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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.

Keywords: Cohesin/Eso1p/fission yeast/Pds5p/sister chromatid cohesion

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; Koshland 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 G_1 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 Separin 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 G_2 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 whereas the Pol η domain presumably catalyzes translesion DNA synthesis when template DNA contains thymine dimers that block regular DNA replication. In addition, the *eso1*$^+$ gene genetically interacts with the *pcn1*$^+$ (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 *ctf7/eco1* mutant were suppressed by high levels of *POL30* (encoding a PCNA), and the *ctf7/eco1* mutant cells combined with the *pol30* mutation or *ctf18* 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 Rcf1p (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 Eso1p family functions in close association with the replication machinery to establish linkages between Cohesin-preloaded nascent sister chromatids. However, it is not known how Eso1p/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 *eso1-H17* (*eso1*^{ts}) 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 *eso1*$^+$ 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), Spo76p in *Sordaria macrospora* (van Heemst *et al*., 1999), BimDp in *Aspergillus nidulans* (Denison *et al*., 1993), AS3p and Pds5p in *Homo sapiens* (Geck *et al*., 1999;

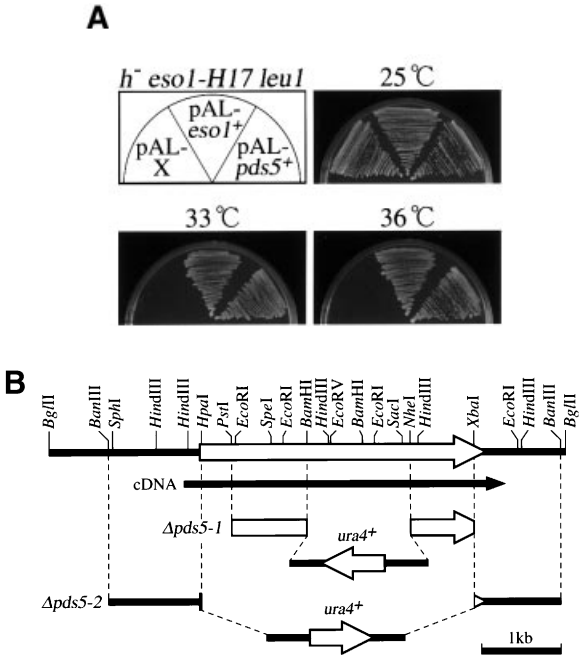


Fig. 1. Isolation of the *pds5*$^+$ gene. (**A**) Suppression of *eso1*^{ts} mutant by the *pds5*$^+$ gene. The *eso1*^{ts} cells stably transfected with the indicated plasmids were incubated on MM plates at the indicated temperatures. pAL-*pds5*$^+$ contains an insert of the initially isolated 6.5 kb *Bgl*II fragment. pAL-X is the pALSK$^+$ vector with no insert and used as a negative control. (**B**) Restriction map of the *pds5*$^+$ gene. The white and black arrows indicate the direction and extent of the *pds5*$^+$ ORF and *pds5*$^+$ cDNA, respectively. The structures of the *Pst*I–*Xba*I (*Δpds5-1*) and the *Ban*III–*Ban*III (*Δpds5-2*) fragments used for the disruption of *pds5*$^+$ gene are also shown. The *pds5*$^+$ sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession No. AB067651.

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 G_2 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 *Δpsd5* gene with this deletion and replacement was inactive as *pds5*$^+$ with no detectable multicopy suppressor activity to the *eso1*^{ts} mutant (data not shown). Diploid cells deleted for one *pds5*$^+$ allele were induced for sporulation to obtain haploid disruptant 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)

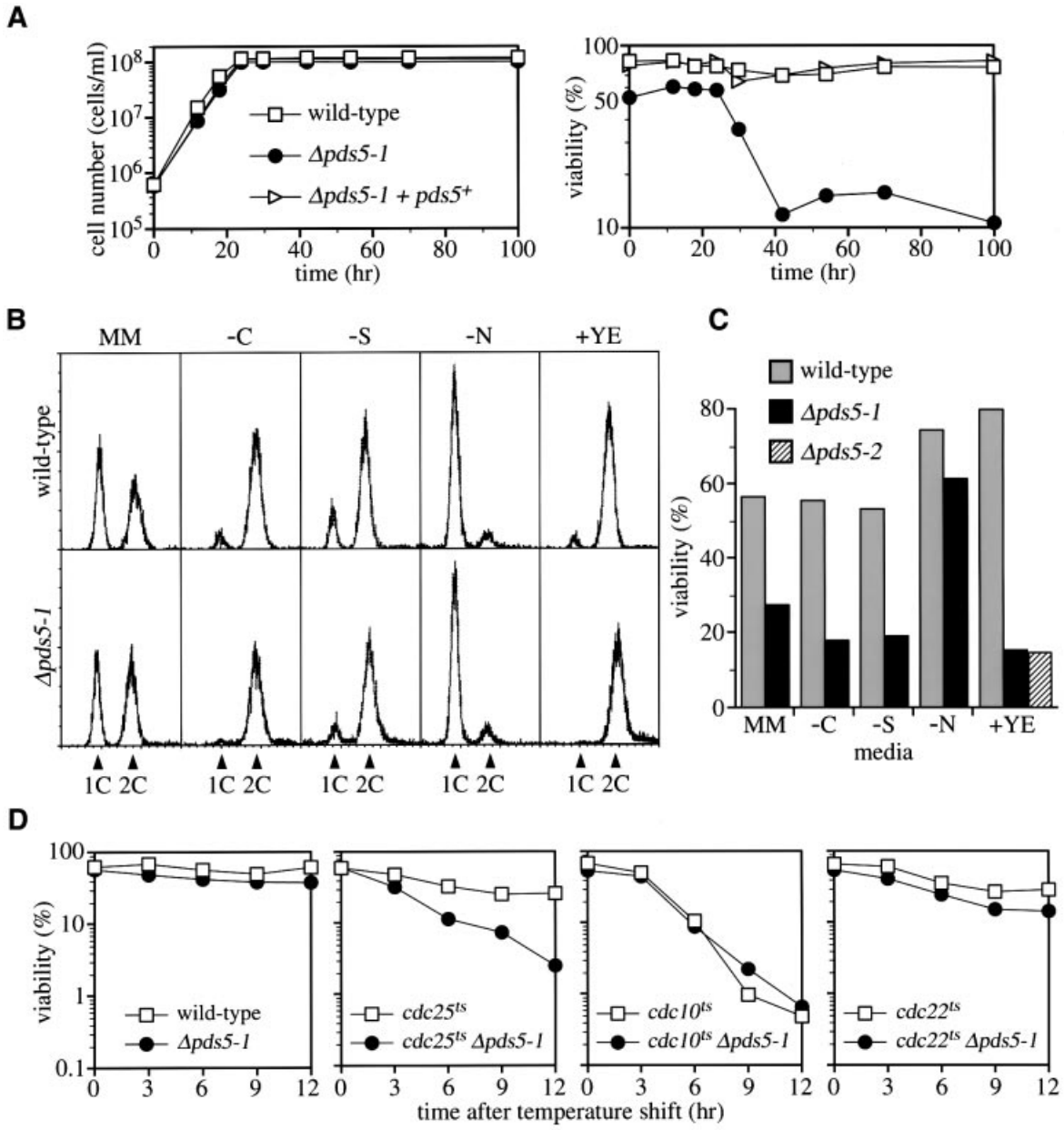


Fig. 2. *Δpds5* cells lose colony-forming ability during G_2 arrest. (**A**) *Δpds5* cells lose colony-forming ability (referred to as viability) after entry into stationary phase. Logarithmically proliferating wild-type, *Δpds5-1* and *Δpds5-1 + pds5*$^+$ (*Δpds5-1* with *pds5*$^+$ integration) cells were cultured to confluence in MM+YE. Cell aliquots were taken at the times indicated and determined for their cell number (left) and viability (right). (B and C) *Δpds5* cells lose viability during stationary phase entry only from G_2. Wild-type and *Δpds5-1* cells were grown to mid-log phase in MM and inoculated in plain MM (MM), MM-C (–C), MM-S (–S), MM-N (–N) or MM+YE (+YE) at a density of 2×10^6 cells/ml. Both wild-type and *Δpds5* cells reached confluence within 15 h. After 55 h incubation, cell aliquots were taken and determined for the cell cycle profile (**B**) and viability (**C**). (**D**) *Δpds5* cells also lose viability during cell cycle arrest at G_2. Wild-type and *Δpds5-1* cells (left), *cdc25*^{ts} and *cdc25*^{ts} *Δpds5-1* cells (second), *cdc10*^{ts} and *cdc10*^{ts} *Δpds5-1* cells (third), or *cdc22*^{ts} and *cdc22*^{ts} *Δpds5-1* cells (right) were grown at 25°C to mid-log phase in MM and then cultured at 36°C. Cells were sampled at indicated times and determined for viability.

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 G_2.

Fission yeast cells arrest in G_2 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 G_2 and entered stationary phase

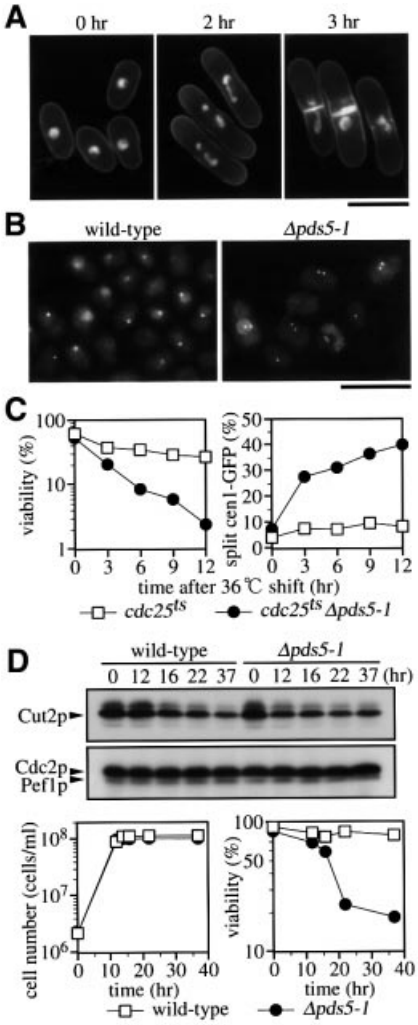


Fig. 3. Sister chromatids prematurely separate in G_2-arrested *Δpds5* cells. (**A**) G_2-arrested *Δpds5* cells are defective in chromosome segregation upon re-entry in the cell cycle. *Δpds5-1* cells were inoculated in MM+YE at a density of 2×10^6 cells/ml and cultured for 70 h to be allowed to enter stationary phase from G_2. 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 μm. (**B**) Sister chromatids are separated in G_2-arrested *Δpds5* cells. cen1–GFP (referred to as wild-type) and *Δpds5-1* cen1–GFP (referred to as *Δpds5-1*) cells were inoculated in MM-S at a density of 2×10^6 cells/ml. Cells divided once to twice in this medium and mostly arrested in G_2 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 μm. (**C**) Sister chromatids prematurely separate during G_2 arrest induced by Cdc25p inactivation. *cdc25ts* cen1–GFP (referred to as *cdc25ts*) and *cdc25ts Δpds5-1* cen1–GFP (referred to as *cdc25ts Δpds5-1*) 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 presence 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 G_2-arrested *Δpds5* cells. *cut2HA+* (referred to as wild type) and *cut2HA+ Δpds5-1* (referred to as *Δpds5-1*) 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 G_2-arrested *Δpds5-1* 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 *Δpds5-2* 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 *Δpds5* cells.

Fission yeast cells can be controlled to enter stationary phase from either G_1 or G_2 by altering nutrient availability in culture medium (Hao *et al.*, 1997). We used this procedure to examine whether *Δpds5* cells lose viability in a cell cycle phase-specific fashion or not. When cultured in MM lacking glucose or sulfate, the majority of *Δpds5-1* cells arrested in G_2 (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 G_1 without a significant viability loss. In plain MM, the cells arrested roughly equally in G_1 and G_2 with partial alleviation of viability loss. These results indicate that *pds5+* was required for G_2-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 G_2, and was beheld also during a cell cycle arrest at G_2 induced by the inactivation of a key G_2–M regulator. At the non-permissive temperature, cells with the thermolabile *cdc25-22* (*cdc25ts*) mutation arrest at G_2 due to the inability to activate Cdc2 kinase (Okayama *et al.*, 1996). When shifted to the non-permissive temperature, *Δpds5-1 cdc25ts* double mutant cells rapidly lost viability, unlike the original *cdc25ts* cells (Figure 2D). In contrast, further confirming the starvation-arrest results, *Δpds5-1* cells combined with the thermolabile *cdc10-129 or cdc22-M45* mutation that arrest at G_1 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 G_2-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 G_2 arrest, we examined the nuclear morphology of the *Δpds5* cells that were arrested in G_2 by growth saturation and then stimulated to resume cell cycling. The G_2-arrested *Δpds5-1* 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 *Δpds5-1 cdc25ts* cells (data not shown). These results indicate that cells lacking Pds5p were viable during G_2 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 G_2-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^{ts}*), *rad21-K1* (*rad21^{ts}*) and *eso1^{ts}* (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 G_2 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 G_2. In this particular experiment, ~90% of both cells arrested in G_2 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 G_2-arrested *Δpds5-1* cells. Similar results were obtained with the *Δpds5-1* cells arrested in G_2 by growth to confluence in MM+YE (data not shown).

To investigate whether or not the lack of sister chromatid cohesion in the G_2-arrested *Δpds5* cells was a consequence of premature sister chromatid separation, we performed the same analysis with the *Δpds5* cells undergoing G_2 arrest upon *cdc25^{ts}* inactivation and examined the time-dependent emergence of two split signals. During proliferation at the permissive temperature, few *Δpds5-1 cdc25^{ts}* cells had two split signals with a frequency comparable to that in *cdc25^{ts}* cells (Figure 3C, right). Upon a shift to 36°C, however, the number of the *Δpds5-1 cdc25^{ts}* cells having two split signals drastically increased with concurrent loss of viability (Figure 3C). In the *cdc25^{ts}* background, the cell's transition from G_2 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 G_2–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 G_2 arrest.

Cut2p is present in G_2-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 G_2-arrested *Δpds5* cells could have been caused by premature degradation of Cut2p during G_2 arrest. To examine this possibility, we determined the level of Cut2p in *Δpds5* cells during G_2 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–Separin cascade was unlikely to be the cause of the chromatid dissociation in the G_2-arrested *Δpds5* cells.

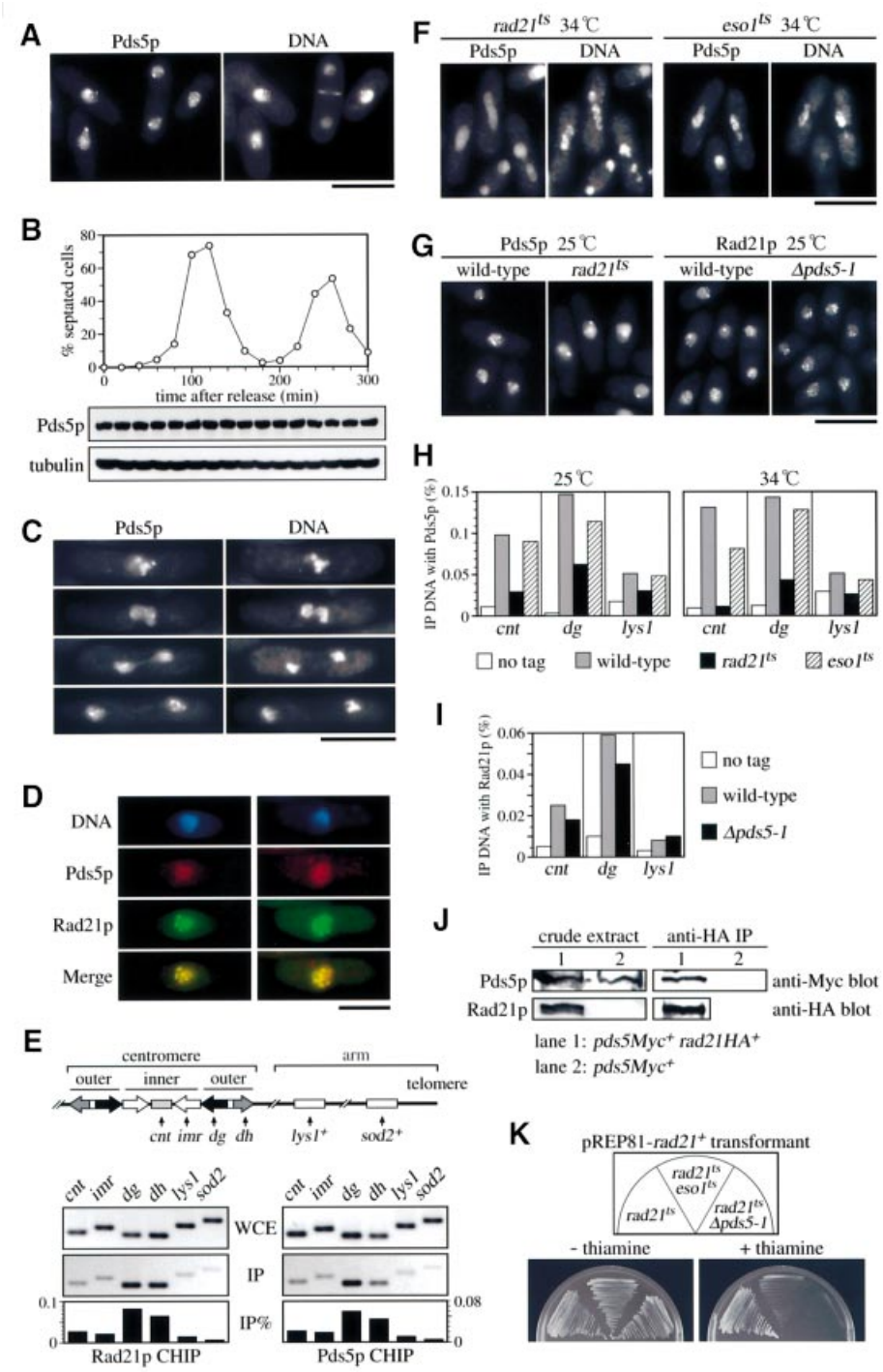
Pds5p is associated with Cohesin

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 (G_1), binucleate septated (S) and uninucleate (G_2) 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 *rad21HA$^+$* 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*^{ts} cells proliferating at the permissive temperature (Figure 4G). Consistently, the CHIP assay



revealed that in *rad21ts* 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 *Δpds5-1* 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. Pds5Mycp 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 Scc2p required for Cohesin loading on chromosome (Furuya *et al*., 1998). *Δpds5* cells were synthetically lethal with the *rad21ts* mutation even at the permissive temperature. Tetrad dissection of the spores obtained by crossing between *Δpds5-1* and *rad21ts* cells yielded no viable double mutant cells even at 23°C. Moreover, the *Δpds5-1 rad21ts* 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 *mis4ts* 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 tetrad dissection of spores from diploid cells with disruption of one *eso1+* allele by *ura4+* yields only two uracil-prototrophic 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 *eso1ts* 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

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 *cdc25ts pds5Myc+* cells were synchronized at late G_2 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. Pds5p 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 PCRs 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. *rad21ts pds5GFP+* and *eso1ts 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 *rad21ts pds5GFP+* (referred to as *rad21ts*) 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), *rad21ts pds5GFP+* (referred to as *rad21ts*) and *eso1ts pds5GFP+* (referred to as *eso1ts*) 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 *pds5Myc+ rad21HA+* and *pds5Myc+*cells were immunoprecipitated with an anti-HA antibody, and the original cell extracts 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 *rad21ts* mutation. The *rad21ts*, *rad21ts eso1ts* and *rad21ts 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 *rad21ts eso1ts* cells are a positive control for synthetic lethality (K.Tanaka *et al*., 2000).

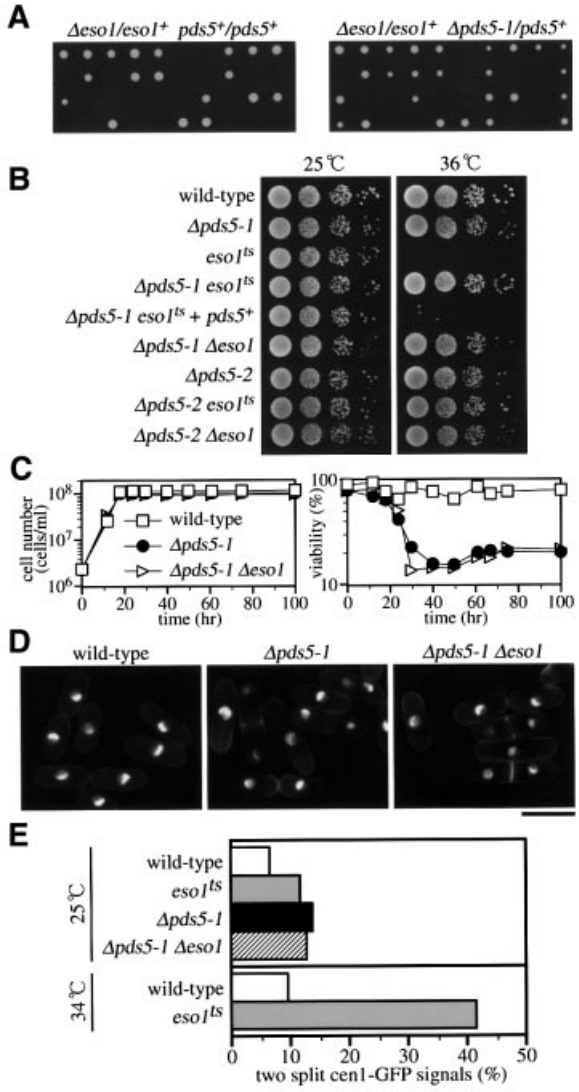


Fig. 5. Eso1p is dispensable for cohesion establishment in the absence of Pds5p. (**A**) Deletion of *pds5*$^+$ suppresses the lethality of *Δeso1* cells. Spores from *Δeso1/eso1*$^+$ *pds5*$^+$*/pds5*$^+$ diploid cells (left) or *Δeso1/eso1*$^+$ *Δpds5-1/pds5*$^+$ diploid cells (right) were tetrad-dissected on YE plates and incubated at 30°C for 4 days. (**B**) Deletion of *pds5*$^+$ allows *Δeso1* cells to grow at 36°C as well as fully suppressing the temperature-sensitive lethality of the *eso1*^{ts} cells. Approximately 10^4, 10^3, 10^2 and 10 wild-type, *Δpds5-1*, *eso1*^{ts}, *Δpds5-1 eso1*^{ts}, *Δpds5-1 eso1*^{ts} + *pds5*$^+$ (*Δpds5-1 eso1*^{ts} integrated with the *pds5*$^+$ gene), *Δpds5-1 Δeso1*, *Δpds5-2*, *Δpds5-2 eso1*^{ts} and *Δpds5-2 Δeso1* cells were spotted on two YE plates and incubated at 25 and 36°C, respectively. The *Δpds5-1 eso1*^{ts} + *pds5*$^+$ strain generated a few colonies that could grow even at 36°C. They were 'pop out' revertants in which the integrated *pds5*$^+$ gene was spontaneously lost from chromosome by homologous recombination. (**C**) *Δpds5 Δeso1* cells are phenotypically indistinguishable from *Δpds5* cells. Logarithmically proliferating wild-type, *Δpds5-1* and *Δpds5-1 Δeso1* cells were cultured in MM+YE. Cells were taken at the times indicated and determined for cell number (left) and viability (right). (**D**) *Δpds5 Δeso1* cells perform proper chromosome segregation. Wild-type, *Δpds5-1* and *Δpds5-1 Δeso1* 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 Δeso1* cells. cen1–GFP (referred to as wild type), *eso1*^{ts} cen1–GFP (referred to as *eso1*^{ts}), *Δpds5-1* cen1–GFP (referred to as *Δpds5-1*) and *Δpds5-1 Δeso1* cen1–GFP (referred to as *Δpds5-1 Δeso1*) 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 *eso1*^{ts} cen1–GFP cells cultured at 34°C for 3 h were used as a control for unestablished sister chromatid cohesion.

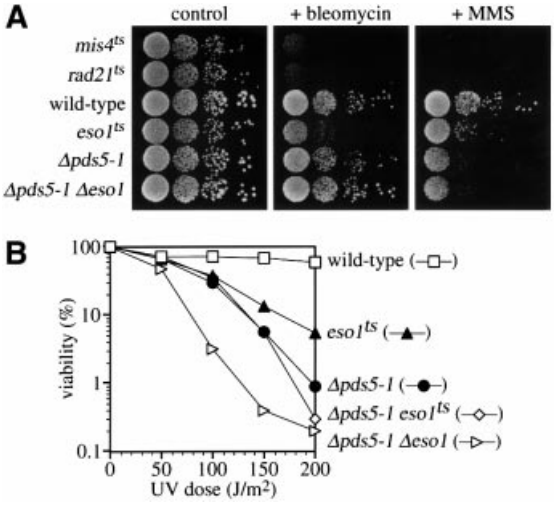


Fig. 6. *Δpds5* cells are sensitive to DNA damage. (**A**) *Δpds5* cells are sensitive to MMS but not to bleomycin. Approximately 10^4, 10^3, 10^2 and 10 *mis4*^{ts}, *rad21*^{ts}, wild-type, *eso1*^{ts}, *Δpds5-1* and *Δpds5-1 Δeso1* cells were spotted on YE plates containing none (referred to as control), bleomycin [0.000025% (w/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, *eso1*^{ts}, *Δpds5-1*, *Δpds5-1 eso1*^{ts} and *Δpds5-1 Δeso1* cells were plated on YE, 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 *Δeso1* 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 Δeso1* cells with two split cen1 signals was comparable to those of *Δpds5-1* cells and permissive temperature-cultured *eso1*^{ts} 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 Δeso1* 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 cen1s during stationary arrest (wild type, 8%; *Δpds1-1*, 49%; *Δpds1-1 Δeso1*, 47%), nuclear morphology during proliferation (Figure 5D), the frequency of cells with two split cen1s 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 *rad21*^{ts}, *mis4*^{ts} and *eso1*^{ts} (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

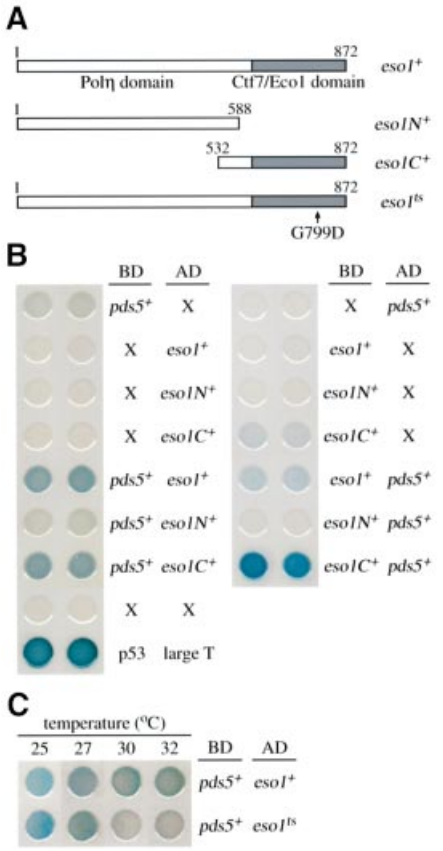


Fig. 7. Physical interaction between Eso1p and Pds5p. (**A**) Schematic representation of *eso1*$^+$ genes used in this experiment. (**B**) Two independent yeast transformants containing the indicated two-hybrid plasmids were grown on the nitrocellulose filters at 30°C and their β-galactosidase activities were assayed. Filters were incubated with X-gal for 2 h (left) or overnight (right) at 30°C. Blue color indicates interaction. X is a vector plasmid with no insert. The pair p53 (pVA3) and SV40 large T-antigen (pTD1) represents a standard positive control. (**C**) Yeast strains containing the indicated plasmids were grown at indicated temperatures and their β-galactosidase activities were assayed.

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 eso1*^{ts} 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 eso1*^{ts} 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-H17* 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 centromere 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 Cohesion-related factors, other eukaryotes contain Pds5p homologues that appear to perform a similar molecular function. Budding yeast Pds5p colocalizes with Scc1p/Mcd1p on chromatin in an Scc1p/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). Spo76p 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 morphogenesis, including sister chromatid cohesiveness and chromatin condensation (van Heemst *et al*., 1999, 2001). Moreover, the conditional lethal phenotype conferred by the *bimD* 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 Eso1p counteracts its hindrance. Consequently, loss of Pds5p totally negated the requirement for Eso1p in establishing cohesion. In addition, Pds5p is essential for stably maintaining established cohesion during prolonged G_2 arrest, irrespective of whether it is induced by cell cycle block at G_2 or stationary phase entry from G_2.

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

Eso1p contains a Pol η domain fused with a Ctf7p/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 chromatin 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 Ctf7p/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-independent formation of cohesion. As already discussed, loss of Pds5p totally negated the Eso1p dependency. Moreover, when the gene dosage of *pds5*$^+$ 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 *eso1*^{ts} mutant, but its molecular mechanism is unknown, although this might be caused by interference of the proper association of Pds5p with Cohesin 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 *rad21GFP*$^+$ strains were described previously (Funabiki *et al*., 1996; Tomonaga *et al*., 2000). The *pds5GFP*$^+$ and *pds5Myc*$^+$ 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 (Bahler *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-*eso1*$^+$ (K.Tanaka *et al*., 2000), pREP81-*rad21*$^+$ (Tatebayashi *et al*., 1998) and pIK1 (Tanaka and Okayama, 2000) plasmids were described previously. The *S.pombe* genomic library was constructed by inserting *Bgl*II-digested wild-type (L972) genomic DNA into the *Bam*HI-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 *Δpds5-1* construct, the 1.3 kb *Bam*HI–*Nhe*I fragment of the *pds5*$^+$ gene that contains ~36% of the ORF was replaced with the 1.8 kb *Hin*dIII fragment of the *ura4*$^+$ gene. Similarly, the 3.4 kb *Hpa*I–*Xba*I fragment that contains ~93% of the ORF was replaced with the *ura4*$^+$ in the *Δpds5-2* construct. The *Pst*I–*Xba*I fragment carrying the *Δpds5-1* construct or the *Ban*III–*Ban*III fragment carrying the *Δpds5-2* construct was used to transform the *ura4-D18* diploid strain and stable ura$^+$ transformants were isolated. The correct replacement of one wild-type allele with *pds5::ura4*$^+$ was confirmed by Southern blot analysis.

For chromosomal integration, a 6.5 kb *Bgl*II fragment containing the *pds5*$^+$ gene was subcloned into the pIK1 vector. This plasmid was digested at the unique *Sph*I site located in the 5′-upstream region of the *pds5*$^+$ ORF and transfected into the *Δpds5-1* or *Δpds5-1 eso1*^{ts} 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 in 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 (2×10^8) 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 microlitres 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 *pds5Myc*$^+$ *rad21HA*$^+$ 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-phenylinodole (DAPI). Cells were examined under the fluorescence microscope (Olympus AX70), and images were captured with a CCD camera (Sensys; Phomometrics).

CHIP assay

CHIP assay was performed as described previously (Saitoh *et al*., 1997). Rad21GFPp and Pds5GFPp 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 *cnt* 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 peritelomic 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 *pds5*$^+$, *eso1*$^+$, *eso1-H17* or the Pol η domain of *eso1*$^+$ (amino acids 1–588) and Ctf7p/Eco1p-homologous domain of *eso1*$^+$ (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*^{ts} mutant block and release method as described previously (Alfa *et al*., 1993).

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