Nuclear shape, growth and integrity in the closed mitosis of fission yeast depend on the Ran-GTPase system, the spindle pole body and the endoplasmic reticulum

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Summary
The double lipid bilayer of the nuclear envelope (NE) remains intact during closed mitosis. In the fission yeast Schizosaccharomyces pombe, the intranuclear mitotic spindle has envelope-embedded spindle pole bodies (SPB) at its ends. As the spindle elongates and the nucleus divides symmetrically, nuclear volume remains constant but nuclear area rapidly increases by 26%. When Ran-GTPase function is compromised in S. pombe, nuclear division is strikingly asymmetrical and the newly synthesized SPB is preferentially associated with the smaller nucleus, indicative of a Ran-dependent SPB defect that interferes with symmetrical nuclear division. A second defect, which specifically influences the NE, results in breakage of the NE upon spindle elongation. This defect, but not asymmetric nuclear division, is partially rescued by slowing spindle elongation, stimulating endoplasmic reticulum (ER) proliferation or changing conformation of the ER membrane.

We propose that redistribution of lipid within the ER-NE network is crucial for mitosis-specific NE changes in both open and closed mitosis. 

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Key words: Ran GTPase, Nuclear division, Fission yeast, Endoplasmic reticulum, Spindle pole body

Introduction
During interphase in all eukaryotic cells the double lipid bilayer of the nuclear envelope (NE) physically separates the chromosomes, and chromosome-related processes, from the cytoplasm and increases in area by 59% (Lim et al., 2007) as the nuclear volume doubles in preparation for mitosis (reviewed by Hetzer et al., 2005; Lim et al., 2007; Winey et al., 1997). In the open mitosis of animal cells, NE breakdown allows the spindle microtubules that are nucleated by the cytoplasmic centrosomes to attach to and then separate the chromosomes. In the closed mitosis of yeast, the centrosome equivalents, called spindle pole bodies (SPBs), are embedded in the NE and nucleate the formation of an intranuclear spindle (Ding et al., 1997). As the spindle elongates in anaphase B, nuclear volume remains constant but division of the roughly spherical nucleus into two smaller spheres, which occurs in less than 5 minutes, requires a rapid increase of 26% in NE area (Lim et al., 2007).

The nucleus, often thought of as a freestanding organelle, is actually a specialized region of the endoplasmic reticulum (ER) (Voeltz et al., 2002): the outer NE is continuous with both the ER and the inner NE (Hetzer et al., 2005), providing a means by which lipids and membrane proteins can move between the sheet form of the ER at the nuclear periphery and the tubular form of the ER, which, in animal cells, extends throughout the cytoplasm and, in yeast, is primarily at the cell periphery (Pidoux and Armstrong, 1993; Voeltz et al., 2002). The Ran GTPase influences many cellular functions (Quimby and Dasso, 2003), including nucleocytoplasmic transport, NE reformation after mitosis in animal cells (Hetzer et al., 2005), and NE structure in fission (Demeter et al., 1995) and budding (Ryan et al., 2003) yeast. However, the mechanism by which the Ran GTPase influences mitosis-specific NE changes during both open and closed mitosis remains unknown. We have previously shown that, when the Ran system is mis-regulated in the fission yeast Schizosaccharomyces pombe, cells arrest with NEs that have lost their integrity, and that this defect is concomitant with and depends on the passage through mitosis (Demeter et al., 1995).

The predictions of a biophysical model of the fission-yeast nucleus (Lim et al., 2007) and experimental observations of abnormal nuclear shapes seen when microtubule bundles lacking SPBs at their ends cause thin tethers to protrude from the spherical nucleus (Khodjakov et al., 2004; Tange et al., 2002; Toya et al., 2007; West et al., 1998; Zheng et al., 2007) raise the possibility that the SPB influences the mechanical properties of the NE to prevent tether formation during normal mitosis and ensure symmetric nuclear division. Because of limitations on the mechanical strength of lipid bilayers and their ability to stretch in response to pressure exerted by the elongating spindle, our computational model incorporates a lipid reservoir (Lim et al.,
Because most cellular lipids are synthesized in the ER, and because the ER is continuous with the NE, it might serve as this lipid reservoir (Lim et al., 2007) that is necessary to accommodate the slow doubling in nuclear volume during interphase in all eukaryotes and the rapid increase in NE area as the spherical nucleus divides into two smaller spheres during closed mitosis.

Because of its multiple functions, conventional genetic screens have identified only a small number of direct Ran targets (Demeter and Sazer, 1998). However, our recent computational model of the fission-yeast nucleus (Lim et al., 2007) made experimentally testable predictions about the possible roles for the ER in NE growth and the SPB in nuclear-shape changes during division.

**Results**

The Ran-GTPase system is required for symmetric nuclear division

To precisely map with respect to cell-cycle events the previously described loss of NE integrity in the temperature-sensitive RanGEF mutant *pim1-d1* (Demeter et al., 1995; Sazer and Nurse, 1994), we monitored nuclear size, shape and integrity in cells producing GFP-Nsp1p and GFP-NLS-β-gal (NLS represents nuclear localization signal) (Yoshida and Sazer, 2004) to visualize the nuclear periphery and the nuclear volume, respectively (see Materials and Methods).

Consistent with previous results using fixed cells (Demeter et al., 1995), a live-cell timecourse analysis showed that, after 4 hours at the restrictive temperature, approximately 50% of *pim1-d1* mutant cells had fragmented NEs (Fig. 1A). Strikingly, approximately 7% of the population had one nucleus that was larger than normal and one smaller than normal at incubation times longer than 2 hours (Fig. 1A).

Time-lapse microscopy revealed that this uneven nuclear division was not a rare terminal phenotype but a transient state that precedes NE breakage: wild-type cells underwent roughly equal nuclear division at mitosis (Fig. 1Ba-h; supplementary material Movie 1) but *pim1-d1* mutant cells (Fig. 1Bi-p; supplementary material Movie 2) consistently underwent an asymmetric nuclear division (Fig. 1Bl) immediately followed by loss of NE integrity (Fig. 1Bm-p). The Ran-GTPase system is therefore necessary for the symmetrical division of the nucleus during mitosis and for the integrity of the NE early in anaphase B.

After asymmetric nuclear division in the *pim1-d1* mutant, the larger and smaller nuclei are dramatically different in volume

We quantified nuclear volume (see Materials and Methods) in the sister nuclei of binucleated wild-type cells and found a small difference of 2.7±1.5 μm³ between the average volumes of the larger (14.1 μm³) and smaller (11.3 μm³) nuclei (Fig. 1C2,D). Because the largest difference in nuclear volume in wild-type cells was 1.8-fold, we defined asymmetric division as a ≥twofold difference in size between the larger and smaller nuclei. Using this criterion to examine asymmetric nuclear division in binucleated *pim1-d1* mutant cells at 36°C, the average volume difference was 12.1±3.4 μm³ (Fig. 1C1,D), indicating a striking asymmetry that is uniform within the population.

In the *pim1-d1* (Ran GEF) temperature-sensitive mutant, the NE breaks immediately after the nucleus divides asymmetrically, the point in the cell cycle at which there is a rapid 26% increase in NE surface area (Lim et al., 2007).

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**Fig. 1.** *pim1-d1* (RanGEF) mutant cells undergo asymmetric nuclear division followed by NE breakage. (A,B) *pim1-d1* and wild-type cells with GFP-Nsp1p and GFP-SV40-NLS-β-gal were grown to log phase at 25°C and shifted to 36°C for the indicated times. (A) The number of *pim1-d1* mutant cells with fragmented NEs (squares, solid lines) or uneven-sized daughter nuclei (diamonds, dashed lines) were counted using fluorescence microscopy of living cells every 30 minutes after a temperature shift to 36°C for 4 hours. (B) Nuclear division was monitored by time-lapse deconvolution microscopy of living cells that were pre-incubated at 36°C for approximately 2 hours and then maintained at 36°C during observation using a heated stage. Wild-type cells (a-h) undergo symmetric division of the nucleus (see supplementary material Movie 1) but *pim1-d1* cells (i-p) (see supplementary material Movie 2) undergo uneven nuclear division (i) and then break, after which the previously nuclear GFP-NLS-β-gal is distributed throughout the cell (m-p). (C) *pim1-d1* (1) or wild-type (2) cells expressing the nuclear reporter GFP-SV40-NLS-β-gal were grown to log phase at the permissive temperature of 25°C and then shifted to the restrictive temperature of 36°C for 4 hours. Large arrowheads indicate the larger nucleus; small arrowheads indicate the smaller nucleus. (D) The nuclear volumes of the larger and smaller nuclei in binucleated cells as shown in C (*n*≥22). Error bars represent s.e.m. Scale bars: 10 μm.
The SPB synthesized in pim1-d1 mutant cells at the restrictive temperature preferentially localizes in the smaller nucleus after asymmetric division.

To test our prediction that the SPB influences nuclear size and shape during division (Lim et al., 2007), we monitored the localization of the new SPB, assembled in pim1-d1 cells at the restrictive temperature, with respect to nuclear size after asymmetric division, using a previously described experimental strategy to distinguish between the new and the old SPB (Grallert et al., 2004). Wild-type (Fig. 2A) and pim1-d1 mutant (Fig. 2B) cells expressing GFP–NLS–β-Gal (Yoshida and Sazer, 2004) and Pcp1p-RFP (Grallert et al., 2004), which encodes the SPB component Pcp1p fused to a slow-folding version of red fluorescent protein (RFP), were starved and then re-fed under conditions that enrich for binucleated cells (see Materials and Methods). We found no correlation between SPB age and nuclear size in wild-type cells (Fig. 2A,E); however, in pim1-d1 cells in which the new and old SPB could be clearly distinguished (Fig. 2B), the new SPB preferentially localized in the small nucleus (Fig. 2B,E). This contrasts with the random distribution of the new SPB between the large and small nucleus in cut6-621 (Fig. 2C,E) or lsd1-H518 (Fig. 2D,E) mutants (Saitoh et al., 1996), which maintain NE integrity. These mutants have defects in fatty-acid metabolism, but the relationship between this biochemical defect and the morphological and other defects in the cells is not known.

Slowing down spindle elongation influences nuclear division in pim1-d1 mutant cells.

To test the hypothesis that the pim1-d1 mutant undergoes NE breakage because it is unable to efficiently increase NE area at mitosis, we slowed its rate of spindle elongation by introducing the temperature-sensitive atb2-983 mutation in α2-

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Fig. 2. After asymmetric division in pim1-d1 mutant cells, the new SPB is preferentially associated with the smaller nucleus. Wild-type (A), pim1-d1 (B), cut6-621 (C) or lsd1-H518 (D) cells with the nuclear reporter GFP–SV40-NLS–β-gal and the SPB reporter Pcp1p-RFP were grown to log phase, starved of nitrogen for 16 hours at the permissive temperature of 25°C, resuspended in complete medium and shifted to the restrictive temperature of 34°C for 4 hours (A,B) or re-suspended in complete medium at 25°C for 30 minutes and shifted at 36°C for 4 hours (C,D). The larger and smaller nuclei were distinguished in binucleated cells in which the difference in RFP fluorescence intensity allowed identification of the new and old SPB. Arrowheads indicate new SPB; arrows indicate old SPB; duplicate cells for each sample are indicated as 1 and 2. (E) The proportion of cells in which the new SPB localized to the larger or smaller nucleus was determined (n≥30).

*P<0.0005. Scale bar: 5 μm.
tubulin, which doubles the length of anaphase B (Asakawa et al., 2006).

When wild-type, \textit{pim1-d1} or \textit{atb2-983} single-mutant, or \textit{pim1-d1 atb2-983} double-mutant strains were incubated at a range of temperatures from 25°C to 36°C, we observed partial suppression of the \textit{pim1-d1} growth defect in the double mutant at 33°C (Fig. 3A). To test whether this rescue correlated with a reduction in NE breakage, we quantified NE integrity in cells incubated at 25°C or 33°C (Fig. 3B). At 25°C, none of the cells in these four strains had broken NEs (Fig. 3C). At 33°C, no wild-type or \textit{atb2-983} cells had broken NEs. For \textit{pim1-d1} cells, 24.2% had broken NEs, but this proportion was decreased by half to 12.6% in the \textit{pim1-d1 atb2-983} mutant (Fig. 3B,C). This rescue was not due to preventing entry into or completion of mitosis, because, at 33°C, the percentage of binucleated cells in \textit{atb2-983} cells was 13.7%, compared with 10.0% in wild-type cells. We also found that introduction of the \textit{atb2-983} mutation did not reduce the frequency of asymmetric nuclear division in \textit{pim1-d1} cells (4.3±0.6% in \textit{pim1-d1}, 4.7±0.4 in \textit{pim1-d1 atb2-983}).

**NE breakage in \textit{pim1-d1} cells is partially rescued by proliferation of the ER membrane**

Because the ER and the NE comprise a single membrane system, the ER is the most likely source of the rapid 26% increase in NE area needed to accommodate the division of the nucleus into two smaller nuclei at anaphase B (Lim et al., 2007). To test the possibility that the ER represents the proposed lipid reservoir necessary for the rapid increase in NE area at mitosis (Lim et al., 2007), we investigated whether artificially increasing the area of ER membrane by overproduction of the ER-membrane protein HMG-CoA reductase (encoded by the \textit{Saccharomyces cerevisiae HMG1} gene) (Lum and Wright, 1995) would protect \textit{pim1-d1} mutant cells from NE breakage at mitosis.

\textit{HMG1} expression decreased viability (Fig. 4A) but did not impact the NE in wild-type or \textit{pim1-d1} cells at 25°C (Fig. 4B,C). It did reduce the proportion of \textit{pim1-d1} cells with fragmented NEs at 36°C from 41.5±4.5% in the vector control to 30.0±5.3% (Fig. 4B,C) but did not increase cell viability when compared to the vector control, as would be expected owing to its toxicity. \textit{HMG1} did not protect the NE by preventing entry into or completion of mitosis; after 27 hours of expression at 25°C, the frequency of binucleated cells was 15.3% compared to 15.8% in the vector control. We also found that \textit{HMG1} overexpression for 30 hours (26 hours at 25°C and 4 hours at 34°C) did not rescue the defect of asymmetric nuclear division in \textit{pim1-d1} cells (7.8±0.2% asymmetric in \textit{pim1-d1}, 7.0±1.0% asymmetric in \textit{pim1-d1 + HMG1}).

To investigate whether the rescue of NE breakage by ER proliferation or by slowing spindle elongation was additive, we overexpressed \textit{HMG1} in \textit{pim1-d1 atb2-983} double-mutant cells using experimental conditions in which we could clearly monitor the influence of \textit{HMG1} overexpression and of the two different temperature-sensitive mutations on NE integrity (\textit{HMG1} was

**Fig. 3.** Slowing spindle elongation partially rescues the defects in growth and NE integrity in \textit{pim1-d1} cells. (A) Wild-type, \textit{atb2-983}, \textit{pim1-d1} and \textit{pim1-d1 atb2-983} double-mutant strains were grown in EMM, spotted onto EMM plates with the vital dye phloxine B, which accumulates in dead cells turning them dark pink, and incubated at the indicated temperatures for 3-5 days. (B) Wild type, \textit{pim1-d1}, \textit{atb2-983} and \textit{pim1-d1 atb2-983} double-mutant strains containing the nuclear reporter GFP-SV40-NLS-β-gal and the NE reporter GFP-Nsp1p were incubated at 35°C for 15 hours on EMM plates and NE integrity monitored by fluorescence microscopy. Star indicates cell with broken nucleus. (C) Quantification of the proportion of cells in which the NE is broken (n=200). The error bars represent s.c.m. Scale bar: 5 μm.
overexpressed for 30 hours, 26 hours at 25°C and 4 hours at 36°C). Consistent with our previous results, we found that, under these conditions, HMG1 overexpression reduced NE breakage in pim1-d1 cells by approximately 10 percentage points, from 46.0±2.1% to 36.6±1.4%. Introduction of the atb2-983 mutation into this strain further reduced the frequency of NE breakage to 20.8±1.0%.

NE breakage in pim1-d1 cells is partially rescued by altering ER-membrane conformation

To more specifically test the possibility that the nuclear-division defects in pim1-d1 mutants are caused by an inability to add sufficient lipid to the NE at mitosis, we sought a way to specifically increase the area of NE membrane. Because reticulon-family proteins stabilize the tubular form of the ER at the expense of the sheet form in vivo and in vitro (Voeltz et al., 2006), we reasoned that they were good candidates for this purpose. We deleted the rtn1 gene [also known as cwl1 (Godoy et al., 1996)], which encodes the only reticulon-like-domain-containing protein in the S. pombe genome (Oertle et al., 2003) and found that it was not essential for viability (Fig. 5B). Deletion of rtn1 did not impair the growth rate at any temperature (Fig. 5B) or alter cell-cycle progression, because the proportion of binucleated cells at 34°C was 16.5% in Δrtn1 and 13.2% in wild-type cells. However, deletion of rtn1 rescued growth of the pim1-d1 mutant at 34°C (Fig. 5B).

Localization of the integral ER-membrane proteins GFP-13g6 (Brazer et al., 2000) in wild-type and pim1-d1 cells at the permissive temperature confirmed the previously described ER localization in wild-type cells (Pidonux and Armstrong, 1993) at the cell cortex, surrounding the nucleus and in a small number of tubules that connect these two ER forms (Fig. 5A, compare cells 1 and 2, 3 and 4): (1) portions of the ER dissociated from the plasma membrane, forming cytoplasmic invaginations; and (2) the reticular organization at the cell periphery was significantly altered. This change is identical to that seen in budding-yeast reticulon mutants (Voeltz et al., 2006) and is consistent with a conversion of tubular ER to sheet ER at the cell periphery.

We then investigated whether deletion of rtn1 influenced the NE in the pim1-d1 background by monitoring the conformation of the ER (Fig. 5A), the integrity of the NE (Fig. 5C,D) and the volume of the sister nuclei (Fig. 5E) in binucleated pim1-d1 Δrtn1 double-mutant cells.

NE breakage in pim1-d1 cells that were incubated at the restrictive temperature was partially rescued by Δrtn1 (Fig. 5C,D). No wild-type, Δrtn1, pim1-d1 or pim1-d1 Δrtn1 cells at the permissive

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**Fig. 4.** Increasing ER membrane by overexpression of HMG1, which encodes the S. cerevisiae ER-membrane protein Hmg1p (HMG CoA reductase), partially rescues the NE-integrity defects of pim1-d1 cells. (A) Wild-type or pim1-d1 cells carrying the plasmid pPL283 (Lum and Wright, 1995) expressing S. cerevisiae HMG1 from the thiamine-repressible nmt1 gene promoter or a vector control were grown in EMM with thiamine to log phase. HMG1 expression was induced by removal of thiamine for a total of 26 hours, the first 22 of which were at the permissive temperature of 25°C and the last 4 of which were at the restrictive temperature of 36°C. Cells were spotted onto EMM plates with the vital dye phloxine B, which accumulates in dead cells turning them dark pink, with or without thiamine and incubated at the indicated temperatures for 3-5 days. (B) The wild-type and pim1-d1 strains described in A with the nuclear reporter GFP–SV40-NLS–β-gal and the NE reporter GFP-Nsp1p were incubated without thiamine at 25°C for 22 hours and then for 4 additional hours at 25°C or 36°C, and NE integrity monitored by fluorescence microscopy. Stars indicate cells with a broken nucleus. (C) Quantification of the proportion of cells in B in which the NE is broken (n=200). The error bars represent s.e.m. Scale bar: 5 μm.
temperature of 25°C and no Δrtm1 or wild-type cells at the restrictive temperature of 36°C had broken NEs (Fig. 5C,D). After 3 hours at 34°C, the temperature at which Δrtm1 partially rescued the loss of viability (Fig. 5B), 20.7% of pim1-d1 mutants but only 4.5% of pim1-d1 Δrtm1 double-mutant cells lost NE integrity. After 2 hours at 36°C (Fig. 5C), Δrtm1 lowered NE breakage from 15.3% to 3.2% (Fig. 5D). At the restrictive temperature, both the absolute and relative sizes of the larger and the smaller nuclei in the pim1-d1 mutant were nearly identical to those of pim1-d1 Δrtm1 double-mutant cells (Fig. 5E).

We investigated the possible relationship between changes in ER conformation and NE integrity by monitoring ER-membrane morphology under conditions in which the Δrtm1 mutation had the greatest influence on NE integrity: 36°C for 2 hours (Fig. 5A, cells 5-8). In pim1-d1 cells, ER conformation resembled that of wild type at both the cell center and periphery (Fig. 5A). However, in pim1-d1 Δrtm1 cells, ER abnormalities, including invaginations from the cell periphery and abnormal sheet-like structures close to or in contact with the NE, were more frequent than in the Δrtm1 strain (Fig. 5A).

Fig. 5. Changing ER-membrane conformation by deletion of rtm1, which encodes a reticulon ortholog, partially rescues the growth and NE breaking in pim1-d1 cells. (A) Wild-type, pim1-d1, Δrtm1 and pim1-d1 Δrtm1 double-mutant strains with the ER-membrane marker GFP-13g6 (Brazer et al., 2000) were incubated at 25°C in EMM for 22 hours (cells 1-4) or incubated at 36°C in EMM for 20 hours and then at 36°C for 2 hours (cells 5-8), and then observed using deconvolution microscopy to visualize ER conformation at the cell center or the cell periphery. Arrowhead indicates ER invagination; arrow indicates abnormal ER structure. (B) Wild-type, pim1-d1, Δrtm1 and pim1-d1 Δrtm1 double-mutant strains were spotted onto EMM plates with the vital dye phloxine B, which accumulates in dead cells turning them dark pink, and incubated at the indicated temperatures for 3-5 days. (C) Wild type, pim1-d1, Δrtm1 and pim1-d1 Δrtm1 double-mutant strains with the nuclear reporter GFP–SV40-NLS–β-gal and the NE reporter GFP-Nsp1p were incubated at the indicated temperatures, and NE integrity was monitored by fluorescence microscopy. Stars indicate cells with a broken nucleus. (D) Quantification of the proportion of cells in C in which the NE is broken (n=200). (E) Wild-type, pim1-d1, Δrtm1 and pim1-d1 Δrtm1 double-mutant strains with the nuclear reporter GFP–SV40-NLS–β-gal were incubated at 36°C for 2 hours, and nuclear volume of the larger and smaller nucleus in binucleated cells (n=20) determined using deconvolution microscopy. The error bars represent s.e.m. Scale bars: 5 μm.
Discussion
The Ran-GTPase system is required for symmetric nuclear division and NE integrity at mitosis
We have previously shown that the Ran-GTPase system in fission yeast is essential for the integrity of the NE and report here that, in the piml-d1 (RanGEF) temperature-sensitive mutant, the NE breaks immediately after the nucleus divides asymmetrically at early anaphase B (Fig. 1), the point in the cell cycle at which there is an increased demand for rapid NE growth (Lim et al., 2007).

The SPB influences nuclear size and shape during nuclear division
Consistent with the possibility that symmetric nuclear division depends on the SPB (Lim et al., 2007), we found that the SPB that is synthesized when Ran-GTPase function is compromised by the piml-d1 (RanGEF) mutation is preferentially associated with the smaller daughter nucleus after asymmetric nuclear division (Fig. 2). The Ran GTPase is therefore directly or indirectly required for proper SPB structure and/or function, and the inability of piml-d1 cells to maintain nuclear size and shape during division is likely to be caused by this as-yet-unidentified SPB defect. The structure and function of animal-cell centrosomes, which are functionally equivalent to yeast SPBs, are also influenced by the Ran-GTPase system, but in those cases reduction of RanGTP promotes loss of centrosome cohesion and thereby the production of multipolar spindles (Di Fiore et al., 2004).

Asymmetric division does not necessarily lead to NE breakdown
In the piml-d1 (RanGEF) temperature-sensitive mutant, the NE breaks immediately after the nuclear divides asymmetrically, the point in the cell cycle at which there is a rapid 26% increase in NE surface area (Lim et al., 2007). However, under conditions in which NE fragmentation is reduced by slowing spindle elongation, increasing ER membrane or changing ER structure, we found no influence on the asymmetry of nuclear division. In contrast to the NE breakage after asymmetric division in piml-d1 cells, the NE remains intact in cut6 and lsd1 single-mutant strains that have defects in fatty-acid metabolism and undergo unequal nuclear division by an unknown mechanism (Saitho et al., 1996). Fission yeast strains with mutations in the kinetochore proteins Mis6p or Mis12p undergo asymmetric nuclear division due to chromosome mis-segregation, but their NEs also remain intact (Goshima et al., 1999). Chromosome mis-segregation is unlikely to account for the asymmetric nuclear division of piml-d1 cells because, at the restrictive temperature, these cells consistently underwent uneven nuclear division (Fig. 1B) but undergo equal chromosome segregation (Sazer and Nurse, 1994).

These data are consistent with the hypothesis that, in fission yeast, the Ran-GTPase system independently influences nuclear division via the SPB, and NE growth and integrity via the ER.

Regulation of NE growth and its coordination with spindle elongation is required for maintenance of NE integrity at mitosis
The observation that the piml-d1 mutant undergoes NE breakage at the point in mitosis when there is a sharp increase in the demand for increased NE area (Lim et al., 2007) suggests that lipid might not be added in sufficient quantity and/or at sufficient speed to keep pace with spindle elongation. Consistent with this possibility, we previously showed that loss of NE integrity correlates temporally with and depends on the passage of cells through mitosis, and we show here that there are three conditions in which the loss of NE integrity and/or loss of viability in piml-d1 mutant cells were partially rescued: slowing spindle elongation by the atb2-983 mutation in α2-tubulin (Fig. 3), increasing ER membrane by overproduction of the ER-membrane protein HMG CoA reductase (Fig. 4) and changing ER morphology from tubular to sheet by deletion of rtn1, which encodes a reticulon-like protein (Oertle et al., 2003) (Fig. 5). Because the Ran GTPase participates in many cellular processes (Quimby and Dasso, 2003) other than NE structure, it is not surprising that these nucleus-specific manipulations result in only partial rescue of piml-d1 viability in the case of atb2-983 and rtn1 deletion, and no rescue in the case of HMG1 overexpression, which is somewhat toxic even at 25°C.

Together, these studies demonstrate the importance of proper regulation of increases in NE area and the coordination of these increases with spindle elongation in mitosis. Furthermore, they suggest that conformational changes in the ER-NE membrane system or lipid redistribution within it are crucial for these processes.

Fission yeast has a single reticulon ortholog, named rtn1, which is not essential for viability or nuclear division. In contrast to the situation in S. cerevisiae, deletion of the single rtn1 gene in fission yeast changes the conformation of the tubular ER at the cell periphery and causes it to detach from the cell periphery (Fig. 5). These conformational changes alter the distribution of lipid between the tubular and sheet forms, thereby promoting lipid incorporation into the NE portion of the ER network. They also facilitate the conversion of tubular to sheet ER at the time of nuclear division during closed mitosis, because deletion of rtn1 partially protects piml-d1 nuclei from breakage upon spindle elongation and partially restores viability (Fig. 5). These data demonstrate that mitotic increases in NE area are normally coordinated with spindle elongation, and they are consistent with the hypothesis that lipid redistribution within the ER-NE network is crucial for these processes. It is notable that Δrtn1 cells do not have nuclei that are larger than normal, suggesting that an as-yet-unknown mechanism that regulates NE growth during interphase functions normally in the absence of Rtn1p.

Is there an evolutionarily conserved role for the Ran-GTPase system in NE structure?
The Ran-GTPase system influences NE structure and assembly in both open and closed mitosis, and is also required for nuclear pore complex (NPC) assembly (Demeter et al., 1995; Hetzer et al., 2005) (reviewed by Quimby and Dasso, 2003), but it is not known whether it does so by means of a common Ran target, because no nucleus-specific targets have been identified either in vivo or in vitro. In meiotic egg-extract systems, the Ran-GTPase system is required for NE vesicle targeting to the chromosomes after mitosis and subsequent fusion to reform the NE in vitro (reviewed by Hetzer et al., 2005; Mattaj, 2004). Ran-mediated vesicle fusion was also proposed to be essential for NPC assembly and NE growth in budding yeast (Ryan et al., 2003), but the kinetics and magnitude of NE growth at mitosis in yeast make it unlikely that it is mediated by vesicle fusion (Hetzer et al., 2005; Ryan et al., 2003). Alternatively, reports that some inner-NE-specific proteins relocalize to the tubular ER upon NE breakdown at mitosis in animal cells (Ellenberg et al., 1997; Yang et al., 1997) are consistent with previously proposed, and recently demonstrated, conversion of the sheet ER of the NE into tubular ER during open mitosis (Hetzer et al., 2005; Mattaj, 2004; Puhka et al., 2007). Conversely, we show...
that changing ER conformation from tubular to sheet in the \textit{pim1-d1} (RanGEF) mutant facilitates the rapid incorporation of large amounts of lipid into the fission-yeast NE during closed mitosis and propose that it might also be crucial for NE reformation after open mitosis in animal cells (Mattaj, 2004). In fact, while this study was in progress, Hetzer’s group reported that tubular ER is required for NE reformation in vitro (Anderson and Hetzer, 2007) in a process mediated by chromatin. By contrast, during nuclear division in fission yeast, the mitotic chromosomes are highly condensed and, because at this stage of the cell cycle they are not in direct contact with the NE, are unlikely to directly mediate the increase in NE area. However, our data on the importance of reticulons in regulating NE growth are consistent with a recent report from Anderson and Hetzer showing that the removal of reticulons from tubular ER is rate limiting for animal-cell NE reformation in vitro (Anderson and Hetzer, 2008). Redistribution of lipid within the NE-ER network is therefore likely to represent a common step in the mitotic-specific changes to the NE during both open and closed mitosis.

Materials and Methods

Yeast strains, strain construction and cell culture

Standard methods and genetic techniques were used (Moreno et al., 1991), and strains are described in Table 1. Spotting experiments were performed by growing cells in Edinburgh minimal medium (EMM) to mid-log phase and spotting 10^6 cells and fivefold dilutions on EMM plates with the pink vital dye phloxine B (Sigma) (that is excluded from living cells), supplements and thiamine as indicated. Gene expression from the \textit{nmt1} gene promoter (Forsburg and Sherman, 1997; Maundrell, 1990) was repressed by growth in EMM with 5 μg/ml thiamine. The \textit{\alpha n}1 (\textit{nmt1-null}) strain was generated by PCR-based targeted gene replacement using a natMX4 drug-resistance cassette (Bahler et al., 1998; Goldstein and McCusker, 1999). \textit{Saccharomyces cerevisiae} HMG1 expression was regulated by the \textit{nmt1} promoter on plasmid pPL283 (Lum and Wright, 1995).

### Table 1. Strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| SS7    | h– leu1-32 | Our stock |
| SS446  | h– leu1-32 ura4-D18 ade6-M210 | Our stock |
| SS482  | h– ad6-M216 pREP3X-SV40NLS-GFP-lacZ | Our stock |
| SS483  | h– pim1-d1 leu1-32 ade6-M216 pREP3X-SV40NLS-GFP-lacZ | Our stock |
| SS749  | h– pim1-d1 ura4-D18 ade6-M210 | Our stock |
| SS777  | h– pim1-d1 ura4-D18 p REP3X-SV40NLS-GFP-lacZ | Our stock |
| SS817  | h– ura4-D18 pREP82X-GFP-nspl int::p REP3X-SV40NLS-GFP-lacZ | Our stock |
| SS1652 | h– ura4-D18 ade6-M210 | Our stock |
| SS1758 | h– pim1-d1 leu1-32 | Our stock |
| SS1876 | h– pcp1.RFP::kan R | This study |

This study

- **Fluorescence microscopy**

- **Nuclear division and -integrity analysis**

Cells expressing the fluorescently labeled NPC protein GFP-Nsp1p, to visualize the NE, and/or a fluorescently labeled soluble nuclear protein GFP-NLS-β-gal, to visualize the nuclear compartment (Yoshida and Sazer, 2004), were examined using either a Zeiss Axioskop fluorescence microscope, from which images were captured by a DVD 1300 black and white CCD camera using QED software (Media Cybernetics, Silver Springs, MD), or a DeltaVision deconvolution microscope system (Applied Precision, Issaquah, WA), to collect images of living cells in a heated cell chamber maintained at 36°C, using a Photometrics CoolSnap HQ camera (Roper Scientific, Tucson, AZ). Time-lapse studies, early mitotic cells were identified on the basis of their oblong-shaped nuclei, and images were collected every 5 seconds until nuclear division was completed. For analysis of nuclear volume and SPB localization, stacks of 0.2 μm z sections were projected two dimensionally using the maximum-intensity protocol and analyzed using SoftWoRx3.3.

- **Nuclear-volume analysis**

To calculate nuclear volume in live cells independent of absolute fluorescence intensity, the distribution of GFP-NLS-β-gal was used to define the periphery of the nucleus by creating two-dimensional (2D) polygons in each z section that were visually verified and then assembled into a 3D model of the nucleus upon which nuclear volume was calculated.

- **Distinguishing the old and the new SPBs**

Using a previously described protocol (Grallert et al., 2004) with minor modifications [including substitution of MSL (Egel et al., 1994) for EMM2 medium], strains with GFP-NLS-β-gal (Yoshida and Sazer, 2004) and Pep1-RFP (the SPB protein Pep1p fused to a slow-folding version of RFP) (Grallert et al., 2004) were grown at the permissive temperature of 25°C to log phase, starved of nitrogen at the permissive temperature of 25°C for 16 hours, then re-fed at the indicated restrictive temperature for 4 hours. Cells in which the old (brightly fluorescent) and new (dimly fluorescent) SPB could be clearly distinguished by differences in RFP signal intensity were used to determine the relationship between SPB inheritance and nuclear size.

- **Statistical analysis**

A chi-square test \( (\chi^2) \) was performed to determine whether the distribution of the new SPB between the large and small nucleus was significantly different from...
random. Because only two phenotypic classes (small or large nucleus) were studied, Yates' correction for continuity was applied. Using 0.05 as the alpha (α) level of significance and one degree of freedom, the probability (P) was calculated for each sample.

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