**Introduction**

The endoplasmic reticulum (ER) is a dynamic membrane system that mediates protein assembly, secretory trafficking, calcium homeostasis, lipid metabolism and the compartmentalization of the nucleus (for review, see Lippincott-Schwartz et al., 2000). The ER consists of structurally distinct domains, including the peripheral and nuclear ER, the ribosome-studded rough ER and smooth ER, and subdomains that contact other organelles in the cell (for review, see Voeltz et al., 2002). Nuclear ER, or the nuclear envelope (NE), is composed of two membrane sheets that surround and compartmentalize the nucleus but remain continuous with the peripheral ER through a network of cytoplasmic tubules (Voeltz et al., 2002). The inner and outer membranes of the NE are connected via fenestrations called nuclear pores that house nuclear pore complexes (NPCs) and mediate the nucleocytoplasmic trafficking of proteins and RNAs. Because the nuclear surface is studded with ribosomes, the outer nuclear membrane is considered to be continuous with the rough ER (for review, see Holmer and Worman, 2001).

The ER intersects with several organelles in the eukaryotic cell, including the Golgi, mitochondria, vacuoles or lysosomes, peroxisomes, late endosomes and plasma membrane (for reviews, see Staehelin, 1997; Voeltz et al., 2002). In particular, ER contacts with peroxisomes and mitochondria have been implicated in the synthesis and exchange of lipids (for reviews, see Voelker, 2000; Voeltz et al., 2002). The nucleus-vacuole junction (NV junction) in *Saccharomyces cerevisiae* is currently the best understood example of how ER membranes associate with other organelles in the cell (for review, see Voeltz et al., 2002). NV junctions are created by specific Velcro-like binding interactions between the vacuole membrane protein Vac8p and the nuclear membrane protein Nvj1p (Pan et al., 2000). The sizes of NV junctions are directly proportional to cellular levels of Nvj1p (Pan et al., 2000; Roberts et al., 2003). The upstream promoter region of *NVJ1* contains two putative stress-response elements (STREs) (Moskvina et al., 1998); consequently, *NVJ1* expression is upregulated and NV junctions expand during stationary phase or nutrient limitation (Gasch et al., 2000; Roberts et al., 2003). NV junctions mediate piecemeal microautophagy of the nucleus (PMN), an autophagic process that involves the blebbing of nonessential portions of the nucleus into invaginations of the vacuole membrane (Roberts et al., 2003). PMN nuclear blebs pinch off as vesicles into the vacuole lumen and are degraded in a manner dependent on vacuolar hydrolases (Roberts et al., 2003).

Recently, two conserved proteins with roles in lipid metabolism have been described at NV junctions in yeast, namely Tsc13p and Osh1p (Kohlwein et al., 2001; Levine and Munro, 2001). Osh1p is a member of the yeast Osh family, which bears structural and functional homology to mammalian oxysterol-binding protein (OSBP) (Levine and...
Munro, 2001). OSBP and OSBP-related proteins (ORPs) are cytoplasmic lipid-binding proteins implicated in the non-vesicular trafficking of specific intracellular lipids (for review, see Olkkonen and Levine, 2004). In particular, OSBP has specific affinity for oxysterols, which are oxygenated derivatives of cholesterol (ergosterol in yeast) that mediate the reduction of excess sterols and function in numerous signaling and regulatory pathways (Olkkonen and Levine, 2004). OSBP and related ORPs share a conserved oxysterol-binding domain but possess additional domains that bind an array of lipids and proteins (Olkkonen and Levine, 2004). OSBP and ORPs appear to regulate lipid metabolism, vesicle transport and various signaling pathways (for review, see Lehto and Olkkonen, 2003) and might specifically mediate lipid exchange at membrane contact sites (Olkkonen and Levine, 2004).

The *S. cerevisiae* genome encodes seven ORPs, OSH1-OSH7 (Beh et al., 2001). Each Osh protein performs both unique and redundant functions that render the entire Osh family essential for cell survival (Beh et al., 2001). The roles of individual Osh proteins are not well defined. However, Osh4p/Kes1p genetically interacts with Sec14p and has been implicated in the secretory function of the Golgi apparatus (Fang et al., 1996; Li et al., 2002). The functional role of Osh1p is linked to the post-synthetic regulation of sterol lipids (Beh et al., 2001). In particular, osh1Δ cells share phenotypic similarities with erg mutants, which are defective in ergosterol synthesis (Jiang et al., 1994; Levine and Munro, 2001). Similar to mammalian OSBP, the subcellular localization of Osh1p in yeast is regulated by several targeting determinants, including a PH domain specific for Golgi membranes (Levine and Munro, 2001) and an FFAT motif (FF in an acidic tract) that targets the ER (Loewen et al., 2003). Additionally, Osh1p contains an ankyrin repeat domain that is necessary and sufficient to target NV junctions in yeast (Levine and Munro, 2001). Consequently, Osh1p localizes to both the Golgi network and NV junctions, although it is targeted exclusively to NV junctions during stationary phase (Levine and Munro, 2001). Scs2p, a protein implicated in vesicular fusion by homology to mammalian VAP (VAMP-associated protein) (Kagiwada et al., 1998), plays a role in the targeting of Osh1p and other proteins to the yeast ER (Loewen et al., 2003) in a manner analogous to VAP-mediated targeting of mammalian OSBP (Wyles et al., 2002). Here, we report that Osh1p interacts specifically with Nvj1p and is a substrate for PMN. A green fluorescent protein (GFP) tagged Osh1p (GFP-Osh1p) is recruited from cytoplasmic and Golgi pools into NV junctions and PMN structures in a manner proportional to the cellular level of Nvj1p. GFP-Osh1p is also targeted in an Nvj1p-dependent fashion when Nvj1p is ectopically mislocalized to extranuclear membranes. Moreover, GFP-Osh1p is sequestered into patches of Nvj1p on the outer nuclear membrane independent of Vac8p and NV-junction formation, thereby ruling out a role for late endosomal or vacuolar membranes in the targeting of Osh1p to the NE. Osh1p appears to bind the NE by physical association with Nvj1p. OSH1 itself is not required for the formation of NV junctions or PMN in starved cells. However, PMN is inhibited in cells depleted of all Osh family proteins, presumably because of the loss of a shared activity.

### Materials and Methods

**Yeast strains, plasmids and growth conditions**

Yeast strains used in this study are listed in Table 1. Plasmids for the expression of *NVJ1* or enhanced yellow fluorescent protein-tagged *NVJ1* (*NVJ1-EYFP*) under CUP1 promoter control (*P*CUP1-NVJ1-EYFP) and *NVJ1-EYFP* under GAL1 promoter control (*P*GAL1-NVJ1-EYFP) were described previously (Roberts et al., 2003; Pan et al., 2000). GFP-Osh1p was expressed under PHO5 promoter control from PS4146-GFP-OSH1, a kind gift from T. Levine (Loewen et al., 2003). *P*CUP1-NVJ1-Fc was created by ligating an *EcoRI*-*NVJ1*-BamHI PCR fragment and the Fc portion of human IgG1 cDNA (including the hinge region, CH2 and CH3 domains) of vector CMD7 (Invitrogen, CA) into pRK2 and pJN40 (Pan et al., 2000; Macreadie et al., 1989). *P*GAL1-NVJ1-myc was constructed by ligating a *BamHI*-*NVJ1*-SalI PCR fragment into the *BamHI* and *SalI* sites of pESC-HIS (Stratagene). An Nvj1p targeting variant, *P*GAL1-GFP-NVJ1, was created by ligating *EcoRI*-GFP-Smal and *SalI*-*NVJ1*-HindIII PCR fragments into the *EcoRI* and *HindIII* sites of pRK2 (Pan et al., 2000; Macreadie et al., 1989). Similarly, *P*CUP1-HA-NVJ1 was constructed by ligating an *EcoRI*-3HA-Smal PCR fragment upstream of *NVJ1* in *P*CUP1-NVJ1 (Pan et al., 2000). All PCR fragments were generated using Taq DNA polymerase (Invitrogen, CA) from appropriate templates, including pEYFP (Clontech, CA), pGTEPI [hemagglutinin (HA) epitope] (Tyers et al., 1992) or genomic yeast DNA. Cells were cultured in standard YPD or synthetic complete medium (SC) with 2% glucose (SCGlu) at 30°C (Sherman, 1991). Nitrogen starvation was cultured in standard YPD or synthetic complete medium (SC) with 2% glucose (SCGlu) at 30°C (Sherman, 1991). Nitrogen starvation medium (SD-N) contained 0.17% yeast nitrogen base (Difco™, VWR International) and 2% glucose without amino acids or ammonium sulfate, unless otherwise indicated.

### Table 1. Strains used in this study

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In vivo Nvj1p-Fc purification

Yeast harboring pRS416-GFP-OSH1 and either P_{CUP1}-NVJ1-Fc or empty vector, respectively, were grown to log phase in 100 ml SCGlu and induced with 0.1 mM CuSO4 for 3 hours. Cells were collected by centrifugation, weighed and overlayed with one volume of acid-washed glass beads and two volumes of extraction buffer (EB) [40 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM dithiothreitol, 0.5 mM EDTA] containing Complete™ protease inhibitors (Roche, Mannheim, Germany) and 2 µg ml⁻¹ pepstatin A, aprotinin and leupeptin (Sigma). Cells were broken by vortexing five times for 2 minutes each, with 2-minute incubations on ice between each vortex. Cell suspensions were solubilized with 1% NP-40, vortexed and cleared by centrifugation (500 g for 10 minutes). The resulting supernatant was reserved and the glass beads were washed with two volumes of EB, vortexed and cleared by centrifugation (as above). This second supernatant was combined with the previous and the protein concentration of the total lysate was measured by the Bradford assay. Approximately 3 mg of total protein was transferred into MicroSpin columns (Amersham) and the final volume was brought up to 0.5 ml with EB. Sixty µl of a 1:1 mix of Protein-A-conjugated agarose beads (Repligen, MA) and 1% bovine serum albumin was incubated with the lysate overnight at 4°C. The beads were subsequently isolated by centrifugation (500 g for 1 minute), washed four times with HNTG (20 mM HEPES pH 7.5, 0.15 M NaCl, 0.1% Triton X-100, 10% glycerol) and transferred to a fresh microcentrifuge tube. Beads were eluted with 40 µl 2× protein gel sample loading buffer (100 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 minutes. Ten-20 µl eluate was analysed by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE).

Immunoblot

With the exception of Nvj1p-Fc lysate preparations, all protein extracts were prepared by trichloroacetic acid (TCA) precipitation as previously described (Roberts et al., 2003). GFP-osh1p and Nvj1p-EYFP were probed by immunoblot with polyclonal BD Living Colors™ A.v. peptide antibodies (Clontech). Nvj1p-myc was probed using mouse anti-c-Myc monoclonal antibodies (Zymed). HA-Nvj1p was probed with mouse anti-HA monoclonal antibodies (Santa Cruz Biotechnology). Precursor and mature forms of aminopeptidase I were probed using antiserum against Ape1p (Klionsky et al., 1992). Nvj1p and Nvj1p-Fc were probed with polyclonal antibodies raised against a portion of Nvj1p, generated as follows. A BamHI-NVJ1 (337-786)-EcoRI PCR fragment encoding the C-terminal segment of Nvj1p between amino acid residues 113 and 262 was inserted into the bacterial glutathione-S-transferase (GST) expression vector pGEX-2TK (Pharmacia). The GST-Nvj1p (113-262aa) fusion protein was affinity purified from BLR Escherichia coli cells (Novagen) using glutathione/Sepharose-4B (Pharmacia). The Nvj1p peptide fragment (residues 113-262) was released from GST by thrombin (Sigma) and used to immunize screened rabbits. Antiserum was prepared by the Pocono Rabbit Farm and Laboratory (Canadensis, PA) and used on immunobLOTS at 1:200 dilution to probe Nvj1p. All immuno-probed proteins were detected with alkaline-phosphatase-coupled goat anti-rabbit antibody (Santa Cruz Biotechnology), horseradish-peroxidase-coupled donkey anti-rabbit antibody (Santa Cruz Biotechnology) or horseradish peroxidase-coupled goat anti-mouse antibody (Santa Cruz Biotechnology) as appropriate, and developed colorometrically or by chemiluminescence using Luminol Reagent (Santa Cruz Biotechnology).

Quantitative degradation analysis of Nvj1p-EYFP and Nvj1p-myc

Cells harboring P_{GAL1}-NVJ1-myc were grown, induced and shifted into SD-N starvation media in a manner analogous to previously published degradation analyses of Nvj1p (Roberts et al., 2003). The Osh depletion strain (osh1Δ-osh7 PAET1-OSH2) and its wild-type parental background (SEY6210) harboring P_{GAL1}-NVJ1-EYFP were treated similarly to the above P_{GAL1}-NVJ1-myc cells except that they were induced overnight in 1% raffinose, 1% galactose medium (lacking methionine) before being shifted into SD-N starvation medium containing 20 µg ml⁻¹ methionine. Two OD600 units of cells were harvested immediately (zero point) from approximately 0.5 OD600 units ml⁻¹ culture and an equivalent volume of culture was collected after 10 hours of starvation. Protein extracts were prepared by TCA precipitation as previously described (Roberts et al., 2003). Samples were separated by SDS-PAGE and immunoprobed as indicated, and the average pixel intensity of Nvj1p-EYFP or Nvj1p-myc was quantified using NIH Image 1.62. The level of Nvj1p-EYFP or Nvj1p-myc remaining after starvation was compared to the level before starvation and expressed in terms of percentage loss.

Cell imaging

Cells were grown to the indicated OD600 or starved in SD-N with or without induction of specific reporters. For P_{CUP1}-NVJ1, P_{CUP1}-NVJ1-EYFP, P_{CUP1}-HA-NVJ1 and P_{CUP1}-NVJ1-Fc, basal levels of expression were achieved by witholding exogenous Cu²⁺ and higher levels of expression were obtained by fortifying media with 0.1 mM CuSO4 for the times indicated. Expression of P_{GAL1}-NVJ1-EYFP was induced as previously described (Roberts et al., 2003). Vacuoles were stained with FM4-64 as described previously (Pan and Goldfarb, 1998). In cells starved of nitrogen (SD-N), vacuoles were first stained with FM4-64 in rich medium (SCGlu), then the cells were washed three times in SD-N and finally grown in SD-N medium for 3 hours. DNA was stained immediately before microscopic analysis with 5 µM Hoechst reagent H-1398 (Molecular Probes).

Confocal microscopy

Confocal microscopy was performed on a Leica TCS NT microscope equipped with a 100× Fluorotar lens and ultraviolet, Ar, Kr/Ar and He/Ne lasers (Leica Microsystems, Chantilly, VA). Laser power and photomultiplier tube (PMT) settings were kept constant for all experiments. All images were processed using Adobe PhotoShop 5.0 (Adobe Systems, CA).

Immunoelectron microscopy

Wild-type cells harboring P_{CUP1}-NVJ1-GFP or P_{CUP1}-GFP-NVJ1 were grown to log phase and induced to express Nvj1p-GFP or GFP-Nvj1p for 1 hour using 0.1 mM CuSO4. Cells were high-pressure frozen, freeze substituted, sectioned and stained as previously described (Giddings et al., 2001) to analyse nucleus-vacuole membrane contacts. GFP-tagged Nvj1p was detected by immunoelectron microscopy with affinity-purified rabbit polyclonal anti-GFP antibody and gold-conjugated anti-rabbit secondary antibody. Serial thin sections were viewed in a CM10 electron microscope (Philips Electronic Instruments, Mahwah, NJ).

Results

Soluble and late-Golgi pools of Osh1p are sequestered into NV junctions in an Nvj1p-dependent manner

Osh1p is a soluble protein that accumulates on the surface of late-Golgi membranes and at NV junctions (Levine and Munro, 2001). During stationary phase, the localization of Osh1p fades at late-Golgi structures, but soluble and NV-junction-associated pools of Osh1p persist (Levine and Munro, 2001). We thought that the depletion of Osh1p at Golgi membranes may be a consequence of the titration of this pool...
into NV junctions, because NV junctions increase in size and frequency during entry into stationary phase (Roberts et al., 2003). The expansion of NV junctions is attributed to the elevated expression of NVJ1 under conditions of nutrient depletion (Pan et al., 2000; Roberts et al., 2003). We monitored the localization of GFP-tagged Osh1p as a function of NVJ1 expression using confocal microscopy. The colocalization of GFP-Osh1p with FM4-64-stained vacuolar membranes was analyzed in wild-type cells expressing elevated levels of ectopic Nvj1p at early log phase, when endogenous Nvj1p levels are normally limiting for NV-junction formation (Pan et al., 2000; Roberts et al., 2003). In rapidly dividing cells, GFP-Osh1p localized chiefly to the cytosol and Golgi, and infrequently at NV junctions (Levine and Munro, 2001). However, overexpression of plasmid-encoded NVJ1 resulted in proportional increases in the amount and intensity of GFP-Osh1p at NV junctions. We quantified cellular levels of GFP-Osh1p by immunoblot and after induction of NVJ1 to exclude the possibility that changes in the expression of GFP-Osh1p could account for its accumulation at NV junctions. As Fig. IC illustrates, GFP-Osh1p levels are constant in cells expressing both low and elevated levels of NVJ1. We conclude NV junctions serve as high-affinity sinks for soluble and Golgi-associated pools of Osh1p.

These results indicate that Nvj1p plays a direct role in recruiting Osh1p to NV junctions. We were interested in determining whether Nvj1p acts as a binding receptor for Osh1p or whether other nuclear-membrane-specific factors are required for sequestering Osh1p into NV junctions. To this end, we monitored the localization of GFP-Osh1p in cells expressing an Nvj1p reporter that is mislocalized to cytoplasmic membranes. The N-terminus of Nvj1p is required for the proper targeting of Nvj1p to the NE (X. Pan, unpublished). Insertion of a GFP tag onto the N-terminus of Nvj1p mislocalizes the GFP-Nvj1p reporter to ER-like cytoplasmic membranes (Fig. 2A), though we cannot rule out the possibility that these structures are abnormal membrane proliferations. Interestingly, these cytoplasmic membranes form ectopic junctions with vacuolar membranes (Fig. 2A), presumably owing to the interaction of the C-terminus of GFP-Nvj1p with Vac8p (Pan et al., 2000). We tested whether Osh1p could also be recruited to these artificial membrane contact sites. A small HA epitope was cloned onto the N-terminus of Nvj1p and co-expressed with GFP-Osh1p in nvj1Δ cells. Strikingly, gradual overexpression of HA-Nvj1p promoted the sequestration of cytoplasmic and Golgi pools of GFP-Osh1p to patches on the vacuolar membrane (Fig. 1B). Unlike NV junctions, these patches were rarely juxtaposed with nuclear chromatin (Fig. 1Biv,vi), consistent with the mislocalization of GFP-Nvj1p (Fig. 2A).

![Fig. 1. Osh1p is sequestered from cytoplasmic and Golgi pools to NV junctions through an Nvj1p-mediated mechanism. (A) Localization of GFP-Osh1p as a function of NVJ1 expression. Cells expressing plasmid-encoded GFP-Osh1p and harboring PCUP1-NVJ1 or empty vector were analyzed at log phase. PCUP1-NVJ1 expression was maintained at a basal level (iii,iv) or induced for 1.5 hours with CuSO4 (v,vi) similar to empty vector control cells (i,ii). Nuclear chromatin (blue) and vacuolar membranes (red) were stained with Hoechst and FM4-64, respectively, and overlayed with GFP-Osh1p (bottom). (B) Localization of GFP-Osh1p as a function of mistargeted HA-NVJ1 expression. Nvj1Δ cells expressing GFP-Osh1p and harboring PCUP1-HA-NVJ1 or empty vector were monitored at log phase. PCUP1-HA-NVJ1 expression was maintained at a basal level (iii,iv) or induced for 1.5 hours with CuSO4 (v,vi) similar to empty vector control cells (i,ii). Nuclear chromatin (blue) and vacuolar membranes (red) were stained with Hoechst and FM4-64, respectively, and overlayed with GFP-Osh1p (bottom). (C) Immunoblot analysis of GFP-Osh1p levels in whole-cell extract as a function of NVJ1 (C) or HA-NVJ1 (D) expression. Wild-type extracts lacking GFP- or HA-tagged proteins are labeled as N.](image-url)
Targeting of Osh1p to nucleus-vacuole junctions

Osh1p purifies with Nvj1p in vivo at NV junctions and Vac8p-independent rafts

The likelihood that Osh1p is recruited to NV junctions via a physical interaction with Nvj1p was tested by affinity co-purification using an Nvj1p reporter fused with the constant region (Fc) of human IgG. When expressed in *nvj1Δ* cells, Nvj1p-Fc rescued the formation of NV junctions and, more relevantly, sequestered GFP-Osh1p into NV junctions (Fig. 3Aii,iii). Additionally, Nvj1p-Fc binds tightly to protein-A-conjugated agarose, allowing its purification from whole cell extracts. Immunoblot analysis revealed that GFP-Osh1p copurified with Nvj1p-Fc from extracts of *nvj1Δ* cells (Fig. 3B). GFP-Osh1p was not isolated from cell extracts lacking Nvj1p-Fc (Fig. 3B), nor did GFP purify with Nvj1p-Fc alone (data not shown).

Importantly, the affinity between Osh1p and Nvj1p proved to be independent of NV-junction formation, because GFP-Osh1p also purified with Nvj1p-Fc when expressed in *vac8Δ* cells (Fig. 3B). Confocal microscopy of GFP-Osh1p in *vac8Δ* cells also supported this finding (Fig. 3Aiii,iv). In the absence of Vac8p, Nvj1p-GFP tends to localize over the entire surface of the NE (Pan et al., 2000). However, Nvj1p-EYFP occasionally accumulates in fluorescent patches on the nuclear surface when overexpressed in *vac8Δ* cells (Fig. 3C) (Pan et al., 2000). Because Vac8p is absent from these cells, these fluorescent patches do not localize with vacuolar membranes (Fig. 3C). These observations suggest that Nvj1p can self-associate laterally in the nuclear membrane in the absence of Vac8p. The clustering activity of Nvj1p provided an opportunity to confirm by microscopy that GFP-Osh1p can associate with Nvj1p on the nuclear surface in the absence of NV junctions. As shown in Fig. 3Aiii,iv, the overexpression of Nvj1p-Fc in *vac8Δ* cells triggered perinuclear patches of GFP-Osh1p to brighten at the expense of cytoplasmic and late-Golgi pools. Similar results were achieved using untagged Nvj1p at moderate or high expression levels (data not shown). In support of biochemical data (Fig. 3B), these results illustrate that Osh1p binds directly to clusters of Nvj1p or Nvj1p-associated factor(s) in the absence of Vac8p and NV junctions.

**Fig. 2.** Nvj1p localizes to the outer leaflet of the nuclear membrane at NV junctions. Immunogold localization of GFP-Nvj1p (A) and Nvj1p-GFP (B) by electron microscopy. Both GFP-tagged Nvj1p reporters were induced in wild-type cells for 1 hour with CuSO₄ before cryofixation and immuno-EM analysis. (A) GFP-Nvj1p, a non-functional reporter, is mislocalized from NV junctions. Colloidal-gold-conjugated antibodies directed against GFP-Nvj1p localize to the surface of cytoplasmic vacuoles away from the nuclear membrane. Nucleus denoted as N, vacuoles labeled as V. (B) Nvj1p-GFP, a functional reporter, localizes specifically to NV junctions. Colloidal-gold-conjugated antibodies directed against Nvj1p-GFP label the surface of nuclei at NV junctions (i). Higher magnification reveals that most gold particles are preferentially concentrated across from the inner nuclear membrane (ii, inner nuclear membrane outlined in white). Nucleus is denoted as N, vacuoles as V. Scale bars, 0.3 μm.

Immunoblot analyses verified that GFP-Osh1p levels did not increase during the induction of HA-Nvj1p (Fig. 1D). These data support the notion that Nvj1p is a binding receptor for Osh1p.

**Nvj1p localizes asymmetrically to the outer membrane of the NE**

The C-terminal domain of Nvj1p extends from the NE into the cytosol, where it interacts with Vac8p displayed on the surface of the vacuole (Pan et al., 2000). The localization of Osh1p to NV junctions is based on an analogous interaction with Nvj1p (see below). We were interested in determining whether Nvj1p existed in the inner nuclear membrane as well, which could provide a means for localizing Osh1p inside the nucleus. Immunoelectron microscopy was used to investigate the localization of Nvj1p-GFP at a resolution sufficient to distinguish between the inner and outer nuclear membranes. As shown in Fig. 2Bi, immunogold-conjugated antibodies directed against the GFP moiety of Nvj1p-GFP localized almost exclusively to interfaces between the nucleus and vacuole. Because Nvj1p-GFP was slightly overexpressed in this experiment, immunogold labeling was occasionally detected elsewhere on the NE. Approximately 90% of the 200 gold particles we scored were clearly associated with the outer face of the NE (Fig. 2Bii). By contrast, only ~10% of the particles localized closer to the inner nuclear membrane (Fig. 2Bii). These results demonstrate that the bulk of Nvj1p, if not all, is localized exclusively to the outer nuclear membrane. By analogy, we conclude that the Nvj1p-dependent accumulation of Osh1p on the NE occurs on the cytoplasmic face.

**Osh1p selectively localizes to PMN structures during stationary phase and nitrogen starvation**

NV junctions are incorporated into PMN vesicles and degraded in the vacuole, so we thought that Osh1p might also be subject to PMN-mediated turnover. To induce maximal PMN frequencies, wild-type cells expressing plasmid-encoded GFP-Osh1p were grown to stationary phase and analysed in SD-N
Osh1p is not essential for NV-junction formation or PMN
The affinity of Osh1p for NV junctions and PMN structures raised the intriguing possibility that Osh1p might function during PMN in a manner similar to the role of Osh4p in Golgi-derived vesicle formation (Fang et al., 1996). Therefore, we tested by confocal microscopy whether NV junctions or PMN were defective in osh1Δ cells. A ‘pulse’ of Nvj1p-EYFP was expressed in osh1Δ cells harboring P<sup>GAL1</sup>-NVJ1-EYFP using galactose medium and the localization of Nvj1p-EYFP was subsequently analysed in SCSu or SD-N medium after glucose repression of Nvj1p-EYFP synthesis. We noted that osh1Δ cells exhibited normal NV junctions and PMN structures in both rich and depleted media (Fig. 4D). The kinetic rate of PMN in osh1Δ cells was measured by analysing the starvation-induced degradation of myc-tagged Nvj1p (Roberts et al., 2003). Consistent with published data, the degradation of Nvj1p-Myc proved to be dependent on VAC8 but independent of ATG7 (Fig. 5A) (Roberts et al., 2003). We observed that Nvj1p-Myc was degraded in osh1Δ cells as efficiently as the wild-type parental strain (Fig. 5B). As a control for this experiment, the degradation of Nvj1p-Myc was largely inhibited in pep4Δ cells (Fig. 5B), which lack the ability to degrade PMN vesicles in the vacuolar lumen (Roberts et al., 2003). We conclude that Osh1p is not required for either the formation of NV junctions or for PMN structures and their degradation.

Cells depleted of all Osh protein accumulate PMN intermediates
Although osh1Δ cells do not exhibit a PMN defect, it is possible that other members of the Osh family share an activity with Osh1p that is required for PMN. A comprehensive deletion analysis concluded that all seven Osh proteins perform a common but essential function that might be related to sterol
lipid homeostasis (Beh et al., 2001). As a result, Olkkonen and Levine proposed that the intracellular targeting of each Osh protein might be sufficiently nonspecific to allow each Osh family member to function ubiquitously in the cell (Olkkonen and Levine, 2004). Owing to the functional redundancy of the Osh family, we analysed NV-junction and PMN morphology as a function of Osh depletion. The localization of Nvj1p-EYFP was analysed in an Osh-depletion strain (osh1Δ-osh7Δ P_{MET3}-OSH2) deleted for the entire OSH gene family but maintained through the plasmid-based expression of OSH2 from the repressible MET3 promoter (Beh et al., 2001). In the absence of exogenous methionine, to promote the expression of P_{MET3}-OSH2, Nvj1p-EYFP localized normally to NV junctions (Fig. 6Ai,ii,iv). However, vacuolar morphology was notably altered in these cells and the incidence of PMN morphology was higher than in wild-type cells (Fig. 6Ai,ii). Strikingly, the frequency of PMN increased dramatically when P_{MET3}-OSH2 expression was repressed upon the addition of methionine to the medium. These cells, depleted of all Osh protein, appeared to accumulate many aberrant Nvj1p-EYFP-labeled PMN structures (Fig. 6Av,vi, arrows). Importantly, the accumulation of these PMN structures occurred under nutrient-rich conditions, when PMN levels are normally low (Fig. 6Ai,ii,v,vi). To measure the kinetics of PMN in Osh-depleted cells, the degradation of Nvj1p-EYFP was analysed by immunoblot in starved oshΔ cells (Roberts et al., 2003). As indicated in Fig. 6B, Nvj1p-EYFP was degraded less efficiently in Osh-depleted cells than in wild-type cells after incubation in starvation medium containing methionine (Fig. 6B, arrows). Interestingly, Osh-depleted cells showed normal maturation of aminopeptidase I precursor (prApe1p) (Fig. 6C), a measure of macroautophagy and cytoplasm-to-vacuole trafficking (Scott et al., 1997; Teter and Klionsky, 2000). These results indicate that the Osh family is not required for all vesicle-forming processes. The PMN defect in cells lacking Osh protein is consistent with a role for Osh1p and other Osh proteins during the formation of PMN vesicles. Because Osh-depleted cells contain perturbed sterol lipid compositions (Beh et al., 2001), we propose that the supply or regulation of

![Fig. 4](image-url) Osh1p accumulates in PMN structures dependent on NV1 but is not required for NV-junction formation or PMN. (A,B) Localization of GFP-Osh1p to PMN structures in wild-type (A) and atg7Δ (B) cells upon nitrogen starvation. Cells were grown to high OD_{600} (~1.0), shifted to SD-N medium and analysed by confocal microscopy after 3 hours of starvation. Vacular membranes (red) and nuclear chromatin (blue) were stained with FM4-64 and Hoechst, respectively. GFP-Osh1p localizes to yellow-labeled PMN structures in the overlay. (C) GFP-Osh1p increasingly accumulates within PMN structures proportional to the expression of NV1. Cells expressing GFP-Osh1p and harboring P_{CUP1-NV1} (ii) or empty vector (i) were grown to low OD_{600} (~0.3), shifted to SD-N medium and analysed by confocal microscopy after 3 hours of starvation. Vacular membranes (red) and nuclear chromatin (blue) were stained with FM4-64 and Hoechst, respectively. GFP-Osh1p localizes to yellow-labeled PMN structures in the overlay. (D) Localization of Nvj1p-EYFP in osh1Δ cells before and after nitrogen starvation. Log-phase osh1Δ cells harboring P_{GAL1-NV1}-EYFP were grown briefly in galactose to induce Nvj1p-EYFP and analysed in synthetic complete (SC) or nitrogen starvation medium (SD-N) after glucose repression. Nuclear chromatin and vacular membranes were stained with Hoechst and FM4-64, respectively. Arrows indicate PMN structures.

![Fig. 5](image-url) PMN-mediated degradation of Nvj1p is unperturbed in osh1Δ cells. (A,B) Starvation-induced degradation of Nvj1p-Myc is dependent on VAC8 and PEP4 but not ATG7 or OSH1. The turnover of a limited pool of Nvj1p-Myc during nitrogen starvation was analysed by immunoblot in isogenic wild-type, atg7Δ and vac8Δ cells (A) or osh1Δ and pep4Δ cells (B). The amount of Nvj1p-Myc remaining after 10 hours in SD-N was digitally quantified and compared with the level before starvation. Bar graphs represent the average percentage loss of Nvj1p-Myc from several independent experiments.
specific lipids at NV junctions plays an important role during the formation of PMN nuclear structures.

**Discussion**

In this study, we report that Nvj1p interacts physically with the oxysterol-binding protein Osh1p and can recruit Osh1p to the NE independent of Vac8p and NV junctions. Thus, we conclude that Nvj1p is a NE receptor for Osh1p. During log phase, Osh1p exhibits dual localization to both late-Golgi membranes and NV junctions, but associates exclusively with NV junctions as cells enter stationary phase (Levine and Munro, 2001). We demonstrate that the reapportionment of Osh1p to NV junctions is explained by increased expression of Nvj1p. Nvj1p levels naturally rise during acute starvation and stationary phase, thereby increasing the size and frequency of NV junctions and PMN structures (Roberts et al., 2003). Extranuclear pools of Osh1p can be sequestered in the absence of NV junctions and Vac8p by patches of Nvj1p on the NE, thereby ruling out a role for the vacuole membrane in the Nvj1p-dependent targeting of Osh1p to the nucleus. Likewise, Osh1p purifies with Nvj1p from both wild-type and vac8Δ cell extracts, suggesting a direct physical interaction. The interaction of Osh1p with Nvj1p is probably mediated by the N-terminal ankyrin repeat of Osh1p (Levine and Munro, 2001). In contrast to our results, others have concluded that the targeting of Osh1p to NV junctions might not result from direct interaction with any of the known components of NV junctions (including Nvj1p) (Olkkonen and Levine, 2004). We cannot explain this discrepancy, although we believe that differing experimental conditions are probably responsible.

Contacts between the ER and other organelles are thought to facilitate lipid biosynthesis and transport (for review, see Olkkonen and Levine, 2004). NV junctions are the only interorganelle contact sites for which the junctional apparatus is known (i.e. Vac8p and Nvj1p). Thus, it is interesting that Osh1p, a lipid-binding protein, is targeted to NV junctions through a physical interaction with Nvj1p, a component of the junction apparatus. We recently found that Tsc13p, an ER-membrane-associated enoyl reductase involved in sphingolipid biosynthesis, is also targeted to NV junctions through a
physical interaction with Nvj1p (E. Kvam, unpublished). It could well be a general rule that factors like Nvj1p that mediate membrane junctions could also serve as docking sites for concentrating enzymes and substrates at their cognate membrane contact sites. Olkkonen and Levine have proposed that the Osh proteins and other ORPs might themselves play a general role in lipid trafficking at membrane contact sites between different organelles (Olkkonen and Levine, 2004).

Although Osh1p is not required for the formation of NV junctions or PMN, an activity that is shared by Osh1p and the other six members of the Osh family is required for late stages of PMN. These results are consistent with a model in which Osh1p plays a general role in a lipid trafficking pathway that passes through NV junctions and also plays a role in the regulation or supply of certain lipids that are required for the formation of PMN nuclear intermediates. Our observation that Osh1p is packaged into PMN vesicles suggests that PMN might play a role in the downregulation of Osh1p during periods of nutrient stress, although more must be learned about the cellular role(s) of Osh1p before physiological relevance can be assigned to this phenomenon.

One of the more curious features of PMN is the tandem exvagination of inner and outer nuclear membranes as if they were physically attached (Roberts et al., 2003). In mammalian cells, blebbing of both nuclear membranes is seen in Bloom’s syndrome (Yankiwski et al., 2000) and tumorigenesis (Ho, 1989), and during apoptosis (for review, see Rogalinska, 2002), although the mediating factors, if any, are not known. In this regard, the role of Nvj1p in NE blebbing is unique. As indicated by immunoelectron microscopy (Fig. 2), Nvj1p localizes exclusively to the outer nuclear membrane and is, therefore, asymmetrically distributed across the yeast NE. This topology excludes the possibility that the inner and outer nuclear membranes at NV junctions are linked via homotypic Nvj1p interactions across the perinuclear lumen. Additionally, these results rule out the likelihood that Nvj1p targets Osh1p to both faces of the NE. A nuclear role for Osh1p is possible because its relative, Osh3p, binds the nuclear DEAD-box RNA helicase Rok1p and is required for karyogamy and normal mitosis (Park et al., 2002; Yano et al., 2004). The sorting signals responsible for targeting Nvj1p to the outer nuclear membrane are not known but probably involve its uncleaved N-terminal signal sequence. Similarly, the N-termini of Pho8p and Suc2p act as uncleavable signal sequences important for their proper sorting to and from the ER (Klionsky and Emr, 1990; Noda et al., 1995). Blocking the N-terminus of Nvj1p with either GFP or HA tags results in the mislocalization of the fusion protein to extranuclear ER-like membranes. Mislocalized Nvj1p retains the ability to interact with vacuoles and sequester Osh1p, thereby supporting our conclusion that Nvj1p is the docking receptor for Osh1p at the NE.

The individual and collective roles of ORPs, including the yeast Osh proteins, are poorly understood. Yeast depleted of all Osh protein accumulate high levels of ergosterol and 22-dihydroergosterol (Beh et al., 2001), yet Osh3p has been shown to play a role in the sensitivity to ISP-1, an inhibitor of sphingolipid synthesis (Yano et al., 2004), and osh4Δ cells bypass the requirement for SEC14, an essential gene encoding a phosphatidylcholine-phosphatidylinositol transfer protein (Fang et al., 1996). Additionally, the oxysterol-binding domain common to all Osh family members associates in some cases with acidic phospholipids (Xu et al., 2001) and phosphoinositides (Li et al., 2002). Thus, yeast ORPs are required for sterol homeostasis but might also affect the synthesis or trafficking of sphingolipids and phosphoinositides. The fact that any one of the seven OSH gene family members, including OSH1, rescues the sterol-accumulation defect of Osh-depleted cells is the basis for the conclusion that all seven proteins share a redundant function in sterol synthesis and/or post-synthetic sterol lipid trafficking (Beh et al., 2001). Owing to the involvement of Osh4p in Golgi secretion, Beh et al. postulated that members of the Osh family might regulate vesicular budding from different membrane compartments, and they predicted that cells depleted of Osh protein would accumulate many vesicles and/or aberrant organelles (Beh et al., 2001). This notion is supported by our observation that Osh-depleted cells accumulate PMN structures and exhibit reduced rates of PMN-mediated degradation. We suggest that the function of Osh1p at NV junctions is linked to the modification or transport of lipids that are required for the formation of PMN blebs and vesicles. Interestingly, human ORP1L (long splice variant), a structural homolog of Osh1p (Olkkonen and Levine, 2004), might play an analogous role at late endosomes. The N-terminal ankyrin repeat of ORP1L targets the protein to late endosomes, where ORP1L plays an undefined role in endosomal structure (Johansson et al., 2003). As we have suggested for PMN, lipids are known to play important roles in the maturation of late endosomes and the formation of multivesicular bodies, which occurs by budding and invagination of the limiting membrane of early endosomes (for review, see Lemmon and Traub, 2000).

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