Brr6 and Brl1 locate to nuclear pore complex assembly sites to promote their biogenesis

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The paralogous Brr6 and Brl1 are conserved integral membrane proteins of the nuclear envelope (NE) with an unclear role in nuclear pore complex (NPC) biogenesis. Here, we analyzed double-degron mutants of Brr6/Br1 to understand this function. Depletion of Brr6 and Brl1 caused defects in NPC biogenesis, whereas the already assembled NPCs remained unaffected. This NPC biogenesis defect was not accompanied by a change in lipid composition. However, Brl1 interacted with Ndc1 and Nup188 by immunoprecipitation, and with transmembrane and outer and inner ring NPC components by split yellow fluorescent protein analysis, indicating a direct role in NPC biogenesis. Consistently, we found that Brr6 and Brl1 associated with a subpopulation of NPCs and emerging NPC assembly sites. Moreover, Brl1 overexpression affected NE morphology without a change in lipid composition and completely suppressed the nuclear pore biogenesis defect of nup116Δ and gle2Δ cells. We propose that Brr6 and Brl1 transiently associate with NPC assembly sites where they promote NPC biogenesis.

Introduction

The nuclear pore complex (NPC) is a large cylindrical structure with multiple copies of more than 30 different proteins named nucleoporins (NUPs; Beck and Hurt, 2017). The NPC is embedded in the nuclear envelope (NE) at sites of inner nuclear membrane (INM) and outer nuclear membrane (ONM) fusion. The NPC promotes the bidirectional nucleocytoplasmic transport of proteins and RNA through the central channel in the NPC lumen that contains NUPs with Phe- and Gly-rich repeats (FG-NUPs; Radu et al., 1995; Strawn et al., 2004; Alber et al., 2007; Wente and Rout, 2010; Ebauer et al., 2015). Other NUPs have a structural role or embed the NPC into the NE. Some of the outer and inner ring complex components bind to the transmembrane (TM) protein Ndc1 (Gerace et al., 1982; Hallberg et al., 1993; Wozniak et al., 1994; Miao et al., 2006; Stavru et al., 2006). Interestingly, yeast Ndc1 has an additional role in inserting the spindle pole body (SPB), the functional equivalent of the human centrosome, into the NE (Winey et al., 1993; Chial et al., 1998). NE embedding of the SPB is a consequence of the closed mitosis in yeast Saccharomyces cerevisiae. It allows the SPB to organize nuclear and cytoplasmic microtubules, with functions in chromosome segregation and spindle positioning, respectively.

Higher eukaryotes have two pathways to assemble NPCs. One pathway assembles NPCs in telophase upon chromat binding of the NUP ELYS/MEL28 (Loïodice et al., 2004; Gillespie et al., 2007; Hetzer and Wente, 2009; Doucet et al., 2010). In contrast, the interphase pathway promotes NPC biogenesis in the intact double membrane of the NE and is independent of ELYS/MEL28, indicating it is mechanistically distinct from the telophase NPC biogenesis (Rexach, 2009; Funakoshi et al., 2011; Vollmer et al., 2015). Recent EM data in human cells visualized membrane intermediates in interphase NPC biogenesis (Otsuka et al., 2016). Interphase NPC assembly follows, at least partially, an inside-out (nuclear to cytoplasm) extrusion of the NE that eventually leads to fusion of the double membrane. Because of the closed mitosis, S. cerevisiae assembles NPCs exclusively by the “interphase” pathway (Winey et al., 1997; Khmelinskii et al., 2010).

The paralogous BRR6 and BRL1 code for two essential integral membrane proteins of the NE in S. cerevisiae, with functions in NPC biogenesis or stability. Brr6, Brl1, and the nonessential Apq12, also an integral membrane protein of the NE, were found to interact with each other by immunoprecipitation (IP; Lone et al., 2015). Interestingly, deformations of the INM, so-called herniations, were observed in conditional lethal brr6 and brl1 cells (de Bruyn Kops and Guthrie, 2001; Hodge et al., 2010). Herniations are also a phenotype of yeast NPC mutants such as nup116Δ cells (Wente and Blobel, 1993). Recently, it was discovered that GLFG repeats in Nup116 stabilize critical interactions with scaffold NUPs during interphase NPC biogenesis. Failure of these interactions, as in nup116ΔGLFG P Một-NUP188 cells, results in the formation of herniations...
of Cu²⁺ on yeast extract, peptone, and glucose (YPD) plates as

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\text{BRR6 BRL1 PGAL1-UBR1}
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cells with assembly defects

Results

teracted with a range of NUPs, and overexpression was NPC biogenesis on the bend of the INM. In addition, Brl1 in- double-degron cells, SPB duplication was only mildly affected. BRR6/BRL1 double-depletion of both proteins to analyze phenotypes. Double depletion of both proteins allows NPC biogenesis. Fission yeast Brr6 (Schizosaccharomyces pombe lacks BRL1) functions in the insertion of the SPB into the NE, indicating membrane-modulating activity of this protein (Tamm et al., 2011). Whether S. pombe Brr6 has an additional role in NPC biogenesis has not been investigated.

Here, we analyzed the functions of Brr6 and Brl1 in S. cerevisiae. Because of their paralogous relationship, we constructed conditional lethal BRR6/BRL1 double-degron mutants to analyze phenotypes. Double depletion of both proteins rapidly affected NPC biogenesis without impairing already assembled NPCs or changing lipid composition. In BRR6/BRL1 double-degron cells, SPB duplication was only mildly affected. The SPB phenotype arose later than the NPC biogenesis de- fect. Brr6 and Brl1 associated with assembly intermediates of NPC biogenesis on the bend of the INM. In addition, Brl1 interacted with a range of NUPs, and BRL1 overexpression was able to bypass the scaffolding function of Nup116 and over- come the NPC biogenesis defect of gle2Δ cells. We propose that Brr6 and Brl1 transiently bind to NPC assembly sites to mediate NPC biogenesis.

Codepletion of Brr6 and Brl1 causes NPC assembly defects

Brr6 and Brl1 are interacting paralogues that may have overlapping functions (Schneider and Cole, 2010). To analyze the full impact of the loss of both gene products, we combined brr6(ts) and brl1(ts) alleles. However, double mutant cells showed a synthetically lethal phenotype (Fig. S1 A). We therefore combined the temperature-inducible degrons (td) td-brr6 and td-brl1 that were under control of the Cu²⁺-inducible PCEPI promoter. Single- or double-degron td-brr6 and td-brl1 cells with P\text{GAL1-UBR1} grew at 23°C or 37°C in the presence of Cu²⁺ on yeast extract, peptone, and glucose (YPD) plates as BRR6 BRL1 P\text{GAL1-UBR1} WT (named BRR6 BRL1 WT) cells but were unable to grow on YPGR plates without Cu²⁺ at 37°C (Fig. 1 A). Galactose-induced expression of the E3 ligase P\text{GAL1-UBR1} promotes degradation of the degron-tagged protein by the proteasome (Kanemaki et al., 2003). Consistently, Brr6 and Brl1 were rapidly degraded upon shifting cells to 37°C in the presence of galactose (Fig. S1 B). The temperature-dependent growth defect was complemented by the corresponding trans-genes (Fig. S1 C).

It has been reported that conditional lethal brr6(ts) or brl1(ts) mutant cells display mislocalization of cytoplasmic NUPs (Hodge et al., 2010). To evaluate whether this phenotype was also observed in the td-brr6 td-brl1 cells and whether mislocalization was a general phenotype for all NUP subgroups, we examined the cellular localization of NUPs after 3 h at 37°C in YPGR medium (Figs. 1 B and S1 D). All NUPs showed a uniform localization along the NE in BRR6 BRL1 WT cells. In contrast, the majority of td-brr6 td-brl1 cells displayed NPC defects upon degradation of Brr6 and Brl1 (Fig. 1, B and C; and Fig. S1, D and E). The signal of Nup82-yeast-enhanced GFP (Nup82-yeGFP) along the NE was reduced in td-brr6 td-brl1 cells compared with WT control. This was also the case for Nup85, a member of the Y-shape complex. In addition, ~40% of td-brr6 td-brl1 cells showed Nup85-yeGFP–positive dots in the cytoplasm (Fig. 1 B, arrowhead) and clustering into several dots on the NE (Fig. 1, B and C). Similar defects were observed for nearly all other NUPs, independently of their localization and function (Fig. 1, B and C; and Fig. S1, D and E). The INM protein Heh2 also showed similar mislocalization (Fig. 1, B and C). Heh2 is a member of the Lap2-emerin-MAN1 family of integral INM proteins, which binds to an early NPC assembly intermediate but not to mature NPCs (Webster et al., 2014). In contrast, we observed only minor localization defects upon Brr6/Brl1 depletion for Heh1 (Fig. S1, D and E), which functions in NPC repair (Webster et al., 2016). No defects were detectable for the ER markers Sec63-yeGFP and Ole1-yeGFP, which are not associated with NPCs (Fig. S1, F and G; Stukey et al., 1990; Delic et al., 2013). Collectively, these data show that the Brr6 and Brl1 proteins are important for proper localization of most NUPs.

We next performed EM to examine the ultrastructure of NPCs in td-brr6 td-brl1 cells. Depletion of Brr6 and Brl1 caused NE abnormalities (Fig. 1 D). In particular, the NE showed many electron-dense inclusions beneath the INM, so-called herniations (Wente and Blobel, 1993), and sheet structures inside the nucleus. Immunostaining indicated that INM inclusions and sheets contained the FG-NUP Nsp1. The Nsp1 label was at the nuclear bottom region of the herniations (Fig. 1 D). In addition, the anti-Nsp1 antibody labeled morphologically proper NPCs in td-brr6 td-brl1 cells (Fig. 1 D, asterisk). Thus, double deple- tion of Brr6 and Brl1 affects NPCs.

To evaluate whether the mislocalization of NUPs in td-brr6 td-brl1 cells was the result of a general NPC destabilization or a defect in new NPC assembly, we compared Nup188-mCherry–marked NPCs that assembled in the presence of Brr6/Brl1 activity with Nup188-yeGFP that was synthesized after Brr6/Brl1 depletion. This experiment was done with the recombination-induced tag exchange (RITE) system (Terweij et al., 2013) that allows rapid switching of tags via Cre-induced recombination from NUP188-mCherry to NUP188-yeGFP (Figs. 1 E and S1, H–J). We inactivated Brr6/Brl1 by shifting td-brr6 td-brl1 cells to 37°C for 1 h. This short time at 37°C was chosen to observe the impact of Brr6/Brl1 inactivation on newly assembled NPCs. With the temperature shift, Cre recombinase was activated by the addition of estradiol. The efficiency of mCherry-to-yeGFP switching during 1 h of Cre induction was ~50–60% (Fig. S1 H). In td-brr6 td-brl1 cells, Nup188-mCherry signal, representing the assembled NPCs, remained equally distributed over the NE during the time-lapse experiment. In contrast, the newly synthesized Nup188-yeGFP accumulated as a dot on the NE (Figs. 1 E and S1 I). This phenotype was observed in ~50% of cells with Cre-induced recombination (Fig. S1 H). In the other 50% of cells, Brr6/Brl1 were probably insufficiently depleted after 1 h at 37°C to cause NPC defects. In a control experiment, we established that BRR6 BRL1 WT cells showed equal distribution of both Nup188-mCherry and Nup188-yeGFP, when Cre recombinase was switched on (Fig. S1, H and J). These data suggest that Brr6 and Brl1 are required for the biogenesis of new NPCs but not for maintenance of already assembled NPCs.
Figure 1. **Loss of Brr6 and Brl1 cause NPC assembly defects.** (A) Serial-dilution growth assay of td-brr6 td-brl1 cells. (B) Images of living cells incubated at 37°C for 3 h. The yeGFP signal along the NE was scanned (enlargement right, yellow circle) for the distribution of the NUPs (graph, bottom). Arrowheads indicate GFP-dots in the cytoplasm. A cartoon of NUPs with GFP-fusions used in B and in Fig. S1 D is shown. Bars: [overview] 5 µm; [enlargements] 1 µm. (C) Percentage of cells with mislocalization of GFP-fusion proteins. Error bars: SD (n > 180); three independent experiments. (D) TEM micrographs of BRR6 BR11 WT and td-brr6 td-brl1 cells incubated for 3 h at 37°C. Anti-Nsp1 staining at normal NPCs (asterisk), herniations (arrowheads), and sheets. N, nucleus; C, cytoplasm. Bars, 100 nm. (E) RITE assay. Estradiol induces a genetic switch of NUP188-mCherry (old) to NUP188-yeGFP (new). td-brr6 td-brl1 cells were imaged at 10-min intervals after addition of estradiol at 37°C. Fig. S1 I shows an additional td-brr6 td-brl1 cell. Bar, 1 µm.
Because of the SPB duplication defect of brr6(ts) cells in S. pombe (Tamm et al., 2011), we asked whether SPB duplication is also affected in td-brr6 td-brl1 cells. Interestingly, mild SPB duplication defects were observed after 4 h at the restrictive condition (Fig. S2, A–E) when the NPC biogenesis defect was already strongly apparent (Fig. 1, B and C; and Fig. S1, D and E). In addition, yeGFP-Brl1 and yeGFP-Brr6 did not accumulate with SPBs during any cell cycle phase, including the time of the insertion of the new SPB into the NE (Fig. S2 F), as is the case in S. pombe (Tamm et al., 2011). Thus, because SPB defects occurred after NPC defects and because of the lack of Br6/Brl1 enrichment at SPBs, the SPB defect in td-brr6 td-brl1 cells may be an indirect consequence of the NPC biogenesis failure. Indeed, we have recently shown that NPCs are important for SPB duplication (Rüthnick et al., 2017).

Depletion or overexpression of Brr6 and Brl1 does not affect cellular lipid composition

Previously, it has been described that conditional lethal brr6(ts) cells change ergosterol and neutral lipid composition when incubated for 6 h at 37°C (Hodge et al., 2010). brr6(ts) and brl1(ts) cells accumulated high levels of monounsaturated and shorter-chain fatty acids after 16 h at 16°C (Lone et al., 2015). This led to the suggestion that both proteins promote NPC biogenesis by regulating lipid homeostasis. To test this model, we analyzed lipid profiles after codepletion of Br6 and Br11 over time and simultaneously followed the appearance of NPC defects by fluorescence microscopy. Although NPC defects were clearly detectable after 3 h at 37°C as indicated by Nup188-yeGFP clustering (Fig. 2 A), the lipid composition of BRR6 BRL1 WT and td-brr6 td-brl1 cells was very similar. The lipid species, the functional lipid subgroups, the chain length, and the number of double bonds were nearly identical in BRR6 BRL1 WT and td-brr6 td-brl1 cells (Fig. 2, B–E). Over the 6-h time course, the tendency of lipid changes was similar for both strains (Fig. 2 F). These data suggest that depletion of Br6/Brl1 affects NPC biogenesis without changing lipid composition.

Recently, it was shown that an additional copy of BRL1 was able to rescue cell viability of nup116Δ or nup57Δ cells (Liu et al., 2015). This rescue was attributed to a change in the lipid composition induced by elevated BRL1, because BA, which modulates membrane fluidity, caused decreased cell viability of nup57Δ cells with an extra copy of BRL1. In our nup116Δ strain background, an additional copy of BRL1 did not allow growth of cells at 37°C (Fig. 3 A). However, expression of Pgal1-yeGFP-BRL1 or PAdh1-yeGFP-BRL1, but not Pgal1-yeGFP Brr6 or PAdh1-yeGFP BRR6, was able to suppress the growth defect of nup116Δ cells at 37°C (Fig. 3 A and see Fig. 7, A and B). Mass spectrometric analysis measurements did not detect changes in lipid profiles in WT cells upon Pgal1-yeGFP BRR6 or Pgal1-yeGFP BRL1 induction after 6 h at 30°C (Fig. 3, B–E), with the exception of a very mild increase in the chain length of certain triacylglyceride (TAG) subclasses in the case of BRL1 overexpression (Fig. 3 D, asterisks). These data support the notion that Br6 and Br11 promote NPC biogenesis without affecting overall lipid composition.

Reduced growth on BA has been used as an argument for a change in lipid composition in brr6(ts) and brl1(ts) cells. To evaluate the broadness of this phenotype, we tested conditional lethal brr6(ts) and brl1(ts) mutant cells together with NPC and SPB mutants for growth on plates with BA. Fig. 3 F shows that only a subset of BRL1 alleles (brl1-56 at 23°C–33°C and brl1-58 at 30°C and 33°C) grow more poorly in the presence of BA in comparison to the cells on the control plate. In contrast, BA did not enhance the growth defect of other mutant cells (br6-19, br6-732, or br6-751). BA even promoted growth of brr6-5001 cells at 33°C. No matter whether BA affected the growth or not, brr6(ts) and brl1(ts) alleles cells showed NPC defects (Fig. 3 G). Reduced growth on BA was also observed for mps2-1, mps2-42, mps2-2, nbl1-2, nbl1-3, pom33Δ, and nup133Δ cells that are defective in the insertion of the new SPB into the NE (mps2(ts), nbl1(ts), and nbl1(ts) or NPC biogenesis (pom33Δ and nup133Δ), but do not have a direct impact on lipid biosynthesis.

Partial NPC localization of Brr6 and Brl1

To better understand the function of Br6 and Br11 in NPC biogenesis, we analyzed the localization and topology of both proteins. Br6 and Br11 have been reported to localize to the NE and ER using plasmid-encoded tagged versions of the genes that were expressed in parallel to the untagged WT gene copy (de Bruyn Kops and Guthrie, 2001; Saitoh et al., 2005). To confirm this localization, we fused BRR6 and BRL1 to yeGFP and moderately overexpressed the gene fusions from the PAdh1 promoter (Fig. 4 A). Br6 and Br11 localized at both the NE and the peripheral ER independently of the position of the yeGFP tag (Fig. 4 A). The intensity of yeGFP-Brr6 and Brr6-yGFP at the cell cortex was stronger than that at the NE. This ratio was reversed for yeGFP-Brl1 and Brl1-yeGFP (Fig. 4 A). The uniform NE localization of yeGFP-Brl1 and yeGFP-Brr6 was disturbed in apq12Δ cells grown at the restrictive temperature. Both proteins showed a punctate pattern in apq12Δ cells (Fig. S3 A, arrowheads). Thus, Br6 and Br11 are uniformly distributed along the NE, in dependence on Apq12.

Tagging of BRR6 and BRL1 at the 3′ end by yeGFP without affecting the promoter confirmed localization of both proteins at the nuclear rim that was marked by Nup188-tdTomato (Fig. 4 B). Scanning of the fluorescence intensities along the NE indicated a fraction of overlapping peak intensities between Brr6/Brl1 and Nup188 (Fig. 4 B, arrowheads). This may indicate localization of Br6/Brl1 to a subpopulation of NPCs. To test this further, we performed immuno-EM using anti-GFP antibodies followed by protein A–gold (Fig. 4, C–E). The membrane NUP yeGFP-Pom34 was used as control for a NE-associated protein that localizes with NPCs. Asi3-yeGFP, a protein of the INM with a function in quality control (Khmelinskii et al., 2014), is at the NE but not at NPCs. Incubation of the yeGFP-tagged cells with only protein A–gold did not result in a NE or NPC signal. In PAdh1-yeGFP-POM34 cells, gold particles reflecting the localization of yeGFP-Pom34 were at the NE and NPCs. For yeGFP-Pom34, ~40% of the gold particles at the NE associated with NPCs (Fig. 4, C and D). In contrast, only 10% of the Asi3-yeGFP signal along the NE overlapped with NPCs. The Asi3-yeGFP signal at NPCs was similar to the NPC occupancy of the NE (Fig. 4 D) and therefore probably reflects Asi3 molecules that were coincidently close to NPCs at the time of fixation. Interestingly, 20–25% of the NE-associated Br6 and Br11 signals were at NPCs. This number was lower than that for Pom34 but clearly higher than the 10% value for Asi3. We therefore conclude that a fraction of Br6 and Br11 associates with NPCs.

We next used the immuno-EM data from Fig. 4 C to quantify the INM and ONM distribution of Br6 and Br11. The Asi3 signal was predominantly associated with the INM, consistent with a model in which Asi3 is a NE-associated protein that localizes with NPCs.
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with published data (Zargari et al., 2007; Fig. 4, C and E). yeGFP-Brr6 and Brr6-yeGFP were equally distributed on both sides of the NE, whereas yeGFP-Brl1 mainly localized to the INM (Fig. 4, C and E). Thus, Brr6 and Brl1 associate with the INM; Brr6 shows additional localization at the ONM.

Membrane topology of Brr6 and Brl1

Brr6 and Brl1 are integral membrane proteins, although their topology is a matter of debate (de Bruyn Kops and Guthrie, 2001; Kim et al., 2006). To elucidate the topology of Brr6 and Brl1, we used the bifunctional complementation (BiFC) assay (Hu et al., 2002). In this assay, C- and N-terminal domains of Venus (VC and VN) that are nonfluorescent are expressed as gene fusions. As soon as VC and VN come close together, because either two fusion proteins interact or N and C termini of an integral membrane protein are on the same side of the membrane, VC and VN interact and the YFP is restored. Following this strategy, VN-BRL1-VC fusions were expressed as the only chromosomal copy. The VN-Brl1-VC signal was detectable at the NE (Fig. 4 F). In contrast, the integral membrane protein VN-Pom152-VC, which only has one membrane-spanning domain (Tcheperegine et al., 1999), did not show a YFP signal (Fig. 4 F). This indicates that N and C termini of Brl1 are located on the same side of the NE. In contrast to VN-BRL1-VC cells, tagging of BRR6 on both sides (VN-BRR6-VC) strongly affected viability of cells. We therefore combined VC-BRR6 with BRL1-VN and BRR6-VN with VC-BRL1 (Fig. S3 B). Both combinations resulted in YFP-positive cells, suggesting that N and C termini of Brr6 are adjacent to the N and C termini of Brl1.

Figure 2. Codepletion of Brr6 and Brl1 does not affect lipid composition. (A) Cells were incubated at 37°C as in Fig. 1 B and analyzed 0, 3, and 6 h after temperature shift with Nup188-yeGFP as NPC marker. Images with maximum-intensity projections are shown. Bar, 5 µm. (B–F) Lipids were extracted from cell cultures used for A with subsequent mass spectrometric analysis by nano-ESI-MS/MS. Samples in B–E were analyzed after 3 h at 37°C. (B) Lipid classes displayed as mol% of measured lipids. (C) Distribution of lipids into functional categories of glycerophospholipids and glycerolipids (GPL), sphingolipids (SP), sterols (ST), and storage lipids. (D) Chain length profiles. (E) Double bond profiles. (F) Changes in the mol% distributions of lipid classes during the time course experiment. Error bars in B–F: SD (n = 3).
Previous data suggest that the C termini of Brr6 and Brl1 are exposed to either the cytoplasm or nucleoplasm (Smoyer et al., 2016). Confirmation of this conclusion came from histidine-biotin-histidine (HBH)-tagged Brr6. The HBH tag of HBH-Brl1, HBH-Brr6, and Brr6-HBH became biotinylated, as indicated by streptavidin detection (Fig. 4 G). Because the biotin modification system is not localized in the nuclear intermembrane space (Huh et al., 2003; Emerman et al., 2010), this result confirms that the N and C termini of Brr6 and the N terminus of Brl1 are localized in either the nucleoplasm or cytoplasm.

Disulfide bonds are important for localization and stability of Brl1
Four conserved cysteine residues are found in all Brr6/Brl1 orthologues (Fig. 4 H). Importantly, Brl1 contains two additional cysteine residues in its second TM domain (Fig. 4 H; TM2). Because the nuclear intermembrane space has an oxidative...
Brr6 and Brl1 localization and the role of disulfide bonds. (A) Localization of yeGFP-tagged Brr6 and Brl1 expressed under control of P\textsubscript{ADH1}. Plot profiles along the yellow lines indicate the distribution of yeGFP signals on the NE and the cortical ER. Bar, 5 µm. (B) Strains expressing NUP188-tdTomato in combination with BRR6-yeGFP, BRL1-yeGFP, or NUP85-yeGFP under the endogenous promoter. The enlargements (top left) show NEs that were used for the plot profiles (right). Black arrowheads in the graph indicate colocalization of Brr6 or Brl1 with Nup188 on the NE. Bars: (overview) 5 µm; (overview and enlargements) 500 nm.
environment (Braakman et al., 1991; Frand et al., 2000), we determined the redox state of the cysteine residues in BrR6 and BrR1 to judge whether they localize in the intermediate space or in the nucleus/cytoplasm.

Analysis of yeGFP-BrR6 and BrR1 by SDS-PAGE under oxidative and reducing conditions excluded the possibility of intermolecular interactions via disulfide bridges (Fig. S3 C), as we failed to detect dimeric or multimeric GFP-BrR6 or BrR1 species in the absence of the reducing agent DTT. Incubation of protein extracts with 24-methyl-polyethylene glycol-maleimide (mmPEG24), which alkylates free cysteine residues and causes an upshift of the protein in SDS-PAGE upon modification, did not affect running behavior of the HBH-tagged version of BrR6 (HBH tag does not contain cysteine; Fig. 4 H, lane 1 and 3). mmPEG24 triggered only a small upshift of BrR1 (Fig. 4 H, lanes 1 and 3). This indicates that most cysteine residues in both proteins were oxidized. To confirm this notion, we preincubated the cell extract with Tris-2-carboxyethyl-phosphine (TCEP) to reduce all cysteine residues followed by mmPEG24 incubation. This scheme resulted in four mmPEG24-modified HBH-BrR6 bands. BrR1 was strongly upshifted into one band (Fig. 4 H, lane 2). This result further indicates that cysteine residues in BrR6 and BrR1 are predominately oxidized in vivo.

To exclude that cysteine residues became oxidized during extract preparation, intact cells were first incubated with the membrane-permeable N-ethylmaleimide (NEM) to block all free cysteines. Cell extracts were then treated with TCEP and mmPEG24. HBH-BrR6 behaved as without NEM incubation (Fig. 4 H, compare lanes 2 and 4), indicating that all cysteine residues of BrR6 are oxidized in cells. The mmPEG24-induced upshift of BrR1 was slightly reduced by NEM (Fig. 4 H, compare lanes 4 and 2). The two cysteine residues in the TM2 domain of BrR1 are probably in a reduced state, and their blockage by NEM slightly reduced the mmPEG24-induced mobility shift.

To test for the importance of the disulfide bonds, we mutated cysteine residues in BrR6 and BrR1 to serine (C-S). Mutating all four central cysteine residues of BrR6 and BrR1 caused cell death (Fig. 4 I, brrR6-4S and brR1-4S). Interestingly, brr1-SCSC supported viability of brr1Δ cells, whereas brl1-SCSC or brl1-SCSCΔ cells failed to form colonies (Fig. 4 I). This suggests that the critical disulfide bond is the most inner one. Indeed, the full upshift of Brl1-SCSC by mmPEG24 was seen only after TCEP reduction, suggesting that the two inner cysteines of Brl1 form a disulfide bond (Fig. S3, D and E).

Why are the cysteine mutants of Brl1 nonfunctional? Analysis of cell extracts by immunoblotting indicated that P\textsubscript{ADH1}-yeGFP-brl1-SCSC was expressed similarly to P\textsubscript{ADH1}-yeGFP-BRL1 (Fig. S3 H). Consistently, the NE signal of yeGFP-brl1-4S and yeGFP-brl1-SCSC was reduced (Fig. S3, F and G). yeGFP-brl1-SCSC was degraded to the level of yeGFP that uniformly stained the cytoplasm. These data indicate that, at least for the overexpressed proteins, the disulfide bonds in Brl1 are important for either protein stability (inner C-C) or proper NE localization (outer C-C). A model for disulfide bond formation in BrR6 and BrR1 is given in Fig. 4 J.

BrR1 interacts with a subset of NUPs

Because BrR6 and BrR1 are not stable components of NPCs, both proteins may only transiently interact with NPCs during biogenesis. We tested the possibility of transient BrR1-NUP interactions using the BiFC assay (Hu et al., 2002). In this assay, transient interactions are preserved after YFP formation (Kerpola, 2008; Khmelinskii et al., 2014). We observed interaction of BrR1 with structural NPCs, including the TM NUPs Ndc1 and Pom33; the outer ring NUPs Nup84, Nup85, and Nup133; and the inner ring NUPs Nup188 and Nup59. BrR1 mildly interacted with Heh1 but not Heh2, which has been indicated to be involved in the quality control of NPC biogenesis (Webster et al., 2016). In contrast, no interaction was observed between BrR1 and the FG-repeat proteins Nup49 and Nup116, the cytoplasmic filament protein Nup82, and nuclear basket NUPs Nup2, Nup60, Mlp1, and Mlp2 (Fig. S5, A and B). Deletion of APQ12 increased Nup84-BrR1 interaction in the BiFC assay but had no impact on other interactions (Fig. S3 I).

To confirm interactions, BrR6-3yeGFP and BrR1-3yeGFP were immunoprecipitated from whole-cell extracts with GFP-Trap beads (Rothbauer et al., 2008). As positive control for IP, we confirmed complex formation between BrR1 and BrR6 (Fig. S3 J; Lone et al., 2015). We detected Ndc1-6HA in the IP of BrR6-3yeGFP (Fig. 5 C). In a similar way, we detected Nup188-6HA and Ndc1-6HA bound to yeGFP-BrR6 and yeGFP-BrR1 (Fig. 5 D). Collectively, these data show that Ndc1 and Nup188 are in relatively stable complexes with BrR6 and BrR1.

Clustered BrR6 and BrR1 proteins recruit NUPs to the NE

To obtain additional evidence for the interaction of BrR6 and BrR1 with NUPs, we asked whether clustering of BrR6 and BrR1 in the NE had the ability to recruit NPCs. We induced BrR6/BrR1 clustering by galactose-induced expression of three tandem copies of the gene coding for the GFP-binding protein (3GBP; Rothbauer et al., 2008) in BRR6-3yeGFP BRL1-3yeGFP (Fig. 6 A). This expression reduced growth of BrR6/BrR1-3yeGFP cells (Fig. S6). In a similar way, we detected Nup188-6HA and Ndc1-6HA bound to yeGFP-BrR6 and yeGFP-BrR1 (Fig. 5 D). Collectively, these data show that Ndc1 and Nup188 are in relatively stable complexes with BrR6 and BrR1.

Clustered BrR6 and BrR1 proteins recruit NUPs to the NE

To obtain additional evidence for the interaction of BrR6 and BrR1 with NUPs, we asked whether clustering of BrR6 and BrR1 in the NE had the ability to recruit NPCs. We induced BrR6/BrR1 clustering by galactose-induced expression of three tandem copies of the gene coding for the GFP-binding protein (3GBP; Rothbauer et al., 2008) in BRR6-3yeGFP BRL1-3yeGFP (Fig. 6 A). This expression reduced growth of BRR6-3yeGFP BRL1-3yeGFP cells but not of BRR6 BRL1 cells (Fig. 6 B). Short P\textsubscript{GAL1}-3GBP expression clustered BrrR6/BrrR1-3yeGFP into a dot-like region at the NE that was stronger in intensity than the BrrR6/BrrR1-3yeGFP signal along the NE (Fig. 6 C, arrowheads). Importantly, Nup188-mCherry was recruited to this BrrR6/BrrR1-3yeGFP dot, as indicated by the colocalization of yeGFP and...
Figure 5. **Brr6 and Brl1 interact with structural NUPs that are required for the early NPC assembly.** (A) BiFC fluorescent signals of cells expressing Brl1-VC and NUP-VN fusions. A cartoon of the NPC with indicated VN-fusions is shown. Bar, 5 µm. (B) Quantification of cells from A. Error bars: SD (n > 100); three independent experiments. (C) Ndc1-6HA coimmunoprecipitates with Brr6-3yeGFP. (D) Nup188-6HA and Ndc1-6HA copurify with yeGFP-Brr6 and yeGFP-Brl1.
mCherry (Fig. 6, C [right] and D). Expression of \( P_{\text{GAL1}}-3\text{GBP} \) in \( \text{NUP188-mCherry} \) cells had no impact on the localization of Nup188-mCherry, excluding binding of the mCherry moiety to 3GBP (Fig. S3, K and L). As a specificity control, we established that expression of \( P_{\text{GAL1}}-3\text{GBP} \) in \( \text{SEC63-3yeGFP or OLE1-3yeGFP} \) cells clustered Sec63-3yeGFP and Ole1-3yeGFP without affecting Nup188-mCherry localization (Fig. 6, E and F; and Fig. S3, M and N). Together with the BiFC, immuno-EM, and IP data, this supports the notion that Br6 and Br11 can interact with NUPs at the NE.

**BRL1 overexpression suppresses the NPC biogenesis defect of \( \text{nup116}\Delta \) and \( \text{gle2}\Delta \) cells**

Cells with a deletion of \( \text{NUP116 or GLE2} \) or carrying the conditional lethal \( \text{gle2-1} \) allele have a growth defect at 37°C that was suppressed by \( \text{BRL1} \) but not \( \text{BR6} \) expressed from the \( \text{GAL1} \) or \( \text{ADH1} \) promoter (Fig. 7, A and B). GLE2 encodes for a NPC component that interacts with Nup116 (Murphy et al., 1996; Suntharalingam and Wente, 2003; Lutzmann et al., 2005). Suppression of the growth defect of \( \text{nup116}\Delta \) cells by Br11 was dependent on the presence of the inner cytoskeletal residues (Fig. 7 B). Recently, it was shown that the GLFG repeats of Nup116 function in a redundant manner with Nup188 in NPC biogenesis (Onischenko et al., 2017). As for \( \text{nup116}\Delta \) cells, the growth defect of \( \text{nup116}\Delta \text{GLFG} \) \( \text{P}_{\text{GAL1}}\text{-NUP116} \) was suppressed by \( \text{P}_{\text{GAL1}}\text{-BRL1} \) but not by \( \text{P}_{\text{ADH1}}\text{-BR6} \) (Fig. 7 C). In contrast, \( \text{P}_{\text{GAL1}}\text{-BRL1} \) did not allow growth of \( \text{nup120}\Delta \) and \( \text{sec13-34} \) cells, which also form herniations at the restrictive temperature (Fig. 7 A). \( \text{NUP120 and SEC13} \) code for components of the Y-shape Nup84 complex that forms an NPC scaffold (Simiосsoglou et al., 2000). In the reverse experiment, we asked whether \( \text{P}_{\text{ADH1}}\text{-NUP116} \) was able to suppress \( \text{br6}\Delta \) and \( \text{br11}\Delta \) mutant alleles. \( \text{P}_{\text{ADH1}}\text{-NUP116} \) allowed growth of \( \text{nup116}\Delta \) cells at 37°C (Fig. 7 D). However, \( \text{P}_{\text{ADH1}}\text{-NUP116} \) did not suppress the growth defect of \( \text{br6}\Delta \) or \( \text{br11}\Delta \) cells (Fig. 7 D). This genetic analysis suggests that \( \text{BRL1} \) overexpression bypasses the functions of \( \text{NUP116 and GLE2} \) in NPC biogenesis.

To understand the suppression of the \( \text{nup116}\Delta \) and \( \text{gle2}\Delta \) phenotypes by \( \text{P}_{\text{ADH1}}\text{-yeGFP-BRL1} \), we analyzed these cells by EM. The herniation phenotype of \( \text{gle2}\Delta \) was accompanied by \( \text{br6}\Delta \Delta \) defects and lipid changes correlated over time in conditional lethal cells. In \( \text{td-brl1} \) cells, yeGFP-Brl1 was also detected at NE evaginations (Fig. 8 E). Consistent with the localization of Br6 to the INM and ONM in WT cells (Fig. 4, C and E), Br6 localized on both membranes of NE evaginations (Fig. 8 E). Because \( \text{BRL1} \) did not suppress the defect of \( \text{nup116}\Delta \) and \( \text{gle2}\Delta \) cells (Figs. 3 A and 7 A), we could use these cells for analysis of the localization of yeGFP-Br6. yeGFP-Br6 localized to the INM and ONM of herniations (Fig. S4, B and C). In summary, Brl1 associates with the INM of NPC biogenesis intermediates, whereas Br6 was at the INM and ONM of these assemblies.

To test whether Br11 has membrane-remodeling activity, we overexpressed \( \text{BRL1} \) from the galactose promoter (\( \text{P}_{\text{GAL1}}\text{-BRL1} \)) and analyzed NE morphology. Indeed, galactose-induced expression of \( \text{P}_{\text{GAL1}}\text{-BRL1} \) affected the morphology of the NE in WT cells (Fig. S4, D–F). In 83% of the \( \text{P}_{\text{GAL1}}\text{-BRL1} \) cells (10 of 12 cells), sheets of NE were detected inside the nucleus by EM (Fig. S4 G). Such defective NE structures were not observed in control cells. This phenotype was not observed for \( \text{P}_{\text{GAL1}}\text{-BR6} \) cells. Thus, Br11 has the potential to remodel the NE.

**Brr6 and Br11 locate to NPC assembly intermediates**

To understand where Br6 and Br11 function during NPC biogenesis, we determined their localization during the NPC assembly. NPC assembly intermediates are transient in nature and have not been described in WT yeast cells. We accumulated such structures using conditional lethal \( \text{td-br6} \) or \( \text{td-brl1} \) cells. In light microscopy images, ~30% of \( \text{td-brr6} \) cells with \( \text{P}_{\text{ADH1}}\text{-yeGFP-BRL1} \) (Fig. 8 A) showed dot-like Nup85-tdTomato signals at the NE, most of which colocalized yeGFP-Br11 puncta (Fig. 8, B and C). This raises the possibility that Br11 associates with NPC assembly intermediates.

Analysis of \( \text{td-br6} \) cells by EM identified INM evaginations that were labeled by the marker Nsp1 (Fig. 8 D). We subgrouped Nsp1-labeled evaginations into three classes according to their depth (Fig. 8 F). With depth less than 30 nm, which was smaller than the INM–ONM distance (Fig. 8 F, right), we defined the INM deformations as NPC assembly intermediates. These small INM evaginations were detected in only one serial section and are therefore not extensions of herniations. The morphological of these structures was similar to NPC assembly intermediates of human cells (Otsuka et al., 2016). NE deformations, which were deeper than the ONM–INM distance, were named small or large herniations, depending on the depth of the deformation (Fig. 8 F). The diameter of herniations was similar to that of NPC (Fig. 8 G). yeGFP-Brl1 was detected at the bend of the INM of assembly intermediates (Figs. 8 D and S4 A). The yeGFP-Brl1 signal localized mostly at the bend of the INM in small and large herniations (Fig. 8 D, middle and right).

In \( \text{td-brr6} \) cells, yeGFP-Br6 was also detected at NE evaginations (Fig. 8 E). Consistent with the localization of Br6 to the INM and ONM in WT cells (Fig. 4, C and E), Br6 localized on both membranes of NE evaginations (Fig. 8 E). Because \( \text{BRR6} \) did not suppress the defect of \( \text{nup116}\Delta \) and \( \text{gle2}\Delta \) cells (Figs. 3 A and 7 A), we could use these cells for analysis of the localization of yeGFP-Br6. yeGFP-Br6 localized to the INM and ONM of herniations (Fig. S4, B and C). In summary, Brl1 associates with the INM of NPC biogenesis intermediates, whereas Br6 was at the INM and ONM of these assemblies.

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**Discussion**

Br6 and Br11 are two integral membrane proteins of the NE with functions in NPC biogenesis (Schneiter and Cole, 2010). It was suggested that both proteins regulate lipid homeostasis, explaining their essential role in NPC biogenesis. Although this model is appealing, indications are missing for how Br6 and Br11 would regulate lipid composition and whether NPC defects and lipid changes correlate over time in conditional lethal \( \text{br6}\Delta \) and \( \text{br11}\Delta \) cells.

Because Br6 and Br11 are paralogues with overlapping functions, as suggested by similar NPC defects and the synthetically lethal phenotype of mutant alleles, we codepleted Br6 and Br11 to see the full range of Br6 and Br11 functions. These double-degron cells showed NPC biogenesis defects, as has been reported for the conditional lethal \( \text{br6}\Delta \) and \( \text{br11}\Delta \) cells (de Bruyn Kops and Guthrie, 2001; Saitoh et al., 2005; Hodge et al., 2010; Lone et al., 2015). However, lipid mass spectrometry analysis did not indicate a change in lipid composition in response to Br6 and Br11 depletion. In addition, \( \text{P}_{\text{GAL1}}\text{-BR6} \) and \( \text{P}_{\text{GAL1}}\text{-BRL1} \) overexpression did not affect lipid composition. The only exception was a very small increase of TAG subspecies with long fatty acids by \( \text{P}_{\text{GAL1}}\text{-BRL1} \) expression. However, it is unlikely that this minute increase has an impact on the NE, because TAGs are mainly components of lipid droplets, which do not have a direct role in membrane curvature...
Figure 6. In vivo cross-linking of Brr6 and Brl1 recruits Nup188. (A) Principle of 3GBP in vivo cross-linking assay. (B) Growth of indicated cells with plasmids p415Gal or p415Gal-3GBP. (C and E) p415Gal or p415Gal-3GBP cells were incubated for 3 h at 30°C with galactose to induce the $P_{\text{GAL1}}$ promoter. The enlargements show NEs that were used for the plot profiles on the right. Arrowheads indicate colocalization of Brr6/Brl1 and Nup188. Bars: (overviews) 5 µm; (enlargements) 1 µm. (D and F) Quantification of cells from C and E. Error bars: SD ($n > 300$ for C and $n > 150$ for E); three independent experiments. Unpaired t test with two-tailed p-value was used to compare the samples. ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, not significant.
Figure 7. Genetic interactions of BRL1. (A) Indicated yeast strains with chromosomal $P_{\text{ADH1}}$-BRR6 or $P_{\text{ADH1}}$-BRL1 or plasmid encoded $P_{\text{GAL1}}$-BRR6 or $P_{\text{GAL1}}$-BRL1 were incubated for 2 d at 37°C. –, no growth; +, growth; –/+ , partial growth; nd, not determined. A cartoon of the NPC with indicated proteins tested in the assay is shown. (B) Serial dilutions of indicated strains with the plasmid-encoded BRL1 alleles on SC-selection plates. (C) Suppression of the growth defect of nup116ΔGLFG $P_{\text{ADH2}}$-NUP116 cells by $P_{\text{ADH1}}$-BRL1. Ten-fold serial dilutions of cells on plates with or without methionine. (D) Growth of brr6(ts), brl1(ts), and nup116Δ cells with $P_{\text{ADH1}}$-NUP116. (E) TEM images of nup116Δ and gle2Δ cells show herniation defects after incubation at 37°C for 3 and 2.5 h, respectively. N, nucleus; C, cytoplasm. Bars: [overview] 500 nm; [enlargements] 100 nm. (F) Immuno-EM analysis with anti-GFP antibody. nup116Δ and gle2Δ cells with $P_{\text{ADH1}}$-yeGFP-BRL1 were incubated for 3 and 2.5 h at 37°C, respectively. Arrowheads, 10-nm gold particles reflecting yeGFP localization. Abbreviations and bars are as in E.
Figure 8. Brr6 and Brl1 accumulate at NPC assembly intermediates. (A) Protein level of yeGFP-Brl1 of the indicated cells represented in B. Tub2 is loading control. (B) Images of cells incubated at 37°C for 3 h. The enlargements (bottom) show NEs that were used for the plot profiles (right). Arrowheads indicate colocalization of yeGFP-Br1 and Nup85-tdTomato. Bars: (overview) 5 µm; (enlargement) 1 µm. (C) Quantification of cells from B. Error bars: SD (n > 230); three independent experiments. (D and E) Immuno-EM of cells incubated for 3 h at 37°C. Localization of Brl1, Brr6 (10 nm gold, anti-Br1, and anti-GFP), and Nsp1 (15 nm, anti-Nsp1) at NPC intermediates and herniations. Cartoons illustrate the morphology of the NE evaginations with gold labeling. N, nucleus; C, cytoplasm. Bars, 100 nm. (F) Deepness of NPC intermediates and herniations (see cartoon) was quantified from td-br6, td-brl1, and td-brr6 td-brl1 cells. The diameter of normal NPCs was measured from BRR6 BRL1 WT cells. *, P ≤ 0.05; ***, P ≤ 0.001; ****, P ≤ 0.0001; ns, not significant.
and fusion (Thiam et al., 2013; McMahon and Boucrot, 2015). In addition, BA affected growth of only a subset of conditional lethal brl1 mutant cells, although all of them showed NPC biogenesis defects. brr6-5001 cells grew even better on BA at 33°C than without the drug. BA also impaired growth of well-studied SPB duplication and NPC biogenesis mutants that are important for the insertion of the SPB or NPC into the NE but are not involved in lipid biogenesis. Thus, a change in growth by BA probably indicates a role of the encoded protein in membrane-related processes but does not necessarily indicate a function in lipid homeostasis.

Previously it was suggested that the C terminus of Brr6 is in the intermembrane space of the NE, whereas the N terminus is exposed into the nucleo- or cytoplasm (de Bruyn Kops and Guthrie, 2001; Saitoh et al., 2005). Our data are most consistent with the model that Br16 and Br11 have two TM domains with N and C termini exposed to the nucleoplasm or cytoplasm (Fig. 4 J). In agreement with this model are membrane orientation predications (Kim et al., 2006; Smoyer et al., 2016) and the observation that Nup116 not only have a function together in one complex. However, it is important to note that Br6 does not always behave like Br11. In contrast to Br11, overexpression of BRL1 suppressed the growth defect of nup116Δ and gle2Δ cells that arises because of a lack of scaffold function during NPC biogenesis (Onischenko et al., 2017). Future in vitro experiments will test whether purified Br6 has membrane-remodeling activity.

Complex formation, genetic interactions, and similar phenotypes of conditional lethal mutants suggest that Br6 and Br11 function together in one complex. However, it is important to note that Br6 does not always behave like Br11. In contrast to BR11, overexpression of BRR6 failed to suppress the growth defect of nup116(ts) and gle2(ts) cells and did not affect NE morphology. Moreover, about half of Br6 associated with the ONM, whereas most of Br11 was at the INM. This may indicate that Br6 and Br11 do not always function together. It will be important to understand when in the NPC biogenesis process both proteins interact and which functions of BRL1 require BRR6.}

Figure 9. Model for Brr6/Br11 function. See Discussion for details.
The *S. pombe* Brr6 becomes enriched at the SPB in early mitosis and with mitotic exit, where it facilitates NE insertion and extrusion of the SPB from the NE, respectively (Tamm et al., 2011). Such function at the SPB was not obvious in *S. cerevisiae*. We detected only relatively mild SPB duplication defects that could be an indirect consequence of the NPC defects in Brr6/Br11-depleted cells (Rüthnick et al., 2017). We were unable to show enrichment of Brr6 and Br11 at the SPB of budding yeast. The difference between *S. pombe* and *S. cerevisiae* is probably an attribute of distinct SPB insertion mechanisms (Cavanaugh and Jaspersen, 2017). In *S. cerevisiae*, the daughter SPB inserts next to the mother SPB that is already embedded in the NE (Seybold and Schiebel, 2013). In *S. pombe*, the mother and daughter SPBs reside on the cytoplasmic face of the NE during interphase, and both become inserted into a fenestra of the NE with mitotic entry (Ding et al., 1997; Bouhlel et al., 2015; Rüthnick and Schiebel, 2016).

In mammalian cells, the membrane curvature–sensing protein Nup133, the Sun domain protein Sun1, and the TM NUP Pom121 have been reported to be involved in interphase NPC assembly (Doucet et al., 2010; Funakoshi et al., 2011; Talamas and Hetzer, 2011). Puzzlingly, Brr6/Br11 homologues are found only in organisms with closed mitosis (Tamm et al., 2011; Jaspersen and Ghosh, 2012; Yang et al., 2017). In these organisms, the interphase NPC pathway is probably the only way to assemble NPCs and as such has to be particularly efficient. This may explain the conservation of Brr6/Br11 in these organisms. Functionally similar proteins most likely fulfill the role of Brr6/Br11 in interphase NPC biogenesis in higher eukaryotes. It will be interesting to test the extended reticulon family (Yang and Strit-}

### Materials and methods

#### Yeast strains and culture conditions

Yeast strains and plasmids used in this study are listed in Table S1. Yeast strains TSA1123, Y12346, and Y12360 were obtained from C. Boone (Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, Canada). The plasmids pUN100-SEC13-ProtA and pUN100-sec13-34-ProtA were obtained from E. Hurt (Heidelberg University Biochemistry Center, Heidelberg, Germany). The plasmid pLPMR2 was obtained from C.P. Lusk (Yale School of Medicine, New Haven, CT). The yeast strain ScEB116 was obtained from G. Rabut (Centre National de la Recherche Scientifique, Rennes, France). The yeast strain SC116 was obtained from S. Wente (Vanderbilt University School of Medicine, Nashville, TN). Gene deletion and epitope tagging of endogenous genes were performed using a PCR-based integration approach (Knop et al., 1999; Janke et al., 2004). Yeast strains were grown in synthetic complete (SC) medium, SC-selection medium (Rose, 1987), YPD, or yeast extract, peptone, and raffinose (YPRAf) with or without 0.1 mM CuSO4 at 16°C, 23°C, 30°C, or 37°C. Galactose was added to a final concentration of 2% to induce expression of genes under a GAL1 promoter. Alkaline lysis and TCA precipitation were used to prepare yeast extracts for analysis of protein levels by immunoblotting (Janke et al., 2004). To test for growth defects, yeast cells were grown overnight in the indicated selection medium before the density was adjusted to OD600 = 1 the next day. The cell suspension was then spotted in a 10-fold serial dilution on the desired plates and incubated at the indicated temperatures.

#### Fluorescence light microscopy

A DeltaVision RT system (Olympus IX71 based; Applied Precision) equipped with the Photometrics CoolSnap HQ camera (Roper Scientific), a 100×/1.4-NA UPlanSAPO objective (Olympus), a mercury arc light source, and softWoRx software (Applied Precision) was used for cell imaging. Imaging was done at 23°C, 30°C, or 37°C using the GFP, YFP, and mCherry channels with different exposure times according to the fluorescence intensity of each protein. For time-lapse experiments, cells were grown and imaged in microfluidic plates (Y04C/CellASIC) within the ONIX microfluidic platform (CellASIC) inserted onto the microscope stage. SC medium with 3% raffinose and 2% galactose was perfused into the microfluidic plate at a pressure of 0.25 psi. Image restoration by 3D deconvolution was performed with softWoRx and processed with ImageJ software (National Institutes of Health). For quantification of SPB signals, the integrated density (IntDen) of the SPB in the brightest stack was measured with a 5 × 5-pixel square and 7 × 7-pixel square for background correction. The following formula was used to calculate the relative fluorescent intensity (RFI): $RFI = \text{IntDen}_{\text{area}} - \left[ \frac{(\text{IntDen}_{\text{area}} - \text{IntDen}_{\text{area}}) \times \text{area}}{\text{area} - \text{area}} \right]$. Quantifications were performed three times, and a combined graph is shown.

#### Lipid analysis

Cells (10 OD) were harvested and homogenized by FastPrep (MP Biomedicals) in 155-mM ammonium bicarbonate buffer (pH 7.5). Homogenized cells were subjected to acidic Bligh–Dyer lipid extraction in the presence of internal lipid standards added from a master mix containing phosphatidylethanolamine (PC; 13:0/13:0, 14:0/14:0, 20:0/20:0; 21:0/21:0; Avanti Polar Lipids), phosphatidylinositol (PI; 17:0/20:4; Avanti Polar Lipids), phosphatidylethanolamine and phosphatidylserine (PE and PS; both 14:1/14:1, 20:1/20:1, 22:1/22:1; semisynthesized as described in Özbalci et al. [2013]), TAG (17:0/17:0; Larodan), TAG (D5-TAG-Mix, and LM-6000/ D5-TAG, 17:0, 17:1, and 17:1; Avanti Polar Lipids), phosphatidic acid (PA; 17:0/20:4; Avanti Polar Lipids), phosphatidylglycerol (PG, 14:1/14:1, 20:1/20:1, 22:1/22:1; semisynthesized as described in Özbalci et al. [2013]), and ceramide (t-Cer; Avanti Polar Lipids). Lids recovered in the organic extraction phase were evaporated by a gentle stream of nitrogen. Before measurements, lipid extracts were dissolved in 10 mM ammonium acetate in methanol and transferred to 96-well plates (Eppendorf Twintec 96). Mass spectrometry measurements were performed in positive ion mode on an AB SCIEX QTRAP 6500+ mass spectrometer, equipped with chip-based (HD-D ESI Chip; Advion Biosciences) nano-electrospray infusion, and ionization (Triversa Nanomate; Advion Biosciences) as described previously (Özbalci et al., 2013). The following precursor ion scanning (PREC) and neutral loss scanning (NL) modes were used for the measurement of the various lipid classes: +PREC 184 (PC), +PREC 282 (t-Cer), +NL 141 (PE), +NL 185 (PS), +NL 277 (PI), +NL 189 (PG), +NL 115 (PA), and +PRE 77 (ergosterol). Ergosterol was quantified after derivatization to ergosterol acetate in the presence of the internal standard (22E-Stigmasta-5,7,22-trien-3-β-ol (R202967; Sigma-Aldrich) using 100 µl acetic anhydride/chloroform (1:12 vol/vol; Eisging et al., 2009). Data evaluation was done using LipidView (ABSciex) and an in-house–developed software (ShinyLipids).
**Bimolecular fluorescence complementation**

The BiFC interaction assay was performed using Brl1 and NUPs tagged with either VC173 or VN155 fragments (VC and VN) of the Venus fluorescent protein (Shyu et al., 2006). All fusions were expressed from their endogenous chromosomal loci. The strains expressing BRL1-VC were constructed on the scEB116 background, and NUPs-VN were constructed by homologous recombination in the BY4741 background. Strains expressing individual BRL1-VC and NUPs-VN fusions were mated to produce an array of yeast strains each expressing a unique combination of tagged BRL1 and NUP, as described previously (Baryshnikova et al., 2010). The resulting strains were cultured overnight at 30°C in SC medium and diluted in SC medium again for 3–4 h at 30°C or 16–18 h at 16°C before imaging.

**Redox-state detection via alkylation shift experiments**

To analyze the redox state of cysteine residues in Brr6 and Brl1 in vivo, cells were grown in YPD medium to exponential phase, followed by treatment with or without 50 mM NEM. The whole-cell lysates were TCA precipitated with the modified protocol as described previously (Keogh et al., 2006). In brief, cells at 3 OD were resuspended in 250 µl of 20% TCA and subjected to glass bead lysis. The precipitated proteins were washed with 1 ml ice-cold acetone and dissolved in modification buffer (Ramesh et al., 2016) with or without 10 mM TCEP for 20 min at 90°C. The resuspensions were modified with 15 mM mmPEG24 for 2 h in the dark.

**Immunoprecipitation**

Cells (25 OD) were harvested and resuspended in lysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, and 10% glycerol) supplemented with 10 mM NaF, 60 mM β-glycerophosphate, 1 tablet/50 ml Roche protease inhibitor cocktail complete (EDTA free), and 1 mM PMSF. Glass beads (BioSpec Products) were added, and cells were lysed in a FastPrep machine (MP Biomedicals). Cell lysate was supplemented with 0.5% Triton X-100 and incubated on ice for 10 min. The soluble proteins were separated from the cell debris by centrifugation and incubated with GFP-Trap agarose beads (Chromotek) at 4°C for 2 h. Beads were washed three times with lysis buffer supplemented with 0.1% Triton X-100 and twice with wash buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 5 mM MgCl₂). Bound proteins were eluted in 50 µl of 2x SDS-PAGE sample buffer, heated to 95°C for 5 min, separated by SDS-PAGE, and transferred to PVDF membrane (Millipore) for Western blotting.

**EM**

Cells were high-pressure frozen, freeze-substituted, sectioned, labeled, and stained for EM. In brief, cells were collected onto a 0.45-µm polycarbonate filter (Millipore) using vacuum filtration and high-pressure frozen with a HPM010 (Abra-Fluid). Cells were freeze-substituted (freeze substitution solution: 0.1% glutaraldehyde, 0.2% uranyl acetate, and 1% water, dissolved in anhydrous acetone) using the EM-AFS2 device (Leica Microsystems) and stepwise infiltrated with Lowicryl HM20 (Polysciences), started by a low temperature of ~90°C. For polymerization, the samples were finally exposed to UV light for 48 h at ~45°C and were gradually warmed up to 20°C. Embedded cells were serially sectioned using a Reichert Ultracut S Microtome (Leica Instruments) to a thickness of 70 nm. Poststaining with 3% uranyl acetate and lead citrate was performed. The sections were imaged on a CM120 BioTwin electron microscope (Philips Electronics) operated at 80–100 kV and equipped with a CCD camera (Keen View; Soft Imaging Systems) or a JE-1400 (Jeol) operating at 80 kV equipped with a 4,000 x 4,000 digital camera (F416; TVIPS). Micrographs were adjusted in brightness and contrast using ImageJ.

**Online supplemental material**

Fig. S1 describes that NPC biogenesis is affected in td-brr6 td-brl1 cells, associated with Fig. 1. Fig. S2 provides information regarding SPB duplication defects in td-brr6 td-brl1 cells. It also describes the localization of Brr6 and Brl1 during the cell cycle with Spc42 as a SPB marker. Fig. S3 shows that mutations in conserved cysteine residues cause mislocalization or destabilization of Brl1, and that NE localization of Nup188-mCherry by 3GBP expression is not affected in OLE1-3GFP cells.

**Acknowledgments**

We thank Drs. C. Boone, E. Hurt, C.P. Lusk, G. Rabut, K. Weis, and S. Wente for plasmids and yeast strains, and Dr. C. Funaya from the EM facility of Heidelberg University for support.

This work is supported by grants of the Deutsche Forschungsgemeinschaft Schi 295/5-2 to E. Schiebel and SFB/TRR83 to C. Lüchtenborg and B. Brügger. The Heinz Götze Memorial Fellowship supported W. Zhang.

The authors declare no competing financial interests.

Author contributions: W. Zhang performed most of the experiments, assembled figures, and helped to write the manuscript. A. Neuner performed the EM together with W. Zhang. D. Rüthnick helped with the microscopic analysis of the data. T. Sachsenhaemer and C. Lüchtenborg performed the lipid mass spectrometry analysis, and B. Brügger analyzed these data. E. Schiebel supervised the project, suggested experiments, and wrote the manuscript.

Submitted: 5 June 2017
Revised: 21 November 2017
Accepted: 10 January 2018
Mukhopadhyay, K. A., Kohli, R., and Prasad. 2002. Drug susceptibilities of yeast cells are affected by membrane lipid composition. *Antimicrob. Agents Chemother.* 46:3695–3705. [https://doi.org/10.1128/AAC.46.12.3695-3705.2002](https://doi.org/10.1128/AAC.46.12.3695-3705.2002)

Murphy, R. J.L., Watkins, and S.R. Wente. 1996. GLE2, a Saccharomyces cerevisiae homologue of the Schizosaccharomyces pombe export factor RAE1, is required for nuclear pore complex structure and function. *Mol. Biol. Cell.* 7:1921–1937. [https://doi.org/10.1091/mbc.7.12.1921](https://doi.org/10.1091/mbc.7.12.1921)

Ossipenko, E. H.H. Tang, K.R. Andersen, and C.M. Gardner. 2014. Analysis of membrane proteins localizing to the inner nuclear envelope in living cells. *J. Cell Biol.* 215:575–590. [https://doi.org/10.1016/j.jcb.201607043](https://doi.org/10.1016/j.jcb.201607043)

Stavrú, F. B.B. Hulsman, A. Spang, E. Hartmann, V.C. Cordes, and D. Görlich. 2006. NDC1: A crucial membrane-integral nucleoporin of metazoan nuclear pore complexes. *J. Cell Biol.* 173:509–519. [https://doi.org/10.1016/j.jcb.200601001](https://doi.org/10.1016/j.jcb.200601001)

Strawn, L.A., T. Shen, D.S. Goldfarb, and S.R. Wente. 2004. Minimal nuclear pore complexes define FG repeat domains essential for transport. *Nat. Cell Biol.* 6:197–206. [https://doi.org/10.1038/nclb0197](https://doi.org/10.1038/nclb0197)

Stuke, J.E., V.M. McDonough, and C.E. Martin. 1990. The OLE1 gene of Saccharomyces cerevisiae encodes the delta 9 fatty acid desaturase and can be functionally replaced by the rat stearyol-CoA desaturase gene. *J. Biol. Chem.* 265:204–2049.

Suntharalingam, M., and S.R. Wente. 2003. Peering through the pore: Nuclear pore complex structure, assembly, and function. *Dev. Cell.* 4:775–789. [https://doi.org/10.1016/S1534-5807(03)00016-X](https://doi.org/10.1016/S1534-5807(03)00016-X)

Talama, J.A., and M.W. Hetzer. 2011. POM121 and Sun1 play a role in early steps of interphase NPC assembly. *J. Cell Biol.* 194:27–37. [https://doi.org/10.1083/jcb.201012154](https://doi.org/10.1083/jcb.201012154)

Tamm, T., A. Grallert, E.P. Grossman, I. Alvarez-Tabares, F.E. Stevens, and I.M. Hagan. 2011. Birth drives the Schizosaccharomyces pombe spindle pole body nuclear envelope insertion/extrusion cycle. *J. Cell Biol.* 195:467–484. [https://doi.org/10.1083/jcb.201106076](https://doi.org/10.1083/jcb.201106076)

Tcheperegin, S.E., M. Marelli, and R.W. Wozniak. 1999. Topology and functional domains of the yeast pore membrane protein Pom152p. *J. Biol. Chem.* 274:5252–5258. [https://doi.org/10.1074/jbc.274.8.5252](https://doi.org/10.1074/jbc.274.8.5252)

Terweij, M., T. van Welsum, S. van Deventer, K.F. Verzijlbergen, V. Menendez-Benito, D. Onsongo, P. San-Segundo, J. Neefjes, and P. van Leeuwen. 2013. Recombination-induced tag exchange (RITE) cassette series to monitor protein dynamics in Saccharomyces cerevisiae. *G3 (Bethesda).* 3:1261–1273. [https://doi.org/10.1534/g3.113.006213](https://doi.org/10.1534/g3.113.006213)

Thiam, A.R., R.V. Faese Jr., and T.C. Walther. 2013. The biophysics and cell biology of lipid droplets. *Nat. Rev. Mol. Cell Biol.* 14:775–786. [https://doi.org/10.1038/nrm3699](https://doi.org/10.1038/nrm3699)

Vollner, B., M. Lorenz, D. Moreno-Andrés, M. Bodenhöfer, P. de Magistris, S.A. Astrudins, A. Schooley, M. Flotenmeyer, S. Leptihn, and W. Antonin. 2015. Nup153 recruits the Nup107-160 complex to the inner nuclear membrane for interphase nuclear pore complex assembly. *Dev. Cell.* 33:717–728. [https://doi.org/10.1016/j.devcel.2015.04.027](https://doi.org/10.1016/j.devcel.2015.04.027)

Webster, B.M., P. Colombi, J. Jäger, and C.P. Lusk. 2014. Surveillance of nuclear pore complex assembly by ESCRT-IDIV/IV. *Cell.* 159:388–401. [https://doi.org/10.1016/j.cell.2014.09.012](https://doi.org/10.1016/j.cell.2014.09.012)

Webster, B.M., D.J. Thaller, J. Jäger, S.E. Ochmann, S. Borah, and C.P. Lusk. 2016. Chm7 and Heh1 collaborate to link nuclear pore complex quality control with nuclear envelope sealing. *EMBO J.* 35:2447–2467. [https://doi.org/10.1525/embj.201649574](https://doi.org/10.1525/embj.201649574)

Wente, S.R., and G. Blobel. 1993. A temperature-sensitive NUP116 null mutant forms a nuclear envelope seal over the yeast nuclear pore complex thereby blocking nucleocytoplasmic traffic. *J. Cell Biol.* 123:275–284. [https://doi.org/10.1083/jcb.123.2.275](https://doi.org/10.1083/jcb.123.2.275)

Wente, S.R., and M.P. Rout. 2010. The nuclear pore complex and nuclear transport, *Cold Spring Harb. Perspect. Biol.* 2:a000562. [https://doi.org/10.1101/cshperspect.a000562](https://doi.org/10.1101/cshperspect.a000562)

Winie, M., M.A. Hoyt, C. Chan, L. Goetsch, D. Botstein, and B. Byers. 1993. NDC1: A nuclear periphery component required for yeast spindle pole body duplication. *J. Cell Biol.* 122:743–751. [https://doi.org/10.1083/jcb.122.4.743](https://doi.org/10.1083/jcb.122.4.743)

Winie, M., D. Yarar, T.H. Giddings Jr., and D.N. Mastronarde. 1997. Nuclear pore complex number and distribution throughout the Saccharomyces cerevisiae cell cycle by three-dimensional reconstruction from electron micrographs of nuclear envelopes. *Mol. Biol. Cell.* 8:2119–2132. [https://doi.org/10.1091/mbc.8.11.2119](https://doi.org/10.1091/mbc.8.11.2119)

Wozniak, R.W., G. Blobel, and M.P. Rout. 1994. POM152 is a integral protein of the pore membrane domain of the yeast nuclear envelope. *J. Cell Biol.* 125:31–42. [https://doi.org/10.1083/jcb.125.1.31](https://doi.org/10.1083/jcb.125.1.31)

Yang, Y.S., and S.M. Strittmatter. 2007. The reticulons: A family of proteins with diverse functions. *Genome Biol.* 8:234. [https://doi.org/10.1186/gb-2007-8-12-234](https://doi.org/10.1186/gb-2007-8-12-234)

Yan, H.J., M. Iwanoto, Y. Hiraoka, and T. Haraguchi. 2013. Function of nuclear membrane proteins in shaping the nuclear envelope integrity during closed mitosis. *J. Biochem.* 141:471–477. [https://doi.org/10.1093/jb/mvx020](https://doi.org/10.1093/jb/mvx020)

Zargari, A., M. Bohan, S. Heessen, C. Andréasson, J. Thiyberg, and P.O. Ljungdahl. 2007. Inner nuclear membrane proteins As1, As2, and As3 function in concert to maintain the latent properties of transcription factors Stp1 and Stp2. *J. Biol. Chem.* 282:794–805. [https://doi.org/10.1074/jbc.M609201200](https://doi.org/10.1074/jbc.M609201200)