The SUN Protein Mps3 Is Required for Spindle Pole Body Insertion into the Nuclear Membrane and Nuclear Envelope Homeostasis

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Abstract

The budding yeast spindle pole body (SPB) is anchored in the nuclear envelope so that it can simultaneously nucleate both nuclear and cytoplasmic microtubules. During SPB duplication, the newly formed SPB is inserted into the nuclear membrane. The mechanism of SPB insertion is poorly understood but likely involves the action of integral membrane proteins to mediate changes in the nuclear envelope itself, such as fusion of the inner and outer nuclear membranes. Analysis of the functional domains of the budding yeast SUN protein and SPB component Mps3 revealed that most regions are not essential for growth or SPB duplication under wild-type conditions. However, a novel dominant allele in the P-loop region, MPS3-G186K, displays defects in multiple steps in SPB duplication, including SPB insertion, indicating a previously unknown role for Mps3 in this step of SPB assembly. Characterization of the MPS3-G186K mutant by electron microscopy revealed severe over-proliferation of the inner nuclear membrane, which could be rescued by altering the characteristics of the nuclear envelope using both chemical and genetic methods. Lipid profiling revealed that cells lacking MPS3 contain abnormal amounts of certain types of polar and neutral lipids, and deletion or mutation of MPS3 can suppress growth defects associated with inhibition of sterol biosynthesis, suggesting that Mps3 directly affects lipid homeostasis. Therefore, we propose that Mps3 facilitates insertion of SPBs in the nuclear membrane by modulating nuclear envelope composition.

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Introduction

The hallmark feature of eukaryotic cells is the nucleus, a double membrane bound organelle that contains the genetic material. The outer nuclear membrane (ONM) of the nucleus is contiguous with the ER membrane while the inner nuclear membrane (INM) is distinct and contains a unique set of proteins that interact with chromatin and other nuclear factors. Embedded in the nuclear membrane are multiple nuclear pore complexes (NPCs) that regulate transport of macromolecules between the cytoplasm and the nucleus [1]. In organisms such as Saccharomyces cerevisiae that undergo a closed mitosis, the centrosome-equivalent organelle known as the spindle pole body (SPB) is present in the nuclear envelope throughout the life cycle [2]. The SPB organizes both cytoplasmic microtubules, which are involved in nuclear positioning, and nuclear microtubules, which are essential for chromosome segregation [3].

Both NPCs and SPBs are composed primarily of soluble proteins that partially assemble into sub-complexes in the nucleus or cytoplasm [reviewed in [1,3]]. Further assembly of both NPCs and SPBs requires insertion into the nuclear membrane at a point where the INM and ONM are joined together. Specific integral membrane proteins interact with soluble components of the NPC and SPB and are thought to anchor the complexes in the nuclear envelope. Ndc1 is essential for insertion of both the NPC and SPB [4–6]. At the NPC, three additional pore membrane proteins, Pom33, Pom34 and Pom152, play partially overlapping roles in NPC assembly [6–8], while Nbp1, Bbp1 and Mps2 are required in addition to Ndc1 for SPB insertion into the nuclear envelope [9–12].

The mechanism of NPC insertion has been extensively studied in both yeast and metazoan systems. Structural studies have shown that five subunits of the NPC (Nup133, Nup120, Nup85, Nup170 and Nup188) contain an ALPS motif (for ARF/GAP1 lipid packing sensor), which targets them to highly curved membranes [13]. These proteins are thought to form a coat complex on the nuclear envelope to facilitate NPC insertion [14–17]. In addition, membrane-bending proteins of the ER such as the reticulons...
Accurate segregation of chromosomes during mitosis is essential to prevent genetic instability and aneuploidy that lead to cancer and other diseases. Centrosomes and spindle pole bodies mediate the assembly of a microtubule-based structure known as the mitotic spindle, which physically separates chromosomes during mitosis so that the two daughter cells contain a complete copy of the genetic material as well as a spindle pole. During every cell cycle, the DNA and the spindle pole must be duplicated exactly once to ensure proper formation of a bipolar mitotic spindle. In yeast cells, the nuclear envelope does not break down, so the spindle pole must be inserted into the nuclear membrane so that it can form both the microtubules involved in the mitotic spindle and those involved in positioning of the nucleus. How a large protein complex such as the spindle pole body is inserted into the lipid layers of the nuclear membrane is not well understood. We show that the evolutionarily conserved SUN protein Mps3 is involved in spindle pole insertion into the nuclear membrane. This likely reflects a function for SUN proteins in controlling nuclear envelope structure by modulating the types of lipids that are present in the nuclear membrane.

have been shown to play a role in de novo NPC assembly [17,18]. Modification of lipids within nuclear membrane leaflets probably also occur at sites of NPC insertion to accommodate membrane curvature and fusion. Several proteins involved in lipid synthesis and membrane fluidity have been genetically linked to NPC assembly [19–21], although their role in NPC insertion is not well characterized. In vertebrates, the SUN (for Sad1-UNC-84 homology) protein Sun1 also is required for NPC assembly [22,23]. A recent study suggested that hSun1 together with Pom121 is required for de novo assembly of NPCs possibly by facilitating membrane fusion [24].

The mechanism of SPB insertion into the nuclear membrane is poorly understood in comparison to insertion of NPCs. It is possible that many of the same events, such as membrane bending, curvature and lipid modification, are needed for SPB duplication since fusion of INM and ONM also must occur during SPB insertion. No specific factors that possess these functions have ever been directly implicated in the SPB duplication process with the exception of a recent report suggesting that the amphipathic alpha-helix of Nbp1 aids SPB insertion [12]. Perhaps one of the best clues as to how the SPB might insert into the nuclear membrane comes from a plethora of genetic interactions that have been identified between genes encoding SPB components and NPC subunits, including suppression of complete deletions of the SPB membrane components, Mps2 and Mps3, by POM34 or POM132 deletion [4,25–27]. While it is possible that the NPC is involved in SPB insertion through its role in nuclear translocation of SPB subunits or in mRNA processing [25,28], the fact that SPB duplication can occur in the absence of certain structural subunits points to a model in which NPCs and SPBs compete for a shared insertion factor, such as Ndc1 [4]. Alternatively, blocking NPC assembly by elimination of POM132 or POM34 could alter some aspect of the nuclear membrane that enables the SPB to duplicate in the absence of otherwise essential components. Consistent with this possibility, deletion of POM132 together with NUP170 results in nuclear membrane abnormalities [29].

In the present study, we further examine the role of the nuclear membrane in SPB duplication and show for the first time that changes in membrane composition are sufficient for SPB duplication in the absence of Mps3. In addition, we demonstrate a role for the SUN protein Mps3 in regulation of membrane architecture and provide evidence that it functions in the insertion step of SPB duplication. This role of Mps3 is distinct from the previously described functions of Mps3 in SPB duplication. As a component of the SPB substructure that templates assembly for the new SPB, known as the half-bridge, Mps3 is required for initiation of SPB duplication and for tethering the half-bridge to the soluble core SPB through its interaction with the membrane protein Mps2 [30–32]. This function is similar to that of other SUN-domain-containing proteins, which have been shown to play a role in accurate chromosome segregation and nuclear positioning due to their role in duplication and membrane tethering of centrosomes, basal bodies and SPBs in a wide variety of systems [33–35]. However, our current data suggest that SUN proteins have a novel function in membrane homeostasis, which is involved in insertion of protein complexes such as the SPB into the nuclear envelope.

Results

Identification of Mps3 domains that are required for SPB duplication

A key feature of all SUN proteins, including Mps3, is the presence of a number of structural motifs, including at least one transmembrane domain, regions of coiled-coils and a C-terminal SUN domain [35]. In addition, Mps3 contains an N-terminal acidic domain, a poly-glutamine region and a putative P-loop [30] (Figure 1A). In order to determine their role in SUN protein localization and function, we created deletion alleles in each domain using site directed mutagenesis. In the case of the poly-glutamine repeat (pQ), we not only deleted this region but also expanded it two-fold since this type of mutation is often linked to disease [36,37]. In the case of the putative P-loop, we mutated key residues anticipated to be involved in nucleotide binding to a residue with different chemical properties [38,39]. To test if the various mutants were functional, we assayed complementation of an mps3ΔA mutant using a plasmid shuffle strategy. Although Mps3 plays multiple non-essential roles in the chromosome organization within the nucleus [28,40–49], the primary function of Mps3 in mitotically dividing yeast cells is at the SPB [30,31], so this test will allow us to identify domains of Mps3 that are essential for SPB duplication.

mps3Δαα1, mps3Δαα2, mps3Δαα1Δαα2 and mps3ΔpQ mutants, which lack the first, second and both coiled-coil domains or the poly-glutamine region, respectively, did not exhibit any obvious growth defect at various temperatures (Figure 1B; data not shown). To further examine their role in Mps3 function at the SPB, we tested for genetic interactions with mutants in other SPB components: mps2−1, cdc31−2, CDC31−16, kar11A17, spe4−211, spe29−3 and sfi1−3. Each of these mutants is either inviable (synthetic lethal) or shows an enhanced growth defect (synthetic sick) with mps3−1, which has serine 472 in the SUN domain mutated to asparagine [30]. Like Mps3, KAR1 and CDC31 encode components of the SPB half-bridge that are required for the initial step of SPB duplication: Kar1 is an integral membrane protein and Cdc31 is a small calcium binding protein that also binds to Sfi1 on the cytoplasmic side of the SPB half-bridge. Spe42 and Spe29 are components of the core SPB and Mps2 is a linker protein that tethers the half-bridge to the core SPB through interactions with Mps3 [3,32]. Surprisingly, only two genetic interactions were discovered with this new panel of mps3 mutant alleles: synthetic lethality between mps3Δαα2 and kar11A17, and suppression...
Figure 1. Essential regions of Mps3 for SPB duplication. (A) Schematic of Mps3 showing the N-terminal acidic region, transmembrane domain (TM), P-loop motif, coiled-coils (cc), poly-glutamine (pQ) repeat and C-terminal SUN domain. Alignment of P-loop consensus sequence with amino acids 186–194 of Mps3 is shown below. (B) The indicated mutants within each Mps3 domain were tested for their ability to rescue a deletion of MPS3.

Table:

| Domain          | rescue | ploidy     | genetic interactions | localization   |
|-----------------|--------|------------|----------------------|----------------|
| Δacidic (Δ75-150) | +      | haploid    | none                 | SPB            |
| ΔTM (Δ157-170)   | -      | ND         | ND                   | diffuse*       |
| S190A (P loop)   | +**    | haploid    | none                 | SPB & INM      |
| G192K (P loop)   | +      | haploid    | ND                   | ND             |
| S194A (P loop)   | +      | haploid    | ND                   | ND             |
| Δcc1 (Δ242-260)  | +      | haploid    | none                 | SPB & INM      |
| Δcc2 (Δ366-390)  | +      | haploid    | SL with kar1Δ17      | SPB & weak INM |
| Δcc1 Δcc2       | +      | haploid    | ND                   | ND             |
| ΔpQ (Δ391-401)   | +      | haploid    | rescues CDC31-16    | SPB & INM      |
| 2xpQ (2x 391-401) | +    | haploid & diploid mix | none | weak SPB & INM |
| ΔSUN (Δ459-616)  | -      | ND         | ND                   | ND             |

C

Mps3-GFP  mps3ΔTM-GFP + Mps3

D

SD-URA  5-FOA

- MPS3
mps3-S190A
2x-mps3-S190A

E

glucose  galactose

wild-type  GAL-MPS3  GAL-MPS3-G186K
and GAL-MPS3-G186K

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fluorobenzoic acid (5-FBA) plates immediately prior to use; because mps3
FOA, which removes the covering plasmid containing wild-type
mps3
Cdc31 and Kar1 localization to the SPB was unaffected in
together with H2B-mCherry (red) in cells grown at 23°C. Bar, 2 μm. The covering plasmid was removed from SLJ2571 by plating cells to 2-amino-5-fluorobenzoic acid (5-FBA) plates immediately prior to use; because mps3

mutations of the covering-coil domains or deletion of the poly-
glutamine region have at most minor effects on the localization
and function of Mps3 during SPB duplication.

Duplication of the poly-glutamine region did not affect cell
growth (Figure 1B). However, analysis of DNA content by flow
cytometry revealed that mps3-2xpQ mutants exhibited an increase
in ploidy (Figure 1B), which is a common phenotype in SPB
mutants, and it has been previously observed in a number of mps3
alleles [30,32,40]. The increase in ploidy was fully recessive as cells
containing mps3-2xpQ and a wild-type copy of Mps3-GFP (Figure 1B). Taken together, our data suggests
mutant affected SPB duplication and spindle assembly. We found
showed that 66% of
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Nucleotide binding within the P-loop region, we found
most alleles were able to complement mps3Δ and serve as the sole
copy of MPS3 in the cell (Figure 1B; data not shown), suggesting
that this domain does not function in ATP-binding in vivo.

However, some mutants in the P-loop region such as mps3-S190A
displayed copy number sensitivity such that cells containing a single
integrated copy of the mutant gene were viable, but cells
containing two or more copies of the P-loop mutant gene were
dead (Figure 1D). This indicates that mps3-S190A is a weak dosage-
sensitive antimorphic allele. We were unable to obtain transform-
ants of one allele [MPS3-G186K] under a wide variety of
conditions (data not shown), suggesting that it is a dominant
mutant that arrests cell growth.

Using the galactose-regulatable GAL1-10 promoter, we set up a
system so that we could examine the effects of MPS3-G186K on
cell growth and SPB duplication. Cells containing a single
integrated copy of GAL-MPS3 or GAL-MPS3-G186K in addition to
the endogenous wild-type copy of MPS3 were analyzed for their
effect on growth in a serial dilution assay at 30°C. Under these
conditions, overexpression of wild-type MPS3 had a slight effect on
cell growth while overexpression of MPS3-G186K significantly
inhibited cell proliferation (Figure 1E). This confirms that MPS3-
G186K is a novel dominant lethal mutant.

MPS3-G186K mutants are defective in SPB duplication

These same strains were examined following a 5 h induction
with 2% galactose by flow cytometry to analyze DNA content and
by indirect immunofluorescence microscopy with anti-alpha-
tubulin and anti-gamma-tubulin antibodies to visualize microtub-
ules and SPBs, respectively, to determine if the MPS3-G186K
mutant affected SPB duplication and spindle assembly. We found
that cells overproducing wild-type MPS3 did not arrest and
underwent SPB duplication to form bipolar mitotic spindles
(Figure 2A, 2B). However, MPS3-G186K overproduction resulted in
an accumulation of large-budded cells with a 2N DNA content,
which is suggestive of a failure in SPB duplication and/or spindle
formation (Figure 2A). Examination of microtubule structures
showed that 66% of MPS3-G186K mutants, but only 14% of GAL-
MPS3 cells, contain monopolar spindles: a single DNA mass
associated with a single SPB and microtubule array (Figure 2B; n>200). Unlike the monopolar spindle phenotype previously
described for mps3 mutants where a single focus of gamma-tubulin
is associated with the nuclear DNA [30,32], 40% of MPS3-G186K
cells contain two foci of gamma-tubulin, one of which does not
nucleate microtubules (Figure 2B). This phenotype is indicative of
a defect in SPB insertion into the nuclear envelope: the SPB that
does not nucleate microtubules has not properly inserted into the
nuclear envelope and assembled inner plaque components, which
are necessary for the formation of a nuclear microtubule array [3].
Although the uninserted SPB may nucleate cytoplasmic microtub-
ules, these are generally difficult to observe due to the small
number that is formed at each SPB. This phenotype is highly
reminiscent of that observed in mutants that are defective in SPB
insertion into the nuclear envelope such as mps2-1, mps1-1, mps1-dg
and bbp1-d [9–11,50] and suggests that like these SPB compo-

of the temperature sensitivity of the dominant CDC31-16 mutant by
mps3ApQ (Figure 1B). However, unlike mps3-1 mutants [30],
Cdc31 and Kar1 localization to the SPB was unaffected in
mps3Δc2 or mps3ApQ mutants (data not shown). Most likely, this is
because mps3Δc1-GFP, mps3Δc2-GFP and mps3ApQ-GFP localize to the SPB and INM in a pattern that is highly similar to that of Mps3-GFP (Figure 1B). Taken together, our data suggests
that these same strains were examined following a 5 h induction
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Mutation of P-loop residues results in a novel dominant

When point mutants were constructed in potential residues
involved in nucleotide binding within the P-loop region, we found
most alleles were able to complement mps3Δ and serve as the sole
copy of MPS3 in the cell (Figure 1B; data not shown), suggesting
that this domain does not function in ATP-binding in vivo.
Figure 2. MPS3-G186K expression causes a mitotic arrest due to SPB duplication defects. (A,B) GAL-MPS3 (SLJ995) and GAL-MPS3-G186K (SLJ1797) cells were grown overnight at 30°C in YEP plus 2% raffinose then 2% galactose was added for 5 h to induce expression. (A) DNA content was analyzed by flow cytometry and budding index was determined using phase contrast microscopy. The percentage of large-budded cells is indicated (n = 300). (B) In addition, spindle morphology was examined using indirect immunofluorescence microscopy using anti-α-tubulin (green), anti-γ-tubulin (red) and DAPI (blue). The percentage of large budded cells (n > 200) with bipolar and both types of monopolar spindles is indicated for each strain. Bar, 5 μm. (C) Schematic of SPB duplication pathway showing the timing of Spc110-GFP (green) and Spc42-mCherry (red) assembly. (D) SPBs were visualized using Spc110-GFP (green) and Spc42-mCherry (red) in GAL-MPS3 (SLJ4859) and GAL-MPS3-G186K (SLJ4864) cells grown for 5 h in SC-HIS + 2% galactose + 2% raffinose at 30°C. Arrows point to unduplicated SPBs that contain only Spc42-mCherry, and an asterisk marks a SPB fragment seen in MPS3-G186K, which contains only Spc110-GFP. This may represent a delaminated SPB similar to that observed in some spc110 mutants [101]. Bar, 5 μm. (E) The number of SPBs containing the indicated number of Spc42-mCherry and Spc110-GFP foci in large budded cells was quantitated in two independent experiments (n > 250). We also counted SPB foci in SLJ4859 cells grown in SC-HIS + 2% galactose + 2% glucose for 5 h at 30°C. (F–H) GAL-MPS3-G186K (SLJ1797) mutants were also processed for thin-section EM to examine SPB morphology following induction for 5 h in YPGR at 30°C. Serial sections through nuclei of 15 cells were collected: (F) 7 nuclei contained a single unduplicated SPB, (G) 4 nuclei contained an SPB as well as electron dense material (arrow) resembling a SPB precursor on the cytoplasmic face of the nuclear envelope, and (H) 4 nuclei contained...
MPS3-G186K cells have a membrane proliferation defect

In addition to the SPB duplication defect, several additional phenotypes were uncovered during the course of our EM analysis of MPS3-G186K cells that shed light onto the possible function of Mps3 in SPB insertion. As depicted in Figure 3C–3F, MPS3-G186K mutants appeared to have undergone massive over-proliferation of the nuclear membrane, resulting in 2–8 layers of nuclear envelope, and nuclei appear to have multiple lobes and extensions. The membrane expansion phenotype was highly specific and penetrant, occurring in 96% (48 of 50) of GAL-MPS3-G186K cells examined (Figure 3C–3F) but in none (0 of 34) of the GAL-MPS3 cells (Figure 3A–3B). Therefore, it is most probably due to an effect of the MPS3-G186K mutant and not a general result due to overexpression of MPS3 or an integral membrane protein (see also [31]). The fact that we observe excess membrane in MPS3-G186K mutant suggests that Mps3 directly or indirectly is involved in membrane homeostasis.

Membrane proliferation in GAL-MPS3-G186K was restricted to the nucleus; no excess membrane was seen on other organelles, including the ER that is contiguous with the ONM in budding yeast (Figure 4). Previous work had suggested that the membrane adjacent to the nucleolus was subject to the formation of membrane flares [53], but we found that all areas of the nuclear membrane underwent expansion, not just the nucleolar membrane. However, we did observe that the nucleolar region was often partitioned away from the main mass of the nucleus either by a membrane (Figure 4B) or by the formation of a lobe (Figure 3C). Interestingly, within an individual nucleus, membrane proliferation was not uniform in that there were regions that contained a single bilayer and other regions containing multiple bilayers (Figure 3C and 3D, Figure 4). Analysis of serial nuclear sections showed that the excess membrane begins as tubules within the nucleus, which then proliferate underneath the existing membrane and then fuse with adjacent tubules (Figure 4A) or fold back upon itself to continue proliferation within the nucleus (Figure 4B). The fact that the excess membrane forms only in tight association with existing nuclear envelope and does not form lamellae within the nucleus suggests that Mps3 is intimately involved in formation of the nuclear envelope layers.

While it is possible that the excess membrane in MPS3-G186K inhibits SPB insertion, we found that SPBs as well as NPCs were often associated with a membrane region containing a single bilayer (Figure 2F–2H and Figure 3C, 3E). However, we also detected NPCs in intermediate and inner layers, although these often appeared to be stacked with NPCs in adjacent layers (Figure 3F), most likely to facilitate nuclear-cytoplasmic trafficking of macromolecules, which is reduced but not completely inhibited in MPS3-G186K (Figure S3). Unlike other mutants that affect the yeast nuclear membrane [18–21,34], we did not observe partially assembled NPCs in the nucleoplasm or cytoplasm of MPS3-G186K mutants that would suggest a defect in NPC insertion, perhaps due to the large number of NPCs present in the nuclear envelope [55] and the relatively short period of time in which the dominant mutant is expressed (Figure 3 and Figure 4). Because both SPBs and NPCs are observed in nuclear membrane areas that contain a single bilayer and there is no apparent defect in NPC insertion, we suspect that the SPB assembly defect associated with MPS3-G186K is the result of membrane composition rather than the excess membrane.
MPS3-G186K mutants have defects in nuclear morphology

Membrane proliferation in MPS3-G186K mutants was also accompanied by an abnormal nuclear morphology. In many cases the nuclear membrane completely encircled regions of the cytoplasm, entrapping vesicles and other components (Figure 4A–4B). Membrane extensions and protrusions were observed in all GAL-MPS3-G186K cells (n = 50) and in 50% of GAL-MPS3 cells (17 of 34), which do not over-proliferate the nuclear membrane (Figure 3B–3D). The remaining 50% of GAL-MPS3 cells had a round or oval nuclear morphology similar to wild-type, depending on the cell cycle stage (Figure 3A).

We were able to follow the formation of nuclear morphology defects in real-time in GAL-MPS3-G186K mutants and GAL-MPS3 control cells using HDEL-dsRed to visualize the nuclear and ER membranes and Pus1-GFP to visualize the nucleus. Time-lapse image analysis showed that the nuclei in MPS3 overexpressing cells underwent minor changes in nuclear shape—virtually all nuclei were round or oval shaped throughout the 3.5 h time-course, with a few extensions and protrusions forming in some nuclei only at later time points (Video S1; Figure 5B). In addition, we observed cells undergoing mitosis as the nucleus and nuclear membrane became elongated and then hour-glass shaped. In contrast, membrane extensions and nuclear deformation were easily observed in most MPS3-G186K cells by 1.5–2 h following addition of galactose (Video S2). At later time points, more severe membrane perturbation occurred in the mutant, often resulting in the formation of several masses of nuclear material within one cell (Figure 5B). Although some mutant nuclei elongated, none completed mitosis to form two distinct masses of DNA. Thus, it appears that membrane proliferation is linked to an abnormal nuclear morphology and inhibition of mitotic progression.

Alteration of membrane composition alleviates the growth defect of MPS3-G186K

To better understand the role that Mps3 plays in SPB insertion and membrane structure, we screened the yeast deletion collection for mutants that rescued the growth defect of GAL-MPS3-G186K. If SPB duplication defects in this mutant are the result of defects in membrane composition as our cytology suggests, then it should be possible to find mutants that alter the lipid composition of the nuclear membrane to compensate for MPS3-G186K expression. In total, 93 mutants were found to grow on SC-URA+2% galactose in the presence of p4UR3-GAL1-MPS3-G186K (Table S2). Because a number of these genes likely affect transcription, translation, post-translational modification or galactose-induction of the dominant allele, we used western blotting as a secondary screen to identify mutants that expressed high levels of Mps3-G186K protein, similar to that observed in wild-type cells (data not shown). 37 mutants met this secondary criterion and are shown in Figure 6A together with a wild-type and gal4A control. GALA encodes a transcription factor that is required for activation of genes in response to galactose, including expression from GAL1 [56]. This mutant was identified in our primary screen, but as expected, failed to pass our secondary test for Mps3-G186K production and serves as a control (Table S2).

Of the 37 deletion mutants that restore growth to MPS3-G186K, a number of genes encode proteins involved in transcription or translation. While these do not affect production of Mps3-G186K, they most likely restore growth to the mutant by affecting expression of an unknown target. One possible candidate target is POM4, which has previously been shown to be regulated by translation and whose levels influence growth of mutants defective in SPB insertion, such as mps2, bbpl1 and ndk1 [4,25,26]. Interestingly, we also found that deletion of POM34, POM152 and several other nucleoporins suppressed MPS3-G186K mutants (Figure 6A). A number of these same deletion mutants were recently shown to rescue mps3A [26]. We proposed that deletion of the nucleoporins may rescue growth of cells lacking Mps3 by blocking NPC assembly, thereby liberating a shared insertion factor involved in both SPB and NPC insertion. Alternatively, their deletion may rescue growth of mps3A cells by changing the physical properties of the nuclear membrane, such as membrane fluidity, to facilitate SPB duplication without Mps3. Our finding that MPS3-G186K mutants, which have defects in nuclear membrane structure and SPB duplication, are suppressed by the many of the same nucleoporin deletions as mps3A strongly suggests that changes in nuclear membrane properties, rather than liberation of an insertion factor, alleviate the growth defect in both cases. The fact that we isolated multiple nucleoporins as MPS3-G186K suppressors suggests a common mechanism of suppression. As we demonstrate below for pom152A and infer for the other nucleoporins, alteration of nuclear envelope properties appears to be responsible for suppression of MPS3-G186K as well as rescue of MPS3 deletion.

In addition to nucleoporin deletions, we also found that deletion of two genes involved in lipid metabolism, FAA3 and DEP1, rescues MPS3-G186K mutants. DEP1 encodes a transcriptional regulator of many metabolic genes, including genes involved in phospholipid biosynthesis [57,58]. FAA3 encodes one of five acyl coA synthetases that catalyze the conversion of fatty acids into activated acyl-coA intermediates in the first step in phospholipid biosynthesis. FAA1, FAA2, FAA3, FAA4 and Fat1 localize to different subcellular compartments and display distinct specificities for medium, long and very-long chain fatty acids in vivo [59]. FAA3 encodes a long chain or very long chain fatty acyl coA synthetase that is believed to be partially redundant with Fat1 in vivo [60–62]. However, we found that deletion of the other acyl coA synthetases, including faa1A, was unable to suppress the growth defect of MPS3-G186K (Figure 6B), suggesting that effects of FAA3 elimination are specific and that it may have a function in regulation of lipid composition at the nuclear membrane.
Changes in lipid composition rescues growth, nuclear morphology, and SPB duplication defects of *MPS3-G186K*

Deletion or overexpression of FAA3 leads to an alteration in cellular lipid content [60,63], suggesting that changes in fatty acid levels or composition are what rescue growth of *MPS3-G186K* cells. To test this idea, we treated cells with chemicals that alter membrane composition or fluidity and examined the effects on cell growth and nuclear envelope morphology. We found that growth of *MPS3-G186K* mutants on YPGR was rescued by addition of the membrane fluidizer benzyl alcohol, the sterol biosynthesis inhibitor terbinafine and the fatty acid oleic acid (Figure 5A). In addition, increasing the temperature to 33°C, which increases membrane fluidity due to the cell’s ability to adjust lipid composition by increasing levels of saturated and long chain fatty acids, also rescued growth of *MPS3-G186K* cells (Figure 5B).
Figure 5. Alteration of membrane properties suppresses MPS3-G186K. (A) Wild-type (SLJ001), GAL-MPS3 (SLJ995) and GAL-MPS3-G186K (SLJ1797) cells were tested for their ability to grow on YPGR, YPGR +0.2% benzyl alcohol, YPGR +5 mM oleic acid and YPGR +2.5 μg/ml terbinafine plates at 30°C in a serial dilution assay. Plates were grown for 2 d. Cells were also stamped to YPGR at 16°C for 7 d, 23°C for 3 d and 33°C for 2 d to test the effect of temperature on colony formation. (B) GAL-MPS3 (SLJ4963) and GAL-MPS3-G186K (SLJ4965) containing HDEL-dsRed (red) and Pus1-GFP (green) were grown overnight in SC-LEU +2% raffinose then 2% glucose, 2% galactose, 2% galactose +5 mM oleic acid, 2% galactose +0.2% benzyl alcohol, or 2% galactose +1.25 μg/ml terbinafine was added for 6 h at 30°C to visualize the effects on nuclear morphology. Bar, 5 μm. (C–D) GAL-MPS3 (SLJ4859) and GAL-MPS3-G186K (SLJ4864) cells were grown in SC-HIS +2% raffinose, then treated as in (B). A sample containing 2% galactose was also
acids and altering the amount of sterols [64,65], also suppressed the growth defect of MPS3-G186K, whereas decreasing the temperature to 23°C and 16°C, which decreases membrane fluidity, not only resulted in toxicity of MPS3-G186K but also exacerbated the phenotype associated with overexpression of wild-type MPS3 (Figure 5A). Therefore, these data are consistent with

**Figure 6. Suppressors of MPS3-G186K toxicity.** (A) Mutants from the yeast deletion collection were transformed with pURA3-GAL-MPS3-G186K, and transformants were tested for their ability to grow on SC-URA+2% galactose at for 3 d at 30°C. Possible hits were then further examined by western blotting to ensure that Mps3-G186K was expressed at high levels, similar to the wild-type control. Shown here are the hits that both rescued the growth defect of MPS3-G186K and expressed wild-type levels of Mps3-G186K. A full list of genes recovered in this screen is presented in Table S2. Also shown are the wild-type strain and the gal4Δ mutant, which we isolated in the primary screen but eliminated in the secondary screen because it does not express MPS3-G186K. Hits are organized by function using GoSlim analysis. In some cases, we isolated a mutant that deletes part of an overlapping gene, which is indicated below in parenthesis. (B) The indicated genes were deleted in GAL-MPS3-G186K (SLJ1797) and growth was tested in a serial dilution assay at 30°C on YPD (glucose) for 2 d and YPGR (galactose) for 3 d.

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the possibility that affecting lipid composition to increase membrane fluidity is sufficient to suppress MPS3-G186K. Our observation that overproduction of wild-type Mps3 is lethal at low temperatures suggests that GAL-MPS3 cells may have an altered membrane composition due to a direct role of Mps3 in membrane homeostasis.

Further examination of cells by live cell imaging revealed that other defects associated with expression of MPS3-G186K, including abnormal nuclear morphology, cell division and SPB duplication, were at least partially suppressed by changing lipid composition. Oleic acid was particularly effective at suppressing defects in nuclear shape. The majority of nuclei appeared to maintain a round shape throughout a 3.5 h time course with few extensions or protrusions; however, few nuclei divided (Figure 5B). A rare example is shown in Video S3. In addition, analysis of DNA content by flow cytometry and examination of SPB insertion showed that most cells remained arrested in mitosis probably due to SPB duplication defects (Figure 5C–5D). It is unclear why oleic acid suppresses growth of MPS3-G186K on plates but does not rescue in liquid culture, although similar effects have been reported for numerous mutants (for example, [66,67]).

Treatment of MPS3-G186K cells with other chemicals did not have as profound of an effect on nuclear morphology as oleic acid, perhaps because these drugs have pleiotropic effects on nuclear envelope structure (Figure 5B, Videos S4 and S5). For example, previous studies have shown that addition of benzy alcohol affects NPC insertion [20,21]. While it may alleviate membrane expansion caused by MPS3-G186K, benzyl alcohol addition might result in defects in NPC structure that lead to changes in nuclear morphology.

Nevertheless, despite terbinafine’s partial rescue of nuclear shape, it eliminated the cell cycle arrest of MPS3-G186K and more than doubled the number of large-budded cells that contained two inserted SBPs (Figure 5C–5D). Although this could be due to a slow growth phenotype associated with terbinafine since no mitotic divisions were observed (Video S5 and Figure 5A), we favor the possibility that treatment with terbinafine affects sterol biosynthesis which in turn affects SPB duplication since we also observed that addition of other sterol inhibitors such as ketoconazole to media rescued growth of MPS3-G186K (Figure S4).

Treatment with benzyl alcohol also only partially rescued the nuclear shape and cell cycle arrest of MPS3-G186K, but it was able to restore a distribution of SPB intermediates similar to that of GAL-MPS3 cells in galactose (Figure 5B–5D), suggesting that it alleviates the SPB duplication defect associated with the dominant mutant. Consistent with this idea, we observed cells undergoing chromosome segregation and cell division in the presence of benzy alcohol (Video S4). Importantly, addition of benzy alcohol had virtually no effect on cell cycle progression or SPB insertion in GAL-MPS3 cells (Figure 5C–D). We also found that addition of chemicals had similar effects on MPS3 and MPS3-G186K expression (Figure S5). Thus, the effects of MPS3-G186K on growth, nuclear morphology and SPB insertion can be rescued by altering the lipid composition of the cell using both genetic and chemical methods.

**mps3-1 mutants have defects in lipid homeostasis**

Analysis of our mps3 deletion and point mutants showed that most are not sensitive to benzy alcohol or sterol synthesis inhibitors (data not shown), suggesting that the synthetic effects we observe as a result of these treatments on MPS3-G186K mutants are due to a specific defect in Mps3 function that may be related to SPB insertion since MPS3-G186K, but not other mutants, display a SPB duplication defect (see Figure 1 and Figure 2). In support of this hypothesis, we found that virtually all mutants in the C-terminal SUN domain, which is required for SPB duplication [30–32], were resistant to terbinafine and found that a subset of these mutants, such as mps3-1, mps3-A540D and mps3-W477A, were also sensitive to benzy alcohol (Figure 7A and 7B). Enhanced growth on terbinafine, which inhibits ergosterol biosynthesis [60], suggests that cells containing the mps3-1, mps3-A540D and mps3-W477A mutations have extremely fluid membranes; growth on membrane fluidizing agents like benzy alcohol is toxic specifically to these mutants since they already have alterations in membrane composition. The more severe phenotype seen in some mps3 alleles presumably reflects a greater alteration in the nuclear membrane, although it is possible that some of these mutants may have other defects as well [32,40]. It is interesting that many, but not all, of the mps3 mutants that are sensitive to benzy alcohol are alleles that spontaneously diploidize. Perhaps the larger size of the nucleus in these mutants requires a greater need for lipid synthesis that cannot be met with two mutant copies of MPS3.

To determine if changes in lipid composition generally affect the SPB duplication process or are specific to mps3 mutants, we compared the growth of different recessive mutants in SPB duplication on plates containing oleic acid, benzy alcohol and terbinafine. Most SPB alleles tested, including mutants that disrupt early (cdc31-2, kar1-A17, sf1-3), intermediate (spec42-11, spec29-3) and late steps (mp2-1, bhp1-1, spec110-220) in SPB duplication and SPB regulators (mps1-1, tub4-1) [3], grew at identical rates to wild-type under all conditions tested (Figure 7B; data not shown). The notable exception was the ndc1-39 mutant, which is defective in both the late step of SPB duplication as well as in NPC assembly [5]. Like mps3-1 mutants, ndc1-39 cells exhibited increased sensitivity to oleic acid and benzy alcohol and showed reduced sensitivity to terbinafine (Figure 7B). This suggests that membranes in ndc1-39, like mps3-1 mutants, have very fluid membranes due to changes in membrane composition. While it is possible that the basis for the membrane defect in ndc1-39 is related to its SPB insertion defect, we suspect that it is due instead to its role at the NPC since other SPB mutants, including mp2-1 and bhp1-1, do not share this property. The fact that multiple mps3 mutants, including alleles that are defective in early steps of SPB duplication and not known to cause defects in NPC assembly or function [30,32], are specifically affected by growth on chemicals affecting lipid composition strongly supports our hypothesis that Mps3 function is intimately linked to membrane homeostasis.

**Cells lacking MPS3 have aberrant lipid profiles**

Our finding that MPS3-G186K mutants exhibit a severe membrane over-proliferation phenotype that is rescued by drugs and mutants that affect lipid synthesis, together with our observations that certain mps3 mutants are sensitive to changes in membrane fluidity, indicates that Mps3 may play a role either directly or indirectly in regulating lipid composition in the cell. If this were the case, then we would anticipate that cells lacking MPS3 would have an altered lipid composition compared to wild-type cells. To test this hypothesis, levels of phospholipids and neutral lipids were examined in whole cell preparations from five biological replicates of wild-type, pom152A and pom152A mps3A cells grown to mid-log phase in YPD at 30°C by electrospray ionization tandem mass spectrometry (ESI/MS/MS). pom152A mps3A mutants do not have an noticeable delay in cell division [26], so analysis of lipid composition in these cells will not be complicated by changes in temperature, growth media or cell cycle arrest that are associated with other mps3 alleles.

In cells lacking MPS3, we found that there was no statistically significant change in the overall levels of polar lipids compared to
However, examination of specific subclasses of phospholipids showed that pom152D mps3D mutants had 2.6 and 3.7 times the level of phosphatidyl serine (PS) and 2.0 and 2.3 times the level of phosphatidyl glycerol (PG) compared to wild-type and pom152D cells, respectively (Figure 8A; Table S3). Levels of phosphatidic acid (PA) and phosphatidyl ethanolamine (PE) were reduced 1.5 fold and increased 1.3 fold, respectively, in pom152D mps3D mutants compared with wild-type, although these values are not quite significant (p = 0.11 for PA, p = 0.37 for PE). The levels of other phospholipids such as phosphatidyl choline (PC) and phosphatidyl inositol (PI) were largely unchanged in wild-type, pom152A and pom152A mps3A samples. Thus, the distribution of polar lipids is affected in cells lacking Mps3 function.

Examination of neutral lipids revealed that cells lacking MPS3 contained 2–3.3 times the level of triacylglycerols (TAGs) compared to wild-type and pom152A cells (Figure 8A; Table S3). These cells also had an altered distribution of the three yeast sphingolipids, including a 1.7 fold decrease in inositol phosphorylceramide (IPC) and a 2 fold increase in mannosylinositol phosphorylceramide (MIPC) compared to controls, although these changes were not quite statistically significant (p = 0.15 for IPC, p = 0.08 for MIPC). Even more importantly, we found that deletion of POM152 alone resulted in an 1.5 fold decrease in ergosterol levels as well as a reduction in diacylglycerol (DAG), which was eliminated by simultaneous deletion of MPS3 (Figure 8B; Table S3). This finding is critical for several reasons. First, it provides biochemical support for our theory that nucleoporin deletions result in changes in the physical properties of the nuclear membrane ([26] and above). Second, the fact that these changes in lipid composition are reversed by deletion of...
MPS3 is compelling evidence that Mps3 directly affects membrane homeostasis. From our lipidomics data, we would predict that Mps3 likely acts at multiple steps in lipid biosynthesis, affecting both neutral lipid and phospholipid levels.

Membrane homeostasis in cells lacking MPS3 can be rescued by FAA3

We would predict that changing properties of the nuclear membrane would result in growth defects in pom152Δ mps3Δ.
mutants if changes in lipid composition are required for SPB duplication and cell proliferation in the absence of MPS3. Indeed, we found that pom152Δ mps3Δ mutants were sensitive to benzyl alcohol, particularly at 37°C (Figure 9A). Although the lipid composition of pom152Δ mutants is altered, as was demonstrated in our lipidomics analysis (Figure 8; Table S3), growth of the single deletion is not affected by benzyl alcohol or other chemicals that affect membrane dynamics (Figure 9A). However, overexpression of FAA3, but not other acyl coA synthetases, partially rescued growth of pom152Δ mps3Δ mutants on benzyl alcohol-containing plates at high temperatures (Figure 9B; data not shown). Therefore, it seems that Faa3 is involved in de novo lipid synthesis required for nuclear membrane homeostasis, in particular, in membrane changes brought about by Mps3 that are essential for SPB insertion.

Discussion

Our analysis of the functional domains of Mps3 has led to new insights into its role in SPB duplication and nuclear envelope homeostasis. The requirement for the transmembrane domain in Mps3 function extends previous data suggesting that Mps3 is a structural component of the half-bridge [30,32]. However, our observation that multiple membrane domains can substitute for the Mps3 transmembrane domain is compelling evidence that no specific intra- or intermolecular interactions occur through this region of the protein, including transport through the NPC to the INM and anchorage at the SPB. We therefore tested the requirement for other conserved domains and were surprised to find that, at least when individually mutated, most were not essential for Mps3 function at the SPB in wild-type cells. Perhaps Mps3 has multiple binding partners and interaction domains due to the critical importance of SPB duplication in the maintenance of genomic integrity.

Through mutagenesis of the putative P-loop region of Mps3, we identified a new dominant allele that revealed a role for Mps3 in insertion of the newly duplicated SPB into the nuclear envelope. Although we could detect binding between Mps3 and ATP in vitro (data not shown) and glycine 186 is a conserved residue in the P-loop, we doubt that a defect in nucleotide binding is the underlying cause of the phenotypes we observed in MPS3-G186K mutants. This is because other mutants in conserved residues in the P-loop that also block ATP binding, such as S190A, G192K and S194A, did not result in defects in SPB duplication and nuclear envelope homeostasis. Also, alignment of Mps3 with other fungal orthologs shows that this motif is not conserved even within the Saccharomyces lineage. Thus, MPS3-G186K is likely a fortuitous antimorphic allele rather than a mutant that disrupts ATP binding per se.

Although overexpression of MPS3 in our strains has few phenotypes at 30°C, its overproduction at 16°C and 23°C inhibits cell growth and leads to subtle changes in nuclear morphology (see Video S1). The simplest explanation for the temperature-dependent effect on cell viability when Mps3 is overproduced is that Mps3 plays a direct role in membrane homeostasis. Decreased amounts of saturated and long change fatty acids and changes in sterol levels, which accompany growth at lower temperatures [64,65], are incompatible with the alterations in membrane composition brought about by overproduction of Mps3. This incompatibility leads to a failure in SPB insertion as well as other changes in nuclear structure. Changes in lipid...
composition and nuclear architecture are exacerbated in the \textit{MPS3-G186K} mutant such that this allele is toxic under most conditions, including growth at 30\(^{\circ}\)C. Moreover, because the membrane perturbations are striking and growth arrest is tight, this allele is amenable to genetic and cell biological analysis.

Membrane over-proliferation in \textit{GAL-MPS3-G186K} but not \textit{GAL-MPS3} strongly suggests that it is not simply due to overproduction of an integral membrane protein, but rather are a unique consequence of overexpressing a mutant version of \textit{MPS3}. We propose that this altered version of Mps3 titrates out key factors required for nuclear envelope homeostasis and/or SPB duplication. The most obvious candidate is the endogenous wild-type copy of Mps3 since SUN proteins are known to oligomerize and because a reduction of Mps3 at the spindle pole leads to defects in SPB duplication [23,32,69–71]. Consistent with this idea, mild overexpression of \textit{MPS3} using a 2-micron plasmid partially rescues the growth defect of \textit{GAL-MPS3-G186K} in cells grown at 30\(^{\circ}\)C (Figure S2). Other candidates include the ribosome biogenesis factors Erg2 and Rrs1 that interact with Mps3 and are required for nuclear morphology [48].

Proliferation of intracellular membranes in response to increased levels of membrane proteins has been previously reported in a wide range of cell types; however, the intranuclear membranes that we observed following expression of \textit{MPS3-G186K} are structurally distinct in several ways. First, unlike the flattened arrays of ER membrane known as karmallae formed following overexpression of \textit{HMG1}, which encodes 3-hydroxy-3-methyl-glutaryl coenzyme A [72–75], the \textit{MPS3-G186K}-dependent membranes appear to be exclusively intranuclear and most likely represent overproliferation of the INM. Second, unlike the intranuclear membranes formed upon overproduction of Nup53 that are devoid of NPCs [54], the \textit{MPS3-G186K} membranes contain NPCs that are at least partially functional. In vertebrate cells, Sun1 localizes to NPCs and appears to play a role in their assembly [22–24], however, this function does not appear to be conserved in yeast despite the fact that highly curved membranes are involved in both NPC and SPB duplication since Mps3 does not co-localize with NPCs [48]. Third, the intranuclear arrays that form following \textit{MPS3-G186K} expression are generally closely associated with the nuclear membrane, suggesting that proteins on the surface of the \textit{MPS3-G186K}-induced membranes are able to interact with other nuclear envelope proteins. This is in contrast to intranuclear membrane arrays that form within the nucleolus and do not appear to associate with the nuclear membrane over large regions [76,77]. Fourth, although the elaborate membrane extensions known as escapades formed by increasing levels of the peripheral membrane protein Esc1 also include NPCs [78], the excess membrane found in escapades originates at the nuclear vacuolar junction and results in predominant cytoplasmic extensions, whereas the \textit{MPS3-G186K} membranes appear to begin as tubules inside of the nucleus that then fuse into intranuclear membranes. Fifth, unlike the membrane flares formed by \textit{MPS3-G186K} mutants, lipidomics analysis shows that the balance of ergosterol and DAG is reset to near wild-type levels, which could explain why SPB duplication and nuclear morphology defects were not observed in other SPB mutants [26]. Other classes of lipids such as TAG are elevated in cells lacking \textit{MPS3}, suggesting that Mps3 plays a direct role in regulation of membrane homeostasis. Our observation of allele specific defects in membrane proliferation and drug sensitivity in \textit{mps3} mutants that was not detected in other \textit{SPB} mutants further supports this hypothesis. The fact that these same alleles of \textit{MPS3} are defective in \textit{SPB} duplication indicates that Mps3-dependent changes in the nuclear envelope are important for formation of the new \textit{SPB}, perhaps at multiple steps in \textit{SPB} assembly. Studies in yeast, \textit{Dicyostelium}, \textit{C. elegans} and mammalian cells have shown that loss of \textit{SUN} protein function leads to aberrant nuclear morphology [30,32,41,48,87–89]. Whether they are directly involved in the synthesis or maintenance of the nuclear membrane or are indirectly involved through binding to the nuclear lamina is not well understood [90]. Perhaps the best hint as to how \textit{SUN}
proteins might function in membrane homeostasis comes from two-hybrid analysis of the fission yeast SUN protein Sad1, which pointed to an interaction with the acetyl coA carboxylase enzyme Cut6 that is needed for de novo biosynthesis of long-chain fatty acids and mitotic progression [91,92]. Much like the binding between SUN proteins and their ONM binding partners, known as KASH proteins, in the perinuclear space [35], we envision that SUN proteins like Mps3 may also associate with factors involved in membrane structure. Mps3 binding to enzymes or other proteins involved in membrane remodeling could be a mechanism to promote membrane bending and fusion at specific sites in the nuclear envelope, such as at the duplicating SPB or the NPC. However, Mps3 could also control membrane synthesis at the transcriptional level, by sequestering factors involved in lipid synthesis at the nuclear periphery. Future studies aimed at identification of Mps3 binding partners will help us better understand the role SUN proteins in membrane dynamics and nuclear morphology as well as elucidate the mechanism by which Mps3-dependent changes in the nuclear membrane enable duplicated SPBs to insert into the nuclear envelope.

Materials and Methods

Yeast strains and plasmids

All strains are derivatives of W303 (ade2-1 his3-11,15 leu2-3,112 ura3-1 his3A1 leu2-3,112 ura3-A10) and are listed in Table S1 except those used in Figure 6 and Table S2, which are in BY4741 (Mata his3A1 leu2-3,112 met15A0 ura3-A10) and were taken from the yeast deletion collection (Open Biosystems). Standard techniques were used for DNA and yeast manipulations.

Deletion of MPS3, fusion of SPC110 and NUP49 to GFP and fusion of HTT2 (which encodes one of the two copies of histone 2B), SPC42 or NET1 to mCherry was done by PCR-based methods [93,94]. Correct integration was confirmed by PCR.

The MPS3 ORF and ~500 bp of promoter were amplified by PCR and cloned into the Xhol and BamHI sites of a pRS306 vector containing GFP to construct pSj650 (pRS306-MPS3-GFP) [41]. Construction of pSj148 (pRS305-MPS3) has been previously described [32]. To overexpress MPS3, the entire open reading frame was inserted immediately adjacent to GALI in pRS306 to create pSj146 (pRS306-GALI-MPS3). Plasmids were digested with BstEII or Apal to target integration to LEU2 or URA3, respectively. The number of copies of MPS3 integrated was determined by Southern blotting. Deletion and point mutants, as well as transmembrane insertions, were generated in these plasmids using the Quick Change Mutagenesis Kit (Stratagene).

Each deletion mutant contains an in-frame deletion of the indicated amino acids; in the case of 2xpQ mutant, an extra copy of the pQ domain was inserted immediately after the pQ coding sequence. Sequencing was performed to confirm correct base pair substitutions or deletions were made.

To test for rescue of MPS3-G186K, plasmids containing the indicated SPB genes were taken from the yeast teling library [95] and transformed into SLJ1797. For dilution assays, 5 OD_{600} of cells were serially-diluted 10-fold in sterile growth media and stamped onto agar plates.YPD has 2% glucose and YPGR has 2% galactose and 2% raffinose as the carbon source. Chemicals were purchased from Sigma and were added to media in the following amounts: 5 mM oleic acid, 0.2% benzy alcohol, 1.25 μg/ml terbinafine and 1 μg/ml ketoconazole.

Cytological techniques

Localization of Mps3-GFP, H2B-mCherry, the nuclear localization sequence (NLS) reporter, HDEL::dsRed and Pus1-GFP were visualized as previously described [41]. Briefly, 1 ml of culture was centrifuged, washed with 1 ml ddH₂O, resuspended in approximately 100 μl of ddH₂O and 10 μl was placed on a 25% gelatin pad for image analysis. Fluorescence was performed using a confocal microscope (LSM-510-META; Zeiss) equipped with a ConforCor 3 module with avalanche photodiode detectors, which allow single photon counting, with a 100× 1.46 NA α-Plan Fluar objective (Zeiss). GFP was excited using a 460-nm Argon laser line, while mCherry was excited with a 561-nm HeNe laser line with the appropriate filter sets. Emitted photons were collected through BP 505–540 nm and LP 580 nm filters, with a pinhole size of 1.03 Airy units according to the green channel. Data was acquired using AIM v4.2 software (Zeiss, Inc.). Images were collected with 8–10 image stacks with a 0.3 micron step size through the cells at room temperature. Images were processed using ImageJ software (NIH). At least two independent transformants of each genotype were analyzed by fluorescence microscopy in at least three independent experiments. N:C ratios were calculated as previously described [28].

Time-lapse image analysis of GAL-MPS3 or GAL-MPS3-G186K strains was performed on cultures grown overnight in YEP plus 2% raffinose at 30°C then diluted back to an OD600 of 0.2 and grown in the same media for 2 h. 2% galactose and nothing, 0.2% benzy alcohol, 5 mM oleic acid, 1.25 μg/ml terbinafine or 1 μg/ml ketoconazole were added at 30°C for 30 min. One ml of culture was centrifuged to concentrate the cells and 5 μl was placed on a 1% agarose pad containing the same media and chemical treatment. Fluorescent images were taken from 1 h to 5 h 30 min after galactose induction using the same microscope configuration but with a 40× 1.3 NA oil objective (Zeiss). Emitted photons were collected through BP 505–540 nm and LP 580 nm filters, with a pinhole size of 1.03 Airy units according to the green channel. Images were collected with 17 image stacks with a 0.7 micron step size through the cells at room temperature. The 512×512 images were binned spatially 2×2 and a max projection was applied for each Z-slice. All data processing and video generation were done in ImageJ (NIH).

For the characterization of the SPB duplication using the red/green foci assay, images were acquired with a 100× 1.4 NA oil objective on an inverted Zeiss 200 m equipped with a Yokagawa CSU-10 spinning disc. 488 nm excitation and 568 nm excitation were used for GFP and mCherry, respectively, and emission was collected through BP 500–530 nm and BP 580–650 nm filters, respectively, on a Hamamatsu EMCCD (C9000-03). For each channel, a Z-stack was acquired using 0.6 or 0.7 micron spacing. 13 total slices were acquired, and a max projection image was created for analysis of foci using ImageJ (NIH).

Analysis of DNA content by flow cytometry, EM, and protein localization by indirect immunofluorescence microscopy were performed as previously described [30,32,96]. Poly(A)+ mRNA was visualized as previously described [41]. Briefly, 1 ml of culture was centrifuged, washed with 1 ml ddH₂O, resuspended in approximately 100 μl of ddH₂O and 10 μl was placed on a 25% gelatin pad for image analysis. Fluorescence was performed using a confocal microscope (LSM-510-META; Zeiss) equipped with a ConforCor 3 module with avalanche photodiode detectors, which allow single photon counting, with a 100× 1.46 NA α-Plan Fluar objective (Zeiss). GFP was excited using a 460-nm Argon laser line, while mCherry was excited with a 561-nm HeNe laser line with the appropriate filter sets. Emitted photons were collected through LP 520 nm and LP 590 nm filters, with a pinhole size of 1.03 Airy units according to the green channel. Data was acquired using AIM v4.2 software (Zeiss, Inc.). Images were collected with 8–10 image stacks with a 0.3 micron step size through the cells at room temperature. Images were processed using ImageJ software (NIH). At least two independent transformants of each genotype were analyzed by fluorescence microscopy in at least three independent experiments. N:C ratios were calculated as previously described [28].

Western blotting

The following primary antibody dilutions were used: 1:1000 anti-Flag (Sigma) and 1:1000 anti-glucose-6-phosphate dehydrogenase (G6PDH; Sigma). Fluorescently conjugated secondary antibodies were used at 1:10000 for analysis and quantification using the Odyssey system (Li-Cor).
ESI-MS/MS lipid profiling

Yeast cells were grown overnight at 30°C in YPD, diluted to OD_{600} of 0.2 in the same media and allowed to grow for 6–8h at 30°C. The wet weight of the sample was recorded and lipids were extracted by the following procedure. Pelleted cells were homogenized using glass beads and water and the aqueous homogenized cells were transferred to 15 ml Teflon-lined glass tubes. To 0.8 parts (1.6 ml) of homogenized cells in aqueous solution, 1 part (2 ml) of chloroform and 2 parts (4 ml) of methanol were added. The slurry was shaken, 1 part (2 ml) of chloroform and 1 part of water (2 ml) were added. After shaking well, the slurry was centrifuged at low speed for 5–10 min and the lower layer was removed. One part of chloroform was added, shaken, centrifuged and the lower layer was again removed. This was repeated, and the combined lower layers were washed once with a small volume of 1 M KCl and once with a small volume of water. The combined and washed chloroform extracts were dried under liquid nitrogen and stored at −20°C in 2 ml Teflon-lined glass vials.

An automated lipid extraction (ESI-MS/MS) approach was used to analyze lipid composition in these samples, and data acquisition and analysis were carried out as described previously [99] with modifications. A 5 to 20 μl of aliquot of extract in chloroform was used from each 1 ml lipid sample. Precise amounts of internal standards, obtained and quantified as previously described [99], were added in the following quantities: 0.3 nmol di12:0-PC, 0.5 nmol di24:1-PC, 0.3 nmol 13:0-lysoPC, 0.3 nmol 19:0-lysoPC, 0.3 nmol di12:0-PE, 0.3 nmol di23:0-PE, 0.3 nmol 14:0-lysoPE, 0.3 nmol 18:0-lysoPE, 0.3 nmol di14:0-PA, 0.3 nmol di20:0-phytyl-PA, 0.5 nmol di14:0-PA, 0.3 nmol di20:0-phytyl-PA, 0.2 nmol di14:0-PS, 0.2 nmol di20:0-phytyl-PS, 0.46 nmol 16:0-18:0-PI, 0.33 nmol di18:0-PI, 3.1 nmol tril17:1 TAG, and 4.6 nmol di15:0 DAG. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.4 ml.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS (4000QTrap, Applied Biosystems). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 μl/min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: PC and lysoPC, [M+H]^+ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); PE and lysoPE, [M+H]^+ ions in positive ion mode with Neutral Loss (NL) of 141.0 (NL 141.0); PG, [M+NH₄]^+ in positive ion mode with NL 189.0 for PG; PI, [M+NH₄]^+ in positive ion mode with NL 277.0; PS, [M+H]^+ in positive ion mode with NL 185.0; PA, [M+NH₄]^+ in positive ion mode with NL 115.0; IPC, [M−H]^- in negative ion mode with Precursor of 259.0; MIPC, [M−H]^- in negative ion mode with Precursor of 421.0; MIP₃C, [M−H]^- in negative ion mode with Precursor of 663.1; lysoPG, [M−H]^- in negative ion mode with Precursor of 152.9 used as a standard for IPC, MIPC, MIP₃C. The collision gas pressure was set at 2 (arbitrary units). The collision energies, with nitrogen in the collision cell, were +28 V for PE, +40 V for PC, +25 V for PA, PI and PS, +20 V for PG, −25 V for IPC, −50 V for MIPC, −57 V for lysoPG. Declustering potentials were +100 V for all positive mode scans, −180 V for IPC, MIPC, and MIP₃C, −100 V for lysoPG. Entrance potentials were +15 V for PE and +14 V for PC, PA, PG, PI, and PS, −13 V for IPC, MIPC, and MIP₃C, −10 V for lysoPG. Exit potentials were +11 V for PE and +14 V for PC, PA, PG, PI, −10 V for IPC, MIPC, and MIP₃C, −14 V for lysoPG. The scan speed was 50 or 100 u per sec. The mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode. A series of neutral loss scans were used to detect ergosterol esters, DAG, and TAG species as [M+NH₄]^+ ions. The scans targeted losses of various fatty acids as neutral amminated fragments: NL 253.2 (17:1, for the TAG internal standard); NL 259.2 (15:0, for the DAG internal standard); NL 271.2 (16:1); NL 273.2 (16:0); NL 299.2 (18:1); and NL 301.2 (18:0). The scan speed was 100 u per sec. The collision energy was +25 V, entrance potential was +14 V, and exit potential was +14 V. Fifty continuum scans were averaged in MCA mode. The source temperature (heated nebulizer) was 100°C, the interface heater was on, +5.5 kV was applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software, and the data were corrected for overlap of isotopic variants (A+2 peaks). The first and typically every 11th set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the two internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the “internal standards only” spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each “internal standards only” set of spectra was used to correct the data from the following 10 samples. Finally, the data were corrected for the fraction of the sample analyzed and normalized to the mg wet weight to produce data in the units nmol/mg, except DAG, TAG, and ergosterol esters. For analysis of these classes, there is variation in ionization efficiency [100]. Thus, the ratio of the MS response of the internal standard does not provide a value that is directly proportional to the content of each species. DAG, TAG, and ergosterol ester amounts are thus expressed as relative mass spectral signal/mg wet weight, where a signal of 1.0 represents a signal equal to the signal of 1 nmol tril17:1 TAG, or 1 nmol di15:0 DAG (the internal standards).

Supporting Information

Figure S1 A specific transmembrane domain sequence is not required for Mps3 localization or function at the SPB. (A) Transmembrane regions from other integral membrane proteins were used to replace that of Mps3, and these proteins were localized as in Figure 1C. In the case of mps3ATM([Heh1TM2]-GFP (SLJ4320), imaging was performed with the covering plasmid since this allele is non-functional. Bar, 2 μm. (B) Sequence of Mps3 transmembrane domain and the transmembrane regions from other membrane proteins that were used to replace that of Mps3. Summarized is the ability of each chimeric construct to complement mps3Δ (SLJ2694), which was scored based on growth on 5-FBA at 30°C. +++, indicates robust growth equal to wild-type Mps3; +, indicates good growth with colony sizes smaller than wild-type and no increase in ploidy; +/-, indicates poor growth and/or increase in ploidy; −, indicates no growth similar to an empty vector. In addition, the localization of each chimeric
protein was determined as in (A). Over 30 cells of each genotype were examined and the predominant localization pattern is summarized. Those marked with an asterisk contained the covering plasmid. Also shown is the predicted topology of Mps3 and the chimeric proteins based on TMPred [102]; here, i-o means inside to outside while o-i means the opposite side in. In some cases topology prediction was ambiguous. In most cases, there was a strong prediction that the chimeric protein would have the i-o topology resembling Mps3, indicating that failure of the chimeric protein to complement is not necessarily due to reversal of membrane orientation. (EPS)

**Figure S2** Suppression of MPS3-G186K toxicity by overexpression of SPB components. GAL-MPS3-G186K (SLJ1797) was transformed with the indicated genes on a 2μ-LEU2 plasmid from the yeast tiling library [95] and growth was examined in a serial dilution assay. Cells grown on SC-URA-LEU4% glucose were incubated for 3 d at 30°C while cells grown on SC-URA-LEU4% galactose+2% raffinose were incubated for 5 d at 30°C. (EPS)

**Figure S3** Nuclear import and export is significantly affected in MPS3-G186K. (A-B) Wild-type (SLJ5384), GAL-MPS3 (SLJ5093) and GAL-MPS3-G186K (SLJ5094) strains containing H2BmCherry and cNLS-GFP were grown in SC-LEU+2% raffinose overnight, then cultures were divided: to one set, 2% glucose was added and to a second set, 2% galactose was added, and cultures were incubated for 2 h at 30°C. The distribution of CNLS-GFP in the nucleus versus cytoplasm was analyzed by live cell imaging (A) followed by quantitation of the nuclear to cytoplasmic ratio (N:C) of CNLS-GFP protein (B). (C-D) GAL-MPS3 (SLJ995) and GAL-MPS3-G186K (SLJ1797) strains were also grown in YEP+2% raffinose overnight, then cultures were divided: to one set, 2% glucose was added and to a second set, 2% galactose was added, and cultures were incubated for 4 h at 30°C. Cells were harvested and polyA localization was analyzed by fluorescent in situ hybridization using Cy3-labelled oligo-dT' (red), DNA was stained using DAPI (blue) (C). The distribution of mRNA in the nucleus vs. cytoplasm was quantitated to generate a N:C ratio (D). n>150 for all samples; statistical significance compared to the glucose sample is shown. Bar, 5 μm. (EPS)

**Figure S4** Sterol biosynthesis inhibitors suppress the growth defect of MPS3-G186K. (A) Wild-type (SLJ001), GAL-MPS3 (SLJ995) and GAL-MPS3-G186K (SLJ1797) cells were tested for their ability to grow on YPD, YPGR and YPGR+1μg/ml ketoconazole plates at 30°C in a serial dilution assay. Plates were grown for 2 d. (B) In addition, GAL-MPS3-G186K (SLJ965) containing HDEL-dsRed (red) and Pus1-GFP (green) grown overnight in SC-LEU+2% raffinose was analyzed at 6 h at 30°C following the addition of 2% galactose+1μg/ml ketoconazole to determine the effects on nuclear morphology. Bar, 5 μm. (C) DNA content was also analyzed by flow cytometry. (EPS)

**Figure S5** Levels of Mps3 and Mps3-G186K following treatment with oleic acid, terbinafine and benzyl alcohol. Quantitative western blotting to examine the levels of Mps3-3xFLAG (SLJ5138) and Mps3-G186K-3xFLAG (SLJ5140) following treatment with different chemicals for 6 h in YPGR or following a shift to 33°C for 6 h in YPGR (as in Figure 5). Blots were probed with anti-FLAG antibodies to detect the level of Mps3 or Mps3-G186K protein and with anti-G6PDH, which serves as a loading control. Cells grown in glucose and galactose were assigned values of 0 and 1, respectively, and levels in treated cells in a representative experiment are shown. (EPS)

**Table S1** Yeast strains used in this study. (XLS)

**Table S2** Suppressors of GAL-MPS3-G186K. (XLS)

**Table S3** Lipidomics analysis of cells lacking MPS3. (XLS)

**Video S1** Growth of GAL-MPS3 in YPGR. HDEL-dsRed marks the nuclear membrane and ER while Pus1-GFP highlights the nucleus in cells containing GAL-MPS3 grown in YEP plus 2% raffinose and 2% galactose at 23°C. From ~75–90 min, the upper left cell undergoes mitosis, where the nucleus is observed to form an hour-glass shape and then elongate between the mother and daughter cell. Following the completion of mitosis, the nucleus becomes round again. Another mitosis was observed in the cell in the bottom right between ~130–160 min. At later time points, a few changes in nuclear morphology were observed but overall, the nuclei largely retained a relatively normal shape throughout the 3.5 h time course. Bar, 5 μm. (MOV)

**Video S2** Growth of GAL-MPS3-G186K in YPGR. HDEL-dsRed marks the nuclear membrane and ER while Pus1-GFP highlights the nucleus in cells containing GAL-MPS3-G186K grown in YEP plus 2% raffinose and 2% galactose at 23°C. At the beginning of the time course, the second cell from the upper right assumes an oval shape, suggesting it is starting mitosis. The nucleus elongates and two masses separate into the mother and daughter cell, but Pus1-GFP signal can still be detected in a thin region between the two nuclei throughout the entire time series. Around 150 min, deformations of the nuclei start to become apparent; in some cases such as the upper right cell, regions of the nucleus appear to form lobes, which can then bud from and fuse with each other. Bar, 5 μm. (MOV)

**Video S3** Growth of GAL-MPS3-G186K in YPGR plus 5 mM oleic acid. HDEL-dsRed marks the nuclear membrane and ER while Pus1-GFP highlights the nucleus in cells containing GAL-MPS3-G186K grown in YEP plus 2% raffinose and 2% galactose at 23°C. In addition, 5 mM oleic acid was added, which suppresses most of the dynamic changes in nuclear shape. A cell in the bottom center enters mitosis at ~195 min. Bar, 5 μm. (MOV)

**Video S4** Growth of GAL-MPS3-G186K in YPGR plus 2% benzyl alcohol. HDEL-dsRed marks the nuclear membrane and ER while Pus1-GFP highlights the nucleus in cells containing GAL-MPS3-G186K grown in YEP plus 2% raffinose and 2% galactose at 23°C. In addition, 2% benzyl alcohol was added. Although the nuclear morphology in these cells is somewhat abnormal, three mitotic divisions were observed during the time-lapse, indicating that the addition of benzyl alcohol suppressed the metaphase arrest associated with expression of the dominant mutant. Bar, 5 μm. (MOV)

**Video S5** Growth of GAL-MPS3-G186K in YPGR plus 1.25 μg/ml terbinafine. HDEL-dsRed marks the nuclear membrane and ER while Pus1-GFP highlights the nucleus in cells containing GAL-MPS3-G186K grown in YEP plus 2% raffinose and 2% galactose at 23°C. In addition, 1.25 μg/ml terbinafine was added. Although...
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