A short perinuclear amphipathic α-helix in Apq12 promotes nuclear pore complex biogenesis

Wanlu Zhang1,†,‡, Azqa Khan1,†, Jlenia Vitale1, Annett Neuner1, Kerstin Rink2, Christian Lüchtenborg2, Britta Brügger2, Thomas H. Söllner2 and Elmar Schiebel1

1Zentrum für Molekulare Biologie der Universität Heidelberg, DFKZ-ZMBH Allianz, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany
2Biochemie-Zentrum der Universität Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany
†Present address: Cell Biology and Biophysics Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany.
‡Both authors contributed equally.

The integral membrane protein Apq12 is an important nuclear envelope (NE)/endoplasmic reticulum (ER) modulator that cooperates with the nuclear pore complex (NPC) biogenesis factors Brl1 and Brr6. How Apq12 executes these functions is unknown. Here, we identified a short amphipathic α-helix (AαH) in Apq12 that links the two transmembrane domains in the perinuclear space and has liposome-binding properties. Cells expressing an APQ12 (apq12-ah) version in which AαH is disrupted show NPC biogenesis and NE integrity defects, without impacting Apq12-ah topology or NE/ER localization. Overexpression of APQ12 but not apq12-ah triggers striking over-proliferation of the outer nuclear membrane (ONM)/ER and promotes accumulation of phosphatidic acid (PA) at the NE. Apq12 and Apq12-ah both associate with NPC biogenesis intermediates and removal of AαH increases both Brl1 levels and the interaction between Brl1 and Brr6. We conclude that the short amphipathic α-helix of Apq12 regulates the function of Brl1 and Brr6 and promotes PA accumulation at the NE possibly during NPC biogenesis.

1. Introduction

The nuclear envelope (NE) is a double membrane consisting of the outer nuclear (ONM) and inner nuclear (INM) membranes that surround and protect the nucleus. The ONM is continuous with the endoplasmic reticulum (ER), contains attached ribosomes, carries attachment sites for cytoskeletal elements and shares components with the ER (reviewed in [1]). In addition, the ER and ONM are the sites of triacylglycerol (TAG) and steryl ester lipid biosynthesis [2]. These lipids are essential for membrane growth, and, in case of TAG, also for energy storage in the form of cytoplasmic lipid droplets [3,4]. The INM is involved in genome stability, chromatin organization and regulation of gene expression [5–7]. A recent publication indicated that TAGs are also synthesized at the INM before being incorporated into nuclear lipid droplets [8]. Consistent with these distinct functions, the INM and ONM are specified by proteins and lipids that vary from one another.

The NPC is a large oligomeric complex containing about 30 different proteins that is embedded at sites at which the INM and ONM fuse. The NPCs facilitate the transport of proteins and ribonucleoproteins between the cytoplasm and the nucleoplasm [6]. In human cells and other eukaryotes undergoing an open mitosis, NPCs assemble via two mechanistically distinct pathways [9]. The post-mitotic pathway promotes the assembly of NPCs on the decondensing chromatin, shortly after anaphase onset [10]. During interphase, NPCs assemble by a distinct inside-out mechanism starting from within the nucleus at the INM, the so-called

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interphase pathway [11–13]. In budding yeast, with its closed mitosis, the interphase pathway is the only mechanism to assemble NPCs.

NPC biogenesis intermediates of the interphase pathway have been described in human cells by electron tomography [11]. In wild-type (WT) yeast cells, NPC intermediates have not been observed, probably because NPC assembly is relatively fast and infrequent (approx. 2 assembly events per minute per cell [13]). However, mutations in genes coding for several nucleoporins (Nups) lead to the accumulation of so-called herniations: deformations of the INM that are probably filled with Nups [14–17]. Recently, we suggested that at least some of these herniations arise from defective steps of NPC biogenesis; for example, the failure of the fusion of the INM with the ONM during the assembly process [5,12].

Mechanistic principles of NPC biogenesis by the interphase pathway are poorly understood. In mammalian cells, early factors involved in interphase NPC biogenesis include Y-complex subunits NUP107 and NUP133, the INM protein SUN1 and the transmembrane nucleoporin POM121 [9,18–20]. In yeast, the two paralogous integral NE proteins Brl1 and Brr6 function in NPC biogenesis [12,21–25]. Loss of function of Brl1 (Brr6 like protein No. 1) or Brr6 (bad response to mitosis) triggers strong over-proliferation of the ONM and the disrupted NE even though the distribution of the nucleoporin PA accumulation at the NE, Brl1–Brr6 interaction and NPC biogenesis.

2. Results

2.1. Apq12 carries a short lipid-binding AcH between the two transmembrane domains

Recently, we have shown that the integral membrane protein Brl1 associates with NPC assembly intermediates and may promote fusion of the ONM with the INM during NPC biogenesis [12]. Brl1 interacts and cooperates with the integral membrane protein Apq12 [21]. In order to gain an understanding of the molecular roles of Apq12 (figure 1a), we sought functional elements within this protein. The AmphipSeeK program predicted an amphipathic α-helix (AcH) with two positively charged amino acids (Lys and Arg) on the hydrophilic side and hydrophobic amino acid residues on the opposite side of the helix (figure 1b; ‘AcH’ [30], between the two TM domains of Apq12 (figure 1a).

The positively charged amino acids of an AcH (figure 1b, marked blue) have the ability to interact with polar–apolar lipid surfaces while the hydrophobic interface (figure 1b, yellow) may interact with the aliphatic chains of the lipids [31]. Consistent with this lipid binding prediction, we found that the Atto488 labelled synthetic Apq12 peptide (AcH) bound to giant unilamellar vesicles (GUVs; 1–10 µm in diameter; figure 1c). As control for the binding, we used the Atto488 dye alone, and introduced amino acid changes in the Atto488-labeled Apq12 peptide (AcH-ah) that abolished the helical hydrophobic moment, as an indication of the amphiphilicity of the helix [32], from 0.595 to 0.054 (figure 1b). The Atto488 dye and the AcH-ah peptide both failed to bind to liposomes (figure 1c).

To analyse the binding efficiency of AcH peptide in a more quantitative manner, SUV (small uni-lamellar vesicles; 80–120 nm; electronic supplementary material, figure S1A) bound peptide was separated from the unbound peptide through a nycodenz gradient. Using SUVs composed of NE and plasma membrane (PM) lipids, we tested the impact of the lipid composition. NE-derived SUVs showed a higher AcH peptide binding efficiency compared to the PM SUVs (electronic supplementary material, figure S1B). The Atto488 dye and the AcH-ah peptide did not bind to the SUVs in this experimental regime (electronic supplementary material, figure S1B). In addition, the peptide without liposomes was unable to float through the nycodenz gradient (electronic supplementary material, figure S1B, control).

Next, SUV binding of the AcH peptide was quantified using microscale thermophoresis (MST) measurements. The KD of AcH peptide binding to NE-lipid derived SUVs was 16 µM (figure 1d). The binding affinity of the AcH peptide to PM derived SUVs was decreased to KD = 59 µM (figure 1d). Importantly, AcH-ah peptide failed to bind to any of these SUVs with a measurable KD. Thus, the AcH peptide binds to liposomes depending on its amphipathic nature and the lipid composition of the liposomes. This result indicates that the AcH of Apq12 directly interacts with membranes.
2.2. The \( \alpha \)H of Apq12 localizes in the perinuclear space and is not required for subcellular localization and topology of Apq12

We analysed whether the amphipathic nature of the \( \alpha \)H is important for the function of Apq12. Since \( \alpha p q12\Delta \) mutants show a growth defect at 16°C [26], we tested \( \alpha p q12\Delta , \alpha p q12\Delta \)-ah and an additional \( \alpha \)H \( \alpha p q12\Delta \) mutant, \( \alpha p q12\Delta ^{F5D6R9N} \), for growth at different temperatures. The helical hydrophobic moment of the \( \alpha \)H was decreased in \( \alpha p q12\Delta ^{F5D6R9N} \) to 0.222 and therefore has an intermediate value between the WT \( \alpha \)H of Apq12 and \( \alpha \)H-ah of Apq12-ah (figure 1b).

Apq12 TM1 TM2

\[ \text{LMNFITLVKRF} \]

\[ \text{DMNRNTLVKRN} \]

\[ \text{LMNDRTLKRF} \]

Atto488+NE Atto488+PM Atto647+NE Atto647+PM merge

GUVs+ Atto488 DMSO GUVs+ A\( \alpha \)H-ah GUVs+ A\( \alpha \)H

Atto488 dye are used as controls. Scale bar: 5 \( \mu \)m. (d) Liposomes with different lipid compositions were titrated against Atto488 labelled \( \alpha \)H, \( \alpha \)H-ah and \( \alpha \)H-ah peptides and the Atto488 dye as control. The data were normalized to the amount of bound peptide and the \( K_D \) value of \( \alpha \)H peptide-liposome interaction was calculated from the Hill equation (PM: \( K_D = 58.90 \pm 5.35 \mu\text{M} \), NE: \( K_D = 15.90 \pm 0.91 \mu\text{M} \)). The \( \alpha \)H-ah peptide and the Atto488 dye (together with NE lipids; same result was obtained with PM lipids) show no or only very weak MST signals. Values are given as means ± s.e.m, \( n = 3 \).

**Figure 1.** Apq12 contains an amphipathic helix in the luminal domain. (a) Domain organization of Apq12. The first transmembrane (TM1) domain, an \( \alpha \)H (blue) and the second transmembrane (TM2) domain are indicated. (b) Helixquest predictions of the \( \alpha \)H, \( \alpha \)H-ah and \( \alpha \)H\(^{F5D6R9N}\) helices along with their hydrophobic moments. Helixquest calculates the physicochemical properties of an \( \alpha \)-helix [29]. The amino acids marked in green indicate amino acid changes introduced in \( \alpha \)H in order to reduce the hydrophobic moment (value given below). The amino acid number corresponding to Apq12 indicates start and end of the \( \alpha \)-helix. The arrow inside the helix indicates the hydrophobic moment. (c) Binding of Atto488 labelled synthetic \( \alpha \)H and \( \alpha \)H-ah peptides to Atto647 labelled GUVs, *in vitro*. DMSO and Atto488 dye are used as controls. Scale bar: 5 \( \mu \)m. (d) Liposomes with different lipid compositions were titrated against Atto488 labelled \( \alpha \)H and \( \alpha \)H-ah peptides and the Atto488 dye as control. The data were normalized to the amount of bound peptide and the \( K_D \) value of \( \alpha \)H peptide-liposome interaction was calculated from the Hill equation (PM: \( K_D = 58.90 \pm 5.35 \mu\text{M} \), NE: \( K_D = 15.90 \pm 0.91 \mu\text{M} \)). The \( \alpha \)H-ah peptide and the Atto488 dye (together with NE lipids; same result was obtained with PM lipids) show no or only very weak MST signals. Values are given as means ± s.e.m, \( n = 3 \).

2.2. The \( \alpha \)H of Apq12 localizes in the perinuclear space and is not required for subcellular localization and topology of Apq12

We analysed whether the amphipathic nature of the \( \alpha \)H is important for the function of Apq12. Since \( \alpha p q12\Delta \) mutants show a growth defect at 16°C [26], we tested \( \alpha p q12\Delta , \alpha p q12\Delta \)-ah and an additional \( \alpha \)H \( \alpha p q12\Delta \) mutant, \( \alpha p q12\Delta ^{F5D6R9N} \), for growth at different temperatures. The helical hydrophobic moment of the \( \alpha \)H was decreased in \( \alpha p q12\Delta ^{F5D6R9N} \) to 0.222 and therefore has an intermediate value between the WT \( \alpha \)H of Apq12 and \( \alpha \)H-ah of Apq12-ah (figure 1b). \( \alpha p q12\Delta \)-ah mutant completely failed to grow at 16°C and showed reduced growth at 23°C, similar to the \( \alpha p q12\Delta \) cells (figure 2a). By contrast, growth of \( \alpha p q12\Delta ^{F5D6R9N} \) mutant cells was only reduced, but not completely abolished, at 16°C (figure 2a). Therefore, all further experiments were performed with \( \alpha p q12\Delta \)-ah cells. Thus, the amphipathic nature of the \( \alpha \)H in Apq12 is important for cell growth at lower temperatures.

We next analysed whether the subcellular localization of Apq12 requires the \( \alpha \)H. Consistent with published data on Apq12 distribution [21], yeGFP-Apq12 localized along the NE and the cell periphery, and a location that is probably the peripheral ER (figure 2b). Based on the resolution provided by the fluorescence microscope, similar localizations were observed for yeGFP-Apq12-ah (figure 2b). Thus, the integrity of the \( \alpha \)H of Apq12 is not important for the subcellular distribution of the protein.
Immuno-electron microscopy of yeGFP-Apq12 and yeGFP-Apq12-ah using GFP antibodies and protein A-gold detected both proteins along the INM, ONM, cytoplasmic and cortical ER (figure 2c; electronic supplementary material, figure S1C). Apq12 was detected with the same frequency at the INM as at the ONM (electronic supplementary material, figure S1C). For Apq12-ah, there was a mild enrichment at the INM over the ONM (electronic supplementary material, figure S1C). In addition, yeGFP-Apq12 and yeGFP-Apq12-ah associated with 10% and 15% of NPCs, respectively, while most NPCs were not labelled (electronic supplementary material, figure S1D). This may indicate a transient association of the proteins with assembling NPCs, as is the case for Brl1 and Brr6 [12]. In summary, Apq12 and Apq12-ah show similar subcellular localizations.

Figure 2. The AαH of Apq12 is located in the perinuclear space and does not influence the subcellular localization and topology. (a) Growth test of WT APQ12, apq12Δ, apq12-ah and apq12F5DI6RV9N mutants at the indicated temperatures. 10-fold serial dilutions were spotted onto YPAD plates. (b) Localization of yeGFP-Apq12 and yeGFP-apq12-ah were analysed by fluorescence microscopy. Scale bar: 5 µm. (c) yeGFP-Apq12 and yeGFP-apq12-ah localization by immuno-EM. Gold particles (10 nm) indicate the localization of yeGFP-Apq12 and yeGFP-apq12-ah at the NE and ER. The rectangles indicate the enlargements that are shown underneath. ER, endoplasmic reticulum; N, nucleus. Scale bars: 200 nm and enlargements 50 nm. (d) Strains carrying C- and N-terminal fusions of Apq12 and Apq12-ah with GFP1-10 were imaged to check for reconstitution of GFP with GFP11 from GFP11-mCherry-Scs2TM (ER reporter; GFP11 in the cytoplasm) and GFP11-mCherry-PUS1 (nuclear reporter). Mps3-GFP1-10 is used as a negative control. Scale bar: 5 µm. (e) Localization and topology model for Apq12.
Apq12 is a protein of the NE and the ER with two predicted membrane spanning regions (TM1 and TM2) that are connected by the AαH (figure 1a). To determine the topology of Apq12 and whether it requires a functional AαH, we measured the accessibility of the N- and C-terminus of Apq12 and Apq12-ah by two approaches. First, we used the split GFP system for assessment of the localization of the N- and C-termini of Apq12 [33]. The overlapping localization of GFP11 and GFP1-10 restores GFP fluorescence. Thus, by combining GFP1-10 tagged versions of a protein with nuclear and cytoplasmic GFP11 localized proteins, we can determine the topology of a protein. As a control, we used the perinuclear space localization of the C-terminus of the SUN-domain protein Mps3-GFP11 that failed to restore a fluorescent GFP signal when co-expressed with the ER and ONM localized GFP11-mCherry-Scs2TM that carries GFP11 at the ONM/ER on the cytoplasmic side and the nuclear GFP11-mCherry Pus1 (figure 2d, top panel) consistent with published data [33]. Interestingly, Apq12-GFP1-10 and GFP1-10-Apq12 combined with GFP11-mCherry Pus1 resulted in a green fluorescent NE signal and, in the case of GFP11-mCherry-Scs2TM, a NE/ER signal. These data show that the N- and C-termini of Apq12 are localized to either the cytoplasm or the nucleoplasm depending on whether Apq12 is at the ONM/ER or INM, respectively (figure 2d, middle). Very similar results were obtained for Apq12-ah (figure 2d, bottom).

As a further probe of Apq12 topology, we took advantage of the fact that biotin ligases are not contained in the perinuclear space, and assessed the ability of the N- or the C-termini of Apq12 and Apq12-ah to be biotinylated when fused with the histidine-biotin-histidine (HBH) tag as a topology marker [12]. Pom152 that contains a short cytosolic N-terminal region, one TM domain and a C-terminal region in the perinuclear space was used as a control [34]. Consistent with the topology of Pom152, only the N-terminal HBH tag but not the C-terminal tag of Pom152 was biotinylated, demonstrating that the HBH biotinylation approach identifies the topology of NE proteins correctly (electronic supplementary material, figure S1E). Both the N- and C-termini of Apq12 and Apq12-ah were biotinylated when fused to the HBH tag that carries a biotin acceptor site (electronic supplementary material, figure S1E), confirming the findings from the split GFP approach.

In conclusion, since N- and C-termini of Apq12 localized to either the cytoplasm or nucleoplasm, the AαH resides in the perinuclear space where it connects the two TM domains (figure 2e). A functional AαH is not required for this topological arrangement of Apq12.

2.3. The AαH of Apq12 is important for NE integrity, NPC biogenesis and lipid homeostasis

In order to understand how loss or impairment of the Apq12 function affects the NE and NPCs, we analysed the phenotypes of APQ12, apq12Δ and apq12-ah cells by electron microscopy (EM). As expected, APQ12 wild-type cells had spherical nuclei with intact NE (figure 3a). Herniations as an indication of a defect in NPC biogenesis were detected as a major phenotype in apq12Δ (figure 3b,c, nucleus marked by red square) and apq12-ah mutants at 37°C (figure 3d,e, nucleus marked by red square). About 70% of the apq12Δ mutants incubated at 23°C showed invaginations of the NE (figure 3c). Herniations were relatively infrequent at this growth temperature. Invaginations of the NE and NE breakdown were the major defects of the apq12-ah mutant at 23°C (figure 3e). At 16°C, the major defects of apq12Δ cells were NE invaginations and NE breakdown followed by herniations (figure 3b,c). apq12-ah mutant showed NE breakdown and extrusions as major defects at 16°C (figure 3d,e). Taken together, apq12Δ and apq12-ah mutants show phenotypic variations indicating that inactivation of the AαH does not cause the complete loss of Apq12 function. In addition, the consequence of Apq12 or AαH loss depends on the temperature. The rupture of the NE explains the lethality of apq12Δ and apq12-ah cells at 16°C. Loss of AαH function triggers a defect in NPC biogenesis at 37°C.

It has been suggested that Apq12 has a function in lipid homeostasis [21], which might then account for the NE and NPC defects of apq12 and apq12-ah mutants. Therefore, we directly asked whether the AαH of Apq12 has an impact on lipid homeostasis, by incubating WT, apq12Δ and apq12-ah cells at 16°C, 30°C and 37°C, and assessing their cellular lipid content by mass spectrometry. Overall apq12Δ and apq12-ah mutants showed comparable lipid changes relative to WT (electronic supplementary material, figure S2). The increase in ergosteryl ester (EE) and TAG, and a decrease in ergosterol (Erg) and most species of glycerophospholipids (GPL), were prominent phenotypes in apq12Δ and apq12-ah mutants (electronic supplementary material, figure S2A and B), indicating a lipid metabolism flow from membrane lipids to storage lipids. In addition, we observed a significant decrease of double bonds in GPL in apq12Δ and apq12-ah mutants (electronic supplementary material, figure S2C). Moreover, the chain length (greater than 34) in GPL was significantly increased in both APQ12 mutants (electronic supplementary material, figure S2D). The reduction of membrane lipids, the decrease in the number of double bonds and the increase of chain length in GPL indicate a decrease in membrane fluidity in apq12Δ and apq12-ah mutants, explaining the defects in NE breakdown at 16°C when the flexibility of membrane lipids will be reduced by the reduction in kinetic energy. These data suggest that the AαH of Apq12 does play a role in maintaining lipid homeostasis.

2.4. Increased Apq12 levels are toxic to cells and cause the mis-localization of the NPC biogenesis factors Brl1 and Brr6 and the ER proteins Sec63 and Ole1

To gain deeper insights into the function of Apq12, we overexpressed APQ12 using the galactose-inducible Pgal promoter and followed the growth of these modified yeast cells. Because we lack an Apq12 antibody, we tagged Apq12 with 6His (Apq12-6His) to support immuno-detection of the fusion protein. Overexpression of APQ12 and APQ12-6His was equally toxic for cells (figure 4a). Such overexpression toxicity was not observed for the partner proteins BRL1 and BRR6, which also encode integral membrane proteins (figure 4b). Thus, of the three proteins in this functional module, only APQ12 has toxic consequences upon overproduction.

We next tested whether overexpression of APQ12 and APQ12-6His impact on NE structure and function. We started...
by analysing the impact of APQ12 over-expression on the distribution of the NPC biogenesis factors Brl1 and Brr6 (figure 4c–h; electronic supplementary material, figure S3A and B). In vector control cells, yeGFP-Br1l and yeGFP-Brr6 exhibited a uniform distribution throughout the NE (electronic supplementary material, figure S3C and D). By contrast, 1 h of induction of the P_{Gal1} promoter to elevate Apq12 levels led to dense clustering of Brl1 and Brr6 on the NE (figure 4c–h; electronic supplementary material, figure S3A and B). These clusters were devoid of the NPC marker Nup85-tdTomato (figure 4c,d,f,g; electronic supplementary material, figure S3A and B). Clustering of yeGFP-Br1l and yeGFP-Brr6 in response to APQ12-6His overexpression was also seen in time-lapse experiments (electronic supplementary material, figure S3C and D).
Figure 4. Overexpression of APQ12 causes the mislocalization of ER proteins. (a) Overexpression of APQ12 is toxic for the cells. WT cells with the vector control, PGal1-APQ12 or PGal1-APQ12-6His were spotted in 10-fold serial dilutions onto YPAD (Glu) and YARaf/Gal (Gal/Raf) plates at 30°C. (b) Of the APQ12, BRL1, BRR6 module only APQ12 overexpression is toxic. WT cells with the vector control, PGal1-APQ12, PGal1-BRR6 and PGal1-BRL1 were spotted in 10-fold serial dilutions onto YPAD (Glu) and YARaf/Gal (Gal/Raf) plates at 30°C. (c) Overexpression of APQ12 causes mislocalization of yeGFP-Brl1. Cells with either the vector control (electronic supplementary material, figure S3C) or PGal1-APQ12-6His were incubated with galactose for the indicated times. The boxed cell at 1 h is a two-fold enlargement of the selected cell. (d) Line scan along the NE of a PGal1-APQ12-6His yeGFP-BRL1 NUP85-tdTomato cell (enlarged boxed cell in (c)) incubated for 1 h with galactose. It shows that yeGFP-Brl1 and Nup85-tdTomato are localized on separate domains along the NE. (e) Immunoblot of PGal1-APQ12-6His yeGFP-BRL1 NUP85-tdTomato cells. The pGal1 promoter was induced by the addition of galactose (t = 0). Samples were taken after the indicated times. Tub2 is a loading control. Apq12-6His was detected by anti-His antibodies. (f) Overexpression of APQ12 causes mislocalization of yeGFP-Brr6 and Nup85-tdTomato. Cells with either the vector control (electronic supplementary material, figure S3D) or PGal1-APQ12-6His were incubated with galactose for the indicated times. The boxed cell at 1 h is a two-fold enlargement of the selected cell. (g) Line scan along the NE of a PGal1-APQ12-6His yeGFP-BRR6 NUP85-tdTomato cell (enlarged cell in (f)) incubated for 1 h with galactose. (h) Immunoblot of PGal1-APQ12-6His yeGFP-BRR6 NUP85-tdTomato cells. The pGal1 promoter was induced by the addition of galactose (t = 0). Samples were taken after the indicated times. Tub2 is a loading control. (i,j) Overexpression of APQ12 causes mislocalization of the ER protein Sec63-yeGFP (i) and Ole1-yeGFP (j). Cells with either the vector control or PGal1-APQ12-6His were incubated with galactose for the indicated time. (c,f,i and j) Scale bars: 5 µm.
supplementary material, figure S3E and F). Because Pgal1-APQ12 and Pgal1-APQ12-6His caused similar defects, we used Pgal1-APQ12-6His in further experiments.

We next asked how APQ12-6His overexpression affected the distribution of proteins at the NE/ER and INM, using the NE/ER proteins Sec63 and Ole1, the INM protein Asi3 and the ER luminal marker dsRed-HDEL as exemplars to test the impact upon NE/ER proteins [35,36]. As seen for yeGFP-Brl1 and yeGFP-Brr6, the smooth distribution of Sec63-yeGFP and Ole1-yeGFP along the NE was transformed into clusters as early as 1 h of Pgal1-APQ12-6His induction (figure 4c,f,i,j). The localization of INM protein Asi3-yeGFP was initially unaffected by APQ12 overexpression, at the 1 h time point, however, by 3 h of Pgal1 induction, modest clustering of Asi3-yeGFP along the NE emerged (electronic supplementary material, figure S4A; white arrows). The ER marker dsRED-HDEL, that in vector control cells uniformly stained the NE and the cortical ER, started to accumulate in clusters within one hour of Pgal1-APQ12-6His induction (electronic supplementary material, figure S4B). Thus, increased Apq12 levels appear to impact upon the subcellular localization of ONM/ER proteins more strongly than the INM protein Asi3.

### 2.5. The luminal AαH of Apq12 contributes to toxicity

We asked whether AαH is important for the deformation of the NE. Pgal1-apatq12-ah-6His overexpression was less toxic than Pgal1-APQ12-6His (figure 5c) even though both proteins were overexpressed to similar levels (electronic supplementary material, figure S5A). Consistent with the strongly reduced toxicity of Pgal1-apatq12-ah-6His, yeGFP-Brl1 and yeGFP-Brr6 clusters formed later and were notably less prominent than in Pgal1-APQ12-6His cells. To confirm this, we expressed Pgal1-apatq12-ah-6His (figure 5b-c). Thus, an intact AαH is a major factor in the toxic impact of APQ12 overexpression.

### 2.6. Apq12 needs a functional AαH for the overproliferation of the ONM and ER

To understand how APQ12 overexpression leads to the changes on the ONM and ER, we performed EM analysis of Pgal1-APQ12-6His and Pgal1-apatq12-ah-6His cells. Before the addition of galactose, the NE had uniform spherical morphology (electronic supplementary material, figure S5B). Induction of Pgal1-APQ12-6His led to a shift in the number of ONM/ER extensions (figure 6a(ii), (iii)) that were, in some cases, connected to the cortical ER (figure 6a(iii)) from 5% to 40% (figure 6b). After 1 h and 3 h of galactose addition, Pgal1-APQ12-6His cells contained ONM encircled vesicles containing granular material (figure 6a(v–xii), red asterisks; and figure 6b). We noticed a slight reduction of the severity of these phenotypes after 3 h of Pgal1-APQ12-6His overexpression. This may be related to adaptation or overexpression inactivation in some cells. By contrast, abnormal morphologies were not detected at the INM until 3 h after the induction of APQ12 overexpression, whereupon the INM formed small buds into the luminal space of the ONM vesicles (figure 6a(ix), arrow). The consequences of apq12-ah-6His overexpression were less severe in comparison to APQ12-6His. Pgal1-apatq12-ah-6His induction for 0.5 h and 1 h had no visible impact on the ONM or the ER in most cells (figure 6a(ii–viii)). Only 3 h of Pgal1-apatq12-ah-6His induction caused proliferation of the ONM in approximately 25% of the cell sections (figure 6a(viii), arrows and figure 6d). This delayed accumulation of phenotypes in apq12-ah-6His cells is consistent with the reduced clustering of the BRL1-yeGFP and BRR6-yeGFP and impact on growth compared to APQ12-6His (figure 5).

Overexpression of APQ12 causes the accumulation of large, granular ONM encircled vesicles. To investigate the origin of the content in these vesicles, we expressed Pgal1-APQ12-6His in cells with yeGFP tagged Brl1 as marker for the vesicles, and NLS-mRFP (mRFP fused to a nuclear localization sequence (NLS)) as nuclear marker. Overexpression of Apq12-6His induced yeGFP-Brl1 areas that were devoid of NLS-mRFP (figure 6c, top, marked by arrow) indicating that these vesicles did not contain nuclear components. In a complementing experiment, we expressed Pgal1-APQ12-6His in RPL25-yeGFP dsRed-HDEL cells with the green fluorescent ribosomal subunit as cytoplasmic marker and dsRed-HDEL as ONM/ER marker (figure 6c, bottom). Indeed, the dsRed-HDEL ONM extrusions co-localized with Rpl25-yeGFP (figure 6c, bottom; arrow), demonstrating that these vesicles contained cytoplasmic components including ribosomes. This was further suggested by EM analysis of Pgal1-APQ12-6His cells showing ONM encircled vesicles containing ribosome-like particles (figure 6d).

In a time-lapse experiment, we analysed the cellular distribution of Apq12-yeGFP and Apq12-ah-yeGFP upon Pgal1 expression (electronic supplementary material, figure S5C). Apq12-yeGFP accumulated most prominently at the INM extensions (electronic supplementary material, figure S5C, 30 min; arrowheads). After 40 min of induction Apq12-yeGFP localized in larger spots (electronic supplementary material, figure S5C; arrowheads), consistent with the accumulation of filled ONM deformations seen by EM (figure 6a). For the first 40 min of Pgal1 induction Apq12-ah-yeGFP showed comparable localizations to Apq12-yeGFP (electronic supplementary material, figure S5C). However, due to its defective AαH, Apq12-ah-yeGFP did not accumulate into larger spots. Analysis of Pgal1-APQ12-yeGFP after 0.5 and 3 h of galactose addition by immunocytochemistry detected Apq12-yeGFP localizing at NE extensions and ONM enriched vesicles (electronic supplementary material, figure S5D). Thus, the enrichment of Apq12 at ONM/ER sites induces membrane proliferation.

When taken together, APQ12 overexpression promotes extension of ER tubules from the ONM (figure 6c; steps 1 and 2). Fusion of these extensions with the NE (step 3) may entrap cytoplasmic content into ONM encircled compartments (step 4). However, the precise order of these events needs to be established in further experiments.

### 2.7. APQ12 triggers PA accumulation at the NE dependent on its AαH

The experiments above indicate that Apq12 promotes overproliferation of the ONM and the ER in an AαH-dependent manner. A mobilization of lipids by Apq12 would explain the ONM/ER expansion. To test for this possibility, we applied lipid mass spectrometry analysis to evaluate how Pgal1-induced overexpression of APQ12 and apq12-ah affected the composition of cellular lipids. Cells carrying the pGal1...
Construct were used as vector control. Samples were analysed at 0, 1 and 3 h of galactose addition (electronic supplementary material, figure S6A–D). Throughout the experiment, the vector control behaved in a similar manner to apq12-ah cells. Interestingly, P Gal1-APQ12-6His overexpression triggered an increase in DAG and TAG after 1 h of promoter induction (electronic supplementary material, figure S6A). PS and EE in the P Gal1-APQ12-6His sample was increased after 3 h induction. In addition, the number of two double bonds in GPL decreased in the P Gal1-APQ12-6His sample after 1 h but with an increase of one double bond after 3 h. Furthermore, P Gal1-APQ12-6His expression also affected the chain length of GPL after 3 h of induction compared to P Gal1-apq12-ah-6His induction (electronic supplementary material, figure S6B–E).

Figure 5. The AαH of Apq12 is important for membrane remodelling. (a) Inactivation of the AαH of Apq12 reduces overexpression toxicity. Growth test of cells containing P Gal1-apq12-ah, in comparison to P Gal1-APQ12 and vector control cells. Ten-fold serial dilutions of cells were spotted onto glucose (Glu) and galactose/raffinose (Gal/Raf) plates at 30°C. (b–e) Impact of APQ12 and apq12-ah overexpression on (b,c) yeGFP-BRL1 NUP85-tdTomato and (d,e) yeGFP-BRR6 NUP85-tdTomato cells. (c,e) Quantifications of the maximum GFP intensity of individual cells from (b) and (d) over time. Two independent experiments were performed, and more than 60 cells were analysed per experiment for each time point. Values are given as means ± s.d. t-test ****p < 0.0001. (b,d) Scale bars: 5 µm.
Thus, PGal1-APQ12-6His expression has a mild impact on the lipid composition of cells. Lipid mass spectrometry analysis did not enable us to make conclusions about local changes in lipid content, for example understanding whether the changes were restricted to one location such as the NE or cytoplasmic membrane systems. Using recently reported lipid sensors [8], we tested whether APQ12 overexpression affects PA accumulation at the INM or ONM, and compared the outcome of overproduction of the wild-type molecule with the impact of apq12-ah

Figure 6. Overexpression of APQ12 triggers hyper-proliferation of the ONM and ER. (a) EM analysis of WT cells expressing PGal1-APQ12-6His (left) or PGal1-apq12-ah-6His (right) by the addition of galactose for 0.5 h, 1 h and 3 h. Arrows are explained in the result section; red asterisks indicate ONMs encircled vesicles containing granular material. N, nucleus; R, ribosome-like particles. Scale bar: 200 nm. (b) Quantification of phenotypes from (a). n = 25 cells were analysed for each time point and strain. (c) PGal1-APQ12 was overexpressed for 3 h by the addition of galactose. Top panel: cells expressing yeGFP-Brl1 along with a nuclear marker NLS-mRFP. Bottom panel: cells expressing yeGFP tagged ribosomal subunit Rpl25 or containing the PGal1 control, and an ER luminal marker dsRED-HDEL. Arrows indicate strong yeGFP-Brl1 and dsRED-HDEL signals at the NE. Scale bar: 3 µm. (d) Electron micrograph showing ribosome-like particles trapped in vesicles formed upon overexpression of APQ12. Scale bar: 50 nm. (e) Model for the formation of membrane structures in response to APQ12 overexpression. See Results for details.
overexpression. These sensors use the PA recognizing domain of the *S. cerevisiae* transcription factor Opi1, with or without an NLS [8]. We used the cytoplasmic PA sensor Q2-mCherry and nuclear PA sensor NLS-Q2-mCherry for the analysis of PA changes at the ONM and INM, respectively. Consistent with published data [8], at $t = 0$ strong Q2-mCherry decoration of the cytoplasmic membrane (figure 7a,b) was accompanied by a weak Q2-mCherry nuclear signal. After 1 h and 3 h of $\text{PGal1-APQ12}$ induction, the Q2-mCherry signal accumulated in a rim-like pattern at the NE and co-localized with developing yeGFP-Brl1 clusters at the NE (figure 7a–c). In $\text{PGal1-apq12-ah}$ cells at $t = 0$, the Q2-mCherry reporter showed similar localization to $\text{PGal1-APQ12}$ cells (figure 7a) and induction for 1 h did not impact the localization of the Q2-mCherry sensor. After 3 h of induction the nuclear Q2-mCherry signal was nearly completely diminished without directing the sensor to the NE (figure 7a–c).

Most interestingly, the nuclear NLS-Q2-mCherry ($t = 0$) was recruited from a nuclear localization at $t = 0$ to a rim-like distribution at the NE 1–3 h after induction of $\text{PGal1-APQ12-6His}$ (figure 7d–f). In addition, NLS-Q2-mCherry strongly co-localized with yeGFP-Brl1 enrichments at the NE (figure 7d). By contrast, $\text{PGal1-apq12-ah}$ expression did not impact the nuclear localization of NLS-Q2-mCherry (figure 7d–f). Thus, the AαH of APQ12 is required to induce PA enrichment at the NE.

### 2.8. The AαH of Apq12 is not required for the association of the protein with herniations

The co-localization of Apq12 and apq12-ah with a small subset of NPCs (electronic supplementary material, figure S1D) raises the possibility that both proteins resemble Brl1 and Brr6 in mutants [26]. We used the cytoplasmic PA sensor Q2-mCherry and was therefore not tested. The Brr6-yeGFP level was increased in apq12-ah mutants compared to the WT (figure 9a–d). Analysis of the localization of Brl1-yeGFP and Brr6-yeGFP in apq12-ah mutants showed cellular distributions of both proteins that were reminiscent of those seen in WT (figure 9c,d). We next analysed whether the increase in protein levels of Brl1 and Brr6 in mutants is also reflected in Brl1–Brr6 interaction in co-IP experiments in which Brr6-yeGFP was immunoprecipitated with anti-GFP antibodies followed by the analysis of the immunoprecipitated proteins with Brl1 and HA antibodies. The Br6-yeGFP immunoprecipitation showed complex formation of Apq12 and Apq12-ah with Brl1 and Brr6 (figure 9f). Quantification of the immunoprecipitated proteins from three independent experiments showed that although Brr6-yeGFP precipitation efficiency was very similar, more Apq12 and Brl1 were co-immunoprecipitated from apq12-ah cells than from *APQ12* cells (figure 9g). Thus, a defect in AαH of Apq12 enhances the interaction between Brr6, Brl1 and Apq12.

### 2.9. Interaction of Brl1 and Brr6 is regulated by AαH of APQ12

Apq12 shows physical and genetic interactions with BRL1 and BRR6 [21,23,26]. A dysfunctional AαH could have an impact on Brl1 and Brr6 behaviour and their interaction with Apq12. Analysis of the Brl1 protein indicated an increase of Brl1 levels in *apq12Δ* and *apq12-ah* mutants (figure 9a,b) compared to WT. This increase was more pronounced in *apq12Δ* mutants (figure 9a,b). Because of the lack of Brr6 antibodies, we tested the abundance of Br6-yeGFP in WT, *apq12Δ* and *apq12-ah* mutants. *apq12Δ* showed lethality in combination with *BRR6-yeGFP* and was therefore not tested. The Br6-yeGFP level was increased in *apq12-ah* mutant compared to the WT (figure 9c,d). Analysis of the localization of Brl1-yeGFP and Brr6-yeGFP in *apq12-ah* mutants showed cellular distributions of both proteins that were reminiscent of those seen in WT (figure 9c,d). We next tested whether the increase in protein levels of Brl1 and Brr6 in mutants is also reflected in Brl1–Brr6 interaction in co-IP experiments in which Brr6-yeGFP was immunoprecipitated with anti-GFP antibodies followed by the analysis of the immunoprecipitated proteins with Brl1 and HA antibodies. The Br6-yeGFP immunoprecipitation showed complex formation of Apq12 and Apq12-ah with Brl1 and Brr6 (figure 9f). Quantification of the immunoprecipitated proteins from three independent experiments showed that although Brr6-yeGFP precipitation efficiency was very similar, more Apq12 and Brl1 were co-immunoprecipitated from *apq12-ah* cells than from *APQ12* cells (figure 9g). Thus, a defect in AαH of Apq12 enhances the interaction between Brr6, Brl1 and Apq12.

### 3. Discussion

Apq12 is important for NPC function and cooperates with the essential NPC biogenesis factors Brl1 and Brr6 [21,23,26]. These data indicate that Apq12 is at the heart of machinery that ensures NPC assembly, raising the important question about its molecular function. Because Apq12 does not have any sequence similarities to proteins with enzymatic activity, we have to assume that it functions either as a
protein scaffold or impacts the shape of the NE as reported for reticulons that bend the ER by two cooperating pairs of trans-membrane domains with an adjacent AαH [37,38]. Following the rationale that a combination of transmembrane domains and AαH could be the basis for the function of Apq12, we analysed Apq12 by the AmphipaSeeK program.

Figure 7. APQ12 overexpression induces AαH dependent PA accumulation at the NE. (a) Localization of the cytoplasmic PA sensors (Q2-mCherry) in response to PGal1-APQ12 and PGal1-apq12-ah induction at 0, 1 and 3 h. (b) Line scans of mCherry signal along the indicated lines across the cells in (a). Arrowheads mark the peaks corresponding to the cytoplasmic membrane (c) and NE (N). (c) Quantification of data from (a) showing percentage of cells with nuclear rim localization after 3 h of Gal induction. 100 cells carrying either PGal1-APQ12 or PGal1-apq12-ah from one representative experiment were analysed for nuclear rim localization of the PA sensor. (d) Localization of the nuclear PA sensors (NLS-Q2-mCherry) in response to PGal1-APQ12 (left) and PGal1-apq12-ah (right) induction at 0, 1 and 3 h. (e) Line scans of mCherry signal along the indicated lines across the nuclei in (d). Arrowheads mark the peaks corresponding to the NE (N). (f) Quantification of data from (d) showing percentage of cells with nuclear rim localization done as in (c). (a,d) Scale bars: 5 µm.
Figure 8. Apq12 associates with NPC biogenesis intermediates. (a) yeGFP-Apq12 localization in WT, td-brr6 and td-brl1 cells carrying the NPC marker NUP85-tdTomato. Cells were incubated for 0 h and 3 h with PGal1-UBR1 induction at 37°C. (b) Line scans along the NE of cells shown in the insets in (a). (c) Scatterplot with Pearson correlation coefficient (r) of GFP and mCherry fluorescence intensities (in arbitrary units, a.u.) along the nuclear rim of 5 cells. (d) yeGFP-Apq12-ah localization in WT, td-brr6 and td-brl1 cells carrying the NPC marker NUP85-tdTomato. Cells were incubated for 0 h and 3 h with PGal1-UBR1 induction at 37°C. (e) Line scans along the NE of cells shown in the insets in (d). (f) Scatterplot with Pearson correlation coefficient (r) of GFP and mCherry fluorescence intensities (in arbitrary units, a.u.) along the nuclear rim of 5 cells. (g, d) Scale bars: 5 µm. (g) td-brl1 and td-brr6 cells with yeGFP-APQ12 or yeGFP-APQ12-ah were incubated for 3 h under restrictive conditions (galactose, 37°C) to induce degradation of Brl1 and Brr6 and the formation of herniations. Fixed and embedded cells were analysed for localization of yeGFP-Apq12 and yeGFP-Apq12-ah by immuno-EM using GFP antibodies and 10 nm gold labelled protein A. Green and blue arrowheads indicate the localization of 10 nm gold particles. Scale bars: 100 nm.
Figure 9. Interaction of Brl1 and Brr6 is regulated by AαHo of APQ12. (a) Immunoblot showing endogenous levels of Brl1 in WT APQ12, apq12Δ and apq12-ah backgrounds, using anti-Brl1 antibody. Tub2 is loading control. (b) Quantification of Brl1 from (a) normalized to Tub2. Error bars are s.d., n = 3. **p < 0.01. (c) Immunoblot showing levels of Brr6-yeGFP in WT (APQ12) and apq12-ah backgrounds using anti-GFP antibody. Tub2 is loading control. (d) Quantification of Brr6 from (c) normalized to Tub2. Error bars are s.d., n = 3. ***p < 0.001. (e) Localization of Brr6-yeGFP and Brl1-yeGFP in WT APQ12 (left) and apq12-ah cells (right) and corresponding line scans along NE of cells shown in the insets. Scale bar: 5 µm. (f) Co-IP of Apq12, Brl1 and Brr6. Brr6-yeGFP was immunoprecipitated with GFP antibodies. Apq12-6HA was detected with anti-HA and Brl1 with Brl1 antibodies. (g) Quantification of the ratio between Apq12 and Brr6-yeGFP and, Brl1 and Brr6-yeGFP in WT APQ12 and apq12-ah backgrounds. Error bars are s.d., n = 3; t-test; *p < 0.05.
which predicted the presence of a short AoH connecting the two transmembrane domains. Consistent with this prediction, the synthetic AoH peptide binds to liposomes dependent on its amphipathic nature and the lipid composition. In addition, topological analysis showed that the AoH resides in the intermembrane space of the NE, while N- and C-terminal regions of Apq12 localize in the nucleus or cytoplasm depending on the INM or ONM localization of Apq12.

In terms of cold sensitivity, NE and NPC biogenesis defects, the apq12-ah mutant behaves similar to complete loss of APQ12 function (this study) [21,23,26]. However, phenotypic differences between apq12-ah and apq12Δ are also detectable (figure 3). In addition, Apq12-ah associates at least as efficiently with Br1l and Br6 as Apq12, has the same topological arrangement as Apq12 and associates with NPC assembly intermediates. This together indicates that apq12-ah is not a loss of function allele. The AoH is required for the overall function of Apq12. However, N- and C-termini of Apq12 fulfill functions without the involvement of AoH. This probably explains why prolonged overexpression of apq12-ah still shows a mild membrane deforming phenotype (figure 5).

apq12-ah and apq12Δ mutants impact cellular lipid composition, independent of the tested growth temperature, in a way that reduces membrane fluidity. This accounts for the cold sensitive growth defect and the NE breakdown phenotype at reduced growth temperatures. In addition, we observed the striking accumulation of PA either through synthesis or re-localization at the NE upon overexpression of APQ12, dependent on the functionality of the AoH. Before, it was shown that PA accumulates at sites of NPC mis-assembly [39] raising the question as to whether PA accumulation at the NE induced by P_{Gal1}-APQ12 overexpression is a consequence of defective NPCs. The observation that P_{Gal1}-APQ12 overexpression merely displaces NPCs into areas that lack ONM expansions without the accumulation of defective NPC assemblies (figure 4c; electronic supplementary material, figure S3A), indicates that overproduced Apq12 has the ability to induce PA accumulation at the NE even when NPCs are intact.

Previously we have shown that INM membrane deformations in td-brl1 and td-brr6 cells reflect emerging NPCs that do not fully assemble because of a defect in one of the steps leading to the generation of functional NPCs [12]. Using cells carrying td-brl1 and td-brr6 we observed strong accumulation of Apq12 with NPC biogenesis intermediates and this localization does not require a functional AoH. By contrast, Apq12 only co-localizes with a small number of NPCs in WT cells. Thus, Apq12 joins Br1l and Br6 [12] in a small subset of proteins required for NPC assembly that transiently interact with assembling NPCs but then dissociate from NPCs as soon as they are fully assembled.

Like Apq12, Br1l and Br6, the Lap2-emerin-MAN1 (LEM) family proteins Heh1 and Heh2 are also nonstructural components of NPCs. However, in contrast to Apq12, Br1l and Br6 that function directly in NPC biogenesis, Heh1 and Heh2 contribute to the surveillance of NPC biogenesis [40]. Upon NE damage caused by an NPC biogenesis defect, Chm7, a component of an ESCRT-III like complex, enters the nucleus where it is activated by nuclear Heh1 to promote the sealing of the disrupted NE by the ESCRT machinery [27,40]. In contrast to Heh1, Heh2 probably functions as a sensor for the assembly state of NPCs [41]. Thus, Apq12, Br1l, Br6, Heh1 and Heh2 fulfill quite distinct functions at NPCs.

Based on our findings and the observation that the role of the Apq12-Brl1/Brr6 module overlaps with that of the nucleoporin Nup116 in scaffolding NPC biogenesis [5,12], we suggest the following stages for the stepwise assembly of NPC assembly. The FG nucleoporin Nup116 was shown to scaffold NPC assembly together with Nup188 on the nuclear side of the INM [5,42]. Like Nup116, Apq12, Br1l and Br6 also associate with NPC intermediates [12,43]. The interaction of Br1l with the integral membrane protein Ndc1 [44] and Nup188 could be the trigger for the recruitment of this protein to assembling NPCs [12]. How Apq12 is recruited to NPC intermediates is not understood. The interacting Br1l and Br6 are not essential for this localization since Apq12 associates with herniations upon induced depletion of Br1l and Br6 (figure 8).

Apq12 probably executes multiple functions, some of which are at least partly mediated by its AoH at NPC biogenesis sites. First, Apq12 inserts through its membrane active AoH into the NE from within the intermembrane space (figure 1). This insertion has the potential to generate membrane curvature that may stabilize membrane deformations that arise during NPC biogenesis. AoH peptide did not deform GUVs under the experimental conditions we applied (figure 1). However, the yeast reticulon Yop1 only deforms liposomes in the context of transmembrane domains and AoH [37]. Thus, in future experiments, it will be important to measure the membrane-deforming ability of the TM-AoH-TM core of Apq12. Second, the enrichment of Apq12 at NPC biogenesis sites may promote local accumulation of PA as indicated by the APQ12 overexpression phenotype. Because of its conical shape, PA could stabilize bent membrane regions at INM bends during NPC biogenesis [45]. In addition, PA has the ability to interact with a range of proteins via short stretches of positively charged amino acid residues [46,47]. Presently, no Nup or NPC biogenesis factor with PA binding activity has been described. However, it was only until recently that the PA-binding activity of the ESCRTIII protein Chm7 was reported [39], raising the possibility that proteins involved in NPC assembly may carry hidden PA binding sites. Third, considering that Apq12 has an impact on Br1l and Br6 levels and the interaction of Apq12, Br1l and Br6 (figure 9), it may coordinate localization of the interacting Br1l and Br6 at NPCs biogenesis sites, a function that would be most important at elevated temperatures when Apq12 most strongly plays a role in NPC assembly. APQ12, BRL1 and NUP116 show genetic interactions and overexpression of BRL1 is able to suppress the NPC biogenesis defect of nup116Δ and nup116ΔGLFG PAH47-NUP188 mutants by promoting the fusion of the INM and ONM [5,12,26]. We therefore suggest that Br1l, in part scaffolded by Apq12, directly or indirectly, facilitates the fusion between the INM with the ONM during NPC biogenesis.

The Apq12-Brl1/Brr6 module is only conserved in organisms with closed mitosis [22]. However, considering the simple domain architecture of Apq12 with two TM domains and a short AoH, proteins with similar organization may substitute for the function of Apq12 in higher eukaryotes without having detectable amino acid homology to Apq12, rather a structural similarity with two closely opposed TM domains.
Br1l and Brr6 have an extended intermembrane space domain that is stabilized by two functionally important disulfide bridges [12,22]. This stabilization principal could be substituted for alternative structural features in functional equivalent proteins in higher eukaryotes.

4. Material and methods

4.1. Yeast strains and plasmids

Plasmids and yeast strains used in this study are listed in electronic supplementary material, table S1. All yeast strains have been derived from ESM356-1 (MATa ura3-52 trp1A63 his3a200 leu2A1). A PCR-based integration approach was used for epitope tagging of endogenous genes and gene deletions [48,49]. Yeast strains were cultured in SC (synthetic complete) medium, SC-selection medium [50], YPD (yeast extract, peptone and glucose) or YPRaf (yeast extract, peptone and raffinose) with or without 0.1 mM CuSO4 and grown at indicated temperatures. To induce extract, peptone and raffinose) with or without 0.1 mM YPD (yeast extract, peptone and glucose) or YPRaf (yeast extract, peptone and raffinose) with or without 0.1 mM CuSO4 and grown at indicated temperatures.

4.2. EM and immuno-EM analysis of yeast cells

High-pressure freezing, freeze substitution, sectioning, labeling and staining of cells was done as described [51]. Briefly, vacuum filtration and then high pressure freezing with an HPM010 (Abra-Fluid, Switzerland) was used to collect cells onto a 0.45 μm polycarbonate filter (Millipore), followed by freeze-substitution using the EM-AFS2 device (Leica Microsystems, Vienna, Austria) (0.1% glutaraldehyde, 0.2% uranyl acetate, 1% water—in anhydrous acetone) and infiltrated stepwise with Lowicryl HM20 (Polysciences, War- rington, PA), started at −90°C. The samples were gradually warmed up to 20°C for polymerization under UV exposure. Sectioning of embedded cells was done using a Reichert Ultracut S Microtome (Leica Instruments, Vienna, Austria) to a thickness of 80 nm. Post-staining with 3% uranyl acetate and lead citrate was performed. Sections were imaged at a Jeol JE-1400 (Jeol Ltd., Tokyo, Japan) operating at 80 kV equipped with a 4 k x 4 k digital camera (F416, TVIPS, Gauting, Germany). Micrographs were adjusted using ImageJ. For image processing and analysis, a 100×/1.4 NA UPlanSAPO objective (Olympus), a mercury arc light source and the soft- WoRx software (Applied Precision) was used for cell imaging. Imaging was done with exposure times depending on the fluorescence intensity of each protein. For time-lapse experiments, cells were immobilized on Concanavalin A (Sigma-Aldrich)—coated 35-mm glass bottomed dishes (P35G-1.5-14C; MatTek Corporation) and kept in their respective media. Images were deconvolved using the soft- WoRx software (Applied Precision) and processed with ImageJ (National Institutes of Health, Bethesda MD). For figure 5c,e, quantification of maximum intensity of individual cells was done by utilising CellProfiler 3.1.9 software (Broad Institute, Cambridge, MA) [52].

4.4. Lipid analysis by mass spectrometry

Cells (10 OD) were homogenized in a FastPrep machine (MP Biomedicals) in 155 mM ammonium bicarbonate buffer pH 7.5 and subjected to acidic Bligh&Dyer lipid extractions in the presence of internal lipid standards containing PC (phosphatidylcholine, 13 : 0/13 : 0, 14 : 0/14 : 0, 20 : 0/20 : 0; 21 : 0/21 : 0, Avanti Polar Lipids), PI (phosphatidylinositol, 17 : 0/20 : 0, Avanti Polar Lipids), PE and PS (phosphatidy- lethanolamine and phosphatidylserine, 14 : 1/14 : 1, 20 : 1/ 20 : 1, 22 : 1/22 : 1, semi-synthesized as described [53], DAG (diacylglycerol, 17 : 0/17 : 0, Laroadan), TAG (TAG, D5-TAG-Mix, LM-6000/D5-TAG-17 : 0/17 : 1, 17 : 1, Avanti Polar Lipids), PA (phosphatic acid, 17 : 0/20 : 4, Avanti Polar Lipids), PG (phosphatidylglycerol, 14 : 1/14 : 1, 20 : 1/20 : 1, 22 : 1/22 : 1, semi-synthesized [53], and Cer (ceramide, Avanti Polar Lipids). Lipids recovered after organic extraction were evaporated by a gentle stream of nitrogen. Extracted lipids were dissolved in 10 mM ammonium acetate in methanol and transferred to 96-well plates (Eppendorf twintec plate 96). 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 (Triverse Nano- mate, Advion Biosciences) as described [53]. The following precursor ion scanning (PREC) and neutral loss scanning (NL) modes were used for the measurement of the lipid classes: +PREC 184 (PC), +PREC282 (t-Cer), +NL141 (PE), +NL185 (PS), +NL277 (PI), +NL189 (PG), +NL115 (PA), +PREC 77 (ergosterol), +PREC379 (ergosterol ester). Ergos- terol was quantified following derivatization to ergosterol acetate in the presence of the internal standard (22E)-Stigmasta-5,7,22-trien-3-beta-ol (Aldrich, R202967) using 100 μl acetic anhydride/chloroform (1 : 12v/v) [54]. Data was analysed using LipidView (ABSciex) and an in-house developed software (ShinyLipids).

4.5. Liposome preparation

All lipids were received from Avanti Polar lipids with the exception of Atto647 N, which was obtained from Atto-Tec. The lipid composition of the PM mix consisted of: 34.8 mol% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 15 mol% 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), 20 mol% 1-hexadecanoyl-2-octadecenoyl-sn-glycero-3-phospho-ethanolamine (POPE), 25 mol% cholesterol (from ovine wool), 5 mol% liver L-α-phosphatidylinositol (PI, from liver) and 0.2 mol% Atto647N-DPPE. The composition of the used NE lipid mix was: 19.8 mol% POPC, 3 mol% DOPS,
42 mol% cholesterol, 7 mol% POPE, 23 mol% PI, 5 mol% PI(4,5)P₂ and 0.2 mol% Atto647 [55]. SUVs (small unilamellar vesicles) were formed as described previously [56] by dissolving the lipid mixtures in Octyl-β-D glucopyranoside (OG) containing buffer, OG dilution below the critical micellar concentration, flow dialysis and SUV isolation using Nycodenz-gradient centrifugation. Subsequently, the concentrated liposomes were extruded 23 times with a 100 nm filter and stored at 4°C.

For preparation of GUVs (giant unilamellar vesicles), the PM mix was used. SUVs were prepared to produce GUVs as described previously [57] with the following modifications: (I) the SUVs were desalted two times employing a PD10 column (GE Healthcare) instead of using a Sephadex-G50 gel filtration column in the second desalting step. (II) Platinum-coated glass slides (GeSiM) were applied instead of ITO-coated glass slides (GeSiM). The recovery of the total lipid was compared to the lipid after preparation.

4.6. Dynamic light scattering of the SUVs

DLS was applied to monitor the size of the liposomes. After adding 2 µM lipid (5 µl final volume) to the quartz cuvette, the particle size was determined in a DynaPor NanoStar (Wyatt Technologies) instrument at room temperature. The buffer composition for size determination was set to PBS (150 mM NaCl, 5 mM MgCl₂) at pH 8.0, 150 mM NaCl, 5 mM MgCl₂ – 10% glycerol, 1 tablet per 50 ml Roche protease inhibitor cocktail complete (EDTA free), and 1 mM β-glycerophosphate, 1 tablet per 50 ml Roche protease inhibitor cocktail complete (EDTA free), and 1 mM β-glycerophosphate. The data were normalized to the ratio of bound/unbound peptide and the KD value was determined according to the Hill equation,

\[ y = \frac{(c)^n}{(c)^n + K_D^n}, \]

where \( n \) = Hill slope.

4.7. Binding studies of SUVs

The binding of the AαH and AαH-ah peptides (from PSL Peptide Specialty Laboratories GmbH, Heidelberg; amino acid sequence: KLLMNFITLVKRFL, KLDMMNRNTLVKRNL) coupled with Atto488 (freshly solved in DMSO and diluted in 25 mM HEPES pH 7.4, 135 mM KCl, 1 mM DTT (fusion buffer)) with SUVs was measured by a Nycodenz gradient centrifugation. 0.45 mM lipid was incubated with 18 µM AαH or AαH-ah peptide or 18 µM Atto488 in 25 mM HEPES pH 7.4, 135 mM KCl, 1 mM DTT and 0.01% Triton X-100 and twice with wash buffer (20 mM Tris – Cl, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol) containing 10 mM NaF, 60 mM β-glycerophosphate, 1 tablet per 50 ml Roche protease inhibitor cocktail complete (EDTA free), and 1 mM PMFS. Lysis was done in a FastPrep machine (MP Biomedicals) by adding glass beads (BioSpec Products). 0.5% Triton X-100 was added to the cell lysate and incubated on ice for 10 min. The soluble proteins and cell debris were separated by centrifugation and incubated with GFP-Trap agarose beads (Chromotek) for 2 h 4°C. Beads were washed thrice with lysis buffer containing 0.1% Triton X-100 and twice with wash buffer (20 mM Tris – Cl, pH 8.0, 150 mM NaCl and 5 mM MgCl₂). Elution of bound proteins was done in 50 µl of 2 × SDS-PAGE sample buffer, heated for 5 min at 95°C, and then used for SDS-PAGE and western blotting.

4.8. Binding studies of GUVs

For the peptide interaction to GUVs, 3.3 µM GUVs were incubated with different amounts of peptide in a ratio of 1:1 to 10:1 in fusion buffer at room temperature for 15 min. As a control analysing the change of the GUV morphology 0.04% DMSO was added to the GUVs without peptide. The morphology of the GUVs was visible in the fluorescence of Atto488 and the interaction of the peptide by the fluorescent signal of Atto488. It was measured in a chambered coverslip (Ibidi, catalogue no. 80826) using a DeltaVision microscope.

4.9. MST

For determination of the KD values via MST 2 µM Atto488 coupled to AαH and AαH-ah peptide or Atto488 as a control was incubated 15 min at room temperature with increasing liposome concentrations in fusion buffer with 0.01% Triton x-100. All samples were filled in Premium capillaries and the thermophoresis was measured in a Monoloth NT.115 (NanoTemper) instrument with a LED power of 40% (green filter) and an MST power of 17%. The MST data were evaluated using a single exponential function in consideration of the initial state calculated by linear regression. The data were normalized to the ratio of bound/unbound peptide and the KD value was determined using the Hill equation,

\[ y = \frac{(c)^n}{(c)^n + K_D^n}, \]

where \( n \) = Hill slope.

4.10. Immunoprecipitation

25 OD of cells were harvested and resuspended in lysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, and 10% glycerol) containing 10 mM NaF, 60 mM β-glycerophosphate, 1 tablet per 50 ml Roche protease inhibitor cocktail complete (EDTA free), and 1 mM PMFS. Lysis was done in a FastPrep machine (MP Biomedicals) by adding glass beads (BioSpec Products). 0.5% Triton X-100 was added to the cell lysate and incubated on ice for 10 min. The soluble proteins and cell debris were separated by centrifugation and incubated with GFP-Trap agarose beads (Chromotek) for 2 h 4°C. Beads were washed thrice with lysis buffer containing 0.1% Triton X-100 and twice with wash buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl and 5 mM MgCl₂). Elution of bound proteins was done in 50 µl of 2 × SDS-PAGE sample buffer, heated for 5 min at 95°C, and then used for SDS-PAGE and western blotting.

4.11. Statistical analysis

For the statistical analyses, PRISM v.7 software (GraphPad) was used. Unpaired t-test with two-tailed p-value was used to compare samples. Normal data distribution was assumed but formally tested.

4.12. Antibodies

Antibodies and their conditions of use are as follows: mouse anti-His (western blot, 1:1000; 34660; Qiagen), anti-His (western blot, 1:1000; 34660; Qiagen),...
rabbit anti-Brl1 (western blot, 1:1000; made in-house), rabbit anti-Tub2 (western blot, 1:1000; made in-house) and rabbit anti-GFP (western blot, 1:1000; Proteintech), rabbit anti-HA (western blot, 1:500; Proteintech), rabbit anti-GFP (immuno-EM, 1:5; gift from M. Seedof, Zentrum für Molekularbiologie, Heidelberg, Germany).

Data accessibility. Additional data are provided in electronic supplementary material [59].

Authors’ contributions. Conceptualization, methodology and project administration were accomplished by E.S., W.Z. and A.K. Investigation and formal analysis were carried out by W.Z., A.K., J.V. and A.N. Data curation and visualization were done by A.K. Lipid analysis was performed by C.L. and B.B. Liposome binding experiments were carried out by K.R. and T.H.S. E.S. wrote the manuscript. W.Z. and A.K. critically revised and edited the manuscript. E.S. ensured funding acquisition and provision of all resources.

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