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# *Schizosaccharomyces pombe* Pep12p is required for vacuolar protein transport and vacuolar homotypic fusion

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**In eukaryotic cells, SNARE proteins are essential for intracellular vesicle trafficking. Several SNARE proteins are required for vacuolar protein transport and vacuolar biogenesis in *Saccharomyces cerevisiae*. Previously we demonstrated that one of the fission yeast SNARE proteins, Pep12p, is not required for vacuolar fusion process in *Schizosaccharomyces pombe*. We have re-examined the function of *S. pombe* Pep12p using the newly created *pep12*$^+$ deletion strain. Deletion of the fission yeast *pep12*$^+$ gene results in pleiotropic phenotypes consistent with the absence of normal vacuoles, including missorting of vacuolar carboxypeptidase Y-and various ion- and drug-sensitivities. GFP-Pep12 fusion protein is mostly localized at the vacuolar membrane and the prevacuolar compartment. The *S. pombe pep12*Δ mutation phenocopies that of *vps33*Δ, suggesting that both Pep12p and Vps33p act at the same membrane fusion step in *S. pombe*, and both mutations cause vacuolar deficiency.**



[**Key words:** *Schizosaccharomyces pombe*; SNARE; Vacuolar protein sorting; Syntaxin; Membrane trafficking]

In eukaryotic cells, properly-folded proteins are transported from the endoplasmic reticulum to the Golgi complex for further packaging and transport. This function is mainly performed through vesicle trafficking which plays a central role in the formation and maintenance of different intracellular compartments as well as in communication between cells and their environment. Several processes including tethering, docking, and fusion mediate the fusion of transport vesicles with target organelles. The docking of vesicles with an organelle occurs through the action of SNARE (soluble NSF attachment protein receptors) proteins leading to membrane fusion and mixing of lipid components. SNAREs were originally isolated from bovine brain (1), and from membrane-anchored proteins that have a coiled-coil region. Subsequent trans pairing of vesicle and target membrane SNAREs (v-SNAREs and t-SNAREs, respectively) docks the vesicles in close apposition which is followed by hemifusion and pore opening (2–4).

A total of 24 SNARE proteins have been identified in *Saccharomyces cerevisiae*(5). In *S. cerevisiae*, several SNARE proteins act not only in protein transport but also homotypic vacuolar fusion. Vam3p (6), Vam7p (7), Pep12p (8), Vti1p (9), and Ykt6p (10) are SNARE proteins that function in vacuolar protein transport and vacuolar membrane fusion (11). Pep12p and Vam3p from budding yeast are both t-SNAREs of the syntaxin family that are components of these SNARE complexes. High expression of *PEP12* suppresses the protein transport and vacuole morphology defects in *vam3*Δ mutants. Similarly, high expression of *VAM3* suppresses the protein transport defect and vacuole morphology defect of *pep12*Δ mutant (6). Thus, two SNARE proteins, Pep12p and Vam3p, are key components in vacuolar protein transport and vacuolar membrane fusion, respectively.

The fission yeast *Schizosaccharomyces pombe* is taxonomically and evolutionarily distant from budding yeast and has a large number of small vacuoles under normal conditions. Osmotic stress caused transitory fusion of vacuoles in *S. pombe*. *S. pombe ypt7*Δ and *vps33*Δ mutant have severe vacuolar morphology defects (12,13). These results indicate that Ypt7p (Rab7 GTPase homolog) and Vps33p (Sec1 family) proteins are also required for vacuolar fusion (14–16), suggesting that SNARE proteins must function in the process of vacuolar membrane fusion in *S. pombe*.

Previously, BLAST searches of the *S. pombe* genome database with the SNARE motif identified a total of 17 ORFs encoding SNARE-related proteins (17). We reported that the SPAC6F12.03c gene (*fsv1*$^+$) is required for delivery of carboxypeptidase (SpCPY) to the vacuole in *S. pombe*. Fsv1p is localized to the Golgi apparatus and prevacuolar membranes in *S. pombe*. However, Fsv1p is not required for vacuolar fusion *in vivo* (17). We also reported that the fission yeast *pep12*Δ (SPBC31E1.04) mutant did not exhibit a vacuolar sorting defect or vacuolar morphologies (17). Therefore, we have not identified which SNARE proteins are required for vacuolar fusion in *S. pombe*. Further, in our inspection of the current genome database, we did not find a fission yeast equivalent of Vam3p.

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During construction of a double mutant carrying disruptions in two SNARE genes required for normal vacuolar morphology in *S. pombe*, we noticed that *pep12* mRNA was still detected in a *pep12*Δ strain by Northern blotting analysis. We also determined that *S. pombe* Pep12p is required for vacuolar transport and normal function by using the reconstructed *pep12*Δ strain. To correct information about function of Pep12p, we report here the phenotypes of *pep12*Δ strains.

## MATERIALS AND METHODS

**Strains, media and genetic method** Wild-type *S. pombe* strains ARC039 (*h*$^+$*leu1 ura4)* and KJ100-7B (*h*$^{90}$*leu1 ura4*) were obtained from Yuko Giga-Hama (Asahi Glass, Japan), Koichi Tanaka (The University of Tokyo, Japan). The mutants *cpy1*Δ (*h*$^+$*leu1 ura4-D18 his2 ade6-M216 cpy1*::*ura4*) and *vps34/pik3*Δ (*h*$^+$*leu1 ura4-D18 his2 ade6-M216 vps34*::*ura4*) were constructed as described (18–20). *S. pombe* cells were transformed by the lithium acetate method (21,22). The standard genetic methods used have been described (23). *Escherichia coli* XL1-Blue (Stratagene, CA, USA) was used for all cloning procedures.

**Disruption of *S. pombe pep12*$^+$** The *pep12*$^+$ locus was disrupted in the wild-type *S. pombe* strain ARC039 by replacing an internal *pep12*$^+$ gene fragment with the *S. pombe ura4*$^+$ gene. To amplify the *pep12*$^+$ gene from *S. pombe* chromosomal DNA by PCR, the following oligonucleotides were synthesized: sense 2-Primer, 5′-TCGAATGTGTCCTATATTAGATTTCAGCC-3′ and antisense 3-Primer, 5′-GACTTAGGTTAT-TAGATTGTATTAGGCGG-3′. A 1.8 kb fragment was recovered, and ligated into Promega pGEM-T EASY vector. A 0.6 kb *Hin*dIII- *Hin*dIII fragment was eliminated from the cloned *pep12*$^+$ open reading frame and a 1.6 kb *ura4*$^+$ cassette was inserted. A linearized DNA fragment carrying this disrupted *pep12*$^+$ gene was used to transform the wild-type haploid ARC039, and *ura4*$^+$ transformants were selected. To confirm that the *pep12*$^+$ gene had been disrupted, *ura4*$^+$ transformants were analyzed by PCR using sense 1-Primer, 5′-TTTGATGCGAGCGTATGAATGGCCCC-3′, N-Primer, 5′-GATGTCTTTTGTT-GACTTGGAGCAAGGAAG-3′, antisense 4-Primer, 5′-CCTGAAATTGAGGAAATTGG-TAAAAGGACG3′, and C-Primer, 5′-GTCAAGTAAAGAAATAATCTGTATCAAGG-3′.

**Plasmid construction** pREP41-GFP-Pep12 was constructed as follows. The *pep12* cDNA clone was amplified from an *S. pombe* cDNA library (a gift from Dr. T. Nakamura, Osaka City University, Japan). *Sal*I and *Bam*HI sites were introduced at the 5′ and 3′ ends, and two oligonucleotides were used to amplify *pep12* by PCR. The corresponding PCR product was digested with *Sal*I and *Bam*HI, and cloned into the corresponding sites in pTN54 derived from pREP41 (24).

**Fluorescence microscopy** Vacuoles within wild-type and mutant fission yeast were labeled with FM4-64 (13). Cells were examined with an Olympus BX-60 fluorescence microscope using a U-MGFPHQ filter (for GFP), and a U-MWIG filter (for FM4-64). Images were captured with a Sensys cooled charge-coupled device (CCD) camera using MetaMorph (Roper Scientific) and saved as Adobe Photoshop files.

**Pulse-chase and immunoblot analysis of the *S. pombe* Cpy1 protein** Pulse-chase and immunoprecipitation of the vacuolar carboxypeptidase Y from *S. pombe* (SpCPY) were carried out as described (18). Antibody incubations were carried out using rabbit polyclonal antibody against SpCPY (18). CPY colony blot assay was performed using cells spotted on a nitrocellulose membrane and grown for 3 days. Immunoblot analysis was performed as described (25).

## RESULTS

**Disruption of *S. pombe pep12*Δ strain** Based on the *S. pombe* genome database, *S. pombe* has an *S. cerevisiae* Pep12p homolog (SPBC31E1.04), but not an *S. cerevisiae* Vam3p homolog (17). In *S. cerevisiae*, overexpression of *PEP12* was found to complement a *vam3*Δ mutant while overexpression of *VAM3* complemented a *pep12*Δ mutant. (6). Moreover, expression of human Syntaxin-7 complemented the vacuolar protein transport defects in *pep12*Δ cells as well as the vacuolar morphology defects in *vam3*Δ cells (26). Therefore, we compared the SpPep12p SNARE motif with that in *S. cerevisiae* Pep12p, Vam3p and *H. sapiens* Syntaxin-7. Adjacent to the C-terminal transmembrane domain is a predicted helical domain of about 60 amino acids with the potential to form a coiled-coil structure (27). This motif is conserved within the syntaxin family such as *S. cerevisiae* Pep12p (46%), Vam3p (38%) and *H. sapiens* Syntaxin-7 (40%) (Fig. 1). SpPep12p has one Gln residue at the center of the SNARE motif (Fig. 1), and is structurally classified into Q-SNARE family (27).

Previously, we reported that deletion of *S. pombe pep12*$^+$ (Sp*pep12*$^+$) did not cause defects in vacuolar protein transport or vacuolar morphology (17). The Sp*pep12*$^+$ gene codes for a syntaxin homolog. Although we sought other syntaxin homologs that act in vacuolar homotypic fusion in *S. pombe*, we did not find any new syntaxin homologs. To determine whether multiple genes are required for normal vacuolar morphology in *S. pombe*, we constructed double SNARE gene disruptants including disruption of Sp*pep12*. We performed Northern blotting to confirm transcription of Sp*pep12* in wild-type and in the Sp*pep12*Δ strain. Transcription of Sp*pep12* was detected in both strains (Supplemental Fig. S1A). We performed PCR to characterize the Sp*pep12* gene in Sp*pep12*Δ strain. The disruption construct and primers used are shown in Supplemental Fig. S1B. Disruption of Sp*pep12* gene was confirmed using primers 2 and 3. Only the expected disruption allele was observed in Sp*pep12*Δ strains using primers 1 and 4. However, both wild-type and the disruption allele were observed when primers N and C were used with DNA from Sp*pep12*Δ cells (Fig. S1C). These results indicate that a homologous gene replacement occurred with chromosomal Sp*pep12*$^+$, but that another copy of wild-type Sp*pep12*$^+$ exists elsewhere in the fission yeast genome. Therefore, the Sp*pep12*Δ strain was re-isolated and the disruption was confirmed by Northern blotting analysis and PCR. Transcription of Sp*pep12* was not detected in new Sp*pep12*Δ strain (Fig. S1A). Only the disrupted allele was detected by PCR (Fig. S1C). This result indicated that the new strain harbored only the deletion allele, and the disruption of Sp*pep12*$^+$ was also confirmed by Southern blot analysis (data not shown). The new strain was then used to analyze Pep12p function.

***S. pombe pep12*Δ is a phenocopy of *vps33*Δ** To analyze *S. pombe* Pep12p function, we measured growth of Sp*pep12*Δ cells. Growth was very slow. In liquid YES medium at 30°C, Sp*pep12*Δ cells had a 3-fold longer doubling time than wild-type (ARC001) cells. Wild-type and Sp*pep12*Δ cells were streaked onto YES plates and incubated at 30°C and 37°C for 3 days. While both wild-type and Sp*pep12*Δ cells grew at 30°C, Sp*pep12*Δ cells exhibited a temperature-sensitive growth defect at 37°C (Fig. 2). Furthermore the Sp*pep12*Δ cells grew poorly on synthetic minimal medium (data not shown) and exhibited a growth defect on YES medium plates containing $CaCl_2$ and $CdCl_2$ (Fig. 2). These phenotypes are similar to those of Sp*vps33*Δ cells (13). SpVps33p is an *S. cerevisiae* Vps33p homolog and is required for vacuolar protein transport and normal vacuolar function.

Sp*vps33*Δ cells also exhibited a severe vacuolar morphology defect and therefore examined vacuolar protein transport in Sp*pep12*Δ cells. We have previously reported the isolation and characterization of a vacuolar marker protein, carboxypeptidase Y (SpCPY) (18). During its

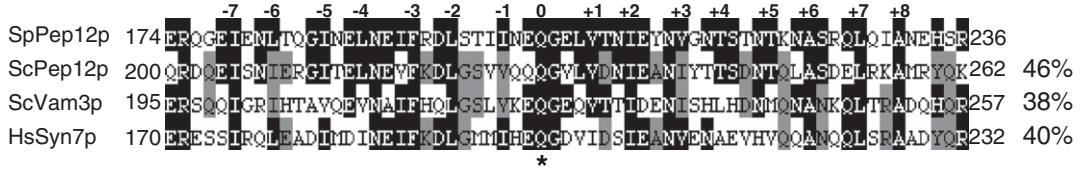


FIG. 1. Sequence alignment of the SNARE motif from *S. pombe* Pep12p, *S. cerevisiae* Pep12p and Vam3p, and *Homo sapiens* Syntaxin-7. Protein names are shown to the left of the lettered amino acid residues. The percentage identity shared between the *S. pombe* Pep12p SNARE motif those of the *S. cerevisiae* and *H. sapiens* proteins is indicated on the right. The star below the residue "Q" indicates the central residue of the predicted coiled-coil. The heptad repeats are numbered above the sequence.