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Non-covalent Interactions with SUMO and Ubiquitin Orchestrate Distinct Functions of the SLX4 Complex in Genome Maintenance

Jian Ouyang[1], **Elizabeth Garner**[3], **Alexander Hallet**[1], **Hai Dang Nguyen**[1], **Kimberly A. Rickman**[3], **Grace Gill**[4], **Agata Smogorzewska**[3,5], and **Lee Zou**[1,2,5]

[1] Massachusetts General Hospital Cancer Center, Harvard Medical School, Charlestown, MA 02129, U. S. A

[2] Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02115, U. S. A

[3] Laboratory of Genome Maintenance, Rockefeller University, New York, NY 10065, U. S. A

[4] Department of Developmental, Molecular & Chemical Biology, Tufts University School of Medicine, Boston, MA 02111, U. S. A

Abstract

SLX4, a coordinator of multiple DNA structure-specific endonucleases, is important for several DNA repair pathways. Non-covalent interactions of SLX4 with ubiquitin are required for localizing SLX4 to DNA-interstrand crosslinks (ICLs), yet how SLX4 is targeted to other functional contexts remains unclear. Here, we show that SLX4 binds SUMO-2/3 chains via SUMO-interacting motifs (SIMs). The SIMs of SLX4 are dispensable for ICL repair, but important for processing CPT-induced replication intermediates, suppressing fragile site instability, and localizing SLX4 to ALT telomeres. The localization of SLX4 to laser-induced DNA damage also requires the SIMs, as well as DNA-end resection, UBC9 and MDC1. Furthermore, the SUMO binding of SLX4 enhances its interaction with specific DNA-damage sensors or telomere-binding proteins, including RPA, MRE11-RAD50-NBS1 and TRF2. Thus, the interactions of SLX4 with SUMO and ubiquitin increase its affinity for factors recognizing different DNA lesions or telomeres, helping to direct the SLX4 complex in distinct functional contexts.

Introduction

The integrity of the genome is protected by the concerted action of a number of DNA repair mechanisms (Ciccia and Elledge, 2010). It has been long believed that different DNA repair pathways have distinct DNA-damage specificities, enabling them to remove different types

[5] Corresponding authors: zou.lee@mgh.harvard.edu. Agata.Smogorzewska@mail.rockefeller.edu.

of DNA lesions from the genome. However, emerging evidence has revealed that different DNA repair pathways may compete and/or cooperate at sites of DNA damage. For example, at DNA double-stranded breaks (DSBs), the choice between homologous recombination (HR) and non-homologous end joining (NHEJ) is intricately regulated by the cell cycle and a subset of DNA repair proteins (Bunting and Nussenzweig, 2013; Chapman et al., 2012). In addition, a number of DNA repair proteins apparently function in more than one pathway. For example, the repair of DNA interstrand crosslinks (ICLs) involves specific factors that function in post-replicative repair (PRR), nucleotide excision repair (NER), and HR (Crossan and Patel, 2012; Kim and D'Andrea, 2012; Kottemann and Smogorzewska, 2013). These new findings suggest that the specificities of DNA repair pathways are not only determined by the biochemical properties of DNA repair proteins themselves, but also by the contexts in which they execute their functions. This emerging concept has raised an important question as to how multifunctional DNA repair proteins are regulated in specific contexts to protect the genome against different types of DNA lesions.

The recently identified SLX4 is a fascinating protein that functions in multiple DNA repair pathways (Svendsen and Harper, 2010). SLX4 associates with several DNA structure-specific endonucleases, including XPF-ERCC1, MUS81-EME1, and SLX1 (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). Furthermore, SLX4 associates with mismatch repair proteins MSH2-MSH3 and the telomere-binding protein TRF2. The unusual ability of SLX4 to form a “tool box” of DNA repair proteins enables SLX4 to regulate and perhaps orchestrate multiple DNA repair processes. For example, SLX4 plays a key role in ICL repair. In fact, SLX4 is also known as FANCP because biallelic inactivating mutations in SLX4 are found in Fanconi Anemia (Kim et al., 2011; Stoepker et al., 2011). XPF-ERCC1, one of the nucleases in the SLX4 complex, is critical for the function of SLX4 in ICL repair (Kim et al., 2013). It was recently shown that SLX4 stimulates the nuclease activity of XPF-ERCC1 and cooperates with XPF-ERCC1 to “unhook” ICLs (Hodskinson et al., 2014; Klein Douwel et al., 2014). SLX4 also plays an important role in Holliday junction (HJ) resolution by coordinating the actions of two other nucleases in the SLX4 complex, MUS81-EME1 and SLX1 (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013). In addition, SLX4 regulates the processing of aberrant DNA replication intermediates and the maintenance of telomeres (Couch et al., 2013; Ragland et al., 2013; Wan et al., 2013; Wilson et al., 2013). MUS81 was recently implicated in the suppression of fragile site-associated genomic instability (Naim et al., 2013; Ying et al., 2013) as well as replication fork progression and restart (Pepe and West, 2014; Sarbajna et al., 2014), suggesting that the SLX4 complex may function in these contexts. How SLX4 recognizes different types of DNA lesions is just beginning to unfold. It has been suggested that SLX4 uses its ubiquitin-binding zinc finger (UBZ) domains to engage the mono-ubiquitylated FANCD2 or other poly-ubiquitylated proteins at ICLs (Lachaud et al., 2014; Yamamoto et al., 2011). Furthermore, a fraction of SLX4 is recruited to telomeres via its interaction with TRF2 (Wan et al., 2013; Wilson et al., 2013). A complete picture of how the SLX4 complex recognizes and processes different DNA lesions is still lacking.

Protein ubiquitylation has a wide range of functions in the DNA damage response (DDR) (Jackson and Durocher, 2013). SUMO, a family of small ubiquitin-like proteins, has also emerged as a key regulator of DNA repair (Gareau and Lima, 2010; Jackson and Durocher,

2013). In response to DNA damage, SUMO rapidly accumulates at sites of DNA damage and promotes the recruitment of DNA repair proteins (Galanty et al., 2009). A growing list of DNA repair proteins, including BRCA1, 53BP1, MDC1, RPA, and BLM, have been shown to be post-translationally modified by SUMO (Dou et al., 2010; Galanty et al., 2012; Galanty et al., 2009; Luo et al., 2012; Morris et al., 2009; Ouyang et al., 2009b; Yin et al., 2012). In addition, many DNA repair proteins interact non-covalently with either ubiquitin or SUMO via specific binding motifs. Interestingly, RAP80, an important regulator of BRCA1, binds to both ubiquitin and SUMO, and these interactions promote the accumulation of BRCA1 at DSBs cooperatively (Guzzo et al., 2012; Hu et al., 2012). However, until now, it was not clear if ubiquitin and SUMO binding could differentially regulate the distinct functions of a single protein.

In this study, we found that SLX4 directly recognizes SUMO-2/3 chains through SUMO-interacting motifs (SIMs). Interestingly, the SIMs and UBZs of SLX4 are functionally distinct. While the UBZs of SLX4 are required for ICL repair, the SIMs are dispensable for this function. On the other hand, the SIMs of SLX4 are important for processing camptothecin (CPT)-induced replication intermediates, suppressing common fragile site-associated genomic instability, and localizing SLX4 to telomeres in ALT (alternative lengthening of telomeres)-positive U2OS cells. The SIMs of SLX4 are also required for targeting SLX4 to sites of laser-induced DNA damage through a process involving DNA end resection, UBC9 and MDC1. Importantly, SLX4 interacts with RPA, the MRE11-RAD50-NBS1 (MRN) complex, and TRF2 in a SIM-enhanced manner, suggesting that the SUMO binding of SLX4 increases its affinity for specific DNA damage sensors and telomere-binding proteins. Our findings provide an intriguing example of how the distinct functions of a single DNA repair protein can be orchestrated by its binding to ubiquitin and SUMO, revealing a potentially widespread mechanism that contributes to the context specificities of multifunctional proteins.

Results

SLX4 binds SUMO-2/3 chains

In a previous study, we have used a nonconjugatable form of GST-SUMO-2 (GST-SUMO-2-GA) to identify SUMO-binding proteins in HeLa nuclear extracts (Ouyang et al., 2009a). From this study, we identified BTBD12, which was subsequently shown to be the ortholog of yeast Slx4p and *Drosophila* MUS312 and renamed human SLX4 (Fig. S1) (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). In addition to SLX4, we also identified a number of proteins that associate with SLX4, including MUS81, EME1, XPF/ERCC4, MSH2, and C20orf94 (Ouyang et al., 2009a), raising the possibility that the whole SLX4 complex associates with SUMO in human cells. Given the importance of the SLX4 complex in DNA repair and the regulatory role for SLX4 in this complex, we sought to determine if SLX4 is a SUMO-binding protein.

We first tested if SLX4 binds GST-SUMO-2 specifically in cell extracts. We expressed HA-SLX4 in HEK293T cells and incubated cell extracts with GST-SUMO-1 or GST-SUMO-2 purified from *E. coli* (Fig. 1A). HA-SLX4 was specifically pulled down by GST-SUMO-2 but not GST-SUMO-1. The basic residues K33 and K35 of SUMO-2 are critical for the

recognition of SUMO-2 by the SIMs of other proteins (Chupreta et al., 2005; Ouyang et al., 2009a). GST-SUMO-2-2KA, which carries the K33A and K35A mutations, was unable to bind HA-SLX4 (Fig. 1A), showing that the interaction of SLX4 with GST-SUMO-2 is specific.

Although SLX4 interacts with GST-SUMO-2 in extracts, a large amount of GST-SUMO-2 is needed to detect this interaction, indicating that the interaction is inefficient. Since SUMO-2/3 forms polymer chains in vivo, we asked if the binding of SLX4 to SUMO-2 is regulated by the formation of SUMO-2 chains. We compared the bindings of SLX4 to purified linear SUMO-2 tetramers (GST-4xSUMO-2) and SUMO-2 monomers (GST-SUMO-2) (Tatham et al., 2008). When small amounts of GST-SUMO-2 and GST-4xSUMO-2 were used, the binding of HA-SLX4 to GST-SUMO-2 was no longer detectable, yet a robust interaction with GST-4xSUMO-2 was detected (Fig. 1B). Similarly, endogenous SLX4 and MUS81 were captured by GST-4xSUMO-2 but not GST-SUMO-2 (Fig. 1C). KU70, an abundant DNA repair protein that binds DSBs, was not captured by either GST-SUMO-2 or GST-4xSUMO-2. Consistent with the GST pulldowns, HA-SLX4 was coimmunoprecipitated by Flag-4xSUMO-2 but not Flag-1xSUMO-2, similarly to RNF4, a protein known to bind SUMO-2/3 chains (Fig. 1D) (Lallemand-Breitenbach et al., 2008; Sun et al., 2007; Tatham et al., 2008). In addition to SUMO-2 tetramers, SLX4 also bound to isopeptide-linked SUMO-2 chains in a length-dependent manner (see Fig. S2C). Finally, immunoprecipitation of SFB (S/Flag/Streptavidin-binding-peptide)-tagged SLX4, but not SFB-GFP, captured endogenous SUMO-2/3 conjugates (Fig. 1E). The amounts of SUMO-2/3 conjugates captured by SFB-SLX4 were much reduced under the denaturing condition (Fig. 1F), suggesting that most of them associate with SLX4 non-covalently. Together, these results suggest that SLX4 is capable of binding SUMO-2/3 chains in vitro and in vivo.

SLX4 directly recognizes SUMO-2/3 chains via a SIM cluster

To understand if the binding of SLX4 to SUMO-2/3 chains is functionally important, we sought to identify the SIMs of SLX4. A set of HA-tagged SLX4 deletion mutants was tested for binding to Flag-1xSUMO-2 and Flag-4xSUMO-2 by coimmunoprecipitation (Fig. 2A, S2A). Three overlapping SLX4 fragments (SLX4-8, 9, and 10) that encompass the central region of SLX4 (amino acids 871-1327) bound to Flag-4xSUMO-2 specifically, whereas other SLX4 fragments encompassing the N or C terminal region of SLX4 did not (Fig. 2A, S2A). SLX4 interacts with XPF, MUS81, and SLX1 through MLR (MUS312-MEI9 interaction-like region), SAP (SAF-A/B, Acinus and PIAS), and SBD (SLX1 binding domain) domains, respectively (Castor et al., 2013; Crossan et al., 2011; Fekairi et al., 2009; Kim et al., 2013; Svendsen et al., 2009). None of these three domains is present in SLX4-8, 9, and 10 (Fig. 2A). The BTB (broad-complex, tramtrack, and bric a` brac) and coiled-coil domains of SLX4 are also absent in SLX4-9 (Fig. 2A). Purified His-SLX4-8 interacted with purified GST-4xSUMO-2 and isopeptide-linked SUMO-2 chains efficiently (Fig. S2B-C), showing that this region of SLX4 binds SUMO-2 chains directly. The TBM (TRF2-binding motif) of SLX4 (Wan et al., 2013; Wilson et al., 2013), which is present in SLX4-8, is dispensable for SUMO binding (see Fig. 7A). Together, these results suggest that the SIMs of SLX4 are located between the coiled-coil and SAP domains and outside of the TBM.

Analysis of the amino-acid sequence of SLX4 between the coiled-coil and SAP domains revealed a cluster of three putative SIMs (referred to as SIM1, 2, and 3; Fig. 2A-B) (Baba et al., 2005; Hecker et al., 2006; Ouyang et al., 2009a; Reverter and Lima, 2005; Song et al., 2004). SIM2 is the most conserved among the three, whereas SIM1 is the least conserved. We disrupted these putative SIMs in His-SLX4-8 either individually or in pairs by replacing the hydrophobic residues with alanines (Fig. 2B). The binding of His-SLX4-8 to 4xSUMO-2 was drastically reduced by disruption of SIM2 and to a lesser extent by loss of SIM3 (Fig. S2D). SIM1 only contributed to the binding slightly in some but not all experiments (data not shown). When all three putative SIMs were removed by a small deletion or disrupted with point mutations, the binding of His-SLX4-8 to 4xSUMO-2 and isopeptide-linked SUMO-2 chains was abolished (Fig. 2C, S2C). These results demonstrate that SLX4 directly recognizes SUMO-2 chains with the SIMs.

To test if the SIMs are critical for the binding of full-length SLX4 to SUMO-2/3 chains, we disrupted them in full-length wild-type SLX4 (HA-SLX4^{WT}) with point mutations (Fig. 2A). The resulting SLX4^{3sim} mutant failed to bind Flag-4xSUMO-2 in cells (Fig. 2D). In contrast, SLX4^{2ubz}, which carries two UBZs disrupted by point mutations (Kim et al., 2013), interacted with 4xSUMO-2 as efficiently as SLX4^{WT}. When the SIMs were mutated in SLX4^{2ubz}, the resulting SLX4^{3sim2ubz} mutant lost its ability to bind 4xSUMO-2. These results suggest that the SIMs but not the UBZs of SLX4 are responsible for the binding to SUMO-2 chains. Immunoprecipitation of SLX4^{WT} and SLX4^{2ubz}, but not SLX4^{3sim} nor SLX4^{3sim2ubz}, captured conjugates of SUMO-2/3 (Fig. 2E), showing that SLX4 uses the SIMs to engage SUMOylated proteins. Both SLX4^{3sim} and SLX4^{3sim2ubz} retained the ability to bind MUS81. Interestingly, SLX4^{2ubz} captured SUMO-2/3 conjugates as efficiently as SLX4^{WT}, suggesting that SLX4 does not use the UBZs to engage hybrid SUMO-ubiquitin chains. Thus, the binding specificities of the SIMs and UBZs of SLX4 are clearly distinct, raising the possibility that they regulate different functions of the SLX4 complex.

The SIMs and UBZs of SLX4 are functionally distinct

SLX4-null cells are highly sensitive to a broad spectrum of DNA lesions as demonstrated by cellular sensitivity to MMC, PARP inhibitor, and chronic treatment with low concentrations of CPT (Crossan et al., 2011; Kim et al., 2011; Kim et al., 2013; Stoepker et al., 2011). To compare the functions of the SIMs and UBZs of SLX4, we expressed SLX4^{WT}, SLX4^{2ubz}, SLX4^{3sim}, or SLX4^{3sim2ubz} in *SLX4*-null (RA3331/E6E7/hTERT) cells by lentiviral transduction (Fig. S3A), and assayed for their ability to suppress the DNA damage sensitivity of *SLX4*-null cells.

Consistent with previous reports (Kim et al., 2013; Lachaud et al., 2014; Yamamoto et al., 2011), SLX4^{WT} but not SLX4^{2ubz} suppressed the sensitivity of *SLX4*-null cells to MMC (Fig. 3A). SLX4^{3sim} was able to suppress MMC sensitivity while SLX4^{3sim2ubz} was not, suggesting that the UBZs but not the SIMs are important for ICL repair. In contrast to MMC sensitivity, the sensitivity of *SLX4*-null cells to PARP inhibitor was similarly suppressed by SLX4^{WT}, SLX4^{2ubz}, SLX4^{3sim}, and SLX4^{3sim2ubz} (Fig. S3B), suggesting that both the UBZs and SIMs are dispensable for the function of SLX4 in HR. Indeed, when assayed for SLX4 Holliday junction resolution activity in BLM-depleted *SLX4*-null cells, both SLX4^{2ubz}

and SLX4^{3sim} functioned as SLX4^{WT} to suppress the formation of segmented chromosomes (Fig. S3C) (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013). Interestingly, *SLX4*-null cells were sensitive to short treatment with high concentrations of CPT (Fig. 3B). Expression of SLX4^{WT} significantly suppressed the CPT sensitivity of *SLX4*-null cells. Compared with SLX4^{WT}, SLX4^{2ubz} and SLX4^{3sim} were defective for the suppression of CPT sensitivity. Furthermore, SLX4^{3sim2ubz} was even more defective than SLX4^{2ubz} and SLX4^{3sim}, suggesting that the SIMs and UBZs of SLX4 have non-redundant roles in the CPT response.

MUS81 and ERCC1 were recently implicated in the processing of replication intermediates at common fragile sites (Naim et al., 2013; Ying et al., 2013), preventing chromosomal instability in the ensuing mitosis and G1 (Mankouri et al., 2013). *SLX4*-null cells displayed several phenotypes indicative of chromosomal instability, including an increase of G1 cells containing multiple 53BP1 nuclear bodies (Fig. 3C, 3D), an increase of micronuclei (Fig. 3E, 3F), and an increase of bulky anaphase bridges (Fig. 3G, 3H) (Chan et al., 2009; Harrigan et al., 2011; Lukas et al., 2011). All indicators of chromosomal instability of *SLX4*-null cells were suppressed by SLX4^{WT} (Fig. 3D, 3F, 3H). SLX4^{SAP} and SLX4^{SBD}, which are unable to bind MUS81 and SLX1, were defective for the suppression of these indictors (Fig. S3D-F). Importantly, SLX4^{3sim} failed to fully suppress all of these indicators (Fig. 3D, 3F, 3H). SLX4^{2ubz} suppressed 53BP1 nuclear bodies, a marker of fragile site instability, but was mildly defective for the suppression of anaphase bridges and micronuclei. SLX4^{3sim2ubz} was more defective than SLX4^{3sim} and SLX4^{2ubz} for the suppression of anaphase bridges and micronuclei. Thus, the SIMs and UBZs of SLX4 have non-redundant roles in the suppression of chromosomal instability, and the SIMs but not the UBZs are important for the function of the SLX4 complex at common fragile sites.

SUMO binding of SLX4 promotes processing of CPT-induced replication intermediates

The implication of the SIMs of SLX4 in the CPT response prompted us to examine this process in more detail. CPT induces slowing and reversal of replication forks at low concentrations and DSBs at high concentrations (Ray Chaudhuri et al., 2012). In CPT-treated cells, MUS81 is required for the efficient formation of DSBs (Regairaz et al., 2011). Similar to observations in MUS81-deficient cells, the CPT-induced phosphorylation of RPA32 and H2AX was significantly reduced in cells treated with two independent SLX4 siRNAs (Fig. 4A, S4A-B). Compared with SLX4 knockdown, depletion of both SLX4 and MUS81 did not further reduce RPA32 phosphorylation (Fig. 4B, S4C), suggesting that SLX4 and MUS81 act in the same pathway during the CPT response.

Next, we tested if the SIMs and UBZs of SLX4 are required for the processing of CPT-induced replication intermediates. As expected, SLX4^{WT} significantly enhanced CPT-induced RPA32 phosphorylation in *SLX4*-null cells (Fig. 4C, S4D). Depletion of MUS81 in the *SLX4*-null cells complemented with SLX4^{WT} reduced RPA32 phosphorylation (Fig. 4D), suggesting that the restored processing of replication intermediates is dependent upon MUS81. Similar to SLX4^{WT}, SLX4^{2ubz} also enhanced RPA32 phosphorylation in *SLX4*-null cells (Fig. 4C, S4D). In contrast, SLX4^{3sim} and SLX4^{3sim2ubz} were unable to enhance RPA32 phosphorylation to the same extent as SLX4^{WT}. The *SLX4*-null cells complemented

with SLX4^{3sim} or SLX4^{3sim2ubz} did not display any defects in DNA synthesis and S-phase progression compared to SLX4^{WT}-complemented cells (Fig. S4E), ruling out indirect effects of cell-cycle alterations. Thus, the SIMs are important for the cleavage of CPT-induced replication intermediates by MUS81, linking the SUMO binding of SLX4 to a specific function of the SLX4 complex.

SUMO binding of SLX4 promotes SLX4 localization to DNA damage and telomeres

The defects of SLX4^{3sim} in specific functions raise the possibility that the SUMO binding of SLX4 is required for the recognition of DNA damage in specific contexts. Although SLX4 is involved in the CPT response, CPT-induced foci of HA-SLX4^{WT} cannot be reliably quantified (data not shown). In cells treated with BrdU, UV laser inflicts multiple types of DNA lesions, providing a sensitive means to follow the localization of DDR proteins to sites of DNA damage (Bekker-Jensen et al., 2006). Consistent with our previous results (Svendsen et al., 2009), HA-SLX4^{WT} was recruited to laser-induced DNA damage stripes in BrdU-treated U2OS cells (Fig. 5A, S5A). The fraction of cells that displayed SLX4^{WT} stripes was enriched for Cyclin A-positive cells (Fig. S5B), suggesting that SLX4 is preferentially recruited to DNA damage stripes in S phase. SLX4^{2ubz}, which was defective for the localization to ICLs (Fig. S5C) (Lachaud et al., 2014), was also recruited to laser-induced DNA damage stripes in BrdU-treated cells (Fig. 5B). In contrast, SLX4^{3sim} and SLX4^{3sim2ubz} failed to localize to the stripes efficiently. The localization defects of SLX4^{3sim} and SLX4^{3sim2ubz} were not due to cell-cycle alterations (Fig. S5D). Knockdown of UBC9, the only SUMO-conjugating enzyme, reduced the levels of SUMO-2/3 conjugates and compromised the localization of SLX4^{WT} to DNA damage stripes (Fig. S5E-F). The localization of SLX4^{3sim}, which is unable to bind SUMO, was not affected by UBC9 knockdown (Fig. S5G). At the time of analysis, UBC9 knockdown did not affect the cell cycle (Fig. S5H), nor did it affect the recruitment of RPA32 (Fig. S5I), suggesting that ssDNA formation was not compromised. In contrast to UBC9 knockdown, depletion of the SUMO-targeted ubiquitin ligase (STUbL) RNF4 did not affect the localization of SLX4^{WT} (Fig. S5J-K), suggesting that SUMO but not SUMO-ubiquitin hybrid chains promote SLX4 recruitment.

A fraction of SLX4 is localized to telomeres via an interaction with TRF2 (Wan et al., 2013; Wilson et al., 2013). In cells with active ALT pathway, a number of telomere-binding proteins are SUMOylated (Potts and Yu, 2007), and telomeres associate with PML bodies, which are enriched for SUMOylated proteins (Yeager et al., 1999). To test if the SIMs of SLX4 contribute to the association of SLX4 with telomeres, we analyzed the localization of SLX4^{WT} and SLX4^{3sim} in ALT-positive U2OS cells. We also tested SLX4^{L1022A}, which carries a disrupted TBM, and SLX4^{3simLA}, which lacks both the SIMs and TBM (Fig. S5L). As previously reported (Wilson et al., 2013), limited colocalization of SLX4^{L1022A} and TRF2 was observed (Fig. 5C, S5M). Although SLX4^{3sim} retained a substantial ability to colocalize with TRF2, the colocalization of SLX4^{3simLA} with TRF2 and telomeres was virtually abolished (Fig. 5C, S5M-N). These results suggest that both the SIMs and TBM contribute to the telomere localization of SLX4 in U2OS cells. Compared with SLX4^{WT}, SLX4^{2ubz} did not display any defect in the colocalization with TRF2 (Fig. 5C). Interestingly, in *SLX4*-null cells immortalized by hTERT expression, SLX4^{L1022A}, which

contains the SIMs, failed to colocalize with telomeres (Fig. S5O). These results raise the interesting possibility that the SIMs may only contribute to the telomere localization of SLX4 in ALT-positive cells.

Regulation of SLX4 recruitment by resection and MDC1

UV laser induces multiple types of DNA lesion that could undergo DNA end resection (Bekker-Jensen et al., 2006; Kong et al., 2009). Recent studies in yeast suggested that DNA end resection promotes the SUMOylation of a number of DNA repair proteins (Cremona et al., 2012; Psakhye and Jentsch, 2012). To test if resection is required for the localization of SLX4 to sites of DNA damage, we analyzed the localization of $SLX4^{WT}$ in cells depleted of CtIP, a protein required for efficient resection (Sartori et al., 2007). Knockdown of CtIP did not alter the cell cycle but significantly reduced the localization of $SLX4^{WT}$ to DNA damage stripes (Fig. 6A, S6A-B). Consistently, when resection was inhibited by Mirin, an inhibitor of the MRE11 nuclease (Dupre et al., 2008; Shibata et al., 2014), the recruitment of $SLX4^{WT}$ to DNA damage stripes was also compromised (Fig. 6B). These results suggest that resection promotes the recruitment of SLX4 to sites of DNA damage.

To investigate if SLX4 is recruited to resected DNA ends, we tested if $SLX4^{WT}$ colocalizes with RPA. $SLX4^{WT}$ was colocalized with RPA32 in DNA damage stripes, and virtually all $SLX4^{WT}$ stripes are positive for RPA32 (Fig. 6C-D). Interestingly, knockdown of MDC1, which recruits a number of DDR proteins to the chromatin flanking DSBs, also reduced the localization of $SLX4^{WT}$ to DNA damage stripes without altering the cell cycle (Fig. 6E, S6B-C). MDC1 knockdown only reduced the recruitment of $SLX4^{WT}$ but not RPA32 (Fig. S6D), suggesting that MDC1 functions independently of resection. Furthermore, double knockdown of CtIP and MDC1 did not further reduce the localization of $SLX4^{WT}$ (Fig. 6F, S6E), suggesting that resection and MDC1 act together to recruit SLX4. It is possible that certain DDR proteins are recruited to DNA damage sites by MDC1 and SUMOylated in a resection-dependent manner, providing an anchor for SLX4 recruitment.

SUMO binding of SLX4 enhances SLX4 interactions with RPA, MRN and TRF2

To understand how SLX4 is regulated by SUMO binding, we sought to determine if SLX4 interacts with select DDR and telomere-binding proteins in a SIM-regulated manner. Because both the TBM and SIMs contribute to the localization of SLX4 to ALT telomeres, we asked if the SIMs contribute to the SLX4-TRF2 interaction. Consistent with previous reports (Wan et al., 2013; Wilson et al., 2013), SFB-$SLX4^{L1022A}$, which carries a disrupted TBM, was unable to bind myc-TRF2 efficiently in U2OS and HEK293T cells (Fig. 7A, S7A-B). Interestingly, $SLX4^{3sim}$ displayed a partial defect in TRF2 binding in ALT-positive U2OS cells (Fig. 7A), but not in telomerase-positive HEK293T cells (Fig. S7A). When SUMO-2 was co-overexpressed in HEK293T cells, $SLX4^{3sim}$ interacted with TRF2 less efficiently than $SLX4^{WT}$ (Fig. S7B). Notably, disruption of the SIMs in $SLX4^{L1022A}$ did not further reduce TRF2 binding in U2OS cells or HEK293T cells overexpressing SUMO-2 (Fig. 7A, S7B). These results suggest that the TBM of SLX4 is the main determinant for the SLX4-TRF2 interaction, whereas the SIMs enhance the interaction in ALT cells or in cells with heightened protein SUMOylation.

Given the colocalization of SLX4 with RPA in DNA damage stripes and the involvement of MDC1 in SLX4 recruitment, we next tested if SLX4 interacts with RPA and MDC1. Immunoprecipitation of SFB-SLX4^{WT} captured RPA70 but not MDC1 (Fig. 7B). Although we were unable to detect an interaction between SLX4 and MDC1, the MRN complex, which is recruited to DNA damage sites by MDC1 (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008), was captured by SFB-SLX4^{WT} (Fig. 7B). In addition to RPA70, SLX4 also interacted with phosphorylated RPA32 (Fig. S7C), suggesting that SLX4 binds the RPA complex at sites of DNA damage. Importantly, the interactions of SLX4 with RPA and MRN, but not the interaction with MUS81, were reduced by UBC9 knockdown (Fig. 7B), suggesting that SLX4 binds RPA and MRN in a SUMOylation-enhanced manner.

Although SLX4 interacted with RPA and MRN in a SUMOylation-enhanced manner, these proteins were captured by SLX4 in the unSUMOylated form (Fig. 7B). RPA70 was recently suggested to be SUMOylated (Dou et al., 2010). A slow-migrating form of RPA70 was reduced by UBC9 knockdown and induced by CPT treatment (Fig. 7B, S7D-E). Compared with unmodified RPA70, the slow-migrating form of RPA70 was more enriched by SFB-SLX4^{WT} immunoprecipitation (Fig. S7E-F), suggesting that SLX4 preferentially binds SUMOylated RPA70. Nonetheless, the majority of SLX4-bound RPA70 was unSUMOylated. Thus, although SLX4 has the ability to bind SUMOylated RPA70 preferentially, the interactions of SLX4 with RPA and MRN are mainly regulated by other SUMOylated proteins.

Finally, we asked if the SIMs of SLX4 contribute to the interactions of SLX4 with RPA and MRN. Consistent with the role for the SIMs in recognizing SUMOylated proteins, SLX4^{WT} interacted with SUMOylated RPA70 more efficiently than SLX4^{3sim} (Fig. 7C). SLX4^{WT} also interacted with unSUMOylated RPA and MRN more efficiently than SLX4^{3sim} (Fig. 7C), suggesting that the SIMs promote the interactions of SLX4 with RPA and MRN indirectly as well. It is possible that the binding of SLX4 to the SUMO-2/3 chains on multiple DDR proteins increases the local concentration of SLX4 at sites of DNA damage, enabling SLX4 to interact with specific unSUMOylated DDR proteins more efficiently.

Independent roles for the SIMs and UBZs in the protein interactions of SLX4

If the SIMs and UBZs of SLX4 regulate the SLX4 complex independently in different contexts, one would expect that the SIMs and UBZs should have distinct roles in the protein interactions of SLX4. Furthermore, the SIMs and UBZs should be able to function independently of each other. Indeed, SLX4^{3sim} but not SLX4^{2ubz} was defective for the interactions with RPA70 and MRN (Fig. 7C). Furthermore, SLX4^{3sim2ubz} was much more defective than SLX4^{2ubz} for the binding to RPA and MRN, showing that the SIMs are indeed functional in the absence of the UBZs. Interestingly, SLX4^{3sim2ubz} was also more defective than SLX4^{3sim} for the interactions with RPA and MRN, suggesting that the UBZs have a secondary role in these interactions in the absence of the SIMs. Together, these results clearly show that the SIMs and UBZs of SLX4 can function independently of each other in the interactions of SLX4 with DDR proteins, providing a mechanistic basis for their distinct functions in the regulation of the SLX4 complex.

Discussion

SUMO and ubiquitin orchestrate distinct functions of the SLX4 complex

In response to DNA damage, cells need to evoke appropriate DNA repair pathways to remove DNA lesions and protect genomic integrity. While different DNA repair pathways have different specificities for DNA lesions, a growing list of DNA repair proteins are found to act in multiple pathways, raising an important question as to how these proteins recognize distinct forms of DNA lesions. As a key coordinator of structure-specific endonucleases, SLX4 participates in DNA repair in several different contexts. In this study, we found that SLX4 executes distinct functions through binding to SUMO or ubiquitin, revealing a novel mechanism that orchestrates the context-specific roles of a single DNA repair protein.

We and others have previously shown that non-covalent binding of SLX4 to ubiquitin via the UBZs is important for ICL repair (Kim et al., 2013; Klein Douwel et al., 2014; Lachaud et al., 2014; Yamamoto et al., 2011). In this study, we found that SLX4 binds SUMO-2/3 chains via the SIMs independently of the UBZs. In contrast to the UBZs, the SIMs of SLX4 are dispensable for ICL repair. On the other hand, the SIMs but not the UBZs are important for efficiently localizing SLX4 to laser-induced DNA damage and ALT telomeres. These findings clearly show that the SIMs and UBZs regulate distinct functions of the SLX4 complex. Interestingly, both the SIMs and UBZs of SLX4 contribute to its functions in the CPT response and the suppression of chromosomal instability. However, the defects of $SLX4^{3sim}$ and $SLX4^{2ubz}$ mutants in these functions are additive, suggesting that the roles for the SIMs and UBZs are not redundant. It is possible that SLX4 interacts with different SUMOylated or ubiquitylated proteins during the processing of aberrant replication intermediates, allowing it to fully execute its functions. It should be noted that even the $SLX4^{3sim2ubz}$ mutant is able to suppress the PARP inhibitor sensitivity of *SLX4*-null cells and the chromosome segmentation in cells lacking SLX4 and BLM, indicating the existence of an additional mechanism that regulates the HR function of SLX4.

How does SUMO binding regulate the SLX4 complex?

In response to laser microirradiation, SUMO-2/3 rapidly accumulates at sites of DNA damage (Galanty et al., 2009). In yeast, numerous DDR proteins are SUMOylated in a resection-dependent manner (Cremona et al., 2012; Psakhye and Jentsch, 2012). In human cells, a number of DDR proteins recruited by MDC1, such as RNF168, HERC2, RAP80, BRCA1, and 53BP1, are SUMOylated (Danielsen et al., 2012; Galanty et al., 2009; Morris et al., 2009; Yan et al., 2007). The localization of SLX4 to laser-induced DNA damage is dependent upon UBC9 and the SIMs, but not the UBZs, suggesting that the binding of SLX4 to the SUMO-2/3 chains at sites of DNA damage is required for efficient SLX4 recruitment. Similar to laser-induced DNA damage stripes, ALT telomeres are enriched for SUMOylated proteins (Potts and Yu, 2007). The colocalization of SLX4 with TRF2 in U2OS cells was compromised by the loss of the SIMs, suggesting that the binding of SLX4 to the SUMO-2/3 chains at telomeres contributes to the localization of SLX4 to telomeres.

While the SIMs promote the localization of SLX4 to laser-induced DNA damage and ALT telomeres, they are unlikely the sole determinant of context specificity. In telomerase-

positive *SLX4*-null cells, SLX4^{3sim} is recruited to telomeres by TRF2. Even in ALT-positive U2OS cells, some SLX4^{3sim} remains colocalized with TRF2. The telomere specificity of SLX4 is largely determined by the interaction between SLX4 and TRF2. The binding of SLX4 to the SUMO-2/3 chains at ALT telomeres may increase the local concentration of SLX4, allowing it to engage TRF2 more efficiently. Similarly, the binding of SLX4 to the SUMO-2/3 chains at laser-induced DNA damage may increase SLX4 local concentration, enhancing its interactions with specific DDR proteins. As exemplified by the preferential binding of SLX4 to SUMOylated RPA70, SLX4 indeed has the ability to recognize SUMOylated DDR proteins. However, RPA70 is unlikely the only SUMOylated DDR protein that is recognized by SLX4. SLX4 binds unSUMOylated RPA and MRN in a UBC9 and SIM-regulated manner, suggesting that other SUMOylated proteins promote these interactions indirectly. It is plausible that SLX4 interacts with the SUMO-2/3 chains on multiple DDR proteins at sites of DNA damage, providing the initial affinity of SLX4 to these loci that allows SLX4 to efficiently engage its context-specific partners. In the absence of the SIMs, multiple interactions of SLX4 with DDR proteins are compromised, impairing the localization of SLX4 to sites of DNA damage.

Our findings suggest that SLX4 recognizes different types of DNA lesions by binding to SUMO or ubiquitin, yet whether and how these lesions are differentially marked by SUMO or ubiquitin is not fully understood. It is possible that the efficiencies of ubiquitylation and SUMOylation of DDR proteins vary in response to different types of DNA damage, making one of these modifications preferentially recognized by SLX4 in a specific context. It is more likely that the recruitment of SLX4 is determined not only by the levels of SUMOylation or ubiquitylation, but also by the specific DDR proteins that interact with SLX4 in a modification-regulated manner. Furthermore, SLX4 itself may be differentially modified in response to different types of DNA damage, modulating its ability to recognize SUMO, ubiquitin, or specific DDR proteins. The differential accumulation of DDR proteins at different DNA lesions, the differential modifications of these proteins, and the differential modification of SLX4 may all influence whether SLX4 recognizes a specific type of DNA lesion via SUMO, ubiquitin, or both.

The concerted action of SUMO and ubiquitin in the DDR

While both ubiquitylation and SUMOylation have been linked to the DDR, how these two modifications work in concert is only partially understood. Previous studies have suggested that ubiquitylation and SUMOylation can function in either a competitive or a cooperative manner. For example, the yeast DNA replication/repair protein PCNA can be ubiquitylated or SUMOylated at the same residue (Hoege et al., 2002). Whereas the ubiquitylation of PCNA promotes PRR (Stelter and Ulrich, 2003), the SUMOylation of PCNA prevents unwanted recombination (Armstrong et al., 2012; Pfander et al., 2005). In human cells, the STUbL RNF4 recognizes SUMOylated DDR proteins including MDC1. RNF4 generates hybrid SUMO-ubiquitin chains and promotes the turnover of MDC1, linking SUMOylation and ubiquitylation during the DDR (Galanty et al., 2012; Guzzo et al., 2012; Luo et al., 2012; Yin et al., 2012). Furthermore, RAP80, a key component of the BRCA1-A complex, recruits BRCA1 to sites of DNA damage by binding to both ubiquitin and SUMO (Guzzo et al., 2012; Hu et al., 2012). In this study, we found that the binding of SLX4 to SUMO or

ubiquitin regulates distinct functions of SLX4, revealing a third mechanism by which SUMO and ubiquitin can act in concert. In this situation, the binding of a single protein to SUMO or ubiquitin helps it acquire context specificities, allowing the protein to switch its roles in a regulated manner. It is tempting to speculate that many multifunctional proteins other than SLX4 have the ability to recognize SUMOylated and ubiquitylated partners, providing a widespread mechanism to help directing multifunctional proteins to different signaling and functional circuitries in cells.

Materials and Methods

Materials and methods are provided in Supplemental Materials and Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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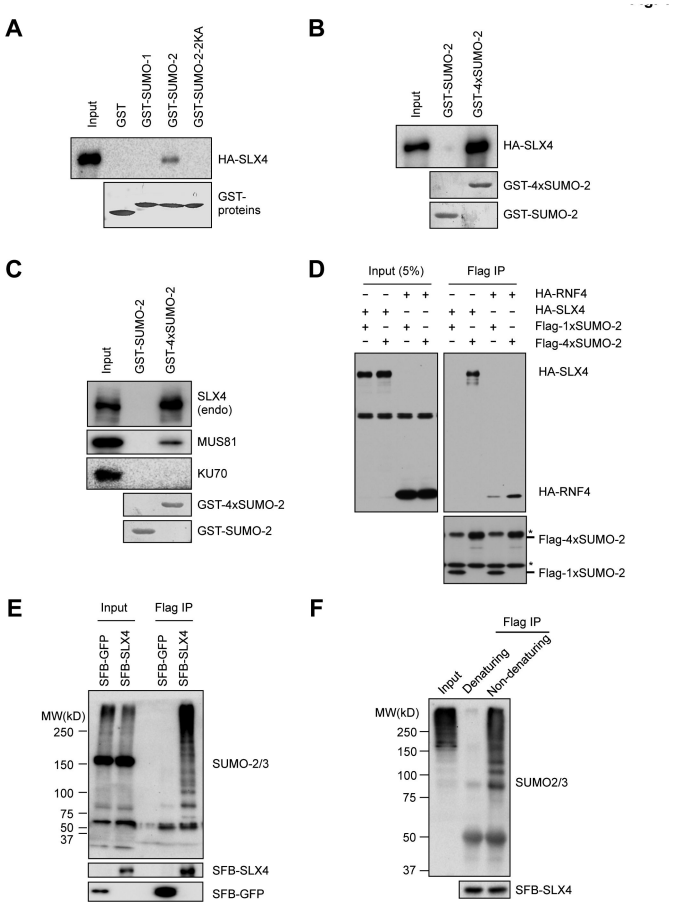

Fig. 1. Binding of SLX4 to SUMO-2/3 chains

(A) Purified GST, GST-SUMO-1, GST-SUMO-2, and GST-SUMO-2-2KA (200 μg of each) were incubated with extracts derived from HEK293T cells expressing HA-SLX4. The HA-SLX4 captured by GST pulldowns was detected by anti-HA antibody. **(B-C)** Purified GST, GST-SUMO-2, and GST-4xSUMO-2 (5 μg of each) were incubated with extracts derived from HA-SLX4-expressing cells (B) or untransfected cells (C). The HA-SLX4 (B) and endogenous SLX4 and MUS81 (C) captured by GST pulldowns were detected by Western blot. **(D)** HA-SLX4 and HA-RNF4 were coexpressed with Flag-1xSUMO-2 or Flag-4xSUMO-2 in HEK293T cells. Flag-tagged SUMO-2 was immunoprecipitated using anti-Flag antibody, and the HA-SLX4 and HA-RNF4 associated with SUMO-2 were detected by Western blot. **(E)** SFB-tagged GFP and SLX4 were immunoprecipitated using anti-Flag antibody under a non-denaturing condition. The SUMO-2/3 conjugates in the immunoprecipitates were analyzed using anti-SUMO-2/3 antibody. **(F)** SFB-SLX4 was immunoprecipitated using anti-Flag antibody under a denaturing or a non-denaturing condition.

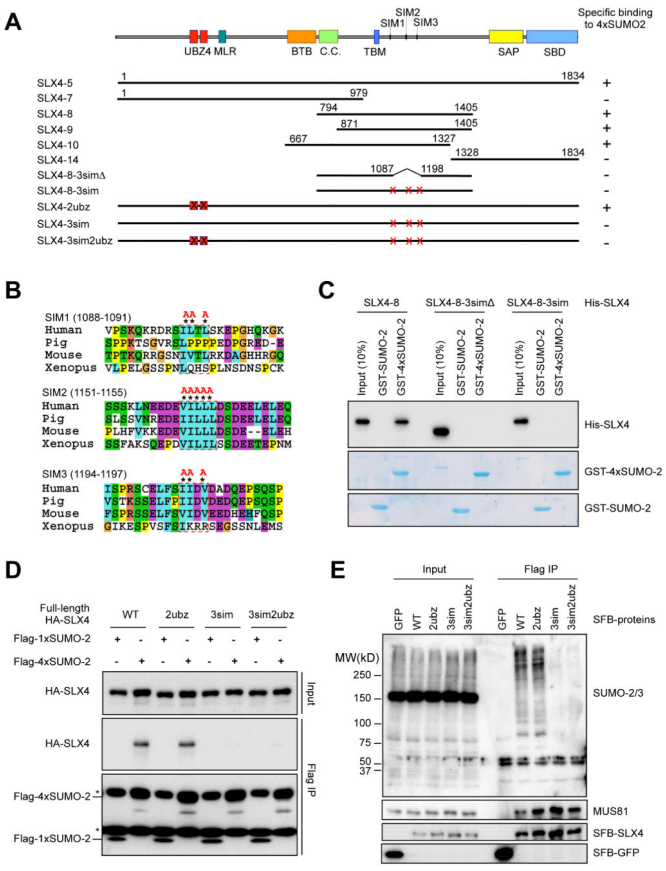

Fig. 2. SLX4 recognizes SUMO-2/3 chains via the SIMs

(A) A schematic representation of the domain structure of SLX4 and the SLX4 fragments tested in this study. A summary of the ability of the SLX4 fragments to specifically recognize SUMO-2 chains is shown on the right. **(B)** Amino acid sequences of the three SIMs of human SLX4 are aligned with the corresponding sequences of SLX4 homologues from higher vertebrates. The hydrophobic residues that are mutated in the SLX4 mutants are marked by *. **(C)** The three SIMs of SLX4-8 were disrupted by an internal deletion (SLX4-8-3simΔ) or point mutations (SLX4-8-3sim). His-SLX4-8 and its mutant derivatives were purified and tested for binding to purified GST-SUMO-2 and GST-4xSUMO-2. **(D)** HA-SLX4^{WT} and the indicated SLX4 mutants were coexpressed with Flag-1xSUMO-2 or Flag-4xSUMO-2 in HEK293T cells. Flag-tagged SUMO-2 was immunoprecipitated, and the HA-SLX4 proteins in the immunoprecipitates were analyzed using anti-HA antibody. *: two proteins non-specifically recognized by anti-Flag antibody. **(E)** SFB-tagged GFP, SLX4^{WT}, and the SLX4 mutants were immunoprecipitated from cell extracts using anti-Flag antibody. The SUMO-2/3 conjugates and MUS81 in the immunoprecipitates were analyzed by Western blot.

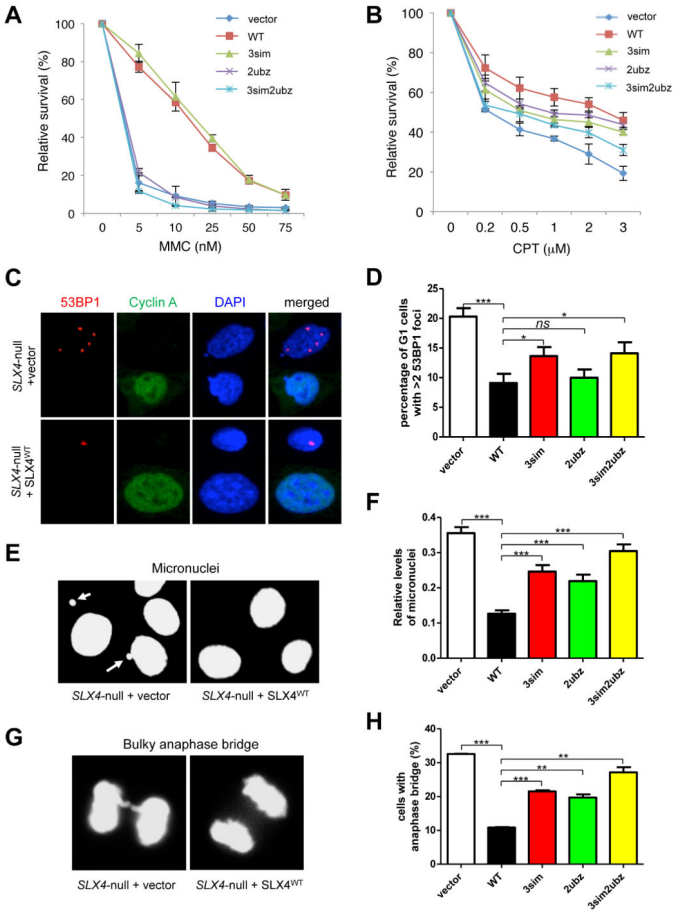

Fig. 3. The SIMs and UBZs of SLX4 are functionally distinct

(A) *SLX4*-null cells complemented with vector, SLX4^{WT}, SLX4^{3sim}, SLX4^{2ubz}, and SLX4^{3sim2ubz} were tested for MMC sensitivity. **(B)** *SLX4*-null cells complemented with vector, SLX4^{WT}, SLX4^{3sim}, SLX4^{2ubz}, and SLX4^{3sim2ubz} were tested for CPT sensitivity. **(C-D)** *SLX4*-null cells complemented with vector, SLX4^{WT}, SLX4^{3sim}, SLX4^{2ubz}, and SLX4^{3sim2ubz} were analyzed by immunostaining of 53BP1 and Cyclin A. Representative images of cells are shown in (C). The fractions of G1 cells (Cyclin A-negative) with multiple 53BP1 foci (>2) were quantified in the indicated cell populations (D). Error bars: S.E.M.; n=40 (>200 G1 cells were analyzed in each population). **(E- F)** DNA of *SLX4*-null cells complemented with vector or SLX4^{WT} was stained with DAPI (E). The levels of micronuclei in the indicated cell populations were quantified and normalized to the numbers of nuclei analyzed (F). Error bars: S.E.M.; n=40 (>1,300 nuclei were analyzed in each population). **(G-H)** The bulky anaphase bridge of an *SLX4*-null cell complemented with vector and the normally segregated chromosomes of an *SLX4*-null cell complemented with SLX4^{WT} (G). The fractions of mitotic cells with bulky anaphase bridges were quantified in the indicated cell populations (H). Error bars: S.D.; n=2 (>50 mitotic cells were analyzed in each population).

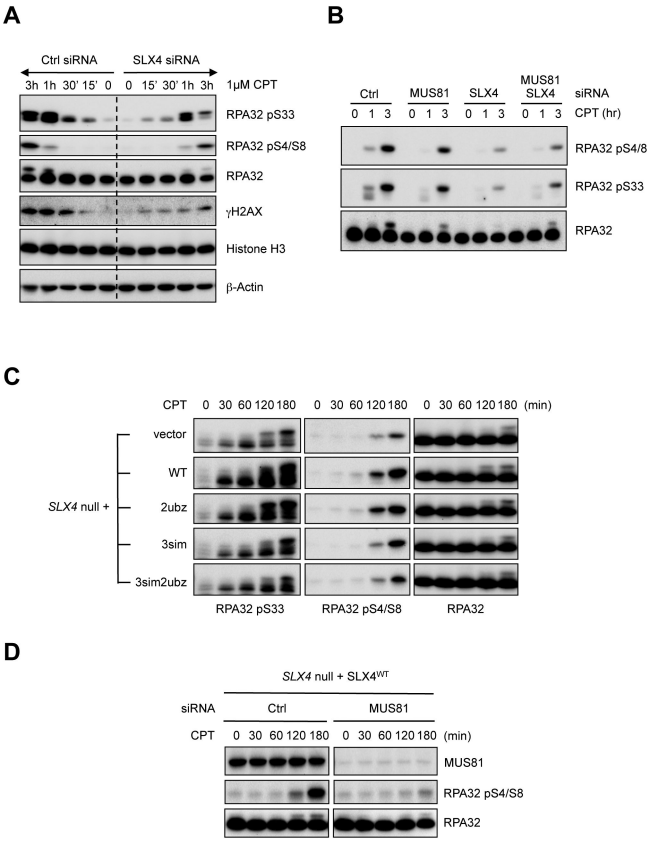

Fig. 4. The SUMO binding of SLX4 is important for processing CPT-induced replication intermediates

(A) U2OS cells were transfected with control or SLX4 siRNA and treated with 1 μM CPT. At the indicated time points, the phosphorylation of RPA32 and H2AX was analyzed with phospho-specific antibodies. Knockdown of SLX4 is shown in Fig. S4A. **(B)** U2OS cells were transfected with the indicated siRNAs. The CPT-induced phosphorylation of RPA32 was analyzed as in (A). Knockdown of MUS81 is shown in Fig. S4C. **(C)** *SLX4*-null cells complemented with vector, $SLX4^{WT}$, $SLX4^{2ubz}$, $SLX4^{3sim}$, and $SLX4^{3sim2ubz}$ were treated with 0.1 μM CPT. The CPT-induced phosphorylation of RPA32 was analyzed at the indicated time points. **(D)** *SLX4*-null cells complemented with $SLX4^{WT}$ were transfected with control or MUS81 siRNA. Cells were treated with 0.1 μM CPT, and the phosphorylation of RPA32 was analyzed.

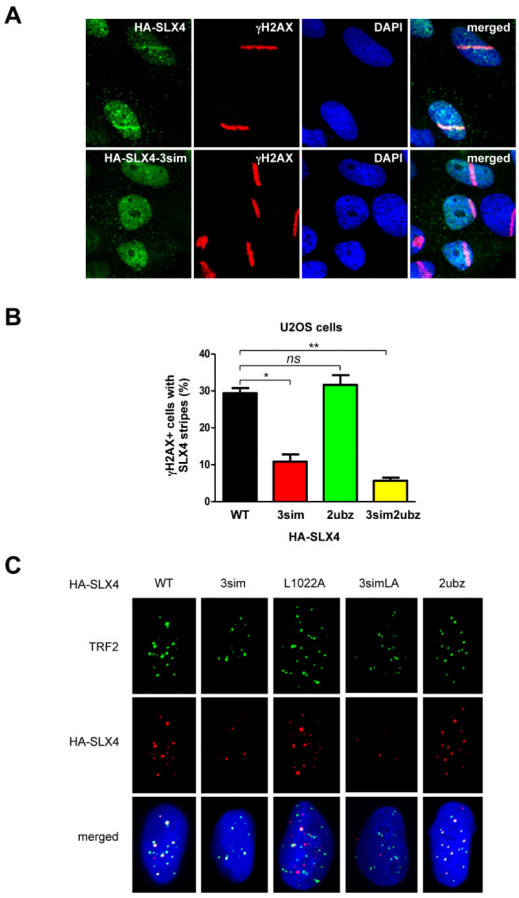

Fig. 5. SUMO binding of SLX4 promotes its localization to laser-induced DNA damage and ALT telomeres

(A) U2OS cells expressing HA-tagged SLX4^{WT} and SLX4^{3sim} were microirradiated with UV laser and analyzed by immunostaining of HA and γH2AX 1 hr later. The expression of SLX4^{WT} and SLX4^{3sim} is shown in Fig. S5A. **(B)** U2OS cells expressing HA-tagged SLX4^{WT}, SLX4^{3sim}, SLX4^{2ubz}, and SLX4^{3sim2ubz} were microirradiated with UV laser and analyzed as in (A). The fractions of γH2AX-positive cells with HA stripes were quantified. Error bars: S.E.M.; n=2. The expression of SLX4^{WT} and the SLX4 mutants and the cell-cycle profiles of various cell populations are shown in Fig. S5A, S5D. **(C)** The colocalization of HA-tagged SLX4^{WT}, SLX4^{3sim}, SLX4^{L1022A}, SLX4^{3simLA}, and SLX4^{2ubz} with endogenous TRF2 was analyzed in U2OS cells. Representative images of each cell line are shown. The expression of SLX4^{WT} and the SLX4 mutants is shown in Fig. S5L.

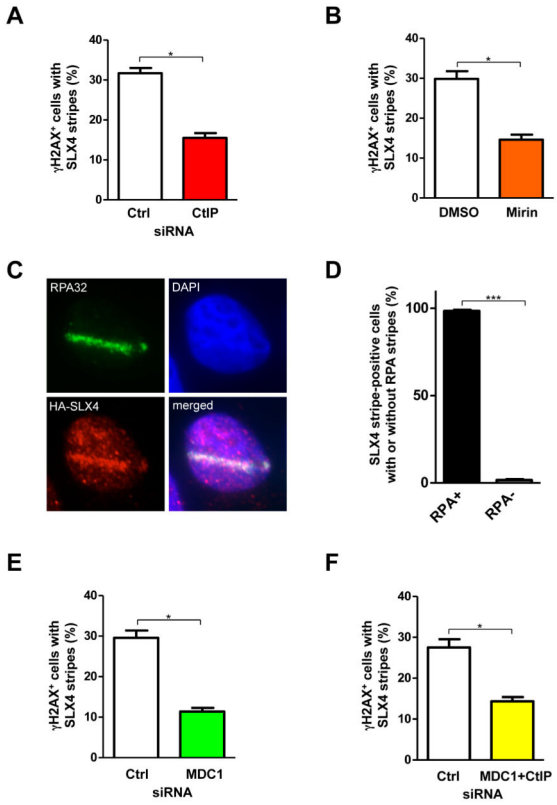

Fig. 6. Localization of SLX4 to laser-induced DNA damage requires DNA-end resection and MDC1

A-B) U2OS cells expressing HA-SLX4^{WT} were treated with control or CtIP siRNA (A), or with 25 μM Mirin (B). The localization of HA-SLX4^{WT} to DNA damage stripes was analyzed 1 hr after microirradiation and quantified as in Fig. 5B. Error bars: S.E.M.; n=2. Knockdown of CtIP and its effects on the cell cycle are shown in Fig. S6A-B. **(C-D)** The localizations of HA-SLX4^{WT} and RPA32 were analyzed 1 hr after microirradiation (C). The fractions of HA-SLX4^{WT} stripe-positive cells with or without RPA32 stripes were quantified (D). Error bars: S.E.M.; n=2. **(E)** U2OS cells expressing HA-SLX4^{WT} were treated with control or MDC1 siRNA. The localization of HA-SLX4^{WT} was analyzed as in Fig. 5B. Error bars: S.E.M.; n=2. Knockdown of MDC1 and its effects on the cell cycle are shown in Fig. S6B-C. **(F)** U2OS cells expressing HA-SLX4^{WT} were treated with control siRNA or both CtIP and MDC1 siRNAs, and analyzed as in Fig. 5B. Error bars: S.E.M.; n=2. Knockdown of CtIP and MDC1 is shown in Fig. S6E.

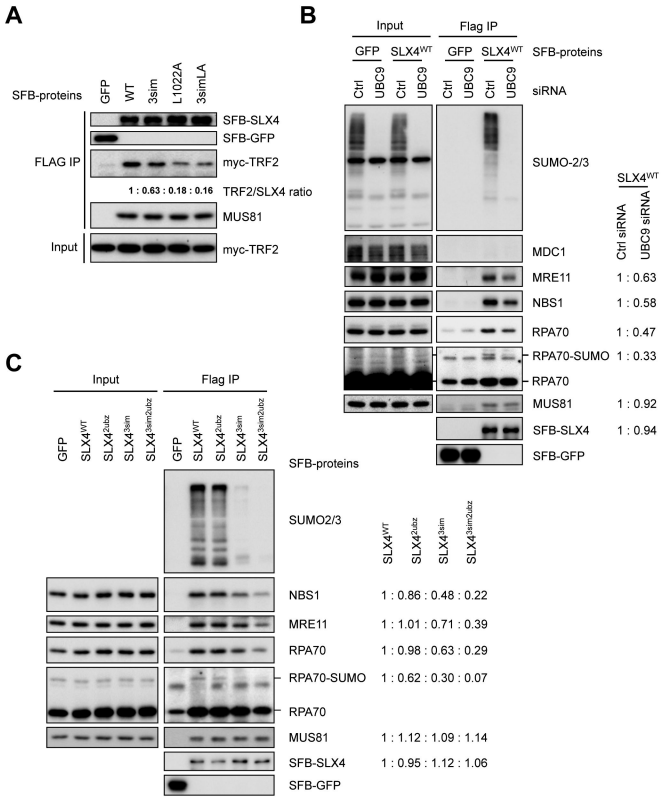

Fig. 7. SLX4 interacts with TRF2, RPA and MRN in a UBC9- and SIM-regulated manner

(A) SFB-tagged GFP, SLX4^{WT}, SLX4^{3sim}, SLX4^{L1022A}, and SLX4^{3simLA} were coexpressed with myc-TRF2 in U2OS cells. SFB-tagged proteins were captured using anti-Flag antibody, and the associated myc-TRF2 and MUS81 were detected by Western blot. The relative abundance of myc-TRF2 pulled down by SFB-SLX4 variants was shown. **(B)** HeLa cells were transfected with control or UBC9 siRNA and plasmids expressing SFB-GFP or SFB-SLX4^{WT} as indicated. SFB-tagged proteins were immunoprecipitated with anti-Flag antibody. The indicated proteins in inputs and immunoprecipitates were analyzed by Western blot. The relative abundance of proteins pulled down by SFB-SLX4^{WT} in control and UBC9 knockdown cells is shown. **(C)** HEK293T cells expressing SFB-tagged GFP, SLX4^{WT}, SLX4^{2ubz}, SLX4^{3sim}, or SLX4^{3sim2ubz} were treated with 1 μM CPT for 1 hr and subjected to immunoprecipitation using anti-Flag antibody. The indicated proteins in the immunoprecipitates were analyzed by Western blot. The relative abundance of proteins pulled down by SFB-SLX4 variants is shown.