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Coordinated regulation by two VPS9 domain-containing guanine nucleotide exchange factors in small GTPase Rab5 signaling pathways in fission yeast

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ABSTRACT

The small GTPase Rab5 is reported to regulate various cellular functions, such as vesicular transport and endocytosis. VPS9 domain-containing proteins are thought to activate Rab5(s) by their guanine-nucleotide exchange activities. Numerous VPS9 proteins have been identified and are structurally conserved from yeast to mammalian cells. However, the functional relationships among VPS9 proteins in cells remain unclear. Only one Rab5 and two VPS9 proteins were identified in the *Schizosaccharomyces pombe* genome. Here, we examined the cellular function of two VPS9 proteins and the relationship between these proteins in cellular functions. Vps901-GFP and Vps902-GFP exhibited dotted signals in vegetative and differentiated cells. *vps901* deletion mutant (*Δvps901*) cells exhibited a phenotype deficient in the mating process and responses to high concentrations of ions, such as calcium and metals, and *Δvps901Δvps902* double mutant cells exhibited round cell shapes similar to *ypt5-909* (Rab5 mutant allele) cells. Deletion of both *vps901* and *vps902* genes completely abolished the mating process and responses to various stresses. A lack of vacuole formation and aberrant inner cell membrane structures were also observed in *Δvps901Δvps902* cells by electron microscopy. These data strongly suggest that Vps901 and Vps902 are cooperatively involved in the regulation of cellular functions, such as cell morphology, sexual development, response to ion stresses, and vacuole formation, *via* Rab5 signaling pathways in fission yeast cells.

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1. Introduction

Small GTPases are guanine nucleotide binding proteins of approximately 21 kDa in various eukaryotic organisms. The Ras subfamily of small GTPase controls cell growth [1], whereas the Rho subfamily regulates cell morphology and migration [2]. The Rab subfamily modulates dynamic inner membrane transport [3], and the Ran subfamily regulates nuclear transport [4]. In the Rab subfamily, Rab5 was first identified as a factor that regulates early endocytic pathways [5] and vacuolar function [6]. Recently, Rab5 was also reported to be involved in various cellular functions, such as phagocytosis [7] and chromosomal alignment [8], in mammalian cells.

The activities of Rab5 are reported to be specifically modulated by VPS9 domain-containing proteins [9–11]. The VPS9 domain activates Rab5 by exchanging GDP bound to Rab5 for GTP. Numerous VPS9 proteins have been identified in species ranging from yeast to plant and mammalian cells, and these proteins are implicated in vesicular transport including endocytic and signal transduction pathways. In baker's yeast, VPS9p is potentially involved in the autophagic pathway [12], and a mutant allele of the vps9 gene rabex-5 in fruit flies was reported to function as a neoplastic tumor suppressor gene [13]. Human VPS9 domain-containing protein Rin1 affects intracellular trafficking of the EGF

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receptor in mammalian cells [14], and mutations in the VPS9 domain in ALS2 are responsible for infantile-onset ascending hereditary spastic paraplegia (IAHSP) [15]. Moreover, *VPS9a* was shown to be essential for the embryogenesis of *Arabidopsis thaliana* [16], suggesting that VPS9 proteins also play important roles in cellular functions. Although more than 10 vps9 genes were identified in the human genome, the mechanism by which they regulate the three Rab5 proteins (Rab5A, B, and C) and their relationship among VPS9 proteins in human cells remain unclear.

To address the question of how multiple VPS9 domain-containing factors could cooperate in the regulation of cellular functions, we studied fission yeast cells. The fission yeast genome contains only one *rab5*$^+$ (*ypt5*$^+$) gene and two *vps9*$^+$ (*vps901*$^+$, *vps902*$^+$) genes, and this organism therefore serves as a simple model for understanding the Rab5 signaling pathway and the relationship among multiple VPS9 factors. Fission yeast with a mutant *rab5* allele (*ypt5-909*) exhibits abnormal phenotypes regarding cell morphology, sexual development, ion-stress response, and vacuole formation [17]. Previously, we reported that Vps901, one of two VPS9 proteins in fission yeast, regulates vacuolar protein transport [18]. Here, we examine the functional role of VPS9 proteins in fission yeast cellular functions using *vps901 vps902* mutant cells and discuss the basis of coordination among VPS9 proteins in Rab5 signal transducing pathways.

2. Materials and methods

2.1. Strains and media

The strains used in this study are summarized in Table 1. The basic strains were provided by the National Bio-Resource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. In addition, deletion mutants and fluorescent protein-tagged integrants were constructed by homologous recombination-mediated transformation. *vps901 vps902* double mutants of YT003, YT017, and YT018 and integrants of YT008, YT011, and YT016 were constructed by crossing. Yeast cells were grown in YE [0.5% yeast extract (Becton, Dickinson and Company), 3% glucose], SSL+N [1% glucose, 3 mM aspartic acid, 14.7 mM KH_2PO_4, 1.4 mM Na_2HPO_4, 25.9 mM ammonium acetate, 0.075 mg/ml adenine, 2 mM $MgSO_4$, 0.68 mM $CaCl_2$, 0.04 μM biotin, 2.1 μM calcium pantothenate, 81.2 μM nicotinic acid, 55.5 μM *myo*-inositol, 8.1 μM H_3BO_4, 0.16 μM $CuSO_4$, 0.6 μM KI, 0.74 μM $FeCl_3$, 2.36 μM $MnSO_4$, 0.83 μM Na_2MoO_4, 1.4 μM $ZnSO_4$], or SSL-N [1% glucose, 1.8 mM KH_2PO_4, 2 mM $MgSO_4$, 0.68 mM $CaCl_2$, 0.04 μM biotin, 2.1 μM calcium pantothenate, 81.2 μM nicotinic acid, 55.5 μM *myo*-inositol, 8.1 μM H_3BO_4, 0.16 μM $CuSO_4$, 0.6 μM KI, 0.74 μM $FeCl_3$, 2.36 μM $MnSO_4$, 0.83 μM Na_2MoO_4, 1.4 μM $ZnSO_4$] with appropriate supplements. Cells were incubated at 30 °C in YE or SSL+N for growth or 28 °C in SSL-N for mating.

2.2. Cell staining and light microscopy techniques

Phalloidin staining of actin was performed using a previous method reported by Marks and Hyams with modifications [19]. Alexa Fluor 546-labeled phalloidin was purchased from Molecular Probes (A22283). Vacuolar membranes of fission yeast cells were stained with FM4-64 (Molecular Probes T-3166) [20] as follows. Logarithmic phase cells grown at 30 °C in YE were incubated with 1.6 μM FM4-64 and maintained on ice for 30 min. The cells were then washed once with YE, resuspended in fresh YE, and incubated for 1 h at 30 °C. Half of the cells were washed twice with distilled water, resuspended in distilled water, and incubated for 2 h to induce vacuolar fusion. pREP41-Ub-GFP-SpCPS was transformed into fission yeast cells for the observation of Ub-GFP-CPS signals [21]. Fluorescence images and differential interference contrast (DIC) images were obtained using a Fluoview FV1000 confocal laser-scanning microscope (Olympus). The objective lens was a water-immersion lens (×60, NA = 1.20).

Table 1
Strains used in this study.

Strain name	Genotype	Source
L968	*h*^{90}	NBRP
TN4	*h*$^-$ *leu1-32*	NBRP
TN8	*h*^{90} *leu1-32*	NBRP
MM59-2B	*h*^{90} *ura4-D18*	NBRP
MM72-1D	*h*$^-$ *leu1-32 ura4-D18*	NBRP
MM79-3C	*h*$^+$ *leu1-32 ura4-D18*	NBRP
TN29	*h*^{90} *leu1-32 ura4-D18*	NBRP
KS2	*h*^{90} *leu1-32 ypt5-909*	M. Yanagida
TM002	*h*$^-$ *leu1-32 ura4-D18 vps901::ura4*$^+$	This study
TM003	*h*^{90} *leu1-32 ura4-D18 vps901::ura4*$^+$	This study
TM006	*h*$^-$ *leu1-32 ura4-D18 vps902::ura4*$^+$	This study
TM007	*h*$^+$ *leu1-32 ura4-D18 vps902::ura4*$^+$	This study
TM005	*h*^{90} *leu1-32 ura4-D18 vps902::ura4*$^+$	This study
YT002	*h*$^-$ *leu1-32 ura4-D18 vps902::LEU2*	This study
YT017	*h*$^-$ *leu1-32 ura4-D18 vps901::ura4*$^+$ *vps902::ura4*$^+$	This study
YT018	*h*^{90} *leu1-32 ura4-D18 vps901::ura4*$^+$ *vps902::ura4*$^+$	This study
YT003	*h*^{90} *leu1-32 ura4-D18 vps901::ura4*$^+$ *vps902::LEU2*	This study
YT004	*h*^{90} *leu1-32 ura4-D18 vps901::vps901-GFP << LEU2*	This study
YT015	*h*^{90} *leu1-32 ura4-D18 vps902::vps902-GFP << LEU2*	This study
YT012	*h*^{90} *leu1-32 ura4-D18 vps902::vps902-mCherry << ura4*$^+$	This study
YT014	*h*^{90} *leu1-32 ura4-D18 ypt5::mCherry-ypt5<<ura4*$^+$	This study
YT008	*h*^{90} *leu1-32 ura4-D18 vps901::vps901-GFP << LEU2 ypt5::mCherry-ypt5<<ura4*$^+$	This study
YT016	*h*^{90} *leu1-32 ura4-D18 vps902::vps902-GFP << LEU2 ypt5::mCherry-ypt5<<ura4*$^+$	This study
YT011	*h*^{90} *leu1-32 ura4-D18 vps901::vps901-GFP << LEU2 vps902::vps902-mCherry << ura4*$^+$	This study

NBRP, National Bio-Resource Project; M. Yanagida (Okinawa Institute of Science and Technology).

2.3. Electron microscopy techniques

Cells were grown to logarithmic phase at 30 °C in YE. Half of the cells were resuspended in fresh YE, whereas the other half of the cells were resuspended in distilled water and incubated at 30 °C for 2 h. Cells were attached to thin formvar films mounted on copper loops (8 mm diameter) and immediately frozen in liquid propane. Frozen cells were transferred to acetone containing 0.2% uranyl acetate and 2% osmium tetroxide (−85 °C). After 2 days, the samples were slowly warmed to room temperature. Then, the samples were washed with acetone and embedded in Spurr resin. Ultrathin sections were obtained using an ultramicrotome (EM UC6, Leica). Ultrathin sections were stained with lead citrate and observed using a transmission electron microscope (JEM-1230, JEOL) at 80 kV.

3. Results and discussion

3.1. Localization of Vps901 and Vps902 in fission yeast cells

To assess the function of VPS9 proteins in fission yeast cells, we first explored VPS9 protein localization in cells using strains in which GFP-tagged VPS9 proteins were designed to be expressed by their native promoters. GFP-tagged fusion genes were integrated into their respective chromosomal locus with maker genes. GFP-tagged VPS9 proteins were thought to be functional shown as below (section 3.3 Fig. S2A). GFP-tagged Vps901 and Vps902 were observed as dotted signals in vegetative growing cells (Fig. 1A). Dotted signals were also observed in mating cells and spores

(Fig. 1B) induced by nitrogen starvation. Similar signals were observed in GFP-tagged Ypt5 (Rab5) integrated cells, suggesting that VPS9 proteins are colocalized with Ypt5 [17]. To examine the colocalization of Vps901, Vps902 and Ypt5, we crossed strains to generate cells in which GFP-tagged VPS9 proteins and mCherry-tagged Ypt5 proteins were coexpressed under control of their own promoters (Materials and Methods). GFP-tagged Vps901 partially colocalized with mCherry-Ypt5 in cells, and GFP-Vps902 also partially colocalized with mCherry-Ypt5 (Fig. 1C upper and middle panels, respectively). In addition, we previously reported that Ypt5 was biochemically detected in the vesicular membrane fraction [17]. Taken together, these observations suggest that Vps901 and Vps902 cooperate with Ypt5 at intracellular vesicles. We next examined the possibility of colocalization of these two VPS9 proteins, and the results showed that fluorescent-labeled Vps901 and Vps902 partially colocalized (Fig. 1C lower panels), suggesting that these VPS9 proteins are colocalized and cooperate in fission yeast cells.

3.2. *vps9* double mutant cells display growth defects and abnormal cell morphology

To examine the cellular functions of VPS9 proteins in fission yeast, *vps9* deletion mutant strains were constructed by homologous recombination. Both *vps901* and *vps902* deletion mutants exhibited no apparent cell growth abnormalities; however, the *Δvps901Δvps902* double mutant exhibited poor growth at 30 °C and was unable to grow at 37 °C on agar plates (Fig. S1A). In liquid media, the double mutant grew slowly and exhibited a lower culture saturation density compared with wild-type cells (Fig. S1B). We next examined the morphology of fission yeast cells, and wild-type cells exhibited a rod-like cell shape, whereas *Δvps901 Δvps902* double mutant cells exhibited a shorter long axis and a longer short axis (Fig. 2A). This round-like cell shape is similar to that of *ypt5-909* cells [17], suggesting that Vps901 and Vps902 regulate cell morphology cooperatively. In fission yeast cell growth, cells elongate along the long axis and actin patches accumulate at the cell tips; this process is

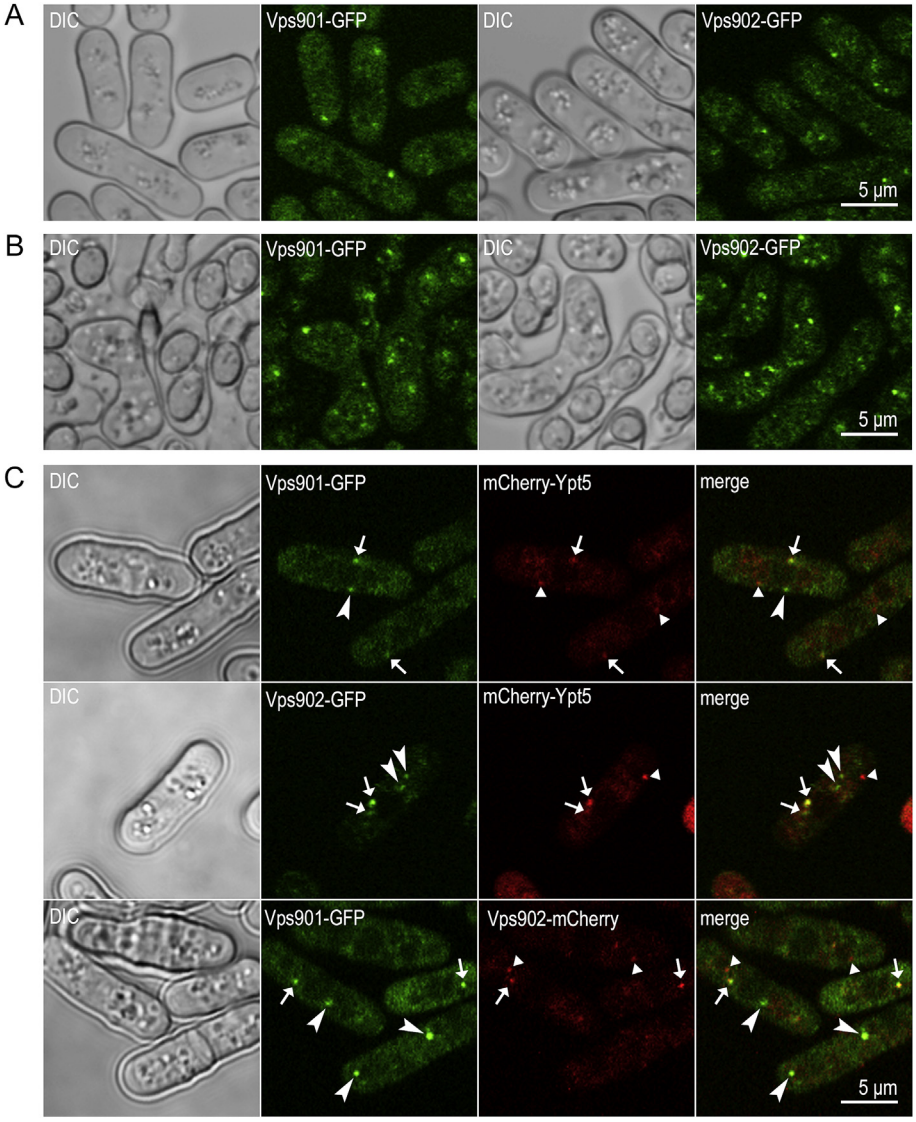

Fig. 1. Localization of Vps901 and Vps902 in fission yeast cells. GFP-tagged *vps9* genes were integrated into their native chromosomal loci. Vps901 and Vps902 were visualized by fluorescent confocal microscopy in vegetative growing cells (A), mating cells, and spores (B) (YT004, YT015). The localization of the two molecules was compared in the same cells using different colors of fused fluorescent proteins (C). In the upper panels, Vps901 was fused with GFP, whereas Ypt5 was fused with mCherry (YT008). Arrows indicate colocalization of Vps901-GFP and mCherry-Ypt5; arrowheads and triangles denote Vps901-GFP and mCherry-Ypt5, respectively. The middle panel presents the localization of Vps902-GFP and mCherry-Ypt5 (YT016), whereas the lower panel presents the localization of Vps901-GFP and Vps902-mCherry (YT011).

indispensable for the formation of cell polarity [19]. Previously, we found that efficient actin patch formation was abrogated in *ypt5-909* cells [17]; however, actin patch formation in *vps9* double mutant cells was normal and similar to that observed in wild-type cells (Fig. 2B). Thus, VPS9 protein cooperativity in the regulation of cell morphology may occur independently of actin patch formation.

3.3. Vps901 and Vps902 roles in the mating process and ion-stress responses

ypt5-909 mutant cells exhibited a phenotype characterized by deficiencies in sexual development, a slow mating rate, and severe spore formation [17]. Therefore, we next measured the mating and sporulation rates of Δ*vps901* and Δ*vps902* mutants as well as double mutant cells in the process of conjugation and spore formation induced by nitrogen starvation (Fig. 3A). Δ*vps901* mutant cells exhibited a reduced mating rate (50% of wild-type) and a reduced sporulation rate (20% of wild-type), whereas Δ*vps902* mutant cells exhibited no apparent differences compared with wild-type cells. Vps901 is known to be involved in the regulation of mating and spore formation processes. Furthermore, Δ*vps901*Δ*vps902* double mutant cells were completely unable to mate and form spores. These results strongly suggest that Vps901 is mainly involved in mating and spore formation and that Vps902 partially cooperates with Vps901 in the regulation of these processes.

Fission yeast cells respond to high concentrations of various ions and osmotic stress, and maintain their homeostasis (Fig. S2A). *ypt5-909* mutant cells were previously shown to be sensitive to high concentrations of ions, such as calcium and potassium [17]. Thus, *vps9* mutant cells were assessed for their sensitivity to high concentrations of various ions and osmotic stress (Fig. 3B). In addition to the sensitivities to high calcium and potassium concentrations, Δ*vps901* mutant cells appeared to be sensitive to copper, cobalt, and zinc ions; by contrast, Δ*vps901* Δ*vps902* double mutant cells did not respond to these ion stresses. With regard to sodium and manganese ions, Δ*vps901* and Δ*vps902* single mutants grew as well as wild-type cells; however, Δ*vps901*Δ*vps902* double mutant cells were unable to grow on the medium, suggesting that Vps901 and Vps902 cooperate in the response to high sodium and manganese ion concentrations. We also found that Vps901 and Vps902 cooperate in the response to osmotic stress induced by 2 M sorbitol. The phenotypes of Δ*vps901*Δ*vps902* double mutant cells were similar to those of *ypt5-909* mutant cells (Fig.S2B).

3.4. Carboxypeptidase S (CPS) transport, vacuolar fusion, and vacuolar structures in vps9 mutants

Previously, we reported that Vps901 is essential for the transport of CPS [18], a vacuolar marker protein that is delivered from the Golgi apparatus to the vacuole lumen *via* an MVB sorting pathway [21]. The ubiquitin-conjugated fusion protein Ub-GFP-CPS was expressed from the nmt promoter, and CPS transport into vacuoles was monitored. Although CPS was not transported to vacuoles in Δ*vps901* cells, transport in Δ*vps902* mutant cells was

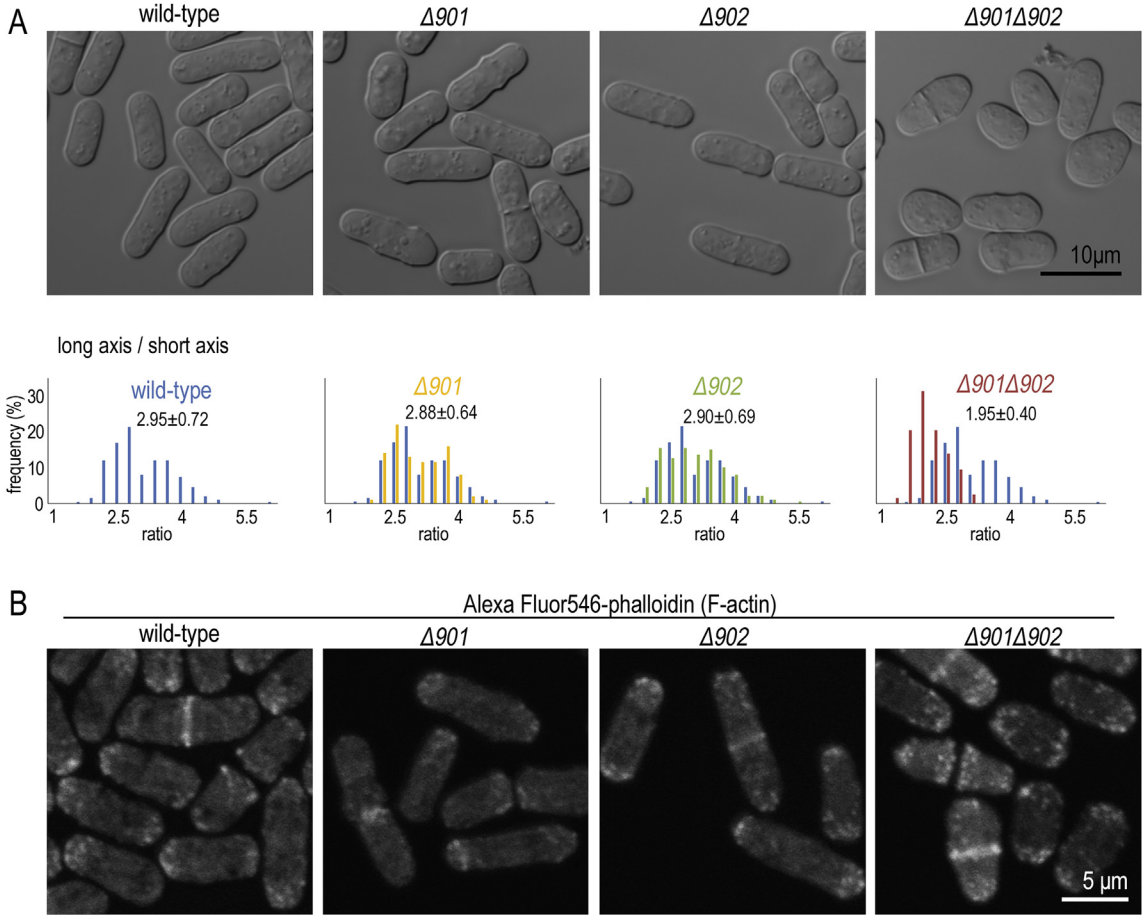

Fig. 2. Morphology of *vps9* mutants. The long and short axis lengths of 200 growing cells were measured, and the ratios of the long and short axis are shown as a bar chart (A) (TN4, TM002, TM006, YT017). F-actin in cells was stained with Alexa Fluor 546-labeled phalloidin and visualized by confocal microscopy (B) (TN4, TM002, TM006, and YT017). Actin rings and actin patches could be observed at the middle of elongated cells and cell tips, respectively.

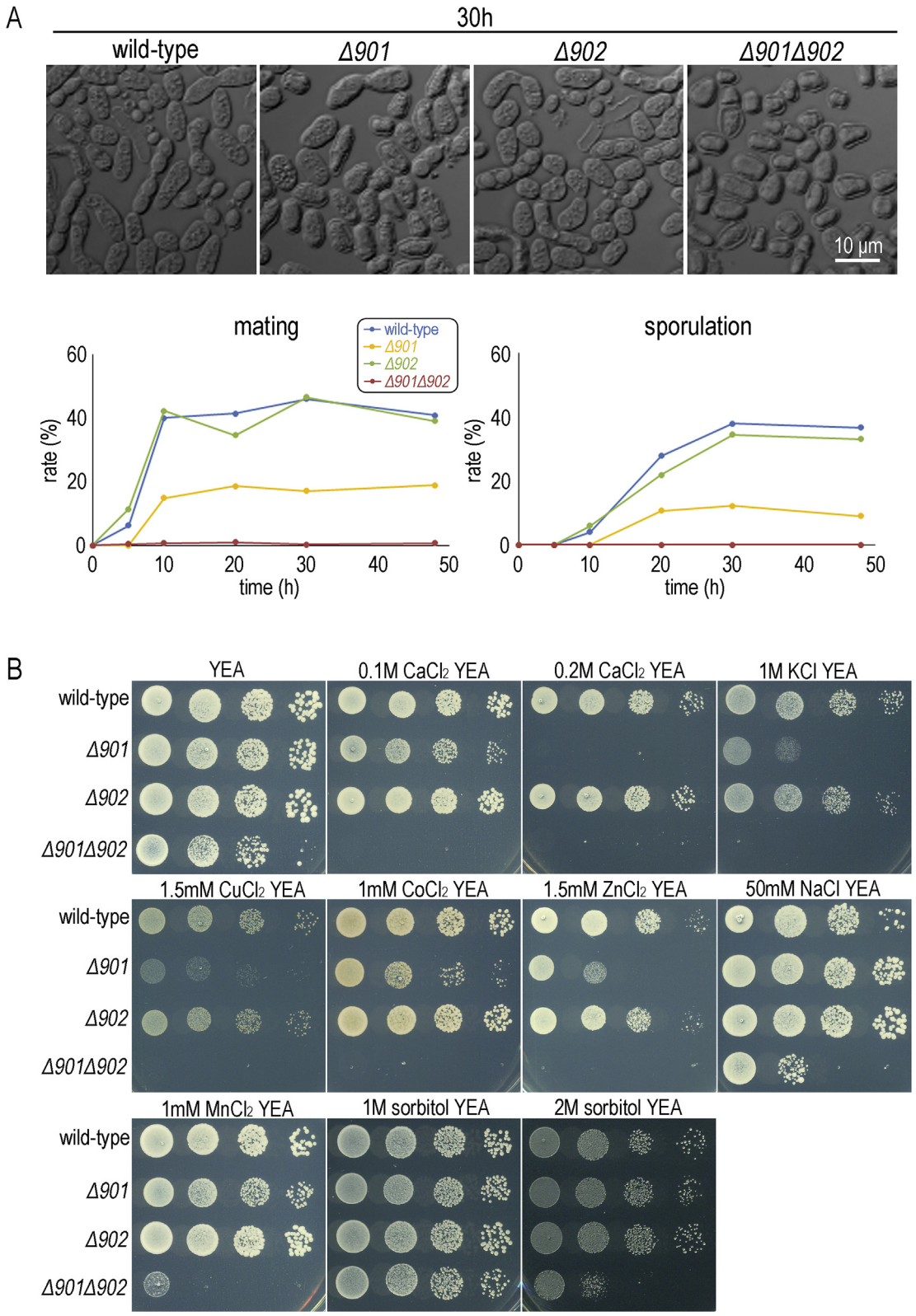

Fig. 3. Vps901 and Vps902 cooperate in regulating sexual development processes and responses to high ion concentrations. Homothallic h^{90} cells were subjected to the mating process in nitrogen-free medium (TN8, TM003, TM005, and YT018). Although the *Δvps901* cell mating rate was approximately 50% of that observed in wild-type cells, *Δvps901Δvps902* double mutant cells were unable to enter the mating process and form spores (A). To determine the responses to high ion concentration and osmotic stresses, wild-type and mutant cells (4×10^4, 4×10^3, 4×10^2, 4×10 cells) were spotted onto agar plates containing various concentrations of ions and sorbitol (B) (TN4, TM002, TM006, YT017).

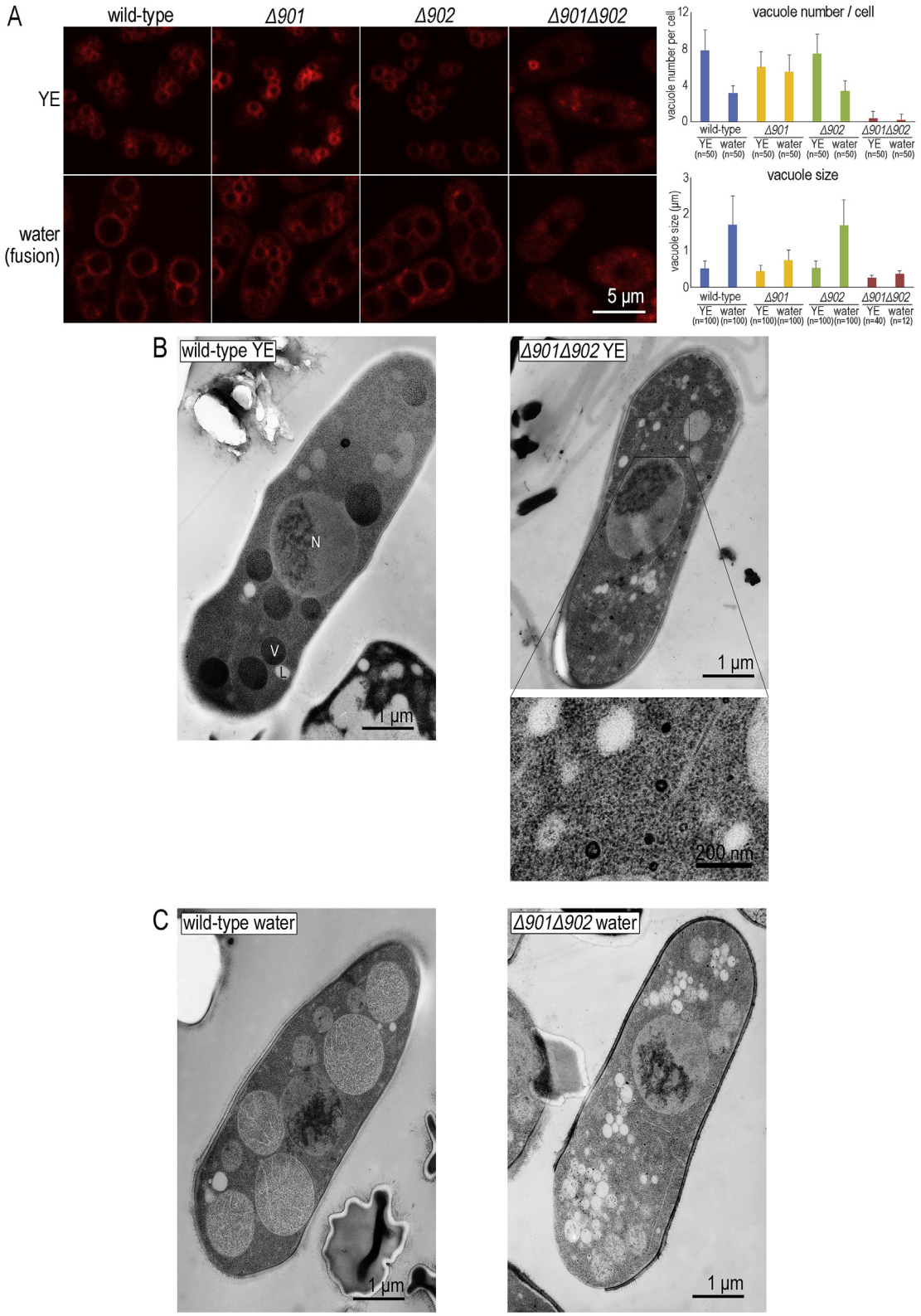

Fig. 4. Vps901 and Vps902 regulate vacuole formation and vacuole fusion. FM4-64 dye was used to stain vacuoles. Cells in the logarithmic phase were collected and incubated with FM4-64 on ice for 30 min. The cells were then chased at 30 °C for 1 h (A) (TN4, TM002, TM006, YT017). Vacuole fusion was induced in water at 30 °C for 2 h. The numbers and sizes of stained vacuoles were counted and measured, respectively. Electron microscopic analyses were performed as described in the Materials and Methods section (L968, YT003). In the electron microscopy photos, the nucleus, vacuole, and lipid body are indicated as N, V, and L, respectively.

indistinguishable from that observed in wild-type cells (Fig. S3), suggesting that Vps902 is not necessary for CPS transport to vacuoles.

We also reported that efficient vacuole fusion did not occur in *Δvps901* cells in response to water [18], suggesting that Vps901 is required for water-induced vacuole fusion *in vivo*. Here, we assessed vacuole fusion in *Δvps902* and *Δvps901Δvps902* double mutant cells (Fig. 4A). In *Δvps902* cells, the vacuole size was normal, and the fused vacuole number and size appeared normal compared with wild-type cells. However, normal-sized vacuoles were not observed in *Δvps901Δvps902* double mutant cells, as assessed by FM4-64 staining, and no change of membrane structure in the response to water treatment was noted.

To examine detailed membrane-bound structures in *Δvps901Δvps902* cells, electron microscopic analysis was performed using freeze-substitution fixation (Fig. 4B). Wild-type cells cultured in rich medium exhibited large electron-dense vacuoles (Fig. 4B left panel). By contrast, *Δvps901Δvps902* cells cultured in rich medium did not exhibit the large vacuoles characteristic of wild-type cells but instead contained smaller, light-density compartments (Fig. 4B right panel). Upon water treatment, the vacuoles fused and became larger and less electron-dense, and clusters of these smaller compartments formed in *Δvps901Δvps902* cells. Interestingly, several particles were noted in *Δvps901Δvps902* cells, irrespective of whether the cells were treated with water; these particles exhibited a hollow core surrounded by a highly electron-dense shell and were relatively uniform in size with diameters of 50–100 nm (Fig. 4B magnified photograph). Most of these particles were observed in the vicinity of the clusters of light-density compartments, whereas some particles were noted around the cell periphery. These particles potentially correspond to the particles noted in the budding yeast mutant strains *ΔVPS9* [22] and *ΔVPS21* (Rab5) [6]. In addition, elongated endoplasmic reticulum was more prominent in *Δvps901Δvps902* cells compared with wild-type cells. These data strongly suggest that Vps901 and Vps902 cooperate in the formation of vacuoles.

Taken together with the data presented above, VPS9 domain-containing guanine nucleotide exchange factors Vps901 and Vps902 cooperatively play important roles in cellular functions in fission yeast, such as cell growth, cell morphology, sexual development, ion-stress responses, vacuolar transport, vacuole formation, and fusion. Moreover, the localization of fluorescent protein-tagged Vps901 and Vps902 suggested that these proteins cooperate with Ypt5 (Rab5) and work on the same vesicles.

Genetic analyses using mutant cells indicated that the phenotypes of *Δvps901Δvps902* cells were similar to those of *ypt5* mutants, which strongly suggests that Vps901 and Vps902 cooperatively transduce Ypt5 signaling pathways. Our results also suggest that Vps901 mainly contributes to mating processes, specific ion-stress responses (Ca^{2+}, K^{+}, Cu^{2+}, Co^{2+}, Zn^{2+}), vacuole transport, and fusion. In addition, Vps902 cooperates in other functions, such as cell growth, cell morphology, responses to Na^{+} and Mn^{2+} ions and osmotic stresses, and vacuolar formation. To elucidate the precise role of Vps901 and Vps902 in cellular functions, other analyses, including biochemical analyses of guanine nucleotide exchange activity, are required.

The *vps9* mutants examined here (*Δvps901*, *Δvps902*, and *Δvps901Δvps902*) exhibited various phenotypes that are similar to other known mutants, and these similarities suggest that these factors could share signaling pathways. With regard to cell growth and cell morphology, the *Δvps4*, *Δvps16*, *Δvps41*, and *Δend4* mutants in vesicular transport also exhibit defects in cell growth and morphology [23]. Recently, we demonstrated that the VPS9 domain-containing factor RIN1 functions as a Rho effector in *C. elegans*, suggesting that the Rho GTPase signaling pathway could also utilize the Rab5 pathway [24]. Furthermore, mutations in the *ypt7* gene, which encodes Rab7, and the *vps33* gene, which is a subunit of the HOPS complex and the CORVET tethering complex, produce a phenotype with defects in spore formation [25,26]. The mutants *Δpep12*, *Δvps33*, and *Δvps34*, which encodes PI3 kinase, are sensitive to high calcium ion concentrations [26–28]. The *ypt7* (Rab7), *wis1* (MAPKK), *sty1* (MAPK), and *atf1* (ATF1) genes are reported to be essential for both vacuole fusion and mating processes [25,29–31]. Furthermore, *Δpep12*, *Δvps33*, *Δvps34*, *Δypt7*, *vps11*$^{-}$, and *vps39*$^{-}$ mutant cells exhibit aberrant vacuole formation phenotypes [25–27,32,33]. Thus, future studies should assess the molecular mechanisms by which Vps901 and Vps902 cooperatively or individually play roles in Ypt5-mediated pathways, as well as the other pathways discussed above, in fission yeast cells.

Conflict of interest

None.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.031.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.031.

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