size in vulva cells by controlling fibrillarin expression. (A) DIC microscopy of the vulva cells of let-7 (n2853) and let-7(n2853); ncl-1(e1942) worms, at the permissive (15°C) or non-permissive (25°C) temperatures. Insets represent enlarged images of the boxed regions in the corresponding figures. Arrowheads point to the nucleoli of the vulva cells. Scale bar, 10 μm. (B) Quantitative representation of the results shown in (A), illustrating the nucleolar sizes in the vulva. Asterisks signify the difference in sizes observed at the indicated temperatures, where ***P < 0.001, n = 22–36 for let-7(n2853). NS = not significant, n = 100–110 for ncl-1(e1942); let-7(n2853). (C) Western blot analysis of FIB-1 and an Actin control in the indicated strains. Numbers below represent the relative levels of FIB-1 protein expression (normalized to the control sample of each pair-wise comparison). Adapted from Yi et al., (2015) with permission from PLoS Genetics.
Fibrillarin was also expressed at consistently higher levels in \textit{let-7(n2853)} worms grown at 15°C compared to those grown at the restrictive temperature (25°C), but only a minor difference in expression was seen in the double mutants grown at each temperature (Fig. 3C). Collectively, our findings showed that \textit{let-7}, \textit{ncl-1}, and \textit{fib-1} function together as a novel genetic pathway to regulate nucleolar size in \textit{C. elegans}.

**Conservation of genetic pathways in regulation of nucleolar size**

While the fibrillarin gene is structurally unique in most metazoans, the homologues of NCL-1 are divergent from each other in various organisms. NCL-1 homologous proteins belong to the TRIM/RBCC/NHL (NCL-1, HT2A, and LIN-41) family characterized by the presence of a RING domain, a B-box zinc finger, and a coil-coiled domain. Proteins in the NHL family are divided into 2 groups (groups 1 and 2) based on their C-terminal domain sequences. The TRIM proteins in group 2 possess a C-terminal SPRY domain and are absent in invertebrates, whereas the proteins in group 1 possess a variety of C-terminal domains, including NHL, PHD, and MATH, and are present in both vertebrates and invertebrates. The NHL domain folds into 6-bladed \(\beta\) propellers, a structure well known for protein binding. Many TRIM-NHL proteins have now been found to bind to single-stranded RNA. A Brat (\textit{Drosophila} homolog of worm NCL) binding sequence motif (UCGUUG designated as an NHL site) that is distinct from the Pumilio (PUM) binding sequence (UGUAUAUA designated as a PUM site) was recently identified, providing initial evidence that Brat/NCL-1 can directly target mRNA transcripts to suppress gene expression.

To this end, we identified adjacent NHL and PUM sites in the \textit{C. elegans fib-1} 3′-UTR (Fig. 4A) as well as in the \textit{Drosophila} and human \textit{fib-1} 3′-UTRs. We also discovered multiple NHL sites and a PUM site in the \textit{C. elegans dao-5} 3′-UTR and in the \textit{Drosophila Nopp140} and human \textit{Nolc1} 3′-UTRs (Fig. 4B). These

![Figure 4](image_url)

**Figure 4.** Evolutionary conservation among \textit{C. elegans}, \textit{Drosophila} and human of the regulatory cascade including \textit{let-7}, TRIM/NHL, and fibrillarin as well as \textit{dao-5/nopp140/Nolc1}. (A and B) The presence of PUM and NHL sites in \textit{fib-1/fib/fbl} 3′-UTR (A) and in \textit{dao-5/nopp140/Nolc1} 3′-UTR (B) is depicted. (C) The \textit{let-7} binding sites in \textit{cel-ncl-1}, \textit{dmel-brat} and \textit{hsa-TRIM71} are indicated. \textit{let-7} binding sites on \textit{hsa-TRIM71} have also been reported previously.
results suggest that dao-5/nopp140 may also be direct targets of NCL-1, expanding the potential role of NCL-1 in translational regulation across species. To further explore the possible parallels between the C. elegans let-7/ncl-1/fib-1 cascade and related genes in Drosophila and human, we analyzed the brat and TRIM-71 3’-UTRs and found let-7 binding sites in both sequences (Fig. 4C). Taken together, we propose that let-7/trim/fib-1 constitutes a conserved genetic network to control nucleolar size.

Interestingly, this pathway is reportedly linked to animal development, lending further support to its functional significance and conservation. During early embryonic development, LIN-28, which is an essential regulator of let-7 biogenesis and a pluripotency factor, mediates nucleolus maturation. The pathway has also been implicated in tumor formation. Phylogenic tree analysis revealed that Drosophila brat is most closely related to C. elegans ncl-1 and then to its ortholog Mei-P26. Flies without brat exhibit enlargement of nucleoli and are prone to brain tumor formation. While the ncl-1 loss-of-function mutant phenocopies the nucleoli enlargement defect and can be rescued by brat, it shows no extra cell proliferation. Based on this observation, we speculate that brat’s effect on nucleolus enlargement takes place prior to tumor formation, or that these 2 events are uncoupled. However, within the context of nucleolar size and cell proliferation, brat and p53 share many important functional attributes. Enlargement of nucleoli is the hallmark of many human tumors and can be primarily attributed to the loss of p53, and brat and p53 are each known as tumor suppressor genes. Perhaps more importantly, both proteins converge on the regulation of fib-1 expression, with p53 reportedly repressing at the level of transcription and Brat suppressing translation. Collectively, these findings imply that the complex process of nucleolar size control is governed by multiple conserved genetic networks, which likely crosstalk to each other to ensure proper cell proliferation.

**Conclusion and perspectives**

Recent studies by our group and others have reported numerous conserved complexes and networks with implications in nucleolar size control, providing important insights into the underlying molecular determinants and further reinforcing the complexity of this process. Genome-wide screens in lower eukaryotic models have facilitated the identification of relevant regulators and should be further exploited for in-depth, mechanistic understanding of its regulation. Although we have provided additional evidence to illustrate the evolutionary conservation of regulatory molecules and networks governing nucleolar size, control mechanisms are likely much more complicated in higher eukaryotes given the greater number of cell types and developmental pathways. Understanding at this level thus poses a daunting challenge, but recent advances in genome editing techniques and imaging tools should expedite our understanding of how different networks connect to one another to establish the size of nucleoli across distinct tissues and functions.

Although many new roles of the nucleolus have been explored, ribosome production and assembly have long been regarded as the most critical function of this organelle. The size of the nucleolus closely correlates with Pol I transcription activity and ribosome biogenesis, and is therefore tightly coordinated with metabolic demands of the cell. Consequently, nucleolar size is impinged on by a number of cellular growth and proliferation signal pathways, including oncogenes, such as Ras, Myc and PI3K, and tumor suppressor genes, such as p53, Rb and PTEN. However, whether nucleolar size is the driving force for tumor formation or rather a consequence of dis-regulation of cellular signaling or Pol I is still unresolved, and should be thoroughly addressed. Intriguingly, while both NCL-1 and Brat are suppressors of nucleolar size, NCL-1 is distinct in its lack of effect on cellular proliferation, as observed with the Drosophila homolog Brat. To further dissect the implications of this functional separation, genetic screens based on the ncl-1 mutant should be performed to identify putative enhancers of proliferation. Ultimately, a better understanding of the role of the nucleolus in maintaining and/or promoting tumorigenesis could have far-reaching implications with regard to the development of new anti-cancer drugs.

**Abbreviations**

NHL a family protein of NCL-1, HT-2A and LIN-41 in C. elegans

TRIM-NHL tripartite motif protein with a C-terminus of NHL- domain

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
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