SUMOylation and PARylation cooperate to recruit and stabilize SLX4 at DNA damage sites

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Abstract

SUMOylation plays important roles in the DNA damage response. However, whether it is important for interstrand crosslink repair remains unknown. We report that the SLX4 nuclease scaffold protein is regulated by SUMOylation. We have identified three SUMO interaction motifs (SIMs) in SLX4, mutating all of which abrogated the binding of SLX4 to SUMO-2 and covalent SLX4 SUMOylation. An SLX4 mutant lacking functional SIMs is not recruited to PML nuclear bodies nor stabilized at laser-induced DNA damage sites. Additionally, we elucidated a novel role for PARylation in the recruitment of SLX4 to sites of DNA damage. Combined, our results uncover how SLX4 is regulated by post-translational modifications.

Introduction

Maintaining the integrity of our genomes is key to avoid DNA damage-induced diseases, including cancer and neurological diseases [1]. Cells are equipped with elaborate repair machineries and signalling pathways to counteract both exogenous and endogenous sources of DNA damage. Combined, these pathways are known as the DNA damage response (DDR).

Post-translational modifications (PTMs) play key roles in maintaining genome integrity by regulating DDR components. PTMs are important to assemble dynamic DNA repair complexes through covalent and non-covalent interactions. These PTMs include phosphorylation, methylation, acetylation, PARylation, ubiquitination and modification by small ubiquitin-like modifiers (SUMOs). Extensive crosstalk exists between these PTMs to strengthen and balance signal transduction [2].

Mice deficient for SUMOylation die early during embryogenesis and show compromised genome integrity [3]. SUMO accumulation at sites of DNA damage is regulated by the SUMO E3 ligases PIAS1 and PIAS4. Examples of covalently modified SUMO target proteins in the DDR include MDC1, 53BP1, BRCA1, PCNA and RNF168 [4].

Moreover, SUMOylation can regulate proteins in a non-covalent manner via SUMO interaction motifs (SIMs) [5]. SIM motifs in DNA repair components enable repair complex assembly [6]. Several ubiquitin E3 ligases were found to contain SIMs, enabling their interaction with and ubiquitination of SUMOylated proteins [7].

Whereas much is known about the role of SUMOylation in DNA double-strand break repair, our understanding of the role of SUMOylation in the repair of other types of DNA damage is limited. As a scaffold for nucleases, SLX4 plays a key role in maintaining genome stability [8]. Mutations in SLX4 cause Fanconi anaemia by defects in interstrand DNA crosslink (ICL) repair [8]. How the function of SLX4 in this repair pathway is regulated by PTMs remains enigmatic.

Here, we report on a novel role for SUMOylation in the DDR, by regulating SLX4. SUMOylation of SLX4 is dependent on three SIM motifs located throughout the protein. A SIM- and consequently SUMOylation-deficient mutant of SLX4 was unable to rescue SLX4-knockout MEFs treated with the DNA-crosslinking agent mitomycin C (MMC). Mechanistically, this can be explained by reduced SLX4 retention at DNA damage sites through loss of SUMO–SIM-mediated interactions. Furthermore, we show that SLX4 binds PARP1 and we show that PARylation and SUMO signalling cooperate to recruit SLX4 to sites of DNA damage.

Results and Discussion

SLX4 SUMOylation is regulated through the cell cycle

SLX4 was identified as a SUMOylation target in two different proteomic screens by our group [9, 10]. First, we decided to study whether the SUMOylation levels of SLX4 were affected by DNA damage. To this end, HIS-SUMO2-expressing U2OS cells were exposed to

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different genotoxic agents including hydroxyurea (HU), campto-thecin (CPT), methyl methane sulphonate (MMS) or MMC. HIS-SUMO2 conjugates were purified and analysed by SDS–PAGE and

immunoblotting to determine the fraction of SUMOylated SLX4 (Fig 1A). While treatment with all these different agents increased the levels of γ-H2AX, a DNA damage marker, only treatment with
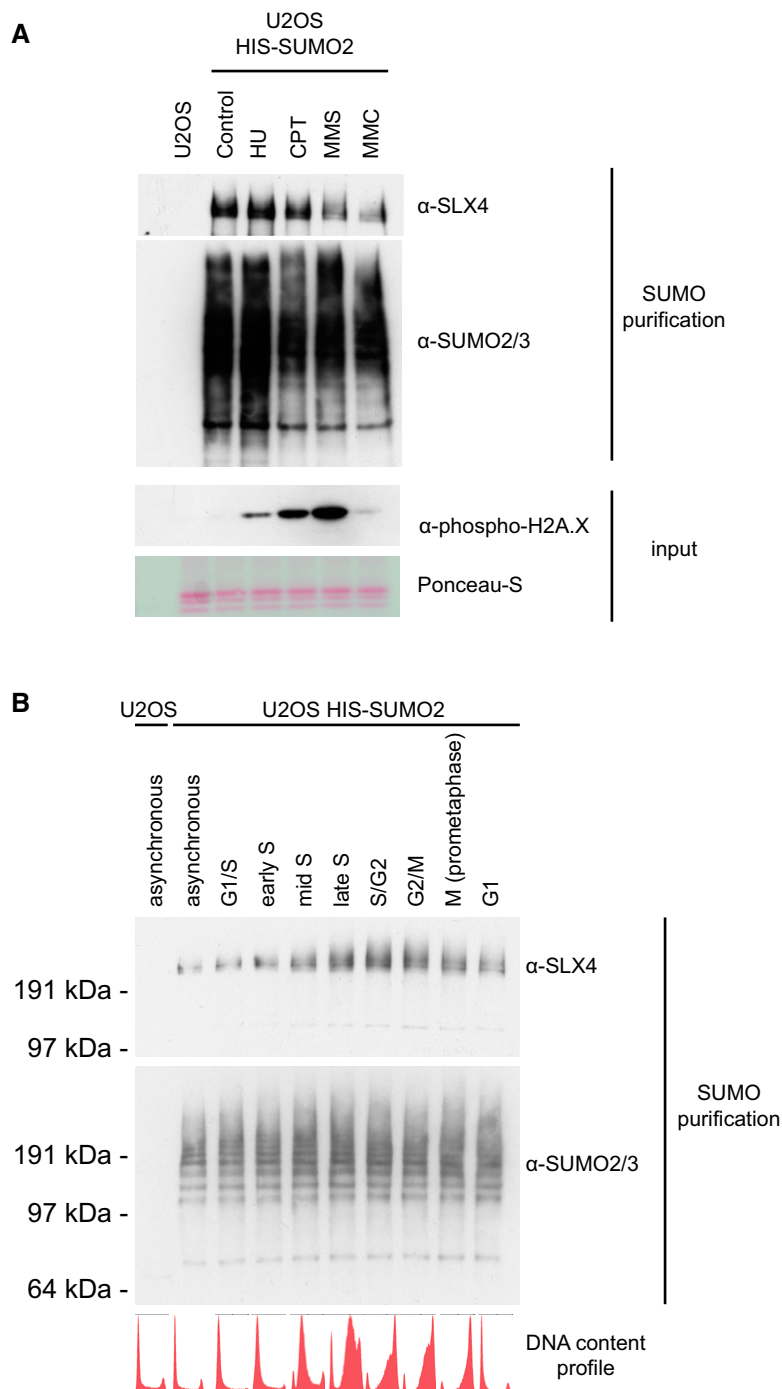

Figure 1.   SLX4 SUMOylation in response to DNA damage and during cell cycle progression.

A   HIS-SUMO2 conjugates were purified from U2OS cells (negative control) or U2OS-HIS-SUMO2 cells after 6 h of exposure to HU (2 mM), CPT (14 μM), MMS (0.01%) or MMC (100 ng/ml). Samples were analysed by SDS–PAGE and immunoblotting using antibodies against SLX4, SUMO-2/3 or phosphorylated H2A.X. Ponceau-S staining is shown as a loading control. This experiment was performed twice.

B   HIS-SUMO2 conjugates were purified from U2OS (negative control) or cell cycle-synchronized U2OS-HIS-SUMO2 cells. Samples were analysed by SDS–PAGE and immunoblotting using antibodies against SLX4 or SUMO2/3. FACS analysis was performed to profile DNA contents and verify cell cycle synchronization. This experiment was performed three times.

MMS and MMC caused a slight reduction in the SUMOylation levels of SLX4. This reduction could potentially be explained by a general reduction in the amount of SUMO2 conjugates in response to these DNA-damaging agents.

Recently, we have found that reducing SUMOylation slowed down cell cycle progression [10]. We have previously used a proteomics approach to identify SUMOylated proteins that are dynamically regulated during cell cycle progression. This approach revealed SLX4 as one of the novel SUMO-2 target proteins. In order to verify SUMOylation of SLX4 during cell cycle progression, we used a thymidine block and release to synchronize HIS-SUMO2-expressing U2OS cells in different stages of the cell cycle. A CDK1 inhibitor and nocodazole were used to obtain G2/M and prometaphase stage-enriched cells, respectively. Subsequently, cells were lysed and SUMO conjugates were enriched. SLX4 SUMOylation levels were analysed by SDS–PAGE and immunoblotting (Fig 1B). We observed an increase in SLX4 SUMOylation levels as the cells progressed through the S and G2 phases, decreasing again upon completion of the cell cycle when the cells re-entered the G1 phase. SUMOylation levels of SLX4 at the G1 stage are probably overestimated due to prolonged arrest of some cells at the G2/M phase by CDK1 inhibition (Fig 1B). Our results show that the SUMOylated fraction of SLX4 increases as cells progress through the cell cycle.

SLX4 is SUMOylated in a SIM-dependent manner

Detailed analysis of the primary mouse SLX4 (mSLX4) structure revealed three SIMs [5]. These potential SIMs in mSLX4 are located at positions (955–964 EVILLLDSDE, 997–1001 VIDVE and 1179–1183 DVVEV) (Fig 2A). To verify whether these SIMs enabled SUMO binding, we created a mutant that lacks all of these motifs, by mutating large hydrophobic residues into alanines (ΔSIM mutant). Subsequently, we studied whether mSLX4 was able to interact with a recombinant SUMO2 tetramer and found that wild-type SLX4 was able to bind SUMO2, whereas binding was abolished by mutating the SIMs (Fig 2B).

Previously, USP25 was found to be SUMOylated in a SIM-dependent manner [11]. To determine whether the SIMs in mSLX4 mediate its SUMOylation in a similar manner, we verified SUMOylation of the mSLX4 ΔSIM mutant (Fig 2C) and found that SLX4 SUMOylation was lost upon disruption of its SIMs, indicating that SLX4 is SUMOylated in a SIM-dependent manner.

The SIM domains in SLX4 enhance DNA ICL repair

Since SLX4 is predominantly SUMOylated during the S/G2 phases of the cell cycle (Fig 1B) and SLX4-deficient cells are sensitive to MMC [8], SLX4 SIMs and SUMOylation might be necessary to overcome replicative damage caused by ICLs. To address this point, we performed rescue experiments of SLX4-deficient (SLX4$^{-/-}$) mouse embryonic fibroblasts (MEFs) with retroviral expression of GFP-mSLX4 fusion constructs (Fig 2D). As shown previously, cells deficient for SLX4 were highly sensitive to MMC. Re-introduction of GFP-mSLX4-wt rescued the SLX4$^{-/-}$ sensitivity. In contrast, expressing GFP-SLX4-ΔSIM resulted only in a partial rescue, demonstrating that SIMs in SLX4 enhance SLX4-mediated ICL repair. However, no difference in rescue efficiency between both SLX4 constructs was observed for CPT sensitivity (Fig 2E). Our results

contrast with two recently published papers [12, 13]. MMC sensitivity differences could potentially be explained by the different systems used. We use a MEF-based knockout and rescue model, while the other studies used either a patient cell line [12] or siRNA-mediated knockdown and rescue human model system [13]. Differences between mouse and human models have been previously described for SLX4 [14]. Considerably lower expression levels are observed in the other studies for the ΔSIM construct, compared to wild-type, potentially explaining the differences in CPT survival, which is only 5% at the highest dose used. We have found virtually equal expression levels for our wild-type and ΔSIM constructs (Fig 2D).

SIM motifs are required for SLX4 localization in nuclear bodies

SLX4 has been described to localize in nuclear bodies [14, 15]. To study whether the SIMs play a role in the subcellular localization of SLX4, we performed microscopy experiments. U2OS cells and SLX4$^{-/-}$ MEFs were infected with retroviral vectors encoding GFP-SLX4-wt or the ΔSIM mutant. Cells were fixed, stained with DAPI to visualize nuclei, embedded and analysed by confocal microscopy. Whereas wild-type SLX4 was located in the nucleoplasm and enriched in nuclear bodies, the SLX4-ΔSIM mutant lost its ability to efficiently accumulate in these nuclear substructures (Fig 3A).

The nucleus contains several different types of nuclear bodies, including Cajal bodies and PML bodies. Previously, it was shown that PML bodies are enriched for SUMOs, with SIM domains in PML playing a major role in recruiting SUMOylated proteins [16]. Consistent with SLX4 SUMOylation, the mSLX4 nuclear bodies were enriched for SUMO-2/3 (97.9% co-localization) (Fig 3B). Similarly, mSLX4 co-localized with PML in these nuclear bodies (97.7% co-localization) (Fig 3C). Overall, these results indicate that it is the SUMOylated fraction of SLX4 that is located in nuclear bodies.

The SIM domains in SLX4 are required for retention of SLX4 at laser-induced DNA damage tracks

Since SLX4 is a scaffold for the nucleases ERCC1-XPF and EME1-MUS81 [17, 18], and SLX4 stimulates the activity of ERCC1-XPF in ICL repair [19, 20], we tested the ability of the mSLX4-ΔSIM mutant to bind these nucleases. To address this point, we transiently expressed HA-tagged wild-type and ΔSIM mSLX4 in U2OS cells and performed a HA-immunoprecipitation in native conditions to co-purify interacting partners. However, no differences were observed between wild-type SLX4 and the ΔSIM mutant in terms of XPF- or MUS81-binding capacity (Fig 4A).

Next, in order to identify potential SIM-dependent SLX4-interacting partners, we performed mass spectrometry analysis of co-immunoprecipitated proteins. Statistical analysis of proteins interacting with wild-type SLX4 versus the ΔSIM mutant revealed SUMO2 as the major difference between both interactomes (Fig 4B). These results were confirmed by immunoblotting. The most prominent SUMOylated bands identified were about 200 kDa in size, possibly representing SUMOylated SLX4.

Local DNA damage can be induced in cells by employing focused lasers [21]. SLX4 was previously shown to accumulate in laser-induced DNA damage [14,18]. Interestingly, SUMO is also recruited
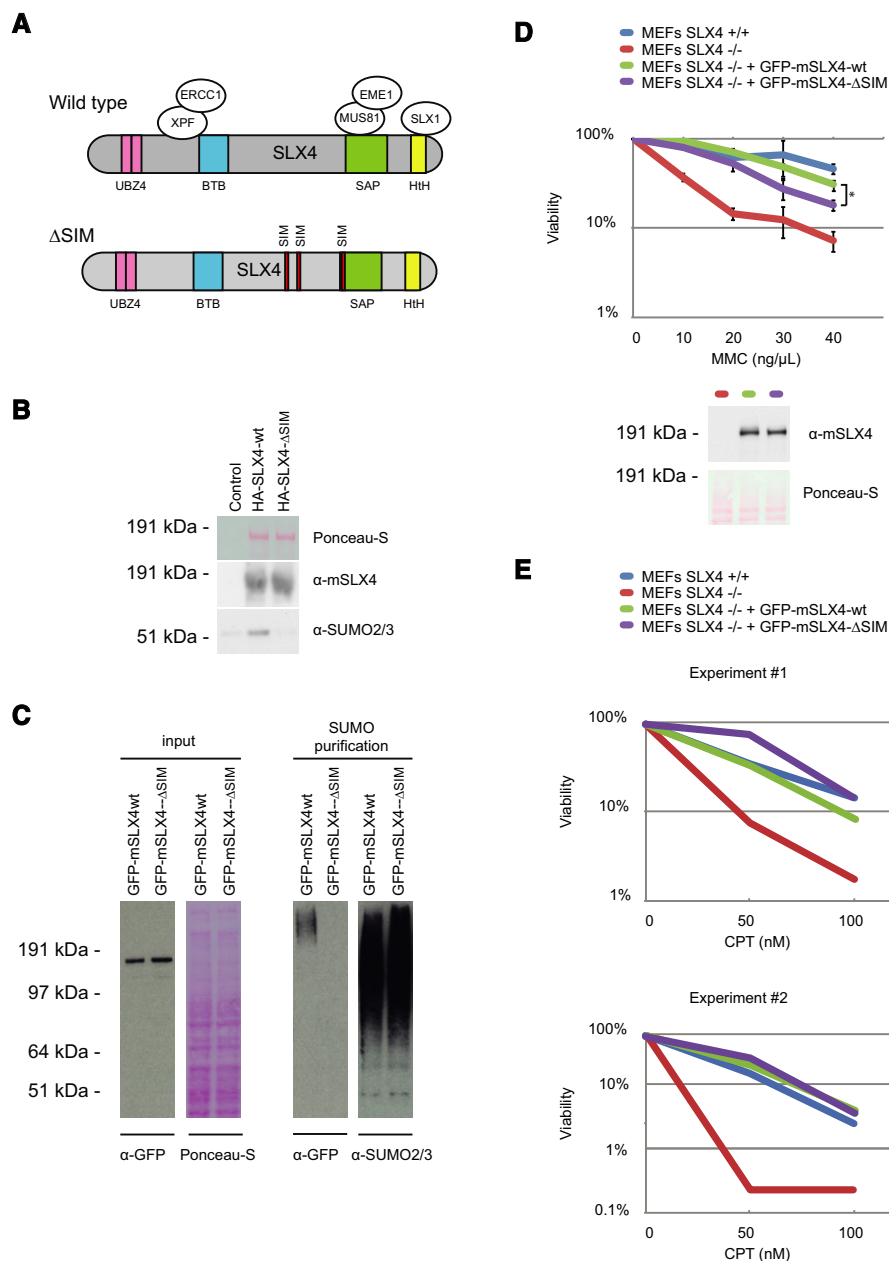
Figure 2. SIMs enhance ICL repair activity of SLX4.

A   Cartoon showing SLX4 domains, binding sites for interaction partners and the SIMs mutated in SLX4-ΔSIM mutant.

B   The SLX4-ΔSIM mutant does not interact with SUMO2 tetramers. HA-tagged mSLX4 constructs were expressed in U2OS cells, purified by immunoprecipitation (IP) and analysed for tetra-SUMO2 binding. IP samples were analysed by SDS–PAGE and immunoblotting using antibodies against mSLX4 or SUMO2/3. This experiment was performed three times.

C   HIS-SUMO2 purification was performed from lysates of U2OS-HIS-SUMO2 cells infected with retroviral vectors encoding GFP-mSLX4-wt or GFP-mSLX4-ΔSIM. Samples were analysed by SDS–PAGE and immunoblotting using antibodies against GFP or SUMO2/3. Ponceau-S staining is shown as a loading control. This experiment was performed three times.

D, E   Cell viability of SLX4$^{-/-}$ and SLX4$^{+/+}$ MEFs, after MMC treatment (D) at the indicated concentrations, and rescue experiments using retroviruses encoding GFP-mSLX4-wt or GFP-mSLX4-ΔSIM. The difference between SLX4$^{-/-}$ MEFs rescued with wt SLX4 or ΔSIM mutant is significant (paired *t*-test; *P* = 0.003). Average and SEM of four independent experiments are shown. Two different experiments are shown for CPT sensitivity (E).

to such DNA damage tracks, indicating that SUMOylated proteins at sites of DNA damage could mediate the recruitment of SLX4 [21].

To address whether the SIMs of SLX4 play a role in its recruitment to DNA damage tracks, we studied the recruitment of

GFP-SLX4-wt or the ΔSIM mutant following multi-photon laser-mediated micro-irradiation in time-course experiments (Fig 4C). As previously described, NBS1-mCherry was rapidly recruited to laser-induced DNA damage tracks [22]. We obtained similar kinetics
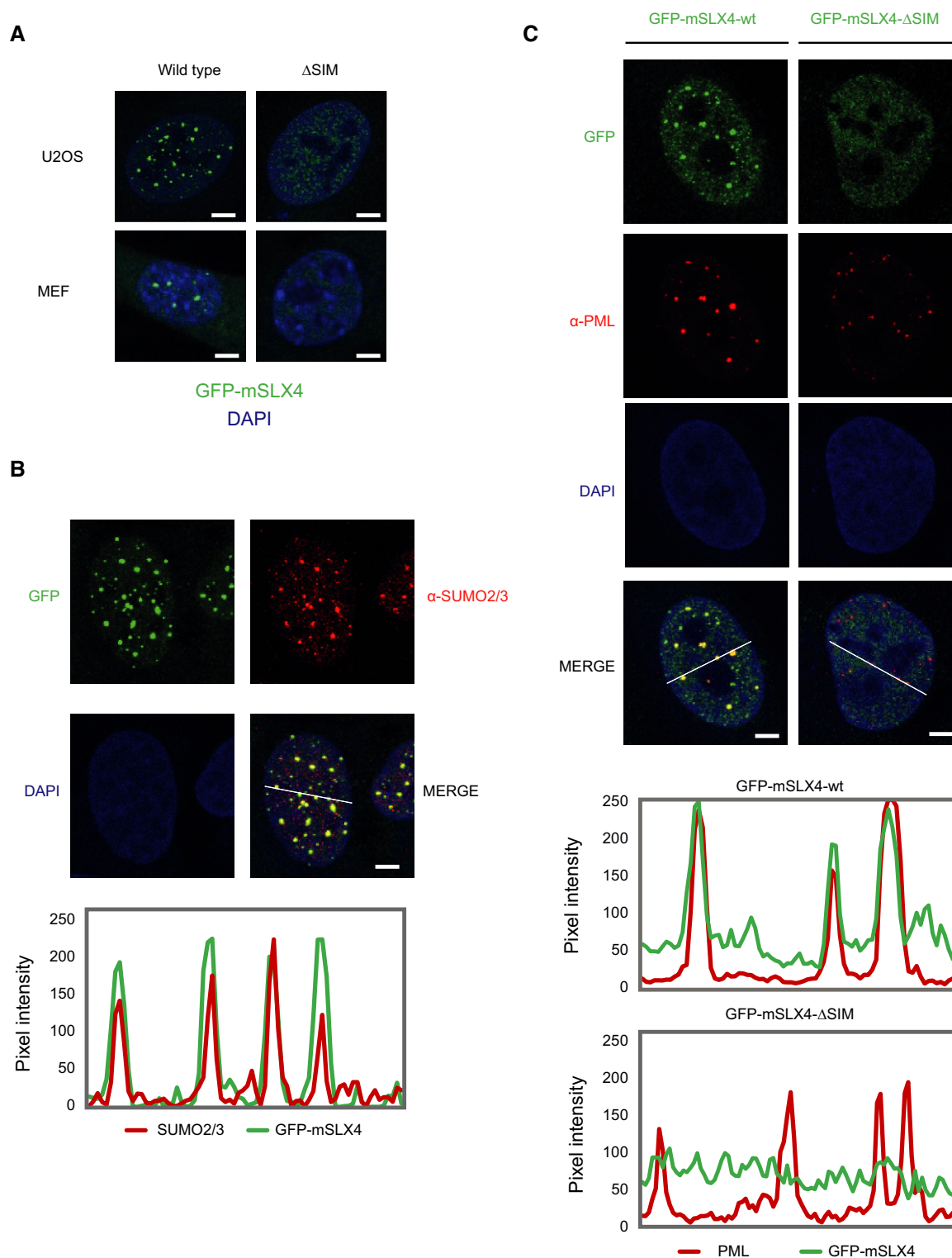
Figure 3. The sub-nuclear localization of SLX4 depends on its SIMs.

A Confocal microscopy image of U2OS and SLX4$^{-/-}$ MEFs, expressing GFP-mSLX4-wt or GFP-mSLX4-ΔSIM retroviral constructs. Scale bars represent 5 μm.

B SLX4 co-localizes with SUMO2/3 in nuclear bodies. Confocal microscopy images of U2OS cells expressing retroviral GFP-SLX4-wt constructs and immunostained for SUMO2/3. DAPI staining was used to visualize nuclei. Co-localization was confirmed by fluorescent intensity overlap. Scale bars represent 5 μm.

C SLX4 co-localizes with PML in nuclear bodies. Confocal microscopy images of U2OS cells expressing retroviral GFP-SLX4-wt or GFP-SLX4-ΔSIM constructs immunostained for PML. DAPI was used to visualize nuclei. All experiments were performed at least twice. Co-localization was confirmed by fluorescent intensity overlap. Scale bars represent 5 μm.
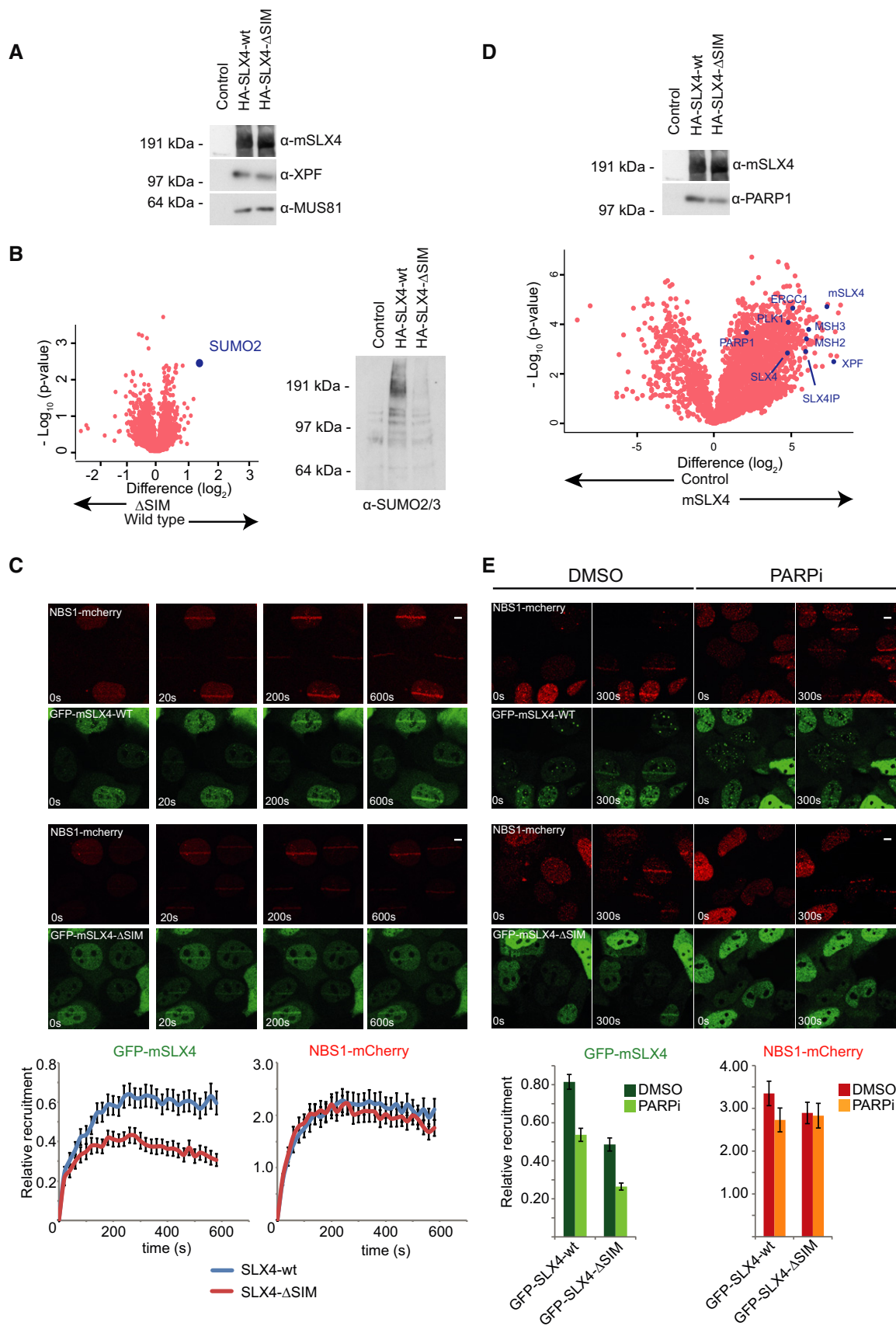
Figure 4.

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Figure 4.   SUMOylation and PARylation regulate the localization of SLX4 at local sites of DNA damage.

A   HA-mSLX4-wt and HA-mSLX4-ΔSIM bind equally well to XPF and MUS81. Transiently overexpressed HA-mSLX4 constructs in U2OS cells were immunoprecipitated under native conditions. Samples were analysed by SDS–PAGE and immunoblotting using the indicated antibodies. This experiment was performed four times.

B   Protein complexes co-purified with HA-SLX4-wt or SIM mutant were analysed by mass spectrometry (volcano plot) or SDS–PAGE and immunoblotting with an antibody against SUMO2/3. Data are derived from three technical repeats of three biological repeats.

C   SIMs stabilize mSLX4 at DNA damage tracks. U2OS cells expressing NBS1-mCherry and either GFP-mSLX4-wt or GFP-mSLX4-ΔSIM were subjected to laser micro-irradiation. Time lapse microscopy was used, and recruitment to the DNA damage tracks was measured at the indicated time points. This experiment was performed twice. Averages and SEMs are shown (*n* = 50). Scale bars represent 5 μm.

D   PARP1 is an interacting partner of SLX4. Protein complexes co-purified with HA-mSLX4-wt were analysed by mass spectrometry (volcano plot) or SDS–PAGE and immunoblotting with the indicated antibodies. Known SLX4 interactors and PARP1 are highlighted in the volcano plot. Data are derived from three technical repeats of three biological repeats.

E   PARP activity enhances SLX4 recruitment to DNA damage tracks. U2OS cells expressing NBS1-mCherry and either GFP-mSLX4-wt or GFP-mSLX4-ΔSIM were subjected to laser micro-irradiation after DMSO or PARPi treatment. GFP-mSLX4 constructs and NBS1-mCherry recruitment 300 s after irradiation are shown. The reduction in recruitment in response to the PARPi was significant (see Supplementary Methods). Averages and SEMs of three independent experiments are shown. Scale bars represent 5 μm.

for GFP-mSLX4, reaching a maximum 5 min after irradiation (Fig 4C). These kinetics were more rapid compared to a previous publication [14], possibly due to different laser systems used in the different studies.

Although wild-type SLX4 was rapidly recruited to laser-induced damage tracks, the ΔSIM mutant was recruited much less efficiently. Quantification of the live-cell imaging data revealed a twofold reduced accrual of the ΔSIM mutant compared to its wild-type counterpart. Moreover, we noticed that wild-type SLX4 was present at sites of DNA damage for a prolonged period of time compared to the ΔSIM mutant, indicating that the SLX4 SIMs play a role in the retention of SLX4 at sites of DNA damage.

Additionally, we observed that GFP-mSLX4-enriched nuclear bodies were not affected by the induction of DNA damage (Fig 4C and E and Supplementary Video S1). This suggests that the nucleoplasmic fraction of SLX4 is recruited to DNA damage tracks, where SIMs enable SLX4 to bind to SUMOylated proteins at damaged DNA. Our results suggest that the SUMO–SIM interactions enhance the localization of SLX4 at sites of DNA damage.

SLX4 recruitment to laser-induced damage tracks is enhanced by PARP activity

Mass spectrometry analysis of SLX4 interactors enabled us to identify known SLX4 interactors and other proteins (Fig 4D). Importantly, one of the enriched proteins which we identified was PARP1. The activity of PARP1 is required for the rapid cellular response to DNA damage [23]. Since recruitment of GFP-mSLX4 to the DNA damage tracks is very fast, we hypothesized that PARylation might play a role in its accrual. To address this point, we studied the recruitment of both wild-type and ΔSIM GFP-mSLX4 constructs in the presence and absence of a PARP inhibitor (PARPi) (Fig 4E). Inhibiting PARylation resulted in a significant decrease in GFP-mSLX4 recruitment both for wild-type SLX4 and the ΔSIM mutant, while NBS1-mCherry recruitment was not affected. Interestingly, recruitment of the SLX4 ΔSIM mutant was almost abolished by the PARPi, suggesting that PARylation and SUMOylation cooperate to recruit SLX4 to DNA damage sites. However, we cannot exclude the possibility that, given the variety of lesions that multi-photon lasers produce, PARylation and SUMOylation contribute to recruit SLX4 to different types of DNA damage.

Interestingly, human SLX4 was co-immunoprecipitated together with HA-mSLX4, indicating that SLX4 can form oligomers (Fig 4D).

We speculate about a model in which SUMO interaction with SIMs in SLX4 would promote SLX4 SUMOylation, facilitating the formation of higher-order SLX4 complexes, which, in turn, would recruit nucleases and repair proteins to stimulate DNA repair. Combined, our report provides new insight in the regulation of the protein scaffold SLX4 by cooperative PTMs within the DDR.

Materials and Methods

HIS-SUMO2 purification

Histidine-tagged SUMO2 conjugates were purified from U2OS cells as described before [9].

Microscopy and multiphoton laser micro-irradiation

Co-localization images were taken with a Leica TCS SP8 confocal microscope equipped with different lasers. Laser micro-irradiation was carried out on a Leica SP5 confocal microscope equipped with an environmental chamber set to 37°C. DNA damage tracks were generated with a Mira modelocked titanium–sapphire laser.

Mass spectrometry

The mass spectrometry data have been deposited to the ProteomeXchange Consortium [24] via the PRIDE partner repository with the data set identifier PXD001681.

For more detailed Materials and Methods, see Supplementary Methods.

Author contributions

RGP performed experimental work, experimental design and wrote the manuscript. SAGC performed experimental work. MSL performed the laser micro-irradiation experiments together with RGP. HvA provided reagents and supervised MSL. ACOV proposed the project, did experimental design and wrote the manuscript. All authors commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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