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Master Thesis

酵母菌 Hsp26 蛋白在原生孢子膜形成時的作用

The role of yeast Hsp26 during prospore membrane formation

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ABSTRACT

Meiosis plays an important role in gametogenesis. A diploid budding yeast cell enters meiosis under nutrient starvation which finally produces four haploid spores. The overall process of meiosis and spore formation is called sporulation. During this process, heat shock protein Hsp26 was strongly induced. Previous studies found that the sporulation efficiency of *hsp26* mutant is higher than that of the wild type, and this increase occurs at the step of spore formation. Also, there is an increase in the percentages of abnormal asci in *hsp26* mutant cells; asci alignment depends on the meiotic spindle functions. In order to specifically disrupt meiotic spindles, low level of benomyl was used. It caused a reduction of spore formation in the wild type, but not in the *hsp26* mutant cells. Thus it was proposed that Hsp26 might be involved in the spindle checkpoint controlling the transition from meiosis II to spore formation. To understand the molecular mechanism of how Hsp26 might be involved in the spindle checkpoint during spore formation, we studied a potential Hsp26-interacting protein, Ady3. Ady3 is a component of the leading edge protein (LEP) coat of the prospore membrane (PSM) and is important for PSM formation and spore wall synthesis. Previous studies found that more Ady3 signals were detected on the LEP coat in the *hsp26* mutant. Taken together, we hypothesized that Hsp26 might be involved in the spindle checkpoint by regulating Ady3 localization onto the leading edge coat, thus

preventing the formation of abnormal asci. In addition, localization dependence study showed that Ady3 binding to the leading edge depends on Ssp1. Therefore, we also hypothesized the other possibility that Ssp1 might act as the regulation target of Hsp26 in controlling spore formation. Upon microtubule-perturbation treatments, we found that the localization of Ssp1 to the LEP coat is affected in wild type cells, but not in *hsp26* mutant cells. Based on these results, we proposed that in response to the spindle checkpoint signaling, Hsp26 might be involved in the regulation of Ssp1 for controlling spore formation.

Keywords:
Hsp26; prospore membrane; leading-edge complex; spindle checkpoint; meiosis

中文摘要

減數分裂在有性生殖中扮演重要的角色。在營養缺乏的環境中，二倍體的酵母菌（*Saccharomyces cerevisiae*) 細胞會經由減數分裂產生四個單倍體的孢子。整個減數分裂和孢子形成 (spore formation) 的過程稱為孢子生殖 (sporulation)。在孢子生殖過程中熱休克蛋白 Hsp26 會大量的表現，過去研究發現 *hsp26* 突變株的產孢率比野生型高，由於減數分裂沒有明顯的差異，表示缺少 Hsp26 是影響孢子形成時期。此外 *hsp26* 突變株中不正常排列的孢子比例較多，孢子的排列受到紡錘體 (spindle) 的控制，因此使用 benomyl 擾亂野生型與 *hsp26* 突變株的紡錘體，發現 *hsp26* 突變株的產孢率沒有受到影響，而野生型則有明顯的降低。根據此結果，之前推測 Hsp26 可能參與在紡錘體相關的檢控點。本論文的主要目的在於更深入探討 Hsp26 調控孢子形成的機制。首先，我們研究可能和 Hsp26 交互作用而且參與在孢子形成時期的蛋白質：Ady3。Ady3 位在原生孢子膜（prospore membrane; PSM) 的前緣蛋白鞘 (leading edge protein coat; LEP coat) 上，與原生孢子膜的形成及孢子壁合成有關。根據之前的研究發現在 *hsp26* 突變株中偵測到較多 Ady3 位在前緣蛋白鞘上，因此推測 Hsp26 可能藉由調控 Ady3 結合到前緣蛋白鞘上，進而控制了產孢過程。此外，過去的研究顯示 Ady3 靠著 Ssp1 結合到前緣蛋白鞘上，故推測出第二種假設，Hsp26 可能經由調控 Ssp1 而影響到 Ady3 與前緣蛋白鞘的結合。以 benomyl 或低溫擾亂紡錘體後，經由免疫螢光觀察，發現野生型中 Ssp1 結合到前緣蛋白鞘上的比例均會降低，但是在 *hsp26* 突變株中則未降低，結果與第二種假設相符。我們推測在酵母菌的產孢過程中，當紡錘體異常時 Hsp26 可能會受到紡錘體相關檢控點的訊號，進而參與在調控 Ssp1 結合到前緣蛋白鞘上的過程。

關鍵詞：Hsp26 蛋白；原生孢子膜；前緣蛋白鞘；紡垂體檢控點；減數分裂

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CHAPTER 1

INTRODUCTION

I. The Importance of Meiosis

Meiosis is a special type of cell division essential for sexual reproduction. It was followed by the subsequent development of the haploid products into gametes. Meiosis and gamete formation must be well coordinated to ensure the formation of viable gametes. Errors in the process of meiosis, such as chromosome missegregation, lack of tension on spindle microtubules, improper spindle assembly, spindle disassembly would lead to the production of inviable or aneuploidy gametes. In maintaining genome integrity, meiotic events are secured by surveillance mechanisms, known as checkpoints, which monitored successful completion of key events at various points throughout the cell cycle (Hartwell and Weinert, 1989).

The budding yeast, *Saccharomyces cerevisiae*, is an ideal model organism to study meiotic event since diploid cells can be induced to undergo meiosis by depletion of nitrogen and the presence of fermentable carbon source. It results in the formation of four haploid progeny, the spores, inside the boundaries of the mother cell. Meiosis in yeast is therefore also called sporulation. This process in yeast is analogous to the formation of germ cells in higher eukaryotes. The major difference to meiosis in higher

eukaryotes is that in yeast meiosis the nuclear envelope does not break down during the divisions and that there is no cytokinesis after the first meiotic division. Instead, the cellularization of the four daughter cells occurs coordinated at the end of meiosis II.

II. The Roles of Heat-Shock Proteins in Meiosis

When a cell experiences environmental stress, it stops or at least slows down most of its original functions, such as transport processes, DNA, RNA and protein synthesis. However, there is a set of proteins which are preferentially expressed under these restrictive conditions, called the stress proteins or heat-shock proteins (Hsps) (Lindquist and Craig, 1988). Most of the Hsps are molecular chaperone which play an essential cytoprotective role in preventing irreversible damage to cellular proteins by binding to unfolded or malfolded peptides to retard thermal denaturation and aggregation of cellular proteins. Additionally, Hsps are also responsible in many normal cellular processes, including folding and assembly of nascent polypeptides, oligomerization, intracellular protein transport, embryonic development and progression through the cell cycle. In particular, some Hsps have unique patterns of expression in meiotic cells of various organisms, e.g. small Hsps (sHsps). This type of Hsps is expressed during sporulation in yeast (Kurtz *et al*., 1986) and in growing oocytes and spermatocytes of *Drosophila* (Zimmerman *et al*., 1983). sHsps are characterized by a conserved

C-terminal α-crystallin domain and subunit masses between 15 and 40 kDa (de Jong *et al*., 1993; Arrigo and Landry, 1994). sHsps are molecular chaperones that bind specifically to unfolded proteins *in vitro* and prevent their aggregations (Jakob *et al*., 1993), Also, it has been showed that sHsps can enhance the cellular resistance to heat shock (Landry *et al*., 1989).

Yeast Hsp26 protein

Hsp26 is a member of the sHsps superfamily. Previous studies of Hsp26 are more focused on its molecular chaperone role. Hsp26 forms large inactive-oligomeric complexes of 24 subunits under physiological temperatures. Hsp26 prevents the aggregation of target proteins by binding of non-native proteins to dissociated Hsp26. To ensure the efficiency of these chaperone activities, Hsp26 needs to be disassembled into dimers under heat shock condition (Haslbeck *et al*., 1999; Stromer *et al*., 2004). Additionally, the phenomenon that Hsp26 inhibits actin polymerization *in vitro* suggests that Hsp26 might be involved in the modulation of cytoskeletal architecture (Rahman *et al*., 1995).

In addition to being induced by high temperatures, Hsp26 accumulates after diauxic shift and during the course of sporulation (Kurtz *et al*., 1986; Petko and Lindquist, 1986). However, Hsp26 is not required for cell growth at high temperatures,

nor for spore development, or germination (Petko and Lindquist, 1986).

During the transition from log phase to stationary phase, as well as during sporulation, yeast Hsp26 protein accumulates to very high levels (Kurtz *et al*., 1986). However, the role of Hsp26 in sporulation remains undiscovered. Until recently, it has been shown that Hsp26 might be involved in the spindle checkpoint machinery during the transition from meiosis II to spore formation (Ho, 2005).

III. Spore Formation in *Saccharomyces cerevisiae*

In *S. cerevisiae*, spores are formed at the end of meiotic event that resulted in four daughter cells, which are formed in boundaries between mother cells. This process starts during meiosis II phase and requires *de novo* synthesis of prospore membranes (PSMs), plasma membrane equivalent, and spore walls.

Formation of PSM relies on secretory processes that are equivalent to the fusion of vesicles with the plasma membranes (Neiman, 1998). The process is started with formation of cytosolic precursors of the PSMs during meiosis I. Its formation is then initiated on the cytoplasmic side of the spindle pole bodies (SPBs), centrosome equivalents of yeast. At the beginning of meiosis II, the specialized meiotic outer plaque (MOP) of the SPB serves as the attachment sites of the all precursors of the PSMs which then assemble into continuous PSMs, one per SPB. Once an initial membrane cap

has been established by fusion of vesicles on the SPB, the membrane grows to engulf the nuclear lobe by continued vesicle fusion. The growth of the membrane must be controlled for ensuring the proper shape and dimensions to enclose a nucleus. Two membrane-associated complexes that are implicated in the control of PSM expansion, such as: the septins and the leading-edge complex (Neiman, 2005).

Upon completion of meiosis II, the nuclear lobes pinch off and the PSMs close to form two membranes stacked on each other (Lynn and Magee, 1970; Moens and Rapport, 1971; Peterson *et al*., 1972; Zickler and Olson, 1975). The closure of the PSM is a cytokinetic event; the molecular mechanism of which is unknown. The closed PSMs then mature to become the spore walls. Spore wall synthesis which consists of four layers occurs in the lumen between the double membranes. The first two inner layers consist predominantly of mannan and beta-1,3-glucans, which will develop into spore plasma membrane. Meanwhile, the last two outer layers, which are specific to the spore consist of chitosan and dityrosine molecules, are essential in spore wall coverage. These outer spore wall layers will confer resistance to environmental stress to the spores. Once spore wall synthesis completes, the mother collapses to form a mature ascus (Neiman, 2005).

The Leading-Edge Complex

The leading edges of the PSMs are covered by a proteinaceous coat termed leading edge protein (LEP) coat (Knop and Strasser, 2000). The LEP coat is discovered by electron micrographs which reveal an electron-dense coat located at the lip of each growing PSM (Moreno-Borchart *et al*., 2001). Three components of the LEP coat have been identified: Ssp1, Ady3, and Don1 (Moreno-Borchart *et al*., 2001; Nickas and Neiman, 2002). Localization dependence studies suggest that Ssp1 may be the most membrane-proximal component, serving to connect Ady3 to the leading edge, and Ady3 in turn anchors Don1.

Ssp1 (Sporulation-specific) is an essential meiosis-specific phospoprotein that has been shown to localize to the secretory vesicles and to recruit the LEP coat proteins to the opening of the PSM. Deletion of the *SSP1* gene causes a dramatic disorganization of the PSM which fails to capture nuclei and cytoplasm organelles (Moreno-Borchart *et al*., 2001). Consequently, it completely abolishes the formation of spores. Moreover, removing Ssp1 from PSM is crucial for underlying membrane closure in yeast sporulation (Maier *et al*., 2007). The *ssp1* phenotype demonstrates the crucial role of the leading edge complex (or at least Ssp1) for proper PSM growth (Neiman, 2005).

Ady3 (Accumulates Dyads) is a component of the leading-edge structures that interacts with SPBs and necessary for recruitment of Don1 into this leading-edge

structure. Ssp1-dependent recruitment of Ady3 to the PSM starts at the level of the precursors. The recruited Ady3 can then promote the binding of the membranes to the SPB. Ady3 might ensure the initial steps of membrane formation pathway, together with the Ssp1-dependent membrane clustering, to proceed efficiently. Also, it was speculated that Ady3 might be required for the structural integrity of the LEP coat, which could be important in promoting the faithful closure of the PSM (Moreno-Borchart *et al*., 2001).

Loss of Ady3 leads to the dyads accumulation (Rabitsch *et al*., 2001). Also, it sporulates poorly due to a failure to synthesize spore wall polymers. Nickas and Neiman (2002) have proposed that Ady3 recruits Don1 to the leading edge of the PSM during meiosis via interactions with SPB components and may facilitate the recruitment of other factors that are required to promote spore wall formation. Deletion of Don1 does not show obvious defects in yeast sporulation (Knop and Strasser, 2000). In *don1Δ* strains no detectable phenotype is observed, PSM assembly and spore formation occur at wild type level (Moreno-Borchart *et al*., 2001; Nickas and Neiman, 2002).

Interestingly, Hsp26 was found to interact with Ady3 in a yeast-two hybrid screen and co-immunoprecipitation analysis (Ito *et al*., 2001; Ho, 2005; Chen, 2007). In here, our further study attempts to unravel the relationship between Hsp26 and Ady3 during prospore membrane formation and how it is linked to the spindle checkpoint upon the completion of meiosis II.

IV. Spindle Checkpoint

Cell cycle checkpoints are surveillance pathways that ensure the accurate order of events within cell cycle by preventing the occurrence of later events until the earlier events have been properly executed (Hartwell and Weinert, 1989). In budding yeast, there are two types of surveillance pathways which monitor mitotic spindle function: spindle assembly and spindle position checkpoint. Spindle assembly monitors bipolar attachment of sister chromatids, while spindle position checkpoint responsible for monitoring the orientation of mitotic spindle along mother-bud axis.

The spindle assembly checkpoint is an elegant regulatory system that delays the onset of anaphase until each and every chromosome has established a bipolar orientation. It was proposed that a detached-microtubule and lack of spindle tension at kinetochore may trigger the spindle assembly checkpoint. If they were not corrected before anaphase, it would result in chromosome mis-segregation, yielding aneuploid germ cells (Lew and Burke, 2003).

In budding yeast, the mother-bud neck determines the cleavage plane, and accurate deposition of one set of chromosomes into the bud and the other set of chromosomes into the mother requires orientation of the mitotic spindle along the mother-bud axis. Therefore, spindle alignment along the polarity axis is crucial for fidelity of chromosome segregation. Misalignment of the mitotic spindle eventually leads to

aneuploidy (Caydasi *et al*., 2010). When the spindle is misaligned, the spindle position checkpoint delays cell cycle exit until the spindle orientation is correct. Several observations suggest that this checkpoint monitors the entry of a spindle pole into the bud as an indicator of correct anaphase spindle orientation (Lew and Burke, 2003).

Little is known about the role of spindle checkpoint in meiosis. Recent paper has reported that in meiosis, the spindle checkpoint proteins not only act in a surveillance system to ensure that chromosomes are properly attached to the spindle, but also involved in ensuring that kinetochores can initially attach to the bipolar spindle. Additionally, other spindle checkpoint proteins are also involved in the timing of the meiotic cell cycle (Maro *et al*., 2003; Murray and Lacefield, 2007). How the meiotic cells sense inappropriate microtubule attachment, signaling the checkpoint and correcting the error remains unresolved and is an important area of future research.

Specific Aim

Hsp26 is induced during sporulation yet little is known about its function. Prior study (Ho, 2005) showed that the *hsp26* mutants display an increase in sporulation which occurs at the step of spore formation. Also, there is an increase of the abnormal asci, such as linear tetrads and linear triads were found in the *hsp26* mutants. Moreover, the sporulation in the *hsp26* mutants is less affected by a minor benomyl treatment, compared to wild type control. These results suggested that there might be a novel Hsp26-dependent spindle-related surveillance mechanism in controlling the transition from meiosis II to spore formation.

In order to further investigating the role of Hsp26 during spore formation, we searched interacting protein from Yeast Genome Database and found a potential candidate gene, *ADY3*. Ady3 is a component of the LEP coat that covers the ring-shaped opening of PSM. This membrane formation is an indispensable *de novo* synthesis process of the spore plasma membrane. Previous report from Chen (2007) found that more Ady3 signals located in the LEP coat in the *hsp26* mutants compared to the wild type. Based on this result, we proposed that Hsp26 might be involved in the spindle checkpoint machinery by regulating the localization of Ady3 to the LEP coat, thus can control the PSM formation. Additionally, based on the knowledge that Ssp1 is required for the recruitment of Ady3 to the LEP coat, we can not role out the possibility that

Hsp26 may control the step of spore formation by regulating the localization of the former protein, Ssp1. Thus, further investigation on the Hsp26 role in either Ssp1 or Ady3 localization as response to microtubule perturbations has become the main interest in this study.

CHAPTER 2

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

The budding yeast *S. cerevisiae* strains and their relevant genotypes used in this study are listed in Table 1. General yeast cell growth condition was performed on YPAD plates (1% yeast extract, 2% peptone, 2% glucose, 0.004% adenine, 1.5% agar) or synthetic drop-out plates (0.15% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.2% amino acids, 2% glucose, 1.5% agar) at 30℃. For sporulation efficieny analysis, independent colonies were patched on the YPAD plate and incubated at 30℃ overnight. These plates were replicated onto solid sporulation media (0.2% yeast extract, 0.1% glucose, 2% KAc, 0.1% amino acids mixture, 2% agar) and incubated at 30℃ for three days. At least 200 cells per patch were counted for the sporulation frequency under phase contrast microscope. To sporulate cells for liquid sporulation test and cytological analyses, cells were pre-grown in the YPAD media at 30℃ overnight rolling at 60 rpm. At the next day, cells were refreshed by dilution of 1:2 into YPAD media for 8-10 h. The cells were then resuspended in liquid sporulation media (2% potassium acetate) and incubated at 30℃ or other indicated temperatures with vigorous shaking at ~200 rpm.

DNA Preparation, Transformation, and Plasmid Constructions

For DNA manipulation, plasmids were prepared according to a modification of alkaline lysis method (Sambrook and Russell, 2001). Restriction enzymes were purchased from New England Biolabs (NEB) and DNA fragment digestions were performed according to the instructions from the manufacturers. Digested DNAs were separated by electrophoresis on 0.8%-1% agarose gels. DNA fragments were cut and eluted from gel slices according to the protocol of GeneClean III kit (Bio101). Ligation reactions were carried out at 16℃ overnight with T4 DNA ligase from NEB.

E. coli transformations were performed by the calcium chloride procedure (Sambrook and Russell, 2001). Yeast transformations were carried out by the lithium acetate procedure (Ito *et al.*, 1983).

Plasmid constructions

Plasmids used in this study are listed in Table 2 and the primers used are listed in Table 3.

pT566 (modified SK(+) without *Not*I restriction site)

The pBluescript SK(+) was digested with *Not*I and then filled-in using the Klenow fragment to abolish the *Not*I site.

pT613 (SK(+)-*SSP1*)

The 2962-bp fragment containing the *SSP1* open reading frame was PCR-amplified from TY178 genomic DNA using SSP1-U1-*Xba*I and SSP1-D1-*Xho*I. The 2930-bp PCR product was cloned into pSK(+) at *Xba*I-*Xho*I site.

pT640 (SK(+) without *Not*I site-*SSP1*-*Not*I)

The 2.9-kb *Xba*I-*Xho*I *SSP1* fragment containing *Not*I site was generated by recombinant PCR in which pT613 was used as templates and SSP1-U1-*Xba*I, SSP1-*Not*I D1, SSP1-*Not*1 U1, and SSP1-D1-*Xho*I as primers and subcloned into pT566 at the *Xba*I-*Xho*I sites.

pT642 (pT566-*3XHA*-*SSP1*)

Three copies of HA fragment from TP174 was inserted into pT640 at the *Not*I site. It was confirmed to be in-frame construction.

pT647 (pRS306-*3XHA*-*SSP1*)

The 3.0-kb *Xba*I-*Xho*I fragment containing *3XHA-SSP1* from pT642 was subcloned into pRS306 at *Xba*I-*Xho*I site, which then cut at *Eco*RI for two-step gene replacement of *3XHA-SSP1*.

pT661 (pRS306-*3Xmyc-ADY3*)

The 1140-bp *Bam*HI-*Sac*I fragment containing *3Xmyc-ADY3* $_{(-264\sim+757)}$ from pT572 (YEp352-*3Xmyc-ADY3*) was subcloned into pRS306 at *Bam*HI-*Sac*I site, which then cut at *Xba*I for two-step gene replacement of *3Xmyc-ADY3*.

pT700 (pGBDU-C1-*SSP1*)

SSP1 (+4 to p717) was cloned by PCR from pT613 (SK(+)-SSP1), using SSP1-U3-*Bam*HI (from -9 to +28), SSP1-D1-*Xho*I (from p732 to p695) primers. In order to create pT700, the 2456-bp of PCR product was cut with *Bam*HI and *Xho*I and was subcloned into *Bam*HI and *Sal*I sites of pT477 (pGBDU-C1 *URA3*).

Yeast Strain Constructions

Constructions of yeast null mutants

Disruption of yeast genes was carried out by the one-step gene disruption method. Endogenous tagging of yeast genes were generated by two-step gene replacement method (Boeke *et al*., 1987). The detailed process is described as follows and the primers used are listed in Table 3.

Epitope tagging of Ssp1

To construct endogenous *3XHA-SSP1* yeast strain, pT647 was used to replace the chromosomal copy of *SSP1* with *3XHA-SSP1* by the two-step gene replacement (pop-in/pop-out) strategy. Yeast haploid cells (TY178**a**) were transformed with pT647 that had been cut with *Eco*RI for integration (pop-in). Ura^+ transformants were selected and patched on the YPAD plates. On the next day, it was replicated onto 5-FOA

(5-fluoroorotic acid hydrate) plates to identify the cells that lost URA3 selection marker by undergoing the intrachromosomal recombination (pop-out). All of the viable colonies were confirmed by whole-cell PCR. The tagged colony of TY178**a** which named K1004 was selected. To generate *α*–mating types which carrying *3XHA-SSP1,* the K1004 cells was crossed with TY179*α* cells. The spores which were carrying the epitope tagging were confirmed by PCR. The tagged colony of TY179*α* which named K1005 was selected. The K1004 and K1005 were crossed to generate diploid cells, which then named as K1007.

Epitope tagging of Ady3

To construct endogenous *3Xmyc-ADY3* yeast strain, pT572 was used to replace the chromosomal copy of *ADY3* with *3Xmyc-ADY3* by the two step gene replacement method. Yeast haploid cells that carrying *3XHA-SSP1* (K1004 and K1005) were transformed with pT572 that had been cut with *Xba*I for integration (pop-in). The pop-out procedure was as described above. Successful replacements were checked by whole cell PCR using ADY3-U3 and ADY3-D4 primers. The PCR product of *3xmyc-ADY3* is 615-bp, and that of *ADY3* is 495-bp. The diploid cells (K1055) were generated by crossing two tagged-haploid cells (K1053 and K1054).

***3XHA-SSP1* and *3Xmyc-ADY3* but *hsp26::URA3* mutant**

pT119 (SK^+-*hsp26::URA3*) was used to replace the chromosomal *HSP26* in yeast strains which carrying both *3XHA-SSP1* and *3Xmyc-ADY3* with *hsp26::URA3*, targeting for substitution by cutting with *Spe*I and *Xho*I. The *hsp26* haploid mutants were confirmed by whole cell PCR using HSP26-U2 and HSP26-D2 primers. The wild-type *HSP26* gene generates a 0.88-kb PCR product and the *hsp26::URA3* destructed allele generates a 1.65-kb PCR product.

Time Course Analyses

Meiotic time course analyses

Yeast cells were grown overnight to saturation in 2 ml YPAD at 30℃. At the next day, 100 μl of saturated cells were reinoculated to 2 ml of fresh YPAD and incubated at 30℃ for 18-20 h. The cultured cells were then refreshed by dilution of 1:2 into YPAD media for 10 h at 30℃. 1.5 ml of cell culture was collected, washed once with 2% KAc and resuspended in 10 ml 2% KAc. Total time course analyses were proceeded from 8 h to 44 h after inoculation into 2% KAc. Every 2 h time intervals, 10 μl of cells were examined under the phase contrast microscope to assess spore formation. At least 200 cells were counted for each time point. At the same time, 180 μl of cells were collected for nuclear division analysis.

Nuclear division analyses

180 μl of cells were collected from sporulation cultures at the indicated time points. Cells were fixed for 30 mins at room temperature with shaking at ~80 rpm by adding 20 μl of 37% formaldehyde (Sigma F-1268). Fixed cells were spun down at 8000 rpm for 1 min. Cells were then washed once with 200 μl of 1X PBS, resuspended in 200 μl of 1X PBS and then stored at 4℃. Polylysine-coated slides were prepared in advance. The stock of 1 mg/ml poly-lysine (Sigma P-1524) was thawed and centrifuged for 10

mins at 13000 rpm. One drop of polylysine was added to each well, stand for 15 mins, and washed with water. The slides were air dried. 30 μl of fixed cells were dropped to each well of polylysine-coated slides and stand for about 15 mins. The excess liquid were sucked off and washed once with 1X PBS before mounting with 20 μl of Vectashield mounting medium (Vector Laboratories) containing 1 μg/ml DNA-binding dye, DAPI (4'-6'-diamidino-2-phenylindole), covered with covered slips and sealed with nail polish. The slides were stored at -20℃ in the black box. The cells were observed with fluorescence microscope (Olympus AX70, UPlan apo 100X/1.35 oil iris). The percentages of bi-/tetranucleated cells were calculated from at least 200 DAPI-stained cells for each time point.

Cytology Analyses

Whole cell staining and Immunofluorescence

4.5 ml of sporulating cells were fixed at indicated time points by adding 0.5 ml of 37% formaldehyde at room temperature for 30 mins with shaking at ~80 rpm. Fixed cells were spun down at 2000 rpm for 3 min and washed twice with 1.5 ml of solution A (1.2 M sorbitol, 50 mM KPO_4 pH 7.0). Cell pellets were resuspended in 1 ml of solution A and stored at 4℃. 500 μl of cells were resuspended in 0.5 ml of solution A containing 0.1% ß-mercaptoethanol and 50 μg/ml zymolase and incubated at 30℃

roller drum (~30 rpm) for 20 mins. Cells were spun down at 2000 rpm for 2 min and gently washed with 1 ml of solution A. Cells were then resuspended in 150 μl of solution A. 30 μl of cells were added to each well of polylysine-coated slides and left on bench for 15 min. The excess liquid was sucked off and cells were gently washed for 3 times with 1X PBS + 1% BSA, 5 min/wash. For analyzing subcellular localization of both Ssp1 and Ady3, the cells were incubated overnight at 4℃ in a humidified box with primary antibodies: mouse monoclonal anti-HA antibody (16B12, Santa Crauz) and rabbit polyclonal anti-myc antibody (A-14, Santa Crauz) diluted 1:100 in 1X PBS + 1% BSA. The cells were washed three times (5 min/wash) with 1X PBS. Next, the cells were incubated at room temperature for 2 h with secondary antibodies: donkey-anti mouse IgG conjugated to TR and goat-anti rabbit IgG conjugated to FITC (Jackson ImmunoReseach) diluted 1:200 in 1X PBS + 1% BSA. To detect spindle, rat monoclonal anti-tubulin, 1/34 YOL (Accurate Chemical, 1:25) and donkey-anti rat IgG conjugated to TR diluted 1:200 in 1X PBS + 1% BSA was used. After incubation with secondary antibodies, the cells were washed three times with 1X PBS (5 min/wash). The slide was mounted with 20 μl of Vectashield mounting medium (Vector Laboratories) containing 1 μg/ml DAPI (4'-6-diamidino-2-phenylindole), covered with the cover slips and the edges of cover glasses were sealed with nail polish. The slides were stored at -20℃ in black box. The cells were observed under fluorescence

microscope (Olympus AX70, UPlan Apo 100X/1.35 oil iris).

Image Analysis

The images of immunofluorescence staining were captured with a cooled charged-coupled device (CCD) camera (Photometrics) and processed with the V for Windows software (Photometrics).

Microtubule Perturbation Treatments

Benomyl Treatment

Benomyl was usually prepared freshly on the day of the experiment, following the methods in the report of Shonn *et al*. (2000). DMSO (dimethyl sulfoxide; Sigma D-8418) or benomyl (10 mg/ml stock in DMSO; Sigma-Aldrich) was dissolved in near-boiling sporulation medium to avoid precipitation of benomyl. The medium was then allowed to slowly cool to room temperature. At the time point of benomyl treatment (12 h), cells were harvested and immediately resuspended in the medium containing benomyl or DMSO.

Cold-Shock Treatment

Cells were grown overnight to saturation in YPAD (yeast extract-peptone plus 1% adenine and 2% glucose). 100 μl of saturated cells were diluted into 2 ml fresh YPAD and grown for 18-20 h at 30℃. At the next day, cells were washed once with 2% KAc, resuspended in liquid sporulation medium (2% KAc), and grown at 30℃. At the indicated time point of cold-shock treatment, cells were sporulated at 10℃ for the indicated time-lengths. Next, cells were continued to grow at 30℃.

Quantification of Ady3 Subcellular Localization

At the indicated time point of sporulation, meiotic II cells were fixed and stained with anti-tubulin, anti-HA and anti-myc antibodies to detect spindle, Ssp1 and Ady3, respectively. Only meiotic II cells with two spindles, four Ssp1 rings, and different number of Ady3 rings were counted for comparing Ady3 localization signals in both wild type and *hsp26* mutant cells. At least 50 meiotic II cells with four Ssp1 rings were counted in each experiment.

Quantification of Ssp1 Subcellular Localization

At the indicated time point of sporulation, meiotic II cells were fixed and stained with anti-tubulin and anti-HA antibodies to detect spindle and Ssp1. Only meiotic II

cells with two spindles and different number of Ssp1 rings were counted for comparing Ssp1 localization signals in both wild type and *hsp26* mutant cells. At least 100 meiotic II cells were counted in each experiment.

Yeast Two-Hybrid Assay

The yeast strain PJ694A (K589), which contains three separate reporter genes *HIS3*, *ADE2*, and *lacZ* under the control of Gal4-inducible promoters, was used in performing yeast-two hybrid assay (James *et al*., 1996).

In order to generate two-hybrid expression vector, nearly full-length fragments of SSP1 were cloned into pGBDU-C1 (pT477). pGBDU vector contains the yeast selectable marker URA3 and allow proteins to be expressed as a Gal4 binding domain (BD) fusion protein. The Gal4 activation domain (AD) domain fusion protein (pT487) was constructed by Ho, 2005.

pGBDU-C1-*SSP1* (pT700) and pGAD-C1-*HSP26* (pT487) were co-transformed into PJ69-4A (K589) using LiAc method (Ito *et al*., 1983) and were plated on the synthetic dropout (SD)-Ura-Leu medium. To test protein-protein interaction, transformant cells were replicated onto SC-ura-leu-ade medium and SC-ura-leu-his (containing 3mM 3-AT).

CHAPTER 3

RESULTS

Subcellular localization of Ssp1 and Ady3

Epitope tagging of Ssp1 and Ady3

In order to monitor the localization of both Ssp1 and Ady3 protein in yeast cells, 3XHA- and 3Xmyc-coding sequence were introduced into the open reading frame of Ssp1 and Ady3 right after the start codon, respectively. This construction had been checked to be in frame. To examine whether the epitope tagging might cause artificial effects, the sporulation efficiency were assessed. As shown in table 4 and 5, the sporulation efficiency and distribution of ascal types in wild type and *hsp26* mutant cells which carrying epitope tagging of Ssp1 and Ady3 were similar with which without epitope tagging. These data indicates that yeast cells carrying epitope tagging of Ssp1 and Ady3 do not affect the function of corresponding proteins.

Ssp1 and Ady3 colocalize in the LEP coat in both wild type and *hsp26* mutant cells

To gain insight into the role of Hsp26 in either Ssp1 or Ady3 localization during PSMs formation, theirs subcellular localization were analyzed. There is no differences

were observed between wild type and *hsp26* mutant cells during the process of PSMs formation. The PSMs formation starts with the appearance of precursors of the PSM in the cytoplasm during meiosis I. At the beginning of meiosis II, all precursors of the PSMs are found at the SPBs which are appeared as a dot-like structure. Next, during meiosis II Ady3 was observed to be colocalized with Ssp1 as a ring-like structure in the LEP coat (Figure 1 and 2). Each ring corresponds to the growing tip of one PSM which is still open and surrounds one spindle axis.

Analysis of *hsp26* Mutant during Prospore Membranes (PSMs) Formation

Colocalization of Ady3 to Ssp1 is not affected by Hsp26

Firstly, we attempt to analyze in normal physiological conditions whether the colocalization of Ady3 to Ssp1 is affected by Hsp26. In this case, when Ssp1 was observed to be localized at the LEP coat, at the same time we expected that Ady3 can not colocalize with Ssp1. Comparing Ady3 signals in both wild type and the *hsp26* mutant cells by judging two meiotic spindles with four Ssp1 rings as the meiotic II cell that was forming PSMs, we can analyze whether Hsp26 can regulate Ady3 localize to the LEP coat. Our result indicates that in both wild type and the *hsp26* mutant cells,

almost all of the meiotic II cells with four Ssp1 rings were concomitantly observed with four Ady3 rings at the LEP coat (Figure 3). It suggests that the localization of Ady3 to the LEP coat seemed not to be affected by Hsp26 under normal physiological conditions.

Localization of Ssp1 to the leading edge of PSM is not affected by Hsp26

The colocalization of Ady3 to Ssp1 is not affected by Hsp26. This was also the case for Ssp1. Quantification of Ssp1 ring's number shows that under normal physiological conditions, the localization of Ssp1 to the leading edge of PSM is not affected by Hsp26 (Figure 4).

Our working hypothesis proposed that Hsp26 might be involved in the spindle checkpoint machinery by regulating the localization of either Ssp1 or Ady3 to the LEP coat, thus can control the PSM formation steps. Therefore, we assumed that without any spindle-related surveillance signals, Hsp26 would not trigger its function. Consequently, we did not observe any discrepancy in the localization of either Ssp1 or Ady3 in both wild type and *hsp26* mutant cells. For further investigating whether Hsp26 may regulate the localization of either Ssp1 or Ady3 to the LEP coat in response to the meiotic spindle perturbations, we performed some microtubule perturbation treatments:

benomyl and cold-shock treatment.

Benomyl, a microtubule inhibitor, does not affect the localization of Ady3 to the LEP coat in both wild type and *hsp26* mutant cells

In order to more specifically perturb meiotic spindle, we treated cells with the microtubule-depolymerizing drug, benomyl. The experimental procedures used for benomyl treatment in this study were adapted from Ho (2005). At 12 h (during meiotic nuclear division) after inoculation into sporulation medium, the cells were resuspended into the sporulation medium containing 20 μg/ml benomyl or 1% DMSO (untreated). The concentrations of benomyl that we used should only disturb but not block the progression of meiotic nuclear divisions. The wild-type cells subjected to 20 μg/ml of benomyl displayed a decreased in the sporulation frequency and a slightly reduction in the percentages of meiosis I and meiosis II cells compared with an untreated culture (1% DMSO). In contrast, the 20 μg/ml benomyl-treated *hsp26* mutant cells were almost unaffected in either meiotic nuclear division or spore formation (Table 6). Next, we performed immunofluorescence analysis to examine Ady3 localization profiles in both wild type and *hsp26* mutant cells under benomyl treatment. The localization of both Ssp1 and Ady3 as a ring-like structure in the *hsp26* mutant cells was as similar as that in the wild type cells; additionaly, it was observed that Ssp1 localizes to the entire PSM in

late meiosis II and not only to the LEP coat (Figure 5). Meanwhile, we calculated the number of meiotic II cells with four Ssp1 rings and different number of Ady3 rings. Finding of this study has shown that almost all meiotic II cells of both wild type and *hsp26* mutant cells displayed four Ssp1 rings at the LEP coat, along with four Ady3 rings (Figure 6). The result suggests that Hsp26 can not regulate the localization of Ady3 to the LEP coat.

The localization of Ssp1 to the LEP coat is affected under benomyl treatment in wild type cells, but not in *hsp26* mutant cells

To address whether the localization of Ssp1 can be regulated by Hsp26, immunofluorescence analysis was performed to compare the number of meiotic II cells with different number of Ssp1 rings in both benomyl-treated wild type and benomyl-treated *hsp26* mutant cells. As shown in figure 7, there were more than 20% meiotic II cells without any Ssp1 rings can be detected in the benomyl-treated wild type cells; whereas, benomyl-treated *hsp26* mutant cells only displayed a minor percentage (5~6%). This finding supports the idea that in response to the meiotic spindle perturbations, Hsp26 might inhibit the localization of Ssp1 and subsequently prevent the LEP coat formations.

Cold-Shock Treatment

Cold-shock treatment conditions for perturbing meiotic spindles

Low temperatures are also known to destabilize microtubules (Gupta *et al*., 2001). Culturing cells at 10℃ during the meiotic cell cycle has been reported to cause disruption of the microtubule cytoskeleton (Amon *et al*., 2005). At first, we attempted to find the suitable conditions (time-length and timing) of cold-shock treatments that should only interfere but not block the progression of meiotic nuclear divisions. At 12 h after inoculation into sporulation medium, both wild type and *hsp26* mutant cells are sporulated at 10℃ at various time lengths to observe the time lengths that are required to make the low temperature effects become the most significant. Cold-shock treatment for 30 mins seemed to significantly delay the sporulation frequency of wild type cells, but not that of *hsp26* mutant cells (Table 7-1). In contrast, nuclear division of both wild type and *hsp26* mutant cells were not affected (Table 7-2). We have found the suitable conditions of cold-shock treatment: 12 h after inoculation into sporulation medium treated cells at 10℃ for 30 mins; however, 12 h is the time point at when cells just about leaving the pachytene stage of meiosis I phase. Therefore, this treatment condition probably can also affect the meiotic I cells. In order to more specifically disturb meiosis II cells, we attempted to find another time points for cold-shock treatment by

assessing the sporulation frequency of wild type cells. At 12, 13, 14 h time point, the sporulation frequency of wild type cells were decreased compared to the untreated cells. However, it was only slightly decreased when the time point was extended to 15 and 16 h (Table 8). Thus, we suggested that 14 h is the suitable time point to perform cold-shock treatment.

To analyze the kinetics of spore formation and nuclear division in the wild type and *hsp26* mutant cells, we performed time course analyses by using above cold-shock treatment condition (time point = 14 h; time length = 30 mins). The kinetics of nuclear divisions in the cold-shock treated wild type cells seemed not to be affected; however, it displayed a decrease in the sporulation frequency compared with an untreated culture (sporulated at 30℃). Meanwhile, the sporulation frequency and nuclear division of cold-shock treated *hsp26* mutant cells seemed not to be affected (Figure 8 and 9). Taken together, these data indicates that cold-shock treatment for 30 mins at 14 h after sporulation induction can affect the sporulation in wild type cells, but not in the *hsp26* mutant cells.

Colocalization of Ady3 to Ssp1 is not affected by Hsp26 in cold-shock treated cells

Disruption of meiotic spindles by cold-shock treatment caused a reduction in spore formation in the wild type cells, but not in *hsp26* mutants; therefore, we proposed that this reduction might be regulated via Hsp26 by controlling the localization of either Ady3 or Ssp1 to the LEP coat, thereby prevent the progression of spore formation of defective spindle daughter cells. To address for the former possibility, we performed immunofluorescence analysis to observe the profiles of Ady3 localization in both cold-shock treated wild type and cold-shock treated *hsp26* mutants. In overall calculations, we pooled together the number of all Ssp1 and Ady3 rings that were observed. As shown in the table 9, the percentage ratio of Ady3 to Ssp1 was 100% which indicated that the number of Ssp1 was similar with that of Ady3. Also, by calculating the percentages of meiotic II cells with four Ssp1 rings and different number of Ady3 rings, we found that all meiotic II cells in both wild type and *hsp26* mutant cells displayed four Ssp1 rings at the LEP coat, along with four Ady3 rings (Figure 10). This result was consistent with our previous result of benomyl treatment which suggested the localization of Ady3 to the LEP coat is not regulated by Hsp26.

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Cold-shock treatment cause effects similar to those of benomyl

To further confirm whether Hsp26 may regulate the localization of Ssp1 in response to the meiotic spindle perturbations, we analyzed the localization profiles of Ssp1 in sporulation cultures which were shifted to low temperatures (10℃) at 14 h after sporulation induction for 30 mins. Low temperature stress in the wild type cells led to the decrease of Ssp1 localization to the LEP coat, but not in the *hsp26* mutant cells (Figure 11). Quantification analysis of Ssp1 subcellular localization under cold-shock treatment has also revealed that there were about 20% of meiotic II wild type cells without any Ssp1 ring can be observed (Figure 12). This result was consistent to that observed in benomyl-treated cells (Figure 7). Taken together, these results indicate that microtubule perturbations somehow led Hsp26 to inhibit the localization of Ssp1 to the LEP coat and subsequently prevent spindle-defective daughter cells to form into mature spore.

Analysis of Hsp26 and Ssp1 Interaction

Our previous result can not distinguish whether Ssp1 is a direct target of Hsp26 regulation through spindle checkpoint signaling, or alternatively whether Ssp1 regulation is a secondary response. To address this question, we performed yeast-two hybrid assay to analyze whether Hsp26 can physically interact with Ssp1. For this study, nearly full length of *HSP26* and *SSP1* were cloned into a plasmid containing the Gal4 activation domain (AD) and into a plasmid containing Gal4 binding domain (BD), respectively. Plasmids of the activation domain and binding domain with TPBP which can form a homodimer were used as a positive control; whereas, vector plasmids of the activation domain (pGAD) and binding domain (pGBDU) were used as a negative control. Yeast GRID database of two-hybrid analysis showed that Hsp26 can interact with Ady3; also, Ady3 can interact with Ssp1. This is consistent with our yeast two-hybrid result in figure 13, cells containing both BD vector with Ady3 and AD vector with Hsp26; or cells containing both BD vector with Ssp1 and AD vector with Ady3 can grow on the selective media (SC-ura-leu-ade medium and SC-ura-leu-his containing 3mM 3-AT). However, cells containing both BD vector with Ssp1 and AD vector with Hsp26 can not grow on the selective media. This result implicates that Ssp1 did not physically interact with Hsp26.

CHAPTER 4

DISCUSSION

Hsp26 Function in the LEP Coat Formations during PSMs Formation

The *hsp26* mutation suppresses the effects caused by microtubule perturbation treatments

During the transition from meiosis II to spore formation, we proposed that Hsp26 might affect the localization process of either Ssp1 or Ady3 to the LEP coat in response to the meiotic spindle perturbations. Amon *et al*. (2005) has reported that the response of meiotic cells to high levels of benomyl or low temperatures is specific and due to microtubule perturbations. Disruption of the microtubule cytoskeleton during the meiotic II phase by exposure to the low level of benomyl or low temperatures (10℃) caused a reduction in spore formation in the wild type cells, but not in the *hsp26* mutant cells. Interestingly, these treatments did not cause a significant effect during meiotic nuclear division (Table 6 and 7). This result was consistent with the benomyl treatment analysis described by Ho (2005). This phenomenon suggests that microtubule defects in the *hsp26* mutant cells could not be normally monitored; therefore, abnormal spores with those defects still initiate the spore formation. In other words, it might further

support our preliminary hypothesis which suggested that in preventing the progression of spore formation of defective spindles daughter cell, there is a novel Hsp26-dependent spindle-related checkpoint which we suspected to be related with the regulation in the localization of the leading edge protein (Ssp1, Ady3) during PSMs formation.

The localization of Ssp1 is affected under microtubule perturbation treatments

Interactions between Hsp26 and Ady3 had been confirmed by yeast two-hybrid and co-immunoprecipitation. Therefore, our speculation was more focused to the regulation of Ady3. However, quantification analyses of Ady3 localization upon microtubule perturbation treatments have revealed that its co-localization with Ssp1 to the LEP coat during PSMs formation is not affected in both wild type and *hsp26* mutant cells.

Localization dependence analysis showed that localization of Ady3 to the PSM precursors and the leading edge depends on Ssp1 (Moreno-Borchart *et al*., 2001). During late meiosis II, Ssp1 not only localizes to the LEP coat, but also to the entire prospore membrane. The phenomenon of Ssp1-dependent recruitment of Ady3 to the PSM led us to speculate that Hsp26 might control the localization of Ssp1 during PSM formation. Ssp1 localization analysis using immunofluorescence microscopy showed that upon microtubule perturbation treatments there were about 20% of wild type

meiotic II cells without any Ssp1 ring can be observed (Figure 7 and 12) which implied that those cells were not allowed to complete the process of PSM formation. These results supported our hypothesis that the localization of Ssp1 in response to meiotic spindle perturbations might be regulated by Hsp26.

The Spindle Checkpoint in Meiosis

Hsp26 might be involved in the regulation of Ssp1 localization for controlling spore formation

Once cells have entered meiosis I, cell cycle events are no longer coupled with the developmental program (Amon *et al*., 2005). When cells sensed an error in microtubules (meiotic spindles), for controlling meiotic cell cycle progression, spindle checkpoint machinery will be activated to halt the cell cycle progression until the defect in microtubules are completely repaired. Concomitantly, for controlling the developmental program progression in response to microtubule perturbations, we purposed there is a novel, as-yet-uncharacterized pathway mediated by Hsp26 to suppress the localization of Ssp1 to the LEP coat, thereby preventing the PSM formation and subsequently the progression of spore formation. Therefore, our analyses revealed a novel Hsp26-dependent regulation pathway via regulation of Ssp1

localization during spore formation progression in budding yeast.

The regulation pathway of Hsp26 in controlling Ssp1 localization

In *S. cerevisiae*, spindle alignment along the polarity axis is particularly crucial for fidelity of chromosome segregation and thus determines the fate of the daughter cells. Accurate chromosome segregation requires bipolar attachment of sister chromatids to the meiotic spindle. Understanding of how cells control the progression of spore formation in defective-spindle daughter cells has been the main interest of our study. The decrease of Ssp1 localization to the LEP coat in microtubule perturbation treated-cells suggested that Hsp26-regulated Ssp1 localization might be involved in the spindle checkpoint signaling. This regulation pathway is important to prevent spore formation progression until all the meiotic spindles defects have been repaired. Furthermore, it will be interesting to determine how Hsp26 in response to the spindle checkpoint inhibit Ssp1 to localize to the LEP coat and whether Ssp1 directly or indirectly involved in this regulation machinery.

The Relationship between Hsp26 and Ssp1

A search from yeast GRID database did not show interaction between Hsp26 and Ssp1. Similarly, we did not observe any direct interaction among them. One possibility is that Hsp26 by interacting with Ady3 might form Hsp26-Ady3/Ssp1 protein complex. At the beginning of meiosis II, the assembly of the LEP coat starts with the formation of cytosolic precursors, which then bind in an Ady3-dependent manner to the SPBs (Moreno-Borchart *et al*., 2001). At this stage, Ssp1 will recruit Ady3 to the leading edge of PSM. Concomitanly, we proposed that in response to spindle checkpoint signaling, Hsp26 might regulate LEP coat formation step by interacting with Ady3, thus inhibit Ady3/Ssp1 protein complex to associate with cytosolic PSM precursors. Under microtubule-perturbation treatment by observing Ssp1 localization profiles in *ady3* mutant strains, we can address for this possibility. Alternatively, the decrease in Ssp1 localization may be an indirect effect of Hsp26 regulation. In other words, Ssp1 might be a downstream target of this regulation pathway.

Our analyses demonstrate that upon microtubule defects Hsp26 regulation in preventing the formation of abnormal daughter cells is important pathway to ensure the normal cell cycle and developmental progression. For further insight, it will be interesting to find out how Hsp26 senses the defect in microtubules and what exactly the direct target of its regulation.

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subset of *D. melanogaster* heat shock mRNAs in normal development without heat shock. Cell 32: 1161-1170.

Table 1. Yeast strains

Strain	Genotype
TY178	*MATa ade2-1 trp1-1 his3-11,15 ura3-1 leu2-3,112 can1-100*
TY179	*MATα ade2-1 trp1-1 his3-11,15 ura3-1 leu2-3,112 can1-100*
W303	TY178 x TY179
	MATa / *MATα* *ade2-1* / *ade2-1* *trp1-1* / *trp1-1* *his3-11,15* / *his3-11,15* *ura3-1* / *ura3-1* *leu2-3,112* / *leu2-3,112* *can1-100* / *can1-100*
K580	TY178 but *hsp26::URA3*
K581	TY179 but *hsp26::URA3*
K582	W303 but homozygous *hsp26::URA3*
K589	PJ69-4A
	MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ
	LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ
K1003	TY178 but *3XHA-SSP1*
K1004	TY179 but *3XHA-SSP1*
K1005	W303 but homozygous *3XHA-SSP1*
K1053	TY178 but *3XHA-SSP1; 3Xmyc-ADY3*
K1054	TY179 but *3XHA-SSP1; 3Xmyc-ADY3*
K1055	W303 but homozygous *3XHA-SSP1; 3Xmyc-ADY3*
K1057	TY178 but *3XHA-SSP1; 3Xmyc-ADY3; hsp26::URA3*
K1060	TY179 but *3XHA-SSP1; 3Xmyc-ADY3; hsp26::URA3*
K1061	W303 but homozygous *3XHA-SSP1; 3Xmyc-ADY3; hsp26::URA3*

Table 2. Plasmids

Plasmid	Characteristics	
pRS306	pBluescript,*URA3*	Integrating plasmid
YEp351	2μm *LEU2*	High-copy number plasmid
YEp352	2μm *URA3*	High-copy number plasmid
TP174	Carry 3XHA encoding sequence	
TP208	Carry 3Xmyc encoding sequence	
T105	SK(+)-*HSP26*	Su and Tung
T119	SK(+)-*hsp26::URA3*	Su and Tung
T408	2 μm pGADU-C1 *LEU2*	AD vector, Amp^r
T445	pGAD-C3-TPBP *LEU2*	AD vector (positive control)
T446	pGBDU-C3-TPBP *URA3*	BD vector (positive control)
T477	2 μm pGBDU-C1 *URA3*	retransform T411 to get T477
T479	2 μm pGBDU-C3 *URA3*	retransform T413 to get T477
T483	pGBDU-C3-*ADY3 URA3*	BD vector, Amp^r ; Ho
T484	pGAD-C3-*ADY3 LEU2*	AD vector, Amp^r ; Ho
T487	pGAD-C1-*HSP26 LEU2*	AD vector, Amp^r ; Ho
T566	SK(+) without *Not*I site	Chen
T572	YEp352-*3Xmyc-ADY3*	Chen
T613	SK(+)-*SSP1*	Tung
T640	SK(+) without *Not*I site-*SSP1-Not*I	Tung
T642	T566-*3XHA-SSP1*	Tung
T647	pRS306-*3XHA-SSP1*	Tung
T661	pRS306-*3Xmyc-ADY3*$_{-264\sim+757}$	
T700	pGBDU-C1-*SSP1*	

Table 3. Primer sequences

Name	Sequences (5’-3’)
SSP1-U1-*Xba*I	CCGCTTTGTCTTTCTAGAATGGGTACAGATCTGAAAG
SSP1-D1-*Xho*I	ATTTCCTGAGTTCACTCGAGCGTTAAGAAGACGACAAC
SSP1-U2	GGGTACAGATCTGAAAGATTGCCAGC
SSP1-D2	GCTTCGACTGCTTTGGGGAAGTTG
SSP1-*Not*D1	CAGAGCTTCTGCGGCCGCCCATTGTTGCTAGCTTTTCTACTC
SSP1-*Not*U1	CTAGCAACAATGGGCGGCCGCAGAAGCTCTGGCACATATGAG
SSP1-U3-*Bam*HI	AGCTAGCAACAATGGGATCCTCTGGCACATATGAGAATG
ADY3-U3	GGGTAGCCGGCCAGCAAGTCTAC
ADY3-D4	CTTCGGGAACGCCTATTCGACAG
HSP26-U2	CTAAACATATAAATAGGACCTCCATTAG
HSP26-D2	TGTTTCAAGCCATATGCAAGCAAC

Table 4-1. Sporulation frequency of 3XHA-tagged Ssp1 and 3Xmyc-tagged Ady3 in wild type and *hsp26* mutant on sporulation plates

Strain	Genotype	Sporulation frequency (%)	% of wild type
W303	Wild type	58 ± 1.4	100.0
K1055	Wild type but *3XHA-SSP1; 3Xmyc-ADY3*	57 ± 2.0	98.3
K582	*hsp26Δ*	72 ± 0.5	124.1
K1061	*hsp26Δ* but *3XHA-SSP1; 3Xmyc-ADY3*	74 ± 0.2	127.6

Wild type cells (W303) without and with epitope tagging (K1055), the *hsp26* mutant (K582) without and with epitope tagging (K1061) were patched on YPAD plate and replicated onto sporulation plates for 3 days at 30℃. At least 400 asci were scored by phase contrast microscopy. Data shown are averages of two independent experiments.

Table 4-2. Ascus types of 3XHA-tagged Ssp1 and 3Xmyc-tagged Ady3 in wild type and *hsp26* mutant on sporulation plates

Strain	Genotype	Distribution of ascal types (%)		
		Monad	Dyad	Triad/Tetrad
W303	Wild type	0.0	7.1	92.9
K1055	Wild type but *3XHA-SSP1; 3Xmyc-ADY3*	0.0	10	90
K582	*hsp26Δ*	0.0	8.6	91.4
K1061	*hsp26Δ* but *3XHA-SSP1; 3Xmyc-ADY3*	0.0	8.2	91.8

Wild type cells (W303) without and with epitope tagging (K1055), the *hsp26* mutant (K582) without and with epitope tagging (K1061) were patched on YPAD plate and replicated onto sporulation plates for 3 days at 30℃. Light microscope was used to determine the types of asci. At least 400 asci were scored. Data shown are averages of two independent experiments.

Table 5-1. Sporulation frequency of 3XHA-tagged Ssp1 and 3Xmyc-tagged Ady3 in wild type and *hsp26* mutant in liquid sporulation medium

Strain	Genotype	Sporulation frequency (%)	% of wild type
W303	Wild type	65 ± 2.3	100.0
K1055	Wild type but *3XHA-SSP1; 3Xmyc-ADY3*	66 ± 2.2	101.5
K582	*hsp26Δ*	75 ± 2.0	115.4
K1061	*hsp26Δ* but *3XHA-SSP1; 3Xmyc-ADY3*	74 ± 0.2	113.8

Wild type cells (W303) without and with epitope tagging (K1055), the *hsp26* mutant (K582) without and with epitope tagging (K1061) were induced for sporulation in 2% KAc for 3 days at 30℃, and scored under light-microscope. At least 400 asci were scored. Data shown are averages of two independent experiments.

Table 5-2. Ascus types of 3XHA-tagged Ssp1 and 3Xmyc-tagged Ady3 in wild type and *hsp26* mutant in liquid sporulation medium

Strain	Genotype	Distribution of ascal types (%)		
		Monad	Dyad	Triad/Tetrad
W303	Wild type	0.0	4.8	95.2
K1055	Wild type but *3XHA-SSP1; 3Xmyc-ADY3*	0.8	4.7	94.5
K582	*hsp26Δ*	0.0	1.3	98.7
K1061	*hsp26Δ* but *3XHA-SSP1; 3Xmyc-ADY3*	0.0	5.4	94.6

Wild type cells (W303) without and with epitope tagging (K1055), the *hsp26* mutant (K582) without and with epitope tagging (K1061) were induced for sporulation in 2% KAc for 3 days at 30℃. Light microscope was used to determine the types of asci. At least 400 asci were scored. Data shown are averages of two independent experiments.

Table 6. Sporulation frequency and nuclear division analysis of wild type and *hsp26* mutant under benomyl treatment

Strain	Genotype	Treatment	Sporulation frequency (%)	Bi-/tetranucleates (%)
K1055	Wild type	1% DMSO	57 ± 6.0	58 ± 2.8
		20 μg/ml Benomyl	43 ± 0.7	49 ± 1.8
K1061	*hsp26Δ*	1% DMSO	62.5 ± 2.1	57 ± 4.6
		20 μg/ml Benomyl	60 ± 5.1	58.5 ± 6.4

Cells were cultured in the liquid sporulation medium to induce meiosis at 30℃. At 12 h after meiotic induction at 30℃, cells were resuspended in sporulation medium containing 1% DMSO or 20 μg/ml of benomyl for 2 days. At least 200 cells were counted to assess the sporulation frequency. The percentages of cells completing meiosis I and II were determined by DAPI staining and at least 200 DAPI-stained cells were counted. Data shown are averages of two independent experiments.

Table 7-1. Sporulation frequency analysis of wild type and *hsp26* mutant under cold-shock treatment (10℃)

Strain	Genotype	Treatment	Sporulation frequency (%)	% of wild type
K1055	Wild type	Control	58.5 ± 2.1	100
		5’	61 ± 1.1	104.3
		10’	59 ± 4.6	100.8
		20’	55 ± 6.7	94
		30’	44 ± 4.6	75.2
		45’	46 ± 0.3	78.6
K1061	*hsp26Δ*	Control	66.5 ± 0.7	100
		5’	62 ± 5.6	93.2
		10’	63.5 ± 3.9	95.5
		20’	65 ± 6.7	97.7
		30’	62 ± 6.0	93.2
		45’	59 ± 2.5	88.7

Cells were cultured in the liquid sporulation medium to induce meiosis at 30℃ for 42 h. At 12 h after sporulation induction, cells were sporulated at 10℃ for the indicated time lengths. At least 200 cells were counted to assess the sporulation frequency. Data shown are averages of two independent experiments.

Table 7-2. Nuclear division analysis of wild type and *hsp26* mutant under cold-shock treatment (10℃)

Strain	Genotype	Treatment	Bi-/tetranucleates (%)
K1055	Wild type	Control	62
		30’	61.5
K1061	*hsp26Δ*	Control	65
		30’	66.5

At 12 h after sporulation induction, cells were sporulated at 10℃ for 30 mins. At 42 h of time point, cells were collected, fixed, and stained with DAPI. The percentages of cells completing meiosis I and meiosis II were determined by DAPI staining and at least 200 DAPI-stained cells were counted.

Table 8. Sporulation frequency analysis of wild type under cold-shock treatment (10℃) at different timing

Strain	Genotype	Timing	Treatment	Sporulation frequency (%)	% of wild type (control)
K1055	Wild type	Control		55.5 ± 0.9	100
		12 h	30’	45 ± 0.2	81.1
		13 h	30’	44.75 ± 1.4	80.6
		14 h	30’	41.75 ± 0.2	75.2
		15 h	30’	46.75 ± 1.2	84.2
		16 h	30’	48.5 ± 0.2	87.4

Cells were cultured in the liquid sporulation medium to induce meiosis at 30℃ for 42 h. At different timing of sporulation, cells were sporulated at 10℃ for 30 mins. At least 200 cells were counted to assess the sporulation frequency. Data shown are averages of two independent experiments.

Table 9. Overall calculation of Ssp1 and Ady3 under cold-shock treatment

Strain	Genotype	Treatment	Number of Ssp1	Number of Ady3	Ady3:Ssp1 percentage ratio
K1055	Wild type	Control	784	784	100
		10℃ 30’	252	252	100
K1061	*hsp26Δ*	Control	421	421	100
		10℃ 30’	329	329	100

At 14 h after sporulation induction, cells were sporulated at 10℃ for 30 mins. The number of Ssp1 and Ady3 rings was observed using immunofluorescence microscopy.

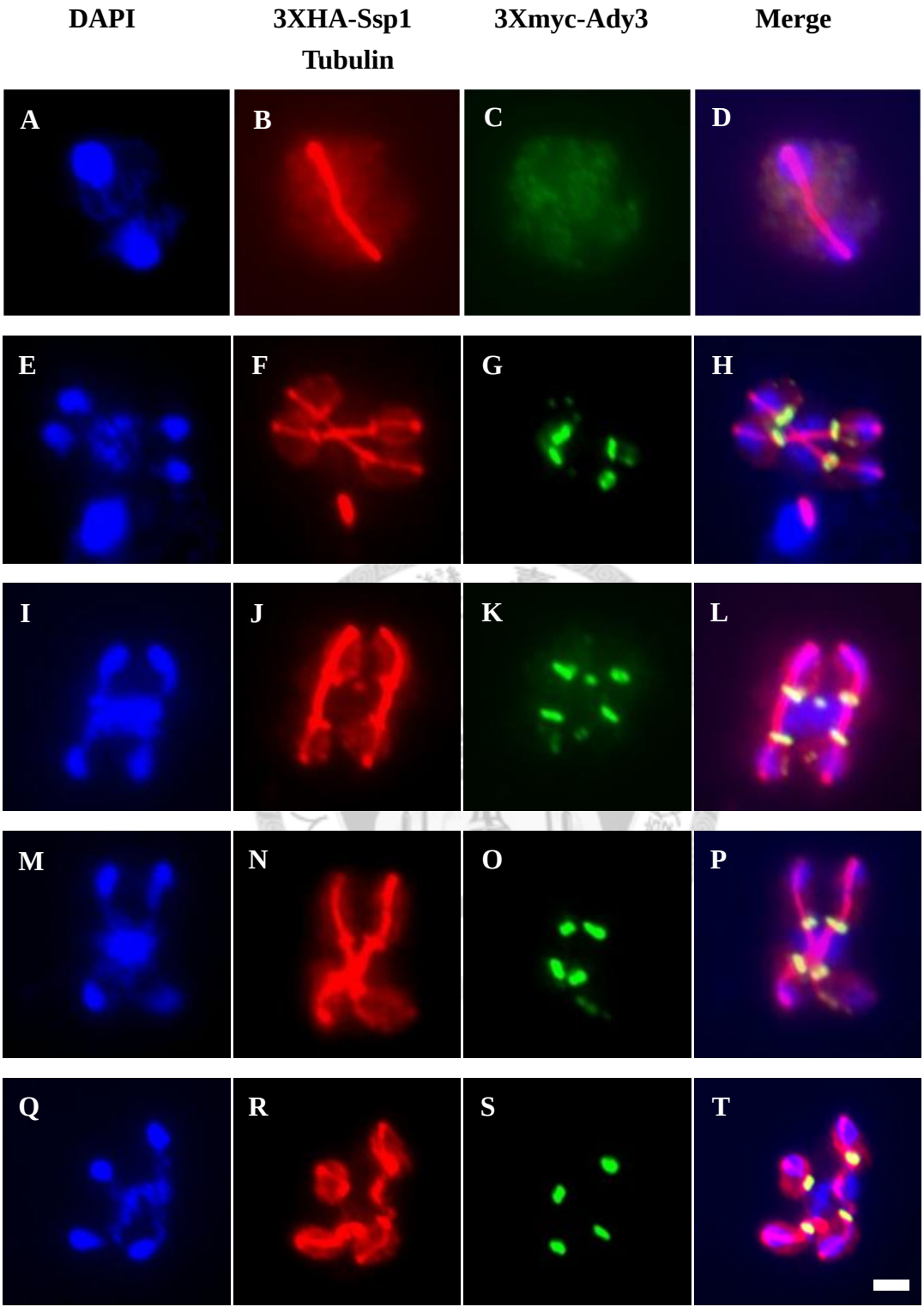

Figure 1. Ady3 and Ssp1 were colocalized on the LEP coat in wild type cells

W303 cells carrying endogenous tagging of 3Xmyc-ADY3 and 3XHA-SSP1 were induced during sporulation for 15 h and stained with a myc-specific antibody (A14), a HA-specific antibody (16B12) and a tubulin-specific antibody (1/34 YOL). Meiosis I (A~D). Meiosis II (E~T). Scale bar: 2 μm.

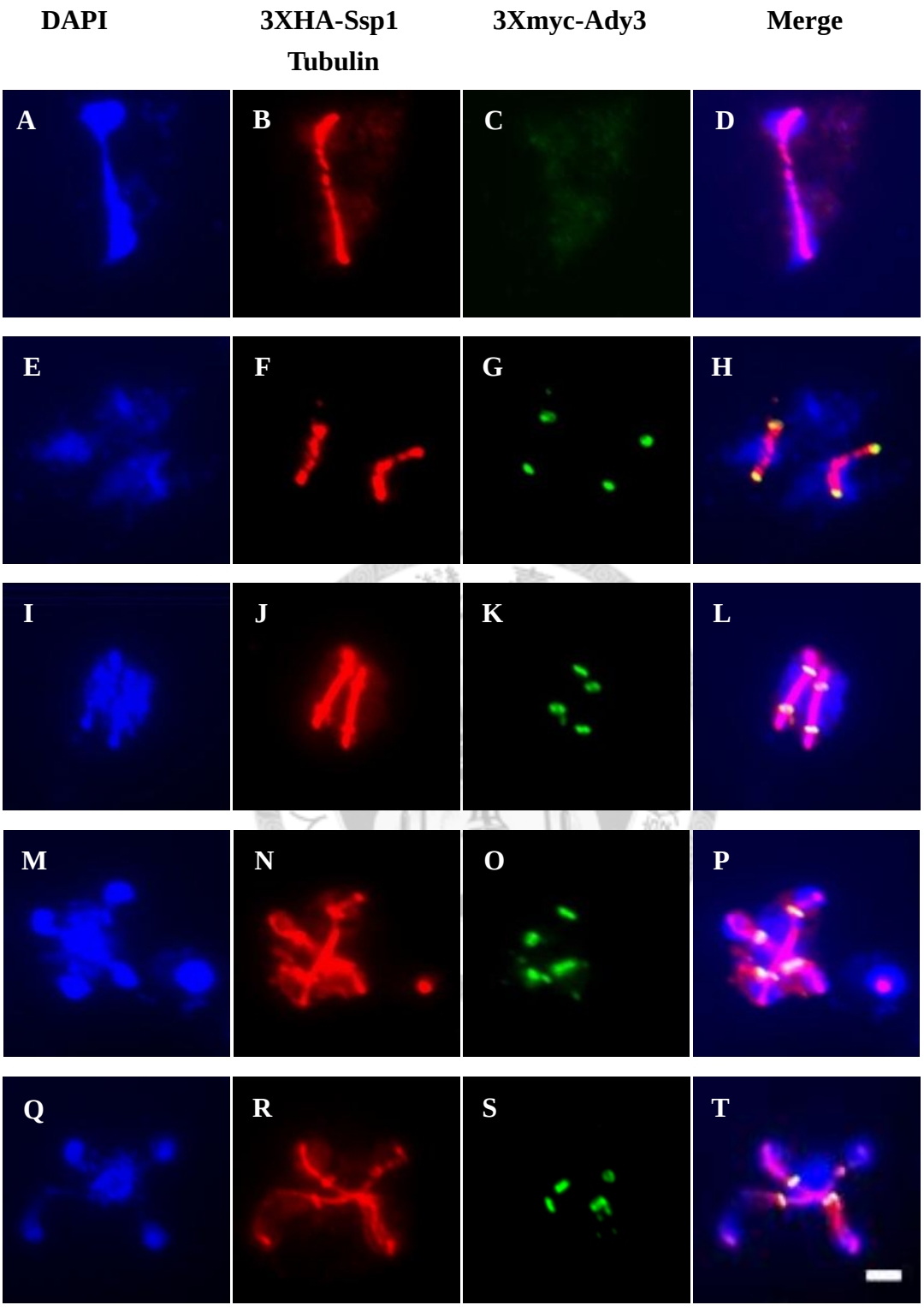

Figure 2. Ady3 and Ssp1 were colocalized on the LEP coat in *hsp26* mutant cells
W303 *hsp26* mutant cells carrying endogenous tagging of 3Xmyc-ADY3 and 3XHA-SSP1 were induced during sporulation for 15 h and stained with a myc-specific antibody (A14), a HA-specific antibody (16B12) and a tubulin-specific antibody (1/34 YOL). Meiosis I (A~D). Meiosis II (E~T). Scale bar: 2 μm.

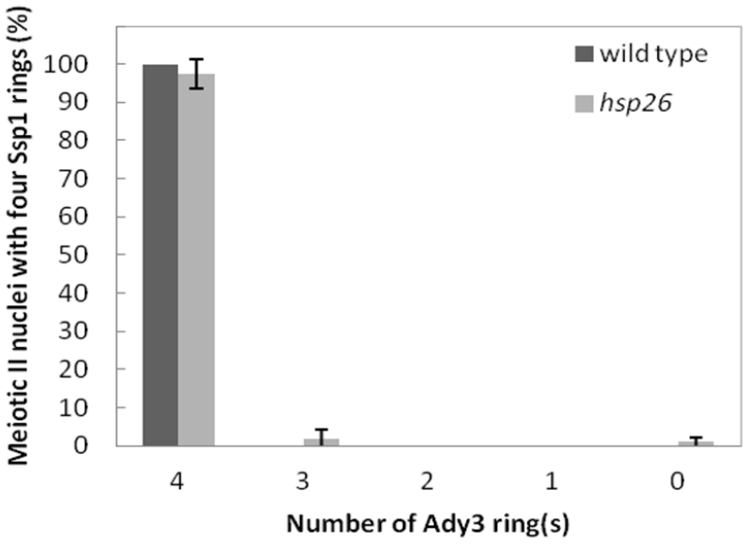

Figure 3. Comparison the number of Ady3 in wild type and *hsp26* mutant cells
Wild type and *hsp26* mutant cells were sporulated for 15 h and the different number of Ady3 rings per cell was calculated using immunofluorescence microscopy. At least 50 meiotic II cells with four Ssp1 rings and different number of Ady3 rings were counted. The standard deviations were derived from two independent experiments.

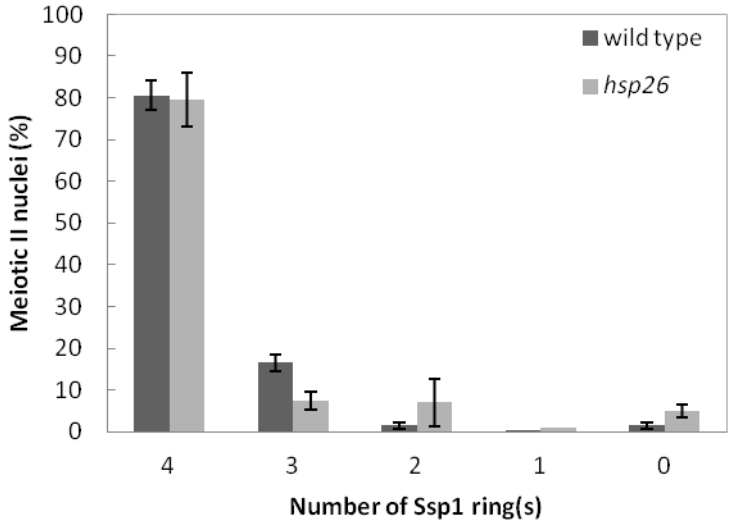

Figure 4. Comparison the number of Ssp1 in wild type and *hsp26* mutant cells
Wild type and *hsp26* mutant cells were sporulated for 15 h and the different number of Ssp1 rings per cell was calculated using immunofluorescence microscopy. At least 50 meiotic II cells with different number of Ssp1 rings were counted. The standard deviations were derived from two independent experiments.

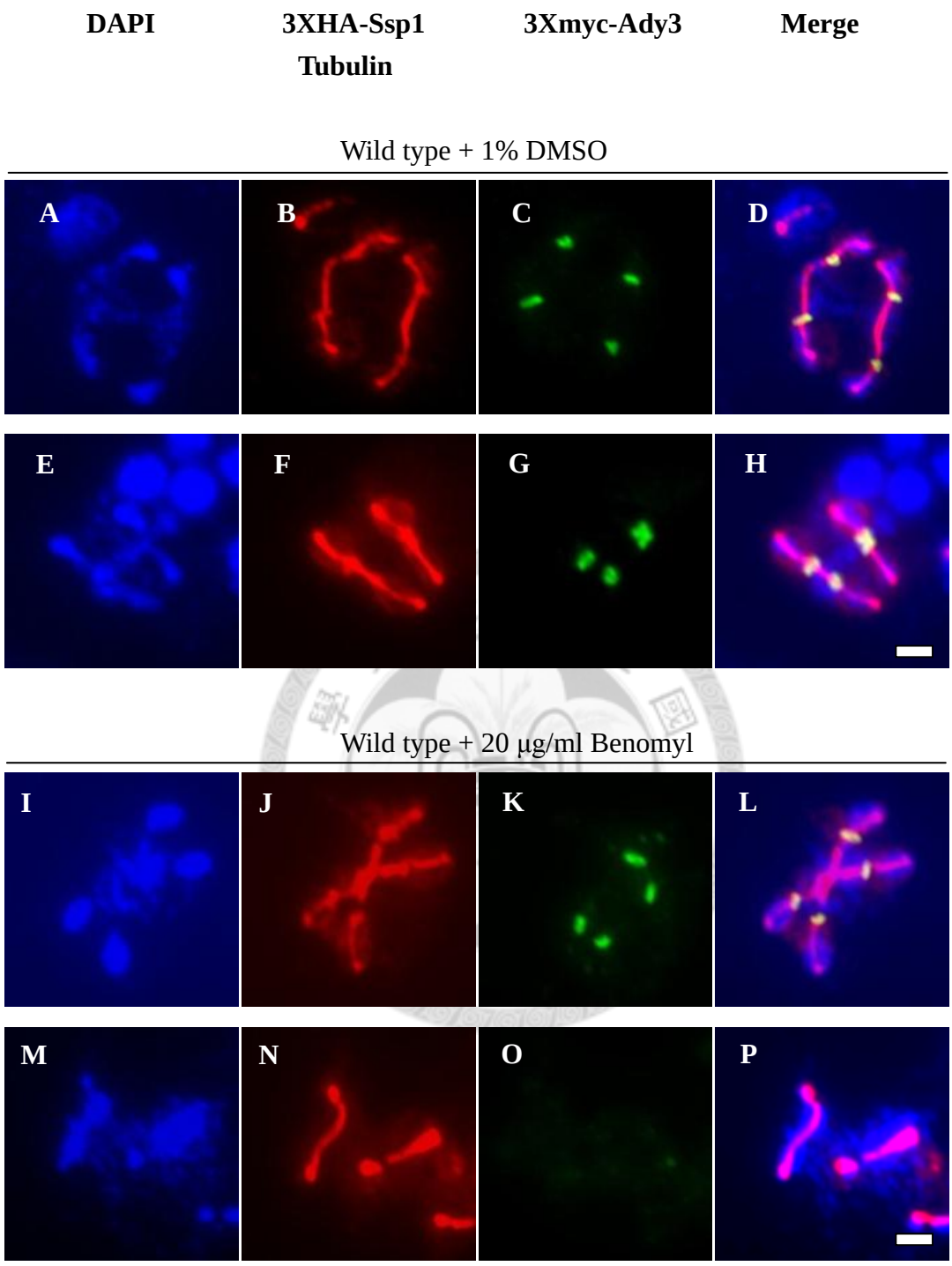

Figure 5-1. Localization of Ady3 and Ssp1 in wild type cells under benomyl treatment

W303 wild type cells carrying endogenous tagging of 3Xmyc-ADY3 and 3XHA-SSP1 were sporulated into 2% KAc containing 1% DMSO (A~H) or 20 μg/ml benomyl (I~P). 16 h-meiotic II cells were fixed and stained with a myc-specific antibody (A14), a HA-specific antibody (16B12) and a tubulin specific antibody (1/34 YOL). Scale bar: 2 μm.

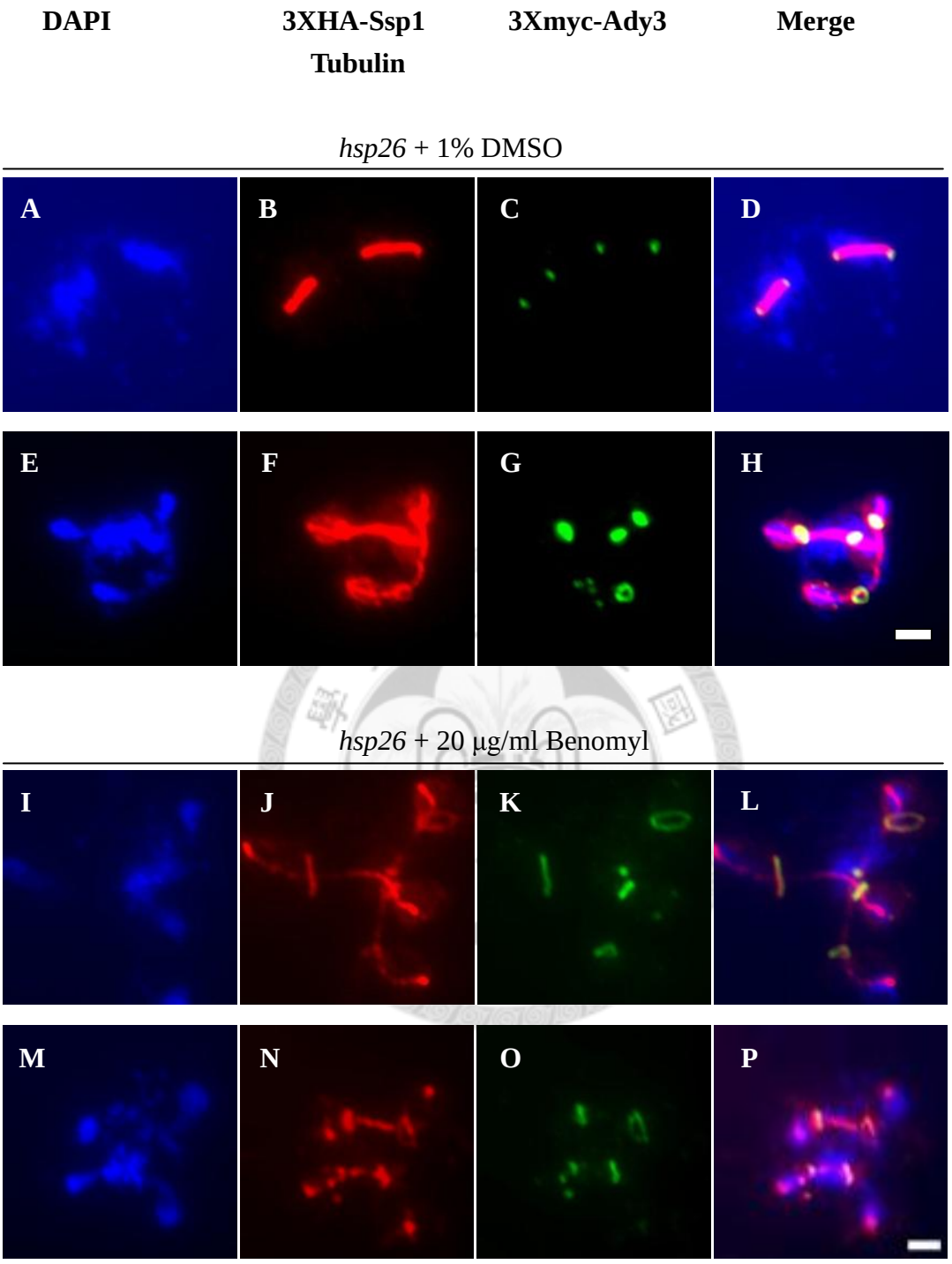

Figure 5-2. Localization of Ady3 and Ssp1 in *hsp26* mutant cells under benomyl treatment

W303 *hsp26* mutant cells carrying endogenous tagging of 3Xmyc-ADY3 and 3XHA-SSP1 were sporulated into 2% KAc containing 1% DMSO (A~H) or 20 μg/ml benomyl (I~P). 16 h-meiotic II cells were fixed and stained with a myc-specific antibody (A14), a HA-specific antibody (16B12) and a tubulin specific antibody (1/34 YOL). Scale bar: 2 μm.

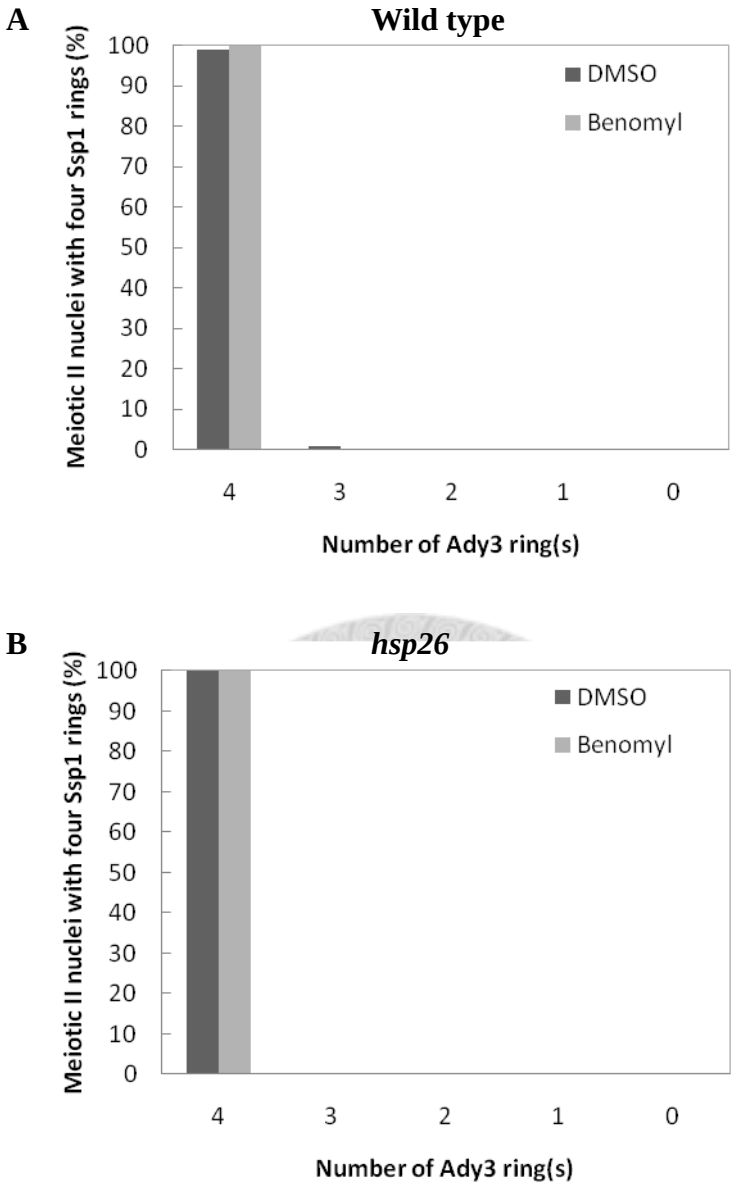

Figure 6. Comparison of Ady3 localization in wild type and *hsp26* mutant cells under benomyl treatment

At 12 h after meiotic induction at 30℃, wild type and *hsp26* mutant cells were sporulated in sporulation medium containing 20 μg/ml benomyl or 1% DMSO. The different number of Ady3 rings per cell was calculated using immunofluorescence microscopy. At least 50 meiotic II cells with four Ssp1 rings and different number of Ady3 rings were counted.

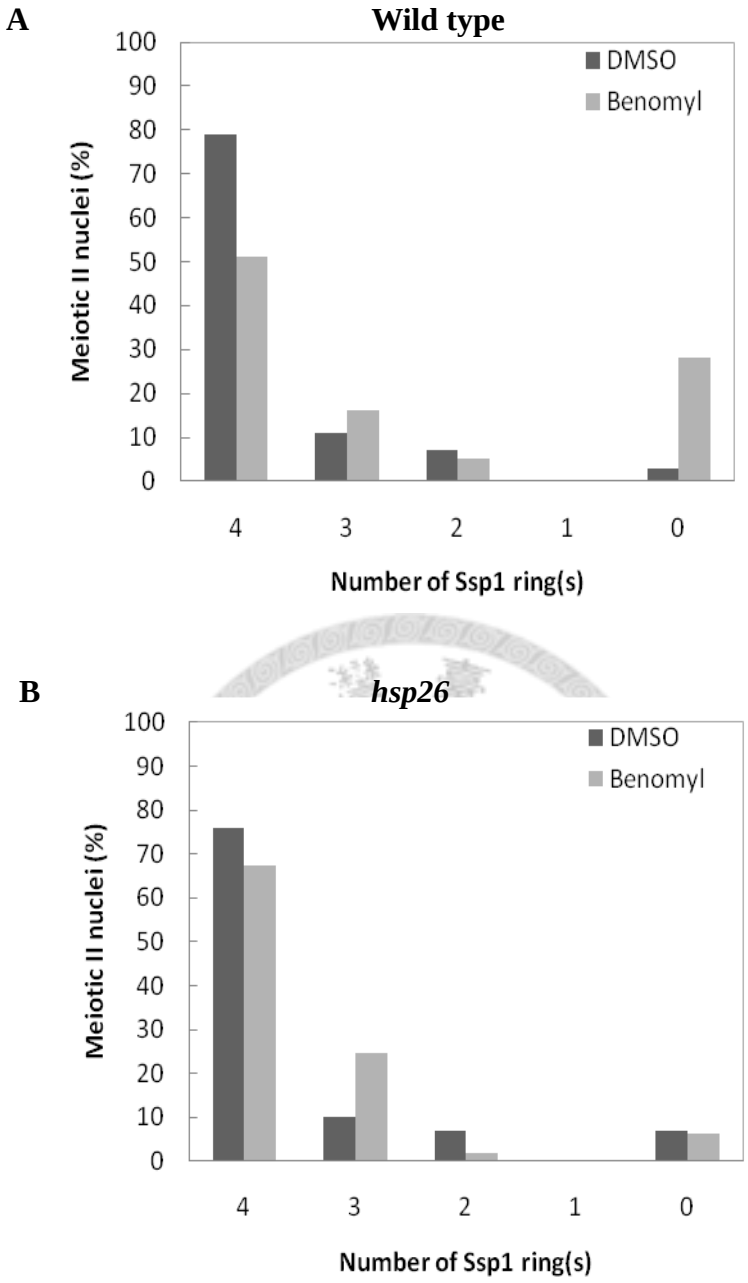

Figure 7. Comparison of Ssp1 localization in wild type and *hsp26* mutant cells under benomyl treatment

At 12 h after meiotic induction at 30℃, wild type and *hsp26* mutant cells were sporulated in sporulation medium containing 20 μg/ml benomyl or 1% DMSO. The different number of Ssp1 rings per cell was calculated using immunofluorescence microscopy. At least 50 meiotic II cells with different number of Ssp1 rings were counted.

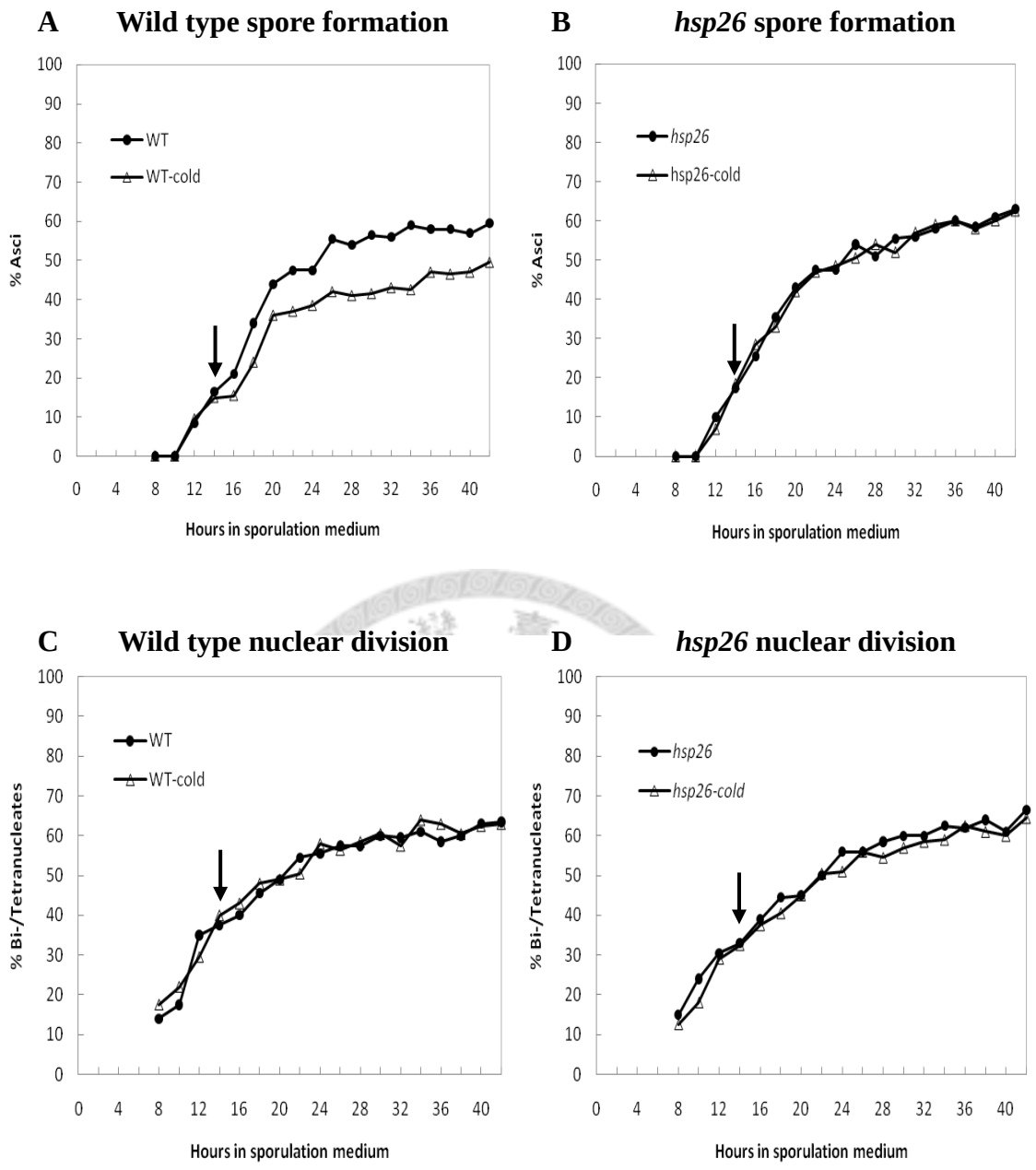

Figure 8. The kinetic of meiosis in wild type and *hsp26* mutant at 30℃ and 10℃

Meiotic time course of spore formation and nuclear division in cold-shock treated wild type cells (A and C) and *hsp26* mutant cells (B and D). At 14 h (shown by the arrow) after sporulation induction at 30℃, cells were incubated in 10℃ for 30 mins.

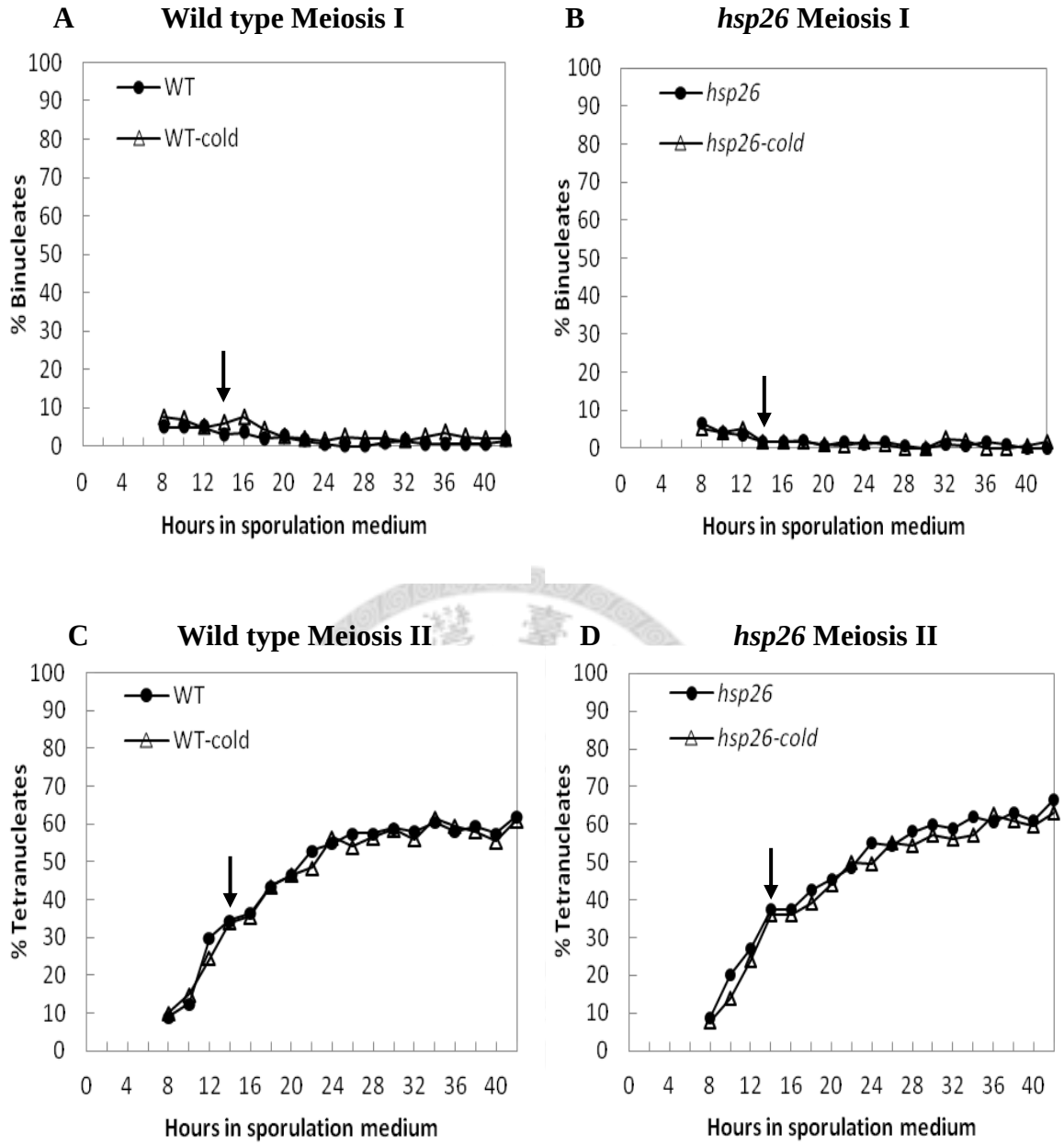

Figure 9. The meiotic nuclear division profiles in cold-shock treated wild type and *hsp26* mutant cells

At 14 h (shown by the arrow) in sporulation medium, cells were sporulated in 10℃ for 30 mins or in 30℃ (control). The percentages of the cells completing meiosis I and meiosis II were determined by DAPI staining and at least 200 cells per time point were scored.

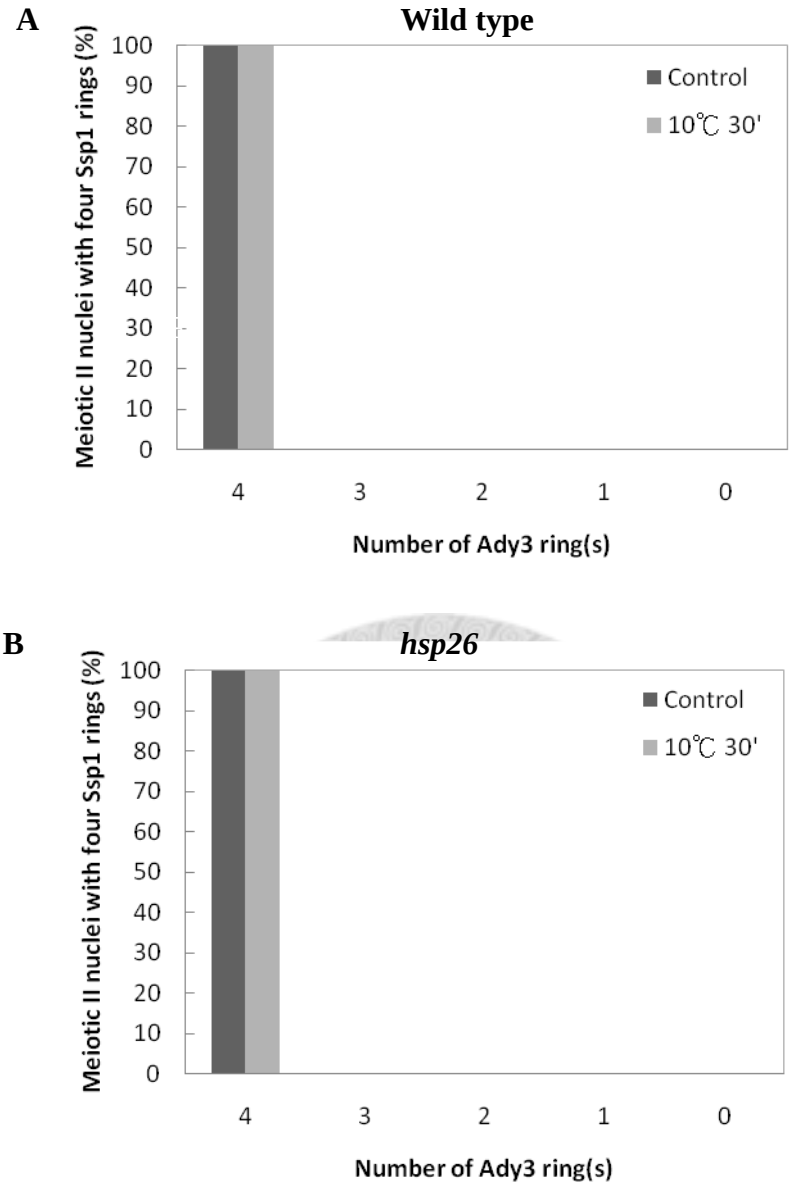

Figure 10. Comparison of Ady3 localization in wild type and *hsp26* mutant cells under cold-shock treatment

At 14 h after meiotic induction at 30℃, wild type and *hsp26* mutant cells were incubated in 10℃ for 30 mins. The different number of Ady3 rings per cell was calculated using immunofluorescence microscopy. At least 100 meiotic II cells with four Ssp1 rings and different number of Ady3 rings were counted.

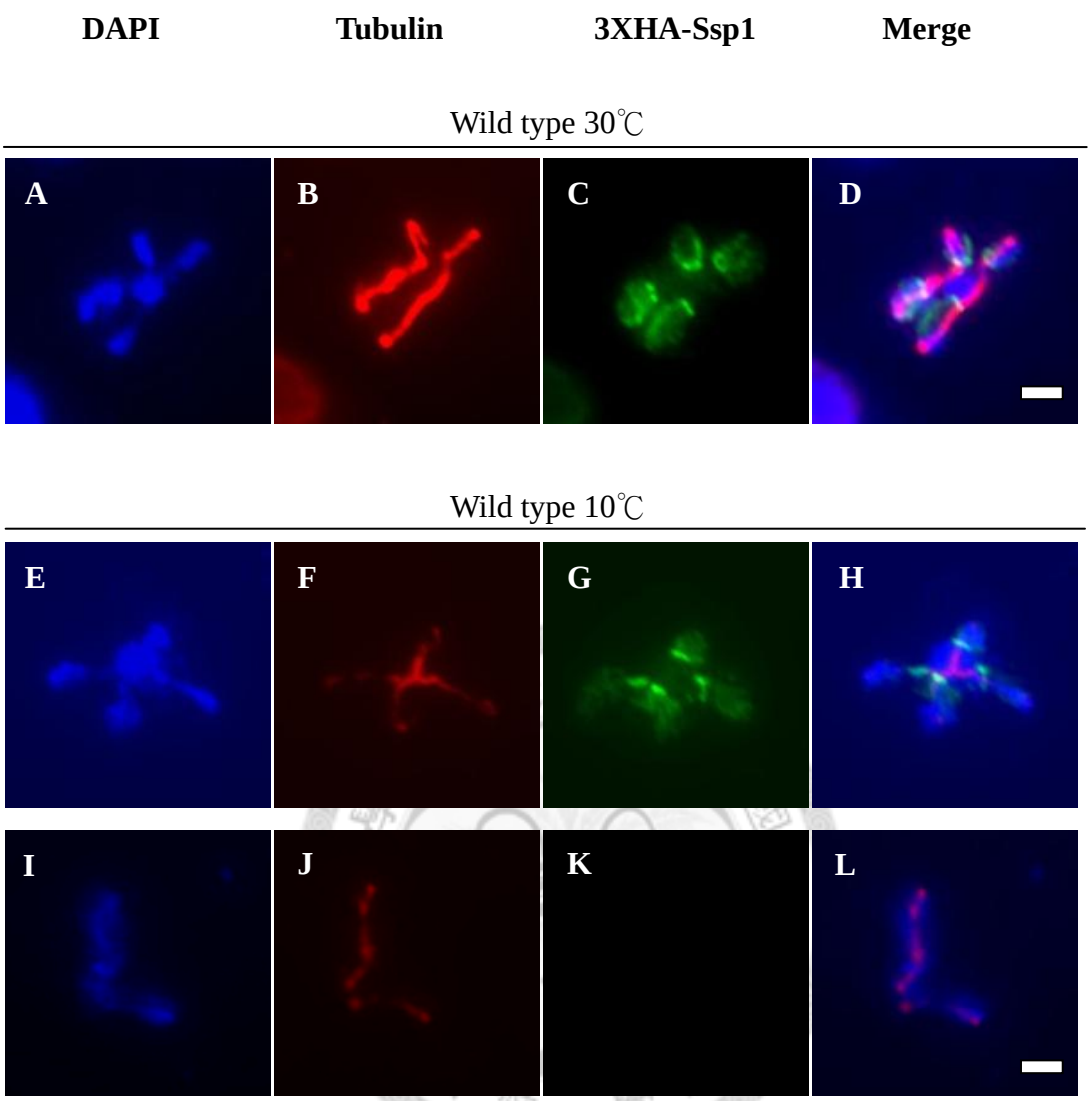

Figure 11-1. Localization of Ssp1 in wild type cells under cold-shock treatment

At 14 h after sporulation induction, W303 wild type cells carrying endogenous tagging of 3Xmyc-ADY3 and 3XHA-SSP1 were sporulated at 30℃ (A~D) or 10℃ for 30 mins (E~L). 16 h-meiotic II cells were fixed and stained with a HA-specific antibody (16B12) and a tubulin-specific antibody (1/34 YOL). Scale bar: 2 μm.

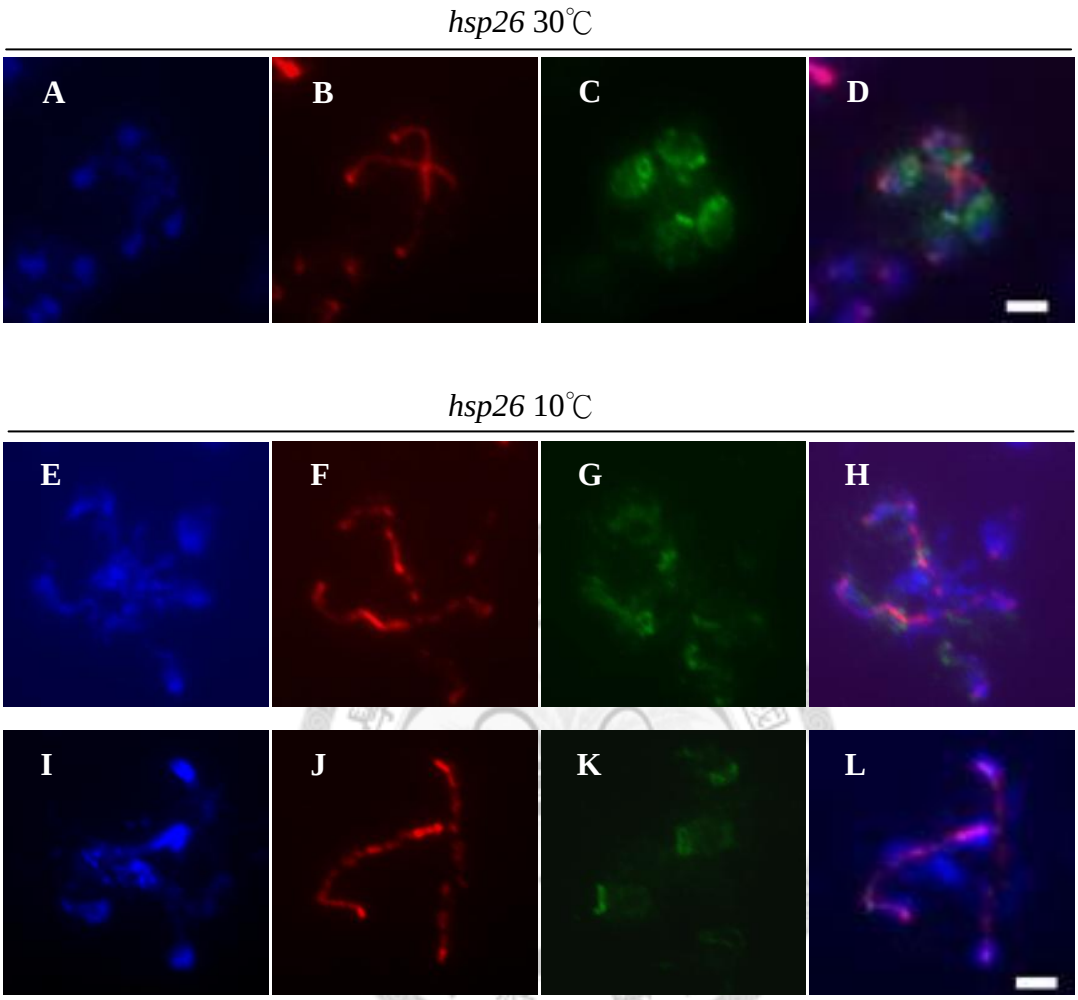

Figure 11-2. Localization of Ssp1 in *hsp26* mutant cells under cold-shock treatment
At 14 h after sporulation induction, W303 *hsp26* mutant cells carrying endogenous tagging of 3Xmyc-ADY3 and 3XHA-SSP1 were sporulated at 30℃ (A~D) or 10℃ for 30 mins (E~H). 16 h-meiotic II cells were fixed and stained with a HA-specific antibody (16B12) and a tubulin-specific antibody (1/34 YOL). Scale bar: 2 μm.

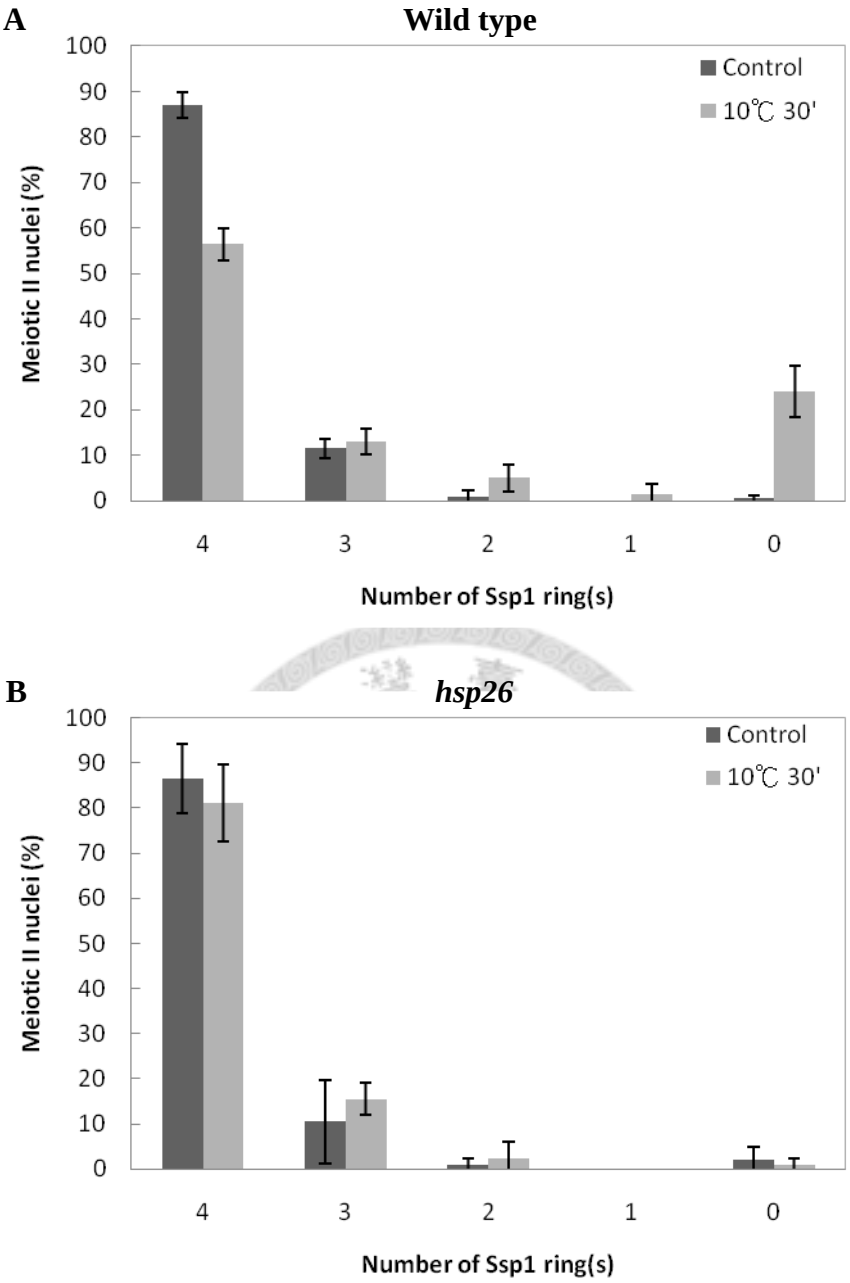

Figure 12. Comparison of Ssp1 localization in wild type and *hsp26* mutant cells under cold-shock treatment

At 14 h after meiotic induction at 30℃, wild type and *hsp26* mutant cells were incubated in 10℃ for 30 mins. The different number of Ssp1 rings per cell was calculated using immunofluorescence microscopy. At least 100 meiotic II cells with different number of Ssp1 rings were counted. The standard deviations were derived from two independent experiments.

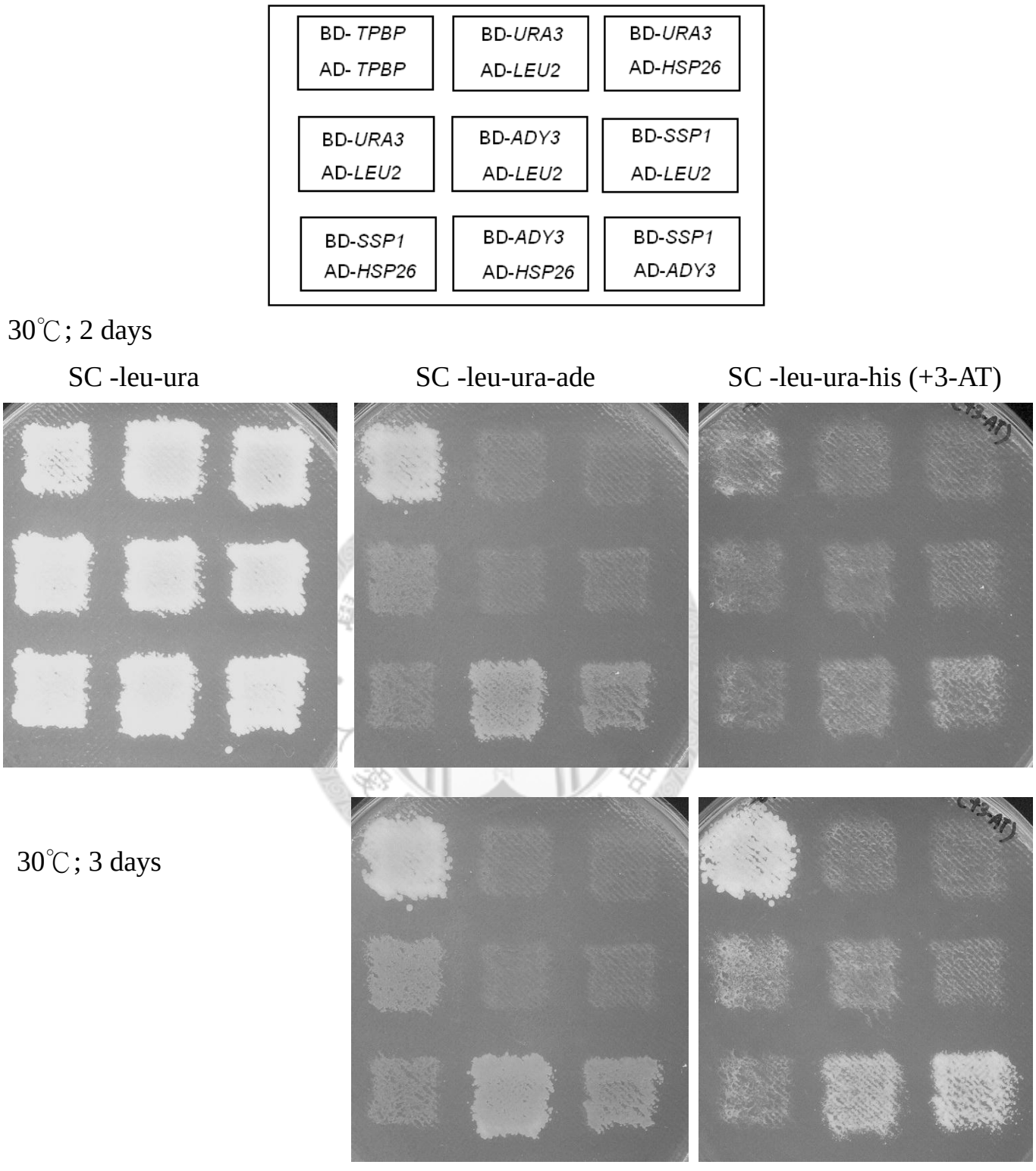

Figure 13. Yeast-two hybrid assay for analyzing the interaction of Hsp26 and Ssp1
Yeast strain PJ69-4A (K589) was co-transformed with AD-derived and BD-derived plasmid, as indicated. Transformed cells were replicated onto the selective plates (SC-leu-ura-ade and SC-leu-ura-his 3 mM 3-AT) to test *ADE2* and *HIS3* reporter genes and incubated at 30℃ for 2-3 days.