Published online 6 January 2016

Nucleic Acids Research, 2016, Vol. 44, No. 5 ***2199–2213***
doi: 10.1093/nar/gkv1523

S. cerevisiae Mre11 recruits conjugated SUMO moieties to facilitate the assembly and function of the Mre11-Rad50-Xrs2 complex

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Received February 24, 2015; Revised December 01, 2015; Accepted December 19, 2015

ABSTRACT

Double-strand breaks (DSBs) in chromosomes are the most challenging type of DNA damage. The yeast and mammalian Mre11-Rad50-Xrs2/Nbs1 (MRX/N)-Sae2/Ctp1 complex catalyzes the resection of DSBs induced by secondary structures, chemical adducts or covalently-attached proteins. MRX/N also initiates two parallel DNA damage responses—checkpoint phosphorylation and global SUMOylation—to boost a cell's ability to repair DSBs. However, the molecular mechanism of this SUMO-mediated response is not completely known. In this study, we report that *Saccharomyces cerevisiae* Mre11 can non-covalently recruit the conjugated SUMO moieties, particularly the poly-SUMO chain. Mre11 has two evolutionarily-conserved SUMO-interacting motifs, Mre11^{SIM1} and Mre11^{SIM2}, which reside on the outermost surface of Mre11. Mre11^{SIM1} is indispensable for MRX assembly. Mre11^{SIM2} non-covalently links MRX with the SUMO enzymes (E2/Ubc9 and E3/Siz2) to promote global SUMOylation of DNA repair proteins. Mre11^{SIM2} acts independently of checkpoint phosphorylation. During meiosis, the *mre11^{SIM2}* mutant, as for *mre11S*, *rad50S* and *sae2*Δ, allows initiation but not processing of Spo11-induced DSBs. Using MRX and DSB repair as a model, our work reveals a general principle in which the conjugated SUMO moieties non-covalently facilitate the assembly and functions of multi-subunit protein complexes.

INTRODUCTION

Small ubiquitin-like modifier (SUMO) is a small regulatory protein found in almost all eukaryotic organisms (1–3). In the yeast *Saccharomyces cerevisiae*, the essential gene *SMT3* encodes the SUMO protein. SUMOylation is a post-translational modification, in which SUMO is covalently attached to a substrate protein including SUMO itself, to form a poly-SUMO chain. SUMOylation is mediated by an enzymatic cascade that is analogous to the one that is involved in ubiquitination. The removal of the SUMO adduct from targets is catalyzed by specific SUMO proteases, e.g. Ulp1 and Ulp2. The conjugated SUMO moieties (CSMs) are recognized by two types of SUMO-binding motifs, short hydrophobic sequences known as SUMO-interacting motifs (SIMs) (4) and the ZZ zinc fingers (5,6). The addition, removal and recognition of SUMO are influenced by and affect a plethora of cellular pathways. Because SUMOylation frequently targets entire groups of physically interacting proteins rather than individual proteins, it has been proposed that protein-group SUMOylation functions to establish new physical interactions between proteins that have SUMO-binding motifs (7). Alternatively, CSMs can covalently or non-covalently prevent premature aggregation by increasing the water solubility of individual protein subunits (8,9) prior to their assembly into a functional protein complex (10–12). A proof-of-concept has been provided by simultaneously expressing three capsid proteins of the foot-and-mouth disease virus (FMDV); these three SUMO fusion proteins formed a stable heterotrimeric complex. The proteolytic removal of SUMO moieties from the ternary complexes resulted in virus-like particles with a size and shape resembling the authentic FMDV, which contains 20 heterotrimers of the capsid proteins (10).

SUMOylation is strongly connected to the repair of DNA double-strand breaks (DSBs) (13). In *S. cerevisiae* vege-

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tative cells, mutations or deletion of SUMOylation genes cause a pronounced sensitivity to DNA damage and genomic instability, including the poly-SUMO chain mutant (*smt3-allR*) (14), E2 conjugating enzyme (*ubc9*), E3 ligase enzymes (*siz1*Δ, *siz2*Δ, *mms21*) (7,15), deSUMOylation proteases ($ulp1^{ts}$, *ulp2*Δ) (16,17) as well as SUMO-targeted ubiquitin ligases (STUbLs; *slx5*Δ, *slx8*Δ) (18,19). In parallel, with checkpoint phosphorylation, vegetative yeast cells also induce SUMOylation of many proteins that are needed for replication and repair in response to DNA damage (7,15). The MRX (Mre11-Rad50-Xrs2)-Sae2/Com1 dsDNA endonuclease complex has been implicated as a positive regulator for DSB-induced global SUMOylation (15). Mre11 exhibits notable two-hybrid interactions with Ubc9 and Siz2, and it has been proposed that the binding of Siz2 might be achieved through Ubc9-catalyzed SUMOylation of Mre11 (7). Sae2 (also called Com1)(20) is a SUMOylated protein during vegetative growth, and SUMOylation of Sae2 increases both soluble Sae2 and the MRX function in DNA end resection (21). Sae2 apparently mediates removal of the MRX complex from the DNA damaged sites during vegetative growth. The MRX complex is retained at DSB ends in *sae2*Δ, thus turning on the DNA damage checkpoint to stall cell cycle progression (22,23). Recently, it has been reported that, following the resection of DSBs, Siz2 also collaborates with the ssDNA binding complex RPA (replication protein A) to enhance global SUMOylation (7,24). The relationship between MRX and RPA in promoting DSB-induced SUMOylation and the molecular mechanism of the interaction between MRX and Sae2 interactions remains unclear.

MRX has multiple functions during *S. cerevisiae* meiosis (25,26). First, it has a unique role in Spo11-induced DSBs independently of its catalytic activity, and the C-terminal portion of Mre11 is specifically required for this function (27,28). Second, the MRX-Sae2 endonuclease complex acts at each 5′-end of DSBs to generate 3′-end ssDNA tails through the removal of a covalently linked Spo11-oligonucleotide complex (29,30). The 3′-end ssDNA tails subsequently assemble into nucleoprotein filaments comprised of two RecA-family recombinases (Rad51 and Dmc1) and their accessory factors to catalyze DSB repair via homologous recombination (31–33). Third, MRX senses DSBs and activates the $Tel1^{ATM}$ checkpoint kinase for target phosphorylation. This checkpoint phosphorylation has dual roles in preventing superfluous Spo11-induced DSBs (34,35) and in promoting interhomolog recombination (12,35,36). Interhomolog recombination is a hallmark of meiotic recombination. A few Spo11-induced DSBs must be repaired using a homologous non-sister chromosome (but not a sister chromatin) as template to generate new combinations of DNA sequences (26).

Accumulating evidence has also revealed that SUMOylation functionally links two groups of *S. cerevisiae* proteins that are essential for interhomolog recombination. The first group includes three meiosis-specific chromosomal proteins Hop1, Red1 and Mek1. These proteins are the axial components of the synaptonemal complex (SC)—a zipper-like proteinaceous structure that mediates chromosome synapsis between homologous chromosomes during meiotic prophase. The SC consists of two dense lateral/axial elements and a central element. To assemble the SC, both the SC central protein, Zip1, and the SC axial protein, Red1, non-covalently interact with conjugated SUMO moieties (CSMs), such as poly-SUMO chains or conjugates. During SC assembly, the SC initiation protein, Zip3, acts as a SUMO E3 ligase that promotes the formation of additional CSMs (11,37). Consistent with these findings, it has been shown that the SUMOylation of Ubc9 promotes the formation of a poly-SUMO chain, which is a key event for SC formation (38). Furthermore, SUMOylation and the ubiquitin-mediated removal of CSMs (e.g. SUMOylated topoisomerase II or Red1) have been implicated in SC-mediated crossover interference (39). Crossover interference is a genetic phenomenon in which crossovers tend to be evenly spaced along any given meiotic chromosomes. SUMOylation is also critical in the regulation of meiotic recombination or chromosomal morphogenesis in other sexually-reproductive organisms, including the fission yeast *S. pombe* (40), the fungus *Sordaria* (41), *Arabidopsis thaliana* (42) and mammals (43).

The DNA damage checkpoint proteins $Mec1^{ATR}$ and $Tel1^{ATM}$ are the second group required for establishing interhomolog bias during meiotic recombination. $Tel1^{ATM}$ is activated by non-resected DSBs via an Xrs2-dependent mechanism (44–47), and $Mec1^{ATR}$ is recruited to RPA-coated ssDNA tails via its binding partner, Ddc2 (48,49). $Mec1^{ATR}$ activation also requires three additional DNA damage sensors: the yeast 9-1-1 complex (Ddc1-Mec3-Rad17), its clamp loader, the Rad24-RFC complex and Dpb11 (48,50–52). These two protein kinases phosphorylate the SC axial protein Hop1 to ensure interhomolog recombination (11,12,36,53,54). Notably, both $Tel1^{ATM}$- and $Mec1^{ATR}$-dependent Hop1 phosphorylation requires Red1 and the Red1-CSM interaction (11,36). Red1 first non-covalently associates with CSMs (11) and then with the 9-1-1 complex (55) to activate $Mec1^{ATR}$ resulting in Hop1 phosphorylation via its binding to the 9-1-1 complex (12). It is still unclear how the Red1-CSM ensemble couples with MRX at the non-resected DSB ends during meiosis.

Here, we investigate the molecular mechanism and physiological impacts of the Mre11-SUMO interaction in response to DSBs during vegetative growth and meiosis. Our results reveal that the yeast *S. cerevisiae* Mre11 can non-covalently recruit CSMs to facilitate both global SUMOylation and DSB repair.

MATERIALS AND METHODS

Yeast strains, two-hybrid assay and physical analysis

All vegetative experiments were performed using haploid cells from isogenic W303 strains as described previously (15,56). All meiotic experiments were performed using diploid cells from isogenic SK1 strains. Quantitative yeast two-hybrid assays, tetrad dissection, fluorescence-activated cell sorting (FACS), pulsed-field gel electrophoresis (PFGE) and Southern hybridization were carried out as previously described (11,36,37).

Antisera, immunoblot, dephosphorylation assay and cytology

The antisera used against Hop1, phosphorylated Hop1-T318, Zip1, phosphorylated Zip1-S75, H2A and phosphorylated H2A-S129 have been described previously (36). Peroxidase-anti-peroxidase (PAP) antibody (Sigma, CA, USA), IgG Sepharose beads (GE Healthcare, Bucks, UK), anti-HA antibody (Roche, Basel, SWZ) and anti-Rad53 antibody (Santa Cruz Biotechnology, TX, USA) were purchased commercially. Western blotting analyses were performed as recently described and repeated 2-4 times (36). The dephosphorylation assay was performed as described previously (54). Cytology analyses were carried out as previously described (11,37).

Immunoprecipitation

Yeast vegetative cultures (20 ml; OD_{600} ≈0.5) were harvested and washed once with ice-cold water. Cells were resuspended in 250 μl of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA pH 8.0, 0.1% Triton X-100) containing an EDTA-free protease inhibitor complete cocktail (Roche) and 20 mM N-ethylmaleimide (Sigma). To prepare the total cell lysates, the cell suspension was mixed with 1/2 volume of 0.5-mm acid-washed glass beads (Sigma), vigorously vortexed (30 s of vortexing and 30 s on ice) five times, and then microcentrifuged (16 000 x g) at 4°C for 10 min. The supernatants were collected and incubated with IgG Sepharose beads (Sigma) or immobilized anti-HA affinity resin (Sigma) at 4°C for 2 h. The precipitants were washed three times with 1 ml of lysis buffer and then resuspended in 100 μl Laemmli loading buffer containing 100 mM dithiothreitol. The proteins were analyzed by SDS-PAGE and immunoblotting as described previously (11).

RESULTS

Identification of two SUMO-interacting motifs (SIMs) in Mre11 that preferentially interacts with the poly-SUMO chain

We identified, *in silico*, two putative SIMs in *S. cerevisiae* Mre11: $Mre11^{SIM1}$ (IRIL, residues 9-12) and $Mre11^{SIM2}$ (ESDKIKVV, residues 154-161) (57). Both SIMs are evolutionarily conserved in *S. pombe* Mre11: $Mre11^{SIM1}$ (IRIL, residues 18-21) and $Mre11^{SIM2}$ (ENDNIVV, residues 163-169) (Figure 1A). Furthermore, they reside at the outermost surface of the *S. pombe* Mre11-Nbs1 complex crystal structure (58) (Figure 1A). Nbs1 is the *S. pombe* homolog of Xrs2. Thus, Mre11 might non-covalently associate with the SUMO monomer or CSMs.

These two distinct possibilities were further examined by two-hybrid assays with either vegetative or meiotic two-hybrid reporter cells (11,59). We found that Mre11 preferentially interacts with CSMs rather than the Smt3 monomer; the hierarchy for two-hybrid interactions with Mre11 was Smt3 > Smt3-allR > Smt3-ΔGG ≅ mock control in both vegetative and meiotic reporter cells (Table 1). Smt3-allR cannot form a polymeric chain because the nine lysine residues in the wild type Smt3 are replaced by arginine, but it remains competent in the SUMO conjugation with all target proteins (including wild-type Smt3) (60). Smt3-ΔGG is a conjugation-incompetent Smt3 mutant that lacks the C-terminal pair of glycines required for E1-mediated Smt3 activation (61). Next, we constructed two SIM mutant proteins ($Mre11^{I9R}$ and $Mre11^{I158R}$), each with a mutation from isoleucine (I) to arginine (R). We found that neither of these two mutants exhibited notable two-hybrid interactions with Smt3, Smt3-allR or Smt3-ΔGG (Table 1).

S. cerevisiae Mre11 exhibits strong two-hybrid interactions with the SUMO E2 enzyme Ubc9 and the SUMO E3 ligase Siz2 (7), and Mre11 is also a SUMOylated protein (7,11). We examined whether the Mre11-Ubc9 and Mre11-Siz2 interactions are mediated via SUMOylated Mre11 or non-covalently via the Mre11-CSM ensembles. We found that the hierarchy for the two-hybrid interaction with Ubc9 or $Siz2_{348-726}$ was Mre11 > $Mre11^{I158R}$ ≅ $Mre11^{I9R}$ ≅ mock control (Table I). $Siz2_{348-726}$ is the C-terminal domain of Siz2 and harbors two SIMs (11). These results suggest that both interactions are likely mediated via the Mre11-CSM ensembles rather than SUMOylated Mre11 (also see below).

To minimize the potential effects of arginine replacement on protein structure or folding, we also constructed additional $Mre11^{SIM1}$ and $Mre11^{SIM2}$ mutants with one, two or three alanine mutations, respectively. All these mutants exhibited reduced or no two-hybrid interactions with Smt3, Smt3-allR or Smt3-ΔGG (Supplementary file, Supplementary Table S1). The hierarchy for the two-hybrid interaction of the $Mre11^{SIM1}$ mutants with Smt3 was Mre11 >> $Mre11^{I11A}$ > $Mre11^{I9A}$ > $Mre11^{I9A,I11A}$ ≅ $Mre11^{I9R}$, and that of the $Mre11^{SIM2}$ mutants with Smt3 was Mre11 >> $Mre11^{I158A}$ > $Mre11^{I158A,V161A}$ ≅ $Mre11^{V160A, V161A}$ ≅ $Mre11^{I158R}$ ≅ $Mre11^{V160A}$ > $Mre11^{V161A}$ ≅ $Mre11^{I158A,V160A}$.

$Mre11^{I9R}$ is defective in MRX assembly

Mre11 displays strong two-hybrid interactions with Xrs2 and Rad50 (62). Using both vegetative and meiotic two-hybrid reporter cells (11,59), we found that $Mre11^{I158R}$ (as for Mre11) exhibited notable two-hybrid interactions with Xrs2 and Rad50. In contrast, $Mre11^{I9R}$ failed to interact with Rad50 and Xrs2 in the same assay (Table 1).

Next, we constructed three W303 strains (*MRE11-6HA*, *mre11^{I9R}-6HA*, *mre11^{I158R}-6HA*) that express an HA-epitope-tagged wild-type and mutant Mre11, respectively. Compared to *MRE11-6HA* and *mre11^{I158R}-6HA*, the *mre11^{I9R}-6HA* mutant displayed a slow vegetative growth phenotype on the YPD plate (Figure 1B). The hierarchy for resistance to the DNA damage agent methyl methanesulfonate (MMS) is *MRE11* ≅ *MRE11-6HA* > *mre11^{I158R}-6HA* >> *mre11^{I9R}-6HA* ≅ *mre11*Δ (Figure 1B). Again, to minimize the potential effects of arginine replacements on protein structure or folding, we also expressed and compared different $Mre11^{SIM1}$-6HA and $Mre11^{SIM2}$-6HA mutants for MMS resistance in a SK1 *mre11*Δ mutant (Figure 1C). For all the mutants examined here, their two-hybrid interactions with Smt3 correlated well with the hierarchy for MMS resistance (Figure 1C): *MRE11-6HA* > *mre11^{I11A}-6HA* > *mre11^{I9A}-6HA* > *mre11^{I9A,I11A}-6HA* ≅ *mre11^{I9R}-6HA* ≅ *mre11*Δ and *MRE11-6HA* >> *mre11^{V160A}-6HA* ≅ *mre11^{V161A}-*