

Role of the fission yeast SUMO E3 ligase Pli1p in centromere and telomere maintenance

Blerta Xhemalce^{1,4,*}, Jacob-S Seeler^{2,4},
Geneviève Thon³, Anne Dejean²
and Benoît Arcangioli^{1,*}

¹Unité de la Dynamique du Génome, Institut Pasteur, Paris Cedex, France, ²Unité Organisation Nucléaire et Oncogénèse, Institut Pasteur, Paris Cedex, France and ³Department of Genetics, Institute of Molecular Biology, University of Copenhagen, Copenhagen K, Denmark

Sumoylation represents a conserved mechanism of post-translational protein modification. We report that Pli1p, the unique fission yeast member of the SP-RING family, is a SUMO E3 ligase *in vivo* and *in vitro*. *pli1Δ* cells display no obvious mitotic growth defects, but are sensitive to the microtubule-destabilizing drug TBZ and exhibit enhanced minichromosome loss. The weakened centromeric function of *pli1Δ* cells may be related to the defective heterochromatin structure at the central core, as shown by the reduced silencing of an *ura4* variegation reporter gene inserted at *cnt* and *imr*. Interestingly, *pli1Δ* cells also exhibit enhanced loss of the *ura4* reporter at these loci, likely by gene conversion using homologous sequences as information donors. Moreover, *pli1Δ* cells exhibit consistent telomere length increase, possibly achieved by a similar process. Point mutations within the RING finger of Pli1p totally or partially reproduce the *pli1* deletion phenotypes, thus correlating with their sumoylation activity. Altogether, these results strongly suggest that Pli1p, and by extension sumoylation, is involved in mechanisms that regulate recombination in particular heterochromatic repeated sequences.

The EMBO Journal (2004) 23, 3844–3853. doi:10.1038/sj.emboj.7600394; Published online 9 September 2004

Subject Categories: chromatin & transcription; genome stability & dynamics

Keywords: centromere; genomic instability; SUMO E3 ligase; telomere

Introduction

Covalent protein modification by SUMO (sumoylation) has been shown to play important roles in such diverse processes as nucleo-cytoplasmic transport, chromosome segregation, DNA metabolism, as well as transcriptional regulation (reviewed in Melchior, 2000; Müller *et al.*, 2001; Seeler and Dejean, 2003). Like the modification with ubiquitin and other ubiquitin-like modifiers (Ubls), sumoylation is achieved

with a distinct but evolutionarily conserved pathway consisting of E1-activating, E2-conjugating and E3 ligase enzymes. Sumoylation of numerous target proteins can be achieved *in vitro* using only E1 and E2 enzymes; however, *in vivo*, it is likely that the substrate specificity and the rate of the reaction depend critically on the activity of E3 ligase-containing complexes, as is also the case for ubiquitylation. To date, three protein families have been implicated as SUMO E3 ligases. The first, or SIZ/protein inhibitor of activated Stat (PIAS) family, was initially shown in budding yeast (*Saccharomyces cerevisiae*) to mediate the modification of septins (Johnson and Gupta, 2001; Takahashi *et al.*, 2001). Subsequently, members of the mammalian PIAS family were shown to be critical for the modification of transcription factors such as p53, nuclear receptors (e.g. AR, PR), Lef1 and Sp3 (Jackson, 2001; Kotaja *et al.*, 2002; Sapetschnig *et al.*, 2002; Gill, 2003). A second type of E3 ligase is given by the nuclear import factor RanBP2, which has been shown to mediate the modification of the nuclear proteins SP100 and HDAC4 (Kirsh *et al.*, 2002; Pichler *et al.*, 2002), and has provided a further link between sumoylation and nucleo-cytoplasmic transport. Finally, a third SUMO E3 ligase was discovered with the polycomb protein Pc2, which was found to enhance the modification of the transcriptional repressor CtBP1 (Kagey *et al.*, 2003).

Studies of sumoylation in mammalian cells have largely been focused on the effects on transcriptional regulation, largely because the majority of the currently characterized SUMO substrates are either transcription factors or co-factors. A growing number of cases supports a model in which the sumoylation of a transcription factor leads to repression (reviewed in Verger *et al.*, 2003). Consistent with this is the finding that the sumoylation target sites often fall within known repression domains of dual-function transcription factors and that mutation of these sites leads to de-repression. While the precise mechanisms involved remain elusive, it has been shown in some cases (Lef1, Sp3) that sumoylation leads to the sequestration of the transcription factor to specific nuclear domains, such as PML nuclear bodies, and thus to the concomitant attenuation of transcriptional activity. That sumoylation may also play a role at the DNA (or promoter) level has been suggested by Shii and Eisenman (2003), who showed that the E2 enzyme (Ubc9) fused to a GAL4 DNA-binding domain could lead to transcriptional repression of a reporter gene that is associated with the appearance of sumoylated chromatin, presumably at histone H4 within nucleosomes.

Analysis of the role of sumoylation in genetically tractable organisms has opened new fields of interest such as chromosome condensation and cohesion, and DNA replication and repair. In the budding yeast, the proliferating cell nuclear antigen (PCNA) was shown to be modified by ubiquitin and SUMO on the same lysine residue (K164), sumoylation occurring during S phase and ubiquitylation upon DNA damage, channeling DNA repair towards the RAD6-dependent post-replication repair mechanisms (Hoegge *et al.*, 2002; Stelter and Ulrich, 2003). It was proposed that sumoylation of

*Corresponding authors. Unité de la Dynamique du Génome, URA1664 du CNRS, Jacques Monod Building, Institut Pasteur, 25, rue du Dr Roux, 75724, Paris Cedex 15, France. Tel.: +33 1 4568 8454;

Fax: +33 1 4568 8960; E-mail: blerta@pasteur.fr; barcan@pasteur.fr

⁴These authors contributed equally to this work

Received: 29 March 2004; accepted: 12 August 2004; published online: 9 September 2004

PCNA has an inhibitory function on repair since abrogation of SUMO modification by mutating both K164 and K127 (the other residue subject to sumoylation) partially rescues the DNA damage sensitivity of the PCNA K164R single mutant. Again, in budding yeast, SMT4, a de-sumoylation enzyme, was found as a high copy suppressor of thermosensitive alleles of the *SMC2* gene, encoding one of the core condensin subunits (Strunnikov *et al*, 2001) and of the *PDS5* gene, required for proper chromatid cohesion maintenance (Stead *et al*, 2003). Furthermore, *SMT4* mutants exhibit severe defects in chromatin condensation (Strunnikov *et al*, 2001) and precocious dissociation of sister chromatids during mitosis, which has been linked to the sumoylation of two major proteins TOP2 and PDS5 (Bachant *et al*, 2002; Stead *et al*, 2003). In *Drosophila melanogaster*, the *Su(var)2-10* gene, which encodes a member of the Siz/PIAS family, is required for viability, and mutants show defects in both chromosome condensation and inheritance (Hari *et al*, 2001). In *Schizosaccharomyces pombe*, mutations in the genes encoding SUMO (Pmt3p; Tanaka *et al*, 1999), E1 (Rad31p; Shayeghi *et al*, 1997), E2 (Hus5p; al-Khodairy *et al*, 1995) in the SUMO pathway produce cells that survive but display severe growth defects, that is, are essentially nonviable, and thus preclude proper genetic analysis.

In this work, we report the characterization of a novel fission yeast nuclear protein, Pli1p, as an E3 ligase in the SUMO pathway. Deletion of the *pli1* gene, while exhibiting only mild or no mitotic phenotypes, leads to increased sensitivity to the microtubule spindle poison TBZ and minichromosome loss, indicating improper centromere and/or kinetochore function. Consistent with this, *pli1Δ* cells display alleviated silencing of an *ura4* reporter gene at the central centromeric core required for kinetochore assembly. Furthermore, we present evidence for a role of Pli1p in protecting heterochromatic repeated sequences (i.e. centromeres and telomeres) from illegitimate recombination. The phenotypes described for *pli1*-deleted cells are totally or partially reproduced by point mutations within the RING finger, in correlation with their E3 ligase activity, thus strongly suggesting that sumoylation via Pli1p is involved in centromeric and telomeric functions in fission yeast.

Results

Isolation of Pli1p, an *S. pombe* Siz1/Siz2/PIAS homologue

Our previous studies of the biological role of the essential chromatin-associated protein switch-activating protein 1

(Sap1p) (De Lahondes *et al*, 2003) prompted us to perform a yeast two-hybrid screen of a *S. pombe* cDNA library with this protein as bait. While the functional relevance of the obtained interactants remains to be elucidated, this screen identified a novel cDNA corresponding to the hypothetical open reading frame (ORF) listed as SPAC1687.05 under the reserved name of *pli1* in the Sanger Institute GeneDB database. The *pli1*⁺ ORF translates into a putative protein of 727 amino acids with a predicted molecular mass of 80.7 kDa. Amino-acid sequence comparisons revealed significant similarity to the SIZ1 and SIZ2 proteins of budding yeast as well as all known mammalian PIAS proteins. Pli1p notably shares with these two well-conserved domains: an amino-terminal SAP (SAF-A/B, Acinus and PIAS) domain and a central SP-RING (Siz/PIAS-RING) domain (Hochstrasser, 2001), essential for the SUMO E3 ligase activity associated with the Siz/PIAS proteins (Seeler and Dejean, 2003) (Figure 1). Western blot analysis of WCE of cells containing an endogenous HA (data not shown) or CFP-tagged version of the protein (Figure 2B) confirmed the existence and size of the Pli1p protein. Further immunofluorescence analysis using an anti-GFP antibody to detect the endogenous Pli1-CFP protein revealed that Pli1p is localized in the nucleus, where it forms numerous spots (Figure 2A).

Pli1p promotes SUMO/Pmt3p conjugation in vivo and in vitro

The homology of Pli1p to the SIZ1/2 budding yeast and mammalian PIAS proteins suggests that Pli1p might act as a SUMO E3 ligase. We therefore first analyzed the effect of *pli1* deletion on the *in vivo* pattern of SUMO conjugates (Figure 3A) Wild-type and *pli1Δ* cells containing or not an N-terminal His₆ tag fused in frame with the endogenous *pmt3* gene were analyzed by Western blotting using a rabbit antibody raised against the *S. pombe* SUMO/Pmt3p protein. *pli1Δ* extracts showed an increase in the intensity of free Pmt3p and an overall severe decrease in the intensity of Pmt3p-specific bands corresponding to Pmt3p conjugates, indicating a significant reduction of global sumoylation in *pli1*-deleted cells.

To demonstrate that Pli1p possesses SUMO E3 ligase activity, we employed a modification system consisting of recombinant Pmt3p and the E1 (Rad31p-Fub2p) and E2 (Hus5p) enzymes to modify Rad22p, a previously described *S. pombe* SUMO substrate (Ho *et al*, 2001). As shown in Figure 3B, ³⁵S-labelled, *in vitro* translated Rad22p is efficiently modified in the absence of Pli1p at high concentrations of E2 enzyme (lane 2). At one-fifth the concentration of

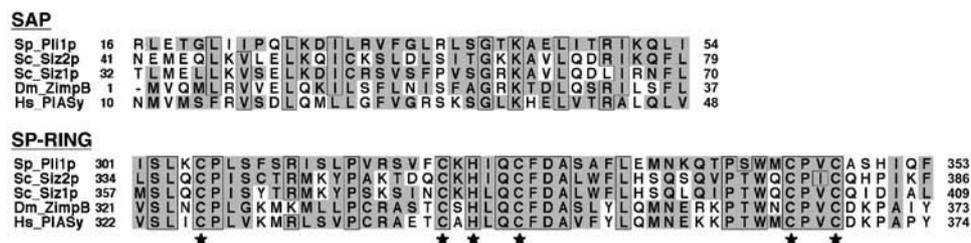


Figure 1 Sequence alignment of the SAP and SP-RING domains of Pli1p and several (putative) SP-RING SUMO ligases. Alignment of *S. pombe* Pli1p, *S. cerevisiae* Siz2p, Siz1p, *D. melanogaster* Zimp-B and *Homo sapiens* hPIASy protein sequences (GenBank accession numbers CAA22599.1, NP_014799, NP_010697, AAD29288, Q8N2W9, respectively), using CLUSTAL X algorithm. Identical residues are boxed and similar residues are shaded. Overall sequence identity of Pli1p with the other four orthologs is 26–27% (33–38% similarity), whereas the SAP domain has 22–35% identical residues (75–68% similarity) and the SP-RING domain 39–51% (70–75% similarity). Asterisks indicate the positions of the cysteiy and histidyl residues forming the C2HC3 conserved SP-RING domain.

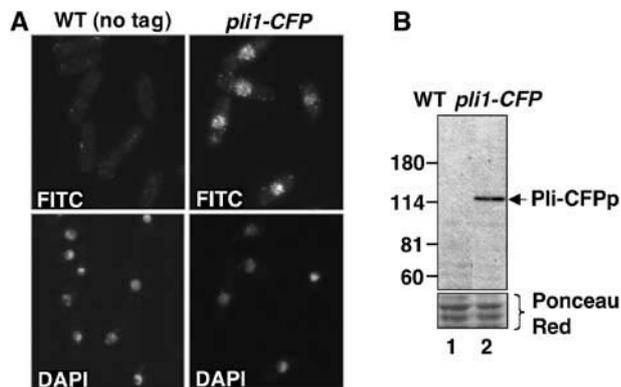


Figure 2 Pli1p is localized in the nucleus and forms numerous foci. (A) WT (PB46) and *pli1-CFP* (BX4) cells grown exponentially to 5×10^6 cells in MM at 33°C were fixed with paraformaldehyde and stained with anti-CFP rabbit polyclonal antibody, followed by FITC-conjugated anti-rabbit IgG secondary antibody and DAPI. Z-scanning of cells every 0.4 μm showed that the CFP-specific spots observed in *pli1-CFP* cells were present throughout the nucleus. (B) Anti-CFP Western blot of denaturing protein whole-cell extracts of WT (PB46) and *pli1-CFP* (BX4) cells grown in the same conditions as above.

E2, Rad22p modification was limited (lane 3) but could be restored by the addition of recombinant Pli1p (lanes 4–7) in a dose-dependent manner. *In vitro* binding assays showed that Pli1p interacts with both the E2 enzyme (Hus5p), as well as with the Rad22p substrate (data not shown), thus demonstrating that Pli1p fulfills the criteria for SUMO E3 ligase function. Furthermore, by introducing point mutations of either C321S or C321S/H323A/C326S within the SP-RING finger (Figure 1), we obtained Pli1 proteins which partially or totally lost the capacity to enhance the modification of Rad22p in the *in vitro* sumoylation system (Figure 3B and C). Indeed, addition of the inactive Pli1p C321S/H323A/C326S mutant to the reaction reduced sumoylation to levels somewhat below those obtained in its absence, possibly because Pli1p itself is a SUMO substrate *in vitro* (data not shown) and, when inactive, behaves as a competitor for sumoylation. When introduced in *S. pombe* cells, the *pli1C321S* WCE showed intermediate levels of global sumoylation between WT and *pli1Δ* cells (Figure 3A), whereas the C321S/H323A/C326S mutant behaved like the *pli1Δ* cells (data not shown).

pli1 is involved in centromeric function

In *S. pombe*, the *pmt3* null mutant displays severe morphological abnormalities and hypersensitivity to various stress conditions (Tanaka *et al*, 1999), while the *hus5* (encoding Ubc9p) null allele is almost inviable (al-Khodairy *et al*, 1995). In contrast, *pli1*-deleted strain grew well and lacked hypersensitivity to DNA-damaging agents like UV, MMS and hydroxy-urea (data not shown). However, *pli1Δ* cells were sensitive to the microtubule-destabilizing drug thiabendazole (TBZ) (Figure 4A). As TBZ sensitivity is frequently associated with defects in centromeric function, we then tested the chromosome stability in *pli1Δ* cells, which indeed showed a 10-fold increase in the frequency of the *Ch16* minichromosome loss (Figure 4B). We further analyzed the effect of *pli1* deletion on the heterochromatic function of *S. pombe* centromeres. In fission yeast, the centromeres are large DNA structures (40–100 kb) composed of outer repetitive regions

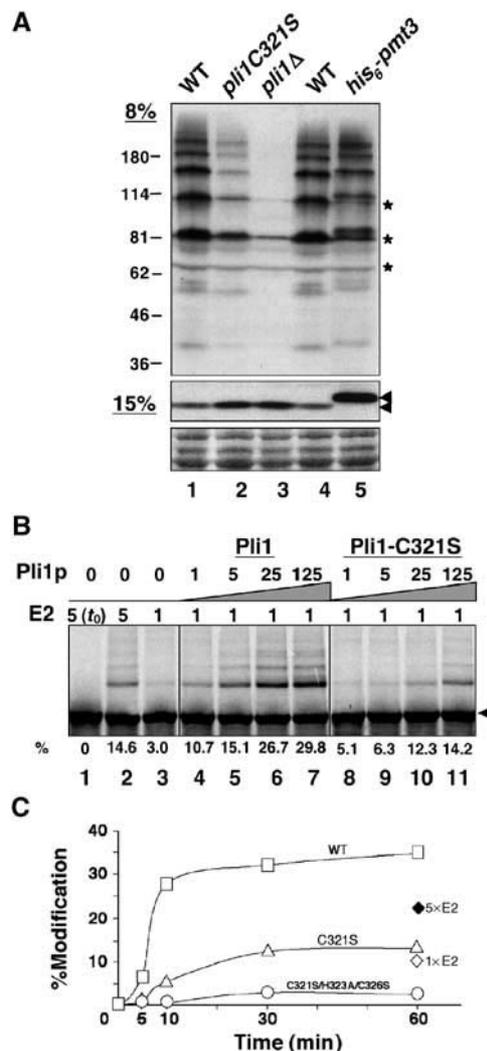


Figure 3 Pli1p promotes SUMO conjugation *in vivo* and *in vitro*. (A) Whole-cell extracts of WT, *pli1C321S* and *pli1Δ* cells were separated on denaturing 8% (top panel) and 15% (middle panel) SDS-polyacrylamide gels and Western blotted with anti-Pmt3p antiserum. The specificity of bands revealed by the anti-Pmt3p polyclonal antibody is shown by their slight upward shift in the *His₆pmt3* strain carrying the endogenous *pmt3* gene fused in frame with an amino-terminal His₆ tag. The arrows indicate unconjugated Pmt3p; asterisks indicate crossreacting bands. Strains used: PB46 (WT); BX6 (*pli1C321S*); BX2 (*Δpli1*); BX5 (*his₆pmt3*). (B) Sumoylation reactions with *in vitro*-translated, ³⁵S-labeled Rad22p substrate (4 μl per reaction in 22 μl total) and recombinant, bacterially produced Pmt3p (Pmt3-GG, 1 μg in lanes 2–7), E1 (GST-Rad31p-Fub2p, 32 ng), E2 (Hus5p, 80 ng in lanes 1–2 and 4–7; 400 ng in lane 3) and WT or Pli1C321S (4.8; 24; 120; 600 ng in lanes 4–7, respectively) proteins. The fastest migrating band of the modified species (i.e. mono-modified Rad22) was quantitated by PhosphorImager analysis and expressed as a percentage of total Rad22p. (C) Reaction time course of Rad22p *in vitro* modification. Standard reactions (4 μl ³⁵S-Rad22p, 1 μg Pmt3-GG, 32 ng E1, 80 ng E2) were assembled, containing 8 ng of WT Pli1p (open squares), Pli1pC321S (open triangles) or Pli1pC321S/H323A/C326S (open circles), and incubated for the indicated times. Open and closed diamonds represent modification without added E3 at 80 and 400 ng of E2 (Hus5p), respectively. Percent total modification was quantitated by PhosphorImager analysis.

(*otr*) corresponding to the pericentromeric heterochromatin and central regions (innermost repeat *imr* and central *cnt*), which form specialized SpCENP-A-dependent chromatin,

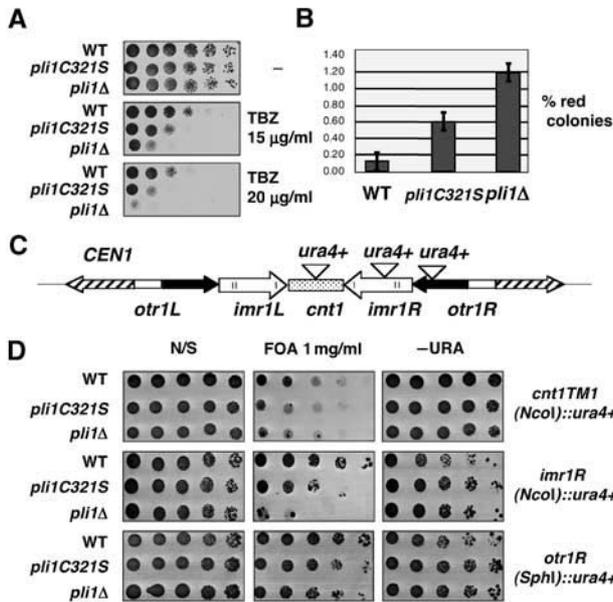


Figure 4 Effects of *pli1* mutants on centromeric function. (A) *pli1* cells are sensitive to the microtubule-destabilizing drug thiabendazole (TBZ): Serial five-fold dilutions of exponential cultures of WT (PB10), *pli1C321S* (BX7) and *pli1Δ* (BX1) cells were spotted to YES and YES plates containing TBZ (17.5 or 20 μg/ml) and incubated for 4 days at 33°C. (B) *pli1* cells show an increased frequency of minichromosome loss. Independent colonies of WT (JS161), *pli1C321S* (BX9) and *pli1Δ* (BX8) strains grown in adenine selective media, to select for the presence of the Ch16 minichromosome, were plated to YE plates at a concentration of 10³ cells/plate and incubated for 4 days at 33°C. The values shown are the average of the percentage of red colonies calculated for 16 independent colonies in two independent experiments. (C, D) *pli1* deletion affects silencing at the central core of centromeres. Expression of the *ura4⁺* gene inserted at three different positions of centromere 1, schematically represented in (C), was assayed by plating efficiency of serial three-fold (*cnt1*) or five-fold (*imr* and *otr*) dilutions of cell suspensions spotted on selective media shown in (D). Strains used: *cnt1*TM1(*NcoI*::*ura4⁺*): WT = FY336, *pli1C321S* = BX10, *pli1Δ* = PG2964; *imr1R*(*NcoI*::*ura4⁺*): WT = FY498, *pli1C321S* = BX12, *pli1Δ* = PG2972; *otr1R*(*SphI*::*ura4⁺*): WT = FY648, *pli1C321S* = BX14, *pli1Δ* = PG2976.

and previous studies have shown that transcription of genes inserted into these domains is silenced (Chikashige *et al*, 1989; Niwa *et al*, 1989; Clarke and Baum, 1990; Allshire *et al*, 1994, 1995; Baum *et al*, 1994; Ekwall *et al*, 1999; Partridge *et al*, 2000). We assayed silencing of the *ura4⁺* gene inserted at three different loci in *CEN1* (Centromere of Chromosome I): the center region (*cnt1*::*ura4⁺*), the innermost repeat (*imr*::*ura4⁺*) and the outer repeat (*otr*::*ura4⁺*) (Figure 4C) in WT and *pli1Δ* strains, by the ability of cells to survive in the presence of 5-fluoro-orotic acid (FOA), which generates a toxic metabolite in (*ura⁺*) strains. In WT cells, the inserted *ura4⁺* variegates between expressed and repressed states as cells can grow both on -URA and FOA plates. However, deletion of *pli1* causes a significant decrease in silencing at the *imr1*::*ura4⁺* and, in a milder way, at the somewhat more expressed *cnt1*::*ura4⁺* insertion sites, as indicated by the lower efficiency of plating on FOA plates of these cells (Figure 4D). These results were also confirmed by Northern blot analysis using an *ura4* probe (data not shown). The use of the *pli1C321S* and *pli1C321S/H323A/C326S* mutants in these experiments showed a strong correlation between SUMO E3 ligase activity and the centromeric phenotypes,

since the *pli1C321S* single mutant showed intermediate levels of TBZ sensitivity, minichromosome loss and silencing at the central region between WT and *pli1Δ* cells (Figure 4) and the triple mutant exactly reproduced the *pli1* deletion phenotypes (data not shown).

Implication of *pli1* in the sequence stability of centromeres

While assaying silencing of the *ura4⁺* gene inserted at *cnt1* and *imr1R*, we noted that *pli1* mutant cells recurrently gave rise to large colonies on FOA plates (Figure 4D) that turned out to be unable to grow on -URA plates. As this does not correspond to a variegation phenotype (Allshire *et al*, 1994), we analyzed them at the DNA level. The central domain of *CEN1* is composed of two 5.6 kb inverted repeats (*imr1R* and *imr1L*) surrounding a 4.1 kb center (*cnt1*), which otherwise partially shares its sequence with the central domain of *CEN3* (Takahashi *et al*, 1992) (Figure 5A). The *ura4* gene was inserted at the *NcoI* sites of *cnt1* and *imr1R* (Allshire *et al*, 1994, 1995). Challenging independent FOA papillae from WT and *pli1Δ* strains carrying the *ura4⁺* gene at *cnt1* and *imr1R* for growth on -URA plates indicated that most, if not all, papillae from *pli1* mutants were stable (*ura4⁻*), whereas papillae from WT could grow both on FOA and -URA plates as expected (Allshire *et al*, 1994). Genomic DNA from 20 independent (*ura⁻*) clones of the *pli1Δ*, *CEN1-imr1R*(*NcoI*::*ura4⁺*) (PG2972) strain was digested with *KpnI* and analyzed by a Southern blot sequentially using a *cnt1* and an *ura4* probe (Figure 5B). This analysis revealed that all isolated clones had deleted the *ura4* gene at *imr1R*. Further Southern blot analysis using an *NcoI* digestion and an *imr1* probe revealed that the deletion of the *ura4* gene had restored the WT sequence, as indicated by the presence of an *NcoI* site at *imr1R* (Figure 5B). Since *imr1R* and *imr1L* are identical over 99% of their sequence, the simplest interpretation would be that the *ura4* gene inserted at *imr1R* was deleted following gene conversion events using the homologous sequence at the inverted repeat *imr1L* as template. A similar result was obtained with 19 over 20 independent (*ura⁻*) clones of the *pli1Δ*, *CEN1-cnt1*(*NcoI*::*ura4⁺*) (PG2964) strain (see Supplementary Figure S1). Since *cnt1* also shares 100% identity over 3.3 kb with the central domain of *CEN3*, these latter clones likely arose from interchromosomal gene conversion events. The frequency of *ura4* loss was evaluated at $\approx 10^{-3}$ for both domains in *pli1Δ* cells and, as stable (*ura⁻*) cells could not be isolated from 10⁴ wild type cells, *ura4* deletion is therefore increased by at least 10-fold in *pli1Δ* compared to WT cells. This effect seems to be specific for the centromeric loci, since an artificial intrachromosomal recombination reporter (van den Bosch *et al*, 2002) at a *euchromatic* locus displayed no difference between *pli1Δ* and WT cells (see Supplementary Figure S2).

Pli1p is involved in the control of telomere length

While examining silencing at three other heterochromatic loci: the *mat2/3* inactive sexual locus, the rDNA and the telomeres, we noticed that *pli1* mutants slightly increased the silencing of the *ura4⁺* gene next to the telomeric repeat of the *Ch16m23::ura4⁺ TEL[72]* minichromosome without affecting silencing at the two other loci (data not shown). As the frequency of transcriptional repression at the telomere can be increased by longer telomeres (Kyrion *et al*, 1993) and,

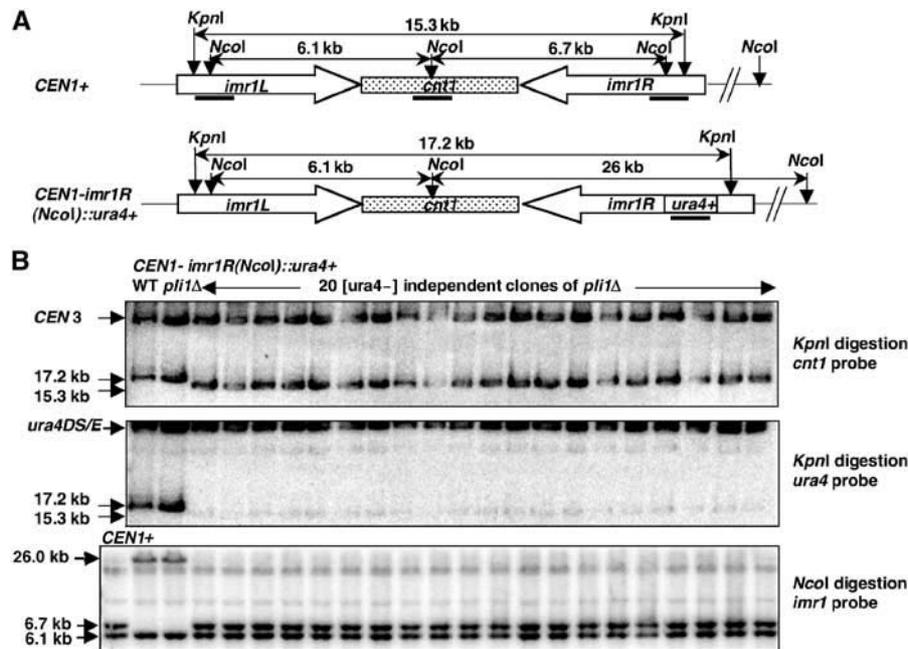


Figure 5 Effect of *pli1* deletion on the stability of *ura4+* gene inserted at *imr1R*. (A) Structure of centromere 1 central domain in *CEN1+* and *CEN1: imr1R(NcoI)::ura4+* strains. *KpnI* and *NcoI* sites, sizes of their respective digestion fragments and positions of the probes used for the Southern blot analysis are indicated. Note that the *cnt1* and *ura4* probes also recognize homologous sequences, respectively, at *CEN3* and *ura4D/SE* loci, thus serving as internal controls. (B) Southern blot analysis of stable [*ura-*] independent clones raising from *CEN1-imr1R(NcoI)::ura4* deleted for *pli1* gene. After digestion with *KpnI*, genomic DNA prepared in agarose plugs was subjected to pulsed field electrophoresis on 1.2% agarose gel and analyzed by a Southern Blot using the *cnt1* probe. The membrane was then stripped and reprobbed with the *ura4* probe. Genomic DNA was also digested with *NcoI*, subjected to electrophoresis on 0.75% agarose gel, transferred to a nylon membrane and hybridized to the *imr1* probe. Sizes or identities of bands are indicated. Strains used: *CEN1+*: PB10; *CEN1-imr1R(NcoI)::ura4+*: WT = FY498, *pli1Δ* = PG2972; and 20 [*ura-*] independent clones isolated from PG2972 strain = BX13_1–20.

further, since deletion of *pmt3* was previously reported to lead to telomere elongation (Tanaka *et al*, 1999), we assessed telomere length in *pli1* cells. Genomic DNA digested with *EcoRI* was analyzed by a Southern blot using a ³²P end-labeled telomeric oligonucleotide (Cooper *et al*, 1997) (Figure 6B). The *EcoRI* site is located ≈0.8 kb away from the telomeric repeats sequences, giving rise to a broad telomere hybridization signal centered at 950 bp in the WT strain (Figure 6A, lane 1). The *pli1Δ* strain showed a clearly upshifted, more intense signal that ranges from 1 to 1.4 kb (lane 3), corresponding to a telomere length intermediate between that of WT and *pmt3Δ* cells (Tanaka *et al*, 1999). The *pli1C321S* single mutant again showed a milder effect on the increase of telomere length (lane 2), while the *pli1C321S/H323A/C326S* triple mutant showed the same effect as the *pli1* deletion (data not shown).

To test whether the increase in telomere length in *pli1* mutants could have resulted from normally repressed gene conversion events, we performed a genetic analysis involving *pli1* and three major genes involved in homologous recombination: *rad22* (fission yeast *RAD52* homolog), *rad50* and *rad51/rhp51*. We therefore produced diploids doubly heterozygous for *pli1* and each of the three *rad* genes by sequential disruption. At least 100 tetrads from microdissected asci were genotyped for each experiment and representative examples are shown in Figure 6A. The *pli1-rad50* double mutants showed a reduced viability (50%) and a significant increase in generation time independent of mating type (data not shown). They also exhibited a significant increase in telomere length compared to the *rad50Δ* single mutant (Figure 6B,

compare lanes 4 and 5), but very similar to that observed in *pli1Δ* cells (lane 3). In contrast, disruptants of *pli1* and either *rad22* or *rad51* displayed synthetic lethality, in that double-mutant clones gave rise to microcolonies of 8–150 cells that contained numerous abnormally elongated cells (data not shown). In order to possibly circumvent the lethality of *pli1-rad51* double mutants, we crossed the *pli1C321S* and *rad51* mutants and found that the obtained *pli1C321S-rad51Δ* double mutants were viable, although they grew slower than the *rad51* single mutants of the same mating type (Figure 6A). When we next analyzed clones from tetra-type tetrads of the *pli1C321S × rad51Δ* cross at the telomere level (Figure 6B, lanes 6–9), we found that *pli1C321S-rad51Δ* clones (lane 6) showed a telomere length similar to the *rad51Δ* single mutant (lane 7) and not the *pli1C321S* single mutant (lane 8). This shows that the increase in telomere length characteristic of the *pli1C321S* single mutant (lane 2 and 8) requires the wild-type *rad51* background, consistent with the hypothesis that telomere elongation of *pli1* mutants is, at least partially, mediated by gene conversion (homologous recombination) type events.

Discussion

In this work, we show that the *S. pombe* homolog of the Siz/PIAS proteins, Pli1p, functions as a SUMO E3 ligase *in vivo* and *in vitro*. *pli1* mutant cells display phenotypes intermediate between SUMO-deficient and WT cells with respect to telomere length and centromeric function, as witnessed by TBZ sensitivity and enhanced minichromosome loss. We

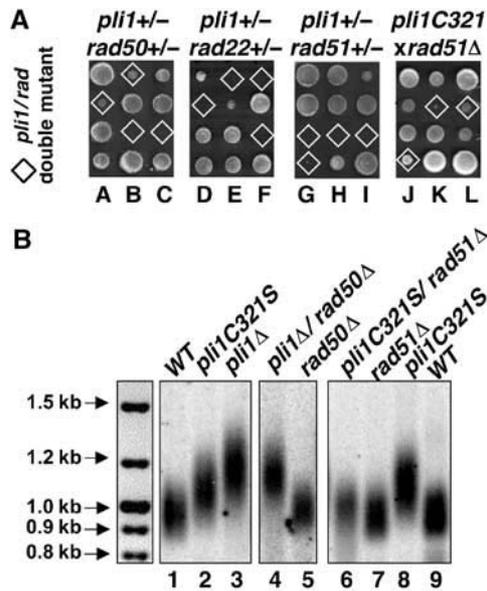


Figure 6 Genetic interactions of *pli1* and recombination genes: effects on telomere length. **(A)** Genetic interaction of *pli1* with homologous recombination mutants. Three representative tetrads from diploids heterozygous for disruption of *pli1* and either *rad50* (BX16), *rad22* (BX18) or *rad51* (BX19), and three representative tetrads from the *rad51*(JAC1/51Δ) × *pli1C321S* (BX7) cross, as indicated. The genotype of each segregant was determined (or inferred) by replica plating on appropriate selective media and/or by PCR; double-mutant clones are indicated by diamonds. Given the close physical linkage of the *rad22* and *pli1* loci (0.05 Morgans), tetrad analysis of the *rad22* +/− *pli1* +/− diploid (250 tetrads) was complemented by extensive random spore analysis (data not shown). **(B)** Southern blot analysis of telomere length in (1) WT (PB10); (2) *pli1C321S* (BX7); (3) *pli1Δ* (BX1); (4) *pli1Δ/rad50Δ* (BX20); (5) *rad50Δ* (BX21); (6) *pli1C321S/rad51Δ* (BX22_1); (7) *rad51Δ* (BX22_2); (8) *pli1C321SΔ* (BX22_3); (9) WT (BX22_4). BX22_1–4 correspond to the four clones of a tetra-type tetrad from the *rad51*(JAC1/51Δ) × *pli1C321S* (BX7) cross. After digestion with *EcoRI*, genomic DNA was subjected to electrophoresis on 1% agarose gel, transferred to a nylon membrane, and hybridized to a telomeric DNA probe (Cooper *et al*, 1997).

furthermore provide evidence that Pli1p and hence SUMO modification might play an important structural role at the centromere domain required for kinetochore assembly (central core), and in the protection of heterochromatic repeated sequences from illegitimate recombination.

***Pli1p* and the centromere**

The centromere is the chromosomal site of kinetochore assembly which allows spindle microtubule attachment and chromosome segregation to daughter cells. Centromeres of fission yeast and higher eucaryotes are composed of large repetitive DNA domains packaged into a heterochromatic structure (Clarke, 1990; Pluta *et al*, 1995; Bjerling and Ekwall, 2002). *S. pombe* centromeres (40–100 kb) exhibit a symmetrical organisation with a central core (*cnt* + *imrs*) flanked by outer repeats (*otrs*). Although the whole centromeric region is refractory to transcription, the molecular mechanisms underlying silencing seem to be quite distinct between the two domains. Indeed, the *clr4* (Su(var)3–9 homolog) and *swi6* (HP1 homolog) mutants affect silencing over the *otr* repeats as well as the *mat2/3* inactive sexual locus, rDNA and the telomeres, but not over the central core.

Moreover, in contrast to the *otrs*, the central core has an unusual chromatin structure lacking regularly spaced nucleosomes, likely due to the presence of the histone H3-variant Cnp1p (CENP-A), believed to form the primary kinetochore-building scaffold. Consistent with this, the central core domain is strictly required for minimal centromeric function (Chikashige *et al*, 1989; Niwa *et al*, 1989; Clarke and Baum, 1990; Allshire *et al*, 1994, 1995; Baum *et al*, 1994; Ekwall *et al*, 1999; Partridge *et al*, 2000). Our results indicate a specific role of Pli1p for silencing at the central domain of centromeres, since, of all known heterochromatic loci tested (*cnt1*, *imr1R*, *otr1R*, *mat2-3*, rDNA, telomeres), only the *imr1R* and *cnt1* exhibited reduced silencing of the inserted *ura4* gene in *pli1* deficient cells. Furthermore, *pli1Δ* cells also exhibit TBZ sensitivity and high rate of minichromosome loss, which might result from a defect in establishment or maintenance of proper central core structure for centromere and/or kinetochore function. Interestingly, the double *pli1Δswi6Δ* mutant showed a striking increase in TBZ sensitivity compared to the single mutants alone (data not shown). This synergistic attenuation of centromere function suggests that Swi6p and Pli1p operate in separable pathways and thus correlates well with the differential effects of Pli1p at the central core versus the outer repeats.

***Pli1p* and recombination**

Like in many organisms, *S. pombe* telomeres are composed of short tracts of repeated sequences organized into a non-nucleosomal chromatin structure by the mediation of the TRF-like protein Taz1p (Cooper *et al*, 1997). Telomere length is maintained by highly regulated mechanisms as mutations affecting telomerase, telomere-specific binding proteins, silencing, replication, DNA repair and checkpoint proteins lead to either lengthening or shortening of telomeres and, furthermore, to senescence (reviewed in Blackburn, 2001; Henson *et al*, 2002). We have shown here that, while *pli1* cells exhibit only a slight increase in reporter gene (*ura4*) silencing, they do manifest a consistent increase of telomere length, for which Rad51p, but not Rad50p, seems to be required. These genetic interactions strongly suggest that the increased telomere length in *pli1* cells might result from normally repressed gene conversion events in which the 3' end of a telomere illegitimately invades a homologous telomeric repeat array (Henson *et al*, 2002). Moreover, we found that the *ura4* variegation reporter gene at the central core of centromeres was lost in the *pli1* genetic background by restoring the wild type *imrR* or *cnt1* sequences. This strongly suggests that *ura4* deletion had occurred upon gene conversion events using the respective homologous *imr1L* and *cnt3* sequences as donors of information. While recombination events at the central core show an increase by at least 10-fold, recombination rates at a euchromatic context seem to be unaffected by the absence of *pli1*. Thus, the effect of Pli1p on recombination may be region specific and could be explained by two, albeit nonmutually exclusive mechanisms. First, Pli1p may exert an indirect effect on replication and/or chromatin structure that leads to an increased number of recombinogenic substrates at the central core and at the telomere in *pli1* cells. Second, Pli1p might directly regulate the recombination machinery by actively inhibiting the activity of recombination proteins at these repeated sequences. The finding of enhanced apparent gene conversion at centromeres and

telomeres is consistent with either hypothesis, while the strong genetic interaction of *pli1* mutants with *rad50*, *rad51* and *rad22* (*RAD52* fission yeast homolog), that range from strongly impaired mitotic growth (*rad50*) to lethality (*rad22*, *rad51*), mostly favors the second hypothesis and suggests that homologous recombination is necessary for survival of *pli1Δ* cells. Similarly, a negative genetic interaction between *ulp1-1615N* and all the *RAD51*-dependent recombination pathway mutants in *S. cerevisiae* has been described recently (Soustelle *et al*, 2004). Given that both Rad22p and Rad51p also represent possible SUMO substrates *in vivo* (Ho *et al*, 2001), it will be interesting to study the centromere and telomere phenotypes of nonmodifiable versions of these proteins.

Pli1p and sumoylation

The Pli1p protein fulfills the criteria for SUMO E3 ligase activity in that it interacts with both the E2 enzyme (Hus5p/Ubc9p) and the substrate and increases the rate of SUMO modification in an *in vitro* reconstituted *S. pombe* sumoylation assay. The *in vivo* significance of Pli1p is witnessed by the drastic reduction of global sumoylation in *pli1*-deficient cells. Despite this striking decrease of SUMO conjugates, *pli1*-deficient cells failed to show severe mitotic growth defects, in contrast to *pmt3*- (SUMO) and *hus5*- (Ubc9) deleted cells (al-Khodairy *et al*, 1995; Tanaka *et al*, 1999), which are almost inviable. This is reminiscent of *S. cerevisiae* *pli1* homologs, *siz1* and *siz2*, in that both single- and double-deletion mutants grow and have few obvious phenotypes (Johnson and Gupta, 2001). Nonetheless, the *pli1* deletion phenotypes recapitulate some of the *pmt3* phenotypes and characteristics, albeit in a milder way. First, *pli1Δ* cells have telomeres of intermediate length between wild-type and *pmt3Δ* cells. Second, *pli1Δ* cells exhibit intermediate levels of minichromosome loss and sensitivity to TBZ. Therefore, the milder pleiotropic effects of *pli1Δ* cells may be due to attenuated sumoylation of target proteins involved in centromeric and telomeric functions and possibly in the recombination process. It remains possible that the phenotypes observed in *pli1Δ* cells stem from a physiological activity of Pli1p that is independent from its role in sumoylation. We consider this unlikely since we observe a very strong correlation between the levels of SUMO E3 ligase activity of Pli1 proteins mutated at the RING domain and the described phenotypes: TBZ sensitivity, minichromosome loss, silencing at the central core, centromere sequence instability and telomere length regulation. In this regard, some of the first evidence implicating SUMO in recombination and/or centromere function was obtained by yeast two-hybrid screens with human Rad51 or Rad52 as baits and from screens for high-copy suppressors of temperature-sensitive alleles of the budding yeast *MIF2* or of its human homolog, the centromeric CENP-C protein (Meluh and Koshland, 1995; Shen *et al*, 1996).

The discovery that Pli1p and, by extension, sumoylation, plays a role in regulating recombination, opens the way to identifying the SUMO targets involved, as well as the mechanisms by which sumoylation controls their activity. The study of such targets and of their nonmodifiable mutant counterparts should provide further valuable insight into the structure and function of centromeres and telomeres.

Materials and methods

Fission yeast strains, plasmids, media and methods

The *S. pombe* strains used in this study are listed in Table I. Growth, maintenance and standard genetic methods for fission yeast strains were as described by Alfa *et al* (1993). Details regarding strain constructions are available upon request.

In vitro sumoylation assays

DNA sequences encoding Pmt3p, the sumoylation E1 (Rad31p/Fub2p heterodimer), E2 (Hus5p) and E3 (Pli1p) enzymes and Rad22p (the sumoylation substrate used here) were obtained by reverse transcription-polymerase chain reaction (RT-PCR) from *S. pombe* total RNA using specific oligonucleotide primers for first-strand synthesis and amplification. A Pmt3GG-encoding cDNA was cloned into pQE-30 (Qiagen) for the bacterial expression of a His₆-Pmt3-GG fusion protein. cDNAs encoding Rad31p and Fub2p were fused in-frame with a short linker and cloned into vector pGEX2T (Pharmacia) to produce a functional GST-Rad31p-Fub2p fusion protein. Hus5p- and Pli1p-encoding cDNAs were cloned into vector pGEXcs that contains a tobacco etch virus (TEV) protease cleavage site. Site-directed mutagenesis (Stratagene QuikChange) was performed to produce Pli1 RING finger point mutations C321S and C321S/H323A/C326S. A Rad22p cDNA was cloned into PING14A (Hagemeyer *et al*, 1993) for *in vitro* transcription/translation using Sp6 RNA polymerase and the TNT-coupled reticulocyte lysate system (Promega). All constructions were verified by sequencing and detailed maps are available upon request. His₆-Pmt3GG, the mature form of *S. pombe* SUMO, was expressed in *Escherichia coli* strain M15 (Qiagen) and purified on Ni-NTA agarose (Qiagen) using the manufacturer's protocol. Glutathione-S-transferase (GST) fusion proteins were expressed in strain BL21(DE3)/pLysS and purified using standard methods. Hus5p and Pli1p were cleaved from GST with GST-TEV protease and separated from free GST and GST-TEV by passage over glutathione (GSH-) agarose beads. All bacterially produced proteins were concentrated, dialyzed extensively against 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, and protein concentrations were estimated colorimetrically against a BSA standard (BioRad). *In vitro* sumoylation reactions were carried out using 4 μl of a standard ³⁵S-methionine-labeled, *in vitro*-translated Rad22p preparation, 1 μg His₆-Pmt3GG and the indicated amounts of E1, E2 and E3 proteins in a 22 μl reaction volume in a buffer containing 50 mM Tris (pH 7.5), 5 mM MgCl₂, 5 mM ATP and incubated for 1 h at 37°C. Reactions terminated by boiling in Laemmli buffer were subjected to SDS-PAGE and autoradiography. Percent modification was determined by PhosphorImager analysis using ImageQuant software.

Fluorescence microscopy

Cells grown in exponential conditions to 5 × 10⁶ cells/ml in MM were fixed with 3.6% paraformaldehyde at room temperature for 45 min. Cells processed as described (Alfa *et al*, 1993) were sequentially stained with an anti-GFP rabbit primary antibody (Molecular Probes), a FITC conjugated anti-rabbit IgG secondary antibody (Jackson Laboratory) and DAPI. Images were acquired with a Hamamatsu ORCAII-ER cooled CCD camera controlled by the Openlab[®] software (version 3.2, Improvion) and processed using Adobe Photoshop[®] software (version 6, Adobe).

Preparation of S. pombe whole-cell extracts and Western blot analysis

In all, 10⁸ exponentially growing cells were harvested, washed with water and TNET + (200 mM NaCl, 0.1% Triton, 0.01% SDS, 50 mM Tris-HCl (pH 8) supplemented with protease inhibitor Cocktail (Dove *et al*, 1998) and 10 mM NEM), resuspended in 200 μl of TNET+, broken with 500 μl of glass beads in a Fastprep[®] Apparatus and clarified by 10 min centrifugation at 13 000 r.p.m. In all, 20 μl of the WCE in 1 × Laemmli buffer and heated for 5 min at 90°C was separated by SDS-PAGE and analyzed by Western blotting with anti-GFP (Molecular Probes) or anti-Pmt3p rabbit primary antibody and a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham).

Determination of minichromosome loss rates

Ch16 is a linear minichromosome containing the *ade6-216* mutation that suppresses the *ade6-210* mutation by intra-allelic complemen-

Table 1 *S. pombe* strains

Strain	Genotype	Reference
PB10	<i>h⁻ (Msmt0) leu1-32 ade6-210</i>	Lab stock
PB46	<i>h⁹⁰ ura4-D18 leu1-32 ade6-216</i>	Lab stock
BX1	<i>h⁻ (MsmtO) pli1Δ::kanMX6 leu1-32 ade6-210</i>	This work
BX2	<i>h⁹⁰ pli1Δ::kanMX6 ura4-D18 leu1-32 ade6-210</i>	This work
BX3	<i>h⁹⁰ pli1-HA::kanMX6 ura4-D18 leu1-32 ade6-210</i>	This work
BX4	<i>h⁹⁰ pli1-CFP::kanMX6 ura4-D18 leu1-32 ade6-216</i>	This work
BX5	<i>h⁹⁰ His₆-pmt3 ura4-D18 leu1-32 ade6-210</i>	This work
BX6	<i>h⁹⁰ pli1C321S leu1-32 ade6-216</i>	This work
BX7	<i>h⁻ (MsmtO) pliC321S ura4-D18 leu1-32 ade6-210</i>	This work
JS161	<i>h⁻ ura4-D18 leu1-32 ade6-210 [Ch16 ade6-216]</i>	Matsumoto <i>et al</i> (1987)
BX8	<i>h³ pli1Δ::kanMX6 ura4-D18 leu1-32 ade6-210 [Ch16 ade6-216]</i>	This work
BX9	<i>h³ pli1C321S ura4-D18 leu1-32 ade6-210 [Ch16 ade6-216]</i>	This work
FY336	<i>h⁻ ura4-DS/E leu1-32 ade6-210 cnt1TM1(Nco1)::ura4</i>	Allshire <i>et al</i> (1994)
PG2964	<i>h³ pli1Δ::kanMX6 ura4-DS/E leu1-32 ade6-216 cnt1TM1(Nco1)::ura4</i>	This work
BX10	<i>h³ pli1C321S ura4-DS/E leu1-32 ade6-216 cnt1TM1(Nco1)::ura4</i>	This work
BX11_1-20	<i>h³ pli1Δ::kanMX6 ura4-DS/E leu1-32 ade6-216 isolated from PG2964</i>	This work
FY498	<i>h⁺ ura4-DS/E leu1-32 ade6-210 imr1(Nco1)::ura4ori1</i>	Allshire <i>et al</i> (1995)
PG2972	<i>h³ pli1Δ::kanMX6 ura4-DS/E leu1-32 ade6-210 imr1(Nco1)::ura4ori1</i>	This work
BX12	<i>h³ pli1C321S ura4-DS/E leu1-32 ade6-210 imr1(Nco1)::ura4ori1</i>	This work
BX13_1-20	<i>h³ pli1Δ::kanMX6 ura4-DS/E leu1-32 ade6-210 isolated from PG2972</i>	This work
FY648	<i>h⁺ ura4-DS/E leu1-32 ade6-210 otr1R(Sph1)::ura4</i>	Allshire <i>et al</i> (1995)
PG2976	<i>h³ pli1Δ::kanMX6 ura4-DS/E leu1-32 ade6-210 otr1R(Sph1)::ura4</i>	This work
BX14	<i>h³ pli1C321S ura4-DS/E leu1-32 ade6-210 otr1R(Sph1)::ura4</i>	This work
FY521	<i>h⁻ ura4-DS/E leu1-32 ade6-210 [Ch16 ade6-216, m23::ura4-Tel[72]]</i>	Nimmo <i>et al</i> (1994)
PG2994	<i>h⁺ pli1Δ::kanMX6 ura4-DS/E leu1-32 ade6-210 [Ch16 ade6-216, m23::ura4-Tel[72]]</i>	This work
BX15	<i>h³ pli1Δ::kanMX6 ura4-DS/E leu1-32 ade6-210 [Ch16 ade6-216, m23::ura4-Tel[72]]</i>	This work
BX16	<i>h⁺/h⁻ (MsmtO) ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-210/ade6-216 pli1 +/-::kanMX6 rad50 +/-::LEU2</i>	This work
BX17	<i>h⁺/h⁻ (MsmtO) ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-210/ade6-216 pli1 +/-::kanMX6 rad22 +/-::LEU2</i>	This work
BX18	<i>h⁺/h⁻ (MsmtO) ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-210/ade6-216 pli1 +/-::kanMX6 rad22 +/-::LEU2</i>	This work
BX19	<i>h⁺/h⁻ (MsmtO) ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-210/ade6-216 pli1 +/-::kanMX6 rad51 +/-::ura4</i>	This work
BX20	<i>h⁺ ura4-D18 leu1-32 ade210 pli1Δ::kanMX6 rad50Δ::LEU2</i>	This work
BX21	<i>h⁺ ura4-D18 leu1-32 ade210 rad50Δ::LEU2</i>	This work
JAC1/51Δ	<i>h⁺ ura4-D18 leu1-32 ade6-704 rad51Δ::ura4⁺</i>	Kim <i>et al</i> (2002)
BX22_1	<i>h⁺ ura4-D18 leu1-32 ade6-? pli1C321S rad51Δ::ura4⁺</i>	This work
BX22_2	<i>h⁻ (Msmt0) ura4-D18 leu1-32 ade6-? rad51Δ::ura4⁺</i>	This work
BX22_3	<i>h⁻ (Msmt0) ura4-D18 leu1-32 ade6-? pli1C321S</i>	This work
BX22_4	<i>h⁺ ura4-D18 leu1-32 ade6-?</i>	This work
SL1	<i>h⁺ ura4-D18 leu1-32 ade6-M26 int::pUC8/ura4/MATa/ade6-L469</i>	Osman <i>et al</i> (1996)
BX23	<i>h⁺ ura4-D18 leu1-32 ade6-M26 int::pUC8/ura4/MATa/ade6-L469 pli1Δ::kanMX6</i>	This work

tation (Matsumoto *et al*, 1987). The rate of Ch16 loss was estimated by a colony color assay described by Tanaka *et al* (1999) with slight modifications. Strains were grown in MM lacking adenine to select for Ch16-harboring cells. Single colonies were dispersed in 1 ml of MM without adenine and cell number was measured in a Coulter[®] cell counter. The cells were then diluted into YE medium and immediately plated onto YE plates at a concentration of $\sim 10^3$ cells/plate. After 4 days growth at 33°C, the proportion of [ade-] (completely red) colonies was determined.

Silencing assays

Series of three- or five-fold dilutions of *S. pombe* strains grown exponentially in YES at 33°C were spotted onto AA, AA lacking uracil or 5'-FOA plates, which were then incubated at 33°C until full growth was achieved.

Isolation of stable independent [ura-] cells

S. pombe strains containing the *ura4* reporter gene inserted at *cnt1* or *imr1* were grown to saturation in YES at 33°C and plated onto

YES plates at a concentration of $\sim 10^3$ cells/plate. After 4 days growth at 33°C, colonies were replica plated onto 5'-FOA. FOA^R papillae arising from independent colonies were consecutively streaked on 5'-FOA and YES plates and replica plated on AA lacking uracil.

Southern blot analyses

S. pombe strains were exponentially grown in MM or YES at 33°C and DNA was prepared either in agarose plugs (Young *et al*, 2002) or by standard methods (Moreno *et al*, 1991). For pulsed field electrophoresis, DNA prepared and digested in agarose plugs was resolved in 1.2% agarose gels with a Pulsafor[™] system (Pharmacia) in 0.15 × TBE at 360 V with a pulse time of 0.5 s for 6 h at 9°C. After electrophoresis, the DNA was transferred to Hybond-N⁺ filters (Amersham) and crosslinked with UV Stratalinker (Stratagene). Following DNA-DNA hybridization, the membranes were exposed on a phosphor screen and the signal detected by PhosphorImager 445SI (Molecular Dynamics) and displayed using ImagequantNT. The sequences of all the probes used in this study are available upon request.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We are grateful to K Tanaka for the *pmt3* +/− strain, A Pastink for the intrachromosomal recombination assay, SD Park for the *rad51Δ* strain, O Gadal, F Feuerbach-Fournier, O Danot, N Joly and M

Gilbert for technical advice and useful comments on the manuscript. This work was supported by a fellowship from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie (MENRT) to BX; the Novo Nordisk Foundation and Danish Research Council to GT; the Pasteur-Negri-Weizmann Council, the EEC 5th Framework Programme to AD, the Human Frontiers in Science Program (HFSP) and the Association pour la Recherche sur le Cancer (ARC) to BA.

References

- al-Khodairy F, Enoch T, Hagan IM, Carr AM (1995) The *Schizosaccharomyces pombe* hus5 gene encodes a ubiquitin conjugating enzyme required for normal mitosis. *J Cell Sci* **108**: 475–486
- Alfa CE, Gallagher IM, Hyams JS (1993) Antigen localization in fission yeast. *Methods Cell Biol* **37**: 201–222
- Allshire RC, Javerzat JP, Redhead NJ, Cranston G (1994) Position effect variegation at fission yeast centromeres. *Cell* **76**: 157–169
- Allshire RC, Nimmo ER, Ekwall K, Javerzat JP, Cranston G (1995) Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. *Genes Dev* **9**: 218–233
- Bachant J, Alcasabas A, Blat Y, Kleckner N, Elledge SJ (2002) The SUMO-1 isopeptidase Smt4 is linked to centromeric cohesion through SUMO-1 modification of DNA topoisomerase II. *Mol Cell* **9**: 1169–1182
- Baum M, Ngan VK, Clarke L (1994) The centromeric K-type repeat and the central core are together sufficient to establish a functional *Schizosaccharomyces pombe* centromere. *Mol Biol Cell* **5**: 747–761
- Bjerling P, Ekwall K (2002) Centromere domain organization and histone modifications. *Braz J Med Biol Res* **35**: 499–507
- Blackburn EH (2001) Switching and signaling at the telomere. *Cell* **106**: 661–673
- Chikashige Y, Kinoshita N, Nakaseko Y, Matsumoto T, Murakami S, Niwa O, Yanagida M (1989) Composite motifs and repeat symmetry in *S. pombe* centromeres: direct analysis by integration of *NotI* restriction sites. *Cell* **57**: 739–751
- Clarke L (1990) Centromeres of budding and fission yeasts. *Trends Genet* **6**: 150–154
- Clarke L, Baum MP (1990) Functional analysis of a centromere from fission yeast: a role for centromere-specific repeated DNA sequences. *Mol Cell Biol* **10**: 1863–1872
- Cooper JP, Nimmo ER, Allshire RC, Cech TR (1997) Regulation of telomere length and function by a Myb-domain protein in fission yeast. *Nature* **385**: 744–747
- De Lahondes R, Ribes V, Arcangioli B (2003) Fission yeast sap1 protein is essential for chromosome stability. *Eukaryot Cell* **2**: 910–921
- Dove JE, Brockenbrough JS, Aris JP (1998) Isolation of nuclei and nucleoli from the yeast *Saccharomyces cerevisiae*. *Methods Cell Biol* **53**: 33–46
- Ekwall K, Cranston G, Allshire RC (1999) Fission yeast mutants that alleviate transcriptional silencing in centromeric flanking repeats and disrupt chromosome segregation. *Genetics* **153**: 1153–1169
- Gill G (2003) Post-translational modification by the small ubiquitin-related modifier SUMO has big effects on transcription factor activity. *Curr Opin Genet Dev* **13**: 108–113
- Hagemeyer C, Cook A, Kouzarides T (1993) The retinoblastoma protein binds E2F residues required for activation *in vivo* and TBP binding *in vitro*. *Nucleic Acids Res* **21**: 4998–5004
- Hari KL, Cook KR, Karpen GH (2001) The *Drosophila* Su(var)2-10 locus regulates chromosome structure and function and encodes a member of the PIAS protein family. *Genes Dev* **15**: 1334–1348
- Henson JD, Neumann AA, Yeager TR, Reddel RR (2002) Alternative lengthening of telomeres in mammalian cells. *Oncogene* **21**: 598–610
- Ho JC, Warr NJ, Shimizu H, Watts FZ (2001) SUMO modification of Rad22, the *Schizosaccharomyces pombe* homologue of the recombination protein Rad52. *Nucleic Acids Res* **29**: 4179–4186
- Hochstrasser M (2001) SP-RING for SUMO: new functions bloom for a ubiquitin-like protein. *Cell* **107**: 5–8
- Hoegge C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**: 135–141
- Jackson PK (2001) A new RING for SUMO: wrestling transcriptional responses into nuclear bodies with PIAS family E3 SUMO ligases. *Genes Dev* **15**: 3053–3058
- Johnson ES, Gupta AA (2001) An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* **106**: 735–744
- Kagey MH, Melhuish TA, Wotton D (2003) The polycomb protein Pc2 is a SUMO E3. *Cell* **113**: 127–137
- Kim WJ, Park EJ, Lee H, Seong RH, Park SD (2002) Physical interaction between recombinational proteins Rhp51 and Rad22 in *Schizosaccharomyces pombe*. *J Biol Chem* **277**: 30264–30270. Epub 32002 Jun 30265
- Kirsh O, Seeler JS, Pichler A, Gast A, Müller S, Miska E, Mathieu M, Harel-Bellan A, Kouzarides T, Melchior F, Dejean A (2002) The SUMO E3 ligase RanBP2 promotes modification of the HDAC4 deacetylase. *EMBO J* **21**: 2682–2691
- Kotaja N, Karvonen U, Janne OA, Palvimo JJ (2002) PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. *Mol Cell Biol* **22**: 5222–5234
- Kyrion G, Liu K, Liu C, Lustig AJ (1993) RAP1 and telomere structure regulate telomere position effects in *Saccharomyces cerevisiae*. *Genes Dev* **7**: 1146–1159
- Matsumoto T, Fukui K, Niwa O, Sugawara N, Szostak JW, Yanagida M (1987) Identification of healed terminal DNA fragments in linear minichromosomes of *Schizosaccharomyces pombe*. *Mol Cell Biol* **7**: 4424–4430
- Melchior F (2000) SUMO—nonclassical ubiquitin. *Annu Rev Cell Dev Biol* **16**: 591–626
- Meluh PB, Koshland D (1995) Evidence that the MIF2 gene of *Saccharomyces cerevisiae* encodes a centromere protein with homology to the mammalian centromere protein CENP-C. *Mol Biol Cell* **6**: 793–807
- Moreno S, Klar A, Nurse P (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol* **194**: 795–823
- Müller S, Hoegge C, Pyrowolakis G, Jentsch S (2001) SUMO, ubiquitin's mysterious cousin. *Nat Rev Mol Cell Biol* **2**: 202–210
- Nimmo ER, Cranston G, Allshire RC (1994) Telomere-associated chromosome breakage in fission yeast results in variegated expression of adjacent genes. *EMBO J* **13**: 3801–3811
- Niwa O, Matsumoto T, Chikashige Y, Yanagida M (1989) Characterization of *Schizosaccharomyces pombe* minichromosome deletion derivatives and a functional allocation of their centromere. *EMBO J* **8**: 3045–3052
- Osman F, Fortunato EA, Subramani S (1996) Double-strand break-induced mitotic intrachromosomal recombination in the fission yeast *Schizosaccharomyces pombe*. *Genetics* **142**: 341–357
- Partridge JF, Borgstrom B, Allshire RC (2000) Distinct protein interaction domains and protein spreading in a complex centromere. *Genes Dev* **14**: 783–791
- Pichler A, Gast A, Seeler JS, Dejean A, Melchior F (2002) The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell* **108**: 109–120
- Pluta AF, Mackay AM, Ainsztein AM, Goldberg IG, Earnshaw WC (1995) The centromere: hub of chromosomal activities. *Science* **270**: 1591–1594
- Sapetschnig A, Rischitor G, Braun H, Doll A, Schergaut M, Melchior F, Suske G (2002) Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *EMBO J* **21**: 5206–5215
- Seeler JS, Dejean A (2003) Nuclear and unclear functions of SUMO. *Nat Rev Mol Cell Biol* **4**: 690–699
- Shayeghi M, Doe CL, Tavassoli M, Watts FZ (1997) Characterisation of *Schizosaccharomyces pombe* rad31, a UBA-related gene required for DNA damage tolerance. *Nucleic Acids Res* **25**: 1162–1169

- Shen Z, Pardington-Purtymun PE, Comeaux JC, Moyzis RK, Chen DJ (1996) UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. *Genomics* **36**: 271–279
- Shiio Y, Eisenman RN (2003) Histone sumoylation is associated with transcriptional repression. *Proc Natl Acad Sci USA* **100**: 13225–13230. Epub 12003 October 13224
- Soustelle C, Vernis L, Freon K, Reynaud-Angelin A, Chanet R, Fabre F, Heude M (2004) A new *Saccharomyces cerevisiae* strain with a mutant Smt3-deconjugating Ulp1 protein is affected in DNA replication and requires Srs2 and homologous recombination for its viability. *Mol Cell Biol* **24**: 5130–5143
- Stead K, Aguilar C, Hartman T, Drexel M, Meluh P, Guacci V (2003) Pds5p regulates the maintenance of sister chromatid cohesion and is sumoylated to promote the dissolution of cohesion. *J Cell Biol* **163**: 729–741. Epub 2003 November 2017
- Stelter P, Ulrich HD (2003) Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature* **425**: 188–191
- Strunnikov AV, Aravind L, Koonin EV (2001) *Saccharomyces cerevisiae* SMT4 encodes an evolutionarily conserved protease with a role in chromosome condensation regulation. *Genetics* **158**: 95–107
- Takahashi K, Murakami S, Chikashige Y, Funabiki H, Niwa O, Yanagida M (1992) A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere. *Mol Biol Cell* **3**: 819–835
- Takahashi Y, Toh-e A, Kikuchi Y (2001) A novel factor required for the SUMO1/Smt3 conjugation of yeast septins. *Gene* **275**: 223–231
- Tanaka K, Nishide J, Okazaki K, Kato H, Niwa O, Nakagawa T, Matsuda H, Kawamukai M, Murakami Y (1999) Characterization of a fission yeast SUMO-1 homologue, pmt3p, required for multiple nuclear events, including the control of telomere length and chromosome segregation. *Mol Cell Biol* **19**: 8660–8672
- van den Bosch M, Zonneveld JB, Vreeken K, de Vries FA, Lohman PH, Pastink A (2002) Differential expression and requirements for *Schizosaccharomyces pombe* RAD52 homologs in DNA repair and recombination. *Nucleic Acids Res* **30**: 1316–1324
- Verger A, Perdomo J, Crossley M (2003) Modification with SUMO. *EMBO Rep* **4**: 137–142
- Young JA, Schreckhise RW, Steiner WW, Smith GR (2002) Meiotic recombination remote from prominent DNA break sites in *S. pombe*. *Mol Cell* **9**: 253–263