Establishment and maintenance of sister chromatid cohesion in fission yeast by a unique mechanism

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During S phase, chromatid cohesion is established only between nascent sisters and with faithful pairing along their entire region, but how this is ensured is unknown. Here we report that sister chromatid cohesion is formed and maintained by a unique mechanism. In fission yeast, Eso1p, functioning in close coupling to DNA replication, establishes sister chromatid cohesion whereas the newly identified Cohesin-associated protein Pds5p hinders the establishment of cohesion until counteracted by Eso1p, yet stabilizes cohesion once it is established. Eso1p interacts physically with Pds5p via its Ctf7p/Eco1p-homologous domain.

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Introduction

In eukaryotes, all the genetic information packaged in a set of chromosomes is duplicated once in each cell cycle and faithfully inherited by daughter cells. The faultless execution of this process depends not only on the error-free replication of each chromosome during S phase, but also on the correct distribution of replicated chromosomes into each daughter cell at mitosis. The two identical copies of chromosomes (sister chromatids) resulting from their replication in S phase are physically linked together along their entire region until mitosis takes place. This linkage, called ‘sister chromatid cohesion’, is crucial for the correct segregation of chromosomes, because without it, cells could not know either sisters among chromatids or the polarity of the sister chromatid kinetochores constructed on the centromeres (Miyazaki and Orr-Weaver, 1994; T.Tanaka et al., 2000). Consequently, defects in sister chromatid cohesion result in chromosome missegregation and aneuploidy, which may cause cell death and oncogenesis to mitotic cells and developmental anomaly and birth defects to germ cells. Sister chromatid cohesion is also important for efficient homologous recombinational repair of DNA double-strand breaks, because the intact sister chromatid paired to the damaged chromatid serves as the repair template (Kadyk and Hartwell, 1992).

Recent studies have revealed the generality and evolutionary conservation of the basic components that constitute sister chromatid cohesion (Biggins and Murray, 1999; Hirano, 2000; Koshisand and Guacci, 2000; Nasmyth et al., 2000). Both the establishment and maintenance of sister chromatid cohesion depend on a multi-subunit protein complex called ‘Cohesin’. In the budding yeast Saccharomyces cerevisiae, Cohesin contains at least four subunits, Scc1p/Mcd1p (homologous to fission yeast Rad21p), Scc3p/Irr1p, Smc1p and Smc3p (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). Similarly, in Schizosaccharomyces pombe and Xenopus laevis, Cohesin is made of homologues of these proteins (Losada et al., 1998, 2000; Tomonaga et al., 2000). In addition, Pds5p, which is also required for the maintenance of sister chromatid cohesion, genetically and physically interacts with the Cohesin complex (Hartman et al., 2000; Panizza et al., 2000; Sumara et al., 2000). In budding yeast, Cohesin molecules are loaded on chromosomes in late G1 to bind discrete sequences of ~200 bp distributed at ~10 kb intervals along the entire length of a chromosome, but more densely at the centromere region, and they remain bound until metaphase (Blat and Kleckner, 1999; T.Tanaka et al., 1999). The loading of Cohesin onto chromosomes depends on a separate complex that contains Scc2p (homologous to fission yeast Mis4p) and Scc4p (Ciosk et al., 2000). At the metaphase-to-anaphase transition, a ubiquitin-dependent proteolysis involving the anaphase promoting complex/cyclosome (APC/C) activates the ‘Separin’ protease Esp1p (Cut1p in fission yeast) by degrading the ‘Securin’ protein Pds1p (Cut2p in fission yeast), an inhibitor of Separin (Zachariae and Nasmyth, 1999). Activated Separins then cleaves Scc1p/Mcd1p/Rad21p, resulting in the disconnection and separation of sister chromatids (Uhlmann et al., 1999; Tomonaga et al., 2000).

The establishment of sister chromatid cohesion occurs only during DNA replication (Uhlmann and Nasmyth, 1998). A protein called Ctf7p/Eco1p in budding yeast or Eso1p in fission yeast is essential for this process, but not for the maintenance of cohesion during subsequent G2 and M phases (Skibbens et al., 1999; Toth et al., 1999; K.Tanaka et al., 2000). In cells lacking Ctf7p/Eco1p, Cohesin binds to chromosomes normally, but the physical linkages between sister chromatids are never established (Toth et al., 1999).

Fission yeast Eso1 protein is composed of two functionally distinct domains: the N-terminal two-thirds homologous to DNA polymerase η (Pol η) and the C-terminal one-third homologous to budding yeast Ctf7p/ Eco1p (K.Tanaka et al., 2000). The Ctf7p/Eco1p-homologous domain is essential for establishing sister chromatid cohesion.
cohesion whereas the Pol η domain presumably catalyzes translesion DNA synthesis when template DNA contains thymine dimers that block regular DNA replication. In addition, the esol+ gene genetically interacts with the pen1+ (encoding a proliferating cell nuclear antigen, PCNA) and cdc20+ (encoding a catalytic subunit of DNA polymerase ε) genes. Similarly, in budding yeast, temperature sensitivity and chromosome loss of the cif7/eco1 mutant were suppressed by high levels of POL30 (encoding a PCNA), and the cif7/eco1 mutant cells combined with the pol30 mutation or cif18 mutation are synthetically lethal even at the permissive temperature (Skibbens et al., 1999). The product of the CTF18 gene shares significant sequence similarity with Rfc1p and forms a complex with Rfc2p, Rfc3p, Rfc4p and Rfc5p in place of Rfc1p (Hanna et al., 2001; Mayer et al., 2001). This alternative replication factor C (RFC) complex is required for sister chromatid cohesion in budding yeast and has been speculated to be responsible for loading DNA polymerase κ (Trf4p), a novel polymerase required for sister chromatid cohesion (Wang et al., 2000). These findings strongly support the idea that sister chromatid cohesion is established at the replication fork, and the Esolp family members are in close association with the replication machinery to establish linkages between Cohesin-preloaded nascent sister chromatids. However, it is not known how Esol1p/Ctf7p/Eco1p establishes cohesion, and more importantly, it is not known how cohesion is ensured to be established only between sister chromatids and with faithful pairing along their entire region.

We recently obtained a clue to resolve these questions by identifying a Cohesin-associated protein with unexpected properties. Here we report the presence of a unique mechanism for the establishment and maintenance of sister chromatid cohesion in fission yeast.

Results

A newly isolated fission yeast gene encodes a budding yeast Pds5 homologue

A new gene was isolated as a multicopy suppressor of the temperature-sensitive esol-H17 (esol+) mutant, which arrests in M phase with a viability loss due to a failure to establish sister chromatid cohesion in S phase (K.Tanaka et al., 2000). In a search for genes that could effectively rescue this mutant, two non-overlapping plasmid clones, S1 and Bg1, were recovered from Schizosaccharomyces pombe genomic libraries (K.Tanaka et al., 2000; Figure 1A). Further analysis revealed that S1 was esol+ whereas Bg1 was a new gene.

Nucleotide sequencing of both Bg1 and a subsequently isolated corresponding cDNA identified a single open reading frame (ORF) (Figure 1B) that is capable of encoding a putative protein of 1205 amino acids with a calculated molecular mass of 139 kDa and with two potential nuclear localization signals (NLSs). A homology search revealed that the predicted Bg1 gene product was a member of a widely conserved family that includes Pds5p in S.cerevisiae (Hartman et al., 2000; Panizza et al., 2000), Sp076p in Sordaria macrospora (van Heemst et al., 1999), BmDp in Aspergillus nidulans (Denison et al., 1993), AS3p and Pds5p in Homo sapiens (Geck et al., 1999; Sumara et al., 2000) and putative proteins from Arabidopsis thaliana and Drosophila melanogaster. Amino acid identities among these proteins spread along their entire region (20–30% identities), but are significantly higher in the N-terminal region that contains a leucine repeat structure (Geck et al., 1999). In addition, multiple HEAT repeats, which are proposed to serve as a flexible scaffold on which other components can assemble, were contained in these proteins (Neuwald and Hirano, 2000; Panizza et al., 2000). Accordingly, the gene in Bg1 was named pds5+ and characterized further.

Δpds5 cells lose viability during G2 arrest

To understand the physiological role of the pds5+ gene, we constructed cells lacking pds5+ (Δpds5-1), in which the central one-third of the ORF was replaced with the ura4+ gene (Figure 1B). The Δpds5 gene with this deletion and replacement was inactive as pds5+ with no detectable multicopy suppressor activity to the esol+ mutant (data not shown). Diploid cells deleted for one pds5+ allele were induced for sporulation to obtain haploid disruptive spores. Haploid Δpds5-1 cells could germinate and propagate well under all the nutritional conditions tested and at all the temperatures tested between 18 and 36°C. This viable phenotype had not resulted from insufficient deletion of the pds5+ ORF because another deletion mutant (Δpds5-2) lacking >90% of the ORF (Figure 1B).
was also viable and grew normally. Thus, fission yeast Pds5p is not essential for proliferation unlike the BimDp of *A. nidulans* and the Pds5p of *S. cerevisiae* (Denison et al., 1993; Hartman et al., 2000; Panizza et al., 2000). However, the Δpds5 mutants lost the proliferative ability after arrest in G2.

Fission yeast cells arrest in G2 if cultured to saturation in nitrogen-rich medium, such as in MM+YE (Costello et al., 1986). Both wild-type and Δpds5-1 cells were inoculated into this medium, grown to saturation with cell sampling at indicated times, and determined for cell number (Figure 2A, left) and colony-forming ability as viability (Figure 2A, right). The Δpds5-1 cells proliferated at the same rate and to the same density as wild-type cells, retaining a full viability during logarithmic growth. However, upon continuation of culture, they quickly lost their colony-forming ability within 20 h after reaching saturation, whereas wild-type cells retained full viability for >70 h. Under this culture condition, both wild-type and Δpds5-1 cells arrested at G2 and entered stationary phase.
Fig. 3. Sister chromatids prematurely separate in G2-arrested \( \Delta \text{pds5} \) cells. (A) G2-arrested \( \Delta \text{pds5} \) cells are defective in chromosome segregation upon re-entry in the cell cycle. \( \Delta \text{pds5} \) cells were inoculated in MM+YE at a density of 2 \( \times \) 10\(^6\) cells/ml and cultured for 70 h to be allowed to enter stationary phase from G2. Cells were then stimulated to re-enter the cell cycle by re-inoculation into fresh YE medium. Cell aliquots were fixed at indicated times and stained with DAPI. Bar, 10 \( \mu \)m. (B) Sister chromatids are separated in G2-arrested \( \Delta \text{pds5} \) cells. cen1–GFP (referred to as wild-type) and \( \Delta \text{pds5} \) cen1–GFP (referred to as \( \Delta \text{pds5} \)–1) cells were inoculated in MM-S at a density of 2 \( \times \) 10\(^6\) cells/ml. Cells divided once to twice in this medium and mostly arrested in G2 within 5 h. After 20 h incubation, cells were examined for the presence of two split cen1–GFP signals under the fluorescence microscope. Bar, 10 \( \mu \)m. (C) Sister chromatids prematurely separate during G2 arrest induced by Cdc25p inactivation. \( \Delta \text{pds5} \) cen1–GFP (referred to as \( \Delta \text{pds5} \)–2) and \( \Delta \text{pds5} \) cen1–GFP (referred to as \( \Delta \text{pds5} \)–1) cells were grown to mid-log phase in MM at 25°C and then shifted to 36°C, with sampling cells at indicated times to determine cell viability (left) and the percentage of two split cen1–GFP signals (right). For the cen1–GFP examination, cells were fixed with methanol at -80°C. (D) Cut2p is present in G2-arrested \( \Delta \text{pds5} \) cells. cut2HA\(^*\) (referred to as wild type) and cut2HA\(^*\) \( \Delta \text{pds5} \)–1 (referred to as \( \Delta \text{pds5} \)–1) cells were inoculated in MM+YE and cultured. Cells were taken at the times indicated and determined for the level of Cut2p, cell number and viability. Cdc2 and Pef1 kinases (Tanaka and Okayama, 2000) detected by anti-PSTAIR mAb were loading controls.

(Figure 2B). The loss of the colony-forming ability of the G2-arrested \( \Delta \text{pds5} \)–1 cells was fully rescued by chromosomal integration of a single copy of the \( pds5^+ \) gene (Figure 2A, right). The same viability loss was observed with \( \Delta \text{pds5} \)–2 cells, in which >90% of the Pds5p ORF was deleted (Figure 2C, +YE column), indicating that loss of Pds5p function caused the viability loss of \( \Delta \text{pds5} \) cells.

Fission yeast cells can be controlled to enter stationary phase from either G1 or G2 by altering nutrient availability in culture medium (Hao et al., 1997). We used this procedure to examine whether \( \Delta \text{pds5} \) cells lose viability in a cell cycle phase-specific fashion or not. When cultured in MM lacking glucose or sulfate, the majority of \( \Delta \text{pds5} \)–1 cells arrested in G2 (Figure 2B) with loss of colony-forming ability to a degree comparable to that obtained by growth to saturation in MM+YE (Figure 2C). On the other hand, in nitrogen-free MM, the majority of the cells arrested in G1 without a significant viability loss. In plain MM, the cells arrested roughly equally in G1 and G2 with partial alleviation of viability loss. These results indicate that \( pds5^+ \) was required for G2-arrested stationary cells to retain viability.

This requirement, however, was not caused by starvation-induced stress or the peculiarity of the stationary phase entered from G2, and was beheld also during a cell cycle arrest at G2 induced by the inactivation of a key G2–M regulator. At the non-permissive temperature, cells with the thermolabile \( cdc25 \)–22 (\( cdc25^\text{*} \)) mutation arrest at G2 due to the inability to activate Cdc2 kinase (Okayama et al., 1996). When shifted to the non-permissive temperature, \( \Delta \text{pds5} \)–1 \( cdc25^\text{*} \) double mutant cells rapidly lost viability, unlike the original \( cdc25^\text{*} \) cells (Figure 2D). In contrast, further confirming the starvation-arrest results, \( \Delta \text{pds5} \)–1 cells combined with the thermolabile \( cdc10 \)–129 or \( cdc22 \)–M45 mutation that arrest at G1 or early S (Okayama et al., 1996) lost viability no more than the original cdc mutant during incubation at the non-permissive temperature. All the double and original single cdc mutants ceased cell cycling within 6 h after a shift to the non-permissive temperature. These results demonstrate that \( pds5^+ \) is required for G2-arrested cells to retain their ability to proliferate subsequently.

Cells lacking \( pds5^+ \) are defective in chromosome segregation upon re-entry in the cell cycle

To understand the reason for the requirement of \( pds5^+ \) for cell viability during G2 arrest, we examined the nuclear morphology of the \( \Delta \text{pds5} \) cells that were arrested in G2 by growth saturation and then stimulated to resume cell cycling. The G2-arrested \( \Delta \text{pds5} \)–1 cells were small and round, a typical phenotype of stationary phase cells (Figure 3A, left). Upon transfer into fresh medium, they elongated, indicating that they were still alive. Two hours later, a number of the cells entered M phase showing condensed chromosomes (Figure 3A, middle), but eventually arrested in M phase with ‘cut’ (septation in the absence of nuclear division) and mis-segregation phenotypes (Figure 3A, right). Comparable abnormalities were also seen in a similar arrest-resume experiment with \( \Delta \text{pds5} \)–1 \( cdc25^\text{*} \) cells (data not shown). These results indicate that cells lacking Pds5p were viable during G2 arrest, but failed to perform proper chromosome segregation after resumption of cell cycling and that this caused the loss of colony-forming ability.
**Sister chromatids are precociously separated in G₂-arrested Δpds5 cells**

The abnormal chromosome segregation seen with Δpds5 cells was typical of the mutants defective in sister chromatid cohesion such as mis4-242 (mis4*), rad21-K1 (rad21*) and eso1* (Furuya et al., 1998; Tatebayashi et al., 1998; K.Tanaka et al., 2000). We therefore hypothesized that pds5* might be required for maintaining sister chromatid cohesion during G₂ arrest and examined this possibility by performing visualization of the cen1 DNA that tells the status of sister chromatid cohesion. The original cen1–green fluorescent protein (GFP) strain was engineered to contain repeats of the Escherichia coli LacO operator sequence and a transcriptional unit expressing a fusion protein between LacI repressor and GFP with the addition of NLS (GFP–LacI–NLS), both integrated at a locus near the cen1 (Goshima et al., 1999).

The cen1–GFP strains having (referred to as wild type) and lacking pds5* (referred to as Δpds5-1) were grown to confluence in sulfate-free minimal medium (MM-S) to induce stationary phase entry from G₂. In this particular experiment, ~90% of both cells arrested in G₂ and entered stationary phase. Fluorescence microscopic examination revealed that a number of the Δpds5-1 cells had two split cen1–GFP signals (Figure 3B). Among >150 nuclei examined for each strain, only 4% of wild-type nuclei contained two split signals whereas 33% of Δpds5-1 nuclei had two split signals, indicating that sister chromatid cohesion was disrupted in many of the G₂-arrested Δpds5-1 cells. Similar results were obtained with the Δpds5-1 cells arrested in G₂ by growth to confluence in MM4+YE (data not shown).

To investigate whether or not the lack of sister chromatid cohesion in the G₂-arrested Δpds5 cells was a consequence of premature sister chromatid separation, we performed the same analysis with the Δpds5 cells undergoing G₂ arrest upon cdc25* inactivation and examined the time-dependent emergence of two split signals. During proliferation at the permissive temperature, few Δpds5-1 cdc25* cells had two split signals with a frequency comparable to that in cdc25* cells (Figure 3C, right). Upon a shift to 36°C, however, the number of the Δpds5-1 cdc25* cells having two split signals drastically increased with concurrent loss of viability (Figure 3C). In the cdc25* background, the cell’s transition from G₂ to M phase is significantly retarded even at the permissive temperature of 25°C, because of insufficient activity of Cdc25 phosphatase (Hudson et al., 1990). Consequently, cells with this mutation were highly synchronized to the G₂–M transition point during their exponential growth, yet there was no obvious premature sister chromatid separation. These results indicate that Δpds5 cells could properly establish and maintain sister chromatid cohesion during their exponential growth. This was confirmed by virtually no enhanced loss of the minichromosome Ch16 (Niwa et al., 1989) in exponentially growing Δpds5-1 cells, whose accurate segregation requires proper sister chromatid cohesion. During a single generation of sister cells, >99.8% of both Δpds5-1 and Δpds5-2 cells maintained Ch16. We therefore concluded that Δpds5 cells could properly establish sister chromatid cohesion in S phase but could not maintain established cohesion without premature separation during prolonged G₂ arrest.

**Cut2p is present in G₂-arrested Δpds5 cells**

At anaphase, the APC/C-dependent degradation of Cut2p/Securin triggers sister chromatid separation (Yanagida, 2000). Consequently, the premature sister chromatid separation in the G₂-arrested Δpds5 cells could have been caused by premature degradation of Cut2p during G₂ arrest. To examine this possibility, we determined the level of Cut2p in Δpds5 cells during G₂ arrest. To ease detection, we used a strain in which the endogenous cut2* gene was tagged at the C-terminus with a hemagglutinin (HA) epitope (Funabiki et al., 1996). Cut2p slightly decreased during growth to confluence in both cells (Figure 3D), but its amount was comparable between the two at each time point, although Δpds5-1 cells steeply lost viability after reaching stationary phase. These results show that premature activation of the Securin–Sepin cascade was unlikely to be the cause of the chromatid dissociation in the G₂-arrested Δpds5 cells.

**Pds5p is associated with Cohesin**

As expected from the presence of potential NLS and the function indicated by the experimental results, Pds5p tagged with GFP, which was fully functional (data not shown), was localized in the nucleus with intense punctate staining (Figure 4A). Pds5p was present at the same level throughout the cell cycle (Figure 4B), and virtually all cells in non-synchronous culture including binucleate unseptated (G₁), binucleate septated (S) and uninucleate (G₂) cells (Figure 4A), and even those arrested at prometaphase by a β-tubulin mutation (Figure 4C, upper panel) contained punctate Pds5p stains on chromosomes. However, punctate stains appeared to weaken at the metaphase-to-anaphase transition at which chromosome separation takes place (Figure 4C, second panel), but quickly re-intensified at anaphase–telophase (Figure 4C, third and bottom panels). Thus, Pds5p seemed to remain bound to chromosomes throughout the cell cycle except for the metaphase-to-anaphase transition where sister chromatid cohesion was dissociated.

Next we examined whether or not Pds5p colocalizes with Cohesin bound to nuclear chromosomes. The chromosomal pds5* gene was tagged at the C-terminus with a Myc epitope. Similarly, the chromosomal rad21* gene that encodes Cohesin subunit was tagged with an HA epitope (Tomonaga et al., 2000). Both constructs were confirmed to be fully functional (data not shown). Logarithmically growing cells having pds5Myc* and rad21HAp* were fixed, and Pds5Mycp and Rad21HAp were detected by indirect immunofluorescence analysis. As shown in Figure 4D, both Pds5p and Rad21p displayed punctate stains in the nuclear chromatin region, and in most cases, their signals were perfectly superimposed on each other, showing that Pds5p and Cohesin colocalized on chromatin as spots. This was confirmed by the following chromatin immunoprecipitation (CHIP) assay with various DNA probes. Exponentially growing rad21GFP* or pds5GFP* cells were fixed for crosslinking of DNA and proteins, and then chromatin-containing cell extracts were immunoprecipitated with anti-GFP antibody. DNAs coprecipitated with Rad21GFPp or Pds5GFPp were amplified by the quantitative PCR method using primer sets whose chromosomal locations are illustrated at the top of Figure 4E. The distribution pattern
of Pds5p and Rad21p bound to each chromosome region was very similar; both Pds5p and Rad21p abundantly bound to the centromere, but most abundantly to repetitive outer centromere regions (Figure 4E) (Tomonaga et al., 2000; Watanabe et al., 2001).

The formation of Pds5p spots on chromatin absolutely depended on the presence of intact Cohesin. Inactivation of thermolabile Rad21p leads to precocious sister chromatid separation, which brings metaphase arrest to cells due to the activation of spindle assembly checkpoint (Tomonaga et al., 2000). Similarly, inactivation of thermolabile Eso1p leads to a failure of establishing sister chromatid cohesion, which similarly brings metaphase arrest to cells due to the activation of the same checkpoint (K.Tanaka et al., 2000). Under these metaphase arrest conditions, we examined chromatin localization of Pds5p. As shown in Figure 4F, unlike the cells lacking Eso1p, those with inactive Rad21p lost Pds5p spots with concomitant homogeneous staining of the nucleus. Furthermore, the number of Pds5p spots was reduced even in rad21<sup>ts</sup> cells proliferating at the permissive temperature (Figure 4G). Consistently, the CHIP assay...
revealed that in rad21 cells the amount of centromere-bound Pds5p was reduced significantly at the permissive temperature and to almost none over the background at the restrictive temperature (Figure 4H). In contrast, localization of Rad21p on nuclear chromatin did not depend on Pds5p. The number and intensity of Rad21p spots, and the amount of coprecipitated DNA with Rad21p were similar between wild-type and Pds5-1 cells (Figure 4G and I).

The Rad21p-dependent colocalization of Pds5p and Rad21p on chromatin and the requirement of Pds5p for the maintenance of sister chromatid cohesion suggest that Pds5p is a Cohesin-associated protein. To investigate this possibility, we performed immunoprecipitation analysis. Logarithmically growing cells containing both pds5Myc+ and rad21HA+ or only pds5Myc+ were lysed and immunoprecipitated for Rad21HAp with an anti-HA antibody. Pds5Myc coprecipitated with Rad21HAp only from the cell lysate containing both pds5Myc+ and rad21HA+ (Figure 4J). In reversed immunoprecipitation, Rad21HAp coprecipitated with Pds5Mycp (data not shown). In this particular experiment, the amount of coprecipitated Pds5p was estimated to be a few percent of total Pds5p in the cells, suggesting that only a small fraction of Pds5p molecules were bound to Rad21p at least in the soluble fraction.

Taken together, these results suggest that Pds5p is physically associated with, or could be an integral component of, Cohesin, although this factor is dispensable for the formation of the Cohesin complex, its loading on chromatin and the establishment of cohesion as already shown.

**pds5+ gene genetically interacts with rad21+ and mis4+**

Supporting the physical interaction, pds5+ showed a genetic interaction with rad21+ and mis4+, the latter encoding a fission yeast homologue of ScScc2p required for Cohesin loading on chromosome (Furuya et al., 1998).

Δpds5 cells were synthetically lethal with the rad21+ mutation even at the permissive temperature. Tetrads dissection of the spores obtained by crossing between Δpds5-1 and rad21 cells yielded no viable double mutant cells even at 23°C. Moreover, the Δpds5-1 Δrad21 cells rescued by the pREP81-rad21+ plasmid could not grow when rad21+ expression in the plasmid was repressed by thiamine addition (Figure 4K). On the other hand, deletion of pds5+ reduced the restriction temperature of mis4+ cells by 3–4°C (data not shown), in which the loading of Cohesin onto chromatin is partially defective (Tomonaga et al., 2000).

**Loss of Pds5p negates Eso1p’s requirement for cohesion establishment**

Eso1p is absolutely essential for the establishment of cohesion (K. Tanaka et al., 2000). But we found that Eso1p became totally dispensable for cohesion establishment when Pds5p lacked. Cells deleted for eso1+ are lethal at any temperature, and tetrads dissection of spores from diploid cells with disruption of one eso1+ allele by ura4+ yields only two uracil-protoprototrophic viable progenies (Figure 5A, left). However, if one pds5+ allele was disrupted simultaneously in this diploid strain, the two-viable:two-lethal segregation was degraded, and more than two progenies came to form colonies (Figure 5A, right). Structural analysis of the disrupted loci revealed that these new colonies were Δpds5-1 Δeso1 segregants, which could propagate even at 36°C (Figure 5B). Δpds5-2 deletion also rescued Δeso1 cells. Moreover, original eso1+ cells were rescued to be able to proliferate at 36°C by deletion of pds5+, but this suppression was completely abolished by chromosomal integration of a single copy of the pds5+ gene (Figure 5B).

During logarithmic growth, Δpds5-1 Δeso1 cells proliferated as rapidly as wild-type and Δpds5-1 cells (Figure 5C, left), being fully viable (Figure 5C, right) with a normal nuclear structure and performing proper

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**Fig. 4.** Pds5p is closely associated with Cohesin. (A) Pds5p is localized as punctate stains in the nucleus throughout the cell cycle. pds5GFP+ cells were grown to mid-log phase in MM and directly stained with HOE to visualize DNA. Bar, 10 μm. (B) Pds5p is constitutively present throughout the cell cycle. The cd25p pds5Myc+ cells were synchronized at late G2 by temperature shift-up and shift-down. Their cell cycle progression was monitored for two generations. Cells were taken at indicated times, and determined for the septation index and the level of Pds5p. α-tubulin was used as a loading control. (C) Punctate Pds5p signals are seen on M phase chromosomes, but not on those at the metaphase-to-anaphase transition. nda3-KM311 pds5GFP+ cells were grown to mid-log phase in MM at 34°C, and then shifted to the non-permissive temperature of 18°C for 8 h to induce prometaphase arrest. Cells were then released from the arrest by a shift back to the permissive temperature and directly stained with HOE to visualize DNA. Images of four independent cells corresponding to metaphase, early anaphase, late anaphase and telophase are shown. Bar, 10 μm. (D) Pds5p colocalizes with Rad21p. Rad21p and Rad21p were detected by indirect immunofluorescence analysis. DNA was stained with DAPI (referred to as DNA). The Pds5p and Rad21p images were merged in the bottom panel (referred to as Merge). Images of two independent cells are shown. Bar, 5 μm. (E) Association of Pds5p with the centromere regions. Exponentially growing rad21GFP+ and pds5GFP+ cells in MM were subjected to CHIP analysis. Representative PCR’s of whole-cell extracts (WCE) and immunoprecipitates (IP) are shown. The percentage of immunoprecipitation (IP%) is indicated on the bottom graph. Schematic representation of chromosome I and the primers used are shown at the top. (F) Punctate nuclear localization of Pds5p depends on intact Rad21p. rad21Δ pds5GFP+ and eso1Δ pds5GFP+ cells were grown to mid-log phase in MM at 25°C, then cultured at 34°C for 3.5 h and examined by fluorescent microscopy followed by direct staining with HOE to visualize DNA. Bar, 10 μm. (G) Punctate nuclear localization of Rad21p is independent of Pds5p. pds5GFP+ (referred to as wild type) and rad21Δ pds5GFP+ (referred to as rad21Δ) cells, or rad21GFP+ (referred to as wild type) and Δpds5-1 rad21GFP+ (referred to as Δpds5-1) cells were grown to mid-log phase in MM at 25°C and directly examined by fluorescent microscopy, Bar, 10 μm. (H) Chromatin localization of Pds5p depends on intact Rad21p. Negative control without GFP tagging gene (referred to as no tag), pds5GFP+ (referred to as wild type), rad21Δ pds5GFP+ (referred to as rad21Δ) and eso1Δ pds5GFP+ (referred to as eso1Δ) cells grown at 25°C or cultured at 34°C for 3.5 h were subjected to CHIP analysis. (I) Chromatin localization of Rad21p is independent of Pds5p. Negative control without GFP tagging gene (referred to as no tag), rad21GFP+ (referred to as wild type) and Δpds5-1 rad21GFP+ (referred to as Δpds5-1) cells grown at 25°C were subjected to CHIP analysis. (J) Pds5p associates with Rad21p. Extracts of pds5GFP+ Δpds5-1 Δaso1+ cells were immunoprecipitated with an anti-HA antibody, and the original cell extract and precipitates (from 15 times more extracts) were analyzed by western blotting. Pds5p and Rad21p were detected with anti-Myc and anti-HA antibodies, respectively. (K) Cells lacking Pds5p are synthetically lethal with the rad21Δ mutation. The rad21Δ, rad21Δ eso1Δ and rad21Δ Δpds5-1 cells transfected with pREP81-rad21+ were inoculated on both an MM plate (+thiamine) and a plate containing 10 μM thiamine (+thiamine) and incubated for 6 days at 23°C. The rad21Δ eso1Δ cells are a positive control for synthetic lethality (K. Tanaka et al., 2000).
Fig. 5. Eso1p is dispensable for cohesion establishment in the absence of Pds5p. (A) Deletion of pds5Δ suppresses the lethality of Δesol cells. Spores from ΔesolΔesol·Δpds5Δ/pds5Δ diploid cells (left) or Δesol·Δpds5·Δpds5Δ diploid cells (right) were tetrad-dissected on YEPD plates and incubated at 30°C for 4 days. (B) Deletion of pds5Δ allows Δesol cells to grow at 36°C as well as fully suppressing the temperature-sensitive lethality of the eso1Δ cells. Approximately 10⁴, 10⁵, 10⁶ and 10 wild-type, Δpds5-1, eso1Δ, Δpds5-1 eso1Δ, Δpds5-1 eso1ΔΔpds5-1 (Δpds5-1 eso1Δ integrated with the pds5Δ gene), Δpds5-1 Δesol, Δpds5-2, Δpds5-2 Δesol and Δpds5-2 Δesol cells were spotted on two YEPD plates and incubated at 25 and 36°C, respectively. The Δpds5-1 eso1ΔΔpds5-1 strain generated a few colonies that could grow even at 36°C. They were ‘pop out’ revertants in which the integrated pds5Δ gene was spontaneously lost from chromosome by homologous recombination. (C) Δpds5·Δesol cells are phenotypically indistinguishable from Δpds5Δ cells. Logarithmically growing wild-type, Δpds5-1 Δesol cells were cultured in MM2-EY. Cells were taken at the times indicated and determined for cell number (left) and viability (right). (D) Δpds5·Δesol cells perform proper chromosome segregation. Wild-type, Δpds5-1 and Δpds5-1 Δesol cells were grown to mid-log phase in YE medium, fixed and stained with DAPI. Bar, 10 μm. (E) Sister chromatid cohesion is established in Δpds5·Δesol cells. cen1·GFP (referred to as wild type), eso1Δ cen1·GFP (referred to as eso1Δ), Δpds5-1 cen1·GFP (referred to as Δpds5-1) and Δpds5-1 Δesol cen1·GFP (referred to as Δpds5-1 Δesol) cells were grown to mid-log phase at 25°C in MM and examined directly for the presence of two split cen1·GFP signals. The eso1Δ cen1·GFP cells cultured at 34°C for 3 h were used as a control for unestablished sister chromatid cohesion.

Fig. 6. Δpds5 cells are sensitive to DNA damage. (A) Δpds5 cells are sensitive to MMS but not to bleomycin. Approximately 10⁴, 10⁵, 10⁶ and 10 mis4Δ, rad21Δ, wild-type, eso1Δ, Δpds5-1 and Δpds5-1 Δesol cells were spotted on YEP plates containing none (referred to as control), bleomycin [0.000025% (v/v)] or MMS [0.004% (v/v)] and incubated at 27°C for 3 days (control) or 4 days (others). (B) Δpds5 cells are sensitive to UV irradiation. Wild-type, eso1Δ, Δpds5-1, Δpds5-1 eso1Δ and Δpds5-1 Δesol cells were plated on YES, irradiated with various doses of UV and incubated at 27°C for 7 days.

chromosome segregation (Figure 5D). These phenotypic features suggest that loss of Pds5p restored to Δesol cells the ability to establish sister chromatid cohesion at a level high enough for the majority to perform proper chromosome segregation. This was confirmed by visualization of the cen1 locus with the cen1-GFP system. The frequency of Δpds5-1 Δesol cells with two split cen1 signals was comparable to that of Δpds5-1 cells and permissive temperature-cultured eso1Δ cells, although slightly higher than that of wild-type cells (Figure 5E). These results indicate that in the absence of Pds5p sister chromatid cohesion seemed to be established properly without requirement for Eso1p.

Eso1p executes function via Pds5p

The negation of Eso1p’s requirement for cohesion establishment by loss of Pds5p suggests that Eso1p may execute function via Pds5p. The striking phenotypic similarity between Δpds5·Δesol cells and Δpds5 cells supported this possibility. Both cells were indistinguishable in the doubling time during logarithmic growth (Figure 5C, left), the profile of time-dependent viability loss during stationary arrest (Figure 5C, right), the frequency of cells with two split cen1Δs during stationary arrest (wild type, 8%; Δpds5-1, 49%; Δpds5-1 Δesol, 47%), nuclear morphology during proliferation (Figure 5D), the frequency of cells with two split cen1Δs during proliferation (Figure 5E) and the DNA damage sensitivity (Figure 6).

The efficient execution of the homologous recombinational repair system requires proper sister chromatid cohesion. Therefore, hypersensitivity to DNA double-strand breaks (DSBs) is a common feature shared by the mutants defective in sister chromatid cohesion, such as rad21Δ, mis4Δ and eso1Δ (Furuya et al., 1998; Tatebayashi et al., 1998; K.Tanaka et al., 2000; Figure 6A). However, Δpds5-1 cells were nearly as resistant as wild-type cells to
bleomycin, a strong DSB-inducing chemical (Figure 6A), providing further evidence for properly established cohesion in this mutant, but were mildly sensitive to methyl methanesulfonate (MMS) (Figure 6A) and UV (Figure 6B). The reason for the elevated sensitivity to the latter two agents is unknown, but it does not involve cell cycle checkpoint defects (data not shown). Pds5p might be required for the efficient repair of base modifications induced by MMS and UV. Nevertheless, Δpds5-1 Δeso1 cells were indistinguishable from Δpds5-1 cells in sensitivities to bleomycin and MMS. In addition, Δpds5-1 eso1Δ cells were similar to Δpds5-1 cells in UV sensitivity (Figure 6B). On the other hand, Δpds5-1 Δeso1 double disruptant cells were more sensitive to UV than Δpds5-1 eso1Δ or Δpds5-1 cells, as expected from the deletion of the Pol η domain in this Δeso1 construct. Taken together, these results suggest that Pds5p is likely to be a critical target for Eso1p to establish cohesion.

**Eso1p interacts physically with Pds5p**

Consistent with the functional relationship between Pds5p and Eso1p, physical interaction of Pds5p with Eso1p was detected by the yeast two-hybrid assay, but not by immunoprecipitation. Pds5p and Eso1p were fused to the Gal4p DNA-binding domain (BD) and Gal4p transcriptional activator domain (AD) in two combinations. Pairwise combinations of the BD and AD protein fusions were then tested for their ability to interact with each other by induction of β-galactosidase activity. A significant interaction between Pds5p and Eso1p was observed in both combinations (Figure 7B). Similar or stronger signals were obtained with both combinations of the N-terminally truncated, but not C-terminally truncated version of Eso1p with Pds5p, indicating that Eso1p interacts with Pds5p through the Ctf7p/Eco1p-homologous domain, which is essential for cohesion establishment.

A key question is whether this interaction is relevant to the functional relationship between Eso1p and Pds5p. We addressed this question by performing the same two-hybrid analysis with the temperature-sensitive eso1-1 allele. As shown in Figure 7C, thermolabile Eso1p interacted with Pds5p at 25 and 27°C as efficiently as wild-type Eso1p, but at semi- and non-permissive temperatures of 30 and 32°C, unlike for the wild-type Eso1p combination, their interaction was greatly reduced. A similar result was obtained with the other fusion combination (data not shown). These results indicate that the physical interaction of Eso1p with Pds5p is essential for Eso1p to establish cohesion.

**Discussion**

**Pds5p is an integral component of Cohesin**

Sister chromatid cohesion, which is essential for faithful transmission of chromosomes from mother to daughter cells, is formed by linking sister chromatids paired along their entire region with molecules of the multi-protein complex called Cohesin. All the experimental data we present indicate that the newly identified fission yeast Pds5p is likely to be an integral component of Cohesin that plays a critical role in the establishment and maintenance of sister chromatid cohesion. Both Pds5p and Rad21p co-immunoprecipitated, colocalized in the nucleus as dots and bound to the centromeres regions in the same distribution pattern. Moreover, punctate localization of Pds5p depended on intact Rad21p. At the metaphase-to-anaphase transition where Rad21p was cleaved (although only a small fraction; Tomonaga et al., 2000), or at the metaphase arrest induced by inactivation of thermolabile Rad21p, Pds5p dots disappeared with their concomitant dispersion in the nucleus. In contrast, punctate localization of Rad21p on chromosomes was independent of Pds5p, indicating that the formation and subsequent chromatin loading of the Cohesin complex do not require Pds5p, which is consistent with the elucidated biological functions of the Cohesin complex and Pds5p as discussed below. Furthermore, supporting their physical relationship, cells lacking Pds5p were synthetically lethal with thermolabile Rad21p at permissive temperatures.

Like other Cohesin-related factors, other eukaryotes contain Pds5p homologues that appear to perform a similar molecular function. Budding yeast Pds5p colocalizes with Sic1p/Mcd1p on chromatin in an Sic1p/Mcd1p-dependent manner, just like fission yeast Pds5p in a
Rad21p-dependent manner, and is required for both sister chromatid cohesion and condensation (Hartman et al., 2000; Panizza et al., 2000). Spo70p in S. macrospora and BimDp in A. nidulans localize to mitotic and meiotic chromosomes except at metaphase and anaphase, and are required for several aspects of chromosome morphology, including sister chromatid cohesiveness and chromat condensation (van Heemst et al., 1999, 2001). Moreover, the conditional lethal phenotype conferred by the bim1D mutation can be suppressed by a mutation in the SUDA gene encoding the Cohesin subunit Smc3p (Holt and May, 1996). This genetic interaction suggests the possible physical interaction between the Pds5p homologue and also Cohesin in this organism. Consistently, it was reported that human and Xenopus homologues of Pds5p are associated with 14S Cohesin complexes (Sumara et al., 2000).

**Pds5p plays dual roles in the establishment and maintenance of sister chromatid cohesion**

Our experimental data show that Pds5p plays critical roles in both the establishment and the maintenance of sister chromatid cohesion. The genetic analyses with gene disruptants demonstrate that Pds5p strongly hinders the formation of sister chromatid cohesion until Esolp counteracts its hindrance. Consequently, loss of Pds5p totally negated the requirement for Esolp in establishing cohesion. In addition, Pds5p is essential for stably maintaining established cohesion during elongated G2 arrest, irrespective of whether it is induced by cell cycle block at G2 or stationary phase entry from G2.

**Eso1p establishes cohesion by suppressing the Pds5p-imposed inhibition of cohesion formation**

Eso1p contains a Pol η domain fused with a Ctt7p/Eco1p-homologous domain, the latter of which is essential for establishing sister chromatid cohesion during S phase, which seems to be performed in a replication-coupled fashion (K. Tanaka et al., 2000). One critical question is: how does Eso1p establish cohesion? As presented, Pds5p is a newly identified Cohesin subunit that colocalizes with at least Rad21p on chromatid throughout the cell cycle except for the metaphase-to-anaphase transition and plays a critical role in the maintenance of established cohesion. Eso1p interacts physically with Pds5p in a perfect correlation with its ability to establish cohesion, but it is no longer required for cohesion establishment in the absence of Pds5p. These results indicate that Pds5p seemingly constitutively bound to Cohesin hinders the formation of cohesion until counteracted by Eso1p via a physical interaction through its Ctt7p/Eco1p domain, but functions to stabilize cohesion once it is formed.

The amount of Pds5p relative to Cohesin seems to be extremely important for this novel factor to hinder the Eso1p-dependent formation of cohesion. As already discussed, loss of Pds5p totally negated the Eso1p dependency. Moreover, when the gene dosage of pds5p was cut in half in diploid cells by deleting one of its alleles, Eso1p dependency of the formation of sister chromatid cohesion was partially suppressed (K. Tanaka, unpublished data). However, contrary to our expectation, not only loss but also overexpression of Pds5p negated the Eso1p dependency and could suppress the lethality of Δeso1 cells (K. Tanaka, unpublished data), implying that overexpression produces the same effect as no expression. We believe that this effect was the basis for the isolation of the pds5+ gene as a multicopy suppressor of the eso1Δ mutant, but its molecular mechanism is unknown, although this might be caused by interference of the proper association of Pds5p with Cohesins by its overproduced molecules themselves.

The biological meanings of the establishment and maintenance of sister chromatid cohesion by the mechanism involving the unexpected relation between Pds5p and Eso1p are unknown. But, one benefit by having such a mechanism would be the prevention of undesired non-sister chromatid or inter-chromatid cohesion while ensuring the formation of cohesion between nascent sister chromatids with faithful pairing along their entire region.

**Materials and methods**

**Strains and growth conditions**

Double mutants were obtained by crossing between appropriate single mutants followed by tetrad analysis and confirmed by checking genetic markers and/or by PCR analysis. The cut2HA+, rad21HA+ and rad21GFPp strains were described previously (Funabiki et al., 1996; Tomonaga et al., 2000). The pds5GFPp and pds5MYCp strains, in which the chromosomal pds5+ gene was tagged at the C-terminus with a GFP and a 13× Myc epitope, respectively, and expressed by their native promoter, were constructed as described previously (Babler et al., 1998). The cen1–GFP system to visualize the in vivo status of sister chromatids was described previously (Goshima et al., 1999), and cells harboring cen1–GFP were maintained in the presence of 10 μM thiamine.

Cells were cultured in the complete yeast extract medium (YE), or in the minimal medium (MM, also called EMM2 or PM) (Alfa et al., 1993). Media containing 1.6% agar (w/v) were used for plating. When necessary, MM was modified by reducing the concentrations of carbon (glucose, referred to as C) or sulfate (sodium sulfate, manganese sulfate, zinc sulfate and copper sulfate, referred to as S) or nitrogen (ammonium chloride, referred to as N). In low-glucose MM (MM-C), glucose concentration was reduced to 0.1% (w/v) [2% (w/v) in normal MM]. In sulfate-free MM (MM-S), the sulfate compounds were replaced with the chloride compounds, and in nitrogen-free MM (MM-N), ammonium chloride was omitted. In MM containing YE (MM+YE), yeast extract was supplemented at a concentration of 0.5% (w/v). Cells were cultured at 30°C unless specified.

**Plasmids and libraries**

The pALSK+, pAL-esol+ (K. Tanaka et al., 2000), pREP11-rad21+ (Tatebayashi et al., 1998) and pK1 (Tanaka and Okayama, 2000) plasmids were described previously. The S. pombe genomic library was constructed by inserting BgIII-digested wild-type (1.972) genomic DNA into the BamHI-digested pALSK+ vector (kindly provided by K. Okazaki). The S. pombe cDNA library has been described previously (H. Tanaka et al., 1999).

**Gene disruption and chromosomal integration of pds5+**

In the pds5Δ-1 construct, the 1.3 kb BamHI–NheI fragment of the pds5+ gene that contains ~36% of the ORF was replaced with the 1.8 kb HindIII fragment of the ura4+ gene. Similarly, the 3.4 kb HpaI–XbaI fragment that contains ~95% of the ORF was replaced with the ura4+ in the pds5Δ-2 construct. The PstI–XbaI fragment carrying the pds5Δ-1 construct or the BamII–BamHI fragment carrying the pds5Δ-2 construct was used to transform the ura4-D18 diploid strain and stable ura4+ transformants were isolated. The correct replacement of one wild-type allele with pds5Δ::ura4+ was confirmed by Southern blot analysis.

For chromosomal integration, a 6.5 kb BglII fragment containing the pds5+ gene was cloned into the pK1 vector. This plasmid was digested at the unique Scal site located in the 5′-upstream region of the pds5+ ORF and transfected into the pds5Δ-1 or pds5Δ-1 eso1Δ+ strains at 25°C. Proper integrants were identified as described previously (Tanaka and Okayama, 2000).
Minichromosome loss assay

The rate of chromosome loss was investigated by using the nonessential 530 kb minichromosome Ch16 (Niwa et al., 1989). Ch16 carries the ade6-M216 allele that complements the ade6-M210 allele. Logarithmically growing ade6-M210 and Δpds5 ade6-M210 cells having Ch16 in adenine minus MM were inoculated into the YE medium supplemented with adenine and propagated for about nine generations. Cells were then plated on low-adenine YE plates. The cells stably maintaining Ch16 form white colonies, whereas those that had lost Ch16 make red colonies. The rates of Ch16 loss were estimated by the frequencies of the emergence of red colonies.

Protein preparation and western blotting

Total boiled protein extracts were prepared as follows. Cells (2 × 10^6) were washed in ice-cold Stop buffer, resuspended in 20 μl of HB (Moreno et al., 1991), and boiled for 5 min. Approximately 0.5 ml of chilled glass beads (~500 μm in diameter) were added and cells were broken by vigorous vortexing at 4°C for 5 min. Forty microliters of HB and 60 μl of 2× denature buffer (100 mM Tris–HCl pH 6.8, 6% SDS) were then added and reboiled for 5 min. A total of 50 μg of protein from each sample were electrophoresed in an SDS–polyacrylamide gel and immunoblotted with anti-HA (12CA5; Boehringer Mannheim), anti-PSTAIR (Yamashita et al., 1991), anti-Myc (9E10; Boehringer Mannheim) or the anti-α-tubulin (Sigma T5168) mouse monoclonal antibodies (mAbs).

Cell extracts for immunoprecipitation were prepared as described previously (Tanaka and Okayama, 2000) by using the HB supplemented with 300 mM NaCl. Immunoprecipitation was performed in the HB+150 mM NaCl with anti-HA rat mAb (3F10; Boehringer Mannheim). Precipitates were electrophoresed in SDS–polyacrylamide gels and immunoblotted with anti-HA (12CA5) or anti-Myc (9E10) mouse mAbs.

Indirect immunofluorescence analysis

For indirect immunofluorescence analysis, logarithmically growing pds5pΔ pdr21HA cells in MM were fixed with formaldehyde and glutaraldehyde for 1 h. Cells were further processed for immunofluorescence as described (Alfa et al., 1993). Pds5Mycp was detected by immunoreactions with anti-Myc mouse mAb (9E10) and subsequently with anti-mouse Cy3-conjugated goat antibody (Amersham Pharmacia Biotech). Rad21HAp was detected similarly with anti-HA rat mAb (3F10) and anti-rat Alexa488-conjugated goat antibody (Molecular Probes). DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were examined under the fluorescence microscope (Olympus AX70), and images were captured with a CCD camera (SenSys; Photometrics).

CHIP assay

CHIP assay was performed as described previously (Saitoh et al., 1997). Rad21p and Pdr2p were immunoprecipitated with anti-GFP rabbit antibody (Clontech). Fission yeast centromeres were organized into two distinct domains: outer regions consisting of highly repeated sequences and an inner region consisting of unique sequences (Takahashi et al., 1992). The cni and imr sequences are the probes for the inner region, and the dg and dh sequences are those for the outer region, respectively. In addition, lys1 and sod2 were used as probes in the pericentromeric and peritelomeric arms, respectively (Watanabe et al., 2001). The linear range of PCR was determined by serial dilutions of template DNA and all assays were performed within this range. PCR products were separated by 2% agarose gel electrophoresis, captured with a CCD camera and quantified with the NIH Image software.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed using the Matchmaker Two-Hybrid system (Clontech). Full-length pds5p, esol1, esol7 HA1 or the Ph domain of esol1 (amino acids 1–588) and Ctf7p/Eco1p-homologous domain of esol1 (amino acids 532–872) were fused to the DNA-binding domain (pGBT9) or the transcriptional activator domain (pGAD424) of Gal4p. These plasmids were co-transfected into the S. cerevisiae reporter strain SFY526 and β-galactosidase activity was assayed with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as substrate.

Other techniques

Flow cytometry, cell number counting and UV irradiation were performed as described previously (K.Tanaka et al., 1992, 2000). DAPI staining was carried out as described previously (Adachi and Yanagida, 1989) (stationary phase cells were fixed for up to 2 h). Hoechst 33342 (Hoe) was added to a final concentration of 50 μg/ml in the culture medium immediately before examination. Cell cycle synchronization was performed by the cdc25 mutant block and release method as described previously (Alfa et al., 1993).

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