

The Furin Cytoplasmic Domain Is Localized to the *trans*-Golgi Network of Yeast

Alison J. Farrell

Biological Sciences, Central Washington University, 400 E. University Way

Ellensburg, WA 98926, USA

Tel: 425-820-2685 E-mail: alison101@comcast.net

Katherine A. Keltner

Biological Sciences, Central Washington University, 400 E. University Way

Ellensburg, WA 98926, USA

Tel: 509-373-0409 E-mail: Katherine_A_Keltner@rl.gov

Kathryn L. Bruce

Biological & Environmental Sciences, CBX081, Georgia College & State University

Milledgeville, Georgia, 31061, USA

Tel: 706-474-1200 E-mail: klynbruce@gatech.edu

Jessica L. Snell

Biological & Environmental Sciences, CBX081, Georgia College & State University

Milledgeville, Georgia, 31061, USA

Tel: 770-365-2018 E-mail: jessica.l.snell@gmail.com

Mark F. Law

Biological & Environmental Sciences, CBX081, Georgia College & State University

Milledgeville, Georgia, 31061, USA

E-mail: markf_law@yahoo.com

Matthew C. Furgerson

Biological & Environmental Sciences, CBX081, Georgia College & State University

Milledgeville, Georgia, 31061, USA

E-mail: mfurgerson@bmb.uga.edu

David B. Nix

Biological & Environmental Sciences, CBX081, Georgia College & State University

Milledgeville, Georgia, 31061, USA

Tel: 404-395-2349 E-mail: dnix@bmb.uga.edu

Michael L. Gleason (Corresponding author)

Biological & Environmental Sciences, CBX081, Georgia College & State University

Milledgeville, Georgia, 31061, USA

Tel: 478-445-0813 E-mail: mike.gleason@gcsu.edu

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Abstract

Kex2p and furin are membrane-bound proteases localized to the *trans*-Golgi network (TGN) of yeast and animal cells. While their N-terminal luminal and catalytic domains are highly homologous, they do not share common sorting signals in their C-terminal cytoplasmic tails. To study furin's localization in yeast we created KfP chimeras with the N-terminal regions of Kex2p and the C-terminal regions of human furin. Our mating, biochemical, and immunofluorescence studies of the chimeras in yeast have demonstrated sorting consistent with furin's trafficking patterns in human cells. Next, to see if furin sorting signals are recognized by yeast sorting machinery, key signals were mutated. For two mutagenized chimeras (KF5p/6p), mating activity was abolished, indicating that the enzyme no longer resides in the yeast TGN. This is surprising since the furin sorting signal that was altered normally binds a sorting protein (PACS-1) not known to exist in yeast.

Keywords: Furin, Kex2p, Protease, PACS-1, Trans-Golgi network, Endosome, Sorting, Localization

1. Introduction

1.1 Kex2p and furin are homologous proteases localized to the trans-Golgi network

Conserved cellular machinery is a common feature of eukaryotes. An interesting example of this is found for two proteases, furin, common to animal cells, and Kex2p, found in *Saccharomyces cerevisiae* and other yeast. Besides sharing significant structural homology, these two serine proteases are also strikingly similar in catalytic specificity and cellular function. Both are type I membrane-bound proteins, principally localized to the *trans*-Golgi network (TGN), that proteolytically process and activate other proproteins passing through the secretory pathway (Fuller *et al.*, 1989b; Takahashi *et al.*, 1995; van den Ouweland *et al.*, 1990).

Kex2p was the first of the two proteases to be characterized genetically and biochemically (Fuller *et al.*, 1989a). Soon followed the discovery of a family of related membrane-bound subtilisin-like serine proteases that began with furin (Fuller *et al.*, 1989b) and then led to seven other animal paralogs (Rockwell *et al.*, 2002; Thomas, 2002). Kex2p and furin are both endoproteases that hydrolyze the carboxyl-side peptide bond of dibasic amino acid sequences. Kex2p cleaves pro-killer toxin, mating factor- α polypeptides 1 and 2, and Ke1p (Julius *et al.*, 1984; Sato *et al.*, 2009; Wickner & Leibowitz, 1976), while over 40 different animal proproteins and animal pathogen proproteins are thought to be cleaved and activated by furin (Thomas, 2002). Both proteases are extensively modified in the endoplasmic reticulum (ER) and Golgi by signal peptidase cleavage, glycosylation, and their own autohydrolysis of the N-terminal pro-region that delivers each as a proteolytically active enzyme to the TGN of their respective cells (Thomas, 2002). Structurally, the domains of the furin and Kex2p enzymes show a high-degree of conservation along their entire length, but significant sequence alignment and amino acid identity is only present in their N-terminal domains (Fig. 1). Conversely the final quarter of each protein (their C-terminal regions) show structural homology, but no significant sequence homology.

1.2 Maintenance of TGN proteins

A major question in membrane trafficking is how resident TGN proteins are maintained against the flow of Golgi contents passing beyond this compartment (Bonifacino & Glick, 2004; Bonifacino & Rojas, 2006). Early studies revealed that retrotransport from the endosomal compartments is the major means by which TGN proteins are maintained in this compartment, and although less understood, TGN retention plays a role as well. This was first proposed for Kex2p based largely on the observations that it is rapidly lost to the vacuole when its cytoplasmic tail is truncated and by pulse-chase experiments that revealed native Kex2p experiences increasing glycosylation with longer chase times (Fuller *et al.*, 1989b). It was later shown that MNN1-dependent mannosylation of N-linked core oligosaccharides on membrane proteins occurs with increased residency within the late Golgi of yeast (Ballou *et al.*, 1990). Other studies by Brickner & Fuller (1997) discovered retrieval and retention signals in the cytoplasmic tail of Kex2p. Laboratories studying the localization of furin, as reviewed by Thomas (2002), confirmed that rapid cycling of furin follows a similar pattern, and that it too contains sorting signals in its cytoplasmic tail. Thus, retrotransport to the TGN is thought to maintain these resident proteins at a high steady-state in the TGN, while their presence in distal compartments is, by comparison, more transitory (Bonifacino & Rojas, 2006). While descriptions of endosomal compartments are varied and complex, it is

generally held that endocytic vesicles fuse with early endosomes (EE) that then mature into late endosomes, also known as the pre-vacuolar compartment (PVC) in yeast. Endosomes exist in equilibrium with the cell surface and the TGN as vesicles transport membrane and contents between them, and by maturation of the late endosome into the lysosome in animal cells, or its analog in yeast, the vacuole.

1.3 Maintenance of Kex2p in the yeast TGN

Retention and retrieval of Kex2p and DPAP A, a related proprotein protease in yeast, involves two well-studied sequence signals within the cytoplasmic tails of these enzymes, the *trans*-Golgi localization signal-1 (TLS-1) and TLS-2 (Brickner & Fuller, 1997; Bryant & Stevens, 1997; Wilcox *et al.*, 1992). Mutational studies strongly implied that a tyrosine in TLS1 (Y⁷¹³XF) in Kex2p is critical for retrieval of the native Kex2p from an endosomal compartment, as yeast expressing fully active A⁷¹³-enzyme rapidly lose the protease to the vacuole (Wilcox *et al.*, 1992). Downstream of TLS1 is TLS2, which under *Soi1p* suppression of the Y⁷¹³→A mutation delays the exit of Kex2p from the TGN (Brickner & Fuller, 1997; Redding *et al.*, 1996a).

Many sorting proteins are involved with the exit of Kex2p from the yeast TGN. Studies of *chc1* clathrin heavy chain mutants and *chc1 sec1* mutants also blocked for secretion demonstrated that any Kex2p that is lost to the cell surface is rapidly internalized by a non-clathrin mediated endocytosis (Payne & Schekman, 1989; Redding *et al.*, 1996b). This and other work established that Kex2p's main route of exit from the TGN is to the PVC. This "direct" pathway is also taken by the well-studied yeast receptor Vps10p (Bowers & Stevens, 2005). However, defects in the phosphoinositide phosphatase, Inp53p, were found to correlate with a slow delivery of Kex2p and Ste13p to the PVC, which indicated that these proteins may also be arriving at the PVC by an "indirect" route from the TGN (Ha *et al.*, 2001), presumably via the early endosome (EE). This was confirmed when the Fuller lab determined that Kex2p partitions its forward transport from the TGN by both the direct and indirect pathways (Sipos *et al.*, 2004). Vesicular transport of Kex2p to the PVC is known to require clathrin, adaptor proteins Gga1p/2p, and the dynamin, Vps1p, with fusion of the Kex2p-containing vesicle at the PVC requiring the t-SNARE Pep12p (Abazeed & Fuller, 2008; Ha *et al.*, 2001). The proteins involved in the forward transport of Kex2p from the TGN to the EE, include clathrin, adaptor protein AP-1, and Inp53p (Sipos *et al.*, 2004; Abazeed & Fuller, 2008). Kex2p delivered by the "indirect" path from the EE will eventually reside in the PVC as *Soi3p* facilitates the acidification of the EE compartment leading to its maturation into a late endosome (Sipos *et al.*, 2004).

Retrotransport pathways from endosomes to the TGN often involve a retromer complex (Seaman, 2005). This process was initially discovered in studies that examined how the membrane-based receptor Vps10p delivers procarboxypeptidase Y (proCPY) to the yeast PVC and is then retrieved to the yeast TGN by a complex of retromer proteins (Seaman *et al.*, 1998). Since then, animal cell homologs, called sorting nexins (SNXs), have been shown to act similarly (Verges, 2007). Indeed, Kex2p is known to require an ortholog of the sorting nexin SNX3, known as Grd19p, for its retrieval from the PVC (Voos and Stevens, 1998). Likewise, Mvp1p, an ortholog of human sorting nexin SNX8, has been implicated in a retromer-based Kex2p retrieval from the PVC (Ekena & Stevens, 1995). Little is directly known about the retrieval of Kex2p from the EE membrane, except that it does require Tlg1p, Tlg2p and Vti1p to form a t-SNARE complex known to mediate fusion of endosomal vesicles with the yeast TGN (Bonifacino & Rojas, 2006).

1.4 Maintenance of furin in the animal cell TGN

The cellular localization of furin in human and animal cells is decidedly within the TGN, and nearly all of its suspected proproteins are activated in this compartment (Molloy *et al.*, 1999; Thomas, 2002). However, a small fraction of furin transits through the cell surface and endosomal compartments where some opportunistic animal pathogen proproteins are activated by proteolysis (Molloy *et al.*, 1994; Bosshart *et al.*, 1994). Furin's transport to the cell surface may be explained by many observations. It has been noted that clathrin and adaptor proteins AP-1 or AP-4 may participate in a small fraction of it being constitutively transported from the TGN to the cell surface (Schäfer *et al.*, 1995; Teuchert *et al.*, 1999a; Rohn *et al.*, 2000). It has also been speculated that, in the absence of sorting signals, single-pass proteins with a somewhat longer TMD, like furin's compared to that of Kex2p, are more likely to be lost from the Golgi and then retained at the cell surface within lipid rafts (Bosshart *et al.*, 1994; Lingwood & Simons, 2010; Rayner & Pelham, 1997; Schäfer *et al.*, 1995). Another proposed pathway to the cell surface involves furin's inclusion in regulated secretory granules (Dittié *et al.*, 1997). The strongest evidence supports a path for furin to the cell surface via an endosomal compartment (Thomas, 2002) or compartments (Molloy *et al.*, 1999) that may parallel Kex2p's presumed pathway for arrival at the cell surface in mutant yeast (Payne & Schekman, 1989). For example, AP-1 is thought to bind to the furin sorting signal Y⁷⁵⁹KGL (Fig. 2, amino acids in green) to facilitate vesicular budding of furin from the TGN to an endosomal or

secretory compartment (Crump *et al.*, 2001). While most the furin is retrotransported to the TGN, a small portion of it reaches the cell surface via a sorting signal-directed EE to cell surface step (Thomas, 2002).

Regardless of how furin gets to the animal cell surface, it is ultimately returned to an endosomal pool of the protease by the sorting signal motif, Y⁷⁵⁹KGL, which interacts with AP-2 to enable clathrin-mediated endocytosis (Teuchert *et al.*, 1999b). Thomas (2002) has proposed a shuttling mechanism in which furin is directly transported to an endosomal compartment, that is then followed by clathrin and AP1 (or AP4) retrotransport to the TGN. Key to furin's retrotransport is a phosphorylated acidic cluster (AC) in its cytoplasmic tail (Fig. 2, amino acids in red), that mutational studies have defined and further studies have indicated must be bound by the phosphofurin acidic cluster sorting protein-1 (PACS-1) during its retrotransport (Schäfer, *et al.*, 1995; Takahashi, *et al.*, 1995; Voorhees *et al.*, 1995; Wan *et al.*, 1998). AC is a cluster of acidic amino acids and two closely-spaced serines that are phosphorylated by casein kinase II. Additionally, adaptor proteins and clathrin are thought to interact with furin through a PACS-1 intermediate.

1.5 Study of furin sorting signals in yeast

In this paper, we report our investigations that ask if C-terminal furin sorting signals in yeast are sufficient to localize Kex2p to the TGN? To study this, we chimerically-fused the N-terminal pre-pro-enzymatic and regulatory domains of the Kex2p coding regions of the yeast *KEX2* gene (K) to the C-terminal regions of furin encoded by the human *fur* cDNA (F) to create chimeric constructs KFJp and KF2p (see Figs. 1 and 2). Intriguingly, we initially observed that the chimeric enzyme (KFp) is well-retained in the yeast TGN by several measures, and our findings support a trafficking pattern in yeast that is typical of furin in animal cells. This led us to wonder whether any of the human furin sorting signals are being recognized by conserved sorting machinery native to the yeast cell, and to our surprise we report mating results for site-specific mutations to the AC that are consistent with a hypothetical PACS-1-like retrieval of the chimeric enzyme from yeast endosomes to the TGN.

2. Experimental Procedures

All standard reagents and culture media used in these experiments were purchased from Sigma-Aldrich, Fisher Scientific, BIO-RAD, or VWR Scientific.

2.1 Bacterial and Yeast Strains and their Growth

Standard bacterial *E. coli* strains used for molecular cloning were derivatives of DH5 α or strains purchased as commercially competent cells such as XL1-Blue or XL10-Gold from Stratagene/Agilent Technologies. Growth of *E. coli* and preparation of competent cells were as described (Sambrook *et al.*, 2000).

The two principle yeast strains used in these studies were BFY106-4D (Pep4⁺) and CB017 (Pep4⁻). These strains were derived by the Fuller lab from a parental W303-1A yeast strain (R. Rothstein, Columbia University, NY) as described by (Wilcox *et al.*, 1992). Both strains are *MATa* and contain a deletion of the gene for Kex2p (*kex2Δ*). The full genotype for each of the two strains is as follows:

BFY106-4D (Pep4⁺): *MATa kex2Δ::HIS3 can1-100 ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1*

CB017 (Pep4⁻): *MATa kex2Δ::TRP1 pep4::HIS3 prb1::hisG prc2::hisG can1-100 ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1*

As indicated above, BFY106-4D is phenotypically wild-type for vacuolar proteases (Pep4⁺), while CB017 is significantly impaired for proteolytic turnover for proteins (Pep4⁻) delivered to its vacuoles due to the deletion of three key vacuolar protease genes: *PEP4*, *PRB1*, and *PRB2*. Two strains used for the mating test were DC14 *MATa his1* and DC17 *MATa his1* which originated in the J. Thorner lab (U.C. Berkeley). Standard growth and maintenance of strains was on rich media (e.g., YPD) and synthetic drop-out media (e.g., SDC-ura) as described by (Sherman, 2002). The addition of 2% D-galactose was used to induce transcription driven by a *GALI* promoter. For some experiments yeast strains were grown by in liquid broths by shaking at 30°C until cell density reached mid-log or late-log phase (OD₆₀₀ 0.5 or 1.0).

2.2 Plasmids, Cloning and Mutagenesis

The original chimeric construct, *KFJ* (Fig. 2) was constructed from genomic *KEX2* DNA and human *fur* cDNA by J. Burchard in the Fuller lab, and was then placed into pBM258 plasmid to create pBM258-*KFJ* used in some of our studies. pBM258 is a standard YCp50 plasmid containing the *GALI10,1* promoter region (Davis, 1989; Johnston & Davis, 1984). Using pBM258-*KX22* described by (Fuller *et al.*, 1989a) and human *fur* cDNA (Open Biosystems), we constructed a new chimera with eleven codons removed from the 5'-end of the C-terminal coding region, we called *KF2* (Fig. 2). Enzymes required for cloning were obtained from New England Biolabs.

We then placed *KF2* under the control of the *GAL1* promoter in pBM258, creating pBM258-*KF2*. From pBM258-*KF2*, we created site-specific mutants in the cytoplasmic tail of furin called *KF3*, *KF5*, and *KF6* (Fig. 2). Mutagenesis was done using a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene/Agilent Technologies). The plasmids pBM258-*KX22* and pCW-*KX20* (Wilcox *et al.*, 1992) are nearly equivalent constructs used in our experiments that both express the positive control, native Kex2p, via a *GAL1* promoter. The plasmid pBM258 is as described, and since it contains no structural gene for *KEX2*, it was also used as a negative control vector for some studies.

2.3 Kex2p specific Proteolytic Assay of Intact Cells

Non-saturating proteolysis reactions were conducted by a one-hour assay at 30°C on Cbz-YKR-pNA (Bachem) at 405 nm as described (Achstetter *et al.*, 1984). Additional Kex2p-specific assay conditions were required (Fuller *et al.*, 1989a) and necessitated the use of the following protease inhibitors to eliminate the action of competing proteases: 1-μM E-64, 25-μM Pepstatin A, 100-μM TPCK, 500-μM PMSF, and 100-μM TLCK. Kex2p-specific activity of intact mid-log cells was determined in 50-mM Tris-HCl, pH 7.5 buffer containing protease inhibitors as above and as described (Wilcox *et al.*, 1992). Lysis of intact yeast was accomplished by a 3X freeze/thaw lysis with liquid nitrogen frozen cells thawed on ice in the above buffer which included 1% Na-deoxycholate and 1% Triton-X-100.

2.4 Immunofluorescence Microscopy

Mid-log yeast Pep4^+ or Pep4^- cells expressing: no enzyme (pBM258), Kex2p (pCW-*KX20*), or KFJp (pBM258-*KFJ*) were prepared for microscopy by fixation in buffered 1% formaldehyde, followed by spheroplasting with Zymolyase and then applied to poly-lysine-coated slides as described (Wilcox *et al.*, 1992). Acetone-permeabilized cells were then pre-treated with pre-immune rabbit serum and then labeled with α -ssKex2p rabbit polyclonal antibody sandwiched to goat- α -rabbit IgG and rabbit- α -goat-FITC. The antigen used to raise the immune serum, ssKex2p, is a secreted soluble version of Kex2p missing the spacer-TMD-tail region C-terminal domains (Brenner & Fuller, 1992), hence it binds to the enzyme moiety of either Kex2p or chimera. The cellular location of Kex2p or chimera was then determined by indirect immunofluorescence on a Leica epifluorescence microscope also capable of differential interference contrast (DIC) microscopy.

From photos of prepared slides, two investigators individually counted the occurrences in which cells exhibited strong fluorescence in punctate structures (Golgi) and halos (cell surface) and, in rare instances, when fluorescence coincided with a vacuole visible by DIC. The presence of multiple labeled structures in a cell (e.g., several punctate structures) was counted as a single occurrence within the cell. The counts of the two investigators were averaged. Cells that exhibited fluorescent labeling of the cell surface (halos) were nearly always (97% of the time) accompanied by punctate fluorescent staining typical of the Golgi. Thus to simplify their scoring, all cells showing surface fluorescence were counted simply as “+ cell surface” in our data.

2.5 Mating Assays

Matings of *MAT α* cells with testing strains DC14 and DC17 were as described (Redding *et al.*, 1996; Sprague, 1991; Wilcox *et al.*, 1992). Briefly, the expression of Kex2p in the TGN is required for α -mating factor production. *MAT α* cells that have mated with *MAT α* DC14 cells will grow as prototrophic diploids on minimal media (SD or SGal), whereas the auxotrophic haploid cells will not. As expected, a consistent lack of mating with DC17 (a *MAT α* strain isogenic to DC14) was regularly demonstrated. All cells grow on YPD, so the fraction of *MAT α* cells capable of mating can be determined in qualitative patch assays or quantitatively. In quantitative tests *MAT α* cells and DC14 cells were grown in broth to mid to late-log and then adjusted in concentration so that 1.0×10^6 cells/ml of *MAT α* tester cells were mated to 1.0×10^7 cells/ml of DC14. For mating, cells were either serially diluted in titer plates and then replicated onto minimal or rich media using a pin-replicating tool or mated in microfuge tubes and then spread uniformly over the surface of a plate. All plates were grown for 2-3 days at 30°C before the data were recorded.

3. Results

3.1 Preliminary biochemical analysis indicates that the KFp chimera localizes to the TGN and cell-surface

The steady-state expression of Kex2p and the KFJp chimera were originally examined by J. Burchard in the Fuller lab (M.S. Thesis, Stanford University, 1992). Importantly, her original experiments determined that both proteins were expressed to the same extent using a western blot analysis. Moreover, when the yeast cells were crudely fractionated, and the membrane fractions expressing KFJp and Kex2p compared, it was observed that the bead-beaten lysates expressing the KFJp chimera had the protein distributed between the ultrahigh-speed microsomal 100S fraction (i.e., Golgi) and a low-speed 10S fraction. By comparison essentially all of the Kex2p in control cells was found in the high-speed microsomal fraction indicative of Kex2p's predominant TGN

localization (Fuller *et al.*, 1989b). The dense cell fraction (10S) obtained by this type of method is consistent with membrane-cell wall fragments (Sipos & Fuller, 2002).

3.2 Quantitation of the physical partitioning of the KFp chimera by assay of its enzymatic domain

In the corresponding author's laboratory we first followed-up on these initial observations by three means. By the direct enzymatic detection of Kex2p activity, we first examined yeast cell membranes that were gently lysed and fractionated on density gradients, and secondly, directly measured the amount of surface Kex2p activity present relative to total Kex2p activity. The localization of the Kex2p enzymatic domain was assessed indirectly by immunofluorescence microscopy as the third means.

The Kex2p-specific proteolytic activity of membrane fractions from gently lysed yeast cells were separated on Percoll density gradients (Fig. 3A). The relatively larger peak of Kex2p proteolytic activity in the denser fractions for the cells expressing KFJp in comparison to Kex2p confirms the earlier findings obtained by bead-beaten lysis. The assay is quite specific, as most of the proteolytic activity measured in this Kex2p assay is due solely to the activity of Kex2p enzymatic domain (Wilcox *et al.*, 1992), with only a small fraction of the proteolysis (1-2%) being due to competing proteases.

Second, the amount of Kex2p-specific proteolytic activity present on the cell surface of living yeast cells expressing KFJp or Kex2p was compared. The technique used was previously employed by the Fuller lab (Wilcox *et al.*, 1992 and Fuller *et al.*, 1989b). The results of intact Pep4⁺ cell studies (Fig. 3B) confirm that a substantial fraction of KFJp chimera was found on the cell surface and that the amount of enzyme detected was significantly larger than assayed for the Kex2p control ($p < 0.04$).

A third technique provided a visual localization of the enzyme by indirect immunofluorescence using a polyclonal antibody against the Kex2p enzymatic domain common to both Kex2p and KFJp. It is worth noting that these studies are well-controlled by the fact that Kex2p was established as one of the earliest markers for the yeast TGN and that it is still among the most widely-used biochemical markers for this compartment in yeast (Bonifacino & Rojas, 2006; Fuller *et al.*, 1989a). Data shown in Fig. 4 demonstrate that the Kex2p enzymatic domain antigen lies in the peripherally distributed Golgi of yeast. This characteristic type of yeast Golgi staining is clearly visible as punctate staining near the plasma membrane as seen for both Pep4⁺ cells expressing the Kex2p control (Fig. 4C) and KFJp chimera (Fig. 4E). Companion DIC photos (Figs. 4D and 4F, respectively) show the location of vacuoles in fluorescing cells that by comparison are devoid of fluorescence. Notably, as seen in Fig. 4E, the KFJp chimera is also localized to the plasma membrane. This is revealed by the dim fluorescent halos encircling the yeast's cell membrane. Scoring the occurrence of these two features provided statistically valid data ($p < 0.001$) as we observed that 39% of the cells expressing the KFJp chimera had a cell-surface distribution in Pep4⁺ cells compared to only 3% in the Kex2p controls (Table 1). In Pep4⁻ yeast, where vacuolar proteolytic turnover of proteins is known to be impaired (Wilcox *et al.*, 1992), our studies demonstrate approximately 60% of the fluorescently labeled cells expressing KFJp had a cell-surface distribution. By comparing these cell surface distributions of the chimera in Pep4⁺ and Pep4⁻ yeast, we deduced that KFJp can enter endosomes from the cell surface leading to its turnover in the vacuole. Importantly, the data also support that the KFJp chimera resides in the yeast Golgi as nearly all the fluorescing cells had evidence of TGN localization.

3.3 Mating activity assays localize the enzymatic domain to the TGN

Our studies have also utilized a unique measure for Kex2p's TGN localization that takes advantage of the enzyme's principal value to the *MATa* yeast, namely the proteolytic maturation of α -mating factor (α F) from its proprotein substrates. In various versions of this assay, the change in the mating activity of *MATa* yeast can be attributed to the loss of a critical component, such as Kex2p enzyme from the yeast's TGN (Sprague, 1991; Wilcox *et al.*, 1992). To underscore this point, strains of *MATa* yeast that have null mutations in the *KEX2* gene or that have been deleted (*kex2Δ*) can no longer mate (Leibowitz & Wickner, 1976). Other seminal studies defined the processing proteases involved as being Kex2p, DPAP A, and another TGN resident proprotein protease, Kex1p (Bryant & Stevens, 1997; Chen *et al.*, 1997; Cooper & Bussey, 1992; Fuller *et al.*, 1988). All three must be co-localized to the TGN so that mature and biologically active 13-amino acid α F is properly processed from the much larger pro-mating-factor- α polypeptides that pass through this compartment. Properly processed biogenically active α F peptides are then secreted to act as pheromones that attract *MATa* yeast for mating. In our studies we employed various mating assays to follow the localization and maintenance of Kex2p and the KFp chimeras in the yeast TGN. Qualitative analysis of mating by velveteen patch transfer clearly reveals in Fig. 5A that furin sorting signals are sufficient to localize the Kex2p enzymatic domain of the chimera to the TGN so that mating with a *MATa* strain (DC14) can occur. Quantitative mating results reveal that cells with normal vacuolar turnover (Pep4⁺) cells (Fig. 5B and Table 2) expressing the chimera have a slightly lower

degree of mating compared to those expressing Kex2p, suggestive of a loss of the furin-targeted enzyme from the TGN to the cell surface. Interestingly, Pep4⁻ cells deficient in the vacuolar turnover of three key proteases (*pep4Δ prc1Δ prb1Δ*) have higher levels of mating in both the Kex2p and chimera expressing cells (Table 2). This reflects the role of the vacuole in turnover and is consistent with results from the Fuller lab demonstrating increased levels of mating when Kex2p is expressed in Pep4⁻ cells. Even more intriguing is the observation from Table 2 that Pep4⁻ cells expressing KFp have higher levels of mating than Pep4⁻ cells expressing Kex2p. As Kex2p traffics by default to the vacuole where it is proteolytically turned-over in Pep4⁻ cells or tends to accumulate in Pep4⁻ cells (Wilcox *et al.*, 1992), it appears that in Pep4⁻ cells KF2p partitioned to a large cell-surface pool can be readily retrieved to endosomes by endocytosis and then by retrotransport to the yeast TGN. Thus a cell surface to endosome to TGN sorting pathway for furin, known to occur in animal cells (Thomas, 2002), appears to be paralleled by a yeast sorting pathway that recognizes furin sorting signals. Although, this is likely to involve a sorting process that does not involve AP-2 as this clathrin adaptin is not known to exist in yeast (Teuchert *et al.*, 1999b).

3.4 Mutagenesis of furin sorting signals disrupts retrotransport of KFp chimeras to the yeast TGN

Previous studies demonstrated that the protein PACS-1 interacts with the AC of furin's cytoplasmic tail and that when the acidic residues or phosphorylatable serines are mutated it eliminates the ability of furin to be retrieved to the TGN (Takahashi *et al.*, 1995; Voorhees *et al.*, 1995; Wan *et al.*, 1998). In the chimeras KF5p and KF6p we have recreated those mutations by substituting the acidic residue sequence E⁷⁷⁵ED with three alanines and by deleting the two phosphorylatable serines and the intervening aspartic acid at S⁷⁷³DS, respectively (Fig. 2). As shown by our qualitative in Fig 5A, semiquantitative mating data in Fig. 5B and quantitative data in Table 2, little detectable mating activity could be elicited by either chimera with AC mutations (KF5p and KF6p). This data is consistent with the loss of the Kex2p enzyme from the TGN to forward compartments (endosomes and then the cell surface or vacuole) from which it can no longer be retrieved by a PACS-1-like mechanism.

A signal defined by the tyrosine in the YKGL motif of the furin cytoplasmic tail was previously shown to interact with clathrin adaptor protein AP-1 in human cells and to be necessary for its exit from the TGN to endosomes (Schäfer *et al.*, 1995; Teuchert *et al.*, 1999a, 1999b). To investigate whether this signal was being recognized in yeast we mutated the KF2 chimera (Y⁷⁵⁹→A). Contrary to our expectations we observed that mating efficiency was slightly decreased in Pep4⁻ cells, indicating the exit of the Y⁷⁵⁹→A chimera (KF3p) from the TGN (Table 2). Though it is an interesting observation, it is difficult to explain based on the parallel experiment in human cells where vesicular transport was impaired. Alternatively it is interesting to speculate that the YKGL signal may act more like the poorly defined TLS2 of Kex2p or DPAP A, because loss of such a signal would be consistent with the mating data for KF3p (Brickner & Fuller, 1997; Bryant & Stevens, 1997; Redding *et al.*, 1996b).

3.5 KFp Chimeras are poorly maintained in the yeast TGN in the absence of steady state expression

The Fuller lab has used a sensitive version of the mating assay, called "onset of impotence" that measures the length of time needed for Kex2p to escape the TGN-endosome retrieval loop and to then be turned-over in the vacuole once its expression by a *GAL1* promoter is repressed (Brickner & Fuller, 1997; Redding *et al.*, 1996; Wilcox *et al.*, 1992). As the steady-state of the enzyme diminishes, the amount in the TGN decreases and so does mating efficiency. In these studies, the kinetics for the onset of impotence were carefully measured for native Kex2p and Kex2p Y⁷¹³→A, the latter of which has a disrupted TLS1 signal. Our Kex2p control studies in Pep4⁻ cells parallel those they previously obtained for onset of impotence. Moreover, we observed that the chimeras with intact AC motifs, KFJp and KF2p, have kinetics for the onset of impotence (Fig. 6C), that parallel those reported for the Kex2p Y⁷¹³→A mutant (Wilcox *et al.*, 1992). For Kex2p this mutation essentially eliminates the TLS1 signal needed for its retrieval from the PVC to the TGN, but leaves intact another, TLS2, that helps retain Kex2p in the TGN (Brickner & Fuller, 1997; Redding *et al.*, 1996; Wilcox *et al.*, 1992). Does this mean then that the chimera only experiences a single, transient passage through the compartment? Several lines of evidence argue against this interpretation, such as the findings concerned with the intracellular distribution of the chimera, but most notably because we have observed the near complete loss of mating when the AC of the furin portion of the chimera is mutated. Thus, we surmise that the intact AC motif in the furin portion of the KFp chimera seems to be weakly involved in its retrotransport.

4. Discussion

While much is known about the trafficking of furin in animal cells and conversely the trafficking of Kex2p in yeast cells, until this study, little was known about how the sorting signals of the human furin protease might be recognized by the yeast's sorting machinery. Since it is the yeast's sorting machinery that seems to be recognizing part of the human protein's sorting signals, it is worth viewing furin's sorting within the context of

Kex2p's well-known trafficking patterns. To this end, a model for the trafficking pathways of the KFP chimera in yeast has been proposed and is compared to those for Kex2p (Fig. 6).

4.1 How does the KFP chimera traffic to the cell surface of yeast?

A large fraction of the KFP chimera is demonstrated to be on the cell surface of yeast by our fractionation, intact cell, and immunofluorescence studies. This is somewhat surprising in that the vacuole is often viewed as the default pathway for membrane proteins in yeast (Roberts *et al.*, 1992). The only apparent exit signal within the chimera for its transport to the cell surface of the yeast is the relatively long TMD of furin. If true, then its delivery might be by bulk-flow, perhaps, within lipid rafts being directed to the cell surface (Wieland *et al.*, 1987; Raynor and Pelham, 1997; Lingwood, 2010). Alternatively it maybe a signaled process (Wang *et al.*, 2006) involving an exomer-like coat (Fig 6B, path a1). At present it is not even possible to ascertain if the KFP chimera is accumulating at the cell surface by passing to it through an early endosomal compartment (Fig 6B, path a2) as the work of many furin investigators might suggest (Thomas, 2002). Moreover, the extent to which inefficient endocytosis may account for the large fraction of chimera at the cell surface is unknown as well.

4.2 How does the KFP chimera undergo retrotransport to the yeast TGN?

Although there is no apparent similarity in the cytoplasmic sorting signals for Kex2p and furin, there are many conserved structural and functional features that we have cited, including the high degree of sequence conservation in their N-terminal domains, shared enzymatic specificity, overall structural homology, and localization to the TGN. While an acidic cluster is not readily apparent in Kex2p, it is present in furin and many other retrotransported proteins (Bonifacino & Rojas, 2006). The discovery of an acidic cluster in a variety of animal proteins prompted studies to find a PACS-1-like molecule in yeast. In part these studies were undertaken because of the striking similarity of the yeast receptor Vps10p to the cation-independent mannose-6-phosphate receptor (CI-M6PR), which also contains an acidic cluster. Interestingly, in animal cells CI-M6PR is known to be retrieved from endosomes via redundant retrotransport pathways to the TGN. These redundant retrotransport pathways include the canonical retromer-based mechanism and two others involving Rab9/TIP47 and PACS-1 (Bonifacino & Rojas, 2006; Krise *et al.*, 2000; Seaman, 2005; Thomas, 2002). Another example is the retromer-based PVC to TGN retrieval of Kex2p that requires the sorting nexin Grd19p (Voos & Stevens, 1998). Hettema *et al.* (2003) found that Snx4p, Snx41p and Snx42p will act as a complex required for the retrieval of the v-SNARE Snc1p from EE to the TGN. However, while Snx4p/41p/42p all have homology to animal sorting nexins, the retrieval of Snc1p by these proteins is poorly understood and apparently a non-retromer-based mechanism. Might one or more of these mechanisms be involved in the retrotransport of KFP? Given the high degree of conserved cellular machinery that typifies diverse eukaryotes, it was somewhat surprising that no structural homologs to PACS-1 have been found in yeast. Might there be a functional PACS-1 homolog that so poorly conserves the animal cell protein's structural features that it cannot be recognized in homology searches? Our data suggests a PACS-1-like molecule might exist in yeast, and so too, a hypothetical retrotransport mechanism.

5. Conclusion

By far, the most compelling finding in our study was the near complete loss of mating when the furin AC site of the KFP chimera had key acidic residues altered or serine residues deleted. While no structural ortholog of PACS-1 has been identified within the yeast genome, our preliminary mating data support a hypothesis that a functional homolog, if not a structural homolog of PACS-1, may exist in yeast. An unbiased or selective screen may serve to identify other genes linked to this hypothetical molecule. One approach might be to use a suppression of the onset of impotence (*soi*) screen similar to that used by the Fuller laboratory (Redding *et al.*, 1996) to discover if there is a yeast sorting protein interacting with the AC motif in the KFP chimera. Another approach would involve studies of the KFP chimera in strains mutant for sorting proteins known to function in post-Golgi vesicular traffic, or to block the function of these sorting proteins, and thereby elucidate the pathways by which KFP traffics within yeast. These types of studies and others may prove key in determining anterograde (Fig. 6, a1 or a2) pathways and retrotransport pathways to the TGN via the EE or PVC (Fig. 6, r1 or r2, respectively).

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Table 1. Site-specific indirect immunofluorescence recorded for Pep4⁺ cells expressing Kex2p and KFJp*

	# Cells Observed	% Fluorescently Labeled Cells	Golgi Only	+ Cell Surface†	Vacuole
Kex2p	71.5	47	95.8%	2.8%	1.4%
KFJp	375.5	32	59.7%	39.4%	0.9%
No Enzyme	215	1	0.0%	0.0%	0.7%

* Two investigators examined the photographs as described in the Materials and Methods.

† 97% of all cells showing cell surface fluorescence also had punctate staining typical of Golgi localization.

Table 2. Quantitative mating trials*

Strain	Protease	Experiment Number											Mean±SEM
		1	2	3	4	5	6	7	8	9	10	11	
	Kex2p	6.5	11.1	1.3	10.6	17	4.1	3.2	3.3	1.6	-	-	6.5±1.8
	KF2p	4.1	2.1	0.9	12.7	8.4	7.5	-	4.1	3.8	-	-	5.4±1.4
Pep4 ⁺	KF3p	-	1.3	0.4	6.6	-	3.4	1.7	-	-	-	-	2.4±1.1
	KF5p	-	-	-	-	-	0.02	-	-	-	-	-	0.02
	KF6p	-	-	-	-	-	0.1	-	-	-	-	-	0.1
	Kex2p	-	-	-	-	-	-	-	7.5	4.6	10.6	10.3	8.2±1.4
	KF2p	-	-	-	-	-	8.9	-	6.9	8.2	14.6	7.4	9.2±1.4
Pep4 ⁻	KF3p	-	-	-	-	-	12.1	-	-	-	11.7	10.3	11.4±0.94
	KF5p	-	-	-	-	-	0.7	-	-	-	0	0	0.23±0.23
	KF6p	-	-	-	-	-	0.4	-	-	-	0	0	0.13±0.13

*Variation of method as previously described (Hartwell, 1980; Sprague, 1991). 200-μl each of late log MATα and DC14 cells were combined and allowed to settle in microfuge tubes for 5 hrs during which time mating could occur. After which tubes were briefly vortexed to suspend diploids and 100-μl of cells was spread onto plates, grown, and analyzed as described.

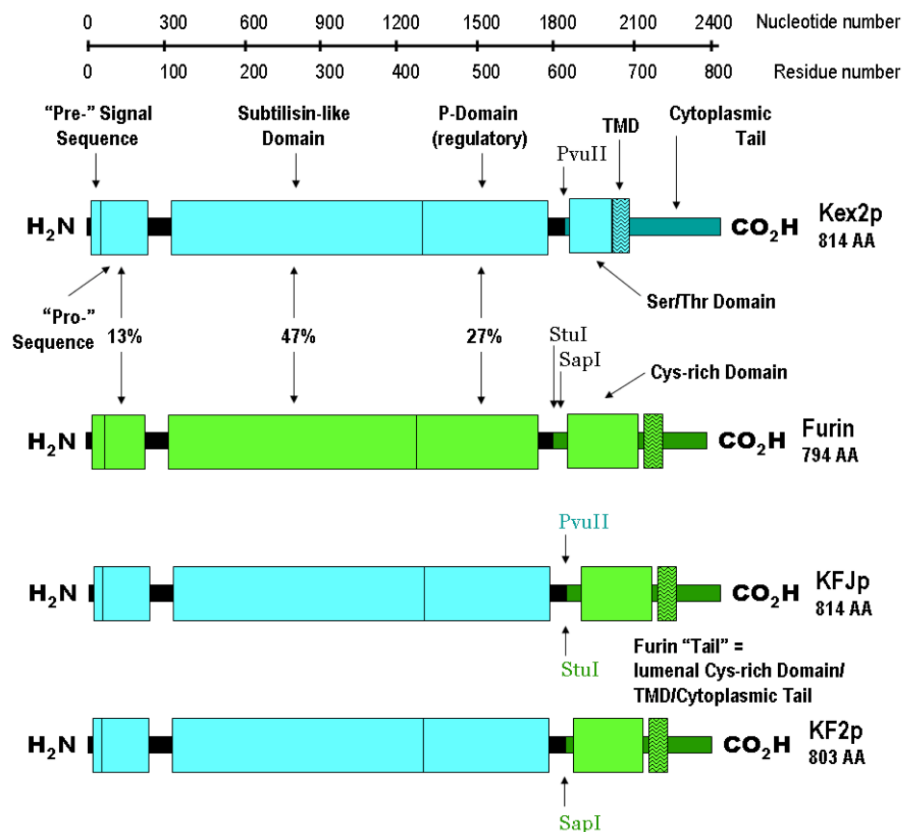


Figure 1. Schematic representations of Kex2p, furin and KFp chimeras. Glycosylation sites are not shown. Adapted from Fuller *et al.* (1991).

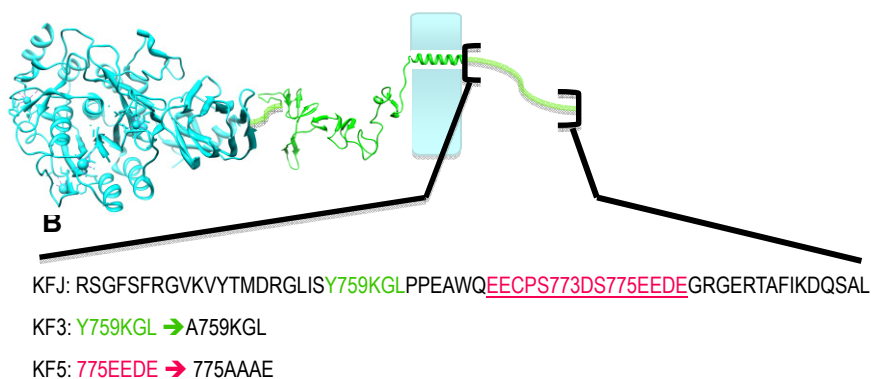
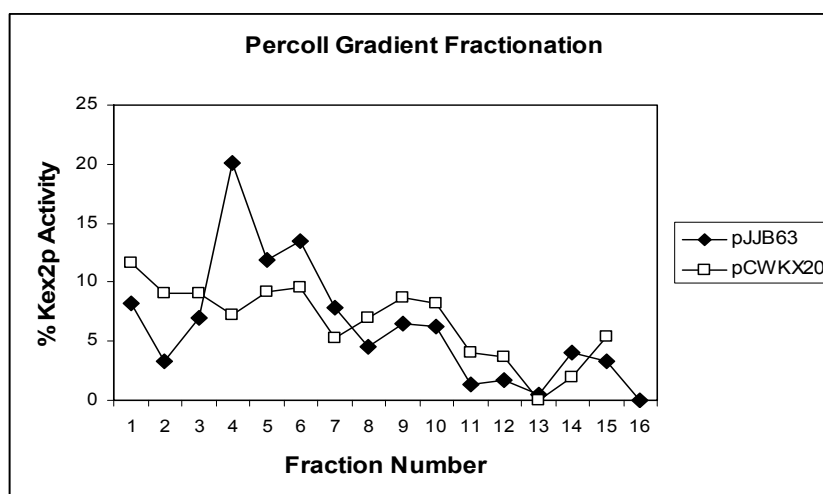


Figure 2. Representations of the KFp Chimera Structure. (A) KFJp and KF2p have an N-terminal Kex2p catalytic and P-domain (blue) fused in frame to furin's cysteine rich region, a single-pass transmembrane domain α -helix, and cytosolic tail of unknown tertiary structure (green). X-ray diffraction studies have solved the catalytic and P domain of Kex2p and it is shown in blue. Structural homology of the luminal Cys-rich spacer of furin with an epidermal growth factor receptor (EGFR) domain was used as a substitute for the Cys-rich domain for representation purposes. The combined image used RCSB files 1P8J, 1MOX, and 1R64 and was manipulated with the UCSF Chimera package. (B) The cytosolic tail sequence of furin (Van den Ouweland *et al.*, 1989) is shown with highlighted residues that correlate to targets of site-directed mutagenesis used in this study. KFJp and KF2p have identical TMDs and cytosolic tails. They differ only in a few amino acids splicing the Kex2p enzyme to the furin C-terminal domains.

A



B

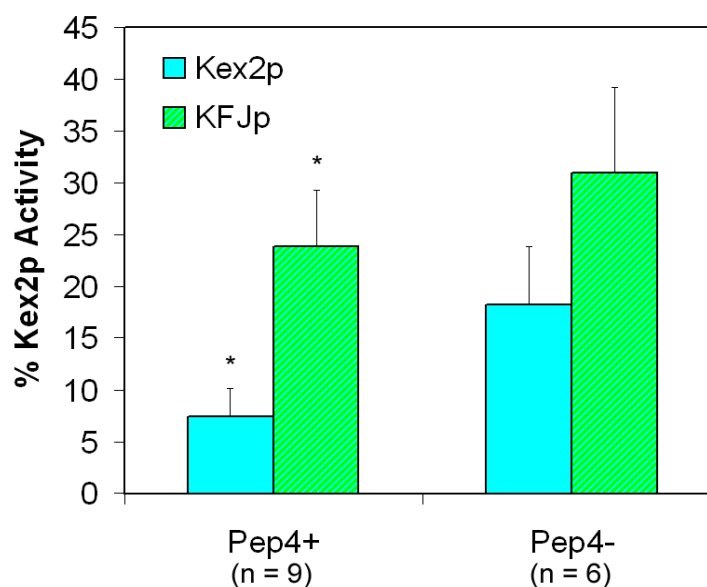


Figure 3. Kex2p activity present in subcellular fractions and on the surface of intact cells. (A) Sphero-plasted cells that were deficient in vacuolar proteases (Pep4⁻ cells) were dounced with a B pestle on ice, and loaded onto Percoll gradients in thin-wall polyallomer tubes and centrifuged for 3 hours at 21,000 rpm in a ss34 rotor using adaptors. Fractions were collected by a drip from the bottom of the centrifuge tube, thus the densest fraction was collected first. Gradient fractions were then tested for Kex2p-specific proteolytic activity as described. The relative activity of each fraction compared to the total activity observed across the gradient has been plotted. The legend indicates the plasmid used to express Kex2p and KFJp in the CB017 strain. pCWKX20 was the control plasmid for the expression of Kex2p and the plasmid designated *pJJB63* is an earlier designation for pBM258-*KFJp* used to express the chimera KFJp. (B) Intact cell assay of Kex2p protease activity was compared to total activity present in the lysate of an equal volume of intact cells. Early log phase cells were induced by the addition of 2% galactose and incubated for 4 hours at 30°C prior to assay. During the assay, intact yeast cells were incubated for 60 minutes with gentle resuspension every 5 minutes. Pep4⁺ cell surface activity for KFJp was significantly different than Kex2p activity ($p < 0.04$). Error bars represent standard error of mean.

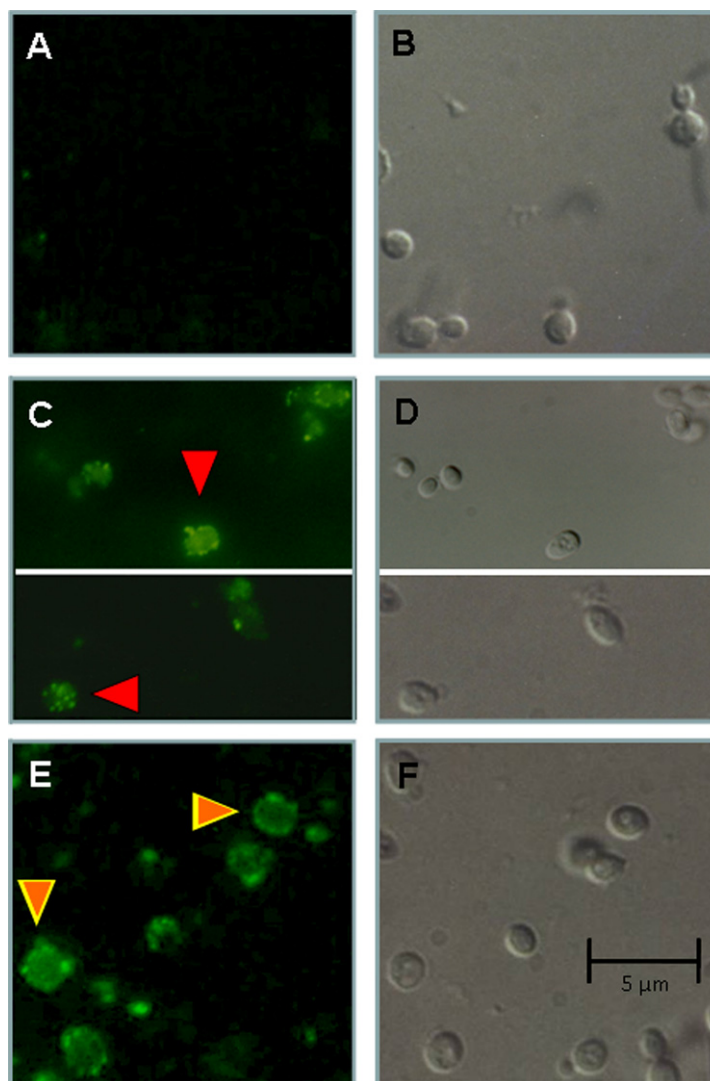


Figure 4. Immunofluorescence studies of the localization of the chimera by furin sorting signals. (A) The Pep4^+ *kex2Δ* cells were grown to mid-log in 2% galactose SC-ura broth and conveyed the plasmids pBM258, pCWKX20, or pBM-KFJp that expressed (A) no enzyme (C) Kex2p or (E) KFJp. All cells were processed in parallel as described earlier. Companion DIC images are to the right (B, D, and F, respectively). (C) Clearly visible is the punctate staining of Golgi typical for Kex2p (red marker) and other resident Golgi proteins (Franzusoff *et al.*, 1991). In (E) the faint halos of fluorescently labeled cell membrane along with some punctate labeling can be seen (orange markers). Bars in the DIC images indicate 5-μm.

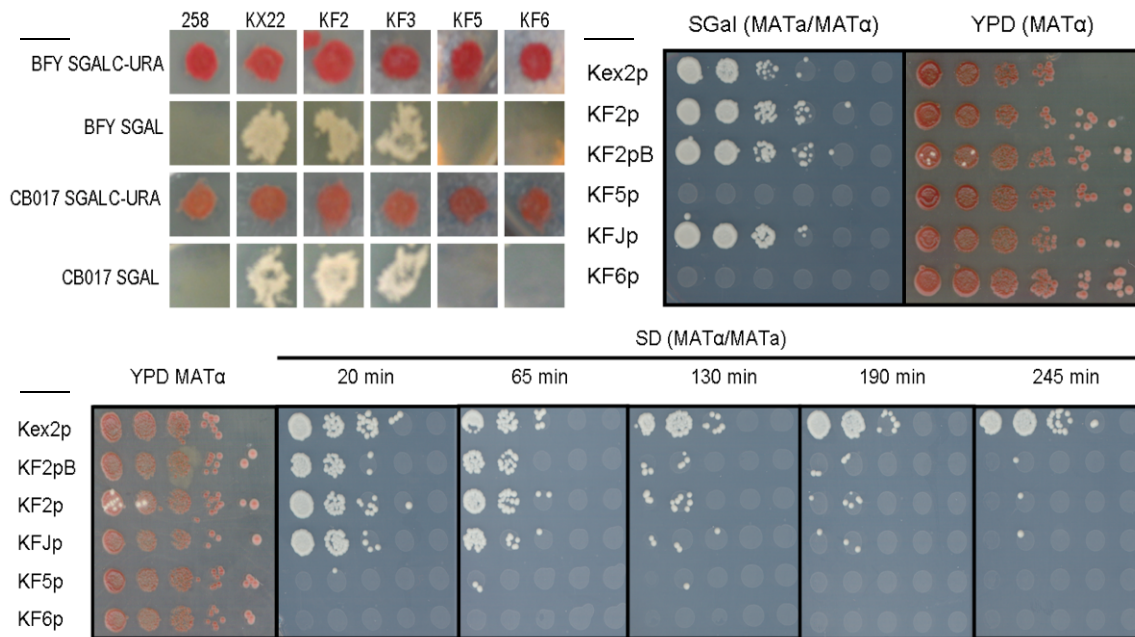


Figure 5. Qualitative and semi-quantitative comparisons of mating. (A) Initial patch mating of BFY106-4D (Pep4⁺) and CB017 (Pep4⁻) expressing Kex2p, KFJp or KF2p, or site-directed mutants of KF2p. (B) Plating of steady state Pep4⁺ cells with a replicating pin tool after 1:10 serial dilutions prior to mating (YPD) and after mating (SGal). (C) Onset of impotence in Pep4⁺ cells was determined by 1:10 dilution and incubation in SDC-ura broth for times shown, which was followed by plating as in B, except that SD plates were used instead of SGal. KF2pB and KF2p represent parallel cultures of BFY106-4D cells expressing pBM258-KF2.

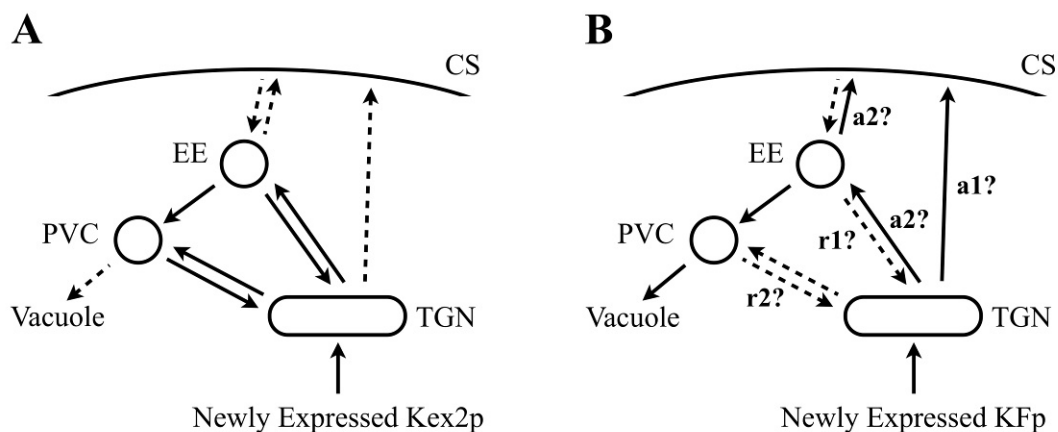


Figure 6. Model for the vesicular trafficking of KfP in yeast. Solid arrows indicate principle pathways of transport. (A) Standard model described for the trafficking of Kex2p in yeast (Sipos *et al.*, 2004). Note that strong retrotransport from both early endosome (EE) and the pre-vacuolar compartment (PVC) is indicated. (B) Proposed trafficking for KfP. Anterograde transport to the cell surface may occur through a TGN to cell surface (CS) pathway (a1) or a pathway through the EE (a2). Also indicated is the relatively weak retrotransport of KfP to TGN from either the EE (r1) or PVC (r2).