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Imp2, the PSTPIP homolog in fission yeast, affects sensitivity to the immunosuppressant FK506 and membrane trafficking in fission yeast

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ABSTRACT

Cytokinesis is a highly ordered process that divides one cell into two cells, which is functionally linked to the dynamic remodeling of the plasma membrane coordinately with various events such as membrane trafficking. Calcineurin is a highly conserved serine/threonine protein phosphatase, which regulates multiple biological functions, such as membrane trafficking and cytokinesis. Here, we isolated *imp2-c3*, a mutant allele of the *imp2*$^+$ gene, encoding a homolog of the mouse PSTPIP1 (proline-serine-threonine phosphatase interacting protein 1), using a genetic screen for mutations that are synthetically lethal with calcineurin deletion in fission yeast. The *imp2-c3* mutants showed a defect in cytokinesis with multi-septated phenotypes, which was further enhanced upon treatment with the calcineurin inhibitor FK506. Notably, electron micrographs revealed that the *imp2-c3* mutant cells accumulated aberrant multi-lamella Golgi structures and putative post-Golgi secretory vesicles, and exhibited fragmented vacuoles in addition to thickened septa. Consistently, *imp2-c3* mutants showed a reduced secretion of acid phosphatase and defects in vacuole fusion. The *imp2-c3* mutant cells exhibited a weakened cell wall, similar to the membrane trafficking mutants identified in the same genetic screen such as *ypt3-i5*. These findings implicate the PSTPIP1 homolog Imp2 in Golgi/vacuole function, thereby affecting various cellular processes, including cytokinesis and cell integrity.

1. Introduction

Cytokinesis is the final step in the cell cycle and is the process that physically separates a mother cell into two daughter cells [1,2]. In order to successfully complete this process, cells have to dynamically organize a multitude of proteins, such as components of the actomyosin contractile ring and of a physical membrane barrier. Recently, the functional link between cytokinesis and other cellular events regulating membrane dynamics, especially membrane trafficking has received much attention [3,4].

We have been using the fission yeast *Schizosaccharomyces pombe* (*S. pombe*) as a model system for studying the cellular functions and the regulatory mechanism of calcineurin, which is an evolutionarily conserved Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase and an important regulator of Ca^{2+} signaling. Ppb1, the *S. pombe* calcineurin, regulates multiple biological processes, such as cytokinesis, membrane trafficking, ion homeostasis and cell wall integrity [5–7]. The immunosuppressive drug FK506 blocks the activation of calcineurin through the formation of complexes with immunophilins, and this drug has been frequently used to delineate various cellular functions mediated by calcineurin [8,9]. In order to identify genes that share an essential function for growth with calcineurin, we performed a chemical genetic screen to isolate mutants that exhibit sensitivity to FK506 [10,11], and identified various membrane trafficking genes, including *ypt3*$^+$ (encoding a Rab 11 homolog) [12], *ryh1*$^+$ (encoding a Rab 6 homolog) [13], *gdi1*$^+$ (encoding a Rab GDI) [14], *apm1*$^+$ (μ1 subunit of the Adaptor–Protein complex 1) [15], and *sip1*$^+$ (AP-1 accessory protein) [16]. Notably, these membrane trafficking mutants displayed defects in cytokinesis besides those in membrane trafficking. Here, we isolated the *imp2-c3*, a mutant allele of the

Abbreviations: PSTPIP1, proline-serine-threonine phosphatase interacting protein 1; YPD, yeast extract-peptone-dextrose; EMM, Edinburgh minimal medium; YES, yeast extract with supplements; GFP, green fluorescent protein; YFP, yellow fluorescent protein; ORF, open reading frame.

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Table 1
S. pombe strains used in this study.

Strain	Genotype	Reference
HM123	*h⁻ leu1-32*	Our stock
HM528	*h⁺ his2*	Our stock
KP201	*h⁻ leu1-32 cis3-1/imp2-c3*	This study
KP630	*h⁻ leu1-32 ura4-D18 apm1::ura4⁺*	[15]
KP208	*h⁻ leu1-32 ura4-D18 pmk1::ura4⁺*	[20]

imp2$^+$ gene, encoding a homolog of the mouse PSTPIP1 [17], that is structurally similar to the Pombe Cdc15 homology (PCH) Proteins, as a new addition to the genes which affect sensitivity to FK506 upon mutation. Imp2 was previously isolated as a component of the actin contractile ring and is structurally similar to the *S. pombe* Cdc15, a founding member of the F-BAR (Fes/Cip4 homology-Bin/Amphisin/Rvsp) domain protein, involved in actin ring organization [18]. In this study, we have characterized a novel role for Imp2 in membrane trafficking and showed that Imp2 was involved in Golgi/vacuole function, suggesting a functional interplay between actomyosin ring-mediated cytokinesis and membrane trafficking events.

2. Materials and methods

2.1. Strains, media, and genetic and molecular biology methods

S. pombe (*S. pombe*) strains used in this study are listed in Table 1. The complete medium (yeast extract-peptone-dextrose; YPD) and

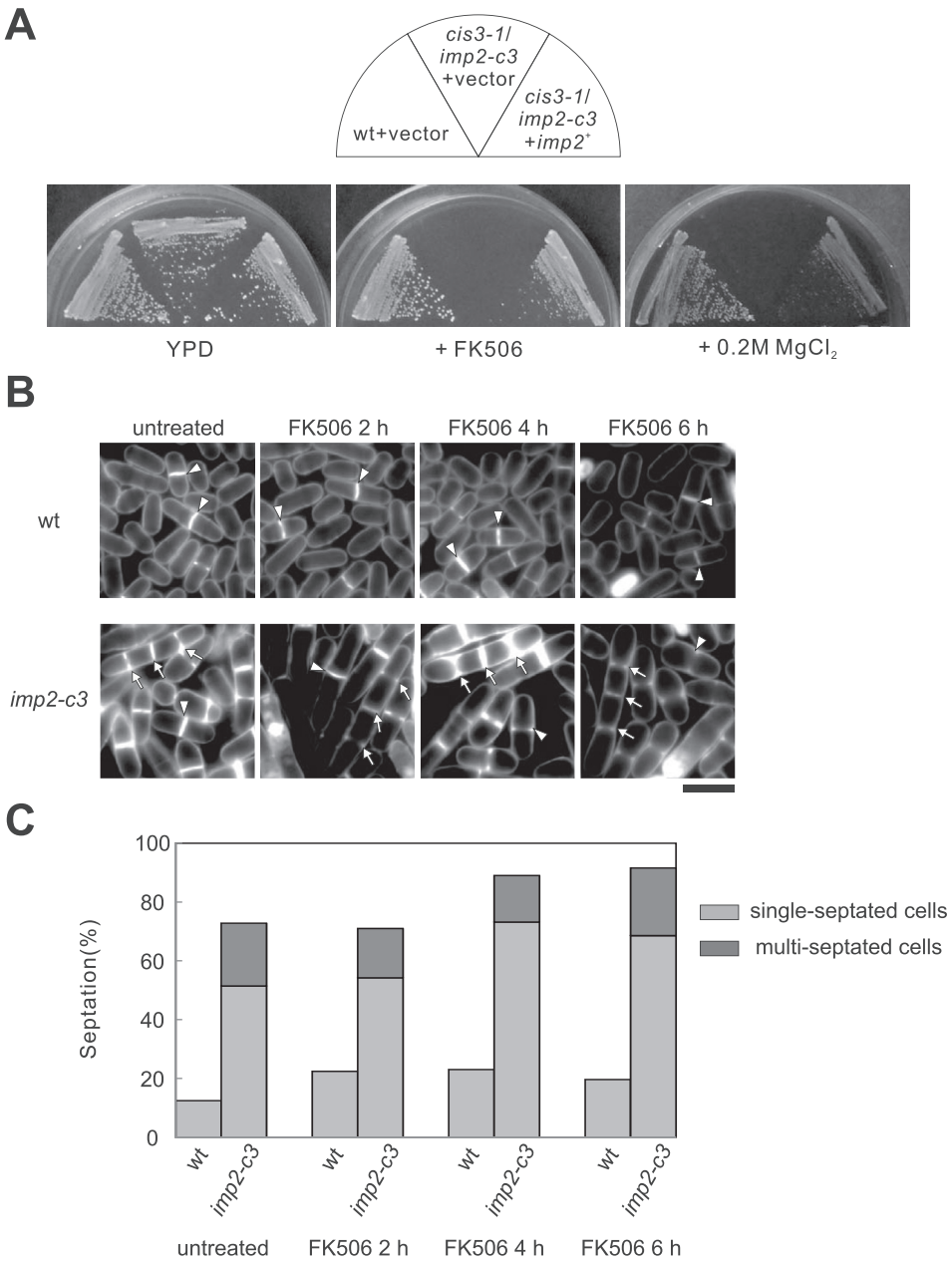

Fig. 1. Mutation in the *cis3*$^+$/*imp2*$^+$ gene causes immunosuppressant- and chloride-sensitive phenotypes and defects in cytokinesis. (A) The immunosuppressant and chloride sensitivities of the *cis3-1/imp2-c3* mutant cells are shown. Cells transformed with the multi-copy vector pDB248 or the vector containing the *cis3*$^+$ gene were streaked on to the plates as indicated, and then incubated for 3 days at 27°C. (B) Fluorescence micrographs of wild-type (wt) and *imp2-c3* mutant cells stained with Calcofluor. Cells were incubated in YPD or YPD plus FK506 at 27°C and then stained with Calcofluor to visualize cell wall and septum. Bar, 10 μm. (C) Percentage of cells forming a division septum at each time point in wild-type (wt) and *imp2* mutant cells after the addition of FK506 at 27 °C. Values are the average of 5 times with 100 cells counted for each time point.

the minimal medium (Edinburgh minimal medium; EMM) have been described previously [19,20]. Standard genetic and recombinant DNA methods [19] were used except where otherwise noted. FK506 was provided by Astellas Pharma, Inc. (Tokyo, Japan).

2.2. Isolation of the cis3-1/imp2-c3 mutant

The *cis3-1/imp2-c3* mutant was isolated in a screen of cells that had been mutagenized with nitrosoguanidine as described previously [11]. Briefly, the mutants were spread on YPD plates to give ~1000 cells/plate and incubated at 27 °C for 4 days. The plates were then replica-plated at 27 °C onto plates containing 0.5 μg/ml FK506 or 0.2 M $MgCl_2$. Mutants that showed both FK506 sensitivity and $MgCl_2$ sensitivity were selected and designated as chloride- and immunosuppressant-sensitive (*cis*) mutants. The original mutants isolated were back-crossed three times to wild-type strains HM123 and HM528.

2.3. Cloning of the cis3$^+$/imp2$^+$ gene

To clone the *cis3*$^+$ gene, the immunosuppressant sensitivity of *cis3-1* mutants (KP201) was utilized. The *cis3-1* mutants were grown at 27 °C and transformed with an *S. pombe* genomic DNA library constructed in the vector pDB248 [21]. Leu$^+$ transformants were replica-plated onto YPD plates containing FK506 and the plasmid DNA was recovered from the transformants that showed plasmid-dependent rescue. These plasmids complemented the immunosuppressant and chloride ion sensitivity of the *cis3-1* mutant. By DNA sequencing, the suppressing plasmids were identified to contain the *imp2*$^+$ gene (SPBC11C.11.02). To investigate the relationship between the cloned *imp2*$^+$ gene and *cis3-1* mutant, linkage analysis was performed as follows: the entire *imp2*$^+$ gene was subcloned into the pUC-derived plasmid containing the *Saccharomyces cerevisiae LEU2* gene and integrated by homologous recombination into the genome of the wild-type strain HM123. The integrant was mated with the *cis3-1* mutant. The resulting diploid was sporulated, and tetrads were dissected. In all cases examined, only parental ditype tetrads were found, indicating allelism between the *imp2*$^+$ gene and the *cis3-1* mutation. Accordingly, we renamed the *cis3-1* mutant as the *imp2-c3* mutant. We also disrupted the *cis3*$^+$/*imp2*$^+$ gene following the method developed by Bahler et al. [22]. The *cis3*-null mutants also displayed the immunosuppressant and chloride-sensitive phenotypes (data not shown). However, the *cis3* null cells were sick, and unstable in the phenotypes presumably due to the occurrence of revertants, we therefore used the *cis3-1/imp2-c3* mutant cells throughout the study.

2.4. Microscopy and miscellaneous methods

Light microscopy methods, such as differential interference contrast and fluorescence microscopy, were performed as previously described [15]. Data from at least 3 individual experiments with a minimum of 50 counted cells were used for quantification.

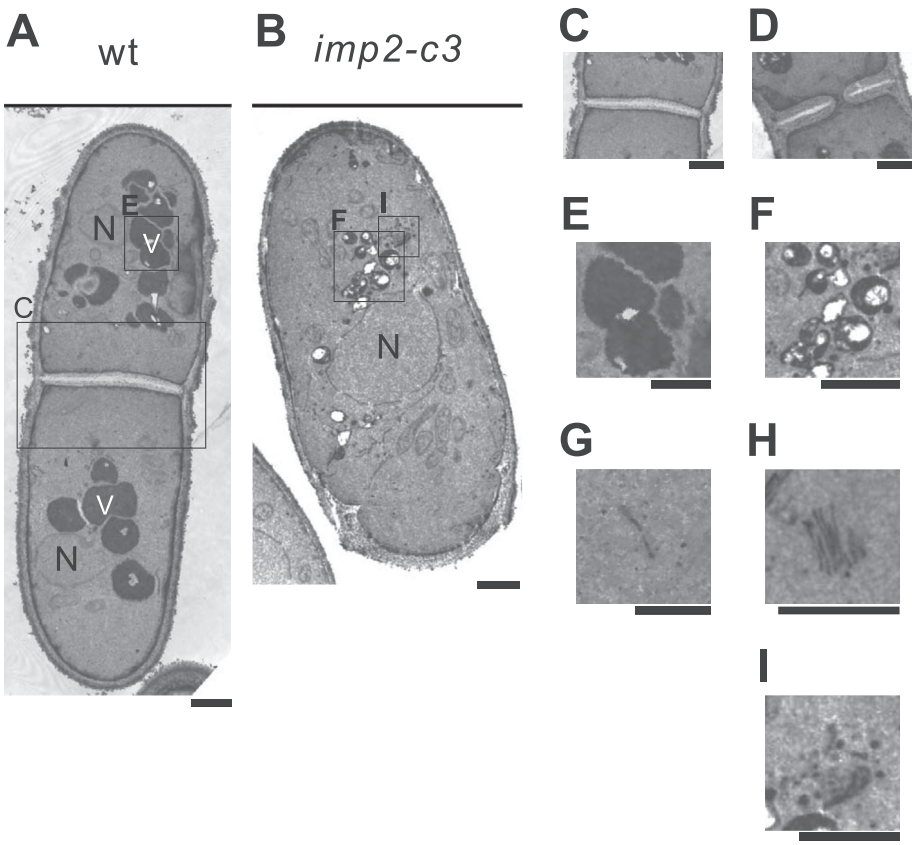

Fig. 2. *imp2-c3* mutant cells exhibited enlarged Golgi structures, accumulated vesicles, and fragmented vacuoles. (A) Wild type cells (wt) were analyzed by electron microscopy at 27°C. The boxed region was shown in (C) and (E). N, nucleus; V, vacuoles. Bar, 1 μm. (B) *imp2-c3* mutant cells were analyzed by electron microscopy at 27°C. The boxed regions were enlarged in F and I.N, nucleus. Bar, 1 μm. (C) Septum structures in the wild-type cells (wt). Bar, 1 μm. (D) Septum structures in the *imp2-c3* mutant cells. Bar, 1 μm. (E) Vacuoles in the wild-type cells (wt). Bar, 1 μm. (F) Fragmented vacuoles in the *imp2-c3* mutant cells. Bar, 1 μm. (G) Golgi structures in the wild-type cells (wt). Bar, 1 μm. (H) Multi-lamellar Golgi structures in the *imp2-c3* mutant cells. Bar, 1 μm. (I) Putative post-Golgi vesicles around Golgi structures in the *imp2-c3* mutant cells. Bar, 1 μm.

Conventional electron microscopy and measurement of the acid phosphatase secretion were performed as described previously [15].

3. Results

3.1. Isolation of the cis3-1/imp2-c3 mutant

Our genetic screen used to search for genes that share an essential overlapping function with calcineurin using FK506, a specific inhibitor of calcineurin, isolated several genes involved in membrane trafficking and cytokinesis [10–16,23,24]. During the screen, we observed that several FK506 sensitive mutants also displayed hypersensitivities to chloride ion, including the *apm1*$^+$/*cis1*$^+$ gene that encodes a homolog of the mammalian μ1A subunit of the clathrin adaptor protein complex1 [15], and the *myp2*$^+$/*cis2*$^+$ gene that encodes a type 2 myosin heavy chain [23]. Here, we isolated and characterized the *cis3-1* mutant cells. As shown in Fig. 1A, the *cis3-1* mutants grew well as compared with the wild-type (wt) cells on rich YPD plate. However, the *cis3-1* mutant cells could not grow on the YPD plate containing FK506 (Fig. 1A +FK506). The *cis3-1* mutant cells also failed to grow in the presence of 0.2 M $MgCl_2$ whereas the wt cells grew well (Fig. 1A +0.2 M $MgCl_2$). As predicted, no double mutant was obtained by the genetic cross between *cis3-1* and calcineurin deletion (Δ*ppb1*) (our unpublished data), indicating *cis3-1* and Δ*ppb1* are synthetically lethal.

3.2. The cis3-1 is an allele of the contractile ring imp2$^+$ gene that encodes a homolog of the proline-serine-threonine phosphatase interacting protein PSTPIP

We cloned the *cis3*$^+$ gene (Materials and Methods) and the isolated *cis3*$^+$ gene complemented all the phenotypes associated with the *cis3-1* mutant (Fig. 1A, *cis3-1/imp2-c3* +*imp2*$^+$). Nucleotide sequencing of the cloned DNA fragment revealed that the *cis3*$^+$ gene is identical to the *imp2*$^+$ gene (SPBC11C.11.02), which encodes a protein of 670 amino acids that exhibits significant sequence similarity to the Cdc15/PSTPIP family of cleavage furrow proteins, including *Homo sapiens* PSTPIP [17] and *S. cerevisiae* HOF1 [25,26]. After confirming the allelism between the *imp2*$^+$ gene and the *cis3-1* mutation (Materials and Methods), we renamed the *cis3-1* mutant as the *imp2-c3* mutant.

We also characterized the mutation site in the *imp2-c3* mutant. For this, genomic DNA from the *imp2-c3* mutant was isolated, and the full-length coding region of the *imp2-c3* was sequenced. Nucleotide sequence analysis revealed a single nucleotide change, from G to A (at position 1835 −1), located at the 3′ end of the conserved 3′ splice site of intron three (Fig. S1). Because the splice acceptor site at the 3′ end of the intron terminates the intron with an almost invariant AG sequence, the identified mutation destroys a splice site in the *imp2*$^+$ gene which produces a mutant allele, presumably due to altered splicing.

3.3. The cis3-1/imp2-c3 mutant showed cytokinesis defects that were exacerbated by FK506

Because Imp2 was shown to be involved in septation in *S. pombe* [18], we examined the morphological phenotypic changes in the *imp2-c3* mutant upon FK506 treatment. Cells grown to mid-log phase at 27 °C in liquid YPD medium were subjected to a shift to a medium containing FK506 at 27 °C. Even in the absence of FK506, 72.8% of the *imp2-c3* mutant cells had division septa as compared with the frequency of 12.4% seen in a wild-type population, and abnormally multi-septated cells were observed in the *imp2-c3* mutant cells (Fig. 1B, C; untreated). Upon shift to the medium containing FK506 for 6 h, the frequency of septated cells in the *imp2-c3* mutant cells significantly increased up to almost 90% (Fig. 1C), whereas the septation index of wt cells moderately increased up to 20% (Fig. 1B, C). It is therefore implied that calcineurin and Imp2 play overlapping functions for cytokinesis.

3.4. Electron microscopic analysis of the imp2-c3 mutant cells revealed membrane trafficking defects in the imp2-c3 mutant cells

In order to gain a detailed insight into the role of Imp2, we analyzed the *imp2-c3* mutant cells with electron microscopy. Wild-type (wt) and the *imp2-c3* mutant cells were cultured at 27 °C and examined by electron microscopy (Fig. 2A, B). As shown in Fig. 2D, septum structures in the *imp2-c3* mutant cells were markedly thick as compared with that of the wt cells (Fig. 2C), consistent with the reported role of Imp2 in septation [18]. We also noted that the vacuoles in the *imp2-c3* mutant cells were highly fragmented (Fig. 2B, F), as compared with those in wt cells (Fig. 2A (V), E). Notably, Golgi structures were thick, swollen, and frequently multi-lamellar in the *imp2-c3* mutant cells (Fig. 2H) as compared with those in wt cells (Fig. 2G) and vesicular structures associated with Golgi stacks were observed (Fig. 2I). These abnormal structures were negligible in the wt cells (Fig. 2A). It should be noted that the findings seen in the electron micrographs of the *imp2-c3* mutant cells are similar to those in the previously identified immunosuppressant-sensitive mutants

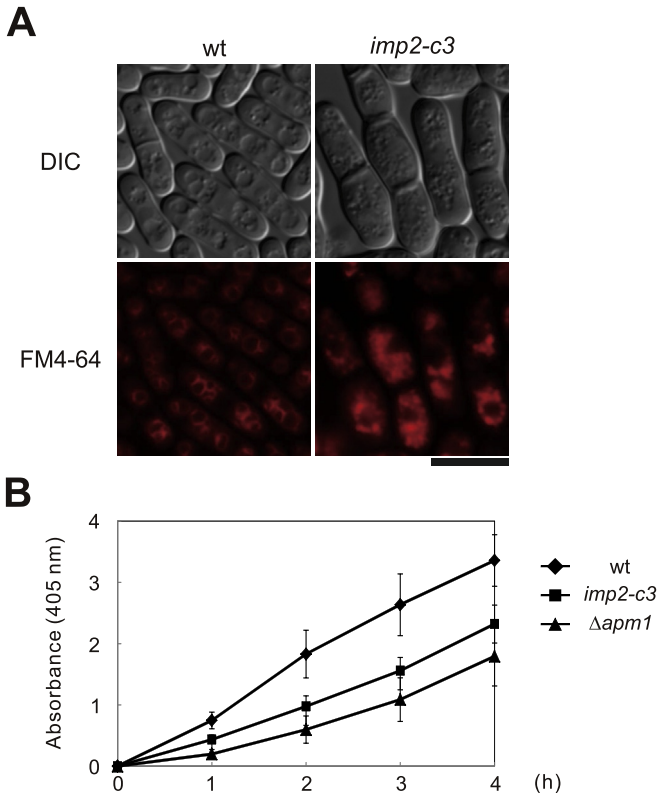

Fig. 3. Imp2 is involved in membrane trafficking. (A) The *imp2-c3* mutant cells are defective in vacuole fusion. Wild type (wt) and *imp2-c3* mutant cells were grown in YPD medium at 27 °C. Cells were harvested, labeled with FM4-64 fluorescent dye, resuspended in water, and examined by fluorescence microscopy. Photographs were taken after 90 min. Bar, 10 μm. (B) Defective secretion of acid phosphatase in *imp2-c3* mutant cells. Wild-type (wt), Δ*apm1* cells and *imp2-c3* mutant cells were assayed for secreted acid phosphatase activity as indicated. The data presented are representative of three independent experiments.

such as the *ypt3-i5* [12], Δ*apm1* [15] and *sip1-i4* mutant cells [16]. In general, electron microscopic analysis of mutants that exhibit defects in membrane trafficking reveals the accumulation of organelles or vesicular intermediates of the compartments that precede the step in which they first function [12,27,28]. These results implicate Imp2 in membrane trafficking presumably at the step of Golgi/endosome and/or vacuoles.

3.5. The *imp2-c3* mutant cells exhibit defects in secretion and vacuolar fusion

The above data prompted us to investigate if the *imp2-c3* mutant cells were defective in membrane trafficking as suggested by electron micrographs. We examined the vacuolar fusion induced by osmotic stress in wt and *imp2-c3* mutant cells. The cells were labeled with FM4-64 for 60 min and when the cells were collected, washed, and resuspended in water for 90 min, the wt cells had evidently large vacuoles that resulted from vacuolar fusion (Fig. 3A, wt), whereas vacuoles remained small and numerous in the *imp2-c3* mutant cells suspended in water, indicating a defect in vacuole fusion (Fig. 3A, *imp2-c3*), consistent with the fragmented vacuoles as observed in electron micrographs.

We further determined the ability of *imp2-c3* mutant cells to secrete acid phosphatase, a protein that follows the classical secretory pathway from the endoplasmic reticulum to the extracellular periplasmic space. As a control strain, we used the Δ*apm1* cells, which was shown to display a reduction in the secretion of acid phosphatase [15]. The *imp2-c3* mutant cells secreted much less acid phosphatase than the wt cells, and the degree of the secretion defect as detected in *imp2-c3* mutant was almost comparable to that in the Δ*apm1* cells (Fig. 3B). These results are consistent with the secretory defects associated with the *imp2-c3* mutant cells.

3.6. Imp2 is required for cell wall integrity

Because previously identified immunosuppressant-sensitive mutants involved membrane trafficking, such as the *ypt3-i5* and Δ*apm1* mutant cells also displayed defects in cell wall integrity [12,15], we examined whether the *imp2-c3* mutant cells display hypersensitivity to the cell wall-damaging agent micafungin, an inhibitor of (1, 3)-β-D-glucan synthase [29]. As a control strain, we used *pmk1*-null cells, in which the MAPK Pmk1, which regulates cell wall integrity was deleted [20]. The *pmk1*-null cells failed to grow in the YPD plates containing micafungin at concentrations higher than 1.0 μg/ml, wherein the wt cells grew well, consistent with the previous report [30] (Fig. 4A). The growth of *imp2-c3* mutant cells was significantly inhibited in the YPD plates containing 1.0 μg/ml micafungin and the *imp2-c3* mutants did not form colonies in the YPD plates containing 2.0 μg/ml micafungin (Fig. 4A). The cell wall integrity defects in the *imp2-c3* mutant cells was further confirmed using a treatment with β-glucanase, an enzyme that cleaves 1,3-β-D-glucan. As shown in Fig. 4B, the *imp2-c3* mutant cells lysed much faster than the wt cells and a little slower than the *pmk1*-null cells, consistent with the data obtained by micafungin. Thus, Imp2 can be assumed to be involved in membrane trafficking and cell wall integrity.

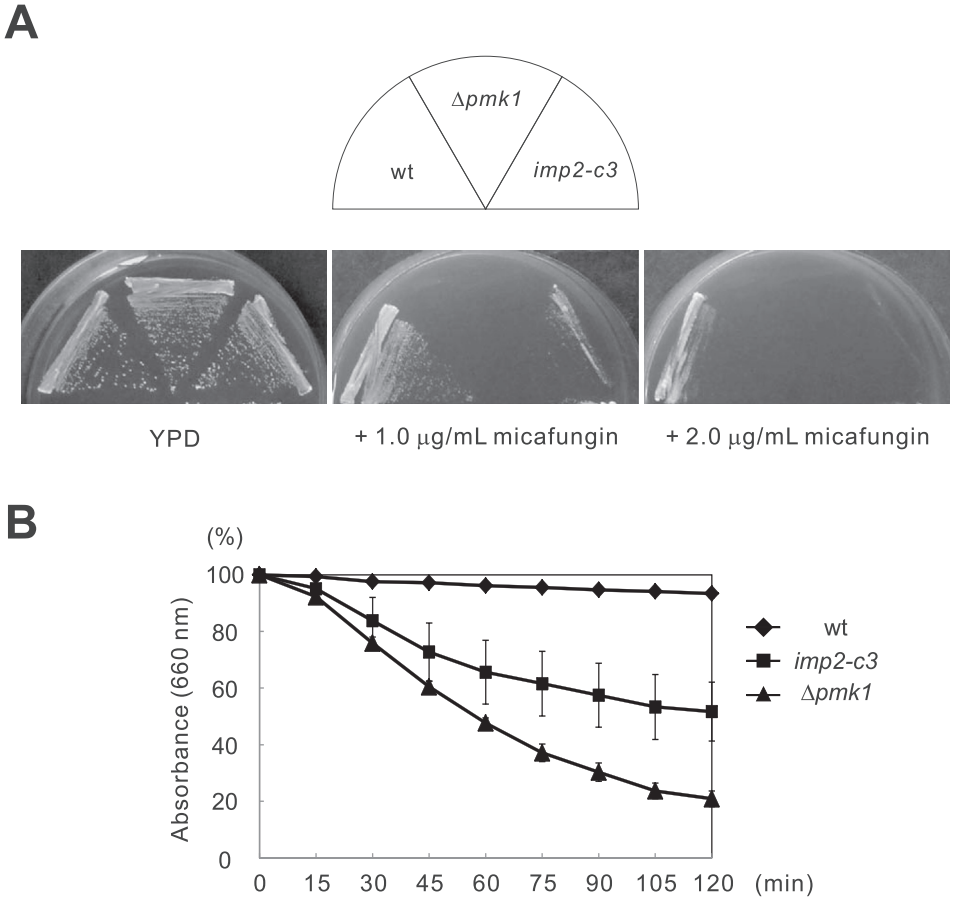

Fig. 4. Imp2 is required for cell wall integrity. (A) The *imp2-c3* mutant cells exhibited micafungin sensitivity. Wild type (wt), Δ*pmk1* cells and *imp2-c3* mutant cells were streaked on to the plate as indicated and then incubated for 3 days at 27 °C. (B) The *imp2-c3* mutant cells are hypersensitive to β-glucanase treatment. Cells as indicated were grown in YPD at 27°C and treated with 0.1 μg/mL Zymolyase-100T for various times, and the OD_{660} of the cell suspension was monitored.

4. Discussion

In summary, our chemical genetic screen using the calcineurin inhibitor FK506 identified the mutation in the *imp2*$^{+}$, a previously characterized septation gene. Importantly, we have presented evidence that Imp2 plays a role in membrane trafficking, in addition to its established role in cytokinesis. These include abnormal Golgi/vacuolar structures as evidenced by electron micrographs and defects in secretion of acid phosphatase and vacuolar fusion. Notably, these membrane trafficking phenotypes as well as cell integrity defects associated with the *imp2-c3* mutation were remarkably similar to those reported in the Golgi membrane trafficking mutants identified in the same chemical genetic screen, such as *ypt3-i5*, the mutation in the Rab11 homolog in *S. pombe* [12]. Cytokinesis requires highly regulated membrane vesicle trafficking activities both in plant and animal cells, and several membrane trafficking genes have attracted much attention in their role in cytokinesis [4,31]. Especially, one of the members of the Rab GTPases Rab11 plays a role in cytokinesis in various organisms by delivering Golgi/endosomal materials to the cleavage furrow and/or dividing sites [32,33]. Our findings that the component of the actin contractile ring Imp2 plays a putative role in Golgi membrane trafficking are quite interesting, providing an additional layer of the functional interplay between cytokinesis and membrane trafficking events. Imp2 is structurally similar to the Pombe Cdc15 protein, a founding member of the F-BAR (Fes/Cip4 homology-Bin/Amphisin/Rvsp) domain protein, which serves as a regulator and a coordinator of actomyosin assembly and membrane dynamics [34]. Mammalian F-BAR proteins, including PSTPIP, bind phospholipids and thereby participate in membrane deformations and affect many physiological functions such as internalization of transmembrane receptors [35]. This property of the phospholipid-binding as well as to self-dimerization associated with the F-BAR domain protein, generates a driving force to induce 3D membrane curvature, thereby contributing to the ingression of cleavage furrow or membrane dynamics [35]. Notably, according to the Pombase ORFeome data, the Imp2 localization revealed dot-like structures in addition to the established localization to the contractile ring (http://www.riken.jp/SPD/Img_page/46_iP/46G01_Loc.html) [36]. It would be intriguing to speculate that Imp2, by localizing not only to the contractile ring but also in the Golgi/endosomal structures regulates Golgi membrane dynamics, affects various cellular functions via its interaction with Golgi-localized proteins through the SH3 domain and/or through its proposed property to bind phospholipid. Given that some members of the F-BAR proteins in higher eucaryotes are implicated in inflammatory or neurodegenerative disorders, such as Pyogenic arthritis with pyoderma gangrenosum and acne (PAPA) syndrome [37], our findings may prove to be useful to clarify the role of the F-BAR domain proteins in the membrane dynamics as well as the molecular basis of diseases linked to these molecules. Furthermore, fission yeast chemical genomic screen to search for the immunosuppressant-sensitive genes may provide a viable system to identify genes associated with membrane trafficking and cytokinesis.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2014.12.100.

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