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Molecular Circuitry of the SUMO (Small Ubiquitin-like Modifier) Pathway in Controlling Sumoylation Homeostasis and Suppressing Genome Rearrangements*[S]

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Small ubiquitin-like modifier (SUMO) E3 ligases are known to have a major role in preventing gross chromosomal rearrangements (GCRs); however, relatively little is known about the role of SUMO isopeptidases in genome maintenance and their role in controlling intracellular sumoylation homeostasis. Here we show the SUMO isopeptidase Ulp2 in *Saccharomyces cerevisiae* does not prevent the accumulation of GCRs, and interestingly, its loss causes subunit-specific changes of sumoylated minichromosome maintenance (MCM) helicase in addition to drastic accumulation of sumoylated nucleolar RENT and inner kinetochore complexes. In contrast, loss of Ulp1 or its mis-localization from the nuclear periphery causes substantial accumulations of GCRs and elevated sumoylation of most proteins except for Ulp2 targets. Interestingly, the E3 ligase Mms21, which has a major role in genome maintenance, preferentially controls the sumoylation of Mcm3 during DNA replication. These findings reveal distinct roles for Ulp1 and Ulp2 in controlling homeostasis of intracellular sumoylation and show that sumoylation of MCM is controlled in a subunit-specific and cell cycle dependent manner.

Protein sumoylation is an essential post-translational modification in eukaryotes (1, 2). Two families of enzymes control reversible sumoylation of specific substrates, including SUMO[3] (small ubiquitin-like modifier) E3 ligases and SUMO isopeptidases. Three SUMO E3 ligases Siz1, Siz2, and Mms21 have been identified in *Saccharomyces cerevisiae* and are shown to have distinct, but partially overlapping roles in catalyzing substrate-specific sumoylation (3–6). Siz1 and Siz2 are paralogs, and they redundantly catalyze the bulk of sumoylation in cells (5, 6). Mms21 catalyzes sumoylation of fewer substrates but plays a more important role in genome maintenance than Siz1 and Siz2 (6, 7). Deletion of *SIZ1* and *SIZ2* is lethal in cells lacking Mms21 E3 ligase activity (4, 5). Moreover, deletion of either *SIZ1* or *SIZ2* causes further accumulation of gross chromosome rearrangements (GCRs) in cells lacking Mms21 E3 ligase activity (6). These findings suggest that the functions of these E3 ligases are partially redundant, which correlates with their partially overlapping roles in catalyzing intracellular sumoylation (5, 6).

Besides SUMO E3 ligases, homeostasis of intracellular sumoylation is also regulated by SUMO isopeptidases, which catalyze the removal of SUMO from its targets. Two SUMO isopeptidases Ulp1 and Ulp2 have been identified in *S. cerevisiae* (8–10). Ulp2 is not required for cell viability; however, its loss causes accumulation of poly-SUMO chains, resulting in pleiotropic effects including slow growth and sensitivity to higher temperature (9, 11). Moreover, overexpression of *ULP2* (also known as *SMT4*) suppresses defects in chromosome condensation and segregation, suggesting its role in regulating chromosome segregation (10, 12). This role of Ulp2 in chromosome segregation appears to be conserved in higher eukaryotes including *Caenorhabditis elegans* and human cells, although its targets are poorly known (13, 14). Consistent with its nuclear function, Ulp2 has been shown to localize throughout the nucleus and occasionally the nucleolus (15, 16). On the other hand, Ulp1 is essential for cell viability and localizes at the nuclear periphery via the nuclear pore complex (NPC) (8, 17–21). Ulp1 has been shown to interact with Kap95 and Kap60 via its N-terminal NPC targeting domain (1–340 amino acids) (17). Removal of the NPC targeting domain of Ulp1 or the loss of NPC components Nup60 and Mlp1/Mlp2 (19–21) attenuates its localization at the nuclear periphery and causes the accumulation of Rad52 foci, which is indicative of endogenous DNA damage and repair (19). These studies suggest that localization of Ulp1 at the nuclear periphery has an important role in protecting genome integrity, possibly by preventing Ulp1 from desumoylating nucleoplasmic proteins yet to be determined. The distinct localization patterns of Ulp1 and Ulp2 likely contribute to their substrate selectivity in cells, which has been poorly understood.

In this study we first characterized the function of Ulp1 and Ulp2 in preventing the accumulation of GCRs and identified a genetic basis for the essential function of Ulp1. To identify the substrates of Ulp1 and Ulp2, we applied quantitative mass spectrometry (MS) to analyze the effect of *ulp1* and *ulp2* mutations on intracellular sumoylation. These studies led to the finding that Ulp2 has highly specific desumoylation activity *in vivo*,

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[S] This article contains supplemental Tables 1–9 and Figs. 1 and 2.

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[3] The abbreviations used are: SUMO, small ubiquitin-like modifier; GCR, gross chromosome rearrangement; NPC, nuclear pore complex; YPD, yeast extract/peptone/dextrose; MCM, minichromosome maintenance; HF, His_6-3XFLAG.

whereas Ulp1 has a broader specificity toward many substrates. Interestingly, loss of Ulp1 or its mislocalization from the nuclear periphery causes specific and aberrant desumoylation of Ulp2 targets, including the essential replicative MCM helicase (22). Furthermore, Mms21, the E3 ligase with a major role in preventing the accumulation of GCRs (6), preferentially catalyzes sumoylation of specific MCM subunits.

Experimental Procedures

Yeast Genetics Methods—All yeast strains used for MS and biochemical experiments were derived from the *HF-SMT3* (*HF-SUMO*) strain, in which a His_6-3×FLAG tag was integrated into the N terminus of *SMT3* in its chromosomal locus (6). The strains used to study GCRs were derived from RDKY6678, which contains the *yel072w*::*CAN1/URA3* assay (23). All tagged MCM strains and the indicated mutations were integrated into the chromosomal locus of the gene of interest (supplemental Table 1) using standard yeast genetic methods unless otherwise noted. The yeast strains used here also have their 2-μm circles removed, according to a previous study (24). Fluctuation analysis of GCRs was performed as previously described (25). At least 16 isolates were examined per mutant to calculate the median rate of accumulating GCRs. Error bars in the graph represent the upper and lower limits determined by the 95% confidence intervals of the median. (epilab Course Materials). *p* values were calculated using the two-tailed Mann-Whitney *U* test as previously described (26).

MS and Biochemical Methods—For quantitative MS analysis, each mutant strain was grown in synthetic media containing either light or heavy stable isotope-labeled lysine and arginine. A 2-liter culture was used for each strain, which was grown to an optical density (600 nm) near 0.5 and then harvested. Cell pellets of the two yeast strains to be compared were combined and used to purify sumoylated proteins under denaturing conditions using methods described previously (6). The methods used in the MS experiments and data analysis have been described previously (6). The complete list of sumoylated proteins and their abundance changes are shown in supplemental Tables 2–9. Each sumoylated protein was quantified based on the median of the abundance ratios of at least three unique peptides per protein.

To detect Net1 and MCM sumoylation, the same denaturing method using nickel-nitrilotriacetic acid and anti-FLAG affinity resins was used to purify sumoylated proteins from the same amount (200 ml) of yeast culture during logarithmic growth (6). After cell lysis, a Bradford assay was used to ensure the same amount of total proteins was used to purify sumoylated proteins. To elute sumoylated proteins from anti-FLAG affinity resins, buffer containing 50 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS was added to the anti-FLAG resins, which were heated to 100 °C for 10 min. The eluted sample was analyzed using an appropriate antibody as indicated. Typically, half of the eluted sample was analyzed to detect sumoylated MCM. To induce DNA replication stress, 0.1 M hydroxyurea was added to a logarithmic growing YPD culture (optical density at 0.5) for 2 h. For cells growing in synthetic media, 0.1 M hydroxyurea was added for 3 h. To analyze Mcm3 and Mcm6 sumoylation during the cell cycle, Mcm6-His_6-3HA cells (Bar1-deficient) were arrested by adding either 30 nM α-factor (G_1 arrest) or 7.5 μg/ml nocodazole (M-phase arrest) for 3 h at 26 °C. To prepare S-phase cells, G_1-arrested cells were washed with fresh YPD and then released into fresh YPD media for 40 min at 26 °C. The G_1, early S, and G_2-M phase cells used for the pulldown experiment were confirmed by FACS analysis. Full-length recombinant Mcm3 purified from bacteria was used to immunize rabbits (Covance), and serum containing anti-Mcm3 polyclonal antibody was used to detect Mcm3 (27).

Results

The Roles of Ulp1 and Ulp2 in Preventing GCRs and Maintaining Viability—Considering the known roles of Ulp1 in SUMO maturation and Ulp2 in disassembly of poly-SUMO chains (8, 11), we first examined the effect of *smt3GG* and *4R-smt3GG* mutations on the rate of accumulating GCRs using the *yel072w*::*URA3/CAN1* assay (23). The *smt3GG* mutation supplies cells with mature SUMO (8), whereas the *4R-smt3GG* mutation additionally eliminates the bulk of poly-SUMO chains (11). Both *smt3GG* and *4R-smt3GG* mutations caused a modest change in the rate of accumulating GCRs (Fig. 1*A*), suggesting that the removal of poly-SUMO chains does not appreciably affect the accumulation of GCRs. Next we examined the effect of *ulp2*Δ mutation and found that the loss of Ulp2 has a minimal effect on the rate of accumulating GCRs in wild type and *smt3GG* and *4R-smt3GG* mutants. Thus, neither the accumulation of poly-SUMO chains in cells lacking Ulp2 nor the lack of poly-SUMO chains appreciably alters the accumulation of GCRs measured by the *yel072w*::*URA3/CAN1* assay.

The essential function of Ulp1 has been attributed to its role in SUMO maturation and desumoylating other proteins in cells whose identities have been unknown (8). We reasoned that the latter function of Ulp1 could be bypassed by mutations that down-regulate intracellular sumoylation. Because Siz1 and Siz2 are known to have a major role in intracellular sumoylation (3, 5, 6), we tested whether loss of Siz1 and/or Siz2 could suppress the lethality of *ulp1*Δ by performing tetrad dissection of diploid cells containing heterozygous *ulp1*Δ, *siz1*Δ, and *siz2*Δ mutations as well as a homozygous *smt3GG* mutation to supply mature Smt3. As shown in Fig. 1*B*, deletion of *SIZ1* and *SIZ2*, but neither one alone, suppresses the lethality of *ulp1*Δ. In contrast, loss of Mms21 E3 ligase activity did not rescue the lethality of *ulp1*Δ (Fig. 1*B*). This suggests that an essential function of Ulp1 is to desumoylate the bulk of intracellular sumoylation, catalyzed by Siz1 and Siz2.

Interestingly, the loss of Ulp1 in the *siz1*Δ *siz2*Δ mutant caused a significant increase in the rate of accumulating GCRs, which was comparable with that of an *mms21-CH* mutant and was considerably higher than that of the *siz1*Δ *siz2*Δ mutant (Fig. 1*C*). By comparison, the loss of Ulp2 in the *siz1*Δ *siz2*Δ mutant caused a smaller increase in the rate of accumulating GCRs. Thus Ulp1 has a more important role than Ulp2 in suppressing GCRs, albeit in cells lacking Siz1 and Siz2. To avoid the complication of deleting Siz1 and Siz2, we chose to analyze the hypomorphic *ulp1* mutant further. The *ulp1-N338*Δ mutation has been shown to compromise the localization of Ulp1 at the nuclear periphery without affecting cell viability

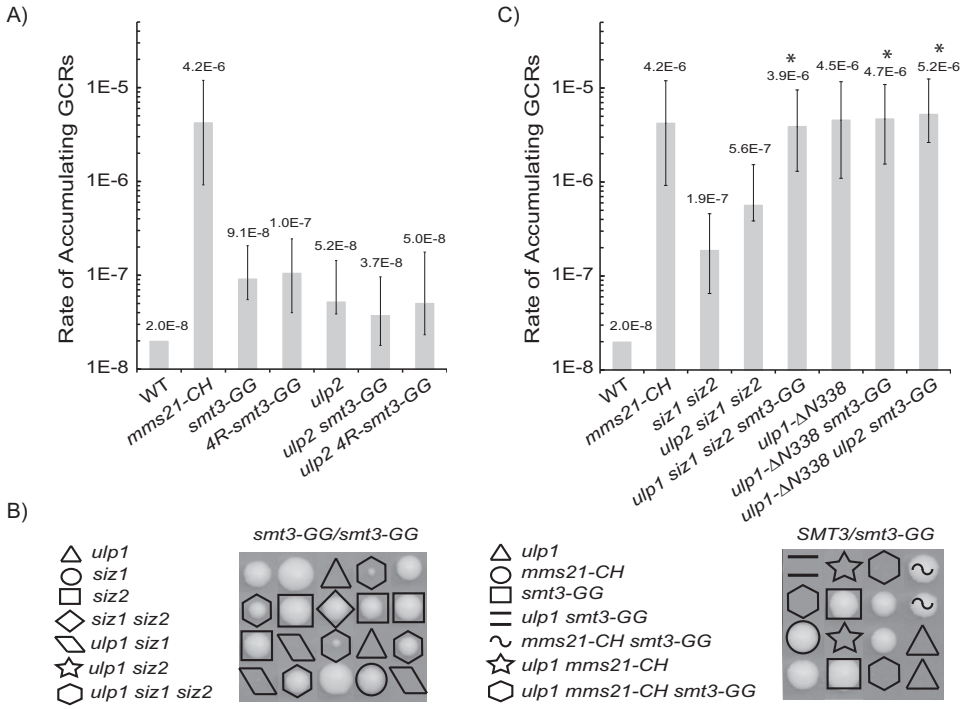

FIGURE 1. **Characterization of the functions of Ulp1 and Ulp2 in genome maintenance and cell viability.** *A*, rate of accumulating GCRs in cells lacking Ulp2 or poly-sumoylation using the *yel072w*::*CAN1/URA3* assay. The *4R-smt3GG* mutation contains a stop codon to remove the last three amino acids of *SMT3* to yield the mature SUMO and the K11R, K15R, K19R, and K27R mutations. *B*, tetrad analysis shows the lethality of *ulp1*Δ is suppressed by the removal of both Siz1 and Siz2, but not the inactivation of Mms21 E3 ligase activity. *C*, rate of accumulating GCRs in cells lacking Ulp1 or the NPC-targeting domain of Ulp1 using the *yel072w*::*CAN1/URA3* assay. *Error bars* represent the upper and lower limits determined by the 95% confidence intervals of the median. The *asterisk* (*) indicates GCR rates of mutants with $\rho < 0.0001$ when compared with the *smt3*-GG strain. Additional information about ρ value is shown in supplemental Fig. 1.

(17, 18, 21). We found this *ulp1-N338*Δ mutation causes a significant increase in the rate of accumulating GCRs comparable with that of the *mms21-CH* mutant and is independent of Ulp2 (Fig. 1*C*). This suggests that mislocalized Ulp1 could either directly desumoylate Mms21 targets or indirectly down-regulate Mms21 activity to cause accumulation of GCRs. Distinguishing these possibilities would require knowledge of Ulp1 and Ulp2 targets and how they are regulated by the localization of Ulp1.

Ulp2 Has a Specific Role in Desumoylating Proteins at the rDNA, Centromere, and Origins of DNA Replication—To identify the *in vivo* targets of Ulp1 and Ulp2, we chose to use our quantitative MS approach as previously described (6). In this approach, after purification of sumoylated proteins, Ulp1 is used to elute sumoylated proteins for MS analysis. This approach provides information on the amount of sumoylated proteins but does not distinguish whether they are poly-sumoylated or mono-sumoylated at one or more lysines, which can be studied using alternative methods. Using this quantitative MS approach, we found that deletion of *ULP2* in the *HF-4R-smt3GG* strain caused substantial increases in three protein complexes located in distinct chromosomal regions, including rDNA (the RENT complex), centromere (inner kinetochore complex), and origins of DNA replication (the MCM complex) (Fig. 2*A* and supplemental Table 2). Among them, loss of Ulp2 caused accumulation of ~20-fold more sumoylated Net1, Cdc14, and Tof2, subunits of the RENT complex in the nucleolus (28, 29). Similarly, the loss of Ulp2 also caused accumulation of ~20-fold more sumoylated Ame1, Mcm21, Okp1, Mcm16, and Mcm22, which are components of the inner kinetochore complex (30–32). This drastic accumulation of sumoylated RENT and inner kinetochore complexes is consistent with them being Ulp2 targets.

Loss of Ulp2 also causes significant accumulations of sumoylated subunits of the MCM complex, the essential replicative DNA helicase (33–36). However, unlike the RENT and inner kinetochore complexes, the effect of Ulp2 removal on MCM sumoylation is subunit-specific. As shown in Fig. 2*A*, loss of Ulp2 resulted in relatively modest changes in the amount of sumoylated Mcm2 and Mcm6 but caused a 4–10-fold accumulation of sumoylated Mcm3, Mcm4, Mcm5, and Mcm7. In addition to these increases in sumoylation, loss of Ulp2 reduced the amount of most other sumoylated proteins (Fig. 2*A* and supplemental Table 2), which is unlikely a direct effect of Ulp2 removal. Because Ulp1 is the remaining SUMO isopeptidase, its activity could be up-regulated to compensate for the loss of Ulp2. Alternatively, loss of Ulp2 may down-regulate the activity of SUMO E3 ligases in cells; although this possibility is difficult to reconcile with the drastic accumulation of sumoylated RENT, kinetochore, and MCM complexes upon Ulp2 removal, which is largely unaffected by the *4R-smt3GG* mutation (supplemental Table 3). Loss of Ulp2 has been shown to cause excessive poly-sumoylation and slow growth (11). However, we found that deletion of *ULP2* in the *HF-SUMO* and *HF-4R-smt3* strains did not cause detectable changes to their cell cycle profile or growth rate (supplemental Fig. 1). One possible explanation is that the N-terminal HF tag on Smt3 in these cells may partially compromise poly-SUMO formation in the absence of

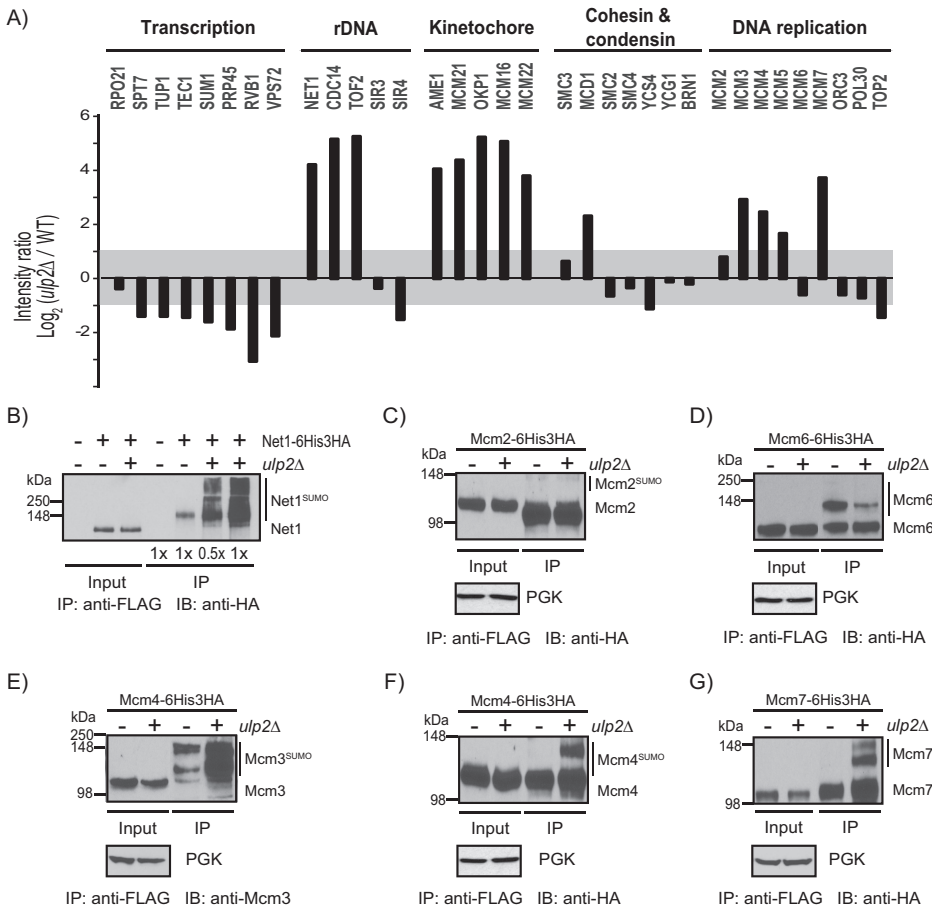

FIGURE 2. **Effect of the loss of Ulp2 on intracellular protein sumoylation.** *A*, effect of loss of Ulp2 on the relative abundance of sumoylated proteins by comparing *ulp2*Δ mutant to the parental strain using quantitative MS. The abundance ratio of each sumoylated protein is shown in log2 scale. *B*, sumoylated Net1-His_6-3×HA in wild type or *ulp2*Δ mutant was purified by nickel-nitrilotriacetic acid and then anti-FLAG affinity resins. The eluted proteins from anti-FLAG resins were probed by anti-HA antibody. Sumoylated Net1 is indicated showing slower electrophoretic mobility compared with un-sumoylated Net1. *IP*, immunoprecipitation; *IB*, immunoblot. *C–G*, sumoylated Mcm2, Mcm3, Mcm4, Mcm6, and Mcm7 in wild type and *ulp2*Δ mutant are analyzed using the same method as in *B*. The strain background used is indicated at the *top of each figure*. In each case sumoylated MCM subunits in the anti-FLAG affinity-purified sample show a slower electrophoretic mobility compared with unmodified MCM subunits, which occasionally appear as a contaminant due to the His_6 tag on MCM subunits and the low stoichiometry of sumoylation of MCM. The same amount of cell lysate was used to purify sumoylated proteins, which was normalized using Bradford assay and confirmed by anti-phosphoglycerate kinase (*PGK*) Western blot.

Ulp2, although this tag has little appreciable effect on cell growth or GCR phenotype in wild type background (6). For this reason all subsequent biochemical experiments were performed using the *HF-SUMO* strain background unless noted otherwise.

To rule out the possibility that the observed changes of sumoylated proteins are due to a change in protein expression and to validate the MS findings, we chose to analyze selected Ulp2 targets further. To this end, we used nickel-nitrilotriacetic acid and anti-FLAG affinity resins to purify total sumoylated proteins from *HF-SUMO* (His_6-3×FLAG-Smt3) strain, which additionally contains Net1-His_6-3×HA, and then probed for the presence of sumoylated Net1 using an anti-HA antibody. As shown in Fig. 2*B*, loss of Ulp2 caused a drastic accumulation of slower migrating and sumoylated species of Net1 in purified sumoylated proteins, whereas the abundance of un-modified Net1 in the cell lysate was unaffected, indicating a specific role of Ulp2 in desumoylating Net1. To study MCM sumoylation, we first evaluated the effect of epitope-tagging of MCM subunits on the rate of accumulating GCRs using the *yel072w*::*CAN1/URA3* assay (6, 23). Because C-terminal tagging of Mcm3 compromises its function (37), a His_6-3×HA tag was introduced to the N terminus of Mcm3 at its endogenous locus, whereas the same His_6-3×HA tag was fused to the C termini of other MCM subunits. We found a modest accumulation of GCRs caused by epitope tagging of most MCM subunits except for Mcm5, whose tagging resulted in a drastic accumulation of GCRs (supplemental Fig. 2). Although this finding implicated a role of MCM in preventing the accumulation of GCRs, sumoylation of Mcm5 was not analyzed further. Moreover, N-terminal tagging of Mcm3 caused a relatively stronger, albeit modest accumulation of GCRs, which prompted us to develop an anti-Mcm3 antibody for its analysis.

After purification of sumoylated proteins in the indicated strains (all containing HF-SUMO), we probed for the presence of sumoylated Mcm2, Mcm3, Mcm4, Mcm6, and Mcm7 using either an anti-HA or an anti-Mcm3 antibody. As shown in Fig. 2*C*, sumoylated Mcm2 in unperturbed wild type cells was not detected in this experiment, and there was no evidence for its accumulation upon the loss of Ulp2. Sumoylated Mcm6 was readily detected in wild type cells and were partially reduced by the loss of Ulp2 (Fig. 2*D*). In contrast, two major sumoylated

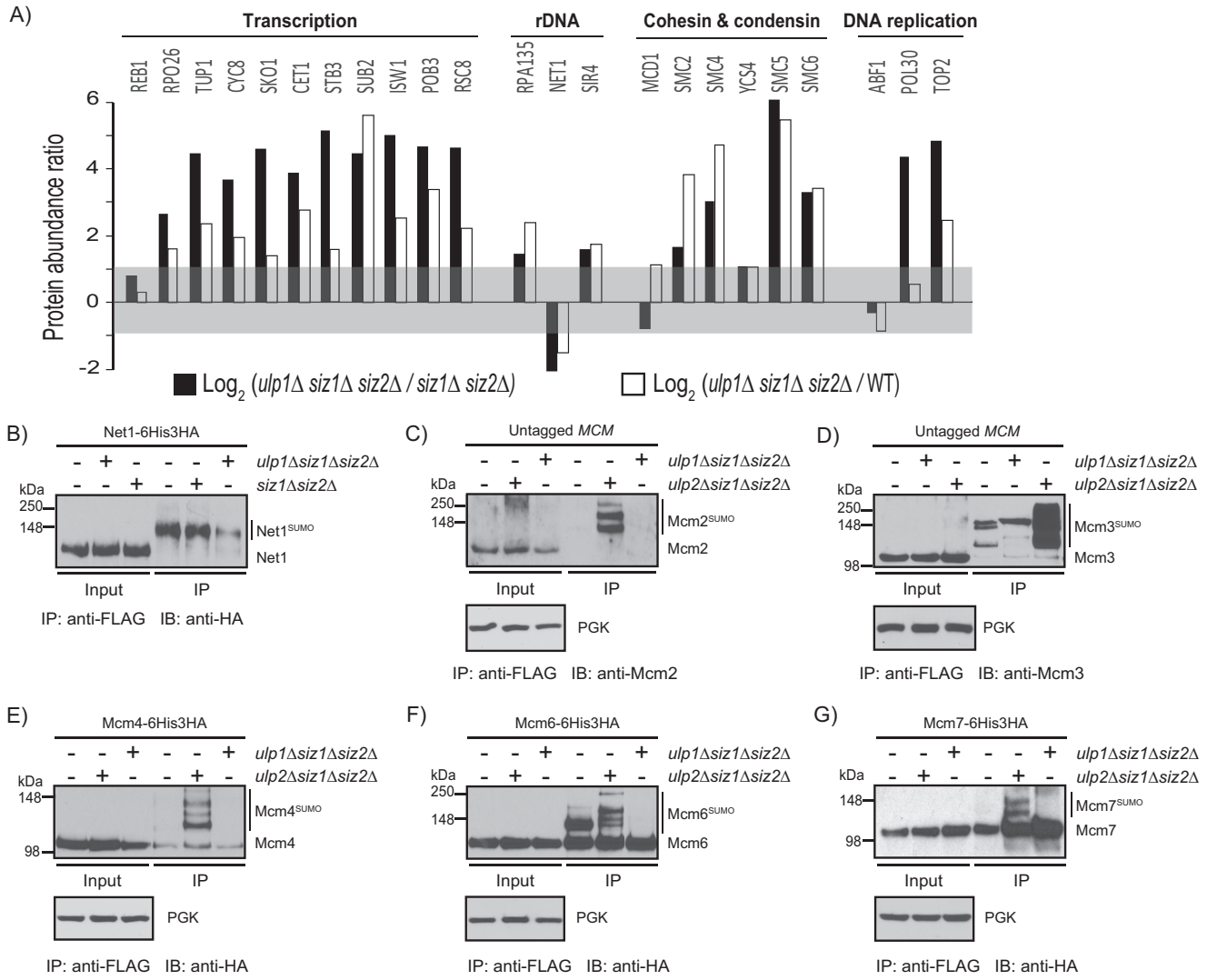

FIGURE 3. **Ulp1 has a broad role in intracellular desumoylation distinct from Ulp2.** *A*, effect of the losses of Ulp1, Siz1, and Siz2 on sumoylated proteins measured by quantitative MS. The abundance ratio of each sumoylated protein is shown in log2 scale. *White bar*, abundance ratio of sumoylated proteins between *ulp1*Δ *siz1*Δ *siz2*Δ and wild type strains. *Black bar*, abundance ratio of sumoylated proteins between *ulp1*Δ *siz1*Δ *siz2*Δ and *siz1*Δ *siz2*Δ mutants. *B*, effects of deleting Ulp1, Siz1, and Siz2 on sumoylated Net1, which was detected as a slower migrating species compared with Net1 in whole cell lysate. *IP*, immunoprecipitation; *IB*, immunoblot. *C–G*, effects of deleting Ulp1 or Ulp2 on sumoylated MCM subunits in cells lacking Siz1 and Siz2. Sumoylated proteins were purified from each strain and detected by various antibodies as indicated using the same method as in Fig. 2*B*. The strain background used is indicated at the *top of each panel*. *PGK*, anti-phosphoglycerate kinase.

species of Mcm3 are present in wild type cells, and both were strongly induced upon the loss of Ulp2 (Fig. 2*E*). Like Mcm2, sumoylated Mcm4 and Mcm7 in wild type cells were not readily detected (36). However, the loss of Ulp2 caused significant accumulations of sumoylated Mcm4 and Mcm7 (Fig. 2, *F* and *G*). In each case, the abundance of each MCM subunit was unaffected by the loss of Ulp2, and sumoylated species of MCM subunits were undetectable in cell lysate without enrichment. Un-sumoylated MCM could still be seen after anti-FLAG immunopurification due to the relatively low stoichiometry of MCM sumoylation. Together, these findings were in general agreement with the MS findings (Fig. 2*A*). It should be noted here that a previous study using C-terminal tagged Mcm3 did not detect sumoylated Mcm3 in unperturbed wild type cells (36). However, C-terminal tagging of Mcm3 has been found to compromise its function (37).

Ulp1 Has a Broad Role in Intracellular Desumoylation Distinct from Ulp2—To identify Ulp1 targets, we applied the same quantitative MS assay to measure the effect of deleting *ULP1* on sumoylated proteins in the *siz1*Δ *siz2*Δ mutant, in which Ulp1 is not essential. As shown in Fig. 3*A* (supplemental Tables 4 and 5), losses of Ulp1, Siz1 and Siz2 caused drastic accumulations of the majority of sumoylated proteins compared with wild type and *siz1*Δ *siz2*Δ mutant, indicating a broad role of Ulp1 for desumoylating most proteins in cells. Interestingly, sumoylation of Net1 was strongly reduced by the loss of Ulp1 (Fig. 3*A*), which we confirmed by Western blotting to detect sumoylated Net1 (Fig. 3*B*). Because Ulp2 has a highly specific role in desumoylating Net1 (Fig. 2*A*), we hypothesized that this reduction of sumoylated Net1 could be explained by elevated Ulp2 activity to compensate for the loss of Ulp1.

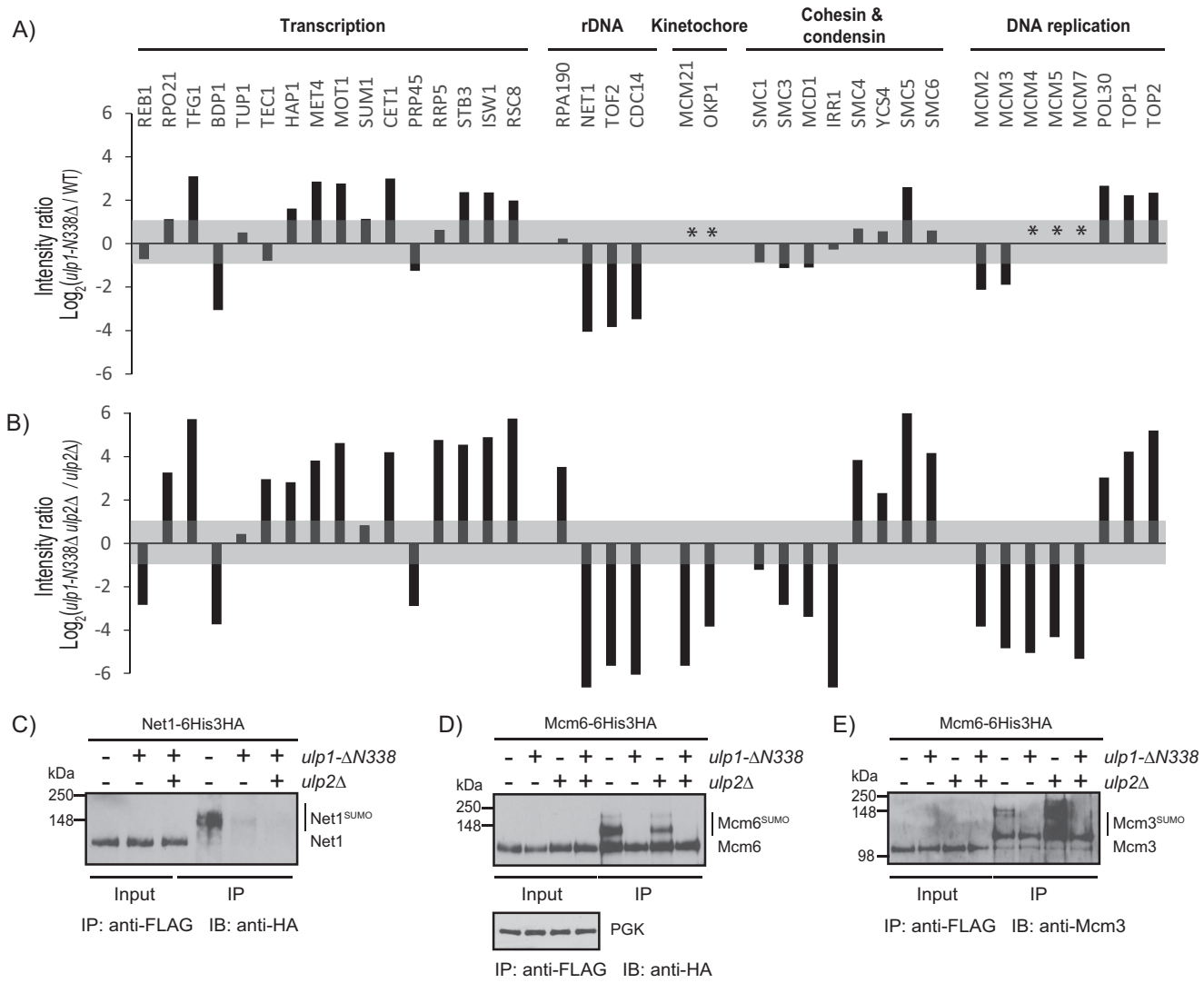

FIGURE 4. **Loss of Ulp1 NPC-targeting domain causes specific desumoylation of Ulp2-specific targets.** *A*, effect of *ulp1-N338*Δ on intracellular protein sumoylation measured by quantitative MS. Asterisk indicates the protein was not identified. *B*, effect of *ulp1-N338*Δ on intracellular protein sumoylation in cells lacking Ulp2 measured by quantitative MS. *C–E*, effect of *ulp1-N338*Δ and *ulp2*Δ mutations on sumoylated Net1, Mcm6, and Mcm3, using the same method as in Fig. 2*B*. The strain background used is indicated at the *top of each panel*. *IP*, immunoprecipitation; *IB*, immunoblot; *PGK*, anti-phosphoglycerate kinase.

In cells lacking Siz1 and Siz2, Mms21 and/or the E2 enzyme Ubc9 are expected to be responsible for the accumulation of sumoylation caused by the loss of Ulp1 (Fig. 3*A*). However, other Ulp2 targets including MCM were below the detection limit of these MS experiments, perhaps due to elevated Ulp2 activity. To investigate the relative specificity of Ulp1 and Ulp2 in desumoylating MCM, we purified total sumoylated proteins and analyzed the presence of sumoylated MCM subunits. As shown in Fig. 3*C*, loss of Ulp2, Siz1, and Siz2 resulted in a drastic accumulation of sumoylated Mcm2, which is below the detection limit in wild type and the *ulp1*Δ *siz1*Δ *siz2*Δ mutant. Similarly, loss of Ulp2, but not Ulp1, caused a more drastic accumulation of sumoylated Mcm3, Mcm4, Mcm6, and Mcm7 (Fig. 3, *D–G*). These findings show that Ulp2 had a more important role than Ulp1 in desumoylating MCM in cells lacking Siz1 and Siz2, whereas Ulp1 had a broad role in desumoylating non-Ulp2 targets in cells.

Localization of Ulp1 Prevents Its Desumoylation of Ulp2-specific Targets—Ulp1 and Ulp2 are known to have different subcellular localizations (15–18), which may contribute to their distinct substrate specificity. To test this we investigated the role of the NPC-targeting domain of Ulp1 (N-terminal 338 amino acid), which directs Ulp1 to the nuclear periphery (17, 18, 21). We found that removal of the Ulp1 NPC-targeting domain caused an ~20-fold reduction of sumoylated Net1 and ~4-fold reduction of sumoylated Mcm2 and Mcm3 (Fig. 4*A* and supplemental Table 6). In contrast, there are significant accumulations of many sumoylated proteins in the *ulp1-N338*Δ mutant, including Pol30, Smc5, and others. This finding shows that mis-localized Ulp1 did not non-specifically desumoylate all nucleoplasmic proteins, but instead specifically reduced the amount of sumoylated Net1 and MCM subunits. The reduction of sumoylated Net1 raises a possibility that Ulp2 could be involved. To test this, we compared sumoylated proteins in

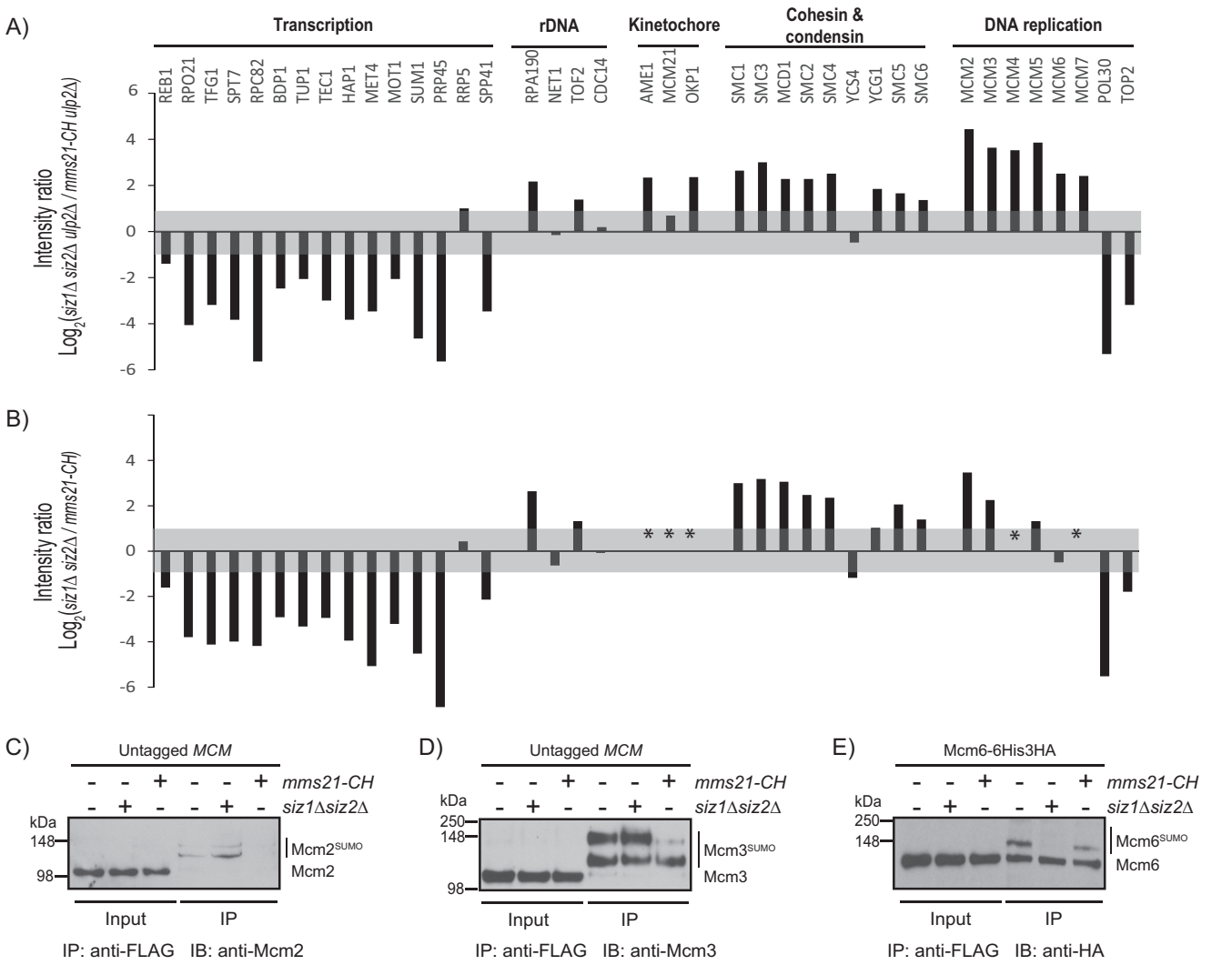

FIGURE 5. **Comparison between *siz1Δ siz2Δ* and *mms21-CH* mutants reveals MCM as a target of Mms21.** *A*, relative abundance of sumoylated proteins in *ulp2Δ siz1Δ siz2Δ* and *ulp2Δ mms21-CH* mutants measured by quantitative MS. *B*, relative abundance of sumoylated proteins in *siz1Δ siz2Δ* and *mms21-CH* mutants measured by quantitative MS. *Asterisks* indicate the protein was not identified. *C–E*, Effect of *siz1Δ siz2Δ* and *mms21-CH* mutations on sumoylated Mcm2, Mcm3, and Mcm6 using the same method as in Fig. 2*B*. The strain background used is indicated at the *top of each panel*. *IP*, immunoprecipitation; *IB*, immunoblot.

ulp1-N338Δ ulp2Δ and *ulp2Δ* mutants using quantitative MS and found that removal of the Ulp1 NPC-targeting domain resulted in significant reductions of sumoylated Net1, Tof2, Cdc14, various kinetochore, and MCM subunits in cells lacking Ulp2 (Fig. 4*B* and supplemental Table 7). The accumulations of many other sumoylated proteins in the *ulp1-N338Δ ulp2Δ* mutant indicate that Ulp1-N338Δ does not non-specifically desumoylate proteins in the nucleoplasm.

In agreement with these MS findings, removal of the Ulp1 NPC-targeting domain largely eliminates sumoylated Net1, and this occurs independent of Ulp2 (Fig. 4*C*). Similarly, sumoylated Mcm6 is strongly reduced upon removal of the Ulp1 NPC-targeting domain, again in an Ulp2-independent manner (Fig. 4*D*). Interestingly, removal of the Ulp1 NPC-targeting domain specifically reduces the amount of slower migrating sumoylated species of Mcm3, which is independent of Ulp2, whereas the faster migrating sumoylated species of Mcm3 is relatively unaffected. Together, these results show that the localization of Ulp1 at the nuclear periphery prevents it from desumoylating Ulp2 targets including Net1 and MCM as well as being necessary for proper desumoylation of other targets.

Roles of E3 Ligases Siz1, Siz2, and Mms21 in Sumoylating Ulp2 Targets—Considering that the accumulations of GCRs in cells lacking Ulp1 or its NPC-targeting domain are comparable with that caused by the loss of Mms21 E3 ligase activity (Fig. 1), we reasoned that these *ulp1* mutations might reduce the amount of sumoylated Mms21 targets. Although the loss of Ulp1 could do so by up-regulating Ulp2 activity to desumoylate Ulp2 targets (Fig. 3), mis-localized Ulp1 could achieve the same task directly (Fig. 4). If so, the question is whether Mms21 preferentially sumoylates Ulp2 targets. To address this, we quantified sumoylated proteins in *ulp2Δ mms21-CH* and *ulp2Δ siz1Δ siz2Δ* mutants using MS. As shown in Fig. 5*A* and supplemental Table 8, sumoylated Septins and Pol30, known Siz1/Siz2 targets, are considerably more abundant in the *ulp2Δ mms21-CH* mutant than in the *ulp2Δ siz1Δ siz2Δ* mutant as expected. On the other hand, known Mms21 targets including the SMC family proteins are more abundant in the *ulp2Δ siz1Δ*

*siz2*Δ mutant than in the *ulp2*Δ *mms21-CH* mutant (6). Among Ulp2 targets, the amount of sumoylated Net1, Cdc14, Tof2, and kinetochore subunits are comparable in cells lacking Siz1/Siz2 or Mms21 E3 ligase activity (Fig. 5*A*). Interestingly, sumoylation of essentially all MCM subunits is considerably more abundant in the *ulp2*Δ *siz1*Δ *siz2*Δ mutant than the *ulp2*Δ *mms21-CH* mutant (Fig. 5*A*), indicating that sumoylation of MCM is preferentially controlled by Mms21 albeit in cells lacking Ulp2.

To investigate whether Mms21 preferentially controls the sumoylation of MCM in cells containing intact Ulp1 and Ulp2, we next quantified sumoylated proteins in *siz1*Δ *siz2*Δ and *mms21-CH* mutants by MS. As shown in Fig. 5*B* and supplemental Table 9, the amount of sumoylated SMCs was more abundant in cells lacking Siz1/Siz2 compared with cells lacking Mms21 E3 ligase activity, whereas sumoylation of Septins and Pol30 are strongly Siz1/Siz2-dependent (6). Interestingly, sumoylation of MCM subunits shows a varying dependence on these E3 ligases. For example, sumoylation of Mcm2 and Mcm3 was more dependent on Mms21, whereas sumoylation of Mcm6 is more specific to Siz1/Siz2 as previously reported (6). In agreement with these MS findings, sumoylated Mcm2 was reduced more by the *mms21-CH* mutation compared with the loss of Siz1 and Siz2 (Fig. 5*C*). Between the two major sumoylated species of Mcm3, the slower migrating species of Mcm3 was specifically reduced by the *mms21-CH* mutation, whereas the loss of Siz1 and Siz2 had little effect (Fig. 5*D*). On the other hand, sumoylated Mcm6 is more strongly reduced by the loss of Siz1 and Siz2 compared with the *mms21-CH* mutation (Fig. 5*E*). These findings revealed that Mms21 more specifically controls the sumoylation of Mcm2 and Mcm3, whereas Siz1 and Siz2 preferentially control the sumoylation of Mcm6.

Regulation of MCM Sumoylation in Response to DNA Replication Stress—A previous study showed that a DNA alkylating agent could induce the sumoylation of MCM, including Mcm2, Mcm4, Mcm5, and Mcm6 but not Mcm3 and Mcm7 (36). To explore a role for MCM sumoylation during DNA replication, we asked whether MCM sumoylation could be induced by hydroxyurea (*HU*) treatment, which caused stalled DNA replication forks without extensive DNA damages. As shown in Fig. 6*A*, hydroxyurea treatment has little effect on sumoylated Mcm2, Mcm4, Mcm6, and Mcm7; however, it caused a significant accumulation of the slower migrating sumoylated species of Mcm3, whereas the faster migrating species of sumoylated Mcm3 is relatively unaffected (Fig. 6*A*). Moreover, Mms21, but not Siz1 and Siz2, was required for the hydroxyurea-induced sumoylated species of Mcm3, detected by anti-Mcm3 and anti-HA antibodies (Fig. 6*B*). It is presently unknown whether this hydroxyurea-induced sumoylation of Mcm3 by Mms21 is a consequence of stalled DNA replication fork or it helps to stabilize stalled replication forks. Nevertheless, this finding suggests that Mms21-dependent sumoylation of Mcm3 might occur during DNA replication. To test this we examined the timing of Mcm3 and Mcm6 sumoylation during the cell cycle. As shown in Fig. 6*C*, the faster migrating species of Mcm3 was relatively unchanged during the cell cycle, whereas the slower migrating species of Mcm3 accumulated during the S phase, and it was reduced in the G_1 and G_2-M phases. In contrast, sumoylated Mcm6 was already present in G_1 cells, persisted as cells entered the early S phase, and disappeared during the G_2-M phase (Fig. 6*C*).

Discussion

Protein sumoylation is essential for cell viability and regulates many nuclear processes (1, 2). Recent studies have revealed a major role for Mms21 in DNA recombination repair and preventing the accumulation of GCRs (6, 7, 36); however, relatively little has been known about the function and substrates of SUMO isopeptidases in genome maintenance. The NPC has been shown to contribute to genome maintenance and regulates the localization of Ulp1, although the mechanism has been insufficiently understood (4, 17–21). Here we report several advances in addressing these questions using *S. cerevisiae* as a model organism.

First, we identified an essential function of Ulp1 in desumoylating the bulk of intracellular sumoylation, which is rescued by the removal of Siz1 and Siz2. Concerning the role of Ulp1 and Ulp2 in genome maintenance, we found that the loss of Ulp2 or elimination of the bulk of poly-sumoylation in cells did not cause appreciable accumulation of GCRs (Fig. 1*A*). Interestingly, either deleting Ulp1 or disrupting its localization at the nuclear periphery caused substantial accumulation of GCRs (Fig. 1*C*). Considering the major role of Mms21 in preventing the accumulation of GCRs (6), reduced sumoylation of certain Mms21 targets might have occurred in these *ulp1* mutants. Although disrupting Ulp1 localization at the nuclear periphery could lead to its aberrant desumoylation of Mms21 targets in the nucleoplasm, reducing sumoylated Mms21 targets by the loss of Ulp1 is expected to be indirect.

Second, understanding the functions of Ulp1 and Ulp2 requires knowledge of their substrates. Here we found that the loss of Ulp2 caused a drastic and specific accumulation of sumoylated proteins at three chromosomal regions, including the RENT complex in the nucleolus and the inner kinetochore complexes at centromeres and specific subunits of the MCM helicase at DNA replication forks (Fig. 2, *A* and *D*). On the other hand, Ulp1 broadly desumoylated many proteins in cells except for these Ulp2 targets (Fig. 3*A*). Interestingly, Ulp1 and Ulp2 appear to compensate for the loss of each other. For example, a broad reduction in many sumoylated proteins caused by the loss of Ulp2 could be explained by elevated Ulp1 activity, whereas reduced sumoylated Net1 in cells lacking Ulp1 is best explained by elevated Ulp2 activity. The distinct substrate specificity of Ulp1 and Ulp2 is further illustrated by their roles in regulating the desumoylation of various MCM subunits (Fig. 3).

Third, the NPC has been shown to regulate Ulp1 localization at the nuclear periphery (4, 17–21). Given the broad specificity of Ulp1 toward many sumoylated proteins, one might speculate that the NPC may restrict the access of Ulp1 toward proteins in the nucleoplasm to prevent aberrant desumoylation. Interestingly, we found that removal of the NPC targeting domain of Ulp1 caused a specific loss of sumoylated Ulp2 targets including MCM in an Ulp2-independent manner, and this was accompanied by the accumulation of many sumoylated proteins in the nucleoplasm (Fig. 4). This observation argues against the model that mis-localized Ulp1 may non-specifically desumoylate all

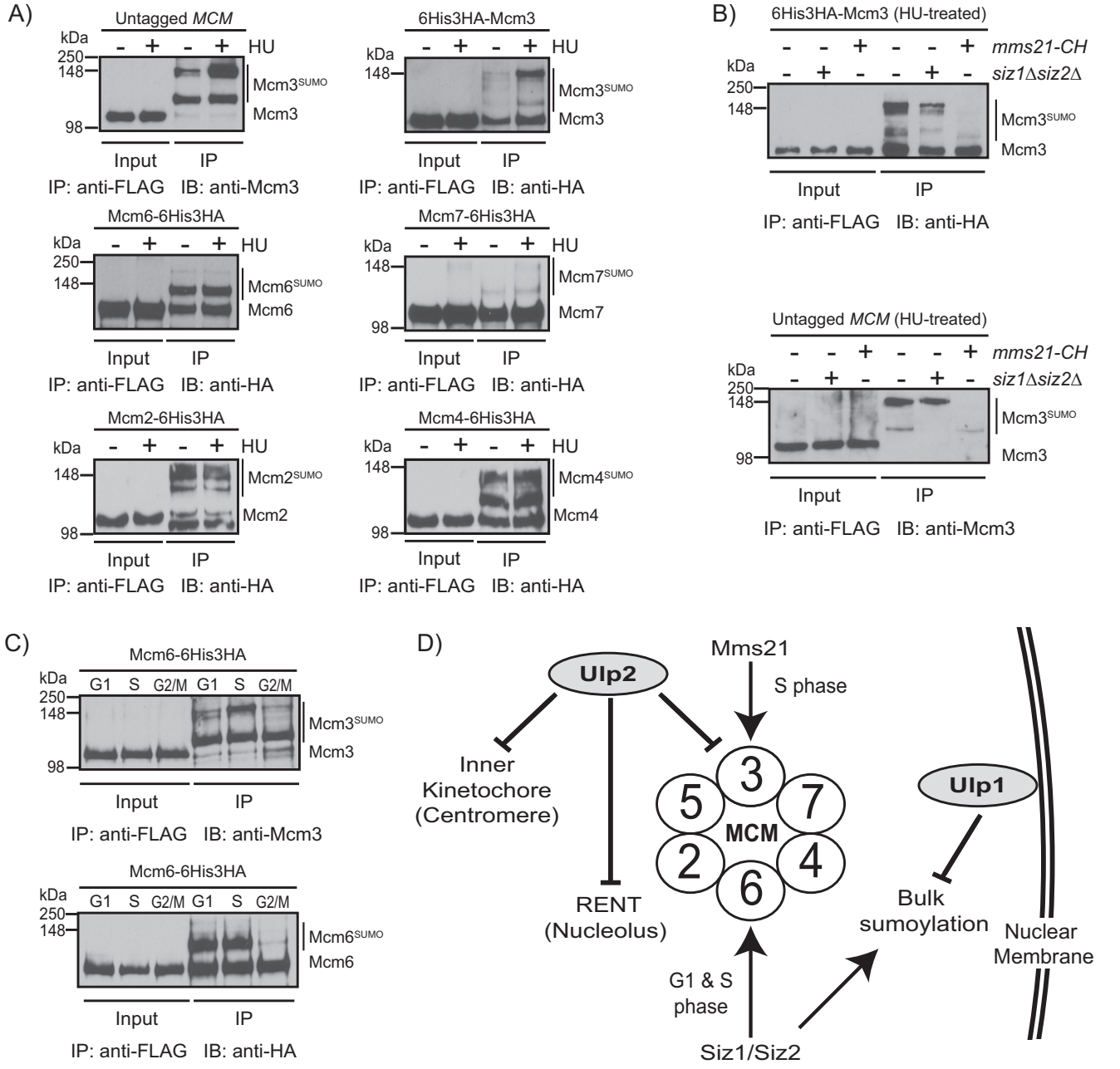

FIGURE 6. **Regulation of MCM sumoylation in response to DNA replication stress and during the cell cycle.** *A*, effect of hydroxyurea (*HU*) treatment on sumoylated MCM subunits. *IP*, immunoprecipitation; *IB*, immunoblot. *B*, effect of *siz1*Δ *siz2*Δ and *mms21-CH* mutations on hydroxyurea-induced sumoylated Mcm3 detected by anti-HA and anti-Mcm3 antibodies. *C*, cell cycle dynamics of sumoylated Mcm3 and Mcm6 after G_1 release into S and G_2/M phase. In each case sumoylated MCM subunits were analyzed using the same method as in Fig. 2*B*. The strain background used is indicated at the *top of each panel*. *D*, regulation of sumoylation of MCM and other Ulp2 targets by the SUMO pathway.

proteins in the nucleoplasm. The accumulation of most sumoylated proteins in cells lacking the NPC-targeting domain could be due to elevated activities of SUMO E3 ligases to counter the activity of mis-localized Ulp1, a characteristic feature of sumoylation homeostasis in which a relatively small number of enzymes could compensate for each other.

Finally, we and others have shown that sumoylation of the SMC family proteins are preferentially controlled by Mms21 (4, 6, 38). Sumoylation of MCM has been reported previously (6, 36), although how the SUMO pathway regulates MCM sumoylation is largely unknown. Interestingly, epitope-tagging of most MCM subunits caused varying degrees of GCR accumulation (supplemental Fig. 2), suggesting a properly functioning MCM is needed to prevent genome rearrangements, which could be compromised by improper epitope tagging. Here we have characterized sumoylation of MCM in greater detail and found that Mms21 preferentially controls the sumoylation of Mcm2 and Mcm3 (Fig. 5). Interestingly, between the two major sumoylated species of Mcm3 found, Mms21 specifically controlled the accumulation of the slower migrating sumoylated species of Mcm3 (Fig. 5), which occurred during S phase and was further induced in response to DNA replication stress (Fig. 6). On the other hand, Siz1 and Siz2 had a major role in regulating sumoylation of Mcm6, which occurred during the G_1 and

early S phases but disappeared during the G_2-M phase. Although Ulp2 had a more important role in desumoylating MCM (Fig. 3), the association of Ulp1 with the nuclear periphery was necessary to prevent its aberrant desumoylation of MCM (Fig. 4), which could contribute to the role of the NPC in genome maintenance (19, 39).

Taken together, our findings here have identified Ulp1 and Ulp2 substrates and revealed that sumoylation of MCM is controlled in a subunit-specific and cell cycle-dependent manner. The observation that reduced sumoylation of MCM is correlated with the accumulation of GCRs raises the hypothesis that sumoylation of Mms21-specific targets including Mcm2 and Mcm3 may contribute to the suppression of genome rearrangements and possibly regulate DNA replication.

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