Lif1 SUMOylation and its role in non-homologous end-joining

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ABSTRACT

Non-homologous end-joining (NHEJ) repairs DNA double-strand breaks by tethering and ligating the two DNA ends. The mechanisms regulating NHEJ efficiency and interplay between its components are not fully understood. Here, we identify and characterize the SUMOylation of budding yeast Lif1 protein, which is required for the ligation step in NHEJ. We show that Lif1 SUMOylation occurs throughout the cell cycle and requires the Siz SUMO ligases. Single-strand DNA, but not double-strand DNA or the Lif1 binding partner Nej1, is inhibitory to Lif1 SUMOylation. We identify lysine 301 as the major conjugation site and demonstrate that its replacement with arginine completely abolishes Lif1 SUMOylation in vivo and in vitro. The lif1-K301R mutant cells exhibit increased levels of NHEJ repair compared with wild-type cells throughout the cell cycle. This is likely due to the inhibitory effect of Lif1 SUMOylation on both its self-association and newly observed single-strand DNA binding activity. Taken together, these findings suggest that SUMOylation of Lif1 represents a new regulatory mechanism that downregulates NHEJ in a cell cycle phase-independent manner.

INTRODUCTION

DNA double-strand breaks (DSBs) are highly toxic lesions that challenge genomic integrity. DSBs arise both endogenously and from exogenous sources. Two distinct repair pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR), have evolved to repair DSBs. Although NHEJ ligates the ends with little or no requirement for homology, HR uses a homologous sequence to copy the lost information (1,2). The pathway used depends on the organism, the type of break and the cell cycle phase. In the yeast *Saccharomyces cerevisiae*, NHEJ is used throughout the cell cycle, whereas HR operates predominantly in S and G2 phases when it is the preferred over NHEJ. In mammals, NHEJ is a dominant pathway in DSB repair and is critical for processing breaks that occur in the immune system during V(D)J recombination (3). Defects in the human NHEJ pathway lead to cancers, immunodeficiences and neurodegeneration, emphasizing the importance of this pathway to human health (4,5). As the core factors of NHEJ are evolutionarily conserved, budding yeast has been used as a model organism for NHEJ studies (5–7). Three protein complexes are fundamentally required for efficient NHEJ in *S. cerevisiae* [reviewed in (6,7)]. The Ku complex, consisting of the Ku70 and Ku80 subunits, binds specifically to DNA ends in a sequence-independent manner (8,9). Besides promoting NHEJ, Ku binding to DSB ends also protects it from nucleolytic degradation (9–12). The second complex, Mre11/Rad50/Xrs2 (MRX), which binds early at DSB sites, can either bridge the broken ends for NHEJ repair or initiate resection to commence HR (13,14). Both Ku and MRX recruit the DNA ligase IV complex (Dnl4/Lif1/Nej1) to DNA ends, where it performs the end-joining reaction as the final step of NHEJ. Lif1 serves several roles in this step. It preferentially binds longer DNA substrates to form oligomeric complexes (5,15,16). This correlates with its recruitment of Dnl4 to chromosomal DSBs and stimulation of Dnl4 ligase activity (16,17).

Finally, Lif1 interacts with three NHEJ proteins, Nej1, Xrs2 and Dnl4 (15,17–21).

How the choice between HR and NHEJ is made and how NHEJ components collaborate remains largely unaddressed questions. CDK and Mec1-checkpoint-mediated phosphorylation have been shown to contribute to repair pathway choice. More recently, SUMOylation has been implicated in DSB repair and the DNA damage response (22). SUMOylation entails the covalent attachment of Small Ubiquitin-like Modifier (SUMO) to target proteins in a three-step process requiring E1 (Aos1/Uba2), E2 (Ubc9) and often E3 ligase enzymes [reviewed in (23)]. In budding yeast, three mitotic E3 ligases, Siz1, Siz2 and Mms21, have been identified (24–26). Although SUMOylation has been shown to be critical for DNA replication and repair, the consequences of SUMO attachment to many target proteins are not known (27–29).

Here, we report that Lif1 is subjected to SUMO modification *in vivo*. We show that Siz1 and Siz2 stimulate this modification, whereas double-strand DNA (dsDNA) or Lif1-interacting partners do not have any effect. We identify K301 as the main SUMOylation site and show that K301R increases Lif1 self-interaction without affecting binding to dsDNA or other NHEJ factors. This correlates with an increased capacity for NHEJ repair in *lif1-K301R* cells. Our results suggest that SUMOylation of Lif1 is involved in downregulation of NHEJ.

MATERIALS AND METHODS

Plasmids and yeast strains

Plasmids for expression of Lif1 and Nej1–Lif1 complex were generous gifts from Dr. Murray Junop (McMaster University, Canada) (5). The construct containing full-length Nej1 was kindly provided by Dr. Stefan Åström (Stockholm University, Sweden). The two-hybrid vectors were generous gifts from Dr. Thomas E. Wilson (University of Michigan, USA) (15). Construction of plasmids pGADT7-*XRS2* and pGADT7-*DNL4* was previously described elsewhere (30). The Lif1-K301R mutant was generated by substituting the lysine codon at position 301 with arginine using site-directed mutagenesis. Primer sequences are available on request.

Yeast strains PJ69-4a and PJ69-4α (*MATa* or *α leu2-3,112 ura3-52 trp1-901 his3-200 gal4Δ gal80Δ GAL-ADE2 lys2::GAL1-HIS3 met2::GAL7-LacZ*) (31,32) were used for two-hybrid analyses. The *lif1* null mutant derivative of PJ69-4A strain was obtained from Dr. Stefan Åström (Stockholm University, Sweden). Yeast strain JKM139 (*MATa hmrΔ::ADE1 hmlΔ::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL-HO*) was kindly provided by Dr. James Haber (Brandeis University, USA). The *lif1::URA3* derivative of JKM139 was prepared by one-step gene replacement method, and the *lif1-K301R* allele was introduced into JKM139 by a two-step gene replacement procedure. All the strains (*LIF1-TAP::HIS, LIF1 K301R-TAP::HIS*) used for in vivo SUMOylation studies are *RAD5* derivatives of W303 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) (33). Yeast strains deleted for *SIZ1* and *SIZ2* were obtained by one step gene replacement.

Recombinant protein expression and purification

Purification of Lif1 and Lif1-K301R

The $(His)_6$-Lif1 protein was expressed in Rosetta-gami (DE3) pLysS using pMJ4102 plasmid (kindly provided by Dr. Murray Junop). An overnight culture (150 ml) was used to inoculate 12 l of 2xTY supplemented with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol. Cultures were grown at 37°C with vigorous shaking to an OD_{600} of 0.5 and induced for 3 h at 37°C in the presence of 1 mM Isopropylthio-β-galactoside (IPTG). Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at −80°C. Extract from 10 g of cell paste was prepared by sonication in 40 ml of Cell Breakage Buffer (CBB) buffer [50 mM Tris–HCl (pH 7.5), 10% sucrose, 2 mM ethylenediaminetetraacetic acid] containing 600 mM KCl, 0.01% NP40, 1 mM β-mercaptoethanol and protease inhibitor cocktail (pepstatin, leupeptin, aprotinin, chymostatin, benzamidine and phenylmethylsulfonyl fluoride). The lysate was clarified by ultracentrifugation, and the resulting supernatant was loaded on SP-sepharose column (7 ml, GE Healthcare). The SP-column was then eluted with a 70 ml gradient of 150–1000 mM KCl in buffer K (20 mM K_2HPO_4, 10% glycerol, 0.5 mM EDTA). The Lif1-containing fractions were pooled and mixed with 1 ml of Ni–NTA agarose (Qiagen). The beads were washed with 10 ml of buffer K containing 500 mM KCl, followed by 10 ml of buffer K containing 500 mM KCl and 10 mM imidazole. The bound proteins were eluted with buffer K supplemented with 500 mM KCl and containing 50, 150, 300 or 500 mM imidazole. The fractions with Lif1 protein eluting from 50 to 300 mM imidazole were pooled and loaded onto a 1-ml Heparin column (GE Healthcare). The column was washed and eluted with a 10 ml gradient of 150–1000 mM KCl in buffer K. The peak fractions were pooled and concentrated in a Centricon-30 (Sartorius Stedim) to 10 mg/ml and stored in small aliquots at −80°C. The same procedure was used to purify the $(His)_6$-Lif1-K301R mutant protein.

Expression and purification of Nej1

Recombinant MBP-tagged Nej1 protein was expressed in Rosetta-gami (DE3) pLysS containing pSTEF446 plasmid (gift from Dr. Stefan Åström). A saturated 150 ml of overnight culture was used to inoculate 12 l of 2xTY containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol. Cultures were grown at 37°C with vigorous shaking to an OD_{600} of 0.5 and induced for 12 h at 16°C in the presence of 0.1 mM IPTG. Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at −80°C. Extract from 10 g of cell paste was prepared by sonication in 40 ml of CBB containing 600 mM KCl, 0.01% NP40, 1 mM β-mercaptoethanol and protease inhibitor cocktail. The lysate was clarified by ultracentrifugation, and the resulting supernatant was loaded on SP-sepharose column (7 ml). The SP-column was then eluted with a 70 ml gradient of 150–1000 mM KCl in buffer K. The Nej1-containing fractions were pooled and mixed with 1 ml of amylose agarose (New England Biolabs). The beads were washed with 10 ml of buffer K containing 200 mM KCl.

The bound proteins were eluted with buffer K containing 1, 5 or 10 mM maltose and 200 mM KCl. The fractions with Nej1 protein eluting from 1 to 10 mM maltose were pooled and loaded onto a 1-ml Heparin column and eluted with a 10 ml gradient of 150–1000 mM KCl in buffer K. The peak fractions were pooled, concentrated in a Centricon-30 to 10 mg/ml and stored in small aliquots at −80°C.

In vitro SUMOylation assay

The Aos1/Uba2 complex, Ubc9 and SUMO proteins were purified as described elsewhere (27). *In vitro* SUMOylation assay was performed accordingly to Kolesar *et al.* (34). Briefly, 10 μl reaction volume contained 0.35 μM Aos1/Uba2, 1.25 μM Ubc9, 2.5 μM Smt3, 0.3 μM Siz1(1–465) or 0.3 μM Siz2, 1.5 μM Lif1 or its K301R derivative, 100 μM ATP, 100–300 mM KCl and the buffer S2 (50 mM HEPES, 10 mM $MgCl_2$). Reactions were incubated for 40–60 min at 30°C and used before further experiments. To assess the SUMOylation efficiency, reaction was stopped by adding 10 μl of sodium dodecyl sulphate (SDS) Laemmli buffer (62.5 mM Tris–HCl, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue), and 10 μl of the mixture was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel. Proteins were visualized by staining with Coomassie Blue and/or blotted using anti-6xHis (Sigma Aldrich), anti-Lif1 (kind gift from Dr. Olivier Fritsch, University of Basel, Switzerland) and anti-Smt3 antibodies (provided by Dr. Helle Ulrich, Cancer Research, UK). The levels of SUMOylated proteins were analysed using ImageJ software.

LC-MS/MS analysis of gel bands

Gel bands were washed and proteins were reduced with dithiothreitol, alkylated by incubation with iodoacetamide and subsequently digested by addition of trypsin (recombinant, proteomics grade, Roche) over night at 37°C. Peptides were analysed on an UltiMate 3000 reversed phase-HPLC system (Thermo Fisher Scientific) connected to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) via a nanoelectrospray ionization source (Proxeon, Thermo Fisher Scientific). Peptides were separated applying a 60-min linear gradient from 2.5% up to 40% acetonitrile. The voltage of the ion source was set to 1500 V. The mass spectrometer was operated in the data-dependent mode: 1 full scan in the orbitrap (m/z: 400–1800, resolution 60 000) with lock mass (m/z 445.120 025) enabled was followed by maximal 20 MS/MS scans. Monoisotopic precursors were selected, and singly charged signals were excluded from fragmentation. The collision energy was set at 35%, Q-value at 0.25 and the activation time at 10 msec. Fragmented ions were excluded from further selection for 90 s.

Peptide identification was performed by the SEQUEST algorithm in the Proteome Discoverer 1.3.0.339 software package (Thermo Fisher Scientific). Spectra were searched against a small database containing the protein sequences of the SUMOylation assay plus proteases, contaminants and a short list of unrelated proteins. To increase the identification rate of the SUMOylated peptides, the proteins of interest were digested *in silico* with trypsin, and the resulting peptides were extended at the N-terminus with amino acids EQIGG and added to the database (35). EQIGG is the part of SUMO that remains attached to the target sequence after tryptic digest.

Search parameters were as follows: tryptic specificity with max. 3 missed cleavages, peptide tolerance of 2 ppm, fragment ions tolerance of 0.8 Da. Carbamidomethylation of Cys was set as static modifications, oxidation of Met and SUMOylation residue (484.2282 Da) at Lys as variable modifications. Modified peptides were checked manually.

Detection of Lif1 SUMOylation *in vivo*

Protein extracts and immunoprecipitates were prepared as described previously (28). In brief, cells were disrupted by bead beating under denaturing conditions, and diluted protein extracts were immunoprecipitated using immunoglobulin G-Sepharose to pull down TAP-tagged Lif1. Immunoprecipitated proteins were then washed and eluted with loading dye before separating by SDS–PAGE and immunoblotting with anti-SUMO or PAP antibodies. As only 5–10% of Lif1 is SUMOylated, this modified form is not detected by the PAP antibody without long exposures but is preferrentially detected by anti-Smt3, which also recognizes ProA on the TAP tag (28). For the analysis of SUMOylation during cell cycle, Lif1-TAP strains were arrested in G1 phase with 5 ug/ml α-mating factor or in G2/M phase with 15 ug/ml nocodazole, then released into the cell cycle. Samples were collected every 15 min for analysis by protein immunoprecipitation and western blotting. Samples from these time points were also examined by fluorescence-activated cell sorting (FACS) analysis. Induction of Lif1 SUMOylation by treatment with methyl methane sulphonate (MMS) was achieved by subjecting logarithmically growing cells to 0.3% MMS for 2 h before harvesting samples.

Electrophoretic mobility shift assay

Indicated amounts of various forms of Lif1 protein were incubated with 600 bp (500 nM) dsDNA substrate at 37°C in 10 μl of buffer B [50 mM Tris–HCl (pH 7.8), 5 mM $MgCl_2$ and 1 mM dithiothreitol] for 10 min. After the addition of gel-loading buffer [60% glycerol, 10 mM Tris–HCl (pH 7.4) and 60 mM EDTA], the reaction mixtures were resolved on 1% agarose gels in TBE buffer [40 mM Tris–HCl, 20 mM boric acid, 2 mM EDTA (pH 7.5)], and the DNA species were analysed by Gel Logic 212 PRO (Kodak). Analyses with shorter DNA substrates were performed in the presence of fluorescently labeled 90-mer single-strand DNA (ssDNA) (270 nM nucleotides), 90-mer dsDNA (540 nM nucleotides) or both ssDNA and dsDNA (270 nM nucleotides and 540 nM nucleotides, respectively) and various forms of Lif1. The reaction mixtures were prepared as described earlier in the text and then separated on 10% native polyacrylamide gel in TBE buffer [20 mM Tris–HCl, 10 mM boric acid, 1 mM EDTA (pH 7.5)]. DNA species were analysed by FLA9000 Starion (Fuji-Film).

Pull-down assay

Amylose resin was incubated with $(His)_6$-Lif1 and $(His)_6$-Lif1-K301R proteins or *in vitro* SUMOylated $(His)_6$-Lif1 together with MBP-Nej1. After extensive washing $(100\,\mu l)$ with immunoprecipitation buffer IP $(20\,mM\ K_2HPO_4, 10\%$ glycerol, $0.5\,mM$ EDTA) containing $50\,mM$ KCl, the bound proteins were eluted from the affinity resin by boiling with $30\,\mu l$ of SDS sample buffer. The samples were resolved on a 10% SDS–PAGE gel and transferred to a Polyvinylidene difluoride (PVDF) membrane. The blot was subjected to analysis with anti-His antibodies (Sigma Aldrich).

Two-hybrid analysis

The analysis was performed as described elsewhere (36). The indicated recombinant vectors were co-transformed into haploid yeast cells and tested for expression of the reporter gene by growth on synthetic complete media lacking histidine, tryptophan and leucine (SD/−His/ −Trp/−Leu) for 4 days at 30°C.

Plasmid-based rejoining assay

The assay was carried out as described previously (37,38). Briefly, plasmid pRS425 containing the *LEU2* gene was digested with the indicated restriction enzymes to completion. Yeast cultures were grown to log phase and transformed with 500 ng of corresponding linearized plasmid using the lithium acetate method (39). Parallel transformation was always performed with an equivalent amount of undigested plasmid to enable normalization for differences in transformation efficiencies between strains and individual experiments. Serial dilutions were plated on selective media, and colonies were counted after 5 days of incubation.

HO-induced DSB repair assay

In contrast to plasmid-based rejoining assay, HO-induced DSB repair assay measures DSB rejoining in chromatinized chromosomal DNA (40,41). Parental JKM139 strain contains no *HML* and *HMR* loci (see genotype above), two silent copies of the *MAT* locus. Therefore, HO-induced DSB at the *MAT* locus can be repaired only by NHEJ in this strain. The HO-encoding gene is placed under the control of a galactose-inducible promoter. In persistent DSB induction experiment (40), yeast cultures grown overnight in media containing 3% glycerol were pelleted, washed with distilled water and directly plated in serial dilutions on glucose- and galactose-containing media in parallel. The efficiency of rejoining of HO-induced DSBs was calculated as the number of colonies on galactose plates normalized to that on glucose. Transient DSB induction experiment as well as persistent DSB induction experiment in G1-, S- and G2/M-arrested cells were perfomed as described previously with asynchronous cells being treated similarly except the addition of arresting agent (42).

Sensitivity to DNA damage-inducing agents

For the purpose of DNA damage sensitivity assays, a spot test was used. Serial 10-fold dilutions of cell suspensions were spotted onto Yeast Peptone Dextrose (YPD) plates containing indicated concentrations of hydroxyurea, MMS or bleomycin. Images were taken 4 days after incubation at 30°C.

RESULTS

Lif1 is a target for SUMOylation and requires both Siz1 and Siz2 SUMO ligases

A previous study has shown that Lif1 is SUMOylated *in vivo* (28). To define requirements for SUMOylation of Lif1, we took advantage of our *in vitro* SUMOylation reaction (27). The reaction was assembled with SUMO (Smt3), E1 (Aos1/Uba2 complex), E2 (Ubc9) and ATP. We directly tested the SUMO ligase required for Lif1 SUMOylation. As shown in Figure 1A, Lif1 SUMOylation was stimulated by Siz1 (lane 3) or Siz2 (lane 4) proteins, indicating that both E3 ligases SUMOylate Lif1 *in vitro*. The modification was also confirmed using anti-Lif1 and anti-Smt3 antibodies (Supplementary Figure S1). To test the role of these E3 ligases *in vivo,* we analysed SUMOylation of endogenous Lif1 protein in *siz1Δ*, *siz2Δ* and *siz1Δ siz2Δ* cells. Although *siz1Δ* and *siz2Δ* did not show a significant effect, the *siz1Δ siz2Δ* double mutant abolished Lif1 SUMOylation (Figure 1B), indicating that both Siz1 and Siz2 are required but are redundant for Lif1 SUMOylation. Quantification revealed that up to 10% of Lif1 was SUMOylated when cells were treated with MMS, although basal level of SUMOylation without DNA damage was also detected (Figures 1B and 6A).

As Lif1 binds to dsDNA (16), we asked whether DNA affects Lif1 SUMOylation. As shown in Figure 1A, addition of neither ssDNA nor dsDNA had any effect on the level of Lif1 SUMOylation in the absence of an E3 ligase. In the presence of Siz1 or Siz2 ligase, although dsDNA had no effect, ssDNA resulted in up to 50% reduction in Lif1 SUMOylation level (Figure 1C). This result was surprising, as Lif1 has not previously been shown to bind to ssDNA. To understand the effects of ssDNA, we tested whether Lif1 is capable of binding to ssDNA. Using electrophoretic mobility shift assay, we found that Lif1 can interact with ssDNA, and direct comparison as well as competition with dsDNA revealed similar albeit weaker, affinity compared with dsDNA (Figure 1D). Thus, ssDNA binding may underlie the reduction in Lif1 SUMOylation in Figure 1C. The fact that Lif1 and Nej1 form a tight complex *in vitro* and *in vivo* (18,20,21) prompted us to test the effect of Nej1 on Lif1 SUMOylation. We found that Nej1 did not have any impact on Lif1 SUMOylation (Figure 1E). Taken together, these data indicate that neither binding to Nej1 nor to dsDNA affects Lif1 SUMOylation, but ssDNA binding is likely inhibitory to its SUMOylation.

Lysine 301 is the main SUMO-conjugation site in Lif1

To determine the SUMOylation sites in Lif1, we analysed the SUMOylated protein using mass spectrometry (MS). This analysis identified lysine at position 301 (K301) as a major conjugation site (Supplementary Figure S2).
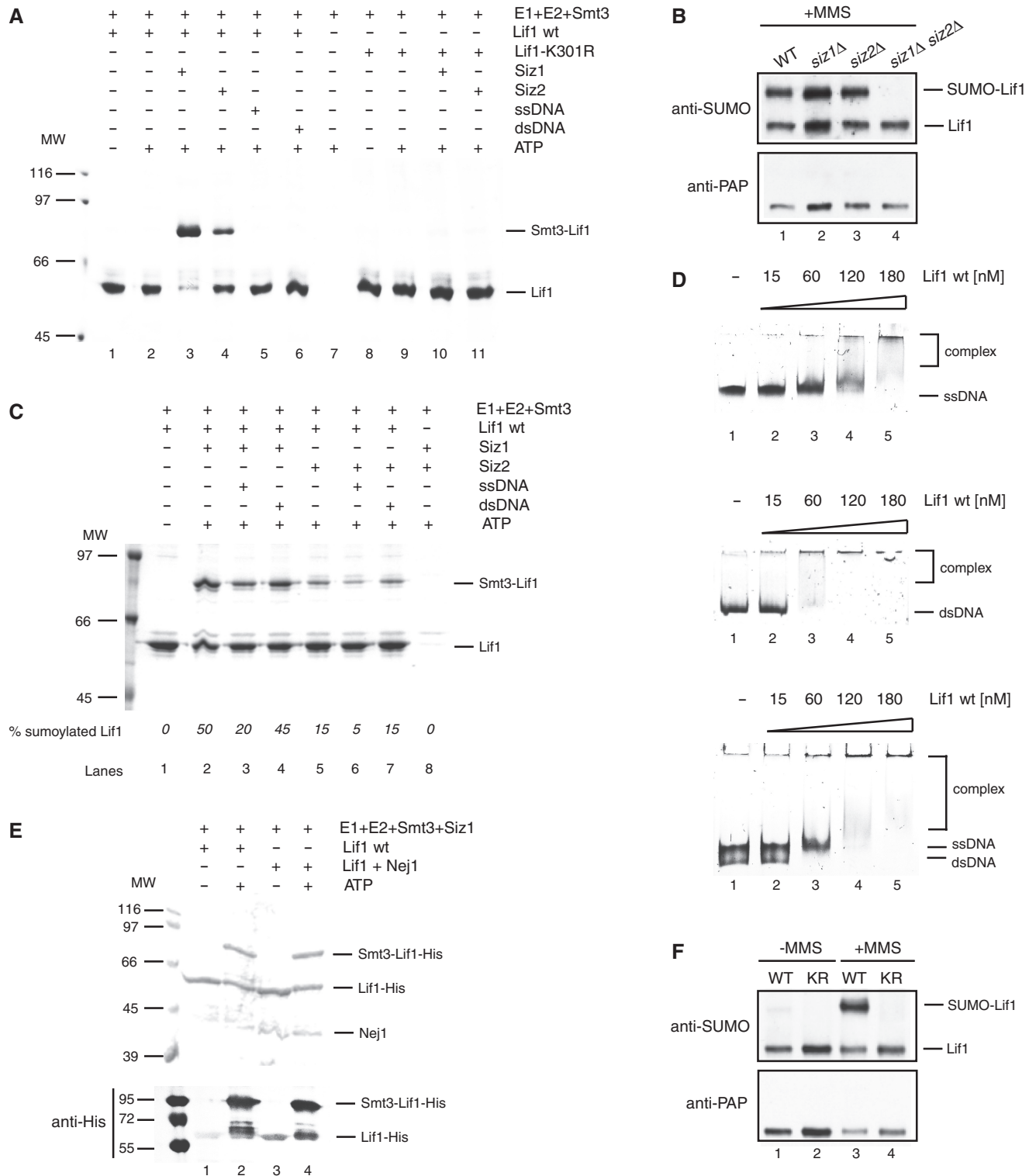
Figure 1. SUMOylation of Lif1 is dependent on Siz1 and Siz2 E3 ligases *in vitro* and *in vivo* and targets the lysine 301. (**A**) Lif1 is a target for SUMOylation *in vitro* and is stimulated by the Siz SUMO E3 ligases. SUMOylation assay was performed using recombinant Aos1/Uba2 (0.35 μM), Ubc9 (1.25 μM), SUMO (2.5 μM) and Lif1 (1.5 μM) proteins in the absence or presence of Siz1(1–465) (0.3 μM; lane 3), Siz2 (0.3 μM; lane 4), 83-mer ssDNA (100 μM nucleotides; lane 5), dsDNA (lane 6) and ATP (100 μM). The same experiment was also performed in the presence of Lif1-K301R (lanes 8–11). Reactions were incubated for 1 h at 30°C and analysed by 10% SDS–PAGE, followed by Coomassie blue staining. (**B**) Lif1 SUMOylation requires the Siz SUMO E3 ligases *in vivo*. Logarithmically growing yeast cells expressing TAP-tagged Lif1 protein in the indicated ligase mutant backgrounds were exposed to 0.3% MMS for 2 h and immunoprecipitated using immunoglobulin G-Sepharose to pull down TAP-tagged Lif1. After SDS–PAGE separation, immunoprecipitated proteins were immunoblotted with anti-Smt3 or anti-PAP antibodies. (**C**) SUMOylation of Lif1 in the presence of DNA *in vitro*. SUMOylation assay was performed in the presence of Lif1 (1.5 μM) and in the absence or presence of Siz1(1–465) (0.1 μM), Siz2 (0.1 μM), 83-mer ssDNA (100 μM nucleotides), dsDNA (100 μM nucleotides) and ATP

(continued)

Therefore, we introduced the K301R substitution into the *LIF1* sequence and purified the mutant protein (Lif1-K301R). As shown in Figure 1A (lanes 8–11), the mutant protein failed to be SUMOylated *in vitro*. This indicates that K301 is essential for this post-translational modification.

To address whether K301 is also targeted for SUMOylation *in vivo*, we fused Lif1 and Lif1-K301R with the TAP tag at the chromosomal locus in yeast. Lysates from these strains were subjected to immunoprecipitation and immunodetection using an antibody recognizing the TAP tag on Lif1 protein and another recognizing SUMO. This analysis revealed stronger SUMOylation of Lif1 under DNA damaging conditions, as seen previously (28). Similarly to *in vitro* conditions, Lif1-K301R failed to be SUMOylated, indicating that K301 is essential for Lif1 modification *in vivo* (Figure 1F).

DNA binding of Lif1-K301R and SUMOylated Lif1 proteins

We asked whether SUMOylation of Lif1 affects its DNA binding activity by electrophoretic mobility shift assay analysis. First, we tested the DNA binding activity of Lif1 wild-type and K301R mutant proteins. No differences in DNA binding were observed, indicating that the K301R substitution does not affect the dsDNA binding properties of the protein (Figure 2A).

Next, we compared DNA binding properties of unmodified and SUMOylated Lif1 proteins. SUMOylated Lif1 protein (~90%, Supplementary Figure S3) did not show any decrease in the ability to bind to dsDNA compared with wild-type. However, although the unmodified protein formed a discrete band with DNA, SUMOylated Lif1 showed a diffused signal, suggesting
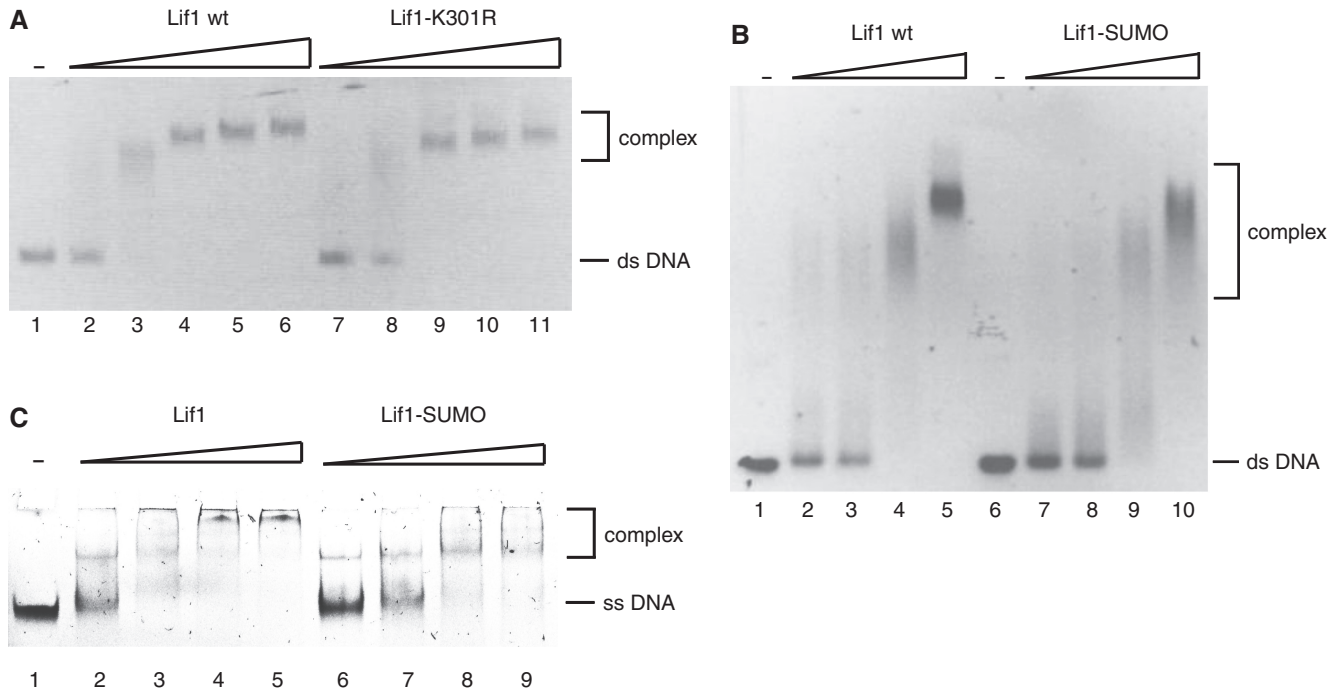

Figure 2. Binding of dsDNA by Lif1-K301R mutant and SUMOylated Lif1 proteins. (**A**) Increasing concentrations (0.1, 0.2, 0.4, 0.6 and 0.8 μM) of the Lif1 wild-type (lanes 2–6) and K301R mutant (lanes 7–11) proteins were incubated in the presence of 600 bp dsDNA (500 nM). (**B**) Increasing concentrations (0.4, 0.5, 1 and 2 μM) of SUMOylated (lanes 7–10) and non-SUMOylated (lanes 2–5) Lif1 proteins were incubated in the presence of 600 bp dsDNA (500 nM). Reaction mixtures were separated on 1% agarose gel and analysed. (**C**) Increasing concentrations (80, 160, 240 and 320 nM) of non-SUMOylated (lanes 2–5) and SUMOylated (lanes 6–9) Lif1 were incubated in the presence of fluorescently labelled 90-mer ssDNA (270 nM). The reaction mixtures were separated on 10% native polyacrylamide gel. DNA–protein complexes were visualized using the FLA-9000 Starion (Fuji-Film).

Figure 1. Continued
(100 μM) as indicated. Reactions were pre-incubated in the absence of ATP for 10 min at 30°C to enable complex formation. Next, ATP was added, and reactions were incubated for 30 min at 30°C. Analysis was performed by 10% SDS–PAGE followed by Coomassie blue staining. (**D**) Lif1 has both ssDNA and dsDNA binding activities. Indicated concentrations of Lif1 were incubated with fluorescently labelled 90-mer ssDNA (270 nM nucleotides), 90-mer dsDNA (540 nM nucleotides) or mixture of both ssDNA and dsDNA (270 nM nucleotides and 540 nM nucleotides, respectively). The reaction were incubated for 10 min at 37°C and separated on 10% native polyacrylamide gel. (**E**) Lif1 SUMOylation is unaffected by the presence of Nej1. Standard *in vitro* SUMOylation assay was performed using Aos1/Uba2, Ubc9, SUMO, Lif1, Siz1 and ATP in the absence (lane 2) or presence (lane 4) of Nej1. Reactions were incubated for 1 h at 30°C and analysed by 10% SDS–PAGE followed by Coomasie blue staining. SUMOylated Lif1 protein was detected by western blotting using anti-His antibodies. (**F**) Lysine 301 of Lif1 is the target for SUMOylation *in vivo*. Yeast cells expressing TAP-tagged wild-type Lif1 (wt) or Lif1-K301R (KR) mutant protein from the endogenous locus were grown in the absence or presence of 0.3% MMS and analysed as in B.

differences in the formation of higher order complexes (Figure 2B). Strikingly, SUMOylated Lif1 exhibited a decrease in ssDNA binding (Figure 2C), again suggesting that the DNA binding properties of Lif1 change after SUMOylation.

Lif1 SUMOylation and the K301R mutation do not affect interaction with Nej1, Xrs2 and Dnl4

Lif1 has been reported to interact with several proteins functioning in the NHEJ pathway, including Nej1, Xrs2 and Dnl4 (15,16,18–21,43). We tested whether SUMOylation of Lif1 impinges on these functions by checking whether SUMOylation at lysine 301 or the K301R substitution in Lif1 alters its ability to interact with Nej1. To ensure that the reaction conditions, as

well as the amounts of proteins in the pull-down reactions, are the same, we took equivalent fractions from the SUMOylation reactions performed in the presence or absence of ATP. As is evident from pull-down experiments, the Lif1–Nej1 interaction was unaffected by SUMOylation status and by the K301R substitution (Figure 3A and B). The ability of the Lif1-K301R mutant to interact with Nej1 was also confirmed using two-hybrid analysis, where Nej1 was fused to the activation domain of Gal4, whereas Lif1 (wild-type or K301R) was fused to the DNA binding domain (Figure 3C). Similarly to the Nej1-Lif1 two-hybrid interaction, neither the Xrs2–Lif1 nor the Dnl4–Lif1 combinations showed any noticeable differences between wild-type and K301R mutant protein (Figure 3C). Taken together,
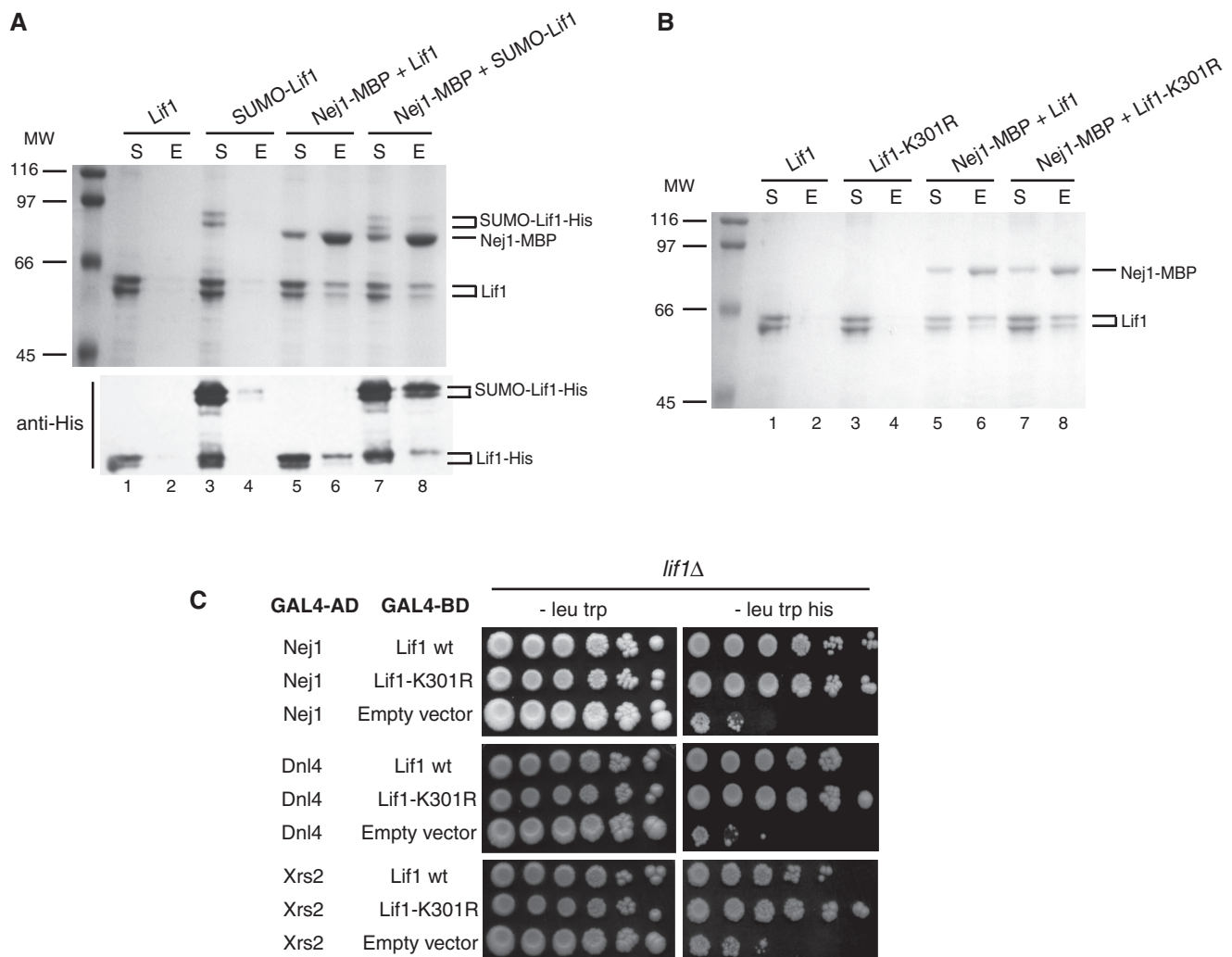

Figure 3. Lif1 SUMOylation and K301R substitution do not affect Lif1's interaction with Nej1, Xrs2 and Dnl4. (**A**) Pull-down of SUMOylated Lif1 and Nej1 proteins. (His)$_6$-Lif1 (5 μg) from the SUMOylation reactions done in the presence or absence of ATP was incubated with MBP-Nej1 (5 μg) and amylose resin. The bound proteins were eluted from the affinity resin by boiling with SDS sample buffer. Samples were resolved on a 10% SDS–PAGE gel, followed by Coomasie blue staining. The same samples were also transferred to a PVDF membrane and blotted with anti-His antibodies. (**B**) Lif1-K301R mutant is proficient for Nej1 interaction. Pull-down experiment with (His)$_6$-Lif1-K301R was performed as described in (A). Supernatant (S) and Elution (E) fractions were analysed. (**C**) Yeast two-hybrid interaction of Lif1 and Lif1-K301R with Nej1, Xrs2 or Dnl4. The *lif1Δ* PJ69-4 A strain transformed with *LIF1* or *LIF1-K301R* fused to the GAL4 DNA-binding domain and *NEJ1*, *XRS2* and *DNL4* fused to the GAL4 transcription activation domain, were spotted as 10-fold serial dilutions on medium lacking leucine and tryptophan or leucine, tryptophan and histidine. Empty vector was included as a negative control.

SUMOylation status and the K301R substitution do not affect Lif1's interactions with Nej1, Dnl4 or Xrs2.

SUMOylation of Lif1 inhibits its self-interaction

As Lif1, as well as its human counterpart, has been shown to self-associate (15,44–48), we asked whether SUMOylation of Lif1 affects this interaction using two-hybrid assays. Interestingly, we detected stronger self-interaction for Lif1-K301R compared with wild-type, indicating that SUMOylation of Lif1 at lysine 301 negatively regulates its self-association (Figure 4A).

To further demonstrate the inhibitory effect of SUMOylation on Lif1 self-association, we generated two-hybrid reporter strains lacking Siz1, Siz2 or both. In agreement with the aforementioned results, down-regulation of Lif1 SUMOylation by deleting the E3 ligases resulted in an enhancement in Lif1 self-association compared with the wild-type two-hybrid strain (Figure 4B).
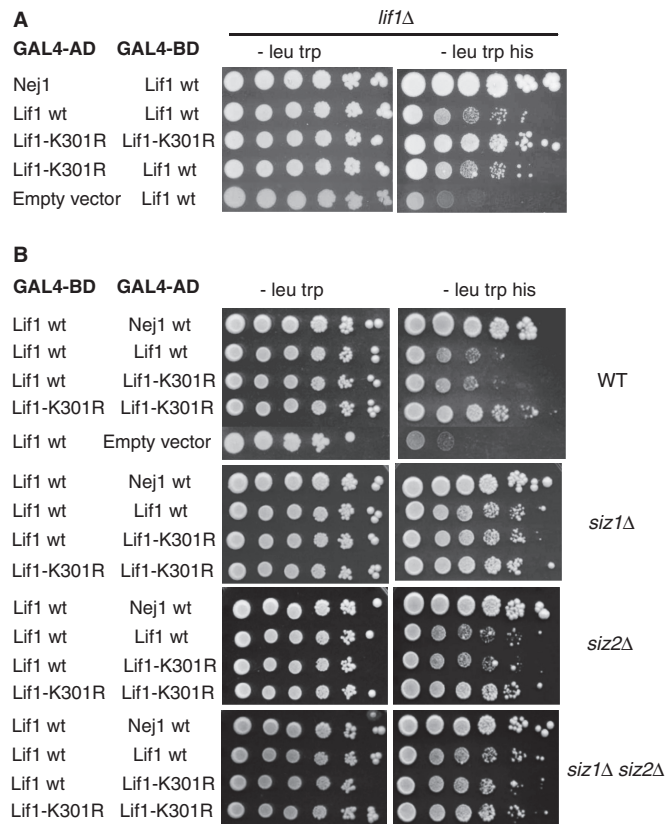

Figure 4. Abolition of Lif1 SUMOylation leads to defective Lif1 self-interaction. (**A**) Yeast two-hybrid analysis of Lif1 self-association. The *lif1Δ* PJ69-4 A strain containing *LIF1* or *LIF1-K301R* fused to the GAL4 DNA-binding domain and *NEJ1*, *LIF1* and *LIF1-K301R* fused to the GAL4 transcription activation domain, were spotted as 10-fold serial dilutions on medium lacking leucine and tryptophan or leucine, tryptophan and histidine. Empty vector was included as negative control. Nej1-Lif1 interaction serves as a positive control. (**B**) Yeast two-hybrid analysis of Lif1 self-association in PJ69-4 A *siz1*, *siz2* and *siz1 siz2* strains. The experiment was performed as in A.

Lif1 SUMOylation inhibits NHEJ repair

To determine the functional effect of the non-SUMOylatable *lif1-K301R* allele, we replaced wild-type *LIF1* with this mutation in yeast and tested sensitivity to various DNA damaging agents. Similarly to *lif1Δ* cells, the *lif1-K301R* mutant did not show significant sensitivity to any type of DNA damaging agent (Supplementary Figure S4) (7). Next, we used a plasmid-based rejoining assay to test whether Lif1 SUMOylation affects DSB repair. In line with previous findings (17), we observed a severe defect of *lif1Δ* cells in rejoining plasmid-based DSBs, regardless of polarity, complementarity, length or sequence content of the cleavage site (Supplementary Figure S5). An exception is the repair of blunt ends that is also markedly reduced in wild-type cells. The *lif1-K301R* cells consistently showed higher efficiency of NHEJ repair for all combinations of linearized plasmids tested compared with wild-type cells, although these were not statistically significant, probably owing to the large margins in the plasmid assays (Supplementary Figure S5).

To further test the effect of Lif1 SUMOylation, we assayed donor-less HO-induced chromosomal rejoining that is strictly dependent on NHEJ (40,41). Under persistent DSB induction, *lif1-K301R* cells exhibited nearly 2-fold increase in rejoining HO-induced breaks compared with wild-type cells (Figure 5, $p = 4.5 \times 10^{-5}$). Analysis of transient DSB induction shows a similar trend; however, the increase was not statistically significant (Supplementary Figure S6). Together, these results suggest that Lif1 SUMOylation is inhibitory to NHEJ repair, particularly under persistent DSB-induction conditions.

As the choice of NHEJ versus HR is regulated differently during the cell cycle, we asked whether Lif1 SUMOylation also varies during cell cycle progression. When cells synchronized in either G1 or G2/M were released, we observed that Lif1 SUMOylation does not show cell cycle phase dependence (Figure 6A and Supplementary Figure S7), suggesting that Lif1 is SUMOylated constantly. This is consistent with the finding that *lif1-K301R* resulted in increased NHEJ repair in G1, S, G2/M arrested or asynchronized cells in the donor-less HO-induced chromosomal rejoining assay (Figure 6B).

DISCUSSION

HR and NHEJ are two major repair pathways for DSBs and are important for genomic stability. How the pathway choice is made and how protein interactions within each pathway are regulated remains unclear. Several types of reversible modifications of proteins can dynamically modulate protein functions, providing possible mechanisms for this regulation (49). Besides CDK and Mec1-checkpoint-mediated phosphorylation, modification by SUMO has emerged as an important regulator in DSB repair. A number of DNA repair proteins have been shown to undergo SUMOylation, primarily after DNA damage (22,50–53). Nevertheless, little is known about the precise role of SUMOylation in regulating each of these substrates and how that contributes to different repair mechanisms.
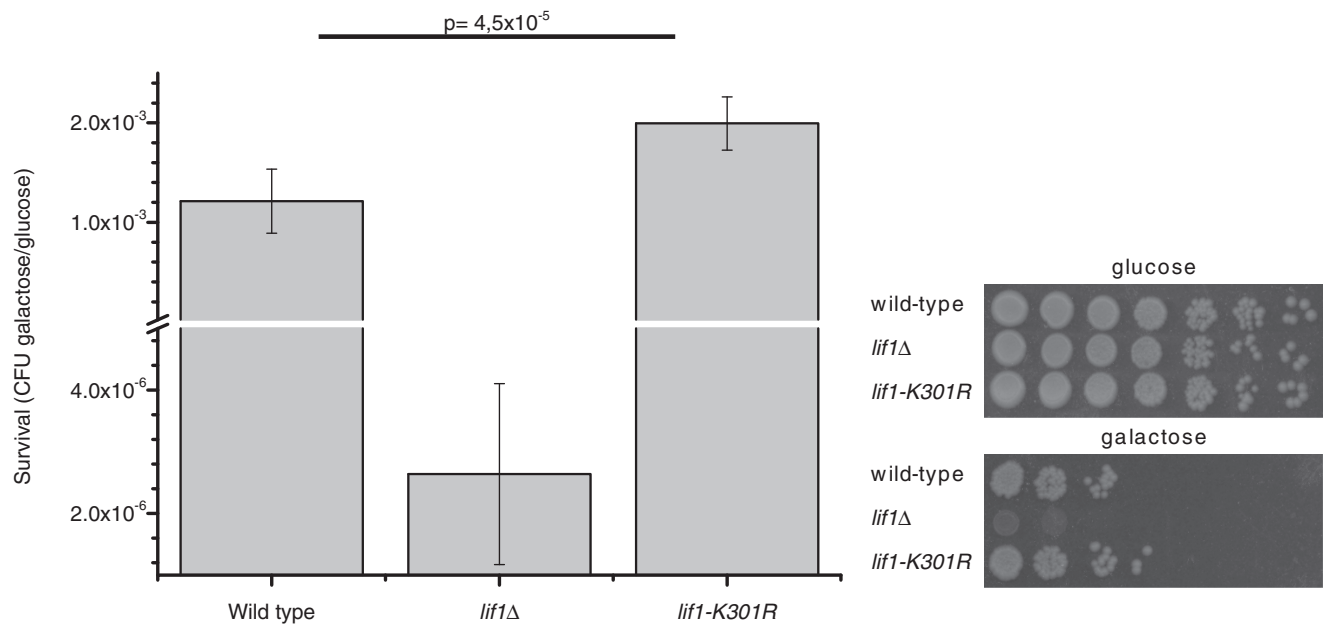
Figure 5. *lif1-K301R* increases efficiency of the chromosomal DSBs repair in persistent donor-less HO-induced assay. Wild-type, *lif1Δ* and *lif1-K301R* cells were grown overnight in 3% glycerol, washed and plated in serial dilutions on glucose- and galactose-containing media. The left panel represents quantification of the efficiency of rejoining of HO-induced DSBs calculated as the number of colonies obtained on plates with galactose normalized to that on glucose. The data represent the average of five independent experiments. The right panel represents spot test with 10-fold serial dilutions of the strains spotted on plates containing either glucose or galactose. For statistical analyses, one tailed paired t-test was used to calculate *p*-value.

SUMOylation of Lif1 involves lysine 301 and is influenced by ssDNA binding

Here, we examined SUMOylation of the NHEJ protein Lif1. We identified lysine 301 as the major target site for SUMOylation, and its substitution with arginine abolished this modification both *in vitro* and *in vivo*. We showed that the Siz1 or Siz2 SUMO E3 ligase was required for Lif1 SUMOylation *in vitro*. This is further supported by the *in vivo* analysis that demonstrated redundancy of Siz1 and Siz2 in Lif1 modification. Redundancy of Siz proteins has been observed for several other substrates (29,52). Whether they can differently affect their substrates is unclear in most cases and will need to be addressed in the future.

Although Nej1 and dsDNA had no impact on Lif1 SUMOylation, ssDNA exerted an inhibitory effect. We further uncovered that Lif1 binds ssDNA with a similar, albeit weaker, affinity than dsDNA. It is tempting to speculate that this newly discovered ssDNA binding activity of Lif1 might be important in joining DNA ends containing ssDNA regions, such as in microhomology-mediated end-joining. This activity may add to its known interaction and stimulation of Fen1 endonuclease (54) and interaction and coordination of Pol4 (55) to promote certain types of end joining. How ssDNA binding inhibits Lif1 SUMOylation requires further study, but we surmise that ssDNA binding may induce conformational changes in Lif1 that either prevent its interaction with SUMOylation enzymes or disfavour proper alignment of K301 with these enzymes. The inhibitory effect of ssDNA on Lif1 SUMOylation is

in contrast to Rad52 whose SUMOylation is stimulated by ssDNA binding (27), highlighting an interesting difference of ssDNA effect on NHEJ versus HR protein SUMOylation.

Lif1 SUMOylation decreases self-association and ssDNA binding without affecting binding to other proteins or dsDNA

Unlike ubiquitination, SUMOylation does not target proteins for degradation, but instead can alter protein–protein interactions or biochemical properties of the proteins (22,52,56). Using *in vitro* pull-down and yeast two-hybrid assays, we determined that SUMOylation of Lif1 does not affect its interaction with Nej1, Xrs2 or Dnl4, indicating that these interactions are not regulated by this modification. Binding of the Lif1-K301R mutant or SUMOylated Lif1 to dsDNA was also indistinguishable from that of wild-type or unmodified protein. On the contrary, ssDNA binding was reduced on Lif1 SUMOylation. Although the biological significance of this reduction is unclear, several possibilities can be envisioned. For example, this may help prevent joining of ssDNA-containing ends or interference with HR steps such as resection and consequent loading of RPA or Rad51 proteins onto ssDNA.

An interesting effect of Lif1 SUMOylation is its influence on self-association of the protein. We observed increased association of the Lif1-K301R mutant compared with wild-type protein in yeast two-hybrid. In addition, deletion of *SIZ1* or *SIZ2* resulted in increased Lif1 self-interaction, and *siz1Δ siz2Δ* led to interaction
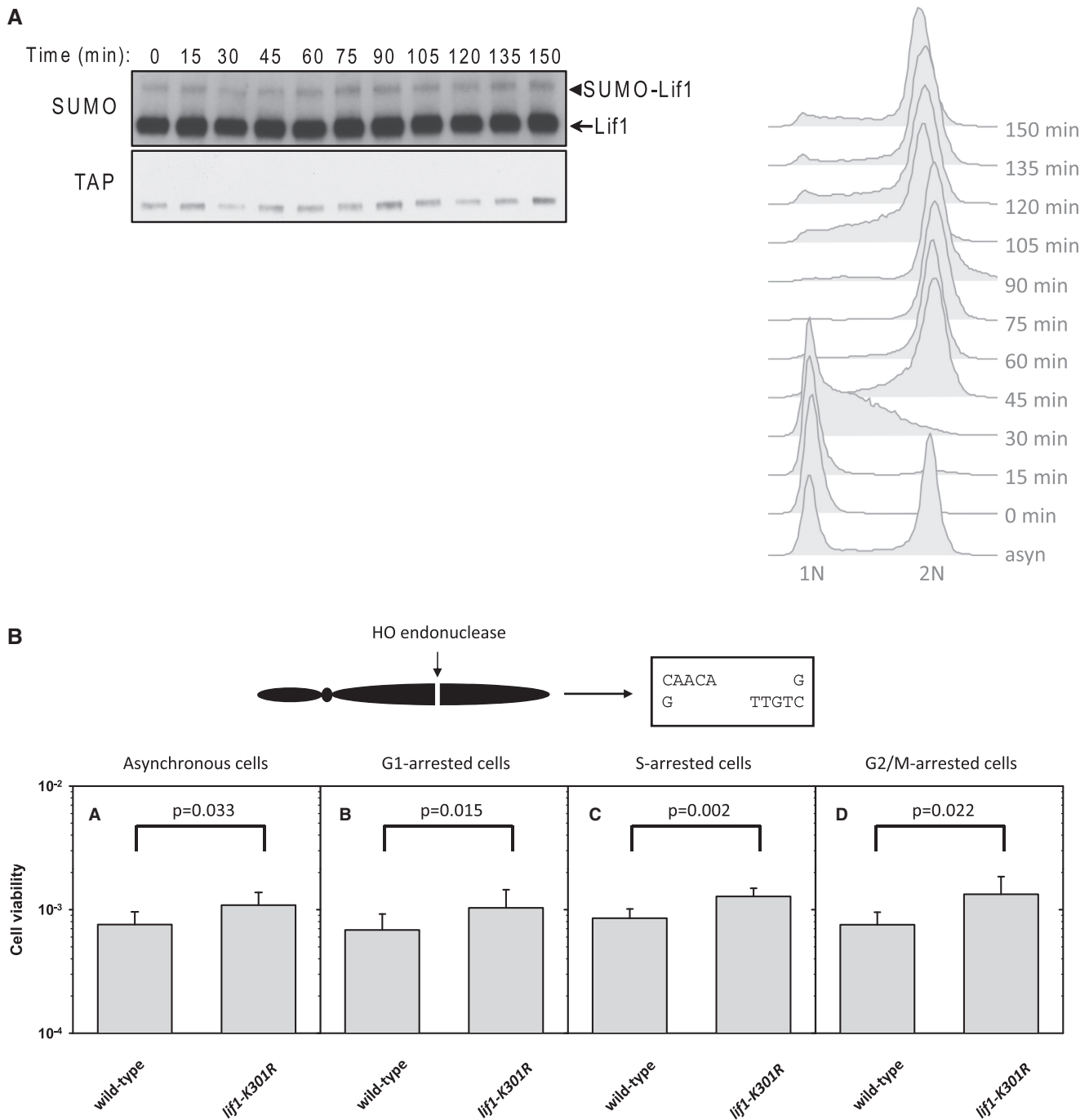
A

B

Figure 6. Lif1 is SUMOylated throughout the cell cycle and *lif1-K301R* repairs DSBs more efficiently than the wild-type cells during the cell cycle. (**A**) Alpha-factor-arrested G1 cells (0 min) containing Lif1-TAP were released into fresh media, and samples were examined at indicated time points for SUMOylation of Lif1 (left) and by FACS analysis (right). (**B**) Survival after persistent induction of HO endonuclease in the asynchronous cells (A), and cells arrested in G1 (B), S (C) and G2/M (D) phases in wild-type and *lif1-K301R* cells. The data represent the average of four independent experiments. For statistical analyses, one tailed paired t-test was used to calculate *p*-value.

levels similar to the non-SUMOylatable Lif1-K301R mutant. Both these results indicate that SUMOylation at lysine 301 negatively regulates Lif1 oligomerization. This may provide an explanation for the differential effects of SUMOylated Lif1 versus unmodified Lif1 in binding to DNA. For example, reduced self-association of Lif1 owing to SUMOylation may alter the assembly of the DNA–protein complexes.

Abolition of Lif1 SUMOylation promotes NHEJ repair

To elucidate the role of Lif1 SUMOylation *in vivo*, we took advantage of two NHEJ-dependent DSB repair assays. First, using a plasmid-based rejoining assay, we observed slightly increased (though not statistical significant) recircularization efficiency for all combinations of linearized plasmids in *lif1-K301R* cells compared with

wild-type. Second, we assayed repair of DSBs induced by HO endonuclease at the *MAT* locus on chromosome *III* in a strain that lacks the donor sequences and thus solely uses NHEJ for repair (41). Here, *lif1-K301R* cells rejoined DSBs more efficiently (statistically significant) than the wild-type cells on persistent HO induction. When HO was induced transiently, *lif1-K301R* cells still repaired HO-induced DSB efficiently than the wild-type cells, but the difference was not statistically significant.

As Lif1 is phosphorylated by cyclin-dependent kinase in S and G2 phases, but not G1 (42), we asked whether SUMOylation of Lif1 is also differently regulated during the cell cycle or is involved in the cell cycle regulation of NHEJ. We observed a constant level of Lif1 SUMOylation throughout the cell cycle. Additionally, *lif1-K301R* increases NHEJ in G1, S, and G2/M phases of the cell cycle. These results suggest that SUMOylation of Lif1 serves as a cell cycle-independent negative regulator of NHEJ. This level of regulation may safeguard the genome by ensuring that NHEJ levels remain low during normal growth.

Lif1 SUMOylation is induced by MMS; the meaning of this increase is unclear, though we provide several possibilities. As *lif1Δ* cells are not MMS sensitive, Lif1 modification may prevent its interference with other repair pathways. For example, SUMOylation of Lif1 results in decreased binding to ssDNA, thus possibly alleviating competition with other ssDNA binding proteins. On the other hand, the lack of sensitivity to MMS and other DNA damaging agents by *lif1Δ* or *lif1-K301R* may be due to the presence of redundant pathways. For example, SUMOylation of Ku, MRX or other NHEJ proteins could compensate for loss of Lif1 SUMOylation (28,57–59). Further studies combining various SUMO-deficient alleles will be able to address this possibility.

Moreover, the effect of Lif1 SUMOylation on its self-interaction could represent a similar mechanism of regulation as is observed for human XRCC4 where binding of the BRCT domain or DNA-PK phoshorylation alters XRCC4 tetramerization (48,60,61). Inhibition of NHEJ could be an efficient strategy in the fight against cancers, particularly those with mutations in HR genes (*BRCA1* and *BRCA2*), because repression of the alternative DSB repair pathway of NHEJ in these cells could lead to a substantial decrease in cancer cell survival. Understanding the molecular mechanisms of NHEJ and its regulation will aid the development of novel therapeutic strategies, such as blocking XRCC4 self-association, that may be used along with DNA-damaging agents in the treatment of HR-deficient cancers.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–7.

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