ABSTRACT

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Nick Zaban / Biochemistry
Author Name/Major

Author Signature

5/17/08
Date

Xiaofeng Wang
Institute for Molecular Virology
Mentor Name/Department

Mentor Signature

COVER SHEET

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AUTHOR'S NAME: Nick Zaban

MAJOR: Biochemistry

DEPARTMENT: Biochemistry

MENTOR: Xiaofeng Wang

DEPARTMENT: Institute for Molecular Virology

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The Effects of Doa4 and Other Multivesicular-Body Proteins on Brome Mosaic Virus Replication in Yeast

Nick Zaban
University of Wisconsin-Madison College of Agricultural and Life Sciences
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Mentor: Xiaofeng Wang, Institute for Molecular Virology

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Abstract

Brome Mosaic Virus (BMV) replication was measured in yeast single and double deletion mutants of Doa4 and other proteins in the multivesicular-body (MVB) pathway in order to determine the role of Doa4 and other MVB proteins in BMV replication. BMV is a positives-strand RNA virus that is studied as a model for many other RNA viruses. The MVB pathway packages proteins into endosomes and designates them for degradation. One MVB protein, Doa4, has been shown to affect BMV replication. Deletion mutants were transformed with plasmids encoding for BMV. Northern blotting and Western blotting were used to detect the accumulation of BMV RNAs and proteins in order to determine the degree of BMV replication in each mutant. One mutant containing the deletion of Doa4 and another MVB protein, Bro1, demonstrated BMV replication 60% of wild-type. This indicates that the deletion of Bro1 partially restores BMV replication when Doa4 is deleted.

Introduction

BMV is a plant virus that infects cereal grains and grasses throughout most of the world. BMV is one of many viruses in the alphavirus superfamily of positive-strand RNA viruses, and is commonly studied as a model for other positive strand RNA viruses such as Hepatitis C, the West Nile virus, foot and mouth disease, the virus responsible for severe acute respiratory syndrome (SARS), and many others (Ahlquist, 1998, Schwartz *et al*., 2002, and Kushner *et al*, 2003). The BMV genome is composed of three single-stranded RNAs. RNA 1 codes for the 1a helicase, RNA 2 codes for the 2a polymerase,

and RNA 3 codes the 3a protein that causes the spread of the virus from cell to cell. RNA 3 also serves as a template for a negative-strand subgenomic RNA 4, which encodes the protein coat of BMV (Ahlquist 1998). Even though BMV is a plant virus, the yeast *Saccharomyces cerevsiae* can serve as a host for BMV replication (Janda and Ahlquist, 1993). BMV has been studied in yeast because yeast grows quickly, has been well studied as a model for many basic cellular processes, and its genome has been completely sequenced (Noueiry and Ahlquist, 2003). BMV is researched in order to understand its replication and many complex molecular virus host interactions, serving as a model for the numerous other positive strand RNA viruses.

BMV replicates in the cell using the host's own components to facilitate the process. BMV protein 1a forms membrane spherules that envaginate from the host perinuclear endoplasmic reticulum in order to replicate the viral RNA. Viral RNA is replicated in these spherules by the 2a polymerase and 1a protein into complementary negative-strand RNA that then serves as a template to make more progeny positive-strand RNA. These membrane spherules provide a surface to collect and sequester replication factors and viral RNA, and protect the RNA from nucleases in the host that would degrade the nucleic acid. These replication spherules or similar structures have been identified in association with viral replication in all the studied positive-strand RNA viruses. (Ahlquist *et al.*, 2003, Noueiry and Ahlquist, 2003).

One host factor that has been shown to affect BMV replication is Degradation of Alpha 4, or Doa4. Doa4 is a protease that cleaves the polypeptide ubiquitin from substrate proteins, thereby deubiquitinating them. It is one of 16 proteins in *S. cerevisiae* that are categorized as ubiqutin-specific proteases, a family with well-conserved regions

shared among all of its members. Doa4 functions in two intracellular protein degradation pathways, de-ubiquitinating soluble proteins prior to their degradation in the proteasome, and de-ubiquitinating membrane-associated proteins in the multivesicular-body (MVB) pathway that are designated for degradation in the vacuole. Doa4 prevents the degradation of ubiquitin, therefore playing an important role in conserving ubiquitin and maintaining ubiquitin homeostasis (Amerik *et. al*, 2000, Swaminathan *et. al*, 1999).

The MVB pathway assembles proteins on the plasma membrane into endosomes that mature into multivesicular-bodies that fuse with either the vacuole in yeast or the lysosome in mammalian cells and degrade the MVB cargo protein. Many proteins are involved in vacuolar proteins sorting. 17 proteins are designated as Class E Vacuolar Protein Sorting (Vps) proteins, and these proteins are essential to the MVB pathway. Mammalian orthologs of Class E Vps proteins function in the MVB pathway in mammals, along the with mammalian Doa4 ortholog, UBPY. Tagging proteins with ubiquitin is important for targeting proteins for entry into and sorting within the MVB pathway. MVB cargos transiently associate sequentially with four ESCRT (Endosomal Sorting Complex Required for Transport) complexes, ESCRT-0, I, II, and III, and then the late endosome fuses with the lumen of the vacuole. Doa4 deubiquitinates MVB cargo proteins associated with ESCRT-III (Katzmann *et al*, 2002, Piper and Katzmann, 2007).

Deletions of Doa4 and other proteins involved in the MVB pathway have been previously shown to reduce BMV replication. BMV replication decreases well below wild-type levels in yeast strains with individual deletions of several Class E Vps proteins, including Bro1, Did2, Vps2, Vps4, Vps24, and Vta1. Vps2 and Vps24 are two of the four proteins that form the core of the ESCRT-III complex, forming a Vps2-Vps-24

subcomplex. The Vps2-Vps24 subcomplex is important for the recruitment and positioning of Doa4 in the MVB pathway (Katzmann *et*, 2002). Bro1 is important in regulating Doa4 activity in the MVB pathway. Bro1 recruits Doa4 to the ESCRT-III complex by associating with its non-catalytic N-terminal region, and also acts as a cofactor for Doa4 by interacting with the Doa4 catalytic domain and stimulating Doa4's de-ubiquitination activity (Richter *et al*, 2007).

Vps4, Did2, and Vta1 function downstream of Doa4 in the MVB pathway. Vps4 is AAA-type ATPase that catalyzes the disassociation of the ESCRT-I, ESCRT-II, and ESCRT-III complexes. The core ESCRT-III protein Vps2 can directly stimulate Vps4. In addition, Did2 and Vta1 are important in facilitating the disassociation of ESCRT-III by Vps4. Did2 is recruited to the ESCRT-III complex by interactions with Vps2 and Vps24. Did2 then recruits Vta1 and Vps4 to the ESCRT-III complex. Vta1 binds directly to Did2 and with Vps60, both ESCRT-III accessory components. Vta1 stimulates Vps4 ATPase activity. Did2 is also required for the disassociation of Bro1 and Doa4. Did2 does not function in the Vps4 mediated disassociation of ESCRT-I or ESCRT-II complexes, only ESCRT-III (Azmi *et al*, 2008, Nickerson *et al*, 2006).

In ΔDoa4 yeast deletion mutants, BMV replication is reduced to less than 10% of the level of replication in wild-type yeast (Kushner *et al*, 2003). ΔDoa4 cells have been shown to have significantly reduced levels of Ole1 Δ9 fatty acid desaturase, which converts saturated fatty acids to unsaturated fatty acids. Ole1 is crucial in providing the lipids to form the membranes necessary for BMV replication. Ole1 is regulated by transcription activators Spt23 and Mga2, both of which must be ubitquitinated in order to be in their active forms. Therefore, the deletion of Doa4 reduces levels of free ubiqutin,

which reduces the expression of Ole1 and affects the cellular lipid composition necessary for BMV replication. BMV replication in ΔDoa4 cells is partially rescued by complementation with Ole1. However, since replication is not fully rescued, it indicates that Doa4 affects other cellular processes besides Ole1 expression that facilitate BMV replication (Wang *et al*, 2007).

In order to determine additional roles of Doa4 in BMV replication and the relationships of Doa4 with other MVB components in BMV replication, the replication of BMV will be analyzed in double deletion mutants of *S. cerevisiae* with deletions of both Doa4 and one of the following Vps proteins: Bro1, Did2, Vps2, Vps4, Vps24, and Vta1. Deletions of Did2, Vps4, and Vps24 have been shown to suppress mutations of Doa4 in *S. cerevesiae* (Amerik *et al*, 2000). This study will investigate if the deletion of Vps proteins suppresses the Doa4 mutation in regard to BMV replication. This will determine the step in the MVB pathway in which BMV replication is affected by Doa4, and the Vps proteins involved in this step. These findings on the role of the MVB pathway in BMV replication will be used to draw conclusions about the role that the MVB pathway plays in the replication of other positive-strand RNA viruses.

Materials and Methods

Yeast and Plasmid Preparation

The wild-type strain of *S. cerevisiae* used was BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The following single deletion mutants were constructed by deleting the indicated gene: ΔDoa4, ΔBro1, ΔDid2, ΔVps2, ΔVps4, ΔVps24, ΔVta1. In addition, the following double deletion mutants were constructed by deleting the two indicated

genes: ΔDoa4ΔBro1, ΔDoa4ΔDid2, ΔDoa4ΔVps2, ΔDoa4ΔVps4, ΔDoa4ΔVps24, ΔDoa4ΔVta1. The strains were grown on YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar), and then competent cells were made and plasmid transformations were performed using a Frozen EZ Yeast Transformation Kit (*Zymo Research*).

Two plasmids were transformed into each strain, pB12AON3, and pB3VG128-H. pB12AON3 contains genes for the expression of the BMV 1a and 2a viral proteins. Both of these genes are regulated by endogenous ADH promoters. In addition, the plasmid contains a leucine selection marker. pB3VG128-H codes for BMV RNA3 regulated by a CUP1 promoter, and also contains a histidine selection marker. The transformations were grown on minimal media plates (0.7% yeast nitrogen base, 2% dextrose, 2% agar, 0.14% each essential amino acid) lacking histidine and leucine in order to screen for the successful transformation of both plasmids. The plates were incubated at 30° C for a period of two to four days. Five colonies from each strain were streaked onto minimal media and then stored at 4° C.

Three of the yeast colonies from each strain were scraped from plates and grown in 3 mL of dextrose minimal media. In addition, 3 colonies of wild-type, ΔDoa4, ΔBro1, and ΔDoa4ΔBro1 strains were grown in 3 mL galactose minimal media. The cells were grown until they reached 1 - 3 OD_{600}/mL. The cells were then re-cultured in 8 mL dextrose or galactose media, and harvested mid-logarithmic phase at 0.5 – 1.0 OD_{600}/mL. 2 or 3 OD_{600} of each culture were harvested for protein extraction, while the rest of the cultures were harvested for RNA extraction. Cells were pelleted and stored at -80° C.

RNA Extraction and Analysis

RNA was analyzed using Northern blotting. RNA was extracted using a hot phenol protocol. Cells were re-suspended in RNA buffer A (50 mM NaOAc pH 5.2, 10 mM EDTA, 1% SDS). 65° C saturated phenol was added, and then cells were incubated for 5 minutes in 65° C. The phenol was used to lyse the cells and denature cellular proteins. The buffer A/phenol mixture was centrifuged, and then the aqueous phase with buffer A was removed and vortexed with a tris-saturated phenol/chloroform solution used to extract the phenol. The aqueous chloroform layer was removed and precipitated in EtOH and NaOAc, pH 5.2, for 15 – 30 minutes at -80° C. The RNA was pelleted, washed with 80% EtOH, and resuspended in ddH_20. The RNA concentration was measured using a *Nanodrop* ND-1000 spectrophotometer. Equal masses of RNA from each sample were resuspended in loading buffer containing ethidium bromide and formaldehyde to prevent RNA degradation. The samples immersed in buffer were incubated at 65° C to denature secondary structures, cooled on ice, and then loaded into agarose gel (1% agarose, 1x MOPS solution, 5% formaldehyde) and run at 150V for approximately two hours. The 18S RNA bands were observed using an *Alpha Innotech* gel imager in order to insure that RNA samples were loaded equally. The RNA was transferred to a 0.45 transfer membrane using a 10x SSC (9% NaCl, 4.5% sodium citrate) transfer buffer. The membranes were cross-linked with UV light in order to protect RNA and then blocked with NorthernMax Pre-hybridization/Hybridization buffer (*Ambion*). Radioactive probes containing $\propto^{32}$P-UTP were used to detect the presence of (+) RNA3, (-) RNA3, and Ole1 RNA on the membranes. The membranes were washed and then placed in a phosphor screen. The phosphor screens were scanned with a Typhoon

Scanner (*GE Healthcare)*, and the intensity of the radioactive bands detected by the scanner were measured to determine the relative RNA concentrations in each sample.

Protein Extraction and Analysis

Proteins were analyzed using Western blotting. Protein was extracted from either 2 or 3 OD_{600} of yeast from each samples. The cells were lysed with glass beads and a yeast lysis buffer containing several protein inhibitors. A *Biospec* mini-beadbeater was used to thoroughly lyse cells. The proteins were extracted from the lysate by adding a SDS lysis buffer, boiling, and then centrifuging. Proteins were separated using SDS-page gel electrophoresis. *Bio-Rad* 5-15% and 5-20% acrylamide pre-cast gels were used for electrophoresis. The proteins were transferred to immobilon polyvniylidene fluoride membranes using an electrophoretic transfer apparatus. Membranes used to detect for ubiquitin were autoclaved for an hour and 20 minutes at 121° C in order to denature deubiquitinases. The membranes were blocked using 2.5% BSA in a 1x TBST buffer. Primary antibodies were added to detect for 1a, 2a, ubiquitin, and PGK proteins. PGK is a host protein used as a standard to normalize the levels of viral proteins and ubiquitin detected. The membranes with primary antibody were incubated overnight at 4° C. Anti-mouse secondary antibodies were used to detect for the presence of 2a, ubiquitin, and PGK primary antibodies, and anti-rabbit secondary antibodies were used to detect for the presence of 1a primary antibody. The secondary antibodies contain a horseradish peroxidase that was used to cleave *Thermo Scientific* chemiluminescent substrate. The chemiluminescene of each sample was detected by a *Bio-Rad* ChemiDoc molecular

imager. The relative chemiluminescene of each sample was used to determine the relative concentrations of 1a 2a, ubiquitin, and PGK.

Results

RNA Analysis

The relative amount of BMV replication was determined by accumulation of BMV (+) 3, (-) 3, and subgenomic (+) 4 viral RNAs in each sample. The volume of RNA in each mutant was divided by the average volume of RNA synthesized in wild-type BY4741, and the amount of replication is expressed as a percentage of wild-type. In yeast strains with deletion of Vps2, Vps24, Vps4, Did2, or Vta1, BMV RNA replication was severely blocked by 3-5 fold. Bro1 supported close to wild type BMV RNA replication. Vps4 deletion mutants did not grow well with BMV components and were not assessed.

To gain additional information on how these mutants affect BMV replication and if they interplay with Doa4 in BMV replication, we made double mutants which all have Doa4 deleted, plus each of the above proteins. All of the double deletion mutants showed much less BMV replication than the corresponding single deletion mutant of a Vps protein without the deletion of Doa4. However, since a single deletion mutant of Vps4 was not analyzed, it is not known whether ΔDoa4ΔVps4 replicates less than ΔVps4. All of the double deletion mutants showed better BMV replication than ΔDoa4. But, with the exception of ΔDoa4ΔBro1 in dextrose, BMV replication was only slightly greater, and many times the percentage difference between ΔDoa4 and double deletion mutants was within the margin of error. The analysis of Ole1 RNA accumulation turned out to be

inconclusive; it did not match previous experimental results. This may be due to the fact that Ole1 RNA degrades fairly the quickly.

The only double deletion mutant to show significant BMV replication was ΔDoa4ΔBro1. ΔDoa4ΔBro1 showed (+) 3 RNA accumulation that was approximately 74% of wild type when cultured in a dextrose medium. However, when ΔDoa4ΔBro1 is cultured in a galactose medium, the RNA accumulation was only approximately 33% of wild type. The (+) 4 and (-) 3 RNA accumulation show about the same level of replication for ΔDoa4ΔBro1 as (+) 3. It is important to note that ΔBro1 had by far the greatest amount of BMV replication. ΔBro1 (+)3 accumulation was 55-100% of wild type RNA accumulation in dextrose. ΔBro1 mutants could not be analyzed in galactose because the mutants exhibited very poor growth and were discarded.

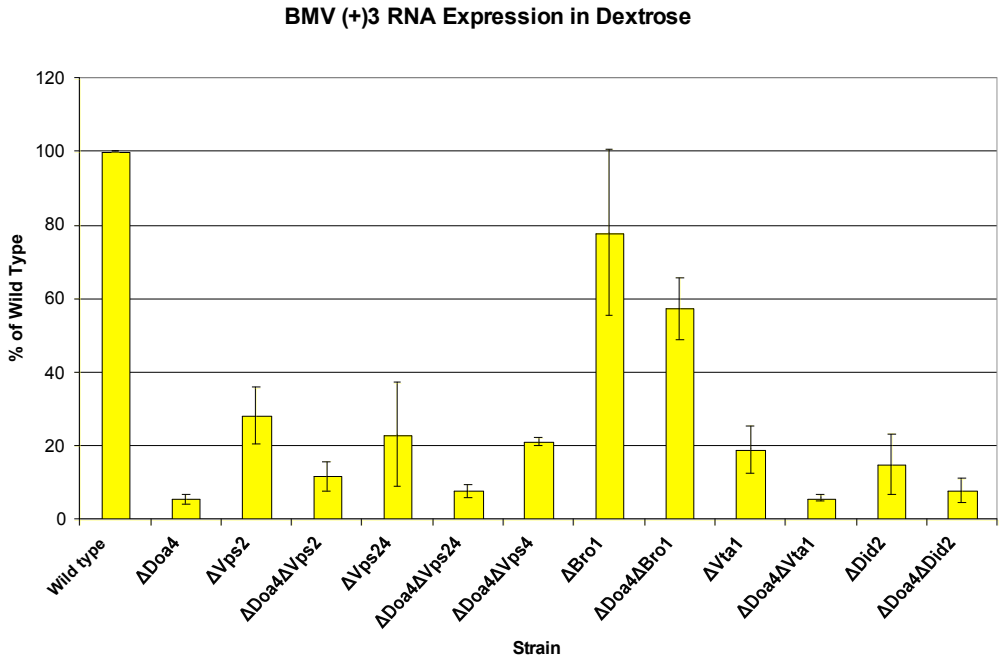

Figure 1: BMV(+3) accumulation in Vps protein single and double deletion mutants in *S. cerevisiae* grown in dextrose.

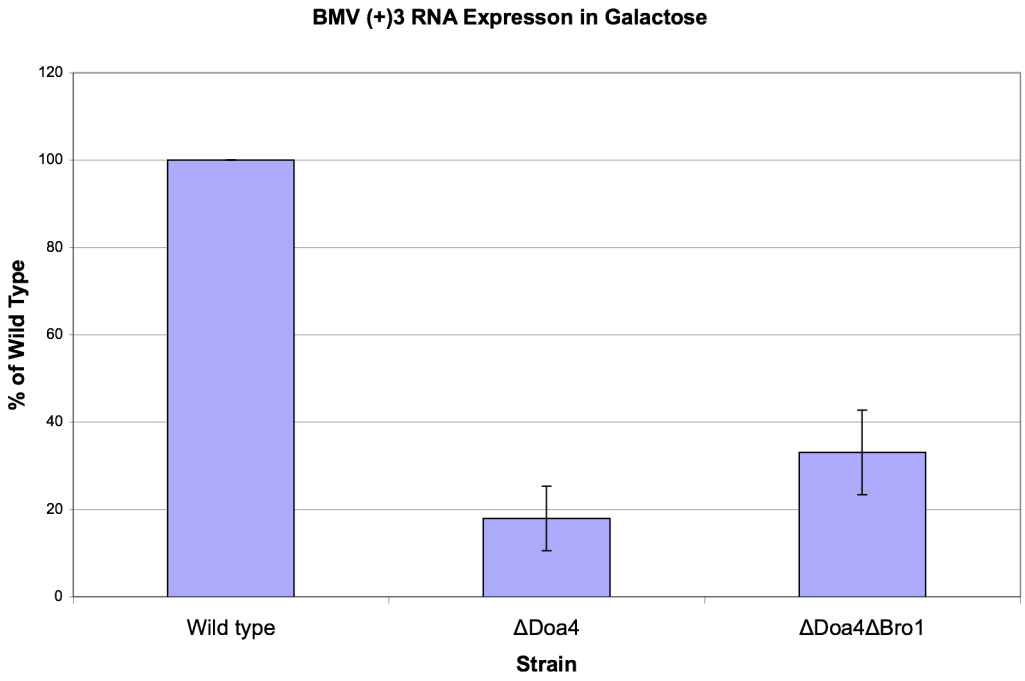

Figure 2: BMV(+3) accumulation in Vps protein single and double deletion mutants in *S. cerevisiae* grown in galactose.

Figure 3: Northern blot images of BMV (+)3 and (-)3 RNA grown in dextrose.

Figure 4: Northern blot images of BMV (+)3 and (-)3 RNA grown in galactose.

Protein Analysis

Western blotting was used to detect the accumulation of BMV 1a and 2a proteins, and the level of free-ubiquitin present in the cell. Except for ΔDoa4 and ΔBro1, protein levels were not obtained for single deletion mutants in this experiment. Many of the protein blots had low resolution and were somewhat inconsistent. In addition, the membrane with PGK proteins had the bands for ΔDoa4 and ΔDoa4ΔBro1 grown in galactose cut off. Therefore, since the other bands could not be normalized by the PGK bands, it was not possible to analyze the PGK samples grown in galactose. Unlike Northern blotting, linear relationships between protein levels cannot be obtained with Western blotting. However, some general trends in terms of protein levels were observed. Almost all of the double deletion mutants had near wild type levels of 1a and 2a proteins. ΔDoa4ΔVps24 was the only strain that had significantly decreased levels of 1a and 2a. ΔDoa4 and all other double mutants had 1a and 2a levels close to wild type.

Previous research shows that ΔDid2, and ΔVps24 deletion mutants have wild-type free-ubiquitin levels, while ΔDoa4 deletion mutants have greatly reduced free-

ubiquitin levels (Amerik, 2000). In this experiment, ΔDoa4 showed a decrease in free-ubiqutin, which is consistent with previous research. ΔBro1 single deletion mutants also showed a decrease in free-ubiquitin compared with wild type. In contrast with ΔDoa4, almost all double deletion mutants with Doa4 and another Vps protein deleted exhibited free-ubiqutin levels at or above wild-type. ΔDoa4ΔDid2, ΔDoa4ΔVps44, ΔDoa4ΔVps24 have previously been shown to have free-ubiquitin levels near wild-type (Amerik, 20000). In this experiment, ΔDoa4Did2, ΔDoa4ΔVps24, and ΔDoa4ΔVta1 had free-ubiquitin levels near wild-type. ΔDoa4ΔVps4 and ΔDoa4ΔBro1 had free-ubquitin levels above wild type. However, since the ΔDoa4ΔVps4 values contradict previous published results, and due to the somewhat inaccurate nature of western blots, it's a more valid conclusion that free-ubiquitin levels for ΔDoa4ΔVps4 and ΔDoa4ΔBro1 are near-wild type, not greater than wild-type. Since ΔDoa4ΔDid2, ΔDoa4ΔVps4, ΔDoa4ΔVps24, ΔDoa4ΔBro1, and ΔDoa4ΔVta1 double deletion mutants with Doa4 and another Vps protein deleted have near wild type ubiquitin levels, while the single deletion of Doa4 alone has reduced free-ubiquitin in the cell, it indicates a recovery in free-ubiquitin in the cell by the deletion of the Vps protein. The exception to this trend is ΔDoa4ΔVps2, which showed reduced levels of free-ubiquitin. Since the amount of free-ubiquitin in ΔVps2 single deletion mutants was not assayed, it is unknown whether the ΔVps2ΔDoa4 double deletion mutant has different levels of free-ubiquitin than the single deletion mutant.

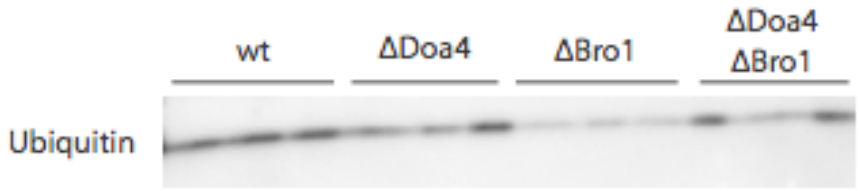

Figure 5: Bands indicate free levels of ubiquitin in cells grown in dextrose. There was one ΔDoa4 colony that had a lot of free-ubiquitin, and a ΔDoa4ΔBro1 that was much lower in ubiqutin. However, these values are most likely outliers that can be disregarded.

Discussion

In this study, it was shown that the Doa4 and the Vps proteins Bro1, Did2, Vps2, Vps24, Vps4, and Vta1 play a role in BMV replication in *S. cerevisiae.* This was proved by the fact that the deletion of each of these proteins caused a decrease in the replication of BMV positive-strand and negative-strand RNA 3. By analyzing the replication of RNA 3 in double deletion mutants, consisting of the deletion of Doa4 and another Vps protein, it was observed that the deletion Bro1 compensates for the deletion of Doa4, restoring BMV RNA 3 replication and cellular levels of free-ubiquitin. Deletions of Did2, Vps2, Vps24, Vps4, and Vta1 were not able to compensate for the deletion of Doa4 and restore BMV replication. BMV RNA 3 replication in ΔDoa4ΔDid2, ΔDoa4ΔVps2, ΔDoa4ΔVps24, ΔDoa4ΔVps4, and ΔDoa4ΔVta1 was very close to RNA 3 replication in ΔDoa4. This probably means that the deletion of Doa4 is the main factor affecting BMV replication in these mutants.

The results lead to the conclusion that a decrease in free-ubiquitin is not the only cause for a decrease in BMV replication in Doa4 deletion mutants. ΔDoa4Did2, ΔDoa4ΔVps24, and ΔDoa4ΔVta1 all had cellular free-ubiquitin levels near wild-type. However, these deletion mutants had BMV RNA 3 replication far below wild-type. Even

though free-ubiquitin levels were restored in the double deletion mutants and significantly greater than the ΔDoa4 single-deletion mutant, BMV replication was not restored. In addition, free-ubiqutitin levels in ΔBro1 were reduced well below wild-type, but RNA 3 replication in this deletion mutant was near wild-type, and greater than any other single or double-deletion mutant. This indicates that there is a component of replication other than free-ubiquitin that is regulated by Doa4 and other Vps proteins. Since the resolution on ubiqutin protein blots was poor, there were some lanes that had results that were large outliers, and the standard deviation of ubiqutin volume was high. Therefore, the analysis of free-ubiqutin in double deletion mutants should be repeated. In addition, protein level analysis should be done on single deletion mutants in order to determine the levels of free-ubiqutin in all of the single-deletion mutants. These results should then be compared with the levels of free-ubiquitin in the double deletion mutants. Also, single and double-deletion mutants could be complemented with free-ubiquitin added to the cell. If double-deletion mutants complemented with ubiquitin exhibited reduced BMV replication, it would further indicate that Doa4 plays an additional role in BMV replication besides reducing the amount of free-ubiquitin in the cell.

It is interesting to note that the deletion of one Vps protein, Bro1, restored BMV replication in Doa4 double-deletion mutants, while deletion of all of the other Vps proteins did not restore replication. This may be due to the difference in the role that Bro1 plays in the MVB pathway in contrast to the roles that Did2, Vps2, Vps24, Vps4, and Vta1 play in the MVB pathway. Did2, Vps2, Vps24, Vps4, and Vta1 are all either core components of the MVB ESCRT-III complex, or they play a key role in disassembling the complex. Bro1, however, does neither. It recruits Doa4 to the

ESCRT-III complex, but does not affect the assembly or disassembly of the complex. Since the deletion of Bro1 restores BMV replication when Doa4 is deleted, while the deletion of ESCRT-III components does not restore BMV replication, it shows that the proper functioning of the ESCRT-III complex is essential in BMV replication. Without the proper formation and disassembly of the ESCRT-III complex, the deletion of Doa4 cannot be overcome and BMV replication is not restored. In order to further test this conclusion, single and double deletion mutants of other components of the ESCRT-III complex could be analyzed.

One protein that could be subject for further analysis is Snf7. Snf7 forms a subcomplex with Vps20 that is part of the ESCRT-III complex (Katzmann *et al*, 2002). The deletion of Snf7 also compensates for the deletion of Doa4 and restores free-ubiquitin levels (Amerik *et al*, 2000). ΔDoa4ΔSnf7 double deletion mutants were constructed and transformed with BMV, but they did not grow in liquid media. Vps4 could also be the focus of further study. Vps4 is the ATPase that disassembles the ESCRT-III complex, and it interacts with Vps2, Did2, and Vta1. Because it has a central role in the functioning of the ESCRT-III complex, studying double-deletion mutants of Vps4 and other Vps proteins could give further insight on the role of the ESCRT-III complex and the MVB pathway in BMV replication.

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