Journal of
Clinical and Experimental Pharmacology

Stein et al., Clin Exp Pharmacol 2014, 4:3
DOI: 10.4172/2161-1459.1000155

Review Article Open Access

The Non-classical Pathway is the Major Pathway to Secrete Proteins in *Saccharomyces cerevisiae*

Kathryn R Stein, Bennett J Giardina and Hui-Ling Chiang*

Department of Cellular and Molecular Physiology, Penn State University College of Medicine, 500 University Drive, Hershey, PA 17033, USA

Abstract

Protein secretion is a fundamental process in all living cells. Proteins that contain the ER signal are secreted by the classical pathway, whereas proteins without the ER sequence are secreted via the non-classical pathway. Recent evidence indicates that a large number of signal-less proteins including metabolic enzymes, transcriptional factors, translation factors, heat shock proteins, and anti-oxidant proteins are secreted by a variety of cells that range from bacteria to human. Furthermore, the secretion of gluconeogenic enzymes has been observed in bacteria, fungi, parasites, and mammalian cells. Gluconeogenic enzymes are secreted into the periplasm when *Saccharomyces cerevisiae* are grown in low glucose. The distribution of gluconeogenic enzymes in the periplasm/extracellular fraction of *Saccharomyces cerevisiae* was observed by immuno-TEM, confirmed with an extraction protocol, and identified in a large-scale proteomic study. It is expected that there are other proteins that lack the ER signal sequence and utilize the non-classical pathway to be secreted. Indeed, 92 proteins were identified that were present in the extracellular fraction using proteomics and more than 95% of the proteins do not have the ER sequence. Thus, the non-classical pathway is the major pathway to secrete proteins in *Saccharomyces cerevisiae*. This review article focuses on the use of multiple techniques including immuno-TEM, an extraction protocol, and proteomics to identify proteins that are present in the extracellular fraction in *Saccharomyces cerevisiae*, and to compare the dataset produced in yeast with other secretomic studies performed on a range of organisms from bacteria to human cells. Common proteins include metabolic enzymes, heat shock proteins, anti-oxidant proteins, and translation factors. Therefore, the secretion of these proteins is widely observed across species.

Keywords: Non-Classical Secretory Pathway; Proteomics/Secretomics; Gluconeogenesis; Vacuole Import and Degradation; Fructose-1,6-Bisphosphatase; Malate Dehydrogenase; Isocitrate Lyase; Phosphoenolpyruvate Carboxykinase; Glyceraldehyde-3-Phosphate Dehydrogenase; Cyclophilin A

Abbreviations: FBPase: Fructose-1,6-Bisphosphatase; MDH2: Malate Dehydrogenase; Icl1p: Isocitrate Lyase; Pck1p: Phosphoenolpyruvate Carboxykinase; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; Cpr1p: Cyclophilin A; Vid: Vacuole Import and Degradation

Signal-less Proteins are Secreted via the Non-classical Pathway from Bacteria to Humans

Protein secretion plays an important role in the physiological processes of many organisms, spanning from bacteria to humans. Secretory proteins are required for cell growth, cell differentiation, proliferation, blood coagulation, and immune defense [1-8]. Secretory proteins are also crucial in cancer angiogenesis, invasion, and metastasis [6,9-13]. As such, proteins that are secreted by cancer cells are promising sources for biomarker discovery [6,9-13]. The secreted carcinoembryonic antigen, α-fetoprotein, and a prostate-specific antigen have each been used as cancer-specific markers [6]. Proteins released from viruses, bacteria, fungi, and parasites are critical for pathogen-host interactions and for their survival within hosts [14-21]. Additionally, fungi have thick cell walls that rely on secretory proteins for nutrient scavenging, cell separation, and the formation and maintenance of cell walls [22-29].

Cells can secrete proteins via multiple pathways [1-3,7,19,30,31]. In the classical pathway, proteins are secreted after they are synthesized and translocated into the ER. They are then transported to the Golgi and subsequently secreted after secretory vesicles fuse with the plasma membrane [30,32,33]. In general, these proteins contain the N-terminal signal sequence. Recent evidence indicates that proteins without the ER sequence are also secreted from a variety of cells. The signal-less proteins include translation factors, transcriptional regulators, metabolic enzymes, chaperones, and mitochondrial proteins [2,5,7,16-20,23,31,34-37]. Viral proteins such as HIV-Tat and the Herpes simplex tegument protein VP22 do not have the conventional signal sequences and are secreted from cells [2,7]. Mitochondrial matrix protein rhodanese is also released into the culture media when over-expressed in HEK-293 cells [38]. Signal-less nuclear proteins such as Engrailed homeoprotein isoform 2 and HMG proteins are exported from cells [2,3,7]. Heat shock proteins Hsp70, Hsp90, and Hsp97 do not have the ER sequence and constitute the largest protein family in the secretome from adult worms of *Schistosoma japonicum* [39]. Glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase have been shown by immuno-TEM to be present on the surface of *Candida albicans, Saccharomyces cerevisiae* [26], and *Listeria monocytogenes* [20]. Importantly, signal-less proteins can be secreted from cells in a controlled manner [1,3,4,8,40,41]. The protein galectin-1, from a family of proteins involved in cell proliferation, differentiation and apoptosis [8,40,41], is secreted when stimulated by the addition of erythropoietin in the leukemia cell line K-562 [40]. Molecules such as FGF-2, IL-1b, and HMGB1 are released from cells upon stimulation [1,4].

Gluconeogenic Enzymes are Secreted from Bacteria, Fungi, Parasites, and Animal Cells

Gluconeogenic enzymes are secreted from various organisms

***Corresponding author:** Hui-Ling Chiang, Department of Cellular and Molecular Physiology, Penn State University College of Medicine, 500 University Drive, Hershey, PA 17033, USA, Tel: 717-531-0860; Fax: 717-531-7667; E-mail: hxc32@psu.edu

Received January 28, 2014; **Accepted** April 25, 2014; **Published** April 30, 2014

Citation: Stein KR, Giardina BJ, Chiang H (2014) The Non-classical Pathway is the Major Pathway to Secrete Proteins in *Saccharomyces cerevisiae*. Clin Exp Pharmacol 4: 155. doi:10.4172/2161-1459.1000155

including bacteria, fungi, parasites, and animal cells [14,18-20,36,42]. Fructose-1,6-bisphosphatase (FBPase) is a key gluconeogenic enzyme and is found in secretomes from *Bacillus anthracis* [14] and *Clonorchis sinensis* [36]. Infection with *Clonorchis sinensis* is an emergent public health problem in many countries in Asia [36]. Adult *Clonorchis sinensis* worms dwell in bile ducts and cause various hepatic and biliary diseases. Secreted FBPase is found in sera from mice infected with *Trichinella spiralis* [36]. Moreover, secreted FBPase is detected in sera in humans infected with *Ascaris lumbricoides, Taenia asiatica, Taenia solium,* and *Taeniarhynchus saginatus* [36]. Malate dehydrogenase is another gluconeogenic enzyme and is detected in the secretomes from *Bacillus anthracis, Clonorchis sinensis* [36], and *Schistosoma mansoni* [18]. Similarly, the enzyme, phosphoenolpyruvate carboxykinase, is identified in the secretomes from *Clonorchis sinensis, Echinostoma caproni,* and *Schistosoma mansoni* [18,19,36]. Additionally, secreted isocitrate lyase is necessary for full virulence of *Mycobacterium tuberculosis* and of the plant-pathogenic fungus *Leptosphaeria maculans* [43,44]. These enzymes were also found in extracellular vesicles released from *Histoplasma capsultatum* [45]. Furthermore, they were present in exosomes isolated from a mouse insulinoma cell line [42]. Thus, the secretion of gluconeogenic enzymes is widely observed from bacteria to animal cells. Because gluconeogenic enzymes do not contain signals for the classical pathway, they are secreted via the non-classical pathway.

In *Saccharomyces cerevisiae*, gluconeogenic enzymes are secreted into the periplasm when cells are grown in media low in glucose [46-55]. These gluconeogenic enzymes include FBPase that converts fructose-1,6-bisphosphate to fructose-6-phosphate, malate dehydrogenase (MDH2) that catalyzes the interconversion of malate and oxaloacetate, isocitrate lyase (Icl1p) that produces succinate and glyoxylate from isocitrate, and phosphoenolpyruvate carboxykinase (Pck1p) which coverts oxaloacetate to phosphoenolpyruvate. The secretion of gluconeogenic enzymes is dependent on the duration of starvation [56,57]. FBPase is not secreted in cells that are starved of glucose for 1 day. However, FBPase secretion increases as cells are starved of glucose for 3 days [56,57]. These results suggest that the secretion of FBPase is related to the depletion of glucose or FBPase substrates in the cells.

Prolonged Glucose-starved Cells Re-fed with Glucose Utilize Vacuole Import and Degradation Pathway to Degrade Gluconeogenic Enzymes

FBPase has been used extensively to study glucose-induced inactivation and degradation in *Saccharomyces cerevisiae* [49,58-61]. FBPase expression is induced when yeast cells are grown in media containing low glucose. Following glucose addition to glucose-starved cells, this protein is inactivated and subsequently degraded [61-70]. FBPase is either ubiquitinated and degraded in the proteasome or phosphorylated and degraded in the vacuole. The site of degradation is dependent on the duration of starvation. When glucose is added to cells that are starved for one day, this protein is degraded in the proteasome [71]. However, when glucose is added to cells that are starved for three days, FBPase is degraded in the vacuole via the Vacuole-import and degradation (Vid) pathway [61-67,71-73]. The other gluconeogenic enzymes MDH2, Icl1p, and Pck1p are also targeted to the vacuole for degradation via the same Vid pathway when glucose-starved cells are re-fed with glucose [64,71].

For the vacuole pathway, glucose induces a transient increase in cAMP levels which activates the RAS2-PKA signaling pathway. This leads to the inactivation and subsequent degradation of FBPase in the vacuole [49,58-61]. Prior to targeting to the vacuole, FBPase is associated with multiple FBPase-containing structures that have been purified to near homogeneity. The first structures to be purified were called Vid vesicles [74]. These round vesicles are 30-50 nm in diameter and exist as free and aggregated forms in the cytoplasm [74]. The second and third FBPase-containing structures were called small and large Vid/endosomes, respectively, because they differed in size [63]. Immuno-TEM studies indicated that Vid/endosomes contained clusters of Vid vesicles [63]. Furthermore, FBPase was associated with numerous Vid vesicles inside these Vid/endosomes [63]. These results suggest that Vid vesicles are used to transport FBPase to different locations for the vacuole import and degradation pathway.

A number of *VID* (vacuole import and degradation) genes are required for the degradation of FBPase in the vacuole. As mentioned above, Vid vesicles exist as free forms and aggregated forms in the cytoplasm. In wild-type cells, Vid vesicle proteins such as Vid24p, Sec28p, and Vid30p are present in these aggregates and associated with actin patches. The *VID28* gene is involved in the formation of Vid vesicle aggregates in the cytoplasm [75]. In cells lacking the *VID28* gene, Vid vesicles failed to aggregate and these Vid proteins were secreted [75]. FBPase is secreted in both wild-type and the *Δvid28* mutant cells.

Immuno-TEM Reveals the Presence of Gluconeogenic Enzymes in the Periplasm in Cells Grown in Low Glucose

Immuno-TEM was used to demonstrate that gluconeogenic enzymes FBPase, MDH2, Icl1p, and Pck1p are secreted into the periplasm in cells grown in low glucose. Wild-type cells co-expressing Icl1p tagged with HA and Pck1p tagged with Myc were grown in media containing low glucose for 3 days and processed for Western blot or immuno-TEM [76]. Sec28p is a COPI coatomer subunit and is distributed mainly in the intracellular fraction. The distribution of Sec28p, FBPase, MDH2, Icl1p-HA, and Pck1p-Myc was examined using polyclonal antibodies directed against Sec28p, FBPase, MDH2, HA, and Myc antibodies. When total lysates from wild-type cells were blotted with polyclonal antibodies against these proteins, they all reacted with a single protein band at the predicted molecular weight (Figure 1A).

The distribution of gluconeogenic enzymes was determined by incubating thin sections of wild-type cells with or without affinity purified primary antibodies followed by goat anti-rabbit secondary antibodies conjugated with 10 nm gold particles (Figure 1B). In the absence of primary antibodies, there were very few gold particles observed between the cell wall and the plasma membrane. Sec28p was not detected in the periplasm. In contrast, substantial amounts of FBPase, MDH2, Icl1p-HA, and Pck1p-Myc were observed in the periplasm (Figure 1B). Quantification of gold particles indicated that 76.7% of FBPase was in the periplasm and 33.3% was in the cytoplasm. Furthermore, 36.9% of MDH2, 46.9% of Icl1p-HA, and 57.6% of Pck1p-myc were also distributed in the periplasm.

Non-gluconeogenic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin A (Cpr1p) are also secreted into the periplasm in glucose-starved cells (Figure 1B). GAPDH is a cell surface protein [77,78], whereas Cpr1p has been identified in culture media in *Saccharomyces cerevisiae* in large-scale secretomic/extraction studies [79,80]. Polyclonal antibodies against GAPDH and Cpr1p recognized single protein bands at the predicted molecular weight (Figure 1A). Moreover, these proteins were found in the periplasm in cells grown in low glucose (Figure 1B). Quantification of gold particles indicated that 33.7% of the GAPDH and 42.7% of Cpr1p were distributed in the periplasm. Taken together, 33.7-76.7% of

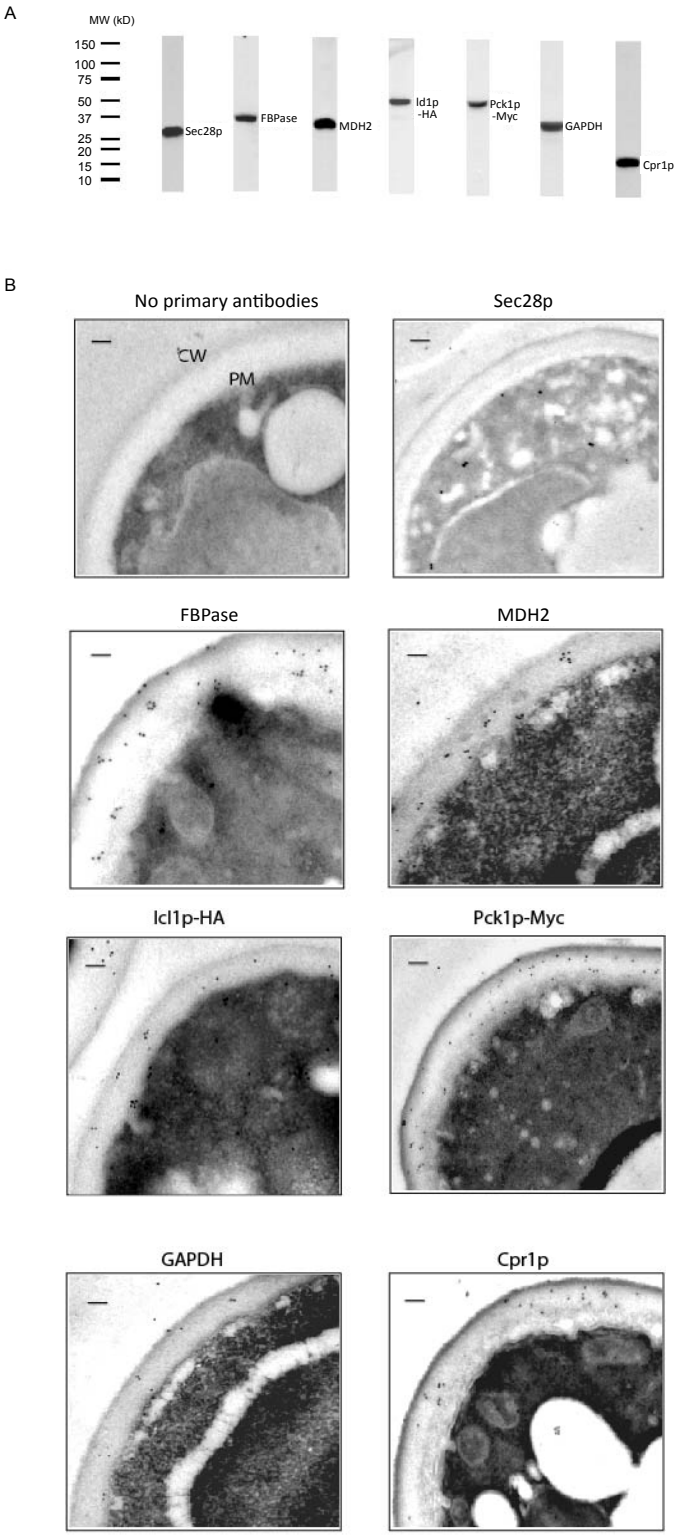

Figure 1: FBPase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p are in the periplasm. (A), Total lysates from wild-type cells expressing Icl1p-HA and Pck1p-Myc were examined for the specificities of antibodies by Western blotting. These antibodies reacted with a single band at the predicted molecular weights for Sec28p, FBPase, MDH2, Icl1p-HA, Pck1p-Myc, GAPDH, and Cpr1p. (B), Wild-type cells expressing Icl1p-HA and Pck1p-Myc were grown in low glucose for 3 days and fixed. Cells were processed and embedded in LR White resin. Thin sections of cells (10 nm) were incubated with or without primary antibodies against Sec28p, FBPase, MDH2, Icl1p-HA, Pck1p-Myc, GAPDH, and Cpr1p in whole cells. Thin sections were then incubated with secondary goat anti-rabbit antibodies conjugated with 10 nm gold particles. Enlargements of the periplasm of the whole cells are shown. PM: Plasma membrane; CW: Cell wall; Bars: 200 nm. (This figure is in a manuscript published in Communicative and Integrative Biology, 2013 [76]).

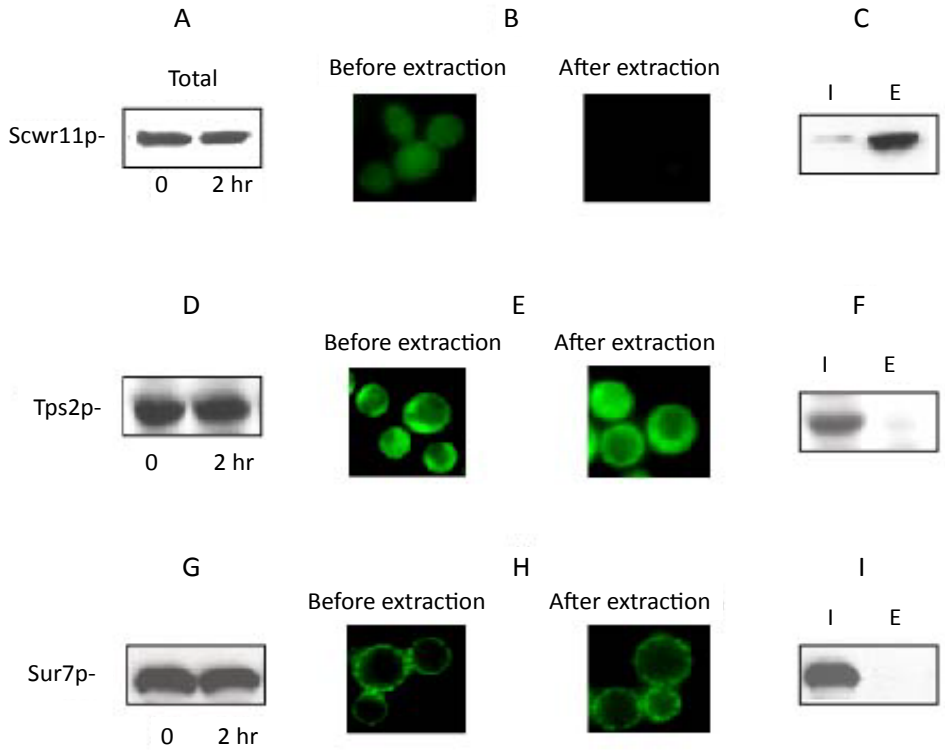

Figure 2: The extraction protocol does not cause non-specific leakage of proteins into the extracellular fraction. (A), Wild-type cells expressing Scw11p-GFP were grown in YPKG for 3 days and examined for expression of proteins following glucose addition for 0 and 2 hours using Western blotting with anti-GFP antibodies. **(B)**, Cells expressing Scw11p-GFP were glucose starved and taken for fluorescence imaging before extraction. Extracellular proteins were extracted and cells were visualized by fluorescence microscopy after extraction. **(C)**, Cells expressing Scw11p-GFP were glucose starved and subjected to the extraction procedure. Following extraction, the distribution of Scw11p-GFP in the Intracellular (I) fraction and the Extracellular fraction **(E)** was examined by Western blotting. Wild-type cells that expressed Tps2p-GFP were examined for protein expression **(D)**, GFP distribution before and after extraction by fluorescence microscopy **(E)**, and the distribution in I and E fractions following extraction by Western blotting. **(F)**, Wild-type cells expressing Sur7p-GFP were examined for expression **(G)**, GFP distribution before and after extraction **(H)**, and the distribution in the I and E fractions after extraction **(I)**.

FBPase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p are secreted into the periplasm in cells grown in low glucose.

The Use of an Extraction Procedure to Extract Periplasmic Proteins from Whole Cells

Because of the distribution of FBPase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p in the periplasm, it is necessary to extract these proteins from this location. Wild-type cells were grown in low glucose for 3 days and pelleted by a low speed centrifugation. Following centrifugation, culture media was removed and cells were incubated with the extraction buffer containing β-mercaptoethanol and Tris with pH 9.4 at 37°C [25,81]. This extraction method was selected, because it is easy to perform and it effectively extracts known periplasmic proteins from whole cells. Furthermore, extracted cells internalize exogenously added molecules and maintain the integrity of the plasma membrane. It has been utilized to study the secretion of a mammalian galectin-1 expressed in *Saccharomyces cerevisiae* [82]. A similar method has also been used to identify proteins associated with the cell wall in *Candida albicans* [28,83]. The following control experiments indicated that proteins that are known to be in the extracellular fraction are extracted effectively from whole cells without causing non-specific leakage of proteins into the extracellular fraction.

Control experiments for these extraction experiments included a soluble cell wall protein Scw11p [84], a cytosolic protein Tps2p [85], and a plasma membrane protein Sur7p [86,87]. If the extraction causes non-specific leakage of proteins, Tps2p or Sur7p may be released into the extracellular fraction. However, if the extraction procedure works, the soluble cell wall protein Scw11p should be extracted, whereas Tps2p and Sur7p should not be extracted. In addition, the distribution of Tps2p and Sur7p should not be altered following the extraction protocol.

Wild-type cells expressing GFP protein that was fused to Scw11p, Tps2p, or Sur7p were grown in low-glucose media for 3 days and then transferred to medium containing high glucose. The expression of these proteins was examined using Western blotting and their distribution before and after extraction was determined using fluorescence microscopy. Scw11p-GFP was expressed in cells grown in low glucose and in cells that were re-fed with glucose for 2 hours (Figure 2A). To extract Scw11p, cells expressing Scw11p-GFP were subjected to the extraction procedure. Following extraction, proteins that were released into the supernatant were precipitated with TCA, washed and solubilized in SDS buffer to form the fraction referred to as the Extracellular (E) fraction. The remaining cell-associated fraction were pelleted and lysed. Proteins were solubilized in SDS-PAGE buffer. This fraction was called the Intracellular (I) fraction. Before extraction, Scw11p-GFP exhibited a diffuse signal throughout the entire cell similar to that reported in the *Saccharomyces cerevisiae* GFP database (Figure 2B). Following the extraction procedure, most of the GFP signal decreased, suggesting that this protein had been extracted from whole cells. Consistent with this

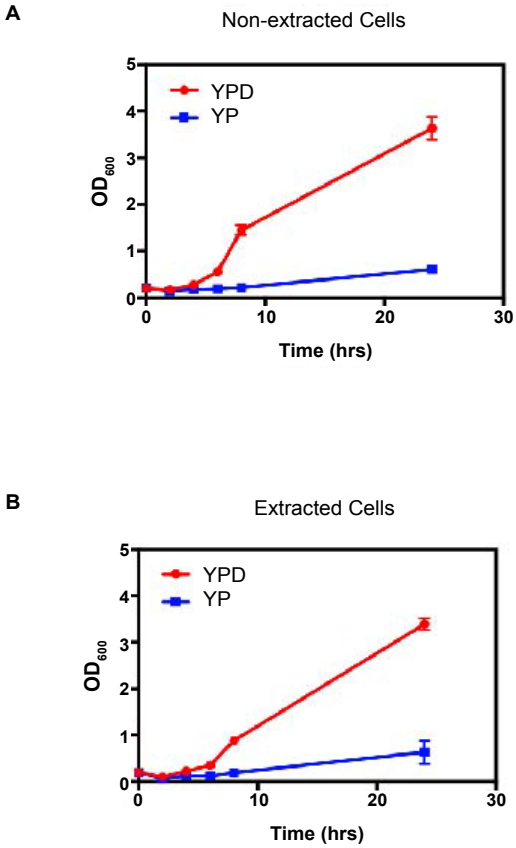

Figure 3: Extracted cells increase cell densities in YPD containing 2% glucose. Cells were grown in YPKG for 3 days until OD_{600}=4-5/ml. Half of the cells were extracted, while the remaining half were not extracted. Both non-extracted (A) and extracted (B) cells were diluted to OD_{600}=0.2/ml in 10 ml YPD containing 2% glucose or 10 ml YP without glucose. Cell densities were measured at OD_{600} for 0, 2, 4, 6, 8, and 24 hours using a Beckman spectrophotometer.

idea, the majority of the Scw11p-GFP was detected in the extracellular fraction following the extraction procedure (Figure 2C).

Tps2p is the phosphatase subunit of the trehalose-6-phosphate synthase/phosphatase complex [85] and is distributed mostly in the cytosol. If the extraction protocol causes non-specific leakage of Tps2p, significant amounts of Tps2p should be detected in the extracellular fraction. Tps2p-GFP was expressed in glucose-starved cells and in cells that were shifted to glucose for 2 hours (Figure 2D). Tps2p-GFP showed a strong cytosolic signal before extraction (Figure 2E). A similar cytosolic distribution was also observed after the extraction. Moreover, the majority of Tps2p-GFP was in the intracellular fraction following the extraction protocol (Figure 2F). Thus, the extraction procedure does not cause non-specific leakage of Tps2p into the extracellular fraction.

Sur7p is a component of eisosomes on the plasma membrane [86,87]. Sur7p-GFP was expressed in cells grown in low glucose, and its levels did not increase following the addition of glucose (Figure 2G). Fluorescence studies indicated that Sur7p-GFP was distributed in punctate structures at the cell periphery before and after extraction (Figure 2H). Finally, following the extraction, Sur7p was detected in the intracellular fraction only (Figure 2I). Thus, this extraction protocol effectively extracted Scw11p into the extracellular fraction. Additionally, this procedure does not alter the distribution of Sur7p and Tps2p in the cell and does not cause the release of these proteins into the extracellular fraction.

Extracted Cells Re-grow in Rich Media and Actively Transport the Vital Dye FM 4-64 to the Vacuole Membrane

Cells grown in low glucose for 3 days are in the G0 stage of the cell cycle. When diluted in YPD media containing high glucose, these cells are able to re-enter the cell cycle and divide. Cells that were extracted also resumed growth and divided in YPD media. To measure cell growth, wild-type cells that were grown in low glucose for 3 days were diluted to OD_{600}=0.2/ml in YPD containing 2% glucose or in YP without glucose. Cell densities were measured at OD_{600} for 0-24 hours using a spectrophotometer. When cells were diluted in YPD containing 2% glucose, cells increased their densities following a lag (Figure 3A). In contrast, when they were diluted in YP without glucose, cells did not increase densities, indicating that the presence of glucose in the media is required for cells to re-grow. Cells that were extracted and then diluted in YPD also increased cell densities following a lag (Figure

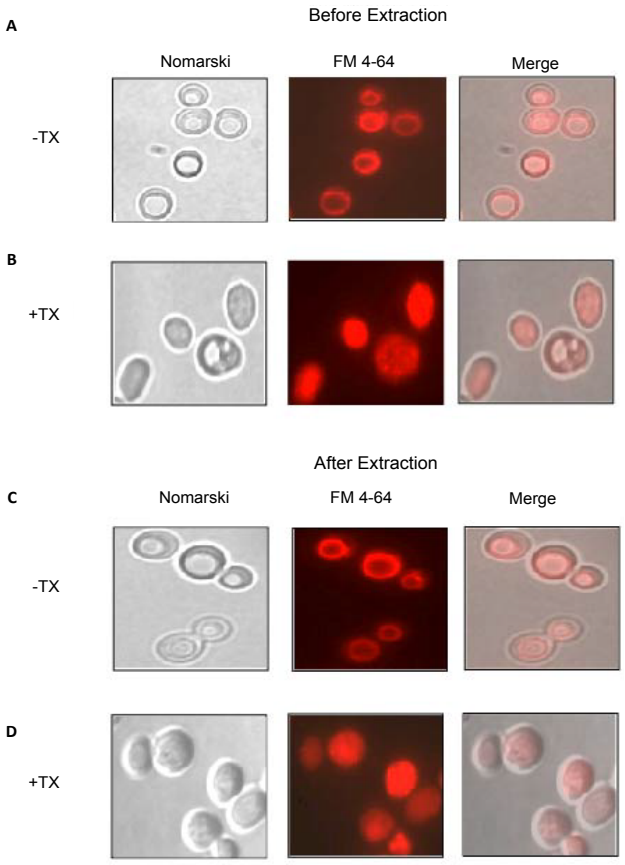

Figure 4: Extracted cells internalize and transport the vital dye FM to the vacuole membrane. Wild-type cells were grown in YPKG for 3 days and divided. Half of the cells were subjected to the extraction procedure, while the other half was not extracted. Non-extracted and extracted cells were then treated with or without Triton X-100 (TX) for 30 min. Cells were centrifuged and resuspended in YP containing 1 µl FM (16 mg/ml) for 3 hours. The distribution of FM in non-extracted cells (A), non-extracted cells treated with TX (B), extracted cells (C), and extracted cells treated with TX (D) was observed by a Zeiss microscope.

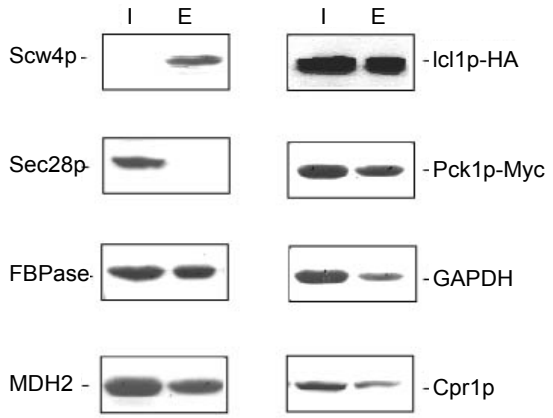

Figure 5: The extraction protocol confirms the presence of FBPase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p in the extracellular fraction. Wild-type cells expressing Scw4p-GFP were grown in YPKG containing low glucose for three days and extracellular proteins were extracted. The distribution of Scw4p-GFP in the intracellular and extracellular fractions was examined by Western blotting using anti-GFP antibodies. Wild-type cells co-expressing Icl1p-HA and Pck1p-Myc were grown in low glucose media for 3 days and extracted. The distribution of Sec28p, FBPase, MDH2, Icl1p-HA, Pck1p-Myc, GAPDH, and Cpr1p in the intracellular and extracellular fractions was examined by Western blotting using anti-Sec28p, anti-FBPase, anti-HA, anti-Myc, anti-GAPDH and anti-Cpr1p antibodies. (This figure is in a manuscript published in Communicative and Integrative Biology, 2013 [76]).

3B). These extracted cells did not increase densities when diluted in YP containing no glucose. Hence, cells that were extracted are able to re-grow in rich media containing high glucose.

In addition, cells that were extracted are able to internalize exogenously added vital dye into the cells. FM 4-64 (FM) is a fluorescent dye that is internalized from the media and transported to the vacuole in live cells [88]. When wild-type cells were grown in YPKG (Yeast extracts-peptone-potassium acetate-0.5% glucose) for 3 days and then incubated with FM for 3 hours, this dye was on the vacuole membrane in ring-like circles as observed by fluorescence microscopy (Figure 4A). However, when these cells were treated with Triton X-100 and then incubated with FM, this dye was not observed on the vacuole membrane (Figure 4B), indicating that the integrity of the plasma membrane is required for cells to internalize FM and transport it to the vacuole membrane. When cells were extracted and then incubated with FM, this dye was transported correctly to the vacuole membrane (Figure 4C). However, when these extracted cells were treated with Triton X-100 and then incubated with FM, this dye was not on the vacuole membrane (Figure 4D). Thus, the extraction procedure does not impair the ability of the cells to internalize and transport this dye to the vacuole membrane. Because internalization of FM requires the integrity of the plasma membrane, this indicates that the plasma membrane is intact in cells that were extracted.

The Extraction Procedure Confirms the Distribution of FBPase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p in the Extracellular Fraction

Having established that this extraction procedure does not cause non-specific leakage of proteins into the extracellular fraction, and does not impair the ability of cells to re-grow and to transport FM to the vacuole, this protocol was used to confirm the presence of FBPase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p in the extracellular fraction. Wild-type cells were grown in low glucose for 3 days and subjected to the extraction procedure. Following extraction, the distribution of proteins in the intracellular and extracellular fractions was determined. Scw4p is another soluble cell wall protein and was used as a control for proteins that are distributed in the extracellular fraction. Sec28p was used as a control for proteins that reside in the intracellular fraction. As expected, most of the Scw4p was in the extracellular fraction, while Sec28p was distributed mainly in the intracellular fraction (Figure 5). Significant amounts of FBPase, MDH2, Icl1p-HA, Pck1p-Myc, GAPDH, and Cpr1p were in the extracellular fraction. Under this experimental condition, Tco89p and Vps34p signaling molecules involved in glucose regulation of gluconeogenic enzymes are distributed mainly in the intracellular fraction [56,75]. Tco89p is a subunit of the TORC1 complex [89] required for the degradation of FBPase [64]. Vps34p is the major phosphatidylinositol 3-kinase in yeast [90].

Identification of Extracellular Proteins Using Proteomics

Given that the extraction method was able to detect the presence of FB Pase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p in the extracellular fraction, this protocol was used to extract extracellular proteins for a large-scale proteomic analysis. Wild-type cells were grown in low glucose for 3 days, extracellular proteins were obtained, precipitated and digested with trypsin. The resulting tryptic peptides were then subjected to SCX (Strong cation exchange) separation followed by reverse phase C18 nano flow-LC separation. Eluant was spotted onto MALDI target plates and analyzed in a data-dependent manner on an ABI 5800 MALDI TOF-TOF. Protein identification was accomplished using the Paragon Algorithm [91] in Protein Pilot TM 4.0 software (AB Sciex). A total of 92 proteins with an estimated local False Discovery Rate (FDR) of less than 5% were identified in the extracellular fraction. These proteins were further classified into different functional groups as defined by the *Saccharomyces* genome database (Table 1). A high percentage of proteins (18%) were involved in carbohydrate metabolism and in amino acid metabolism (14%). Proteins that have roles in the generation of precursor metabolites, in protein folding, in translation and in stress were also identified. Gluconeogenic enzymes MDH2, Icl1p, and Pck1p were identified in the extracellular fraction. Likewise, non-gluconeogenic enzymes GAPDH and Cpr1p were also identified in the extracellular fraction. Heat shock proteins Ssa1p and Ssa2p found in the cell wall of *Saccharomyces cerevisiae* using immunofluorescence microscopy [27] were also identified as extracellular proteins (Table 1). Proteins involved in the metabolism of alcohol, amino acids, purines/pyrimidines, fatty acids, glycerol, and acetate were identified as well (Table 1). Additionally, several of the heat shock proteins, anti-oxidant proteins, ribosomal proteins, translation initiation factors, translation elongation factors, and proteins with unknown functions have been identified (Table 1).

Comparisons with Secretomic Studies from Other Organisms

Proteins that were identified in other secretomic studies from different organisms were compared with those identified in *Saccharomyces cerevisiae*. *Bacillus anthracis* is a Gram-positive, rod-shaped bacterium. It is the etiologic agent of anthrax and is a disease of wildlife, livestock, and humans [14]. *Listeria monocytogenes* is a Gram-positive rod-shaped bacterium that causes severe food-borne infections in animals and humans. *Listeria monocytogenes* is able to spread directly from cell to cell, thus evading the immune system [20]. *Staphylococcus aureus* is a Gram-positive bacterium and is an opportunistic pathogen that causes a range of life-threatening

Table 1: Comparison of proteins identified using the extraction procedure with those identified in secretomic studies from different organisms.

Name	*B. anthracis (14)*	*L. monocytogenes (20)*	*S. aureus (17)*	*C. albicans (95)*	*C. albicans (28)*	*S. cerevisiae (28)*	*S. cerevisiae (29)*	*S. cerevisiae (94)*	*K. lactis (22)*	*C. sinensis (36)*	*B. malayi (16)*	*S. japonicum (39)*	*E. caproni (19)*	*S. mansoni (18)*	ASMC (34)	HepG2 *(12)*	HNOSCC *(9)*	Glioblastoma *(11)*
A. Glycolysis/Gluconeogenesis																		
Pgi1p, Phosphoglucose isomerase								+				+						
Fba1p, Fructose 1,6-bisphosphate aldolase		+	+	+	+	+		+	+			+	+	+	+		+	+
Tpi1p, Triose phosphate isomerase	+		+			+		+	+	+	+	+	+			+	+	
Tdh1p, Glyceraldehyde-3-phosphate dehydrogenase	+	+		+	+	+	+	+				+	+	+	+		+	
Tdh3p, Glyceraldehyde-3-phosphate dehydrogenase	+	+		+	+	+	+	+				+	+	+	+		+	
Pgk1p, 3-Phosphoglycerate kinase		+	+	+	+	+	+	+	+			+	+	+	+	+		+
Gpm1p, Tetrameric phosphoglycerate mutase	+	+		+	+	+		+	+			+		+	+	+		+
Eno1p, Enolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eno2p, Enolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cdc19p, Pyruvate kinase				+	+	+	+	+				+			+		+	+
Pck1p, Phosphoenolpyruvate carboxykinase										+			+	+				
Mdh2p, Malate dehydrogenase cytosolic isozyme	+			+										+				+
Icl1p, Isocitrate lyase	+																	
B. TCA Cycle																		
Lpd1p, Dihydrolipoamide dehydrogenase		+								+								
Aco1p, Aconitase																		+
Idh1p, Isocitrate dehydrogenase																		+
Idh2p, Isocitrate dehydrogenase																		+
Idp1p, NADP-specific isocitrate dehydrogenase																		
Lsc1p, Alpha subunit of succinyl-CoA ligase																		
Sdh1p, Subunit of succinate dehydrogenase																		+
Sdh2p subunit of succinate dehydrogenase					+													
Fum1p, Fumarase																		
Mdh1p, Mitochondrial malate dehydrogenase	+			+								+						+
C. Electron Transport																		
Cor1p, Subunit of the ubiquinol-cytochrome c reductase				+														
Qcr2p, Ubiquinol-cytochrome C reductase subunit 2				+														
Atp1p, Alpha subunit of mitochondrial F1F0 ATP synthase					+					+								+
Atp2p, Beta subunit of mitochondrial F1F0 ATP synthase				+						+				+	+			+
Atp14p, Subunit of ATP synthase			+															
D. Pentose Phosphate Pathway																		
Tkl1p, Transketolase					+			+				+		+				
E. Purine/Pyrimidine Metabolism																		
Adk1p, Adenylate kinase								+										+
Rnr4p, Ribonucleotide-diphosphate reductase							+											
Ura1p, Dihydroorotate dehydrogenase																		
F. Fermentation																		
Pdc1p, Pyruvate decarboxylase				+	+	+	+	+										
Adh1p, Alcohol dehydrogenase I				+	+	+	+	+										+
Adh2p, Alcohol dehydrogenase II					+	+												+
Adh3p, Mitochondria alcohol dehydrogenase isozyme III																		+
Ald4p, Mitochondria aldehyde dehydrogenase										+		+	+					+
G. Metabolism																		
Ach1p, CoA transferase																		
Etr1p, 2-Enoyl thioester reductase																		
Rhr2p, DL-glycerol-3-phosphatase				+														
Sec14p, PtdIns/PtdCho transfer protein																		
H. Amino Acid Metabolism																		
Aro8p, Aromatic aminotransferase I																		
Met17p, Methionine and cysteine synthase	+	+																

Met6p, Methionine Synthase				+				+									
Mmf1p, Mitochondrial transamination of isoleucine																	
I. Heat Shock Proteins																	
Hsp12p, 12KD heat shock protein									+								
Hsp26p, Small heat shock protein (sHSP)																	
Hsp60p, Tetradecameric mitochondrial chaperonin				+									+		+		
Ssa1p, HSP70 family		+			+	+	+	+	+		+	+		+	+			+
Ssa2p, HSP70 family		+			+	+	+	+	+		+	+		+	+			+
Ssa4p, HSP70 family		+			+	+	+	+	+		+	+		+	+			+
Ssb1p, Member of HSP70 family			+	+	+	+	+	+	+									+
Ssb2p, Member of HSP70 family			+	+	+	+	+	+	+									+
Hsc82p, Hsp90 family (nearly identical to Hsp82)					+		+	+					+				+	+
Sba1p, Co-chaperone that regulates Hsp90 family								+										
Sse1p, Co-chaperone that regulates Hsp90 family				+			+	+										
Hsp104p, Heat shock protein, HSP100 gene family																		
Sis1p, Type II HSP40 co-chaperone, DnaJ family								+										+
Sti1p, Hsp90 co-chaperone																		
Mge1p, Mitochondrial matrix co-chaperone																		
J. Protein Folding																		
Cpr1p, Cytoplasmic cyclophilin									+			+				+	+	+
Cpr3p, Mitochondrial cyclophilin																		+
Fpr1p, FK506-binding protein proline rotamase																+		
K. Oxidation Response																		
Ahp1p, Thiol-specific peroxiredoxin			+			+		+						+		+		+
Ctt1p, Cytosolic catalase T																		
Oye2p, Conserved NADPH oxidoreductase								+										
Prx1p, Mitochondrial peroxiredoxin				+											+			
Sod1p, Superoxide dismutase		+	+				+	+			+	+				+		+
Sod2p, Superoxide dismutase		+	+				+	+			+	+				+		+
Tsa1p, Thioredoxin peroxidase				+	+			+			+	+		+		+	+	
L. Transcription																		
Wtm1p, Transcriptional modulator																		
M. Translation																		
Rps31p, Ribosomal Protein of the Small subunit 40S									+									
Rpl25p, Large (60S) ribosomal subunit																		
Tif2p, Translation initiation factor							+											
Efb1p, Elongation factor		+		+											+			+
Eft2p, Translation elongation factor 2 (EF-2)		+													+		+	+
Tef2p, Translational elongation factor EF-1 alpha		+			+			+				+		+	+			
N. Cytoskeleton																		
Act1p, Actin, structural protein				+			+	+		+		+	+	+	+	+	+	+
O. Other																		
Asc1p, G-protein beta subunit				+				+										+
Bmh1p, 14-3-3 gene family					+	+	+	+			+	+	+	+	+		+	+
Bmh2p, 14-3-3 gene family					+	+	+	+			+	+	+	+	+		+	+
Cdc48p, ATPase					+													+
Dug1p, Cys-Gly metallo-di-peptidase																		+
Inh1p, ATPase Inhibitor																		+
Pet9p, ADP/ATP carrier, mitochondrial membrane																		
Ptr3p, Plasma membrane amino acid sensor system																		
Shs1p, Seventh Homolog of Septin																		+
Tfp1p, Vacuolar Membrane ATPase				+											+			
Uba1p, Ubiquitin activating enzyme																		
Ynk1p, Nucleoside diphosphate kinase	+			+														
Gre1p, Hydrophilin of unknown function																		
Om45p, Mitochondrial outer membrane protein																		
Ypr127wp, Putative pyridoxal reductase																		

diseases [17,92]. These pathogens secrete proteins that are required for their adaptation to the environment, for adhesion, and for invasion [17,20,92]. The dimorphic fungus *Candida albicans* is an opportunistic pathogen of humans and is capable of switching its cell morphology from yeast to hyphal form [28,83,93]. *Kluyveromyces lactis* grows as a yeast form and is often haploid [22,37]. *Saccharomyces cerevisiae* is non-

pathogenic and can grow as haploid or diploid forms [29,94]. Parasites such as *Clonorchis sinensis, Brugia malayi, Schistosoma japonicum, Echinostoma caproni,* and *Schistosoma mansoni* enter a wide range of invertebrate and vertebrate hosts [15,16,18,19,36,39]. They secrete a number of proteins during infection that cause a down-modulation of the host immune response in favor of the maintenance of their biological cycles [15,16,18,19,36].

The yeast results were also compared with those reported for animal cells such as the Arterial Smooth Muscle Cells (ASMC) that secrete a large number of proteins involved in maintaining tone in vessel walls, repairing wounds, and development [34]. Finally, identified proteins from yeast were compared with those reported for cancer cell lines. These included the HepG2 human hepatoma, the Head and Neck/Oral Squamous Cell Carcinoma (HNOSCC), and glioblastoma (LN18, U98, U118 and U87) cell lines [9-12]. These cancer cells secrete a large number of proteins into their surroundings during growth and invasion [9-12]. As shown in Table 1, common proteins that are secreted from other organisms include glycolytic enzymes, heat shock proteins, elongation factors, oxidative enzymes, cyclophilins, and actin [6,10,12,18-20,36,93]. Among glycolytic enzymes, enolase is the most frequently identified protein followed by 3-phosphoglycerate kinase, fructose-1,6-bisphosphate aldolase, phosphoglucose isomerase, phosphoglucose mutase and GAPDH. Among the heat shock proteins, the Hsp70p family is the most frequently identified. Thus, the secretion of these signal-less proteins is widely observed across species.

Conclusion Remarks

Multiple techniques including immuno-TEM, an extraction procedure, and proteomics have been used to study extracellular proteins that are secreted in *Saccharomyces cerevisiae*. Using immuno-TEM, significant amounts of gluconeogenic enzymes (FBPase, MDH2, Icl1p, and Pck1p), as well as non-gluconeogenic enzymes (GAPDH and Cpr1p), were observed to be secreted into the periplasm. Because of their distribution in the periplasm, an extraction protocol is needed to release these proteins from this location.

Control experiments indicated that the extraction protocol does not cause non-specific leakage of Tps2p and Sur7p into the extracellular fraction, while successfully extracting most of the Scw11p from whole cells. Fluorescence studies indicated that the distribution of Tps2p and Sur7p was not altered by the extraction procedures. Cells that were extracted were also able to re-grow and increase cell densities following a dilution in YPD with a growth curve similar to non-extracted cells. In addition, cells that were extracted retained the ability to internalize the fluorescent dye FM and actively transport it to the vacuole membrane. Because the integrity of the plasma membrane is needed for FM to be internalized and transported to the vacuole, the plasma membrane should be intact in cells that were extracted. Most importantly, this extraction protocol was able to detect the presence of FBPase, MDH2, Icl1p, Pck1p, GAPDH, and Cpr1p in the extracellular fraction. Under the same conditions, other molecules such as Sec28p, Vid24p, Vid30p, Vps34p, and Tco89p were distributed mainly in the intracellular fraction [56,57,89]. Therefore, this extraction method is effective for extracting periplasmic proteins from whole cells. For proteins that are secreted directly into the culture media, they can be collected from the culture media without the use of this method.

Among the extracellular proteins that were identified by proteomics, fewer than 5% of the identified proteins contain an N-terminal signal sequence. Since more than 95% of the extracellular proteins lack the signal sequence, the non-classical pathway is the major pathway to secrete proteins and the classical pathway is the minor pathway. This conclusion is consistent with a previous *Saccharomyces cerevisiae* study in which 99 proteins were identified in the secretome and only 17 proteins contained a signal sequence [94].

It is interesting that extracellular proteins that are secreted from cells are involved in so many diverse functions. The secretion of metabolic enzymes may be dependent on the physiological state of the cells. In addition, metabolic enzymes may be secreted as enzymatically active forms [23,77,83]. When GAPDH from *Candida albicans* is fused with invertase from *Saccharomyces cerevisiae*, activities of both GAPDH and invertase are detected on the cell surface of *Saccharomyces cerevisiae*. Secreted proteins may perform different functions outside the cells. Glycolytic enzymes have been identified as immunogens during infection by *Candida albicans* [28,83,95]. GAPDH and enolase bind to various mammalian proteins such as lysozyme, fibronectin, actin, myosin and plasmin [28,83,93]. These secreted glycolytic enzymes may be involved in invasion by pathogens during infection.

In summary, immuno-TEM, the extraction protocol, and proteomics have been instrumental in producing a large amount of information needed to study extracellular proteins that are secreted in *Saccharomyces cerevisiae*. Many of the identified extracellular proteins from *Saccharomyces cerevisiae* are also secreted from other organisms, suggesting that secretion of these proteins is conserved across species. As more research tools become available in the future, more genes and proteins that play important roles in the secretion of extracellular proteins can be identified.

References

1. Jackson A, Friedman S, Zhan X, Engleka KA, Forough R, et al. (1992) Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells. Proc Natl Acad Sci U S A 89: 10691-10695.
2. Nickel W (2003) The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. Eur J Biochem 270: 2109-2119.
3. Cleves AE (1997) Protein transports: the nonclassical ins and outs. Curr Biol 7: R318-320.
4. Shin JT, Opalenik SR, Wehby JN, Mahesh VK, Jackson A, et al. (1996) Serum-starvation induces the extracellular appearance of FGF-1. Biochim Biophys Acta 1312: 27-38.
5. Jacobs JM, Waters KM, Kathmann LE, Camp DG 2nd, Wiley HS, et al. (2008) The mammary epithelial cell secretome and its regulation by signal transduction pathways. J Proteome Res 7: 558-569.
6. Pavlou MP, Diamandis EP (2010) The cancer cell secretome: a good source for discovering biomarkers? J Proteomics 73: 1896-1906.
7. Nickel W (2010) Pathways of unconventional protein secretion. Curr Opin Biotechnol 21: 621-626.
8. Hughes RC (1999) Secretion of the galectin family of mammalian carbohydrate-binding proteins. Biochim Biophys Acta 1473: 172-185.
9. Ralhan R, Masui O, Desouza LV, Matta A, Macha M, et al. (2011) Identification of proteins secreted by head and neck cancer cell lines using LC-MS/MS: Strategy for discovery of candidate serological biomarkers. Proteomics 11: 2363-2376.
10. Shin YK, Yoo BC, Hong YS, Chang HJ, Jung KH, et al. (2009) Upregulation of glycolytic enzymes in proteins secreted from human colon cancer cells with 5-fluorouracil resistance. Electrophoresis 30: 2182-2192.
11. Formolo CA, Williams R, Gordish-Dressman H, MacDonald TJ, Lee NH, et al. (2011) Secretome signature of invasive glioblastoma multiforme. J Proteome Res 10: 3149-3159.
12. Yamashita R, Fujiwara Y, Ikari K, Hamada K, Otomo A, et al. (2007) Extracellular proteome of human hepatoma cell, HepG2 analyzed using two-dimensional liquid chromatography coupled with tandem mass spectrometry. Mol Cell Biochem 298: 83-92.

13. Jimenez CR, Knol JC, Meijer GA, Fijneman RJ (2010) Proteomics of colorectal cancer: overview of discovery studies and identification of commonly identified cancer-associated proteins and candidate CRC serum markers. J Proteomics 73: 1873-1895.

14. Lamonica JM, Wagner M, Eschenbrenner M, Williams LE, Miller TL, et al. (2005) Comparative secretome analyses of three Bacillus anthracis strains with variant plasmid contents. Infect Immun. 73: 3646-3658.

15. Bernal D, Carpena I, Espert AM, De la Rubia JE, Esteban JG, et al. (2006) Identification of proteins in excretory/secretory extracts of Echinostoma friedi (Trematoda) from chronic and acute infections. Proteomics 6: 2835-2843.

16. Hewitson JP, Harcus YM, Curwen RS, Dowle AA, Atmadja AK, et al. (2008) The secretome of the filarial parasite, Brugia malayi: proteomic profile of adult excretory-secretory products. Mol Biochem Parasitol 160: 8-21.

17. Hempel K, Pane-Farre J, Otto A, Sievers S, Hecker M, et al. (2010) Quantitative cell surface proteome profiling for SigB-dependent protein expression in the human pathogen Staphylococcus aureus via biotinylation approach. J Proteome Res. 9: 1579-1590.

18. Cass CL, Johnson JR, Califf LL, Xu T, Hernandez HJ, et al. (2007) Proteomic analysis of Schistosoma mansoni egg secretions. Mol Biochem Parasitol 155: 84-93.

19. Sotillo J, Valero ML, Sánchez Del Pino MM, Fried B, Esteban JG, et al. (2010) Excretory/secretory proteome of the adult stage of Echinostoma caproni. Parasitol Res 107: 691-697.

20. Schaumburg J, Diekmann O, Hagendorff P, Bergmann S, Rohde M, et al. (2004) The cell wall subproteome of Listeria monocytogenes. Proteomics 4: 2991-3006.

21. Morphew RM, Wright HA, LaCourse EJ, Woods DJ, Brophy PM (2007) Comparative proteomics of excretory-secretory proteins released by the liver fluke Fasciola hepatica in sheep host bile and during in vitro culture ex host. Mol Cell Proteomics 6: 963-972.

22. Swaim CL, Anton BP, Sharma SS, Taron CH, Benner JS (2008) Physical and computational analysis of the yeast Kluyveromyces lactis secreted proteome. Proteomics 8: 2714-2723.

23. Delgado ML, Gil ML, Gozalbo D (2003) Candida albicans TDH3 gene promotes secretion of internal invertase when expressed in Saccharomyces cerevisiae as a glyceraldehyde-3-phosphate dehydrogenase-invertase fusion protein. Yeast 20: 713-722.

24. Levin DE (2005) Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiol Mol Biol Rev 69: 262-291.

25. Mrsa V, Seidl T, Gentzsch M, Tanner W (1997) Specific labelling of cell wall proteins by biotinylation. Identification of four covalently linked O-mannosylated proteins of Saccharomyces cerevisiae. Yeast 13: 1145-1154.

26. Gozalbo D, Gil-Navarro I, Azorin I, Renau-Piqueras J, Martinez JP, et al. (1998) The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of Candida albicans is also a fibronectin and laminin binding protein. Infect Immun 66: 2052-2059.

27. López-Ribot JL, Chaffin WL (1996) Members of the Hsp70 family of proteins in the cell wall of Saccharomyces cerevisiae. J Bacteriol 178: 4724-4726.

28. Nombela C, Gil C, Chaffin WL (2006) Non-conventional protein secretion in yeast. Trends Microbiol 14: 15-21.

29. Braconi D, Amato L, Bernardini G, Arena S, Orlandini M, et al. (2011) Surfome analysis of a wild-type wine Saccharomyces cerevisiae strain. Food Microbiol 28: 1220-1230.

30. Duden R (2003) ER-to-Golgi transport: COP I and COP II function (Review). Mol Membr Biol 20: 197-207.

31. Bendtsen JD, Jensen LJ, Blom N, Von Heijne G, Brunak S (2004) Feature-based prediction of non-classical and leaderless protein secretion. Protein Eng Des Sel 17: 349-356.

32. McMahon HT, Mills IG (2004) COP and clathrin-coated vesicle budding: different pathways, common approaches. Curr Opin Cell Biol 16: 379-391.

33. Price A, Wickner W, Ungermann C (2000) Proteins needed for vesicle budding from the Golgi complex are also required for the docking step of homotypic vacuole fusion. J Cell Biol 148: 1223-1229.

34. Dupont A, Corseaux D, Dekeyzer O, Drobecq H, Guihot AL, et al. (2005) The proteome and secretome of human arterial smooth muscle cells. Proteomics 5: 585-596.

35. Dowell JA, Johnson JA, Li L (2009) Identification of astrocyte secreted proteins with a combination of shotgun proteomics and bioinformatics. J Proteome Res 8: 4135-4143.

36. Zheng M, Hu K, Liu W, Hu X, Hu F, et al. (2011) Proteomic analysis of excretory secretory products from Clonorchis sinensis adult worms: molecular characterization and serological reactivity of a excretory-secretory antigen-fructose-1,6-bisphosphatase. Parasitol Res 109: 737-744.

37. Madinger CL, Sharma SS, Anton BP, Fields LG, Cushing ML, et al. (2009) The effect of carbon source on the secretome of Kluyveromyces lactis. Proteomics 9: 4744-4754.

38. Sloan IS, Horowitz PM, Chirgwin JM (1994) Rapid secretion by a nonclassical pathway of overexpressed mammalian mitochondrial rhodanese. J Biol Chem 269: 27625-27630.

39. Liu F, Cui SJ, Hu W, Feng Z, Wang ZQ, et al. (2009) Excretory/secretory proteome of the adult developmental stage of human blood fluke, Schistosoma japonicum. Mol Cell Proteomics 8: 1236-1251.

40. Lutomski D, Fouillit M, Bourin P, Mellottée D, Denize N, et al. (1997) Externalization and binding of galectin-1 on cell surface of K562 cells upon erythroid differentiation. Glycobiology 7: 1193-1199.

41. Lutomski D, Joubert-Caron R, Lefebure C, Salama J, Belin C, et al. (1997) Anti-galectin-1 autoantibodies in serum of patients with neurological diseases. Clin Chim Acta 262: 131-138.

42. Lee HS, Jeong J, Lee KJ (2009) Characterization of vesicles secreted from insulinoma NIT-1 cells. J Proteome Res 8: 2851-2862.

43. Idnurm A, Howlett BJ (2002) Isocitrate lyase is essential for pathogenicity of the fungus Leptosphaeria maculans to canola (Brassica napus). Eukaryot Cell 1: 719-724.

44. McKinney JD, Höner zu Bentrup K, Muñoz-Elías EJ, Miczak A, Chen B, et al. (2000) Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 406: 735-738.

45. Albuquerque PC, Nakayasu ES, Rodrigues ML, Frases S, Casadevall A, et al. (2008) Vesicular transport in Histoplasma capsulatum: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. Cell Microbiol 10: 1695-1710.

46. Belinchón MM, Gancedo JM (2007) Glucose controls multiple processes in Saccharomyces cerevisiae through diverse combinations of signaling pathways. FEMS Yeast Res 7: 808-818.

47. Carlson M (1998) Regulation of glucose utilization in yeast. Curr Opin Genet Dev 8: 560-564.

48. Carlson M (1999) Glucose repression in yeast. Curr Opin Microbiol 2: 202-207.

49. Gancedo C (1971) Inactivation of fructose-1,6-diphosphatase by glucose in yeast. J Bacteriol 107: 401-405.

50. Gancedo JM (1992) Carbon catabolite repression in yeast. Eur J Biochem 206: 297-313.

51. Gancedo JM (1998) Yeast carbon catabolite repression. Microbiol Mol Biol Rev 62: 334-361.

52. Gancedo JM (2008) The early steps of glucose signalling in yeast. FEMS Microbiol Rev 32: 673-704.

53. Gancedo JM, Gancedo C (1979) Inactivation of gluconeogenic enzymes in glycolytic mutants of Saccharomyces cerevisiae. Eur J Biochem 101: 455-460.

54. Minard KI, McAlister-Henn L (1992) Glucose-induced degradation of the MDH2 isozyme of malate dehydrogenase in yeast. J Biol Chem 267: 17458-17464.

55. Minard KI, McAlister-Henn L (1994) Glucose-induced phosphorylation of the MDH2 isozyme of malate dehydrogenase in Saccharomyces cerevisiae. Arch Biochem Biophys 315: 302-309.

56. Alibhoy AA, Giardina BJ, Dunton DD, Chiang HL (2012) Vps34p is required for the decline of extracellular fructose-1,6-bisphosphatase in the vacuole import and degradation pathway. J Biol Chem 287: 33080-33093.

57. Giardina BJ, Chiang HL (2013) The key gluconeogenic enzyme fructose-1,6-bisphosphatase is secreted during prolonged glucose starvation and is internalized following glucose re-feeding via the non-classical secretory and internalizing pathways in Saccharomyces cerevisiae. Plant signaling & behavior 8: e24936.

58. Pohlig G, Holzer H (1985) Phosphorylation and inactivation of yeast fructose-1,6-bisphosphatase by cyclic AMP-dependent protein kinase from yeast. J Biol Chem 260: 13818-13823.

59. Rittenhouse J, Moberly L, Marcus F (1987) Phosphorylation in vivo of yeast (Saccharomyces cerevisiae) fructose-1,6-bisphosphatase at the cyclic AMP-dependent site. J Biol Chem 262: 10114-10119.

60. Jiang Y, Davis C, Broach JR (1998) Efficient transition to growth on fermentable carbon sources in Saccharomyces cerevisiae requires signaling through the Ras pathway. EMBO J 17: 6942-6951.

61. Brown CR, Chiang HL (2009) A selective autophagy pathway that degrades gluconeogenic enzymes during catabolite inactivation. Commun Integr Biol 2: 177-183.

62. Brown CR, Cui DY, Hung GG, Chiang HL (2001) Cyclophilin A mediates Vid22p function in the import of fructose-1,6-bisphosphatase into Vid vesicles. J Biol Chem 276: 48017-48026.

63. Brown CR, Dunton D, Chiang HL (2010) The vacuole import and degradation pathway utilizes early steps of endocytosis and actin polymerization to deliver cargo proteins to the vacuole for degradation. J Biol Chem 285: 1516-1528.

64. Brown CR, Hung GC, Dunton D, Chiang HL (2010) The TOR complex 1 is distributed in endosomes and in retrograde vesicles that form from the vacuole membrane and plays an important role in the vacuole import and degradation pathway. J Biol Chem 285: 23359-23370.

65. Brown CR, McCann JA, Chiang HL (2000) The heat shock protein Ssa2p is required for import of fructose-1, 6-bisphosphatase into Vid vesicles. J Cell Biol 150: 65-76.

66. Brown CR, McCann JA, Hung GG, Elco CP, Chiang HL (2002) Vid22p, a novel plasma membrane protein, is required for the fructose-1,6-bisphosphatase degradation pathway. J Cell Sci 115: 655-666.

67. Brown CR, Wolfe AB, Cui D, Chiang HL (2008) The vacuolar import and degradation pathway merges with the endocytic pathway to deliver fructose-1,6-bisphosphatase to the vacuole for degradation. J Biol Chem 283: 26116-26127.

68. Horak J, Regelmann J, Wolf DH (2002) Two distinct proteolytic systems responsible for glucose-induced degradation of fructose-1,6-bisphosphatase and the Gal2p transporter in the yeast Saccharomyces cerevisiae share the same protein components of the glucose signaling pathway. J Biol Chem 277: 8248-8254.

69. Horak J, Wolf DH (2005) The ubiquitin ligase SCF(Grr1) is required for Gal2p degradation in the yeast Saccharomyces cerevisiae. Biochem Biophys Res Commun 335: 1185-1190.

70. Regelmann J, Schule T, Josupeit FS, Horak J, Rose M, et al. (2003) Catabolite degradation of fructose-1,6-bisphosphatase in the yeast Saccharomyces cerevisiae: a genome-wide screen identifies eight novel GID genes and indicates the existence of two degradation pathways. Mol Biol Cell 14: 1652-1663.

71. Hung GC, Brown CR, Wolfe AB, Liu J, Chiang HL (2004) Degradation of the gluconeogenic enzymes fructose-1,6-bisphosphatase and malate dehydrogenase is mediated by distinct proteolytic pathways and signaling events. J Biol Chem 279: 49138-49150.

72. Chiang HL, Schekman R (1991) Regulated import and degradation of a cytosolic protein in the yeast vacuole. Nature 350: 313-318.

73. Chiang HL, Schekman R, Hamamoto S (1996) Selective uptake of cytosolic, peroxisomal, and plasma membrane proteins into the yeast lysosome for degradation. J Biol Chem 271: 9934-9941.

74. Huang PH, Chiang HL (1997) Identification of novel vesicles in the cytosol to vacuole protein degradation pathway. J Cell Biol 136: 803-810.

75. Giardina BJ, Dunton D, Chiang HL (2013) Vid28 protein is required for the association of vacuole import and degradation (Vid) vesicles with actin patches and the retention of Vid vesicle proteins in the intracellular fraction. J Biol Chem 288: 11636-11648.

76. Giardina BJ, Chiang HL (2013) Fructose-1,6-bisphosphatase, Malate Dehydrogenase, Isocitrate Lyase, Phosphoenolpyruvate Carboxykinase, Glyceraldehyde-3-phosphate Dehydrogenase, and Cyclophilin A are secreted in Saccharomyces cerevisiae grown in low glucose. Communicative & integrative biology 6: e27216.

77. Delgado ML, Gil ML, Gozalbo D (2003) Starvation and temperature upshift cause an increase in the enzymatically active cell wall-associated glyceraldehyde-3-phosphate dehydrogenase protein in yeast. FEMS Yeast Res 4: 297-303.

78. Delgado ML, O'Connor JE, Azorin I, Renau-Piqueras J, Gil ML, et al. (2001) The glyceraldehyde-3-phosphate dehydrogenase polypeptides encoded by the Saccharomyces cerevisiae TDH1, TDH2 and TDH3 genes are also cell wall proteins. Microbiology 147: 411-417.

79. Rowe JD, Harbertson JF, Osborne JP, Freitag M, Lim J, et al. (2010) Systematic identification of yeast proteins extracted into model wine during aging on the yeast lees. J Agric Food Chem 58: 2337-2346.

80. Oliveira DL, Nakayasu ES, Joffe LS, Guimarães AJ, Sobreira TJ, et al. (2010) Characterization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis. PLoS One 5: e11113.

81. Klis FM, de Jong M, Brul S, de Groot PW (2007) Extraction of cell surface-associated proteins from living yeast cells. Yeast 24: 253-258.

82. Cleves AE, Cooper DN, Barondes SH, Kelly RB (1996) A new pathway for protein export in Saccharomyces cerevisiae. J Cell Biol 133: 1017-1026.

83. Chaffin WL, López-Ribot JL, Casanova M, Gozalbo D, Martínez JP (1998) Cell wall and secreted proteins of Candida albicans: identification, function, and expression. Microbiol Mol Biol Rev 62: 130-180.

84. Cappellaro C, Mrsa V, Tanner W (1998) New potential cell wall glucanases of Saccharomyces cerevisiae and their involvement in mating. J Bacteriol 180: 5030-5037.

85. De Virgilio C, Burckert N, Bell W, Jeno P, Boller T, et al. (1993) Disruption of TPS2, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in Saccharomyces cerevisiae, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity. Eur J Biochem 212: 315-323.

86. Walther TC, Brickner JH, Aguilar PS, Bernales S, Pantoja C, et al. (2006) Eisosomes mark static sites of endocytosis. Nature 439: 998-1003.

87. Young ME, Karpova TS, Brugger B, Moschenross DM, Wang GK, et al. (2002) The Sur7p family defines novel cortical domains in Saccharomyces cerevisiae, affects sphingolipid metabolism, and is involved in sporulation. Mol Cell Biol 22: 927-934.

88. Vida TA, Emr SD (1995) A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J Cell Biol 128: 779-792.

89. Reinke A, Anderson S, McCaffery JM, Yates J 3rd, Aronova S, et al. (2004) TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in Saccharomyces cerevisiae. J Biol Chem 279: 14752-14762.

90. Schu PV, Takegawa K, Fry MJ, Stack JH, Waterfield MD, et al. (1993) Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. Science 260: 88-91.

91. Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, et al. (2007) The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics 6: 1638-1655.

92. Dreisbach A, van Dijl JM, Buist G (2011) The cell surface proteome of Staphylococcus aureus. Proteomics 11: 3154-3168.

93. Martínez-Gomariz M, Perumal P, Mekala S, Nombela C, Chaffin WL, et al. (2009) Proteomic analysis of cytoplasmic and surface proteins from yeast cells, hyphae, and biofilms of Candida albicans. Proteomics 9: 2230-2252.

94. Insenser MR, Hernáez ML, Nombela C, Molina M, Molero G, et al. (2010) Gel and gel-free proteomics to identify Saccharomyces cerevisiae cell surface proteins. J Proteomics 73: 1183-1195.

95. Ebanks RO, Chisholm K, McKinnon S, Whiteway M, Pinto DM (2006) Proteomic analysis of Candida albicans yeast and hyphal cell wall and associated proteins. Proteomics 6: 2147-2156.