Saccharomyces cerevisiae Expresses Three Functionally Distinct Homologues of the Nramp Family of Metal Transporters

MATTHEW E. PORTNOY,[1] XIU FEN LIU,[2]† AND VALERIA CIZEWSKI CULOTTA[1,2]*

Departments of Biochemistry and Molecular Biology[1] and Environmental Health Sciences,[2]
Johns Hopkins University School of Public Health, Baltimore, Maryland 21205

The baker's yeast *Saccharomyces cerevisiae* expresses three homologues of the Nramp family of metal transporters: Smf1p, Smf2p, and Smf3p, encoded by *SMF1*, *SMF2*, and *SMF3*, respectively. Here we report a comparative analysis of the yeast Smf proteins at the levels of localization, regulation, and function of the corresponding metal transporters. Smf1p and Smf2p function in cellular accumulation of manganese, and the two proteins are coregulated by manganese ions and the *BSD2* gene product. Under manganese-replete conditions, Bsd2p facilitates trafficking of Smf1p and Smf2p to the vacuole, where these transport proteins are degraded. However, Smf1p and Smf2p localize to distinct cellular compartments under metal starvation: Smf1p accumulates at the cell surface, while Smf2p is restricted to intracellular vesicles. The third Nramp homologue, Smf3p, is quite distinctive. Smf3p is not regulated by Bsd2p or by manganese ions and is not degraded in the vacuole. Instead, Smf3p is down-regulated by iron through a mechanism that does not involve transcription or protein stability. Smf3p localizes to the vacuolar membrane independently of metal treatment, and yeast cells lacking Smf3p show symptoms of iron starvation. We propose that Smf3p helps to mobilize vacuolar stores of iron.

Nramp (for natural resistance-associated macrophage protein) represents a family of evolutionarily conserved membrane proteins that facilitate the transport of heavy metal ions (5, 6, 15, 17, 31). Members of the Nramp family have been found in mammals, birds, insects, plants, fungi, and bacteria (3, 5, 9, 15, 16, 22, 40). Among the best studied are the Nramp1 and Nramp2 transporters of rodents. Although these proteins share 61% homology at the amino acid level, they exhibit distinct functions. Mouse Nramp1 plays an important role in the control of infection against intracellular parasites and is exclusively expressed in monocytes/macrophages and polymorphonuclear leukocytes (2, 15). Nramp2 (also known as DCT1 or DMT1) is more ubiquitously expressed in most tissues (17) and acts as a divalent metal transporter capable of transporting iron, manganese, copper, zinc, cadmium, and lead (17). Mutations in *Nramp2* have been associated with defects in duodenal iron uptake and cellular iron utilization in the *mk* mouse and the Belgrade rat models of anemia (13, 14).

The baker's yeast *Saccharomyces cerevisiae* expresses three closely related Nramp homologues, Smf1p, Smf2p, and Smf3p, encoded by *SMF1*, *SMF2*, and *SMF3*, respectively (6, 7, 29, 43). Like mammalian DMT1, the Smf proteins exhibit a somewhat broad substrate specificity. Smf1p was originally defined as a high-affinity manganese transporter (39) and was later shown to contribute to cellular accumulation of cadmium and copper (28). Smf2p can affect cobalt levels in yeast (28) and may also participate in manganese trafficking (43). In more recent studies, Chen and coworkers demonstrated that both Smf1p and Smf2p can stimulate iron uptake into *Xenopus* oocytes (7). The role of Smf3p in metal homeostasis has not been defined.

Studies on Smf1p have revealed a novel method of regulating Nramp transport activity in response to metals. Specifically, treatment of yeast cells with manganese triggers the rapid degradation of the Smf1 protein. When cells are replete with manganese, the bulk of Smf1p is targeted to the yeast vacuole for degradation, and this vacuolar targeting involves the *S. cerevisiae BSD2* gene product (26). Bsd2p is a membrane protein localized to the endoplasmic reticulum that helps direct Smf1p to the vacuole in response to manganese treatment (26, 28). When cells are starved for manganese, Smf1p fails to enter the vacuole, and the transporter arrives at the plasma membrane, where metal ion uptake is thought to occur (25, 26). This plasma membrane localization of Smf1p is independent of Bsd2p (26). Such a simple switch in localization of the metal transporter allows for rapid changes in metal uptake without the need for new protein synthesis.

By comparison, nothing is known regarding the cellular localization or regulation of the other yeast Nramp homologues, Smf2p and Smf3p. Are these proteins functionally redundant with Smf1p, or do they act in unique pathways of metal transport? To address this issue, we have comparatively analyzed the three yeast Nramp homologues at the levels of cellular localization, transporter regulation, and function. We report here that like Smf1p, Smf2p is regulated at the posttranslational level by manganese ions and the *BSD2* gene. By comparison, Smf3p shows no regulation by manganese; however, Smf3 protein levels are controlled by iron through a posttranscriptional mechanism. We additionally found that the three Smf proteins are housed at distinct cellular locations: Smf1p at the cell surface, Smf2p in intracellular vesicles, and Smf3p at the vacuolar membrane. Hence, as is the case with mammals, fungi have evolved with diverse Nramp isoforms that perform unique functions in metal metabolism.

MATERIALS AND METHODS

Yeast strains and growth conditions. The *S. cerevisiae* strains used in this study are presented in Table 1. The isogenic set of wild-type (YR98), *bsd2Δ* (XL115), *pep4Δ* (XL126), *bsd2Δ pep4Δ* (XL125), *smf1Δ* (XL112), and *smf2Δ* (XL117) strains has been previously described (26, 28). The *smf1Δ smf2Δ* strain XL131 was created by replacing the *SMF2* gene of XL112 with *HIS3* using the pSMF2-HIS3 plasmid (28). The *smf3Δ* (MP112) and *smf1Δ smf2Δ smf3Δ* (MP113)

* Corresponding author. Mailing address: Johns Hopkins University School of Public Health, 615 N. Wolfe St., Room 7032, Baltimore, MD 21205. Phone: (410) 955-3029. Fax: (410) 955-0116. E-mail: vculotta @jhsph.edu.
† Present address: Digene Corporation, Gaithersburg, Md.

TABLE 1. Strains used in this study

Strain	Genotype	Reference or source
YR98	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52	27
XL115	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52 bsd2Δ::HIS3	27
XL126	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52 pep4Δ::URA3	27
XL125	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52 pep4::URA3, bsd2Δ::HIS3	27
XL112	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52 smf1Δ::URA3	27
XL117	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52 smf2Δ::HIS3	27
XL131	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52 smf1Δ::URA3 smf2Δ::HIS3	This study
MP112	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52 smf3Δ::LEU2	This study
MP113	$MAT\alpha$ ade2 his3Δ200 leu2-3 112 lys2Δ201 ura3-52 smf1Δ::URA3 smf2Δ::HIS3 smf3Δ::LEU2	This study
YPH250	MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1	23
YPH250Δaft1	MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 aft1Δ::TRP1	23
CM3260	MATa ura3-52 trp1-63 leu2-3 gcn4-101 his3-609	23
M2P	MATa ura3-52 trp1-63 leu2-3 gcn4-101 his3-609 AFT1-up	23
SM2561	MATa his3 leu2 ura3 lys2 trp1	S. Michaelis
SM3397	MATa his3 leu2 ura3 lys2 trp1 ubc7Δ::LEU2	S. Michaelis
SM2898	MATa ura3 leu2-3,113 his3-11,15	S. Michaelis
SM2899	MATa ura3 leu2-3,113 his3-11,15 pre1-1 pre2-1	S. Michaelis

strains were constructed by disrupting *SMF3* in YR98 and XL131 using the *smf3*Δ::*LEU2* plasmid pJS409. The *aft1*Δ (YPH250Δaft1), *AFT1-up* (M2P), *ubc7*Δ (SM3397), and *pre1-1^{ts} pre2-1^{ts}* (SM2899) strains and their corresponding wild-type parents (YPH250, CM3260, SM2561, and SM2898, respectively) were kind gifts of A. Dancis, D. Kosman, and S. Michaelis. Stocks of strains were maintained on standard yeast extract-peptone-dextrose (YPD) media. Cultures for experimental analysis were obtained by growth in a synthetic minimal medium containing dextrose (SD) (36) or in a metal-depleted minimal defined medium (MDM) prepared through the use of an ion-exchange resin (10, 26). As needed, 10 μM ZnCl$_2$, 10 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 1.0 μM CuSO$_4$, 10 μM MnSO$_4$, 1.0 μM CoSO$_4$, or a combination of these metals was added to the MDM.

Molecular biology. An *SMF3* deletion plasmid, pJS409, was constructed by amplifying *SMF3* sequences from -493 to $+116$ and from $+1313$ to $+1862$ by PCR using primers designed to introduce *Hin*dIII and *Bam*HI sites or *Sal*I and *Hin*dIII sites on the upstream and downstream fragments, respectively. After digestion at these sites, the fragments were simultaneously ligated into the *Bam*HI and *Sal*I sites of pRS305 (*LEU2*) (37). Linearization of this plasmid with *Hin*dIII and transformation of yeast cells resulted in a deletion in chromosomal *SMF3* sequences from $+116$ to $+1313$ that was verified by PCR. The *CEN* Smf1-hemagglutinin (HA)-expressing plasmid pSF4 was previously described (26). A multicopy Smf1-HA plasmid, pSF5, was constructed by inserting the *Apa*I-to-*Nde*I fragment of pSF4 into the 2μ *LEU2* plasmid pVC36 (23). The Smf2-HA-expressing plasmid pSF6 was constructed by amplifying *SMF2* sequences from -257 to the stop codon employing a primer that replaced the termination sequence with *Nde*I and by using these sequences to replace the *Nde*I-*Apa*I *BSD2*-containing fragment of plasmid pXL36 (*CEN LEU2*) (27). As a result, Smf2p was fused at the C terminus to two copies of the HA epitope. A multicopy Smf2-HA plasmid, pSF7, was obtained by inserting the *Apa*I-*Nde*I fragment of pSF6 into the 2μ *LEU2* plasmid pVC36 (23).

Two Smf3-HA plasmids were constructed: one whose product contained the HA tag at the C terminus and the other whose product had HA integrated at amino acid position 424. The *SMF3* construct (pMP043) whose product had a C-terminal tag was obtained in a manner identical to that of pSF6 using *SMF3* sequences from -578 to the stop codon. The internally tagged Smf3-HA plasmid (pMP054) was constructed by first amplifying *SMF3* sequences from -578 to $+1824$ and inserting these sequences into the *Bam*HI and *Xho*I sites of pRS316 (*CEN URA3*) (37). Dual tandem copies of the sequence encoding the HA epitope were then introduced in frame at position $+1272$ by two successive rounds of site-directed mutagenesis (Quikchange; Stratagene).

For RNA blot analysis of *SMF2* and *SMF3* expression, 30 and 10 μg, respectively, of total RNA was subjected to formaldehyde-agarose gel electrophoresis, followed by transfer to a nylon membrane as described previously (35). Detection of specific RNA transcripts employed *SMF2* (sequences from -257 to $+1664$) and *SMF3* ($+418$ to $+1429$) DNA probes amplified by PCR and radiolabeled with ^{32}P.

Biochemical analyses and immunodetection techniques. Measurements of manganese ion accumulation were obtained through atomic absorption spectrophotometry. Cells were grown to an optical density at 600 nm (OD$_{600}$) of 1.0 in 10 ml of MDM supplemented with 150 nM CuSO$_4$ and 10 μM ZnCl$_2$. Cells were then harvested, washed, resuspended in 500 μl of distilled water, and subjected to atomic absorption spectrophotometry as described earlier (27).

To monitor iron regulation of the *FET3* promoter, cells transformed with a 2μ *URA3 FET3-lacZ* reporter plasmid (kind gift of D. Winge) were grown overnight in selecting SD medium, diluted into YPD to an OD$_{600}$ of 0.5, and grown for an additional 5 h until mid-log phase. Cells were harvested and washed, and crude extracts were prepared by glass bead homogenization in Z buffer (0.06 M

Na$_2$HPO$_4$, 0.04 M Na$_2$H$_2$PO$_4$, 0.01 M KCl, 0.001 M MgSO$_4$ [pH 7.0]). A total of 100 μg of whole-cell extract in 400 μl of Z buffer was then combined with 100 μl of a 4-mg/ml solution of *o*-nitrophenyl-β-D-galactopyranoside. Following a 1-h incubation at 30°C, *lacZ* activity was measured as a function of absorption at 420 nm.

For Western blot analysis, yeast cells expressing the Smf1-HA, Smf2-HA, or Smf3-HA fusion protein were grown to mid-logarithmic phase (OD$_{600}$ = 1.0) in selecting SD medium or MDM as needed. Extracts were prepared by either alkaline lysis (30) or glass bead homogenization (32), with similar results. Samples (10 to 20 μg) were subjected to sodium dodecyl sulfate gel electrophoresis on precast 12% polyacrylamide gels (Invitrogen) and were analyzed by Western blotting using a mouse anti-HA antibody (BABCO) as previously described (27).

Immunofluorescence microscopy analysis was conducted essentially as described previously (26), using strains transformed with a *CEN* plasmid for the expression of either Smf3-HA, Smf2-HA, or Smf1-HA. Cells were grown to a mid-logarithmic stage in selecting SD medium or MDM, fixed with formaldehyde, digested with Zymolyase, and stained with a mouse anti-HA antibody for 2 h. The secondary antibody consisted of a goat anti-mouse antibody coupled to fluorescein isothiocyanate (FITC) (Boehringer Mannheim), and staining proceeded for 1 h. Nucleic acids were stained by incubation with 1 μg of 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma) per ml for 5 min. FITC and DAPI staining were monitored by fluorescence microscopy, whereas visualization of yeast vacuoles used Nomarski optics.

RESULTS

Regulation of Smf2p by the *BSD2* gene and metal ions. Yeast Smf1p is known to be regulated at the posttranslational level by manganese ions and Bsd2p; however, the regulation of other yeast Nramp homologues had not been explored. To monitor expression and regulation of Smf2p, the protein was tagged at the C terminus with an HA epitope and was expressed from its native promoter on a *CEN* vector. The corresponding protein complemented a manganese trafficking defect associated with loss of *SMF2* (E. Luk and V. C. Culotta, unpublished data). To examine whether Smf2-HA is subject to the same pathway of protein turnover seen with Smf1p, we used a set of isogenic strains containing mutations in *BSD2* or in *PEP4*. *PEP4* is necessary for vacuolar proteolysis (19). As shown in Fig. 1A, Smf2-HA protein levels showed a dramatic increase in the *bsd2*Δ strain compared to those of the isogenic wild-type strain. Smf2-HA also accumulated to a high level in the *pep4*Δ mutant, and there was no additive effect seen with *bsd2* and *pep4* mutations. Thus, as is the case with Smf1p (26), Smf2p is subject to degradation by vacuolar proteases in a Bsd2p-dependent fashion. We additionally examined whether Smf2p falls under the negative control of metal ions. As shown by immunoblot analysis, depletion of the heavy metals zinc, copper, iron, and manganese from the growth medium resulted
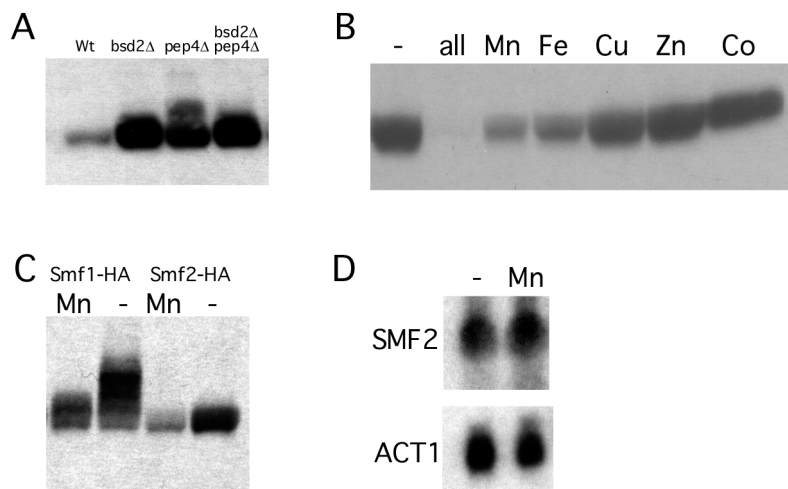
FIG. 1. Role of *BSD2*, vacuolar degradation, and metal ions in the control of Smf2p expression levels. (A) The indicated yeast strains were transformed with pSF6 expressing Smf2-HA, and the corresponding cell lysates were analyzed by Western blot analysis using an anti-HA antibody. Strains used: wild type (Wt), YR98; bsd2Δ, XL115; pep4Δ, XL126; pep4Δ bsd2Δ, XL125. (B) Strain YR98 transformed with the Smf2-HA plasmid pSF6 was grown in MDM depleted of metal ions (−) or in the same medium supplemented with the individual metals [i.e., 10 μM ZnCl₂, 10 μM Fe(NH₄)₂(SO4)₂, 1.0 μM CuSO₄, 10 μM MnSO₄, or 1.0 μM CoSO₄] or a combination of all the metals. Smf2-HA expression was monitored by Western blotting as for panel A. (C) Expression of Smf1-HA (pSF4) and Smf2-HA (pSF6) proteins in strain YR98 was analyzed by Western blotting as for panel A. (D) YR98 cells grown in MDM (−) or in MDM supplemented with 10 μM MnSO₄ were subjected to Northern blot analysis with sequential hybridization to an *SMF2* probe and an *ACT1* probe as a control.

in a dramatic increase in Smf2-HA protein levels in a wild-type strain (Fig. 1B). The individual addition of manganese and iron back to the medium partially suppressed Smf2-HA levels, whereas supplementation with zinc, copper, or cobalt had no effect (Fig. 1B). This pattern of negative regulation by metal ions is very similar to what has been previously reported for Smf1p (26). As shown in Fig. 1C, both Smf1p and Smf2p are repressed by the addition of manganese to the growth medium, although the accumulation of Smf1-HA under metal starvation conditions appears to be approximately two- to threefold higher than that obtained with Smf2-HA.

To test whether gene transcription played any role in metal regulation of *SMF2*, RNA prepared from cells grown in metal-depleted or manganese-replete medium was analyzed by Northern blotting. As shown in Fig. 1D, *SMF2* mRNA levels were not changed by metal depletion, demonstrating that metal ions increase Smf2p expression only through an increase in protein accumulation, as has been reported previously for Smf1p (26).

Localization of Smf2p. Indirect immunofluorescence microscopy was utilized to determine the subcellular localization of Smf2p. Cells expressing Smf2-HA from its native promoter were probed with a mouse anti-HA antibody and with a secondary antibody coupled to FITC. Under metal-replete conditions, the steady-state levels of Smf2p were too low to be detected by immunofluorescence microscopy (not shown). Yet since the bulk of Smf2p is degraded by vacuolar proteases under these conditions, we could readily detect Smf2-HA localization in a *pep4*Δ mutant. In this case, very intense staining of Smf2-HA was observed within the lumen of the vacuole identified by Nomarski optics (Fig. 2A). A quite different staining pattern was found in an isogenic *pep4*Δ *bsd2*Δ double mutant. In this case, there was no vacuolar staining, and the Smf2-HA localization was restricted to punctate bodies reminiscent of Golgi-like vesicles. Hence, as has been reported for Smf1p (26), Smf2p is normally targeted to the vacuole for degradation by *PEP4*-dependent proteases, and this delivery of Smf2p to the vacuole is dependent on the *BSD2* gene product.

However, contrary to what has been observed for Smf1p (26), we failed to detect any cell surface staining of Smf2p in *bsd2* mutants.

We additionally compared the localization of Smf1p and Smf2p under metal starvation conditions. Consistent with our earlier studies, the majority of Smf1-HA exhibited a cell surface staining pattern when cells were starved for manganese
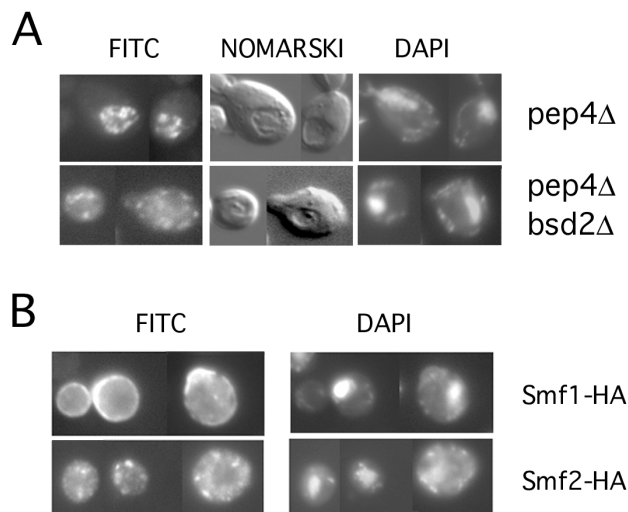

FIG. 2. Immunofluorescence microscopy of Smf2-HA. (A) Strains XL126 (*pep4*Δ) and XL125 (*pep4*Δ *bsd2*Δ) transformed with pSF6 expressing Smf2-HA were grown in MDM supplemented with all five essential metal ions as described in the legend to Fig. 1B. Cells were probed with anti-HA and an FITC-conjugated anti-mouse antibody and doubly stained with DAPI for nucleic acid detection. Cells were analyzed by epifluorescence at a magnification of ×1,000 or by Nomarski optics for visualization of vacuoles seen as indentations. (B) Strain YR98 expressing either Smf1-HA (from pSF4) or Smf2-HA (pSF6) grown in MDM without metal supplementation was subjected to immunofluorescence microscopy as for panel A.
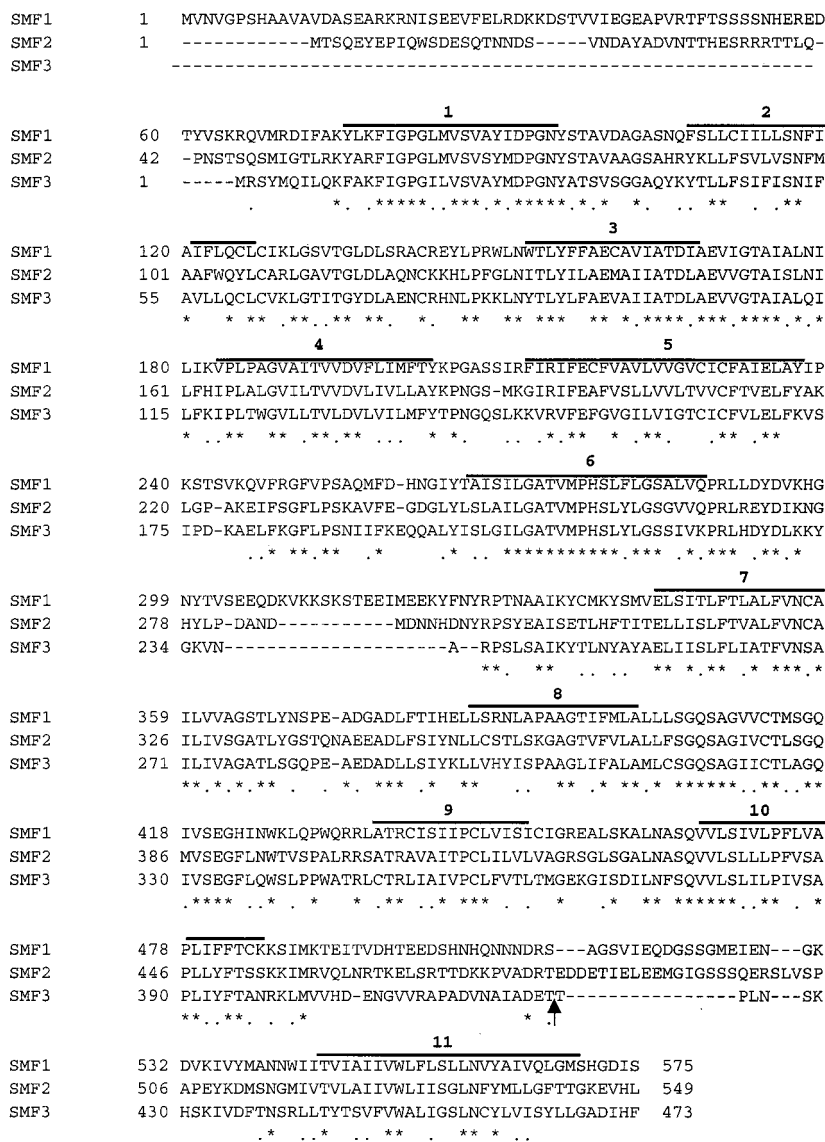
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                                                              1                                2
SMF1    1   MVNVGPSHAAVAVDASEARKRNISEEVFELRDKKDSTVVIEGEAPVRTFTSSSSNHERED
SMF2    1   ------------MTSQEYEPIQWSDESQTNNDS-----VNDAYADVNTTHESRRRTTLQ-
SMF3        ------------------------------------------------------------

                                                      1                               2
SMF1    60  TYVSKRQVMRDIFAKYLKFIGPGLMVSVAYIDPGNYSTAVDAGASNQFSLLCIILLSNFI
SMF2    42  -PNSTSQSMIGTLRKYARFIGPGLMVSVSYMDPGNYSTAVAAGSAHRYKLLFSVLVSNFM
SMF3    1   -----MRSYMQILQKFAKFIGPGILVSVAYMDPGNYATSVSGGAQYKYTLLFSIFISNIF
                .   *. .*****,.***.*.*****.*.*   *.  ,. .**  .*   .**
                                                              3
SMF1    120 AIFLQCLCIKLGSVTGLDLSRACREYLPRWLNWTLYFFAECAVIATDIAEVIGTAIALNI
SMF2    101 AAFWQYLCARLGAVTGLDLAQNCKKHLPFGLNITLYILAEMAIIATDLAEVVGTAISLNI
SMF3    55  AVLLQCLCVKLGTITGYDLAENCRHNLPKKLNYTLYLFAEVAIIATDLAEVVGTAIALQI
                *   * ** .**..**.**..*.  *.  ** .** ***  ** *.****.***.****.*.*
                                  4                                 5
SMF1    180 LIKVPLPAGVAITVVDVFLIMFTYKPGASSIRFIRIFECFVAVLVVGVCICFAIELAYIP
SMF2    161 LFHIPLALGVILTVVDVLIVLLAYKPNGS-MKGIRIFEAFVSLLVVLTVVCFTVELFYAK
SMF3    115 LFKIPLTWGVLLTVLDVLVILMFYTPNGQSLKKVRVFEFGVGILVIGTCICFVLELFKVS
                *  ..**  ** .**.**  ...   * *.    .. .*.** *  *.**.   .** .**
                                                            6
SMF1    240 KSTSVKQVFRGFVPSAQMFD-HNGIYTAISILGATVMPHSLFLGSALVQPRLLDYDVKHG
SMF2    220 LGP-AKEIFSGFLPSKAVFE-GDGLYLSLAILGATVMPHSLYLGSGVVQPRLREYDIKNG
SMF3    175 IPD-KAELFKGFLPSNIIFKEQQALYISLGILGATVMPHSLYLGSSIVKPRLHDYDLKKY
                 ..* **.**  .*     .* ..  *********** .*** .*.*** .**.*
                                                                   7
SMF1    299 NYTVSEEQDKVKKSKSTEEIMEEKYFNYRPTNAAIKYCMKYSMVELSITLFTLALFVNCA
SMF2    278 HYLP-DAND-----------MDNNHDNYRPSYEAISETLHFTITELLISLFTVALFVNCA
SMF3    234 GKVN-------------------A--RPSLSAIKYTLNYAYAELIISLFLIATFVNSA
                                      **.  ** .. .. ** *.** .* ***.*
                                              8
SMF1    359 ILVVAGSTLYNSPE-ADGADLFTIHELLSRNLAPAAGTIFMLALLLSGQSAGVVCTMSGQ
SMF2    326 ILIVSGATLYGSTQNAEEEADLFSIYNLLCSTLSKGAGTVFVLALLFSGQSAGIVCTLSGQ
SMF3    271 ILIVAGATLSGQPE-AEDADLLSIYKLLVHYISPAAGLIFALAMLCSGQSAGIICTLAGQ
                **.*.*.**  . .  *. *** .* **   .. ** .* **.* ******.**..**
                                  9                                10
SMF1    418 IVSEGHINWKLQPWQRRLATRCISIIPCLVISICIGREALSKALNASQVVLSIVLPFLVA
SMF2    386 MVSEGFLNWTVSPALRRSATRAVAITPCLILVLVAGRSGLSGALNASQVVLSLLLPFVSA
SMF3    330 IVSEGFLQWSLPPWATRLCTRLIAIVPCLFVTLTMGEKGISDILNFSQVVLSLILPIVSA
                .****..*. *  *  * .**  .* *** .    .*  ** ****** .** .  *
SMF1    478 PLIFFTCKKSIMKTEITVDHTEEDSHNHQNNNDRS---AGSVIEQDGSSGMEIEN---GK
SMF2    446 PLLYFTSSKKIMRVQLNRTKELSRTTDKKPVADRTEDDETIELEEMGIGSSSQERSLVSP
SMF3    390 PLIYFTANRKLMVVHD-ENGVVRAPADVNAIADETT---------------PLN---SK
                **..**. ..*              * .|
                                                              11
SMF1    532 DVKIVYMANNWIITVIAIIVWLFLSLLNVYAIVQLGMSHGDIS   575
SMF2    506 APEYKDMSNGMIVTVLAIIVWLIISGLNFYMLLGFTTGKEVHL   549
SMF3    430 HSKIVDFTNSRLLTYTSVFVWALIGSLNCYLVISYLLGADIHF   473
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FIG. 3. Amino acid compositions of the *S. cerevisiae* Nramp homologues. Amino acid alignment of Smf1p, Smf2p, and Smf3p using the ClustalW alignment program. Asterisks represent amino acid identity and dots represent amino acid similarity. Bold lines above sequences represent predicted transmembrane domains based on hydropathy analysis and on homology to other members of the Nramp family (6, 17). The arrow represents the position in Smf3p where two copies of HA were introduced by site-directed mutagenesis (see Materials and Methods).

(Fig. 2B). However, under precisely the same conditions, Smf2-HA localization was restricted to intracellular punctate bodies. Therefore, although Smf1p and Smf2p are coregulated by manganese ions and Bsd2p, they exhibit distinct cellular localizations.

The *SMF3* gene. Upon inspection of the *S. cerevisiae* genome sequence, we identified an open reading frame whose product exhibited extensive homology to yeast Smf1p and Smf2p (YLR034C); this gene was entered in the *Saccharomyces cerevisiae* Genome Database as *SMF3*. Overall, the three yeast Smf proteins show ~26% identity to human Nramp2 (data not shown) and ~48% identity to each other at the amino acid level (Fig. 3). This homology extends over most of the proteins' sequences, with three notable exceptions: Smf3p lacks an amino-terminal extension present on Smf1p and Smf2p, Smf1p harbors additional intervening sequence separating transmembrane domains 6 and 7, and Smf3p has a comparatively short

sequence separating transmembrane domains 10 and 11 (Fig. 3).

Regulation of *SMF3* by iron. Expression of Smf3p was monitored through introduction of an HA epitope either at the C terminus of the protein (as was used for Smf1p and Smf2p in the experiment shown in Fig. 1) or at an internal region between transmembrane segments 10 and 11 (as was used for Smf1p in other studies [43]). To address whether Smf3p is negatively regulated by *BSD2*, the epitope-tagged versions of Smf3-HA were expressed in an isogenic set of wild-type and *bsd2Δ* mutant strains. As seen in Fig. 4A, loss of *BSD2* increased Smf1-HA and Smf2-HA protein expression, but no similar increase was observed with Smf3-HA. Identical results were obtained with Smf3p regardless of whether the HA tag was placed at the C terminus (Fig. 4A) or at the internal site (not shown). We next tested whether *SMF3* is regulated by metals. As seen in Fig. 4B, levels of the Smf1-HA protein were
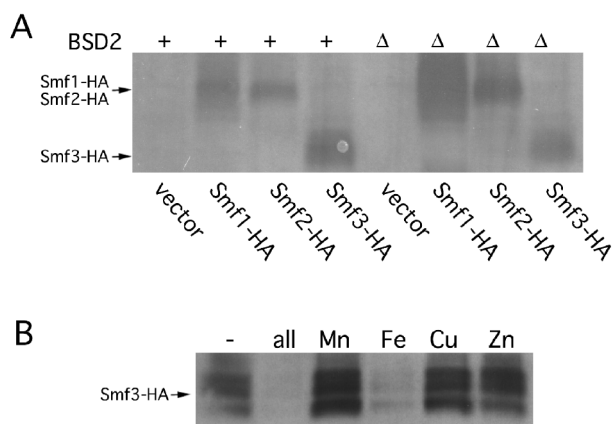
FIG. 4. Regulation of Smf3 protein levels. (A) The isogenic wild-type YR98 (+) and *bsd2Δ* mutant strain XL115 (Δ) were transformed with plasmids for the expression of Smf1-HA (pSF5), Smf2-HA (pSF7), or Smf3-HA (pMP043) or with the empty vector pRS425. Cells were grown in selecting SD medium, and lysates were prepared and analyzed by Western blotting as described for Fig. 1A. Arrows mark the positions of the 65-kDa Smf1-HA, 62-kDa Smf2-HA, and 54-kDa Smf3-HA polypeptides; molecular masses were confirmed by comigration of standards (not shown). (B) Strain YR98 expressing Smf3-HA from plasmid pMP054 was grown in MDM that was supplemented with the indicated metal ions as described for Fig. 2B. Cells lysates were prepared and subjected to Western analysis as for panel A.

greatly increased in medium depleted of manganese, iron, copper, and zinc, demonstrating that like Smf1p and Smf2p, the Smf3 polypeptide is negatively regulated by heavy metals. However, while Smf1p and Smf2p are down-regulated by manganese (Fig. 1C), addition of manganese ions to the growth medium had no effect on Smf3-HA (Fig. 4B). Instead, Smf3-HA was strongly repressed by iron (Fig. 4B). Again, the results with Smf3p were independent of the position of the HA epitope tag.

Upon inspection of the 5′ flanking region of *SMF3*, we noted a consensus sequence for Aft1p, an iron-sensing transcription factor that activates iron metabolism genes in yeast (44, 45) (Fig. 5A). No such sequence was noted for *SMF1* or *SMF2* (data not shown), suggesting that iron regulation of *SMF3* may reflect transcription control by Aft1p. To address this, *SMF3* mRNA levels were monitored by Northern blotting. Under the same iron starvation and iron-replete conditions that modulate Smf3 protein (Fig. 4B), there was no substantial effect on *SMF3* mRNA (Fig. 5B, lanes 1 and 2). By comparison, transcription of the *FET3* gene, involved in iron uptake (1, 11, 21), was completely repressed by iron. *FET3* is Aft1 regulated (4, 45), and accordingly the gene was strongly induced in a strain expressing a constitutively active *AFT-up* allele (Fig. 5B, lanes 5 and 6) and repressed in an *aft1Δ* null strain (Fig. 5B, lanes 3 and 4). By contrast, *SMF3* transcription was not decreased by the *aft1Δ* mutation and was only modestly increased in the *AFT-up* strain (Fig. 5B, lanes 3 through 6). Moreover, a null mutation in *AFT1* had no effect on the dramatic iron repression of Smf3p seen at the level of protein expression (Fig. 5C). Therefore, iron regulation of Smf3p does not involve either Aft1p or *SMF3* gene transcription.

We next tested whether iron regulates Smf3p at the level of protein turnover. A null mutation in *PEP4* did not alter the levels of Smf3p tagged with HA at amino acid 424 (Fig. 6A), demonstrating that this protein is not subject to vacuolar degradation. (While Smf3p tagged with HA at position 424 is resistant to proteolysis, the corresponding tag at the C terminus is degraded in a *PEP4*-dependent manner, as this peptide

is predicted to lie within the lumen of the vacuole (see Fig. 10). It is likely that the short hydrophilic C terminus of native Smf3p (≈6 amino acids) is normally resistant to vacuolar proteases, but addition of the 18-amino-acid HA tag exposes the C terminus to proteolytic digestion.) Another major protein degradation pathway in yeast involves the 26S proteasome, where proteins destined for degradation are tagged with ubiquitin via the action of ubiquitin-conjugating enzymes, such as Ubc7p (8, 20). It has previously been shown that Smf1p is partially stabilized in a *ubc7* mutant (26). However, as seen in Fig. 6A, the steady-state levels of Smf3-HA were only modestly affected by a *ubc7Δ* mutation, and the same results were obtained regardless of the position of the HA epitope. To more definitively address the role of the proteasome in Smf3p degradation, we monitored Smf3-HA expression in a strain containing temperature-sensitive mutations in *PRE1* and *PRE2*, two essential genes encoding components of the proteasome (18). In this experiment, cells were grown at the permissive temperature prior to shifting to the nonpermissive temperature for 3 h to inactivate the proteasome. As seen in Fig. 6A, Smf3-HA levels were not substantially affected by the *pre1-1 pre2-1* mutation, indicating that proteasome degradation does not account for the large effects of iron on Smf3p expression. In parallel with these genetic studies, the effect of iron on Smf protein turnover was also tested by monitoring degradation rates of the protein under metal-starved or iron-replete conditions. For these studies, the time course of Smf3 stability was examined in wild-type cells treated with cycloheximide to inhibit new protein synthesis. As shown in Fig. 6B, Smf3-HA was extremely stable. The protein levels remained largely constant over 5 h, even in iron-treated cells. By comparison, the same cycloheximide treatment resulted in rapid degradation of Smf1-HA in cells replete with manganese (Fig. 6B, bottom). Overall, these studies strongly indicate that iron regulates Smf3 protein levels by a novel mechanism that does not appreciably involve either transcription or protein stability.

Localization of Smf3p. The subcellular localization of Smf3p was examined by indirect immunofluorescence microscopy. Since Smf1p (26) and Smf2p (Fig. 2B) exhibit shifts in localization with metal treatment, we analyzed Smf3-HA localization under metal-replete and iron starvation conditions. As seen in Fig. 7, Smf3-HA exhibited rim staining coincident with the vacuolar membrane. The vacuoles were identified as indentations under Nomarski optics. It is noteworthy that this rim or membrane staining of the vacuole is quite distinct from the luminal or internal staining of the vacuole seen with Smf2p (Fig. 2A) or Smf1p (26) under metal-replete conditions. Furthermore, unlike that of Smf1p and Smf2p, Smf3p localization did not change under metal starvation conditions, and these results were obtained with Smf3p containing the HA epitope either at the C terminus (not shown) or at position 424 (Fig. 7). It thus appears that Smf3p is a constant resident of the vacuolar membrane.

Role of Smf3p in iron homeostasis. Since yeast Smf1p and Smf2p are specifically regulated by manganese, it is not surprising that these transporters participate in manganese uptake and distribution (39, 43; Luk and Culotta, unpublished). As expected, a combined deletion of *SMF1* and *SMF2* results in a dramatic decrease in steady-state accumulation of cellular manganese as measured by atomic absorption spectroscopy (Fig. 8A). However, a corresponding mutation in *SMF3* had no effect on manganese accumulation. A single *smf3Δ* mutant displayed wild-type cellular levels of manganese, and there was no further decrease in manganese accumulation in the *smf1Δ smf2Δ smf3Δ* triple mutant compared to that of the *smf1Δ smf2Δ* strain (Fig. 8A).

A

```
FET3  -257 AAGTGCACCCATTTG -243
ATX1  -107 CTTTGCACCCCCAAA -121
SMF3  -420 TAGTGCACCCGATCA -434
      CONSENSUS   TPuCACCC
```
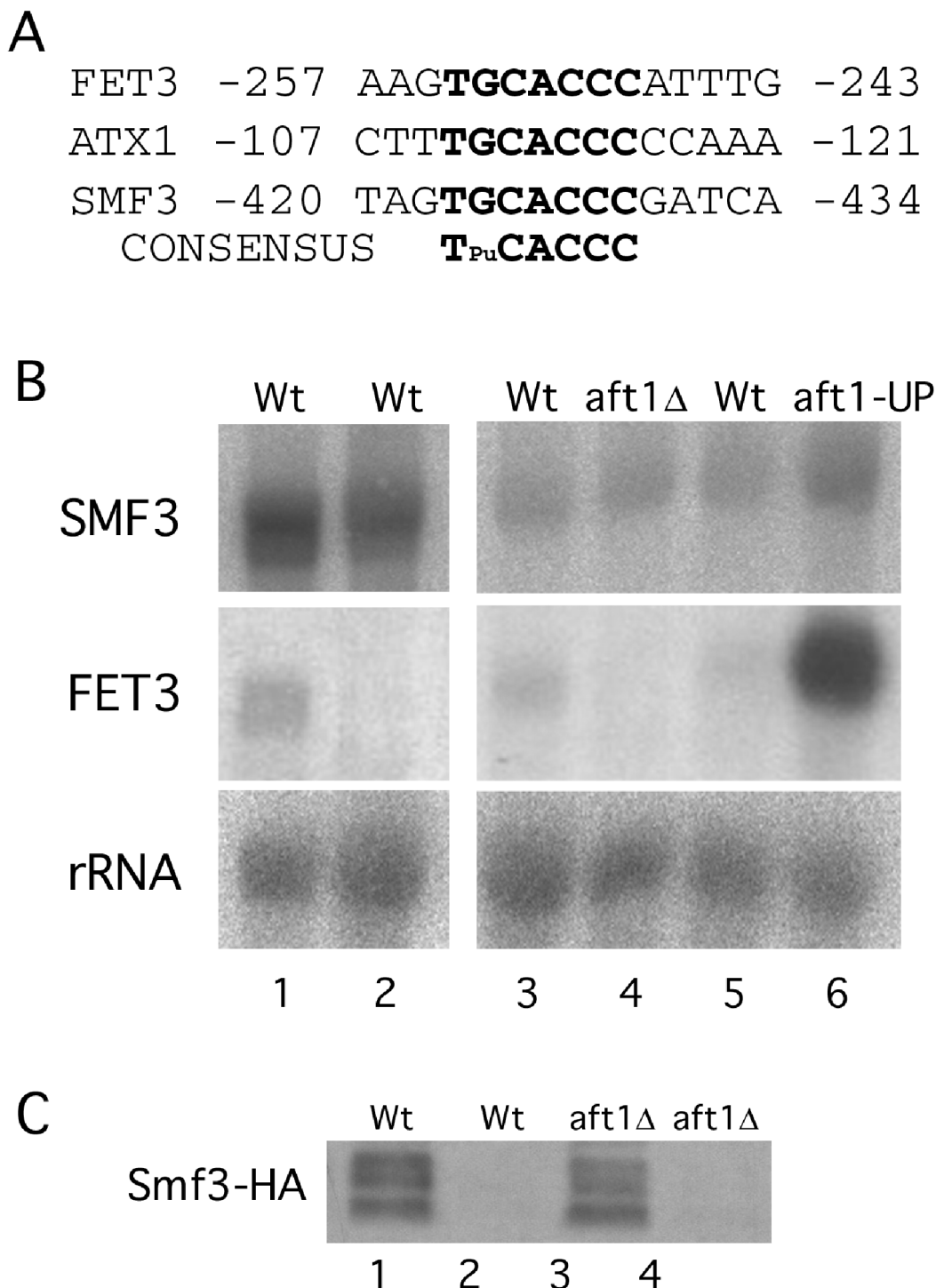
B

C

FIG. 5. *AFT1* and *SMF3* gene expression. (A) Marked in bold are the Aft1p consensus binding sequences for *FET3* (45), *ATX1* (24), and *SMF3*. Numbers indicate sequences with respect to the translational start site. (B) The indicated strains were grown in MDM that was depleted of heavy metals (lane 1), MDM supplemented with 10 μM Fe(NH$_4$)$_2$(SO4)$_2$ (lane 2), or YPD medium (lanes 3 through 6). Total RNA was subjected to Northern analysis with sequential hybridization to *SMF3* and *FET3* probes. The following strains were utilized: wild type (Wt), YR98 (lanes 1 and 2), YPH250 (lane 3, and CM3260 (lane 5); aft1Δ, YPH250aft1Δ; and aft1-UP, M2P. (C) The indicated strains expressing Smf3-HA (from pMP054) were grown in MDM that was either iron depleted (lanes 1 and 3) or supplemented with iron (lanes 2 and 4) as for panel B. Cell lysates were subjected to Western blot analysis as for Fig. 4. Wt, YPH250; aft1Δ, YPH250aft1Δ.

Based on the tight regulation of Smf3p by iron (and not manganese), it seemed possible that this Nramp isoform functions in iron homeostasis. To address whether Smf3p affects intracellular iron stores, we used a reporter construct in which the *FET3* gene promoter was fused to *lacZ*. Through regulation involving Aft1p, this promoter is strongly induced by iron starvation conditions (4, 45). As shown in Fig. 8B, the *smf3*Δ strain exhibited a large induction of *lacZ* reporter activity sug-

A

B
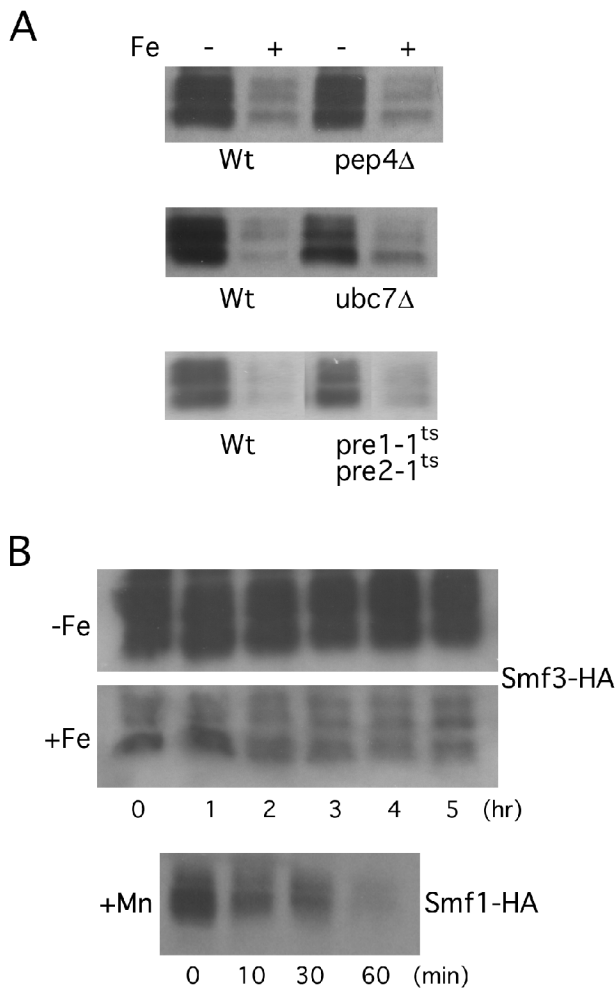
FIG. 6. Stability of the Smf3 polypeptide. (A) The designated strains were transformed with the Smf3-HA-expressing vector pMP054 and were grown in MDM that was supplemented with iron where indicated as for Fig. 5B. In the experiment with the *pre1-1ts pre2-1ts* mutant, cells were cultured at 25°C prior to shifting to 37°C for 3 h. All cells were subjected to Western blot analysis as for Fig. 4. The following strains were utilized: wild type (Wt), YR98 (upper panel), SM2561 (middle panel), and SM2898 (lower panel); pep4Δ, XL126; ubc7Δ, SM3397; and pre1-1ts pre2-1ts, SM2899. (B) Strain YR98 expressing either Smf3-HA (on pMP054) or Smf1-HA (on pSF5) were grown in MDM alone (−Fe) or in MDM supplemented either with 10 μM iron (+Fe) or with 10 μM manganese (+Mn) as described in the legend to Fig. 1B. Following growth to an OD_{600} of 0.6, 100 μg of cycloheximide per ml was added and aliquots of cells were removed at the indicated time points for Western analysis.

gestive of an iron starvation status. By comparison, an *smf2Δ* strain and an *smf1Δ smf2Δ* strain showed no induction of reporter activity, and an *smf1Δ smf2Δ smf3Δ* strain exhibited the same level of induction as seen with the single *smf3Δ* mutant (Fig. 8B). Therefore, Smf3p appears to play a role in controlling the intracellular availability of iron.

DISCUSSION

Here we have compared and contrasted the three isoforms of yeast Nramp proteins based on their regulation by metals, cellular localization, and function. Our major finding is that yeast Smf1p, Smf2p, and Smf3p are nonredundant. Smf1p and Smf2p are regulated by manganese and *BSD2*, and both are targeted to the vacuole for degradation when manganese is plentiful. However, Smf1p responds to manganese starvation

by moving to the plasma membrane, whereas Smf2p redistributes to intracellular vesicles (Fig. 9). Smf3p is even more disparate. The Smf3 polypeptide is uniquely down-regulated by iron in a manner that does not involve protein turnover. Moreover, while Smf1p and Smf2p shift their localization upon metal starvation, Smf3p appears to be a constant resident of the vacuolar membrane (Fig. 9). We propose that Smf1p is primarily involved in the uptake of manganese (and possibly other metals) from the extracellular environment, whereas Smf2p's role may be to mobilize metals from vesicular stores. The nature of the vesicles in which Smf2p resides is unknown, but they appear to be important for delivering manganese to the mitochondria (Luk and Culotta, unpublished). We hypothesize that the primary role of Smf3p is to help mobilize iron stores of the vacuole (see below).

The divergent patterns of regulation and localization observed with Smf1p, Smf2p, and Smf3p presumably reflect unique features of the corresponding polypeptide sequences. Of the three Nramp isoforms, Smf1p and Smf2p are most closely related in overall topology, although Smf1p contains a somewhat extended N-terminal region compared to that of Smf2p, and this may facilitate a cell surface localization. The Smf3p sequence is the most different of the three proteins. Smf3p is completely devoid of the 5- to 7-kDa N-terminal extensions that are present in Smf1p and Smf2p and also exhibits a gap between transmembrane segments 10 and 11. It is possible that one or more of these missing sequences in Smf3p accounts for its lack of regulation by *BSD2*. Our preliminary studies suggest that the N-terminal regions of Smf1p and
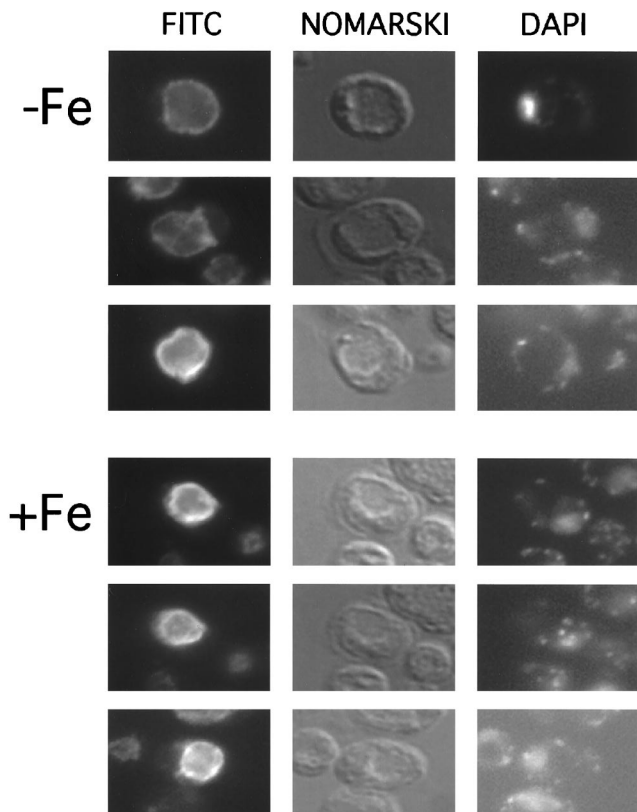

FIG. 7. Immunofluorescence microscopy localization of Smf3p. Strain YR98 expressing Smf3-HA (from pMP054) was grown in MDM supplemented with 10 μM iron where indicated. Cells were fixed and subjected to immunofluorescence microscopy as for Fig. 2.
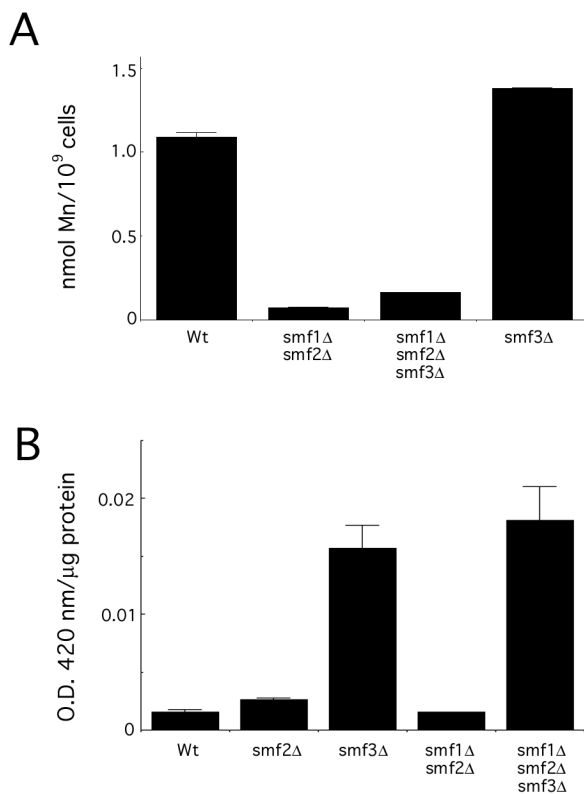
FIG. 8. Effects of *smf* null mutations. (A) The indicated strains were grown in MDM supplemented with 150 nM $CuSO_4$ and 10 mM $ZnCl_2$ (the medium was depleted of iron and manganese to maximize expression of the Smf proteins). Cells were washed and prepared for atomic absorption spectroscopy analysis of manganese content. The following strains were analyzed: wild type (Wt), YR98; smf2Δ, XL117; smf3Δ, MP112; smf1Δ smf2Δ, XL131; and smf1Δ smf2Δ smf3Δ, MP113. (B) The indicated strains were tested for expression of an *FET3-lacZ* reporter plasmid as described in Materials and Methods. Beta-galactosidase activity is reported as units of absorption at 420 nm per microgram of lysate protein. Strains utilized are as described in the legend to panel A.

Smf3p are involved in the response to manganese versus iron; however, other, as-yet-unidentified sequences are needed for metal-specific regulation (data not shown). Work to identify these sequences is under way.

How is Smf3p regulated by iron? We found that the Smf3 polypeptide is not degraded in response to iron, and furthermore, iron does not appreciably regulate transcription of the *SMF3* gene. While *SMF3* does contain in its promoter a consensus site for the iron-sensing Aft1p transcription factor, Aft1p does not significantly modulate *SMF3* expression. The effects seen are similar to the modest regulation of the *S. cerevisiae ATX1* gene by *AFT1* (24). Since mRNA and protein stability effects cannot account for the strong regulation of Smf3p by iron, Smf3p may be regulated at the level of protein translation. A limited degree of secondary structure in the 5′ untranslated region of *SMF3* has been noted (data not shown), and work to determine the role of these sequences in modulating *SMF3* expression is under way. Iron-mediated control of polypeptide translation has been described for mammalian proteins, such as ferritin (34, 41); however, the mechanism is likely to be different in the case of yeast Smf3p, as the relevant mammalian iron regulatory factor (i.e., cytosolic aconitase) is not expressed in this fungus.

Our data are consistent with a model in which Smf3p helps to mobilize iron from stores in the vacuole. Smf3p is always

localized to the vacuolar membrane, whereas Smf1p and Smf2p are targeted to the vacuolar lumen for degradation. The vacuole in yeast has been hypothesized to contain a large reservoir of iron (33), and indeed *smf3* mutants exhibit signs of iron starvation, as seen through induction of a *FET3* reporter construct. A comparison of the predicted topology maps for Smf3p and the mammalian Nramp transporters is consistent with the transport of metal from the lumen of the vacuole to the cytosol (6) (Fig. 10). Smf3p lacks the most C-terminal transmembrane domain found in the mammalian Nramp transporters, and therefore, the C terminus of Smf3p is predicted to lie on the luminal face of the vacuole membrane. As evidence for the predicted topology, the HA epitope at the C terminus of Smf3p is removed by vacuolar proteases (susceptible to *PEP4*-dependent degradation), whereas the HA epitope placed at the predicted cytoplasmic side is resistant to *PEP4* proteolysis (see above) (summarized in Fig. 10).

Recent work by Urbanowski and Piper (42) has identified a pair of proteins (Fth1p and Fet5p) on the vacuolar membrane that are homologous to the high-affinity iron uptake proteins, Ftr1p and Fet3p, at the cell surface. Based on topology analysis and homology to Ftr1p and Fet3p, the authors suggest that Fth1p and Fet5p may act to mobilize the iron stores of the vacuole (42). It is therefore possible that multiple pathways exist for moving iron out of the vacuole. Indeed, multiple systems operate at the cell surface for the uptake of iron (e.g., high-affinity Ftr1p and low-affinity Fet4p iron transporters [12, 38]), and Smf3p may be somewhat analogous to the Fet4p transporter, which operates on a variety of metals, including iron.

Although Nramp transporters can potentially recognize a broad range of metal substrates (e.g., Cu, Cd, Mn, and Fe) (17, 28, 40), biological specificity may come from their regulation. For example, *SMF1* and *SMF2* are expressed only under manganese or iron starvation conditions, whereas *SMF3* is predominantly expressed under iron starvation. Overall, the Smf family of Nramp metal transporters in yeast is providing useful insight into metal-regulated gene expression and the complexity of metal ion homeostasis within a single cell.
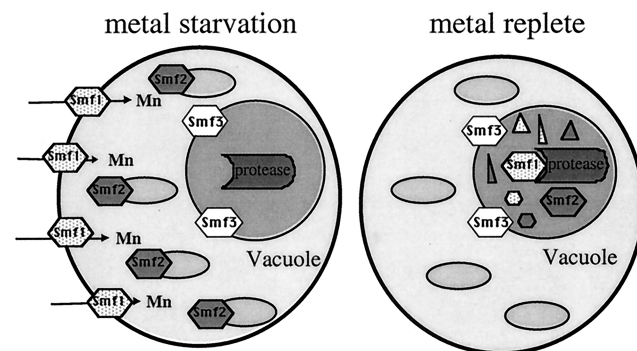

FIG. 9. Model for yeast Nramp localization. Depicted is a diagram of a yeast cell, illustrating the distinct localizations of Smf1, Smf2, and Smf3. With metal starvation, Smf1 localizes to the cell surface (arrows indicate direction of manganese uptake), Smf2 localizes to intracellular vesicles (represented by small ovals), and Smf3 localizes at the vacuolar membrane (represented by inner circle). Upon metal-replete conditions (treatment with manganese and iron), both Smf1 and Smf2 are targeted to the vacuole lumen for degradation by proteases (small triangles and hexagons represent products of Smf degradation). Smf3 remains at the vacuolar membrane and is not degraded.
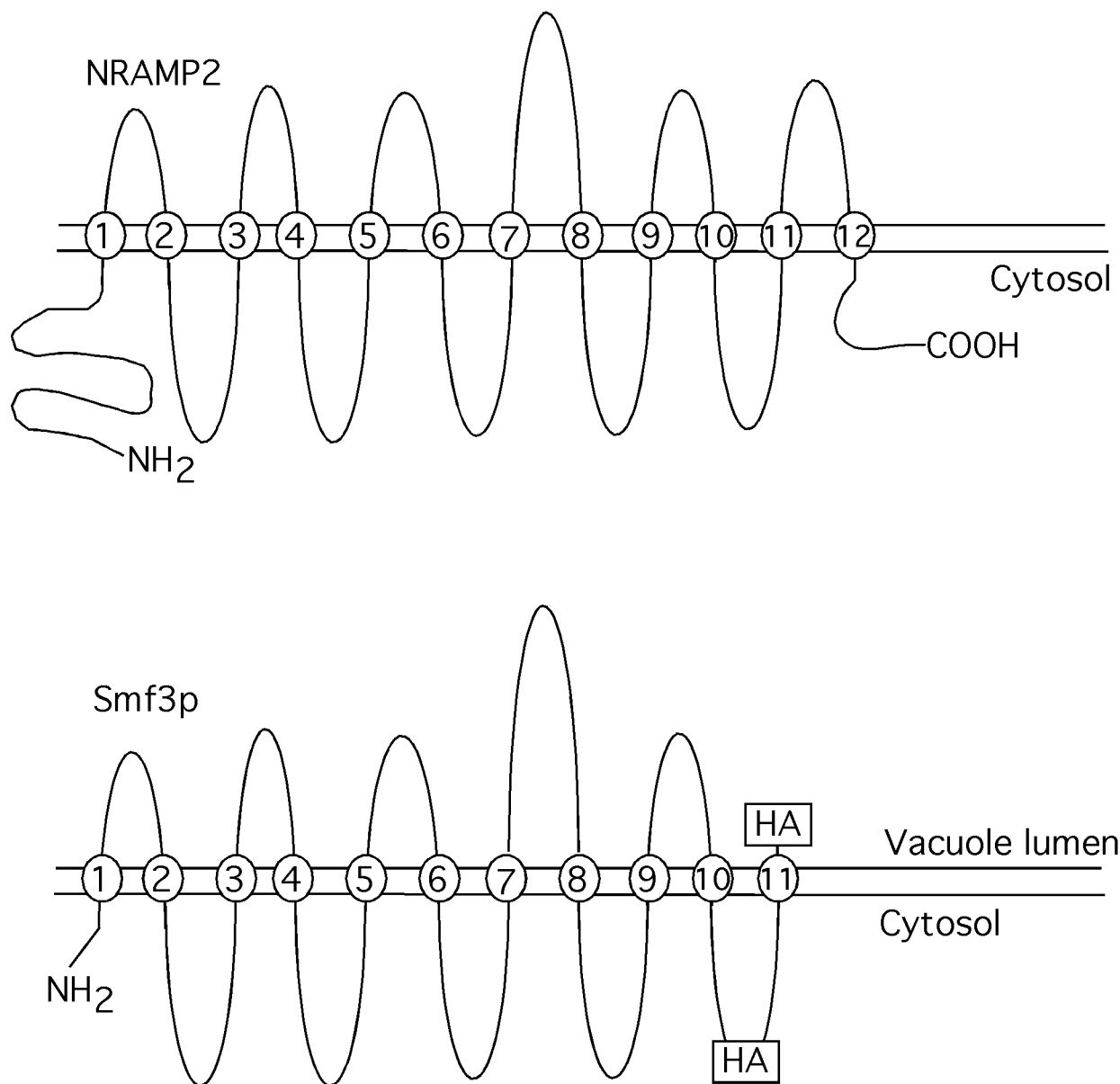
FIG. 10. Membrane topology of Smf3p. Shown are the predicted membrane topologies for mammalian DMT1 (6, 17) and yeast Smf3p. Positions at which HA epitopes were introduced into Smf3p are indicated.

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