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# The Vacuole Import and Degradation Pathway Converges with the Endocytic Pathway to Transport Cargo to the Vacuole for Degradation

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Additional information is available at the end of the chapter

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## 1. Introduction

In eukaryotes, pathways concerned with protein synthesis and those involved in protein degradation serve to maintain the levels of proteins in a cell [1]. The degradation of proteins occurs by two major pathways, the proteasomal degradation pathway and the lysosomal degradation pathway [2]. In the proteasomal degradation pathway, target proteins are ubiquitinated by a system of E1, E2 and E3 enzymes [2,3]. Thereafter, the ubiquitinated proteins are delivered to the proteasome for degradation [2,3]. In contrast, the lysosome, which contains many hydrolytic enzymes, serves as the site of degradation for a multitude of pathways. One such pathway is the macroautophagy pathway [4]. This indiscriminating catabolic process, comprising of approximately 30 *ATG* genes, helps cells to endure phases of nutrient starvation and other stresses by degrading proteins and organelles in the lysosome [5-7]. In disparity, chaperone-mediated autophagy is a selective autophagy pathway that targets specific cargo proteins (having the KFERQ amino acid sequence) to the lysosome for degradation via cytosolic chaperone proteins [8-11]. Therefore, vital processes such as cell development, growth and homeostasis require autophagy and its absence or deregulation can result in diseases such as cancer, and even neurodegeneration [12,13].

Vesicular transport facilitates the delivery of proteins to the different organelles of the cell, with the exception of transport to the nucleus, peroxisomes, endoplasmic reticulum etc [14]. These intermediate carriers of proteins range from the endosomes to the coat protein complex I (COPI) vesicles, COPII vesicles and clathrin-coated vesicles [14-16]. The anterograde transport (from ER to Golgi) of proteins is mediated by the COPII vesicles while the retrograde transport (from Golgi to ER) is mediated by the COPI vesicles [14-16]. In addition, the transport of proteins from the plasma membrane to the early endosomes, and

from the Golgi to endosomes is facilitated by the clathrin-coated vesicles [14]. In yeast, the organelle that is homologous to the mammalian lysosome is the vacuole [17-18]. This organelle is essential for cellular processes such as maturation of vacuolar resident proteins, protein degradation, and for osmoregulation [18]. The transport of vacuole resident proteins into the vacuole is essential for the function of this organelle. For example, the Vps pathway transports carboxypeptidase Y (CPY) from the Golgi to the vacuole for maturation [19]. This pathway enlists the involvement of approximately 40 *VPS* genes [19]. Moreover, endocytosis is another pathway that delivers proteins from the plasma membrane and other extracellular molecules to the vacuole [18-20]. In addition, proteins can also be delivered from the cytoplasm into vacuole. For instance, the Cvt pathway delivers enzymes such as aminopeptidase I (API) and  $\alpha$ -mannosidase from the cytoplasm to the vacuole [5,21].

Transport of proteins and organelles to vacuole can be affected by alterations in nutrient stimuli [5,21]. Upon starving *Saccharomyces cerevisiae* of nitrogen, proteins are sequestered in autophagosomes and then transported to the vacuole for degradation by the macroautophagy pathway. The target of rapamycin 1 protein (Tor1p) is a component of TORC1 that functions to regulate gene expression, ribosomal synthesis and nutrient transport [22,23]. Intriguingly, Tor1p inhibits the macroautophagy pathway. Rapamycin induces the macroautophagy pathway even in the absence of nitrogen starvation. In another instance, when yeast is replenished from growth in media containing oleic acid to that containing glucose, the peroxisomes are transported to the vacuole for degradation [24].

## 2. Regulation of gluconeogenic enzymes in yeast

In *Saccharomyces cerevisiae*, essential regulatory enzymes in the gluconeogenesis pathway such as fructose-1,6-bisphosphatase (FBPase), malate dehydrogenase (MDH2), phosphoenolpyruvate carboxykinase (Pck1p) and isocitrate lyase (Icl1p) are induced when cells are grown in media depleted of glucose [25-27]. These enzymes function to synthesize glucose from non-carbohydrate carbon sources such as pyruvate or acetate. Upon supplying cells with media containing fresh glucose, these enzymes are inactivated [26-28]. This is referred to as catabolite inactivation [26-28]. FBPase is the best-studied example of catabolite inactivation [26,27]. From previous investigations, it has been determined that there may be many contributing factors; however protein degradation is the principal mechanism that inactivates FBPase.

FBPase is a suitable candidate for degradation studies for two reasons. First, expression of FBPase can be induced in response to specific stimuli [25-27]. And secondly, following glucose replenishment, FBPase is promptly degraded and exhibits a half-life of approximately 20-40 min. A key factor in targeting FBPase for degradation may be protein modification. To better illustrate this, it has been suggested that phosphorylation of FBPase may be a regulatory factor in this protein's degradation [29]. There is evidence that FBPase is phosphorylated at serine 11 and that this phosphorylation increases following glucose replenishment [30]. Protein kinase A (PKA) and the Ras2 signaling pathway mediate phosphorylation of FBPase [29-31].

### 3. The site of degradation of gluconeogenic enzymes

The site of degradation of FBPase is dependent on the duration of starvation. From studies conducted by the Wolf lab, it has been demonstrated that following glucose replenishment, FBPase is inactivated by ubiquitination [32-35]. There is evidence that the N-terminal proline residue is essential for the polyubiquitination of FBPase following glucose replenishment [36]. Thereafter, ubiquitinated FBPase is then delivered to the proteasome for degradation [32-36]. This is because the mutations in genes involved in the proteasome pathway such as *CIM3* results in the inhibition of FBPase degradation [32-36]. In contrast, after glucose starvation of yeast cells for 3 days, it has been determined that FBPase is phosphorylated and inactivated by PKA [29-31]. Following inactivation, FBPase is delivered to the vacuole for degradation [37-39]. For instance, the degradation of FBPase was examined using a *Δpep4Δprb1Δprc1* vacuole mutant. This mutant strain contains deletion of proteinases A, B and C. In the absence of these genes, there is retardation in the degradation of proteins that are delivered to the vacuole [38,41]. In this study, upon replenishing cells with fresh glucose following one day starvation it was observed that FBPase was degraded normally. However, following glucose replenishment after 3 days glucose starvation, FBPase degradation was inhibited. This suggests that FBPase degradation following 3 days glucose starvation is dependent on the presence of vacuolar proteinases. More recently, our lab has also demonstrated that other gluconeogenic enzymes such as MDH2, Pck1p and Icl1p also share the same degradation characteristics as FBPase. Furthermore, the re-distribution of these enzymes from the cytosol to vacuole following glucose replenishment has been validated by immunofluorescence and immunoelectron microscopy studies. At present, it is suggested that differential modification of FBPase following glucose replenishment dictates whether the protein is degraded in the vacuole or the proteasome. Such a disparate degradation behavior has been previously ascribed to the degradation of the fatty acid synthase subunit  $\beta$  [42]. Depending on growth conditions, fatty acid synthase subunit  $\beta$  is degraded either in the vacuole or the proteasome.

### 4. The vacuole import and degradation pathway

The gluconeogenic enzymes (FBPase, MDH2, Pck1p and Icl1p) are transported to the vacuole for degradation by a selective autophagy pathway [37-41]. This pathway is called the vacuole import and degradation (Vid) pathway. The genes involved in this pathway are cumulatively called *VID* genes [37-41]. For the purposes of characterizing this pathway, FBPase was selected as a marker for associated studies. By using a myriad of mutagenesis assays, our lab has identified many genes that play a role in the Vid pathway. For instance, mutants, created by subjecting cells to UV mutagenesis, have been studied for their ability to degrade FBPase. A colony blotting procedure was utilized to screen for mutants defective in FBPase degradation following glucose replenishment [40]. The results from these experiments were further validated by performing pulse-chase experiments. It was determined that while FBPase was degraded with a half-life of 20-40 min in wild-type cells, mutants degraded FBPase with a half-life ranging from 120-400 min. Moreover, all *vid* mutations were recessive as these mutants were complemented for the FBPase degradation defect upon mating with wild-type cells.

Another strategy to identify genes involved in the Vid pathway was by transposon mutagenesis. For this strategy, a transposon-lacZ/LEU2 library was transformed into wild-type cells. These mutants were then screened for FBPase degradation defects using a colony blotting procedure [43]. The identities of the mutated genes were ascertained by extracting the genomic DNA and the subsequent amplification of the nucleotide sequences adjoining the transposon insertion site via PCR. The product from the PCR was sequenced and analyzed using gene sequence alignment software from the National Center for Biotechnology Information (NCBI). Moreover, the degradation defect attributed to these mutants was confirmed by using yeast null mutants for the corresponding genes. Furthermore, the FBPase degradation phenotype was rescued upon transforming the corresponding *VID* genes into these mutants. The *vid* mutants are distinct from those affecting protein secretion (*sec*), vacuolar proteolysis (*pep*) and vacuolar protein sorting (*ups*). Upon studying the distribution of FBPase in cells of these mutants, it was inferred that the mutants can be classified into two categories. After replenishing cells with fresh glucose, some mutants depicted a more cytosolic staining of FBPase (Class A mutants) while other mutants showed FBPase to be distributed in punctate structures (Class B mutants) [40].

## 5. Vid vesicles: Intermediate carriers of the Vid pathway

From fractionation analysis, it was proposed that in the Vid pathway, FBPase was delivered to the vacuole for degradation via intermediate vesicles. This hypothesis was investigated by isolation and purification of FBPase-associated vesicles to near homogeneity [44]. In this investigation, wild-type cells were shifted to glucose for 30 min at 22°C and vesicles were purified. At this temperature, there is a delay in the delivery of FBPase to the vacuole [44]. Following homogenization and subsequent centrifugation at 100,000 × g of cells, the intracellular organelles were separated by size via fractionation on a Sephacryl S-1000 column. Immunoblotting with antibodies against FBPase and organelle markers enabled in assessing the purity of the isolated FBPase-associated vesicles. FBPase was detected in two distinct peaks from the S-1000 fractionation [44]. The first peak was enriched in both the vacuole membrane marker CPY and the plasma membrane marker Pma1p [44]. Interestingly, the second FBPase peak was enriched in a number of intracellular organelle markers. These include markers for the ER (Sec62p), Golgi (Mnn1p), vacuole (CPY), mitochondria (cytochrome C), and the ER-derived COPII vesicles (Sec22p) [44]. Owing to the enrichment of the second FBPase peak with numerous intracellular organelle markers, this peak was purified by further fractionation on sucrose density equilibrium gradients. From this fractionation, it was ascertained that FBPase was present in only one peak that corresponded to a density of 1.18 – 1.22 g/ml [44]. As this density did not correspond to any of the above intracellular organelle markers, this indicated that FBPase might be contained in distinct intracellular structures. Upon examining the FBPase containing peak using electron microscopy, a uniform population of vesicles (35-50 nm in diameter) was observed [44].



An understanding of how the Vid vesicles facilitate delivery of FBPase to the vacuole is vital to understanding the degradation kinetics of the Vid pathway. As such, it was first hypothesized that if the Vid vesicles serve as intermediate carriers in the Vid pathway, then FBPase will be associated with these vesicles prior to their delivery into the vacuole. In that endeavor, studying the distribution of FBPase at 22°C aided in examining the kinetics of FBPase association with Vid vesicles in wild-type cells [44]. In this experiment, wild-type cells were replenished with fresh glucose for various times at 22°C. It was determined that FBPase was associated with the Vid vesicle fraction at t=30 min and was then distributed in both the Vid vesicle and vacuole fractions by 60 min [44]. Moreover, FBPase was associated with the vacuole by 90 min [44]. These results indicate that glucose induces FBPase to be distributed in Vid vesicles and that this occurs prior to delivery of this protein to the vacuole. CPY, which was used as a control in this experiment, was not affected by glucose under these same conditions [44]. In order to ascertain whether FBPase was sequestered into the lumen of the Vid vesicles, the vesicles were purified and then incubated in the presence or absence of proteinase K [44]. The underlying principle of this assay is that FBPase that is sequestered into the lumen of the Vid vesicles will be unaffected by proteinase K digestion and that FBPase that is peripherally associated with the vesicles will be digested by proteinase K. It was determined that FBPase was stable when incubated with proteinase K, which indicated that this protein was sequestered in the lumen of Vid vesicles [44]. Addition of 2% Triton X-100 to permeabilize the Vid vesicle membrane resulted in digestion of FBPase by proteinase K. Thus, a portion of FBPase is sequestered inside Vid vesicles. However, these observations do not rule out the prospect of low amounts of FBPase being associated with the vesicles peripherally [44].

## 6. The biogenesis and trafficking of Vid vesicles to the vacuole

Owing to the unique nature of the Vid vesicles, innumerable questions need to be answered. Questions ranging from elucidating the origin of the vesicles to characterizing the mechanism by which FBPase is sequestered are imperative for better understanding the Vid pathway. In addition, if Vid vesicles are intermediary carriers of cargo protein in the Vid pathway, the vesicles should contain proteins that are essential for the import of FBPase into the vesicles and also for transport of FBPase from the vesicles to the vacuole. In that endeavor, *VID24* was characterized as a gene involved in the degradation of FBPase in the Vid pathway. This gene was identified by chromosomal walking [45].

The *VID24* gene encodes a protein with a molecular weight of 41 kDa. Vid24p has been characterized as a peripheral protein that is distributed to the Vid vesicles [45]. Under glucose starvation conditions, Vid24p is expressed at low levels in wild-type cells. Following glucose replenishment, Vid24p is detected at increased levels from 20 to 120 min. It has been suggested that glucose induces *de novo* synthesis of Vid24p as addition of cycloheximide with glucose was determined to inhibit induction of this protein. Furthermore, during glucose starvation, Vid24p produced weak fluorescence upon studying the distribution of Vid24p by immunofluorescence microscopy. In contrast, Vid24p produced a stronger

fluorescent signal following glucose replenishment for 30 to 60 min. Interestingly, Vid24p was mostly distributed in punctate structures within cells. This suggested that Vid24p was associated with intracellular organelles, which were later determined to be the Vid vesicles. This indicates that Vid24p is a structural protein for the Vid vesicles. Furthermore, this also suggests that the *vid24-1* mutant belongs to the Class B category of mutants that accumulate FBPase in punctate structures. The above results highlight the requirement of Vid24p for the transport of FBPase from the Vid vesicles to the vacuole for degradation.

The next question pertains to the origin of the Vid vesicles. It has been proposed that the Vid vesicles may be derived from existing organelles and that they may be synthesized in cells even prior to glucose replenishment. Investigations surrounding the origins of the Vid vesicles have been hindered by the fact that Vid24p is only induced following 20-30 min of glucose replenishment. Therefore, events detailing the biogenesis of Vid vesicles during the first 20-30 min of glucose replenishment are difficult to examine with Vid24p. To circumvent this issue, an alternative strategy was designed that entailed the screening of mutants that failed to form Vid vesicles. This strategy would facilitate in assigning functions to mutants that were involved in specific steps of Vid vesicle biogenesis. In this manner, it was ascertained that the *UBC1* gene was required for Vid vesicle biogenesis [46]. As a matter of fact, the rate of FBPase degradation was observed to decrease in the null mutant of *UBC1*. Moreover, there was a diminished import of FBPase into the Vid vesicle fractions in the  $\Delta ubc1$  mutant. As such, it could be inferred that in the  $\Delta ubc1$  strain, there is a decrease in the level of Vid vesicles. For instance, Vid24p levels were enriched in the pellet fraction that was representative of Vid vesicles in wild-type cells. However, Vid24p levels were diminished in the pellet fraction in the  $\Delta ubc1$  mutant, indicative of an impaired production of the Vid vesicles. At present, the mechanism by which *UBC1* is involved in the biogenesis of Vid vesicles has not been elucidated. Moreover, the formation of multi-ubiquitin chains has also been implicated in the degradation of FBPase in the Vid pathway. As such, yeast strains expressing the R48K/R63K ubiquitin mutant, which blocks multi-ubiquitin chain formation, resulted in inhibiting the degradation of FBPase in the Vid pathway. Interestingly, there was also a diminished amount of FBPase that was associated with the Vid vesicle fraction. Thus, these observations suggest that the *UBC1* gene and the formation of polyubiquitin chains are involved in the biogenesis of the Vid vesicles.

Another question is to understand how FBPase is imported into the Vid vesicles. To elucidate this, an *in vitro* system was developed to investigate the sequestration of FBPase into isolated Vid vesicles in the presence of the wild-type cytosol [47]. A wild-type strain in which the endogenous *FBP1* gene had been deleted for used for this *in vitro* assay. The Vid vesicles were isolated from this strain by differential centrifugation. Thereafter, the isolated Vid vesicles were incubated with a defined amount of purified FBPase in a reaction mixture that also contained wild-type cytosol, ATP and an ATP regenerating system. Proteinase K was added to the reaction mixture to degrade non-sequestered FBPase after 20 min of incubation. It was determined that 20-40% of the purified FBPase was protected from proteinase K digestion *in vitro*. Interestingly, addition of 2% Triton X-100 to permeabilize the membrane facilitated in the digestion of FBPase by proteinase K. As such, it can be inferred

that FBPase is imported inside Vid vesicles, and that this import requires ATP and cytosol. In addition, our lab has also identified the cytosolic heat shock proteins Ssa1p and Ssa2p as being required for the import of FBPase into Vid vesicles [47].

Vid22p is a plasma membrane protein that was also determined to regulate FBPase sequestration into the Vid vesicles indirectly via the action of Cpr1p [43]. By using a transposon mutagenesis strategy, our lab identified the *VID22* gene. Following its synthesis in the cytosol, Vid22p is then targeted to the plasma membrane in a manner that is independent of the ER-Golgi transport pathway. It was determined that the null mutant of *VID22* inhibited the degradation of FBPase following glucose replenishment. Interestingly, FBPase was found to accumulate in the cytosol of the  $\Delta vid22$  mutant strain. This indicates that *VID22* may be required for the import of FBPase into the Vid vesicles. It was ascertained that FBPase sequestration into the Vid vesicles was inhibited upon combining the  $\Delta vid22$  mutant cytosol with the wild-type Vid vesicles using *in vitro* analysis. However, the wild-type FBPase import phenotype was rescued by incubating the wild-type cytosol with Vid vesicles from the  $\Delta vid22$  mutant. From these experiments, it can be inferred that the  $\Delta vid22$  mutant may contain functional Vid vesicles but have a defective cytosolic environment. It has been determined that Vid22p, through its role in regulating the levels of Cpr1p, influences the degradation of FBPase. This is supported by the fact that the levels of Cpr1p in total lysates are diminished in the  $\Delta vid22$  mutant when compared to that observed in wild-type cells. However, this defect that is attributed to the absence of the *VID22* gene is rescued by the addition of purified Cpr1p *in vitro* or by overexpressing Cpr1p *in vivo*. As such, the Cpr1p protein, whose levels are regulated by Vid22p, directly promotes FBPase import into the Vid vesicles. At present, the mechanism by which Vid22p regulated Cpr1p levels has not been elucidated.

The peptidylprolyl isomerase cyclophilin A (Cpr1p) was identified as being required for the import of FBPase into Vid vesicles [48]. This cytosolic protein serves as a receptor for the immunosuppressant drug cyclosporin A. Our lab identified Cpr1p owing to its role as a mediator for the Vid protein Vid22p. By fractionating the wild-type cytosol by purification using ammonium sulfate precipitation, Superose 6 and G75 sizing chromatography, and DEAE ion exchange chromatography, our lab was able to isolate and identify Cpr1p. The role of Cpr1p in the degradation of FBPase was determined by using the  $\Delta cpr1$  mutant strain. It was ascertained that *in vitro* FBPase import and the subsequent degradation of FBPase was inhibited in the null mutant of *CPR1*. Furthermore, it was determined that the sequestration of FBPase into the wild-type Vid vesicles was impeded by the cytosol from the  $\Delta cpr1$  mutant. In contrast, import of FBPase into the Vid vesicles from  $\Delta cpr1$  mutants was not impaired when supplied with the wild-type cytosol. The role of Cpr1p in the involvement of FBPase import into the Vid vesicles was verified by adding increasing amounts of purified Cpr1p to an *in vitro* reaction mixture containing the Vid vesicles and cytosol from the null mutant of *CPR1*. A control experiment comprising of addition of BSA to the *in vitro* reaction mixture containing the Vid vesicles and cytosol from the null mutant of *CPR1* did not stimulate FBPase import. This suggests that Cpr1p has a direct involvement in the import of FBPase into the Vid vesicles.



## 7. The Vid pathway merges with the endocytic pathway to deliver cargo to the vacuole

In order to facilitate a better understanding of the biogenesis of Vid vesicles, Vid vesicles were isolated, purified and interacting proteins or those serving as structural components were identified using MALDI analysis. Interestingly, constituents of COPI vesicles such as Ret1p, Ret2p, Sec21p and Sec28p were identified on purified Vid vesicles [49]. As described previously, the COPI vesicles mediate transport of proteins from the Golgi to the ER [15,50]. It has been previously reported that COPI proteins have also been identified as components of endocytic compartments in both mammalian cells and in yeast [15,50]. Moreover, COPI proteins are involved in multivesicular body sorting in yeast and in endosomal trafficking in mammalian cells [15,50]. Our lab has demonstrated that COPI proteins associate with Vid vesicles [49]. This suggests that the COPI proteins may play a role in FBPase degradation. The *RET1*, *RET2*, *RET3*, *SEC26*, *SEC27*, *SEC21* and *SEC28* genes encode the different coatamer proteins in yeast. With the exception of *SEC28*, all the other genes are essential. As such, the role of the essential COPI genes in FBPase degradation was studied using temperature sensitive mutants. Following glucose replenishment of the null mutant of *SEC28* and the COPI temperature sensitive mutants, it was ascertained that FBPase degradation was impaired. Moreover, the  $\Delta sec28$  mutant and all of the temperature sensitive mutants of COPI genes inhibited the import of FBPase into the Vid vesicles. The  $\Delta vam3$  mutant served as a control in these experiments. The *VAM3* gene encodes a vacuolar t-SNARE that mediates fusion of intermediary vesicles with the vacuole. As such, the  $\Delta vam3$  mutant blocks FBPase degradation following its import into the Vid vesicles. These results suggest that the COPI genes are required for the import of FBPase into the Vid vesicles. The above results were verified by studying the distribution of FBPase in COPI mutants using sucrose density gradients. It was determined that FBPase distribution was enriched in the cytosolic fractions in these mutants and its levels were diminished in Vid vesicle fractions when compared to the  $\Delta vam3$  mutant. Intriguingly, the FBPase distribution in COPI mutants was similar to that observed in the  $\Delta ubc1$  mutant. As these mutants inhibit the formation of Vid vesicles, this indicates that the COPI genes are also involved in Vid vesicle biogenesis. During glucose starvation, COPI proteins were observed to localize with the Vid vesicle marker Vid24p and the cargo FBPase. Interestingly, levels of COPI proteins in the Vid vesicle fractions displayed a transient increase and decrease following glucose replenishment. Furthermore, it was determined that COPI proteins associated with Vid24p forming a complex. This association was increased following glucose replenishment and was required for recruiting Vid24p to the Vid vesicles.

As the COPI genes have been previously reported to be involved in endocytosis in mammalian cells, it was important to determine whether endocytosis may be involved in our degradation pathway [49]. As a preliminary study, the kinetics of the uptake of the lipophilic dye FM4-64 was examined under our growth conditions. In wild-type cells, after its internalization, the FM4-64 dye stains the endocytic compartments before finally staining

the vacuole membrane. Interestingly, the uptake of the FM dye differed upon studying its distribution in mutants that inhibited the degradation of FBPase in the Vid pathway. While mutants such as *Δvph1* displayed large FM circular distributions, other mutants such as *Δvam3* produced small FM-containing circles. Having identified Sec28p (COPI subunit of coatomer) as a structural protein of Vid vesicles, the distribution of this protein was studied as a means to monitor Vid vesicle trafficking. Sec28p was distributed in punctate structures following glucose replenishment of wild-type cells for 20-30 min. Following glucose replenishment, it was observed that Sec28p was localized to FM-containing endosomes in wild-type cells. In contrast, Sec28p failed to localize to FM-containing structures in the *Δvam3* mutant. As such, it can be inferred that the *VAM3* gene is required for the distribution of Sec28p to endosomes.

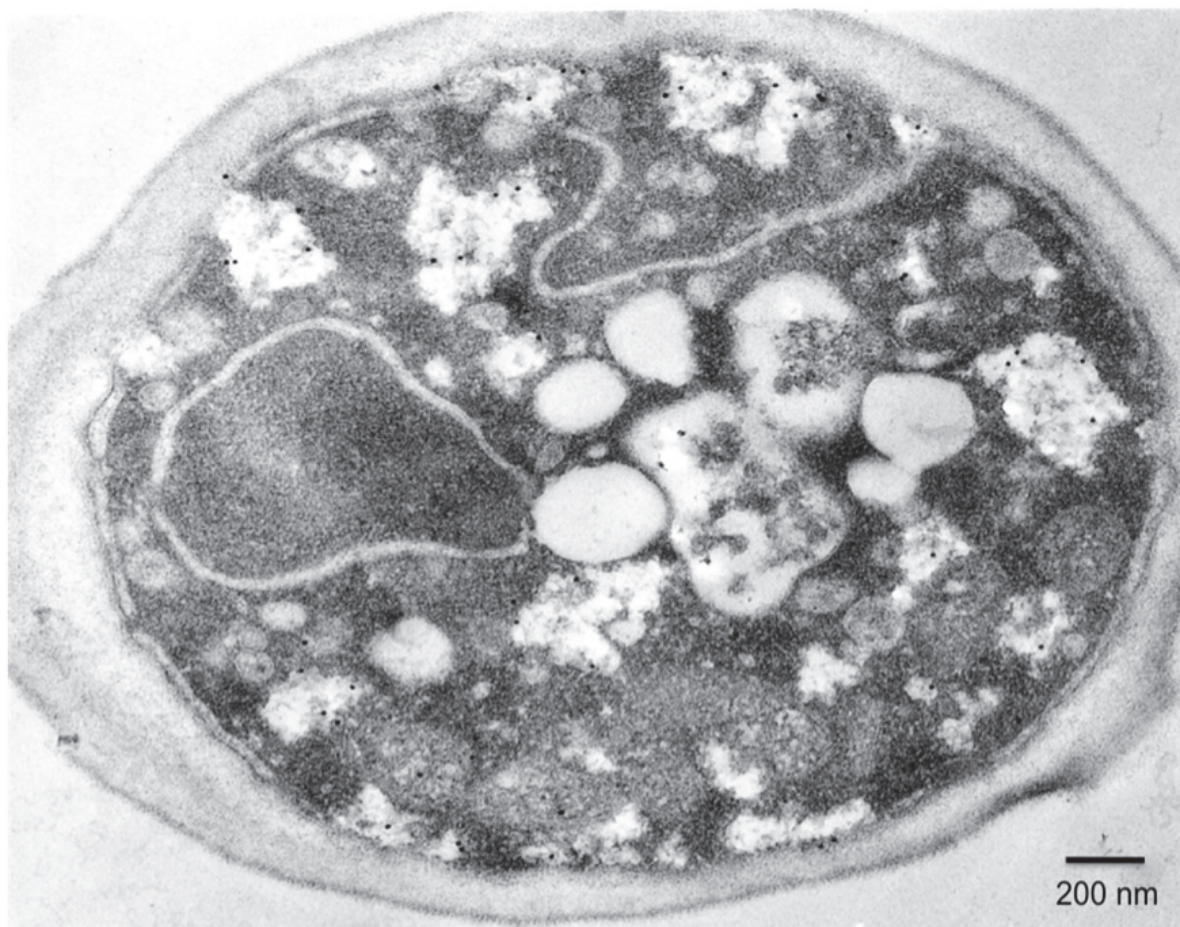
It has been previously determined that the *UBC1* gene is required for the biogenesis of Vid vesicles. In the null mutant of *UBC1*, FBPase is enriched in the cytosol and levels of Vid vesicles are diminished. The trafficking of Sec28p was also studied using a *Δubc1* mutant [49]. It was postulated that if COPI genes are involved in Vid vesicle biogenesis, then COPI proteins such as Sec28p may be discerningly distributed to structural precursors of Vid vesicles in the *Δubc1* mutant. Following glucose replenishment of the *Δubc1* mutant, it was observed that at the earlier time points, Sec28p was distributed at compartments that were stained by the FM dye. However, at later time points, Sec28p was distributed to the FM stained vacuole membrane. These results suggest that the *UBC1* gene is not required for the anterograde transport of Sec28p to the vacuole. As such, it can be inferred that the step following the delivery of Sec28p to vacuole membrane may require *UBC1*. It has been previously established that the biogenesis and budding of the COPI vesicles requires the assembly of COPI proteins at the budding site. Therefore, mutations of the COPI genes should result in altering the distribution of Sec28p to sites where the COPI vesicle buds from a precursor structure. Similarly, it was hypothesized that as Sec28p is a structural component of Vid vesicles, mutations of other COPI genes should affect the distribution of Sec28p to sites where the Vid vesicle is formed. To test this, the distribution of Sec28p was examined in a *ret2-1* mutant. In this mutant, the *ret2-1* gene encodes for a temperature sensitive protein which comprises the  $\delta$  subunit of the COPI complex. Shortly after glucose replenishment, it was determined that Sec28p localized to FM containing endosomes in the *ret2-1* mutant. Interestingly, by 180 min following glucose replenishment, while FM had stained the vacuole membrane, Sec28p was observed as punctate dots near or on the vacuole membrane. This suggests that either Sec28p is a component of vesicles that are in the process of fusing with the vacuole or that Sec28p is budding from the vacuole as a component of retrograde vesicles. This was clarified by studying the distribution of Sec28p after pre-labeling the vacuole membrane with FM dye in the *ret2-1* strain. It was ascertained that Sec28p was distributed to buds that were forming on the vacuole membrane following glucose replenishment. Based on our results, it can be inferred that Sec28p containing vesicles are involved in both transport to and from the vacuole.

## 8. Early steps of endocytosis and actin polymerization are required for degradation of cargo to the Vid pathway

It has been previously ascertained that the Vid pathway merges with the endocytic pathway. An elucidation of this site of merger would afford a better understanding of the Vid pathway. According to one postulation, the Vid vesicles may originate from the plasma membrane or the vacuole. Alternatively, Vid vesicles may converge with endocytic vesicles that are forming on the plasma membrane. This may suggest that FBPase is also distributed near the plasma membrane. This was studied by examining, at the ultra-structural level, the distribution of FBPase in wild-type and *Δpep4* strains [51]. In these studies, following prolonged glucose starvation, the yeast strains were replenished with media containing fresh glucose for 20 min. Immuno-electron microscopy using affinity purified FBPase antibodies followed by secondary antibodies conjugated with 10 nm colloid gold particles facilitated in visualizing the FBPase distribution (Figure 1). It was determined that in both wild-type and *Δpep4* strains, a significant percentage of FBPase was distributed in irregularly shaped intracellular structures in the cytoplasm following glucose replenishment. Interestingly, FBPase was also found near the plasma membrane. This suggests that the early steps of the endocytic pathway are involved in the vacuole dependent degradation of FBPase. These irregularly shaped intracellular structures (containing FBPase) were purified by high speed centrifugation and passing the re-suspended pellet over a S-1000 column. In this manner, it was ascertained that these intracellular structures were enriched for the Vid vesicle marker Vid24p and the endosomal marker Pep12p. From this, it can be inferred that following glucose replenishment, Vid vesicles may associate with the endosomes to form large aggregates of FBPase containing structures.

Owing to the distribution of FBPase near the plasma membrane, this suggests that the early steps of endocytosis may be required for the Vid pathway. In yeast, it has been previously ascertained that the early steps of endocytosis is facilitated by actin polymerization [52-63]. Proteins involved in actin polymerization are recruited to the plasma membrane in a specific and ordered sequence (Figure 2). At the site of cargo internalization, coat module proteins and nucleation promotion factor (NPF) module proteins are recruited at the same time for shaping the membrane and for regulating actin assembly. Coat module proteins comprise of Sla1p, Lsb3p, Pan1p, and End3p. The NPF module proteins consist of Las17p, type I myosins Myo3p and Myo5p, and Vrp1p, Bzz1p and Bbc1p. With the exception of the type I myosins, it should be noted that the coat module proteins and the NPF module proteins are recruited independent of F-actin. Thereafter, the actin module proteins (consisting of 20 proteins) are recruited by F-actin to sites of actin assembly. The actin module proteins are involved in the organization and dynamics of the actin network. This module comprises of proteins such as Act1p, Arp2/3 protein complex, Abp1p, Cap1p, Cap2p, Sac6p and Aim3p among others. The Arp2/3 protein complex is involved in the nucleation of branched actin filaments. This protein complex is comprised of Arp2p, Arp3p, Arc15p, Arc18p, Arc19p, Arc35p and Arc40p. Additionally, the Las17p, Pan1p and Abp1p proteins are required for the activation of the Arp2/3 complex. Finally, the amphiphysin module proteins are recruited by F-actin to mediate scission of endocytic vesicles. This module comprises of Rvs161p and Rvs167p.

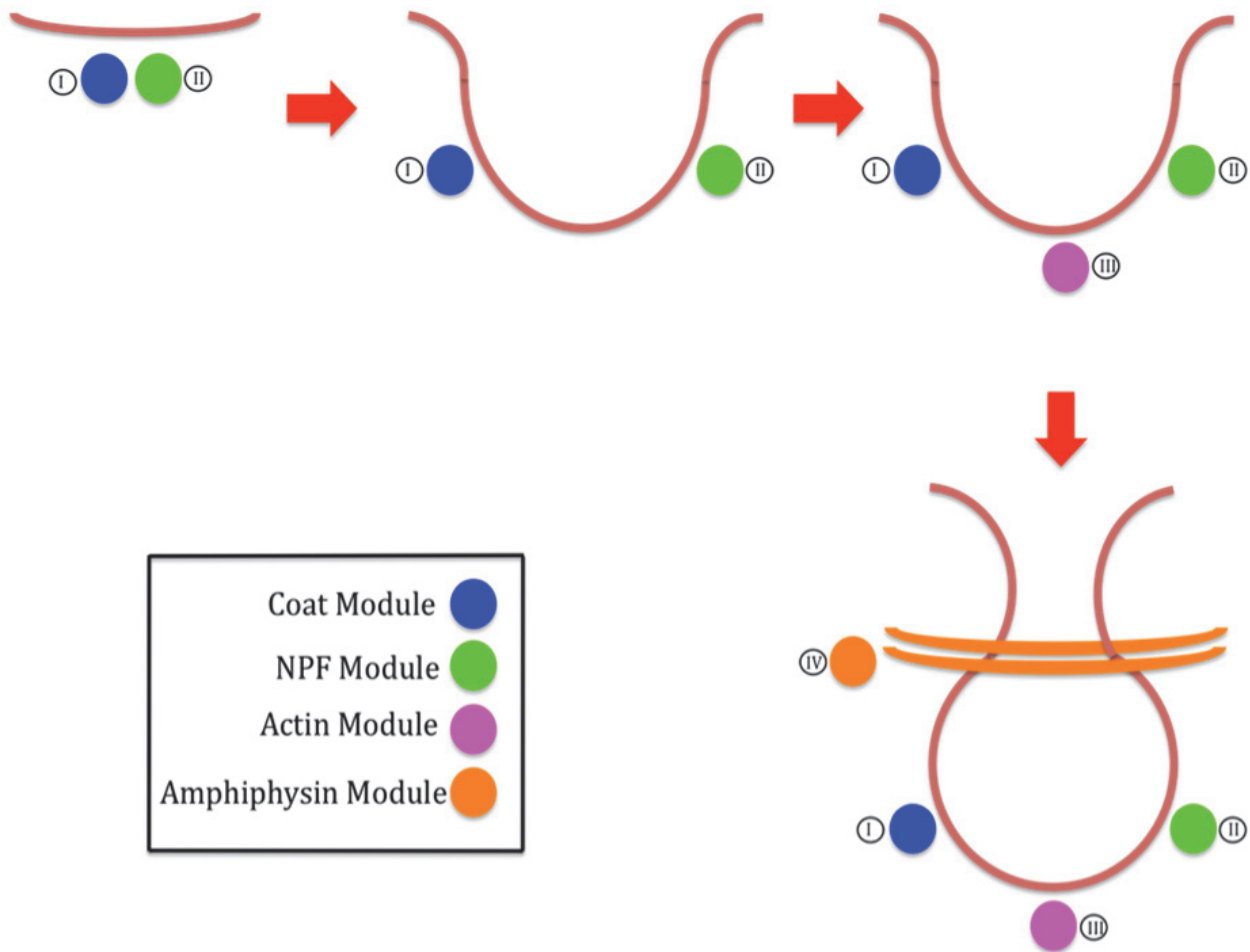




**Figure 1.** Ultra-structural distribution of FBPase in *Apep4* cells following glucose replenishment for 20 min. FBPase was visualized using a purified primary antibody against FBPase and a secondary antibody conjugated with 10 nm colloid gold particles. This research was originally published in The Journal of Biological Chemistry (2010, vol. 285(2), pgs. 1516-1528). © the American Society for Biochemistry and Molecular Biology.

Under our growth conditions, upon examining the distribution of FBPase in a *Δend3* strain, it was determined that the distribution of FBPase in the plasma membrane, endosome and Vid vesicle fractions was diminished in comparison to the control *Δvph1* mutant [51]. This indicates that the early steps of endocytosis may be required for the association of FBPase with Vid vesicles. As it has been previously reported that actin polymerization facilitates the scission of endocytic vesicles from the plasma membrane, the degradation kinetics of FBPase were examined in mutants that blocked the different steps of actin polymerization. In this manner, it was ascertained that the null mutants of *END3* and *SLA1* served to inhibit the degradation of FBPase. Thus, it can be inferred that the actin polymerization genes are required for the association of FBPase with Vid vesicles. Next, fluorescent analysis was used to examine the distribution of proteins to actin patches (sites of actin polymerization). During glucose starvation, it was ascertained that there was a low percentage of co-localization of FBPase to actin patches in wild-type cells (Figure 3) [51,64]. Following glucose replenishment for up to 30 min, FBPase produced a high percentage of co-localization to actin patches. Interestingly, after 60 min of glucose replenishment, FBPase

showed less co-localization to the actin patches. The distribution of MDH2 to actin patches also produced similar results. This indicates that the cargo proteins of the Vid pathway are targeted to the sites of actin polymerization on the plasma membrane.

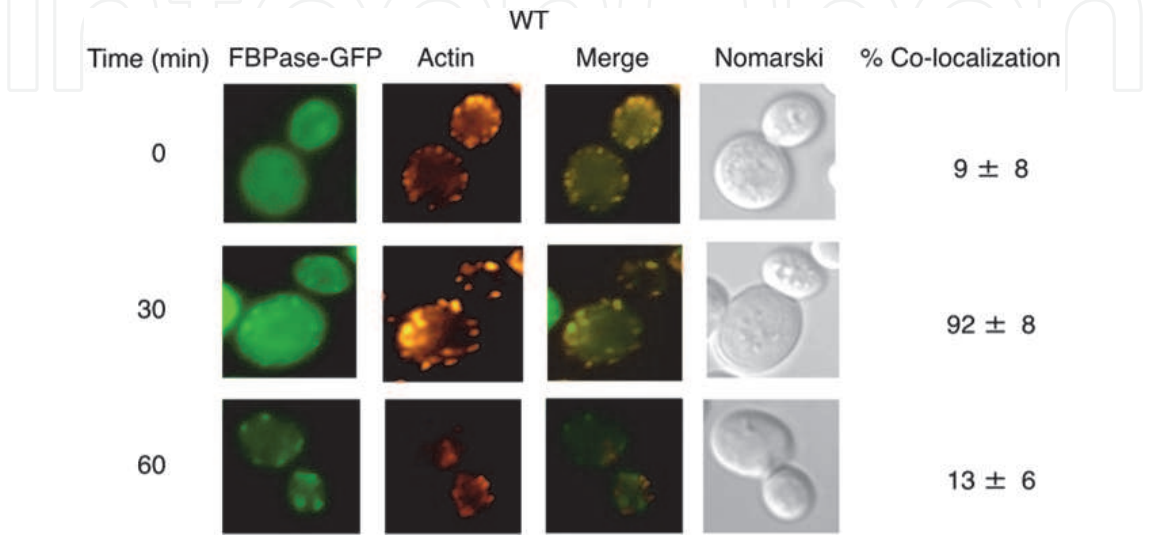


**Figure 2.** Actin polymerization assembly in yeast. (I, II) At the site of internalization, actin polymerization assembly recruits the coat module and nuclear promotion factor (NPF) module proteins for shaping the membrane. (III) The actin module proteins are then recruited for maintaining the integrity and the dynamics of actin assembly. (IV) The amphiphysin module proteins facilitate the scission of endocytic vesicles.

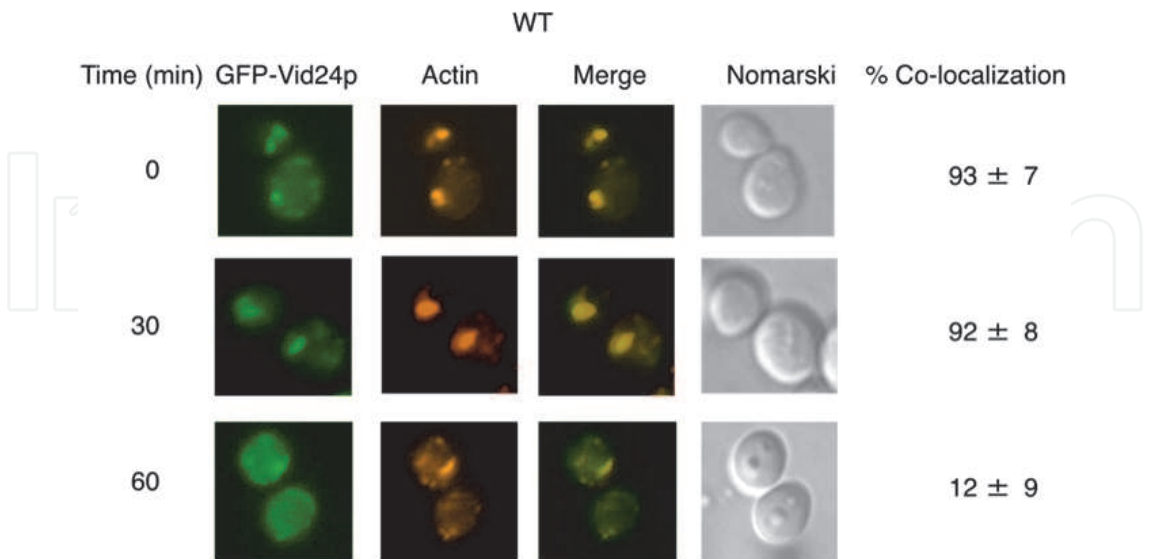
The distribution of the Vid24p to actin patches was next studied in wild-type cells as a means to determine whether the Vid vesicles are distributed to actin patches (Figure 4) [51,64]. During glucose starvation and following replenishment for up to 30 min, Vid24p was observed to be co-localized with actin patches. Intriguingly, by the 60 min time point, Vid24p demonstrated less co-localization with the actin patches. The distribution of Sec28p to actin patches also produced similar results. This suggests that during glucose starvation and following replenishment for up to 30 min, Vid vesicles associate with actin patches. In addition, in the *Arvs167* strain, there is a prolonged association of Vid24p and Sec28p with actin patches. As such, it can be inferred that the actin patches mediate the scission of the Vid-endocytic vesicles from the plasma membrane.



To summarize the above results, we assert that Vid24p and Sec28p are distributed at the sites of actin polymerization (involved in the early steps of endocytosis) during glucose starvation. The gluconeogenic enzymes, FBPase and MDH2 are sequestered into free Vid vesicles and Vid vesicles that are aggregated at the site of actin polymerization. Following the scission of Vid-endocytic vesicles into the cytoplasm as small Vid-endosomes, these vesicles cluster and form large asymmetrically shaped structures. Therefore, the Vid-endosomes serve as intermediary carriers of cargo destined for degradation in the vacuole.



**Figure 3.** FBPase co-localizes with actin patches in wild-type cells. FBPase displays a low percentage of co-localization with actin patches in wild-type cells during glucose starvation. Following glucose replenishment for up to 30 min, FBPase displays a high percentage of co-localization with actin patches. Co-localization of FBPase with actin patches diminishes by the 60 min time point. This research was originally published in *Autophagy* (2012, vol. 8(1), pgs. 29-46). © Landes Bioscience.



**Figure 4.** Vid24p co-localizes with actin patches in wild-type cells. Vid24p co-localizes to actin patches in wild-type cells during glucose starvation and for up to 30 min following glucose replenishment. Co-localization of Vid24p to actin patches diminishes by the 60 min time point. This research was originally published in *Autophagy* (2012, vol. 8(1), pgs. 29-46). © Landes Bioscience.

## 9. The association of Vid vesicles and actin patches requires *VID30*

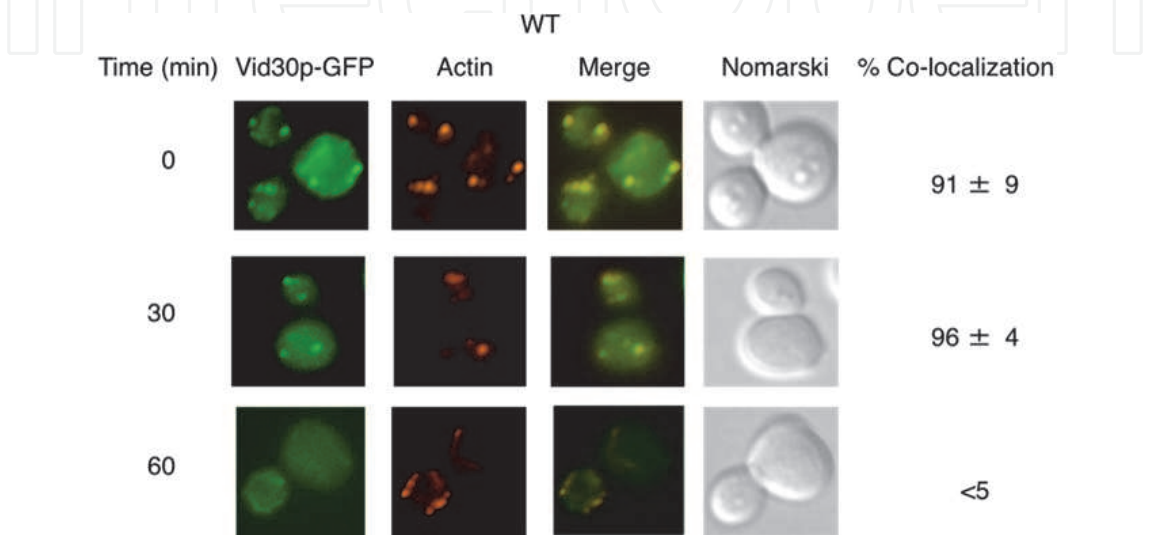
As it has been previously determined that the Vid pathway merges with the endocytic pathway, one could propose that association of Vid vesicles and actin patches may be a pivotal point of this integration. In that endeavor, the *VID30* gene was identified as a putative candidate involved in the Vid pathway using a transposon library screen [64]. This gene encodes a protein that has been previously reported to be involved in the proteasomal degradation of FBPase [65]. Vid30p forms a complex with Vid24p and serves as an E3 ligase in the ubiquitination of FBPase. The requirement of *VID30* in the Vid pathway was verified by examining FBPase degradation in both wild-type and  $\Delta vid30$  cells [64]. After glucose starvation for 3 days and following replenishment, FBPase was degraded in wild-type cells. In contrast, there was an inhibition of FBPase degradation in the  $\Delta vid30$  cells. This indicates that *VID30* is required for the vacuole dependent degradation of FBPase. In order to determine whether Vid30p was distributed to Vid vesicles, wild-type cells expressing Vid30p were glucose starved for 3 days followed by replenishment for up to 20 min. The cells were then subjected to differential centrifugation. Vid30p levels were enriched in the Vid vesicle enriched fraction. This infers that Vid30p is distributed to Vid vesicles.

Using pulldown assays, it was determined that Vid30p interacts with Vid24p and Sec28p under our growth conditions. Moreover, FBPase does not associate with this Vid30p-Vid24p complex. This further supports the notion that FBPase and Vid24p exist in topologically different environments. Thereafter, the effect of the absence of *SEC28* on the interaction of Vid30p and Vid24p was examined using pulldown assays. In this study, Vid30p was pulled down and the levels of Vid24p was examined the bound and unbound fractions. In the  $\Delta sec28$  mutant, the level of Vid24p in the bound fraction was diminished in comparison to that observed in wild-type cells. This indicates that Sec28p is required for the association of Vid30p with Vid24p. Furthermore, the absence of *VID24* also resulted in diminishing the interaction of Vid30p with Sec28p.

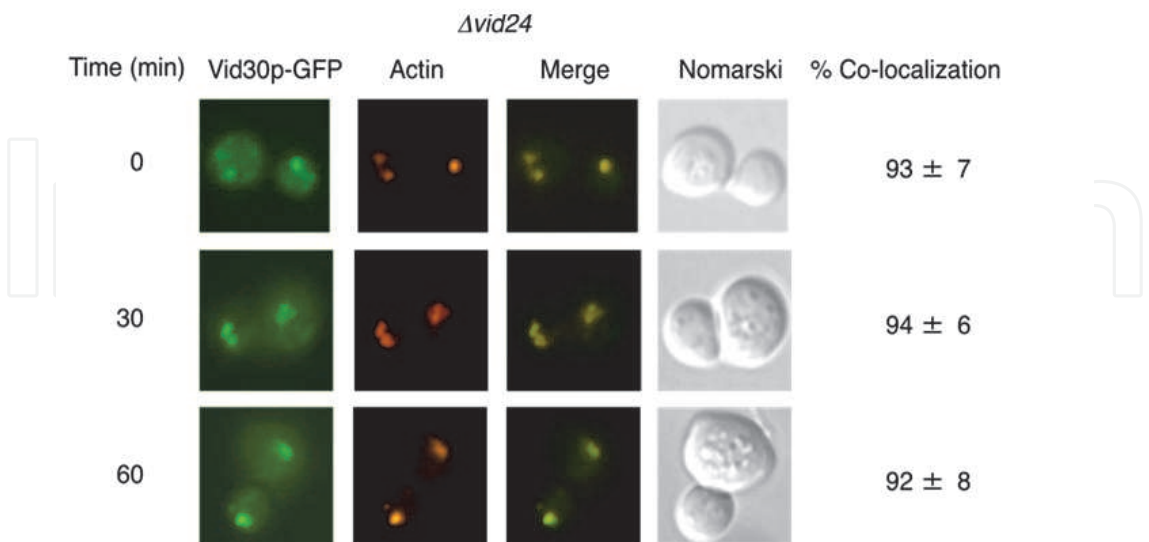
The co-localization of Vid30p with actin patches was studied using fluorescent microscopy. In wild-type cells, it was ascertained that Vid30p was co-localized with actin patches during glucose starvation and following glucose replenishment for up to 30 min (Figure 5) [64]. By the 60 min time point, the localization of Vid30p to actin patches began to diminish. In the absence of *VID24*, Vid30p co-localization with actin patches was prolonged following glucose replenishment (Figure 6) [64]. The absence of *SEC28* also prolonged the Vid30p co-localization to actin patches. This suggests that *SEC28* and *VID24* mediate the dissociation of Vid30p and actin patches. Interestingly, deletion of genes involved in the later steps of actin polymerization, such as *RVS161*, also resulted in prolonging the co-localization of Vid30p with actin patches.

Differential centrifugation was used to determine the step of the Vid pathway that requires the *VID30* gene. In this study, wild-type and  $\Delta vid30$  cells were glucose starved and replenished with glucose for 20 min. By differential centrifugation, it was determined that

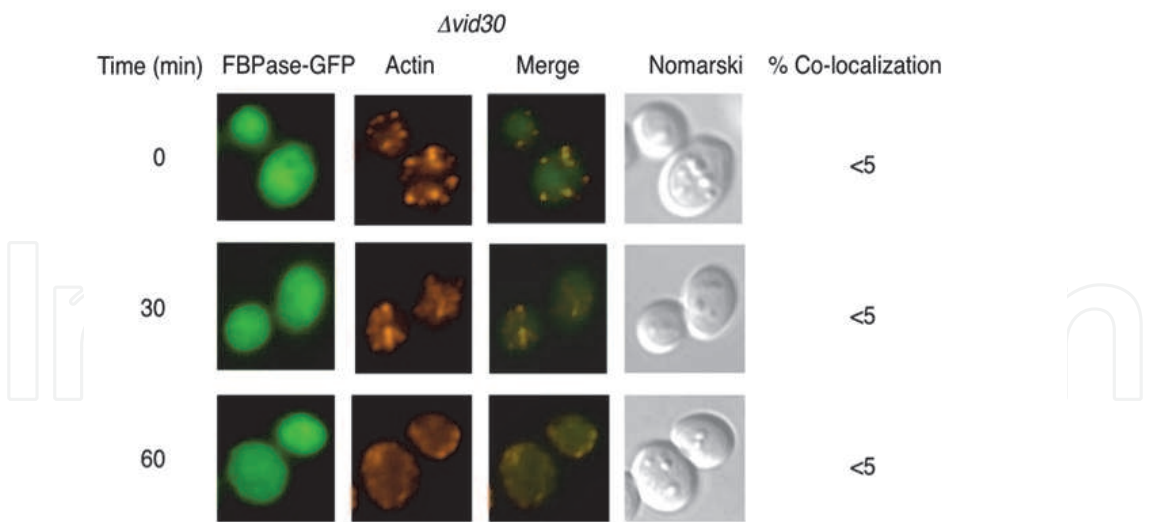
FBPase was detected in both the Vid vesicle and cytosolic fractions in *Δvid30* cells. This is similar to what was observed for wild-type cells. Moreover, most of Vid24p was detected in the Vid vesicle fraction in the *Δvid30* mutant. This suggests that Vid vesicle formation occurs in the absence of the *VID30* gene. Even though Vid30p is distributed to multiple compartments, the deletion of this gene has no impact on the levels of FBPase and Vid24p in the Vid vesicle fraction. Moreover, it was determined that FBPase (Figure 7) and Vid24p (Figure 8) failed to co-localize with actin patches in *Δvid30* cells [64]. This suggests that Vid30p is required for the association of Vid vesicles and actin patches.



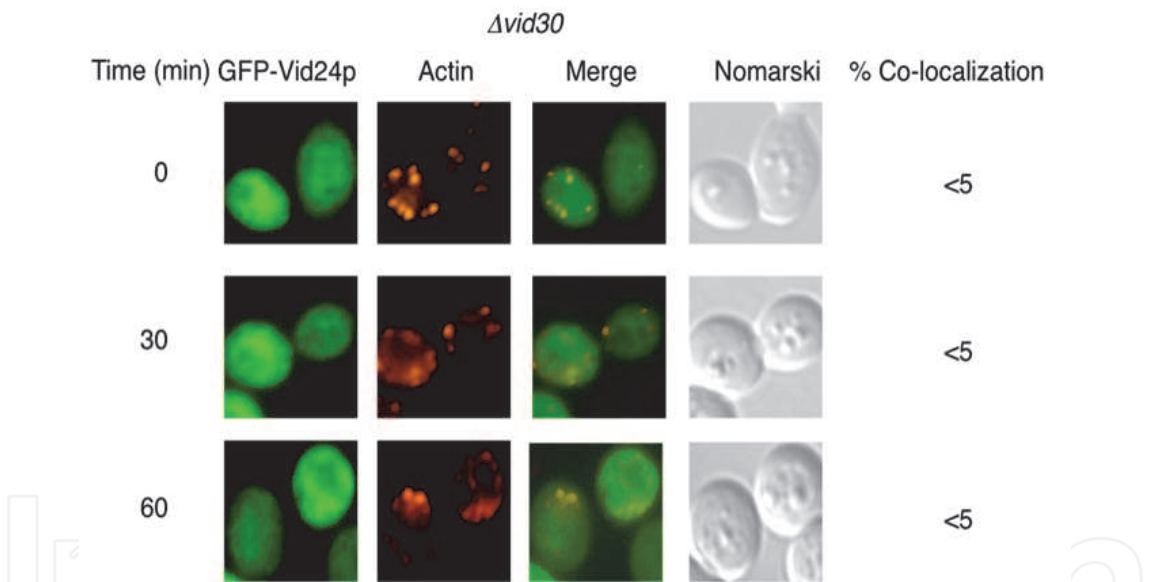
**Figure 5.** Vid30p co-localizes with actin patches in wild-type cells. Vid30p co-localizes to actin patches in wild-type cells during glucose starvation and for up to 30 min following glucose replenishment. Co-localization of Vid30p with actin patches diminishes by the 60 min time point. This research was originally published in *Autophagy* (2012, vol. 8(1), pgs. 29-46). © Landes Bioscience.



**Figure 6.** Vid30p co-localization with actin patches is prolonged in the null mutant of *VID24*. Vid30p is co-localized with actin patches during glucose starvation and for up to 60 min following glucose replenishment in the *Δvid24* strain. This research was originally published in *Autophagy* (2012, vol. 8(1), pgs. 29-46). © Landes Bioscience.



**Figure 7.** FBPase fails to co-localize with actin patches in *Δvid30* cells. During glucose starvation and following glucose replenishment for up to 60 min, FBPase fails to co-localize to actin patches in *Δvid30* cells. This research was originally published in *Autophagy* (2012, vol. 8(1), pgs. 29-46). © Landes Bioscience



**Figure 8.** Vid24p fail to co-localize with patches in *Δvid30* cells. During glucose starvation and following glucose replenishment for up to 60 min, Vid24p fails to co-localize to actin patches in *Δvid30* cells. This research was originally published in *Autophagy* (2012, vol. 8(1), pgs. 29-46). © Landes Bioscience.

Vid30p contains two domains, a LisH (lissencephaly type 1-like homology) and CTLH (C-terminal to the LisH) domain. It has been previously reported that the *LIS1* gene is mutated in Miller-Dieker lissencephaly, a medical condition that contributes to retardation and premature mortality. In addition, the CTLH domain has been postulated to be involved in microtubule function. It was next determined if these domains play a role in the vacuole dependent degradation of FBPase. In either deletion of LisH or CTLH domain in the *VID30* gene, FBPase degradation was inhibited. Deletion of either domain also resulted in

diminishing the association of Vid30p with Vid24p, and also with Sec28p. Vid30p, in which either LisH or CTLH domain had been deleted, was observed to be distributed to Vid vesicles and actin patches. Unlike in wild-type cells, the mutant Vid30p failed to be distributed to the vacuole membrane and was observed to aggregate in punctate structures. Similarly, upon deleting either domain, FBPase was also observed to localize to punctate structures. This indicates that the LisH and CTLH domains of Vid30p are involved in the later steps of the Vid pathway. Thus, in summation, *VID30* is required for the association of Vid vesicles and actin patches, and that the LisH and CTLH domains also required at a later step in the Vid pathway.

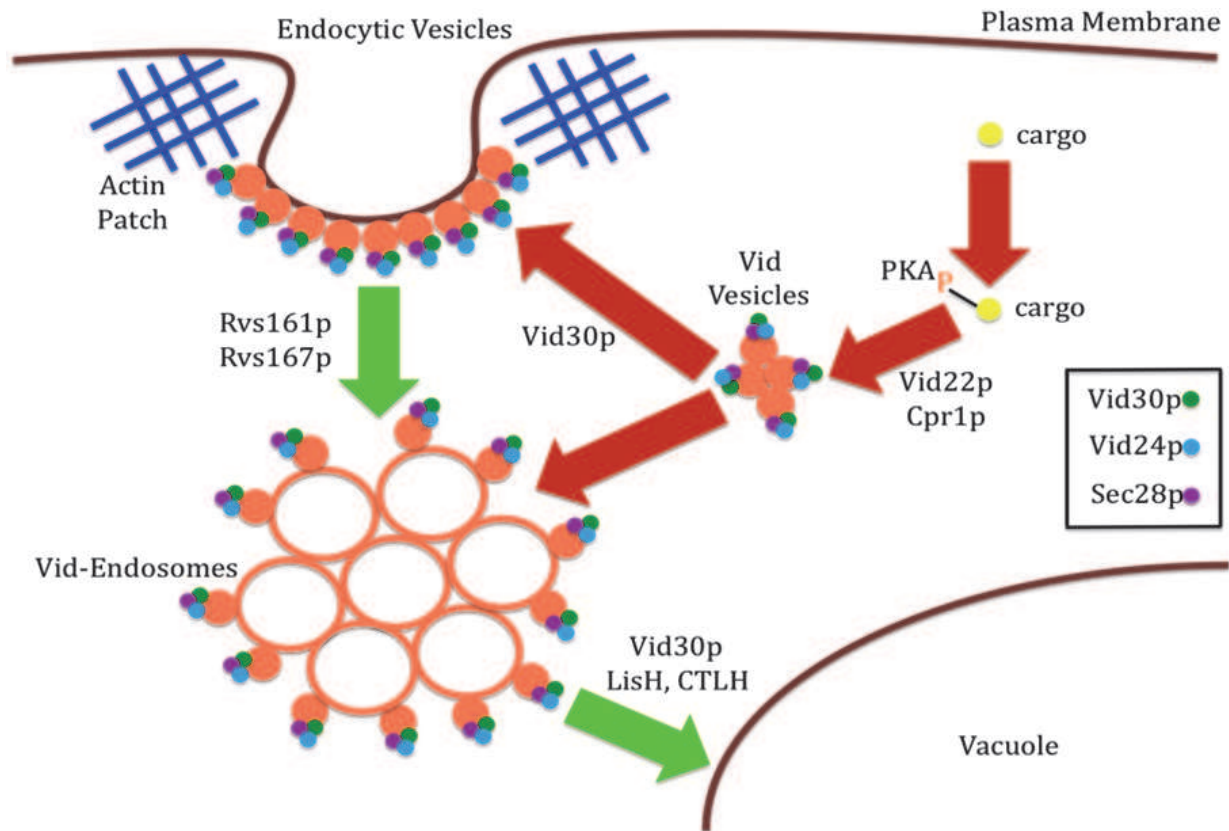
## 10. Proposed model for the Vid pathway

Based on the above findings, we postulate the following model for the vacuole import and degradation pathway (Figure 9). When *Saccharomyces cerevisiae* are grown under glucose starvation conditions, this induces synthesis of gluconeogenic enzymes such as FBPase, MDH2, Pck1p and Icl1p. Vid30p, Vid24p and Sec28p are present as a complex and are distributed on free Vid vesicles and on Vid vesicles aggregating around endocytic vesicles at the actin patch sites. Ubc1p has been implicated in the biogenesis of Vid vesicles. Moreover, Vid30p is required for the association of actin patches to Vid vesicles. In response to glucose, PKA facilitates in the phosphorylation of cargo proteins. Vid vesicles are distributed freely in the cytoplasm and also aggregate around the endocytic vesicles forming from the plasma membrane. The cargo proteins are sequestered into Vid vesicles and this step requires Vid22p and Cpr1p. Vid30p facilitates in the association of free Vid vesicles and actin patches. The amphiphysin module proteins (Rvs161p and Rvs167p) mediate the scission of Vid-endocytic vesicles and these are released as Vid-endosomes into the cytoplasm. Thereafter, the free Vid vesicles also accumulate around the Vid-endosomes to form large Vid-endosome clusters. The LisH and CTLH domains of Vid30p are required for the delivery of Vid-endosome clusters to the vacuole for degradation of cargo proteins. Overall, our current model highlights the association of Vid vesicles to actin patches as mediated by Vid30p as a crucial step in the degradation of gluconeogenic enzymes in the vacuole.

## 11. Future directions

Many questions remain to be answered concerning the degradation of cargo proteins by the Vid pathway. A pivotal question surrounding the mechanism that regulates the degradation of gluconeogenic enzymes by the proteasomal pathway versus the Vid pathway requires further elucidation. Previously, differential modification of cargo proteins following glucose replenishment has been attributed to dictate the site of degradation. For instance, proteins that are degraded in the proteasome are ubiquitinated prior to their degradation. In contrast, cargo proteins that are degraded in the vacuole are subject to phosphorylation by PKA before degradation [66]. Intriguingly, what signaling stimulus regulates this transition from degradation in the proteasome versus that in the vacuole? This warrants further elucidation.





**Figure 9.** Current model of the Vid pathway. Growth of yeast cells under glucose starvation conditions induces the synthesis of gluconeogenic enzymes (cargo). Vid30p, Vid24p and Sec28p are present as a complex on free Vid vesicles and on those that are clustered around the endocytic vesicles at the site of actin patches. Following glucose replenishment, cargo proteins are phosphorylated by PKA and are subsequently imported into the Vid vesicles. Import of cargo into Vid vesicles requires Vid22p and Cpr1p. Vid30p mediates the association of free Vid vesicles and actin patches. Thereafter, Rvs161p and Rvs167p facilitate the scission of Vid-endocytic vesicles that are released into the cytoplasm as Vid-endosomes. The free Vid vesicles also aggregate with the Vid-endosomes to form larger clusters of Vid-endosomes. Finally, the Vid-endosome clusters deliver their cargo to the vacuole for degradation, and this step requires the LisH and CTLH domains of Vid30p.

A second concern pertains to the origin of Vid vesicles. According to one proposal, Vid vesicles may be derived from the plasma membrane or the sites of internalization. Alternatively, Vid vesicles may originate from the vacuole membrane as retrograde vesicles. Deletion of genes involved in plasma membrane internalization, such as *Arvs161* or *Arvs167*, contributed in prolonging the association of Vid vesicles to actin patches [51]. Perhaps Vid vesicles are components of endosomes. It is interesting to note that the Vid-endosome clusters that are formed following glucose replenishment share morphological similarities to multivesicular bodies. Moreover, the importance of the early steps of endocytosis and actin polymerization for the vacuole dependent degradation of cargo proteins requires further analysis. This could imply that cargo proteins are secreted out of the cells and then internalize at actin patch sites. As cargo proteins do not contain the ER-Golgi secretory signal sequence, this could facilitate in the understanding of the non-classical secretory pathway.

A comprehensive understanding of the Vid pathway could have significant implications in studying the etiology of diseases associated with abnormal gluconeogenesis in humans. And this could aid in developing therapeutics that regulate gluconeogenesis and treat the subsequent malady. For example, it has been previously reported that patients afflicted with Type II diabetes also suffer from an increase in levels of gluconeogenesis [67]. And an FBPase inhibitor called managlinat dialanetil has proven to be relatively successful in the treatment of Type II diabetes [67]. Another example is that FBPase may be attributed to cause clonorchiasis-associated hepatic fibrosis owing to the protein's secretion along with excretory products from *Clonorchis sinensis* adult worms [68]. Moreover, studies aimed at evaluating deterioration of the proximal renal tubules have identified FBPase as a crucial marker in this determination [69]. As such, seeking answers to the above questions will enable us in the development of treatments that will help improve the quality of life for the general public.

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