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Report

The SUMO-targeted ubiquitin ligase subunit Slx5 resides in nuclear foci and at sites of DNA breaks

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The Slx5/Slx8 protein complex, a heterodimeric SUMO-targeted ubiquitin ligase, plays an important role in genomic integrity. Slx5/Slx8 is believed to interact with sumoylated proteins that reside in the nuclei of budding yeast cells. In this complex, Slx5, owing to at least two SUMO interacting motifs (SIMs), has been proposed to be the targeting subunit of the Slx8 ubiquitin ligase. However, little is known about the exact subnuclear localization and targets of Slx5/Slx8. In this study we show that Slx5, but not Slx8, forms prominent nuclear foci. The formation of these foci depends on SUMO and a SIM in Slx5. Therefore, we investigated the subnuclear localization and potential chromatin association of Slx5. Using co-localization studies in live cells and fixed chromatin, we were able to localize Slx5 to DNA damage induced foci of Rad52 and Rad9, two proteins involved in the cellular response to DNA damage. Subsequent chromatin immunoprecipitation (ChIP) studies revealed that Slx5 is associated with HO endonuclease induced chromosome breaks. Surprisingly, real-time PCR analysis of Slx5 ChIPs revealed that the level of Slx5 at HO breaks in an *slx8* deletion background is reduced about 4-fold. These results indicate that the DNA-damage targeting of Slx5/Slx8 depends on formation of the heterodimer and that this occurs at a subset of nuclear foci also containing DNA damage repair and checkpoint factors.

Introduction

Ubiquitin and SUMO are small, conserved proteins that can conjugate to lysine residues of specific cellular proteins. These modifications, termed ubiquitylation and sumoylation, respectively, modulate the fate, function and interactions of target proteins.[1] Ubiquitin, as well as SUMO, is attached to protein substrates in a multi-step process involving activating (E1), conjugating (E2) and ligating (E3) enzymes.[1] Multiple rounds of this conjugation process result in formation of ubiquitin and SUMO chains that show specific monomer-monomer linkages. For example, ubiquitin chains linked via lysine 48 (K48) are best known for their role in targeting modified proteins to the proteasome. However, in its monomeric and lysine 63 (K63)-linked forms, ubiquitin mediates other nondegradative functions, including signaling and protein relocalization. Until recently, there was little evidence suggesting that SUMO also played a role in targeting proteins to the proteasome. Rather, protein sumoylation was found primarily to alter protein interactions, localization or activity.[1]

A function for SUMO in proteolytic targeting was recently uncovered by studies of a novel class of SUMO targeted ubiquitin ligases termed STUbLs. STUbLs comprise a conserved family of ubiquitin ligases that interact with sumoylated proteins and use their intrinsic ubiquitin ligase activity to modify them with ubiquitin. This makes STUbLs important enzymes at the cross-roads between the two modification systems.[2] Though only a few putative STUbL targets have been described, the absence of STUbLs leads to accumulation of many sumoylated proteins within the cell.[3-6] It is therefore likely that at least in some cases, ubiquitylation of SUMO-modified proteins leads to proteasomal targeting and destruction.[7]

The founding members of the STUbL family, Slx5 and Slx8, were identified as a complex of proteins required for the viability of *S. cerevisiae* cells lacking *SGS1*, a gene encoding a RecQ DNA helicase involved in genomic integrity.[8] Soon evidence accumulated that cells lacking Slx5 and/or Slx8 are sensitive to genotoxic insults and exhibit high levels of gross chromosomal rearrangements.[9] Furthermore, Slx5 and Slx8 play a role in recombinational DNA repair,[4,10] modulate senescence of telomerase mutants,[10] and affect transcriptional regulation.[11] However, it was not until Slx5 and Slx8 were purified and subjected to in vitro ubiquitylation assays that their role as ubiquitin ligases was realized.[6,12,13]

So far STUbL proteins have been characterized in yeast, including *S. cerevisiae* (Hex3/Slx5, Slx8) and *S. pombe* (Rfp1,

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Rfp2, spSlx8) and also in humans (RNF4).[3,5,7,12-15] In yeast these STUbLs function as heterodimeric proteins (e.g., *S. cerevisiae* Slx5/Slx8). Presently, a single protein (RNF4) appears to take on STUbL functions in human cells. RNF4 localizes diffusely to the nucleus, forms speckles and is also recruited to PML nuclear bodies.[7,14,16,17] The degree of functional conservation is underscored by the finding that *RNF4* can complement both *slx5* and *slx8* deletions in budding yeast and loss of Rfp1, Rfp2 and spSlx8 in fission yeast.[3,12,13] All STUbLs contain RING domains, consistent with a functional role as E3 ligases. These RING domains play a role in the interaction of heterodimeric STUbLs and are essential for ubiquitylation of STUbL substrates in in vitro ubiquitylation assays.[6] Furthermore, Slx5 and its orthologs also contain several SIMs (SUMO-interacting motifs), which are believed to play a role in the targeting and recruitment of sumoylated proteins. This suggests that Slx5 is the primary substrate-recognition subunit of the heterodimeric Slx5/Slx8 STUbL. SIMs form binding pockets for SUMO and have been identified in a variety of proteins with functions including DNA repair, transcriptional activation, nuclear body formation and protein turnover.[2,18]

To date, only a few SUMO modified STUbL substrates have been identified. In vitro studies suggest that sumoylated Rad52, a homologous recombination protein involved in DNA repair, is a better substrate for the heterodimeric Slx5/Slx8 STUbL than unmodified Rad52.[6] Similarly, in vitro, RNF4 has been shown to mediate the ubiquitylation of SUMO-2-modified promyelocytic protein, PML.[7] Slx5 and its orthologs also interact with proteins involved in chromosomal maintenance (Nse5), silencing (Sir2), kinetochore function (Ndc10) and DNA repair (spRad60), amongst other proteins, but the relevance of these interactions still remains unclear.[12,13,19,20]

One important avenue for identifying STUbL functions and substrates is the determination of their localization within the cell. Slx5, Slx8 and their various orthologs have been found to reside in the nucleus. However, varying observations have been made regarding the subnuclear localization of STUbLs. In live budding yeast cells, Slx8 was reported to reside in nucleolar replication foci formed by the proliferating cell nuclear antigen (PCNA).[4] In contrast, deconvolution of immunofluorescence images suggested multiple Slx8 foci and an overlap of Slx8 with nuclear pore complexes (NPCs).[21] In fission yeast, however, Slx8 only displayed a diffuse nuclear localization without foci.[12] In contrast Slx5 orthologs reside in nuclear foci. These foci may be equivalent to speckles formed by hsRNF4 which can also be recruited to PML bodies.[7,12-14,16,17,19,21,22] In yeast Slx5 foci do not overlap with Sir2, telomeres or nucleoli and may partially overlap with NPCs.[19,21] Furthermore, Slx5 foci do not appear to increase in number after genotoxic insults.[21] A heterodimeric complex of Slx5 and Slx8 was also able to interact with double-stranded DNA as shown by in vitro gel-shift assays with recombinant proteins. However, the Slx5/Slx8/double-stranded DNA (dsDNA) interaction does not appear to be sequence specific.[22] Recently, it has been shown in vivo that Slx8 does interact with specific dsDNA breaks.[21]

In the work presented here, we aim to clarify the subnuclear localization of Slx5 and the factors required for it. We show that Slx5 forms distinct nuclear foci that depend on functional SUMO in the cell and the presence of at least one SIM in Slx5. Since Slx5 function has been implicated in the cellular response to genotoxic stress, we investigated the presence of Slx5 at nuclear DNA repair foci formed by Rad52 and Rad9 and found a partial overlap. Furthermore, using chromatin immunoprecipitation (ChIP) assays, we found association of Slx5 with a specific dsDNA break. Interestingly, the association of Slx5 with dsDNA breaks requires Slx8, but is not inhibited by overexpression of conjugation competent SUMO. Based on our findings, we propose a model in which Slx5 is recruited, in an Slx8-dependent fashion, to sites of recombinational DNA repair.

Results

***S. cerevisiae* Slx5-GFP localizes to the nucleus and forms distinct nuclear foci.** To understand the molecular roles of Slx5 and Slx8, we ectopically expressed *SLX5-GFP* and *SLX8-YFP* constructs in budding yeast cells (see Materials & Methods). Both a *slx5* deletion strain expressing *SLX5-GFP* and an *slx8* deletion strain expressing *SLX8-YFP* grew similarly to congenic wild-type cells after UV irradiation. Growth of cells lacking *SLX5* and/or *SLX8* is inhibited after DNA damage by UV irradiation (Fig. 1A) and on media containing hydroxyurea (HU) (Fig. 3C and data not shown). This is consistent with our previous finding that *slx5*Δ and *slx8*Δ DNA damage sensitivity can be fully complemented by epitope-tagged versions of *SLX5* and *SLX8*, respectively.[6]

Next we analyzed the subcellular localization of Slx5-GFP and Slx8-YFP by fluorescence microscopy of logarithmically growing cells. Both Slx5-GFP and Slx8-YFP exhibit a diffuse intranuclear GFP or YFP signal. We also noted that in ~80% of cells (YOK851), Slx5-GFP formed between 1–5 intranuclear foci (Fig. 1B). Furthermore, Slx5-GFP foci overlapped nuclear DNA in DAPI stained cells (Fig. 1C). Our observation that Slx5, but not Slx8, formed intranuclear foci led us to extend our analysis of the subnuclear localization of Slx5.

Slx5-GFP foci are SUMO dependent. The live-cell imaging data revealed both distinct foci and diffuse staining for Slx5-GFP in yeast nuclei. To test if Slx5-GFP foci exhibited a cell cycle-specific distribution, we examined cells after alpha-factor arrest (G_1) and subsequent release of the synchronized cells into the cell cycle (Fig. 2A). We determined that foci-formation appeared most prevalent during S (61%) and G_2/M phase (58%) with overall weaker, less defined and less frequent foci in G_1-phase (25%) of the cell cycle. Cells exiting mitosis frequently showed well defined foci but the overall incidence of foci was slightly reduced (44%). Analysis of Slx5-GFP protein levels at various times before and after alpha factor arrest revealed that foci reduction was not due to reduced levels of the fusion protein (Suppl. Fig. S1).

Next we investigated if Slx5 localization was dependent on its binding partner Slx8. As previously shown, Slx5 can exists in a stable protein complex with Slx8 or by itself.[22] The Slx5-GFP plasmid was introduced into *slx5*Δ and *slx5*Δ *slx8*Δ strains and the distribution of the Slx5-GFP fusion protein was examined in logarithmically growing cells. We noted that in untreated *slx8*Δ cells, Slx5-GFP foci were substantially brighter (Fig. 2B top right) than in the strain containing wild-type *SLX8* (Fig. 2B top left).

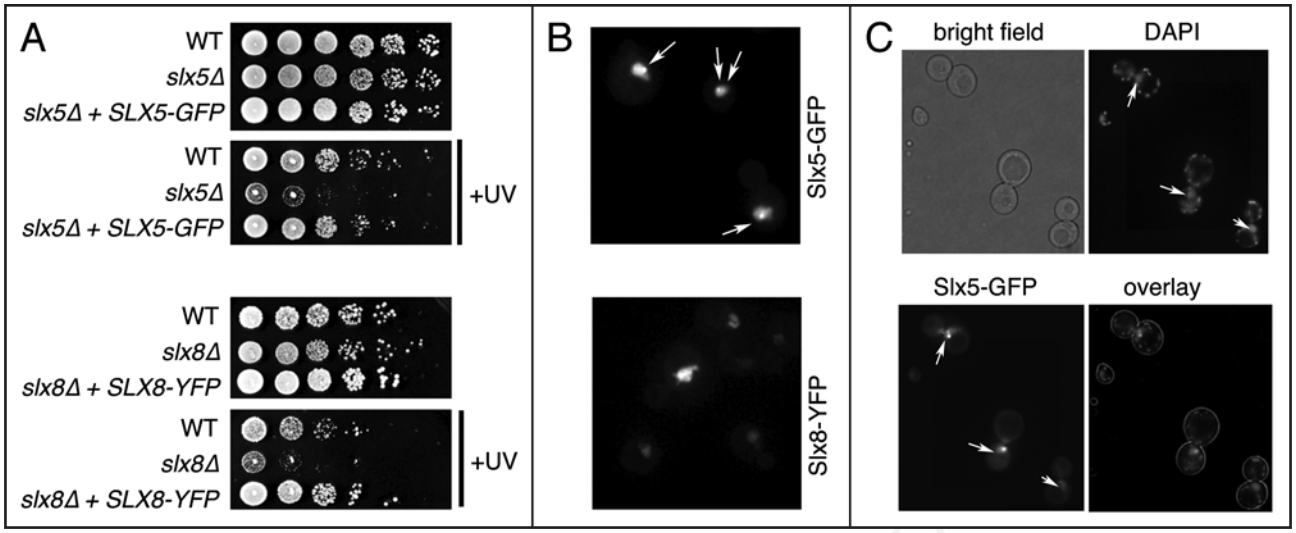

Figure 1. Slx5-GFP localizes to the nucleus and forms distinct foci in *S. cerevisiae*. (A) Strains expressing Slx5-GFP or Slx8-YFP are not sensitive to sub-lethal doses of UV irradiation. Growth of the parental wild-type (WT) strain (MHY500) was compared to that of *SLX5-GFP* (YOK851), and *slx5*Δ (MHY3712) strains (Top 2). Similarly growth of the parental wild-type (WT) strain (MHY500) was compared to that of *SLX8-YFP* (YOK850), and *slx8*Δ (MHY3716) strains (bottom 2). All strains were grown to logarithmic phase in YEPD at 30°C, diluted, spotted in 10-fold increments on YPD, and incubated for 3 days at 30°C. Duplicate YEPD plates (+UV) were prepared as above except that spotted cells were UV irradiated with a dose of 100 J/m^2 using a UV cross-linker. (B) Slx5-GFP and Slx8-YFP exhibit diffuse nuclear staining and Slx5-GFP can localize to distinct foci. Strains expressing Slx5-GFP (YOK851) and Slx8-YFP (YOK850) were grown to logarithmic phase at 30°C and examined under a fluorescence microscope (Slx5-GFP and Slx8-YFP). (C) Slx5 foci overlap with nuclear DNA. DNA of live Slx5-GFP expressing cells (bright-field) was stained with DAPI (upper right) to confirm our observation that Slx5-GFP foci (lower left) overlap nuclear DNA (overlay). Arrows indicate DAPI stained nuclei with Slx5-GFP foci.

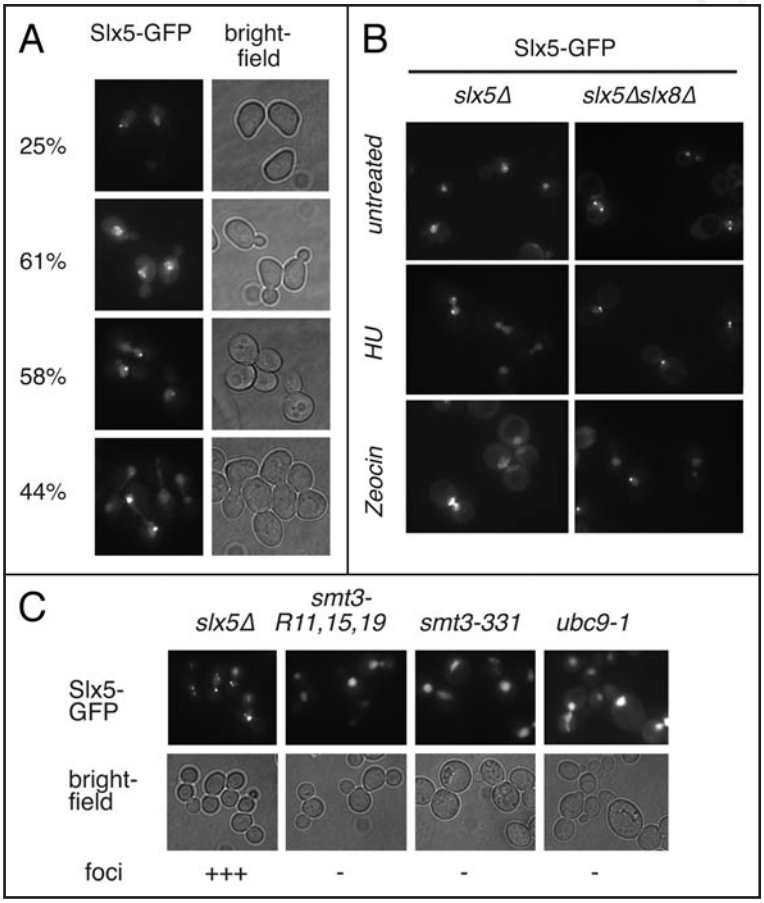

Figure 2. Slx5 foci are modulated. (A) Cell cycle modulation of Slx5-GFP foci. A strain expressing Slx5-GFP (YOK898) was synchronized in G_1 by treatment with α-factor (top row) and released into fresh medium. Samples were analyzed by fluorescence microscopy (Slx5-GFP). Prevalent cell morphologies (bright-field) and percent of Slx5-GFP foci observed are indicated. Foci after time (min) of relase: 25 min: 25%, 50 min: 61%, 75 min: 58%, 100 min, 44%. At least 100 cells were counted at each stage. (B) Modulation of Slx5 foci due to absence of *SLX8*. Slx5-GFP foci were observed in a *slx5*Δ single mutant (YOK851) and a *slx5*Δ *slx8*Δ double mutant (YOK853). All strains were grown to logarithmic phase in YPD and then grown for an additional 3 hours in fresh media (untreated), media containing 0.1 M Hydroxyurea (HU), or media containing 0.05 mg/ml phleomycin D 1 (Zeocin™). Samples were harvested after 3 hours and analyzed by fluorescence microscopy. Slx5-GFP foci were evaluated by dividing average foci intensity by average foci area, revealing a ~50% increase in overall Slx5 foci intensity. Area and intensity measurements were collected using i-vision software. (C) Modulation of Slx5-GFP foci due to mutations in *SUMO* (*SMT3*) and SUMO conjugation. Slx5-GFP was observed in *slx5*Δ (YOK898), two SUMO (*SMT3*) mutants, *smt3-331* (SBY331) and *smt3-R11,15,19* (GBY1), and a *ubc9-1* (YOK847) mutant. Transformants were grown to log phase at 30°C and then analyzed by fluorescence microscopy. Prevalent cell morphologies (bright-field), Slx5-GFP foci and their presence (+++) or reduction (-) are indicated.

Indeed, our measurements revealed that foci in *slx8*Δ cells were on average 50% brighter than those in *SLX8* cells (see Fig. legend for Fig. 2B). Also, while we observed a few cells with a dramatically increased number of Slx5-GFP foci, the number per cell did not increase appreciably for most cells. The same was true when genotoxic stressors were applied. Both strains were observed in the presence of either the DNA-damaging drug Zeocin™ (phleomycin D1) or hydroxyurea (HU), a ribonucleotide reductase inhibitor that leads to stalled replication forks. After complete cell cycle arrest by Zeocin (G_2/M phase) or high levels of HU (S phase), most Slx5-GFP-expressing *slx8*Δ cells contained 1–2 highly defined bright Slx5-GFP foci (Fig. 2B, middle and bottom). In HU-treated *SLX8* cells, brighter foci were also sometimes observed, but the diffuse nuclear staining was not decreased relative to untreated cells. Due to the enhancement of Slx5-GFP foci in *slx8*Δ cells, we decided to re-evaluate the localization of Slx8 both in wildtype and *slx5*Δ strains. However, using our strains and growth conditions, we found little or no evidence of foci formation of Slx8-YFP (Suppl. Fig. S2). Unlike Slx5-GFP, nuclear Slx8-YFP staining remained bright and diffuse in our untreated, Zeocin-treated and HU-treated samples. Therefore, our localization studies raised two major questions: (1) can Slx5 and Slx8 exist in separate pools and (2) what are the requirements for Slx5 foci formation? As *slx8*Δ strains contain elevated levels of sumoylated proteins,[5,6] we hypothesized that SUMO conjugates may be a factor in Slx5-GFP foci formation.

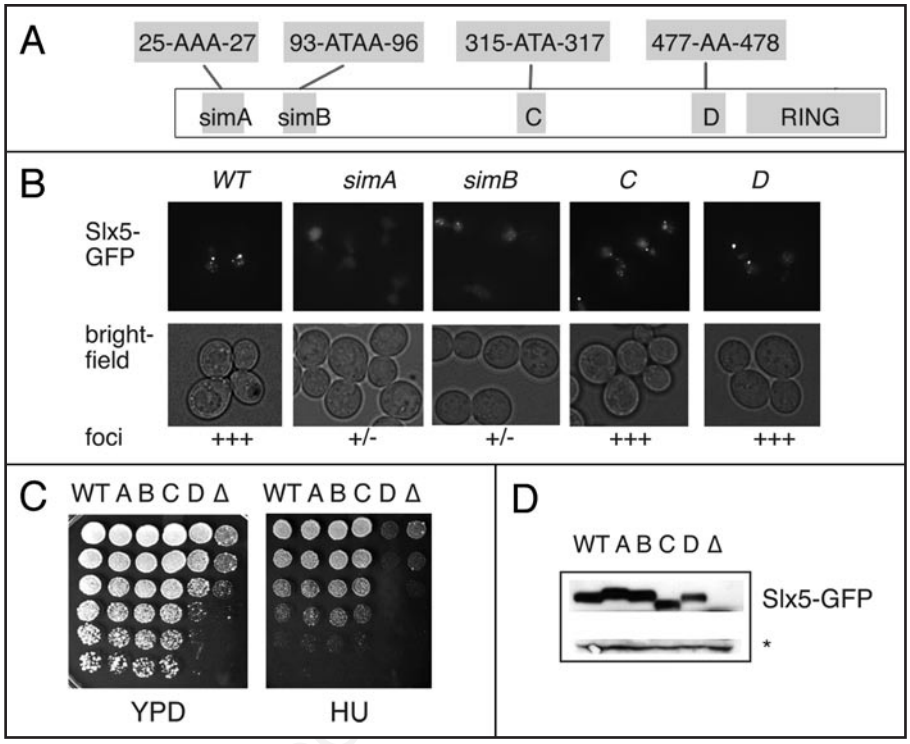

Figure 3. Slx5 foci depend on SUMO-interacting motifs (SIMs) in Slx5. (A) Diagram of Slx5 with four mutations and RING domain highlighted (not to scale). The position and amino-acid identity of each mutant (*simA*, *simB*, and two additional mutants C and D) is indicated (Xie et al. 2007). (B) Analysis of foci in Slx5-GFP mutants. Individual mutants (Table 1) and a wild-type Slx5-GFP clone were transformed into a *slx5*Δ strain (YOK720), grown to logarithmic phase, and then analyzed by fluorescence microscopy. Prevalent cell morphologies (bright-field), Slx5-GFP foci, and the presence or absence of foci (+/-) are indicated. (C) Analysis of growth properties of Slx5-GFP mutants. Individual mutants (see B above) and a wild-type Slx5-GFP clone were transformed into a *slx5*Δ strain (YOK720), grown to logarithmic phase, diluted, spotted in 10-fold increments on YPD or YPD containing 0.1 M hydroxyurea (HU). Also included in this analysis is the untransformed *slx5* deletion strain that fails to grow in the presence of HU. Note that mutant D can form foci (see B above) but fails to grow on HU containing media. (D) Analysis of wild-type and mutant Slx5-GFP proteins. Total protein was harvested from cells expressing the individual mutants (A–D), a wild-type Slx5-GFP clone (WT), or the untransformed slx5Δ strain (Δ). Slx5-GFP proteins were visualized using our anti Slx5 antibody (see materials & methods). A non-specific band (labeled *) is shown as a loading control.

Based on our previous finding that Slx5 contains at least two SIMs,[6] we reasoned that perturbation of SUMO dynamics in the cell may alter the distribution of Slx5-GFP foci. Therefore, we examined Slx5-GFP foci in cells expressing a mutant SUMO protein (*smt3-331*). The *smt3-331* temperature-sensitive mutant was previously shown to cause a delay in sister chromatid separation.[23] The Slx5-GFP plasmid was transformed into *smt3-331* cells (as well as a wild-type *slx5*Δ control strains) and logarithmically growing cells were examined. Notably, Slx5-GFP foci were absent or greatly reduced in *smt3-331* cells at permissive (30°C) and non-permissive temperature (37°C—data not shown) (Fig. 2C—3rd column). We made a similar observation in a strain expressing the *smt3-R11,15,19* mutant (Fig. 2C—2nd column) that is unable to form polySUMO chains.[24] Slx5-GFP foci were absent or greatly reduced in *smt3-R11,15,19* cells at all temperatures assayed. This observation might be related to the previous finding that polySUMO chain formation is important for Slx5 interaction and Slx5/Slx8-mediated ubiquitylation.[5,15] Finally, we also tested *ubc9-1*, a mutant of the SUMO conjugating enzyme E2 which impairs SUMO conjugation.[25] Consistent with our data on the SUMO mutants, Slx5-GFP foci were absent or greatly reduced in *ubc9-1* cells. In all three mutants we were able to detect a diffusely staining Slx5-GFP signal in yeast cell nuclei. In summary, these data suggest that Slx5 foci formation depend on protein sumoylation, particularly formation of polySUMO chains.

Slx5-GFP foci are SIM dependent. To corroborate the inference that Slx5 is recruited to foci by binding SUMO or polySUMO (Fig. 2C), we generated SIM mutants in our Slx5-GFP plasmid and assayed their ability to generate foci in *slx5*Δ cells. SUMO binds a hydrophobic core containing 3–4 aliphatic residues in the SIM. Altogether, we generated four single mutants replacing key hydrophobic residues with alanines in two known SIMs (A & B) and potential SIMs that match the consensus less well (C & D) (Fig. 3A).[6] Each mutant (simA, simB, C and D) was transformed into the *slx5*Δ strain and foci formation was assessed. We found

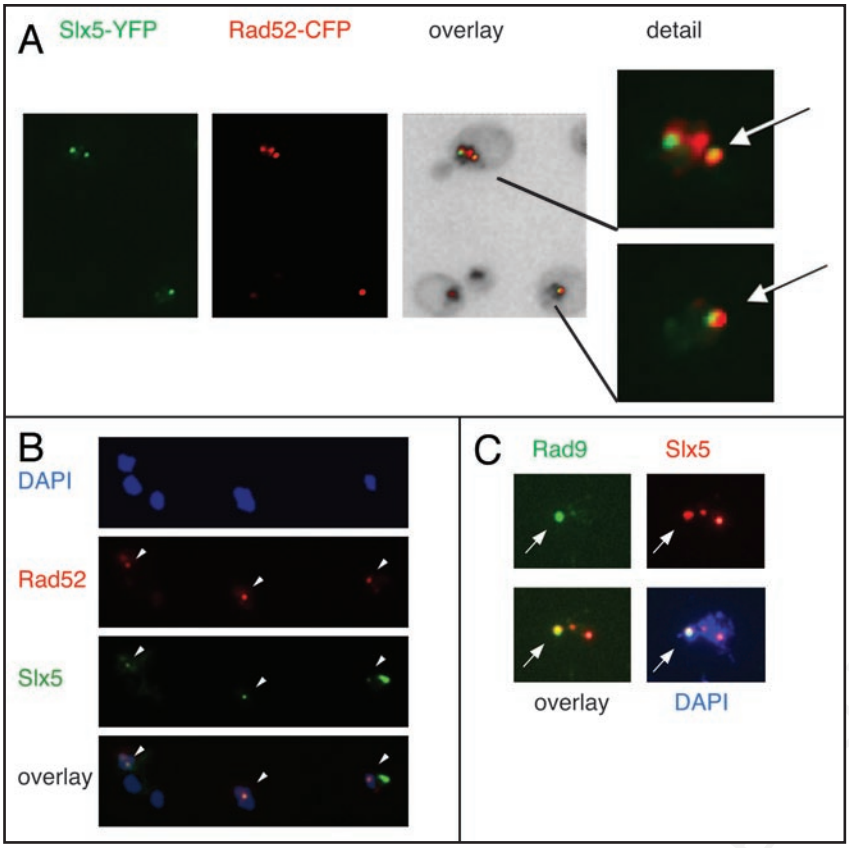

Figure 4. Slx5 foci can colocalize with DNA repair centers. (A) A strain coexpressing Slx5-YFP (green) and Rad52-CFP (red) (YOK510) was grown to logarithmic phase at 30°C, irradiated with 75 J/m^2 of UV light, allowed to recover for 60 minutes in YPD media, and then observed under a fluorescence microscope. Indicated are pseudo-colored Slx5-YFP labeled foci (left) and prominent Rad52-CFP foci (middle). Localization of Slx5 and Rad52 foci is also shown as merged (overlay) and magnified images (detail). Note that Slx5-YFP foci may exist as separate entities away from or in distinct overlay with Rad52 foci (white arrows). (B) A strain coexpressing Slx5-GFP (green) and HA tagged Rad52 (red) (YOK4183) was grown to logarithmic phase at 30°C, and then transferred to media containing 0.05 mg/ml phleomycin D 1 (Zeocin™) for an additional 3 hours. Chromosome spreads of these cells were probed with anti-GFP or anti-HA antibodies (see materials and methods). The arrows in the pseudo-colored panels indicate colocalization of HA-Rad52 and Slx5-GFP as yellow foci (overlay). (C) A single HO-mediated dsDNA break was induced in strain YOK947 coexpressing Slx5-GFP (red) and HA tagged Rad9 (green). Chromosome spreads of arrested cells were probed with anti-GFP or anti-HA antibodies (see materials and methods). The arrows in the pseudo-colored panels indicate colocalization of HA-Rad9 and Slx5-GFP as yellow foci (overlay).

that mutations in the confirmed SIMs A & B resulted in loss of and reduced Slx5-GFP foci, respectively, whereas mutations in C and D had no effect (Fig. 3B). This correlated with the previously reported SUMO-binding defects of these mutants, with *simA* causing a strong reduction by two-hybrid analysis and *simB* having little if any effect. Neither the *simA* nor *simB* mutations reduced overall Slx5-GFP levels based on anti-Slx5 immunoblotting (Fig. 3D). These two mutant Slx5 derivatives retained the ability to promote growth of *slx5*Δ cells on HU (Fig. 3C), suggesting that Slx5 foci formation is not an essential requirement for the cellular response to the DNA damage caused by stalled replication forks. Note that mutant D fails to thrive on media containing HU but displays robust Slx5-foci. Since this mutant also retains the ability to interact with SUMO (Xie et al. 1997) and resides at the C-terminus of Hex3, it may perturb function of the Hex3 RING domain.

Slx5 foci colocalize with Rad52 DNA damage foci. Since Slx5 foci depend on cellular SUMO function and the presence of a Slx5 SIM (Figs. 2C and 3B), we hypothesized that nuclear proteins that are subject to sumoylation may help recruit Slx5 into these foci. One Slx5 target may be Rad52, a homologous recombination and DNA repair protein that can be sumoylated in vivo and forms distinct nuclear foci.[26,27] We previously showed in vitro that a Rad52-SUMO protein is a preferred target for Slx5/Slx8-mediated ubiquitylation compared to the unmodified Rad52.[6]

To determine if Slx5 and Rad52 colocalize in vivo, we transformed cells expressing Rad52-CFP with a plasmid encoding Slx5-YFP. Logarithmically grown cells of this strain were subjected to UV irradiation and allowed to recover for 60 min in fresh growth media. Cells were then harvested and imaged by fluorescence microscopy. As expected, Rad52-CFP formed distinct Rad52 repair centers.[26] In 20% of the stained cells, Rad52 foci were in close proximity or overlapped with Slx5-YFP foci (Fig. 4A). Increasing the recovery time up to 3 hours did not enhance the overlap between Slx5-YFP and Rad52-CFP foci. However, in chromatin spreads of fixed cells, Hex3 foci colocalized with DNA repair foci 90% of the time, reflecting the different sensitivities of these two techniques (see below). These localization data suggest that a fraction of Slx5 concentrates at Rad52 DNA repair centers. Since not all of the Slx5 and Rad52 foci overlap, Slx5-YFP foci formation is not limited to sites of Rad52 accumulation.

Rad52 associates with DNA and DNA repair proteins at sites of DNA damage.[28] Correspondingly, the partial overlap of Slx5 foci with Rad52-CFP suggests that at least some Slx5 protein could be chromatin associated. Therefore, we employed the chromatin spreading technique to assess if Slx5 colocalized with chromatin or DNA bound Rad52. Cells expressing Rad52-HA and Slx5-GFP fusion proteins were subjected to Zeocin-induced DNA damage, spheroplasted and then fixed to glass slides. After detergent washes, only the chromatin remained on slides. Chromatin-bound proteins were detected with fluorescein-labeled antibodies to the HA epitope tag (Rad52) and GFP (Slx5). We found that the majority of brightly staining Rad52 foci (pseudo-colored red) colocalized with Slx5-GFP foci (pseudo-colored green) on fixed chromatin (Fig. 4B). About 10% of Rad52 foci did not overlap with Slx5 foci. Diffusely staining Slx5 appeared to be absent from the chromatin spreads, suggesting that a fraction of Slx5 is not tightly chromatin bound.

To extend these observations, we repeated the chromatin spread analysis using a yeast strain (SKY2965) in which galactose induction of the HO endonuclease results in a single doubled-stranded DNA (dsDNA) break at the HO cut-site in the *MAT* locus.[29] SKY2965 cells also express HA-tagged Rad9, a DNA damage-dependent checkpoint protein that interacts with chromatin at HO endonuclease-specific cleavage sites and forms foci that colocalize with Rad52 after DNA damage.[28,29] After transformation of Slx5-GFP into SKY2965 and induction of the HO dsDNA break, we prepared chromatin spreads and assayed for co-localization of Rad9 and Slx5. As was true for Rad52, chromatin spreads contained distinct single Rad9 foci (pseudo-colored green) that overlapped with Slx5 foci (pseudo-colored red) (Fig. 4C). Often spread chromatin contained more Slx5 foci than Rad9 foci. This observation strengthened our hypothesis that Slx5 can accumulate at various sites within the nucleus to form foci, which include but are not limited to DNA repair centers.

Slx5 associates with dsDNA breaks. To directly test the inference that Slx5 interacts with chromatin at sites of DNA damage, we used ChIP to determine whether Slx5 could associate with sequences proximal to sites of dsDNA breaks. The HO-specific dsDNA break was induced in the same SKY2965 strain expressing Slx5-GFP (YOK947) that we had used for the chromatin spreads (Fig. 4C). DNA damage-induced cell cycle arrest was confirmed by the appearance of large budded cells with a single nucleus at the neck, reflecting the expected pre-anaphase arrest induced by the DNA damage checkpoint. Arrested cells also contained one or more bright Slx5 foci inside the nucleus (data not shown). Crosslinked and sheared chromatin was prepared and immune complexes containing HA-Rad9 and Slx5-GFP were immunoprecipitated using anti-HA (Rad9) or anti-Slx5 antibodies, respectively. We were able to detect an enrichment of DNA flanking the HO cut site in both samples (Fig. 5A). In contrast, we did not detect amplification products with *CENIII*-specific primers, which were used as a specificity control. These data suggest that at least some Slx5 foci, like those containing Rad9, form on or near dsDNA breaks.

Slx5 association with dsDNA breaks is enhanced by Slx8. In the absence of *SLX8*, yeast cells show markedly increased DNA damage sensitivity, elevated levels of sumoylated proteins,[6] and more intensely stained Slx5 foci (Fig. 2D). Therefore, we sought to determine the effect of an *slx8* deletion on the association of Slx5 with dsDNA breaks. Following deletion of *SLX8* in the SKY2965 Slx5-GFP strain described above, HO endonuclease was induced to generate a dsDNA break at the *MAT* locus in both the resulting *slx8*Δ derivative and the parental *SLX8* cells. As above (Fig. 5A), anti-Slx5 ChIP analysis of these strains was used to measure Slx5 binding at the dsDNA break site. Rad9 could associate with dsDNA breaks in both the wild-type and *slx8*Δ cells (Fig. 5B, lanes, 5 and 6). Surprisingly, little Slx5 associated with the HO-induced dsDNA break in *slx8*Δ cells (Fig. 5B, lanes, 7 and 8).

To quantify the contribution of Slx8 to Slx5 association with dsDNA breaks, we used quantitative real-time PCR (RT-PCR). From the RT-PCR analysis, we determined that loss of Slx8 results in a ~4 fold decrease (4.2 ± 0.474) of Slx5 association with dsDNA

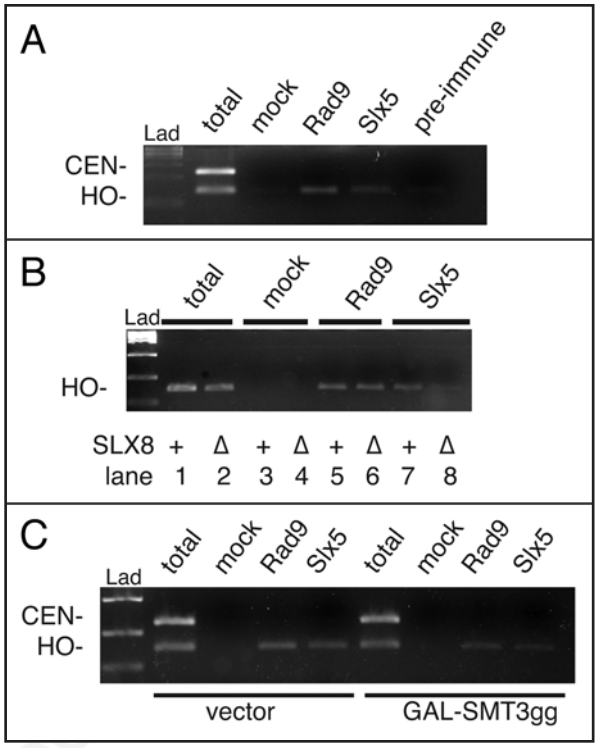

Figure 5. Slx5 associates with sites of dsDNA breaks. (A) Multiplex PCR analysis of ChIPs from *GAL-HO* Rad9-HA strain SKY2965. Chromatin of SKY2965 was prepared after induction of an HO endonuclease-induced dsDNA break (see materials and methods), and ChIPs of SKY2965 were derived using anti Slx5 and anti HA (Rad9) antibodies. Specific association of Slx5 and Rad9 with an HO specific dsDNA break was assessed using break-site specific HO primers and CEN control primers. Total chromatin (total), mock precipitated chromatin (mock), and a ChIP using pre-immune serum are also included as control templates. The *CENIII* specific PCR product runs as a 243 bp fragment and the HO specific PCR product, situated 60 bp from the HO cut site, runs as a 143 bp fragment on a 3% GTG Agarose gel with a 100 base-pair ladder (Lad) in the left-most lane. (B) Reduced association of Slx5 with dsDNA breaks in the absence of Slx8. PCR analysis as above except that ChIPs from a wildtype (+) SKY2965 strain are compared to ChIPs from an isogenic *slx8*Δ strain (Δ) YOK978. As above (A), Rad9 and Slx5 ChIPs were prepared using anti HA (Rad9) and anti Slx5 antibodies, respectively. Included as controls are total chromatin (total) and mock precipitated wildtype and *slx8*Δ chromatin (mock +, mock Δ). 100 base-pair ladder shown in the leftmost lane (Lad). Difference between Slx5 HO break-site association in wildtype and *slx8*Δ cells was quantitated using real-time PCR Taqman assays. Analysis of triplicate samples revealed an average 4.2 (±0.474) -fold decrease of Slx5 association with dsDNA break sites in the *slx8*Δ strain. (C) Unaltered association of Slx5 with dsDNA breaks in the presence of conjugation competent SUMO (GAL-Smt3gg) overexpression. PCR analysis as above except that ChIPs from a SKY2965 strain (vector) are compared to ChIPs from the same strain overexpressing conjugation- competent Smt3 (GAL-Smt3gg). As above (A), Rad9 and Slx5 ChIPs were prepared using anti HA (Rad9) and anti Slx5 antibodies, respectively. Included as controls are total chromatin (total) and mock precipitations.

break sites. By the same analysis, the association of Rad9 with dsDNA breaks remained virtually unaltered. For our calculations the loss of Slx5 occupancy from the HO break-site was normalized to that of Rad9 in *slx8*Δ and *SLX8* cells (Fig. 5B). These data suggest that directly, or indirectly, Slx8 plays an important role in

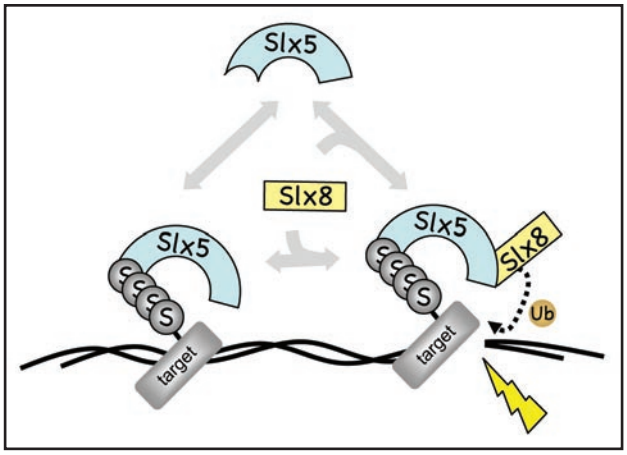

Figure 6. Model of Slx5 localization and interactions in the nucleus. Nuclear localized Slx5 (blue arched shape) can exist either tethered to chromatin (wavy lines) or in the nucleoplasm (top, center). As has been previously shown, soluble Slx5 may be bound to Slx8 (yellow rectangular shape) (Yang et al. 2006). Arrows indicate how Slx5 may change its association with Slx8 and cycle on and off chromatin. The association of Slx5 with DNA is mediated via SUMO or SUMO chains on DNA bound or chromatin associated proteins (shape labeled "target"). SUMO may not be required for all Slx5 or Slx5/Slx8 interactions with chromatin. In our model the localized accumulation of Slx5 on chromatin may result in SUMO-dependent observable foci. These foci are not required for DNA repair (Fig. 3B and C). In this study we show that Slx5 associates with sites of dsDNA breaks (yellow flash shape) and that Slx8 is required for this interaction. It may be that productive Slx5/Slx8 STUbLs ubiquitylate their targets (Ub—bottom right) and then quickly cycle on and off sites of DNA repair without forming foci. Slx5 may accumulate on sumoylated and ubiquitylated proteins after DNA repair is completed. Therefore, Slx5 foci may (1) mark accumulations of sumoylated proteins including those with important functions in chromatin integrity or (2) represent nuclear "disposal-sites" for sumoylated proteins.

the association of Slx5 with dsDNA breaks. They also suggest that Slx5 foci in *slx8*Δ cells are (1) either reduced at DNA repair centers or (2) are less closely associated with break site-specific DNA.

An indirect mechanism by which loss of Slx8 might reduce Slx5 binding to DNA damage sites, is by increasing the levels of SUMO and sumoylated proteins, which might bind Slx5 and limit its ability to associate with dsDNA breaks and DNA repair centers. However, ChIP analysis of Slx5 and Rad9 from cells that expressed elevated levels of SUMO (YOK1184) revealed that the ability of both proteins to interact with dsDNA breaks was only slightly reduced if at all (Fig. 5C). Collectively, our data suggest that Slx5 can form SUMO-dependent foci in the absence of Slx8 but close association of Slx5 with dsDNA breaks requires Slx8.

Discussion

Our observations regarding the subnuclear localization of Slx5, an evolutionarily conserved SUMO-targeted ubiquitin ligase subunit, can be summarized as follows: First, in live-cell studies we find that Slx5 is a nuclear-localized protein that can concentrate in foci. Our findings suggest that focal accumulation of Slx5 requires a SIM within the Slx5 protein and the ability of cells to synthesize polySUMO chains. SIMs mediate the ability of Slx5 to interact with SUMO and SUMO-modified proteins. Second, we show that Slx5 interacts with chromatin and partially localizes to DNA damage-induced DNA repair centers. Third, we use ChIP assays to demonstrate the Slx8-dependent association of Slx5 with a dsDNA break. Our data are consistent with a model in which SUMO-modified proteins recruit Slx5 to specific regions of chromatin, including those requiring STUbL function for DNA damage repair.

Fluorescence microscopy of live cells revealed a diffusely nucleoplasmic localization of both subunits of the heterodimeric Slx5/Slx8 STUbL. However, Slx5 also showed distinct nucleoplasmic foci that were not observed with Slx8. Therefore, nuclear Slx5 may exists in at least two distinct pools that may represent chromatin bound and unbound Slx5 (Fig. 6). Since recombinant Slx5 can exist both in a complex with Slx8 and by itself,[22] we hypothesize that a fraction of nuclear Slx5 may not be bound to Slx8 in vivo. Analyzing the nuclear distribution of YFP tagged Slx8 we and others[12] were unable to detect distinct Slx8 foci. This suggests that the Slx8 subunit either cycles on and off Slx5 at sites of Slx5 enrichment or distributes more evenly across chromatin (Fig. 6). It is also possible that standard epi-fluorescence imaging of live cells is unable to detect more subtle intranuclear enrichment sites of Slx8. The latter may be the case with respect to the recent report that in fixed cells a fraction of Slx5 and Slx8 foci localize to the nuclear pore complex.[21]

Slx5 forms clearly discernible intra-nuclear foci in most cells. Such foci may mark conjugated SUMO or SUMO chains on chromatin-associated proteins (Fig. 6). Indeed, our data with a SUMO mutant that is unable to form chains (smt3-R11,15,19) suggests that Slx5 requires SUMO chains to form foci. Consistent with our observation, polySUMO chain modification of a target protein appear to enhance the ability of Slx5/Slx8 to ubiquitylate it.[15] The absence of Slx5-GFP foci in the smt3-R11,15,19 mutant also underscores the inference that Slx5 foci are not merely aggregates of an overexpressed Slx5 fusion protein. However, we cannot exclude the possibility that Slx5 foci represent sites where highly modified SUMO conjugates are sequestered and the association with Slx5 is incidental. SUMO-modified protein aggregates in the nucleus have been described as a hallmark of several neurodegenerate diseases including Huntington's disease.[30] Nevertheless, at least a fraction of intranuclear Slx5 foci may represent a functional association of Slx5 at sites of target protein accumulation. Rad52 is a putative Slx5/Slx8 target[6] and becomes highly sumoylated upon DNA damage.[27] In live cell analysis, we found that a subset of Slx5 foci overlapped with DNA repair centers formed by Rad52. We also found that Slx5 could colocalize with Rad52 and Rad9 in chromatin spreads. Rad9 is a DNA damage-dependent checkpoint protein that localizes to Rad52 foci.[28] Moreover, we could show by ChIP analysis that Slx5, like Rad52 and Rad9, is recruited to sites of dsDNA breaks. A similar observation was recently reported by Nagai and co-workers who showed that Slx8, the binding partner of Slx5, binds to double-strand DNA breaks by performing ChIP with Slx8-Myc.[21] Therefore, our independent analysis confirms and extends the data of Nagai et al. Our data suggest that Slx5

can interact with SUMO and sumoylated proteins that specifically localize at sites of dsDNA breaks.

We extended these observations by analyzing the ability of Slx5 to associate with dsDNA breaks in the absence of Slx8. It has previously been shown that a deletion of *SLX8* increases foci formed by DNA repair proteins such as Ddc2 and Rad53 even in the absence of DNA damaging drugs,[4,9] and our work reveals a ~50% increase in intensity of Slx5-GFP foci in *slx8*Δ cells. Therefore, we expected that absence of *SLX8* would result in an increased association of Slx5 with dsDNA breaks. To our surprise, the deletion of *SLX8* resulted in the opposite: a four-fold decrease in the association of Slx5 with dsDNA breaks. This implies that efficient recruitment of Slx5 to dsDNA breaks requires Slx8. This could be because formation of a Slx5/Slx8 heterodimer is required for stable Slx5 association with the damaged DNA site or because increased accumulation of polysumoylated proteins in cells lacking Slx8,[5,6] sequesters Slx5 away from newly formed dsDNA breaks. In an attempt to mimic the effect of increased SUMO conjugates in *slx8*Δ cells, we overexpressed mature SUMO in the strain used for ChIP analysis. However, after induction of a dsDNA break both Slx5 and Rad9 association with DNA at the break-site was largely unaffected. Therefore, our in vivo data is consistent with data from in vitro studies in which Slx5-DNA association is dependent on Slx8.[22]

The function and localization of Slx5 almost certainly extends to other sub-nuclear domains beyond DNA repair centers. Several Slx5/Slx8 interactors and potential targets, including kinetochore proteins (Ctf19, Ndc10), spindle-pole body proteins (Spc24) and genomic maintenance/replication fork-associated factors (Sgs1, Srs2, Rad27, Pol32) have been identified.[21,31-33] The identification of additional substrates and sites of Slx5/Slx8 accumulation in the nucleus will be important for understanding the function of Slx5/Slx8 and other STUbLs.

Why are STUbLs recruited to dsDNA breaks or other sites within the nucleus? The human RNF4 STUbL, a nuclear protein that can form speckles and also localizes to PML nuclear bodies, has been show to affect the regulation of transcription factors and play a role in arsenic-induced PML degradation.[7,14,16,17] Potentially, RNF4 and other STUbLs could also help to fine-tune the choreography of DNA repair proteins at sites of DNA damage. Notably, RNF4, the human ortholog of Slx5/Slx8, maps to a chromosomal locus associated with neoplastic diseases,[34] may regulate cell division in germ cells,[17] and could play an important role in promyelocytic leukaemia.[14] The role of STUBLs in the ubiquitylation of SUMO-tagged proteins was first realized in yeast, and further studies of Slx5 and Slx8 will allow us to understand additional details of STUbL involvement in chromosomal maintenance.

Materials and Methods

Yeast strains, media and plasmids. Yeast strains and plasmids used in this study are listed in Table 1. Yeast media preparation and manipulation of yeast cells was performed as previously published.[35] Standard gene names according to the *Saccharomyces Genome Database* are used. Where indicated DNA damage stress was induced using either 50 μg/ml Zeocin (Invitrogen), 0.1 M HU (Sigma-Aldrich), or 100 Joules/m^2 of UV irradiation (Spectronics Spectrolinker). YOK510 is based on a commercially available Rad52-GFP/HIS3 strain (Invitrogen) and was converted to Rad52-CFP by transformation of *Msc1* cut pDH3(*CFP/KAN*) (The Yeast Resource Center). YOK677 is a segregant of the cross between *slx5*Δ*::kanMX* strain MHY3712 and *pdr5*Δ*::kanMX* strain YOK661. Strain MHY4183 is isogenic with YOK677 but expresses HA-tagged Rad52.[36] Similarly, YOK720 is isogenic with YOK677 but contains His6-tagged Rad52. All *slx5* deletion (*slx5*Δ) strains used in this study are sensitive to HU exposure or UV irradiation, can be complemented with a *SLX5-GFP* plasmid and show Slx5-GFP foci. Appearance and number of Slx5-GFP foci formed in *slx5*Δ and *SLX5* cells are similar or close to identical. DNA fragments containing *SLX5* or *SLX8* under the control of their respective promotors were amplified from yeast genomic DNA and placed in-frame with a carboxy-terminal *GFP* tag in the *CEN/LEU2* plasmid pAA3.[37] The coordinates of amplified *SLX5* and *SLX8* fragments are listed below. Furthermore, all *SLX5* and *SX8* GFP fusions fully complement their respective deletions. GFP variant fusions of *SLX5* and *SLX8* were constructed by replacing *GFP* cassettes with *YFP* or *CFP* derived from plasmids pDH3 and pDH5, respectively (the Yeast Resource Center) as previously reported.[38] The *LEU2* backbone of pAA3 based plasmids was changed to *URA3* by homologous recombination with *CEN/URA3* plasmid pRS316.[39] Primer pairs used for *SLX5* amplification were OOK103A (*SLX5* (-280 to -263)) and OOK104A (*SLX5* (+1821 to 1838)) and primer pairs for *SLX8* amplification were OOK198 (*SLX8 (-289 to -273)*) and OOK199 (*SLX8* (+806 to 822)). Site-directed mutagenesis of SIMs (and similar domains) in *SLX5* was performed as previously reported except that plasmid *SLX5-GFP/LEU2* served as the template for mutagenesis.[6] The *GAL-FLAG-SMT3gg 2* μ*/URA3* plasmid was constructed and confirmed by Mary Kroetz (Yale University). All other plasmid inserts were confirmed by sequencing and complementation assays. Expression of Slx5-GFP was confirmed using an anti-Slx5 antibody raised against a synthetic peptide (REANLPVRLYPDRRVGRR) (OpenBiosystems) and an anti-GFP antibody (JL-8: Clontech 632381).

Chromosome spreads. Chromosome spreads were performed as described by Loidl and coworkers.[40] Chromatin spread on glass slides was visualized using DAPI (4',6- diamidino-2-phenylindole). HA-tagged Rad52 was detected using ab9110 (Abcam Inc., Cambridge, MA) conjugated to fluorescein (Thermo Scientific (Pierce) kit 51006). HA-tagged Rad9 was detected using Alexa488 conjugated anti-HA antibody (Invitrogen # A-21287) and Slx5-GFP was detected using Alexa594-conjugated anti-GFP antibodies (Invitrogen # A-21312).

Fluorescence microscopy. Images of live cells and chromatin spreads were collected using a Zeiss Axioskop fitted with, a Retiga SRV camera (Q-imaging), i-Vision software (BioVision Technologies) and a Uniblitz shutter assembly (Rochester, NY). Pertinent filter sets for the above applications include CZ909 (GFP), XF114-2 (CFP), XF104-2 (YFP) (Chroma Technology Group).

Table 1 Yeast strains

Name	Pertinent genotypes or parent strain	Plasmids (*CEN*)	Reference
MHY500	*Matα his3-Δ200 leu2-3,112 ura3-52 lys2-801trp1-1 gal2*		Li and Hochstrasser, 2003
MHY3716	*Matα his3-Δ200 leu2-3,112 ura3-52 lys2-801trp1-1 gal2 slx8Δ::kanMX4*		Xie et al. 2007
MHY3712	*Matα his3-Δ200 leu2-3,112 ura3-52 lys2-801trp1-1 gal2 slx5::kanMX4*		Xie et al. 2007
MHY3861	*Matα his3-Δ200 leu2-3,112 ura3-52 lys2-801trp1-1 gal2 slx5::KAN slx8Δ::kanMX4*		Xie et al. 2007
YOK850	*(slx8Δ) MHY3716*	*SLX8-YFP/URA3*	This study
YOK907	*(slx8Δ) MHY3716*	*SLX5-GFP/LEU2*	This study
YOK851	*(slx5Δ) MHY3712*	*SLX5-GFP/LEU2*	This study
YOK852	*(slx5Δ slx8Δ) MHY3861*	*SLX8-YFP/URA3*	This study
YOK853	*(slx5Δ slx8Δ) MHY3861*	*SLX5-GFP/LEU2*	This study
YOK898	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 slx5Δ::kanMX4 (BY4741)*	*SLX5-GFP/LEU2*	Winzeler et al. 1999
SBY331	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1 lys2 smt3-331*		Biggins et al. 2001
GBY1	*MAT**a** trp1-1 ura3-52 his3-200 leu2-3,112 lys2-801 smt3-R11,15,19::TRP1*		Bylebyl et al. 2003
YOK847	*ubc9-1*	*SLX5-GFP/LEU2*	Betting and Seufert, 1996
YOK720	*Mat**a** ura3 lys2 his3 trp1 leu2 pdr5::kanMX4 slx5::kanMX4 RAD52-HIS^6/HIS3*		This study
YOK726	*slx5Δ (YOK720)*	*SLX5(A)-GFP/LEU2*	This study
YOK731	*slx5Δ (YOK720)*	*SLX5(B)-GFP/LEU2*	This study
YOK716	*slx5Δ (YOK720)*	*SLX5(C)-GFP/LEU2*	This study
YOK718	*slx5Δ (YOK720)*	*SLX5(D)-GFP/LEU2*	This study
YOK510	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 (S288C) RAD52-CFP/HIS3*	*SLX5-YFP/LEU2*	This study
MHY4183	*Mat**a** ura3 lys2 his3 trp1 leu2 pdr5::kanMX4 slx5::kanMX4 RAD52-HIS^6/HIS3*		This study
SKY2965	*Mat**a** ho hml(del)::ade1, hmr(del)::ade1-110 leu2,3-112 lys5, trp1::hisG ura3-52, ade3::GAL1,10:HO, RAD9-HA::kanMX6*		Javaheri et al. 2006
YOK947	SKY2965	*SLX5-GFP/LEU2*	This Study
YOK978	*(slx8Δ::NATMX4) SKY2965*	*SLX5-GFP/LEU2*	This Study
YOK 1184	SKY2965	*GAL-Flag-SMT3gg CEN/URA3*	This Study
YOK 1185	SKY2965	*SLX5-GFP/LEU2*	This Study

Chromatin immunoprecipitation (ChIP assays). Strains for chromatin immunoprecipitations (ChIP) were grown in SD media containing 2% sucrose to an OD_{600} of ~0.4 then transferred to fresh media containing 2% raffinose. At OD_{600} of ~0.7, 3x YEP + 6% galactose was added for GAL-HO endonuclease induction. About 5 hours after galactose induction (OD_{600} of ~1.2), 80% of the cells showed a large budded arrest phenotype with Slx5-foci containing nuclei at the bud-neck. Cells were then crosslinked by addition of paraformaldehyde to 1%. Fixation times varied from 30 to 60 minutes at room temperature. ChIP analyses were performed as previously reported[38,41,42] with the following modifications. HA-tagged proteins were precipitated using the HA-specific ChIP-grade ab9110 (Abcam Inc., Cambridge, MA), Slx5 was precipitated using an anti-Slx5 specific antibody raised against a synthetic peptide (REANLPVRLYPDRRVGRR) and protein-G agarose (Roche 11243233001) was used instead of protein-A sepharose. Immunoprecipitated DNA was analyzed by multiplex PCR with primers specific to the HO break-site[29] and *CENIII*:

OOK295 for HO: HO LIGHT REV (5'-GTG GTG ACG GAT ATT GGG AA-3') and OOK296 for HO: HO LIGHT FWD (5'-GGG AAC AAG AGC AAG ACG AT-3') OOK322 for *CEN3* PM22 (5'-GAT CAG CGC CAA ACA ATA TGG-3') and OOK323 for *CEN3* PM48 (5'-AAC TTC CAC CAG TAA ACG TTT C-3')

HO-specific TAQMAN probes used to quantitate the difference in Slx5 binding in SKY2965 were designed by Applied Biosystems and are available upon request. Taqman reactions were run in a BioRad iCycler.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/CookCC8-7-Sup.pdf

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