The size of the membrane-bound nucleus scales with cell size in a wide range of cell types but the mechanisms determining overall nuclear size remain largely unknown. Here we investigate the role of fission yeast inner nuclear membrane proteins in determining nuclear size, and propose that the Lap2-Emerin-Man1 domain protein Lem2 acts as a barrier to membrane flow between the nucleus and other parts of the cellular membrane system. Lem2 deletion increases membrane flow into and out of the nuclear envelope in response to changes in membrane synthesis and nucleocytoplasmic transport, altering nuclear size. The endoplasmic reticulum protein Lnp1 acts as a secondary barrier to membrane flow, functionally compensating for lack of Lem2. We propose that this is part of the mechanism that maintains nuclear size proportional to cellular membrane content and thus to cell size. Similar regulatory principles may apply to other organelles in the eukaryotic subcellular membrane network.
The size of the nucleus scales with cell size in cell types ranging from yeast to animal cells\textsuperscript{1-3}. The nucleus exists as part of a subcellular membrane network, with the nuclear envelope being continuous with the endoplasmic reticulum (ER)\textsuperscript{4,5}. Despite much study of the molecular mechanisms of membrane growth in eukaryotic cells\textsuperscript{6}, the controls that regulate the overall size of the nucleus remain largely unknown.

In the fission yeast \textit{Schizosaccharomyces pombe}, the nuclear volume to cell volume ratio (N/C ratio) is maintained constant as cells increase in size both throughout the cell cycle and across a 35-fold range of cell volumes\textsuperscript{1}. Similar N/C ratios are observed in widely divergent yeast species\textsuperscript{3} suggesting that maintenance of a particular ratio is important for cell physiology. Studies of multinucleate cells indicate that the amount of cytoplasm surrounding a nucleus determines its size\textsuperscript{1,7,8}. In vitro experiments with \textit{Xenopus} egg extracts\textsuperscript{9,10} and a genetic screen in fission yeast\textsuperscript{11} have implicated nuclear lamina components, nucleocytoplasmic transport, and overall lipid biosynthesis in nuclear size control. Nuclear lamin proteins which are lacking in yeasts have been implicated in nuclear size control in metazoans\textsuperscript{9,10} and

\begin{figure}[h]
\begin{center}
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\end{center}
\caption{Lem2 restricts nuclear size enlargement independently of its chromatin-binding activity. \textbf{a} N/C ratio of wild type (WT), \textit{rae1-167}, \textit{rae1-167 lem2Δ}, and \textit{rae1-167 lem2\textsuperscript{lehel}} cells grown at 25 °C then shifted to the indicated temperature for 4 h (\textit{n} > 30 cells). Two-tailed \textit{t}-tests: **** \textit{P} < 0.0001, \textit{rae1-167} (\textit{n} = 64 cells) against \textit{rae1-167 lem2Δ} (\textit{n} = 35 cells), \textit{t} = 4.299, degrees of freedom = 97; \textit{rae1-167 lem2\textsuperscript{lehel}} (36 °C) (\textit{n} = 31 cells) against \textit{rae1-167} (36 °C) (\textit{n} = 64 cells), \textit{P} = 0.0863, \textit{t} = 1.741, degrees of freedom = 93. In \textit{lem2\textsuperscript{lehel}} cells, the N-terminal helix-extension-helix chromatin-binding region of Lem2 is deleted. In box-and-whiskers diagrams, boxes indicate median and upper and lower quartile and whiskers indicate range of data. The corresponding dot plot is available in Supplementary Fig. 9a. \textbf{b} Images of the nuclear envelope (Cut11-GFP, green) of wild type (WT), \textit{rae1-167} and \textit{rae1-167 lem2Δ} cells grown at 25 °C then shifted to the indicated temperature for 4 h. Maximum intensity projections shown. Scale bar: 5 μm. \textbf{c} Images of the nuclear envelope (Cut11-GFP, green) and chromatin (Hht1-mRFP, magenta) of \textit{rae1-167 lem2Δ} cells grown at 25 °C then shifted to the indicated temperature for 2 h. Maximum intensity projections shown. Scale bar: 5 μm.}
\end{figure}
underlie the nuclear envelope, but the roles of other proteins associated with the nuclear membrane in this process have not been examined.

Here, we assess the contribution of inner nuclear membrane proteins to the maintenance of the N/C ratio in fission yeast. We demonstrate that deletion of Lap2-Emerin-Man1 (LEM) domain protein Lem2, but not that of other inner nuclear membrane proteins, augments nuclear size enlargement phenotypes resulting from perturbation of nucleocytoplasmic transport. We show that Lem2 deletion leads to nuclear shrinkage, accompanied by nuclear envelope blebbing, following perturbation of membrane synthesis. We propose that Lem2 forms part of a nuclear size...
control mechanism, acting as a barrier to membrane flow into and out of the nuclear envelope and that the ER protein Lnp1 acts as a secondary barrier, compensating for lack of Lem2.

Results

Lem2 deletion augments nuclear size enlargement phenotypes. The N/C ratio phenotypes of fission yeast cells with mutations in genes encoding inner nuclear membrane proteins were determined using the deletion mutants ima1Δ, lem2Δ, man1Δ, and kms1Δ12 and the temperature-sensitive mutant sadt.13 None of these mutants displayed a significantly aberrant N/C ratio (Supplementary Fig. 1a). However, deletion of Lem2 augmented the previously characterised nuclear size enlargement of rac1-167 temperature-sensitive mutant cells (Fig. 1a, b)11. Rac1-167 cells have altered nucleocytoplasmic transport11,14. This augmentation was not observed with double mutants of rac1-167 with mutants of the other inner nuclear membrane proteins (Supplementary Fig. 1a) or other nucleus-localised and organelar membrane-localised proteins tested (Supplementary Fig. 2). Lem2 contains a conserved LEM domain that has been shown to anchor chromatin to the nuclear periphery15. We disrupted the chromatin association of Lem2 by deleting its N-terminal helix-extension-helix (HEH) chromatin-binding region15. The Lem2 HEH deleted protein failed to augment the rac1-167 nuclear size enlargement (Fig. 1a), indicating that the role of Lem2 in restricting nuclear enlargement is not dependent on its chromatin binding activity. We also showed that chromatin only occupied part of the enlarged nucleus and thus the extent of chromatin compaction is not affected by the nuclear size changes in rac1-167 lem2Δ cells (Fig. 1c). Additionally, we observed that deletion of Lem2 increases the nuclear enlargement observed when nuclear protein export is inhibited by leptomycin B (LMB) (Supplementary Fig. 1b and c). These data indicate that Lem2 functions to restrict the changes in nuclear size that occur following various perturbations, and that these effects are independent of the association of Lem2 with chromatin.

Lem2 prevents interphase nuclear shrinkage. Cerulenin is an inhibitor of fatty acid synthetase which thereby reduces cellular membrane availability, and can lead to aberrant mitoses16. Treatment of wild type cells with cerulenin results in a slight reduction in the N/C ratio due to these aberrant mitoses but has no effect on the N/C ratio of interphase cells (Fig. 2a–c, Supplementary Fig. 3a, Supplementary Movies 1 and 2). Strikingly, treatment of lem2Δ cells with cerulenin leads to rapid shrinkage of interphase nuclei (Fig. 2b, c, Supplementary Movies 3 and 4), resulting in a major reduction in the N/C ratio (Fig. 2a, Supplementary Fig. 3a). This nuclear shrinkage is accompanied by an increase in concentration of the nuclear envelope marker protein Ctin1-GFP within the nuclear envelope (Fig. 2b), and apparent membrane blebbing of the envelope (Fig. 2d, e, Supplementary Fig. 3b, Supplementary Movie 7). Staining cellular electron microscopy images of lem2Δ cells treated with 10 μg/ml cerulenin for 90 min at 32 °C. Apparent nuclear envelope blebbing events (Fig. 2g, h), the presence of intranuclear membranous structures (I) and membranous compartments (possibly vesicles) in close proximity to the nuclear envelope (J) indicated by black arrowheads. Further examples in Supplementary Fig. 5. Scale bars: 250 nm.

Lem2 acts as a barrier to membrane flow. Given these results we hypothesised that Lem2 acts as a barrier to membrane flow between the nuclear envelope and other parts of the cellular membrane system. This hypothesis was further supported by the observation that the nuclear shrinkage of lem2Δ cells treated with cerulenin was suppressed by treatment with brefeldin A (Fig. 3a, b). Brefeldin A inhibits traffic within the golgi and retrograde traffic from the golgi to the ER by blocking COP1 recruitment. This results in fusion of golgi and ER membranes and ER expansion17. Furthermore, increasing the copy number of Lem2 was found to partially suppress the nuclear size enlargement phenotypes13 of rac1-167 and nem1Δ cells (Fig. 3c, d), indicating that quantitatively increasing the amount of Lem2 enhances its barrier function. Nem1 is the catalytic subunit of a phosphatase responsible for the dephosphorylation and activation of the lipid family phosphatidic acid phosphatase Ned118,19, and its deletion leads to constitutive proliferation of nuclear and ER membranes in interphase. Increasing the copy number of Lem2 suppressed the enlarged nuclear size and aberrant nuclear shape phenotypes of
Fig. 3 Lem2 acts as a barrier preventing inappropriate membrane flow between the nuclear envelope and ER. a Images of the nuclear envelope (Cut11-GFP, green) of lem2Δ cells grown at 25 °C untreated (+EtOH) or treated with 10 µg/ml cerulenin (+Cer) and/or 100 µg/ml brefeldin A (+BFA) for 3 h. Maximum intensity projections shown. Cerulenin: 10 µg/ml, BFA: 100 µg/ml. Scale bar: 5 µm. b N/C ratio of lem2Δ cells (25 °C) untreated (−) or treated with 10 µg/ml cerulenin (+Cer) and/or 100 ng/ml brefeldin A (+BFA) for 3 h (n > 30 cells). Two-tailed Mann–Whitney test: ****P < 0.0001, lem2Δ+Cer (n = 31 cells), lem2Δ+Cer+BFA (n = 32 cells). The corresponding dot plot is available in Supplementary Fig. 9c.

c Images of the nuclear envelope (Cut11-GFP, grayscale) of wild type (WT) and nem1Δ cells containing one or two copies of the gene encoding Lem2 grown at 25 °C. Scale bar: 5 µm.

d N/C ratio of wild type (WT), nem1Δ, and rae1-167 cells containing one or two copies of the gene encoding Lem2 (n > 30 cells). Two-tailed t-tests: ***P = 0.001, nem1Δ+1×lem2 (n = 31 cells) against nem1Δ+2×lem2 (n = 40 cells), t = 4.129, degrees of freedom = 69; **P = 0.0015, rae1-167+1×lem2 (n = 43 cells) against rae1-167+2×lem2 (n = 35 cells), t = 3.297, degrees of freedom = 76. WT and nem1Δ cells grown at 25 °C. Temperature-sensitive rae1-167 cells grown at 25 °C then shifted to 36 °C for 4 h before imaging. The corresponding dot plot is available in Supplementary Fig. 9d.

e Representative images of the ER lumen (GFP-ADEL) of wild type (WT) cells and nem1Δ cells containing one or two copies of genes encoding Lem2 or Lnp1 grown at 25 °C. Scale bar: 5 µm.

f N/C ratio of wild type (WT) cells overexpressing lem2+ or lnp1+ (under control of the nmt1 promoter) grown in minimal medium (EMM) at 28 °C for 0 or 18 h (n > 30 cells). Two-tailed Mann–Whitney test: ****P < 0.0001, lem2+ (0 h) (n = 42 cells) against lem2+ (18 h) (n = 35 cells); lnp1+(0 h) (n = 48 cells) against lnp1+ (18 h) (n = 48 cells). The corresponding dot plot is available in Supplementary Fig. 9e. In all box-and-whiskers diagrams, boxes indicate median and upper and lower quartile and whiskers indicate range of data.
nem1Δ cells (Fig. 3e). Lem2 localisation is not affected by increasing Lem2 copy number although the intensity of Lem2 staining in the nuclear envelope is increased (Supplementary Fig. 6a). Additionally, overexpression of Lem2 in wild type cells was sufficient to lead to a decrease in N/C ratio (Fig. 3f). We observed a further example of nuclear shrinkage in a lem2Δ cell after it was born with an enlarged N/C ratio. It underwent rapid nuclear shrinkage, accompanied by what appeared to be an accumulation of vesicles in the cytoplasm, resulting in a rapid reduction of the N/C ratio from 0.138 to 0.116 within 30 min (Supplementary Fig. 6b). Overall these data indicate that Lem2 acts as a barrier to membrane flow between the nuclear envelope and other parts of the cellular membrane system, and that this influences nuclear size.

ER protein Lnp1 functionally compensates for lack of Lem2. Lunapark/Lnp1 is an ER protein conserved from yeast to humans that stabilises three-way ER junctions. Overexpression of Lnp1 has been shown to suppress the slow growth phenotypes of lem2Δ, suggesting functional compensation. We therefore tested whether Lnp1 acts as a secondary barrier to membrane flow. Deletion of lnp1 did not affect nuclear size when Lem2 was
present, but lem2Δ lnp1Δ cells displayed an enlarged N/C ratio (Fig. 4a, c), and deletion of Lnp1 exacerbated the N/C ratio enlargement phenotype of lem2Δ rae1-167 cells (Fig. 4b, c). Additionally, increasing the copy number of Lnp1 partially suppressed the nuclear size decrease observed in lem2Δ cells following inhibition of fatty acid synthesis (Supplementary Fig. 7). As for Lem2, increasing the copy number of Lnp1 suppressed the nuclear size and shape phenotypes of nem1Δ cells (Fig. 3e), and overexpression of Lnp1 significantly reduced the N/C ratio of wild-type cells (Fig. 3f). These data indicate that Lnp1 is able to partially compensate for Lem2, acting to prevent both over-proliferation and shrinkage of the nuclear envelope by regulating membrane flow within the organelar membrane system.

We propose that the regulation of the flow of membrane within the ER affects membrane flow into and out of the nuclear envelope, thus influencing nuclear size.

Discussion

Our data implicate the inner nuclear membrane protein Lem2 in maintaining an appropriate nuclear size. Lem2 acts as a barrier to membrane flow between organelar compartments, preventing excessive nuclear envelope growth and shrinkage. The ER protein Lnp1 can partially compensate for Lem2, preventing inappropriate membrane flow between the nuclear envelope and other membranous structures. Our study highlights the interconnectedness of cellular membrane compartments, and identifies Lem2 and Lnp1 as barriers to membrane flow, regulating transfer of membrane between compartments, and thus contributing to the control of nuclear size.

This regulation of membrane flow can provide an explanation for the maintenance of nuclear volume as a fixed proportion of cell volume. The different membrane-bound organelles in eukaryotic cells are interconnected to form an overall cellular membrane system. We postulate that as cells increase in size, the overall membrane amount within the cellular membrane system increases proportionally. The association of ribosomes with ER membranes could play a role in ensuring this proportionality, given that ribosome number generally scales with overall cell size. Barrier proteins such as Lem2 and Lnp1 could act like valves regulating membrane flow through the cellular membrane network, such that as cellular membrane content increases with cell size, a proportion of the membrane enters the nucleus at a rate determined by the Lem2 barrier protein. If Lem2 is compromised and membrane synthesis is inhibited then membrane flows out of the nucleus (Fig. 2b, c). Thus Lem2 acts as a valve regulating membrane flow into and out of the nucleus contributing to the maintenance of a constant N/C ratio, a control that is influenced by altering membrane components, membrane synthesis, and nucleo-cytoplasmic transport. When the influence of Lnp1 in the ER on nuclear size, similar controls may act throughout the cellular membrane system, maintaining balance in size between different membrane-bound organelles. These controls could also act to maintain a balance between the size of the nucleus and the ER coordinating transcription in the nucleus with translation by the ribosomes resident in the ER.

Methods

Yeast general methods. S. pombe strains used are listed in Supplementary Table 1. Standard S. pombe media and methods were used. Cells were grown in YM6 medium unless otherwise indicated. Nuclear size phenotypes following LMB and cerulenin treatment were also assessed in EMM medium (Supplementary Fig. 8). Gene deletion mutants from an S. pombe genome-wide haploid gene deletion collection and an S. pombe kinase deletion library were used in genetic crosses to generate double mutant strains. Gene tagging was performed by PCR and homologous recombination. The following primers were used for tagged strain constructions: C-terminal GFP tagging of lem2+ forward, OKK131, reverse, OKK132, AATAAATATTATACTTTCTCAGCTCTTTTGCATTTGAGAGCATTGACTGAATTTAATACACCACTGGCAGTTTGCAATTTAATATTTAAGTTCCAGCTGGCTTTTTCAGCTTTACTGAGTTGGAATTTGTTTTTCCGTATTCTTTCGAAGTTAGGGTCCTCCCAATTG. The lem2+ construct or the additional copy of the lem2+ or lnp1+ gene, with its own promoter and terminator, was subcloned into the pU1+ plasmid pKl46, and integrated into the genomic lem1 locus. For drug treatment, LMB (Sigma-Aldrich, 5.5 µg/ml in MeOH), cerulenin (Sigma-Aldrich, 1 mg/ml in EtOH) and/or Brefeldin A (Sigma-Aldrich, 10 µg/ml in EtOH) was added to cultures at the indicated concentration.

Microscopy and image analysis. Fluorescence imaging was carried out using a Leica DMi8 Elite microscope (Applied Precision) or a Zeiss Axio Observer.Z1 wide-field inverted microscope, an Olympus Plan Apo ×60l.4 NA oil objective and a Photometrics CoolSNAP HQ2 camera (Roper Scientific) in an IMSOL ‘incubator’ Environment Control System. Imaging was carried out at 25 °C unless otherwise indicated. Images were acquired in 0.2, 0.3, or 0.4 µm z-sections over 2.4, 4.4, or 5.4 µm, with a brightfield reference image in the middle of the sample, and deconvoluted using SoftWorx (Applied Precision). Images shown are maximum intensity projections of deconvoluted images unless otherwise indicated. For DiOC6 staining, 5 µg/ml 3,3′-dihexyloxacarbocyanine iodide (DiOC6) was added to 1 ml of exponential cell culture and incubated at room temperature for 3 min before imaging.

Electron microscopy. Ten micrograms per millilitre of cerulenin was added to exponentially growing S. pombe cells at 32 °C. Ninety minutes after drug addition, N/C ratio phenotype was confirmed by light microscopy (Axioskop, Zeiss). Aliquots of S. pombe culture were concentrated via pressure filtration through a 0.45 µm pore membrane filter (Merck Millipore). Cell content was loaded into a flat specimen carrier (Leica Microsystems) and high-pressure frozen using an EM PACT2 high-pressure freezer (Leica Microsystems). For both lem2Δ and wild type cell samples, freezing occurred at between 99 and 109 min after drug addition. Vitrified samples were added to a freeze substitution mix (2% osmium tetroxide, 0.1% uranyl acetate, 5% water, in acetone) and heated at 80 °C in a freeze substitution unit (EM AF52, Leica Microsystems) for 1 h. Temperature was increased to −25 °C over 14 h. Samples were incubated in a 1:1 resin (Epon 812, TAAB Laboratories) and acetone mix for 41 h followed by a 2:1 resin and acetone mix for 5 h. Temperature was increased from −25 to 0 °C over 10 h and four changes of 100% Epon 812 resin were performed over the following 72 h. Temperature was increased to 20 °C over 48 min. Samples were removed from the freeze substitution unit for polymerisation by incubation at 60°C for 2 days.

Resin embedded cells were cut into ~70 nm thin sections using a diamond knife (ultra 45°, Diatome) in a Powertome ultramicrotome (RMC Boeckler). Sections were collected onto formvar-coated copper slot grids (EM Resolutions) and stained with 2% aqueous uranyl acetate, followed by Reynolds’s lead citrate solution. Electron microscopy imaging was performed on a Tecnai G2 Spirit BiotWIN transmission electron microscope (ThermoFisher Scientific) equipped with an Orius CCD camera (Gatan).
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**Author contributions**

K.K., H.C. and P.N. conceived and designed the experiments for this study and wrote and revised the manuscript. K.K., H.C. and A.B. carried out the data analysis. K.K. and P.N. acquired the funding and P.N. supervised the project.

**Additional information**

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