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The small GTPase Rab5 homologue Ypt5 regulates cell morphology, sexual development, ion-stress response and vacuolar formation in fission yeast

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ABSTRACT

Inner-membrane transport is critical to cell function. Rab family GTPases play an important role in vesicle transport. In mammalian cells, Rab5 is reported to be involved in the regulation of endosome formation, phagocytosis and chromosome alignment. Here, we examined the role of the fission yeast Rab5 homologue Ypt5 using a point mutant allele. Mutant cells displayed abnormal cell morphology, mating, sporulation, endocytosis, vacuole fusion and responses to ion stress. Our data strongly suggest that fission yeast Rab5 is involved in the regulation of various types of cellular functions.

1. Introduction

Small GTPases form a huge superfamily consisting of the Ras, Rho, Rab and Ran subfamilies. Each subfamily plays specific roles in cell functions. The Ras subfamily is thought to be involved in the regulation of cell division and growth. The Rho subfamily functions in cell morphology and migration, and the Rab subfamily regulates vesicle trafficking.

Many Rab small GTPases are thought to regulate vesicle transfer in various cell types. Rab5 is one of the best-studied members of the Rab family. In mammalian cells, Rab5 was first reported to control the early endocytic pathway [1–3]. Bilder and their colleagues identified the *rab5* gene as a neoplastic tumor suppressor in *Drosophila* development [4]. Recently, the role of Rab5 in autophagosome formation was clarified [5,6]. In contrast to the anticipated roles of Rab GTPases, Rab5 has been reported to control chromosome alignment [7]. However, mammals have 3 Rab5 GTPase isoforms (Rab5A–C), and their signal transduction pathways have been difficult to analyze.

In the fission yeast *Schizosaccharomyces pombe*, Ypt5 was identified as the only Rab5 homologue, enabling simple genetic analyses. Disruption of *ypt5* gene results in a lethal phenotype, suggesting that Ypt5 is essential for cell growth and is involved in the regulation of vesicle formation and transport [8]. Here, we examined the role of the Rab5 homologue Ypt5 in the cellular function using a point mutant allele of *ypt5*. The multiple functions of Ypt5 in fission yeast cell will be discussed.

2. Materials and methods

2.1. Strains and culture conditions

The strains used in this work are listed in Table 1. *ypt5-909* strains were gifts from M. Yanagida (Okinawa Institute of Science and Technology). The strains h^{90} *leu1-32* and h^{90} *leu1-32 ura4-D18* were provided by the National Bio-Resource Project (NBRP) of MEXT, Japan. GFP-tagged *ypt5*$^+$ and *ypt5–909* integrant strain were constructed as follows. The GFP-tagged *ypt5* fusion genes harboring *ura4*$^+$ marker gene fragments were used to homologous recombination reactions with the *ypt5*$^+$ native allele in a *ura*$^-$ wild-type strain (TN29 in strain list).

Yeast cells were grown in YPD [1% yeast extract (Becton, Dickinson and Company), 2% peptone (Becton, Dickinson and Company), 2% glucose], YE [0.5% yeast extract (Becton, Dickinson and Company), 3% glucose], EMM [14.7 mM potassium hydrogen phthalate, 15.5 mM Na_2HPO_4, 93.5 mM NH_4Cl, 2% glucose, 5.2 mM $MgCl_2$, 99.8 μM $CaCl_2$, 13.4 mM KCl, 282 μM Na_2SO_4, 4.20 μM pantothenic acid, 81.2 μM nicotinic acid, 55.5 μM myoinositol, 40.8 nM biotin, 8.09 μM boricacid, 2.37 μM $MnSO_4$,

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Table 1
Strains used in this study.

Strain Name	Genotype	Source
SA21	h^+	M. Yanagida
909	h^+ *ypt5-909*	Sajiki et al. [19]
TN8	h^{90} *leu1-32*	NBRP
TN29	h^{90} *leu1-32 ura4-D18*	NBRP
KS2	h^{90} *leu1-32 ypt5-909*	M. Yanagida
CK002	h^{90} *ypt5::GFP-ypt5 << ura4⁺ leu1-32 ura4-D18*	This study
CK003	h^{90} *ypt5::GFP-ypt5-909 << ura4⁺ leu1-32 ura4-D18*	This study

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

1.39 μM $ZnSO_4$, 740 nM $FeCl_2$, 247 nM molybdic acid, 602 nM KI, 160 nM $CuSO_4$, 4.76 μM citric acid] or ME [3% malt extract (Becton, Dickinson and Company)] with appropriate supplements. Wild-type strains were basically grown at 30 °C, while the *ypt5-909* strains were grown at the permissive temperature of 26 °C.

2.2. Cell staining and microscopic techniques

Phalloidin staining was performed as described by Marks and Hyams [9]. Alexa Fluor 546-labeled phalloidin was purchased from Molecular Probes (A22283). Vacuolar membranes of fission yeast were visualized with FM4-64 (Molecular Probes T-3166) [10]. The microscopes used in this study were a confocal laser scanning microscopy Fluoview FV1000 (Olympus) and a Delta-Vision System. Images of the actin cytoskeleton images were acquired with a computer-assisted fluorescence microscope system (Delta-Vision, Applied Precision). The objective lens was an oil-immersion lens (100×, NA = 1.35). When necessary, image deconvolution was performed with an image workstation (SoftWorks; Applied Precision).

2.3. Biochemical analysis and cell volume measurement

Subcellular fractionation was performed essentially as described by Sato et al. [11], with some modifications. A total of 2×10^9 cells in logarithmic phase were collected and subjected to fractionation. Fractionated proteins were detected with an anti-GFP (Roche, 11 814 460 001) monoclonal antibody followed by anti-mouse IgG-HRP (Santa Cruz, sc-2055) as the secondary antibody.

The volume of the fission yeast cells was measured with a Beckman Coulter Multisizer 3.

3. Results and discussion

3.1. Localization of Ypt5 in fission yeast cells

Ypt5 comprises 211 amino acids, with a lipid modification site in its carboxyl-terminal region (Fig. 1A). To examine the cellular localization of the Ypt5 GTPase, we constructed a strain in which the *gfp-ypt5* fusion gene was integrated into the native *ypt5* allele. In vegetative cells, Ypt5-GFP was observed as dotted or small ring-like signals, suggesting that Ypt5 could localize at vesicles (Fig. 1B, left panels). In poor environments, such as nitrogen starvation, fission yeast cells enter the mating process and form spores. Dotted signals of GFP-Ypt5 were also observed in mating cells and asci (Fig. 1C). Ypt5 localized to vesicle membranes in wild-type cells.

The strain *ypt5-909* was isolated as a mutant, which had a defect in the entry into and maintenance of cell quiescence [12]. The *ypt5-909* allele encodes a mutant form of Ypt5 in which C209 in the carboxyl terminus (CXC motif) is substituted by arginine (Fig. 1A). We also constructed a mutant strain in which a *gfp-ypt5-909* mutant fusion gene was integrated into the wild-type *ypt5* allele. However, GFP-Ypt5-909 signals were not observed in a dotted pattern but were diffusely localized in the cytoplasm, with the exception of some vacuole-like structures (Fig. 1B, right panels).

Magee and colleagues reported that Ypt5 containing the C209S mutation in the CXC motif failed to target membrane fractions efficiently [13]. We determined if GFP-tagged Ypt5 localized to biochemical fractions in wild-type and mutant cells. In wild-type cells, Ypt5 was mainly detected in the P13 membrane fraction and soluble fractions (Fig. 1D). The P13 membrane fraction corresponds to the endoplasmic reticulum and vacuole membranes in *Saccharomyces cerevisiae* [11]. However, in *ypt5–909* mutant cells, the amount of P13 fraction significantly decreased, and the S100 soluble fraction increased. Our data suggest that C209 in the lipid modification motif is critical for the proper localization of Ypt5 to membranes, resulting in a cellular quiescence phenotype in *ypt5-909* cells.

3.2. ypt5-909 mutant cells display abnormal cell growth and morphology

To address the functions of Ypt5 in cells, we examined the phenotype of *ypt5-909* mutant cells. During vegetative growth, *ypt5-909* cells grew slightly slowly. The doubling time of *ypt5-909* cells in logarithmic phase was 3.2 h, whereas that of wild-type cells was 2.9 h at 26 °C in rich medium. Likewise, in the synthetic medium, the doubling time of mutant cells was 4.2 h, whereas that of wild-type cells was 3.9 h. In stationary phase, the saturated cell density of *ypt5-909* cells was 28% lower than that of wild-type cells (Fig. S1). Taken together, these data suggest that Ypt5 plays a role in cell growth.

Fission yeast cells display rod-like cell morphology. The cell shape of *ypt5-909* was different from that of wild-type cells (Fig. 2A); logarithmic growth phase cells were slightly longer in the short axis, whereas the long axes appeared unchanged (Fig. 2B upper panels). The differences in cell morphology were more notable in stationary phase. *ypt5-909* cells were spherical in stationary phase (Fig. 2A), i.e., the long axis was much shorter and the short axis was longer than that of wild-type cells (Fig. 2B, lower panels). To assess the difference in cell morphology between wild-type and *ypt5–909* cells, we measured the cell volume with a Coulter counter. Unexpectedly, the volumes of wild-type cells and *ypt5-909* cells were nearly identical (Fig. 2C), although the population of *ypt5-909* cells with a volume of 120–160 μm^3 was slightly increased compared to wild-type cells.

Fission yeast cells grow in a polarized manner, which is regulated by actin cytoskeleton re-organization. Thus, we examined the actin cytoskeleton in *ypt5-909* mutant cells. Actin cytoskeletons were stained with fluorescently labeled phalloidin. Actin rings formed normally in *ypt5-909* and wild-type cells; however, actin patches at the cell growth site were reduced in *ypt5-909* cells (Fig. 2D). Actin patch formation is thought to play an important role in polarized cell growth and endocytosis [14]. The abnormal cell morphology of the *ypt5–909* mutant could be due to the reduced actin patch formation in the cell tips.

3.3. ypt5-909 cells fail to enter sexual development and respond to ion stress

In a nitrogen-starved environment, fission yeast cells mate with each other, form spores and enter the sexual developmental process. *ypt5-909* mutant cells were grown in nitrogen-starved media, and the developmental process was monitored. The mating

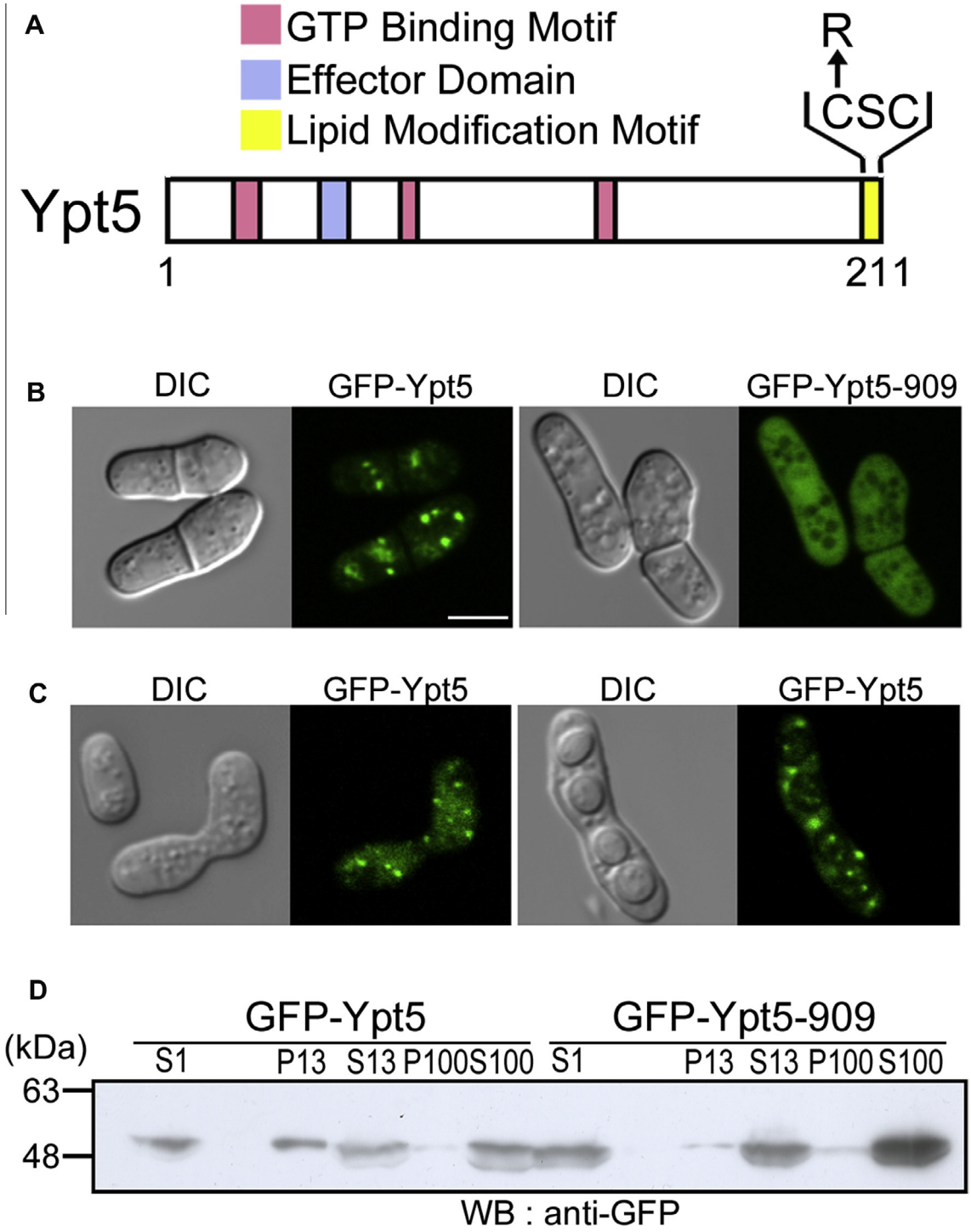

Fig. 1. Ypt5 localized to inner membranes, however, Ypt5-909 mutant protein was unable to localize to membranes. (A) Ypt5 has CXC motif in its carboxyl terminus. *ypt5-909* mutant allele encodes C209R substituted Ypt5 molecule in CXC motif. (B) Dotted signals of GFP-Ypt5 in wild-type vegetative cells. Signals spread to cytoplasm in *ypt5-909* cells. Scale bar: 5 micrometer (C) Dotted signals were also observed in wild-type conjugated cells (left panel) and a spore-forming ascus (right panel). In (B) and (C), fluorescent images were obtained by a confocal laser scanning microscopy. (D) GFP-Ypt5 was detected in P13 membrane fraction in wild-type cells. However, mutant GFP-Ypt5-909 molecule was not detected in P13 fraction.

efficiency of *ypt5-909* mutant cells was significantly low, and spores were not formed (Fig. 3A). Ypt5 has been proposed to play a crucial role in the fission yeast mating process. Ypt7, a Rab7 homologue that is necessary for trafficking from the endosome to the vacuole and homotypic vacuole fusion, play a crucial role in the formation of mature spores [15]. Ypt5 might precede Ypt7 in the sexual development of fission yeast by regulating the mating process.

The response of mutant cells to ions and osmotic stress was evaluated. *ypt5-909* cells were sensitive to high concentration of calcium and potassium ions (Fig. 3B). Sensitivity to high concentrations of calcium has been observed in vesicle transport mutants such as *vps34* [16], *vps33* [17], and *pep12* [18] and the *end4* [19] mutant. The relationship between Ypt5 and these factors in the regulation of calcium response should be addressed.

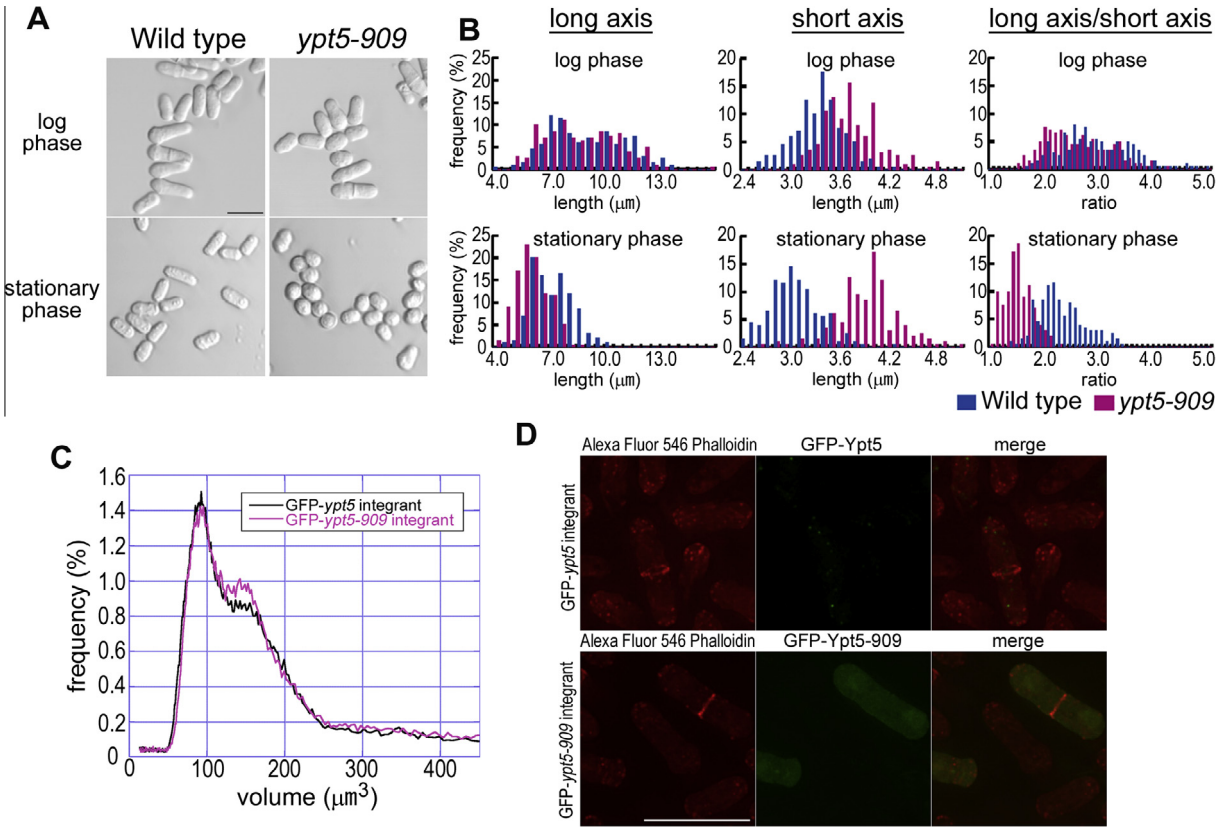

Fig. 2. *ypt5-909* mutant cells displayed short and round cell morphology. (A) The fission yeast cells display the rod-like cell shape. *ypt5-909* cells were longer in the short axis than wild-type cells in logarithmic growth phase, and spherical in stationary phase. (B) 200 cells were measured about long axis and short axis, and the ratio of long axis and short axis for each cell was calculated. (C) Comparison of cell volume between wild-type and *ypt5-909* mutant cells. 100,000 cells were analyzed with a coulter counter. (D) Actin cytoskeletons in cells were stained with Alexa Fluor 546-labeled phalloidin. Actin patches were observed at cell tips of wild-type cells. Microscopic data were obtained and deconvolved with a Delta-Vision system. Scale bar: 10 micrometer.

3.4. *ypt5-909* cells display abnormal endocytosis and vacuole fusion

Mammalian Rab5 is involved in the regulation of early endosome formation. To examine the role of Ypt5 in endosome formation, FM4-64 dye was incorporated into cells, and membrane transport was monitored via fluorescence. FM4-64 dye is incorporated into cells through the plasma membrane, the prevacuolar compartment (PVC), and, finally, vacuole membranes. FM4-64 staining of vacuole membranes was detected within 1 h in wild-type cells, whereas *ypt5-909* vacuoles began to stain only in 3 h, with nearly all vacuoles stained after 4–5 h (Fig. 4A). Endosome formation was significantly delayed. Moreover, the sizes of vacuoles in *ypt5-909* cells were extremely heterogeneous, including large vacuoles, such as those observed during vacuole fusion induced by low osmotic stress.

We next examined the process of vacuole fusion. During conditions of low osmotic stress such as exposure to water, vacuoles within cells fuse with each other to form large vacuoles (Fig. 4B,

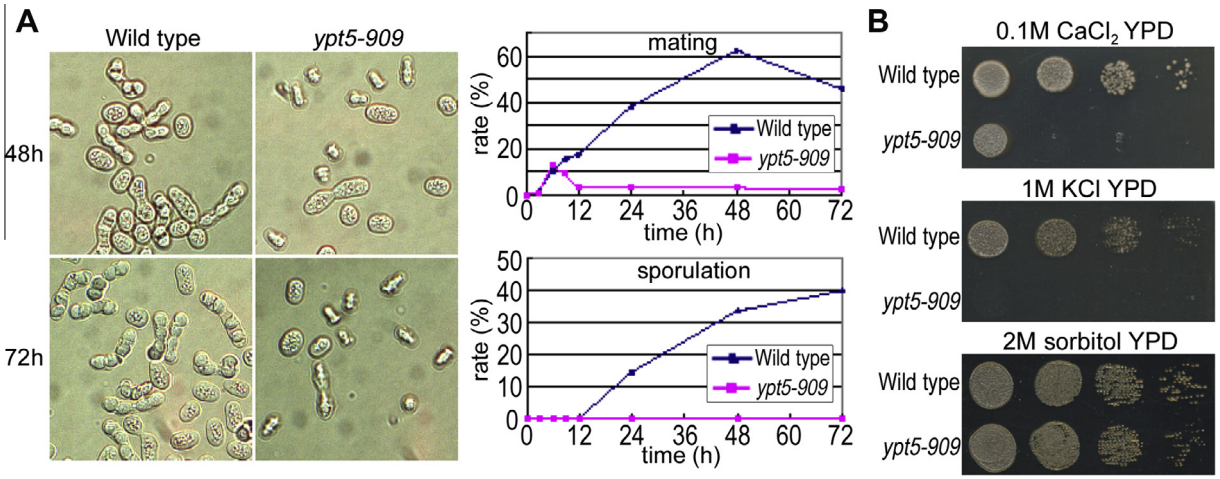

Fig. 3. Phenotypes of *ypt5-909* mutant cell in sexual development and ion-stress responses. (A) Cells were subjected to spore-forming media. Wild-type cells mate and form spores efficiently. However, mutant cells were unable to mate. 500 cells were counted and analyzed for wild-type and *ypt5-909* mutant cells. (B) High sensitivity of *ypt5-909* cells to calcium and potassium ions.

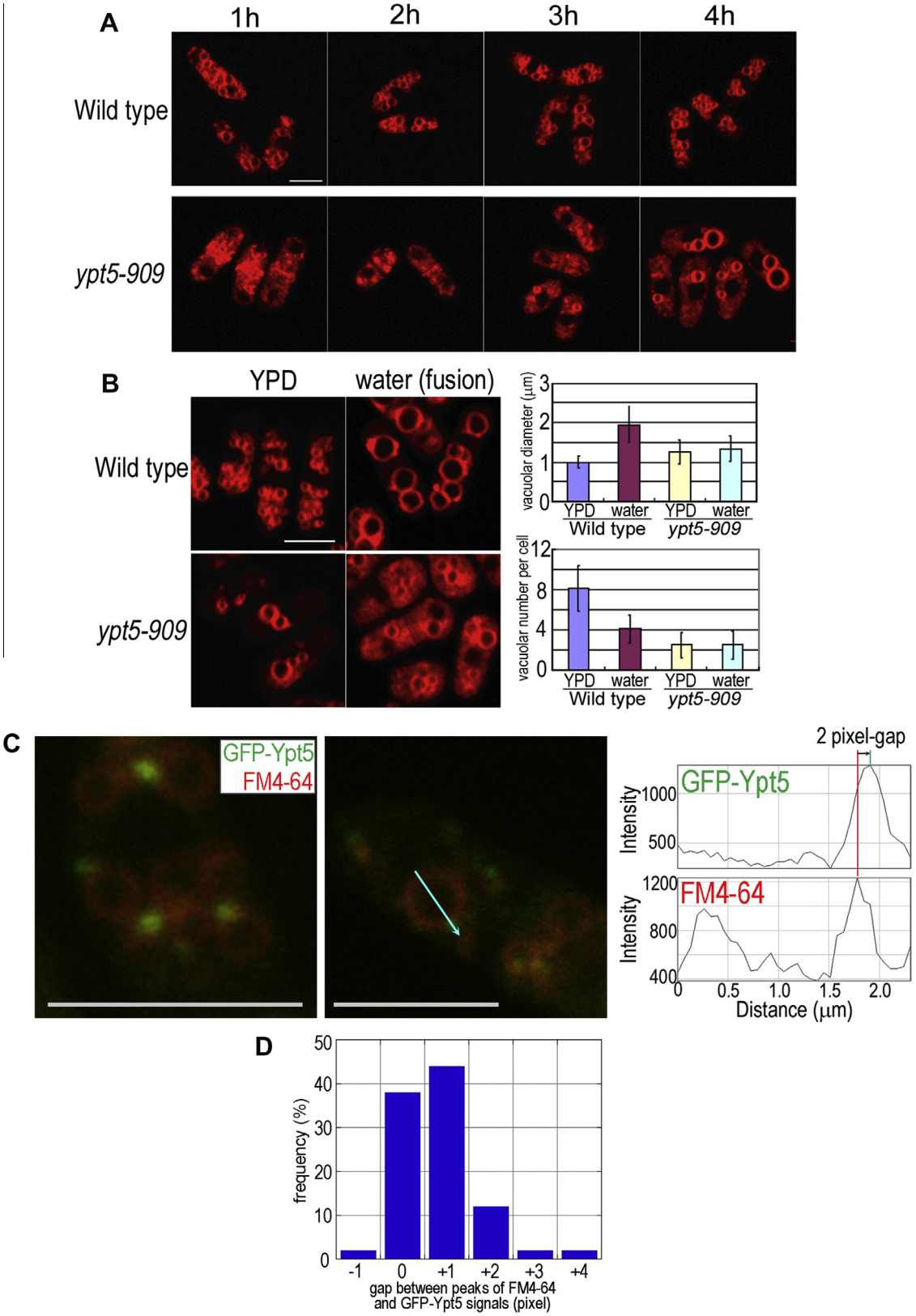

Fig. 4. *ypt5-909* cells had defects in endosome formation and vacuole fusion. (A) Delay of endosome formation and heterogeneous vacuoles in mutant cells. (B) Vacuoles were not fused in mutant cells in response to low osmotic stress. The diameter of vacuole was measured for 100 wild-type and mutant cells. The number of vacuoles was counted for 30 cells. (C) GFP-Ypt5 signals were detected mainly on the vacuole membranes and on the cytoplasmic side of vacuoles. The GFP-Ypt5 signal and FM4-64 membrane signal were compared along with the arrow across the vacuole. Intensity of each signal was analyzed by using ImageJ software. The size of pixel is 0.066 μm (D) Distribution of the distance between GFP-Ypt5 peak signal and FM4-64 membrane signal. Scale bar: 5 micrometer.

wild type). However, vacuoles in *ypt5-909* cells failed to fuse in response to low osmotic stress. These phenotypes seem to partly resemble those of *ste12* [20,21], *ypt7*, *ypt71* [22], *pep12* [18], and *end4* [19] mutants and the *Saccharomyces cerevisiae* mutants *vps21*(*ypt51*) [23], *ypt52*, and *ypt53* [24]. Recently, we identified the candidate activators of Ypt5, Vps901, and Vps902. Vps901 contains the VPS9 domain, which might have guanine-nucleotide exchange activity for Rab5 [25] and is required for vacuolar protein transport and vacuolar fusion [26]. To understand the mechanism of vacuole formation and fusion, the spatio-temporal regulation and interactions between the factors described above should be examined.

We observed GFP-Ypt5 signals in the vacuole membrane and examined the site of localization on the vacuole membrane in detail. The intensity of the GFP-Ypt5 signal was measured across vacuoles and compared with FM4-64 membrane signals (Fig. 4C). The peak signals for GFP-Ypt5 and FM4-64 corresponded well, although slight differences were observed (Fig. 4D). These data suggest that Ypt5 could localize on the cytoplasmic side of the vacuole membrane. Ypt5 might function at this site in the regulation of vacuole fusion.

In this study, we examined a mutant of the fission yeast Rab5 homologue and observed various cell morphology, sexual development, ion-stress response and vacuolar formation and fusion phenotypes. Vesicle transport is involved in mating, the ion-stress response and vacuole formation and fusion, and these phenomena as well as others in which vesicle transport is involved should be analyzed. The unexpected cell morphology phenotype of the *ypt5* mutant led us to consider the relationship between Rab5 and the actin cytoskeleton. Rab5 has been reported to play an important role in RTK and TPA-induced cell morphology in mammalian cells [27,28]. We recently identified the protein VPS9 (RIN1) as a Rho family Rac effector that regulates neuronal cell migration and axon guidance in *Caenorhabditis elegans* [29]. The Rab5 signaling pathway could regulate the actin cytoskeleton. The molecular mechanism by which Rab5 reorganizes the actin cytoskeleton in fission yeast cells should be elucidated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.158.

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