Opposing Activities of the Snx3-Retromer Complex and ESCRT Proteins Mediate Regulated Cargo Sorting at a Common Endosome

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Endocytosed proteins are either delivered to the lysosome to be degraded or are exported from the endosomal system and delivered to other organelles. Sorting of the *Saccharomyces cerevisiae* reductive iron transporter, composed of the Fet3 and Ftr1 proteins, in the endosomal system is regulated by available iron; in iron-starved cells, Fet3-Ftr1 is sorted by Snx3/Grd19 and retromer into a recycling pathway that delivers it back to the plasma membrane, but when starved cells are exposed to iron, Fet3-Ftr1 is targeted to the lysosome-like vacuole and is degraded. We report that iron-induced endocytosis of Fet3-Ftr1 is independent of Fet3-Ftr1 ubiquitylation, and after endocytosis, degradation of Fet3-Ftr1 is mediated by the multivesicular body (MVB) sorting pathway. In mutant cells lacking any component of the ESCRT protein-dependent MVB sorting machinery, the Rsp5 ubiquitin ligase, or in wild-type cells expressing Fet3-Ftr1 lacking cytosolic lysyl ubiquitin acceptor sites, Fet3-Ftr1 is constitutively sorted into the recycling pathway independent of iron status. In the presence and absence of iron, Fet3-Ftr1 transits an endosomal compartment where a subunit of the MVB sorting receptor (Vps27), Snx3/Grd19, and retromer proteins colocalize. We propose that this endosome is where Rsp5 ubiquitylates Fet3-Ftr1 and where the recycling and degradative pathways diverge.

INTRODUCTION

The endocytic pathway is initiated with the internalization of protein and lipid cargo from the plasma membrane. After endocytosis, internalized molecules transit through the endosomal system, a network of interconnected organelles that process and sort cargo molecules back to the plasma membrane, to other organelles, or to lysosomes for degradation. In mammalian cells, early endosomes are pleiomorphic organelles composed of vacuolar domains that contain luminal vesicles and tubular domains that lead into and emanate from the vacuolar portion. This distinct architecture has functional significance. The tubular elements of the organelle are regions where the majority of internalized plasma membrane proteins and lipids are exported and recycled from the endosomal system via bulk flow and cargo-specific processes (Mayor *et al.*, 1993; Maxfield and McGraw, 2004; Bonifacino and Rojas, 2006). Cargo proteins that are not exported are delivered to lysosomes and many of these are degraded. This occurs via an active, signal-mediated event in which these proteins are targeted to the vacuolar region of the endosome where they are incorporated into vesicles

that bud from the limiting membrane into the lumen of the organelle (Piper and Katzmann, 2007). During endosome maturation luminal vesicles accrue until fusion of the late endosome with the lysosome results in degradation of these vesicles and their contents. A central sorting function of the endosomal system is thus to segregate cargo to be exported from the endosome from cargo to be degraded via lysosomal proteolysis.

The core components of the recycling (retrograde) and degradative sorting pathways have been characterized, and some of the signals that mediate sorting into each of these pathways have been identified (Bilodeau *et al.*, 2002; Seaman, 2005; Bonifacino and Rojas, 2006; Piper and Luzio, 2007; Seaman, 2007). Ubiquitylation of cytosolic lysine residues has emerged as a key signal for entry of proteins into the intraluminal vesicles (ILVs) of the degradative pathway, known as the multivesicular body (MVB) sorting pathway (Hicke and Dunn, 2003). Ubiquitylated proteins are recognized by MVB sorting receptors that directly bind ubiquitin and associate with downstream components of the MVB machinery. One of the best-characterized MVB sorting receptors is a complex of Vps27-Hse1, or Hrs-STAM in mammals, but other ubiquitin-binding proteins function in MVB cargo selection as well (Katzmann *et al.*, 2001; Bilodeau *et al.*, 2003; Slagsvold *et al.*, 2005). Downstream of the cargo selection step, three protein complexes, designated ESCRT (endosomal sorting complex required for transport) -I, -II, and -III act together to target ubiquitylated proteins into the MVB-sorting pathway (Babst *et al.*, 2002; Babst, 2005; Nickerson *et al.*, 2007; Piper and Katzmann, 2007; Sakkena *et al.*, 2007; Hurley, 2008). The ESCRT machinery is proposed to mediate inward

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Abbreviations used: ESCRT, endosomal sorting complex required for transport; MVB, multivesicular body.

invagination of the limiting membrane of the endosome, giving rise to intraluminal vesicles containing the cargo to be degraded.

With regard to the cargo-specific nature of retrograde trafficking, multiple retrograde sorting signals and receptors have been identified (Bonifacino and Rojas, 2006). A protein complex called "retromer" is localized to the cytosolic face of endosomal membranes where it mediates retrograde endosome-to-Golgi trafficking of several proteins (Seaman, 2005). Retromer was originally identified as essential in yeast for the proper retrieval of the vacuolar hydrolase receptor Vps10 from late endosomes back to the *trans*-Golgi network (Horazdovsky *et al.*, 1997; Seaman *et al.*, 1997) and was later shown to function in an analogous manner in mammalian cells in regard to retrieval of the cation-independent mannose-6-phosphate receptor (Arighi *et al.*, 2004; Seaman, 2004). Since then, retromer has been implicated in a broad range of protein-sorting pathways and has most recently been shown to be required for the maintenance of proper Wnt secretion in the development of both *Caenorhabditis elegans* and *Drosophila melanogaster* (Belenkaya *et al.*, 2008; Franch-Marro *et al.*, 2008; Pan *et al.*, 2008; Port *et al.*, 2008; Yang *et al.*, 2008), underscoring its importance in retrograde transport. In yeast retromer is composed of five proteins that assemble into two subcomplexes: Vps5 and Vps17 (Snx1/Snx2 and Snx5/Snx6 in mammals) and Vps26, Vps29, and Vps35 (and the homologous proteins in metazoans; Seaman *et al.*, 1998; Haft *et al.*, 2000; Wassmer *et al.*, 2007). Vps5 and Vps17 are members of the sorting nexin family of proteins and are responsible for localizing the retromer complex to tubular endosomal membranes via the concerted action of their BAR (Bin/Amphiphysin/RVS) and PX (phox homology) domains (Carlton *et al.*, 2004; Seet and Hong, 2006). Vps26p, Vps29, and Vps35 comprise the cargo-recognition subcomplex of retromer, and the structure of the Vps29-Vps35 interface has recently been solved (Hiero *et al.*, 2007). We and others have shown that another sorting nexin, Snx3/Grd19, associates with retromer and is necessary for retromer-dependent trafficking of a subset of retrograde cargo (Hettema *et al.*, 2003; Restrepo *et al.*, 2007; Storchlic *et al.*, 2007).

The fate of protein cargo within the endosomal system arises from the interplay of the cargo-sorting machineries of the retrograde and degradative pathways. Importantly, sorting of some proteins into the recycling versus degradative pathway is regulated such that signaling induces a switch from one pathway to the other. To investigate the basis of regulated sorting within the endosomal system, we use trafficking of the yeast high-affinity reductive iron transporter, composed of a heterodimeric complex of Fet3 and Ftr1, as a paradigm. An interesting and useful feature of Fet3-Ftr1 is that its sorting is impacted by environmental and nutritional signals. When cells are starved for iron, Fet3-Ftr1 is unusually stable because it is maintained at the plasma membrane due to retromer-dependent endocytic recycling (Storchlic *et al.*, 2007). However, when cells are exposed to a high concentration of free iron in the culture medium, endocytosed Fet3-Ftr1 is transported to the vacuole and degraded (Felice *et al.*, 2005).

In a prior study, we showed that the sorting nexin, Snx3, directly recognizes a cytosolic signal in Ftr1 that is required for retromer-mediated retrograde transport (Storchlic *et al.*, 2007). Snx3 and retromer physically associate, and they cooperate to prevent vacuolar degradation of Fet3-Ftr1 when cells are starved for iron. In this study, we have investigated the mechanism by which iron shock induces vacuolar targeting and turnover of Fet3-Ftr1. We provide evidence that

the Rsp5 ubiquitin ligase, the Vps27-Hse1 MVB-sorting receptor, and ESCRT proteins are required to sort Fet3-Ftr1 into the MVB pathway. We further show that the MVB sorting receptor (Vps27), Snx3, retromer, and Fet3-Ftr1 all colocalize to a common endosome. Our data suggest that the cargo receptors for the retrograde and degradative sorting pathways survey cargo at a common endosome that is the point of divergence of these two pathways.

MATERIALS AND METHODS

Antibodies and Reagents

Enzymes used in DNA manipulations were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Standard molecular biological and microbiological techniques were used throughout. Polyclonal anti-green fluorescent protein (GFP) antiserum for use in immunoprecipitations was a gift of Dr. Michael Matunis (Johns Hopkins University, Baltimore, MD). Primary mouse monoclonal antibodies used in these studies included: anti-GFP (1:2500; Covance Laboratories, Madison, WI), anti-HA (1:1000; Covance), and anti-PGK (1:10,000; Invitrogen, Carlsbad, CA). Secondary sheep anti-mouse HRP-conjugated antibodies (GE Healthcare, Waukesha, WI) were used at 1:5000. The 2 μ plasmid encoding hemagglutinin (HA)-ubiquitin (pRH990) was a gift of Dr. Randy Hampton (University of California-San Diego, La Jolla, CA) and has been described previously (Gardner *et al.*, 1998). The CEN plasmid encoding GFP-Vps27 (pEE27-4) was a gift of Dr. Markus Babst (University of Utah, Salt Lake City, UT) and has been described previously (Katzmann *et al.*, 2003).

Yeast Strains, Media, and Growth Conditions

Unless otherwise indicated, all yeast strains (Table 1) were constructed by recombination of gene-targeted, PCR-generated DNAs using the method of Longtine *et al.* (1998) to ensure expression from native loci. Primer sequences are available upon request. The strain background expressing the *rsp5* mutations is SEY6210 (*MAT α ura3-52, his3-200, trp1-901, lys2-801, suc2-9, leu2-3*) or SEY6210.1 (*MAT α ura3-52, his3-200, trp1-901, lys2-801, suc2-9, leu2-3*). All other yeast strains were constructed in the BY4742 background (*MAT α his3-1, leu2-0, met15-0, ura3-0*).

To generate the yeast strain expressing Fet3-Ftr1 22K/R, the following method was used. The *FTR1::GFP::HIS MX* cassette was amplified from genomic DNA of strain TSY36 by PCR with primer-encoded SmaI/SacI sites and was cloned into the corresponding restriction sites of vector pRS416. All lysine residues within cytoplasmic loops 1, 2, and 3 (K33, K46, K112, K116, K120, K132, K137, and K205) were mutated to arginines by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit; Stratagene, La Jolla, CA). A vector was then constructed by gapped plasmid repair (Muhlrad *et al.*, 1992) in which the sequence encoding the C-terminal cytosolic tail of *FTR1* was deleted and the remaining open reading frame (ORF; containing only the cytosolic lysine to arginine-mutated loops) was tagged with GFP. The plasmid was recovered from yeast and sequenced, and transformation of this plasmid into a wild-type yeast strain confirmed expression of a GFP-tagged protein of the expected size. This plasmid was digested with SmaI and PmeI, and the restriction fragment was used to transform a *FTR1 Δ ::NATMX* yeast strain, which allowed for integration into the *FTR1* locus. The C-terminal tail of Ftr1 (residues 318–404) in this new strain was then replaced by recombination using a synthetically constructed PCR tail fragment containing the desired lysine-to-arginine mutations within this sequence along with a C-terminal GFP tag. All of the lysine residues in the cytosolic tail of Fet3 were mutated to arginines using a similar strategy. This form of Fet3 also contained a 3xHA epitope tag added to the C-terminus. All mutant loci were amplified by PCR and sequenced to ensure that only the desired mutations were present.

To induce iron starvation, cells were grown to OD₆₀₀ $\approx$ 1.0 in synthetic media containing 50 μ M of the iron chelator bathophenanthroline disulfonic acid (BPS), as previously described (Storchlic *et al.*, 2007). For iron-induced down-regulation assays (i.e., iron shock), late log-phase cells were washed once with water and once with synthetic medium and were then resuspended in synthetic medium containing 1 mM sodium ascorbate and 500 μ M ferric ammonium sulfate and incubated at 30°C. For all other experiments, cells were grown in either yeast extract/peptone/dextrose (YPD) or yeast nitrogen base supplemented with the appropriate nutrients as necessary for the maintenance of plasmids. Cells were grown at 30°C unless a different temperature is specifically indicated.

Pulse-Chase Assays and Immunoprecipitation

For pulse-chase assays, cells were grown overnight in minimal medium lacking iron. The following morning midlog phase cells were pulse-labeled for 15 min at 30°C with ³⁵S-Met/Cys followed by a 40-min chase period at 30°C with cold excess Met/Cys. Cells were then rapidly pelleted, washed once with water, and resuspended in medium containing 1 mM sodium

Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference
TSY36	BY4742/ <i>FTR1-GFP::HISMX</i>	Strohlic <i>et al.</i> (2007)
TSY45	BY4742/ <i>SNX3Δ::KANMX; FTR1-GFP::HISMX</i>	Strohlic <i>et al.</i> (2007)
TSY48	BY4742/ <i>VPS29Δ::KANMX; FTR1-GFP::HISMX</i>	Strohlic <i>et al.</i> (2007)
ASY14	BY4742/ <i>VPS27Δ::KANMX; FTR1-GFP::HISMX</i>	This study
BSY35	BY4742/ <i>VPS24Δ::KANMX; FTR1-GFP::HISMX</i>	This study
BSY38	BY4742/ <i>VPS36Δ::KANMX; FTR1-GFP::HISMX</i>	This study
BSY41	BY4742/ <i>VPS23Δ::KANMX; FTR1-GFP::HISMX</i>	This study
BSY89	BY4742/ <i>VPS27Δ::NATMX; SNX3Δ::KANMX; FTR1-GFP::HISMX</i>	This study
TSY133	BY4742/ <i>FTR1(18 K/R)-GFP::KANMX; FET3-3HA::HISMX</i>	This study
TSY136	BY4742/ <i>FTR1(18 K/R)-GFP::KANMX; FET3(4 K/R)-3HA::HISMX</i>	This study
TSY138	BY4742/ <i>FTR1(18 K/R)-GFP::NATMX; FET3(4 K/R)-3HA::HISMX</i>	This study
TSY140	BY4742/ <i>FTR1-GFP::KANMX; FET3(4 K/R)-3HA::HISMX</i>	This study
TSY141	TSY138/ <i>SNX3Δ::KANMX</i>	This study
TSY142	TSY138/ <i>VPS23-mCherry::KANMX</i>	This study
TSY143	TSY138/ <i>VPS17-mCherry::KANMX</i>	This study
TSY145	TSY138/ <i>SNX3-mCherry::KANMX</i>	This study
TSY146	TSY138/ <i>VPS29Δ::KANMX</i>	This study
TSY147	TSY138/ <i>SNX3Δ::KANMX; VPS29Δ::URA3</i>	This study
TSY148	TSY136/ <i>end4-1(13myc::URA3)</i>	This study
TSY149	TSY138/ <i>PMA1-mCherry::KANMX</i>	This study
TSY156	BY4742/ <i>VPS27Δ::KANMX; VPS17-mCherry::HISMX</i>	This study
TSY159	BY4742/ <i>VPS27Δ::KANMX; VPS26-mCherry::HISMX</i>	This study
TSY161	BY4742/ <i>VPS23-GFP::URA3; SNX3-mCherry::HISMX</i>	This study
TSY163	BY4742/ <i>VPS27Δ::KANMX; SNX3-mCherry::HISMX</i>	This study
TSY165	BY4742/ <i>VPS27Δ::KANMX; VPS23-mCherry::HISMX</i>	This study
TSY169	BY4742/ <i>VPS23-GFP::URA3; VPS17-mCherry::HISMX</i>	This study
TSY192	BY4742/ <i>FTR1(18 K/R)-mCherry::KANMX; FET3(4 K/R)-3HA::HISMX</i>	This study
JPY71	SEY6210/ <i>rsp5Δ::HIS3 + pDsRED415-RSP5</i>	This study
JPY88	SEY6210.1/ <i>rsp5Δ::HIS3 + pDsRED415-rsp5^{G753I}</i>	This study
JPY98	SEY6210/ <i>rsp5Δ::HIS3 + pDsRED415-rsp5^{L733S}</i>	This study
TSY181	JPY71/ <i>FTR1-GFP::URA3</i>	This study
TSY182	JPY88/ <i>FTR1-GFP::URA3</i>	This study
TSY183	JPY98/ <i>FTR1-GFP::URA3</i>	This study
TSY185	TSY181/ <i>VPS29Δ::TRP1</i>	This study
TSY186	TSY182/ <i>VPS29Δ::TRP1</i>	This study
TSY187	TSY181/ <i>VPS29Δ::TRP1</i>	This study

ascorbate and 500 μ M ferric ammonium sulfate (iron shock medium) or 1 mM sodium ascorbate (mock treatment). Samples containing 5 OD₆₀₀ were precipitated with trichloroacetic acid (TCA) on ice for 30 min, washed twice with acetone, and dried in a SpeedVac (Savant Instruments, Farmingdale, NY). The dried pellets were resuspended by sonication in 100 μ l of urea boiling buffer (2 M urea, 50 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS) followed by vortexing with an equal volume of acid-washed glass beads at room temperature for 1 min. One milliliter of Tween-20 immunoprecipitation (IP) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween-20, and 0.1 mM EDTA) was added to each tube, mixed, and centrifuged for 5 min at room temperature at 10,000 $\times$ g. The supernatant was removed, and 4 μ l of polyclonal anti-GFP antiserum was added to it along with 50 μ l packed volume washed protein A-agarose beads (Invitrogen). Immunoprecipitations were rocked overnight at 4°C. Beads were gently pelleted the next morning and washed two times with 1 ml Tween-20 IP buffer and two times with 1 ml of Tween-20 urea buffer (2 M urea, 100 mM Tris, pH 7.5, 200 mM NaCl, and 0.5% Tween-20). All liquid was removed with a needle and syringe, and the beads were eluted by the addition of 50 μ l IP sample buffer (20% glycerol, 10% β -mercaptoethanol, 6% SDS, 125 mM Tris, pH 6.8, and 0.1% bromophenol blue). Eluted material was resolved by 10% SDS-PAGE. After SDS-PAGE, gels were dried on a gel dryer (Bio-Rad, Hercules, CA) and were exposed to phosphorimaging screens (Molecular Dynamics, Sunnyvale, CA) for at least 72 h. Visualization was performed using a Molecular Dynamics Storm 860 PhosphorImager (GE Healthcare), and quantitation was performed using ImageQuant software version 5.2 (GE Healthcare). Protein levels were normalized to the amount present at the zero time point, and the data were plotted in graphical form using Excel version 11.3.6 (Microsoft, Redmond, WA). Experiments were performed three independent times.

For IP to assess ubiquitylation status, cells expressing wild-type or mutant Ftr1-GFP, either transformed or not transformed with pRH990, were grown overnight in minimal medium lacking iron. Cells were then either exposed to iron shock or a mock treatment as described previously, with the exception that cells were only exposed to iron-containing medium for 10 min. Samples containing 12 OD₆₀₀ were removed, washed once with water containing

10 mM *N*-ethylmaleimide (NEM), and then precipitated by the addition of TCA. The method for preparing whole cell lysates as described in the preceding paragraph was then followed with the exception that 5 mM NEM was added to both the IP and wash buffers. Immunoprecipitated material was resolved by 8% SDS-PAGE followed by Western blotting with anti-GFP and anti-HA antibodies.

Protease Protection Assay

This method is based on a published protocol (Davis *et al.*, 1993). Briefly, strain TSY136 was grown overnight at 26°C to OD₆₀₀ $\approx$ 1.0 in 100 ml of synthetic (–His) medium plus 50 μ M BPS. The next morning the culture was split into two new flasks each containing 50 ml. The cells were washed once with water and once with synthetic medium and were resuspended in synthetic medium either containing or lacking 50 μ M BPS. Sodium ascorbate (1 mM) was added to both cultures, and 500 μ M ferric ammonium sulfate was added to the culture to which BPS was not added, and both cultures were incubated at 26°C. Five-milliliter aliquots were removed from both cultures at 0, 15, 30, and 60 min after iron shock or mock treatment. Cells were collected by centrifugation (14,000 $\times$ g for 1 min), resuspended in 5 ml of –His medium containing 20 mM Na₃ and 20 mM NaF, and then incubated on ice for 20 min. The cells were then collected by centrifugation, resuspended in 0.5 ml digestion buffer (DB; 1.4 M sorbitol, 25 mM Tris-HCl, pH 7.5, 10 mM Na₃, 10 mM NaF, 2 mM MgCl₂, 0.5% β -mercaptoethanol) and incubated at 37°C for 30 min. Next, 125 μ l of Pronase (2500 U/ml) prepared freshly in water (or 125 μ l of water for the no protease samples) was added to one of the two aliquots taken for each time point. Tubes were incubated at 37°C for 60 min with mixing by inverting the tubes every 10 min during the incubation. The cells were then spun down and washed two times with 200 μ l of DB with 1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). The samples were then precipitated by the addition of 10% TCA on ice for 30 min, washed twice with acetone, and dried in a SpeedVac (Savant). The dried pellets were resuspended in 2 $\times$ SDS-PAGE sample buffer, and samples were

Figure 1. Pulse-chase analysis of Ftr1 degradation rates. Cells of the indicated genotype were grown overnight in iron-free medium and then pulse-labeled at 30°C with ^{35}S -Met/Cys for 15 min followed by a 40-min chase period with unlabeled excess Met/Cys to allow transport of Fet3-Ftr1-GFP to the plasma membrane. Cells were then washed and switched to iron-shock medium or mock iron-free medium. At the indicated times (in minutes), aliquots were harvested and precipitated with TCA, and whole cell extracts were prepared. Ftr1-GFP was immunopurified with an anti-GFP antiserum and resolved by 10% SDS-PAGE. The amount of full-length Ftr1-GFP in each lane was quantified using a phosphorimager and expressed as a proportion of the amount present at time zero. Each line represents the mean of data collected from three independent experiments with the indicated SDs. All strains expressed Ftr1-GFP from its native locus. WT, wild-type.

resolved by 10% SDS-PAGE followed by immunoblotting with anti-HA and anti-PGK antibodies.

Iron Uptake Assay

The ^{55}Fe uptake assay to assess high-affinity ferric iron uptake was performed as described (Dancis *et al.*, 1990).

Microscopy

Epifluorescence microscopy of Ftr1-GFP was performed on live cells using a microscope (Eclipse E800; Nikon, Melville, NY) fitted with a cooled, high-resolution charge-coupled device camera (model C4742-95; Hamamatsu, Bridgewater, NJ). Images were acquired using Phase 3 Imaging software (Phase 3 Imaging Systems, Milford, MA) and were subsequently manipulated using Photoshop Elements version 2.0 (Adobe, San Jose, CA).

For colocalization studies of Fet3-Ftr1 22K/R with fluorescently labeled endosomal proteins and with Pma1-mCherry, microscopy was performed on live cells using a spinning disk confocal scanner (Perkin Elmer-Cetus, Norwalk, CT) combined with an inverted microscope (TE2000E; Nikon), equipped with a 100 $\times$ /1.45 NA PlanApo objective (Nikon) electronically controlled filter wheels (Sutter Instruments, Novato, CA), and a camera (ORCAII-ERG; Hamamatsu). In confocal mode, the 488- and 568-nm laser lines of an argon/krypton laser (Melles Griot, Rochester, NY) were used for sequential excitation of GFP and mCherry (0.4- and 1.0-ms exposures, respectively, captured at optical sections of 0.5 μm) in combination with a triple bandpass dichroic mirror. Microscope control, image acquisition, and image analysis and manipulation were done using MetaMorph software version 7.0 (Universal Imaging, West Chester, PA). All imaging was done at room temperature ($\sim 24^\circ\text{C}$).

For colocalization analysis of GFP-Vps27 or Vps23-GFP with mCherry-tagged endosomal proteins, microscopy was performed on live cells using a spinning disk confocal scanner (Yokogawa, Shenandoah, GA; CSU-10) combined with an inverted microscope (IX71; Olympus, Melville, NY) equipped with a 60 $\times$ /1.42 NA PlanApo objective and fitted with a ORCA-AG cooled CCD camera (Hamamatsu; model C-4742-80-12AG). In confocal mode, the 488- and 561-nm laser lines of a LMM5 4-line laser launch (Spectral Instruments, Irvine, CA) were used for simultaneous excitation of GFP and mCherry (2.0-ms exposures captured at optical sections of 0.5 μm) in combination with a DualView system (Optical Insights, Santa Fe, NM) containing a dichroic beamsplitter and emission filters (Chroma Technology, Brattleboro, VT). Microscope control and image acquisition were done using IPLab software (Scanalytics, Billerica, MA). Image analysis and manipulation were done using ImageJ software version 1.38s (NIH; <http://rsb.info.nih.gov/ij/>). All imaging was done at room temperature ($\sim 24^\circ\text{C}$).

Statistical Analysis

For analyzing the distribution of Fet3-Ftr1 22K/R-GFP puncta in various yeast strains, a log of likelihood ratio was calculated for each data set and used in a standard χ^2 regression analysis with 1 degree of freedom to generate a p value.

RESULTS

The retromer complex and the sorting nexin Snx3 function together in an endocytic recycling pathway to maintain the Fet3-Ftr1 high-affinity iron transporter at the plasma membrane at steady state when cells are starved for iron (Strochlic *et al.*, 2007). Snx3 functions as a cargo-specific

adapter in this pathway, binding both to a recycling signal in Ftr1 and to the retromer complex. In contrast, when cells are exposed to a high concentration of extracellular iron, the transporter is diverted to the lysosome-like vacuole for degradation (Felice *et al.*, 2005). The mechanism governing the switch between endocytic recycling and iron-induced degradation is unknown. As a first approach to dissecting the mechanism of the switch between sorting pathways, we considered two possible mechanisms. One possibility is that when iron is present, the recycling signal in Ftr1 is inactivated and the transporter is degraded by default transport to the vacuole. In this case, a loss of recycling would be kinetically equivalent to degradation. An alternative possibility is that when iron is present, Fet3-Ftr1 is actively sorted into the degradative pathway via a signal-mediated process that overrides the recycling pathway. A prediction of the first scenario is that the rate of iron transporter turnover in a recycling null strain will be the same when cells are grown in either the presence or absence of iron, whereas the latter model predicts that the rate of transporter turnover in a recycling null strain will be greater when iron is present. To distinguish between these two models, we monitored the rate of turnover of ^{35}S pulse-labeled Ftr1-GFP in wild-type, *snx3Δ*, and *vps29Δ* cells in a pulse-chase assay performed in either the presence or absence of iron shock. In wild-type cells starved for iron, the half-life ($t_{1/2}$) of Ftr1-GFP is greater than 90 min (estimated to be ~ 120 min), but after cells are shocked with iron the $t_{1/2}$ falls to 22 min, a sixfold difference (Figure 1). In both *snx3Δ* and *vps29Δ* cells that are starved for iron, the $t_{1/2}$ of Ftr1-GFP is ~ 45 min. Thus, deletion of a component of the recycling machinery (i.e., Snx3 or Vps29) results in a degradation rate in the absence of iron that is intermediate to the degradation rates in wild-type cells grown in the presence or absence of iron. Furthermore, when recycling mutant cells are shocked with iron, the $t_{1/2}$ of Ftr1-GFP is identical to wild-type cells, 22 min (Figure 1). Taken together, these results suggest that iron-induced degradation of the transporter is not simply due to a lack of recycling and, given the rapid degradation kinetics, is likely an active, signal-mediated process. In addition, the data suggest that recycling does not antagonize degradation, implying that the degradative pathway overrides the recycling pathway when cells are exposed to iron shock.

Constitutive Endocytic Recycling of Fet3-Ftr1 in Degradative Pathway Mutants

Ubiquitin is the best characterized sorting signal that mediates targeting of integral membrane proteins to the lysosome

Figure 2. Ftr1-GFP localization in multivesicular body pathway mutants. Localization of Ftr1-GFP was examined by epifluorescence microscopy in the indicated yeast deletion strains grown at 30°C either in the absence (top panels) or presence (bottom panels) of iron shock for 60 min. The ESCRT complex to which the deleted gene products belong are indicated in parentheses. White arrowheads indicate the “class E” compartment that is adjacent to the vacuole. All strains expressed Ftr1-GFP from its native locus. Note that in the *vps27Δ snx3Δ* double mutant, Ftr1-GFP, is localized to the class E compartment and the limiting membrane of the vacuole, in addition to the plasma membrane. The *snx3Δ* recycling mutant is shown for comparison. Scale bar, 2 μ m.

or vacuole for degradation via the MVB pathway (Hicke and Dunn, 2003; Piper and Luzio, 2007), and a previous report concluded that Ftr1, but not Fet3, is ubiquitinated in response to iron shock (Felice *et al.*, 2005). In yeast and mammalian cells, the ESCRTs are required to incorporate ubiquitinated cargo proteins into the vesicles budding into the lumen of MVBs (Babst, 2005; Nickerson *et al.*, 2007; Piper and Katzmann, 2007; Saksena *et al.*, 2007). Several ESCRT proteins bind ubiquitin, most notably the Vps27-Hse1 MVB pathway cargo receptor (sometimes referred to as “ESCRT-0”) that mediates entry of proteins destined for degradation into this pathway (Bilodeau *et al.*, 2002; Shih *et al.*, 2002). Cells with a deletion of *VPS27*, or any other core component of the MVB machinery, contain an aberrant, multilamellar late endosomal compartment juxtaposed to the vacuole referred to as the “class E compartment.” (Rieder *et al.*, 1996). In these mutants, some cargo proteins accumulate and become trapped in this compartment, whereas other cargo appears to be recycled to the plasma membrane (Raymond *et al.*, 1992; Davis *et al.*, 1993; Bugnicourt *et al.*, 2004). To investigate a possible role for the ESCRT pathway in iron-regulated turnover of Fet3-Ftr1, we determined the localization of Ftr1-GFP by epifluorescence microscopy in wild-type cells and all known ESCRT mutants; representative examples are shown in Figure 2. In every ESCRT mutant, Ftr1-GFP is localized predominantly to the plasma membrane (Figure 2). Importantly, a GFP signal is not observed on the limiting membrane of the vacuole or in intracellular compartments even in cells exposed to iron shock (Figure 2, bottom panels), indicating that Fet3-Ftr1 is diverted from the vacuolar targeting pathway when the MVB sorting machinery is nonfunctional.

The results suggest that either ESCRT mutants are defective for iron-induced internalization of Fet3-Ftr1, or that the iron transporter is efficiently sorted into the recycling pathway even when iron is present in these strains. To distinguish between these possibilities, we generated a double mutant in which we deleted a component of the Fet3-Ftr1 recycling pathway, *SNX3*, in the *vps27Δ* mutant background (*vps27Δ snx3Δ*). In this strain Ftr1-GFP is localized to the class E compartment and to the limiting membrane of the vacuole in the presence and absence of iron shock (Figure 2). Because Snx3 functions to recycle Fet3-Ftr1 after endocytosis (Strohlic *et al.*, 2007), this result implies that ESCRT mutants are not generally defective in endocytosis and that Fet3-Ftr1 is constitutively recycled (i.e., even in the presence of iron) when the MVB pathway is nonfunctional. An important conclusion that follows from these results is that the recycling

machinery is functional in the recognition and sorting of Fet3-Ftr1 in the presence of iron.

In yeast, the HECT (homologous to E6 AP C-terminus)-domain containing E3 ligase Rsp5 is the only ubiquitin ligase that has been shown to be involved in regulated ubiquitylation of proteins at the plasma membrane. Among the substrates for Rsp5-mediated ubiquitylation are the copper transporter Ctr1p (Liu *et al.*, 2007), the zinc transporter Zrt1p (Gitan and Eide, 2000), the general amino acid permease Gap1p (Hein *et al.*, 1995), the uracil permease Fur4p (Galan *et al.*, 1996; Blondel *et al.*, 2004), and the tryptophan permease Tat2p (Beck *et al.*, 1999). Rsp5 is encoded by an essential gene, so we investigated the localization of Ftr1-GFP by epifluorescence microscopy in two different *rsp5* strains that each contain a point mutation within the Rsp5 catalytic HECT domain (*rsp5-1* and *rsp5-smm1*; Fisk and Yaffe, 1999), and in control cells expressing a wild-type copy of *RSP5*. These experiments were conducted with cells grown at 26°C, which is a permissive growth temperature, although Rsp5 activity is severely compromised (Krsmanovic and Kolling, 2004). In both mutant strains, Ftr1-GFP is localized to the plasma membrane in the presence and absence of iron shock, whereas in isogenic wild-type control cells, it is trafficked to the lumen of the vacuole in the presence of iron (Figure 3A). Thus, *rsp5* mutants essentially phenocopy the ESCRT deletion mutants, raising the possibility that Fet3-Ftr1 is constitutively recycled in the *rsp5* mutants as well. To substantiate the results of the microscopy using a biochemical approach, we performed a pulse-chase assay in the presence of iron shock with both the wild-type control strain and the *rsp5-smm1* strain to determine degradation kinetics of Ftr1-GFP as described in Figure 1. In the presence of iron, the $t_{1/2}$ for Ftr1-GFP in wild-type control cells is 29 min, but in the *rsp5* mutant it is greater than 90 min (estimated to be ~120 min; Figure 3B). These results confirm the microscopy-based results and definitively implicate the Rsp5 ubiquitin ligase in targeting of Fet3-Ftr1 to the lumen of the vacuole.

As with the ESCRT mutants, an important issue to address was whether the iron transporter simply fails to be internalized in the *rsp5* mutant strains because of a possible role for the ligase in the initial steps of endocytosis. To test this, we deleted a component of the endocytic recycling machinery, the retromer protein Vps29, in both the wild-type *RSP5* control strain and the *rsp5-1* mutant strain and analyzed the localization of Ftr1-GFP in these strains under iron-limiting (i.e., recycling) growth conditions. The data show that the transporter is localized to the lumen of the vacuole in the *RSP5 vps29Δ* control strain, as expected, but to

Figure 3. The Rsp5 ubiquitin ligase is required for iron-induced degradation of Fet3-Ftr1. (A) Localization of Ftr1-GFP by epifluorescence microscopy in a wild-type *RSP5* strain and isogenic *rsp5* mutants, *rsp5-1* (L733S) and *rsp5-smm1* (G753I), grown at 26°C in the absence of iron or 60 min after iron shock. Scale bar, 2 μ m. (B) Pulse-chase analysis of Ftr1-GFP turnover in wild-type *RSP5* and *rsp5-smm1* strains in the presence of iron. The analysis was carried out as described in Figure 1. The cells were grown at 26°C. (C) Steady-state localization of Ftr1-GFP by epifluorescence microscopy of *vps29Δ* cells (wild-type *RSP5*) and *rsp5-1 vps29Δ* double mutant cells grown at 26°C in iron-free conditions. Note that Ftr1-GFP is localized to the limiting membrane of the vacuole in *rsp5-1 vps29Δ* cells but to the lumen of the vacuole in *RSP5 vps29Δ* cells. Scale bar, 2 μ m.

the limiting membrane of the vacuole in the *rsp5-1 vps29Δ* strain (Figure 3C). Thus, Rsp5 is not required for endocytosis of the iron transporter per se, but it is required at some point downstream of internalization to target Fet3-Ftr1 to the vacuole. Collectively, the results demonstrate that Fet3-Ftr1 is recycled regardless of iron status when the functions of

either Rsp5 or the ESCRT-dependent MVB sorting pathway are compromised.

Constitutive Endocytic Recycling of Fet3-Ftr1 Lacking Cytosolic Lysine Residues

The Fet3-Ftr1 iron transporter appears to be recycled in ESCRT and *rsp5* mutants even in the presence of iron. Because these mutations perturb the overall organization and function of the endosomal system, it is not clear if constitutive recycling of Fet3-Ftr1 is a specific feature of this cargo. Thus, we sought to address the possible role of Fet3-Ftr1 ubiquitylation with respect to the recycling versus degradation sorting decision by generating *cis*-acting mutations within the cargo itself. The Ftr1 subunit of the iron transporter is reported to be ubiquitylated in response to iron shock (Felice *et al.*, 2005), so we mutated every lysine residue to arginine within the cytosolic segments of Fet3 (Fet3-K/R), Ftr1 (Ftr1-K/R), or both Fet3 and Ftr1, hereafter referred to as Fet3-Ftr1 22 K/R (Figure 4A and Supplementary Figure S1A). The mutant genes were integrated at their native chromosomal loci to ensure expression from their native promoters, and so that they are the sole copies of *FET3* and *FTR1* in the cell. The functionality of these mutant transporters was confirmed by a ^{55}Fe uptake assay that demonstrated that cells expressing the mutant transporters import as much iron as a wild-type strain (Supplementary Figure S1B). In addition, steady-state analysis of protein levels of each of these different mutants, determined by immunoblotting for Ftr1-GFP, revealed that both the Fet3-K/R and Ftr1-K/R mutants are expressed at similar levels to wild-type; however, the Fet3-Ftr1 22 K/R mutant is expressed at slightly increased levels (Supplementary Figure S1C), perhaps indicating enhanced stability. That the lysine-less form of Ftr1 (in the context of Fet3-Ftr1 22 K/R, with Ftr1 tagged with GFP) is not ubiquitylated was confirmed by immunoprecipitation of Ftr1-GFP from cells expressing HA epitope-tagged ubiquitin followed by anti-HA immunoblotting (Figure 4B); a ubiquitylated form of Ftr1-GFP was observed for wild-type Ftr1, but not for the cytosolic lysine-less form. As first reported by Felice *et al.* (2005), when epitope-tagged ubiquitin is overexpressed, detection of ubiquitylated Ftr1-GFP does not require, and is not enhanced by, iron shock. In addition, overexpression of HA-tagged ubiquitin does not alter the steady-state distribution of wild-type Ftr1-GFP: when cells expressing this construct are grown under iron-limiting (recycling) conditions, the iron transporter is localized exclusively to the plasma membrane (Figure 4C).

We next analyzed steady-state localization and trafficking of the lysine-less mutant transporters in the presence and absence of iron shock by epifluorescence microscopy. In these experiments, localization of the iron transporter was determined by visualizing GFP-tagged Ftr1. Both Fet3-K/R and Ftr1-K/R, in which only one subunit of the transporter contained cytosolic lysine residues, displayed iron-induced vacuolar targeting that was indistinguishable from the wild-type transporter (Figure 4D), indicating that lysine residues in either Ftr1 or Fet3 can serve to target the transporter into the degradative pathway. This suggests a hierarchy for ubiquitylation whereby lysines in Ftr1, the permease, are preferential sites for ubiquitylation, but if these are unavailable (i.e., due to mutation to arginines), then lysine residues in Fet3 may serve as ubiquitin acceptor sites. In contrast, the mutant lacking all cytosolic lysine residues, Fet3-Ftr1 22K/R, was not targeted to the vacuole, even after 60 min of incubation in iron-containing medium (Figure 4D). Collectively, these results suggest that the cytosolic lysine-less

Figure 4. A mutant form of the iron transporter that lacks cytosolic lysine residues, Fet3-Ftr1 22K/R, is not ubiquitylated and exhibits altered trafficking. (A) A schematic diagram depicting the lysine to arginine changes (red dots) introduced into Fet3 and Ftr1. Fet3 contains four lysine residues in its cytoplasmic C-terminal tail, and Ftr1 contains 18 lysine residues in all of its cytosolic regions. The cytosolic lysine-less iron transporter, Fet3-Ftr1 22K/R, contains 22 mutated residues. In each case, Fet3 is tagged at its C-terminus with a 3xHA epitope, and Ftr1 is tagged at its C-terminus with GFP. (B) Immunoblot analysis of immunopurified wild-type or cytosolic lysine-less Ftr1-GFP from iron-starved cells grown at 30°C and subjected to iron shock for 10 min. To facilitate detection of ubiquitin, cells expressed an HA epitope-tagged form of ubiquitin from a multicopy 2 μ plasmid. Ftr1-GFP was immunopurified as described in the legend to Figure 1 and resolved by SDS-PAGE. Top panels, anti-GFP blots. Approximately 2% of the total input for the immunopurification is shown in the lanes marked “in,” and purified Ftr1-GFP is shown in the lanes marked –Fe and +Fe. Note that a prominent band, indicated by the black arrowhead, is present in the lanes containing Ftr1-GFP from cells that express HA-ubiquitin, regardless of iron status. Bottom panels, anti-HA immunoblots of the same material. The position of molecular mass standards are indicated to the left of the gel panels. (C) Localization of wild-type Ftr1-GFP in cells expressing HA-ubiquitin from a multicopy 2 μ plasmid. The cells were grown in iron-free medium at 30°C. Scale bar, 2 μ m. (D) Localization of wild-type, Ftr1 K/R only, Fet3 K/R only, and Fet3-Ftr1 22 K/R transporters in cells grown at 30°C in the absence of iron or 60 min after iron shock. For each construct, Ftr1-GFP was visualized. Scale bar, 2 μ m.

transporter is recycled regardless of iron status, just as in the ESCRT and *rsp5* mutants.

A striking difference in cells expressing Fet3-Ftr1 22K/R-GFP is the presence of one to five large puncta per cell that are rarely observed in cells expressing the wild-type transporter (Figure 4D). This could be due to accumulation of a biosynthetic cohort of Fet3-Ftr1 22K/R-GFP that is not delivered to the plasma membrane efficiently or an endocytosed cohort that is not recycled efficiently. To distinguish between these possibilities, we examined the localization of Fet3-Ftr1 22K/R-GFP in endocytosis-defective *end4-1* cells by epifluorescence microscopy. In this strain, no puncta were observed even after prolonged incubation in iron-containing medium (Figure 5A). Thus, the internal pool of the mutant iron transporter is derived from the plasma membrane by endocytosis. Importantly, the accumulation of Fet3-Ftr1 22K/R within endosomes is a specific property of

this cargo and does not reflect a general deficiency in the endosomal system; an unrelated, highly expressed, plasma membrane protein, the Na⁺/H⁺ ATPase Pma1 (tagged with mCherry) is localized at steady state both to the plasma membrane and to the lumen of the vacuole, with no intracellular localization to the endosomes decorated with Fet3-Ftr1 22K/R-GFP (Supplementary Figure S2).

The accumulation of internalized Fet3-Ftr1 22 K/R within the cell without transport to the vacuole suggests that this mutant transporter is recycled (albeit likely inefficiently) even in the presence of iron. To directly test the role of the recycling pathway in Fet3-Ftr1 22K/R localization, the genes encoding Snx3 or Vps29 were deleted in cells expressing Fet3-Ftr1 22K/R-GFP, and the localization of the transporter was determined. In the *snx3* Δ and *vps29* Δ mutants, Fet3-Ftr1 22K/R-GFP was now observed to localize, in part, to the limiting membrane of the vacuole in the presence (data not

Figure 5. The Snx3 and retromer recycling machinery retains Fet3-Ftr1 22K/R in the endosomal system. (A) Localization of Fet3-Ftr1 22K/R-GFP in wild-type (WT) cells and endocytosis-defective (*end4-1*) cells grown at 26°C, 60 min after iron shock. Note that the large puncta (endosomes) observed in wild-type cells are not present in *end4-1* cells. Scale bar, 2 μ m. (B) Localization of Fet3-Ftr1 22K/R-GFP in wild-type (WT), *snx3Δ*, and *vps29Δ* strains by epifluorescence microscopy grown at 30°C in the absence of iron. White arrowheads denote the limiting membrane of the vacuole. Scale bar, 2 μ m. (C) Quantification of cells with endosomes labeled by Fet3-Ftr1 22K/R-GFP in WT, *snx3Δ*, *vps29Δ*, and *snx3Δvps29Δ* double mutant strains. Cells were grown overnight in iron-free medium, switched to mock (-Fe) or iron shock (+Fe) medium, and then incubated for 60 min at 30°C. The cultures were then coded for blind analysis, and cells were examined by epifluorescence microscopy to determine the presence or absence of internal puncta (endosomes). For each data set, 200 cells (100 cells each in two independent experiments) were scored. The proportion of cells in each culture that contained puncta is plotted. To determine if the observed differences between the strains were significant, we performed statistical analyses to compare the -Fe data between wild-type and *snx3Δ* and *vps29Δ*. In both cases the differences were statistically different (** $p < 0.001$). Comparison of the +Fe data also indicated statistical significance (* $p < 0.01$). Statistical comparison of the *snx3Δ vps29Δ* double mutant data with all other strains showed that these data were significantly different (** $p < 0.001$). (D) Fet3-HA protease protection assay. Wild-type (TSY136) or *end4-1* (TSY148) cells expressing Fet3-Ftr1 22K/R were grown at 26°C and harvested before and at the indicated times (in minutes) after iron shock. Cells were then incubated with or without Pronase to proteolyze the extracellular portions of cell surface proteins. Fet3 contains an intracellular C-terminal 3xHA epitope tag and was detected by anti-HA immunoblotting. The positions of full-length Fet3-HA (~150 kDa) and the major Pronase cleavage product (~20 kDa) are indicated with arrows to the left of the blot. Note that in wild-type cells iron shock results in protection of up to ~35% of Fet3 (60-min time point), but in endocytosis defective (*end4-1*) cells, no Fet3 is protected. Pgk1 is visualized in the panels below the HA blot; it serves as a loading control and as an indication of cell integrity. The position of molecular mass standards are indicated to the right of the gel panels.

shown) and absence of iron shock (Figure 5B). These results suggest that the lysine-less form of the transporter is constitutively recycled via the Snx3-retromer pathway and that cytosolic lysine residues are required for targeting into the MVB pathway, presumably because they are acceptors for ubiquitin.

We also noted that the proportion of cells with labeled endosomes appeared to decrease when either component of the recycling machinery was deleted. In a single blind study, we determined that in a population of iron-starved wild-type cells expressing Fet3p-Ftr1p 22K/R-GFP, ~55% of the cells contain labeled endosomes, whereas in the *snx3Δ* or *vps29Δ* mutants, this value was ~20% (Figure 5C). Although this difference was determined to be statistically significant ($p < 0.001$), we were surprised that deletion of just a single component of the recycling machinery did not affect endosomal accumulation of Fet3-Ftr1 22K/R more substantially because Snx3 and retromer function together to recycle Fet3-Ftr1 (Strohlic *et al.*, 2007). To determine if this residual localization to endosomes required the remaining components of the recycling machinery, we expressed Fet3-Ftr1 22K/R-GFP in *snx3Δ vps29Δ* double mutant cells, and observed that the proportion of cells with labeled endosomes decreased to <10% in the presence and absence of iron shock (Figure 5C). These results indicate that Fet3-Ftr1 22K/R-GFP accumulates in endosomes from where it is recycled to the plasma membrane by the combined activities of both Snx3 and retromer. This result hints at the existence of another, possibly retromer-dependent, sorting signal that is required for efficient recycling (in addition to the Snx3-dependent recycling signal), and it suggests that one or more of the lysine-to-arginine mutations diminishes the efficiency of Fet3-Ftr1 recycling.

Iron Shock Triggers Endocytosis of Fet3-Ftr1 Independently of Ubiquitylation

Ubiquitin has been implicated as a signal for endocytosis of integral membrane proteins (Hicke and Dunn, 2003), and an interesting question arising from these studies is whether ubiquitylation of the iron transporter is required for its endocytosis. The availability of the cytosolic lysine-less form of the iron transporter provided us with an opportunity to investigate this issue directly because Fet3-Ftr1 22K/R is not ubiquitylated (Figure 4B); if iron-dependent ubiquitylation of Ftr1 triggers endocytosis, then endocytosis of Fet3-Ftr1 22K/R should not be regulated by iron shock. As a first test of this, we quantified the proportion of wild-type cells (expressing Fet3-Ftr1 22K/R-GFP) containing one or more labeled endosomes before and after 60 min in iron-containing medium. Before iron shock, ~55% of the cells contained at least one labeled endosome, and this value increased to 72% after iron shock (Figure 5C). Statistical analysis indicated that this difference was significant ($p < 0.01$). We next used an established protease protection assay (Davis *et al.*, 1993) to measure the proportion of Fet3-Ftr1 22K/R that is internalized after iron shock. Wild-type cells expressing Fet3-Ftr1 22K/R were incubated with or without iron shock, aliquots of cells were withdrawn at various times, and trafficking was halted by disruption of metabolism with NaN_3 and NaF. The cells were then incubated with protease to degrade the exposed portions of cell surface proteins, and the extent of cleavage of Fet3 was determined by semiquantitative immunoblotting. Proteolysis of Fet3-HA (in the context of Fet3-Ftr1 22K/R) was monitored in this assay because it contains a single, large extracellular domain that is readily cleaved to produce a small (~20 kDa) fragment containing the C-terminal epitope tag (Figure 5D). In the absence of iron

Figure 6. Comparison of Fet3-Ftr1 22K/R localization with endosomal markers. (A) Colocalization with recycling machinery. Live cells coexpressing Fet3-Ftr1 22K/R-GFP and Vps17-mCherry or Snx3-mCherry grown at 30°C were incubated for 60 min in iron shock medium and then imaged by spinning disk confocal microscopy. Only one image from the Z series is shown. Magnified areas of the merged images are displayed as insets, and colocalization is indicated in yellow. Scale bar, 2 μm . (B) Colocalization with MVB machinery. Live cells coexpressing Fet3-Ftr1 22K/R-mCherry and GFP-Vps27, or Fet3-Ftr1 22K/R-GFP and Vps23-mCherry, were grown at 30°C and incubated for 60 min in iron shock medium and then imaged by spinning disk confocal microscopy. Only one image from the Z series is shown. Magnified areas of the merged images are displayed as insets, and colocalization is indicated in yellow. Scale bar, 2 μm .

shock, <2% of full-length Fet3-HA was protected from proteolysis, likely corresponding to the recycling cohort and newly synthesized Fet3-HA that had not yet been delivered to the plasma membrane. In contrast, ~35% of Fet3-HA was protected from proteolysis after 60 min in iron-containing medium. Importantly, when this assay was performed using *end4-1* cells, no Fet3-HA was protected from proteolysis, indicating that protection of Fet3-HA requires endocytosis. We conclude that iron shock triggers endocytosis of Fet3-Ftr1 independently of its ubiquitylation.

Colocalization of Fet3-Ftr1, Vps27, and Snx3-Retromer on Endosomes

Given the observation that Snx3 and retromer appear to retain Fet3-Ftr1 22K/R within the endosomal system, we hypothesized that these recycling factors should localize to the endosomal compartments in which this cargo accumulates. To test this, we performed colocalization analysis with Fet3-Ftr1 22K/R-GFP and mCherry-labeled endosomal marker proteins of the recycling pathway using spinning-disk confocal microscopy. Nearly all the Fet3-Ftr1 22K/R-labeled endosomes are positive for both Snx3 and the retromer component Vps17 (Figure 6A). This result indicates that Fet3-Ftr1 22K/R is recycled inefficiently, and we suggest

that it accumulates in the endosomal compartment from which it is exported.

In human cells, retromer is localized predominantly to early endosomes, especially the tubular endosomal network, with a small proportion localized to late endosomes (Arighi *et al.*, 2004; Seaman, 2004). This distribution has been interpreted to indicate that retromer-mediated export from the endosomal system occurs along the entire early-to-late endosome maturation pathway. In yeast, however, the best characterized role for retromer is in the retrieval of the Vps10 sorting receptor from late endosomes to the Golgi apparatus, leading to the suggestion that yeast retromer functions mainly in the retrieval of cargo from late (prevacuolar) endosomes (Seaman, 2005; Bonifacino and Rojas, 2006). Our recent work showing partial colocalization of retromer subunits and Snx4, a sorting nexin that is generally considered to reside on early endosomes (Hettima *et al.*, 2003), led us to suggest that retromer is in fact more broadly localized within the endosomal system (Strochlic *et al.*, 2007), akin to the situation in human cells.

The accumulation of Fet3-Ftr1 22K/R in endosomes from which it is sorted by retromer into the recycling pathway afforded us an opportunity to investigate where along the endosomal maturation pathway retromer exports cargo, specifically in relation to the degradative MVB pathway. To this end, we used spinning-disk confocal microscopy to image live cells expressing tagged forms of the mutant recycling cargo Fet3-Ftr1 22K/R and two components of the MVB pathway, Vps27 and Vps23 (Figure 6B). These ESCRT subunits were chosen because Vps27 functions as a sorting receptor at the gateway to the MVB pathway, and Vps23 functions downstream of this point of divergence, as a component of ESCRT-I. In addition, Vps27 and Vps23 are among the few ESCRT subunits that are functional as fusions to fluorescent proteins (Katzmann *et al.*, 2001, 2003). In the presence of iron shock, endosomes labeled by Fet3-Ftr1 22K/R are positive for GFP-Vps27, but they do not contain Vps23-mCherry. Moreover, minimal colocalization is observed with GFP-Vps27 and Vps23-mCherry (Supplementary Figure S3), which is consistent with the localizations of the mammalian homologues of Vps27 and Vps23 and of Hrs and Tsg101, respectively, in cultured human cells (Bache *et al.*, 2003). We next compared the localizations of GFP-Vps27 and Vps23-mCherry directly with components of the recycling machinery by dual-view spinning disk confocal microscopy; GFP-Vps27 colocalized extensively with mCherry-tagged retromer subunits Vps17 and Vps26p as well as with Snx3-mCherry (Figure 7A). In contrast, Vps23-GFP did not colocalize with Vps17-mCherry or Snx3-mCherry (Figure 7B). Collectively, the data show that Fet3-Ftr1 22K/R accumulates in a compartment where the sorting receptors for the degradative (Vps27) and the recycling (Snx3 and retromer) pathways are enriched, suggesting that the two pathways diverge from or at this compartment.

DISCUSSION

When cells are starved for iron, Fet3-Ftr1 is maintained on the plasma membrane via an endocytic recycling pathway (Strochlic *et al.*, 2007), but when iron is abundant, Fet3-Ftr1 is shunted into the MVB pathway and degraded in the vacuole (Felice *et al.*, 2005; this study). We have investigated the mechanism by which sorting of Fet3-Ftr1 is switched from a recycling pathway in the absence of iron, to a degradative pathway upon addition of iron to the growth medium. The results indicate that there are two relevant points where iron impacts Fet3-Ftr1 sorting in the endosomal system. First,

Figure 7. Comparison of localization of components of the recycling and MVB machineries. (A) Live cells expressing GFP-Vps27 and Vps17-mCherry, Vps26p-mCherry, or Snx3-mCherry were grown at 30°C and imaged by dual view spinning disk confocal microscopy. One image from the Z series is shown. Colocalization of puncta is indicated in yellow. Scale bar, 2 μm. (B) Live cells expressing Vps23-GFP and Vps17-mCherry or Snx3-mCherry were grown at 30°C and imaged by dual view spinning disk confocal microscopy. Only one image from the Z series is shown. Colocalization of puncta is indicated in yellow. Scale bar, 2 μm.

iron shock stimulates Fet3-Ftr1 endocytosis, and second, iron-regulated sorting occurs on endosomes to effect sorting into the MVB pathway which ultimately leads to vacuolar degradation. In principle, both iron-regulated sorting events could be “programmed” at the plasma membrane, so long as the modification in/on Fet3-Ftr1 that results from binding or transport of iron persists during transport within the endosomal system.

Iron-induced vacuolar degradation of Fet3-Ftr1 requires the Rsp5 ubiquitin ligase, the ubiquitin-dependent MVB sorting machinery, and lysine residues in the cytosolic domains of Fet3-Ftr1. These observations suggest a role for ubiquitin in degradative sorting of Fet3-Ftr1. Work by Felice *et al.* (2005) demonstrated that Ftr1 is ubiquitylated in response to iron shock and that endocytosis of Fet3-Ftr1 is not inhibited in a *rsp5-1* ubiquitin ligase mutant. We find that mutations in *RSP5* nonetheless inhibit Fet3-Ftr1 turnover and that endocytosis of a form of Fet3-Ftr1 that cannot be ubiquitylated is still regulated by iron. These results indicate

Figure 8. Model of regulated Fet3-Ftr1 sorting in the endosomal system. Cargo is internalized by endocytosis and is delivered to the early endosome, which is depicted to contain a vacuolar domain and a tubular domain that emanates from the vacuolar portion. In the absence of iron, Fet3-Ftr1 is captured in the vacuolar domain by Snx3 and retromer and sorted into tubules from where cargo buds. After fission of a tubule, the cargo is transported to the Golgi apparatus for “resecretion.” Iron stimulates sorting of Fet3-Ftr1 into the MVB pathway (“stimulated MVB sorting”), which requires the Rsp5 ubiquitin ligase and the Vps27-Hse1 MVB sorting receptor. Sorting of Fet3-Ftr1 into intraluminal vesicles in the presence of iron is efficient and rapid, resulting in a relatively short half-life ($t_{1/2}$ = 22 min at 30°C). In contrast, when the Fet3-Ftr1 recycling machinery is disabled (e.g., by deletion of one component), Fet3-Ftr1 is sorted into intraluminal vesicles inefficiently (“basal MVB sorting”) in the absence of iron and is degraded relatively slowly ($t_{1/2}$ = 45 min at 30°C). We suggest that efficient, stimulated MVB sorting occurs primarily within the vacuolar domains of the early endosome, whereas basal MVB sorting occurs along the endosome maturation pathway.

that the role for Rsp5 and ubiquitin in turnover of Fet3-Ftr1 is after internalization, although the data do not formally rule out that ubiquitin might augment another endocytic signal. A similar finding was reported for the yeast *a*-factor receptor, Ste3, where *a*-factor stimulates endocytosis of a mutant version of Ste3 that lacks lysyl ubiquitin acceptor sites (Chen and Davis, 2002). The increases in the rates of Fet3-Ftr1 and Ste3 endocytosis, which are independent of cargo ubiquitin modification, may reflect a more widespread feature of regulated endocytosis than is currently appreciated. For Fet3-Ftr1, binding of iron to the extracellular domain, and/or transport of iron into the cell, elicits recognition of the cytosolic portions of Fet3-Ftr1 by the endocytosis machinery. The intracellular segments of Ftr1 contain multiple potential endocytic signals, and it will be important in future studies to determine if any of these are required for iron-regulated endocytosis.

Although ubiquitylation of Fet3-Ftr1 is not required for iron-regulated endocytosis, our data definitively show that the Rsp5 ubiquitin ligase is required for subsequent MVB sorting, and we speculate that Rsp5 functions at the early endosome after Fet3-Ftr1 has been internalized to effect sorting into the MVB pathway. Rsp5 is localized in part to endosomes (Wang *et al.*, 2001; Katzmman *et al.*, 2004) where it associates with Hse1, a subunit of the MVB cargo-sorting receptor, raising the possibility that cargo ubiquitylation at the endosome and MVB sorting is coupled. A straightforward model that incorporates these observations posits that Rsp5 ubiquitylates the endosomal cohort of Fet3-Ftr1, thereby promoting efficient MVB sorting due to the local coincidence of ubiquitylated cargo and the Vps27-Hse1 sorting receptor. The role of iron could be either to facilitate recognition and ubiquitylation of Fet3-Ftr1 by Rsp5 or to facilitate recognition of ubiquitylated Fet3-Ftr1 by the MVB sorting machinery, after Rsp5 acts (or both). Surprisingly, ubiquitin appears to be necessary but not sufficient to confer iron-stimulated MVB sorting to Fet3-Ftr1, as iron shock is still required for MVB sorting when Fet3-Ftr1 is ubiquitylated as a consequence of ubiquitin overexpression (Figure 5C). One possible mechanism that might augment the ubiquitin signal is iron-induced oligomerization of the trans-

porter. This could occur initially at the plasma membrane where the transporter first encounters iron and would serve to consolidate a cohort of ubiquitylated Fet3-Ftr1 at the plasma membrane to facilitate endocytosis and in endosomes to increase the efficiency of Rsp5-mediated ubiquitylation and/or the avidity with which the Vps27-Hse1 MVB-sorting receptor captures the cargo (Piper and Luzio, 2007; Traub and Lukacs, 2007). An alternative possibility is that iron shock induces association of Fet3-Ftr1 with another protein that confers MVB sorting (and possibly endocytosis).

The broad goal of this work is to elucidate how iron shock shunts Fet3-Ftr1 out of the endosomal recycling pathway and into the endosomal degradative pathway. A portion of Fet3-Ftr1 colocalizes with Snx3, retromer, and the Vps27 subunit of the MVB sorting receptor (Figure 6). Pairwise comparison of Vps27, Snx3, and retromer (Vps17 and Vps26) localization (Figure 7A) shows that all of these factors colocalize on a subset of endosomes, but that the later-acting ESCRT-1 subunit, Vps23, is largely absent from these endosomes (Figure 7B). The data therefore suggest that the point of divergence for the degradative and recycling pathways is this common endosomal compartment that we speculate is the yeast equivalent of the early endosome of human cells. In support of this, the human homologue of yeast Vps27, Hrs, has been localized to clathrin-coated microdomains of the vacuolar portions of early endosomes where it is proposed to concentrate ubiquitylated cargo (Raiborg *et al.*, 2002, 2006; Raiborg and Stenmark, 2002). Human retromer proteins and Snx3 also localize to the vacuolar domains of these endosomes in addition to the tubular endosomal network and early endosomal export sites (Xu *et al.*, 2001; Arighi *et al.*, 2004). Furthermore, the mammalian retromer component Vps26 has been localized to clathrin-containing exit sites on early endosomes in cultured cells (Popoff *et al.*, 2007), and a recent report has localized Snx1 to early endosomal buds that give rise to specific endosome-to-TGN carriers (Mari *et al.*, 2008). A model that incorporates all of these data (Figure 8) posits that after cargo is internalized from the plasma membrane, it enters the vacuolar domain of the early endosome. Here, Snx3-retromer and the ESCRT-dependent MVB machinery survey cargo. For Fet3-Ftr1 in the presence

of iron, Vps27-Hse1 and the MVB machinery preferentially sort the cargo into intraluminal vesicles of endosomes as they mature. Ultimately, these vesicles are degraded in the lysosome-like vacuole. In the absence of iron, Snx3-retromer preferentially recognizes Fet3-Ftr1 and sorts it into nascent tubules that emanate from the vacuolar portion of the endosome. Based on the requirement for the Ypt6 Golgi Rab GTPase in Fet3-Ftr1 recycling (Strohlic *et al.*, 2007) and the established roles of Snx3 and retromer in endosome-to-Golgi trafficking, this export pathway is predicted to direct Fet3-Ftr1 back to the Golgi for "resecretion" (Figure 8).

Our work directly demonstrates that the lifetime of Fet3-Ftr1 is established by the opposing outcomes of the ESCRT-dependent degradative pathway and the Snx3- and retromer-dependent recycling pathway (Figure 8). Although it is generally appreciated that sorting into each of these pathways results in opposite outcomes for cargo, an important contribution of the work herein is that it directly and rigorously addresses the hypothesis that the interplay of these sorting machineries determines the fate of endosomal cargo using iron-regulated sorting of Fet3-Ftr1 as a paradigm. By pulse-chase analysis, we observe three distinct fates for the iron transporter (Figure 1 and diagrammed in Figure 8). In iron-starved cells, Fet3-Ftr1 is sorted efficiently by Snx3 and retromer into tubular domains of endosomes from where it is exported. Under these conditions the iron transporter is unusually stable ($t_{1/2} > 90$ min), and we predict that other similarly long-lived plasma membrane proteins, such as the Zrt1p zinc transporter ($t_{1/2} \sim 180$ min in zinc starved cells; Gitan *et al.*, 2003), are likely to be cargos of endocytic recycling pathways. At the other end of the spectrum, the stability of the iron transporter is reduced sixfold ($t_{1/2} \sim 22$ min) in the presence of iron because it is sorted efficiently into the MVB pathway (Figure 8, "stimulated MVB sorting"). A third fate is an intermediate rate of iron transporter turnover in recycling-deficient cells ($t_{1/2} \sim 45$ min), which is intriguing because it matches the turnover rates of proteins such as Gal2p and Ste2p in the absence of their substrate/ligand (Horak and Wolf, 1997; Hicke *et al.*, 1998). Under these conditions, Fet3-Ftr1, Gal2p, and Ste2p are not recycled, and their similar slow turnover rates suggest that they are sorted into the MVB pathway less efficiently, in a manner that we term "basal MVB sorting" (Figure 8). Basal ubiquitylation of endosomal cargo proteins could serve to prevent their accumulation within endosomes by maintaining flux into the MVB pathway. Although the rates of turnover via the "stimulated" and "basal" MVB-sorting reactions are quite distinct, both require Rsp5 and ESCRT proteins, indicating that the reactions are intrinsically the same. According to our hypothesis, the Snx3-retromer recycling machinery rescues Fet3-Ftr1 from "basal" MVB sorting, and in future experiments it will be important to determine if the recycling machinery directly antagonizes basal ubiquitylation, promotes de-ubiquitylation of cargo, or if it simply exports particular cargos from the endosome before they are ubiquitylated.

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