Mlp-dependent anchorage and stabilization of a desumoylating enzyme is required to prevent clonal lethality

Xiaolan Zhao, Chia-Yung Wu, and Günter Blobel

Laboratory of Cell Biology, The Rockefeller University, New York, NY 10021

Myosin-like proteins 1 and 2 (Mlp1 and Mlp2) form filaments attached to the nucleoplasmic side of the nuclear pore complexes via interaction with the nucleoporin Nup60. Here, we show that Mlps and Nup60, but not several other nucleoporins, are required to localize and stabilize a desumoylating enzyme Ulp1. Moreover, like Mlps, Ulp1 exhibits a unique asymmetric distribution on the nuclear envelope. Consistent with a role in regulating Ulp1, removal of either or both *MLP*s affects the SUMO conjugate pattern. We also show that deleting *MLP*s or the localization domains of Ulp1 results in DNA damage sensitivity and clonal lethality, the latter of which is caused by increased levels of 2-micron circle DNA. Epistatic and dosage suppression analyses further demonstrate that Mlps function upstream of Ulp1 in 2-micron circle maintenance and the damage response. Together, our results reveal that Mlps play important roles in regulating Ulp1 and subsequently affect sumoylation stasis, growth, and DNA repair.

Introduction

The budding yeast myosin-like protein 1 (Mlp1), myosin-like protein 2 (Mlp2), and their mammalian homologue, translocated promoter region (TPR), form fibers extending from the nuclear pore complexes (NPCs) to the nucleoplasm (Cordes et al., 1997; Strambio-de-Castillia et al., 1999). Their unique localization and structural properties suggest that they play important roles in nuclear organization and transport. However, the functions of these proteins were not fully understood. Previous studies showed that lacking Mlps slightly slows the rate of protein import but does not affect mRNA or protein export, suggesting that their roles in nucleocytoplasmic transport are limited (Strambio-de-Castillia et al., 1999; Galy et al., 2004). Recently, it was found that Mlp1, but not Mlp2, is required for the nuclear retention of unspliced mRNAs (Galy et al., 2004). However, these available data do not explain the two major defects associated with deletion of *MLP*s. The first is that *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ are all sensitive to DNA damaging agents, such as the radiation-mimicking drug bleomycin (Galy et al., 2000; Kosova et al., 2000). Second, and most noticeably, colonies of *mlp1*Δ *mlp2*Δ, but not *mlp1*Δ or *mlp2*Δ strains, exhibit numerous indentations reflecting clonal lethality (Galy et al., 2000). This phenotype is referred to as "nibbled colony". It has not been clear how Mlps affect cell growth and the DNA damage response.

Clonal lethality and DNA damage sensitivity are also exhibited by mutants defective in sumoylating and desumoylating enzymes (see below). Small ubiquitin-like modifier (SUMO) provides a reversible, post-translational modification mostly of nuclear proteins and regulates localization, activity, and binding properties of target proteins (Melchior, 2000; Muller et al., 2001; Seeler and Dejean, 2003). Attachment of SUMO requires the activating enzyme (E1), the conjugating enzyme (E2), and the ligases (E3s), whereas removal of SUMO requires desumoylating enzymes (Melchior, 2000). Interestingly, defects in the sumoylating E2 and E3s (unpublished data), as well as two desumoylating enzymes, Ulp1 and Ulp2 (Holm, 1982; Li and Hochstrasser, 2000; Dobson, M., personal communication), can all lead to nibbled colonies and DNA damage sensitivity. Although the underlying mechanism is not clear, these observations suggest that the proper balance of sumoylation stasis is important for growth and DNA repair. Among these enzymes, Ulp1 is localized to the NPCs (Li and Hochstrasser, 2000; Schwienhorst et al., 2000), likely in a complex with karyopherins, the transporters that carry cargo proteins through NPCs (Panse et al., 2003). However, components of NPCs that are required for the docking of Ulp1 have not been identified.

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Correspondence to Günter Blobel: blobel@rockefeller.edu

C.-Y. Wu's present address is Dept. of Biology, Massachusetts Institute of Technology, 31 Ames St. 68-135, Cambridge, MA 02139.

Abbreviations used in this paper: Mlp, myosin-like protein; NPC, nuclear pore complex; SUMO, small ubiquitin-like modifier.

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In this report, we show that Mlps and Nup60 are required to dock and stabilize Ulp1. We also show that defects in this regulation result in disruption of sumoylation stasis, clonal lethality, and DNA damage sensitivity.

Results and discussion

Clonal lethality of *mlp1*Δ *mlp2*Δ is caused by increased levels of 2-micron circle

Our initial genetic analysis revealed that the nibbled phenotype of *mlp1*Δ *mlp2*Δ is not stably inherited, suggesting that it is likely caused by extrachromosomal or epigenetic factors (see Online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200405168/DC1). It was reported that high levels of 2-micron circle, an extrachromosomal plasmid found in most strains of *Saccharomyces cerevisiae*, can result in nibbled colonies (Broach and Volkert, 1991). 2-micron circle is packed into nucleosomes, and replicates and segregates using the same proteins as chromosomal DNA (Broach and Volkert, 1991; Mehta et al., 2002). It normally neither benefits nor harms the host when maintained at 50–100 copies per cell. Nevertheless, overproduction of 2-micron circle by overexpressing proteins unique to the plasmid's amplification system causes clonal lethality and nibbled colonies (Reynolds et al., 1987; Rose and Broach, 1990). A plausible explanation is that increased levels of 2-micron circle can titrate out essential replication and segregation machineries because even a twofold increase can give rise to DNA equivalent to two small yeast chromosomes. To test whether the nibbled phenotype of *mlp1*Δ *mlp2*Δ strains is related to 2-micron circle, we first removed the plasmid and observed that *mlp1*Δ *mlp2*Δ colonies are completely smooth and uniform in size (Fig. 1 A). In fact, the growth of *mlp1*Δ *mlp2*Δ cells that lack 2-micron circle (cir^0) is indistinguishable from that of wild-type cells. Next, we reintroduced 2-micron circle to *mlp1*Δ *mlp2*Δ cir^0 strains. The resulting *mlp1*Δ *mlp2*Δ strains containing the plasmid (cir^+) regained the nibbled colony morphology (Fig. 1 A). Finally, we found that the copy number of 2-micron circle in *mlp1*Δ *mlp2*Δ, but not in either single mutants, is ~2.5-fold higher than in the wild type (Fig. 1, B and C). These results reveal that an elevated level of 2-micron circle is the cause for the nibbled colony morphology of *mlp1*Δ *mlp2*Δ.

Mlps and Nup60 are required to tether a desumoylating enzyme at the nuclear periphery

To understand how Mlps affect 2-micron circle levels, we looked for other mutants that also exhibit nibbled colony morphology. As mentioned earlier, strains defective in a desumoylating enzyme, Ulp1, display a similar phenotype. The similar localization pattern of Mlps and Ulp1, as well as the common defects in colony morphology of their mutants, suggest that they may function in the same pathway. One possibility is that Mlps are required to dock Ulp1 at NPCs. Fluorescence microscopic examination of live cells showed that the localization pattern of Mlps differs from that of nucleoporins. Consistent with a recent report (Galy et al., 2004), nucleoporins are dis-

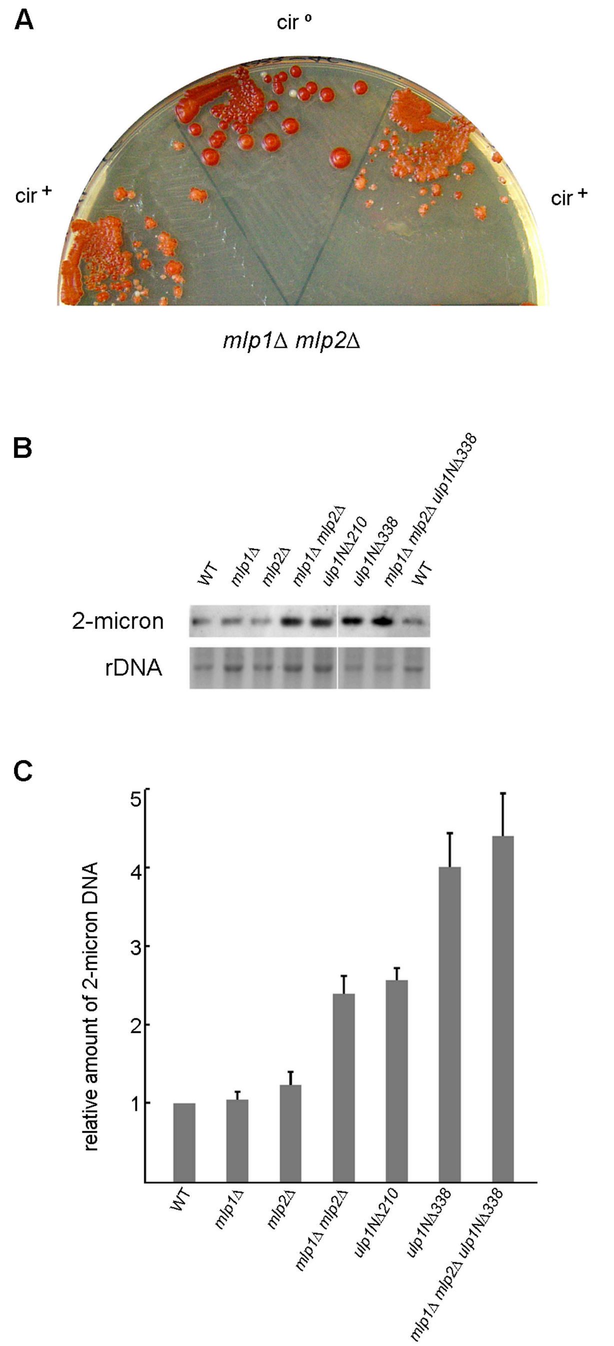

Figure 1. ***mlp1*Δ *mlp2*Δ and *ulp1* mutants contain elevated levels of 2-micron circle.** (A) 2-micron circle causes the nibbled colony morphology in *mlp1*Δ *mlp2*Δ strains. An *mlp1*Δ *mlp2*Δ strain exhibits the nibbled colony morphology (left). This defect is cured by removal of 2-micron circle (middle). Colonies become nibbled again after reintroduction of 2-micron circle to the *mlp1*Δ *mlp2*Δ cir^0 strain (right). (B and C) *mlp1*Δ *mlp2*Δ and *ulp1* mutants contain elevated levels of 2-micron circle. (B) Genomic blot of total DNA extracted from indicated strains was hybridized with a probe specific to the 2-micron circle DNA sequence. rDNA was used as a loading control. (C) 2-micron circle DNA and rDNA bands in B and two additional blots were quantified. The relative amount of 2-micron circle DNA was calculated as the amount of 2-micron circle DNA signal divided by that of rDNA. The relative amount of 2-micron circle DNA in wild-type strains was considered to be 1.

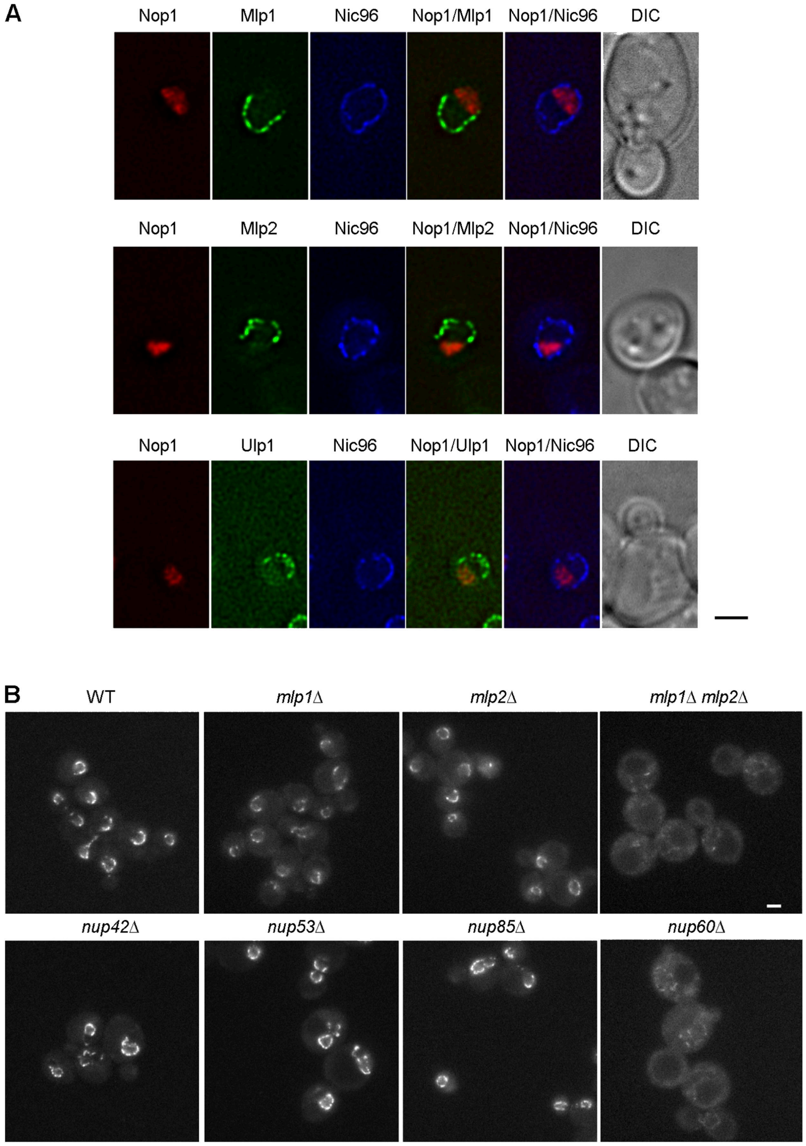

Figure 2. **Mlps and Nup60, but not Nup42, Nup53, or Nup85, are required to dock Ulp1 at the nuclear periphery.** (A) The distribution of Mlps and Ulp1 is different from that of Nic96. Mlp1, Mlp2, and Ulp1 were tagged with YFP, whereas Nic96 was tagged with CFP. A nucleolar protein Nop1 was tagged with mRFP. Representative live-cell images of CFP, YFP, and mRFP fusion proteins as well as their merged pictures are shown. In all panels, mRFP fusion proteins are pseudocolored as red, YFP fusion proteins as green, and CFP fusion proteins as blue. Signals of Mlp1, Mlp2, and Ulp1, but not Nic96, are absent from the region of the nuclear rim juxtaposed to the nucleolus. (B) Mlps and Nup60 are required to dock Ulp1 at NPCs. Fluorescent images of Ulp1-YFP in live cells were taken at 14 Z-sections (step size = 0.3 μm), and the center sections are shown. All the images were acquired using the same settings for the camera and the microscope. Bars, 2 μm.

tributed all around the nuclear envelope, whereas Mlps are excluded from the region juxtaposed to the nucleolus (Fig. 2 A). We reasoned that if Mlps provide docking sites for Ulp1, Ulp1 should exhibit the same asymmetric distribution. To test this idea, Ulp1-YFP fusion protein was expressed in cells containing CFP-tagged nucleoporin Nic96 and mRFP-tagged nucleolar protein Nop1. Examination by fluorescence microscopy revealed that Ulp1-YFP is also excluded from the nucleolus-proximal region of the nuclear envelope (Fig. 2 A). Therefore, the localization pattern of Ulp1 resembles that of Mlps.

To explore further the relationship between Mlps and Ulp1, we examined the localization of Ulp1-YFP in *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ cells. We observed that the signal intensity of Ulp1-YFP at the nuclear rim decreases moderately in *mlp1*Δ or *mlp2*Δ strains compared with wild-type strains (Fig. 2 B). Because the protein level of Ulp1-YFP in these mutants is the same as in wild-type strains (Fig. 3 A), the reduction of nuclear rim signals suggests that some Ulp1 proteins are delocalized. In *mlp1*Δ *mlp2*Δ double mutants, Ulp1-YFP signals at the nuclear rim are greatly reduced (Fig. 2 B). In addition, this is associated with a fivefold reduction of Ulp1 protein levels (Fig. 3 A). These results indicate that lacking both Mlp1 and Mlp2 synergistically affects Ulp1 protein levels and localization. The simplest explanation is that severe delocalization of Ulp1 due to the lack of both Mlps leads to the reduced protein levels.

It has been shown that perturbation of Ulp1 localization by deleting its localization domain (see below) can lead to accumulation and diminution of different SUMO conjugates without affecting its SUMO precursor processing activity (Li and Hochstrasser, 2003). We suspected that loss of either or both Mlps would affect the sumoylation stasis similarly. To assess the global effect on SUMO conjugates, we examined sumoylated protein patterns in various mutants. As shown in Fig. 3 B, two sumoylated proteins accumulate in *mlp1*Δ *mlp2*Δ, but not in *mlp1*Δ, *mlp2*Δ, or wild-type strains, suggesting that severe reduction of Ulp1 at the nuclear rim impedes desumoylation. In addition, two sumoylated protein bands disappear in all three mutants, consistent with the idea that delo-

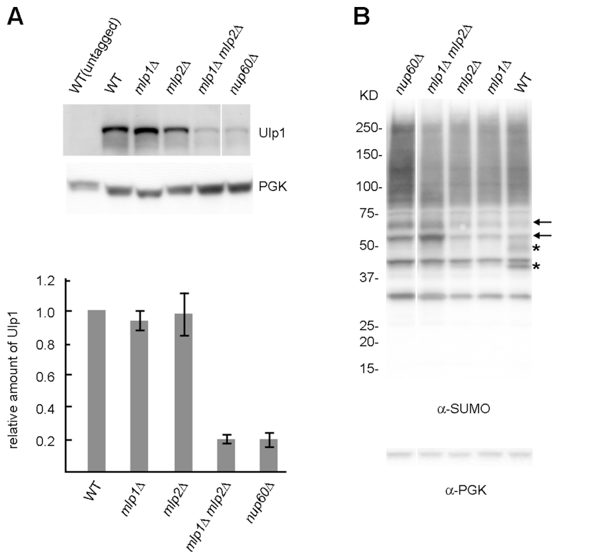

Figure 3. **Ulp1 levels and the SUMO conjugate pattern are changed in cells lacking Mlps or Nup60.** (A) Total protein was extracted from indicated strains and subjected to immunoblot analysis using anti-GFP antibody. *ULP1-YFP* was used to replace the endogenous *ULP1* in all strains, except in WT (untagged). The same blot was stripped and reprobed with anti-PGK antibody to check loading consistency. In the bottom panel, Ulp1-YFP and PGK bands were quantified and the relative amount of Ulp1 was calculated as the amount of Ulp1-YFP signals divided by PGK signals. The value for wild-type strains was considered to be 1. Error bars indicate SDs from three similar blots. (B) Immunoblot analysis of yeast lysates prepared from indicated strains was performed using anti-SUMO antibody. The same blot was stripped and reprobed with anti-PGK antibody to check loading consistency. Arrows indicate the SUMO conjugates whose levels increase in *mlp1Δ mlp2Δ* and *nup60Δ* strains. The asterisks mark the SUMO conjugates whose levels diminish in *mlp1Δ*, *mlp2Δ*, *mlp1Δ mlp2Δ*, and *nup60Δ* strains.

calized Ulp1 gains access to deconjugate noncanonical substrates. Therefore, like mutations in Ulp1 localization domains, mutations in Mlps can affect sumoylation bidirectionally. It is likely that SUMO conjugates, whose level changed in the *mlp1Δ mlp2Δ* strain, play important roles in cellular processes that are defective in this strain.

Mlps are anchored to NPCs via interaction with the nucleoporin Nup60 (Feuerbach et al., 2002). If Mlps are required to localize and stabilize Ulp1, Nup60 should also be involved in regulating Ulp1. Consistent with this idea, we observed an attenuation of nuclear rim signals of Ulp1-YFP in *nup60Δ* strains (Fig. 2 B). This defect is specific to *nup60Δ*, as Ulp1 localization is not affected by deletions of several other nucleoporins, such as Nup53, Nup85, and Nup42 (Fig. 2 B). Moreover, similar to *mlp1Δ mlp2Δ* strains, the level of Ulp1 protein is reduced about fivefold, and the sumoylated protein pattern is also similarly altered in the *nup60Δ* strains (Fig. 3, A and B). These results suggest that an ordered assembly, in which Mlps bridge between Nup60 and Ulp1, likely occurs in cells. Interestingly, *nup60Δ* strains also exhibit the nibbled colony morphology, which can be rescued by removal of 2-micron circle (unpublished data). This finding is in concordance with observations made in the *mlp1 mlp2* double mutant, strengthening the correlation between delocalization/destabilization of Ulp1 and increased levels of 2-micron circle.

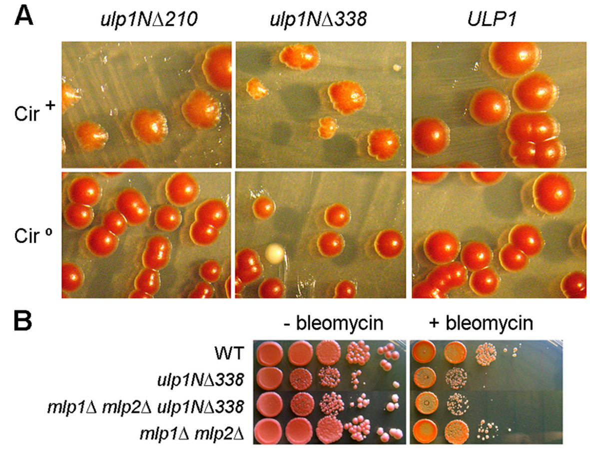

Figure 4. ***ulp1* mutants exhibit the nibbled colony phenotype and *ulp1NΔ338* is epistatic to *mlp1Δ mlp2Δ* for bleomycin sensitivity.** (A) *ulp1NΔ210* and *ulp1NΔ338* strains grow as nibbled colonies on agar (top). This defect is cured by removal of 2-micron circle (bottom). (B) Mid-log phase YPD-grown cells were spotted at 10-fold serial dilutions (from 10^5 to 10) onto plates with or without bleomycin.

Deleting the localization domains of Ulp1 can lead to elevated levels of 2-micron circle and nibbled colony morphology

To investigate the relationship between Ulp1 and 2-micron circle directly, we tested if delocalization of Ulp1 with its anchoring proteins intact can result in elevated levels of 2-micron circle and nibbled colonies. Ulp1 contains two separate domains: the COOH-terminal catalytic domain (residues 403–612) and the NH_2-terminal regulatory domain (residues 1–340), which is necessary and sufficient for NPC localization (Li and Hochstrasser, 2003; Panse et al., 2003). It was previously shown that gradual deletions of the NH_2-terminal domain lead to increased delocalization of Ulp1 from the nuclear rim without affecting its enzymatic activity (Li and Hochstrasser, 2003; Panse et al., 2003). Therefore, we examined the effect of two NH_2-terminal deletion constructs, *ulp1NΔ210* (residues 1–209 deleted) and *ulp1NΔ338* (residues 1–337 deleted), on 2-micron circle and colony morphology. Ulp1NΔ210 contains some sequences required for nuclear rim localization, whereas ulp1NΔ338 lacks all sequences for docking (Panse et al., 2003). As shown in Fig. 4 A, *ulp1NΔ210* and *ulp1NΔ338* colonies are nibbled, and this defect is rescued by removal of 2-micron circle. Furthermore, the copy number of 2-micron circle in *ulp1NΔ210* and *ulp1NΔ338* mutants increases ∼2.5- and ∼4-fold, respectively (Fig. 1, B and C). Therefore, delocalization of Ulp1 by deleting its NH_2 terminus results in higher levels of 2-micron circle, and the severity of delocalization is positively correlated with the levels of 2-micron circle.

Ulp1 functions downstream of Mlps in 2-micron circle maintenance and DNA damage response

The results described above raise the possibility that increased levels of 2-micron circle and the nibbled phenotype of *mlp1Δ mlp2Δ* strains are due to impaired function of Ulp1. However, it was formally possible that other defects of *mlp1Δ mlp2Δ* strains, unrelated to Ulp1, may have resulted in

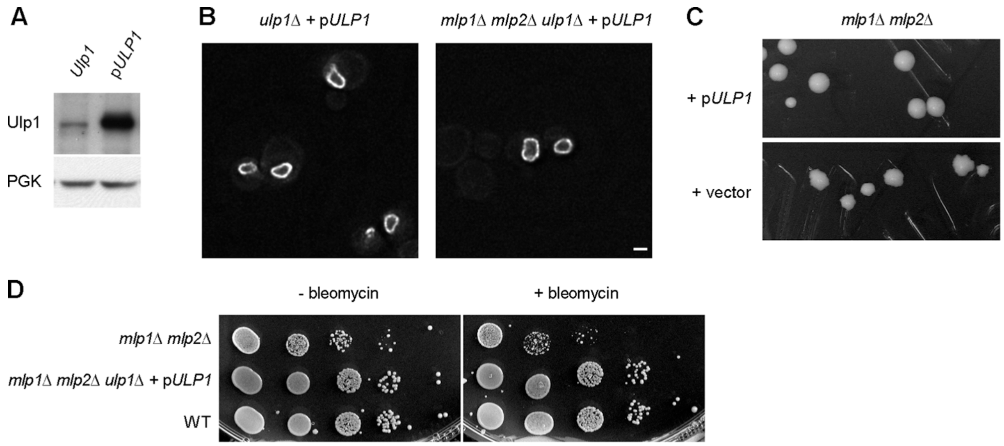

Figure 5. **Overexpression of Ulp1 restores its localization at the nuclear envelope and rescues the nibbled colony morphology as well as the bleomycin sensitivity of *mlp1*Δ *mlp2*Δ strains.** (A) Ulp1 proteins, tagged with GFP, were expressed from the *ULP1* promoter (Ulp1) or from the *NOP1* promoter on a CEN plasmid (p*ULP1*). In the latter case, the endogenous *ULP1* gene was deleted. Total protein was extracted and subjected to immunoblot analysis using polyclonal anti-GFP antibody. The same blot was stripped and reprobed with anti-PGK antibody to check loading consistency. (B) Ulp1 tagged with GFP was expressed from the *NOP1* promoter in *ulp1*Δ or *mlp1*Δ *mlp2*Δ *ulp1*Δ strains. Fluorescent images of Ulp1-GFP in live cells were taken at 14 Z-sections (step size = 0.3 μm), and the center sections are shown. Bar, 2 μm. (C) The *mlp1*Δ *mlp2*Δ strain was transformed with p*ULP1* or with a vector. Transformants were streaked out on selective medium and grown at 30°C for 3 d before being photographed. (D) Sensitivity to bleomycin (5 μg/ml) was tested for the *mlp1*Δ *mlp2*Δ *ulp1*Δ strain containing p*ULP1*, the *mlp1*Δ *mlp2*Δ strain containing the vector, and the wild-type strain containing the vector. Mid-log phase SC-Leu–grown cells were spotted at 10-fold serial dilutions (from 10^5 to 10 cells) onto plates with or without bleomycin.

such a phenotype. To distinguish these possibilities, we took advantage of the differences in degrees of delocalization and amounts of 2-micron circle seen in *mlp1*Δ *mlp2*Δ and *ulp1N*Δ*338* mutants. If the first possibility is correct, a *ulp1* mutation that completely abolishes its nuclear rim localization, such as *ulp1N*Δ*338*, should be epistatic to *mlp1*Δ *mlp2*Δ; i.e., an *mlp1*Δ *mlp2*Δ *ulp1N*Δ*338* triple mutant should resemble *ulp1N*Δ*338*. As shown in Fig. 1 (B and C), *mlp1*Δ *mlp2*Δ *ulp1N*Δ*338* and *ulp1N*Δ*338* strains contain similar amounts of 2-micron circle, which are higher than that of *mlp1*Δ *mlp2*Δ strains. Interestingly, a similar epistatic relationship between *mlp1*Δ *mlp2*Δ and *ulp1N*Δ*338* was also seen for the sensitivity to the DNA damaging agent bleomycin (Fig. 4 B). These results suggest that Ulp1 functions downstream of Mlps in pathways that control 2-micron copy number and DNA repair.

Overexpression of Ulp1 suppresses clonal lethality and DNA damage sensitivity of *mlp1*Δ *mlp2*Δ

It is noteworthy that other tethering sites for Ulp1 exist, as residual Ulp1-YFP signals were seen at the nuclear rim in cells lacking Mlps (Fig. 2 B). The fact that these sites cannot substitute for Mlps to dock Ulp1 when Ulp1 is expressed at the endogenous level suggests that they might be less efficient. Should this be the case, they might be able to dock a sufficient amount of Ulp1 when Ulp1 is overexpressed. We found that Ulp1 exhibits continuous nuclear envelope localization, including the region juxtaposed to the nucleolus when it is moderately overexpressed from the *NOP1* promoter, (Fig. 5, A and B). This is different from the Mlp-dependent localization pattern, which is punctuate and excluded from the nucleolus, indicating that alternative tethering of Ulp1 occurs. Consistently, this localization pattern is independent of Mlps (Fig. 5 B). The restoration of Ulp1 localization under overexpression conditions can rescue the nibbled colony morphology and bleomycin sensitivity of *mlp1*Δ *mlp2*Δ strains (Fig. 5, C and D). This result confirms the conclusion from our epistatic analysis and strongly supports the notion that the nibbled colony morphology and bleomycin sensitivity in *mlp1*Δ *mlp2*Δ strains are due to defects in regulating Ulp1.

In summary, our results demonstrate that Mlps and Nup60 are required to anchor and stabilize Ulp1. We also show that other binding sites for Ulp1 exist and can provide sufficient anchoring when Ulp1 is overexpressed. Although the nature of these alternative sites is not clear, we speculate that they can be nucleoporins or other proteins located at the nuclear envelope. Tethering of Ulp1 could be mediated by Kap60, Kap95, and Kap123, as these karyopherins interact with nucleoporins, Mlps, and Ulp1 (Kosova et al., 2000; Allen et al., 2001; Panse et al., 2003). The last interaction is RanGTP-insensitive and different from the transient transporter–cargo interaction, suggesting that it can mediate the tethering (Panse et al., 2003). However, other forms of interaction are also possible, as the mammalian homologue of Ulp1 can bind directly to the nucleoporin Nup153 (Hang and Dasso, 2002; Zhang et al., 2002). Further work will be required to understand the biochemical interactions used in Ulp1 anchoring. Our data also show that decreased levels of Ulp1 are associated with a severe reduction of its nuclear rim signals. One possible explanation is that drastically delocalized Ulp1 desumoylates and activates proteins involved in its own degradation. This may be a means to protect cells from the catastrophic consequence of excessive and unregulated desumoylating activity.

The existence of multiple pathways for tethering Ulp1 illustrates the importance of its localization at the nuclear periphery. Ulp1 exhibits a stronger desumoylating activity in vitro than the nucleoplasm-localized Ulp2 (Li and Hochstrasser, 2000). Sequestration can prevent Ulp1 from competing with Ulp2 and from desumoylating proteins in an unregulated manner. On the other hand, the localization at the NPC may also play an active role, as Ulp1 can remove SUMO from sumoylated cargo proteins during their passage through the NPC channel. Thus, releasing Ulp1 from the NPCs can perturb the sumoylation stasis by eliciting undesired desumoylation as well as abolishing normal desumoylation. This is exemplified by the reduction and accumulation of different SUMO conjugates in *mlp1Δ mlp2Δ* strains. Observations consistent with this view were made previously using various deletion constructs of Ulp1 (Li and Hochstrasser, 2003). As detachment of SUMO can change properties of target proteins involved in various pathways (Melchior, 2000; Muller et al., 2001; Seeler and Dejean, 2003), impairment of Ulp1 function can undermine many cellular functions, such as 2-micron circle maintenance and the DNA damage response as seen in *ulp1* and *mlp1Δ mlp2Δ* strains. Identification of proteins whose sumoylation status is affected in *mlp1Δ mlp2Δ* strains will help to elucidate the molecular pathways downstream of Mlps and Ulp1.

Materials and methods

Removal and reintroduction of 2-micron circle

To remove 2-micron circle DNA, we used the method described by Tsalik and Gartenberg (1998). To reintroduce 2-micron circle, we mated a cir^0 haploid to a cir^+ haploid. The resulting diploid contains a normal amount of 2-micron circle due to amplification of 2-micron circle contributed from the cir^+ parental strain (Broach and Volkert, 1991). Consequently, spore clones obtained from such a diploid strain inherit a normal amount of 2-micron circle.

Measurement of the level of 2-micron circle

Total yeast DNA was digested with HindIII and separated on a 0.8% agarose gel. DNA fragments were transferred to the nitrocellulose membrane using a standard genomic blot protocol. The membrane was subjected to hybridization using a 500-bp 2-micron–specific probe generated by PCR with the primer pairs "2μm D proteinF" (AATCTGTCCATTGAATGCCT) and "2μm D proteinR" (ATATACTATCTGTTTCAGGGA). Hybridization and detection were performed using the North2South direct HRP labeling and detection kit following the instructions from the manufacturer (Pierce Chemical Co.). Hybridization bands corresponding to 2-micron circle were scanned using a densitometer and quantified using ImageQuant software. The intensity of rDNA bands stained with ethidium bromide was also quantified and was used as a loading control.

Fluorescence microscopy

Cells were prepared and images were taken as described previously (Lisby et al., 2001). In brief, cells were grown at 25°C in synthetic complete (SC) or synthetic complete without leucine (SC-Leu) medium to the log phase and then were processed for imaging at 25°C in the SC or SC-Leu medium. Images were captured with a CoolSNAP CCD camera mounted on a DeltaVision microscope (Applied Precision) at the Bio-Imaging Center (The Rockefeller University, New York, NY). All images were captured at 100-fold magnification using a Plan-Apochromat objective lens (100×, 1.35 NA). Images were acquired and processed using softWoRx software (Applied Precision). Selected images were pseudocolored for presentation using Adobe Photoshop.

Protein extraction and immunoblot analysis

Yeast proteins were extracted as described previously (Yaffe and Schatz, 1984). Total yeast lysates were separated on SDS-PAGE gels and subjected to immunoblot analysis using anti-GFP antibody in Fig. 3 A and Fig. 5 A or anti-SUMO antibody (Johnson et al., 1997) in Fig. 3 B. Membranes were stripped and reprobed with anti-PGK antibody (Santa Cruz Biotechnology, Inc.) to check the consistency of loading.

Online supplemental material

Supplemental material includes the result that the nibbled colony morphology of *mlp1Δ mlp2Δ* mutants is not stably inherited. It also contains yeast strains, plasmids, and growth condition. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200405168/DC1.

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References

Allen, N.P., L. Huang, A. Burlingame, and M. Rexach. 2001. Proteomic analysis of nucleoporin interacting proteins. *J. Biol. Chem.* 276:29268–29274.

Broach, J.R., and F.C. Volkert. 1991. Circular DNA plasmids of yeasts. *In* The Molecular and Cellular Biology of the Yeast *Saccharomyces*. Vol. 1. J.R. Broach, J.R. Pringle, and E.W. Jones, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 297–331.

Cordes, V.C., S. Reidenbach, H.R. Rackwitz, and W.W. Franke. 1997. Identification of protein p270/Tpr as a constitutive component of the nuclear pore complex-attached intranuclear filaments. *J. Cell Biol.* 136:515–529.

Feuerbach, F., V. Galy, E. Trelles-Sticken, M. Fromont-Racine, A. Jacquier, E. Gilson, J.C. Olivo-Marin, H. Scherthan, and U. Nehrbass. 2002. Nuclear architecture and spatial positioning help establish transcriptional states of telomeres in yeast. *Nat. Cell Biol.* 4:214–221.

Galy, V., J.C. Olivo-Marin, H. Scherthan, V. Doye, N. Rascalou, and U. Nehrbass. 2000. Nuclear pore complexes in the organization of silent telomeric chromatin. *Nature.* 403:108–112.

Galy, V., O. Gadal, M. Fromont-Racine, A. Romano, A. Jacquier, and U. Nehrbass. 2004. Nuclear retention of unspliced mRNAs in yeast is mediated by perinuclear Mlp1. *Cell.* 116:63–73.

Hang, J., and M. Dasso. 2002. Association of the human SUMO-1 protease SENP2 with the nuclear pore. *J. Biol. Chem.* 277:19961–19966.

Holm, C. 1982. Clonal lethality caused by the yeast plasmid 2 mu DNA. *Cell.* 29:585–594.

Johnson, E.S., I. Schwienhorst, R.J. Dohmen, and G. Blobel. 1997. The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *EMBO J.* 16:5509–5519.

Kosova, B., N. Pante, C. Rollenhagen, A. Podtelejnikov, M. Mann, U. Aebi, and E. Hurt. 2000. Mlp2p, a component of nuclear pore attached intranuclear filaments, associates with Nic96p. *J. Biol. Chem.* 275:343–350.

Li, S.J., and M. Hochstrasser. 2000. The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. *Mol. Cell. Biol.* 20:2367–2377.

Li, S.J., and M. Hochstrasser. 2003. The Ulp1 SUMO isopeptidase: distinct domains required for viability, nuclear envelope localization, and substrate specificity. *J. Cell Biol.* 160:1069–1081.

Lisby, M., R. Rothstein, and U.H. Mortensen. 2001. Rad52 forms DNA repair and recombination centers during S phase. *Proc. Natl. Acad. Sci. USA.* 98:8276–8282.

Mehta, S., X.M. Yang, C.S. Chan, M.J. Dobson, M. Jayaram, and S. Velmurugan. 2002. The 2 micron plasmid purloins the yeast cohesin complex: a mechanism for coupling plasmid partitioning and chromosome segregation? *J. Cell Biol.* 158:625–637.

Melchior, F. 2000. SUMO–nonclassical ubiquitin. *Annu. Rev. Cell Dev. Biol.* 16:591–626.

Muller, S., C. Hoege, G. Pyrowolakis, and S. Jentsch. 2001. SUMO, ubiquitin's mysterious cousin. *Nat. Rev. Mol. Cell Biol.* 2:202–210.

Panse, V.G., B. Kuster, T. Gerstberger, and E. Hurt. 2003. Unconventional tethering of Ulp1 to the transport channel of the nuclear pore complex by karyopherins. *Nat. Cell Biol.* 5:21–27.

Reynolds, A.E., A.W. Murray, and J.W. Szostak. 1987. Roles of the 2 microns gene products in stable maintenance of the 2 microns plasmid of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7:3566–3573.

Rose, A.B., and J.R. Broach. 1990. Propagation and expression of cloned genes in yeast: 2-microns circle-based vectors. *Methods Enzymol.* 185:234–279.

Schwienhorst, I., E.S. Johnson, and R.J. Dohmen. 2000. SUMO conjugation and deconjugation. *Mol. Gen. Genet.* 263:771–786.

Seeler, J.S., and A. Dejean. 2003. Nuclear and unclear functions of SUMO. *Nat. Rev. Mol. Cell Biol.* 4:690–699.

Strambio-de-Castillia, C., G. Blobel, and M.P. Rout. 1999. Proteins connecting the nuclear pore complex with the nuclear interior. *J. Cell Biol.* 144: 839–855.

Tsalik, E.L., and M.R. Gartenberg. 1998. Curing *Saccharomyces cerevisiae* of the 2 micron plasmid by targeted DNA damage. *Yeast.* 14:847–852.

Yaffe, M.P., and G. Schatz. 1984. Two nuclear mutations that block mitochondrial protein import in yeast. *Proc. Natl. Acad. Sci. USA.* 81:4819–4823.

Zhang, H., H. Saitoh, and M.J. Matunis. 2002. Enzymes of the SUMO modification pathway localize to filaments of the nuclear pore complex. *Mol. Cell. Biol.* 22:6498–6508.