Mec1/Tel1-independent role of protein phosphatase 4 in Hop1 assembly to promote meiotic chromosome axis formation in budding yeast

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Abstract

Dynamic changes in chromosomal structure that occur during meiotic prophase play an important role in the progression of meiosis. Among them, meiosis-specific chromosomal axis-loop structures are important as a scaffold for integrated control between the meiotic recombination reaction and the associated checkpoint system to ensure accurate chromosome segregation. However, the molecular mechanism of the initial step of chromosome axis-loop construction is not well understood. Here, we showed that, in budding yeast, a Tel1/Mec1-related protein phosphatase 4 (PP4) is required to promote the assembly of a chromosomal axis components Hop1 and Red1 onto meiotic chromatin via interaction with Hop1. PP4 did not affect Rec8 assembly. Notably, unlike the previously known function of PP4, this novel function was independent of Tel1/Mec1 kinase functions. The defect in Hop1/Red1 assembly in the absence of PP4 function was not suppressed by Pch2 dysfunction. Since Pch2 is a conserved AAA+ ATPase and facilitates eviction of Hop1 protein from the chromosome axis, suggesting that PP4 is required for the initial step of chromatin loading of Hop1 rather than stabilizing Hop1 on the chromosome axis. These results indicate phosphorylation/dephosphorylation-mediated regulation of Hop1 recruitment onto chromatin during chromosome axis construction before meiotic double-strand break formation.

(194 words)

Introduction

Meiotic recombination is essential for proper segregation of homologous chromosomes during the first meiotic division (meiosis-I) and is initiated by Spo11-dependent programmed meiotic DNA double-strand break (DSB) formation at meiotic recombination hot spots (KEENEY 2001). Meiotic DSB formation and subsequent meiotic recombination require a specific scaffold, an axis-loop structure of chromatin (SHINOHARA *et al.* 2000; BLAT *et al.* 2002; PANIZZA *et al.* 2011). A highly conserved meiotic chromosome axis structure contains core factors Red1, Hop1, and meiosis-specific cohesin complexes, including the meiosis-specific subunit Rec8 in budding yeast (ROCKMILL AND ROEDER 1988; HOLLINGSWORTH AND BYERS 1989; KLEIN *et al.* 1999). After meiotic DSB formation, recombinases Rad51/Dmc1 are recruited to single-stranded DSB ends (BISHOP *et al.* 1992; BISHOP 1994; SHINOHARA *et al.* 1997). The single-stranded DSB ends promote the assembly of Zip-Msh-Mer/synapsis initiation complex (ZMM/SIC) proteins, including the transverse element component Zip1, to construct the synaptonemal complex (SC) and promote crossover (CO) formation (SYM *et al.* 1993; NOVAK *et al.* 2001; BORNER *et al.* 2004; SHINOHARA *et al.* 2008; SHINOHARA *et al.* 2015; DE MUYT *et al.* 2018). In the early leptotene, Zip1 with Zip3 localizes to the centromere and mediates centromere pairing as an initial process to promote chromosome pairing (TSUBOUCHI AND ROEDER 2005; FALK *et al.* 2010).

Hop1 is an axis-associated HORMA-domain protein with multiple roles during meiotic recombination. Hop1 is required for efficient meiotic DSB formation, inter-homolog bias in meiotic recombination, and meiotic prophase checkpoint activation and inactivation (HOLLINGSWORTH *et al.* 1990; SCHWACHA AND KLECKNER 1997; NIU *et al.* 2005; CARBALLO *et al.* 2008; PANIZZA *et al.* 2011; WEST *et al.* 2018). Hop1 together

with Red1 promotes DSB formation. Hop1 is phosphorylated by Tel1/Mec1 (yeast ATM/ATR) kinases, which are sensor kinases of DSBs that activate the meiotic prophase checkpoint signaling pathway via Mek1/Mre4 kinase in response to meiotic recombination as well as synapsis defects (NIU *et al.* 2005; BORNER *et al.* 2008). Hop1, Red1, and Mek1/Mre4 form the RHM complex involved in forming a stable axis structure as a scaffold to integrate recombination and the required regulatory responses associated with it. After meiotic DSB repair, Zip1-mediated SC formation and a conserved AAA+ ATPase Pch2 promote Hop1 removal from the synapsed chromosome axis and attenuate the checkpoint signal (BORNER *et al.* 2008). Recently, Pch2 was demonstrated to be involved in the activation as well as the inactivation of meiotic prophase checkpoint through Hop1 (RAINA AND VADER 2020). Thus, Hop1 phosphorylation is a key step to complete proper meiotic recombination.

Tel1/Mec1 is also required for the integrated meiotic recombination and checkpoint activation. Mec1/Tel1 kinase is involved in intra-chromosomal regulation of the distribution of meiotic DSB formation sites (ANDERSON *et al.* 2015; JOSHI *et al.* 2015; CHALLA *et al.* 2019) and signal transduction of the positive/negative feedback loop of meiotic DSB formation through Rec114 phosphorylation (SASANUMA *et al.* 2007; CARBALLO *et al.* 2008; ZHANG *et al.* 2011). In addition Mec1/Tel1 mediates DSB interference as well as CO interference which is required for non-random distribution (ZHANG *et al.* 2011; GARCIA *et al.* 2015; SHINOHARA *et al.* 2019).

Protein phosphatase 4 (PP4) is a conserved putative serine/threonine protein phosphatase that functions specifically in response to DNA damage by attenuating the phosphorylation signal by Mec1 and Tel1 during mitosis and meiosis (HOFFMANN *et al.* 1994; KEOGH *et al.* 2006; O'NEILL *et al.* 2007; FALK *et al.* 2010; LAI *et al.* 2011;

HUSTEDT *et al.* 2015). Mec1/Tel1 phosphorylates many target proteins including Rad53, an ortholog of Chk2 kinase in budding yeast. In the mitotic DNA damage response pathway, PP4 dephosphorylates these targets in the process of recovery from DNA damage-induced signal transduction (O'NEILL *et al.* 2007). Several studies have revealed the meiotic DSB-associated function of PP4 during meiotic recombination (FALK *et al.* 2010; CHUANG *et al.* 2012). During meiosis, centromere pairing is regulated by the balance of phosphorylation and dephosphorylation of Zip1 through Mec1/Tel1 kinase and PP4 phosphatase (FALK *et al.* 2010).

Here, we focused on the novel function of PP4 which is independent of Mec1/Tel1 function in early prophase-I, especially in a proper meiotic chromosome axis construction via physical interaction with Hop1 protein.

Results

PP4 activity is required for timely meiotic DSB formation and efficient CO formation

Pph3 is a catalytic subunit of the yeast PP4 complex. Pph3 contains several serine/threonine phosphatase motifs (HOFFMANN *et al.* 1994). We constructed a catalytic mutation in the yeast *PPH3* gene by substitution of the conserved histidine residue 112 required for phosphate binding with asparagine (SHI 2009) to generate the *pph3-H112N* mutant (Figure 1A). The mitotic phenotypes of cells with this mutation were analyzed. Pph3 is required for Rad53 dephosphorylation and mitotic DNA damage repair (O'NEILL *et al.* 2007). The *pph3-H112N* mutation did not affect the expression of another PP4 subunit Psy2, with conjugation of a 13-repeated Myc epitope tag (Psy2-13Myc) (Figure S1A). In addition, we analyzed the assembly of Psy2-13Myc on to meiotic chromatin in pachytene cells (Figure 1B). Consistent with a previous study (FALK *et al.* 2010), we detected Psy2-13Myc signals as punctate foci on meiotic chromatin. Importantly, the *pph3-H112N* mutation did not affect the Psy2-13Myc localization. Pph3 and Psy2 form a very stable PP4 complex (O'NEILL *et al.* 2007), this suggesting that the catalytic activity of PP4 is not required for its meiotic chromatin localization. Folk *et al.* reported the meiotic phenotypes of the *pph3*-null (*pph3Δ*) mutant (FALK *et al.* 2010). Therefore, we characterized two *pph3* mutants, *pph3Δ* and *pph3-H112N*, to understand the function of PP4 and its phosphatase activity during meiosis in more detail. First, it was revealed that both *pph3* mutants showed similarly delayed meiosis progression compared to the wild type (Figure S1B). This result was consistent with a previous study on meiosis progression in the *pph3Δ* and *psy2Δ* mutants (FALK *et al.* 2010).

To confirm whether the delayed meiosis progression in *pph3* mutants is caused by meiotic recombination defects, we analyzed programmed meiotic DSB formation as an initial event of meiotic recombination by Southern blotting at the *HIS4-LEU2* hot spot in chromosome III in the *pph3* mutants, although meiotic crossover formation defects in *pph3Δ* have already been reported (FALK *et al.* 2010). The *HIS4-LEU2* hot spot is a well-analyzed artificial meiotic DSB hotspot that includes two known sites, DSB I and II (Figure 1C). We detected and quantified DSB I and showed its frequencies at each time point after meiosis entry (Figure 1D). Meiotic DSBs became detectable 3 h after meiosis entry, and showed a peak at 4 h, and disappeared by 6 h in the wild type (Figure 1D). On the other hand, both *pph3-H112N* and *pph3Δ* showed an approximately 1 h delay in DSB formation compared to the wild type at the time when the DSB reached half of the peak height (11%; Figure 1D, dashed line). In addition, the *pph3* mutants showed an accumulation of meiotic DSBs with hyper-resected ends until 6 h after meiosis entry, resulting in an approximately 3 h delay in DSB disappearance (Figure 1D). These observations indicated the necessity of PP4 phosphatase activity for efficient DSB formation and turnover. PP4 is a Mec1/Tel1-related protein phosphatase. Previous studies revealed that PP4 is required for centromere pairing through Zip1 dephosphorylation for Mec1-mediated phosphorylation at S75, or dephosphorylation of many other Mec1/Tel1 targets during meiosis (KEOGH *et al.* 2006; FALK *et al.* 2010; HUSTEDT *et al.* 2015). Thus, we constructed mitotic cyclin *CLB2* promoter-driven *MEC1* (*pCLB2-MEC1*) *tel1Δ* allele to determine the effect of Mec1 and Tel1 on delayed DSB formation and/or turnover in the *pph3* mutants. *pCLB2* induces mitosis-specific gene expression. Thus, *pCLB2-MEC1* causes meiosis-specific shutdown of *MEC1* transcription (LEE AND AMON 2003). The

pCLB2-MEC1 tel1Δ cells showed a reduced level of steady-state meiotic DSB formation ($7.7 \pm 1.9\%$ of total DNA) compared to that in the wild type ($22.7 \pm 3.1\%$). *pCLB2-MEC1 tel1Δ* and *pCLB2-MEC1 tel1Δ pph3Δ* triple mutants showed more severe reduction of DSB formation ($5.3 \pm 0.45\%$ and $7.7 \pm 3.2\%$, respectively; Figure 1D, graph). At later time points, in contrast to the *pph3* mutants, the *pCLB2-MEC1 tel1Δ* and *pCLB2-MEC1 tel1Δ pph3Δ* mutants showed the timely disappearance of meiotic DSBs by 6 h in meiosis (Figure 1D). These findings suggested that the accumulation of DSBs and the resulting delay in DSB repair, which was observed in the *pph3* mutants at later time points (at 5–6 h) was dependent on Mec1/Tel1 (Figure 1E). In contrast, in the initiation of DSB formation, the *pCLB2-MEC1 tel1Δ pph3Δ* triple mutant showed a peak at 4 h, whereas the *pCLB2-MEC1 tel1Δ* double mutant peaked between 3 and 4 h after onset of meiosis entry (Figure 1E, graph). Since a delay in meiotic DSB formation in the absence of Tel1/Mec1 activity was observed, the delayed DSB formation caused by *pph3* mutations appeared to be independent of Mec1/Tel1.

Next, we analyzed hetero-duplex (HD) formation using Southern blot to analyze meiotic inter-homolog recombination products by using heteroallelic restriction enzyme sites *MluI* and *BamHI* located near the DSB I site and polymorphic *XhoI* sites around the *HIS4-LEU2* hot spot (Figure 1C) (STORLAZZI *et al.* 1996). Corresponding to the delayed meiotic DSB appearance and disappearance at the hot spot, the formation of both HDs corresponding to intermediates for crossover (CO) and non-crossover (NCO) were delayed by 3 h in the *pph3* mutants relative to that in the wild type (Figure 1E). In addition, an increase of NCO intermediates (HD1 and 4) at 8 h and a slight reduction of CO intermediates (HD2 and 3) compared to wild type were observed not

only in the *pph3Δ*, as reported previously (FALK *et al.* 2010), but also in the *pph3-H112N* mutant (Figure 1E).

After DSB end resection, meiotic DSBs are recognized by recombinases Rad51 and Dmc1 to facilitate strand exchange for the formation of COs and NCOs. The cytological analysis revealed that Rad51 appear transiently as punctate foci on meiotic chromatin structure in the wild type (SHINOHARA *et al.* 2000). Thus, we next examined the kinetics of Rad51 assembly and disassembly in the *pph3* mutants using cytological analysis (Figure 1F). In the *pph3* mutants there was a 1 h delay in the Rad51 assembly compared to the wild type. In addition, accumulation of Rad51 foci was evident by 5 h after meiotic entry (Supplemental Figure 1C). On the other hand, the number of Rad51 foci per nucleus at an early time point (3 h) in the *pph3Δ* and *pph3-H112N* mutants was significantly lower than that in the wild type (64 ± 2.7 , 56 ± 2.7 , and 49 ± 3.3 , respectively; Figure 1F). This also indicated the involvement of PP4, as well as its phosphatase activity, for efficient DSB formation. In the disassembly phase of Rad51 foci, a significant reduction in the number of Rad51 foci per nucleus was observed in the wild type. However, continuous accumulation of Rad51 foci was observed in the *pph3* mutants corresponding to inefficient meiotic DSB repair.

PP4 is involved in efficient Hop1 assembly onto chromatin in a Mec1/Tel1-independent manner

Hop1 is a meiosis-specific phosphorylation target of Mec1 and Tel1, and PP4 is involved in the dephosphorylation of these targets (CARBALLO *et al.* 2008; FALK *et al.* 2010). Furthermore, Hop1 is a HORMA-domain protein and a component of the axis structure as a complex with Red1 to form the SC. Hop1 is essential for partner choice

of meiotic recombination (known as inter-homolog bias) and for efficient DSB formation (HOLLINGSWORTH *et al.* 1990; NIU *et al.* 2005; PANIZZA *et al.* 2011; SUBRAMANIAN *et al.* 2016). Since it was observed (Figure 1C and D) that PP4 is required for efficient initiation of meiotic DSB formation, we analyzed the kinetics of Hop1 assembly during meiotic prophase-I (Figure 2A). In wild type Hop1, assembly began 2 h after the onset of meiosis, corresponding to leptotene, and occurred prior to DSB formation.

We also analyzed the localization of Hop1 and Hop1 phosphorylated at threonine 318 (Hop1-pT318) on meiotic chromatin. Hop1 phosphorylation is dependent on Mec1/Tel1 kinase activation by Spo11-induced meiotic DSB formation (CARBALLO *et al.* 2008). Hop1-pT318 has been observed as punctate foci at the end of short patches of Hop1 signal in the wild type (CARBALLO *et al.* 2008) (Figure 2A and B). In the wild type, 50% of the cells displayed Hop1 foci 2 h after meiosis entry, but low levels of Hop1-pT318 were observed at the same time. Hop1-pT318 foci began to appear after 3 h (Figure 2A and B). Hop1-pT318 focus formation requires single-stranded region at meiotic DSB ends (IWASAKI *et al.* 2016). The time lag of approximately 2 h that was evident between the Hop1 loading and the Hop1-pT318 appearance in the present study indicated that Hop1 assembly occurs prior to the DSB formation and/or DSB-end resection in wild type. In the *pph3* mutants, Hop1 assembly was rarely seen at 2 h compared to the wild type. After 2 h, Hop1 staining gradually increased and reached the wild-type level frequency (80%) at 5 h. Notably, pT318 staining appeared at almost the same time (3 h) as the appearance of Hop1 foci (Figure 2A and B) and then gradually increased, similar to Hop1. Hop1 loading seemed to be the rate-limiting factor for Hop1 phosphorylation. These results showed that Pph3 and its activity can promote

the efficient loading of Hop1 on chromosomes. We confirmed that this was not due to delayed expression of Hop1 in the mutant (see Figure 2C).

Next, we examined the effect of Mec1 and Tel1 on Hop1 assembly in the *pph3Δ* mutant. While the *pCLB2-MEC1 tel1Δ* mutant cells did not show any defects in the Hop1 assembly, the *pCLB2-MEC1 tel1Δ pph3Δ* mutant displayed delayed Hop1 assembly, similar to the *pph3* mutants (Figure 2A and B). In contrast, very few Hop1-pT318 foci were observed in the *pCLB2-MEC1 tel1Δ* mutation background.

Next, we analyzed the phosphorylation status of target proteins of PP4 during meiosis using western blot. PP4 is known to be involved in dephosphorylation of phosphorylated S129 of histone H2A (γH2A) after DNA damage in vegetative growth cells (KEOGH *et al.* 2006), and in Hop1-pT318 during meiosis (CHUANG *et al.* 2012). Thus, we first confirmed that γH2A signal accumulated in the *pph3* mutants during meiosis in a Mec1/Tel1 dependent manner, similar to mitotic cells, with markedly reduced γH2A signals in the *pCLB2-MEC1 tel1Δ pph3Δ* mutant (Figure 2C). Moreover, accumulation of Hop1-pT318 signal was also observed until 8 h in the *pph3Δ* mutant. This phosphorylation was dependent on Mec1/Tel1 (Figure 2C). Although Hop1 protein expression was detectable from 2 h after meiosis entry in both wild type and *pph3* mutants, initiation of Hop1 phosphorylation at T318 was delayed by 1 h in the *pph3* mutants compared to the wild type to reach maximum levels. Notably, despite the timely expression of Hop1 protein in the *pph3* mutants (Figure 2D), the appearance of Hop1 foci on meiotic nuclear spread was significantly delayed in the *pph3* mutants (Figure 2A). This result suggested that PP4 might not be involved in Hop1 expression, but rather acted to promotes Hop1 loading onto chromatin. Moreover, the expression kinetics of Hop1 protein were also normal in the *pCLB2-MEC1 tel1Δ* or *pCLB2-MEC1*

tel1Δ pph3Δ mutants. In contrast to the γH2A phosphorylation, residual Hop1-pT318 signal was not detected in the *pCLB2-MEC1 tel1Δ* background, consistent with a previous report showing that Hop1 phosphorylation largely depends on Mec1 and Tel1 activities (CARBALLO *et al.* 2008). Overall, the findings indicate a defect in Hop1 assembly on chromatin, even though a sufficient amount of Hop1 protein is expressed.

PP4 is involved in meiotic chromosome axes formation

Next, we examined the Red1 and Rec8 meiotic chromosome axis components (ROCKMILL AND ROEDER 1988; KLEIN *et al.* 1999). Red1 forms a complex with Hop1 (NIU *et al.* 2005) and Rec8 is a component of the meiosis-specific sister chromatid cohesin complex. Both proteins are required for the proper formation of axial elements in meiosis and a highly-ordered specific chromosome structure, termed the synaptonemal complex (SC) (KLEIN *et al.* 1999). Red1 and Rec8 are recruited to the chromatin independent of each other (SUN *et al.* 2015). We examined Red1 and Rec8 staining of the meiotic chromatin (Figure 3A). In the wild type, Red1 and Rec8 colocalized with each other and formed linear structures along the 4',6-diamidino-2-phenylindole (DAPI) nuclear signal on meiotic chromatin 3 h after meiosis entry. In contrast, in the *pph3* mutants, Red1 and Rec8 were observed as many fine signals throughout the chromatin spreads. The number of Rec8 signals was clearly higher than that of Red1. Time course experiments revealed the significant delay in the assembly of Red1 protein in the *pph3* mutants, similar to the assembly of Hop1 shown in Figure 2A and 2B. In contrast, the timing of Rec8 assembly, despite of its unique structure, was not affected in the presence or absence of Pph3 activity (Figure 3B). In addition, a delay in the disassembly of both Red1 and Rec8 was observed in the *pph3* mutants.

Next, we analyzed Red1 and Rec8 protein expression during meiosis using western blot (Figure 3C). Rec8 phosphorylation via Dbf4-dependent kinase (DDK) and polo-like kinase 1 (PLK1) is associated with cleavage-independent cohesion release from chromatin during late prophase-I (CHALLA *et al.* 2019). While the expression of Rec8 occurred at almost the same time in the *pph3* mutants as that in the wild type, the phosphorylation of Rec8 was delayed in the *pph3* mutants compared to that in the wild type. Corresponding to the delayed phosphorylation of Rec8, Rec8 cleavage that occurs during the meiotic divisions was also delayed in the *pph3* mutants. These results were consistent with the delayed meiosis progression of meiosis in *pph3* mutants (Figure S1). In addition, Red1 protein expression was quite normal in the *pph3* mutants (Figure 3C). These results suggest that PP4 promotes Hop1-Red1 loading but not Rec8-cohesin, which is required for the construction of a proper axis structure.

PP4 physically interacts with Hop1 protein

We next examined the physical interaction between the Hop1-Red1 and PP4 using a co-immunoprecipitation assay (Figure 4A). Psy2-13Myc protein was precipitated using anti-Myc antibody in meiotic cell extracts of *PSY2-13MYC* derivative *PPH3* (wild type) cells. The Hop1 signal was detected in the precipitate from the cells at 2 to 6 h during meiosis, indicating the interaction of Pys2 with Hop1. The Hop1 signal was also detected in the precipitates of *pph3-H112N* mutant cells, indicating that the interaction did not involve the phosphatase activity of PP4. However, the Hop1 signal was weaker at 4 h than that in the *PPH3* background and was undetectable at 6 h in meiosis. This might be caused by reduced amounts of the Psy2-13Myc protein signal in the precipitates. However, the total amount of Psy2-13Myc protein in the *pph3-H112N*

mutant whole cell extracts of the *pph3-H112N* mutant from trichloroacetic acid-fixed cells was indistinguishable from that in wild type-cells (Supplemental Figure 1A). A mutation in *PPH3* affected the interaction between Hop1 and Psy2-13Myc, indicating the interaction between Hop1 and the PP4 complex, rather than Psy2 alone (as an independent complex).

Since Hop1-PP4 physical interaction was detected, we analyzed other axis components Red1 and Rec8 proteins in the precipitates using western blot. After immunoprecipitation in *PSY2-13MYC* cell extracts, as described above, the Hop1 protein signal was observed at 2 and 4 h after meiosis entry. However, the signal was not evident at 6 h, even though Hop1 protein was expressed in the extract. In contrast, Red1 and Rec8 were not observed in the precipitates, even though both proteins were present in whole cell extracts (WCE). The findings indicates that PP4 can interacts with Hop1 mainly, but not Red1 or Rec8. Given that Hop1 binds to Red1 as a complex (Niu *et al.* 2005), there might be two distinct populations of Hop1, Hop1-PP4 and Hop1-Red1.

Defective Hop1 assembly onto chromosome axes in the *pch2Δ* background in the *pph3* mutant

Pch2 is a AAA+ ATPase that removes Hop1 protein from meiotic chromosome axes to stabilize the transverse element Zip1 during formation of the SC structure (CHEN *et al.* 2014). Pch2 is also involved in a PP4-independent Hop1 dephosphorylation (CHEN *et al.* 2014). Since we observed a defect in Hop1 assembly and delayed phosphorylation of Hop1 in the *pph3* mutants, we analyzed the effects of the *pph3* mutations in the

Hop1 assembly on the meiotic chromosome axis in the *pch2Δ* background, which features the over-loading of Hop1.

First, spore viability and distribution of viable spores were analyzed in the *pph3Δ pch2Δ* double mutant by using tetrad analysis. Spore viability in the wild type, *pph3Δ*, and *pch2Δ* was 97%, 88%, and 91%, respectively. Both *pph3Δ* and *pch2Δ* had slightly reduced spore viability, but there was no significant bias in the distribution of viable spores in asci, as reported previously (SAN-SEGUNDO AND ROEDER 1999; FALK *et al.* 2010). In contrast, the pore viability of the *pph3Δ pch2Δ* double mutant was only 25%, with distributions of 0-, 2-, and 4-viable spores. This bias was indicative of the non-disjunction of homologous chromosomes in the first division of meiosis.

In Hop1 and Hop1-pT318 assembly/disassembly kinetics, Hop1-pT318 assembly was delayed in the *pch2Δ* mutant, consistent with a delayed meiotic DSB formation (FARMER *et al.* 2012). This delay was similar to that in the *pph3Δ* and the *pph3Δ pch2Δ* double mutant (Figure 4D and E), indicating the absence of additive defects to *pph3Δ* in Hop1 assembly/disassembly in the *pch2Δ* mutation. The findings suggest that Pch2 and PP4 function in the same pathway in Hop1 assembly/disassembly.

Next, we examined the effect of the *pch2Δ* mutation on Hop1 localization by using cytological analysis. As indicated previously (IWASAKI *et al.* 2016) and in Figure 2A, Hop1-pT318 foci were observed as punctate foci in the wild type and were affected in the *pph3Δ* in early prophase-I (approximately 3 h in meiosis) (Figure 4E). In the *pch2Δ* mutant, Hop1 aggregates colocalized with Hop1-pT318 signals. Similar aggregates were also observed in the *pph3Δ pch2Δ* double mutant at an early time point.

Notably, at the later time point (6 h during meiosis), Hop1-pT318 signals were observed as a beaded chain on the fully elongated Hop1 signal on meiotic chromosomes in the *pch2Δ* mutant cells (Figure 4E). In the *pph3Δ pch2Δ* double mutant, some Hop1 signals were observed as foci of dots and some colocalized with the Hop1-pT318 signal, similar to that in *pph3Δ*. These observations were evidence of the upstream location of the *pph3Δ* mutation relative to the *pch2Δ* mutation in Hop1 localization process. This further suggests that PP4 may function in the promotion of Hop1 assembly onto the meiotic chromatin to construct the chromosome axis before Pch2 function in unloading of Hop1 from the chromosome axis. In contrast, in the *pph3Δ pch2Δ* double mutant, some Hop1-pT318 signals were observed as beaded chain on fully elongated Hop1. This indicates that the *pch2Δ* mutation stabilized Hop1 protein in the *pph3Δ* on some chromosome axis, but not on others. Therefore, there could be other pathways that promote Hop1 assembly in the absence of PP4.

Multiple pathways to promote Hop1 assembly

On the meiotic chromosome axes, Hop1 is dynamic and is removed from chromosomes through Pch2 activity. Finally, Hop1 is replaced by the transverse element component Zip1 in the pachytene (BORNER *et al.* 2008). Given these facts, we examined Zip1 localization in the *pph3Δ pch2Δ* double mutant. In wild type cells, as previously reported, Zip1 was observed as dotty foci in the leptotene (2 h) and Zip1 begins to elongate after DSB-end resection in the zygotene, followed by the extension of Zip1 throughout each chromosome in the pachytene (SYM *et al.* 1993; STORLAZZI *et al.* 1996; SHINOHARA *et al.* 2015). Significant delays in both the appearance of dotty Zip1 signals and the elongation of Zip1 were evident for *pph3Δ* (Figure 5A), as

reported previously (FALK *et al.* 2010). The *pch2Δ* mutant showed a delay in the Zip1 elongation and also dissociation from the chromosomes after full elongation. However, the elongated Zip1 became the major population at 5 h after meiosis. In the *pph3Δ pch2Δ* double mutant, after the delayed appearance of dotted Zip1 signals, similar to *pph3Δ* (2 to 3 h), compromised elongation of Zip1 was observed. Complementary localization of Zip1 and Hop1 on chromosomes from the zygotene to the pachytene was observed in the wild type. A similar localization was observed in the *pph3Δ* mutant. In contrast, *pch2Δ* mutant cells showed uniform colocalization of Zip1 and Hop1 (Figure 5B), as previously reported (BORNER *et al.* 2008). *Saccharomyces cerevisiae* haploid cells have 16 chromosomes. Theoretically, 16 bivalents should be observed in the pachytene stage: however, the number of linear Zip1 signals per cell was 12 ± 2.7 (mean $\pm$ 95% confidence interval) in the *pch2Δ*. The number was significantly reduced in the *pph3Δ pch2Δ* double mutant in 68% of *pch2Δ* (8.2 ± 2.6) (Figure 5B and C). While several elongated Zip1 signal colocalized with Hop1 in *pph3Δ pch2Δ*, Hop1-Zip1 free DAPI signal was also often observed in *pph3Δ pch2Δ* (Figure 5B). Next, we compared the length of Zip1-Hop1 positive signals in *pch2Δ* with in *pph3Δ pch2Δ* to determine whether there is chromosome size bias in the residual elongation of Zip1-Hop1 observed in *pph3Δ pch2Δ*. No significant difference was evident ($P = 0.19$, Figure 5D).

Since the *pph3Δ* mutation showed a chromosome-dependent effect in Hop1-Zip1 elongation in the *pch2Δ* mutant background, we analyzed Rec8 and Red1 localization, as other axis components, to determine the status of the Hop1-Zip1 free region observed in the *pph3Δ pch2Δ* double mutant. Unlike the Hop1 protein on the axes, Red1 is not removed from the axis by Pch2. Rec8 is recruited to chromatin

independent of Hop1-Red1 (SUN *et al.* 2015). Furthermore, the Rec8 focus formation was not affected in the *pph3* mutants (Figure 3A). In the *pch2Δ* mutant cell, Rec8 and Red1 obviously colocalized on the chromosome axes (Figure 5E). On the other hand, in the *pph3Δ pch2Δ* double mutant, Rec8 and Red1 were colocalized only on the synapsed chromosome axes, and many of the Rec8 were observed as foci located in the Red1 free region. This indicates the possibility that 68% of the chromosomes axes with linearized Hop1 in the *pph3Δ pch2Δ* harbored a defect in the Pch2 sensitive step following Hop1 loading, while the defect in other axes was prior to Hop1 assembly.

Delayed meiosis progression in the *pph3Δ* occurs independent of Pch2

Hop1 is an adaptor of meiosis-specific prophase checkpoint. Hop1 phosphorylation is affected in the *pch2Δ* mutant (LO *et al.* 2014) and Mec1/Tel1-dependent phosphorylation of Hop1 affects Hop1 activity (CARBALLO *et al.* 2008). In the wild type, Hop1 phosphorylation was detectable at 3 h during meiosis, continued until 5 h, and then disappeared (Figure 5F). The timing of Hop1-pT318 disappearance matched the timing of the initiation of meiosis-I (Figure 5G). This was also observed with *pph3Δ* mutant cells, in which the Hop1-pT318 signal disappeared at 8 h with the simultaneous appearance of post-meiotic cells. In *pch2Δ* mutant cells, consistent with previous findings, Hop1 protein was accumulated until 10 h and Hop1 phosphorylation was compromised. On the other hand, in the *pph3Δ pch2Δ* double mutant, Hop1 protein accumulated until 10 h and Hop1-pT318 signal also accumulated until 8 h and then disappeared. Notably, corresponding to the delayed disappearance of the Hop1-pT318 signal, the *pph3Δ pch2Δ* double mutant showed a severe delay in meiosis progression (Figure 5E and F). The *pch2Δ* mutation can suppress pachytene arrest in

414 the *zip1* mutant (SAN-SEGUNDO AND ROEDER 1999; BORNER *et al.* 2008). Since the
 415 *pch2Δ* mutation cannot suppress the delayed progression of meiosis in the *pph3Δ*
 416 mutant, this delay in the *pph3Δ* would be caused by a different mechanism from that in
 417 the *zmm* mutant.
 418
 419

Discussion

During meiotic recombination, the chromosome axis is an essential scaffold. Some components, including Mer2 and Rec8, are associated with pre-meiotic DNA replication. On the other hand, the molecular mechanism underlying the formation of a highly organized chromosomal axis structure, especially, the initial step of Hop1 recruitment onto meiotic chromatin, is not well understood. The results of the present study support the suggestions that PP4 may function in Hop1 recruitment onto chromatin in the initiation step of chromosome axis formation in early meiotic prophase-I and that PP4 promotes Hop1 recruitment.

PP4 is involved in Hop1 assembly independent from Tel1/Mec1 kinase activity

During the formation of the meiotic SC structure, PP4 is required for centromere-mediated chromosome pairing through Zip1 phosphorylation in a Mec1/Tel1-dependent manner (FALK *et al.* 2010). We also observed reduced dotty Zip1 signal, which is known to localize to the centromere (TSUBOUCHI AND ROEDER 2005). The reduction was not suppressed even in the *pch2Δ* background (Figure 5A). Both *pph3* mutants, *pph3Δ* and *pph3-H112N*, displayed significant delays in Hop1 assembly onto chromatin, but this delay was Tel1/Mec1-independent (Figure 2A and B). Therefore, this delay in Hop1 assembly would not be caused by compromised centromere pairing. In *pph3* mutants, the appearance of γH2A, which is phosphorylated by Mec1/Tel1 kinase in response to programmed meiotic DSBs, was observed at the same time as that in wild type. The Hop1 phosphorylation (Hop1-pT318) by Tel1/Mec1 was delayed as well. This result supports the idea that delayed Hop1 phosphorylation is caused by the delayed

assembly of Hop1 onto the axes. Hop1 is one of the rate-determining factors that promotes meiotic DSB formation (PANIZZA *et al.* 2011). In addition, we revealed the physical interaction between the Hop1 and PP4 components also in the *pph3-H112N*, a catalytic mutation, and Hop1 protein in the precipitates was not always phosphorylated (Figure 4A and B). These findings suggest that the Hop1-PP4 interaction might not occur only for enzymatic activity, but may also regulate Hop1 assembly.

There is still another possibility that Hop1 localization on the chromosome axes in the absence of PP4 is unstable and is rapidly removed from the axes. To assess this, we examined Hop1 localization in the absence of Pch2. Proper Hop1 assembly did not occur in 32% of the chromosomes in each nucleus of *pph3Δ* cells. This strongly suggests that PP4 is involved in the assembly phase of Hop1 and not in the stabilization of Hop1 on the chromosome axes. Conversely, 68% of chromosomes in the same nucleus displayed stable Hop1 assembly by *pch2Δ*, even in *pph3Δ*. These observations indicate that there are multiple pathways other than the PP4 that promote Hop1 assembly.

What is the molecular mechanism to promote Hop1 assembly?

The molecular mechanism by which PP4 promotes Hop1 assembly through physical interactions is unknown. Hop1 is a HORMA domain protein. The dynamics of C-terminal “closure-motif” may be important for the chromosome axis assembly and disassembly through Red1 interaction. In addition, substitution of K593 of Hop1 with alanine (*hop1-K593A*) results in severe defects in the Red1 interaction (WEST *et al.* 2018). We performed phospho-proteomics analysis in extracts from yeast cells arrested in meiotic prophase-I. The finding revealed the phosphorylation of S595 of

Hop1 during meiosis, possibly by Mek1/Mre4 kinase (Shinohara M., unpublished result). We previously analyzed Hop1 assembly onto chromatin in the unphosphorylated mutation background of *hop1-S595L*. Normal Hop1 assembly was observed in the *hop1-S595L* mutant.

In addition, a delay in Hop1 assembly in the *pph3Δ* mutant was also observed in the *pCLB2-MEC1 tel1Δ* background, and Pph3 catalytic activity was required for the timely assembly of Hop1 (Figure 2A and B). These observations indicate that dephosphorylation of the target protein through PP4 is required for efficient assembly of Hop1, that Tel1/Mec1 is dispensable from the phosphorylation of the target(s), and that this phosphorylation may occur before meiotic DSB formation (Figure 6). Hop1 may be the most probable target for dephosphorylation by PP4. On the other hand, the candidate protein kinase(s) that regulate Hop1 assembly are DDK and/or CDK. However, the ATP analog sensitive *cdc7* allele (*cdc7-as3*) reportedly showed normal Hop1 expression but no Hop1 phosphorylation in the presence of an ATP analog (WAN *et al.* 2006). The yeast Cdc28, a component of CDK, focus formation on meiotic chromatin are dependent on Hop1, Red1 and Rec8, but not on Mek1/Mre4. The analog sensitive allele *cdc28-as1* reportedly displayed compromised Rad51 focus formation and severe defects in Zip1 elongation, but did not affect Red1 loading in the presence of an ATP analog (ZHU *et al.* 2010). These findings indicate that CDK and DDK functions are associated with Hop1 function. However, direct phosphorylation of Hop1 by CDK and/or DDK has not been reported. There are two predicted CDK phosphorylation motifs (S/T-P) in Hop1: S494 and S531 (ZHU *et al.* 2010). The presence of sequential phosphorylation by CDK followed by DDK in the sequence of serine/threonine residues in addition to the CDK phosphorylation motif has been

described for related target proteins such as Mer2 (WAN *et al.* 2006) and Mps3 (RAO *et al.* 2020), and the S494 of Hop1 overlaps this motif (T-S-⁴⁹⁴S-P).

PP4 has an independent role from ZMM/SIC components in the prophase checkpoint

Pch2 activates the meiotic prophase checkpoint when meiotic DSB repair or chromosome synapsis is compromised in *zip1*, *zip2*, or *dmc1* mutant cells (SAN-SEGUNDO AND ROEDER 1999; BORNER *et al.* 2008). It has been reported that the PP4 mutant shows a *zmm* mutant-like phenotype in meiotic recombination, NCO/CO ratio aberration, temperature-sensitive meiosis progression, CO formation, and compromised CO interference (BORNER *et al.* 2004; FALK *et al.* 2010). In addition, defective CO formation in *pph3Δ* is epistatic to *msh4Δ* (FALK *et al.* 2010). We also observed compromised CO and elevated NCO formation through HD analysis (Figure 1C). These are often observed in *zmm* mutants (SHINOHARA *et al.* 2008). *pch2Δ* could not suppress the delayed meiosis progression of the *pph3Δ* mutant. These findings suggest that delayed meiosis progression is caused by a different mechanism from that of *zmm* or *dmc1* mutants. Recently, it was reported that Pch2 in collaboration with Hop1 mediates the meiotic prophase checkpoint (RAINA AND VADER 2020). In the *pph3* mutants, compromised synapsis as well as inefficient meiotic recombination easily activated the meiotic prophase checkpoint because of enhanced Mec1/Tel1 signaling, but the checkpoint signal could not be restored even in the absence of Pch2. This could be caused by insufficient Hop1 loading on the chromosome axis or accumulation of unbound inactive form of Hop1 protein (Figure 6).

Materials and Methods

Yeast strains and media

All the yeast strains and their genotypes are listed in Supplemental table 1. We used isogenic *S. cerevisiae* SK1 background NKY1551 derivatives (STORLAZZI *et al.* 1996). *PPH3-13MYC* was constructed using a PCR-based tagging method (BAHLER *et al.* 1998). *pph3-H112N* was constructed via two-step gene replacement. YPAD (1% yeast extract, 2% Bacto peptone, 0.01% adenine, 2% glucose), SPM (0.3% potassium acetate, 0.03% raffinose), and DISS plates (YPAD medium containing 2.4% Bacto Agar) were used.

Yeast meiosis time course

The experiments were performed as described previously (SHINOHARA *et al.* 2003). Meiosis progression was analyzed by counting the number of nuclei in each ascus at each time point. Nuclei were visualized by staining with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) and observed under an epifluorescence microscope (AxioSkop2, Carl Zeiss). More than 100 nuclei were analyzed at each time point.

Cytological analysis

Immunostaining of yeast meiotic nuclear spreads was performed as previously described (SHINOHARA *et al.* 2015). Stained samples were observed under epifluorescence using the Axioskop 2 microscope equipped with light-emitting diode fluorescence light sources (X-Cite; Excelitas Technologies) and a 100× objective (AxioPlan, NA1.4, Carl Zeiss). Images were captured with a CCD camera (Retiga; Qimaging) and processed using iVision (BioVision Technologies) and Photoshop

(Adobe). The antibodies used in these assays were anti-Hop1 (IWASAKI *et al.* 2016) (guinea pig, 1:500) and anti-Hop1-pT318(IWASAKI *et al.* 2016) (rabbit, 1:500), anti-Red1 (chicken, 1:500) (SHINOHARA *et al.* 2008) and anti-Rec8 (rabbit, 1:1000) (ZHU *et al.* 2010). Nuclei containing more than five foci were counted as focus-positive.

Southern blot to detect meiotic DSBs and recombination intermediates

Genomic DNAs prepared from yeast meiosis time course experiments were digested with *MluI*, *BamHI*, and *XhoI* for heteroduplexes (HDs), and *PstI* for meiotic DSBs. Probes for Southern blotting were Probe 155 for HD and Probe 291 for DSB detection (STORLAZZI *et al.* 1996; SHINOHARA AND SHINOHARA 2013). They were labeled with α -³²P- dATP (NEG512H, PerkinElmer) using the random primer method (Random Primer 6, NEB S1230S, Klenow fragment (3'-5' exo-, M0210) and visualized by using FLA7000 (Cytiva). The visualized signals were analyzed by using Image Quant TL software (Cytiva).

Western blotting

Western blotting was performed as described previously (SHINOHARA *et al.* 2015). Meiotic yeast cell extracts were prepared by cell disruption using a Multi-Beads Shocker (YASUIKIKAI) after trichloroacetic acid treatment. The primary antibody signal was visualized using Alexa Fluor 680–labeled secondary antibodies (Thermo Fisher Scientific) or IRDye 800CW–labeled secondary antibodies (LI-COR Biosciences) using an Odyssey infrared imaging system (LI-COR Biosciences). The primary antibodies used in these assays were anti- γ H2A (anti- γ H2A, AF2288, R&D Systems, 1:1000), anti-Myc (MC045, Nacalai Tesque, 1/1000), anti-Hop1 (guinea pig, 1/5000)(IWASAKI *et*

al. 2016), anti-Hop1-pT318 (rabbit, 1/5000) (IWASAKI *et al.* 2016), anti-Rec8 (rabbit, 1/10000) (ZHU *et al.* 2010), anti-Red1 (chicken, 1/3000) (SHINOHARA *et al.* 2008) and anti- α -tubulin (YL1/2, Santa Cruz Biotechnology, 1/3000). Tubulin signals were visualized using the LI-COR Bioscience system or alkaline phosphatase stain (BCIP-NBT solution kit, Nacalai Tesque) on the same membrane on which other meiotic proteins were detected.

Immunoprecipitation

The immunoprecipitation assay was performed as previously described with minor modifications (SHINOHARA *et al.* 2008). Psy2-13Myc was immunoprecipitated by using anti-Myc antibody (MC045, Nacalai Tesque) antibody which were bound to Dynabeads Protein G (Thermo Fisher Scientific) from meiotic yeast cell lysate prepared using a Multi-Beads shocker (YASUIKIKAI) in lysis buffer (50mM HEPES-NaOH, pH7.5, 150mM NaCl, 20% glycerol, 1mM Sodium orthovanadate, 60mM β -glycerophosphate, 0.1% Nonidet P-40, 1:100 dilution of protease inhibitor cocktail (SIGMA-Aldrich) at the indicated time points in Figure 4A and 4B. Psy2-13Myc, Hop1, Red1 and Rec8 proteins in the precipitates and WCEs were detected as described in the western blot section.

Tetrad analysis

Tetrad analysis was performed as described previously (CHALLA *et al.* 2019). Parental haploid strains were mated for 12 h on YPAD plates at 30°C and then transferred onto SPM plates. After incubation at 30°C for 48 h, tetrads were dissected onto DISS plates and incubated for 48 h.

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Figure legends

Figure1. PP4 dephosphorylation activity is required for proper meiotic recombination.

(A) Schematic of the functional domain structure of yeast Pph3 (scPph3) protein.

Amino acid sequences contributing to phosphate binding and metal coordination are shown in pale blue and red, respectively. The position of the *pph3-H112N* and the substitution of histidine 112 with asparagine is shown.

(B) Localization of Psy2-13Myc was analyzed in *PPH3* (MSY6198/6190) and the *pph3-H112N* (MSY6242/6244) background by immunostaining with anti-Myc antibody (green). Representative images at 6h in meiosis are shown. Scale bar indicates 2 μ m.

(C) Schematics of the *HIS4-LEU2* recombination hotspot. Diagnostic P: *Pst*I, X: *Xho*I, B: *Bam*HI and M: *Mlu*I restriction sites are shown. The size of meiotic DSBs (DSB I, DSB II), and parental (Pa), and heteroduplexes (HDs) that are associated with COs (HD2 and HD3) or NCOs (HD1 and HD4), Parental (Pa1 and Pa2) and the positions of detectable probes 291 and 155 are shown.

(D) Representative Southern blotting of DSB detection in wild type (black, NKY1303/1543), *pph3* Δ (red, MSY5632/MSY5634), *pph3-H112N* (blue, MSY6219/6220), pCLB2-*MEC1 tel1* Δ (purple, MSY5544/5546) and pCLB2-*MEC1 tel1* Δ *pph3* Δ (orange, MSY5760/5762) using probe 291. The graph shows the mean and standard error of the quantified DSB I signals from more than three times trials.

(E) Representative Southern blotting of HD-associated COs and NCOs formed at

the *HIS4-LEU2* hotspot using probe155. The upper graph shows the mean and standard error of the quantified HD-associated COs (HD2 and HD3). The lower graph shows the mean and standard error of the quantified HD-associated NCOs (HD1 and HD4) of wild type, *pph3Δ*, and *pph3-H112N*.

(F) Representative immunostaining images of Rad51 (green) at 3 h on meiotic nuclear spreads in wild type (NKY1303/1543), *pph3Δ* (MSY5632/MSY5634), and *pph3-H112N* (MSY6219/6220). Scale bar indicates 2 μm. The dot plot graph shows the distribution of the number of Rad51 foci in each Rad51 focus positive nucleus at the indicated time points in wild type (black, NKY1303/1543), *pph3Δ* (red, MSY5632/MSY5634), and *pph3-H112N* (blue, MSY6219/6220). The data are presented as the medians and 95% confidence interval. Asterisks indicate statistically significant differences between the indicated strains determined using the Mann-Whitney U-test (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$) using Prism9 software.

Figure 2. Hop1 assembly is delayed in the *pph3* mutant in early prophase-I.

(A) Graphic depiction of the kinetics of Hop1 focus (upper) and Hop1-pT318 focus (lower) assembly on meiotic nuclear spreads in the indicated strains, wild type (black, NKY1303/1543), *pph3Δ* (red, MSY5632/MSY5634), *pph3-H112N* (blue, MSY6219/6220), *pCLB2-MEC1 tel1Δ* (purple, MSY5544/5546) and *pCLB2-MEC1 tel1Δ pph3Δ* (orange, MSY5760/5762). More than 100 cells were analyzed in each time point. Error bars show the mean ± standard deviation from more than three times trials.

(B) Representative cytological immunostaining images of Hop1 (green) and Hop1-

pT318 (red) on meiotic nuclear spreads at 3 h in wild type (NKY1303/1543), *pph3Δ* (MSY5632/MSY5634), and *pph3-H112N* (MSY6219/6220). Scale bar indicates 2 μ m.

(C) Representative western blot images of phosphorylation of histone H2A (γ H2A, red) and α -tubulin expression (green) at the indicated time point during meiosis (upper panel). Expression of Hop1 (green, top), phosphorylation of Hop1 at T318 (Hop1-pT318, red, middle), merged images of Hop1 and Hop1-pT318, and α -tubulin expression (bottom) at the indicated time points during meiosis. M denotes molecular weight markers.

(D) Graphic depiction of the average of the relative Hop1 protein signals from western blot at each time point. The numerical values are shown as a percentage of the peak amount of signal in the wild type. Error bars show the mean and $\pm$ standard deviation from two independent trials.

Figure 3. Rec8 expression is normal in the *pph3* mutant in early prophase I.

(A) Representative cytological immunostaining images of Rec8 (green) and Red1 (red) on meiotic nuclear spreads at 3 h in wild type (NKY1303/1543), *pph3Δ* (MSY5632/MSY5634), *pph3-H112N* (MSY6219/6220), wild type (NKY1303/1543), *pch2Δ* (MSY6446/6448), *pph3Δ* (MSY5632/MSY5634) and *pph3Δ pch2Δ* (MSY6417/6419)

(B) Graphs show the kinetics of Rec8 (upper) and Red1 (lower) focus assembly on meiotic nuclear spreads in the indicated strains, wild type (black, NKY1303/1543), *pph3Δ* (red, MSY5632/MSY5634), and *pph3-H112N* (blue, MSY6219/6220). More than 100 cells were analyzed at each time point. Error

bars show the mean $\pm$ standard deviation from more than three times trials.

(C) Representative western blot images of expression of Rec8 (upper) and Red1 (lower) with α -tubulin expression images as internal controls at the indicated time points during meiosis. M denotes molecular weight markers. Asterisks indicate non-specific protein signals.

Figure 4. PP4 interacts with Hop1 during meiosis.

(A) Immunoprecipitation (IP) analysis of Psy2-13Myc from *PPH3* (non-tag, NKY1303/1543), *PPH3* (*PSY2-13MYC*, MSY6188/6190), *pph3-H112N* (MSY6219/6220) and *pph3-H112N* (*PSY2-13MYC*, MSY6242/6244) cells at the indicated time points during meiosis. Immunoprecipitation products were probed with anti-Myc and anti-Hop1 antibodies.

(B) Immunoprecipitation (IP) analysis of Psy2-13Myc from *PPH3* (non-tag, NKY1303/1543) and *PPH3* (*PSY2-13MYC*, MSY6188/6190) cells at the indicated time points during meiosis. Whole cell extracts and immunoprecipitation products were probed with anti-Myc, anti-Hop1, anti-Red1 and anti-Rec8 antibodies.

(C) Tetrad analysis of spore viability in wild type (NKY1303/1543), *pph3* Δ (MSY5632/MSY5634), *pch2* Δ (MSY6446/6448) and *pph3* Δ *pch2* Δ (MSY6417/6419). The graphs show the fraction of tetrads containing 0-, 1-, 2-, 3- and 4-viable spores. The overall spore viability is shown in parentheses. n: number of asci analyzed.

(D) Graphic depiction of the kinetics of Hop1 (left) and Hop1-pT318 (right) focus assembly on meiotic nuclear spreads in the indicated strains, wild type (black,

NKY1303/1543), *pch2Δ* (gray, MSY6446/6448), *pph3Δ* (red, MSY5632/MSY5634) and *pph3Δ pch2Δ* (orange, MSY6417/6419). More than 100 cells were analyzed at each time point. Error bars show the mean $\pm$ standard deviation from more than three times trials.

(E) Representative cytological immunostaining images of Hop1 (green) and Hop1-pT318 (red) on meiotic nuclear spreads at 3 h (left panel) and 6 h (right panel) in wild type (NKY1303/1543), *pch2Δ* (MSY6446/6448), *pph3Δ* (MSY5632/MSY5634) and *pph3Δ pch2Δ* (MSY6417/6419). Scale bar indicates 2 μ m.

Figure 5. *pch2Δ* does not completely suppress the defect in Hop1 assembly of *pph3Δ*.

(A) Zip1 and Hop1 elongation was analyzed in wild type (NKY1303/1543), *pch2Δ* (MSY6446/6448), *pph3Δ* (MSY5632/MSY5634), and *pph3Δ pch2Δ* (MSY6417/6419) via immunostaining at each time point. The graphs show the percentages of each class of Zip1 morphologies; linear (dark gray, pachytene), partial (pale gray, zygotene) and dotted (black, leptotene). Error bars show the mean $\pm$ standard deviation from more than three trials. More than 100 cells at each time point were analyzed.

(B) Representative cytological immunostaining images of Hop1 (green) and Zip1 (red) on pachytene-phase nuclear spreads at 4 h in wild type (NKY1303/1543), and 6 h in the *pch2Δ* (MSY6446/6448), *pph3Δ* (MSY5632/MSY5634) and *pph3Δ pch2Δ* (MSY6417/6419). Scale bar indicates 2 μ m.

(C) The dot plot graph of the distribution of the number of linearized Hop1 signals in

each pachytene stage nucleus in *pch2Δ* (N = 112) and *pph3Δ pch2Δ* (N = 109) at 6 h during meiosis from three independent trials. Error bars show the mean with 95% confidence interval. Statistically significant differences between the *pch2Δ* and *pph3Δ pch2Δ* were determined using the Mann-Whitney U-test (**** $P < 0.0001$) using Prism9 software.

(D) The dot plot graph of the distribution of lengths of linearized Hop1 signal of pachytene stage nucleus in *pch2Δ* (gray, N = 718) and *pph3Δ pch2Δ* (orange, N = 780) at 6 h in meiosis from three independent trials. Error bars show the mean with 95% confidence interval. Statistically significant differences between the *pch2Δ* and *pph3Δ pch2Δ* were determined using the Mann-Whitney U-test (ns; not significant) using Prism9 software.

(E) Representative cytological immunostaining images of Rec8 (green) and Red1 (red) on pachytene-stage nuclear spreads in wild type (3 h, NKY1303/1543), *pch2Δ* (6 h, MSY6446/6448), *pph3Δ* (6 h, MSY5632/MSY5634) and *pph3Δ pch2Δ* (6 h, MSY6417/6419). Scale bar indicates 2 μ m.

(F) Representative western blot images of expression of Hop1 expression (green) and phosphorylation of Hop1 at pT318 (Hop1-pT318, red) and α -tubulin expression (bottom) at the indicated time points during meiosis. M denotes molecular weight markers.

(G) Meiosis progression in wild type (black, NKY1303/1543), *pch2Δ* (gray, MSY6446/6448), *pph3Δ* (red, MSY5632/MSY5634) and *pph3Δ pch2Δ* (orange, MSY6417/6419) mutants are shown. Frequencies of nuclei with more than two DAPI-staining bodies at each time point are plotted. More than 100 cells were analyzed at each time point. Error bars show the means and standard deviation

from two independent trials.

Figure 6. Model for PP4 involvement in Hop1 loading onto meiotic chromatin.

PP4 plays multiple roles during meiotic prophase. First, PP4 promotes Hop1 loading in the early phase of meiotic prophase in a Mec1/Tel1-independent manner. Until the chromatin loading, Hop1 exists in an inactive form, which might prevent meiosis progression. After meiotic DSB formation, Hop1 is phosphorylated at the DSB site by Tel1/Mec1, and PP4 is involved in dephosphorylation of the phosphorylated Hop1. Meiotic recombination promotes homolog synapsis by exchanging Hop1 to Zip1 through the function of AAA+ Pch2 function.

Supplemental figure 1. Supplemental data for Figure 1.

(A) Representative western blot images showing Psy2-13myc and α -tubulin expression at the indicated time points during meiosis in wild type (non-tag, NKY1303/1543), *pph3* Δ (non-tag, MSY5632/5634), PSY2-13myc (MSY6188/6190), *pph3-H112N* (non-tag, MSY6219/6220) and PSY2-13MYC *pph3-H112N* (MSY6242/6244).

(B) Meiosis progression in wild type (NKY1303/1543), *pph3* Δ (MSY5632/5634) and *pph3-H112N* (MSY6219/6220) mutants. Frequencies of nuclei with more than two DAPI-stained bodies are plotted.

(C) The graph of the kinetics of Rad51 focus assembly on meiotic nuclear spreads in wild type (black, NKY1303/1543), *pph3* Δ (red, MSY5632/MSY5634), and *pph3-H112N* (blue, MSY6219/6220). More than 100 cells were analyzed at

924 each time point. Error bars show the mean $\pm$ standard deviation from more than
925 three times trials.

926

927 Supplemental Figure 2. **Supplemental data presentation for Figure 4.**

928 (A) Whole membrane images of western blot of immunoprecipitates (IP) are
929 shown in Figure 4A and western blot of whole cell extracts (WCEs) before the
930 immunoprecipitation.

931 (B) Whole membrane images of western blot of immunoprecipitates (IP) and
932 whole cell extracts (WCEs) exhibited in Figure 4B,

933

A

B

C

Li et al/ Figure 5

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Li *et al*, Figure 6

Supplemental Table-1 Strain list

Strain	Genotype	Reference
NKY1303	<i>MATa, ho::LYS2, lys2, ura3, leu2::hisG, his4B-LEU2(MluI), arg4 bgl</i>	(STORLAZZI <i>et al.</i> 1996)
NKY1543	<i>MATa, ho::LYS2, lys2, ura3, leu2::hisG, his4X-LEU2(BamHI)-URA3, arg4-nsp</i>	(STORLAZZI <i>et al.</i> 1996)
MSY5632	NKY1303 with <i>pph3::Hyg^R</i>	This study
MSY5634	NKY1543 with <i>pph3::Hyg^R</i>	This study
MSY6219	NKY1303 with <i>pph3-H112N::MluI</i>	This study
MSY6220	NKY1543 with <i>pph3-H112N::MluI</i>	This study
MSY6198	NKY1303 with <i>PSY2-13MYC::TRP1</i>	This study
MSY6190	NKY1543 with <i>PSY2-13MYC::TRP1</i>	This study
MSY6242	NKY1303 with <i>PSY2-13MYC::TRP1, pph3-H112N::MluI</i>	This study
MSY6244	NKY1543 with <i>PSY2-13MYC::TRP1, pph3-H112N::MluI</i>	This study
MSY5544	NKY1303 with <i>pCLB2-MEC1, tel1::TRP1</i>	This study
MSY5546	NKY1543 with <i>pCLB2-MEC1, tel1::TRP1</i>	This study
MSY5760	NKY1303 with <i>pCLB2-MEC1, tel1::TRP1, pph3::Hyg^R</i>	This study
MSY5762	NKY1543 with <i>pCLB2-MEC1, tel1::TRP1, pph3::Hyg^R</i>	This study
MSY6446	NKY1303 with <i>pch2::TRP1</i>	This study
MSY6448	NKY1543 with <i>pch2::TRP1</i>	This study
MSY6417	NKY1303 with <i>pph3::Hyg^R, pch2::TRP1</i>	This study
MSY6519	NKY1543 with <i>pph3::Hyg^R, pch2::TRP1</i>	This study

Storlazzi, A., L. Xu, A. Schwacha and N. Kleckner, 1996 Synaptonemal complex (SC) component Zip1 plays a role in meiotic recombination independent of SC polymerization along the chromosomes. Proc Natl Acad Sci U S A 93: 9043-9048.

Supplemental Figure 1, Li et al

A

B

C

Supplemental Figure 2 Li et al