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End-joining inhibition at telomeres requires the translocase and polySUMO-dependent ubiquitin ligase Uls1

Rachel Lescasse[1,2], Sabrina Pobiega[1,2], Isabelle Callebaut[3] and Stéphane Marcand[1,2,*]

[1]CEA, Direction des sciences du vivant/Institut de radiobiologie cellulaire et moléculaire/Service instabilité génétique réparation recombinaison/Laboratoire télomère et réparation du chromosome, Fontenay-aux-roses, France, [2]CNRS, UMR 217, Fontenay-aux-roses, France and [3]CNRS, UMR 7590, Institut de minéralogie et de physique des milieux condensés, Université Pierre et Marie Curie, Paris, France

In eukaryotes, permanent inhibition of the non-homologous end joining (NHEJ) repair pathway at telomeres ensures that chromosome ends do not fuse. In budding yeast, binding of Rap1 to telomere repeats establishes NHEJ inhibition. Here, we show that the Uls1 protein is required for the maintenance of NHEJ inhibition at telomeres. Uls1 protein is a non-essential Swi2/Snf2-related translocase and a Small Ubiquitin-related Modifier (SUMO)-Targeted Ubiquitin Ligase (STUbL) with unknown targets. Loss of Uls1 results in telomere–telomere fusions. Uls1 requirement is alleviated by the absence of poly-SUMO chains and by *rap1* alleles lacking SUMOylation sites. Furthermore, Uls1 limits the accumulation of Rap1 poly-SUMO conjugates. We propose that one of Uls1 functions is to clear non-functional poly-SUMOylated Rap1 molecules from telomeres to ensure the continuous efficiency of NHEJ inhibition. Since Uls1 is the only known STUbL with a translocase activity, it can be the general molecular sweeper for the clearance of poly-SUMOylated proteins on DNA in eukaryotes.

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Introduction

Inhibition of the non-homologous end joining (NHEJ) repair pathway at telomeres ensures that chromosome ends do not fuse (Jain and Cooper, 2010). The necessity to be continuously efficient is a key feature of NHEJ inhibition at telomeres since a temporary lapse at two telomeres could result in a telomere fusion. Although this accident is sometimes reversible (Pobiega and Marcand, 2010), it poses a major challenge to genome stability. Protection against telomere fusions depends mostly on preventing fusions from occurring. Proteins bound to telomeric repeated sequences establish this strong *cis*-inhibition. Its strength and reliability are the result of in part the multiplicity of DNA-bound molecules at each telomere and in part the synergy between several pathways of inhibition. In fission yeast, telomere repeats are bound by multiple Taz1 proteins that recruit Rap1 proteins. Both factors are required for NHEJ inhibition (Miller *et al*, 2005; Fujita *et al*, 2012). In mammals, NHEJ inhibition is established by the binding of multiple TRF2 proteins to the double-stranded telomere repeats (van Steensel *et al*, 1998; Celli and de Lange, 2005; Bae and Baumann, 2007; Sfeir and de Lange, 2012). Mammalian RAP1, recruited to telomeres by TRF2, is not essential for NHEJ inhibition (Sfeir *et al*, 2010). Nevertheless, an artificial targeting of RAP1 to telomeres lacking TRF2 is sufficient to inhibit NHEJ, suggesting that TRF2 establishes several redundant pathways including one involving RAP1 (Sarthy *et al*, 2009). In the budding yeast *Saccharomyces cerevisiae*, Rap1 binds telomeric DNA directly and establishes three genetically separable pathways to inhibit NHEJ (Marcand *et al*, 2008). Rap1 also protects telomere ends from 5′ to 3′ degradation and checkpoint signalling, and limits telomere elongation by telomerase (Teixeira *et al*, 2004; Negrini *et al*, 2007; Bonetti *et al*, 2010; Vodenicharov *et al*, 2010; Anbalagan *et al*, 2011; McGee *et al*, 2011; Ribeyre and Shore, 2012). In addition to its role at telomeres, Rap1 binds a large number of gene promoters where it plays an essential role in transcription (Lickwar *et al*, 2012).

In S. cerevisiae, the loss of the protein Uls1 results in a negative synthetic interaction with a *rap1* hypomorphic allele, suggesting a link between Uls1 and Rap1 functions (Costanzo *et al*, 2010). Uls1 (also called Ris1, Dis1 or Tid4) is a non-essential Swi2/Snf2-related translocase exhibiting a DNA-dependent ATPase activity (Zhang and Buchman, 1997; Shah *et al*, 2010). It interacts genetically with several genes required for homologous recombination (Collins *et al*, 2007; Costanzo *et al*, 2010; Shah *et al*, 2010; Cal-Bakowska *et al*, 2011) and homologous recombination was proposed to be a target for Uls1 translocase (Chi *et al*, 2011). Uls1 is also a Small Ubiquitin-related MOdifier (SUMO)-Targeted Ubiquitin Ligase (STUbL) (Uzunova *et al*, 2007). SUMOylation is a regulatory reversible post-translational modification linking the SUMO carboxy-terminus and the ε-amino group of a lysine in the target protein. Poly-SUMO chains can be formed through the SUMOylation of SUMO monomers (Bylebyl *et al*, 2003; Ulrich, 2008; Sun and Hunter, 2012). In budding yeast, two STUbLs Uls1 and Slx5/Slx8 recognize and ubiquitinylate for proteosomal degradation proteins conjugated to poly-SUMO chains (Uzunova *et al*, 2007; Xie *et al*, 2007; Mullen and Brill, 2008; Nagai *et al*, 2008; Ulrich, 2008). Proteins targeted by Uls1 remain to be identified.

*Corresponding author. Institut de radiobiologie cellulaire et moléculaire, CEA, Service instabilité génétique réparation recombinaison, CEA/Fontenay, Fontenay-aux-roses 92265, France. Tel.: +33 1 46 54 82 33; Fax: +33 1 46 54 91 80; E-mail: stephane.marcand@cea.fr

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The genetic interaction between *uls1* and *rap1* encouraged us to address a potential function of Uls1 at telomeres. Several studies have linked SUMO and telomere functions (Askree *et al*, 2004; Zhao and Blobel, 2005; Potts and Yu, 2007; Xhemalce *et al*, 2007; Rog *et al*, 2009; Ferreira *et al*, 2011). For instance, SUMOylation of yeast telomere single-strand binding protein Cdc13 regulates its interaction with its partner Stn1 to limit telomere elongation by telomerase during S phase (Hang *et al*, 2011). Since cells lacking Uls1 display normal telomere length (Askree *et al*, 2004), we addressed a defect in NHEJ inhibition. Here, we show that Uls1 activities are required for the maintenance of NHEJ inhibition at telomeres through the clearance of non-functional poly-SUMOylated Rap1 molecules.

Results

Loss of Uls1 causes telomere fusions by NHEJ

To address a defect in NHEJ inhibition at telomeres in cells lacking Uls1, we looked at the appearance of telomere fusions, which can to some extent be amplified by PCR despite their palindromic structures (Mieczkowski *et al*, 2003; Pardo and Marcand, 2005). All *S. cerevisiae* chromosome ends display a conserved X subtelomeric element. About half the chromosome ends contain one or several Y′ subtelomeric elements inserted between the X element and the telomere. We used two primers to amplify fusions between Y′ and X-only telomeres (Figure 1A). Cells were grown exponentially and then allowed to reach stationary phase. As shown in Figure 1B (top panel, 30 PCR cycles), telomere fusions are not detected in wild-type cells nor in *uls1*-Δ exponentially growing cells but are detected in stationary cells lacking Uls1. Telomere length being heterogeneous, amplified telomere fusions appear as a smeared signal. The lengths of the PCR products indicate that the mean length of telomeric repeats in the fusions is ∼500 bp, a length consistent with fusions occurring between telomeres close to the mean telomere length (∼300 bp). Dilution of the template DNA provides a semi-quantitative estimation of the method's sensitivity (Figure 1B, second panel from top, 34 PCR cycles; rarer fusions are amplified as discrete bands). The relative lack of fusions in growing *uls1*-Δ cells could either be due to a continuous counter-selection of fused chromosomes or a reduced fusion frequency or both. In the following experiments, the presence of telomere fusions was tested in cells in stationary phase.

To determine which repair pathway fuses telomeres, we combined the *uls1*-Δ mutant with an NHEJ defective *lif1*-Δ mutant. Lif1 loss suppresses the fusions caused by Uls1 loss (Figure 1C), indicating that fusions are produced by NHEJ. Thus, Uls1 is required for NHEJ inhibition at telomeres. To estimate how severely Uls1 loss challenges NHEJ inhibition, we compared the frequency of telomere fusions between *uls1*-Δ and *rap1*-(Δ) cells. The *rap1*-(Δ) mutant is a degron allele of *RAP1*. In *rap1*-(Δ) cells reaching stationary phase, Rap1 level drops and telomeres fuse at an approximate frequency of one fusion per cell (Pardo and Marcand, 2005; Pobiega and Marcand, 2010). As shown in Figure 1D, telomere fusions are detected in *rap1*-(Δ) cells with a lower number of PCR cycles than in *uls1*-Δ cells. A semi-quantitative estimation indicates that fusions are about a hundred times less frequent in *uls1*-Δ cells than in *rap1*-(Δ) cells in stationary phase, indicating that, relative to Rap1 loss, Uls1 loss causes a mild defect of NHEJ inhibition at telomeres.

Uls1 translocase and ubiquitin ligase activities are involved in NHEJ inhibition at telomeres

We next asked which activities of Uls1 are involved in NHEJ inhibition at telomeres (Figure 2A). The Uls1 protein possesses an ubiquitin E3 ligase RING domain and a Swi2/Snf2-like ATPase/translocase domain in its carboxy-terminal part (Zhang and Buchman, 1997; Uzunova *et al*, 2007; Shah *et al*, 2010). At least four SUMO-interacting motifs (SIM) are present in the amino-terminal region of the protein (Uzunova *et al*, 2007). An allele mutated within these SIMs still interacts with SUMO in a yeast two-hybrid assay (data not shown), suggesting that Uls1 may possess additional, less canonical SIMs that remain to be located (Sun and Hunter, 2012; Vogt and Hofmann, 2012). In the central region, we identified a new tandem domain that we propose to call REPULS for '*Rep*eats in *Uls*1'. Variable number of this domain is present in Uls1 sequences from other hemisascomycetes including *Kluyveromyces lactis* and *Ashbya gossypii* but is missing in the putative Uls1 orthologues from the more distantly related species *Debaromyces hansenii* and *Schizosaccharomyces pombe* (Figure 2A; Supplementary data).

To address the contribution of Uls1 ubiquitin E3 ligase activity, the second cysteine of the RING domain at position 1333 was mutated to a serine (Uzunova *et al*, 2007). To knocked down Uls1 translocase activity, the catalytic lysine at position 975 was mutated to an arginine. In cells carrying either mutation at the endogenous *uls1* locus, telomere fusions are detected. Although an effect of these point mutations on protein folding cannot be ruled out, this result suggests that both enzymatic activities of Uls1 are required for efficient NHEJ inhibition at telomeres (Figure 2B). The slightly lower frequencies of fusions observed in both mutants relative to the frequency observed in *uls1*-Δ cells indicate that each knock-down activity may not be systematically employed by Uls1 or that the point mutants conserve some enzymatic activity. To investigate REPULS domains involvement in NHEJ inhibition at telomeres, an *uls1-Δ677-855* allele lacking the tandem domains was created and integrated at the endogenous *uls1* locus. This mutant displays normal growth and telomere length (data not shown) and telomere fusions remain undetectable (Figure 2C). The simplest interpretation of this result is that the REPULS domains are not involved or are facultative in Uls1 function at telomeres. A more complicated possibility we cannot rule out is that the truncated uls1-Δ677-855 protein displays an aberrant activity that suppresses the occurrence of telomere fusions indirectly.

To further address the phenotype of these *uls1* alleles, we tested their ability to complement an *uls1* deletion. The wild-type and mutant *uls1* genes were inserted at an ectopic position in an *uls1*-Δ strain. The presence of a wild-type or an *uls1-Δ677-855* sequence abolishes the telomere fusions caused by the *uls1* deletion (Figure 2D). By contrast, the presence of an *uls1-K975R* or *uls1-C1333S* sequence has no obvious effect on the frequency of telomere fusions (a two-fold difference or less would be below the assay sensitivity). A synergy between these two point mutants and a mild

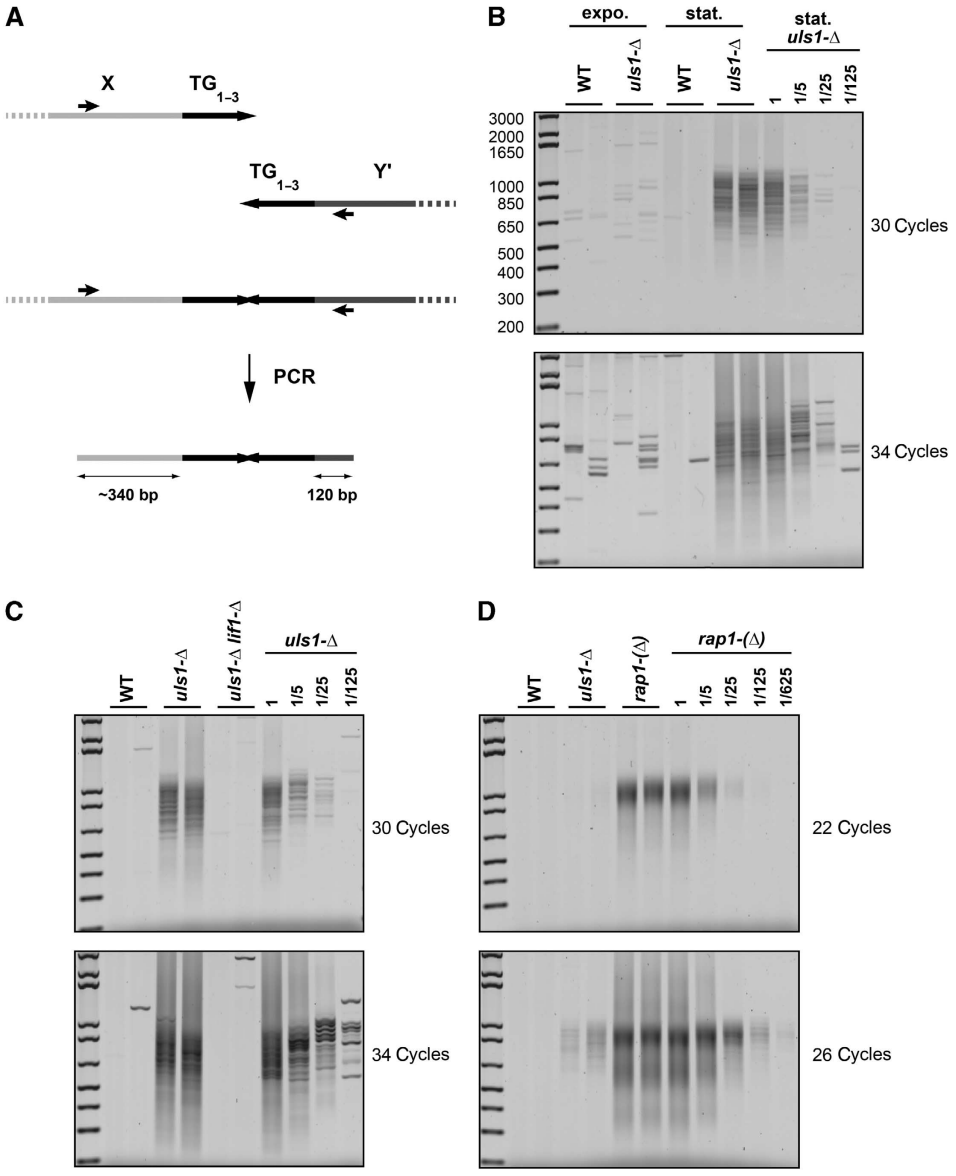


Figure 1 Loss of Uls1 causes telomere fusions by NHEJ. (**A**) Schematic representation of the relative positions of the primers used for PCR amplification. The average wild-type telomere length is ~300 bp. (**B**) Telomere fusions accumulate in stationary *uls1*-Δ cells. Two independent cultures of strains Lev346 (WT) and RL71 (*uls1*-Δ) were grown exponentially in rich medium (expo.) and allowed to reach stationary phase in 6 days (stat). Fusions between X and Y′ telomeres were amplified by PCR with 30 and 34 cycles. Genomic DNA from RL71 (*uls1*-Δ) cells in stationary phase was diluted serially to provide a semi-quantitative estimation of the method sensitivity. (**C**) Telomere fusions in *uls1*-Δ cells are NHEJ dependent. Strains 212-12a and 213-4a (WT), 213-7b and 213-9c (*uls1*-Δ) and 195-28a (*uls1*-Δ *lif1*-Δ) were grown to stationary phase. Telomere fusions were amplified with 30 and 34 cycles. Genomic DNA from 213-7b (*uls1*-Δ) cells was diluted serially. (**D**) Telomere fusions in *uls1*-Δ are less frequent than in *rap1*-(Δ) cells. Strains 210-3d and 211-1a (WT), 211-8d and 211-10b (*uls1*-Δ) and 209-1c and 209-2b (*rap1*-(Δ)) were grown to stationary phase. Telomere fusions were amplified with 22 and 26 cycles. Genomic DNA from 209-1c (*rap1*-(Δ)) cells was diluted serially to provide a semi-quantitative estimation of the relative difference of fusion frequencies.

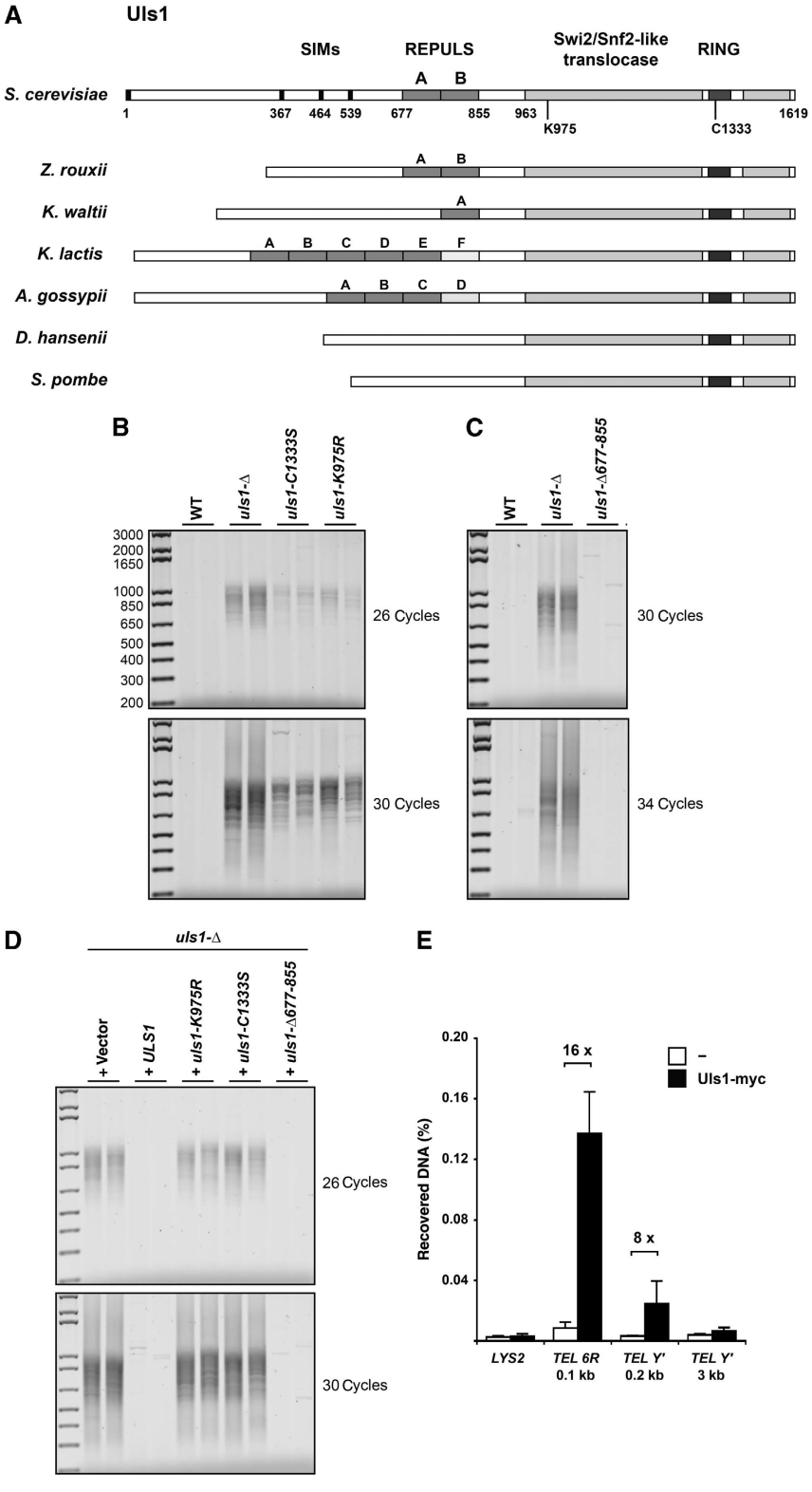
expression defect caused by the ectopic position might explain the stronger phenotype observed in this situation compared to the situation where the same mutants are integrated at the endogenous *ULS1* locus. Together, these results support the hypothesis that Uls1 function at telomeres involves its translocase and ubiquitin ligase activities and that the REPULS domains are facultative for this function.

Uls1 requirement for NHEJ inhibition suggests that the protein might be present at telomeres. We tested this possibility by cross-linked Chromatin Immuno-Precipitation (ChIP) directed against an epitope-tagged variant of Uls1. As shown in Figure 2E, sequences immediately adjacent to the X telomere *TEL 6R* and to the Y′ telomeres are specifically pulled down with Uls1. Sequences at an internal locus (*LYS2*) and 3 kb away from telomere within the Y′ subtelomeric elements are not significantly enriched. This shows that Uls1 is present at the tips of the chromosomes and suggests that Uls1 activities may target other proteins at telomeres.

The absence of poly-SUMOylation bypasses Uls1 requirement

In cells lacking Uls1, unidentified poly-SUMOylated proteins that would normally be degraded become detectable (Uzunova *et al*, 2007). We asked whether the role of Uls1 in NHEJ inhibition at telomeres is linked to its ability to downregulate poly-SUMOylated proteins. In

S. cerevisiae, a single gene, *SMT3*, encodes SUMO. SUMO polymerization occurs on three amino-terminal lysines of Smt3 at positions K11, K15 and K19. In the *smt3-3R* allele, these SUMOylatable lysines of SUMO are mutated to arginine, preventing poly-SUMO chains but preserving the essential functions of SUMO (Bylebyl *et al*, 2003; Ulrich, 2008). Since the *smt3-3R* allele suppresses the accumulation of poly-SUMOylated proteins in cells lacking Uls1 (Uzunova *et al*, 2007), we looked at telomere fusions in cells carrying this allele, either alone or in combination with an *uls1* deletion. As shown in Figure 3A, telomere fusions remain undetectable in *smt3-3R* cells. However, the *smt3-3R* allele abolishes the fusions caused by Uls1 loss. The *smt3-3R* allele could down-regulate NHEJ globally. To address this possibility, we introduced the *smt3-3R* mutant in a *rap1*-(Δ) strain. As shown in Figure 3B, telomere fusions still occur in *rap1*-(Δ) *smt3-3R*



double mutant cells. This indicates that NHEJ remains active in the *smt3-3R* context and that *smt3-3R* must suppress *uls1* deletion by restoring NHEJ inhibition at telomeres. Hence in the absence of poly-SUMO chains Uls1 is no longer needed for NHEJ inhibition. This suggests that poly-SUMOylated proteins are in part responsible for the NHEJ inhibition defect caused by Uls1 loss.

SUMOylation requires an E1 activating enzyme, an E2 conjugating enzyme and one of several SUMO E3 ligases (Ulrich, 2008). We tested whether the SUMO E3 ligases Siz1 and Siz2 might be part of the activities counteracted by Uls1. As shown in Figure 3C, Siz1 loss suppresses the occurrence of telomere fusions in cells lacking Uls1. Siz2 loss causes a more limited suppression. Thus, a SUMOylation defect bypasses the need for Uls1 at telomeres, further suggesting that SUMOylated proteins perturb NHEJ inhibition in the absence of Uls1.

A synthetic negative interaction between uls1-Δ and a rap1 hypomorphic allele is suppressed by smt3-3R

A systematic study revealed previously a negative synthetic interaction between an *uls1* deletion and a hypomorphic allele of *rap1* created by the 'Decreased Abundance by mRNA Perturbation' (DAmP) method (Yan *et al*, 2008; Costanzo *et al*, 2010). In this *rap1-DAmP* allele, *RAP1* coding sequence is separated from its native 3′ UTR. As shown in Figure 3D, *rap1-DAmP* and *uls1*-Δ single mutant cells grow as well as wild-type cells but double mutant *rap1-DAmP uls1*-Δ cells grow slightly slower, reproducing in our W303 genetic background the negative synthetic interaction initially observed in a BY4147 background. This growth defect is unaffected by an NHEJ-defective *lif1*-Δ mutant but is suppressed by the *smt3-3R* allele. Thus, the negative interaction between *uls1* and *rap1* requires poly-SUMOylated proteins. The *rap1-DAmP* allele does not significantly reduced relative Rap1 protein level (Figure 3E). Loss of the native 3′ UTR may alter translation quality. We checked the occurrence of telomere fusions in these cells once they reached stationary phase (Figure 3F). Telomere fusions remain undetectable in *rap1-DAmP* single mutant cells and the frequency of fusions caused by Uls1 loss is not increased by the *rap1-DAmP* allele. Their occurrence is suppressed by Lif1 loss and by the *smt3-3R* allele. The average telomere length remains unchanged in these mutant cells (data not shown).

The negative interaction between *uls1*-Δ and *rap1-DAmP* is not a consequence of telomere fusions since fusions only occur in a small fraction of cells in stationary phase. Furthermore, this negative interaction is not suppressed by a loss of NHEJ. Its cause might be an unidentified telomere defect or a perturbation of transcription from one or several of the many essential genes whose promoter is bound and regulated by Rap1 (Lickwar *et al*, 2012). In both scenarios and in agreement with the telomere fusions caused by Uls1 loss, Rap1 function might be perturbed in the absence of Uls1 either directly or indirectly through poly-SUMOylation. Rap1 is SUMOylated at a low level in normal growth condition (Hang *et al*, 2011). This encouraged us to address the hypothesis of a direct perturbation of Rap1 by SUMO.

Suppression of uls1-Δ by rap1 alleles

If the poly-SUMOylation of Rap1 is responsible of NHEJ inhibition failure at telomeres in cells lacking Uls1, then a non-SUMOylatable allele of *rap1* should suppress this defect. Rap1 displays three distinct and conserved domains: a BRCT domain in its amino-terminal region, a central DNA-binding domain (DBD) and a carboxy-terminal domain called RCT that is unique to Rap1 and its orthologues in other species (Figure 4A) (Chen *et al*, 2011; Matot *et al*, 2012). Only Rap1 DBD and RCT domain are required for NHEJ inhibition at telomeres (Marcand *et al*, 2008). The function of its amino-terminal region, which includes the BRCT domain, remains to be identified. We first tested whether a deletion of this non-essential part of Rap1 could suppress Uls1 loss. Two truncation mutants were introduced in *uls1*-Δ cells: *rap1-Δ2-228* and *rap1-Δ2-309*. Position 228 is the edge of the BRCT domain and position 309 is the last lysine residue before the essential DBD. In *uls1*-Δ cells where the first 228 amino acids of Rap1 are missing, telomere fusions still occur but extension of the deletion to amino acid 309 strongly reduces the frequency of telomere fusions (Figure 4B). The average telomere length is shortened by about 50 bp in these *rap1* mutant cells (data not shown).

Between positions 228 and 309 of Rap1, a sequence close to the most common SUMOylation consensus ΨKXE/D (Ψ: bulky hydrophobic residue) is present around lysine 246 (Figure 4A). The position of this lysine suggests that it could be a SUMOylation site whose loss in the *rap1-Δ2-309* mutant contributes to *uls1*-Δ suppression. To test this hypothesis, lysine 246 was mutated to a non-SUMOylatable arginine in full-length Rap1. This *rap1-1R* allele strongly reduces the frequency of telomere fusions caused by Uls1 loss (Figure 4C). A few telomere fusions are still detected in *uls1*-Δ *rap1-1R* cells. The additional mutation of nearby lysine

Figure 2 Mutations in Uls1 translocase and ubiquitin ligase catalytic domains cause telomere fusions. (**A**) Schematic representation of *S. cerevisiae* Uls1 and Uls1 from other representative yeast species. REPULS domains are only present in *Saccharomycetaceae*, in tandem, isolated or in multiple copies, some of which being degenerated (light grey, see the multiple alignment in Supplementary Figure S1). REPULS domains are not present in hypothetical proteins likely corresponding to the Uls1 orthologues in *D. hansenii* (UniProt Identifier Q6BMG3) and in *S. pombe* (two hypothetical orthologues: UniProt Identifier O60177 and O13762). (**B**) Telomere fusions occur in *uls1-C1333S* and *uls1-K975R* cells. Strains 206-2b and 205-9a (WT), 206-1d and 205-14c (*uls1*-Δ), 199-3a (*uls1-C1333S*) and 200-2c and 200-5d (*uls1-K975R*) were grown to stationary phase. Telomere fusions were amplified with 26 and 30 cycles. (**C**) Telomere fusions are undetectable in *usl1-Δ677-855* cells. Strains 196-11c and 196-13b (WT), 196-5a and 196-6a (*uls1*-Δ) and Lev791 and Lev792 (*uls1-Δ677-855*) were grown to stationary phase. Telomere fusions were amplified with 30 and 34 cycles. (**D**) Complementation of *uls1*-Δ by *ULS1* wild-type and mutant alleles. Strain 210-5b (*uls1*-Δ) was transformed with *Stu*I-digested plasmids pRS406, pRS406-*uls1-K975R*, pRS406-*uls1-C1333S* and pRS406-*uls1-Δ677-855* and with *Mfe*I-digested plasmid pRS404-*ULS1*. Cultures of two independent transformants were grown to stationary phase in rich medium. Telomere fusions were amplified with 26 and 30 cycles. (**E**) Uls1 is present at telomeres. Uls1 was tagged with 13 myc epitopes in the C-terminal position (Uls1-myc). A wild-type strain with untagged Uls1 was used as a control (−). Distances of the primer pairs from the terminal TG_{1-3} repeats are indicated. *LYS2* is an internal non-telomeric locus. ChIP assay was performed as described by Guglielmi *et al* (2007). Immunoprecipitated and input DNA were quantified by qPCR. ChIP signal was normalized to input DNA. Data shown are the mean and standard deviation of three independent experiments.

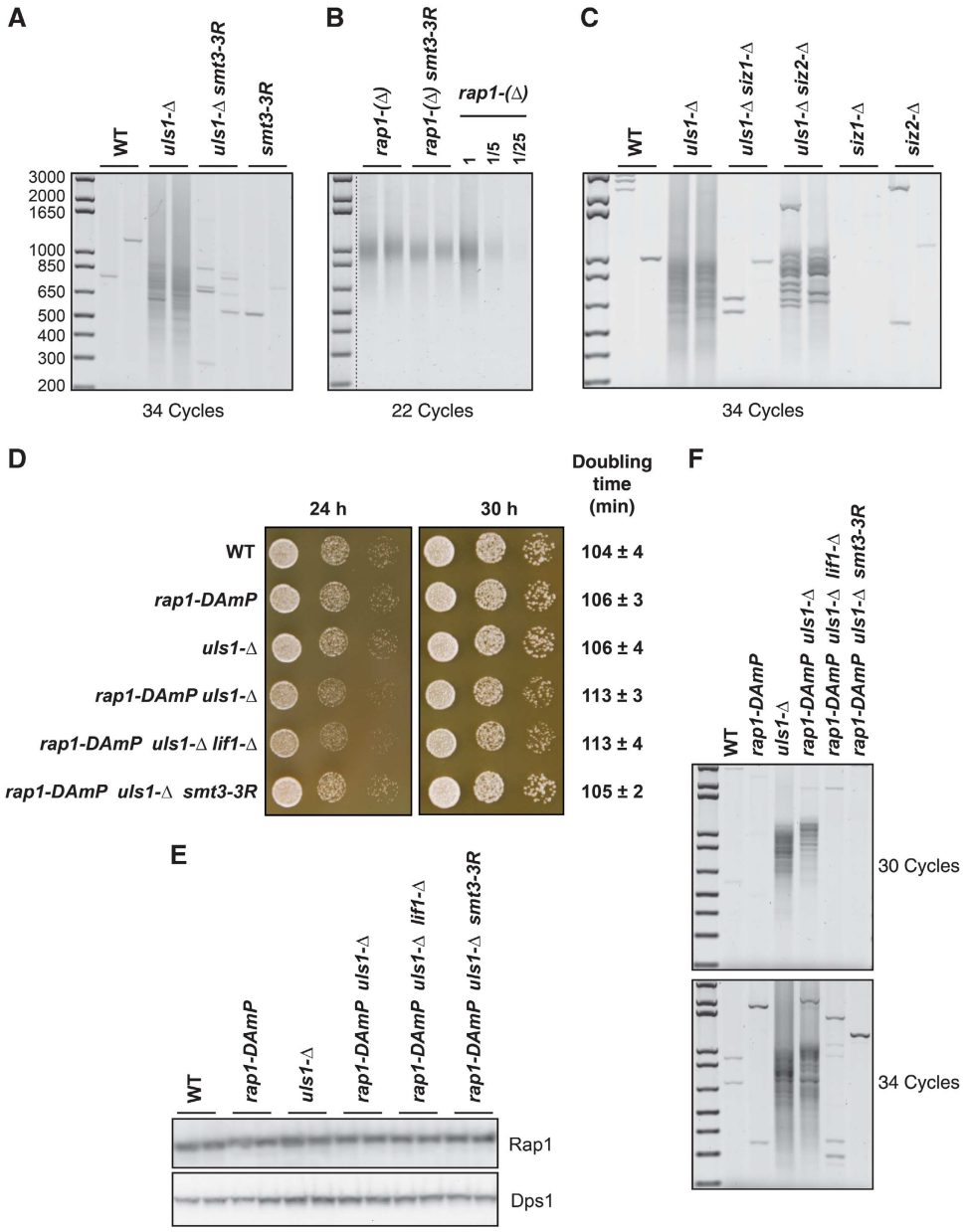


Figure 3 Suppression of *uls1*-Δ by *smt3-3R*. (**A**) Telomere fusions are undetectable in *usl1*-Δ *smt3-3R* cells. Two independent cultures of strains RL179 (WT), RL183 (*uls1*-Δ), RL185 (*uls1*-Δ *smt3-3R*) and RL181 (*smt3-3R*) were grown to stationary phase. Telomere fusions were amplified with 34 cycles. (**B**) Telomere fusions remain frequent in *rap1*-(Δ) *smt3-3R* cells. Strains 169-1c and 169-15c (*rap1*-(Δ)) and 169-6b and 169-11a (*rap1*-(Δ) *smt3-3R*) were grown to stationary phase. Telomere fusions were amplified with 22 cycles. Genomic DNA from 169-1c *rap1*-(Δ) cells was diluted serially to provide a semi-quantitative estimation of the method sensitivity. (**C**) Uls1 suppression by Siz loss. Two independent cultures of strains 168-3d (WT) and 168-3d (*uls1*-Δ) and strains RL266-1 and RL266-2 (*uls1*-Δ *siz1*-Δ), RL267-1 and RL267-2 (*uls1*-Δ *siz2*-Δ), RL268-1 and RL268-2 (*siz1*-Δ) and RL269-1 and RL269-2 (*siz2*-Δ) were grown to stationary phase. Telomere fusions were amplified with 34 cycles. (**D**) Slow growth of *rap1-DAmP uls1*-Δ cells is suppressed by *smt3-3R*. Freshly growing cells of strains 195-19d (WT), 195-3a (*rap1-DAmP*), 195-7b (*uls1*-Δ), 195-8a (*rap1-DAmP uls1*-Δ), 195-6b (*rap1-DAmP uls1*-Δ *lif1*-Δ) and 195-1d (*rap1-DAmP uls1*-Δ *smt3-3R*) were serially diluted by 10-fold in water and spotted on a rich medium plate. Pictures were taken after 24 and 30 h at 30°C. Doubling times are from exponential growth in liquid-rich medium at 30°C. (Data shown are the mean and standard deviation of five independent samples). (**E**) Cultures from the same strains were grown exponentially and total urea-extracted proteins were analysed by western blotting with polyclonal antibodies directed against Rap1 (upper panel) and against RNA synthetase Dps1 (an internal control, lower panel). (**F**) Cultures from the same strains were grown to stationary phase and telomere fusions were amplified with 30 and 34 cycles.

240 to arginine (*rap1-2R*) further reduces this very low level of fusions (Figure 4C; data not shown), suggesting that SUMOylation at lysine 240 might contribute to the weak NHEJ inhibition defect observed in *uls1*-Δ *rap1-1R* cells. The average telomere length remains unchanged in these mutant cells (data not shown). Although these results do not rule out SUMOylation at other lysines in Rap1 or that SUMOylation of other proteins contributes to the *uls1*-Δ phenotype, they indicate that NHEJ inhibition failure in the absence of Uls1 is in part due to Rap1 SUMOylation at lysines 240 and 246.

Increased level of poly-SUMOylated Rap1 in uls1-Δ cells

A prediction of the previous results is that SUMOylated Rap1 molecules accumulate in *uls1*-Δ cells. SUMOylated proteins are

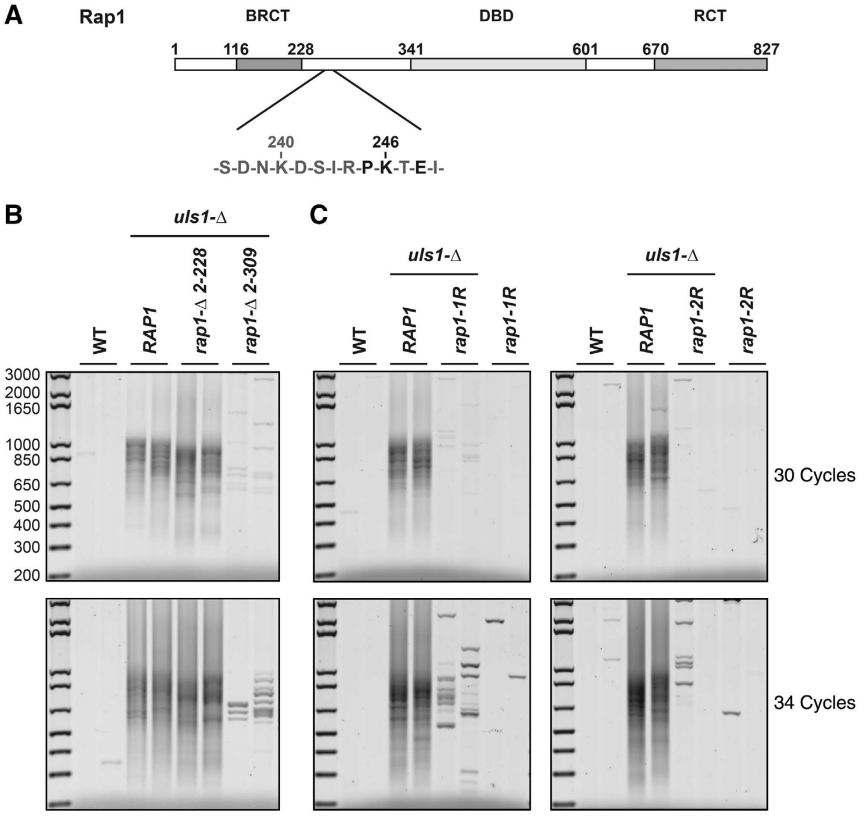


Figure 4 Suppression of *uls1*-Δ by *rap1* alleles. (**A**) Schematic representation of *S. cerevisiae* Rap1. The sequence surrounding lysine 246 is indicated. (**B**) Suppression of *uls1*-Δ by *rap1*-Δ*2-309*. Strains 196-11c and 196-13b (*WT*), 196-5a and 196-6a (*uls1*-Δ), 198-16a and 198-19a (*uls1*-Δ *rap1*-Δ*2-228*) and 196-1a and 196-7c (*uls1*-Δ *rap1*-Δ*2-309*) were grown to stationary phase. Telomere fusions were amplified with 30 and 34 cycles. (**C**) Suppression of *uls1*-Δ by mutation of Rap1 lysine 246. *rap1-1R* is *rap1-K246R* and *rap1-2R* is *rap1-K240R*, *K246R*. Strains 210-2d and 210-3d (*WT*), 210-5b and 210-4c (*uls1*-Δ), 210-10b and 210-5d (*uls1*-Δ *rap1-1R*), 210-1b and 210-7b (*rap1-1R*), 212-2d and 212-10b (*WT*), 212-1a and 212-8a (*uls1*-Δ), 212-5b and 212-17d (*uls1*-Δ *rap1-2R*), 212-3b and 212-4c (*rap1-2R*) were grown to stationary phase. Telomere fusions were amplified with 30 and 34 cycles.

rare and their detection often requires an enrichment step. To detect SUMOylated Rap1, we used a method described by Ulrich and Davies (2009). In short, His-tagged SUMO expressed *in vivo* allows the bulk purification of SUMOylated proteins under denaturing conditions by Ni-NTA pull-down. The presence of a particular SUMO conjugate can then be detected by western blot analysis. Rap1 SUMOylation was tested in exponentially growing cells (Figure 5, upper panel) and in stationary cells (lower panel). As previously observed (Hang *et al*, 2011), Rap1 is SUMOylated in wild-type cells, mostly as mono-SUMO conjugates (Figure 5; filled circle). In cells lacking Uls1, the level of SUMOylated Rap1 increases, mostly as poly-SUMO conjugates (stars), indicating that Uls1 is required to limit the accumulation of poly-SUMOylated Rap1 molecules. In *rap1-2R* cells, poly-SUMOylated Rap1 molecules are less frequent, indicating that a large fraction of the Rap1 poly-SUMO conjugates are linked to lysines 240 and 246. The persistence of mono- and some poly-SUMO conjugates in *rap1-2R* cells reveals that, in addition to lysines 240 and 246, other Rap1 lysines can be SUMOylated. Together, these observations reinforce the notion that Uls1 addresses the consequences of poly-SUMOylated Rap1 molecules by eliminating these conjugates.

Synergy between Uls1 and Sir4

Several pathways synergize to inhibit NHEJ at telomeres. The Rap1 C-terminal domain establishes two parallel inhibitory pathways through the proteins Rif2 and Sir4. In addition, the essential central part of Rap1 inhibits NHEJ independently of Rif2 and Sir4 (Marcand *et al*, 2008). As shown previously, fusions are detected in *rif2*-Δ *sir4*-Δ double mutant cells (Figure 6A, lower panel). They are detected as longer PCR products than in *uls1*-Δ cells, a consequence of the telomere elongation caused by Rif2 loss (Supplementary Figure S2). To know whether Uls1 is linked to these pathways, we tested the genetic interactions between deletions of *ULS1*, *RIF2* and *SIR4*. As shown in Figure 6A, in *uls1*-Δ *sir4*-Δ cells and in *usl1*-Δ *sir4*-Δ *rif2*-Δ cells, telomeres fuse more frequently than in *uls1*-Δ cells. By contrast, fusions occur slightly less frequently in *uls1*-Δ *rif2*-Δ cells. Thus in the absence of Uls1, NHEJ inhibition at telomeres still relies on Sir4 but inhibition by Rif2 is lost. Since a *rif2* deletion alone is insufficient to cause a detectable level of fusions and since fusions are more frequent in *usl1*-Δ *sir4*-Δ *rif2*-Δ cells than in *sir4*-Δ *rif2*-Δ cells, the pathway independent of Rif2 and Sir4 is also at least partially defective in the absence of Uls1. The slight decrease in fusion frequency observed in *uls1*-Δ *rif2*-Δ cells relative to *uls1*-Δ cells might be a consequence of telomere elongation and increased Sir4 recruitment caused by Rif2 loss (Wotton and Shore, 1997; Luo *et al*, 2002; Feeser and Wolberger, 2008; Marcand *et al*, 2008; Chen *et al*, 2011).

Next, we asked whether telomere fusions occurring in *uls1*-Δ *sir4*-Δ cells are still in part linked to Rap1

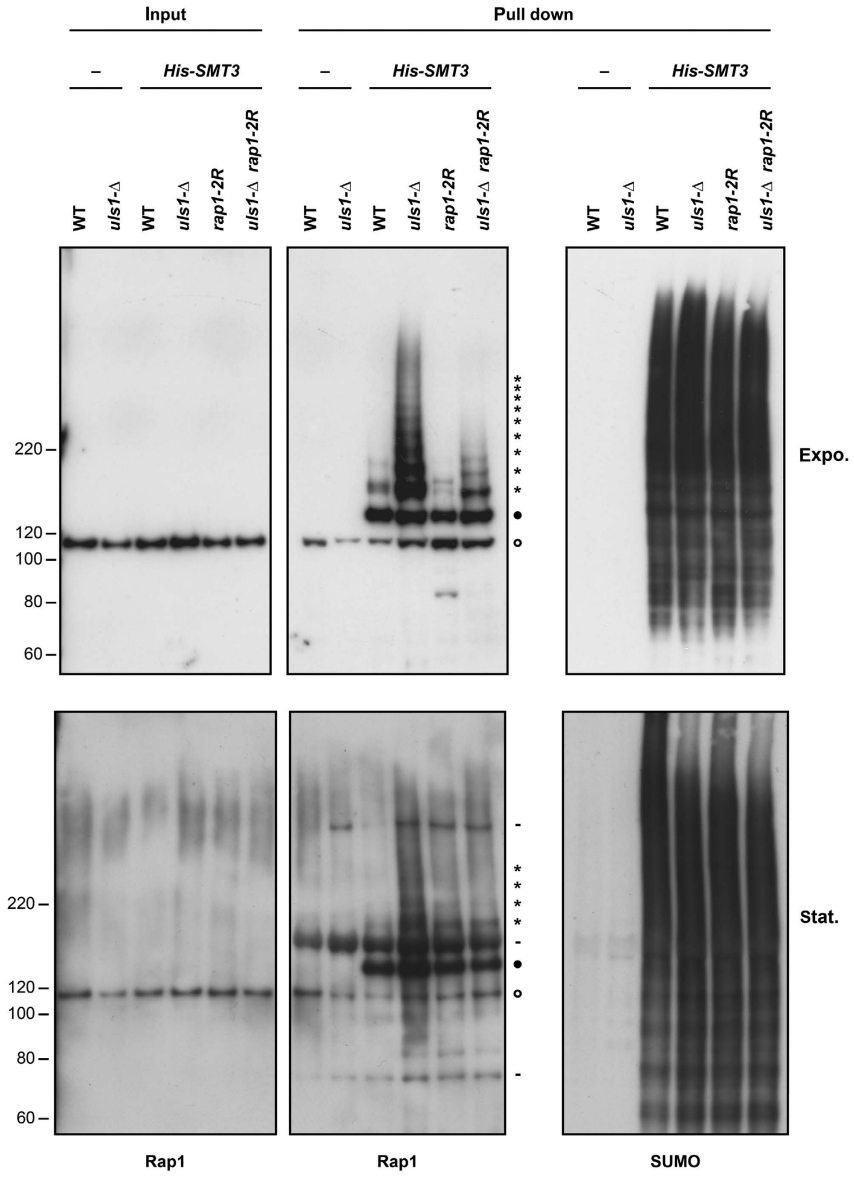


Figure 5 Accumulation of SUMOylated Rap1 molecules in *uls1*-Δ cells. Strains 210-2d (*WT*) and 210-5b (*uls1*-Δ) transformed with empty vector pRS316 and strains 210-2d (*WT*), 210-5b (*uls1*-Δ), 210-3b (*rap1-2R*) and 210-13c (*uls1*-Δ *rap1-2R*) transformed with YEp195-CUP-His-Smt3 were grown exponentially (upper panel) or allowed to reach stationary phase (lower panel). Protein extraction and pull-down were done under denaturing conditions. Input sample is 1/2000th of the total extract subjected to the pull down. Rap1 was detected with a polyclonal antibody (left and central panels). The membrane was then rehybridized with a second polyclonal antibody against SUMO (right panel; the antibody sensitivity did not allow us to detect SUMO conjugates in the input samples (data not shown)). Position of mono-SUMOylated Rap1 is marked with a filled circle. Positions of poly-SUMOylated Rap1 are marked with stars. A background of unmodified Rap1 is detected in all pull-down samples and its position is marked with an empty circle. Total protein extraction from stationary cells was less efficient. Non-specific signals are marked with minus signs.

poly-SUMOylation. The *smt3-3R*, *rap1-2R* and *rap1*-Δ*2-309* alleles were combined with the *uls1*-Δ *sir4*-Δ double mutant. As shown in Figure 6B, all three alleles suppress the telomere fusions caused by Uls1 loss in the absence of Sir4, indicating that the bulk of telomere fusions remains a consequence of Rap1 poly-SUMOylation. However, suppression by *rap1-2R* and *rap1*-Δ*2-309* is only partial. It is possible that the loss of one NHEJ inhibition pathway sensitizes telomeres to SUMOylation at other sites than Rap1 lysines 240 and 246. Sir4 loss may also expose other SUMOylation sites within Rap1 but this remains to be addressed.

Discussion

In this study, we observed that the SUMO-dependent ubiquitin ligase and translocase Uls1 is required for efficient NHEJ inhibition at telomeres. This requirement is bypassed by the absence of poly-SUMO chains and by Rap1 mutants lacking SUMOylation sites. In addition, Uls1 is required to avoid the accumulation of poly-SUMOylated Rap1 molecules. Together, these results show that the failure to inhibit NHEJ at telomeres in the absence of Uls1 is at least in part the consequence of the accumulation of poly-SUMOylated Rap1 that are normally eliminated by Uls1. Rap1 poly-SUMOylation seems to cause a loss of Rap1 function. A simple model is

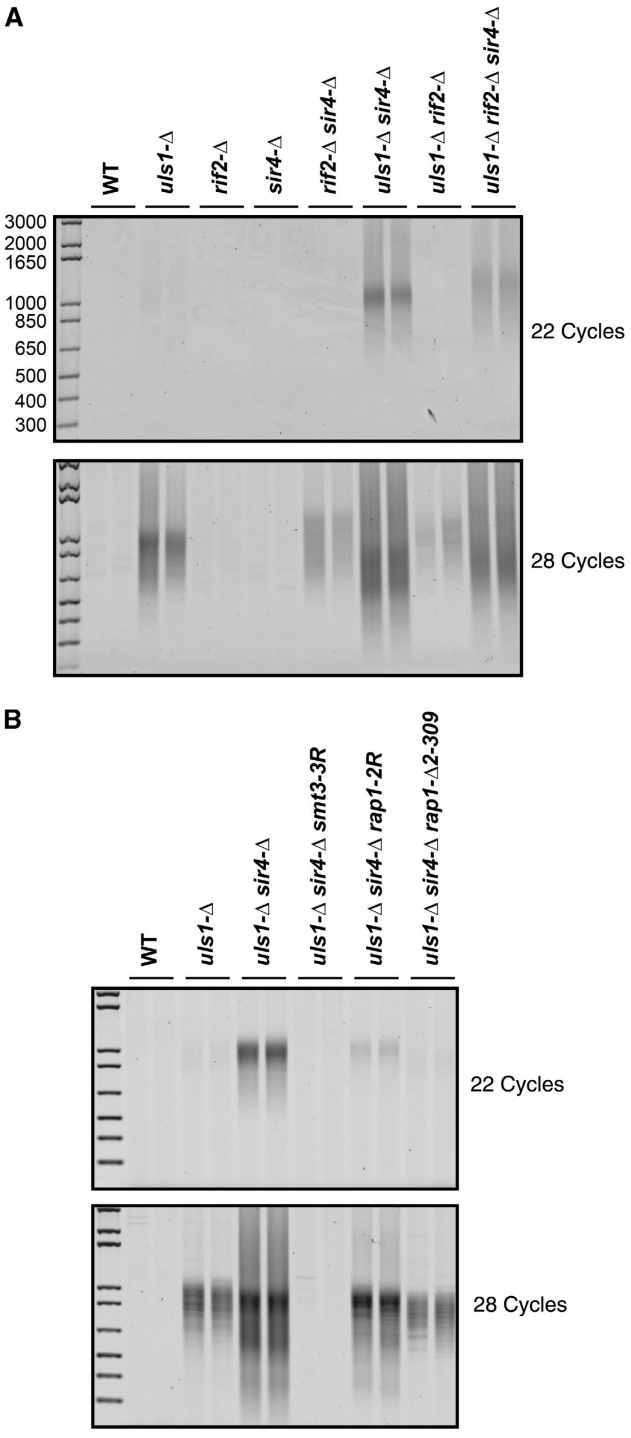


Figure 6 Synergy between Uls1 and Sir4. (**A**) Two independent cultures of strains Lev346 (WT), 183-30d (*rif2*-Δ), 183-10c (*uls1*-Δ *sir4*-Δ) and 183-2d (*uls1*-Δ *rif2*-Δ), and strains 184-48c and 184-9d (*uls1*-Δ), Lev575 and Lev576 (*sir4*-Δ), 184-14c and Lev601 (*rif2*-Δ *sir4*-Δ) and 183-25a and 184-49a (*uls1*-Δ *rif2*-Δ *sir4*-Δ) were grown to stationary phase. Telomere fusions were amplified with 22 and 28 cycles. (**B**) Two independent cultures of strains 205-9a (WT), 205-14c (*uls1*-Δ), 205-16a (*uls1*-Δ *sir4*-Δ *smt3-3R*) and 207-14d (*uls1*-Δ *sir4*-Δ *rap1*-Δ*2-309*) and strains 212-3c and 212-4b (*uls1*-Δ *sir4*-Δ), 212-1b and 212-2a (*uls1*-Δ *sir4*-Δ *rap1-2R*) were grown to stationary phase. Telomere fusions were amplified with 22 and 28 cycles.

that when Rap1 poly-SUMOylation occurs on one or several Rap1 molecule(s) bound to DNA at a telomere, it cripples its ability to inhibit NHEJ (Figure 7). Uls1 recognizes poly-SUMO

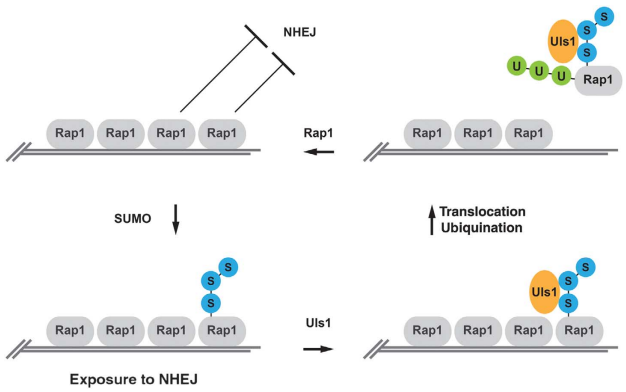


Figure 7 A schematic model for Uls1 function at telomeres. SUMO is depicted in blue and ubiquitin in green. The proposed scenario is described in the main text.

chains conjugated to Rap1. Its translocase activity dissociates these molecules from DNA and ubiquitinylation targets them for degradation by the proteasome (Uzunova *et al*, 2007). Unmodified Rap1 from the pool of free Rap1 molecules rapidly replace the freed binding sites, re-establishing full protection against NHEJ.

Once bound to DNA, Rap1 detaches slowly *in vitro* (Williams *et al*, 2010). *In vivo* Rap1 residence time on DNA is estimated to be in the 30–60 min range at telomeres (Lickwar *et al*, 2012). If this slow turnover was the only way to lose SUMOylated Rap1 molecules, a telomere could be exposed to NHEJ long enough to create a reasonable probability that another telomere is simultaneously exposed at some point, allowing telomere fusions. Uls1 ATP-dependent translocation can prevent it by accelerating the dislodging of these molecules from DNA. Their subsequent ubiquitination and degradation prevent these molecules from rebinding DNA at telomeres or at other sites. In addition, because telomere fusions occurring in non-dividing cells are cumulative events, even low steady levels of SUMOylated Rap1 and relatively infrequent and transient telomere exposure to NHEJ could result in a significant accumulation of fusions over time.

How poly-SUMOylation perturbs Rap1 function is unknown but the requirement for Uls1 translocase activity indicates that Rap1 DNA binding ability must remain significant. Rap1 poly-SUMOylation antagonizes NHEJ inhibition by Rif2 and by the pathway independent of Rif2 and Sir4 but not by the Sir4 pathway, which remains proficient in the absence of Uls1. One possibility is that once at telomeres Sir4 can remain anchored through the silent chromatin and thereby be partially insensitive to a transient Rap1 loss of function. The Sir4-Rap1 complex or the mechanism of NHEJ inhibition by Sir4 may also be specifically insensitive to Rap1 SUMOylation. In all scenarios, the independence between Sir4 and Uls1 adds a level of redundancy that further protects telomeres from NHEJ. The lack of telomere elongation in *uls1*-Δ cells despite a loss of Rif2 function in NHEJ inhibition is surprising. Whether Rap1 SUMOylation can perturb Rif2 function in telomere length homeostasis during replication remains unknown. Perhaps in the absence of Uls1 poly-SUMOylated Rap1 molecules are too rare to influence most telomeres at each replication cycle, thus allowing telomere length homeostasis by functional Rap1 molecules to buffer for the drift of a few telomeres stochastically affected by Rap1

SUMOylation. By contrast, the static nature of telomere fusions may allow the dominance of Rap1 SUMOylation over Rif2 function to manifest itself by the progressive accumulation of telomere fusions. It would be interesting to test this model.

A different issue is whether Rap1 mono- and poly-SUMO conjugates have a positive role in cells. *rap1-1R* and *rap1-2R* mutants display normal growth, normal telomere length and no detectable telomere fusion (Figure 4B; data not shown). This indicates that SUMOylation at lysines 240 and 246 is not essential for the core Rap1 functions. It does not rule out a positive function for Rap1 SUMOylation that would be redundant with another mechanism or only relevant in contexts that do not often occur in normal growth conditions, for instance situations where Rap1 poly-SUMOylation could rapidly and transiently inactivate Rap1 function. Interestingly, DNA damage induces the hyper-SUMOylation of many proteins including Rap1 (Cremona *et al*, 2011; Hang *et al*, 2011). Maybe the modulation of Rap1 function at telomeres or at promoters helps the cell to cope with DNA damage-induced stress. An alternative possibility is that Rap1 poly-SUMOylation has no biological function and is part of a background of SUMOylation in the cell. In this model, Uls1 role would be to keep low the steady level of unselected poly-SUMO conjugates, Rap1 being one target among others.

Uls1 is the only known STUbL with a translocase activity. It may have a large number of targets and therefore can be in principle the general molecular sweeper for the clearance of poly-SUMOylated proteins on DNA in eukaryotes. Uls1 has previously been implicated in homologous recombination at breaks and at replication forks, functions for which its targets remain to be identified (Zhang and Buchman, 1997; Shah *et al*, 2010; Cal-Bakowska *et al*, 2011; Chi *et al*, 2011). In addition, the new and yeast-specific REPULS domain we identified in Uls1 and whose functions remain unknown suggests that additional modes of Uls1 regulation or mobilization are likely to exist.

Materials and methods

Yeast strains and plasmids

The strains used in this study are listed in Supplementary Table 1. Mutations within *ULS1* and *RAP1* were created by overlapping multiple PCRs whose products were assembled to a plasmid by single-strand annealing in yeast. Mutant alleles were recovered, sequenced, cloned into pRS406 and integrated at the endogenous locus by pop-in pop-out selection. Correct integrations were identified by PCR and sequencing. The *smt3-3R::TRP1* and *rap1-DAmP::KAN*^{r} alleles were amplified by PCR from strains GBY1 (a gift from Erica Johnson) and YNL216 (a gift from Jesse Platt and Bradley Johnson) respectively and integrated at their endogenous locus in a W303 wild-type strain.

Amplification of the telomere–telomere fusions by PCR

Cells were streaked on rich medium plate (YPD) at 30°C for 24 h and then grown in liquid-rich medium at 30°C to saturation for 6 days (excepted in Figure 1B where exponentially growing cells were from cultures whose $OD_{600\,nm}$ was maintained below 1 for 24 h). Genomic DNA was extracted with phenol/chloroform and 800–1200 μm acid-washed glass beads, ethanol-precipitated and resuspended in TE pH 8.0 with RNAse A (~10 ng/μl).

Fusions between X and Y′ telomeres were amplified with primers X2 5′-TGTGGTGGTGGGATTAGAGTGGTAG-3′ and Y′2 5′-TTAGGGCT ATGTAGAAGTGCTG-3′. PCRs (30 μl) contained genomic DNA ~10 ng, Phusion HF buffer 1×, DMSO 3%, dNTP 200 μM each, primers 0.5 μM each, HotStart Phusion polymerase 0.6 unit (Finnzymes). Reaction mixes were prepared at room temperature. Amplification conditions were 98°C 30 s, then 22, 26, 30 or 34 cycles of 98°C 10 s, 65°C 20 s, 72°C 1 min, followed by 72°C 5 min, using GeneAmp PCR System 9700 (Applied Biosystems). The products (10 μl) were separated on a 1% agarose gel containing 1× Gel Red nucleic acid stain (Biotium). Fluorescence was analysed on a Typhoon imager (GE).

Detection of SUMO conjugates

Cells were transformed with plasmids pRS316 or YEp195-CUP-His-Smt3 (*URA3*) (Ulrich and Davies, 2009). Cells were grown exponentially in synthetic medium lacking uracil followed by 2 h (expo.) or 5 days (stat.) in rich medium (YPD). Extraction and Ni-NTA pull-down of SUMO conjugates were done as described by Ulrich and Davies (2009). For stationary cells, lysis in NaOH/BME on ice was improved with 0.8 mm zirconium beads and four pulses of 30 s vortex at 4°C. Input aliquots and pull-downs were separated in a NuPage Tris-Acetate 3–8% gel (Novex Life Science) and transferred overnight onto a PVDF membrane at 25 V at 4°C in 25 mM Tris, 192 mM Glycine, 10% v/v Ethanol, 0.01% SDS. Anti-Rap1 rabbit polyclonal antibody (1/500) is from Santa Cruz (Y-300; ref sc-20167). Anti-Smt3 rabbit polyclonal antibody (1/500) is from Abcam (ref ab14405).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: RL, IC and SM designed the experiments. RL, SP and SM performed the yeast genetics experiments. IC performed the *in silico* studies. RL, SP, IC and SM interpreted the data. RL, IC and SM wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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