Cell

Protein Group Modification and Synergy in the SUMO Pathway as Exemplified in DNA Repair

Ivan Psakhye[1] and Stefan Jentsch[1,*]
[1]Department of Molecular Cell Biology, Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany
*Correspondence: jentsch@biochem.mpg.de
http://dx.doi.org/10.1016/j.cell.2012.10.021

SUMMARY

Protein modification by SUMO affects a wide range of protein substrates. Surprisingly, although SUMO pathway mutants display strong phenotypes, the function of individual SUMO modifications is often enigmatic, and SUMOylation-defective mutants commonly lack notable phenotypes. Here, we use DNA double-strand break repair as an example and show that DNA damage triggers a SUMOylation wave, leading to simultaneous multisite modifications of several repair proteins of the same pathway. Catalyzed by a DNA-bound SUMO ligase and triggered by single-stranded DNA, SUMOylation stabilizes physical interactions between the proteins. Notably, only wholesale elimination of SUMOylation of several repair proteins significantly affects the homologous recombination pathway by considerably slowing down DNA repair. Thus, SUMO acts synergistically on several proteins, and individual modifications only add up to efficient repair. We propose that SUMOylation may thus often target a protein group rather than individual proteins, whereas localized modification enzymes and highly specific triggers ensure specificity.

INTRODUCTION

Posttranslational modifications (PTMs) greatly expand the range of functions of proteins. The majority of such modifications are attached functional groups (like phosphate, acetate, or lipids), which change the activities or localization of proteins or induce structural alterations. Moreover, because PTMs are usually reversible and thus have switch-like properties, they are instrumental for the regulation and directionality of cellular pathways and signaling cascades. PTMs typically target individual proteins, and thus, PTM enzymes usually act highly selectively.

A special PTM class is protein modification by covalent attachment of proteins of the ubiquitin family. For ubiquitylation, the principles of substrate selectivity and the mechanistic conse-

quences of the modification are well understood (Kerscher et al., 2006). High specificity toward individual substrates is achieved by a great diversity of ubiquitin ligases and numerous deconjugation enzymes. In the case of ubiquitin's proteolytic role, the ubiquitin modification (usually in the form of a lysine (K)-48 or K6 and K29 polyubiquitin chain) is recognized by dedicated ubiquitin receptors, which escort the polyubiquitylated protein to the proteasome (Finley, 2009; Richly et al., 2005). In cases when ubiquitin does not function as a proteolytic tag (e.g., monoubiquitin; K63-linked polyubiquitin chain), the modified proteins are recognized by different kinds of ubiquitin receptors (e.g., receptors engaged in protein sorting) or binding partners that possess ubiquitin-binding domains. Notably, numerous ubiquitin-binding domains exist, some of which exhibit specificity for certain types of polyubiquitin linkages or even the length of the polyubiquitin chain.

Much less is known regarding how modification by the ubiquitin-related protein SUMO affects the function of substrates and how specificity is provided. Surprisingly, even though SUMOylation targets a large number of cellular proteins as well, its enzymatic machinery, both for conjugation and deconjugation, is much more simply organized compared to the elaborate ubiquitin pathway (Geiss-Friedlander and Melchior, 2007). In fact, only a handful of enzymes have been identified that mediate SUMOylation and de-SUMOylation, and their specificity in vitro seems rather promiscuous. Likewise, the recognition of the SUMO modification is surprisingly simple, as only two SUMO-binding motifs, a short hydrophobic sequence known as SUMO-interacting motif (SIM) and a specific zinc finger (ZZ zinc finger), have been identified (Danielsen et al., 2012; Song et al., 2004). Some additional selectivity may come from substrate or SIM modification by other PTMs (Stehmeier and Muller, 2009; Ullmann et al., 2012), but overall, the question of specificity in the SUMO pathway remains largely unexplained.

SUMO is essential for viability for most eukaryotes, and consequently, mutants defective in components of the SUMO pathway are either lethal or have strong pleiotropic phenotypes (Geiss-Friedlander and Melchior, 2007). Judging from these phenotypes, SUMO plays numerous cellular roles, ranging from signal transduction to DNA transactions, e.g., DNA repair. Indeed, the majority of known SUMO substrates are nuclear proteins, but the significance of the SUMO modification is known for a few substrates only. SUMOylation of the DNA excision repair enzyme thymine-DNA glycosylase (TDG) is a rare

example of a conformational change induced by the SUMO modification (Steinacher and Schär, 2005). This enzymatically important structural alteration is achieved through an intramolecular interaction of conjugated SUMO with a SIM of TDG. Another well-understood example is SUMOylation of proliferating cell nuclear antigen (PCNA) (Hoege et al., 2002; Papouli et al., 2005; Pfander et al., 2005). In this case, SUMOylation recruits Srs2, a recombination-inhibiting enzyme, which removes the recombinase Rad51 from chromatin. For PCNA interaction, Srs2 possesses a PIP (PCNA-interacting protein) box, but crucial additional affinity for SUMOylated PCNA is provided by a SIM, located near the PIP box in Srs2's carboxyl (C)-terminal tail (Armstrong et al., 2012; Pfander et al., 2005). However, PCNA SUMOylation might be a special case as the same residue in PCNA (K164) is also alternatively targeted by mono- and K63-linked polyubiquitylation for other repair functions (Hoege et al., 2002). Another singular case is SUMOylation of RanGAP of higher eukaryotes as this protein is not only a substrate but is also part of multisubunit SUMO ligase (Werner et al., 2012).

For the vast majority of currently identified SUMO substrates, the biological significance and the mechanistic consequences of SUMOylation are not known. Surprisingly, in the few cases in which the SUMOylation acceptor lysine residues had been identified, the respective mutants (lysine to arginine replacements; KR) often barely exhibit deleterious phenotypes (Sacher et al., 2006; Silver et al., 2011). In fact, this paradoxical discrepancy between strong phenotypes of SUMO pathway mutants and the scarceness of phenotypes of mutants deficient in specific SUMO modifications seems characteristic for the SUMO pathway. Because of this feature, one could even argue that several in vivo SUMOylation events are "silent" modifications caused by a promiscuous specificity of a simple organized enzymatic pathway.

Here, rather than by analyzing phenotypes of SUMO pathway mutants, we address SUMOylation specificity and function at the substrate level. We focused on DNA double-strand break (DSB) repair by homologous recombination (HR) as this pathway is well characterized and shows strong ties to the SUMO system (Altmannova et al., 2010; Branzei et al., 2006; Cremona et al., 2012; Dou et al., 2011; Galanty et al., 2009; Maeda et al., 2004; Morris et al., 2009; Ohuchi et al., 2008; Sacher et al., 2006; Torres-Rosell et al., 2007). SUMO pathway mutants exhibit robust sensitivities toward reagents that generate DSBs in vivo, and several HR proteins are known to be SUMOylated. Nonetheless, in the rare cases in which the SUMO acceptor sites have been studied, the corresponding mutants exhibit only very mild phenotypes and mainly only when the demand for HR is very high (Ohuchi et al., 2008; Sacher et al., 2006). Prompted by this finding and because the greater part of HR proteins is SUMO modified, we speculated that perhaps the SUMOylated proteins act in concert. Indeed, we found that SUMOylation targets a protein group rather than a specific protein and found that individual modifications act synergistically. We finally discuss a model in which regulated and highly spatially restricted SUMOylation provides glue-like properties to nearby substrates in order to foster their physical interactions and to stabilize cellular protein complexes.

RESULTS

A Synchronous DNA Damage-Triggered SUMOylation Wave Targets a Whole Set of HR Proteins

To address the enigma of specificity in the SUMO pathway, we selected SUMOylation of HR proteins as a model for our study. DSB repair is initiated by the heterotrimeric MRX complex (Mre11, Rad50, and Xrs2), which recognizes and stabilizes the broken chromosome ends (Lisby et al., 2004). These ends are prepared for DSB repair via HR by nuclease-mediated single-stranded DNA (ssDNA) formation (resection), followed by its rapid coating with the heterotrimeric RPA complex (Rfa1, Rfa2, and Rfa3). Subsequently, RPA is removed from ssDNA and replaced by the recombinase Rad51 with the help of Rad52 and Rad59. The formed Rad51 nucleoprotein filament is then engaged in homology search, followed by strand annealing and final repair (Lisby et al., 2004).

Previous work has shown that the greater part of HR proteins is modified by SUMO (Cremona et al., 2012; Dou et al., 2010; Sacher et al., 2006). We first aimed to complement these findings by screening for SUMO conjugates that accumulated upon DNA damage. We treated cells with the DNA-alkylating agent methyl methanesulfonate (MMS), which primarily causes replication stalling and DSBs, and compared the level of SUMO-conjugates from MMS-treated and untreated cells by using a SILAC-based mass spectrometry protocol (Mann, 2006) (Figure 1A, left). This screen identified 844 different potential SUMO conjugates; the abundance of most of them did not change upon DNA damage. Strikingly, among those that were strongly enriched in the sample derived from MMS-treated cells were specifically HR proteins (Figure 1A, right). When we studied these proteins individually, we noticed that, in practically all cases, SUMOylation yielded multiple species, suggesting that the proteins are perhaps SUMO modified at multiple sites (Figures S1A–S1C available online). Neither the mass spectrometric approach nor a direct analysis identified SUMOylation of Rad51, indicating that the filament-forming recombinase is not a SUMO target. Notably, SUMOylation of the HR proteins occurred predominantly after exposure to MMS (Figures S1A–S1C). MMS-induced SUMOylation was also found for the checkpoint proteins Rad9 and Mrc1 and checkpoint kinases Mec1 and Tel1 (yeast homologs of mammalian ATR and ATM) (Figures S1D–S1G). However, their SUMOylation was already considerable in the absence of MMS, suggesting that perhaps endogenous DNA damage (e.g., caused by replication errors) is already sufficient to trigger their SUMOylation.

The observed activity of the SUMO system to target a whole set of HR factors seems surprising given that PTMs typically target individual proteins to alter their functions. We thus wondered whether this behavior might be special for HR factors or perhaps typical for many SUMOylation events. Evidence for the latter assumption comes from findings that numerous proteins of the ribosome (Finkbeiner et al., 2011a, 2011b; Panse et al., 2006), several septins (Johnson and Blobel, 1999; Johnson and Gupta, 2001; Takahashi et al., 2001), and many proteins involved in nucleotide excision repair (NER) (Silver et al., 2011) are SUMO modified. Indeed, when we repeated the SILAC approach mentioned above, but this time after exposure to UV light, which creates bulky DNA lesions, we found that proteins specifically involved
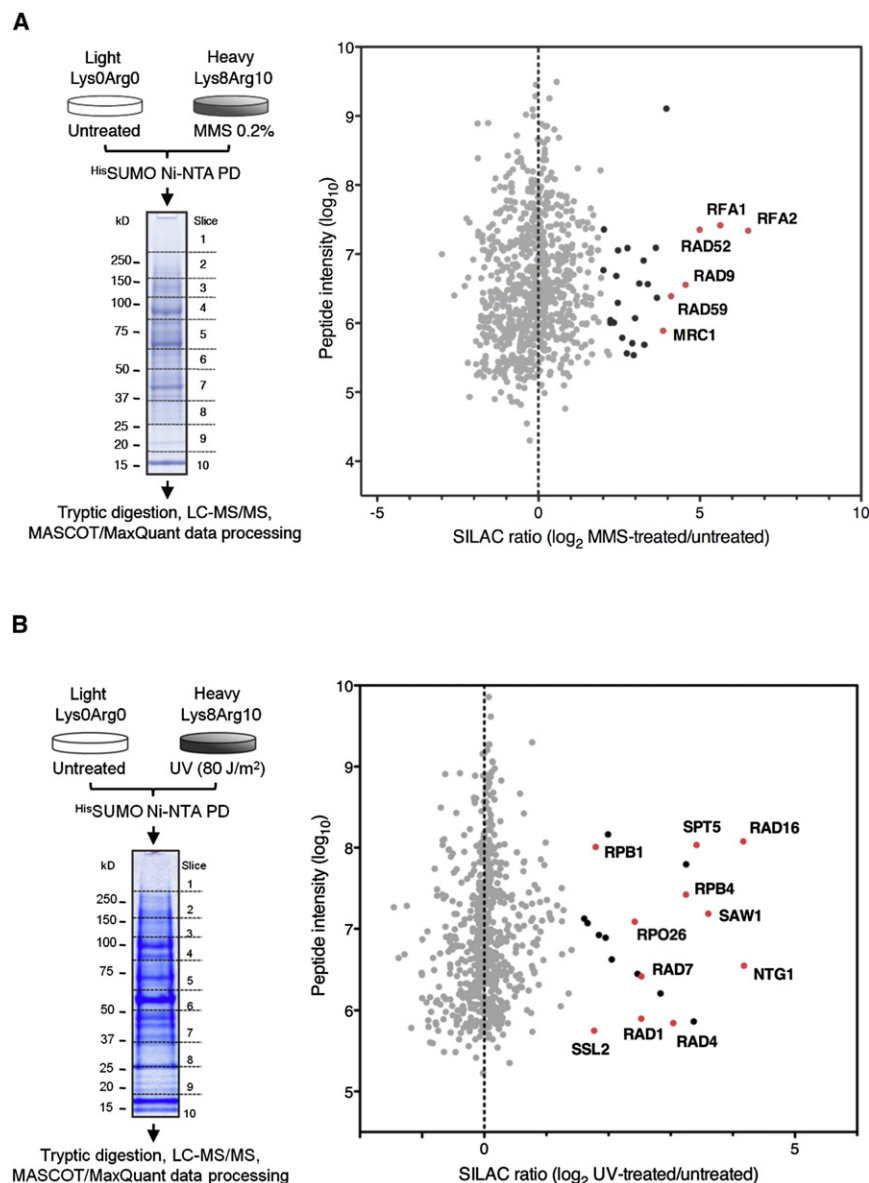
Figure 1. Proteins Acting in the Same DNA Repair Pathway Are Collectively SUMOylated upon a Specific Stimulus

(A) Outline of SILAC experiment performed to detect SUMOylated substrates enriched after MMS-induced DNA damage (left). SILAC ratios (MMS treated versus untreated) for 844 quantified proteins plotted against the sum of the relevant peptide intensities (right). Proteins are colored according to values of MaxQuant Significance(B) (gray, Significance(B) > 10^{-2}; black, SUMOylated proteins enriched after DNA damage with Significance(B) $\leq$ 10^{-2}; red, proteins with Significance(B) < 10^{-4} that are involved in HR and checkpoint activation).

(B) Following UV light treatment, specifically factors implicated in nucleotide excision repair (NER) become increasingly SUMOylated. Same as in (A), but cells grown in heavy media were UV irradiated (80 J/m^2) instead of MMS treatment. SILAC ratios (UV treated versus untreated) for 717 quantified proteins plotted against the sum of the relevant peptide intensities. Proteins are colored according to values of MaxQuant Significance(B) (gray, Significance(B) > 10^{-7}; black, SUMOylated proteins enriched after UV irradiation with Significance(B) $\leq$ 10^{-7}; red, proteins with Significance(B) $\leq$ 10^{-8} that are involved in NER [both transcription-coupled and global genome repair] and base-excision repair).

See also Figure S1.

HR proteins collectively. Moreover, steady-state SUMOylation of RPA even increased in cells deficient in the downstream-acting factors Rad52 and Rad51, most likely because RPA remains associated with damaged DNA as the pathway is blocked.

Extended ssDNA Acts as SUMOylation Wave Trigger

We next asked how the SUMOylation wave is induced and what the primary trigger is. In cycling cells, SUMOylation of the HR proteins was strongly induced not only by MMS but also by the DSB-inducing agent Zeocin (Figure S2D). However, exposure to UV light and 4-nitroquinoline 1-oxide (4NQO), which both cause direct DNA damage, and treatment with the replication inhibitor hydroxyurea (HU) induced SUMOylation only moderately (Figure S2D). This suggests that, in particular, reagents that primarily cause DSBs are potent triggers.

We previously noticed that efficient DNA damage-induced SUMOylation of Rad52 requires the MRX complex (Sacher et al., 2006). Indeed, individual deletions of all three genes encoding MRX subunits strongly reduced SUMOylation of other HR proteins as well (Figure 2A). However, because SUMOylation still weakly occurred, MRX potentiates the induction but does not seem to be the primary trigger. MRX acts early in the pathway and is connected to the formation of ssDNA at the DSB by

in NER become increasingly SUMO modified (Figure 1B). This suggests that proteins acting within the same pathway are indeed often collectively SUMO modified upon a specific stimulus.

Because the HR proteins act together functionally and physically, we speculated that the modifications may serve a common purpose and are perhaps synchronously triggered. In fact, we found that the MMS dose dependency of SUMOylation induction was very similar for all HR proteins analyzed and coincided with the activation of checkpoint signaling (Figure S2A). Likewise, the kinetics of SUMOylation after MMS treatment was nearly identical for the proteins, suggesting a synchronous burst of SUMOylation, which happens seemingly in parallel to the checkpoint activation wave (Figure S2B). Notably, in mutant strains deficient in single HR proteins (Δrad51 or Δrad52), SUMOylation of other HR proteins still occurred (Figure S2C). This suggests that the DNA damage-induced synchronous SUMOylation wave targets
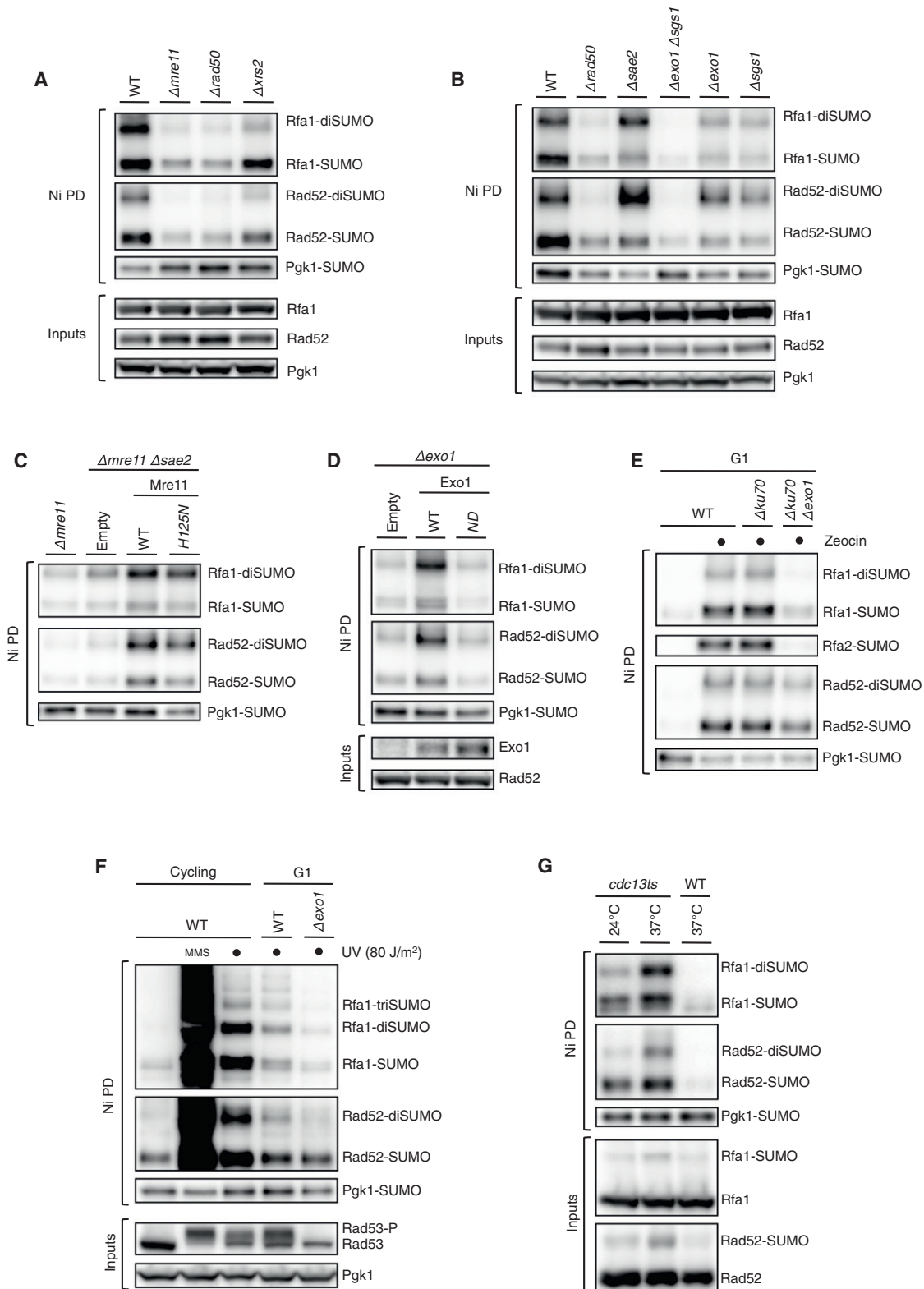
Figure 2. DNA Resection and Exposure of ssDNA Triggers SUMOylation of DSB Repair Proteins
(A) Disruption of MRX complex by deleting individual subunits results in pronounced decrease of SUMOylation of HR proteins following DNA damage. Denaturing Ni-NTA pull-down (Ni PD) was performed to isolate [His]SUMO conjugates from MMS (0.2%) -treated cells lacking Rad50, Mre11, or Xrs2.

resection (Mimitou and Symington, 2011). This reaction is usually initiated by the formation of short 3′ ssDNA overhangs created by the Mre11 subunit of MRX in collaboration with the nuclease Sae2. After this resection-stimulating (but not essential) first step, long tracts of ssDNA are formed by the nuclease Exo1 and also by a parallel pathway involving the Sgs1 helicase acting along with the nuclease Dna2 (Mimitou and Symington, 2008; Zhu et al., 2008). Interestingly, in the absence of Sae2 (and also in cells additionally defective in the nuclease activity of the MRX subunit Mre11 [*mre11-H125N Δsae2*]), HR protein SUMOylation was barely affected (Figures 2B and 2C). In contrast, in cells deficient in the Exo1 protein (Figure 2B; *Δexo1*) or its nuclease activity (Figure 2D; *exo1-ND*) and also in cells lacking Sgs1 (Figure 2B), HR protein SUMOylation was strongly reduced. Indeed, only in the absence of both long-range resection pathways (*Δexo1* and *Δsgs1*), SUMOylation of the HR proteins was virtually absent (Figure 2B). Exo1 deficiency or absence of its activity also strongly diminished the SUMOylation wave in G1 cells (Figures 2E, 2F, and S2F), emphasizing the importance of long-range resection for the trigger.

To exclude the possibility that perhaps the process of resection or the formation of DSBs, rather than extended ssDNA, is the primary SUMOylation trigger, we induced ssDNA accumulation by other means that do not rely on DSB formation. First, we exposed G1-arrested cells to UV light (Figure 2F), which creates extended ssDNA gaps in DNA via Exo1 activity (Giannattasio et al., 2010). Second, we used mutants deficient in the nonessential DNA polymerase subunit Pol32 (Figure S2E), which, when grown at low temperatures (14°C), undergo faulty replication, leading to the accumulation of ssDNA gaps behind replication forks (Karras and Jentsch, 2010). Finally, ssDNA at telomeres can be exposed in mutants defective in the telomeric ssDNA-binding protein Cdc13 (Garvik et al., 1995) (*cdc13ts*; Figure 2G). Because we observed HR protein SUMOylation (parallel to checkpoint activation) under all these different conditions (Figures 2F, 2G, and S2E), we conclude that, indeed, extended ssDNA—not a DSB formation per se—is the crucial signal for the SUMOylation wave.

Crosstalk between DNA Damage Response Pathways

Exo1 is phosphorylated at multiple sites by checkpoint kinases, and this modification appears to inhibit its activity, thereby limiting ssDNA accumulation and DNA damage checkpoint activation (Morin et al., 2008). When we used a phosphorylation-defective *exo1* mutant (*exo1-SA*), which is expected to be irresponsive to checkpoint kinases, we observed that HR protein SUMOylation upon MMS treatment was substantially increased compared to wild-type (WT) cells (Figure S2F). This suggests that inactivation of the checkpoint response may lead to increased SUMOylation of HR proteins (as previously observed for Rad52 [Ohuchi et al., 2009]) not only due to increased DNA damage but perhaps also because Exo1-dependent DNA resection is not repressed. Indeed, inactivation of the checkpoint pathway by removing the genome integrity checkpoint kinase Mec1 (*Δmec1* and *Δsml1*; the used *Δsml1* mutant suppresses the otherwise lethal phenotype of *Δmec1)* or its adaptor protein Ddc2 (*Δddc2* and *Δsml1*) pronouncedly induced HR protein SUMOylation even in the absence of exogenous DNA damage (MMS; Figure S2G). Similarly, absence of the checkpoint transducer Rad9 (*Δrad9*) also strongly induced the SUMOylation wave (Figure S2H). Lastly, mutants deficient in subunits of the 9-1-1 checkpoint clamp (*Δrad17* or *Δmec3*) triggered the response (Figure S2I). Notably, absence of checkpoint signaling caused significant HR protein SUMOylation even in the absence of long-range resection (*Δexo1* and *Δsgs1*) (Figure S2J), probably because ssDNA accumulates in checkpoint mutants at collapsed replication forks. Furthermore, reagents like HU and 4NQO that barely induce HR protein SUMOylation in WT cells strongly triggered the SUMOylation wave if checkpoint signaling is defective (Figure S2K). We thus conclude that the SUMOylation wave acts parallel to the checkpoint-signaling cascade; however, checkpoint kinases keep ssDNA formation and hence the SUMOylation trigger in check.

The SUMOylation Wave Is Catalyzed by the DNA-Bound SUMO Ligase Siz2

We next asked how ssDNA might trigger HR protein SUMOylation mechanistically. We first speculated that perhaps the responsible SUMO ligase might bind ssDNA specifically. By analyzing single mutants of the genes of the three known SUMO ligases of mitotic cells (*Δsiz1*, *Δsiz2*, and *mms21-11* catalytically inactive allele), we observed almost complete absence of DNA damage-induced HR protein SUMOylation in cells deficient in Siz2 (Figures 3A and S3A). As HR protein SUMOylation was normal in the other two mutants, we conclude that Siz2,

(B) Long-range resection by the action of Exo1 and Sgs1/Dna2, but not short-range processing of DNA ends by Sae2 endonuclease, triggers SUMOylation of DNA repair proteins. Similar to (A), but with MMS-treated cells lacking Sae2, Exo1, and Sgs1 or both Exo1 and Sgs1. Both branches of long-range resection contribute to induction of SUMOylation wave in DNA repair pathway.

(C) Short-range processing of DNA DSB ends by Sae2 and Mre11 is not required for the induction of SUMOylation wave following DNA damage. Similar to (A), but with MMS-treated *Δmre11 Δsae2* cells expressing either WT or nuclease-dead (*mre11-H125N*) Mre11.
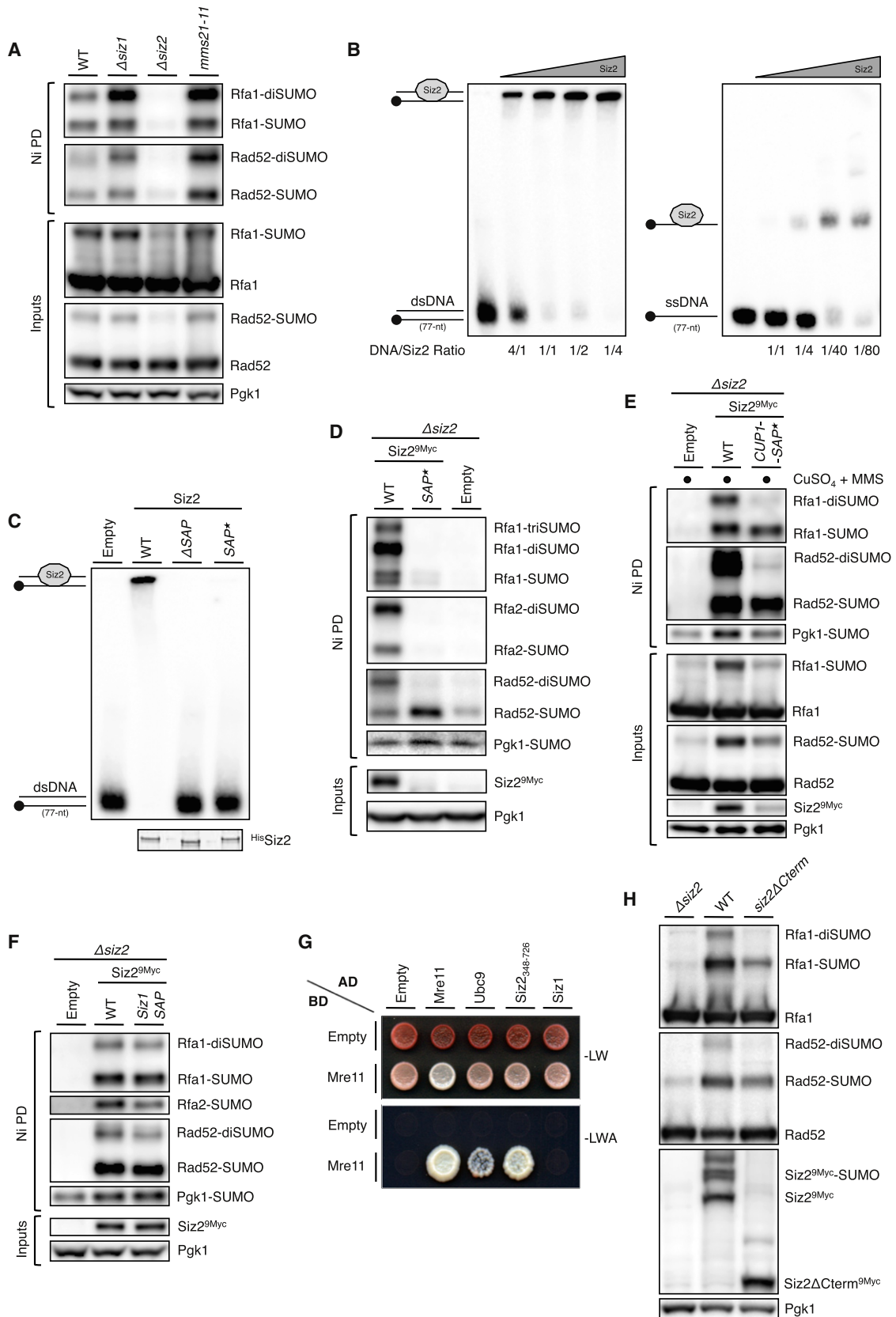
(D) Nuclease activity of Exo1 is required for SUMOylation induction in response to DNA damage. Similar to (A), but with MMS-treated cells expressing either WT or nuclease-dead (*exo1-D173A*, *ND*) Exo1.

(E) SUMOylation of repair proteins can be efficiently triggered by Exo1-resected DNA DSB ends in cells arrested in G1. Similar to (A), but with G1-arrested and Zeocin-treated WT cells or cells lacking nonhomologous end-joining factor Yku70 (*Δku70*) or Yku70 and Exo1 (*Δku70* and *Δexo1*).

(F) ssDNA gaps generated after UV light treatment by Exo1 in G1-arrested cells are sufficient to trigger SUMOylation of repair proteins. Similar to (A), but with cycling or G1-arrested WT or Exo1-deficient (*Δexo1*) cells, which were treated either with MMS or UV light (80 J/m^2). Hyperphosphorylated Rad53 indicates checkpoint activation triggered by resection following DNA damage.

(G) Uncapping of telomeres and exposure of ssDNA in temperature-sensitive *cdc13ts* mutants triggers HR protein SUMOylation. ^{His}SUMO Ni PD from WT and *cdc13ts* cells that were grown to an OD_{600} of 0.7 at 24°C and then shifted to 37°C.

See also Figure S2.

A

WT Δsiz1 Δsiz2 mms21-11

Ni PD

Rfa1-diSUMO
Rfa1-SUMO
Rad52-diSUMO
Rad52-SUMO

Inputs

Rfa1-SUMO
Rfa1
Rad52-SUMO
Rad52
Pgk1

B

Siz2

Siz2

dsDNA
(77-nt)

ssDNA
(77-nt)

DNA/Siz2 Ratio 4/1 1/1 1/2 1/4   1/1 1/4 1/40 1/80

C

Empty WT ΔSAP SAP*

Siz2

Siz2

dsDNA
(77-nt)

^{His}Siz2

D

Δsiz2

Siz2^{9Myc}

WT SAP* Empty

Ni PD

Rfa1-triSUMO
Rfa1-diSUMO
Rfa1-SUMO
Rfa2-diSUMO
Rfa2-SUMO
Rad52-diSUMO
Rad52-SUMO
Pgk1-SUMO

Inputs

Siz2^{9Myc}
Pgk1

E

Δsiz2

Siz2^{9Myc}

Empty WT CUP1--SAP*

• • • CuSO$_4$ + MMS

Ni PD

Rfa1-diSUMO
Rfa1-SUMO
Rad52-diSUMO
Rad52-SUMO
Pgk1-SUMO

Inputs

Rfa1-SUMO
Rfa1
Rad52-SUMO
Rad52
Siz2^{9Myc}
Pgk1

F

Δsiz2

Siz2^{9Myc}

Empty WT Siz1 SAP

Ni PD

Rfa1-diSUMO
Rfa1-SUMO
Rfa2-SUMO
Rad52-diSUMO
Rad52-SUMO
Pgk1-SUMO

Inputs

Siz2^{9Myc}
Pgk1

G

AD
BD

Empty Mre11 Ubc9 Siz2$_{348-726}$ Siz1

Empty
Mre11
-LW

Empty
Mre11
-LWA

H

Δsiz2 WT siz2ΔCterm

Rfa1-diSUMO
Rfa1-SUMO
Rfa1
Rad52-diSUMO
Rad52-SUMO
Rad52
Siz2^{9Myc}-SUMO
Siz2^{9Myc}
Siz2ΔCterm^{9Myc}
Pgk1

a SUMO-ligase of the conserved PIAS family, is the key enzyme responsible for HR protein SUMOylation.

Siz2 indeed binds DNA in vitro, however, only weakly to ssDNA (Figure 3B). Furthermore, Siz2 has no discernable selectivity toward structures resembling resected DNA or replication forks (Figure S3B). Siz2 possesses an SP-RING domain required for its ligase activity, a PINIT domain linked to substrate binding, and an N-terminally located SAP domain, which mediates DNA binding in other proteins (Palvimo, 2007; Yunus and Lima, 2009) (Figure S3C, scheme #1). This SAP domain is indeed crucial as its deletion (Figure S3C, scheme #2) or replacement of highly conserved residues of this domain by alanine (Siz2-G64A,K66A,L69A; termed Siz2-SAP*; Figure S3C, scheme #3) abolished DNA binding of the purified recombinant proteins in vitro (Figure 3C). When Siz2-SAP* was expressed under control of its endogenous promoter, the HR protein SUMOylation wave was severely reduced, but the enzyme was present only at very low intracellular steady-state levels (Figure 3D). Nonetheless, the mutant protein was not enzymatically inactive as expression of this variant via a strong promoter (*pCUP1-SIZ2-SAP**) partially restored MMS-induced HR protein SUMOylation (Figures 3E and S3D). Notably, replacement of the SAP domain by the sequence-specific DNA-binding domain of the transcription factor Gal4 (Figure S3C, scheme #4) restored the Siz2 protein level, yet without supporting HR protein SUMOylation (Figure S3E). In contrast, replacing the Siz2 SAP domain by the SAP domain of the related enzyme Siz1 (Figure S3C, scheme #5) not only led to a normal enzyme level in vivo but also supported HR protein SUMOylation (Figure 3F). We thus conclude that DNA damage-induced HR protein SUMOylation requires SAP-domain-mediated localization of Siz2 on DNA; however, Siz2 binds DNA broadly and not specifically ssDNA.

Because the PINIT domain of the Siz1 SUMO ligase appears to contribute to substrate recognition (Yunus and Lima, 2009), we also constructed a chimeric Siz2 variant in which we replaced its PINIT domain by the analogous domain of the related enzyme Siz1 (Figure S3C, scheme #6). However, this Siz2 variant supported HR protein SUMOylation upon DNA damage (Figure S3F), indicating that the two enzymes must differ in other aspects. Indeed, a distinguishing feature of Siz2 is that it interacts specifically with the Mre11 subunit of the MRX complex in two-hybrid

assays (Figure 3G). Mre11 binding is mediated through the C-terminal domain of Siz2, which harbors two SUMO-binding motifs (Figures 3G and S3C). Deletion of the C-terminal SIM of Siz2 (SIM$_2$) or amino acid replacements within this motif (Figure S3C, scheme #7; V720A and V721A) not only abolished interaction with Mre11 (Figure S3G) but strongly affected HR protein SUMOylation in vivo (Figure S3H). Because Mre11 also binds Ubc9 in two-hybrid assays (Figure 3G), it seems plausible that binding of Siz2 to Mre11 is achieved through Ubc9-catalyzed SUMOylation of Mre11. Notably, binding of Siz2 to Mre11 is crucial for the full HR protein SUMOylation wave response, but not for HR protein SUMOylation per se, as a Siz2 variant lacking its C-terminal tail (Figure S3C, scheme #8; aa 416–726, siz2Δ Cterm) is still able to SUMOylate HR proteins, albeit weakly (Figure 3H). Furthermore, in the related enzyme Siz1, replacing Siz1's C-terminal tail with the C-terminal tail of Siz2 (Figure S3C, scheme #9) does not create an enzyme capable of DNA damage-induced SUMOylation of HR proteins (Figure S3I). This indicates that the two enzymes not only differ in their tails but differ in other aspects as well. Together, these data suggest that the SAP domain brings Siz2 onto chromatin generally, where it can act on DNA-bound HR proteins, whereas the SIM-containing tail, uniquely present in Siz2, fixes and restricts the enzyme on chromatin to SUMOylated Mre11 in order to promote the full SUMOylation wave.

Artificial DNA Targeting of HR Proteins Is Sufficient to Trigger Their SUMOylation

After we ruled out the possibility that the SUMOylation trigger (ssDNA) operates through recruitment of the SUMO ligase specifically to ssDNA, we considered an alternative model. Because HR proteins are assembled on resected DNA, we hypothesized that just recruitment of the HR proteins to DNA is sufficient for their SUMOylation and that only DNA-bound HR proteins are substrates for DNA-localized Siz2 SUMO ligase. To test this idea, we artificially tethered HR proteins to DNA by fusing them to the DNA-binding domain (aa 1–147, BD) of the transcription factor Gal4. Indeed, a BD-Rad52 fusion became efficiently SUMO modified, however, without causing SUMOylation of the earlier-acting HR protein RPA (Rfa1) (Figures 4A, S4A, and S4B). Importantly, this SUMOylation reaction was

Figure 3. DNA Damage-Triggered SUMOylation Wave Is Catalyzed by the DNA-Bound SUMO Ligase Siz2

(A) SUMO ligase Siz2 stimulates HR protein SUMOylation after DNA damage. Denaturing Ni-NTA pull-down (Ni PD) was performed to isolate [His]SUMO conjugates from MMS-treated WT cells, mutants lacking SUMO ligases Siz1 (*Δsiz1*) or Siz2 (*Δsiz2*), or *mms21-11* mutant cells.

(B) Recombinant [His]Siz2 protein binds dsDNA and weaker ssDNA. Increasing amounts of [His]Siz2 were added to 5′ end biotin-labeled 77-nucleotide-long dsDNA and ssDNA (20 fmol), and binding was detected by using chemiluminescent-based electrophoretic mobility shift assay (EMSA).

(C) Recombinant [His]Siz2 variants either lacking the DNA-binding SAP domain (*ΔSAP*; #2 of Figure S3C) or having three highly conserved residues replaced by alanine residues (*SAP**; #3 of Figure S3C) are unable to bind DNA. EMSA with 5′ end biotin-labeled 77-nucleotide-long dsDNA.

(D) DNA-binding-deficient Siz2 is destabilized in vivo and does not stimulate SUMOylation of HR proteins after DNA damage. [His]SUMO Ni PD from *Δsiz2* cells expressing either Myc-tagged WT Siz2 or DNA-binding-deficient Siz2 (*SAP**; #3 of Figure S3C) following MMS treatment.

(E) Expression of DNA-binding-deficient Siz2 by the strong *CUP1* promoter (*CUP1-SAP**) partially restores its protein level and HR protein SUMOylation after MMS treatment.

(F) Chimeric Siz2 carrying the N terminus of Siz1 harboring Siz1's SAP domain (*Siz1 SAP*; #5 of Figure S3C) is expressed to a similar level as WT Siz2 and is fully functional in stimulating HR protein SUMOylation.

(G) SUMO-conjugating enzyme Ubc9 and SUMO ligase Siz2, but not Siz1, interact with Mre11 in the two-hybrid system. C-terminal tail of Siz2 (aa 348–726) is sufficient for Mre11 interaction.

(H) A Siz2 variant Siz2ΔCterm with a deleted C-terminal tail (aa 416–726; #8 of Figure S3C) supports SUMOylation of HR proteins with reduced activity.
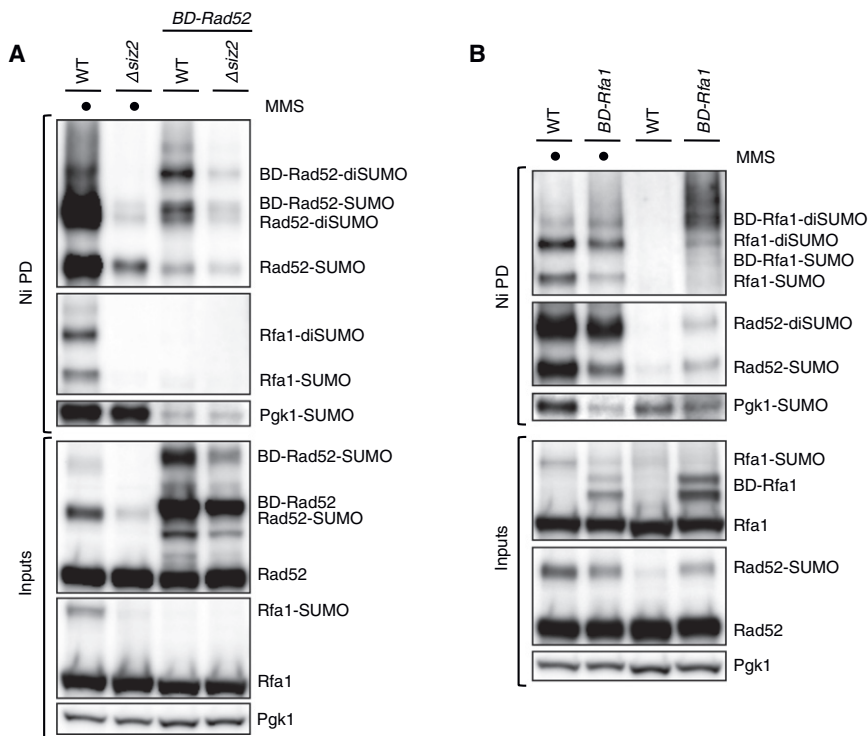
See also Figure S3.

Figure 4. Artificial DNA Targeting of HR Proteins Is Sufficient to Trigger Their SUMOylation

(A) N-terminal Gal4 DNA-binding domain (aa 1–147, BD) fusion protein BD-Rad52 ectopically expressed from high-copy pGBD vector becomes strongly SUMOylated in a Siz2-dependent manner in the absence of DNA damage.

(B) Expression and subsequent SUMOylation of BD-Rfa1 stimulates SUMO modification of both endogenous RPA (Rfa1) and Rad52 in the absence of DNA damage.

See also Figure S4.

independent of DNA damage and bypassed the requirement of ssDNA for the trigger. In contrast, when we used a BD-Rfa1 fusion, not only did Rfa1 become SUMO modified in the absence of DNA damage, but Rad52 did as well (Figure 4B). Notably, SUMOylation of BD-Rad52 occurred on the same lysine residues as for WT Rad52 and required Siz2 harboring its SAP domain (Figures S4A and S4B). Together, these findings therefore suggest that, indeed, DNA binding of the HR proteins is crucial for triggering their Siz2-dependent SUMOylation. We hence conclude that ssDNA operates as SUMOylation wave trigger by bringing the substrates in vicinity to the DNA-bound SUMO ligase.

Multisite SUMOylation of HR Proteins Promotes Physical Interactions

In agreement with the above findings, we found that the SUMOy-lated HR proteins are located on chromatin (Figure 5A). Because the HR proteins not only function in the same pathway but also physically interact, we next asked whether the SUMO modifications influence HR protein complex formation. Indeed, immuno-precipitation of Rad59 after DNA damage coisolated proportion-ally much more of the SUMOylated forms of RPA (Rfa1) and Rad52 compared to the respective unmodified species (Figure 5B). Similarly, RPA (Rfa1^9Myc) immunoprecipitation brought down with stronger preference the SUMOylated forms of Rad52 (Figure S5A). Notably, this preference was further increased when Rad51, the protein that is exchanged for RPA by Rad52, is lacking (Figure S5A). In the absence of check-point-mediated repression (Δmec1 and Δsml1), preferential binding of RPA (Rfa2^9Myc) to SUMOylated Rad52 is already significant without exogenous DNA damage but is strongly

further increased by DNA damage-induced SUMOylation (Figure S5B). Moreover, also in the absence of the SUMO ligase Siz2, binding of RPA to Rad52 was reduced (Figure S5B).

To address the in vivo relevance of these modifications, we first aimed to identify the target residues for SUMOyla-tion. The strategy we used is based on a mass spectrometric analysis of peptide pools obtained after trypsin or thermoly-sin digestion of purified SUMO conju-gates. Because these proteinases cleave SUMO (Smt3) close to its C-terminal tail, we expected to get "branched" peptides corresponding to HR-protein-derived fragments that had an additional mass of the SUMO-derived fragment at acceptor lysine (EQIGG in case of trypsin and IGG in case of thermolysin). This approach identified two SUMOylation sites in Rfa1 and identified one each in Rfa2, Rfa3, Rad59, and Rad52 (Figure 5C). To confirm these sites, we mutated the corresponding genes in the genome in order to replace the acceptor lysine residues by arginine residues. Indeed, SUMOylation in vivo was either abol-ished (Rfa2 and Rfa3) or strongly reduced (Figures 5D–5G), indi-cating that our approach identified the major attachment sites. Rfa1 and Rad59 possess additional minor SUMOylation sites because the elimination of additional SUMOylation consensus sites reduced modification of these proteins in vivo even further. Notably, expression of a lysine-less SUMO variant (SUMO-KRall) as the only source of SUMO resulted in the disappearance of the di- and tri-SUMOylated species of Rfa2 and Rfa3 (Figures 5D and 5E). Because this SUMO variant cannot form lysine-linked poly-SUMO chains, Rfa2 and Rfa3 seem to be partially modified by poly-SUMOylation. Thus, DNA damage-induced SUMOyla-tion of HR proteins is excessive in that a whole set of repair proteins undergoes multisite modification by SUMO and also by poly-SUMO chains. Importantly, abolishing SUMOylation at individual target sites did not negatively influence SUMOylation at other sites or other proteins, indicating that SUMOylation occurs independently and in parallel.

Because conjugated SUMO typically interacts with SIMs, we next asked whether the HR proteins possess these motifs. Indeed, bioinformatic searches predicted multiple SIMs in basi-cally all repair factors (Table S1). When we probed peptides of

a length of 12 aa harboring these motifs flanked by their natural sequences for binding to poly-SUMO chains in vitro, we found that, indeed, a significant number had SUMO-binding properties (Figure S5C). This indicates that the HR proteins are not only excessively SUMOylated at multiple sites but are also decorated with amino acid motifs that have SUMO-binding properties.

HR Protein SUMOylation Accelerates DNA Repair

Because mutants defective in SUMOylation of individual HR proteins did not exhibit sensitivities toward DNA-damaging agents (data not shown), we asked whether wholesale elimination of SUMOylation sites of multiple HR factors would affect DSB repair. We constructed a mutant strain (termed *KR* mutant) in which we changed at their endogenous genomic loci the codons of 11 SUMOylation target lysine residues to arginine codons of the core repair proteins RPA, Rad52, and Rad59 (*rfa1-K133R,K170R,K427R rfa2-K199R rfa3-K46R rad52-K10R,K11R,K220R rad59-K207R,K228R,K238R*). The obtained *KR* strain grew at WT rates (Figure 6A, left) and showed no sensitivity to DNA-damaging agents in plating assays (data not shown). However, when growth curves were measured, the *KR* strain exhibited a substantial delay of roughly 4 hr upon chronic exposure to MMS compared to WT cells (Figure 6A, right). Moreover, spontaneous and MMS-induced interchromosomal recombination rates were reduced in this mutant (Figure 6B). The finding that the *KR* mutant and the strain deficient in Siz2 (*Δsiz2*) have largely identical phenotypes and are epistatic (Figures 6A and 6B) revealed that, in the tested DNA repair pathway, Siz2 acts primarily via SUMOylation of HR proteins.

A crucial step in HR-mediated DSB repair is the loading of Rad51 onto resected DSB ends, which is governed by the formation of the RPA-Rad52-Rad59 complex (Davis and Symington, 2003). Because Siz2-mediated SUMOylation fosters physical interactions within this complex, we tested whether Siz2 affects the loading of Rad51 to a single DSB created by HO endonuclease at the yeast mating type (*MAT*) locus (Figure 6C). Although much less potent than MMS-induced DNA damage, a single DSB was sufficient to trigger the SUMOylation wave both in cells lacking (donor deficient; *Δhml Δhmr*) or harboring homologous donor sequences (*HMLα*) for repair (Figures S6A and S6B). When we monitored by chromatin immunoprecipitation (ChIP) the recruitment of repair factors to sites 0.2 kb and 5.7 kb distal to the persistent DSB (donor-deficient strain), we found that RPA (Rfa1[9Myc]) was recruited rapidly and with similar kinetics in WT and *Δsiz2* cells (Figure 6C, right top). In contrast, loading of Rad51 was delayed in the absence of HR protein SUMOylation (Figure 6C, right bottom). Moreover, when we directly assayed for repair of the HO-induced DSB at the *MAT* locus (mating type switching), we found that repair was substantially delayed in the *KR* mutant compared to WT (Figure 6D). We thus conclude that the SUMOylation wave accelerates DSB repair by fostering HR protein complex formation, which in turn stimulates the loading of the recombinase Rad51 to the sites of repair.

DISCUSSION

Ever since its discovery 15 years ago, the SUMO modification pathway remained enigmatic because of its unorthodox features. Particularly puzzling—especially by comparison to the ubiquitin system—is the small set of enzymes responsible for the modification of a large number of SUMO substrates. This discrepancy, the lack of distinctive modification signals in substrates equivalent to ligase-binding "degrons" in the ubiquitin pathway, plus the apparent promiscuity of the SUMOylation enzymes in vitro, have raised the question of how substrate specificity is provided. Equally peculiar is the phenomenon that SUMOylation affects most frequently only a very small fraction of a given substrate and that cells expressing SUMOylation-deficient mutant substrates often barely exhibit deleterious phenotypes.

Protein Group Modification

By using SUMOylation of HR proteins as a case study, we discovered that the SUMO pathway operates surprisingly different from typical protein modification systems. Whereas most PTMs are highly specific for selected individual proteins in order to endow them with new functions or properties, SUMOylation appears to frequently target a protein group rather than an individual protein. In the case of the HR pathway, multiple repair proteins are synchronously modified by a SUMOylation wave. Strikingly, most HR proteins affected by SUMOylation are modified at multiple sites, and these sites are not conserved in homologs of other species. A similar scenario is found for several other protein groups like proteins involved in nucleotide excision repair (Figure 1B; Silver et al., 2011), yeast septins (Johnson and Blobel, 1999; Johnson and Gupta, 2001; Takahashi et al., 2001), ribosomal proteins (Finkbeiner et al., 2011a, 2011b; Panse et al., 2006), and proteins of snoRNPs (Westman and Lamond, 2011). In all these cases, the SUMOylation machinery seems to target several proteins of the respective complexes synchronously and often at multiple sites. From these findings, we thus assume that "protein group modification" is a typical feature of the SUMO pathway. Notable known exceptions from this rule are SUMOylation of RanGAP (Werner et al., 2012)—as the protein is itself a SUMO ligase—and PCNA. Indeed, PCNA SUMOylation (catalyzed by Siz1) is highly special as SUMOylation targets the same conserved acceptor site in PCNA (K164) as ubiquitylation (to conduct different repair pathways) (Hoege et al., 2002). Notably, protein group modification in the SUMO pathway also differs substantially from other PTM waves like phosphorylation reactions, as most of the SUMO modifications are functionally additive or redundant and do not proceed by a reaction cascade.

SUMOylation Specificity

Protein group modification requires different rules for substrate specificity. Whereas selective modification of an individual substrate involves a highly specific interaction between a modifying enzyme and its substrate, protein group modification may only require close proximity of a promiscuous enzyme to its multiple substrates, plus suitable modification sites, probably within flexible protein domains. Indeed, SUMOylation sites are frequently not conserved and hence are outside of conserved functional domains. Specificity toward a protein group is provided by two principles: a highly specific trigger and topological specificity. As detailed above, the specific SUMOylation
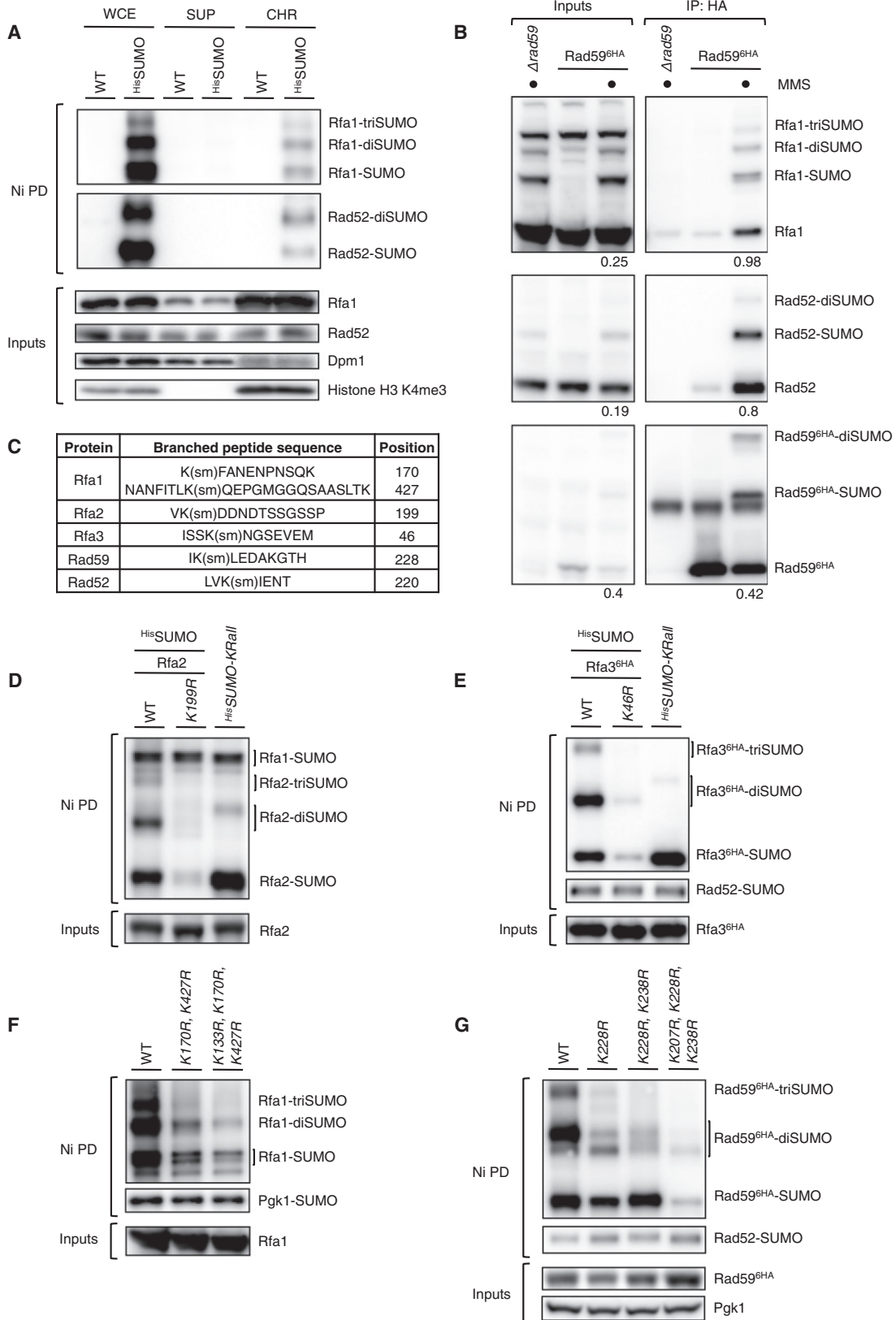
trigger for HR proteins is the formation of ssDNA, which serves as a binding platform for the substrates. As we have shown for Rfa1 and Rad52, artificial DNA targeting is sufficient to trigger their SUMOylation (Figure 4) as Siz2 is already on chromatin due to its SAP domain. Thus, topological specificity is provided by locally concentrated substrates (on ssDNA) and a highly localized SUMO ligase. Whereas protein group modification of septins involves localized Siz1 (Johnson and Gupta, 2001; Reindle et al., 2006), HR protein modification requires Siz2 of the same protein family. Overall, Siz1 and Siz2 have similar domain organizations and DNA-binding properties; however, they differ in Siz2's ability to selectively bind Mre11 of the MRX complex. Because specificity is provided by these two basic principles, there is no need for a large set of different ligases in the SUMO pathway. Importantly, protein group modification also solves the puzzle of why usually only a very small fraction of a given substrate is SUMO modified. In the case of HR proteins, this small fraction apparently represents the DNA-bound population engaged in repair and that is at reach of the Mre11-associated and DNA-bound SUMO ligase.

SUMO as a Protein Glue

Our systematic analysis of SUMOylation sites of HR proteins revealed that individual SUMO modifications act synergistically, which explains why single acceptor site mutants usually exhibit barely any deleterious phenotype. Because SUMOylation of HR proteins fosters protein-protein interactions, the most logical explanation for the observed functional synergy is that individual SUMO-SIM interactions add up affinities for complex formation. Three important features appear to be crucially relevant for this activity: the proteins in question possess low affinities toward their neighbors on their own, even in the absence of SUMOylation; they are modified at multiple sites; and they are decorated with multiple SIMs. Because SIMs are very short sequence motifs, our finding that basically all HR proteins possess multiple in vitro functional SIMs may not be surprising. However, it suggests that they represent widely scattered latent "adhesive surfaces" for potential SUMO interactions. We thus like to propose that SUMO functions comparable to a glue. In this model, upon a specific signal, a DNA-bound, concentrated SUMO ligase catalyzes a local "SUMO spray" on nearby proteins, thereby potentiating physical interactions and in that way accelerating overall repair. Because the SUMO ligases are enzymatically largely promiscuous, they predictably also modify sites that do not contribute to complex stabilization. In other words, although SUMO modifications (e.g., in the HR pathway) act synergistically and add up for repair, not all modifications are expected to be equally important. Furthermore, superfluous SUMO modifications, not protected by interaction with a SIM-containing partner, might be rapidly removed by de-SUMOylation enzymes (Geiss-Friedlander and Melchior, 2007). Because many biological pathways take place within "nuclear bodies" or "foci," it seems attractive to speculate that several of these large protein assemblies are stabilized by the mechanism we describe here. An interesting remaining question is how the SUMO-bridged complexes become disassembled once their activities are no longer needed. Attractive possibilities are that disassembly might be promoted by regulated de-SUMOylation, by proteolytic removal involving SUMO-targeted ubiquitin ligases (STUbLs; Galanty et al., 2012; Prudden et al., 2007; Yin et al., 2012), or perhaps by the activities of "segregases" such as Cdc48/p97 of the ubiquitin pathway (Dantuma and Hoppe, 2012; Jentsch and Rumpf, 2007).

EXPERIMENTAL PROCEDURES

Yeast Strains
Yeast (*S. cerevisiae*) strains used in this study are listed in Table S2.

Biochemical and Molecular Biology Techniques
The biochemical and molecular biology techniques used in this study are standard procedures. Detailed descriptions for individual methods are provided in Extended Experimental Procedures.

SILAC Mass Spectrometry
For the detection of SUMO conjugates enriched upon MMS and UV light treatment (Figures 1A and 1B), SILAC was used. Yeast cells deficient in biosynthesis of lysine and arginine (Δlys1and Δarg4) expressing His-tagged SUMO (^{His}SUMO) were grown for at least ten divisions in synthetic complete (SC) media supplemented either with unlabeled (Lys0 and Arg0; light) or heavy-isotope-labeled amino acids (Lys8 and Arg10; heavy) from Cambridge Isotope Laboratories. Exponentially dividing cells grown in heavy media were treated with 0.2% MMS for 90 min, harvested, and combined with equal amount of untreated cells grown in light media. Alternatively, cells grown in heavy media

Figure 5. Multisite SUMOylation of HR Proteins on Chromatin after DNA Damage Fosters Physical Interaction and Promotes Complex Formation
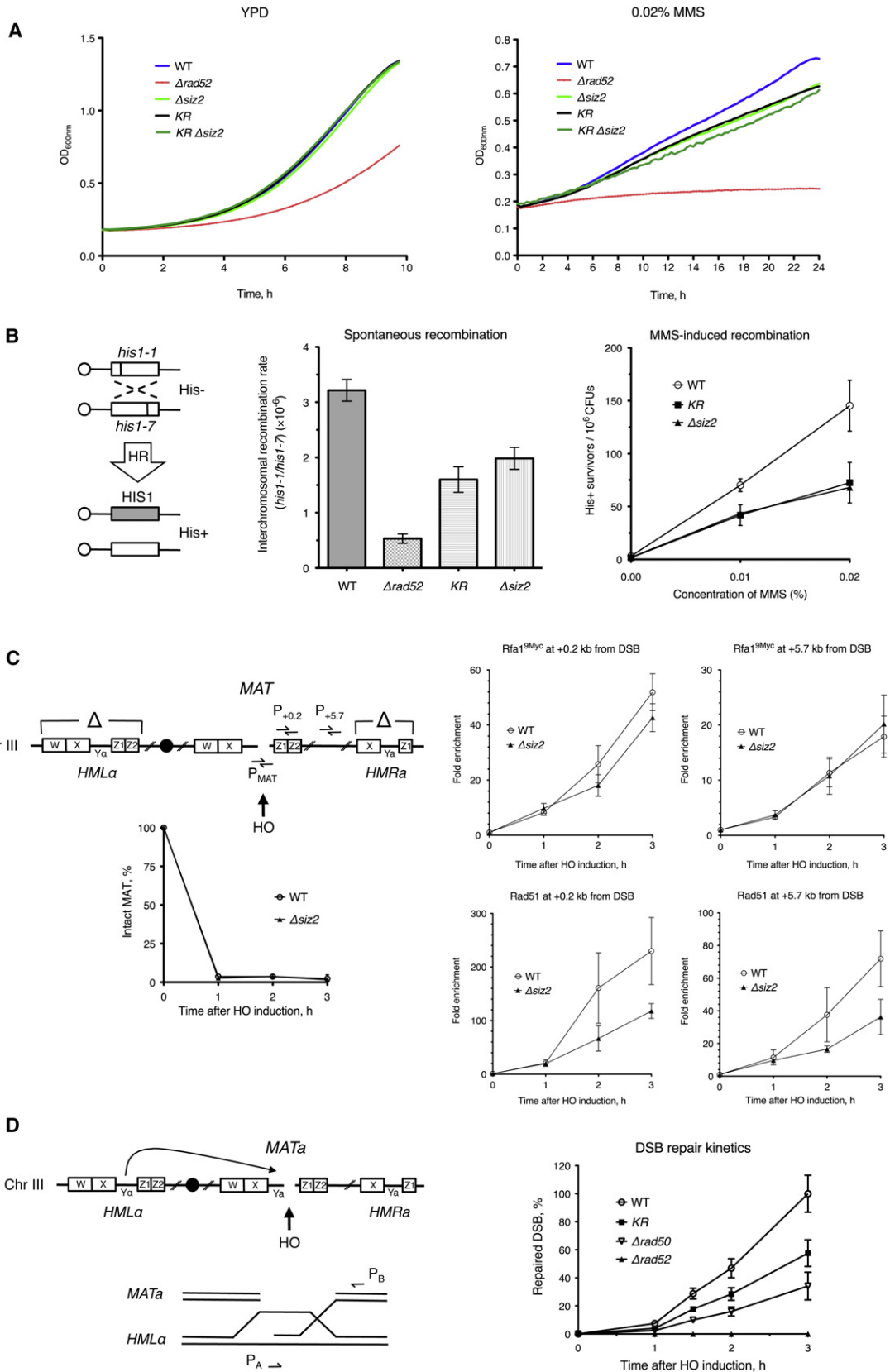
(A) SUMOylated HR proteins are enriched in chromatin after DNA damage. Following subcellular fractionation of MMS-treated WT cells or cells overexpressing ^{His}SUMO into soluble (SOL) and chromatin-enriched (CHR) fractions, denaturing Ni-NTA pull-down (Ni PD) of ^{His}SUMO conjugates was performed. SUMOylated forms of Rfa1 and Rad52 were specifically enriched in the chromatin fraction. To control chromatin fractionation efficiency, the levels of histone H3 lysine-4 trimethylation and the ER membrane protein Dpm1 were detected in fractions prior to Ni PD.

(B) SUMOylation fosters interaction between HR proteins and promotes complex formation. RPA-Rad52-Rad59 complexes, which assemble following MMS-induced DNA damage, were coimmunoprecipitated (co-IP) from extracts by immunoprecipitation of C-terminally HA-tagged Rad59. The preference for SUMOylated species is reflected by the ratios of SUMO-modified/unmodified HR protein fractions in co-IPs versus inputs (quantified by ImageJ software).

(C) Identification of SUMO attachment sites in HR proteins by using proteomic mass spectrometry. Following Ni-NTA pull-down after MMS-induced DNA damage, ^{His}SUMO conjugates were digested either with trypsin or thermolysin, and branched peptides with characteristic SUMO remnants attached to target lysines were detected by LC-MS/MS.

(D–G) HR proteins are SUMOylated independently at multiple sites following DNA damage. ^{His}SUMO Ni PD from MMS-treated cells expressing WT Rfa2 (D), C-terminally HA-tagged Rfa3 (E), Rfa1 (F), C-terminally HA-tagged Rad59 (G), or their various *KR* mutant variants. In addition, cells expressing a ^{His}SUMO variant in which all lysine residues were replaced by arginines (^{His}SUMO-KRall) as the only source of SUMO were used for pull-down to distinguish poly-SUMOylation from multisite SUMOylation (D and E).

See also Figure S5 and Table S1.

A

YPD

0.02% MMS

WT
Δrad52
Δsiz2
KR
KR Δsiz2

OD$_{600nm}$

Time, h

B

his1-1

His-

his1-7

HR

HIS1

His+

Spontaneous recombination

Interchromosomal recombination rate ($his1\text{-}1/his1\text{-}7$) ($\times10^{-6}$)

WT   Δrad52   KR   Δsiz2

MMS-induced recombination

His+ survivors / 10^6 CFUs

WT
KR
Δsiz2

Concentration of MMS (%)

C

MAT

Chr III

W X   Z1 Z2   W X   Z1 Z2   X   Z1

Δ   P$_{+0.2}$ P$_{+5.7}$ Δ

HMLα   P$_{MAT}$ HMRa

HO

Intact MAT, %

WT
Δsiz2

Time after HO induction, h

Rfa1^{9Myc} at +0.2 kb from DSB

Fold enrichment

WT
Δsiz2

Time after HO induction, h

Rfa1^{9Myc} at +5.7 kb from DSB

Fold enrichment

WT
Δsiz2

Time after HO induction, h

Rad51 at +0.2 kb from DSB

Fold enrichment

WT
Δsiz2

Time after HO induction, h

Rad51 at +5.7 kb from DSB

Fold enrichment

WT
Δsiz2

Time after HO induction, h

D

MATa

Chr III

W X   Z1 Z2   W X   Z1 Z2   X   Z1

HMLα   HO   HMRa

MATa   P$_B$

HMLα   P$_A$

DSB repair kinetics

Repaired DSB, %

WT
KR
Δrad50
Δrad52

Time after HO induction, h

were UV irradiated (80 J/m^2) instead of MMS treatment. Then, ^{His}SUMO conjugates were isolated by using denaturing Ni-NTA pull-down and separated on 4%–12% Bis-Tris gel. The whole lane was excised in ten slices and proteins were digested with trypsin and analyzed by LC-MS/MS.

Detection of SUMOylation Sites

For the detection of SUMOylation sites, proteins were digested with trypsin (Shevchenko et al., 1996) or thermolysin. Extracted peptides were analyzed by Orbitrap mass spectrometry (Olsen et al., 2005) and identified by using MaxQuant software (Cox and Mann, 2008). The data set was searched for peptides harboring extra masses on lysines (branched peptides) corresponding to proteolytic remnants of the C-terminal tail of SUMO. In the case of trypsin digestion, extra masses corresponding to a SUMO remnant with the sequence EQIGG were expected, and in the case of thermolysin digestion, with the sequence IGG.

Detection of SUMO-Interacting Motifs

For the detection of SIMs, 12-mer peptides corresponding to in-silico-predicted SIMs and flanking sequences were synthesized by a MultiPep instrument (INTAVIS Bioanalytical Instruments AG) on a membrane, incubated with poly-SUMO-3 chains (UCL-310; BostonBiochem), and probed analogous to western blotting with antibodies directed against SUMO-2/3. For details, see Extended Experimental Procedures (Peptide Array Immunoblotting).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.10.021.

REFERENCES

Altmannova, V., Eckert-Boulet, N., Arneric, M., Kolesar, P., Chaloupkova, R., Damborsky, J., Sung, P., Zhao, X., Lisby, M., and Krejci, L. (2010). Rad52 SUMOylation affects the efficiency of the DNA repair. Nucleic Acids Res. 38, 4708–4721.

Armstrong, A.A., Mohideen, F., and Lima, C.D. (2012). Recognition of SUMO-modified PCNA requires tandem receptor motifs in Srs2. Nature 483, 59–63.

Branzei, D., Sollier, J., Liberi, G., Zhao, X., Maeda, D., Seki, M., Enomoto, T., Ohta, K., and Foiani, M. (2006). Ubc9- and mms21-mediated sumoylation counteracts recombinogenic events at damaged replication forks. Cell 127, 509–522.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372.

Cremona, C.A., Sarangi, P., Yang, Y., Hang, L.E., Rahman, S., and Zhao, X. (2012). Extensive DNA damage-induced sumoylation contributes to replication and repair and acts in addition to the mec1 checkpoint. Mol. Cell 45, 422–432.

Danielsen, J.R., Povlsen, L.K., Villumsen, B.H., Streicher, W., Nilsson, J., Wikström, M., Bekker-Jensen, S., and Mailand, N. (2012). DNA damage-inducible SUMOylation of HERC2 promotes RNF8 binding via a novel SUMO-binding Zinc finger. J. Cell Biol. 197, 179–187.

Dantuma, N.P., and Hoppe, T. (2012). Growing sphere of influence: Cdc48/p97 orchestrates ubiquitin-dependent extraction from chromatin. Trends Cell Biol. 22, 483–491.

Davis, A.P., and Symington, L.S. (2003). The Rad52-Rad59 complex interacts with Rad51 and replication protein A. DNA Repair (Amst.) 2, 1127–1134.

Dou, H., Huang, C., Singh, M., Carpenter, P.B., and Yeh, E.T.H. (2010). Regulation of DNA repair through deSUMOylation and SUMOylation of replication protein A complex. Mol. Cell 39, 333–345.

Dou, H., Huang, C., Van Nguyen, T., Lu, L.S., and Yeh, E.T. (2011). SUMOylation and de-SUMOylation in response to DNA damage. FEBS Lett. 585, 2891–2896.

Finkbeiner, E., Haindl, M., and Muller, S. (2011a). The SUMO system controls nucleolar partitioning of a novel mammalian ribosome biogenesis complex. EMBO J. 30, 1067–1078.

Finkbeiner, E., Haindl, M., Raman, N., and Muller, S. (2011b). SUMO routes ribosome maturation. Nucleus 2, 527–532.

Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu. Rev. Biochem. 78, 477–513.

Galanty, Y., Belotserkovskaya, R., Coates, J., Polo, S., Miller, K.M., and Jackson, S.P. (2009). Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. Nature 462, 935–939.

Figure 6. SUMOylation of DSB Repair Proteins Accelerates the Repair

(A) Cells deficient in HR protein SUMOylation either due to the absence of Siz2 (Δsiz2) or the lack of 11 SUMO acceptor sites in the core HR proteins RPA, Rad52, and Rad59 (KR) show delayed growth upon chronic exposure to DNA damage. Growth curves for WT cells, cells deficient in Rad52 (Δrad52) or Siz2 (Δsiz2), and cells deficient in SUMOylation sites of HR proteins (KR) and also in combination with Δsiz2 (KR and Δsiz2) were measured in YPD medium or YPD containing 0.02% MMS.

(B) Deficiency in HR protein SUMOylation results in reduction of spontaneous and MMS-induced recombination. The interchromosomal recombination rates between chromosomal his1 heteroalleles were measured by fluctuation analysis in WT cells, cells lacking Siz2 (Δsiz2), and mutant cells deficient in SUMOylation sites of HR proteins (KR). The results are the average of at least three independent studies, and error bars represent SD.

(C) Rad51 loading at the DSB is affected in the absence of Siz2. A single unrepairable DSB was induced by HO endonuclease at MAT of cells lacking HML and HMR (donor-deficient strain). DSB induction was monitored in WT and Siz2-deficient cells (Δsiz2) by real-time quantitative PCR with primers (P$_{MAT}$) spanning the HO cut site. ChIP directed against Rad51 and C-terminally Myc-tagged Rfa1 at 0.2 and 5.7 kb distal from DSB was performed 1, 2, and 3 hr after HO induction to compare loading of RPA and Rad51 in the absence of HR protein SUMOylation. The results are the average of at least three independent studies, and error bars represent SEM.

(D) The speed of DSB repair is slowed down in mutants deficient in SUMOylation of RPA, Rad52, and Rad59 (KR mutant). A single repairable DSB (arrow indicates repair reaction) was transiently induced by HO at MATa in WT cells, cells lacking the MRX subunit Rad50 (Δrad50) defective in DSB-resection initiation, or the Rad52 protein (Δrad52), and the KR mutant. Repair kinetics was measured by real-time quantitative PCR (with primers P$_A$ and P$_B$), following the appearance of repaired product Y$_α$ at MAT, 1, 2, and 3 hr after inactivation of HO (shift from galactose to glucose-containing media). The results are the average of at least three independent studies, and error bars represent SEM.

See also Figure S6.

Galanty, Y., Belotserkovskaya, R., Coates, J., and Jackson, S.P. (2012). RNF4, a SUMO-targeted ubiquitin E3 ligase, promotes DNA double-strand break repair. Genes Dev. 26, 1179–1195.

Garvik, B., Carson, M., and Hartwell, L. (1995). Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol. Cell. Biol. 15, 6128–6138.

Geiss-Friedlander, R., and Melchior, F. (2007). Concepts in sumoylation: a decade on. Nat. Rev. Mol. Cell Biol. 8, 947–956.

Giannattasio, M., Follonier, C., Tourrière, H., Puddu, F., Lazzaro, F., Pasero, P., Lopes, M., Plevani, P., and Muzi-Falconi, M. (2010). Exo1 competes with repair synthesis, converts NER intermediates to long ssDNA gaps, and promotes checkpoint activation. Mol. Cell 40, 50–62.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature 419, 135–141.

Jentsch, S., and Rumpf, S. (2007). Cdc48 (p97): a "molecular gearbox" in the ubiquitin pathway? Trends Biochem. Sci. 32, 6–11.

Johnson, E.S., and Blobel, G. (1999). Cell cycle-regulated attachment of the ubiquitin-related protein SUMO to the yeast septins. J. Cell Biol. 147, 981–994.

Johnson, E.S., and Gupta, A.A. (2001). An E3-like factor that promotes SUMO conjugation to the yeast septins. Cell 106, 735–744.

Karras, G.I., and Jentsch, S. (2010). The RAD6 DNA damage tolerance pathway operates uncoupled from the replication fork and is functional beyond S phase. Cell 141, 255–267.

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu. Rev. Cell Dev. Biol. 22, 159–180.

Lisby, M., Barlow, J.H., Burgess, R.C., and Rothstein, R. (2004). Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. Cell 118, 699–713.

Maeda, D., Seki, M., Onoda, F.T., Branzei, D., Kawabe, Y., and Enomoto, T. (2004). Ubc9 is required for damage-tolerance and damage-induced interchromosomal homologous recombination in S. cerevisiae. DNA Repair (Amst.) 3, 335–341.

Mann, M. (2006). Functional and quantitative proteomics using SILAC. Nat. Rev. Mol. Cell Biol. 7, 952–958.

Mimitou, E.P., and Symington, L.S. (2008). Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature 455, 770–774.

Mimitou, E.P., and Symington, L.S. (2011). DNA end resection—unraveling the tail. DNA Repair (Amst.) 10, 344–348.

Morin, I., Ngo, H.P., Greenall, A., Zubko, M.K., Morrice, N., and Lydall, D. (2008). Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response. EMBO J. 27, 2400–2410.

Morris, J.R., Boutell, C., Keppler, M., Densham, R., Weekes, D., Alamshah, A., Butler, L., Galanty, Y., Pangon, L., Kiuchi, T., et al. (2009). The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. Nature 462, 886–890.

Ohuchi, T., Seki, M., Branzei, D., Maeda, D., Ui, A., Ogiwara, H., Tada, S., and Enomoto, T. (2008). Rad52 sumoylation and its involvement in the efficient induction of homologous recombination. DNA Repair (Amst.) 7, 879–889.

Ohuchi, T., Seki, M., Kugou, K., Tada, S., Ohta, K., and Enomoto, T. (2009). Accumulation of sumoylated Rad52 in checkpoint mutants perturbed in DNA replication. DNA Repair (Amst.) 8, 690–696.

Olsen, J.V., de Godoy, L.M.F., Li, G.Q., Macek, B., Mortensen, P., Pesch, R., Makarov, A., Lange, O., Horning, S., and Mann, M. (2005). Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol. Cell. Proteomics 4, 2010–2021.

Palvimo, J.J. (2007). PIAS proteins as regulators of small ubiquitin-related modifier (SUMO) modifications and transcription. Biochem. Soc. Trans. 35, 1405–1408.

Panse, V.G., Kressler, D., Pauli, A., Petfalski, E., Gnädig, M., Tollervey, D., and Hurt, E. (2006). Formation and nuclear export of preribosomes are functionally linked to the small-ubiquitin-related modifier pathway. Traffic 7, 1311–1321.

Papouli, E., Chen, S., Davies, A.A., Huttner, D., Krejci, L., Sung, P., and Ulrich, H.D. (2005). Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. Mol. Cell 19, 123–133.

Pfander, B., Moldovan, G.L., Sacher, M., Hoege, C., and Jentsch, S. (2005). SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. Nature 436, 428–433.

Prudden, J., Pebernard, S., Raffa, G., Slavin, D.A., Perry, J.J., Tainer, J.A., McGowan, C.H., and Boddy, M.N. (2007). SUMO-targeted ubiquitin ligases in genome stability. EMBO J. 26, 4089–4101.

Reindle, A., Belichenko, I., Bylebyl, G.R., Chen, X.L., Gandhi, N., and Johnson, E.S. (2006). Multiple domains in Siz SUMO ligases contribute to substrate selectivity. J. Cell Sci. 119, 4749–4757.

Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell 120, 73–84.

Sacher, M., Pfander, B., Hoege, C., and Jentsch, S. (2006). Control of Rad52 recombination activity by double-strand break-induced SUMO modification. Nat. Cell Biol. 8, 1284–1290.

Shevchenko, A., Jensen, O.N., Podtelejnikov, A.V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H., and Mann, M. (1996). Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc. Natl. Acad. Sci. USA 93, 14440–14445.

Silver, H.R., Nissley, J.A., Reed, S.H., Hou, Y.M., and Johnson, E.S. (2011). A role for SUMO in nucleotide excision repair. DNA Repair (Amst.) 10, 1243–1251.

Song, J., Durrin, L.K., Wilkinson, T.A., Krontiris, T.G., and Chen, Y.A. (2004). Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. Proc. Natl. Acad. Sci. USA 101, 14373–14378.

Stehmeier, P., and Muller, S. (2009). Phospho-regulated SUMO interaction modules connect the SUMO system to CK2 signaling. Mol. Cell 33, 400–409.

Steinacher, R., and Schär, P. (2005). Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. Curr. Biol. 15, 616–623.

Takahashi, Y., Kahyo, T., Toh-E, A., Yasuda, H., and Kikuchi, Y. (2001). Yeast Ull1/Siz1 is a novel SUMO1/Smt3 ligase for septin components and functions as an adaptor between conjugating enzyme and substrates. J. Biol. Chem. 276, 48973–48977.

Torres-Rosell, J., Sunjevaric, I., De Piccoli, G., Sacher, M., Eckert-Boulet, N., Reid, R., Jentsch, S., Rothstein, R., Aragón, L., and Lisby, M. (2007). The Smc5-Smc6 complex and SUMO modification of Rad52 regulates recombinational repair at the ribosomal gene locus. Nat. Cell Biol. 9, 923–931.

Ullmann, R., Chien, C.D., Avantaggiati, M.L., and Muller, S. (2012). An acetylation switch regulates SUMO-dependent protein interaction networks. Mol. Cell 46, 759–770.

Werner, A., Flotho, A., and Melchior, F. (2012). The RanBP2/RanGAP1* SUMO1/Ubc9 complex is a multisubunit SUMO E3 ligase. Mol. Cell 46, 287–298.

Westman, B.J., and Lamond, A.I. (2011). A role for SUMOylation in snoRNP biogenesis revealed by quantitative proteomics. Nucleus 2, 30–37.

Yin, Y., Seifert, A., Chua, J.S., Maure, J.F., Golebiowski, F., and Hay, R.T. (2012). SUMO-targeted ubiquitin E3 ligase RNF4 is required for the response of human cells to DNA damage. Genes Dev. 26, 1196–1208.

Yunus, A.A., and Lima, C.D. (2009). Structure of the Siz/PIAS SUMO E3 ligase Siz1 and determinants required for SUMO modification of PCNA. Mol. Cell 35, 669–682.

Zhu, Z., Chung, W.H., Shim, E.Y., Lee, S.E., and Ira, G. (2008). Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell 134, 981–994.