RESEARCH PAPER

Nucleus 5:4, 352–366; July/August 2014; © 2014 Landes Bioscience

Requirement of the spindle pole body for targeting and/or tethering proteins to the inner nuclear membrane

Greetchen Diaz-Muñoz[1,2,†], Terri A Harchar[3,‡], Tsung-Po Lai[2,§], Kuo-Fang Shen[2,‖], and Anita K Hopper[1,2,*]

[1]Molecular, Cellular and Developmental Biology Interdisciplinary Program; The Ohio State University; Columbus, OH USA; [2]Department of Molecular Genetics; The Ohio State University; Columbus, OH USA; [3]Department of Biochemistry and Molecular Biology; H171; Pennsylvania State University College of Medicine; Hershey, PA USA

Current affiliation: [†]Nebraska Center for Virology; Morrison Research Center; Lincoln, NE USA; [‡]Axiom; Huntsworth Health; Yardley, PA USA; [§]Department of Cell Biology; UT Southwestern Medical Center; Dallas, TX USA; [‖]Molecular & Computational Biology Section; University Southern California; Los Angeles CA USA

Keywords: yeast, INM targeting, SPB, live cell imaging, nuclear membrane organization

Appropriate targeting of inner nuclear membrane (INM) proteins is important for nuclear function and architecture. To gain new insights into the mechanism(s) for targeting and/or tethering peripherally associated proteins to the INM, we screened a collection of temperature sensitive *S. cerevisiae* yeast mutants for defects in INM location of the peripheral protein, Trm1-II-GFP. We uncovered numerous genes encoding components of the Spindle Pole Body (SPB), the yeast centrosome. SPB alterations affect the localization of both an integral (Heh2) and a peripheral INM protein (Trm1-II-GFP), but not a nucleoplasmic protein (Pus1). In wild-type cells Trm1-II-GFP is evenly distributed around the INM, but in SPB mutants, Trm1-II-GFP mislocalizes as a spot(s) near ER-nucleus junctions, perhaps its initial contact site with the nuclear envelope. Employing live cell imaging over time in a microfluidic perfusion system to study protein dynamics, we show that both Trm1-II-GFP INM targeting and maintenance depend upon the SPB. We propose a novel targeting and/or tethering model for a peripherally associated INM protein that combines mechanisms of both integral and soluble nuclear proteins, and describe a role of the SPB in nuclear envelope dynamics that affects this process.

Introduction

The nuclear envelope (NE) is formed by two lipid bilayers, the outer nuclear membrane (ONM), which faces the cytoplasm and is contiguous with the endoplasmic reticulum (ER),[1] and the inner nuclear membrane (INM), which faces the nucleoplasm. The two bilayers connect at the nuclear pore complexes (NPCs). Although there are proteins that are proposed to reside in both the ONM and the INM,[2,3] the general protein composition of the two membranes is distinct. The two membranes serve to ensure proper nuclear function and architecture. INM protein targeting is of special interest because the INM contains a number of proteins that assist processes such as DNA replication and gene expression.[4] Furthermore, inappropriate location of INM proteins causes human envelopathies.[5]

INM integral proteins are translocated from the ONM to the INM. A class of INM transmembrane proteins is proposed to access the interior by a diffusion-retention model,[6] which predicts that proteins attached at the ONM are passively translocated through the NPC and retained at the INM by other proteins or protein complexes such as lamina.[7] However, INM targeting for other proteins is more complex and incorporates different factors and/or multiple steps.[8] For example, a nuclear localization signal (NLS) facilitates the translocation to the INM of the yeast proteins Heh1 and Heh2.[9,10] The *C. elegans* SUN protein, UNC-84, contains multiple targeting sequences and is actively transported.[11] Human Sun2 INM location is dependent on a NLS, a Golgi retrieval signal, and a perinuclear domain.[12] Also, the yeast INM SUN protein, Mps3, binds the histone variant Htz1 for translocation through the NPC.[13]

Another group of proteins are peripherally associated to the INM. Compared with integral INM proteins, information of how they are targeted to the membrane is limited.[14] Most of the information derives from studies of the lamin proteins which reach the nucleoplasm via Ran-dependent nuclear import machinery and then associate with the INM by specific modifications of either the N or C-termini, which confer the ability to bind membranes.[15] The specific targeting of lamin proteins, and perhaps other peripheral INM proteins to the NE and not to other membranes is likely due to the NLS, which delivers the protein specifically to the nuclear interior.

Here we describe studies to investigate the INM targeting mechanism for the peripheral *S. cerevisiae* protein, Trm1, a tRNA methyltransferase.[16] There are two isoforms of the

*Correspondence to: Anita K Hopper; Email: hopper.64@osu.edu
Submitted: 04/23/2014; Revised: 06/27/2014; Accepted: 07/01/2014; Published Online: 07/03/2014
http://dx.doi.org/10.4161/nucl.29793

protein that are generated by alternative translation starts. The form initiating at the first AUG (Trm1-I), localizes exclusively to the mitochondria whereas the form initiating at the second AUG (Trm1-II), localizes to both the mitochondria (10%) and the nucleus (90%).[16,17] Mitochondrial localization of Trm1-I and Trm1-II is achieved by a mitochondrial targeting signal (MTS), while nuclear localization of Trm1-II is driven by a NLS. Endogenous Trm1-II and tagged Trm1-II-GFP are both peripherally associated throughout the INM.[17-21] Mutational analysis of *TRM1* revealed that a region composed of amino acids 133 to 151 is necessary and sufficient for NE targeting.[20]

A genome-wide screen of non-essential yeast genes identified factors required for Trm1-II-GFP INM location.[18] This screen identified *ICE2* and the NatC N-terminal acetylase genes (*MAK3*, *MAK10*, and *MAK31*) as determinants for appropriate INM location. Deletion of these genes causes mislocation of Trm1-II-GFP to the nucleoplasm, sometimes with a residual pool at the nuclear membrane. However, Ice2 and the NatC complex seem to have an indirect role in Trm1-II INM location as Ice2 is an ER protein and NatC N-acetylation is not sufficient for its INM location.[18] The Ran pathway is required for Trm1-II import, but not for its INM maintenance.[20] The cumulative data of *cis* and *trans* acting components led to a model that Trm1-II is imported into the nucleus by a similar mechanism to soluble nucleoplasmic proteins and then it is delivered to the INM.[20] However, as previous attempts did not elucidate the identity of Trm1-II's INM tether, the exact targeting and/or tethering mechanism is still unclear.

In this work we utilized genetic and cell biology approaches to achieve an understanding of targeting and/or tethering of INM peripherally associated proteins. We screened essential *Saccharomyces cerevisiae* genes for the location of galactose-inducible Trm1-II-GFP using an ordered collection of temperature-sensitive (ts) mutants.[22] Surprisingly, we found that multiple components of the spindle pole body (SPB) are required for Trm1-II-GFP INM location. To elucidate the role of the SPB in INM targeting and/or tethering, we utilized a microfluidics perfusion system for live cell imaging to study the dynamics of newly synthesized Trm1-II-GFP. Our data support a model in which Trm1-II-GFP is transported to the nuclear interior by at least two different mechanisms, one of which combines features from the soluble import pathway and the mechanism for targeting integral proteins. Additional studies showed that the SPB is important for appropriate location of an integral INM protein, but not for a soluble nucleoplasmic protein, suggesting a role of the SPB in nuclear architecture that affects membrane proteins.

Results

Trm1-II-GFP is mislocalized in yeast cells with altered SPB structure

To identify mutations of essential genes that affect INM targeting, we screened an ordered collection of ts mutations of essential genes (740 ts alelles, ~500 genes, comprising 45% of the essential proteome)[22] for altered INM targeting of galactose-inducible (newly synthesized) Trm1-II-GFP after shift to the non-permissive temperature (NPT, 37 °C) (**Fig. 1A**). We uncovered a total of 65 ts mutant alleles (54 genes) for which Trm1-II-GFP was mislocated. Prominent among the mutants was a group of strains affecting the SPB (**Table 1**). Upon galactose induction at the NPT in the SPB ts mutants, Trm1-II-GFP locates as a predominant spot, rather than being evenly distributed around the INM as in wild-type (wt) cells (**Fig. 1B**). More detailed analysis revealed that although Trm1-II-GFP mislocation is more severe at the NPT, it also occurs at the permissive-temperature (PT).

The SPB is the microtubule-organizing center and it is inserted in the NE throughout the entire cell cycle. The SPB contains three plaques, outer, central, and inner, that are connected to the nuclear membrane by SPB membrane proteins.[23] During cell division, the SPB is duplicated and forms a half bridge and a satellite where the new SPB is assembled. The SPB proteins that affect the distribution of Trm1-II-GFP at the INM correspond to all parts of the structure (**Fig. 1C**). Thirteen out of the 19 known SPB core components were represented in the ts collection (**Fig. 1C**). Nine of those 13 SPB genes with ts mutations affected the location of galactose-inducible Trm1-II-GFP at the INM (**Table 1**). With the exception of *NDC1*, the ts SPB genes in the collection were represented by multiple ts alleles. However, not all the mutated alleles present in the collection for a particular gene affected Trm1-II-GFP INM location (**Table 1**). Among the other six core components that were not available in the collection, only three are essential (Spc97, Spc98, and Kar1), while proteins Cnm67, Nud1, and Spc72 are unessential. The non-essential SPB proteins did not result in INM mislocation of constitutively expressed Trm1-II-GFP.[18] In addition to the mutations of the nine genes encoding SPB core components, several ts alleles of the genes that encode kinases Mps1 and Cdc28 were identified among the ts mutants affecting Trm1-II-GFP localization (**Table 1**). Mps1, phosphorylates Spc42, Spc98, and Spc110[24-26] and it is involved in many steps of SPB duplication.[27] Cdc28 has a role in SPB duplication via phosphorylation of Spc42, Spc110, and Mps1 when in complex with other proteins.[28,29] Trm1-II-GFP also demonstrates an aberrant location in yeast strains when the SPB components Mps1, Ndc1, Spc29, and Spc110 (also represented in the ts collection) and Kar1 (which was absent from the ts collection) are inactivated by tetracycline off regulation (**Table S1**).[30] In summary, using two different approaches to alter SPB function, (ts collection and tetracycline-regulated strains) we uncovered that the majority of essential SPB or SPB-related proteins have an apparent role in the location of galactose-inducible Trm1-II-GFP to the INM.

One caveat of our screen was that Trm1-II-GFP overexpression may have contributed to its mislocation. To address this concern, we tested whether low levels of Trm1-II-GFP also mislocate in the SPB ts mutants by expressing Trm1-II regulated by its own promoter in a centromere-containing (low copy) plasmid. Previous work had shown that in wt cells containing endogenous and untagged-Trm1-II, the location of Trm1-II expressed from plasmids (even high copy numbers) was not altered.[18,20] Similar

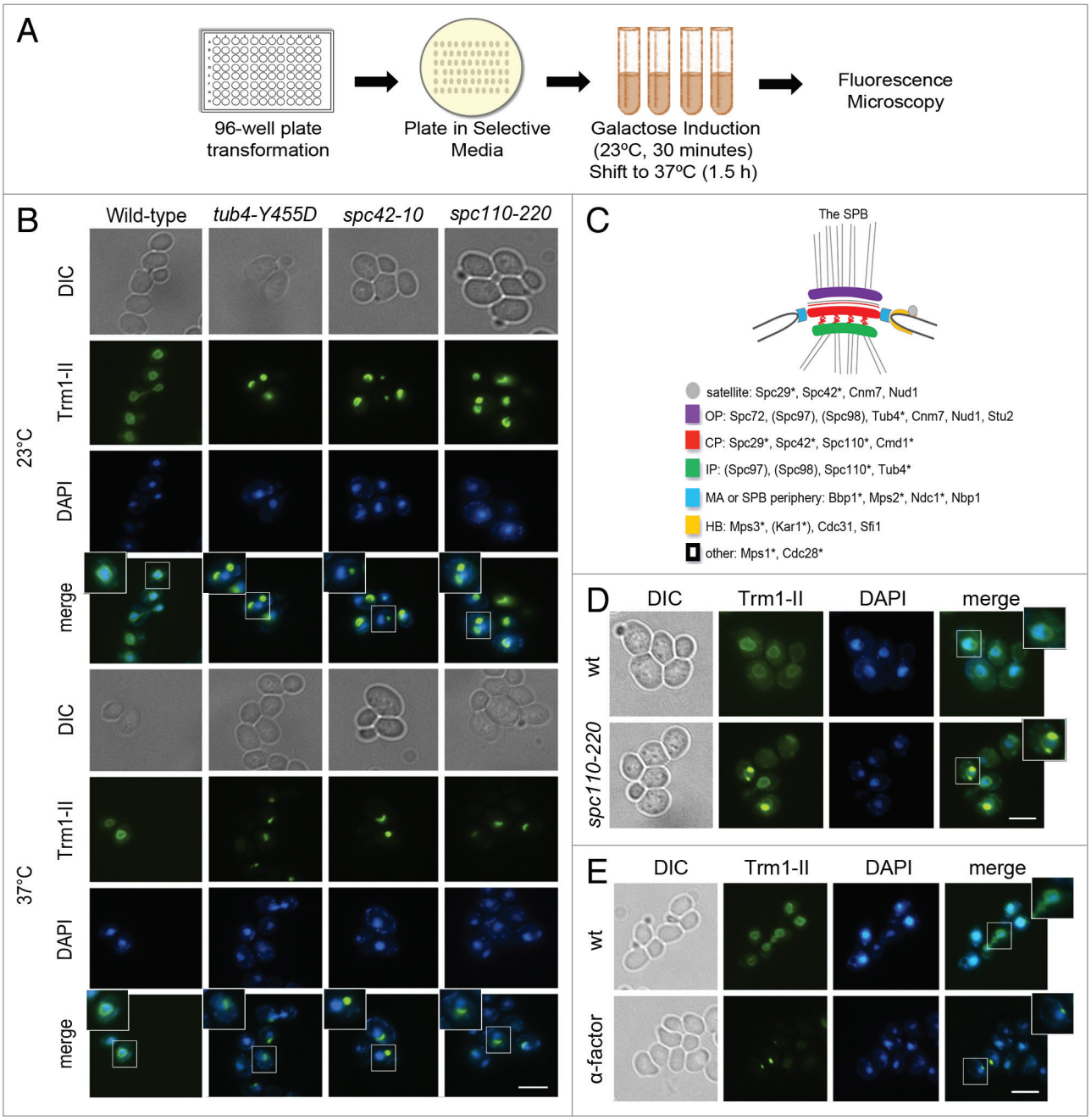

Figure 1. Galactose-inducible Trm1-II-GFP is mislocated in cells with altered SPB. (**A**) General strategy to screen for essential genes affecting Trm1-II-GFP localization. (**B**) Representative DIC and fluorescence images of cells after 2 h of induction at PT (23 °C, top panels) or NPT (37 °C, bottom panels) (**C**) Schematic structure of the SPB. Components are color coded according to their location. Gene products with an asterisk (*) affected Trm1-II-GFP INM location (results from ts collection and by Tet-off depletion). Gene products in parenthesis were not represented in the ts collection. (**D**) Representative DIC and fluorescence images of Trm1-II-GFP (centromeric plasmid) controlled by its own promoter after ~2 h at NPT. (**E**) Galactose-inducible Trm1-II-GFP in wt cells with and without α-factor treatment. Bar = 5 μm.

to galactose-inducible Trm1-II-GFP, the protein accumulated as a spot at the NPT in the majority (65%) of the cells observed (**Fig. 1D**). Thus, mislocation of Trm1-II-GFP in the SPB ts mutants is not a result of high expression. Another caveat is that the Trm1-II-GFP phenotype could be caused by the temperature sensitivity of the mutants tested. However, only a minority of strains in the ts collection affects Trm1-II-GFP location. Moreover, Trm1-II-GFP was mislocated in yeast strains with tetracycline-regulated SPB genes. We conclude that the SPB plays role in targeting or tethering Trm1-II-GFP throughout the INM.

To learn whether alterations of the SPB that naturally occur also affect the distribution of Trm1-II-GFP, we studied the distribution of Trm1-II-GFP in wt cells treated with α-factor. The SPB structure is altered and its duplication is prevented

Table 1. ts mutant alleles represented in the collection for SPB genes that affect the location of galactose-inducible Trm1-II-GFP

Gene	Allele (s) in ts collection	Affects Trm1-II-GFP location: percentage	Gene product function (from SGD)
BBP1 (YKR037C)	*bbp1–1* *bbp1–2*	Yes: 84% cells w/mislocation Yes: 90% cells w/mislocation	SPB duplication
CDC28 (YBR160W)	*cdc28–1* *cdc28–4* *cdc28–13* *cdc28-td*	Yes: 63% cells w/mislocation Yes: 60% cells w/mislocation NO NO	Phosphorylates Spc42, Spc110 and Mps1
CMD1 (YBR109C)	*cmd1–1* *cmd1–3* *cmd1–8*	Yes: 80% cells w/mislocation Yes: 79% cells w/mislocation Yes: 60% cells w/mislocation	Ca^{2+} binding protein that regulates mitosis
MPS1 (YDL028C)	*mps1–1* *mps1–6* *mps1–417* *mps1–3796*	Yes: 88% cells w/mislocation Yes: 62% cells w/mislocation NO NO	SPB duplication
MPS2 (YGL075C)	*mps2–1* *mps2–2*	Yes: 87% cells w/mislocation Yes: 85% cells w/mislocation	SPB insertion and duplication
MPS3 (YJL019W)	*mps3–1* *mps3–7*	Yes: 80% cells w/mislocation Yes; % not determined	Insertion of newly duplicated SPB into the NE
NDC1 (YML031W)	*ndc1–4*	Yes: 40% cells w/mislocation	NPC assembly and SPB duplication
SPC29 (YBR160W)	*spc29–3* *spc29–20*	NO Yes: 58% cells w/mislocation	SPB duplication
SPC42 (YKL042W)	*spc42–10* *spc42–11*	Yes: 90% cells w/mislocation Yes: 67% cells w/mislocation	SPB duplication
SPC110 (YDR356W)	*spc110–220* *spc110–221*	Yes: 90% cells w/mislocation NO	Connects nuclear microtubules to SPB
TUB4 (YLR212C)	*tub4-Y455D* *tub4-ΔDSY*	Yes: 65% cells w/mislocation Yes: 64% cells w/mislocation	Microtubule nucleation

SGD, Saccharomyces Genome Database.

in *MATa* cells treated with the pheromone, α–factor. The SPB in these cells is smaller and at least one component, Spc110, is reduced.[31,32] We reasoned that wt cells arrested with α-factor would mislocate Trm1-II-GFP in a similar fashion to the SPB ts mutants if this phenotype is a consequence of an alteration of the SPB structure. Indeed, galactose-inducible Trm1-II-GFP mislocated similarly to the SPB ts mutants identified when we treated wt cells with α-factor (**Fig. 1E**) (79%). Thus, Trm1-II-GFP mislocation in the SPB ts mutants is likely a consequence of an alteration of the SPB that, directly or indirectly, affects its INM location. As addition of α-factor pheromone arrests the cell cycle at $G_{1,}$ and also is known to cause some indirect changes in the yeast nuclear envelope,[33] one possibility is that Trm1-II-GFP normally localizes to a focus in G_1. However, galactose-inducible Trm1-II-GFP was appropriately distributed in most of the ts mutants for proteins involved in G_1 arrest, (cdc proteins with the exception of Cdc28, Cdc1, and Cdc48 under heat stress, above 38 °C). Moreover, Trm1-II-GFP was mislocated in SPB ts mutants with different cell cycle effects that are not necessarily restricted to G_1.

Role of the SPB in targeting and/or tethering Trm1-II-GFP throughout the INM

To address the location of galactose-induced Trm1-II-GFP with respect to the nuclear envelope, we performed indirect immunofluorescence (IF) in wt and *spc110–220* ts cells using the NPC protein, Nsp1, to mark the NE and the NPC (**Fig. S1A**). In both wt and *spc110–220* ts cells, Nsp1 exhibited a punctuate pattern characteristic of NPC proteins. Upon galactose induction at NPT, Trm1-II-GFP in wt cells showed the expected smooth ring distribution (85%). In the majority of the SPB ts mutant cells (75%) Trm1-II-GFP located as a spot close to or at the NE; interestingly, the Trm1-II-GFP spot often located to a pore-less region. In the remaining 25% of the cells, the localization of Trm1-II-GFP was unclear as it appeared to be excluded from the NE. We also addressed the location of galactose-induced Trm1-II-GFP with respect to the SPB and other subnuclear structures. The Trm1-II-GFP spot did not co-localize with the SPB, the nucleolus or nucleus-vacuole junctions (NVJ) (**Fig. S1B-D**).

We previously proposed that Trm1-II is imported into the nucleoplasm before it is tethered to the INM.[20] However, since the Trm1-II-GFP spot was located close to, or at, the NE in most cells, but in some cells it appeared far from the NE, we explored whether the Trm1-II-GFP spot co-located with a region of the NE that coincides with ER and/or the ER-nucleus junctions. ER-nucleus junctions are ER extensions connecting the perinuclear ER (pER) to the cortical ER (cER).[34] We followed the dynamics of Trm1-II-GFP in both wt and *spc110–220* cells containing endogenously–tagged Sec63-mCherry to locate the ER. Since in *spc110–220* cells Trm1-II-GFP exhibits the spot phenotype at both PT and NPT (**Fig. S2A-B**), we were able to

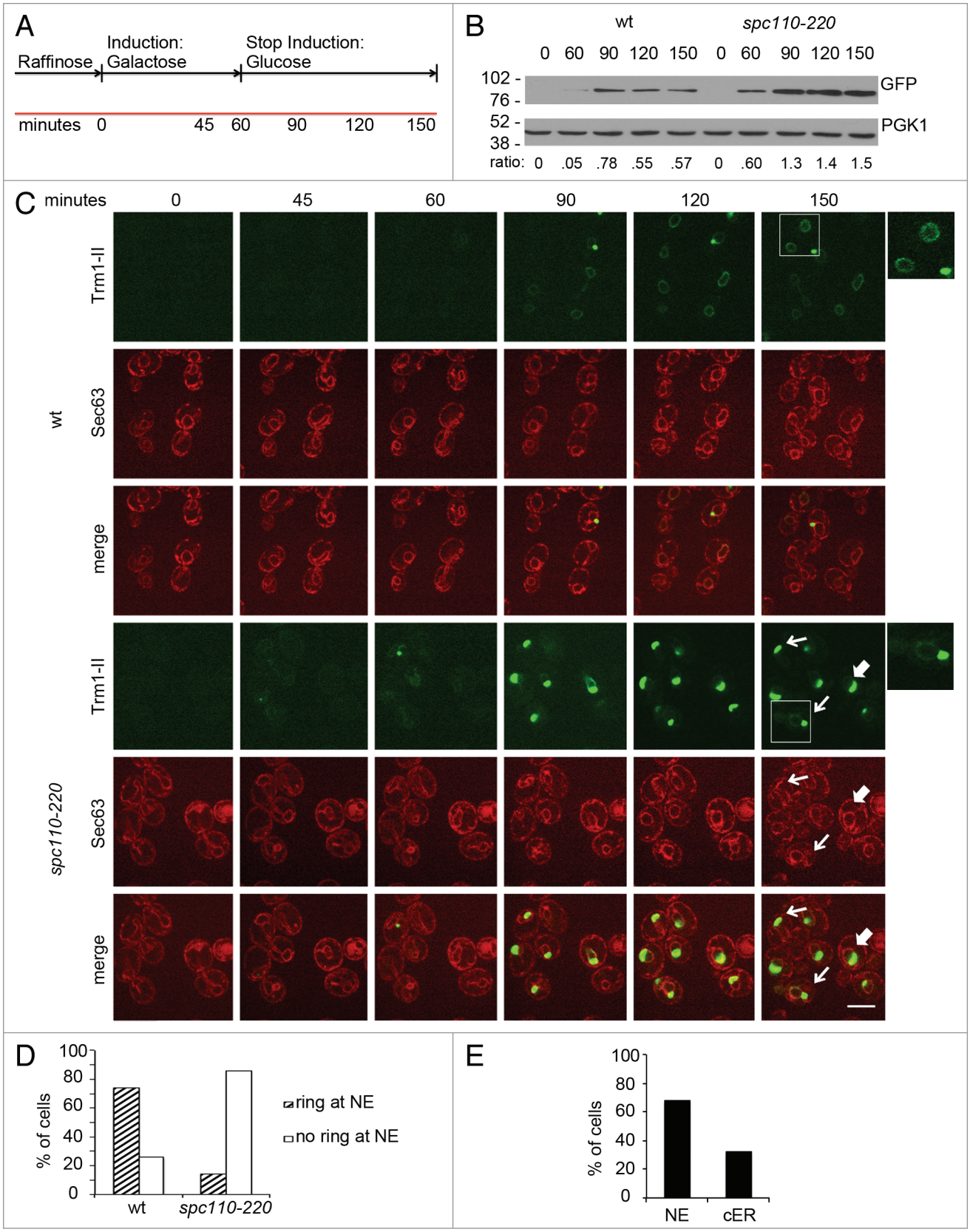

Figure 2. For figure legend, see page 357.

Figure 2 (See previous page). Live imaging using a microfluidics perfusion system to study the subcellular dynamics of galactose-inducible Trm1-II-GFP in wt and SPB ts mutant *spc110–220*. (**A**) Strategy for monitoring Trm1-II-GFP dynamics. Minutes (min) refer to times when images were captured (**B**) Western Blot analysis showing galactose-inducible Trm1-II-GFP expression. Pgk1, loading control. Ratio: quantitation of Trm1-II-GFP to Pgk1. (**C**) Location of galactose induced Trm1-II-GFP in wt and mutant cells; accumulation at the NE (wide arrows) and ER (narrow arrows). Bar = 5 μm. (**D**) Quantitation of phenotypes; n = 272 (wt cells), 324 (ts cells). (**E**) Distribution of mislocated Trm1-II-GFP signal in *spc110–220* cells with respect to the NE (INM-ONM) or cER, n = 278 cells.

study the location of Trm1-II-GFP at room temperature. We employed a microfluidics perfusion system for live cell imaging, which permitted appropriate aeration and media exchange, thus allowing tracking of living cells in optimal growth conditions over extended time. However, the number of cells that can be tracked in a single experiment is limited. Therefore, each experiment was repeated multiple times and, at the last time point, the phenotypes of additional cells in the same microfluidic plate were assessed and calculated. We induced Trm1-II-GFP synthesis for 60 minutes (min) after shifting cells grown in raffinose media to galactose media, at which time further synthesis of Trm1-II-GFP was terminated by introduction of glucose media (**Fig. 2A**). By western analysis of a separate culture we verified that Trm1-II-GFP accumulation stopped 30 min after glucose addition (**Fig. 2B**). The pool of Trm1-II-GFP monitored after glucose introduction is unlikely to be influenced by new protein synthesis because the half-life of endogenous Trm1 mRNA is only 8–15 min.[30,35,36]

Movement of Trm1-II-GFP was followed for 150 min. At 45 and 60 min, there was no detectable signal in wt cells, whereas there was a weak location to the NE in *spc110–220* cells. After 90 min, Trm1-II-GFP in wt cells localized to the nuclear rim in the majority of the cells (**Fig. 2C**). In contrast, in the majority of the *spc110–220* cells, Trm1-II-GFP accumulated as a spot close to the NE or co-localized with the ER-nucleus junction (**Fig. 2C**). We did not detect nucleoplasmic pools of Trm1-II-GFP at any time point in either wt or *spc110–220* cells. Thus, the nucleoplasmic step in the Trm1-II-GFP targeting mechanism either is very transient or it does not exist. Interestingly, in a sub-population of *spc110–220* cells, the Trm1-II-GFP spots located at the cER. The Trm1-II-GFP signal (spots) in *spc110–220* cells appeared earlier than in wt cells probably because Trm1-II-GFP expression in *spc110–220* was detected earlier and was higher than in wt cells (**Fig. 2B**).

Trm1-II-GFP location in additional wt and SPB mutant cells monitored at the 150 min endpoint was consistent with the data collected following Trm1-II-GFP in individual cells. For 74% of wt cells, Trm1-II-GFP was evenly distributed around the NE while in 26% of the cells it locates as a spot (**Fig. 2D**). In contrast, for the *spc110–220* cells 86% showed abnormal Trm1-II-GFP accumulation whereas for 14% of cells, Trm1-II-GFP was distributed around the NE. For 68% of those *spc110–220* cells that showed Trm1-II-GFP mislocation, the signal co-localized with the ER marker close to the NE and ER-nucleus junctions (**Fig. 2E**). Because the ER-nucleus junctions were not visible in all cells, it was not possible to determine the exact location in many of the cells. For the remaining 32% of *spc110–220* cells, Trm1-II-GFP accumulation occurred at the cER (**Fig. 2E**). Therefore, in cells with an altered SPB structure and/or function, Trm1-II-GFP is located preferentially to specific regions or sub-domains close to the NE that often coincides with the ER-nucleus junctions.

One interpretation of the above data is that Trm1-II-GFP contacts the ER/ONM before it is targeted to the INM, perhaps indicating that the initial tether is located at the ER/ONM, and that the SPB functions in Trm1-II-GFP movement from the ER/ONM and tethering to the INM. To further explore the role of the SPB we employed the microfluidics perfusion system to follow the dynamics of galactose-inducible Trm1-II-GFP from its first appearance until it forms a ring at the INM in wt cells arrested with α-factor. The cells were arrested by addition of raffinose media containing α-factor (**Fig. 3A**). After the majority of the cells were arrested as observed by the formation of "shmoo," Trm1-II-GFP was induced with galactose for 60 min, and further Trm1-II-GFP synthesis was repressed by substitution of glucose media in the continued presence of α-factor for an additional 60 min. At 120 min Trm1-II-GFP demonstrated the predominant spot phenotype in the majority of wt shmoo cells (**Fig. 3C**). Then, the shmoo cells were released from the cell cycle arrest by elimination of α-factor from the media flowed to the cells. After 180 min (300 min of the experiment) in the absence of α-factor, we observed that in 40% of the monitored cells Trm1-II-GFP redistributed from a localized region of the NE or the cER throughout the entire INM. Most of the cells initiated bud formation documenting the release from the G_1 arrest by α-factor. However, other cells maintained the "shmoo" shape. The data indicated that the cells re-arrange the nuclear membrane while the changes in the cell wall recovery are in progress, in agreement with previous studies reporting that cytoplasmic re-arrangement and nuclear re-organization in α-factor arrested cells are independent.[33] As the level of Trm1-II-GFP is stable after 90 min (**Fig. 3B**), and the Trm1 mRNA half-life is short, the INM localization of Trm1-II-GFP after release from the cell cycle arrest is most likely a result of redistribution of protein transiently located at discrete regions on the ER/NE to the INM. The data for the location of Trm1-II-GFP in additional wt arrested cells monitored at the 300 min endpoint (180 min after release), demonstrated that in 76%, Trm1-II-GFP was located at the INM, while only 30% of the wt cells maintained the spot phenotype The results obtained by using the microfluidics system for live imaging, support the hypothesis that Trm1-II-GFP is transiently located at the ER/NE before it locates/tethers to the INM. Thus, INM "spreading" from a discrete region of the ER/NE is likely part of the targeting mechanism for Trm1-II-GFP.

Changes of the SPB affect targeting of peripheral and integral INM proteins, but not a nucleoplasmic protein

We reasoned that if the SPB defect affects targeting of Trm1-II-GFP, which was proposed to follow the classical import pathway, then the import of other soluble proteins might also

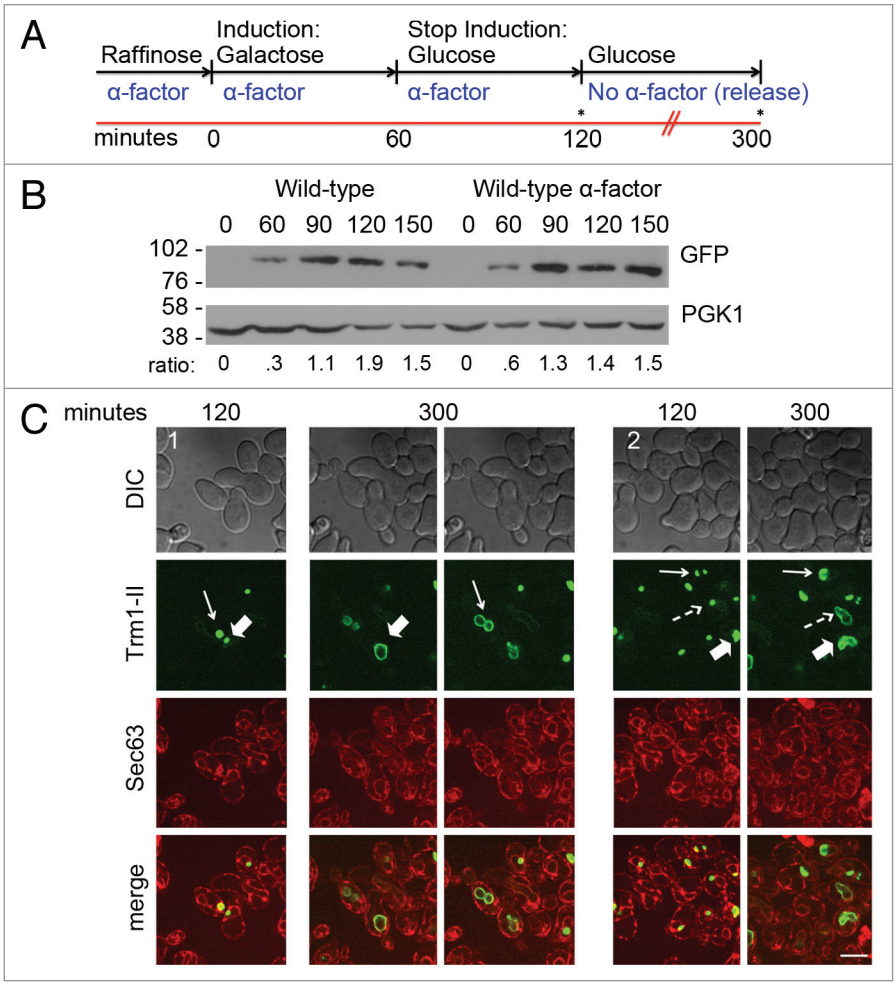

Figure 3. Mislocated Trm1-II-GFP upon α-factor arrest relocates throughout the INM when α-factor is removed. (**A**) Strategy for monitoring Trm1-II-GFP dynamics. Asterisks indicate time points of the images shown. (**B**) Western Blot analysis for galactose-inducible Trm1-II-GFP in wt cells with and without α-factor over time. (**C**) Panel 1: Galactose-inducible Trm1-II-GFP is located at the cER (narrow arrow) or at a discrete region of the NE (wide arrow) before translocation to the INM. Two different single-plane confocal images are shown for 300 min. Panel 2: Three additional cells showing relocation of Trm1-II-GFP (narrow, wide and dashed arrows). Bar = 5 μm.

be affected. We compared the dynamics of galactose-inducible Trm1-II-GFP to the soluble nuclear protein, Pus1-GFP in wt and *spc110–220* cells. In contrast to Trm1-II-GFP, galactose induced Pus1-GFP located in the nucleoplasm in both wt and *spc110–220* cells (**Fig. 4A and B**). Similar results were obtained for Pus1-GFP in wt cells arrested with α-factor (**Fig. S3A**). Thus, the SPB does not appear to play a role in the targeting of at least one nucleoplasmic protein, Pus1-GFP.

We monitored the dynamics of the integral protein Heh2-GFP in wt and *spc110–220* cells. In wt cells galactose-inducible Heh2-GFP localizes as a ring at the NE, as previously reported[9] (**Fig. 4C**). We observed that part of the Heh2-GFP pool accessed the NE in the SPB ts mutant. However, a pool of Heh2-GFP co-localized with the ER marker that is part of the cER (**Fig. 4C**). We also noted that in the SPB ts mutant a pool of Heh2-GFP was located to small extensions that partially co-localized with the ER marker. It was unclear whether these extensions are nuclear or part of the ER. When we analyzed additional cells, the aberrant localization of Heh2-GFP was observed in the majority (70%) of the induced *spc110–220* cells (**Fig. 4D**). Thus the integral INM protein, Heh2-GFP, has an aberrant distribution in the SPB mutant. That the SPB is important for the appropriate distribution of an integral and a peripherally-associated INM protein was further supported by data showing that Heh2-GFP INM location was aberrant in wt cells treated with α-factor (**Fig. S3B**). The results indicate that a defect at the NE in cells with altered SPB affects both a peripherally-associated and an integral INM but not a nucleoplasmic protein. The data support the idea that Trm1-II may utilize an INM targeting mechanism that shares aspects of the mechanism for targeting integral proteins to the INM.

Trm1-II-GFP is alternatively transported via classical pathway when inefficiently tethered

Our data indicate that the Trm1-II INM targeting mechanism does not strictly require that the protein access the nucleoplasm before being located at the INM and that alteration of the SPB appears not to affect access to the nuclear interior. One possible explanation for the nucleoplasmic location of Trm1-II-GFP in mutants defective for the NatC N-acetylation proteins and in mutated forms of Trm1-II reported in previous work[18,20] is that the protein is able to utilize exclusively the classical import pathway for soluble proteins when it is defective in binding to its tether. Therefore, we monitored the dynamics of a galactose-inducible version of Trm1-II-GFP containing a point mutation (Trm1-II(A147D)-GFP) in its INM targeting and/or tethering motif; this version of Trm1-II-GFP was previously shown to be nucleoplasmic in wt cells.[20] In wt cells and the SPB ts mutant *spc110–220*, the majority of Trm1-II(A147D)-GFP was located in the nucleoplasm (87% and 83%, respectively) (**Fig. 5A and B**). However, in a small number of cells some Trm1-II(A147D)-GFP accumulated at the ER or NE in addition to the nucleoplasmic pool that we attribute to some residual INM binding. One interpretation of the results is that if Trm1-II-(A147D)-GFP is unable to bind to its membrane tether, it utilizes the soluble import pathway to access the nuclear interior, and it is therefore not affected by the SPB alteration. This interpretation is in agreement with our data showing that the SPB defect does not affect nuclear targeting of soluble a protein, Pus1-GFP. The results also suggest that in normal conditions, a pool of Trm1-II-GFP may access the nucleoplasm by using the classical import pathway for soluble proteins, but that most of the

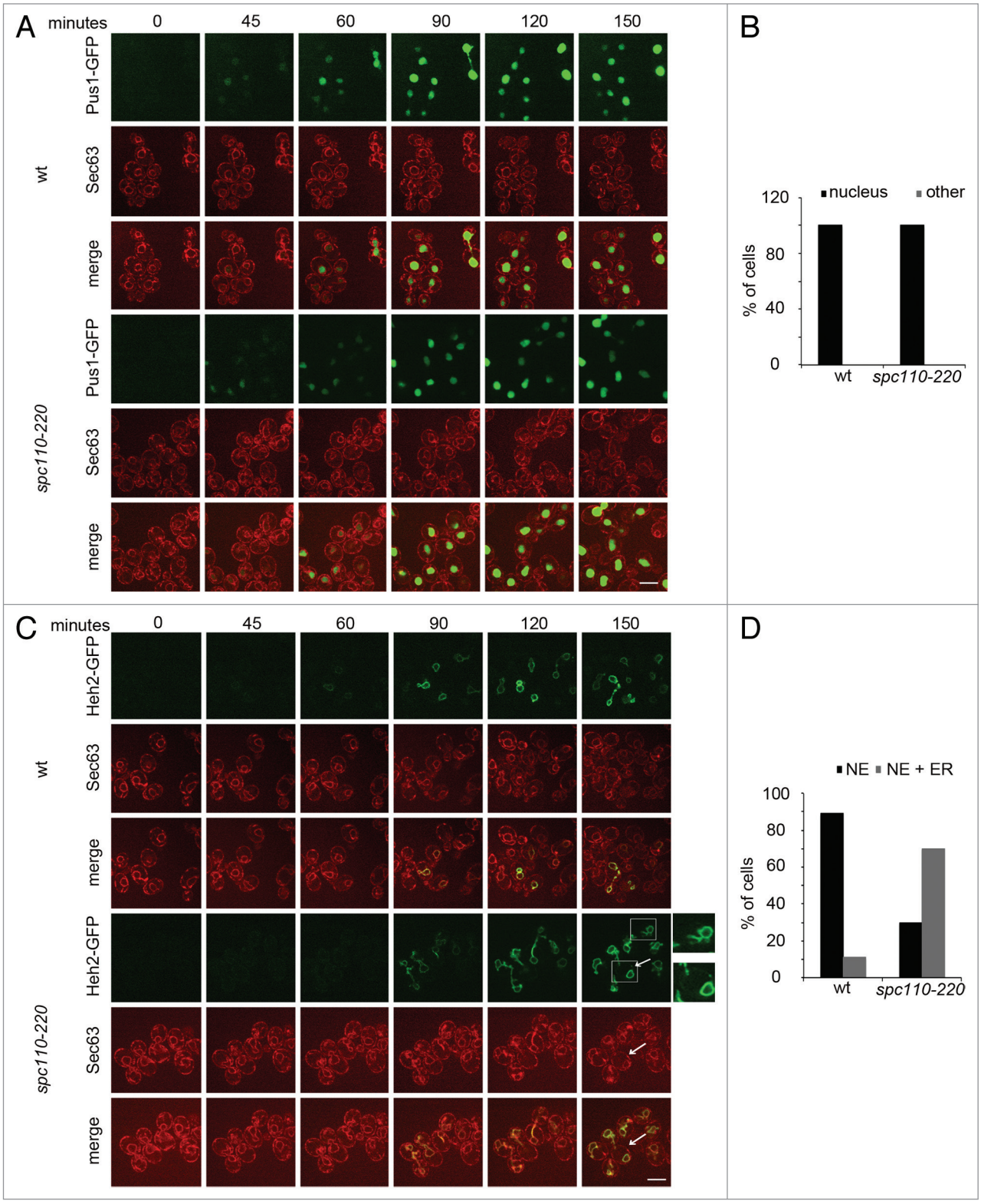

Figure 4. SPB mutation affects INM proteins, but not a soluble nucleoplasmic protein. Strategy as shown in **Figure 3**. (**A**) Galactose-inducible Pus1-GFP localizes to the nucleoplasm in both wt and *spc110–220*. (**B**) Percentage of cells showing Pus1-GFP nucleoplasmic localization. n = 256 (wt cells), 208 (ts cells). (**C**) Galactose-inducible Heh2-GFP shows typical ring localization in wt cells, whereas in the *spc110–220* mutant additional Heh2-GFP start to accumulate at the ER (arrows). Bar = 5 µm. (**D**) Percentage of cells with galactose-inducible Heh2-GFP at the NE vs. NE + ER. n = 310 (wt cells), 280 (ts cells).

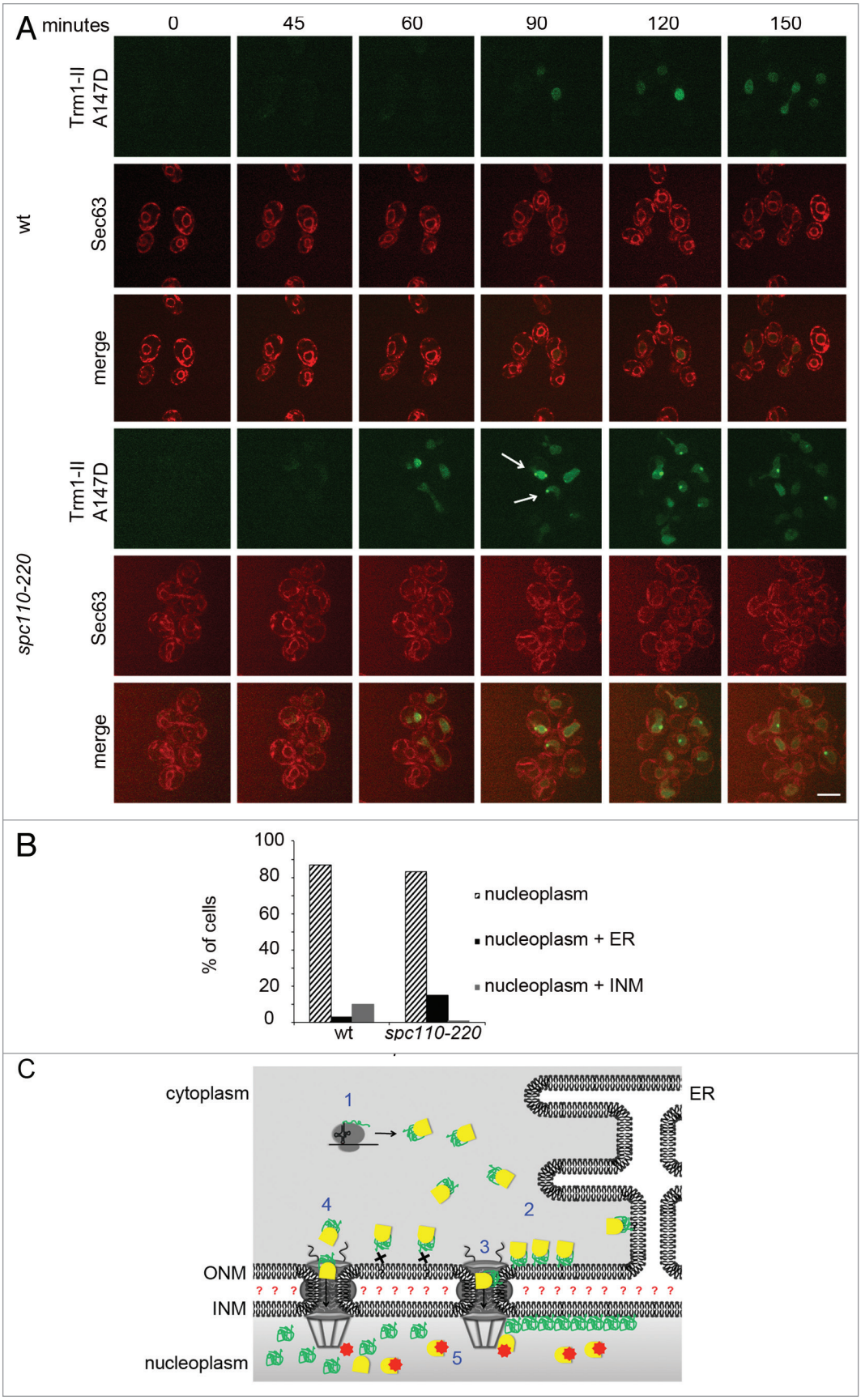

Figure 5. For figure legend, see page 361.

Figure 5 (See previous page). Trm1-II INM targeting and/or tethering employs features of both integral INM targeting and the classical pathway. (**A**) Galactose-inducible Trm1-II(A147D)-GFP localizes to the nucleoplasm in both wt and the ts mutant *spc110–220*. Strategy as shown in **Figure 3**. In both wt and SPB ts mutant, the majority of the mutant protein is located at the nucleoplasm. The nucleoplasmic pool was sometimes accompanied by a pool of protein at the cER or the NE (arrows). Bar = 5 μm. (**B**) Quantitation of phenotypes; n = 100 (wt cells), 164 (ts cells). (**C**) Right: Model for INM targeting for Trm1-II. (1) Trm1-II (green) is translated on polysomes. (2) in a Ran-dependent manner via the NLS (Karyopherin in yellow), Trm1-II is directed to the NE, where it is in transient contact with the ER/ONM. (3) translocation to the INM (4) Trm1-II follows an alternative import mechanism (when unable to tether), which follows the classical import pathway. (5) Trm1-II is released from the importin (GTP = red star) complex and evenly distributed around the INM by interaction with its unknown INM tether (?) or is nucleoplasmic (no tethering).

INM located protein is targeted and/or tethered after its initial interaction with ONM, prior to reaching its final destination (**Fig. 5C**).

Trm1-II maintenance at the INM is dependent on the SPB

Assessment of Trm1-II-GFP subcellular dynamics in SPB ts mutants and wt cells treated with α-factor showed that the INM targeting process is interrupted as a consequence of a SPB alteration. One interesting possibility is that the Trm1-II INM tether at the NE is limited or re-arranged in cells with altered SPB. If so, then Trm1-II-GFP located at the INM might relocate elsewhere upon SPB reorganization or disruption. To test this hypothesis, Trm1-II-GFP was induced in wt cells prior to arrest with α-factor (**Fig. 6A**). Then, location of Trm1-II-GFP was followed. Surprisingly, we observed that the Trm1-II-GFP forming an initial ring at the INM collapsed to a single region of the NE after 120 to 180 min of arrest with α-factor (240–300 min) (**Fig. 6B and C**). Calculations of the number of cells with spots (in additional cells after 3 h of arrest) showed an INM location for Trm1-II-GFP spots in the majority of the cells (93%). A low number (7%) had signal at the cER (**Fig. 6C**). The effect of the SPB in Trm1-II-GFP targeting and/or tethering is consistent with a possible role of the SPB in NE dynamics during the cell cycle. If so, then one should be able to observe Trm1-II-GFP change in location through multiple rounds of mitosis in wt cells. To address this possibility we observed wt cells first synchronized with α-factor and then released for extended times (**Fig. 7A**). The results showed that Trm1-II-GFP INM location is dynamic and once it is tethered to the INM it is subject to changes in distribution (**Fig. 7B**).

Discussion

A new mechanism for targeting and/or tethering peripherally-associated proteins to the INM

We previously proposed that Trm1-II first enters the nucleoplasm and then associates with the INM.[20] However, our data monitoring the movement of Trm1-II in wt cells, α-factor treated cells and cells with defects of the SPB do not support this idea. Rather, they support a modified mechanism by which Trm1-II-GFP first locates to the ER before "spreading." In agreement with this idea, in cells with altered SPB, galactose-inducible Trm1-II-GFP does not locate uniformly around the INM. Rather, in the majority of the cells, Trm1-II-GFP accumulates as one or a few spots mainly located to a discrete pore-less region of the NE that predominantly coincide with the ER-nucleus-junctions. Employing the microfluidics perfusion system for live cell imaging, we confirmed that even at permissive temperature, newly synthesized Trm1-II-GFP and Heh2-GFP did not achieve their normal INM location (but not quite in an identical manner) in *spc110–220* cells and cells treated with α-factor, while the location of the nucleoplasmic protein Pus1-GFP, was not affected. We also observed that the galactose-inducible Trm1-II-GFP spot was able to "spread" from a discrete region at the NE or from the ER-nucleus junctions throughout the entire INM. Our results indicate that the SPB affects INM targeting of Trm1-II-GFP only if the protein is capable to bind its membrane tether and that the protein lacking binding ability accesses the nuclear interior regardless of the SPB defect by using the soluble import pathway. The evidence suggests that the INM targeting mechanism for Trm1-II possesses similarities to the Ran-dependent targeting mechanism of an INM integral protein.[9] In support of this idea, both INM proteins Trm1-II-GFP (peripheral) and Heh2-GFP (integral), but not Pus1-GFP (nucleoplasmic), were affected by the SPB defect.

We propose a revised model of the mechanism for targeting of a peripheral protein to the INM (**Fig. 5C**): after translation (1), Trm1-II is directed to the NE in a Ran-dependent manner via the NLS, where it contacts its initial tether at the ER/ONM (2), similarly to the proposed mechanism for Heh1/Heh2 yeast proteins.[9] Translocation occurs by assistance of the importin complex while Trm1-II remains attached to the membrane (3). Alternatively, when Trm1-II is unable to bind its membrane tether, it follows the classical import pathway as the protein is already bound to the importin complex (4). After translocation, Trm1-II is released from the importin and is evenly distributed throughout the INM or is nucleoplasmic (5). We are investigating whether the INM tether is a protein or lipid.

Integral and peripheral INM proteins using the active transport machinery may share features of their targeting process. However, we do not propose that their mechanisms are identical as when Trm1-II targeting to the INM is prevented, it accumulates as a discrete spot(s)—but when Heh2 INM targeting is affected, it accumulates throughout the ER (in addition to the INM) in a characteristic pattern of ER proteins (this work and ref. 9). Different lipid requirements or preferences could explain why there is always a pool of Heh2 protein at the INM, regardless of the SPB defect. Future studies are needed to investigate how generalizable is this trafficking/targeting pathway for peripherally associated INM proteins.

The SPB role in INM targeting and/or tethering

Our work suggests a role for the SPB in nuclear organization during the cell cycle that also influences how a peripherally-associated protein, Trm1-II, is targeted to the INM. More specifically, it supports the idea of a possible role of the SPB in NE homeostasis that is not restricted to a particular SPB protein,

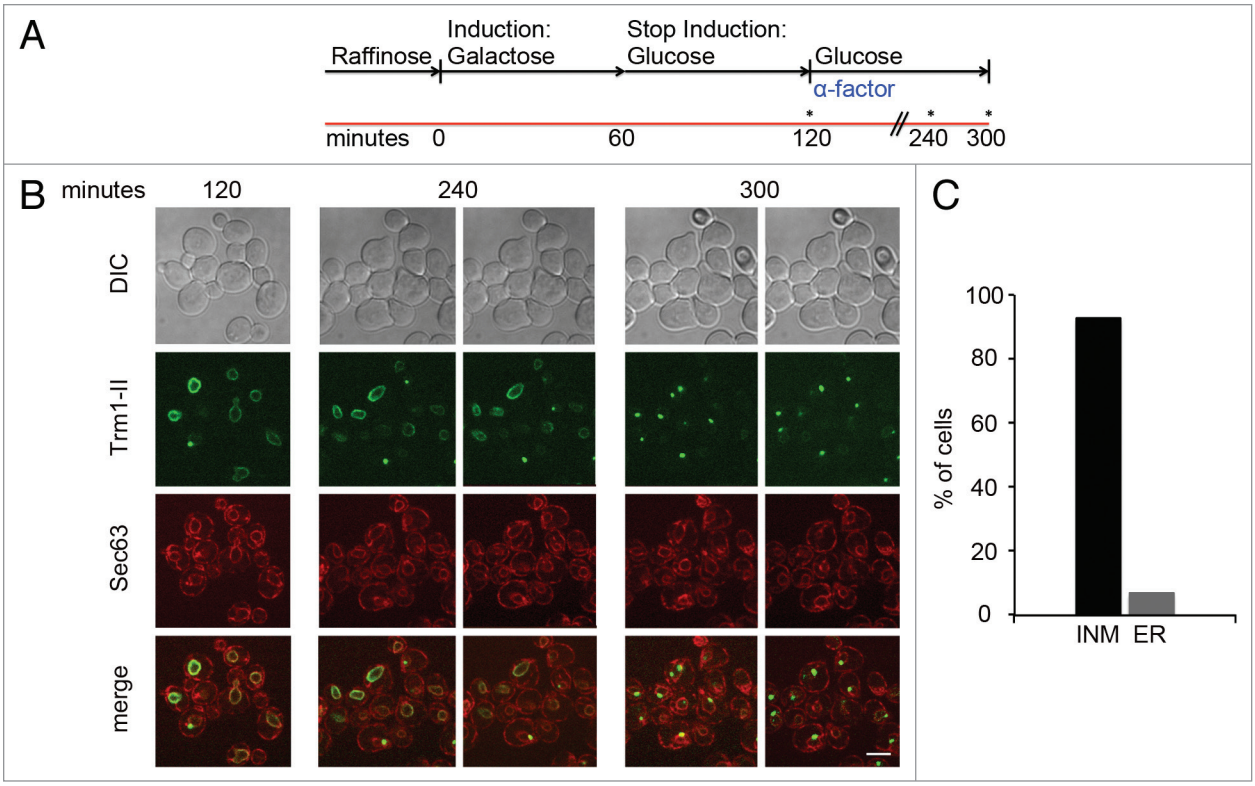

Figure 6. Galactose-inducible Trm1-II-GFP maintenance at the INM is affected in α-factor arrested cells. (**A**) Strategy for monitoring Trm1-II-GFP dynamics. The asterisks represent the time points of the images shown. (**B**) Most rings at the NE "collapse" to a single region of the INM after 300 min of arrest. Bar = 5 μm. (**C**) The majority of the spots after galactose-inducible Trm1-II-GFP "collapse" are located to the NE and not the ER. n = 205 cells.

but is a general effect that occurs when the SPB dynamics are compromised. This conclusion is supported by three lines of evidence that independently alter the SPB and affect Trm1-II INM location: (1) ts mutations of numerous independent SPB components, (2) depletion of several SPB gene products by tetracycline-regulation, and (3) treatment of cells with α-factor.

Finding that the SPB function could affect INM targeting was unanticipated. The SPB is the yeast equivalent of the centrosome. However, it is known that there are some components of the SPB that are also components of the NPC and the nuclear membrane where they play different roles.[37,38]

The SPB may directly or indirectly affect INM targeting and/or tethering. For a direct role, a component(s) of the SPB could interact with the Trm1-II membrane tether. Alternatively, SPB alterations may indirectly affect a cellular process that causes mislocation of INM proteins. One possibility that we tested is that nuclear import is affected in cells with an altered SPB but our data do not support this because galactose-inducible Pus1-GFP and the INM binding mutant Trm1-II(A147D)-GFP, were efficiently transported into nucleus of the SPB ts mutant and wt cells arrested with α-factor.

Another mechanism by which SPB defects might indirectly affect INM targeting and/or tethering is by affecting the cell cycle.[33,39] Indeed, previous work suggests a connection between cell cycle arrest and re-arrangement of the NE that results in formation of distinct NE domains, especially the membrane area that surrounds the nucleolus.[33,40] However, this possibility is unlikely for several reasons. First, the majority of the SPB proteins encoded in the ts collection affected galactose-inducible Trm1-II-GFP INM localization regardless of their role and the stage of the cell cycle where they function. Second, among the *cdc* mutants present in the ts collection (~40) only those encoding Cdc1, Cdc28 and Cdc48 proteins caused galactose-inducible Trm1-II-GFP INM mislocation. In addition to its role in the cell cycle, Cdc1 is a lipid phosphatase of the ER.[41] Cdc28 participates directly in SPB dynamics and lipid homeostasis,[28,29] and Cdc48 has multiple roles in the cell and its effect may also be indirect. Third, our co-localization analysis of galactose-inducible Trm1-II-GFP revealed that the protein accumulates at a discrete region that does not coincide with the nucleolus or its associated membrane in the majority of the *spc110–220* cells examined. Thus, Trm1-II-GFP mislocation is unlikely to be related to those changes in the membrane surrounding the nucleolus.[33,40] As we did not observed a direct relationship of the INM targeting defect of galactose-inducible Trm1-II-GFP with changes at the NE initiated by cell cycle delays, we propose that the phenotype observed in SPB defective cells is caused by alterations of the NE directly related to SPB dynamics.

In addition to targeting, INM tethering was affected as INM-located Trm1-II-GFP "collapses" to a discrete region of the NE after α-factor arrest. The effect of the SPB in INM protein "spreading" and maintenance suggests that it plays a role in INM targeting and/or tethering by influencing NE membrane dynamics. Perhaps, the SPB has a role in controlling

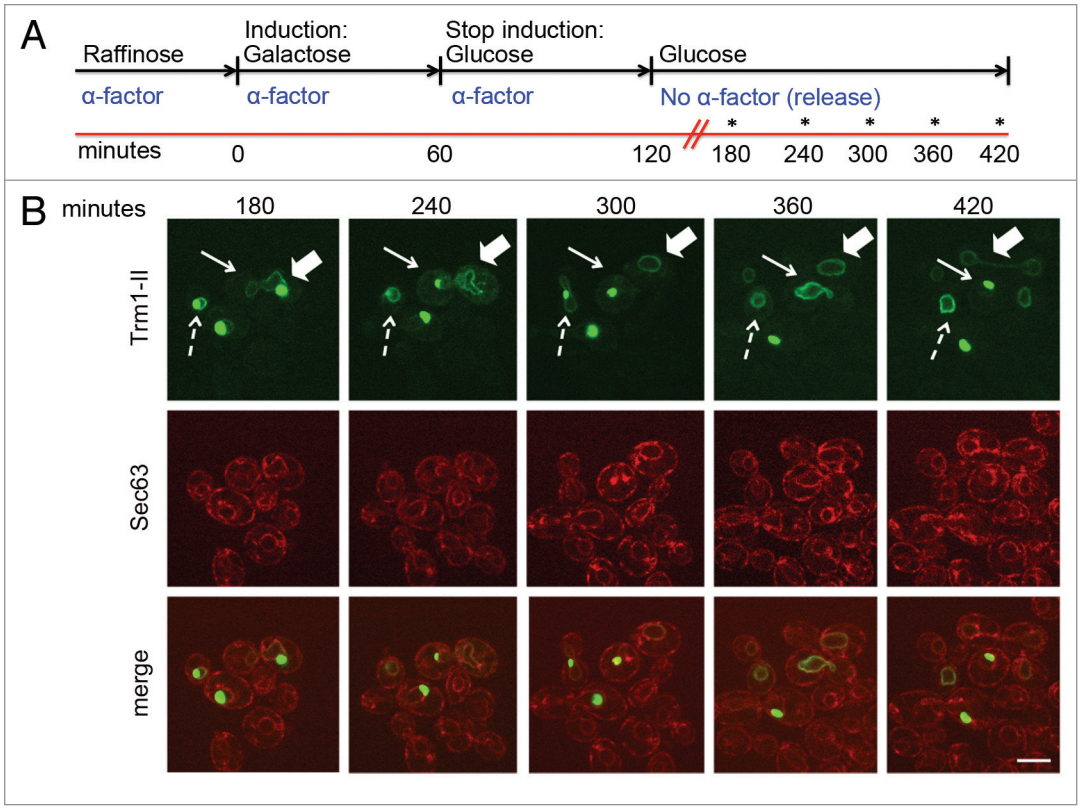

Figure 7. Dynamic spreading and collapse of Trm1-II-GFP during the cell division in wt cells. (**A**) Strategy for monitoring Trm1-II-GFP dynamics. The asterisks (*) represent the time points of the images shown. (**B**) Upon release from α-factor cell cycle inhibition, galactose-inducible Trm1-II-GFP changes in distribution at the INM. Dividing cells (narrow and wide arrows) exchange Trm1-II-GFP pools before division is completed. After division, in one cell Trm1-II-GFP (narrow arrow) remains as a spot at the NE at 300 min and is later distributed around the NE (360 min) when the cell initiates a new division cycle. At 420 min, the mother cell possesses a spot phenotype for Trm1-II-GFP. Another cell (dashed arrow) also demonstrates changes in galactose-inducible Trm1-II-GFP distribution at the INM during cell division. Bar = 5 μm.

lipid homeostasis in the NE as part of a normal coordination of events during cell division. Previous work indicates that the SPB protein Mps3 is involved in NE homeostasis in addition to its role in SPB duplication.[37,42] Although we found that two Mps3 ts mutations, *mps3–1*-and *mps3–7*, also affect Trm1-II-GFP location, our work suggests a broader role for the SPB in NE homeostasis. A role of the SPB in nuclear shape and size in *Schizosaccharomyces pombe* was also proposed.[34] Remarkably, the ER-nucleus junctions seem to play an important role for coordinated changes at the NE. Although there are important differences between the SPBs in *S. pombe* and *S. cerevisiae*, it is interesting to consider the possibility of a conserved role in NE homeostasis in budding yeast.

Further studies of the role of the SPB in membrane dynamics are warranted. Continued analysis of NE dynamics in cells with altered SPB will help elucidate the exact function of this structure. In addition, we were able to uncover that INM targeting and tethering of peripherally associated proteins might be more complex and may incorporate different factors and steps to achieve its location. More information is required to elucidate the targeting and/or tethering of Trm1-II (and other peripheral INM proteins) to the INM. Additional studies on Trm1-II INM tether and detailed characterization of its INM binding motif will help to better understand the dynamics of this peripheral protein and perhaps similar proteins.

Materials and Methods

Yeast strains and plasmids

The strain BY4741 (*MATa his3*Δ *leu2*Δ *met15*Δ *ura3*Δ) was used for most of the experiments. BY4741 is the parent strain for the yeast ts collection.[22] Yeast strains were maintained in YEPD media with or without G418 (0.2 mg/ml) or in synthetic defined media (SC) lacking the appropriate nutritional ingredients for selection. The strain for the Tet-Promoter Collection is R1158 (*URA3::CMV-tTA MATa his3–1 leu2–0 met15–0*); it is derived from BY4741.[30]

Plasmid pGP54a-Trm1-II-GFP,[20] encodes a galactose-inducible Trm1-II-GFP in pRS416. Plasmids pGP54a-Pus1-GFP and pGP54a-Heh2-GFP were generated by inserting the *PUS1* or *HEH2* open reading frames (ORF) into the polylinker region of pGP54a-GFP.[18] Plasmid pGP54a-Trm1-II-GFP-b, containing galactose-inducible Trm1-II-GFP (*LEU2* selection marker) was generated by restriction digestion and subsequent ligation of pGP54a-Trm1-II-GFP into pRS415. Scs2-MORF plasmid

contains a galactose-inducible MORF-tagged Scs2.[43] Plasmid pTPL7 encodes Trm1-II-GFP regulated by its endogenous promoter. To generate this plasmid, the *TRM1* promoter sequence and ORF were amplified by PCR to change the first ATG to ATC, so that the translation ensues only at the 2nd AUG and encodes only Trm1-II. The PCR products were digested with *SacI* and HindIII and then ligated into a *SacI* and HindIII digested pRS416 plasmid that contains a GFP and *TRM1* terminator sequence. Plasmid pFJ35, contains the C-terminus mCherry-tagged H2B protein regulated by the *ADH2* promoter.[44]

Standard techniques were utilized for yeast transformations and DNA manipulation in order to endogenously tag the SPB protein Spc97 and the ER protein Sec63 at the C-terminus with mCherry.

Genetic screen and cytological analyses

96-well plate yeast transformation and galactose induction: Transformation of pGP54a-TRM1-II-GFP into the yeast collection of ts mutants[22] was performed using a method based in the LiAc transformation protocol[45] and adapted for the 96-well plate format[18] with the exception that heat shock was performed using an Inca personal plate incubator (Mikura) at 45 °C for 15 min. Then, 10 μl of cells were transferred to solid selection media and incubated at 23 °C for approximately one week.

To determine the subcellular distribution of Trm1-II-GFP in the ts mutants, galactose to a final concentration of 2% was added to individual log phase cultures propagated at 23 °C in selective media with raffinose as the carbon source. After 30 min, the cultures were transferred to the NPT (37 °C) and were incubated for additional 1.5 h. To view the location of Trm1-II-GFP, live cells were placed on glass slides containing a 1.2% (in media) agarose pad and were maintained at 37–38 °C by the use of the microscope stage warmer (Nikon). The ts mutants for which Trm1-II-GFP showed an aberrant subcellular location were verified (two additional rounds). 740 yeast strains, representing about 99% (750 strains total) of the ts collection,[22] were examined in this way.

Further analyses of Trm1-II-GFP in wt and ts mutants was conducted on cells that were first fixed with formaldehyde, resuspended in DAPI solution and washed with water before epifluorescence microscopy on agarose-containing slides. To analyze the localization of galactose-inducible Trm1-II-GFP in wt cells treated with yeast pheromone, 2 μg/ml of α-factor (Sigma) and incubated with shaking at 23 °C until shmoos were formed. Then, Trm1-II-GFP was induced by addition of galactose and cells were collected after 2 h. To examine the location of endogenous levels of Trm1-II-GFP at the NPT, cells containing plasmid pTPL7 were incubated in an air shaker at 37 °C for 2 h. The location of galactose-inducible Trm1-II-GFP at PT and NPT was analyzed by addition of galactose for 1 h. Then, glucose was added to cells that were incubated an extra hour and collected.

For analysis of tetracycline-regulated SPB genes, individual yeast strains obtained from the Tet-Promoter Hughes Collection[30] were transformed with the plasmid pGP54a-Trm1-II-GFP-b. Individual transformants were grown to log phase in SC-LEU media with raffinose as the carbon source. A final concentration of 10 μg/ml of doxycycline was added to the culture and incubated an extra 16 h. Galactose was then added to the culture to a final concentration of 2%. Cells were visualized using fluorescence microscopy after 18, 36, and 42 h post-addition of doxycycline.

Co-localization experiments

Co-localization experiments were performed using wt and SPB ts mutants *spc110–220* or wt cells arrested with α-factor containing galactose-inducible Trm1-II-GFP. Log phase cultures on raffinose media were induced with 2% of galactose and incubated at 37 °C for 2 h then processed.

Microfluidics perfusion system for live cell imaging

We employed a microfluidics perfusion system (Cell ASIC, EMD Millipore) to study the intracellular dynamics of galactose-inducible Trm1-II-GFP, Pus1-GFP and Heh2-GFP Experiments were conducted at room temperature. At least two independent experiments for each protein were conducted. Changes in the media and incubation times were programmed using the ONIX™ FG Software. The pressure used in all the experiments was 2 psi. First, 50 μl of log phase cells were loaded into a Y04C plate. Immediately, SC media lacking uracil and containing 2% raffinose was flowed through the chamber while the cells were focused and selected for imaging. Then, the carbon source was switched to galactose for 60 min for protein induction. The carbon source was switched to glucose to prevent further induction until the end of the experiment. Images were collected at 0, 45, 60, 90, 120, and 150 min after galactose induction. Additional images of different cells were collected after 150 min.

Dynamics of galactose-inducible Trm1-II-GFP, Pus1-GFP, and Heh2-GFP in wt cells arrested with α-factor was monitored as described above, but with the following changes: raffinose media was switched to raffinose media containing 2 μg/ml of α-factor pheromone and media was flowed until the majority of the cells were arrested. Then, the carbon source was switched to galactose for 60 min using media containing α-factor. Galactose was switched to glucose for 60 min using media containing α-factor. To monitor Trm1-II-GFP after release from the α-factor arrest, the glucose media with α-factor was switched to glucose media without α-factor until the end of the experiment (210 or 420 min after induction).

To monitor INM maintenance of galactose-inducible Trm1-II-GFP after α-factor arrest, raffinose media was switched galactose media for 60 min. Next, galactose media was switched to glucose media for an additional 120 min. Finally, media was switched to glucose media containing α-factor pheromone until a large number of cells were arrested as assessed by acquisition of the shmoo shape.

Microscopy and imaging

All studies (except live imaging using the microfluidics perfusion system), were visualized using a Nikon 90i microscope (60× Plan Apo VC/1.4 NA objective lens) equipped with Differential Interference Contrast (DIC), fluorescein isothiocyanate (FITC), DAPI and Texas red filter sets. Imaging was performed using a CoolSNAP HQ2 (Photometrics) camera and either METAMORPH or Nis-Elements software. Monitoring of live cells in the microfluidics chamber was performed using a Nikon microscope equipped with a spinning disk confocal

apparatus (UltraView, PerkinElmer Life and Analytical Science). Cells were visualized using 488 nm (green) and 568 nm (red) argon ion lasers and a 100×/1.4 NA objective lens. Imaging was performed using a cooled charged coupled device camera (ORCA-AG) and UltraView ERS software (3.1). Image analyses of single optical 0.4 μm optical sections (11 through the cells) were performed using Image J (National Institute of Health). Adobe Photoshop was used for image assembly.

Protein analysis

Western Blot analysis was used to determine levels of galactose-inducible Trm1-II-GFP in wt, SPB ts mutant *spc110–220,* and wt cells arrested with α-factor. Briefly, cells were grown to OD600 = 0.3, then induced with 2% galactose, and incubated at 23 °C for 60 min.[46,47] Cells were collected by centrifugation and washed twice with water. Extracts were obtained and cells lysate were analyzed by SDS-PAGE (10% polyacrylamide gel) followed by western blotting. GFP-tagged protein detection employed primary antibody mouse anti-GFP (1:1000, Roche) and, anti-mouse horseradish peroxidase conjugated antibody (1:3000, Amersham). The loading control Pgk1 was detected using primary antibody rabbit anti-Pgk1 (1:3000) followed by secondary anti-rabbit horseradish peroxidase conjugated antibody (1:3000, Amersham).

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

We thank members of A. K. H. laboratory for their advice and Dr J-Q Wu and members of his laboratory for assistance with confocal microscopy and image analysis. We also thank Drs O Egriboz, JE Hopper, HO Parks, PK Herman, EM Phizicky and A Caplan for plasmids and reagents. Special thanks to Dr C Boone who kindly provided the ordered ts collection prior to publication. This work was supported by an NIH grant (GM27930) to A.K.H. a NIH Research Supplement to Promote Diversity in Health-Related Research to G.D., and the 2009–11 American Heart Association Fellowship (09PRE2080008) to T.P.L.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/nucleus/article/27973

References

1. English AR, Zurek N, Voeltz GK. Peripheral ER structure and function. Curr Opin Cell Biol 2009; 21:596-602; PMID:19447593; http://dx.doi.org/10.1016/j.ceb.2009.04.004
2. Siniossoglou S, Santos-Rosa H, Rappsilber J, Mann M, Hurt E. A novel complex of membrane proteins required for formation of a spherical nucleus. EMBO J 1998; 17:6449-64; PMID:9822591; http://dx.doi.org/10.1093/emboj/17.22.6449
3. Deng M, Hochstrasser M. Spatially regulated ubiquitin ligation by an ER/nuclear membrane ligase. Nature 2006; 443:827-31; PMID:17051211; http://dx.doi.org/10.1038/nature05170
4. Strambio-de-Castillia C, Blobel G, Rout MP. Proteins connecting the nuclear pore complex with the nuclear interior. J Cell Biol 1999; 144:839-55; PMID:10085285; http://dx.doi.org/10.1083/jcb.144.5.839
5. Tapley EC, Starr DA. Connecting the nucleus to the cytoskeleton by SUN-KASH bridges across the nuclear envelope. Curr Opin Cell Biol 2013; 25:57-62; PMID:23149102; http://dx.doi.org/10.1016/j.ceb.2012.10.014
6. Smith S, Blobel G. The first membrane spanning region of the lamin B receptor is sufficient for sorting to the inner nuclear membrane. J Cell Biol 1993; 120:631-7; PMID:8381121; http://dx.doi.org/10.1083/jcb.120.3.631
7. Soullam B, Worman HJ. Signals and structural features involved in integral membrane protein targeting to the inner nuclear membrane. J Cell Biol 1995; 130:15-27; PMID:7790369; http://dx.doi.org/10.1083/jcb.130.1.15
8. Ohba T, Schirmer EC, Nishimoto T, Gerace L. Energy- and temperature-dependent transport of integral proteins to the inner nuclear membrane via the nuclear pore. J Cell Biol 2004; 167:1051-62; PMID:15611332; http://dx.doi.org/10.1083/jcb.200409149
9. King MC, Lusk CP, Blobel G. Karyopherin-mediated import of integral inner nuclear membrane proteins. Nature 2006; 442:1003-7; PMID:16929305; http://dx.doi.org/10.1038/nature05075
10. Liu D, Wu X, Summers MD, Lee A, Ryan KJ, Braunagel SC. Truncated isoforms of Kap60 facilitate trafficking of Heh2 to the nuclear envelope. Traffic 2010; 11:1506-18; PMID:20846261; http://dx.doi.org/10.1111/j.1600-0854.2010.01119.x
11. Tapley EC, Ly N, Starr DA. Multiple mechanisms actively target the SUN protein UNC-84 to the inner nuclear membrane. Mol Biol Cell 2011; 22:1739-52; PMID:21411627; http://dx.doi.org/10.1091/mbc.E10-08-0733
12. Turgay Y, Ungricht R, Rothballer A, Kiss A, Csucs G, Horvath P, Kutay U. A classical NLS and the SUN domain contribute to the targeting of SUN2 to the inner nuclear membrane. EMBO J 2010; 29:2262-75; PMID:20551905; http://dx.doi.org/10.1038/emboj.2010.119
13. Gardner JM, Smoyer CJ, Stensrud ES, Alexander R, Gogol M, Wiegraebe W, Jaspersen SL. Targeting of the SUN protein Mps3 to the inner nuclear membrane by the histone variant H2A.Z. J Cell Biol 2011; 193:489-507; PMID:21518795; http://dx.doi.org/10.1083/jcb.201011017
14. Burns LT, Wente SR. Trafficking to uncharted territory of the nuclear envelope. Curr Opin Cell Biol 2012; 24:341-9; PMID:22326668; http://dx.doi.org/10.1016/j.ceb.2012.01.009
15. Nigg EA, Kitten GT, Vorburger K. Targeting lamin proteins to the nuclear envelope: the role of CaaX box modifications. Biochem Soc Trans 1992; 20:500-4; PMID:1397650
16. Ellis SR, Morales MJ, Li JM, Hopper AK, Martin NC. Isolation and characterization of the TRM1 locus, a gene essential for the N2,N2-dimethylguanosine modification of both mitochondrial and cytoplasmic tRNA in Saccharomyces cerevisiae. J Biol Chem 1986; 261:9703-9; PMID:2426253
17. Rose AM, Joyce PB, Hopper AK, Martin NC. Separate information required for nuclear and subnuclear localization: additional complexity in localizing an enzyme shared by mitochondria and nuclei. Mol Cell Biol 1992; 12:5652-8; PMID:1448094
18. Murthi A, Hopper AK. Genome-wide screen for inner nuclear membrane protein targeting in Saccharomyces cerevisiae: roles for N-acetylation and an integral membrane protein. Genetics 2005; 170:1553-60; PMID:15911569; http://dx.doi.org/10.1534/genetics.105.043620
19. Li JM, Hopper AK, Martin NC. N2,N2-dimethylguanosine-specific tRNA methyltransferase contains both nuclear and mitochondrial targeting signals in Saccharomyces cerevisiae. J Cell Biol 1989; 109:1411-9; PMID:2677019; http://dx.doi.org/10.1083/jcb.109.4.1411
20. Lai T-P, Stauffer KA, Murthi A, Shaheen HH, Peng G, Martin NC, Hopper AK. Mechanism and a peptide motif for targeting peripheral proteins to the yeast inner nuclear membrane. Traffic 2009; 10:1243-56; PMID:19602197; http://dx.doi.org/10.1111/j.1600-0854.2009.00956.x
21. Rose AM, Belford HG, Shen WC, Greer CL, Hopper AK, Martin NC. Location of N2,N2-dimethylguanosine-specific tRNA methyltransferase. Biochimie 1995; 77:45-53; PMID:7599275; http://dx.doi.org/10.1016/0300-9084(96)88103-X
22. Li Z, Vizeacoumar FJ, Bahr S, Li J, Warringer J, Vizeacoumar FS, Min R, Vandersluis B, Bellay J, Devit M, et al. Systematic exploration of essential yeast gene function with temperature-sensitive mutants. Nat Biotechnol 2011; 29:361-7; PMID:21441928; http://dx.doi.org/10.1038/nbt.1832
23. Jaspersen SL, Winey M. The budding yeast spindle pole body: structure, duplication, and function. Annu Rev Cell Dev Biol 2004; 20:1-28; PMID:15473833; http://dx.doi.org/10.1146/annurev.cellbio.20.022003.114106
24. Pereira G, Knop M, Schiebel E. Spc98p directs the yeast γ-tubulin complex into the nucleus and is subject to cell cycle-dependent phosphorylation on the nuclear side of the spindle pole body. Mol Biol Cell 1998; 9:775-93; PMID:9529377; http://dx.doi.org/10.1091/mbc.9.4.775
25. Castillo AR, Meehl JB, Morgan G, Schutz-Geschwender A, Winey M. The yeast protein kinase Mps1p is required for assembly of the integral spindle pole body component Spc42p. J Cell Biol 2002; 156:453-65; PMID:11827982; http://dx.doi.org/10.1083/jcb.200111025
26. Friedman DB, Kern JW, Huneycutt BJ, Vinh DBN, Crawford DK, Steiner E, Scheiltz D, Yates J 3rd, Resing KA, Ahn NG, et al. Yeast Mps1p phosphorylates the spindle pole component Spc110p in the N-terminal domain. J Biol Chem 2001; 276:17958-67; PMID:11278681; http://dx.doi.org/10.1074/jbc.M010461200

27. Schutz AR, Winey M. New alleles of the yeast MPS1 gene reveal multiple requirements in spindle pole body duplication. Mol Biol Cell 1998; 9:759-74; PMID:9529376; http://dx.doi.org/10.1091/mbc.9.4.759
28. Jaspersen SL, Huneycutt BJ, Giddings TH Jr., Resing KA, Ahn NG, Winey M. Cdc28/Cdk1 regulates spindle pole body duplication through phosphorylation of Spc42 and Mps1. Dev Cell 2004; 7:263-74; PMID:15296722; http://dx.doi.org/10.1016/j.devcel.2004.07.006
29. Huisman SM, Smeets MFMA, Segal M. Phosphorylation of Spc110p by Cdc28p-Clb5p kinase contributes to correct spindle morphogenesis in S. cerevisiae. J Cell Sci 2007; 120:435-46; PMID:17213332; http://dx.doi.org/10.1242/jcs.03342
30. Mnaimneh S, Davierwala AP, Haynes J, Moffat J, Peng W-T, Zhang W, Yang X, Pootoolal J, Chua G, Lopez A, et al. Exploration of essential gene functions via titratable promoter alleles. Cell 2004; 118:31-44; PMID:15242642; http://dx.doi.org/10.1016/j.cell.2004.06.013
31. Page BD, Snyder M. CIK1: a developmentally regulated spindle pole body-associated protein important for microtubule functions in Saccharomyces cerevisiae. Genes Dev 1992; 6:1414-29; PMID:1644287; http://dx.doi.org/10.1101/gad.6.8.1414
32. Yoder TJ, Pearson CG, Bloom K, Davis TN. The *Saccharomyces cerevisiae* spindle pole body is a dynamic structure. Mol Biol Cell 2003; 14:3494-505; PMID:12925780; http://dx.doi.org/10.1091/mbc.E02-10-0655
33. Stone EM, Heun P, Laroche T, Pillus L, Gasser SM. MAP kinase signaling induces nuclear reorganization in budding yeast. Curr Biol 2000; 10:373-82; PMID:10753745; http://dx.doi.org/10.1016/S0960-9822(00)00413-9
34. Gonzalez Y, Meerbrey K, Chong J, Torii Y, Padte NN, Sazer S. Nuclear shape, growth and integrity in the closed mitosis of fission yeast depend on the Ran-GTPase system, the spindle pole body and the endoplasmic reticulum. J Cell Sci 2009; 122:2464-72; PMID:19571115; http://dx.doi.org/10.1242/jcs.049999
35. Wang Y, Liu CL, Storey JD, Tibshirani RJ, Herschlag D, Brown PO. Precision and functional specificity in mRNA decay. Proc Natl Acad Sci U S A 2002; 99:5860-5; PMID:11972065; http://dx.doi.org/10.1073/pnas.092538799
36. Munchel SE, Shultzaberger RK, Takizawa N, Weis K. Dynamic profiling of mRNA turnover reveals gene-specific and system-wide regulation of mRNA decay. Mol Biol Cell 2011; 22:2787-95; PMID:21680716; http://dx.doi.org/10.1091/mbc.E11-01-0028
37. Friederichs JM, Ghosh S, Smoyer CJ, McCroskey S, Miller BD, Weaver KJ, Delventhal KM, Unruh J, Slaughter BD, Jaspersen SL. The SUN protein Mps3 is required for spindle pole body insertion into the nuclear membrane and nuclear envelope homeostasis. PLoS Genet 2011; 7:e1002365; PMID:22125491; http://dx.doi.org/10.1371/journal.pgen.1002365
38. Chial HJ, Rout MP, Giddings TH, Winey M. Saccharomyces cerevisiae Ndc1p is a shared component of nuclear pore complexes and spindle pole bodies. J Cell Biol 1998; 143:1789-800; PMID:9864355; http://dx.doi.org/10.1083/jcb.143.7.1789
39. Khalfan W, Ivanovska I, Rose MD. Functional interaction between the PKC1 pathway and CDC31 network of SPB duplication genes. Genetics 2000; 155:1543-59; PMID:10924456
40. Witkin KL, Chong Y, Shao S, Webster MT, Lahiri S, Walters AD, Lee B, Koh JLY, Prinz WA, Andrews BJ, et al. The budding yeast nuclear envelope adjacent to the nucleolus serves as a membrane sink during mitotic delay. Curr Biol 2012; 22:1128-33; PMID:22658600; http://dx.doi.org/10.1016/j.cub.2012.04.022
41. Losev E, Papanikou E, Rossanese OW, Glick BS. Cdc1p is an endoplasmic reticulum-localized putative lipid phosphatase that affects Golgi inheritance and actin polarization by activating Ca2+ signaling. Mol Cell Biol 2008; 28:3336-43; PMID:18332110; http://dx.doi.org/10.1128/MCB.00567-07
42. Friederichs JM, Gardner JM, Smoyer CJ, Whetstine CR, Gogol M, Slaughter BD, Jaspersen SL. Genetic analysis of Mps3 SUN domain mutants in Saccharomyces cerevisiae reveals an interaction with the SUN-like protein Slp1. G3 (Bethesda) 2012; 2:1703-18; PMID:23275891; http://dx.doi.org/10.1534/g3.112.004614
43. Gelperin DM, White MA, Wilkinson ML, Kon Y, Kung LA, Wise KJ, Lopez-Hoyo N, Jiang L, Piccirillo S, Yu H, et al. Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. Genes Dev 2005; 19:2816-26; PMID:16322557; http://dx.doi.org/10.1101/gad.1362105
44. Jiang F, Frey BR, Evans ML, Friel JC, Hopper JE. Gene activation by dissociation of an inhibitor from a transcriptional activation domain. Mol Cell Biol 2009; 29:5604-10; PMID:19651897; http://dx.doi.org/10.1128/MCB.00632-09
45. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. J Bacteriol 1983; 153:163-8; PMID:6336730
46. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979; 76:4350-4; PMID:388439; http://dx.doi.org/10.1073/pnas.76.9.4350
47. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 1981; 112:195-203; PMID:6266278; http://dx.doi.org/10.1016/0003-2697(81)90281-5