Fission yeast Lem2 and Man1 perform fundamental functions of the animal cell nuclear lamina

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Abbreviations: BAF, barrier to autointegration factor; EM, electron microscopy; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; HEH, helix-extension-helix; HEH Domain, HEH-fold containing domain of yeast MSC family proteins; HEH Fold, DNA binding fold, present in LEM domain of higher eukaryotic and HEH domain of yeast MSC family proteins; INM, inner nuclear membrane; LAP, lamin associated protein; LEM, Lap2 Emerin Man1; LEM Domain, HEH-fold containing domain of higher eukaryotic MSC family proteins; MSC, Man1/Src1-C-terminal protein family; NE, nuclear envelope; NPC, nuclear pore complex; SPB, spindle pole body

In animal cells the nuclear lamina, which consists of lamins and lamin-associated proteins, serves several functions: it provides a structural scaffold for the nuclear envelope and tethers proteins and heterochromatin to the nuclear periphery. In yeast, proteins and large heterochromatic domains including telomeres are also peripherally localized, but there is no evidence that yeast have lamins or a fibrous nuclear envelope scaffold. Nonetheless, we found that the Lem2 and Man1 proteins of the fission yeast Schizosaccharomyces pombe, evolutionarily distant relatives of the Lap2/Emerin/Man1 (LEM) sub-family of animal cell lamin-associated proteins, perform fundamental functions of the animal cell lamina. These integral inner nuclear membrane localized proteins, with nuclear localized DNA binding Helix-Extension-Helix (HEH) domains, impact nuclear envelope structure and integrity, are essential for the enrichment of telomeres at the nuclear periphery and by means of their HEH domains anchor chromatin, most likely transcriptionally repressed heterochromatin, to the nuclear periphery. These data indicate that the core functions of the nuclear lamina are conserved between fungi and animal cells and can be performed in fission yeast, without lamins or other intermediate filament proteins.

Introduction

The hallmark of a eukaryotic cell is the nucleus, a specialized region of the endoplasmic reticulum (ER) delineated by the double membranes of the nuclear envelope (NE) (reviewed in refs. 1 and 2). These possibly interrelated properties that distinguish the nucleus from the ER in all eukaryotes are the flattened sheet conformation of its membranes, the specialized set of inner nuclear membrane (INM) localized proteins, and the sequestration of chromosomes within its confines (reviewed in refs. 2–5). However, fundamental questions about structural and functional differences between the nuclei of higher and lower eukaryotes, including nuclear organization and cell cycle changes in NE area and stability, remain largely unanswered.

These differences are most strikingly seen at mitosis, when animal and plant cells undergo nuclear envelope breakdown (open mitosis) but in most lower eukaryotes, the NE remains intact (closed mitosis) (reviewed in ref. 6). Open mitosis allows the spindle microtubules, which are nucleated by cytoplasmic localized centrosomes, to physically attach to and then separate the chromosomes. In the closed mitosis of most lower eukaryotes, such as the fission yeast S. pombe and the budding yeast S. cerevisiae, the centromere equivalents, named spindle pole bodies (SPBs), are embedded in the NE and nucleate formation of an intra-nuclear spindle7 (reviewed in ref. 8) that changes nuclear shape as it elongates and separates the chromosomes.7

In animal cells, cell cycle dependent changes in NE stability are governed in large part by the nuclear lamina that underlies the INM and forms the structural scaffold for the NE. The nuclear lamina consists of the lamin family of intermediate filament proteins and the transmembrane LAPs (Lamin Associated Protein) that anchor them to the INM. A subset of LAPs have an N-terminal HEH (Helix-Extension-Helix) fold-containing LEM domain (Lap2/Emerin/Man1) that binds to chromatin indirectly by means of its interaction with the non-sequence specific DNA binding protein Barrier to Autointegration Factor (BAF).10–12 The lamina anchor proteins, heterochromatin and non-transcribed genes to the predominantly transcriptionally...
repressive environment near the nuclear periphery and provides a structural scaffold for the NE.2 The critical role of the animal cell lamina for NE structure and chromatin organization is underscored by the observations that mutations in lamins or LAPs cause NE fragility and global changes in gene expression associated with human diseases collectively called laminopathies (reviewed in refs. 13–15). Interestingly, however, lamins are not essential for nuclear envelope structure or proliferation in embryonic stem cells.20

During mitosis in all eukaryotes chromatin must dissociate from the NE to allow for the reorganization and anchorage of telomeres and centromeres to the nuclear periphery.3 In yeast cells the nuclear lamina is required for NE structure and chromatin organization,39–41 but in higher eukaryotic cells, the nuclear lamina including anchoring telomeres and other chromosome loci, is not essential for NE structure or telomere anchoring.39–41 However, in animal cells the nuclear lamina including anchoring telomeres and other chromosome loci is required for NE structure and chromatin organization.39–41

During mitosis in animal cells lamins dissociate from the NE to allow for the reorganization and anchorage of telomeres and centromeres to the nuclear periphery.3 In these cells the nuclear lamina, including anchoring telomeres and other chromosome loci, is required for NE structure and chromatin organization.3,39–41

In contrast to the genome organization of metazoans in which small heterochromatic domains are interspersed throughout the chromosomes, yeast heterochromatin is predominantly present in the centromere, telomere, rDNA repeats and mating type loci, all of which are enriched near the nuclear periphery.51,52 This telomere anchoring at the NE depends on the telomere-repeat specific constitutive binding protein Tel1, the Tel1-binding protein Rap1, and the INM-associated proteins Bqt3 and the Bqt4.43 In the absence of Bqt4, the distance of telomeres from the NE increases yet they remain enriched near the nuclear periphery, leading to the suggestion that other proteins may be involved in interphase telomere anchoring of S. pombe.43

We report the characterization of two INM-specific fusion yeast transmembrane proteins, Lem2 and Man1, that share limited domain organization and have divergent evolutionary relationships but very limited amino acid sequence similarity with the LEAG subfamily of animal cell LAPs or BAF, lamins and other intermediate filament proteins that are essential components of the nuclear lamina.

**Results**

Identification of Lem2 and Man1 as *S. pombe* proteins that perform some functions of the animal cell lamina. The starting point of our candidate approach to finding proteins involved in nuclear organization were those shown by the *S. pombe* ORFeome project to localize to the NE47 that also contained at least one predicted transmembrane domain. We then screened for genes
that, like animal cell laminas, altered the conformation of the NE when overexpressed, and then focused on two genes, SPAC14C4.05c and SPAC18G6.10, which met these criteria. Both were also identified\(^7\) as distantly related to the family of animal cell LEM-domain containing proteins (reviewed in ref. 55) (Fig. 1A). Characterization of several members of this protein family indicates a common membrane topology: the N- and C-terminal domains lie in the nucleoplasm and are separated by two transmembrane domains that flank an NE-lumenal domain.\(^8\) The N-terminus contains an HEH fold-containing domain (Pfam Clan C10306).\(^9\) The C-terminus contains the winged helix-turn-helix DNA-binding fold-containing Man1/Ssc1p C-terminal (MSC)\(^9\) domain (Pfam PF09402).\(^9\) Taken together, these data suggest that the proteins encoded by SPAC14C4.05c and SPAC18G6.10 might participate in functions performed by the animal cell nuclear lamina. These S. pombe genes are named man1 and lem2 (SPAC14C4.05c/leh2) and lem2 (SPAC18G6.10/leh1).

NE structure and integrity depend on Lem2 and Man1. Null mutants of lem2 and man1 (lem2Δ and man1Δ) were constructed and tetrad analysis revealed that the mutant spores could germinate and form colonies (Fig. 1B) indicating that neither gene was essential for vegetative growth. The Δlem2 strain was slightly temperature sensitive at 36°C as indicated by the slightly darker pink color of the colony in the presence of the pink vital dye phloxine B, which accumulates in dead cells. The Δlem2 strain and Δman1 mutations were not synthetically lethal and the temperature sensitivity of the double mutant was similar to that of the Δlem2 strain (Fig. 1C). To determine if Lem2 and/or Man1 influence chromatin organization, DNA in wild type, Δlem2, Δman1, and Δlem2Δman1 cells was visualized with DAPI, but no differences were observed at either 25°C or 36°C (Fig. 1D).

We first assessed the influence of Lem2 and Man1 on NE integrity by visualizing nuclear compartmentation using a previously described fluorescence assay\(^50\) based on monitoring the localization of two exclusively nuclear localized proteins (the NE protein GFP-Nsp1p and the soluble nucleoplasmic protein SV40 -galactosidase) that become uniformly distributed in nearly 100% of wild type, but not lem2, Δman1, and Δlem2Δman1 cells incubated at 25°C or wild type and Δman1 cells at 36°C, both GFP reporters localized exclusively to the nucleus (Fig. 1E; see also Fig. S1C), which is indicative of nuclear compartmentation and normal nucleocytoplasmic transport. Consistent with its slight temperature sensitivity (Fig. 1C), in Δlem2 cells incubated at 36°C nuclear compartmentation was disrupted in 8.9 ± 1.8% of cells, whereas deletion of both lem2 and man1 (Δlem2Δman1) disrupted compartmentation in 8.7 ± 1.0% of cells (Fig. 1E; see also Fig. S1C). Taken together these data indicate that nuclear compartmentation is disrupted in the absence of Lem2 but not Man1.

To confirm that this lack of nuclear compartmentation was caused by loss of NE integrity, we examined these strains by transmission electron microscopy (TEM) (Fig. 1F). Consistent with the fluorescence assay results, the NE of Δman1 cells (Fig. 1F, 2) was intact and morphologically similar to that of wild type cells (Fig. 1F, 1). However, in the absence of Δlem2 there were abnormal bulges (Fig. 1F, 3 and 4), indicative of disrupted NE structure, and gaps in the NE (Fig. 1F, 4), indicative of a loss of NE integrity, similar to those in the Δlem2 Δman1 double mutant (Fig. 1F, 5 and 6). The distribution of NPCs (nuclear pore complexes) is unaffected by deletion of lem2, man1 or both (Figs. 1F and 6A).

In S. pombe the RanGTPase Pim1, is essential for viability and NE integrity.\(^10\) To ask whether lem2 and/or man1 function in the pim1 pathway we examined interactions with the temperature sensitive pim1-d1 mutation (Fig. 1E; see also Fig. S1D). Consistent with our previous studies,\(^11\) nuclear compartmentation was intact in 100% of pim1-d1 cells at 25°C but 45.2 ± 2.0% of cells lost compartmentation at 36°C (Fig. 1E). Introduction of the Δman1 mutation did not significantly alter this frequency (46.1 ± 1.1%) (Fig. 1E; see also Fig. S1D). However, 97.4 ± 0.3% of pim1-d1 Δlem2Δman1 cells and 92.5 ± 2.8% pim1-d1 Δlem2 Δman1 cells lost nuclear compartmentation after 4hrs at 36°C (Fig. 1E; see also Fig. S1D). These data provide evidence that pim1-d1 interacts genetically with Lem2 but not Man1. The lem2 null mutation enhances the NE defects in the pim1-d1 mutation indicating that these genes likely destabilize the NE by distinct mechanisms.

Lem2 and Man1 localize independently to the NE but only Lem2 accumulates at the SPB. To confirm the previously described localization of Lem2\(^12\) and determine the localization of Man1, each gene at its endogenous chromosomal locus was fused to the gene encoding GFP, causing no change in cell viability (Fig. S2A). Man1-GFP and Lem2-GFP localized exclusively to the NE during all stages of the cell cycle (Fig. 2A, 1 and 3). The chromosomal DNA distribution in these two strains (Fig. 2A, 2 and 4) was the same as that of the negative control wild type cells with no GFP-fusion protein (Fig. 2A, 5).

At this endogenous level of expression Lem2-GFP (Fig. 2A, 1) but not Man1-GFP (Fig. 2A, 3) accumulated in one or two bright spots at or near the NE in most cells (Fig. 2A, 2) in a pattern that resembled that of SPBs in wild type cells,\(^12\) consistent with previous reports.\(^6,10,11\) The SPB association of Lem2 was confirmed by observing co-localization of Lem2-GFP and the RFP-tagged SPB protein Pcp1\(^51\) in 97% of cells (n = 30) (Fig. 2B). In the absence of Lem2, Man1-GFP remained localized to the NE and in the absence of Man1, Lem2-GFP still localized to the NE and was enriched at the SPB (Fig. 2C), indicating that these two proteins localize to the NE independently of one another. Because of the Lem2 SPB localization, the previously reported interaction of Man1 with the SUN1-domain-containing SPB component Sad1,\(^13\) and the possibility that Lem2 and/or Man1 mediate the interaction between the centromere and SPB, we asked whether either protein influenced the fidelity of chromosome segregation. Following the segregation of Chromosome 1 as previously described\(^14\) we found no chromosome mis-segregation in Δlem2, Δman1 or wild type cells (n = 100).

Increased levels of Lem2 or Man1 caused NE membrane proliferation. Although protein overexpression studies must be interpreted with caution (i.e., overproduction of membrane proteins such as HMG-CoA and animal cell lamin A and B...
**A**

| Strain | 25°C | 30°C |
|--------|------|------|
| Δman1Δlem2 | 1.3+/−0.4 | 8.7+/−1.0 |
| Δlem2 | 0.3+/−0.3 | 8.9+/−1.8 |
| Δman1 | 0 | 0 |

**B**

| Strain | 25°C | 30°C |
|--------|------|------|
| Δman1 Δlem2 | 5.2+/−0.5 | 92.5+/−2.3 |
| pim1-d1 Δman1Δlem2 | 8.7+/−0.3 | 97.4+/−3.8 |
| pim1-d1 Δlem2 | 46.1+/−1.1 | 45.2+/−2.0 |
| pim1-d1 Δman1 | 0 | 0 |

**E**

| Strain name | 25°C | 30°C |
|-------------|------|------|
| Δman1Δlem2 | 1.3+/−0.4 | 8.7+/−1.0 |
| Δlem2 | 0.3+/−0.3 | 8.9+/−1.8 |
| Δman1 | 0 | 0 |

| Wild Type | 0 | 0 |
| pim1-d1 Δman1Δlem2 | 5.2+/−0.5 | 92.5+/−2.3 |
| pim1-d1 Δlem2 | 8.7+/−0.3 | 97.4+/−3.8 |
| pim1-d1 Δman1 | 46.1+/−1.1 | 45.2+/−2.0 |
| pim1-d1 | 0 | 0 |
cause proliferation of the ER and the NE respectively, in the case of Lem2 and Man1 they served three purposes: (1) They demonstrated that Lem2 and Man1 stimulate NE membrane proliferation; (2) they showed that Lem2 and Man1 influence NE conformation in morphologically different ways suggesting that they are functionally and/or structurally different in their membrane interactions; and (3) most importantly, they provided an experimental system in which to characterize the putative DNA binding ability of Lem2 and Man1. When overexpressed in the nuclear compartmentation reporter strain background (Fig. S1A), no vector control containing cells but 15.2% of lem2 cells and 12.9% of man1 cells (n = 300) lost nuclear compartmentation, compared with 0% of wild type cells (Fig. 3A). Overexpression of neither lem2 nor man1 influenced NPC distribution; however, Lem2 and Man1 caused some NPC components, GFP-Npl1 to accumulate in the ER at the cell periphery (Fig. 3A, 5) that is continuous with the NE and co-localized with a fluorescent ER reporter (unpublished observations). In addition, 35.0% of man1 cells had what appeared to be GFP-containing spherical structures in the cytoplasm (Fig. 3A, 3) with GFP-Npl1 localized to their periphery (Fig. 3A, 6). Overexpression of either man1 or lem2 in wild type cells was toxic (Fig. S2B). For this reason, all overexpression studies were performed by first growing cells to log phase with the man1 gene promoter off, then turning the promoter on and monitoring the consequent phenotypes.

When Lem2-YFP was overproduced for 30 h, the protein localized to the nuclear interior in very brightly fluorescent curvilinear or circular patterns (Fig. 3B, 1). Using identical conditions, overproduced Man1-YFP localized to the periphery of spherical cytoplasmic structures and accumulated in the peripheral ER (Fig. 3B, 2). To determine whether these were NE-derived membranes we investigated their protein composition. Man1-GFP and Lem2-GFP expressed from their endogenous promoters localized to the nuclear periphery as previously shown (Fig. 2A, 1 and 3). However, both localized to the periphery of the cytoplasmic spheres and/or the intranuclear membrane stacks induced by overexpression of either untagged man1 or untagged lem2 (Fig. S3) which are similar in morphology to the membranes seen upon overexpression of fluorescently tagged proteins (Fig. 3B). These data indicate that the protein composition of the membranes that form upon lem2 or man1 overexpression are similar to that of the NE, and that Lem2 and Man1 do not compete with one another for membrane association.

To determine if the localization of overexpressed Man1-YFP and Lem2-YFP corresponded to an underlying sub-cellular structure, cells were examined using transmission electron microscopy. In contrast to wild type cells (Fig. 3C, 1), cells overexpressing lem2 had multi-layered stacked membrane structures at the nuclear periphery (Fig. 3C, 2) and in spheres and swirls within the nucleus (Fig. 3C, 3 and 4), continuous with or derived from the inner NE. Overexpression of man1 also resulted in the appearance of some stacked membrane structures within the nucleus (Fig. 3D, 1 and 2). However, only man1 overexpression caused the formation of small cytoplasmic nucleus-like spheres attached to the nucleus, that had double lipid bilayer membranes (Fig. 3D, 1–4) and structures resembling nuclear pores (Fig. 3D, 4), both characteristic of the NE. It is for these reasons we refer to these small NE-derived “mini-nuclei” and in the connections among them and between them and the nucleus (Fig. 4B) indicates that they are derived from the nucleus and are the product of deformation of both the inner and outer nuclear membranes. Taken together these data indicate that overexpression of Lem2 or Man1 deforms the NE in morphologically different ways and that these two proteins directly or indirectly anchor chromatin to the NE membrane.
Figure 2. Endogenously expressed Man1-GFP and Lem2-GFP localize to the NE independently of one another and Lem2 accumulates at the SPB.

Wild type cells with endogenously produced Lem2-GFP (A1, A2), Man1-GFP (A3, A4), or wild type control cells with no GFP-tagged protein (A5) were grown in YE to log phase at 25°C and live cells were stained with the DNA-binding dye Hoechst 33342. The protein (GFP) and DNA (Hoechst) localization were monitored in live cells by deconvolution microscopy. Arrow indicates Lem2-GFP NE localized foci. (B) Cells with endogenously produced Lem2-GFP and the SPB reporter Pcp1-RFP were grown to log phase at 25°C. Protein localization was monitored in live cells. Arrow indicates Lem2 and Pcp1 co-localization. (C) \( \Delta \text{man1} \) cells with endogenously produced Lem2-GFP (C-1) or \( \Delta \text{lem2} \) cells with endogenously produced Man1-GFP (C-2) were grown to log phase at 25°C and protein localization was monitored in live cells. Scale Bar = 5 μm. Boxed insets are twice the size of the original image.
Figure 3. Overexpression of *man1* or *lem2* disrupts NE integrity and alters nuclear membrane structure. Expression of *lem2* or *man1* from the *nmt1* gene promoter in plasmid pHET75 or an empty vector control was repressed and the cells grown to log phase, then derepressed for 30 h at 25°C. (A-1, A-2, A-3) Expression in cells with the nucleoplasmic reporter SV40 NLS-GFP-β-gal and the NE reporter GFP-Nsp1. Nuclear compartmentation was monitored in live cells. Star indicates cell without nuclear compartmentation; arrow indicates GFP-excluding nuclear structure; bracket indicates GFP-containing cytoplasmic spheres. Cell outlines are shown in white. (A-4, A-5, A-6) Expression in cells with only GFP-Nsp1. Protein localization was monitored in live cells. Arrowhead indicates GFP-Nsp1 at the cell periphery; bracket indicates GFP-Nsp1 at the periphery of cytoplasmic spheres. Scale bar = 5 μm. Boxed insets are twice the size of the original image. (B) Expression in wild type cells, of (B-1) *lem2*-YFP or (B-2) *man1*-YFP from the *nmt1* gene promoter in an integrated pHET75 plasmid, was derepressed for 30 h at 25°C, and protein localization was monitored in live cells. Brackets indicate cytoplasmic spheres. Cell outlines are shown in white. Scale bar = 5 μm. (C) Wild type cells (C1) or wild type cells in which expression of *lem2* (C2, C3, C4) or *man1* (D1 to D4) from the *nmt1* gene promoter in pHET75 was derepressed for 30 h at 25°C were high pressure frozen, fixed, stained and visualized using electron microscopy. Arrow indicates intranuclear membrane stack; arrowhead indicates cytoplasmic membrane-bound spheres; black and white bar indicates NPC-like structure. Scale bars are as indicated on individual panels.
DNA co-localization with Lem2 or Man1 was dependent on the HEH domain and overexpression of the HEH domain alone caused chromosome hyper-compaction. To ask if the influence of Lem2 or Man1 on chromatin depended on the putative DNA-binding HEH domain, truncated versions of Man1-YFP or Lem2-YFP lacking the HEH domain (Man1\textsuperscript{DHEH}-YFP or Lem2\textsuperscript{DHEH}-YFP respectively) (See Fig. 1A) were overexpressed in wild type cells. Protein localization and DNA morphology (Fig. 5A) were compared with those of cells overexpressing the full-length proteins (Fig. 4A) or expressing the proteins from their respective endogenous promoters (Fig. 2A). A small proportion of full length and truncated Man1-YFP and truncated Lem2 localized to the ER at the cell periphery. ER localized full length Lem2-YFP cannot be clearly visualized, in part because of the very strong fluorescence signal from the stacked NE membranes. Lem2\textsuperscript{DHEH}-YFP and Man1\textsuperscript{DHEH}-YFP proteins had the same NE association as their full-length counterparts, although the morphology of the proliferated membrane was somewhat different when visualized by fluorescence microscopy (compare Fig. 4A and Fig. 3B to Fig. 5A) and electron microscopy (compare Fig. 5B, 1 and 2 with Fig. 3C, 2–4; compare Fig. 5B, 3 with Fig. 3D, 1–4). In contrast to their full-length versions (Fig. 4A, 1 and 4), DNA did not co-localize with overexpressed Lem2\textsuperscript{DHEH}-YFP or overexpressed Man1\textsuperscript{DHEH}-YFP at the periphery of the nucleus (Fig. 5A, 2 and 4). Because protein gel blot analysis showed that the levels of full-length and truncated proteins are similar (data not shown), these data show that the influence of Lem2 and Man1 on the NE and chromatin anchoring were dependent on their respective HEH domains and not to differences in protein levels.
In an alternative approach to determining the in vivo function of the HEH domain of Man1 and Lem2 (HEH\textsuperscript{Man1}, HEH\textsuperscript{Lem2}) (see Fig. 1A), each domain or the vector control was over-expressed in wild type cells and the DNA visualized using DAPI (Fig. 5C, 1–3). This led to a striking hypercompaction of the chromosomes in 72.0% of cells with HEH\textsuperscript{Man1}, 12.0% with HEH\textsuperscript{Lem2} but 0% with the vector control (n = 200). The proportion of binucleated cells in the vector control, HEH\textsuperscript{Man1} or HEH\textsuperscript{Lem2} overexpressing strains was similar (13.0%, 8.9% and 10.4% respectively). In contrast, the proportion of cells that underwent closed mitosis and lacked the animal cell BAF protein, essential functions of the animal cell nuclear lamina, was not affected. Overexpression of HEH\textsuperscript{Man1}, HEH\textsuperscript{Lem2}, or vector control in cells with the nuclear pore protein Nup107-RFP showed that the compacted DNA localized to a single focus at the NE (Fig. 5C, 4–6). Overexpression of HEH\textsuperscript{Man1}, HEH\textsuperscript{Lem2}, or the vector control in cells with the SPB protein Sid4-GFP\textsuperscript{44} revealed that the hypercompacted DNA foci co-located with the SPB in 96% of HEH\textsuperscript{Man1} cells and 98% of HEH\textsuperscript{Lem2} cells (n = 200) (Fig. 5C, 7–9)

Lem2 and Man1 are required for anchoring of telomeres at the nuclear periphery. To ask whether Lem2 or Man1 participate in telomere anchoring, we monitored the intranuclear position of the telomere-binding protein Tel1-GFP\textsuperscript{50} with respect to the nuclear periphery delineated by the nuclear pore complex (NPC) protein Nup107-RFP in wild type and null mutant strains (Fig. 6A). Using a previously described method,\textsuperscript{48} the relative position of each telomere with respect to the nuclear diameter was used to assign it to one of three zones of equal area within an optical section of the nucleus: Zone I at the periphery, Zone II near the periphery and Zone III in the middle (Fig. 6B). If telomeres were randomly positioned in the nucleus, equal proportions of telomere spots would be found in each zone, but that was not the case in wild type cells: 64% of telomeres were found in zone I, 20% in zone II and 16% in zone III, a distribution skewed toward the nuclear periphery and significantly different from random (p < 0.0001) (Fig. 6C). The distribution of telomeres into the three zones was significantly different from the distribution in wild type cells for both the \textalpha\textsubscript{2} and \textalpha\textsubscript{1} strains (p < 0.0001, p < 0.0002 respectively). The percentage of telomeres at the nuclear periphery decreased to 47.9% in the \textalpha\textsubscript{2} strain, (Fig. 6D) and to 57.8% in the \textalpha\textsubscript{1} strain (Fig. 6E). Telomere distribution in the absence of both Lem2 and Man1 was also significantly different from that of wild type cells (p < 0.0002) but did not differ from that of \textalpha\textsubscript{2} (p > 0.1) indicating that the defects caused by the \textalpha\textsubscript{2} and \textalpha\textsubscript{1} mutations are not additive.

Discussion

Despite substantial differences in nuclear structure and organization between higher and lower eukaryotes, we find that S. pombe Lem2 and Man1, INM localized proteins distantly related to the animal cell LEM-domain containing subfamily of LAPs, perform essential functions of the animal cell nuclear lamina. Although yeast undergo closed mitosis and lack the animal cell BAS protein, that mediates the interaction of LEM proteins with chromatin, and the lamin intermediate filament protein, that is a key component of the animal cell nuclear scaffold, we show that these two fusion yeast proteins influence nuclear structure and integrity, anchor chromatin to the NE via their HEH-domains, and are previously unknown components of the telomere anchoring system.

Lem2 and Man1 are integral proteins of the INM with nuclear N-terminal DNA-binding HEH domains. Previous characterization of several members of the LEM-domain protein family\textsuperscript{3,14,15,15,44,45} indicates that they are integral INM proteins with two transmembrane domains, a cysteine-rich luminal domain that is similar in amino-acid spacing of the INM and ONM. In cells with NE gaps, the DNA-free membrane is seen when lamins are overproduced in animal cells,\textsuperscript{62,63} or when vertebrate lamin B receptor is overexpressed in human\textsuperscript{45} or budding yeast cells.\textsuperscript{66} Overexpression of the S. cerevisiae protein Sic1/Heh1 or Heh2 also changes chromatin organization, but alteration of NE organization was not reported.\textsuperscript{63} The Man1 induced tethered “mini-nuclei” in S. pombe are morphologically distinct from the nuclear blebs seen in animal cells with mutant lamins,\textsuperscript{46,51} or when vertebrate lamin B receptor is overexpressed in human cells\textsuperscript{45} or budding yeast cells\textsuperscript{66} or the nuclear morphology of budding yeast cells overexpressing the INM-associated protein Esc1.\textsuperscript{27}

It has recently been shown that the laminal domain of S. cerevisiae Sic1/Heh1 but not Heh2 interacts with the membrane-associated nucleoporin Pnm152p and in certain mutant backgrounds influences NPC distribution in the NE and causes nucleoporin mislocalization to the cytoplasm.\textsuperscript{69} In contrast, mutation of neither Lem2 nor Man1 influences NPC distribution or function, although they do have the fungal-specific cysteine-rich luminal domain that is similar in amino-acid composition but not sequence to that of the S. cerevisiae Heh1 and Heh2 proteins.

NE integrity depends on Lem2. In S. pombe, the NE remains intact throughout the cell cycle, and we show that Lem2 is essential for this nuclear compartmentation. The NE gaps in \textalpha\textsubscript{2} cells likely result from destabilization of the NE at the sites of NE lumen dilution. These data suggest the possibility that Lem2 may directly or indirectly interact with proteins in the NE lumen or the ONM (Outer Nuclear Membrane) that tether the two membranes to each other and/or maintain the uniform spacing of the INM and ONM. In cells with NE gaps, the spherical structure of the nucleus is maintained, a morphology similar to that seen when a transient NE hole arises from a defect in the SPB insertion into the membrane.\textsuperscript{27} But, it is strikingly different from the morphology of cells lacking a functional Ran-GTPase system\textsuperscript{70} in which the NE fragments due to its inability to sufficiently increase NE area during elongation of the
intraneur spindle. We find that lem2 but not man1 interacts genetically with the temperature sensitive Ran GEF pim1-d1 mutant and significantly exacerbates its previously characterized NE defects, suggesting that they influence the NE by independent mechanisms. In contrast, deletion of man1 does not destabilize the NE either alone or in combination with the Aem2 or pim1-d1 temperature sensitive mutations.

Localization to the INM of the S. cerevisiae HEH-domain proteins Heh1 and Heh2 requires Ran GTPase dependent nuclear protein import, but in that organism neither disruption of the Ran system nor disruption of HEH1 or HEH2 destabilizes the NE. The possibility that NE breakage in the pim1-d1 mutant is caused by the inability to transport Lem2 to the INM is not consistent with our observations that NE breakage is significantly greater in pim1-d1 cells than in Aem2 cells and that breakage in the Aem2 pim1-d1 double mutant cells is significantly greater than that of the pim1-d1 single mutant. These observations raise the possibility that another RanGTPase-dependent function is necessary for NE stability.

Lem2 and Man1 anchor chromatin to the nuclear periphery via their HEH-domains. In the animal cell lineage a HEH DNA-binding fold lies within the LE domain of a subset of LAPs, and it interacts indirectly with DNA by binding to the animal cell-specific DNA-binding protein BAF.\(^5\) Phosphorylation, and perhaps other cell cycle dependent protein modifications of components of the lamina, releases chromatin from the NE at mitosis and promotes NEBD (reviewed in refs. 5, 17 and 18).

In single celled eukaryotes, which do not have BAF, the HEH domain has been predicted to bind directly to chromatin.\(^19\) and promotes NEBD (reviewed in refs. 5, 17 and 18).

The observation that chromatin associates with the over-expression-induced proliferated NE, specifically at the periphery of the Man1-induced "mini-nuclei" and the ability of excess HEH domain to release chromatin from the nuclear periphery, are consistent with a model in which Lem2 and Man1 anchor heterochromatin to the nuclear periphery. This possibility is consistent with the recent work from Karl Ekwall’s laboratory\(^69\) showing that Man1 binds to multiple chromatin loci that are characterized by their association with the heterochromatin-specific binding protein Swi6. Man1 is associated with nearly 30% of the genome at loci that are distributed throughout the three S. pombe chromosomes including the centromere and subtelomeric regions. In these respects, Man1 functions similarly to the animal cell nuclear lamina in anchoring transcriptionally repressed genes to the nuclear periphery. It will be interesting to now determine the loci to which the Lem2 protein binds.

Lem2 and Man1 anchor specific heterochromatin domains, including telomeres, to the NE. S. cerevisiae Heh1 binds to telomeric and sub-telomeric repeats and when mutated changes the expression of a small number of sub-telomeric genes,\(^33\) but does not alter the intraneur distribution of telomeres (subtelomeric genes) or telomeric silencing, which is correlated with the NE association of telomeres in this organism.

Our preliminary data for Lem2 and the data of Karl Ekwall\(^69\) for Man1 indicate that these two S. pombe proteins also bind to telomeric and sub-telomeric regions of the chromosomes. Although Lem2 and Man1 are not essential for global chromatin organization, they are each essential for telomere anchoring at the NE, a characteristic they share with the constitutive telomere-binding proteins Bq3 and Bq4.\(^44\) Telomere distance from the NE increases in the absence of Bq3 or Bq4, but their distribution remains skewed toward the nuclear periphery, which suggested the possibility that other anchoring proteins exist, and Lem2 and Man1 are two such proteins.\(^45\) In S. pombe, loss of telomeric nuclear envelope localization does not alter telomeric silencing or telomere length.\(^45\)

Lem2, but neither Man1 nor the related Heh1 or Heh2 proteins of S. cerevisiae\(^45\) (see Fig. 1A), accumulates at the SPB to which centromeric heterochromatin is anchored during interphase but not mitosis of the cell cycle, yet its SPB-specific function remains unknown: it does not influence mitotic chromosome segregation and although excess HEH-domain peptide dissociates chromatin from the nuclear periphery, it does not disrupt the interaction between the SPB and centromeric chromatin. Like Lem2, Im1 is inner NE localized fusion yeast
Figure 6. Lem2 and Man1 are each required for tethering telomeres to the nuclear periphery. (A) Wild type, \(\Delta\)lem2, \(\Delta\)man1 and \(\Delta\)man1\(\Delta\)lem2 cells with the telomere-binding protein Taz1-GFP (to visualize the telomere) and the NPC component Nup107-RFP (to visualize the nuclear periphery) were grown to log phase at 25°C in YE. Telomere localization within the nucleus relative to the nuclear periphery was monitored using deconvolution microscopy. Scale bar = 5 \(\mu\)m. (B) Three zones of equal area were designated and each telomere was assigned to a zone based on its distance from the nuclear periphery relative to the nuclear diameter as previously described. Zone I is the outermost layer representing telomeres at or near the nuclear periphery. Zone II is the intermediate layer, and zone III is the inner layer containing only telomeres near the center of the nucleus. (C–F) Comparison of percentage of telomeres found in each zone for wild type cells to (C) expected distribution of randomly distributed spots, (D) \(\Delta\)lem2 cells, (E) \(\Delta\)man1 cells and (F) \(\Delta\)man1\(\Delta\)lem2. \(n = 200\) for each strain. For comparative purposes, the same wild type distribution in shown in each graph. Asterisk indicates statistically significant difference between wild type and mutant distribution of telomeres calculated using the chi-square test.
protein enriched at the SPB,\textsuperscript{14,15} but the SPB localization of these 2 proteins is mutually exclusive\textsuperscript{16} suggesting that they may play distinct roles at the SPB which remain to be determined.

Evolution of nuclear organization. It has been proposed that proteins structurally related to Lem2 and Man1, with primitive HEH DNA binding folds and transmembrane domains, may have been present in the last Eukaryotic Common Ancestor.\textsuperscript{14-16} They may have played an important role in tethering nucleic acids to membranes at the time of emergence of the first eukaryote, thereby stabilizing these metabolites. Other data indicate that the same may be true of S. pombe Lem2 and Man1. They influence nuclear structure and organization in the absence of two key components of the animal cell lamina: lamin, the intermediate filament proteins that form the NE scaffold, and BAF, the protein that mediates the binding of LEM-domain containing lamin-associated proteins to chromatin. Consistent with the possibility that HEH-domain containing proteins represent the foundation upon which the animal cell lamina was built, it has been postulated that the presence of the BAF protein in the animal cell lineage allowed for the proliferation and specialization of members of the LEM-domain protein family in animal cells.\textsuperscript{17}

Comparative studies of nuclear organization in yeast, plants and animals will lead to a better understanding of the principles of nuclear organization as they relate to nuclear structure and function in both open and closed mitosis and to the evolution of nuclear organization since the emergence of the first nucleated cells.

Materials and Methods

Yeast cell culture. Standard methods and genetic techniques were used\textsuperscript{18} and strains are described in Table 1. Transformations were performed by lithium acetate.\textsuperscript{71,72} Spotting experiments were performed by Nucleus Volume 3 Issue 1

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S. pombe \textsuperscript{147}, each domain was PCR amplified from the \textit{S. pombe} cDNA library AACT (generous gift of Dr. Steve Elledge), the products digested with BsmHI and Smal and cloned into the multicopy pDS473\textsuperscript{a} plasmid.

To construct C-terminal GFP-tagged versions of \textit{man1} or \textit{lem2} at their chromosomal loci, the C-terminal domain was PCR amplified and cloned between the BsmHI-Smal sites of the pREP3X,\textsuperscript{74} plasmid. The resulting plasmid was linearized using the NdeI site in the C-terminal domain of \textit{man1} or \textit{lem2} and transformed into haploid wild-type cells (SS446).

The \textit{Δman1} (\textit{man1} null) and \textit{Δlem2} (\textit{lem2} null) strains were generated by PCR-based targeted gene replacement of the open reading frame with a KanMX drug resistance cassette\textsuperscript{78} and identified by their ability to grow on YE plates with GIBCO \textsuperscript{TM} Geneticin (G418) (Invitrogen).

Fluorescence microscopy. A DeltaVision Deconvolution Microscope System (Applied Precision, Issaquah, Wash.), with a Nikon TE2000 inverted microscope and a Nikon Plan APO 100X 1.4 N.A. lens and a Photometrics CoolSnap HQ Camera (Roper Scientific) was used to collect images of the whole cell, by analyzing stacks of 0.2 μm Z sections, that were projected two dimensionally using the maximum intensity protocol, using SoftWoRx 3.5 (Applied Precision, Inc.) software. In some cases, as indicated, cells were examined using a Zeiss Axiovert fluorescence microscope, with a Zeiss Plan-NEO FLUAR 100X 1.3 N.A. lens and a Photometrics CoolSnap HQ Camera (Media Cybernetics). Individual images were extracted into Photoshop (Adobe) to generate the panels for the figures.

Live cells producing the NE localized NPC component Nsp1p fused to GFP (GFP-Nsp1p) to visualize the nuclear periphery and the soluble protein β-galactosidase fused to GFP and targeted to the nucleus by the SV40 nuclear localization signal (SV40 NLS-GFP-β-gal) were used to visualize the nuclear interior and monitor NE integrity, as previously described.\textsuperscript{19} In cells with intact NEs these GFP signals are exclusively nuclear, but in cells with broken NEs the SV40 NLS-GFP-β-gal signal localizes throughout the cell. The percent of cells with broken NEs was determined by counting at least 200 cells.

Telomere localization was determined by assigning each to one of 5 zones equal in area within the nucleus as previously described.
| Table 1. Strains used in this study |
|-----------------------------------|
| **Strain Name** | **Genotype** | **Source** |
| SS2171 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2172 | h· man1: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2173 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2192 | h· lem2-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2193 | h· lem2-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2194 | h· man1-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2198 | h· man1-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2199 | h· man1-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2200 | h· man1-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2234 | h· leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2236 | h· leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2268 | h· leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2352 | tac1-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2357 | h· tac1-GFP: KanMX6 lem2 Natm4 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2358 | h· tac1-GFP: KanMX6 lem2 Natm4 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2394 | h· rpm107-tomato Natm4 leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2395 | h· rpm107-tomato Natm4 leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2396 | h· rpm107-tomato Natm4 leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2407 | h· leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2408 | h· leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2409 | h· leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2410 | h· sad4-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2411 | h· sad4-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2412 | h· sad4-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 pDS473a | This study |
| SS2418 | h· rpm107-tomato Natm4 tac1-GFP: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2420 | h· rpm107-tomato Natm4 tac1-GFP: KanMX6 lem2 Natm4 leu1-32, ura4-D18, ade6-M210 | This study |

| Table 1. Strains used in this study (continued) |
|-----------------------------------------------|
| **Strain Name** | **Genotype** | **Source** |
| SS2015 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2016 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2017 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2040 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2044 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 | This study |
| SS2045 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2046 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2047 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2048 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2049 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2050 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2058 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2089 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2090 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2126 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2127 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2159 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2169 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
| SS2170 | h· lem2: KanMX6 leu1-32, ura4-D18, ade6-M210 pDUAL-m1-YPF | This study |
in S. cerevisiae except that: the S. pombe telomeres were localized with Tac1-GFP; the nuclear pores were visualized with Nup107-RFP; live cells were grown in YE liquid cultures instead of on agar; and 20 stacks of images (exposure, 1,000 ms; step size, 200 nm) were taken using a deconvolution microscope instead of a wide-field microscope.

To monitor the fidelity of chromosome segregation, wild type, Δman1 and Δlem2 strains were constructed that carried a tandem array of 256 lac operator (lacO) repeats integrated at the man1 and Dα locus that is tightly linked to the centromere of chromosome I, and expressed a Lac1-GFP chimera that binds to the lacO repeats. These strains were used to monitor chromosome segregation during mitosis, as previously described by determining whether binucleated cells have one GFP dot in each nucleus (equal segregation) or two GFP dots in one nucleus and none in the other (mis-segregation).

Electron microscopy. Cells were harvested by vacuum filtration onto 0.45 μm Millipore filters and the resulting wet cell paste was loaded into aluminum sample holders with a 100 or 200 um well (Technotrade International) for high pressure freezing in a Bal-Tec HPM 010 (Leica, Inc.). Frozen samples were freeze-substituted in 2% osmium tetroxide and 0.1% uranyl acetate in acetone at −80°C for 4 d, warmed to −20°C overnight, then to 4°C for 3hr and room temperature for 1 h, followed by infiltration and embedding in Epon/Araldite resin. Thin sections (60–70 nm) were stained with 2% uranyl acetate dissolved in 70% methanol, 30% water, rinsed, and stained in lead citrate. Images were obtained with a Philips CM10 or CM100 (FEI, Inc., Hillsboro, OR) equipped with a Gatan BioScan digital camera.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no financial interests in relation to the submitted work.

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Supplemental Material

Supplemental material may be downloaded here: http://www.landesbioscience.com/journals/nucleus/article/11882/.

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| Strain Name | Genotype | Source |
|-------------|----------|--------|
| SS2425 h-lep107-tamato Matα64 Tac1-GFP::KanMX6 man1-1 Nat804 lec1-32, ura4-D18, ade6-M216 | This Study |
| SS3565 h- man1 lec1-32 int::pREP82X-GFP::KanMX8 0418 ura4-D18 ade6-M216 | This Study |
| SS3566 h- man1 lec1-32 int::pREP82X-GFP::KanMX8 0418 ura4-D18 ade6-M216 | This Study |
| SS2628 h- pgl1-2 man1 nsp1 leu1-32 int::pREP82X-GFP::KanMX8 0418 ura4-D18 ade6-M216 | This Study |
| SS3635 h- nap107-tamato Matα64 Tac1-GFP::KanMX6 man1-1 Nat804 lec1-32, ura4-D18, ade6-M216 | This Study |

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Supplemental Material

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