Biochemical Characterization of the Wilson Disease Protein and Functional Expression in the Yeast Saccharomyces cerevisiae

(Received for publication, April 30, 1997, and in revised form, June 16, 1997)

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Wilson disease is a disorder of copper metabolism characterized by hepatic cirrhosis and neuronal degeneration due to inherited mutations in a gene encoding a putative copper-transporting P-type ATPase. Polyclonal antisera generated against the amino terminus of the Wilson protein detected a specific 165-kDa protein in HepG2 and CaCo cell lysates. Further analysis revealed that this protein is synthesized as a single-chain polypeptide and localized to the trans-Golgi network under steady state conditions. An increase in the copper concentration resulted in the rapid movement of this protein to a cytoplasmic vesicular compartment. This copper-specific cellular redistribution of the Wilson protein is a reversible process that occurs independent of a new protein synthesis. Expression of the wild-type but not mutant Wilson protein in the ccc2Δ strain of Saccharomyces cerevisiae restored copper incorporation into the multicopper oxidase Fet3p, providing direct evidence of copper transport by the Wilson protein. Taken together these data reveal a remarkable evolutionary conservation in the cellular mechanisms of copper metabolism and provide a unique model for the regulation of copper transport into the secretory pathway of eucaryotic cells.

Copper is an essential trace element that plays a fundamental role in biochemistry, permitting facile electron transfer reactions in diverse metabolic pathways. Despite this essential role, copper is highly reactive and potentially toxic, thus specialized pathways have evolved for the trafficking of this metal within cells (1). This is dramatically illustrated by the genetic disorders Wilson and Menkes disease as well as by recent studies revealing copper-dependent neuronal degeneration in amyotrophic lateral sclerosis and Alzheimer’s disease (2, 3). Although little information is currently available about these pathways, the recent cloning of the genes involved in several of these disorders provides the opportunity to elucidate such events at the molecular level.

Wilson disease is an inherited disorder resulting in hepatic cirrhosis and neuronal degeneration due to a marked impairment in biliary copper excretion. The Wilson disease gene has been cloned and shown to encode a protein with homology to the cation-transporting P-type ATPase family (4–6). This family includes a number of membrane proteins that utilize ATP-dependent phosphorylation of an invariant aspartate residue to derive energy for cation transport across membranes (7). Gene disruption studies of homologous transporters in prokaryotes and yeast have shown that these genes encode proteins required to maintain cellular copper homeostasis (8, 9). Despite these sequence data there is currently no information available about the structure or function of the Wilson disease protein. This current study was undertaken to directly address these issues.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—Cell lines were obtained and cultured as described previously (10). Copper concentration in the media was altered by adding CuSO4, and copper chelation was accomplished with 40 μM bathocuproine disulfonate. Cycloheximide was added at a concentration of 10 μg/ml and shown to inhibit ~95% protein synthesis. To generate polyclonal antisera to the Wilson protein, oligonucleotide primers were used to amplify a cDNA encoding amino acids 325–635 as described (10, 11). Murine monoclonal antibodies to γ-adaptin-1 (AP-1)1 were purchased from Sigma, a rabbit polyclonal antibody to TAP-1 was obtained from Ted Hansen (Washington University School of Medicine), rabbit polyclonal antibodies to the human asialoglycoprotein and transferrin receptors were obtained from Alan Schwartz (Washington University School of Medicine), and murine monoclonal antibodies to the bovine cation-independent mannose 6-phosphate receptor and AP-2 were obtained from Stuart Kornfeld (Washington University School of Medicine). Secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) were from Jackson ImmunoResearch.

Immunoblotting, Immunoprecipitation, and Gradient Fractionation—Cells were grown to confluence, lysed in 0.25% Nonidet P-40, 100 mM Tris-HCl, pH 8.0, at 4 °C for 30 min, and proteins in a postnuclear supernatant were separated by SDS-PAGE under reducing conditions followed by electrophoretic transfer to nitrocellulose membranes. Membranes were analyzed by immunoblotting as described (10). Cells were labeled by supplementation of the medium with [35S]methionine and [35S]cysteine at 500 μCi/ml for specific pulse periods followed by chase in serum-free medium as indicated. In some experiments tunicamycin was added 4 h before the addition of the radiolabel to a final concentration of 10 μg/ml. Immunoprecipitation was performed using protein A-Sepharose beads (12). For gradient fractionation, cells were lysed, homogenized, and subjected to isopycnic density gradient centrifugation as described previously (10, 13). Fractions were removed, subjected to SDS-PAGE on 5–12% gradient gels, and analyzed by immunoblotting as described above.

Immunofluorescence—Cells were plated on glass coverslips, grown for 24 h, fixed in 4% paraformaldehyde in phosphate-buffered saline for 20 min, quenched with 0.1% ethanolamine, and permeabilized in 0.2% Triton X-100 in phosphate-buffered saline for 10 min. Nonpecific binding was blocked by incubation in 3% bovine serum albumin in phosphate-buffered saline for 30 min followed by incubation with primary and secondary antibodies as indicated. After staining, coverslips were mounted on Mowiol 4-88 and analyzed using an Olympus BX-60 micro-

† The abbreviations used are: AP-1, γ-adaptin-1; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; PAGE, polyacrylamide gel electrophoresis.
Characterization and Expression of the Wilson Disease Protein

Fig. 1. Immunoblot analysis of Wilson protein in human cell lines. Cells were lysed, and 75 μg of protein was separated by SDS-PAGE, transferred to nitrocellulose, incubated with Wilson antibody, and analyzed by chemiluminescence. Lane 4 (Lymph) represents Epstein-Barr virus-transformed peripheral blood lymphocytes.

scope. To obtain through-focus images of cells, some samples were analyzed by laser confocal scanning microscopy (Molecular Dynamics) using a Nikon planapo (63 ×) oil-immersion lens (na 1.4). Argon-krypton laser emission wavelengths were 488 nm for FITC and 508 nm for TRITC.

Construction and Mutagenesis of the Wilson cDNA—The open reading frame of the human Wilson protein was constructed as follows. A Xhol-EcoRV fragment encoding amino acids 540–829 was obtained by screening a human liver cDNA library (4); HindIII-XhoI (amino acids 20–540), EcoRV-SacI (amino acids 829–1255), and SacI-Kpnl (amino acids 1255–1465) fragments were obtained by polymerase chain reaction of human liver cDNA using oligonucleotides corresponding to known Wilson sequence. Fragments were cloned into the pCRII vector (Invitrogen) followed by digestion with the designated restriction enzymes. To create the full-length cDNA, recovered fragments were reintegrated into the pCRII vector by four-part ligation. The 5’-most region of the Wilson protein was amplified using oligonucleotides corresponding to this region (14), and a 228-base pair Bsp120I fragment containing the first 156 nucleotides of the coding region was inserted into the full-length cDNA following digestion with Bsp120I. The resulting plasmid was designated pHWD. To subclone this cDNA for expression in Saccharomyces cerevisiae, pHWD was digested with XhoI and Kpnl, and the 4.5-kilobase insert was gel-purified, treated with Klenow polymerase to create blunt ends, and ligated into pYES2 (Invitrogen). Site-directed mutagenesis was performed using Klenataq polymerase (CLONTECH) and the ExSite mutagenesis kit (Stratagene) according to manufacturer specifications. Oligonucleotide primers corresponding to the H1070Q or P1038A mutations were synthesized and used to amplify pHYES2/hWD. The presence of the specific mutations as well as the fidelity of the entire cDNA sequence was confirmed by dideoxynucleotide sequencing (15).

Yeast Strains, Growth Conditions, and Transformations—S. cerevisiae strains used in this study were as follows: ccC2: MATa, his3–200, trp1–101, ural3–52, ade2, ade5, CCC2Δ::LEU2 (9); fet3Δ: MATa, ura3–52, lys2–801, ade2–101, his3–200, leu2–Δ1, GAL; HIS4: MATa, his3–Δ1, trp1–289, ura3–52, leu2, GAL; HY5: MATa, his3(his3–200 or his3–Δ1), trp1–Δ1 or trp–289), ura3–52, leu2, CCC2Δ::LEU2, GAL. To create HY5, a ccC2 strain capable of growth on galactose, HIS4 was crossed with cc2A using standard techniques for yeast manipulations (16). All strains were grown at 30 °C in appropriate dropout media (Bio101) supplemented with 2% indicated sugars. Yeast cells were transformed by the lithium acetate method (17), and uracil-based selection was used to screen for transformants. To induce expression of the Wilson protein, cells were grown in appropriate dropout media containing 2% raffinose at 30 °C to an optical density of 1.5 at 600 nm. Subsequently, galactose was added to a final concentration of 2%, and growth continued for an additional 24 h. Total yeast cell extracts were prepared as described (18), except that samples were not heated before analysis. Fet3p oxidase activity in crude membrane extracts and 64Cu incorporation into newly synthesized Fet3p were analyzed as described previously (19).

RESULTS

As can be seen in Fig. 1, a single 165-kDa protein was observed in HepG2 and CaCo cell lysates analyzed by immuno blotting with an antibody specific to the human Wilson protein. To further characterize this protein, HepG2 cells were pulse-labeled for 1 h with [35S]methionine and [38S]cysteine, and cell lysates were immunoprecipitated and subjected to SDS-PAGE. In this analysis, a single 165-kDa band was detected (Fig. 2A, lane 1) that was not observed when preimmune serum or antisera preincubated with the Wilson glutathione S-transferase fusion protein was used (data not shown). The derived amino acid sequence of the Wilson protein contains several potential consensus sites for N-linked glycosylation; however, preincubation of HepG2 cells with tunicamycin before immunoprecipitation did not alter the size of this protein (Fig. 2A, lanes 1 and 2). This result was not due to a lack of an effect of tunicamycin in these cells, as immunoprecipitation of these same lysates with antibody to human ceruloplasmin demonstrated the expected differences in mobility for this protein following inhibition of glycosylation (Fig. 2A, lanes 3 and 4). Consistent with these results, following an initial 10-min metabolic pulse, the Wilson protein was identified by immunoprecipitation as a 165-kDa band that did not change size during a prolonged chase period and was not detected in media (data not shown).

These biosynthetic studies reveal that the Wilson protein is synthesized in HepG2 cells as a single-chain intracellular protein. To determine the steady state location of this protein within these cells, isopycnic density gradient centrifugation was performed. Following ultracentrifugation of HepG2 cell lysates, individual fractions were separated by SDS-PAGE and analyzed by immunoblotting. The results of such an experiment are shown in Fig. 3, where the Wilson protein is observed in the later gradient fractions (lanes 9–11). To determine the location of specific organelles in these fractions, immunoblotting was performed using antibodies that recognize proteins with well defined subcellular locations. TAP-1, a protein that transports peptides into the endoplasmic reticulum and is confined to this compartment, and the cis-Golgi (20) were detected in the earliest fractions (Fig. 3, lanes 1–4). In contrast, AP-1 and the cation-independent mannose 6-phosphate receptor, located in the trans-Golgi network and late endosomes (21, 22), were detected in fractions overlapping with the Wilson disease protein (Fig. 3, lanes 7–11).

The results of the fractionation studies suggest that the Wilson protein is localized to an intracellular compartment in the later portion of the secretory pathway of the cell. However, considerable overlap among these compartments is observed in the fractionation experiments. To further characterize the location of the Wilson protein, double-label immunofluorescence microscopy was performed. In these studies, the Wilson protein was consistently detected in a concentrated perinuclear location in HepG2 cells that showed considerable overlap with the

![Fig. 2. Immunoprecipitation of Wilson protein from HepG2 cells. After a 1-h pulse with [35S]methionine and [38S]cysteine, cells were lysed and immunoprecipitated with antisera to human Wilson protein (lanes 1 and 2) or human ceruloplasmin (lanes 3 and 4). For some experiments, cells were incubated with tunicamycin (tm) (4 μg/ml) for 2 h before immunoprecipitation (lanes 2 and 4).](image-url)
FIG. 3. Immunoblot analysis of HepG2 cell lysates after isopycnic density gradient centrifugation in a continuous linear sucrose gradient. 100 μg of protein from each fraction was separated by SDSPAGE, transferred to nitrocellulose, and immunoblotted with antibody to Wilson protein (WD), the cation-independent mannose 6-phosphate receptor (MPR), AP-1, or TAP-1.

FIG. 4. Double-label immunofluorescence microscopy of HepG2 cells. Cells were fixed, permeabilized, and incubated with rabbit antibody to the Wilson protein (WD) followed by incubation with FITC-conjugated donkey antibodies to rabbit IgG (A, C, E) and murine antibody to either the mannose 6-phosphate receptor (MPR) (B), AP-1 (D), or AP-2 (F) followed by incubation with TRITC-conjugated donkey antibodies to murine IgG. Bar, 10 μm.

The detection of Fet3p oxidase activity in the membrane fraction of the ccc2Δ mutant transformed with the wild-type lacking the Wilson/Menkes gene homologue, CCC2, are deficient in high affinity iron uptake due to a failure to incorporate copper into the ceruloplasmin homologue, Fet3p (9). To examine expression of the Wilson protein in these yeast strains, immunoblot analysis was performed on equivalent amounts of protein from total cell lysates of wild-type (see Fig. 8, lane 2) or ccc2Δ mutants transformed with vector alone (Fig. 7, lane 3) or plasmids containing wild-type or mutant human Wilson cDNA (Fig. 7, lanes 4–6). As can be seen in this analysis, a single 185-kDa protein was observed in each of the transformants, equivalent in size to that observed in HepG2 cells (Fig. 7, lane 1).

The function of the Wilson protein in these transformants was examined by analyzing the activity of Fet3p. Consistent with previous studies (9), ccc2Δ mutants transformed with vector alone were devoid of Fet3p oxidase activity (Fig. 8A, lane 2). In contrast, transformation of this strain with the human Wilson cDNA resulted in detectable Fet3p activity equivalent to or greater than that found in the parent strain (Fig. 8, lanes 1 and 3). Despite equivalent amounts of protein expression in the transformed ccc2Δ strains (Fig. 7), neither of the mutant Wilson proteins were able to restore Fet3p oxidase activity. These results were not due to alterations in the amount of Fet3p among these transformants, as revealed by immunoblot analysis of Fet3p using equivalent amounts of membrane protein (Fig. 8B). Under these conditions, the protein is devoid of copper and migrates more slowly than the holoprotein (19) and is detected as a doublet due to differences in glycosylation (24).

The detection of Fet3p oxidase activity in the membrane fraction of the ccc2Δ mutant transformed with the wild-type

location of the mannose 6-phosphate receptor and AP-1 (Fig. 4, A–D). These findings suggest that a considerable portion of the Wilson protein localizes to the trans-Golgi network. In contrast, no overlap was observed when antibodies to the plasma membrane AP-2 complex were used in these same experiments (Fig. 4, E and F). No specific signal was observed in HeLa cells incubated with the Wilson antisera, and no signal was detected in HepG2 cells when this antisera was preincubated with Wilson glutathione S-transferase fusion protein (data not shown).

During the course of these immunofluorescence studies, a variable degree of Wilson-specific signal was also observed in a punctate pattern in HepG2 cells (Fig. 4C). To determine if this localization was influenced by specific metabolic demands of the cell, HepG2 cells were preincubated with varied concentrations of copper and then subjected to immunofluorescence microscopy. The results of these studies indicated that 2 h after incubation of HepG2 cells in media containing 200 μM copper, the Wilson protein was redistributed from the trans-Golgi network to a peripheral compartment characterized by a punctate staining pattern (Fig. 5B). Chelation of excess copper by the addition of bathocuproine disulfonate to the medium for 2 h resulted in redistribution of the Wilson protein to a concentrated perinuclear location identical to that found in control cells (Fig. 5C). These same observations were made when total protein synthesis was inhibited by cycloheximide (Fig. 5, D–F), indicating that copper induced a relocation of the Wilson protein from the trans-Golgi network to the peripheral compartment. The effect of copper on the subcellular localization of the Wilson protein was initiated within 15 min after the addition of excess copper to the medium and occurred at a minimal concentration of 40 μM CuSO4 (data not shown).

The effect of copper was saturable with respect to copper concentration and time, with movement from the trans-Golgi network detected within 15 min and found in >98% of all cells within 2 h. These effects were specific for copper, as no change in the location of the Wilson protein was observed when cells were incubated with increasing concentrations of zinc, iron, cadmium, or cobalt (data not shown). Copper did not result in a general reorganization of the structure and distribution of subcellular organelles as evidenced by an undisturbed location of the mannose 6-phosphate receptor and AP-1 (Fig. 4). In contrast to recent studies on the copper-dependent degradation of the plasma membrane copper transporter Ctr1 in yeast (29), no qualitative or quantitative differences in the Wilson protein were observed with changes in copper concentration as revealed by immunoblot analysis and metabolic labeling (data not shown).

To directly analyze function of the Wilson protein, a cDNA encoding the open reading frame was constructed as detailed under “Experimental Procedures.” Site-directed mutagenesis was utilized to create missense mutations, and the wild-type and mutant constructs were transformed into the ccc2Δ mutant of S. cerevisiae. Previous studies have shown that yeast strains
Wilson protein prompted a more direct examination of the role of this protein in copper transport in this strain. After metabolic labeling with $^{64}$Cu, equivalent amounts of crude membrane fractions from Wilson transformants and control cells were separated by SDS-PAGE and analyzed by autoradiography. As can be seen in Fig. 9, a single 80-kDa radioactive band corresponding to holoFet3p was observed under these conditions in membrane fractions from the $ccc2^D$ mutant transformed with wild-type Wilson protein (lane 3), directly demonstrating copper transport to newly synthesized Fet3p.

**DISCUSSION**

These studies indicate that the Wilson disease protein is a 165-kDa single-chain polypeptide localized to the trans-Golgi network of HepG2 cells under steady state conditions. As each of the N-linked glycosylation consensus sites are located within
with 64Cu, and membrane fractions were analyzed by nonreducing or mutant Wilson cDNAs (observed here is different from recent studies of the hamster in this sense, the copper-induced trafficking of the Wilson protein from intact cells, and pulse-chase studies performed with exosome, immunoprecipitation of iodinated cell surface proteins of several methods of permeabilization that preserved detection. This process was independent of a new protein synthesis and sucrose gradient studies did not detect Wilson protein in the cytosol, the failure to detect such glycosylation in the tunicamycin studies lends support to the model of this protein developed from hydropathy plots of the primary sequence (5, 6, 14). The cell-specific expression is consistent with previous RNA blot analysis in human tissues and cell lines (4–6). These findings are also compatible with clinical observations in patients with Wilson disease that suggest a primary role for this protein in biliary copper excretion (25) as well as with recent data on the localization of the homologous Menkes disease protein in human and rodent cell lines (10, 26, 27).

The variation in the location of Wilson protein in HepG2 cells under steady state conditions (Fig. 4) prompted further analysis, including alterations in the metal content of the media. These studies revealed a copper-induced redistribution of the Wilson protein from the trans-Golgi network to a dispersed punctate-staining pattern suggestive of a vesicular compartment. This process was independent of a new protein synthesis specific for the Wilson protein and dependent upon the copper concentration in the media. Under conditions of copper excess, sucrose gradient studies did not detect Wilson protein in the endoplasmic reticulum fractions. Additionally, immunofluorescence microscopy revealed no colocalization with endosomal or lysosomal markers, including lamp-1, transferrin receptor, and FITC-dextran (data not shown). Thus, this dispersed staining pattern may represent a unique compartment for copper transporter and sequestration analogous to that observed in recent studies on the accumulation of HLA-DM in the MIIC intracellular compartment, where loading of class II molecules occurs before antigen presentation at the cell surface (28, 29). Given the known limitations of immunofluorescence microscopy, further analysis using electron microscopic methods are currently being undertaken to further define this compartment.

The Wilson protein was not observed on the cell surface despite prolonged incubation of cells in excess copper. The use of several methods of permeabilization that preserved detection of the asialoglycoprotein receptor on the plasma membrane, immunoprecipitation of iodinated cell surface proteins from intact cells, and pulse-chase studies performed with excess Wilson antibody in the media also failed to detect the Wilson protein on the plasma membrane (data not shown). In this sense, the copper-induced trafficking of the Wilson protein observed here is different from recent studies of the hamster homologue of the Menkes protein in copper-resistant cell lines, where copper was found to induce a relocalization of the Menkes protein from the trans-Golgi network to the plasma membrane (26). In those studies, plasma membrane signal was detected only in cells expressing a 10–70-fold excess of the Menkes protein, raising concern that the plasma membrane localization may represent missorting of the overexpressed protein into a default or bulk flow pathway as observed with overexpression of other proteins where sorting signals use saturable pathways to effect localization (30). Further studies utilizing polarized cells as well as in vivo analysis may help to resolve these uncertainties.

Despite these caveats, the results of both studies suggest a unique mechanism of copper-induced trafficking for copper-transporting ATPases, which may be an essential component of cellular copper homeostasis. It is unclear at this point if intracellular copper is directly involved in this process or serves as the mediator of subsequent events such as phosphorylation, which are then transduced as signals for protein movement. Somewhat analogous processes have been observed with lysosomal enzymes on trafficking of the mannose 6-phosphate receptor and insulin on the localization of the GLUT4 glucose transporter (31, 32). In these cases, sorting is dependent, at least in part, on signals in the cytoplasmic domains of the target protein and components of organelar protein coats (33), providing a direction for future studies on the mechanisms of copper-induced trafficking of the copper-transporting ATPases.

To address the putative copper transporting function of the Wilson protein, the yeast S. cerevisiae was utilized as a model system. Previous studies demonstrated that CCC2 gene, which is a yeast Wilson/Menkes homologue (34), encodes a protein essential for the transfer of copper from the cytosol to a compartment for incorporation into Fe(III), a ceruloplasmin homolog necessary for high affinity iron uptake (9). Expression of the human Wilson protein in ccc2Δ yeast restored copper incorporation into newly synthesized Fet3p, providing direct evidence of copper transport. A conserved CPC-motif within the putative sixth transmembrane domain has been proposed to play an essential role in copper transport (35). Expression of a Wilson protein with a point mutation in this domain (CAC) failed to restore oxidase activity of Fet3p in the ccc2Δ mutant (Fig. 9). Furthermore, expression of a Wilson protein encoded by the most common disease allele, an H1070Q mutation (13, 36), also failed to restore Fet3p oxidase activity in ccc2Δ yeast. These data are consistent with the marked reduction of holo-ceruloplasmin observed in patients with Wilson disease and with previous studies on the cellular site of copper incorporation into newly synthesized ceruloplasmin in hepatocytes (37). Thus this system permits delineation of the structural determinants of eukaryotic copper transporters as well as providing an assay to analyze patient mutations.

In mammals, the cytoplasm of the hepatocyte is the central storage site of copper, and the biliary system is the sole mechanism of copper excretion (38). The data in this study provide a molecular explanation for this physiology, where the copper concentration in the hepatocyte cytosol is the primary determinant of movement of the Wilson transporter to a compartment necessary for copper sequestration and export. An implication of this model is that some Wilson mutations may impair copper homeostasis by disturbing trafficking of the transporter rather than function per se, analogous to what has been observed for the cystic fibrosis transmembrane regulator (39). The combined analysis of function and localization as shown in this current study will be essential to delineate among these possibilities. The influence of environmental or genetic factors on this process may underlie the observation of marked clinical variability...
in Wilson disease among affected family members with the identical mutation (40).

The data presented here provide the first direct evidence of how the Wilson protein functions to maintain hepatic copper homeostasis. The toxicity of copper, as evidenced by the hepatic and neuronal degeneration in patients with Wilson disease, demands a rigorous, tightly controlled mechanism to handle the intracellular transport of this metal. The current study indicates that altered copper concentrations in the cellular milieu result in the redistribution of the Wilson protein from the trans-Golgi network to a cytoplasmic compartment, which may serve to sequester copper before exocytosis. The ability of the Wilson protein to functionally complement the yeast Ccc2p directly demonstrates that the Wilson gene product is capable of copper transport. Taken together these data indicate that the primary function of this protein in hepatocytes is to transport copper from the cytosol to the secretory compartment of the cell for incorporation into newly synthesized apoceruloplasmin and for excretion into the bile. These findings reveal a remarkable evolutionary conservation of the mechanisms of cellular copper metabolism and suggest that further utilization of the tractable systems of yeast genetics may permit identification of additional components of this pathway. Given the increasing recognition of the role of copper in inherited and acquired neurodegenerative disorders as well as the importance of antioxidant defenses in human disease, such studies are likely to provide additional insights into this area of biology.

Acknowledgments—We thank Stuart Kornfeld and Susan Wente for valuable advice and discussions, Andy Dancis, Ted Hansen, Stuart Kornfeld, and Alan Schwartz for yeast strains and antibodies, Michael Welsh for 64Cu, and Leo Klomp, Aimee Payne, and David Wilson for critical review of the manuscript.

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