ABSTRACT

Nuclear shape and size are cell-type specific. Change in nuclear shape is seen during cell division, development and pathology. The nucleus of Saccharomyces cerevisiae is spherical in interphase and becomes dumbbell shaped during mitotic division to facilitate the transfer of one nucleus to the daughter cell. Because yeast cells undergo closed mitosis, the nuclear envelope remains intact throughout the cell cycle. The pathways that regulate nuclear shape are not well characterized. The nucleus is organized into various subcompartments, with the nucleolus being the most prominent. We have conducted a candidate-based genetic screen for nuclear shape abnormalities in S. cerevisiae to ask whether the nucleolus influences nuclear shape. We find that increasing nucleolar volume triggers a non-isometric nuclear envelope expansion resulting in an abnormal nuclear envelope shape. We further show that the tethering of rDNA to the nuclear envelope is required for the appearance of these extensions.

KEY WORDS: Nucleolus, Nuclear envelope, rDNA, Heh1

INTRODUCTION

Eukaryotic cells contain their genetic material in a membrane-bound compartment called the nucleus. The nuclear membrane, which separates the nucleoplasm and the cytoplasm, is a double lipid bilayer comprising the inner nuclear membrane (INM) and the outer nuclear membrane (ONM). The ONM is continuous with the endoplasmic reticulum (ER) and shares several proteins with it. Nuclear pore complex (NPC) proteins embedded in the nuclear envelope (NE) facilitate the selective transport between the nucleus and cytoplasm (Newport and Forbes, 1987; Ungricht and Kutay, 2017). In metazoans, a meshwork of intermediate filaments, the LINC complex, RNA processing and phospholipid biosynthesis also affect nuclear morphology (Imbalzano et al., 2013; Titus et al., 2010). The nuclear envelope remains intact throughout the cell cycle. The pathways that regulate nuclear shape are not well characterized. The nucleus is organized into various subcompartments, with the nucleolus being the most prominent. We have conducted a candidate-based genetic screen for nuclear shape abnormalities in S. cerevisiae to ask whether the nucleolus influences nuclear shape. We find that increasing nucleolar volume triggers a non-isometric nuclear envelope expansion resulting in an abnormal nuclear envelope shape. We further show that the tethering of rDNA to the nuclear envelope is required for the appearance of these extensions.

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insights into this process (Casolari et al., 2004; Murthi and Hopper, 2005; Montpetit et al., 2005; Kume et al., 2017; Wang et al., 2016). Budding yeast undergoes closed mitosis, and the nuclear membrane remains intact throughout the cell cycle. Consequently, nuclei remain spherical for most of the cell cycle. At mitosis, nuclei elongate and squeeze through the bud neck, carrying one set of chromosomes to the daughter cell. At this stage, the nucleus is dumbbell shaped and divides at the end of mitosis resulting in two spherical nuclei. Mutations that lead to deviations from this round morphology have been reported in multiple studies. The absence of proteins that regulate phospholipid biosynthesis, such as Spo7, Pah1 or Nem1, leads to NE extensions in the cytoplasm referred to as ‘nuclear flares’ (Siniossoglou et al., 1998). Overexpression of the INM-associated protein Esc1 leads to nuclear bleb formation (Hattier et al., 2007). Additionally, nuclear flares have been observed in mutants that impair DNA repair, recombination, spindle function and chromosome segregation (Witkin et al., 2012).

In yeast, NE extensions, or nuclear flares, are found to occur in limited regions of the nucleus, primarily near the nucleolus. It has been proposed that the NE near the nucleolus serves as a membrane sink, because this region is particularly prone to flares and blebs (Witkin et al., 2012) Yeast cells appear to have inbuilt mechanisms to prevent the occurrence of NE distortions. The polo-like kinase Cdc5 functions to prevent the formation of nuclear flares (Walters et al., 2014). Vesicle trafficking genes, such as ARL3 and YPT6, have an indirect role in preventing nuclear flares by interacting with known nuclear-flare-preventing genes such as SPO7 and NEM1 (Siniossoglou et al., 1998; Webster et al., 2010). However, when cell expansion is constrained, the nucleus becomes bi-lobed, with bulk DNA in one lobe and the nucleolus in the other (Walters et al., 2019). It has been proposed that changes in nuclear size may manifest as an altered nuclear shape in order to maintain a constant N/C ratio (Walters et al., 2012; Mukherjee et al., 2016).

Although studies have shown that nuclear flares appear on the NE at the site of the nucleolus, it has not been investigated whether the internal nuclear compartments such as the nucleolus contribute to nuclear shape. In this study, we asked whether perturbing nucleolar function affects the NE. We find that multiple mutations that increase the nucleolar volume also perturb nuclear shape and induce flares and blebs. We find that reducing nucleolar volume reverses this phenotype. We also show that rDNA anchoring to the NE is required for the appearance of flares. Our findings imply that nuclear shape changes could be a consequence of attempts by the cell to increase nuclear volume.

RESULTS
Loss of nucleolar proteins affects nuclear envelope morphology
To ask whether the nucleolus influences nuclear architecture, we designed a focused screen for nuclear shape defects using *Saccharomyces cerevisiae*. Genes encoding nucleolar proteins in *S. cerevisiae* were shortlisted based on the presence of the keyword ‘nucleolus’ in the description of genes in SGD (https://www.yeastgenome.org/). Among a total of 35 genes, 12 non-essential genes were chosen for the screen. Strains carrying knockout mutations of these genes were screened for nuclear shape changes using live-cell imaging. Briefly, plasmids encoding fluorescently-tagged INM protein Esc1 and nucleolar protein Nop1 were transformed into wild-type (Wt) and selected deletion strains of the BY4741 background. Live-cell imaging was performed to check for abnormalities in the shape of the nucleus in these strains. Fig. 1 summarizes the scheme of the screen. In the Wt strain (BY4741),

![Fig. 1. Schematic representation of methodology employed for the screen.](image-url)

(A) Schematic depicting the mutant screen, see Materials and Methods for details. Plasmids expressing fluorophore-tagged nuclear envelope (NE) and nucleolar (NO) proteins were transformed into the nucleolar gene deletion mutants. Transformants were screened for NE shape changes by live-cell imaging. The observed NE abnormalities were scored manually and quantified, and those in which more than 15% of the cells showed changes were considered positive. (B) The number of genes screened for nuclear organization changes, and the functional classification of the genes screened positive are shown.
Esc1 appeared as a ring and Nop1 as a spot in the nucleus (Fig. 2A). However, some Wt and mutant cells showed distorted shapes and extensions in the NE and an enlargement of the nucleolus. In Wt, less than 15% of cells showed NE shape alterations and less than 10% had nucleolar enlargement (Fig. 2B; Fig. S1D). These percentages were set as thresholds, and the mutants that were scored above the threshold were considered ‘positive’ for NE or nucleolar shape irregularities. Of the 12 non-essential genes screened, mrt4Δ, ybl028cΔ, rsa3Δ and spo12Δ mutants were found to have significant irregularities (Fig. 2A). The percentage of cells that showed NE defects in each of the mutants is presented in Fig. 2B. In order to rule out that this change in shape was specific to Esc1, the localization of an NPC protein, Nup49, was also checked using live-cell imaging (Fig. 2C). The four mutants that screened positive for nuclear shape changes showed NE irregularities with both Esc1 and Nup49, suggesting that the NE shape in these mutants is abnormal.

Because only four mutants were found to have NE shape abnormalities, we asked whether a specific nucleolar function was associated with the shape changes observed. Therefore, the physical interactors of the proteins deleted in these mutants were also tested in the NE shape change mutant screen. The interactors were obtained from the BioGRID database (BioGRID Release 3.4.125). Of the physical interactors obtained, 71 were non-essential and were

**Fig. 2. Nuclear shape is affected in nucleolar mutants.** (A) Wild-type and indicated deletion strains carrying plasmids expressing GFP–Esc1 and mRFP–Nop1 were imaged for nuclear and nucleolar morphology. AiryScan images representing the maximum intensity projections of the planes with Esc1 or Nop1 signal are shown. Nuclear shape changes and nucleolar size alterations in the mutants as compared to the wild-type cells can be observed. Scale bar: 1 μm. (B) Quantification of the percentage of cells showing NE abnormalities for the Wt and mutant strains. For each strain, ~200 cells were counted from three independent experiments. Data are mean±s.d. Statistical analysis was performed for triplicates (**P<0.001; one-way ANOVA). (C) Wild-type and indicated deletion strains carrying plasmids expressing GFP–Nup49 and mRFP–Nop1 were imaged for NE morphology. NE morphology changes were observed in the nucleolar deletion mutants, as shown in the representative images. Scale bar: 1 μm.
screened for nuclear architecture defects, and 14 of the 71 scored positive. The NE morphology changes were confirmed in these mutants using both Mat a and Mat α strains of the knockout collection. The results from all the interactors are summarized in Table S1. Three of the interactor mutants, rpl1aΔ, rpl8aΔ, and ssf1Δ, are shown in Fig. 2A, and additional data for other interactors are shown in Fig. S1E–H. We found multiple deviations from the generally circular shape of the NE. Sometimes the nuclei were not spherical but rather irregularly shaped; some nuclei had extensions of the NE that projected outwards like flares, whereas other projections appeared to form blebs (Fig. 3A). In many mutants, the NE proteins appeared to cluster on the NE, as opposed to the uniform distribution seen in Wt cells, and many mutants displayed multiple types of abnormalities. Of note, the first four positive mutants and their interactors exhibited similar NE defects. We analysed the nuclear shape in these mutants using parameters including circularity and surface area to establish deviations from wild-type nuclear shape (Fig. 3B; Fig. S1). We found that the circularity of the NE was lesser than that of Wt cells for most mutants and that the NE surface area was larger than that of Wt in several mutants.

The live-cell imaging described above involved introduction of an extra copy of the genes encoding the marker proteins used to visualize the nuclear morphology. To rule out the possibility that the change in NE shape was due to the additional copies of the protein, we performed immunofluorescence to detect the NE organization using endogenously expressed marker proteins. An epitope-tagged Esc1 (Esc1–13×Myc) at the genomic locus was introduced in the

![Fig. 3. Multiple shape abnormalities were seen in nucleolar mutants.](image)

(A) Representative images of individual nuclei showing various NE shape abnormalities observed in the nucleolar mutants (GFP–Esc1 in green and mRFP–Nop1 in magenta). The region represents an area of 1 µm² containing the nucleus. (B) The circularity index for the NE in wild-type and mutant strains is shown. Data are mean±s.d. of n>50 cells, except ssf1, where n=25 cells. ***P<0.001; one-way ANOVA. (C) Frames from time-lapse live-cell imaging of wild-type and mutant cells expressing GFP–Esc1 (green) and mRFP–Nop1 (magenta) are shown. The yellow arrowheads represent the cells with abnormal nuclear morphology, as seen during division. Dotted lines indicate cell outlines. Scale bar: 5 µm. (D) The percentage of cells showing NE abnormalities in the different stages of budding was estimated for each mutant strain. A representation of the budding stage is indicated (orange, unbudded; purple, small budded; pink, large budded). For each strain, over 100 cells displaying abnormal NE were counted. Cells with abnormalities were classified based on bud size, and the data was plotted. (E) The occurrence of extensions at the NE relative to the nucleolar position was analysed in the nucleolar mutants. The percentage of the cells showing NE abnormalities found at the nucleolar region and away from the nucleolar region were quantified. For each strain, over 100 cells displaying abnormal NE shape were counted.
gene deletion background, and the localization of Esc1 and Nop1 were checked using specific antibodies. Immunofluorescence results were in accordance with the live-cell imaging results (Fig. S2A). In order to rule out the possibility of DAPI or glucose deprivation leading to NE shape changes during live-cell imaging, we also imaged in medium containing glucose and lacking DAPI (Fig. S2B). No difference was seen between the two imaging methods for these mutants. Among the proteins deleted in the 18 positive mutants we identified components involved in various nucleolar processes, including ribosome biogenesis, ribosome export and rDNA condensation (Table S2).

NE defects in the mutants of genes encoding nucleolar proteins are predominantly found at the nucleolar region and in large budded cells

We first measured the growth rate of the mutant strains with NE abnormalities and did not find any significant change in growth rate compared to that of Wt cells (data not shown). We further investigated whether the NE abnormalities were associated with a specific cell cycle stage. We scored the cell cycle stage of the nuclei that displayed NE shape abnormalities and scored the bud size in these. We found that in most mutants, the cells with abnormal NEs were least commonly unbudded cells and most commonly large budded cells (Fig. 3C,D). We excluded large budded cells with dividing nuclei from the analysis. We also confirmed this by observing the mutants through the cell division cycle in live cells, where extensions of NE and NE blebs were seen in various stages of cell division (Fig. 3C, yellow arrowheads). In order to see whether these abnormalities were associated with specific regions of the NE, the region of the NE extension was compared with the nucleolar position as visualized by Nop1 staining. We found that regions with abnormal NE were more often associated with the nucleolus than with other regions of the NE (Fig. 3E). The association of NE extensions with the nucleolar region of the NE has been reported previously for certain other mutants (Campbell et al., 2006; Witkin et al., 2012).

Nuclear envelope abnormalities correlate with nucleolar enlargement

Although the NE abnormalities associated with the mutants tested were diverse and differed between strains, we noticed that in a majority of these mutants the nucleolus was larger than those of Wt cells. To test whether the nuclear and nucleolar size had indeed increased, we transformed cells with Pus1–GFP and mRFP–Nop1. Pus1–GFP occupied the entire nucleus and mRFP–Nop1 stained the nucleolus (Fig. 4A). First, we measured the nucleolar volume of the mutants to determine whether there were any measurable differences compared to the nucleolar volume of wild-type cells. Using mRFP–Nop1, we found that the nucleolar volume was much higher in the mutant strains than in the wild type (Fig. 4B). To test whether there was a corresponding increase in nuclear volume, the Pus1–GFP signal was measured. The nucleolar volume of the mutants (except ybl028cΔ) was not significantly larger than that of the wild-type cells (data not shown). As a consequence, the nucleolar to nuclear volume ratio was higher in these mutants compared to that of wild-type cells, which was close to 0.3 as expected (Fig. 4C). We also noticed that Pus1–GFP often did not stain the NE flares (although it occupied the blebs), and therefore we reasoned that the NE extensions could be more accurately represented by considering the surface area of the NE. We used GFP–Esc1 and GFP–Nup49 in the mutants to measure the nuclear surface area. We found that the area of the NE in the mutants was larger than that of wild-type cells (Fig. 4D). Interestingly, the nuclear surface area to cell surface area ratio remained constant (Fig. 4E). Because the increased nucleolar volume correlated with increased surface area of the NE, it
is possible that the enlarged nucleolus was leading to the formation of NE extensions.

In order to test directly whether the defects observed were due to nucleolar enlargement, we generated nucleolar mutant strains with fewer copies of rDNA repeats (Kobayashi et al., 2001; Takeuchi et al., 2003). The copy number of rDNA was measured in all the mutants to confirm the reduction in rDNA copies (Fig. 5A). The nucleolar mutants by themselves did not have an altered copy number of rDNA (Fig. S3A). The size of the nucleolus in reduced rDNA copy number strains was determined by measuring the volume of the nucleolus. It was found that the nucleolar volume of wild-type cells carrying fewer copies of rDNA repeats was significantly less than the wild-type cells with an unaltered copy number of rDNA (Fig. 5B,C). Using a different strain and method of estimation, it was recently shown that the nucleolar volume in yeast strains having reduced copy number is not less than in strains with normal copy number (Dauban et al., 2019). It is possible that using a plasmid-encoded rDNA copy in our strains rather than a chromosome-engineered strain, as used to obtain reduced copy numbers of rDNA in that study, could be the reason for this difference. In an earlier study, using plasmid-encoded reduced rDNA copies, a reduction in the size of the nucleolus was observed (Campbell et al., 2006). When the rDNA copy number was reduced in the nucleolar mutants, a significant reduction in the nucleolar volume was observed. There was no difference in nucleolar volume between the mutants and wild-type strain with reduced rDNA copy number (Fig. 5C). Concurrently, the percentage of cells showing NE defects was significantly reduced (Fig. 5B,D). We measured the nuclear surface area of strains with reduced rDNA copies in wild-type cells and nucleolar mutants. In the strains with wild-type rDNA copy number, the nuclear surface area was larger in the mutants than in the wild type (as reported above); however, the nuclear surface area of mutants with reduced copies of rDNA was similar to that of the wild-type strain with reduced rDNA copy number (Fig. 5E). The restoration of nuclear shape in the reduced rDNA copy number background was further confirmed by transmission electron microscopy in two of the nucleolar mutants (Fig. 5F) and by immunofluorescence of endogenous proteins (Fig. S4A,B). Although the nucleolar to nuclear volume ratio was variable, the nuclear surface area to cell surface area ratio was unchanged (Fig. S3C,E). The strong correlation between nucleolar volume and NE extensions suggests that the NE abnormalities observed in these mutants could be a consequence of the enlarged nucleolus.

rDNA tethering to the NE is required for the NE extensions observed

The nucleolus of S. cerevisiae is usually observed as a crescent or dot-like structure near the NE and occupies about a third of the total nuclear volume. The rDNA is anchored to the NE by the cohibin complexes, which in turn connect with the INM proteins Heh1 (also known as Src1) and Nur1 (Mekhail and Moazed, 2010; Chan et al., 2011). The localization of the nucleolus towards the nuclear periphery is dependent on Heh1 and Nur1. We asked whether the NE tethering of the rDNA was required for the NE extensions seen. We, therefore, introduced the heh1Δ mutation into the wild-type strain and the nucleolar mutants. We observed that, in many cells with the heh1Δ mutation, the nucleolus was no longer at the nuclear periphery, even in otherwise wild-type cells (Fig. 6A). In the nucleolar mutants carrying heh1Δ, we noticed that the nuclei were circular, and hardly any extensions could be detected (Fig. 6A,B; Fig. S4C). Upon measuring the nuclear area and nucleolar volumes, we saw that both were significantly higher in heh1Δ cells compared to those of wild-type cells. The nucleolar mutants in the heh1Δ background also had nuclear area and nucleolar volumes (Fig. 6C,D; Fig. S4D) similar to those of the heh1Δ single mutants without any further expansion of the NE. We measured the cell surface area and found that the ratio of the cell surface to the nuclear surface area was maintained in all wild-type and heh1Δ cells (Fig. 6E). We also performed the same experiments in the nur1Δ mutant background and found similar results: restoration of nuclear shape with no further expansion of the NE (Fig. S5A,B). These results suggest that nucleolar tethering to the NE is required for the NE extensions. Of note, the nucleolar volume and nuclear surface area of heh1Δ and nur1Δ were significantly higher than those of the wild-type cells, and no further increase was seen when nucleolar mutations were introduced. We measured the copy number of rDNA in all the strains (single and double mutants) used in the study to see whether the copy number correlated with the nucleolar volume. We found that, as reported earlier, heh1Δ and nur1Δ did have increased and variable copy number compared to that of the wild-type strain (Mekhail et al., 2008; Saka et al., 2016). The rDNA copy number in the double mutants was not altered beyond that observed in the single mutants (Fig. S3B). This suggests that the NE expands isometrically to accommodate the enlarged nucleolus in heh1Δ and nur1Δ cells, and because there is no further increase in nucleolar volume upon introduction of the nucleolar mutations, no further increase in nuclear surface area is seen (Fig. S3D).

NE abnormalities arise due to increased phospholipid biosynthesis

We next tested whether the NE abnormalities observed in the mutants were due to elevated phospholipid synthesis. To this end, we treated the mutants with cerulenin, a known inhibitor of fatty acid synthase (Inokoshi et al., 1994). Remarkably, when we treated the nucleolar mutants with cerulenin, the majority of nuclei were spherical (Fig. 7A,B). This result suggests that the NE abnormalities observed in the mutants are due to an increase in phospholipid synthesis. Taken together, these data indicate that NE abnormalities arise in these mutants due to increased NE biosynthesis that primarily occurs near the nucleolar region.

Yeast cells regulate the biosynthesis of phospholipids depending on the requirement. CHOI1 and PAH1 are two key regulatory genes involved in the phospholipid biosynthesis pathway (Fig. 7C). The transcription of CHOI1 is regulated by the transcription factor Opi1 and is required for membrane biogenesis, whereas PaH1 promotes the synthesis of storage lipids (Adeyo et al., 2011). PaH1 negatively regulates phospholipid synthesis by reducing the expression levels of CHOI1 (Han and Carman, 2017). To check whether the observed NE extensions in the nucleolar mutants were due to increased phospholipid biosynthesis, we measured the transcript levels of CHOI1 and PAH1. It was observed that the transcript levels of CHOI1 in the nucleolar mutants were significantly increased (Fig. 7D), whereas the transcript levels of PAH1 were significantly lower in these mutants (Fig. 7E). These levels correlate with the increased NE expansion in the mutants. If the change in transcript levels of PAH1 and CHOI1 observed indeed correlate with the NE expansion seen in these mutants, then, in conditions that restored the NE shape, these transcript levels should remain unchanged. We therefore asked whether reduced rDNA copy number or heh1Δ mutation restored PAH1 and CHOI1 transcript levels. Indeed, we found that in the reduced rDNA copy number strains, CHOI1 transcript levels were reduced and PAH1 transcript levels increased compared to levels in the wild type strain and, importantly, they remain at those levels in the nucleolar mutants as well (Fig. 7F,G). Similarly, in the heh1Δ
Fig. 5. Reducing the rDNA copy number in the nucleolar mutants restores the nuclear shape abnormalities. (A) rDNA copy number in the wild type strain and in wild-type and nucleolar mutant strains with reduced rDNA copy number. rDNA copy number was checked using qPCR and plotted as fold change compared to Wt. The *rrn10*Δ mutant, a mutant known to have a high rDNA copy number, is shown as a control. A minimum of five individual colonies were used for each strain. Data are mean±s.d. (**P ≤ 0.01; one-way ANOVA). (B) Live-cell imaging of Wt and nucleolar mutants with reduced rDNA copy number was performed. GFP–Esc1 was used as the marker for NE and mRFP–Nop1 used as the marker for the nucleolus. Representative images are shown. Scale bar: 2 µm. (C) Nucleolar volume in Wt and mutant strains with or without reduced rRNA. Statistical analysis was performed for 100 individual cells for each strain, and mean±s.d. is shown (**P ≤ 0.01; one-way ANOVA). (D) For each of the indicated strains, over 200 cells were scored for NE abnormalities from three independent experiments and plotted as the mean±s.d. Statistical analysis was done for triplicates (**P ≤ 0.01; one-way ANOVA). (E) Nuclear surface area of the indicated strains. Data are mean±s.d. of 100 individual cells for each strain (**P ≤ 0.01; **P ≤ 0.01; one-way ANOVA). (F) Representative electron micrographs for the indicated strains are shown (yellow arrows indicate abnormalities; N, nucleus; C, cytoplasm; V, vacuole). Scale bar: 500 nm.
cells, CHO1 transcript levels were higher and PAH1 transcript levels were lower than wild-type levels, as expected. Of note, the single and double mutants had similar levels of expression, correlating with our observation of no further increase in the NE in the double mutants (Fig. 7H,I). Similar results were also obtained in the nur1Δ background (Fig. S5C,D).

Taken together, these data indicate that increased nucleolar volume triggers phospholipid biosynthesis leading to expanded NE (Fig. 8). The increased phospholipid biosynthesis leads to the formation of extensions, blebs and irregular shape of the nucleus. However, when the rDNA is not anchored to the NE, the nuclear membrane expands isometrically.

**DISCUSSION**

In this work, we performed a candidate-based screen for NE abnormalities induced by the loss of nucleolar proteins. We report that the loss of many nucleolar proteins alters the NE morphology leading to the appearance of flares and blebs. There is also an increase in nucleolar size in these mutants. We show that this altered morphology can be suppressed by reducing the nucleolar size, by reducing the rDNA copy number, suggesting that the enlarged nucleolar size causes the extended NE phenotypes. We also demonstrate that the NE flares and blebs arise as a consequence of enhanced phospholipid biosynthesis. We show that these non-isometric extensions of NE require the tethering of the rDNA to the NE. Lastly, in all conditions, cells maintain a constant ratio of cell to nuclear surface area.

We have shown that the deletion of several of the nucleolar and/or ribosomal proteins leads to NE extensions and other shape abnormalities. In a majority of these mutants, there is an accumulation of ribosomal subunit components in the nucleolus, thus causing an increase in nucleolar size (Neumuller et al., 2013). This can lead to pleiotropic effects in the cell, including altered protein synthesis, and the NE abnormalities could be due to these
reasons rather than the direct consequence of nucleolar expansion. However, this is unlikely because, in the reduced rDNA copy number strains carrying these nucleolar mutations, NE extensions were very close to wild-type levels. This suggests that the loss of these specific nucleolar proteins is not leading to NE abnormalities. Another argument could be that there is slow growth in the nucleolar mutants, which leads to NE abnormalities. We found that some of these strains do grow more slowly than the wild-type strain, whereas others don’t show the slow-growth phenotype. However, there was no correlation between slow growth and NE abnormality. Many mutants that had wild-type growth also had high levels of NE abnormality, for example csm1Δ, rpl11aΔ and rsa3Δ.

Flares and extensions have been reported in mutants that impair the phospholipid biosynthesis pathway (Neumuller et al., 2013; Siniossoglou et al., 1998; Campbell et al., 2006; Schneiter and Cole, 2010) and in those with mitotic delay (Walters et al., 2014). Although we cannot rule out the possibility that the nucleolar mutants tested activate phospholipid biosynthesis, this is unlikely, because in the nucleolar mutant strains with reduced rDNA copy number and in heh1Δ and nur1Δ mutants carrying the nucleolar mutations, the phospholipid biosynthesis was not elevated. This suggests that the effects on the NE morphology are linked to the nucleolar size. Mitotic delay induced through mutations in regulators of the cell cycle also produces flares. It has been shown that Cdc5, the Polo-like kinase that regulates multiple proteins in

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Fig. 7. Nucleolar mutants affect the regulation of phospholipid biosynthesis. (A) Representative live-cell images for nucleolar mutants showing NE abnormalities with or without cerulenin treatment are shown. DMSO indicates vehicle control treatment. GFP–Esc1 (green) is used as a marker for the NE. Scale bar: 5 µm. (B) The percentage of cells showing NE abnormalities in the indicated nucleolar mutants with and without cerulenin treatment is shown. Nearly 200 cells were counted across three independent experiments to calculate percentages. Data are mean±s.d. (***P<0.001; one-way ANOVA). (C) Schematic diagram of phospholipid biosynthesis in yeast. PA (phosphatidic acid) is converted to either lipid droplets or is used for phospholipid synthesis. Red colour indicates downregulation, and green colour indicates upregulation of phospholipid synthesis. CDP-DAG, cytidine diphosphate diacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol. (D–I) CHO1 (D,F,H) and PAH1 (E,G,I) RNA levels in Wt (D,E), reduced rDNA (F,G) and heh1Δ mutant (H,I) backgrounds. RNA was quantified in wild type and nucleolar mutants by reverse transcriptase qPCR (RT-qPCR) and the fold change relative to wild type is shown. All the RT-qPCR experiments were performed with three or more independent cDNA samples for each strain. In all graphs, a log2 scale is used for the y-axis. Significance tests were performed to compare the levels between the nucleolar mutants and the respective wild types (ns, not significant; *P≤0.05; **P≤0.01; ***P≤0.001; one-way ANOVA).
mitosis, including condensins that compact the rDNA during mitosis, has a specific role in preventing these extensions (Walters et al., 2014). We believe the extensions observed in the nucleolar mutants arise due to a different reason than that causing the extensions seen in cdc5 mutants. First, in our study, we excluded large budded cells with two nuclei and dumbbell-shaped nuclei. Among the cells that had abnormalities, we saw the flares in all stages of the cell cycle, although we saw more large budded cells with abnormalities. Secondly, no mitotic delay was observed in any of these mutants, although we did find more cells in the G0 phase compared to wild type. Thirdly, the extensions observed in the cdc5 mutants are unaffected by both rDNA copy number and rDNA anchoring (Walters et al., 2014), whereas the flares seen in this study were suppressed by both rDNA copy number reduction and nucleolar anchor mutants. Therefore, the flares and extensions observed in these nucleolar mutants are different from the flares induced by mitotic delays. We propose these flares are induced by the sensing of increased nucleolar volume by the cell, and NE extensions are an outcome of the increased phospholipid biosynthesis induced in response to the increased nucleolar volume. However, isometric expansion is prevented by the rDNA association with the NE, resulting in flares and blebs. We suggest that if the rDNA were not anchored, the NE synthesis triggered by the increased nucleolar volume would allow the nuclei to expand isometrically and produce larger nuclei of normal shape.

Many studies have linked nuclear transport to nuclear size in yeast and other organisms (Kume et al., 2017; Jorgensen et al., 2007; Levy and Heald, 2010). In Schizosaccharomyces pombe, the inhibition of nuclear export leading to the accumulation of mRNA and protein in the nucleus results in an increased nucleolar volume (Kume et al., 2017). This study also uncovered that perturbations to phospholipid synthesis alter nuclear volume and shape and, importantly, that the nuclear volume increase induced in the RNA export mutants requires phospholipid biosynthesis. The authors also speculate that the increased pressure of the accumulated bulk nucleoplasmic contents could trigger NE biosynthesis. In another recent study in S. cerevisiae, it was shown that the nucleoplasm could be the critical determinant of nuclear size (Walters et al., 2019). These authors have speculated that it could be either the bulk nucleoplasm or some specific factor that triggers NE expansion. Our work supports this idea of bulk nucleoplasm being a key regulator of NE biosynthesis, because we observed that the bulk nucleolar volume increases in the mutants tested, which in turn increases the volume of nucleoplasm, and this seems to trigger the expansion of the NE. Although we cannot rule out that a specific factor in the nucleoplasm may be involved, bulk nucleoplasm may be a key for triggering NE expansion. Future studies will examine how the nucleolar expansion is communicated to the phospholipid biosynthesis pathway.

MATERIALS AND METHODS

Yeast methods

All the strains used in this study were derived from BY4741 unless otherwise stated and are listed in Table S3. Standard procedures were followed for yeast manipulations. To generate the strains with reduced rDNA copy number, the plasmid pRD1-hyg::URA3 (Michel et al., 2005; Wai et al., 2000) (gift from Dr David Shore, University of Geneva, Switzerland) was transformed, and transformants were selected on SC–Ura plates containing hygromycin as described previously (Takeuchi et al., 2003). The reduced copy number was confirmed using quantitative PCR, and cells were maintained on selection medium to keep the copy numbers low. To make GFP–Esc1, GFP was inserted in-frame at the Xba1 site towards the N-terminus of ESC1 in pDZ45 plasmid (a gift from Dr Rolf Sternglanz, Stony Brook University, New York, USA). The functionality of the construct was confirmed through the telomere position effect assay as shown in Fig. S1A–C (Pasupala et al., 2012). pUN100-GFP-NUP49 was a gift from the Doye laboratory (Belgareh and Doye, 1997) and mRFP-Nop1 was from Dr Emmanuelle Fabre (Ulbrich et al., 2009). For Pus1–GFP, full-length PUS1 with its promoter was amplified and ligated in-frame to GFP in pUG23 (U. Guedener and J. H. Hegemann, Heinrich-Heine-Universität, Düsseldorf, Germany).

Screen for NE morphology changes

To screen for NE morphology changes, the deletion strains from EUROSCARF were streaked on a YPD plate. A single colony of each
Electron microscopy

For transmission electron microscopy, samples were prepared as described previously (Wright, 2000), with some modifications. In brief, yeast cell cultures were grown in YPD medium and harvested at mid-log phase (0.8 OD at A600). Cells were fixed in ice-cold fixing solution (2% glutaraldehyde, 1% paraformaldehyde and 1 mM MgCl₂ in 50 mM potassium phosphate buffer, pH 6.8) for 2 h at 4°C. After washes, samples were resuspended in 4% KmnO₄ for 1 h at room temperature. After secondary fixing, cells were resuspended in 1 ml of freshly prepared 2% uranyl acetate for 1 h at room temperature and subsequently washed. Dehydration of cells was followed by clearing with propylene oxide and infiltration with a combination of 100% propylene oxide and 100% Spurr low-viscosity resin (TED PELLA, INC, USA). Finally, samples were embedded in 100% resin and kept in a vacuum oven at 45°C for 12 h, then shifted to 68°C for 3 days. For the electron microscopy examination, thin sections of greysilver colour interference (60–70 nm) were cut and mounted onto 300-mesh copper grids. The mounted sections were stained with alkaline lead citrate, washed gently with distilled water and allowed to dry for 1 h. Dried sections were observed under a Talos S200 transmission electron microscope (FEI, The Netherlands) at an operating voltage of 200 KV. Images were digitally acquired by CetaCMOS camera using TIA (TEM Imaging & Analysis) software.

Quantitative PCR for mRNA and rDNA copy number estimation

RNA isolation was performed using the hot acid phenol method (Abraham and Mishra, 2018). Briefly, mid-log phase (OD 0.6-1.0) yeast culture was pelleted, and the pellet was resuspended in 400 μl of 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% SDS buffer. 400 μl of preheated acid phenol was added, and the mixture was incubated at 65°C for 60 min. Samples were chilled on ice for 5 min and centrifuged at 15,000 g for 5 min. The aqueous layer was collected in a separate tube, and an equal amount of chloroform was added and centrifuged at 15,000 g for 5 min. RNA was precipitated by adding 1/10th volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of chilled 100% ethanol to the collected aqueous layer and spinning at 15,000 g for 5 min. RNA was resuspended in nuclease-free water. DNase digestion was performed using RNase-free DNase, and genomic DNA contamination was checked by PCR. 1 μg of RNA was converted into cDNA using a cDNA synthesis kit (Thermo Scientific Verso cDNA Synthesis Kit), and the quality of cDNA was tested by PCR. The gene expression of CHO1 and PAH1 relative to ACT1 was calculated using the ΔΔCt method. RNA samples were isolated from at least three different colonies for each strain and tested. The fold change was plotted and error bars indicate standard deviation. Significance tests were done on fold-change using a one-way ANOVA test.

To estimate the rDNA copy number, genomic DNA was isolated from overnight cultures by the glass bead method (Philippesen et al., 1991). For checking the rDNA copy number, real-time PCR was performed using 50 ng of DNA template and primers from the non-transcribed spacer regions (NTS2). ACT1 was used as the control housekeeping gene. Copy number was compared between wild-type and reduced rDNA copy number strains.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


