Altering Sphingolipid Metabolism in *Saccharomyces cerevisiae* Cells Lacking the Amphiphysin Ortholog Rvs161 Reinitiates Sugar Transporter Endocytosis

Jeanelle Morgan,1,2‡ Paula McCourt,1,2† Lauren Rankin,1 Evelyn Swain,2§ Lyndi M. Rice,1,2 and Joseph T. Nickels, Jr.1,2*

Pharmacogenomics Division, Medical Diagnostics Laboratories, L.L.C., Hamilton, New Jersey 08690,1 and Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, Pennsylvania 191022

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Amphiphysins are proteins thought to be involved in synaptic vesicle endocytosis. Amphiphysins share a common BAR domain, which can sense and/or bend membranes, and this function is believed to be essential for endocytosis. *Saccharomyces cerevisiae* cells lacking the amphiphysin ortholog Rvs161 are inviable when starved for glucose. Altering sphingolipid levels in *rvs161* cells remediates this defect, but how lipid changes suppress remains to be elucidated. Here, we show that the sugar starvation-induced death of *rvs161* cells extends to other fermentable sugar carbon sources, and the loss of sphingolipid metabolism suppresses these defects. In all cases, *rvs161* cells respond to the starvation signal, elicit the appropriate transcriptional response, and properly localize the requisite sugar transporter(s). However, Rvs161 is required for transporter endocytosis. *rvs161* cells accumulate transporters at the plasma membrane under conditions normally resulting in their endocytosis and degradation. Transporter endocytosis requires the endocytosis (endo) domain of Rvs161. Altering sphingolipid metabolism by deleting the very-long-chain fatty acid elongase *SUR4* reinitiates transporter endocytosis in *rvs161 and rvs161 endo−* cells. The sphingolipid-dependent reinitiation of endocytosis requires the ubiquitin-regulating factors Doa1, Doa4, and Rsp5. In the case of Doa1, the phospholipase A2 family ubiquitin binding motif is dispensable. Moreover, the conserved AAA-ATPase Cdc48 and its accessory proteins Shp1 and Ufd1 are required. Finally, *rvs161* cells accumulate monoubiquitin, and this defect is remediates by the loss of *SUR4*. These results show that defects in sphingolipid metabolism result in the reinitiation of ubiquitin-dependent sugar transporter endocytosis and suggest that this event is necessary for suppressing the nutrient starvation-induced death of *rvs161* cells.

The budding yeast *Saccharomyces cerevisiae* gene *RVS161/END6* encodes a helical protein of 265 amino acids that is a member of the N-BAR (for Bin, amphiphysin, Rvs) family of proteins (61). Rvs161 regulates cell polarity (20), actin cytoskeleton polarization (69), endocytosis (50), and secretory vesicle trafficking (7, 25). *rvs161* mutant cells die during stationary phase, have mating defects, are sensitive to high concentrations of NaCl, have endocytosis and actin defects, and are unable to grow on nonfermentable carbon sources (8, 13, 15, 50, 63, 69). Mutational studies have revealed two functionally independent Rvs161 domains: an NH2-terminal/BAR domain involved in endocytosis and actin organization and a COOH-terminal domain required for cell fusion during haploid cell mating (8).

The N-BAR family of proteins is constantly growing and includes yeast Rvs161 and Rvs167; human *BIN1*, *BIN2*, and *BIN3*; *hob1*+ and *hob3*+ from *Saccharomyces pombe*; murine Alp1; and human amphiphysins, which are a family of multidomain proteins that are involved in the late steps of clathrin-coated vesicle scission (24, 30, 58, 61). Two genes encode amphiphysin in humans, one expressed in the brain (amphiphysin I) and a second (amphiphysin II) with a broad tissue distribution and a wide array of alternatively spliced isoforms. Both have an N-terminal BAR domain and a C-terminal SH3 domain. The BAR domain interacts with phospholipids, which induces membrane curvature and tubulation, whereas the distal SH3 domain associates with proteins having proline-based motifs. Human amphiphysin has been implicated in endocytosis due to its interaction with dynamin and because of its homology to Rvs161 and Rvs167. It may be important in recycling the plasma membrane at synaptic terminals (14, 28, 42, 68).

The crystal structures of several N-BAR domains have been solved (9, 45, 58, 71, 72). The domain is present in proteins that are critical for the recycling of synaptic vesicles and T-tubule formation in muscle, such as amphiphysins, endophilin, nadrin, beta-centaurins, arfaptin, and oligophrenins. Peter et al. (58) have shown that an N-BAR domain (the BAR domain plus an adjacent amphipathic helix) is capable of inducing three-dimensional membrane curvature. Some BAR proteins have additional interactions with lipids through pleckstrin homology or phox homology domains. These domains may target a protein to a specific membrane compartment, while the BAR domain simultaneously detects or initiates membrane curva-
ture. There are two other BAR domains, F-BAR and I-BAR, which bind membranes and in some cases induce membrane tubulation (10, 35).

rsv161 and rsv167 mutants have common phenotypes (2, 7, 63, 69), and Rvs161 and Rvs167 physically interact (70). However, they have distinct nonoverlapping cell functions and physical interactions with other proteins (8, 26). The BAR domains of each cannot be interchanged, and the overexpression of RVS161 or RVS167 cannot cross-suppress the other’s phenotype (2, 70). Defects of rvs proteins, including salt sensitivity, cell death during starvation, and the lack of growth on nonfermentable carbon sources, are suppressed by mutations altering the expression of genes encoding inositolphosphorylceramide mannosyltransferase, respectively, and are required for the biosynthesis of sphingosine (18, 29, 51) and suppress yeast complex sphingolipids (Fig. 1) (17, 19). Recessive mutations of RVS161 and RVS167 suppress the growth defects we observed and restores sugar transporter endocytosis and degradation. Doa1, Doa4, and Rsp5 are required for sur4-dependent suppression and for transporter endocytosis and degradation, as is the conserved AAA-ATPase Cdc48 and its accessory factors, Shp1 and Ufd1.

**FIG. 1.** Sphingolipid biosynthetic pathway in *S. cerevisiae*. Genes are in italics. FA, fatty acid; VLCFA, very long chain fatty acids.

rsv161 cells die under conditions of glucose starvation. Here, we show that they harbor starvation defects on other fermentable carbon sources and are unable to thrive when galactose, maltose, or melibiose is the available carbon source. Mutant cells can sense a glucose starvation signal, derepress glucose-repressed genes, initiate Snf3- and Rgt2-dependent *HXT* transcription, and properly localize high- and low-affinity glucose transporters. They also express and properly localize the Gal2 galactose and Mal61 maltose permeases. However, rsv161 cells are unable to endocytose and degrade these sugar transporters. The loss of function of *SUR4* suppresses all carbon source growth defects we observed and restores sugar transporter endocytosis and degradation. Doa1, Doa4, and Rsp5 are required for sur4-dependent suppression and for transporter endocytosis and degradation, as is the conserved AAA-ATPase Cdc48 and its accessory factors, Shp1 and Ufd1.

**MATERIALS AND METHODS**

Media and miscellaneous microbial techniques. Yeast strains were grown in YEP (1% yeast extract, 2% Bacto-peptone) containing the indicated concentrations of the indicated carbon source in YPG (1% yeast extract, 2% Bacto-peptone, 3% glycerol) or in synthetic minimal medium containing 0.67% yeast nitrogen base (Difco) supplemented with the appropriate amino acids and adenine. Yeast transformations were performed using the procedure described previously (43). For the routine propagation of plasmids, *Escherichia coli* XL1-Blue cells were used and grown in Luria broth supplemented with ampicillin (200 mg/ml).

**Strain and plasmid construction.** The yeast strains used are derived from W303 (YIN17) (MATa ura3-52 leu2 his3 2 α ade2 α), hxt1::HXT1-GFP-TRP1, hxt2::HXT2-GFP::TRP1, hxt4::HXT4-GFP::TRP1, gal2::GAL2-GFP::TRP1, and mlb1::MAL61-GFP::TRP1 alleles were generated as described previously (43) by using the pFA6a-GFP(S65T) module. cdc48::kanr ura3-52::cdc48-3::URA3 and ufd1::kanr ura3-52::ufd1-1::URA3 strains were generated using the diploid strain YH1 (MATa ura3-52 ura3-52 his3/α his3/2 leu2/2 leu2/2 TRP1/1 lys2/2 ade2/2 ade2/2). As the deletion of CDC48 or UFD1 is lethal in haploid strains. First, cdc48::kanr and ufd1::kanr alleles were synthesized by the PCR amplification of the cdc48::kanr or ufd1::kanr allele from heterozygous CDC48/cdc48::kanr and UFD1/ufd1::kanr strains (Research Genetics), respectively. These alleles were transformed into YH1, and integrants were selected on yeast extract-peptone-dextrose (YPEP) plates containing 250 μg/ml G418. Proper integration was determined by PCR. Ylp-cdc48-3 and Ylp-ufd1-1 were digested with StuI and integrated at the ura3-52 locus of the CDC48/cdc48::kanr and UFD1/ufd1::kanr strains, respectively. Cells were sporulated, and haploid cdc48::kanr ura3-52::cdc48-3::URA3 and ufd1::kanr ura3-52::ufd1-1::URA3 strains were obtained by prototrophic amino acid and temperature-sensitive selections. The cdc48-3 and ufd1-1 alleles used to construct Ylp-cdc48-3 and Ylp-ufd1-1 were generated by the PCR amplification of YIN315 and YIN318, respectively, doa1::His3, doa1::Asp1::TRP1, and doa1::Asp1::LEU2 were digested with StuI and integrated at the ura3-52 locus of the CDC48/cdc48::kanr and UFD1/ufd1::kanr strains, respectively. Cells were sporulated, and haploid cdc48::kanr ura3-52::cdc48-3::URA3 and ufd1::kanr ura3-52::ufd1-1::URA3 strains were obtained by prototrophic amino acid and temperature-sensitive selections. The cdc48-3 and ufd1-1 alleles used to construct Ylp-cdc48-3 and Ylp-ufd1-1 were generated by the PCR amplification of YIN322, YIN323, and YIN325, respectively, doa1::His3, pgal1::Rps5-1 was used to express the dominant-negative rps5-1 allele.

**Total RNA isolation.** All solutions were prepared with diethyl pyrocarbonate-treated water. Cells were harvested, centrifuged, and pellets for 30 s. Cell pellets were resuspended in 200 μl of YRL buffer (200 mM Tris, pH 7.5, containing 500 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and 200 μl of PCIAA (phenol, chloroform, isoamyl alcohol). Two hundred microliters of nitric acid-washed beads was added, and cells were vortexed for 2.5 min. Three hundred microliters of YRL buffer and 200 μl of PCIAA were added, and cells were vortexed for 2.5 min. Cells were centrifuged for 5 min, and the resulting clear lysate was removed and added to 400 μl of PCIAA, vortexed for 2.5 min, and centrifuged for 5 min. The resulting aqueous layer was added to 500 μl of 100% ethanol, and total RNA was precipitated overnight at −20°C. A total RNA pellet was obtained by centrifugation at 13,000 rpm for 15 min at 4°C, washed twice with 70% ethanol, vacuum dried, and resuspended in water. RNA was stable at −20°C for several weeks.
Northern analysis. Total RNA was resolved using 6% formaldehyde agarose gel electrophoresis. Total RNA (20 μg) in loading buffer (20 mM morpholinepropanesulfonic acid, pH 7.0, containing 10 mM sodium acetate, 2 mM EDTA, 45% formamide, 6% formaldehyde, 1% ethidium bromide, 0.003% bromophenol blue, 0.03% xylene cyanol FF, 1.5% Ficoll) was analyzed. RNA was blotted onto Hybond-N nitrocellulose (Amersham, Arlington Heights, IL) overnight at room temperature using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Churches buffer (10 mM sodium-phosphate buffer, 1 mM EDTA, 1% bovine serum albumin, 7% SDS) was used for all hybridization procedures. Hybridization was performed overnight at 65°C. Gel-purified radiolabeled probes were boiled in 200 μl salmon sperm DNA prior to use. After hybridization, blots were washed twice in 2× SSC at room temperature, twice in 2× SSC-0.5% SDS at 65°C, and twice in 0.1× SSC at room temperature. Gene expression was determined by autoradiography using Kodak X-OMAT film. The specificity of each HXT probe was checked by Northern analysis using hxt1, hxt2, hxt4, and hxt6 strains. U2 expression was used as a loading control.

Fluorescence microscopy. Cultures were grown to exponential phase in YEP containing 6% glucose. Cells then were shifted to YEP containing the indicated concentrations of glucose for the specified time. Hxt1-green fluorescent protein (Hxt1-GFP), Hxt2-GFP, and Hxt4-GFP localization was visualized using a Leica DRBM Fluorescence microscope, fluorescein isothiocyanate optics, and a PlanAPO 100 objective. Images were obtained using Open Labs software (version 2.1). Final fluorescence images were generated using Adobe Photoshop (version 7.0).

Western analysis of Hxt1-GFP and Hxt2-GFP stability. Cultures were grown to exponential phase (density at 600 nm of 0.5 to 1.0) in YEPD and then shifted to the appropriate medium to regulate HXT expression and protein synthesis. Twenty-milliliter cultures were collected at various times, and a total cell protein extract was obtained. Briefly, cells were pelleted, washed once with distilled water, and lysed in buffer A [200 mM Tris-HCl, pH 7.9, containing 390 mM (NH₄)₂SO₄, 10 mM MgSO₄, 20% (vol/vol) glycerol, 1 mM EDTA] using glass beads. Lysis buffer also contained 15 mM mercaptoethanol, 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride], 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 10 μl of Sigma protease inhibitor solution (Sigma Chemicals, St. Louis, MO). A hundred micrograms of total cell protein extract was resuspended in Laemmli buffer (12), and proteins were resolved using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes.

All steps for Western analysis were performed at room temperature. Membranes were blocked for 1 h with 5% nonfat dry milk in buffer B (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.05% Tween-20). Incubations with primary and secondary antibodies were performed for 1 h in buffer C. Membranes were washed six times after antibody incubations with buffer C. Blots were incubated with mouse anti-GFP monoclonal antibodies (1:2,000 dilution) (Clontech, Mountain View, CA) and anti-mouse horseradish peroxidase (HRP)-conjugated monoclonal antibodies (1:5,000 dilution) (Amersham Corp., Arlington Heights, IL). Anti-actin polyclonal antibodies (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA) were used to determine protein loading. Hxt1-GFP and Hxt2-GFP were detected using chemiluminescence and autoradiography. Anti-Gal2 and anti-Mal61 polyclonal antibodies were used at a 1:500 dilution. GS-800 densitometer and Quantity One software (4.6.6). All relative densitometry values were normalized to total actin levels. The values in Fig. 9 are the averages from three experiments ± standard deviations.

RESULTS

**rsv161** cells harbor pleiotrophic carbon source growth defects that can be suppressed by altering sphingolipid levels. Cells lacking **RVS161** cannot grow under conditions of glucose starvation (Fig. 2A). As previously described (15), this starvation defect could be suppressed by altering sphingolipid levels through the loss of **SUR4**. We asked whether starvation-induced death extended to other fermentable carbon sources. **rsv161** cells were unable to grow when starved for fructose, sucrose, or raffinose (Fig. 2A). Interestingly, mutant cells also were incapable of growing on 2% galactose, 2% maltose, or 2% melibiose (Fig. 2B). Deleting **SUR4** remediated all carbon source defects we observed (Fig. 2). To uncover the molecular basis for these carbon source defects and their sphingolipid-dependent suppression, we asked whether **rsv161** cells could sense changes in glucose levels or distinguish what carbon source they were grown on and regulate gene transcription accordingly.

**rsv161** cells are able to derepress glucose-repressed genes as well as activate carbon source-dependent transcription. Glucose represses the expression of genes that are dispensable under rich growth conditions (e.g., **GAL**, **SUC**, and genes encoding cytochromes and triglyceric acid cycle enzymes) (54). When glucose levels are depleted, the expression of these and other genes are induced or derepressed, and this transcriptional response is required for sustained growth. We asked whether **rsv161** cells were capable of derepressing glucose-repressed genes. As a model for glucose derepression, we examined the expression levels of the inveratase gene **SUC2**.

Cells were grown in YEPD and shifted to glycerol-containing medium to induce **SUC2** expression. All strains tested were able to derepress and induce the expression of **SUC2** (Fig. 3A). We also tested if mutant cells could induce **SUC2** expression in raffinose-grown cultures. We reasoned that raffinose utilization (2% raffinose) through inverterase-dependent hydrolysis would more closely mimic low-glucose conditions while being less deleterious to cells than examining expression in glucose-starved conditions. We asked how these genes could be derepressed under conditions of glucose deprivation. We asked how these genes could be derepressed under conditions of glucose deprivation.
**FIG. 3.** Transcriptional responses of *rvs161* cells to growth on multiple carbon sources and carbon source starvation are intact. In all cases, cells were grown to exponential phase in YEPD at 30°C prior to shifting cells to the various carbon sources. Gene expression levels were determined by Northern analysis. (A) Cells were shifted to medium containing 2% glycerol–0.05% glucose. (B) Cells were shifted to medium containing 2% raffinose. Cells were shifted to medium containing 2% galactose for GAL2 expression (C) or 2% maltose for MAL61 expression (D). (E) Lane 1, cells were shifted to 2% glucose, and *HXT* expression levels were determined after 2 h; lane 2, cells were shifted to 0.05% glucose, and *HXT* expression levels were determined after 2 h; lane 3, cells were shifted back to 2% glucose and *HXT* expression levels were determined after 2 h. *U2* expression was used as a loading control.

The induction of *SUC2* expression in raffinose has been demonstrated previously (57). *SUC2* expression was induced in all strains tested and was sustained to similar levels (Fig. 3B). *rvs161* cells were incapable of growing on galactose and maltose (Fig. 2B). Thus, we examined the galactose- and maltose-induced expression of the *GAL2* and *MAL61* permease genes, respectively. We found no differences in expression between wild-type and *rvs161* cells (Fig. 3C). Finally, the sucrose-induced extracellular excretion of Suc2 invertase that is required for raffinose hydrolysis was normal in *rvs161* mutants (data not shown). Based on these results, we conclude that *rvs161* cells do not harbor defects in derepressing glucose-repressed genes, in their transcriptional response to growth on alternative fermentable carbon sources, or in secreting enzymes that are required to grow on various carbon sources.

**rvs161** cells activate the Snf3- and Rgt2-dependent expression of *HXT* genes. Glucose utilization in *S. cerevisiae* begins with the transport of glucose into the cell by specific high- and low-affinity glucose transporters. Hxt2 and Hxt4 are high-affinity glucose transporters and are expressed when glucose levels are low, while the expression of the low-affinity glucose transporter Hxt1 is induced in high glucose concentrations. The expression of *HXT3* is moderately induced at all glucose concentrations (55). The glucose-dependent expression of *HXT* transporters is initiated and terminated by the Snf3 and Rgt2 glucose sensors. We asked whether the lack of proper *HXT* expression contributed in any way to starvation-induced death by asking if *rvs161* cells were capable of initiating and terminating Snf3- and Rgt2-dependent *HXT* expression. Glucose concentration-dependent *HXT* expression levels were determined using Northern analysis. The expression patterns of *HXT1* to *HXT4* in wild-type and *rvs161* cells grown under various glucose concentrations were identical (Fig. 3D). Thus, *rvs* cells are able to initiate Snf3- and Rgt2-dependent transcriptional signaling, resulting in the proper expression of *HXT* genes.

**rvs161** cells are defective in the endocytosis and degradation of multiple sugar transporters. Normal *HXT* gene expression observed in *rvs161* cells prompted us to determine whether Hxt transporter mislocalization and/or protein instability contributed to starvation-induced death. *rvs161* mutants do accumulate vesicles at the cytoplasmic side of the plasma membrane (7, 25). We constructed strains harboring an endogenous GFP-tagged allele of *HXT2*. Various glucose levels regulated *HXT2* gene expression, and fluorescence microscopy was used to visualize localization.

As expected based on our expression data, plasma membrane-associated Hxt2 was not seen in wild-type cells grown in rich medium (data not shown). It localized to the plasma membrane after cells were shifted to starvation media for 2 h (0.05% Glu), and it disappeared after a shift back to rich medium for 5 h (Fig. 4). Thus, wild-type cells properly localized Hxt2 in response to changes in glucose levels. We found that Rvs161 was dispensable for the plasma membrane-associated localization of Hxt2 but was absolutely required for its disappearance (Fig. 4). Hxt2-GFP levels accumulated at the plasma membrane in mutant cells even after being shifted to rich medium for 5 h and persisted for up to 16 h. Importantly, we could reinitiate the loss of Hxt2 at the plasma membrane if we deleted *SUR4* in mutant cells (Fig. 4). Interestingly, cells lacking *SUR4* showed a fluorescent fragmented vacuolar morphology rather than the large single fluorescent organelle seen in cells with normal *Sur4* function. This has been observed previously (39).

To determine whether the disappearance of Hxtp from the plasma membrane was representative of their endocytosis and degradation, the kinetics of glucose transporter degradation were determined using Western analysis. The high-affinity glu-
cose transporters Hxt2 and Hxt4 were examined first. Hxt2 was detected in wild-type cells after 2 h in 0.05% glucose-containing medium and was almost completely endocytosed and degraded after a 3-h shift to rich medium (Fig. 5A). sur4 mutants showed similar kinetics (Fig. 5A). In contrast, rvs161 cells accumulated Hxt2 (Fig. 5A), while degradation was restored if we deleted SUR4 (Fig. 5A). Identical results were obtained when examining Hxt4 degradation (data not shown).

The endocytosis and degradation of low-affinity glucose transporters also were defective in rvs161 cells, as evidenced by the aberrant accumulation of Hxt1. We found that Hxt1 was plasma membrane localized in wild-type cells grown in medium containing 2% glucose (Fig. 5B). After 1 h of being shifted to medium containing 0.05% glucose, wild-type cells completely endocytosed and degraded this transporter (Fig. 5B). rvs161 cells accumulated Hxt1 under these same conditions (Fig. 5B). The loss of SUR4 in rvs161 cells restored Hxt1 endocytosis and degradation levels to those seen in sur4 cells (Fig. 5B). Fluorescence microscopy showed that Hxt1-GFP localized to the plasma membrane in rvs161 cells (data not shown).

rs161 cells also were defective in endocytosing and degrad-

FIG. 4. Aberrant plasma membrane accumulation of Hxt2 in rvs161 cells can be suppressed by the loss of SUR4. Cells were grown to exponential phase at 30°C in YEPD and then sequentially shifted to medium containing the indicated sugar concentrations for the indicated times. Hxt2-GFP localization was visualized by live-cell fluorescence microscopy. WT, wild type; Glu, glucose; hr, hours.

FIG. 5. Sugar transporter endocytosis defects of rvs161 cells can be suppressed by the loss of SUR4. Cells were grown to exponential phase at 30°C in YEPD (A and B) or YEP containing 2% galactose (C). They then were sequentially shifted to medium containing the indicated sugar concentrations for the times indicated. Cell lysates were resolved by SDS-PAGE, and sugar transporter levels were determined by Western analysis using anti-GFP polyclonal antibodies (A and B) or anti-Gal2 polyclonal antibodies (C). Act1 (actin) levels were used as a loading control. WT, wild type; Glu, glucose; hr, hours. (A) Hxt2-GFP; (B) Hxt1-GFP; (C) Gal2.

The endocytosis domain of Rvs161 is required for growth under glucose starvation and for the endocytosis of glucose transporters. The fact that Rvs161 is required for growth on multiple carbon sources and for the endocytosis of multiple sugar transporters prompted us to determine the Rvs161 do-
main(s) responsible for these functions. Brizzio et al. (8) isolated strains harboring recessive \textit{rsv161} alleles giving rise to either endocytosis (End$^-$ Fus$^-$) or cell fusion/mating (End$^+$ Fus$^-$) defects, thus delineating the functional domains of \textit{Rvs161}. \textit{R35C}, \textit{R113K}, and \textit{P158S} alleles cause endocytosis defects, while \textit{A175P} and \textit{P203Q} are defective in cell fusion/mating based on several criteria (Fig. 6A) (8).

We tested whether the two cell fusion and three endocytosis mutants (\textit{P158S}, \textit{R35C}, and \textit{R113K}) could grow when starved of glucose. Surprisingly, none of these mutants harbored defects at 30°C (data not shown). Only when we grew cells at 37°C did we observe a phenotype (Fig. 6B). \textit{rsv161} cells harboring \textit{P158S}, \textit{R35C}, or \textit{R113K} (End$^-$ Fus$^-$) alleles were unable to grow. \textit{A175P} cells (End$^+$ Fus$^-$) grew as well as wild-type cells, but \textit{P203Q} cells consistently showed an intermediate starvation defect (Fig. 6B). We obtained similar results with the carbon sources and various sugar starvation conditions tested in Fig. 2 (data not shown).

We next asked if the \textit{endo} domain was required for glucose transporter endocytosis. We examined the localization and endocytosis of Hxt2-GFP by using \textit{rsv161} \textit{R113K} as a representative \textit{endo}– mutant (Fig. 7). At 30°C, mutant cells localized, endocytosed (with a slight delay), and degraded Hxt2-GFP like wild-type cells. However, at 37°C, \textit{rsv161} \textit{R113K} cells were incapable of endocytosing Hxt2-GFP from the plasma membrane. The loss of \textit{SUR4} suppressed this defect (data not shown). Cells harboring the \textit{A175P} allele were not defective in endocytosis (data not shown). Similar results were observed for Hxt1-GFP, Gal2-GFP, and Mal61-GFP localization and degradation (data not shown). Thus, the endocytosis domain of \textit{Rvs161} is required for growth under glucose starvation and for the endocytosis and degradation of multiple sugar transporters.

\textbf{Reinitiation of Hxt2 endocytosis in \textit{rsv161 sur4} requires Doa1, Doa4, and Rsp5 functions.} We next asked if factors required for ubiquitin-mediated endocytosis were needed for the \textit{sur4}-dependent reinitiation of sugar transporter endocytosis. To address this, we determined if Hxt2-GFP endocytosis in \textit{rsv161 sur4} cells required Doa1 (which regulates the cellular ubiquitin concentration), Doa4 (ubiquitin hydrolase), and/or Rsp5 (ubiquitin ligase). Fluorescence microscopy revealed a requirement for all three proteins. \textit{rsv161 sur4 doa1}, \textit{rsv161 sur4 doa4}, and \textit{rsv161 sur4} cells harboring the dominant-negative \textit{rsp5-1} allele all accumulated Hxt2-GFP under conditions of high glucose growth (Fig. 8). Thus, the reinitiation of glucose transporter endocytosis in \textit{rsv161} cells by the loss of \textit{SUR4} requires several factors for ubiquitin-mediated endocytosis.

The Cdc48 binding domain of Doa1 is required for the reinitiation of glucose transporter endocytosis in \textit{rsv161 sur4} cells. Doa1 contains a ubiquitin binding domain (PFU) and a second carboxyl-terminal domain (PUL), which binds to the conserved AAA-ATPase Cdc48 (49). Both domains link ubiquitylated substrates to Cdc48 and are thought to be required for Doa1 function (49, 62). We asked if one or both of these...
domains were required for the sur4-dependent reinitiation of sugar transporter endocytosis in rvs161 cells. We also determined whether Cdc48 itself, and/or the Cdc48 accessory factors Shp1 and Ufd1, also were needed. Shp1 and Ufd1 bind directly to Cdc48 (46). Both Shp1 and Ufd1 bind ubiquitin. Shp1 is necessary for Cdc48 function in membrane fusion and proteosomal degradation, while Ufd1 facilitates endoplasmic reticulum (ER)-dependent degradation and the activation of membrane-associated transcription factors. We examined the glucose concentration-dependent appearance/disappearance of Hxt2-GFP from the plasma membrane, and the data are presented in Table 1.

The Cdc48 binding PUL domain of Doa1 (doo1ΔC allele) was required to reinitiate endocytosis by the loss of SUR4, while the PFU domain (doo1F417D F434D allele) was dispensable. rvs161 sur4 doa1ΔC cells accumulated Hxt2 to the same extent as rvs161 cells shifted from 0.05 to 6% glucose for 5 h, while the accumulation of this transporter in rvs161 sur4 (doo1F417D F434D) cells was similar to that seen in rvs161 sur4 suppressor cells (Table 1). Cdc48 itself also was required, as rvs161 sur4 cells harboring a cdc48-3 ts allele were incapable of endocytosing Hxt2 at the restrictive temperature. In fact, rvs161 sur4 cdc48-3 ts cells showed a defect in endocytosis and displayed the characteristic elongated phenotype of cdc48 alleles even at the permissive temperature (62). Both of the Cdc48 binding factors, Shp1 and Ufd1, also were required. rvs161 sur4 cells deleted for SHP1 or harboring a temperature-sensitive ufd1-1 allele were defective in Hxt2 endocytosis (Table 1). Based on these results, we conclude that the sphingolipid-dependent reinitiation of sugar transporter endocytosis in rvs161 cells requires the function of multiple Cdc48 complexes.

Loss of SUR4 reinitiates the monoubiquitin hyperaccumulation defect of rvs161 cells. The gene products that are required for the sur4-dependent suppression of rvs161 endocytosis defects bind to and/or regulate ubiquitin levels. Moreover, our attempt to suppress the endocytosis defects of rvs161 cells by overexpressing ubiquitin failed. Thus, we asked whether rvs161 cells have an altered ubiquitin metabolism, and if so, does the loss of SUR4 suppress these defects. rvs161 cells hyperaccumulated monoubiquitin (1.3-fold), and deleting SUR4 in these cells did decrease the level of this ubiquitin species to nearly that seen in wild-type cells (~80%) (Fig. 9). Interestingly, sur4 cells had a drastically lower level of mono-ubiquitin than the wild type, but their levels of high-molecular-weight conjugates were normal (Fig. 9). When we looked at those mutations in rvs161 sur4 cells causing the loss of glucose concentration-dependent Hxt2 endocytosis (Table 1), the overall observation was that they all altered ubiquitin metabolism. The common phenotype seen was a decrease in very-high-molecular-weight ubiquitin conjugate levels and the accumulation of several faster-migrating polyubiquitin species, although this phenotype was more subtle in rvs161 sur4 shp1 and rvs161 sur4 ufd1-1 cells. In some cases, the level of monoubiquitin was altered (rvs161 sur4 doa1, rvs161 sur4 doa1ΔC, and rvs161 sur4 cdc48-3 cells). Based on these results, we believe that the synthesis of monoubiquitin, its conversion to high-molecular-weight conjugates, and the subsequent turnover of these conjugates all are necessary for the sur4-dependent suppression of rvs161 endocytosis defects.

**DISCUSSION**

The rate of glucose transporter endocytosis is drastically reduced in rvs161 cells, and this defect correlates with starvation-induced death under low-glucose conditions. These results suggest that the glucose starvation defect is linked to the improper regulation of hexose transporter trafficking and/or degradation. Viability under starvation is linked to the endo domain of Rvs161, as endo− mutants are inviable when starved and are defective in endocytosing glucose transporters from the membrane. In addition, the loss of SUR4 suppresses the starvation defect and reinitiates glucose transporter endocytosis.

How might the reinitiation of endocytosis remediate starvation-induced death? One possibility is that the accurate endo-

**TABLE 1.** Glucose concentration-dependent appearance/disappearance of Hxt2-GFP from the plasma membrane

| Strain                  | % of cells with plasma membrane- | % of cells with plasma membrane- | % of cells with plasma membrane- |
|-------------------------|---------------------------------|---------------------------------|---------------------------------|
|                         | associated Hxt2 at glucose conc. | associated Hxt2 at glucose conc. | associated Hxt2 at glucose conc. |
| Wild type               | 1 ± 0.5                         | 95 ± 3                          | 14 ± 5                          |
| rvs161                  | 2 ± 1                           | 97 ± 3                          | 95 ± 4                          |
| rvs161 sur4             | 1 ± 1                           | 97 ± 5                          | 21 ± 4                          |
| rvs161 sur4 doa1        | 3 ± 2                           | 92 ± 4                          | 94 ± 6                          |
| rvs161 sur4 doa1ΔC      | 1 ± 0.5                         | 93 ± 3                          | 18 ± 4                          |
| rvs161 sur4 doa1ΔC      | 2 ± 0.5                         | 94 ± 3                          | 92 ± 4                          |
| rvs161 sur4 doa1ΔC      | 6 ± 4                           | 88 ± 5                          | 94 ± 6                          |
| rvs161 sur4 ufd1-1      | 1 ± 0.5                         | 99 ± 3                          | 97 ± 4                          |
| rvs161 sur4 shp1        | 1 ± 0.5                         | 95 ± 5                          | 89 ± 5                          |

*Cells were sequentially shifted from 6% glucose to 0.05% glucose for 1 h, and then back to 6% glucose for 3 h. Values are the averages from three independent experiments.

*Cells were assayed at 30°C.*
cytosis/degradation of sugar transporters acts as a regulatory step that is necessary to maintain proper stoichiometric transporter ratios, which are critical for growth under nutrient stress conditions; rvs161 mutants accumulate high-affinity transporters in response to glucose starvation, and they lack the ability to endocytose/degrade low-affinity transporters. Glucose transporters in mammalian cells, specifically Glut1, exist in dimeric and tetrameric states, but these oligomerizations are not essential for glucose uptake (48). A nonfunctional chimera consisting of yeast Hxt1 and Hxt4 transporters inhibits the function of wild-type glucose transporters (67). Whether this chimera functions as a dominant-negative mutant, directly interacting with and inhibiting specific glucose transporters, has not been studied. Therefore, is maintaining proper ratios of homo- and heterodimeric transporters necessary for viability under various growth conditions? With that said, we cannot rule out that the reinitiation of global endocytosis itself remediates the starvation defect rather than the specific reinitiation of proper stoichiometric transporter ratios. rvs161 cells are defective for growth under sulfur and nitrogen starvation conditions, and the loss of SUR4 remediates these growth defects as well (15).

An alternative scenario is that rvs161 cells internalize and metabolize too much glucose due to defects in endocytosis and the constitutive accumulation of sugar transporters at the plasma membrane, which depletes cellular ATP stores. The first step in glucose utilization after internalization is a phosphorylation event by hexose kinase, resulting in glucose-6-phosphate production and shunting through the glycolytic pathway (27). rvs161 cells accumulate the high-affinity glucose transporter Hxt2 and may accumulate others, such as Hxt6 and Hxt7. The concentration of glucose (2.7 mM) under starvation conditions is within the $K_{m}$ ranges of Hxt6 and Hxt7 ($K_{m} = 1$ to 2 mM) as well as those of Hxt2 ($K_{m} = 1.5$ mM) and Hxt4.
(K_m − 10 mM) (34). Here again the general reinitiation of endocytosis/degradation would act as a balance in conjunction with the rate of biosynthesis in order to maintain proper transporter ratios at the plasma membrane. Preliminary studies examining the rate of glucose binding and internalization in rvs161 cells thus far have been inconclusive (E. Swain and J. T. Nickels, unpublished data).

In addition to its role in endocytosis, Rvs161 is required for actin repolarization following osmotic salt stress. Wild-type cells depolarize actin following salt stress and repolarize after a period of adaptation, whereas rvs161 mutant cells depolarize actin but are unable to repolarize afterwards (1, 69). The loss of SUR4 suppresses the actin polarization defect of rvs161 cells under conditions that are semipermissive for viability (3.4% NaCl) (1). However, it does not suppress the actin polarization defect of mutant cells grown under glucose starvation conditions (26) or under high salt stress, which results in inviability (6% NaCl) (45a). Thus, how the loss of SUR4 suppresses rvs defects cannot be explained solely through its effects on the actin cytoskeleton. Interestingly, the glucose starvation defect of the endo-deficient Rvs161 point mutants correlates with their ability to form an Rvs161-Rvs167 complex, as the rvs161 R113K allele cannot bind Rvs167, as determined by two-hybrid analyses (P. McCourt, J. Morgan, and J. T. Nickels, unpublished data). These results seem reasonable, as the loss of SUR4 is able to remediate defects of rvs161 rvs167 cells (15). Thus, suppression can occur in the absence of any Rvs161- or Rvs167-associated multiprotein complexes.

The Cdc48 binding domain of Doa1 was required for sugar transporter endocytosis in rvs161 sur4 cells. Moreover, direct roles for Cdc48-Shp1 and Cdc48-Ufd1 were demonstrated. Cdc48-Shp1 regulates several membrane fusion events, including nuclear envelope growth and reforming the ER and Golgi assembly after mitosis (46, 47, 64). During ER-dependent degradation, Cdc48-Ufd1 extracts ubiquitylated substrates from membranes for their degradation. In yeast, Cdc48-Ufd1 mobilizes the transcription factors Spt23 and Mga2 from membranes, as they are drawn to nascent buds destined for endocytosis (33, 59, 66). Based on these and other observations, Meyer and Popp (46) suggest that the fundamental activity of Cdc48 is the energy-dependent removal of ubiquitylated proteins from membranes. Once removed, these proteins are free to be degraded or deubiquitylated. How Cdc48 regulates sugar transporter endocytosis needs to be explored; understanding how it helps in reinitiating sphingolipid-dependent endocytosis should be an excellent model for study.

The level of monoubiquitin was increased in rvs161 cells, and the deletion of SUR4 remediated this defect to some extent. Monoubiquitin serves as a regulatory signal for the intracellular transport of proteins through the late secretory and endocytic pathways (reviewed in references 31 and 32). In yeast, membrane proteins such as amino acid permeases and mating factor receptors are ubiquitylated, and this modification acts as a signal for internalization and/or endosomal sorting (reviewed in references 38 and 65). The mammalian sugar transporters GLUT1 and GLUT4 are modified with ubiquitin as well as the ubiquitin-like protein SUMO (22, 41). Whether yeast glucose transporters exhibit a similar fate is not known. However, the sphingolipid-dependent reinitiation of endocytosis has a strict requirement for factors regulating ubiquitin pools, pointing to the ubiquitylation of glucose transporters being an important regulatory step in their turnover.

The N-BAR domain of the BAR family of proteins plays a role in initiating and/or sensing membrane curvature (58). If yeast Rvs161 and/or Rvs167 sense and are drawn to nascent buds destined for endocytosis, they may act in the formation and function of a large membrane-associated multiprotein assembly complex that is involved in bud fission and subsequent endocytosis (21). Rvs167 does associate with the fission machinery; however, this is a late event in the scission process (37). sur4 cells accumulate the sphingoid LCB phytosphingosine (51). The endogenous addition of this LCB reinitiates endocytosis in cells deficient in sphingolipid biosynthesis (23, 73), and its transient accumulation may be required for ubiquitin-dependent proteolysis following heat stress (11). LCB accumulation may constitutively alter membrane curvature, causing invaginations within specific microdomains. These may give rise to putative pseudonascent buds that normally are initiated or stabilized by Rvs161-Rvs167 complexes, which then can attract factors that are required for fission. An important question remaining is whether LCB-dependent endocytosis requires the general fission machinery to initiate bud scission and release, as it does require ubiquitylation.

Defects in sphingolipid biosynthesis also could activate signaling pathways required to remediate rvs defects; activating these pathways would circumvent the need for any Rvs-dependent events during endocytosis. Sphingolipid intermediates in yeast, as in mammalian cells (16, 36, 52), are important signaling molecules, particularly the sphingoid base phytosphingosine. The accumulation of LCBs in yeast activates the Pkc1-MAP cell wall integrity pathway (74). The accumulation of the mammalian LCB sphingosine 1-phosphate in yeast stimulates Ca^{2+} accumulation, possibly initiating the LCB-dependent activation of Ca^{2+}/calmodulin-dependent signaling cascades (5).

Whether the sphingolipid mutations suppressing rvs defects all function by activating some LCB-dependent event(s) is not known. sur4 mutants accumulate phytosphingosine but also accumulate C_{24} fatty acids, are devoid of C_{26} fatty acids, produce complex sphingolipids that have shorter fatty acid moieties, and hyperaccumulate an inositolphosphorylceramide species (51). Whether any of these changes in lipid metabolism contribute to suppression is not known. Moreover, sur2 cells do not make phytosphingosine; thus, all complex sphingolipids are derived from the LCB dihydrosphingosine, and they lack C_{4} hydroxylation (29). Whether the loss of SUR2 and the accumulation of dihydrosphingosine activates pathways identical to those regulated by phytosphingosine is not known.

A number of sphingolipid mutations suppressing rvs161 defects alter calcium homeostasis (75). Csg2 and Sur1 are required for the synthesis of mannose inositolphosphorylceramide, and csg2 and sur1 mutants are calcium sensitive (3, 4). Recessive mutations in SUR2 and SUR4 remediate the calcium sensitivity of csg2 and sur1 cells (75). Interestingly, the addition of exogenous phytosphingosine alone to csg2 cells remedies their calcium-sensitive phenotype (75). We have been unsuccessful in suppressing rvs defects through calcium remediation or phytosphingosine supplementation. The endocytosis defect of rvs cells may preclude using exogenous methods; thus, more detailed studies are warranted.
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