

BRIEF REPORT



Meiosis-specific localization of the exocytic Rab Ypt2 in fission yeast

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ABSTRACT

Fission yeast Ypt2, an orthologue of the mammalian small GTPase Rab8, is responsible for post-Golgi membrane trafficking. During meiosis, Ypt2 localizes at the spindle pole body (SPB), where it regulates *de novo* biogenesis of the spore plasma membrane. Recruitment of Ypt2 to the SPB is dependent on its meiosis-specific GDP/GTP exchange factor (GEF), the SPB-resident protein Spo13. Here we have examined the SPB recruitment of Ypt2 by Spo13. The GEF activity of Spo13 was required, but not essential for recruitment. Furthermore, Ypt2 recruitment was regulated in a meiosis-specific manner and partially regulated by the nuclear Dbf2-related (NDR) kinase Sid2, indicating the existence of a novel regulatory mechanism for localization of Rab GTPases during meiosis.

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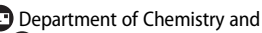
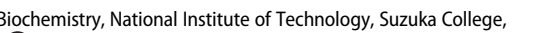
Introduction, results and discussion


In eukaryotic cells, there are various membrane-bound organelles that frequently exchange part of their membranes. Each portion of membrane is transported via a regulated process and is fused accurately to the target membrane. This specific membrane trafficking is mainly regulated by Rab family small GTPases.^{1–3} Each Rab GTPase cycles between the surface of the membrane structure and the cytoplasm. Active Rab in its GTP-bound form mainly associates with the membrane via its geranylgeranylated C-terminus, whereas inactive GDP-bound Rab becomes soluble in the cytoplasm when a GDP-dissociation inhibitor (GDI) shields the geranylgeranyl group. Rab is essentially activated by a specific guanine nucleotide exchange factor (GEF), which is often responsible for its precise localization.^{4,5} Localization and activation of Rab are followed by the recruitment of Rab effectors to the membrane, ensuring various processes of membrane transport.^{2,6,7}

Exocytosis is the process of membrane transport from the Golgi/endosome to the plasma membrane to secrete molecules out of the cell. Regulation of exocytosis by Rabs has been elucidated in detail, especially in the budding yeast *Saccharomyces cerevisiae*. Two conserved Rab proteins, *S. cerevisiae* (Sc) Ypt31/Ypt32 and ScSec4,

which are the respective orthologues of mammalian Rab11 and Rab8, are involved in the exocytic pathway. Activated ScYpt32 recruits ScSec2, a GEF for ScSec4, as its effector, which in turn recruits and activates ScSec4.^{7,8} This regulatory mechanism, termed the “Rab GEF cascade”, is conserved throughout evolution.^{9–13} In the fission yeast *Schizosaccharomyces pombe*, Ypt3 and Ypt2 are orthologues of mammalian Rab11 and Rab8, respectively. As in other organisms, Ypt3 and Ypt2 are involved in the exocytic pathway, because post-Golgi membrane trafficking is severely inhibited in *ypt3* and *ypt2* mutants.^{14–16} During vegetative growth, Ypt3, Ypt2 and the ScSec2 orthologue Sec2 are all localized at the growing sites, cell poles and division sites of cells,^{14,17} and localization of Sec2 and Ypt2 at these sites is diminished in the temperature-sensitive mutant *ypt3-ki8*.¹⁷ In addition, Sec2 preferentially associates with both Ypt3 in its GTP-bound form and Ypt2 in its GDP-bound form.¹⁷ Together, these data suggest that the corresponding Rab-GEF cascade (*i.e.*, Ypt3–Sec2–Ypt2) also functions in the *S. pombe* exocytic pathway.

Sporulation is a cell specialization process that culminates in ascospore formation.^{18,19} During sporulation, a double unit membrane, termed the forespore membrane (FSM), is newly formed within the cytoplasm of the

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diploid mother cell.²⁰⁻²³ Electron microscopy has shown that, prior to FSM formation, thick multilayered disc-shaped structures are added to the cytoplasmic side of the spindle pole body (SPB), the microtubule-organizing center equivalent to the centrosome of animal cells.²⁴ FSM biogenesis then initiates on this modified SPB at metaphase in meiosis II. The FSM expands by membrane vesicle fusion to capture the haploid nuclei produced by meiosis. After meiosis, the edge of the FSM closes to form a daughter cell, in which the separated inner layer of the FSM becomes the plasma membrane of the nascent spore.^{20,21,24,25} Our previous study revealed that Ypt2 and Ypt3 play an important role in the initiation of FSM formation. During metaphase I, Ypt2 relocates to the SPB dependent on Spo13, its meiosis-specific GEF. Spo13 is recruited to the SPB prior to Ypt2 and is also essential for the initiation of FSM formation.^{17,26,27} These data suggest that the meiotically modified SPB serves as the nucleation site for the FSM via Spo13 and Ypt2. In addition, after the recruitment of Ypt2 by Spo13, the constitutive GEF Sec2 also relocates to the SPB independent of Spo13.¹⁷ Therefore, two GEFs for Ypt2 are recruited to the SPB, but the difference in the roles of Spo13 and Sec2 remains unresolved. Here we have focused on the recruitment of Ypt2 to the SPB during sporulation and have examined its molecular mechanism in more detail.

Although the GEF activity of Spo13 is much lower than that of ScSec2 *in vitro*, it is required for the initiation of FSM formation.²⁷ A phenylalanine residue around the center of the GEF domain of Spo13 is essential for activity, and replacement of phenylalanine 77 with alanine (Spo13^{FA}) eliminates it.²⁷ To examine the function of Spo13 in more detail, here we investigated a *spo13-FA* mutant strain, which lacks GEF activity. First, we examined FSM formation by using green fluorescent protein (GFP)-tagged Psy1, an FSM resident SNARE protein.²³ The microtubules were marked by α -tubulin Atb2 N-terminally tagged with mCherry, a monomeric red fluorescent protein (mCherry-Atb2), to monitor the progression of meiosis. During meiosis II in wild-type cells, GFP-Psy1 was observed as four cup-like structures, indicating initiation of the FSM at each end of the spindle microtubules. By contrast, FSM formation was severely inhibited in the *spo13-FA* mutant (Fig. 1A), as reported previously.²⁷ Next, we observed localization of GFP-Ypt2 at the SPB in the *spo13-FA* mutant. In wild-type cells, the GFP-Ypt2 signal appeared as dots at both ends of the spindle microtubule during meiosis I, and was subsequently detected at the FSM during meiosis II (Fig. 1C and D).¹⁷ In the *spo13-FA* mutant, however, the GFP-Ypt2 signal was mainly diffused in the cytoplasm throughout meiosis I and II, similar to observations in

the *spo13* deletion (*spo13* Δ) mutant (Fig. 1C).¹⁷ Given that the Spo13-FA mutant protein (Spo13^{FA}) can localize at the meiotic SPB,²⁷ these results indicate that the defect in initiation of FSM formation in the *spo13-FA* mutant is due to a depletion of Ypt2 recruitment to the SPB by Spo13. Notably, we found that a small fraction of *spo13-FA* cells did initiate FSM formation. About one-third of the *spo13-FA* mutant cells initiated FSM formation during meiosis II (Fig. 1A). In the *spo13-FA* mutant, 5% of diploid cells formed four spores in an ascus. In contrast, spore-like structures were not observed in the *spo13* Δ mutant (Fig. 1B). Consistent with this observation, the GFP-Ypt2 signal was occasionally detected at the end of spindle microtubules in the *spo13-FA* mutant (Fig. 1C), suggesting the possibility that Spo13^{FA} retains some ability to recruit Ypt2 *in vivo*. We therefore examined the behavior of Ypt2^{S18V}, a GDP-locked mutant of Ypt2 that is not activated by Spo13, but binds to it in a yeast two-hybrid assay.¹⁷ GFP-Ypt2^{S18V} was expressed in wild-type cells and its localization was observed. As shown in Fig. 1D and Supplementary Fig. 1, the fluorescent signal of GFP-Ypt2^{S18V} was mainly dispersed throughout the cytoplasm, but showed a slightly higher concentration at the spindle poles during meiosis. This signal at the SPB was detected at a higher frequency as compared with GFP-Ypt2 in *spo13-FA* mutant cells (Fig. 1C and D). Considering that the affinity of Spo13^{FA} for ScSec4 is significantly lower than that of Spo13,²⁷ these data suggest that the binding affinity between Ypt2 and Spo13 is important for recruitment of Ypt2 to the SPB.

These results also suggest that other mechanisms are likely to be involved in Ypt2 recruitment. One possibility is that Spo2, another meiotic SPB resident protein that recruits Spo13 to the SPB, participates in the Ypt2 recruitment. Therefore we confirmed the localization of Ypt2 in both *spo2* Δ and *spo2* Δ *spo13-FA* mutant cells (Supplementary Fig. 2B). GFP-Ypt2 was completely diffused in the cytoplasm in both mutants, probably due to the lack of Spo13 localization at the SPB (Supplementary Fig. 2A).²⁶ Furthermore, overexpression of Spo2-mCherry, which localized at the SPB, did not suppress the defect in recruitment of Ypt2 in *spo2* Δ *spo13* Δ cells (Supplementary Fig. 3). Thus, these data suggest that Spo2 regulates Ypt2 recruitment by acting as a scaffold for Spo13 on the SPB. Another possibility is that the constitutively expressed GEF Sec2 might have role in Ypt2 recruitment. Several lines of evidence suggest that the GEF domains of Sec2 and Spo13 form homodimeric coiled-coil structures.²⁷⁻³⁰ Sec2 at the meiotic SPB probably lacks the ability to recruit Ypt2 in *spo13* Δ cells, although it is recruited to the SPB at late meiosis I independent of Spo13.¹⁷ It is possible that the GEF domains of Sec2 and Spo13^{FA} might form a heterodimeric coiled-

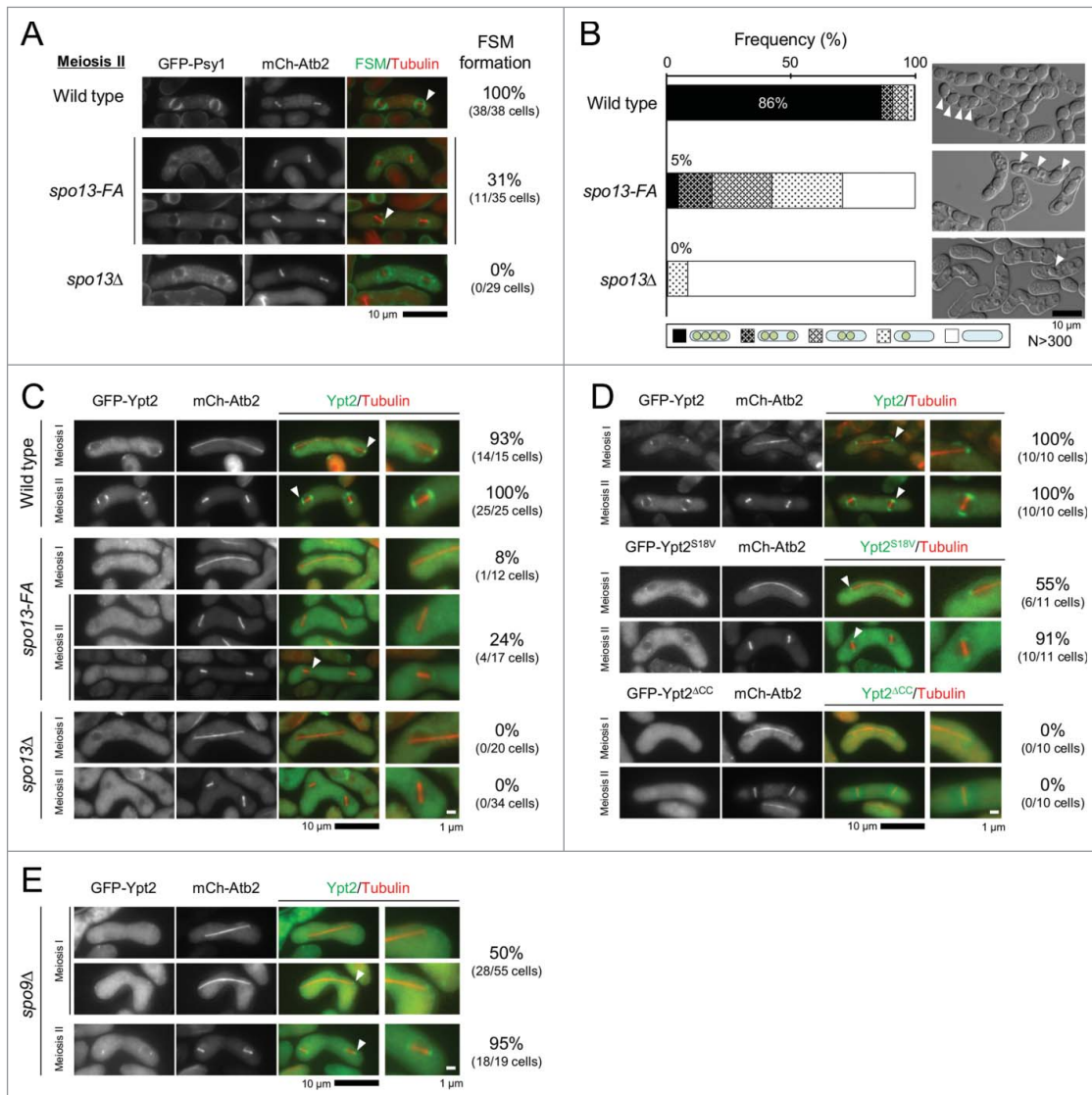


Figure 1. Recruitment of Ypt2 to the SPB by Spo13 requires binding affinity and its prenylation. (A) The early stage of FSM assembly is disrupted in a *spo13* mutant defective in GEF activity. Wild-type (KI173), *spo13-FA* (KI472) and *spo13Δ* (KI495) cells expressing the FSM marker GFP-Psy1 and the microtubule marker mCherry-Atb2 were sporulated on ME plates at 28°C for 1 day. GFP-Psy1 (green) and mCherry-Atb2 (red) are overlaid in the merged images. Arrowheads indicate the FSM formation. The frequency of the cells forming the FSM during meiosis II is also indicated. (B) Spore formation in the *spo13-FA* mutant. Wild-type (KI109), *spo13-FA* (KI470) and *spo13Δ* (KI97) cells were sporulated on ME plates at 28°C for 2 days. Diploid cells were classified by the number of spores or spore-like structures formed in an ascus. Arrowheads indicate spores or spore-like structures. The frequency of asci containing four spores are indicated. N > 300. (C) Ypt2 recruitment to the SPBs in the *spo13-FA* mutant. Wild-type (KI109), *spo13-FA* (KI470) and *spo13Δ* (KI97) cells expressing GFP-Ypt2 and mCherry-Atb2 were sporulated on ME plates at 28°C for 1 day. GFP-Psy1 (green) and mCherry-Atb2 (red) are overlaid in the merged images. Arrowheads indicate GFP-Ypt2 signal at the end of spindle microtubules. High-magnification images are shown on the right. The frequency of diploid cells in which GFP-Ypt2 signal is observed at the end of spindle microtubules during meiosis II was indicated. (D) Localization of a GDP-locked Ypt2 mutant protein (Ypt2^{S18V}) and a Ypt2 mutant protein without the two C-terminal cysteine residues to be geranylgeranylated (Ypt2^{ΔCC}). Wild-type cells expressing mCherry-Atb2 and intact GFP-Ypt2 (KI95), GFP-Ypt2^{S18V} (KI513) or GFP-Ypt2^{ΔCC} (KI452) were sporulated on ME plates at 28°C for 1 day. GFP-Ypt2 (green) and mCherry-Atb2 (red) are overlaid in the merged images. Arrowheads indicate GFP-Ypt2 signal at the end of spindle microtubules. High-magnification images are shown on the right. The frequency of diploid cells in which GFP-Ypt2 signal is observed at the end of spindle microtubules was indicated. (E) Ypt2 recruitment to the SPB in the *spo9Δ* mutant. *spo9Δ* (KI443) cells expressing GFP-Ypt2 and mCherry-Atb2 were sporulated on ME plates at 28°C for 1 day. GFP-Ypt2 (green) and mCherry-Atb2 (red) are overlaid in the merged images. Arrowheads indicate GFP-Ypt2 dots at the end of spindle microtubules. Arrowheads indicate GFP-Ypt2 signal at the end of spindle microtubules. High-magnification images are shown on the right. The frequency of diploid cells in which GFP-Ypt2 signal is observed at the end of spindle microtubules was indicated. Wild type (KI109) is shown in (Fig. 1C and 2B).

coil to recruit and activate Ypt2, ensuring formation of the FSM in the *spo13-FA* mutant. Indeed, in the *spo13-FA* mutant, GFP-Ypt2 localization was mainly observed during meiosis II (Fig. 1C), when Sec2 would have been accumulated at the SPB.¹⁷ Interestingly, the GFP-Ypt2^{S18V} signal persisted at the SPB at meiosis II, whereas wild-type GFP-Ypt2 moved to the expanding FSM (Fig. 1D and Supplementary Fig. 1).¹⁷ These data suggest that activation of Ypt2 is required for its relocalization from the SPB to the FSM, but is not essential for its recruitment to the SPB. Furthermore, these observations suggest that the membrane association of Ypt2 is not necessary for its localization to the SPB.

Generally, Rab proteins are post-translationally modified by one or two geranylgeranyl groups attached at cysteine residues at the C-terminus. The geranylgeranyl moiety of Rab is crucial not only as a lipophilic anchor for membrane association, but also for the interaction with a GDP dissociation inhibitor (GDI). GDI binds to the geranylgeranyl moiety of inactive Rab, which makes Rab soluble in the cytoplasm; subsequently, Rab is brought back to the membrane via a GDI displacement factor (GDF) and GEF.^{7,11,13} GDIs are highly conserved throughout evolution, and *S. pombe* has a single GDI protein, Gdi1. The GDP-bound form of Ypt2 (Ypt2^{S18V}) showed accumulation at the SPB as mentioned above, whereas the hydrolysis-defective GTP-bound form of Ypt2 (Ypt2^{Q68L}) was not observed at the SPB but was diffused uniformly throughout the cytoplasm (Supplementary Fig. 1). From previous studies, Ypt2^{Q68L} is predicted to be defective in geranylgeranylation.¹¹ Therefore these facts suggest that inactive geranylgeranylated Ypt2 is recruited to the SPB during meiosis I. To confirm the requirement for geranylgeranylation of Ypt2, we eliminated the two cysteine residues at the C-terminus of GFP-Ypt2 that are predicted to undergo geranylgeranylation. The resulting truncated protein GFP-Ypt2^{ΔCC} was completely diffused in the cytoplasm (Fig. 1D), indicating that the geranylgeranyl group of Ypt2 is required for its recruitment to the SPB.

In *S. pombe*, the sporulation-related gene *spo9*⁺ encodes a subunit of geranylgeranyl diphosphate synthase.³¹ In the *spo9Δ* mutant, therefore, Rab proteins are predicted to show defective geranylgeranylation modification due to a shortage or absence of geranylgeranyl diphosphate. Indeed, the *spo9Δ* mutation has been shown to affect membrane association and geranylgeranylation of the vacuolar Rab protein Ypt7.³¹ To further confirm the requirement of geranylgeranylation of Ypt2 for its recruitment to the SPB, therefore, we observed the subcellular localization of GFP-Ypt2 in *spo9Δ* cells. We found that the GFP-Ypt2 signal was mainly diffused in the cytoplasm. In half of the *spo9Δ*

cells during anaphase I, the GFP-Ypt2 signal was detected at the SPB but its signal was very weak (Fig. 1E). The GFP-Ypt2 signal was also detected at the FSM during meiosis II (Fig. 1E), suggesting that Ypt2 might be geranylgeranylated slightly and that Ypt2 is associated with the membrane at a reduced level in *spo9Δ*. The *spo9Δ* mutant shows a severe defect in FSM expansion, but not in the initiation of FSM formation.³¹ Thus, it is possible that the defective membrane association of Rab proteins, including Ypt2 and Ypt7, might result in the *spo9Δ* mutant phenotype. It is also consistent with the fact that both Ypt2 and a Rab geranylgeranyl transferase are essential for cell viability, whereas Spo9 is not.³¹ During meiosis I, however, localization of GFP-Ypt2 to the SPB was largely delayed and its fluorescent intensity was remarkably reduced even in *spo9Δ* cells (Fig. 1E). Taking these observations together with the completely abolished localization of Ypt2^{ΔCC} (Fig. 1D), the geranylgeranyl moiety might be important for Ypt2 recruitment to the SPB especially during meiosis I. What is the role of the geranylgeranyl moiety in localization of Ypt2 at the SPB during meiosis I? It is possible that geranylgeranylation affects the membrane association of Ypt2; alternatively, interaction with GDI. Our previous study has shown that, in prophase I, GFP-Ypt2 is observed as many dots in the cytoplasm and is then gradually diffused throughout the cytoplasm, followed by the appearance of its intense dots at the spindle poles during metaphase I.¹⁷ We hypothesized that the GDI Gdi1 might release Ypt2 from the secretory vesicles and transfer it to the SPB. However, the Gdi1-GFP signal was diffused in the cytoplasm and not concentrated at the SPB throughout meiosis I (data not shown). Thus, these observations could not verify the involvement of GDI in Ypt2 relocalization and this hypothesis will need further investigation. In cells at meiosis I, membrane structures around the SPB have not been observed by electron microscopy,^{21,24} whereas GFP-tagged Syb1, the SNARE protein on secretory vesicles, accumulates at the spindle poles at the end of meiosis I.¹⁷ As shown above, the recruitment of GFP-Ypt2^{S18V}, which is predicted to show unstable membrane association, was delayed in meiosis I (Fig. 1D). Taken together, these observations indicate that membrane association of Ypt2 might also be important for recruitment of Ypt2 to the SPB, especially in meiosis I.

As mentioned above, the localization of Spo13 at the SPB is dependent on Spo2. Both Spo2 and Spo13 are localized at the SPB immediately after expression at prophase I.²⁶ During this phase, however, GFP-Ypt2 is not recruited to the SPB despite its constitutive expression.¹⁷ Therefore, we considered that the recruitment of

Ypt2 by Spo13 and its scaffold Spo2 might require an additional regulatory mechanism. To address this possibility, we observed GFP-Ypt2 recruitment by Spo13 during vegetative growth. In vegetative cells expressing GFP-Ypt2, we simultaneously expressed Spo13-mCherry and Spo2 to anchor Spo13 on the mitotic SPB.²⁵ Notably, Ypt2 was not recruited to the SPB by this ectopically-expressed Spo13 (Fig. 2A). Thus, these data indicate that the Ypt2 is recruited to the SPB in a meiosis-specific manner.

In the secretory pathway in *S. cerevisiae*, a nuclear Dbf2-related (NDR) serine/threonine kinase, ScCbk1, phosphorylates ScSec2 and regulates its localization to the secretory vesicles, thereby affecting the recruitment of ScSec4.³² Moreover, it has been proposed that phosphorylation of ScSec2 by other unknown kinases affects its binding specificity.³³ Furthermore, in mammals, NDR2 phosphorylates the Sec2 orthologue Rabin8, causing a change in binding specificity, which is required for ciliary membrane formation.^{34,35} NDR kinases are highly conserved throughout evolution, and *S. pombe* possesses three NDR kinase homologues, Sid2, Orb6 and Mug27.³⁶⁻³⁸ Sid2 and Orb6 are involved in two conserved cross-talking signaling pathways that ensure cell proliferation: namely, the septation initiation network (SIN) and the morphogenesis Orb6 network (MOR), respectively.^{39,40} The SIN pathway triggers the initiation of cytokinesis and comprises an upstream GTPase (Spg1), two scaffolds (Cdc11 and Sid4) and three kinases (Cdc7, Sid1 and Sid2) that form a cascade, whereas the MOR pathway is required for polarized growth and cell separation after cytokinesis, and comprises an upstream regulator (Pmo25), a scaffold (Mor2) and two kinases (Nak1 and Orb6).^{39,41} Mug27 (also known as Ppk35 and Slk1) is a meiosis-specific NDR kinase that is closely related to the SIN kinase Sid2.³⁷ Intriguingly, both Mug27 and the SIN components accumulate at the SPB during meiosis I and have a role in FSM expansion.^{37,42} Mug27 functions in the same pathway as Sid2 because Sid2 rescues the sporulation defect in *mug27Δ*, but Mug27 is not thought to be a component of the SIN pathway because its localization to the SPB is independent of the SIN scaffold Cdc11.³⁷ Moreover, Mug27 is recruited to the SPB at metaphase of meiosis I, but not during vegetative growth.³⁷ We hypothesized that Mug27 and/or the SIN pathway might regulate the recruitment of Ypt2 to the meiotic SPB. We therefore examined Ypt2 localization in mutants of Mug27 (*mug27Δ*) and the SIN pathway (*sid2-250* and *cdc7-24*). To evaluate the precise timepoint of Ypt2 localization, the length of spindle microtubules was measured and the cells were classified into three types based on the following behavior of GFP-Ypt2: i) GFP-Ypt2 was not detected

at either end of the spindle microtubule; ii) GFP-Ypt2 was observed at one end of the spindle microtubule; iii) GFP-Ypt2 was observed at both ends of the spindle microtubule. In wild-type cells, GFP-Ypt2 was observed at the SPB at metaphase I or early anaphase I. In contrast, recruitment of Ypt2 was delayed in the SIN mutants *cdc7-24* and *sid2-250*, but not in *mug27Δ* cells (Fig. 2B and C), indicating that Cdc7 is likely to activate Sid2, which meiotically regulates the recruitment of Ypt2. To examine whether this delay in Ypt2 localization was due to the delay in Spo13 localization at the SPB, we observed the recruitment of Spo13-GFP to the SPB in the SIN mutants. As shown in Fig. 2D, Spo13-GFP was expressed and localized at the SPB at prophase I similar to wild-type cells. These results indicate that recruitment of Ypt2 to the SPB is partially regulated by the SIN pathway. In addition, the *mug27Δsid2-250* double mutant showed more significant delay in Ypt2 localization (Fig. 2B and C), suggesting that Mug27 might also be involved in the regulation of Ypt2 recruitment.

Lastly, we observed FSM formation. The SIN mutants showed a moderate defect in FSM formation (Fig. 2E) and formed aberrant spores (Fig. 2F). As compared with the SIN mutants, the *mug27Δ* mutant showed a severer defect in sporulation, whereby much smaller spore-like structures were observed in the ascus.³⁷ (Fig. 2F) Consistent with a previous report indicating that Mug27 and Sid2 have redundant roles in FSM expansion,³⁷ the *mug27Δsid2-250* double mutant showed a significant defect in FSM expansion (Fig. 2E) and formed no spore-like structures (Fig. 2F). Taken together, the two related-NDR kinases Mug27 and Sid2 have different functions on the meiotic SPB: Mug27 regulates mainly FSM expansion, whereas Sid2 is mainly responsible for the efficient recruitment of Ypt2.

These meiotic functions of NDR kinases might be mediated via the phosphorylation of unknown target proteins, because the protein kinase activity of Mug27 is required for sporulation.³⁷ The consensus sequence of phosphorylation by Sid2 kinase is RXXS.⁴³ One possibility is that Sid2 phosphorylates a GEF specifically at meiosis, as in the case of ScCbk1 in the budding yeast exocytic pathway. However, neither Spo13 nor its binding partner Spo2 contains the RXXS motif, suggesting that they are not a direct target of phosphorylation by NDR kinases. Ypt2 has an RXXS sequence at its C-terminus, but immunoblotting bands of GFP-Ypt2 do not shift during meiosis,¹⁷ suggesting that Ypt2 might not be modified in a meiosis-specific manner. Therefore, an unknown phosphorylation target of the SIN pathway might exist and mediate the regulation of Ypt2 recruitment by Spo13.

Taken together, our results enable us to propose a model in which Ypt2 regulates FSM formation during

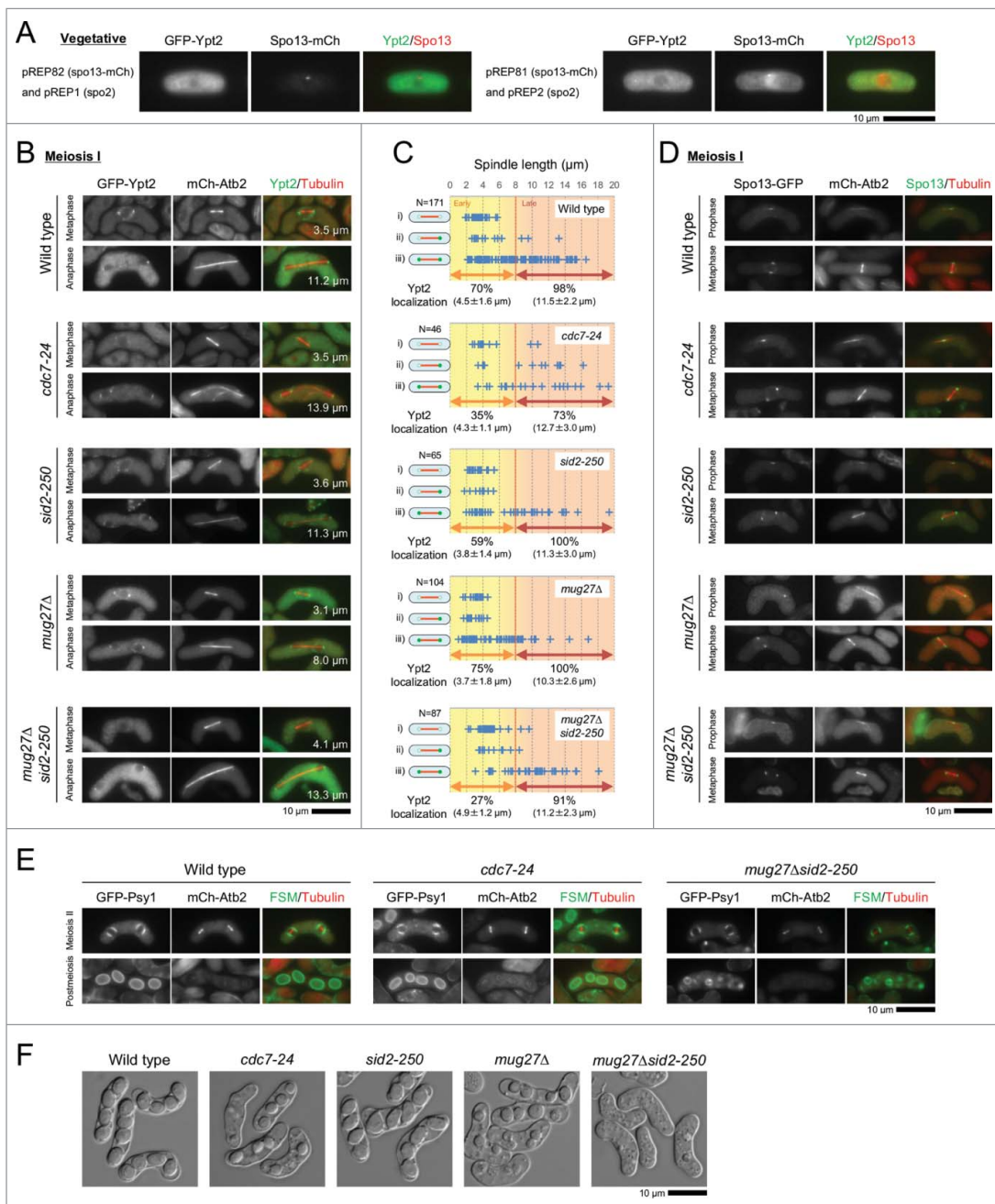


Figure 2. Meiosis-specific recruitment of Ypt2 is partially regulated by NDR kinases. (A) Ypt2 is not recruited by SPB-anchored Spo13 during vegetative growth. Wild-type cells (KI81) were transformed by two plasmids to express Spo13-mCherry and Spo2 ectopically during vegetative growth. Left, cells transformed by pKI98 and pYN177. Right, cells transformed by pKI97 and pYN183. Transformed cells were grown on MM+N plates at 28°C for 1 day. GFP-Ypt2 (green) and Spo13-mCherry (red) are overlaid in the merged images. GFP-Ypt2 was not detected at the SPB where Spo13-mCherry localized. $N > 10$. (B, C) Ypt2 recruitment is delayed in the SIN mutants. Wild-type (KI109), *cdc7-24* (KI426), *sid2-250* (KI512), *mug27Δ* (KI500) and *mug27Δ sid2-250* double mutant (KI447) expressing GFP-Ypt2 and mCherry-Atb2 were sporulated on ME plates at 28°C for 1 day. (B) GFP-Ypt2 (green) and mCherry-Atb2 (red) are overlaid in the merged images. The spindle length is shown at the bottom right of the merged images. (C) Mutant cells were classified into three types by GFP-Ypt2 behavior: i) GFP-Ypt2 was not detected at either spindle pole (C, top of each graph); ii) GFP-Ypt2 was observed at one spindle pole (C, middle of each graph); iii) GFP-Ypt2 was observed at both spindle poles (C, bottom of each graph). The percentage of Ypt2 localization is shown on the bottom of each graph. (D) Spo13 is recruited normally in the *mug27* and SIN mutants. Wild-type (KI112), *cdc7-24* (KI479), *sid2-250* (KI508), *mug27Δ* (KI503), and *mug27Δ sid2-250* double mutant (KI496) cells expressing Spo13-GFP and mCherry-Atb2 were sporulated on ME plates at 28°C for 1 day. Spo13-GFP (green) and mCherry-Atb2 (red) are overlaid in the merged images. In all mutant cells during metaphase I, Spo13-GFP was detected at the both ends of the spindle microtubules. $N > 10$. (E) FSM formation in the *mug27* and SIN mutants. Wild-type (KI173), *cdc7-24* (KI430) and *mug27Δ sid2-250* double mutant (KI449) cells expressing GFP-Psy1 and mCherry-Atb2 were sporulated on ME plates at 28°C for 1 day. GFP-Psy1 (green) and mCherry-Atb2 (red) are overlaid in the merged images. (F) Spore formation in the *mug27* and SIN mutants. Wild type (KI112), *cdc7-24* (KI479), *sid2-250* (KI508), *mug27Δ* (KI503), *mug27Δ sid2-250* double mutant (KI496) cells were sporulated on ME plates at 28°C for 2 days.

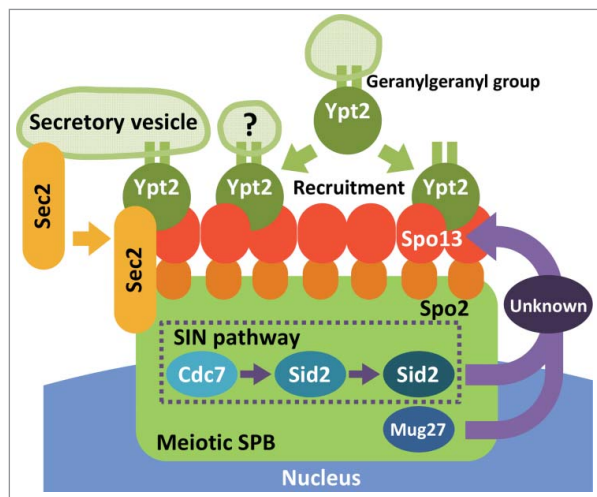


Figure 3. Model of Ypt2 recruitment. In prophase I, Spo13 and its scaffold protein Spo2 are expressed and localized at the SPB, when Ypt2 is dispersed throughout the cytoplasm. In metaphase I, Ypt2 is recruited to the SPB by Spo13. It requires geranylgeranyl moiety of Ypt2. The SIN pathway is meiotically activated at the SPB. Activated Sid2 and its homologue Mug27 phosphorylate an unknown target that allows Spo13 to recruit Ypt2 efficiently at metaphase I or early anaphase I. At late anaphase I, exocytosis-related components including Sec2 accumulate at the SPB. Ypt2 is likely to be activated mainly by Sec2, which ensures its stable membrane association.

meiosis (Fig. 3). At prophase of meiosis I, Spo13 is expressed and localized at the SPB via Spo2 (Fig. 2D and Supplementary Fig. 2A),²⁶ whereas Ypt2 proteins are localized in vesicles dispersed throughout the cytoplasm.¹⁷ Next, Ypt2 may be extracted from the vesicles by GDI, which in turn may mediate the recruitment of Ypt2 to the SPB by Spo13 at metaphase I.¹⁷ The NDR kinase Sid2 is meiotically activated by the SIN cascade, including Cdc7, and localized at the SPB. Activated Sid2 phosphorylates an unknown target that allows Spo13 to recruit Ypt2 efficiently at metaphase I or early anaphase I (Fig. 2B and C).³⁷ Subsequently, at late anaphase I, exocytosis-related components including Sec2 accumulate at the SPB.¹⁷ Considering that *spo13-FA* mutant cells were observed to occasionally form the FSM and that inactive Ypt2 (Ypt2^{S18V}) remained localized at the SPB (Fig. 1C and D), Ypt2 is likely to be activated mainly by Sec2, which ensures its stable membrane association. Why does Sec2 at the SPB not recruit Ypt2 in the *spo13Δ* mutant? We found that the Spo2-fusion protein Spo13-Spo2-mCherry was anchored on the SPB and rescued the defects in Ypt2 recruitment and sporulation in the *spo2Δspo13Δ* double mutant (Supplementary Fig. 3). We also examined the behavior of the Sec2-Spo2-mCherry fusion protein in the *spo2Δspo13Δ* mutant. The fluorescent signal of Sec2-Spo2-mCherry was weak, but present at the SPB.

The SPB-anchored Sec2-Spo2-mCherry occasionally recruited GFP-Ypt2 in the *spo2Δspo13Δ* cells at meiosis II, but sporulation was still defective (Supplementary Fig. 3). These data suggest that the Sec2 homodimer remains able to recruit Ypt2 during meiosis, but its function might be inhibited by unknown mechanisms, at least in the *spo13Δ* mutant. As mentioned above, it is possible that a Sec2-Spo13 heterodimer might activate Ypt2 on the meiotic SPB. For elucidation of the meiotic regulation of Ypt2 recruitment and the functions of its GEFs, further studies will be required.

Materials and methods

Yeast strains and culture conditions

The fission yeast strains used in this study are listed in Supplementary Table S1. Strains expressing tagged proteins were constructed by crossing two strains and/or by integration of the plasmid DNA listed in Supplementary Table 2. Vegetative cultures were propagated in complete (YE) or synthetic (SD, MB or MM+N) medium supplemented with nutrients essential for auxotrophic strains. For sporulation, cells precultured on YE or SD plates were incubated on malt extract medium (ME) or synthetic sporulation medium (SSA) plates, respectively.

The *spo13* mutant strains defective in GEF activity were constructed as follows. The *spo13-FA* mutation was introduced by site-directed mutagenesis by PCR using primers incorporating the mutation. The resulting PCR product was introduced into *spo13Δ* cells, in which the *spo13* gene was disrupted by the *ura4⁺* gene (*spo13::ura4⁺*). The cells were spread on YE plates containing 15 mg/ml of 5-fluoroorotic acid (5-FOA) to screen for transformants in which the *spo13::ura4⁺* locus was chromosomally replaced by *spo13-FA*. Sequencing analysis of the *spo13* locus verified that the transformants obtained were *spo13-FA* mutants.

Plasmids

The plasmids used in this study are listed in Supplementary Table 2. A GTP- or GDP-locked mutation (Q68L or S18V, respectively) was introduced into the *ypt2promoter-GFP-ypt2* fragment by PCR using pKI17 as a template and primers containing the respective mutation. Elimination of the two cysteine residues at the C-terminus of Ypt2 that are geranylgeranylated was conducted similarly. The resulting PCR products were digested by *ApaI* and *SacI*, and subsequently cloned into integration vectors. Further information on the plasmids constructed in this study is given on the National

BioResource Project (NBRP) Yeast website (<http://yeast.lab.nig.ac.jp/yeast/>).

Fluorescence microscopy

Proteins were visualized by fusion to GFP or mCherry. Living cells were observed under a fluorescence microscope (model BX51; Olympus, Tokyo, Japan), and images were obtained by using a cooled charge-coupled device (CCD) camera (ORCA-R2; Hamamatsu Photonics, Hamamatsu, Japan) controlled by AQUACOSMOS software (Hamamatsu Photonics). Images were processed and analyzed by using Image J software.

Abbreviations

FSM forespore membrane
GDI GDP dissociation inhibitor
GEF guanine nucleotide exchange factor
SPB spindle pole body

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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