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Microautophagy of the Nucleus Coincides with a Vacuolar Diffusion Barrier at Nuclear–Vacuolar Junctions

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Nuclei bind yeast vacuoles via nucleus-vacuole (NV) junctions. Under nutrient restriction, NV junctions invaginate and release vesicles filled with nuclear material into vacuoles, resulting in piecemeal microautophagy of the nucleus (PMN). We show that the electrochemical gradient across the vacuolar membrane promotes invagination of NV junctions. Existing invaginations persist independently of the gradient, but final release of PMN vesicles requires again V-ATPase activity. We find that NV junctions form a diffusion barrier on the vacuolar membrane that excludes V-ATPase but is enriched in the VTC complex and accessible to other membrane-integral proteins. V-ATPase exclusion depends on the NV junction proteins Nvj1p,Vac8p, and the electrochemical gradient. It also depends on factors of lipid metabolism, such as the oxysterol binding protein Osh1p and the enoyl-CoA reductase Tsc13p, which are enriched in NV junctions, and on Lag1p and Fen1p. Our observations suggest that NV junctions form in two separable steps: Nvj1p and Vac8p suffice to establish contact between the two membranes. The electrochemical potential and lipid-modifying enzymes are needed to establish the vacuolar diffusion barrier, invaginate NV junctions, and form PMN vesicles.

INTRODUCTION

PMN is a selective autophagic process in yeast cells that targets parts of the nucleus for degradation. Like other autophagic processes PMN is induced by nitrogen or carbon starvation (Roberts *et al*., 2003; Levine and Klionsky, 2004). The response to starvation is regulated by the rapamycin-sensitive TORC1 complex (De Virgilio and Loewith, 2006). Because rapamycin treatment reproduces many characteristic features of starved cells, such as growth arrest and autophagy, this drug is frequently used to study autophagic processes and also PMN.

Nuclei and vacuoles closely associate via nucleus-vacuole (NV) junctions which involve interactions between the outer nuclear membrane protein Nvj1p and the vacuolar membrane protein Vac8p (Pan *et al*., 2000; Roberts *et al*., 2003). NV junctions invaginate toward the vacuolar lumen and evolve into a tear drop–like bleb which releases a vesicle into the vacuolar lumen. This vesicle contains nuclear material and is degraded inside vacuoles (Roberts *et al*., 2003). Using vacuolar degradation of an Nvj1-GFP fusion as a criterion, PMN was found to depend on numerous other proteins. Among these are core components of the autophagic and CVT machineries (Atg genes), phosphatidyl-inositol-3-kinase complex I, and components of the vacuolar fusion machinery (Krick *et al*., 2008). Deletion of Atg18 does not disrupt formation of NV junctions, suggesting that the Atg machinery might affect PMN at later stages. They might assist in the closure of the PMN vesicle, similarly as shown for their role in microautophagy of peroxisomes, where they are concentrated in the micropexophagy-specific membrane apparatus (Mukaiyama *et al*., 2004; Sakai *et al*., 2006).

NV junctions contain proteins involved in lipid biosynthesis and trafficking. One of these, the enoyl-CoA reductase Tsc13p, is an essential ER membrane protein that catalyzes the terminal step of very-long-chain fatty acid biosynthesis (Kohlwein *et al*., 2001). Pharmacological inhibition of fatty acid synthesis and elongation, as well as mutations in *TSC13*, decrease the size of PMN blebs and vesicles (Kvam *et al*., 2005). Osh1p is another protein related to lipid metabolism that is recruited to the ER and outer nuclear membrane by Nvj1p (Kvam and Goldfarb, 2004). Osh1p belongs to the oxysterol-binding protein (OSBP) family which has seven members in yeast (Beh *et al*., 2001; Levine and Munro, 2001). OSBPs are thought to mediate nonvesicular lipid trafficking, and some have been implicated in cell signaling (Levine, 2004). Yeast mutants lacking certain combinations of Osh proteins exhibit defects in endocytosis, vesicular transport, and altered vacuolar structure (Beh and Rine, 2004).

The vacuolar membrane contains a proton-pumping V-ATPase which is composed of the peripheral V_1 sector (subunits A–H) and the membrane-integral V_0 sector (subunits a, c, c′, c″, d, and e). ATP-hydrolysis by the V_1 sector drives proton translocation through V_0 (Nishi and Forgac, 2002). This acidifies the vacuolar lumen and generates an electrochemical potential over the vacuolar membrane (Kane, 2006). The low pH stimulates the activity of vacuolar hydrolases that are required to efficiently degrade macromolecules which are transferred into the vacuolar lumen via endocytosis, autophagy, and other trafficking routes. Besides their

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Table 1. Vph1p exclusion in various strains (% of Nop1-DsRed positive cells showing Vph1 exclusion next to the nucleolus)

Mutant	Function	PMN activity	Vph1p exclusion in HC	Vph1p exclusion in rapamycin
WT		100%	23%	46%
Datg7	Autophagy core machinery; conjugates Atg12p to Atg5p and Atg8p to phosphatidylethanolamine	0–20%	12%	51%
Δatg12	Autophagy core machinery; conjugated to Atg5p to form a complex involved in Atg8p lipidation	0–20%	20%	51%
Δatg18	Autophagy; binds phosphatidylinositol (3,5)-bisphosphate and phosphatidylinositol 3-phosphate	0–20%	16%	39%
Δypc1	Alkaline ceramidase that also has reverse (CoA-independent) ceramide synthase activity	100%	16%	38%
Δlag1	Ceramide synthase component; synthesis of ceramide from C26(acyl)-CoA and dihydrosphingosine or phytosphingosine, functionally equivalent to Lac1p	20–40%	9%	18%
Δlcb3	Long-chain base-1-phosphate phosphatase; regulates ceramide and long-chain base phosphates levels	40–60%	15%	29%
Δerg5	C-22 sterol desaturase; formation of the C-22(23) double bond in the sterol side chain in ergosterol biosynthesis	0–20%	8%	15%
Δfat1	Fatty acid transporter and very long-chain fatty acyl-CoA synthetase, may form a complex with Faa1p or Faa4p that imports and activates exogenous fatty acids	100%	18%	38%
Δscs7	Sphingolipid alpha-hydroxylase; alpha-hydroxylation of sphingolipid-associated very long chain fatty acids	40–60%	18%	31%
Δfen1	Fatty acid elongase, involved in sphingolipid biosynthesis; acts on fatty acids of up to 24 carbons in length; regulatory effects on 1,3-beta-glucan synthase, v-ATPase, and the secretory pathway	40–60%	18%	26%
Δsur4	Elongase; synthesizes very long chain fatty acids from C18-CoA primers; regulates sphingolipid synthesis	40–60%	19%	29%

crucial role in intracellular pH regulation, V-ATPases have been implicated in numerous vesicular trafficking steps, such as vacuole fusion in yeasts (Peters *et al*., 2001; Bayer *et al*., 2003), exocytosis of multivesicular bodies in worms (Liegeois *et al*., 2006), regulated fusion of synaptic vesicles in flies (Hiesinger *et al*., 2005), insulin secretion in mammalian cells (Sun-Wada *et al*., 2006), and phagosome-lysosome fusion (Peri and Nusslein-Volhard, 2008). In all these systems, membrane fusion requires physical presence of the V-ATPase complex, but not its proton translocation activity. Whereas V-ATPase pump activity is not essential for vacuole fusion, it is necessary for the opposing reaction of vacuole fragmentation. Therefore, it influences vacuole size and number that depend on the vacuolar fusion-fission equilibrium (Baars *et al*., 2007). The proton pump activity of V-ATPases can be inhibited by bafilomycin A and concanamycin A. These inhibitors bind to V_0 and are effective at nanomolar concentrations (Bowman and Bowman, 2002; Huss *et al*., 2002). The physical role of V-ATPases or V-ATPase subunits in vacuole fusion is not abolished by low concentrations of these inhibitors which suffice to completely suppress proton pump activity (Peters *et al*., 2001; Bayer *et al*., 2003; Muller *et al*., 2003).

We performed a screen for factors necessary for PMN (Dawaliby *et al*., in preparation) which identified numerous subunits of the V-ATPase, seven of the eight V_1 sector subunits (A, C, D, E, F, G, and H) and the V_0 sector subunits Vph1 (subunit a) and Vma3 (subunit c). Therefore, we explored the role of the V-ATPase in PMN in detail. We analyzed which steps of PMN depend on V-ATPase activity and, in testing V-ATPase localization during PMN, we discovered a novel diffusion barrier at NV junction sites.

MATERIALS AND METHODS

Yeast Strains and Plasmids

Yeast strains used are listed in Table 2, and the primers used for generating mutations are listed in Table 3. BJ3505 is a deletion strain for Pep4 and Prb1 (Jones *et al*., 1982). Nvj1 was deleted in BJ3505 by one step PCR-mediated gene disruption with *natNT2* cassette from the pFA6a-natNT2 plasmid (Janke *et al*., 2004). Vph1-$(Gly)_6$-eGFP (*kanMX*) was generated by genomic integration of a PCR fragment coding for EGFP and the auxotrophic marker using the pYM27 plasmid (Janke *et al*., 2004). N-terminal tagging of Nvj1 was performed similarly using pYM-N16 and pYM-N17 plasmids, respectively (Janke *et al*., 2004), creating BJ3505 pGPD-eGFP-Nvj1 (*natNT2*) and BJ 3505 pGPD-HA-Nvj1 (*natNT2*). Nvj1-eCFP and DsRed-Nop1 strains were obtained by transformation of yeast cells with p416-pADH-Nvj1-eCFP and pRS314-pNopDsRed-Nop1 (Gadal *et al*., 2001).

BJ3505 pGPD-HA-Nvj1 Vph1-GFP and BJ3505 pGPD-eGFP-Nvj1 Vph1-GFP resulted from transformation of p416-pVph1-GFP into the corresponding strains. Strains BY4741 and BY4741 Δvph1 were purchased from Euroscarf. BY4741 GFP-Nop1, BY4741 Δvph1 GFP-Nop1 and BY4741 Δvph1 GFP-Nop1 were obtained by transforming BY4741 and BY4741 Δvph1 with pRS315-pNop-GFP-Nop1.

Plasmid p416-pADH-Nvj1-eCFP was obtained by amplifying the Nvj1-eCFP cassette from genomic DNA and inserting it into p416-ADH using BamHI and HindIII restriction sites.

Plasmid p416-pVph1-Vph1-GFP was generated first by integrating the endogenous Vph1 promoter between XbaI and SacI restriction sites into pRS416. Genomically tagged Vph1-GFP was amplified by PCR and inserted into the resulting p416pVph1 plasmid using SpeI and SmaI restriction sites. pRS314-pNop-DsRedNop1 and pRS315-pNop-GFP-Nop1 were kindly provided by Ed Hurt.

General Methods

Yeast strains were grown (30°C, 150 rpm) in HC medium or in HC dropout medium lacking an amino acid or uracil corresponding to the auxotrophic marker of the plasmid carried by the strain. Cells were harvested by centrifugation at an OD_{600} of 0.4–0.8. Precultures were grown under the same conditions to saturation.

Reagents

Rapamycin, concanamycin A, bafilomycin A_1, and oligomycin were purchased from Alexis Biochemicals (Plymouth Meeting, PA), PMSF from

Table 2. Yeast strains used in the study

Yeast strain	Genotype	Source or reference
BJ3505	MATa pep4::HIS3 prb1-Δ1.6R lys2-208 trp1-Δ101 ura3-52 gal2 can	(Jones *et al.*, 1982)
BJ3505 Δnvj1	BJ3505, nvj1::natNT2	This study
BJ3505 Δnvj1 Vph1-GFP Vac8-YFP DsRed-Nop1	BJ3505 nvj1Δ, Vph1-6gly-GFP-G418, pRS314-pNop- DsRed-Nop1 (TRP)	This study
BJ3505 Vph1-6gly-GFP	BJ3505, Vph1-6gly-GFP-G418	(Peters *et al.*, 2001)
BJ3505 Vph1-6gly-GFP DsRed-Nop1	BJ3505 Vph1-6gly-GFP, pRS314-pNop-DsRed-Nop1 (TRP)	This study
BJ3505 Vph1-6gly-GFP Nvj1-eCFP	BJ3505 Vph1-6gly-GFP, p416-pADH-Nvj1-eCFP	This study
BJ3505 Vph1-6gly-GFP DsRed-Nop1 Nvj1-eCFP	BJ3505 Vph1-6gly-GFP DsRed-Nop1, p416-pADH-Nvj1-eCFP (URA)	This study
BJ Vac8-eYFP	BJ3505, Vac8-eYFP-G418/hphNT1	This study
BJ pGPD-eGFP-Nvj1	BJ3505, pGPD-eGFP-Nvj1-natNT2	This study
BJ pGPD-HA-Nvj1	BJ3505, pGPD-HA-Nvj1-natNT2	This study
BJ pGPD-HA-Nvj1 DsRed-Nop1 Vph1-GFP	BJ pGPD-HA-Nvj1, pRS314-pNop-DsRed-Nop1, p416-pVph1-Vph1-GFP (URA)	This study
BY4742 GFP-Vtc1 DsRed-Nop1	BY4742 GFP-Vtc1p-Nat, pRS316-pNop-DsRed-Nop1 (URA)	This study
BY4742 GFP-Vtc4 DsRed-Nop1	BY4742 GFP-Vtc4p-Nat, pRS316-pNop-DsRed-Nop1 (URA)	This study
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	(Brachmann *et al.*, 1998)
BY4741 Nop1-GFP	BY4741, pRS315-pNop-GFP-Nop1 (LEU)	This study
BY4741 Δvma1 Nop1-GFP	BY4741 vma1::kanMX (euroscarf), pRS315-pNop-GFP-Nop1 (LEU)	This study

Roche (Indianapolis, IN), FM4-64 (Synaptored TMC2) from VWR (Darmstadt, Germany), ClonNAT (nourseothricin) from Werner BioAgents (Jena, Germany), G418 sulfate from Calbiochem (La Jolla, CA) and low melting point agarose from Sigma-Aldrich (St. Louis, MO).

PMN Induction and Addition of Inhibitors

Cells were shaken overnight at 30°C in logarithmic phase ($OD_{600} < 1$) in HC or HC dropout medium lacking an amino acid or uracil, corresponding to the auxotrophic marker of the plasmid carried by the strain. Cultures were adjusted to $OD_{600} = 0.5$ and 0.2 μM rapamycin was added from a 20 μM stock in DMSO. Cells were incubated 3h at 30°C. For strains that did not carry a *PEP4* deletion, 1 mM PMSF was added to the culture from a freshly prepared 200 mM stock in 70% ethanol every hour. Where indicated, 1 μM concanamycin A, bafilomycin A_1, or 5 μM oligomycin were added after 2h of rapamycin treatment and the cells incubated for one more hour. Alternatively, rapamycin and one of the other inhibitors were added simultaneously and the cells incubated for 3 h.

FM4-64 Staining

To visualize vacuolar membranes in vivo, cells were stained with the lipophilic dye N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl)-pyridinium dibromide (FM4-64) as previously described (Müller *et al.*, 2000). Cells were grown overnight at 30°C to logarithmic phase ($OD600 < 1$) in HC or selective medium. Cultures were adjusted to OD600 = 0.4, and 10 μM FM4-64 was added from a 10 mM stock solution in DMSO. Cells were incubated for 1 h at 30°C, harvested (1 min, 3000 × *g*, RT), washed twice in fresh medium, resuspended in medium to OD600 = 0.4, and shaken for 1 h at 30°C. Further treatment of the cells with inhibitors was begun after this chase period.

Assay of PMN

PMN was monitored via the transfer of nucleolar material to vacuoles. Cells expressing the nucleolar marker Nop1p-GFP were stained with FM4-64. Cells were pelleted (15 s, 8000 × *g*, room temperature), resuspended in medium to OD600 = 5, supplemented with 0.2% low melting point agarose from a 1% stock solution in water (kept liquid at 40°C), and immediately analyzed by confocal microscopy using an LSM 510 microscope (Zeiss) with a ×100 Zeiss Plan Apochromat objective (numerical aperture 1.4). Excitation wavelengths of 488 nm for GFP and of 543 nm for FM4-64 were used. Alternatively, a standard fluorescence microscope (Leica DMI6000) with a Hamamatsu Orca R2 camera was used, equipped with a ×100, 1.4 NA lens

Table 3. Primers used in the study

Purpose	Forward primer	Reverse primer
Nvj1 disruption by *natNT2* cassette from pFA6a-natNT2	TCGTGGAATCTTTTCGTTAGGTCTTTCTGTA GCCGTTTTGCGTACGCTGCAGGTCGAC	CATGAGAGTATGCTTGTTCAGTGTTCACTT GTGCATCTAGATATCGATGAATTCGAGCTCG
Nvj1 N-terminal HA or GFP genomic tag	ATATCAAAAAAGCTACAAATATATAATTGTAAA ATATAATAAGCATGCGTACGCTGCAGGTCGAC	ACCTAACGAAAAGATTCCACGAACC AATGGGGGAGTCATCGATGAATTCTCTGTCG
Nvj1-eCFP C-ter genomic tag	GCACAAGTGAACACTGAACAAGCATACTCTCA ACCATTTAGATACCGTACGCTGCAGGTCGAC	TGTAAGTGACGATGATAACCGAGATGACGG AAATATAGTACATTAATCGATGAATTCGAGCTCG
Nvj1-eCFP amplification from genome for insertion in p416-pADH-Nvj1-eCFP	CGCGGATCCATGACTCGTCCCCCATTGGTTCGTGGAATC	GGGAAGCTTTTACTTGTA CAGCTCGTCCATGCCGAGAGT
Vph1-6-Gly-GFP C-ter genomic tag	GACATGGAAGTCGCTGTTGCTAGTGCAAGCTCTTCC GCTTCAAGCGGTGGTGGTGGTGGTGGTATGTCTAAA	GGTGGATTGGATTGCAAGTCTAACGTTTTC ATGAGATAAGTTTGGGCATAGGCACTAGTGGATCT
Vph1-6 Gly -GFP amplification from genome for insertion in p416-pVph1-Vph1-GFP	CGCCGACCAGAGGTATTTAGAAGTG	GCGCTCTAGATATTTTCCTTGTGTAACC

and driven by Volocity software. The number of cells carrying both PMN structures and GFP stained vesicles in their vacuolar lumen was counted.

Statistical Analysis

To quantify PMN, NV junctions, or exclusion sites, photos of at least 10 random fields were taken for each condition. At least 200 cells showing the respective markers were evaluated per condition and experiment. Data from three or more independent experiments was averaged, standard deviations were calculated and are shown as error bars.

Vph1 Exclusion Analysis

Cells were grown and analyzed as for the assay of PMN. Photos of at least 10 random fields were taken for each condition. The number of cells, of 200 cells per condition, presenting Vph1p exclusion sites in the vicinity of Nop1p and/or Nvj1p was counted. For random exclusion of Vph1p we scored 200 cells per condition for Vph1p exclusion sites that were not adjacent to Nop1p. Data from three or more independent experiments was averaged and the corresponding standard deviations calculated.

RESULTS

PMN Requires V-ATPase Pumping Activity

Screening of the yeast deletion collection for mutants defective in PMN has identified many V-ATPase subunits (Dawaliby *et al*., in preparation). Because an increasing number of V-ATPase activities become apparent that do not depend on the H^+ pump function of the enzyme, we tested the effect of acute pharmacological inhibition of V-ATPase activity. We investigated the effect of concanamycin A and bafilomycin A, two potent and highly specific inhibitors that leave the V-ATPase physically intact yet abolish its pumping activity. PMN was measured by confocal fluorescence microscopy, using the transfer of the nucleolar marker GFP-Nop1p into vesicular or tubular structures inside vacuoles (Figure 1A). We quantified the fraction of cells whose vacuoles contained PMN vesicles or structures bearing the fluorescent nucleolar marker. Under normal growth conditions cells showed only basal PMN activity, whereas treatment with rapamycin induced PMN fivefold (Figure 1B) (Roberts *et al*., 2003). When V-ATPase inhibitors were added to the medium at the time of induction of PMN, PMN was reduced to the levels observed in noninduced control cells (Figure 1, A and B). Oligomycin, an inhibitor of the mitochondrial F-ATPase applied in the same solvent, did not affect PMN but the protonophore FCCP inhibited PMN as potently as the V-ATPase inhibitors (Figure 1A). The same effects were observed when Nvj1p-GFP was used as a marker for PMN activity (not shown). Analysis of PMN in knockout mutants of the V_1 sector subunit Vma1 confirmed these results. PMN was observed in only 15% of the induced Δvma1 cells but in more than 50% of the wild-type cells (Figure 2). This suggests that the pumping activity of the V-ATPase is necessary for efficient PMN.

V-ATPase Pump Activity Affects Two Steps of PMN

Because PMN can be microscopically subdivided into the stages of membrane association, membrane invagination, bleb formation, and final release of a vesicle (Kvam and Goldfarb, 2006b), we tried to determine which of these steps depends on V-ATPase activity. We quantified the influence of concanamycin A when added together with the induction of PMN by rapamycin and, for comparison, when added after an initial phase of induction (2h). At this time, most invaginations had already been formed and further incubation until the end of the 3h incubation period can then serve to test the persistence of these structures in the absence of V-ATPase pump activity. If V-ATPase activity were only required for an initial step of PMN, the process should acquire resistance to V-ATPase inhibition once this early

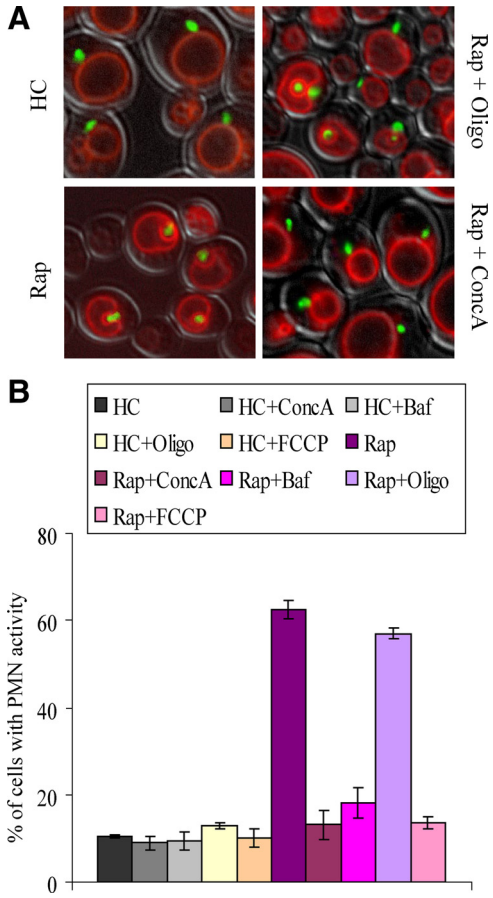

Figure 1. The V-ATPase inhibitor concanamycin A reduces PMN. (A) Wild-type cells expressing GFP-Nop1 under its endogenous promotor were stained with FM4-64 in HC media to visualize vacuoles. Cells were then incubated for a total of 3 h with 0.2 μM rapamycin and/or 1 μM concanamycin A, 5 μM oligomycin, or 5 μM FCCP. Cells were analyzed by fluorescence microscopy. (B) The percentage of cells carrying PMN vesicles or PMN structures was determined for each condition. For each experiment, 200 cells per condition were analyzed by confocal microscopy. Three independent experiments were averaged and standard deviations calculated.

step has been completed. To distinguish early and late phases of PMN we differentiated between cells showing PMN vesicles and cells showing only PMN structures (i.e., NV junctions and their invaginations into the vacuolar lumen). Use of concanamycin A from the beginning of the rapamycin treatment decreased the frequency of PMN structures and PMN vesicles equally (Figure 3). When added two hours after rapamycin, concanamycin A reduced the fraction of cells with PMN vesicles to the same low levels as observed in the samples that had received concanamycin A from the beginning (Figure 3B). By contrast, the frequency of PMN structures remained significantly higher, suggesting that PMN structures remained relatively stable after inactivation of V-ATPase. Thus, V-ATPase activity appears to contribute to two different steps of PMN. First, it is needed to form invaginations at NV junctions. Once formed, the PMN structures persist even after inhibition of V-ATPase. However, the scission of vesicles from these structures into the vacuolar lumen remains dependent on V-ATPase activity. This suggests that invagination and vesicle formation can be uncoupled.

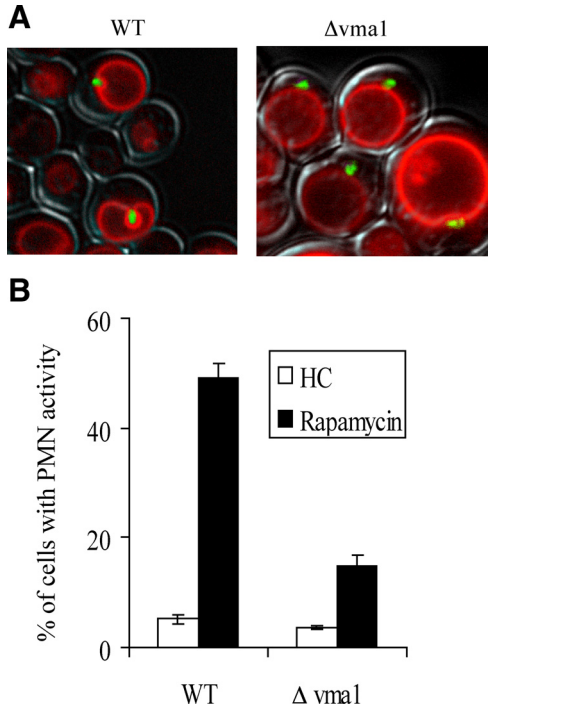

Figure 2. V-ATPase cells are defective in PMN. (A) Wild-type or Δvma1 cells expressing GFP-Nop1p under its endogenous promotor were stained with FM4-64 in HC media to visualize vacuoles. Cells were then treated with 0.2 μM rapamycin, incubated for a total of 3 h, and analyzed by fluorescence microscopy. (B) Cells carrying PMN vesicles or PMN structures were counted as in Figure 1.

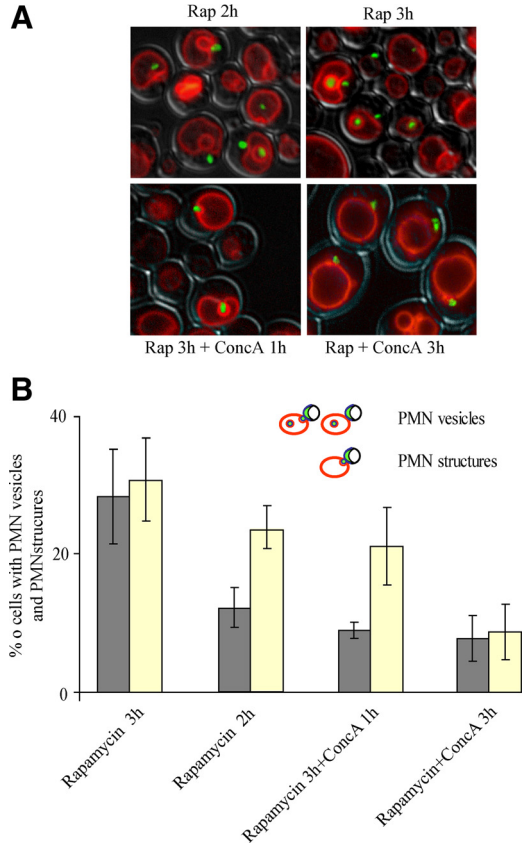

Figure 3. Effect of concanamycin A on PMN structures and vesicles, depending on the time of addition. (A) Cells expressing GFP-Nop1 under its endogenous promotor were stained with FM4-64 in HC and were then treated for a total of 3 h as follows: With 0.2 μM rapamycin for 3 h; with rapamycin and concanamycin A for 3 h; with rapamycin for 2 h before adding 1μM concanamycin A and continuing the incubation for another 1h; with rapamycin for 2h. (B) For PMN activity the number of cells with PMN vesicles or PMN structures inside the vacuoles was counted. The scheme illustrates the different PMN phenotypes monitored.

NV Junctions Form a Diffusion Barrier at the Vacuolar Membrane

To further investigate the role of the V-ATPase in PMN we analyzed the localization of a GFP-tagged version of the a-subunit (Vph1p) of the V-ATPase V_0 sector (Peters *et al.*, 2001), the nucleolar marker DsRed-Nop1p, and Nvj1p-eCFP. The tagged proteins are functional because the cells were viable and showed normal growth and PMN activity. They did not display phenotypes associated with loss of V-ATPase activity, such as inviability at pH 7.5, diminished quinacrine accumulation, or defective vacuole fragmentation upon hypertonic shock (Peters *et al.*, 2001; Baars *et al.*, 2007; Ryan *et al.*, 2008). The cells were grown in rich Hartwell's synthetic complete (HC) medium and analyzed by fluorescence microscopy. Vph1p-GFP was homogeneously distributed over the vacuolar rim in >80% of the cells that showed Nvj1p-eCFP in the optical section. On PMN induction by rapamycin, the distribution of Vph1-GFP changed strikingly. The protein became excluded from sharply defined stretches of the vacuolar membrane. These sites faced the nucleolar marker DsRed-Nop1p and the NV junction marker Nvj1p (Figure 4). 50% of cells in which DsRed-Nop1 was visible in the optical section showed an exclusion of Vph1p-GFP from the adjacent vacuolar membrane. These zones of Vph1p exclusion corresponded in all cases to Nvj1p-eCFP localization. Experiments with a GFP-fusion of the V_1 subunit B (Vma2) yielded similar results, suggesting that the effect concerns the entire V-ATPase rather than only a specific subunit (Figure 4B; Supplemental Figure 1). In Δnvj1 background, exclusion of Vph1p was observed with the same low frequency as in wild-type cells that were noninduced, treated with concanamycin A or FCCP (Figure 5). Thus, the induction of PMN and the increase in the number of NV junctions, which depend on Nvj1p, correlate to the exclusion of V-ATPase from vacuolar membrane patches facing the nucleus. This suggests that NV junctions might form a diffusion barrier that prevents access of V-ATPase to this region.

To test whether there is selectivity in the exclusion from NV junctions we analyzed GFP fusions of other vacuolar membrane proteins. As a small protein we chose alkaline phosphatase (Pho8p) which is entirely oriented toward the vacuolar lumen. The Pho8p fusion protein exposes only its GFP tag to the cytosol (Cowles *et al.*, 1997). As a complex that exposes large hydrophilic domains to the cytosol we chose the VTC complex. VTC is a vacuolar polyphosphate polymerase of approx. 500 kDa that is completely oriented toward the cytosol. It can be labeled by N-terminal tagging of Vtc4p or Vtc1p (Muller *et al.*, 2003; Uttenweiler *et al.*, 2007; Hothorn *et al.*, 2009). The cytoplasmic parts of VTC are of similar molecular mass as those of V-ATPase. In contrast to Vph1p-GFP, GFP-Pho8p was homogeneously distributed over the vacuoles in presence and absence of rapamycin (Figure 6). Likewise, GFP fusions of the vacuolar transporters Zrc1p and Ycf1p and of the vacuolar SNARE Vam3p were not excluded from NV junctions (Figure 6B; Supple-

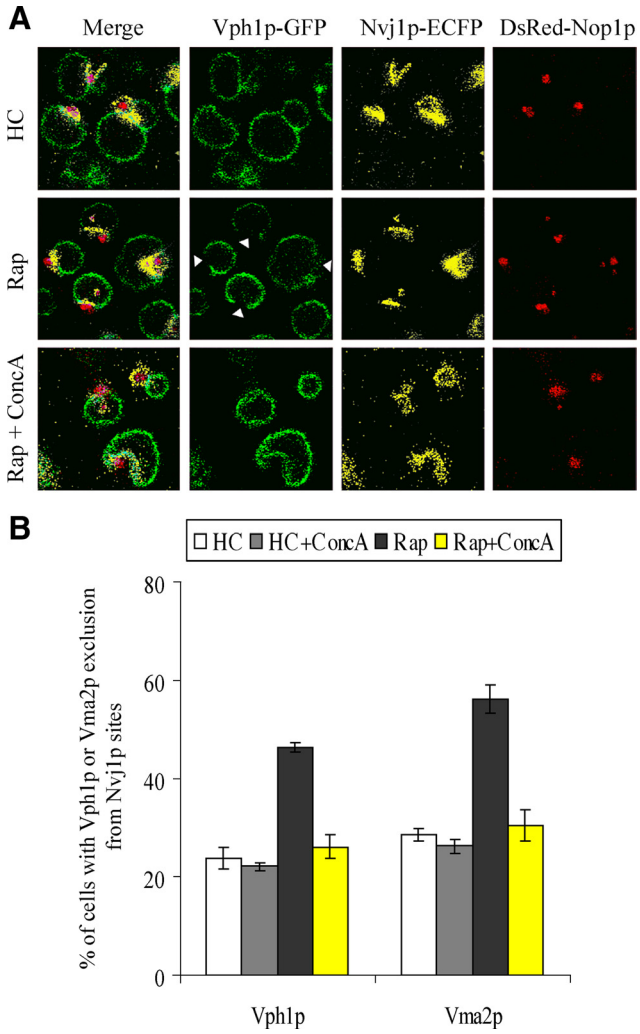

Figure 4. Vph1p exclusion is abolished by concanamycin A. (A) Wild-type yeast cells carrying Vph1p-GFP, DsRed-Nop1p, and Nvj1p-ECFP were treated in HC medium with 0.2 μM rapamycin and/or 1 μM concanamycin A for 3 h, or left untreated. Samples were analyzed by confocal microscopy. Arrowheads show Vph1p exclusion sites. (B) The percentage of wild-type cells in A that presented Vph1p exclusion from Nvj1p-CFP–positive sites was quantified. In a parallel experiment, we performed the same quantifications for a strain carrying Vma2-GFP instead of Vph1-GFP.

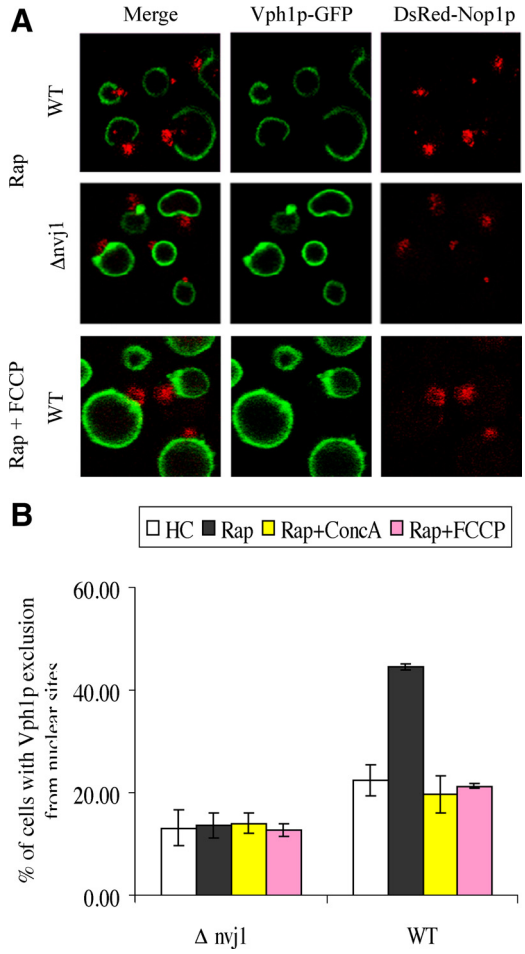

Figure 5. Vph1p exclusion depends on Nvj1. (A) Wild-type and Δnvj1 mutants carrying Vph1p-GFP and DsRed-Nop1p were treated with 0.2 μM rapamycin for 3 h. Samples were analyzed by confocal microscopy. (B) Wild-type cells carrying Vph1p-GFP and DsRed-Nop1p were treated together with 0.2 μM rapamycin and 5 μM FCCP for 3 h. Samples were analyzed by confocal microscopy. (C) The percentage of wild-type and Δnvj1 cells presenting Vph1p exclusion at DsRed-Nop1p-positive sites was determined.

mental Figure 2). The exclusion sites were also accessible to the fluorescent lipid dye FM4-64, which inserts into the vacuolar boundary membrane (Supplemental Figure 2). GFP-Vtc1p was even concentrated at NV junctions and PMN structures (Figure 6, A and B). 55% of the cells accumulated GFP-Vtc1p at NV junctions upon rapamycin treatment while only 24% of the untreated cells showed similar accumulation. GFP-Vtc4p fusions behaved similarly. Quantification with ImageJ revealed that the GFP-Vtc fluorescence intensity at NV junction sites was more than twofold higher than in other parts of the vacuolar membrane of rapamycin-treated cells. These observations suggest that the exclusion zone is continuous with the vacuolar membrane and that exclusion of proteins from PMN sites is not general but selective for Vph1p. It is unlikely to be a simple consequence of the size of the protein parts exposed to the cytosol because this parameter is similar for the VTC and V-ATPase complexes, yet only V-ATPase shows exclusion. In line with a previous proposal (Kvam *et al.*, 2005) we speculate that NV junctions might induce a phase separation in the plane of the vacuolar membrane, creating a membrane zone that favors enrichment of certain proteins while providing an unfavorable environment for others.

Vph1p Exclusion from NV Junctions Depends on the Electrochemical Potential of the Vacuole

Because V-ATPase activity promotes PMN, we tested whether H^+ pumping influenced the Vph1p diffusion barrier. We investigated the effect of concanamycin A on NV junction frequency and Vph1p-GFP exclusion in cells carrying DsRed-Nop1p and Nvj1p-eCFP (Figure 7). In rapamycin-treated cells, concanamycin A decreased the percentage of cells presenting Vph1p exclusion and the percentage of cells with PMN structures to values seen in the absence of PMN induction. By contrast, Nvj1p patch frequency was only slightly affected. These data suggest that NV junctions form a diffusion barrier in the vacuolar membrane that is promoted by the electrochemical potential of the vacuole.

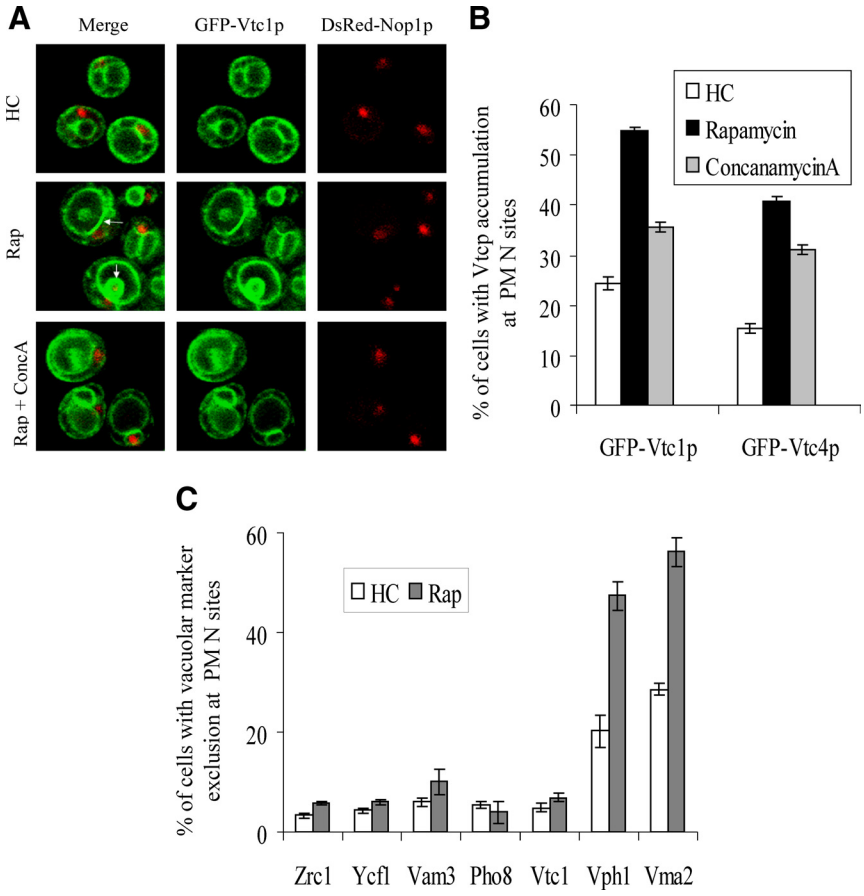

Figure 6. Presence of vacuolar proteins at PMN sites. (A) Wild-type cells expressing DsRed-Nop1 and GFP-Vtc1p or GFP-Vtc4p were incubated in HC medium with or without 0.2 μM rapamycin for 3 h before being analyzed by confocal microscopy. Only GFP-Vtc1p pictures are shown as examples. (B) Quantification of DsRed-Nop1p–positive sites showing an enrichment of GFP-Vtc1p or GFP-Vtc4p when compared with the rest of the vacuolar boundary membrane. (C) Cells expressing DsRed-Nop1 and GFP fusions of the indicated proteins were incubated and analyzed as in A. The frequency of cells showing exclusion of the respective GFP fusion from DsRed-Nop1–positive sites was determined.

V-ATPase Exclusion Is Independent of PMN and Recruitment of the Nucleolus to NV Junctions

In the course of a screening project (Dawaliby *et al*., manuscript in preparation) we noted that the nucleolus is preferentially degraded by PMN, consistent with electron microscopic observations that suggested that the nucleolus is often present in PMN structures (Roberts *et al*., 2003).We hence tested whether some characteristics of Vph1p exclusion are distinct from those of PMN. We analyzed whether the nucleolus needs to be present at a NV junction and whether components of the Atg machinery, which are also involved in PMN (Krick *et al*., 2008), are required for Vph1p exclusion. Deletion mutants in core components of the autophagy machinery, Δatg12 and Δatg7, showed normal levels of Vph1p exclusion but strong defects in PMN (Table 1; Supplemental Figure 4). To test whether the nucleolus or other nuclear material needs to be positioned next to the NV junction to induce Vph1p exclusion we exploited the observation that N-terminal tagging and overexpression of Nvj1p creates Nvj1p patches not only at NV junctions but also along ER-like cytoplasmic membranes. This produces a surplus of ectopic patches of Nvj1p which do not face the nucleus. (Kvam and Goldfarb, 2006b). We tested Vph1p-GFP exclusion relative to the nucleolus in cells that carried the nucleolar marker Nop1p-DsRed and overexpressed Nvj1p from the strong GPD promoter (Figure 8, A and B). We separately scored Vph1p exclusion at sites distant from NV junctions. We refer to those as random exclusion sites, in contrast to restricted exclusion sites that are adjacent to the nucleus. Restricted exclusion of Vph1p did not increase by overexpression of Nvj1p. Upon rapamycin-induction, however, their frequency increased up to threefold, confirming that Nvj1p overexpression alone does not determine the full onset of PMN (Figure 8B). Nvj1 overexpression increased the frequency of random exclusion from 4 to 20% in rapamycin-treated cells, indicating that an exclusion site can form in the absence of nuclear material. Also this increase was rapamycin-sensitive, suggesting that both restricted and random exclusion sites may be under the control of TORC1 signaling. In a similar experiment we tested the morphological relationship of ectopic Nvj1p patches to the exclusion sites in strain that carried Vph1p-CFP and overexpressed GFP-Nvj1p (Figure 8, C and D). The random exclusion sites were always adjacent to a patch of GFP-Nvj1p. In sum, these experiments suggest that ectopic patches of Nvj1p suffice to induce phase separation on the vacuolar membrane. Thus, Vph1 exclusion is a membrane differentiation that arises already from an association of the ER and the vacuolar membrane. It is independent of the recruitment of nuclear or nucleolar material to this site and can be distinguished from PMN by its independence of the autophagy core machinery.

Diffusion Barrier Formation at NV Junctions Depends on Lipid Metabolism

Two observations link PMN to lipid metabolism. First, oxysterol-binding (OSH) proteins are necessary for PMN (Kvam and Goldfarb, 2004) and are enriched at NV junctions. Second, Tsc13p, which is involved in the synthesis of very long chain fatty acids and sphingolipids, is found in PMN blebs and influences the size of PMN vesicles (Kvam *et al*., 2005). This suggests that NV junctions might coincide with and depend on a special lipid composition. Because

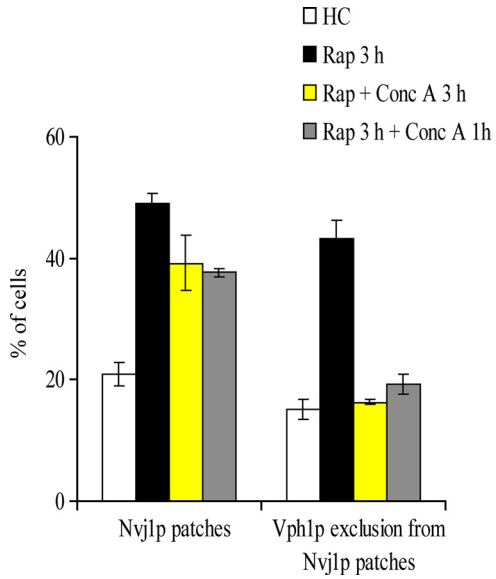

Figure 7. Concanamycin A inhibits Vph1p exclusion but not formation of Nvj1p patches. Wild-type cells carrying Vph1p-GFP and Nvj1p-CFP had been stained with FM4-64 in HC and were then treated for a total of 3 h as follows: With 0.2 μM rapamycin for 3 h; with rapamycin for 2 h before adding 1 μM concanamycin A and continuing the incubation for another 1 h; with rapamycin and concanamycin A for 3 h. Samples were analyzed by confocal microscopy. The percentage of cells showing Nvj1p patches adjacent to vacuoles and the percentage of cells showing Vph1p exclusion next to Nvj1p patches was determined.

such lateral heterogeneity of the vacuolar membrane might underlie the exclusion of Vph1p from NV junctions we tested several mutants of lipid metabolism for their effect on Vph1p exclusion (Table 1). Δosh1 and Δosh2 mutants showed a phenotype very similar to the one observed in Δnvj1 mutants (i.e., strongly reduced Vph1p-GFP exclusion in rapamycin-treated cells) (Supplemental Figure 3). Because Tsc13 is essential and a conditional mutant was not available, we tested nonlethal mutants in the same metabolic pathway, such as fat1, scs7, fen1, and sur4 (Mitchell and Martin, 1997; Oh *et al*., 1997; Watkins *et al*., 1998). Fen1 and Sur4 interact genetically and physically with Tsc13 (Kohlwein *et al*., 2001; Miller *et al*., 2005). Δfen1 and Δsur4 cells showed >50% defect in PMN activity and Vph1p exclusion was reduced to a similar degree (Table 1). This result suggests a requirement for very long chain fatty acids in the formation of NV junctions and supports a role of Tsc13 partners in this process.

Because very-long chain fatty acids are incorporated into phytoceramide, we also tested mutants in biosynthesis of this sphingolipid (Δypc1, Δlag1, Δlcb3) (D'Mello N *et al*., 1994; Qie *et al*., 1997; Mao *et al*., 2000). These mutations significantly decreased PMN activity and Vph1p exclusion under rapamycin treatment (Table 1). The strongest phenotype was observed in the Δlag1 mutant. Even after rapamycin induction, Vph1p exclusion was reduced to the basal level observed with untreated wild-type cells. Because sphingolipids and sterols are associated and coordinated in yeast cells (Guan *et al*., 2009), we also tested a mutant in ergosterol biosynthesis (Δerg5) (Ashman *et al*., 1991; Kelly *et al*., 1997). The C-22 sterol desaturase mutant Δerg5 showed severely reduced PMN activity and Vph1p exclusion upon rapamycin treatment. A caveat for the evaluation of this mutant is that the vacuoles in Δerg5 were fragmented, rendering quantification difficult.

DISCUSSION

The NV-junction is a zone of contact between vacuoles and the nuclear envelope. It affects membrane organization on the nuclear side because nuclear pore complexes are not found in vicinity of the vacuolar membrane (Severs *et al*., 1976; Pan *et al*., 2000). Here we show that Vph1p-GFP is excluded from the vacuolar membrane sections that form an NV junction. This barrier is specific for V-ATPase and the exclusion does not affect the VTC complex, which has a cytosolic domain of similar size. It has been speculated that NV junctions might have a special lipid composition (Kvam and Goldfarb, 2006a). The results we obtained with the mutants in Osh genes and in ceramide metabolism support this notion and suggest a role of lipids in NV junction formation and function. An observation that strengthens this view is that the accumulation of the VTC complex at NV junctions is abolished by inhibition of phosphatidylinositol-3-kinase (C. Dangelmayr, R. Dawaliby *et al*., unpublished results). NV junctions might hence form a specialized lipid zone on the vacuolar membrane that excludes certain vacuolar proteins by providing an unsuitable membrane environment for them. In that sense they resemble lipid rafts, specialized membrane microdomains that have been extensively characterized. Rafts are rich in cholesterol and sphingolipids (Simons and Ehehalt, 2002; Korade and Kenworthy, 2008) and serve as organizing centers for the assembly of signaling molecules. They influence membrane fluidity and membrane protein trafficking and regulate neurotransmission and receptor trafficking (Simons and Ehehalt, 2002; Pichler and Riezman, 2004). Similarly, NV junctions recruit selected proteins such as Osh1p, Tsc13p, and VTC and exclude others.

V-ATPase exclusion is reverted by concanamycin A. This suggests that NV junctions form a barrier that requires the electrochemical potential of the vacuolar membrane. This potential depends to a large extent on the proton translocation activity of the V-ATPase which acidifies vacuoles (Forgac, 2007). Numerous studies demonstrated roles of the V-ATPase in membrane homeostasis and vesicular traffic. Only some of these depend on proton pump activity, such as autophagosome-lysosome fusion, passage through endosomes, or the fragmentation of yeast vacuoles (Schmid *et al*., 1989; Yamamoto *et al*., 1998; Liu *et al*., 2005; Baars *et al*., 2007; Kawai *et al*., 2007). An example for a function independent of proton-translocation is the physical role of the V_0 sector during membrane fusion at various membrane trafficking steps (Peters *et al*., 2001; Bayer *et al*., 2003; Hiesinger *et al*., 2005; Liegeois *et al*., 2006; Sun-Wada *et al*., 2006; Peri and Nusslein-Volhard, 2008). A purely physical role of the V-ATPase in formation of PMN vesicles is unlikely because the process is sensitive to concanamycin A and because V-ATPase is excluded from PMN structures and NV junctions that give rise to these vesicles. Thus, one should rather consider effects via the membrane potential. Vesicle formation and fission can depend on lateral inhomogeneities within membranes, induced either by spontaneous phase separations of lipids (Julicher and Lipowsky, 1993; Sackmann and Feder, 1995; Julicher and Lipowsky, 1996; Munn *et al*., 1999; Proszynski *et al*., 2005; Falguieres *et al*., 2009), or by the concentration of membrane-apposed or membrane-integral proteins (Wenk and De Camilli, 2004; Lee *et al*., 2005; Ramos *et al*., 2006). Such lateral phase separations can be strongly influenced by the membrane potential, as exemplified by the

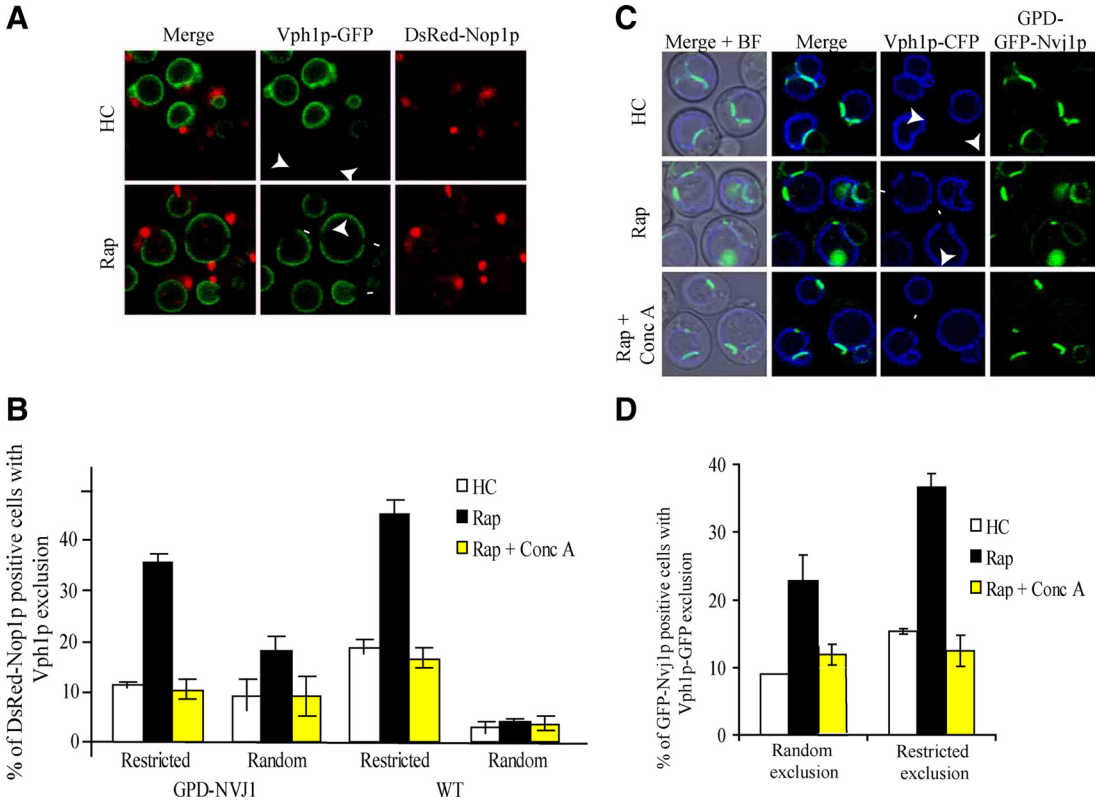

Figure 8. Random exclusion of Vph1p upon overexpression of Nvj1p. (A) Cells overexpressing HA-Nvj1 from the GPD promoter and carrying Vph1p-GFP and DsRed-Nop1p were treated in HC medium for 3 h with 0.2 μM rapamycin and 1 μM concanamycin A as indicated, or left without additions. Cells were analyzed by confocal microscopy. Arrows indicate random exclusion sites. (B) Quantification of random and restricted exclusion of Vph1p in the cells from A. (C) Cells with GFP-Nvj1 overexpressed from the GPD promoter and carrying Vph1p-CFP were treated and analyzed as in A. Arrows indicate random exclusion sites. BF: bright field. (D) Quantification of random and normal exclusion of Vph1p in the cells from C.

massive compartmentation of the yeast plasma membrane into two clearly distinct zones which are occupied either by the plasma membrane ATPase Pma1p or the arginine symporter Can1p (Malinska *et al*., 2003). These two zones mix when the membrane is depolarized (Grossmann *et al*., 2007). Also, phase separation in synthetic membrane systems can depend on the membrane potential (Herman *et al*., 2004; Schaffer and Thiele, 2004). Thus, our observations and those from previous studies are consistent with the hypothesis that the full differentiation of an NV junction may comprise lipid phase separations depending on the electrochemical potential across the vacuolar membrane.

A comprehensive study systematically tested vacuole- and autophagy-related genes for a role in PMN (Krick *et al*., 2008). This confirmed numerous known PMN factors and identified a novel requirement for Atg genes in PMN. Whereas our microscopic observations on atg mutants agree with this study, there is a discrepancy concerning the V-ATPase which Krick *et al.* (2008) found not to be required for PMN. This discrepancy could be due to the different assays used. We visualized transfer of the nucleolar protein Nop1p-GFP into vacuoles microscopically. This microscopic assay is independent of the actual degradation of the PMN vesicles or the reporter. In their tests of V-ATPase mutants Krick *et al.* (2008) used the proteolytic degradation of a GFP-Osh1p fusion protein as a measure for PMN. The assay is based on the fact that GFP is more protease-resistant than the linker between GFP and Osh1p and hence accumulates in vacuoles. The parameters influencing this assay are complex. On induction of PMN, the GFP-Osh1p fusion is transferred into vacuoles, the PMN vesicle membranes must be degraded, and then the vacuolar proteases cleave GFP-Osh1p and GFP. Although the GFP domain is more protease-resistant than the GFP-Osh1p fusion, it is nevertheless degradable by vacuoles, which can easily be visualized by pulse-chase experiments in which expression of new GFP is prevented by Gal-shut-off experiments (our unpublished results). The relative protease-resistance of GFP leads to its accumulation inside vacuoles to a certain level. This level, however, depends on multiple parameters, such as the protease and lipase activities inside the vacuoles, the time that the fusion protein is exposed to vacuolar hydrolases, the kinetics with which the GFP-Osh1p linker and the released GFP domain are degraded, and the level of PMN activity. Mutations can reduce the activities of vacuolar lipases and proteases to varying degrees (Kane, 2006; Forgac, 2007), which can influence the half-lives of GFP-Osh1p and its GFP fragment differently. V-ATPase mutants still show some proteolytic activity in their vacuoles but at much lower levels than wild-type cells. Thus, V-ATPase mutants might still be able to cleave the sensitive GFP-Osh1p linker but degrade the more resistant GFP more slowly than wild types. This can hide a defect in PMN since even if less GFP-Osh1p is transferred into vacuoles delayed degradation of its GFP fragment could lead to its overproportional accumulation. The data of Krick *et al.* (Figure 5C) are consistent with this notion because they show two important differences between V-ATPase mutants and the wild-type: Wild-type cells contain virtu-

ally no fragment before induction of PMN. Induction dramatically enhances the transfer of GFP-Osh1p into wild-type cells, producing the fragment. In contrast, the V-ATPase knockout Δvma2 shows high levels of GFP fragment already before induction of PMN and these remain unaltered upon PMN induction. Furthermore, Δvph1 mutants, which retain limited vacuolar acidification due to the presence of the Stv1p-containing V-ATPase complexes (Manolson *et al*., 1994), show low levels of GFP fragment before induction. On induction of PMN, GFP fragment accumulates but with a significant delay relative to wild-type cells. The interpretation of the proteolysis-based assay of PMN is hence not straightforward for mutants influencing the hydrolytic capacity of the vacuoles. In these cases the assay must be validated by determining the half-lives of the GFP-fusion and the GFP-fragment in each mutant.

Cells overexpressing N-terminally tagged Nvj1 excluded Vph1p from vacuolar sites that were not adjacent to the nucleus. These random exclusion sites were next to Nvj1p patches but far from the nucleus. This confirms that contact with nuclear material is dispensable for making contacts between vacuoles and the ER or outer nuclear membrane (Roberts *et al*., 2003). Furthermore, it shows that differentiation of the contact sites into "NV" junctions (or their ER-equivalents) is independent of the nucleus. That this "differentiation" can be regarded as a separate step in the establishment of NV junctions is supported by the fact that inhibition of V-ATPase pump activity by concanamycin A barely reduces the frequency of nuclear-vacuolar contacts but strongly reduces Vph1p exclusion from these sites (Figure 7).

Integrating our results with those of earlier studies (Kvam and Goldfarb, 2007; Krick *et al*., 2008), we can hence extend the model of NV junction formation and PMN by a new step. Whereas V-ATPase activity is not required for forming nuclear-vacuolar contact sites, further evolution into NV junctions that generate a diffusion barrier on the vacuolar side does depend on it. This diffusion barrier likely depends on a specific lipidic environment. The deformation of NV junctions into PMN sites and the subsequent formation of vesicles from them requires the electrochemical potential and completion of the process depends on Atg genes.

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