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Fission Yeast Dma1 Requires RING Domain Dimerization for Its Ubiquitin Ligase Activity and Mitotic Checkpoint Function*

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Background: Dma1 inhibits cytokinesis during a mitotic checkpoint by ubiquitinating Sid4, a major scaffold for the Polo-like kinase Plo1.
Results: Dma1 RING domain dimerization is required for proper localization, E3 activity, and checkpoint function.
Conclusion: Dma1 dimerization is required to efficiently ubiquitinate its substrates.
Significance: Dma1 is related to the human checkpoint proteins CHFR and RNF8, which might have similar structural properties as Dma1.

In fission yeast (*Schizosaccharomyces pombe*), the E3 ubiquitin ligase Dma1 delays cytokinesis if chromosomes are not properly attached to the mitotic spindle. Dma1 contains a C-terminal RING domain, and we have found that the Dma1 RING domain forms a stable homodimer. Although the RING domain is required for dimerization, residues in the C-terminal tail are also required to help form or stabilize the dimeric structure because mutation of specific residues in this region disrupts Dma1 dimerization. Further analyses showed that Dma1 dimerization is required for proper localization at spindle pole bodies and the cell division site, E3 ligase activity, and mitotic checkpoint function. Thus, Dma1 forms an obligate dimer via its RING domain, which is essential for efficient transfer of ubiquitin to its substrate(s). This study further supports the mechanistic paradigm that many RING E3 ligases function as RING dimers.

E3 ubiquitin ligases facilitate the final step in protein ubiquitination by promoting transfer of ubiquitin (Ub)[2] from the E2 enzyme to a target lysine residue on the substrate (1). Two distinct classes of E3 ligases exist, which are classified by the presence of either a RING or HECT domain. RING domain E3 ligases have been thought to act mainly as scaffolds to bring the E2∼Ub complex in proximity to the substrate (2); however, recent evidence suggests that RING domains might also allosterically activate their cognate E2 (3). In contrast, HECT domain E3 ligases first ligate ubiquitin to an active cysteine residue on themselves before actively catalyzing ubiquitination of the substrate (4). In addition to either a HECT or RING domain, many E3 ligases also contain a substrate recognition motif that provides substrate specificity.

The *Schizosaccharomyces pombe* checkpoint protein Dma1 is a RING E3 ligase that inhibits cytokinesis if chromosomes are not segregated properly (5, 6). Dma1 contains a C-terminal RING domain and an N-terminal phospho-binding FHA domain, which is required for its localization to spindle pole bodies (SPBs) and the cell division site and likely confers substrate specificity to Dma1 (5). During a mitotic checkpoint arrest, Dma1 ubiquitinates Sid4, a major scaffold for the Polo-like kinase Plo1, and thereby impedes Plo1 localization to SPBs (7). Because SPB-localized Plo1 is required for activation of the septation initiation network (SIN), which triggers cytokinesis, ubiquitination of Sid4 restricts the ability of Plo1 to promote cytokinesis (7).

Many RING domain E3 ligases can multimerize, and this has proven to be important for their function; however, the nature of these oligomer complexes varies widely. Some RING E3 ligases, such as RNF4 (8), cIAP (9), and Siah (10), self-interact to form homodimers, whereas others interact with distinct RING E3 ligases to form heterodimers, such as Mdm2-MdmX (11), Ring1b-Bmi1 (12), and BRCA1-BARD1 (13). In addition to these smaller complexes, some RING E3 ligases can also form higher order oligomers either with themselves or with other RING E3 ligases (14). Although it is not completely clear why many RING E3 ligases need to multimerize for their function, recent studies suggests that two RING domains might be required to spatially accommodate the E2∼Ub conjugate (15).

In this study, we found that Dma1 forms a homodimer via its RING domain and that dimerization is required for its E3 ligase activity. Accordingly, mutant cells expressing a constitutively monomeric form of Dma1 are defective in their mitotic checkpoint response, and Sid4 ubiquitination is abolished, demonstrating that Dma1 requires dimerization *in vivo*. Furthermore, in the absence of dimerization, Dma1 has reduced localization at SPBs and the cell division site, suggesting that dimerization is required for proper Dma1 localization. Because Dma1 is functionally related to human checkpoint proteins CHFR and RNF8

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[2] The abbreviations used are: Ub, ubiquitin; SPB, spindle pole body; SIN, septation initiation network; aa, amino acid(s); SVAU, sedimentation velocity analytical ultracentrifugation.

TABLE 1
Yeast strains used in this study
RFP, red fluorescent protein.

Strain	Genotype	Ref.
KGY7811	h$^-$ *dma1*-V5::*kan*^{R} *ade6-M210 ura4-D18 leu1-32*	This study
KGY12765	h$^+$ *dma1*-3-FLAG::*kan*^{R} *ade6-M216 ura4-D18 leu1-32*	This study
KGY13101	h$^-$/h$^+$ *dma1*-V5::*hyg*^{R}/*dma1*-3-FLAG::*kan*^{R} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study
KGY12781	h$^-$ *dma1(L241A,V245A)*-V5::*kan*^{R} *ade6-M210 ura4-D18 leu1-32*	This study
KGY12709	h$^+$ *dma1(L241A,V245A)*-3-FLAG::*kan*^{R} *ade6-M216 ura4-D18 leu1-32*	This study
KGY13526	h$^-$/h$^+$ *dma1(L241A,V245A)*-V5::*kan*^{R}/*dma1(L241A,V245A)*-3-FLAG::*kan*^{R} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study
KGY11605	h$^+$ *dma1*-GFP::*kan*^{R} *sid4*-RFP::*kan*^{R} *ade6-M21X ura4-D18 leu1-32*	7
KGY13085	h$^+$ *dma1(L241A,V245A)*-GFP::*kan*^{R} *sid4*-RFP::*hyg*^{R} *ade6-M216 leu1-32 ura4-D18*	This study
KGY13370	h$^+$ *dma1(F206A)*-GFP::*kan*^{R} *sid4*-RFP::*hyg*^{R} *ade6-M216 leu1-32 ura4-D18*	This study
KGY13112	h$^-$ *dma1(L241A,V245A)*-GFP::*kan*^{R} *cdc7*-3-mChery::*kan*^{R} *ade6-M21X leu1-32 ura4-D18*	This study
KGY13110	h$^+$ *sid4-SA1 dma1(L241A,V245A)*-GFP::*kan*^{R} *sad1*-RFP::*hyg*^{R} *ade6-M21X leu1-32 ura4-D18*	This study
KGY13366	h$^+$ *dma1(R64A,L241A,V245A)*-GFP::*kan*^{R} *ade6-M216 ura4-D18 leu1-32*	This study
KGY13124	h$^-$ *nda3-KM311 dma1(L241A,V245A)*-GFP::*kan*^{R} *ade6-M21X ura4-D18 leu1-32*	This study
KGY13387	h$^-$ *nda3-KM311 dma1*::*kan*^{R} *ade6-M21X leu1-32 ura4-D18*	This study
KGY5637	h$^-$ *nda3-KM311 ade6-M210 leu1-32 ura4-D18*	lab stock
KGY1296	PJ69–4A MATα *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	20
KGY13966	h$^-$ *nda3-KM311 dma1(F206A)*-GFP::*kan*^{R} *ade6-M21X ura4-D18 leu1-32*	This study
KGY14036	h$^-$/h$^+$ *dma1(F206A)*-V5::*hyg*^{R}/*dma1(F206A)*-3-FLAG::*kan*^{R} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study

(16), it is likely that these proteins have similar structural properties.

EXPERIMENTAL PROCEDURES

General Yeast Methods—S. pombe strains (Table 1) were grown in yeast extract medium supplemented with the appropriate amino acids (17). For diploid construction, strains of opposite mating type and complementary adenine markers (*ade6-M210* and *ade6-M216*) were crossed at 25 °C on sporulation medium and then plated onto minimal medium lacking adenine 24 h later to select for diploids at 32 °C. For *dma1* mutant constructions, a pIRT2 plasmid containing the *dma1* open reading frame plus 500 bp of 5′- and 3′-flanking sequences was mutated using a site-directed mutagenesis kit (Agilent Technologies). Gene replacements at the endogenous *dma1*$^+$ locus were carried out as described previously (7), and stable integrants were selected based on resistance to 5′-fluoroorotic acid. We validated all mutants by colony PCR using primers outside of the *dma1* 5′- and 3′-flanking sequences.

Protein Methods—S. pombe cell pellets were lysed by bead disruption as described previously (18), with the exception that a complete protease inhibitor mixture (Calbiochem) was added to the Nonidet P-40 buffer prior to lysis. For immunoprecipitation experiments, 2 μg of anti-GFP (Roche Applied Science), anti-FLAG (Sigma), or anti-V5 (Invitrogen) antibody was used with protein G-Sepharose (GE Healthcare). Immunoblotting was performed with anti-GFP, anti-FLAG, or anti-V5 antibody and a fluorescent mouse secondary antibody (LI-COR Biosciences) at the manufacturers' recommended concentration.

For *in vitro* ubiquitination assays, Dma1-GFP immunoprecipitates were washed three times with Nonidet P-40 buffer and twice with reaction buffer (50 mM Tris, 2.5 mM $MgCl_2$, and 0.5 mM DTT, pH 7.5). Washed beads were then incubated with recombinant E1 (Boston Biochem), the Ubc13-Uev1 E2 complex (Boston Biochem), 15 μM ubiquitin (Sigma), and 2 mM ATP in reaction buffer. Reactions were incubated on a VorTemp shaker for 90 min at 25 °C. Proteins were run on a 3–8% Tris acetate gel (Invitrogen) and immunoblotted with anti-GFP antibody.

Analytical Ultracentrifugation—Recombinant His_6-Dma1-(187–end) fusion protein was produced in BL21 cells by isopropyl β-D-thiogalactopyranoside induction overnight at 18 °C. Bacterial cells were lysed by sonication, and proteins were affinity-purified on His·Bind resin (Novagen) in His·Bind buffer (500 mM NaCl, 40 mM Tris-HCl, and 5 mM imidazole, pH 7.9). The resin was washed with 20 volumes of His·Bind buffer, and protein was eluted with elution buffer (500 mM NaCl, 20 mM Tris-HCl, and 1 M imidazole, pH 7.9). His_6-Dma1(187–end) was then purified by gel filtration using an S-200 column (GE Healthcare) in buffer containing 10 mM Tris-HCl, pH 7.4, and either 150 mM NaCl or 500 mM NaCl. Peak elution fractions were visualized on a Coomassie Blue-stained gel (Sigma). Fractions were combined and concentrated using a Microcon column (3,000 molecular weight cutoff, Millipore). His_6-Dma1(187–end) recombinant protein (~1 mg/ml) was run on an Optima XLI ultracentrifuge (Beckman Coulter) equipped with a four-hole An-60 Ti rotor at 42,000 rpm at 4 °C. Samples were loaded into double-sector cells (path length of 1.2 cm) with charcoal-filled Epon centerpieces and quartz windows. Sedfit (version 12.0) (19) was used to analyze velocity scans using every eight scans from a total of 393 scans. Approximate size distributions were determined for a confidence level of $p = 0.95$, a resolution of $n = 300$, and sedimentation coefficients between 0 and 10 S.

Yeast Two-hybrid Methods—Dma1 fragments were cloned into bait and prey plasmids pGBT9 and pGAD424, respectively (20). Point mutations were made in pGBT9-*dma1(187–end)* and pGAD424-*dma1(187–end)* plasmids using the site-directed mutagenesis kit and were sequence-verified. Bait and prey plasmids were simultaneously transformed into strain KGY1296 (Table 1) using a standard LiAc transformation procedure, and transformants were selected for growth on synthetic medium supplemented with methionine, uracil, histidine, and adenine but lacking leucine and tryptophan. Two-hybrid interactions were tested on synthetic medium supplemented with methionine and uracil but lacking leucine, tryptophan, histidine, and adenine.

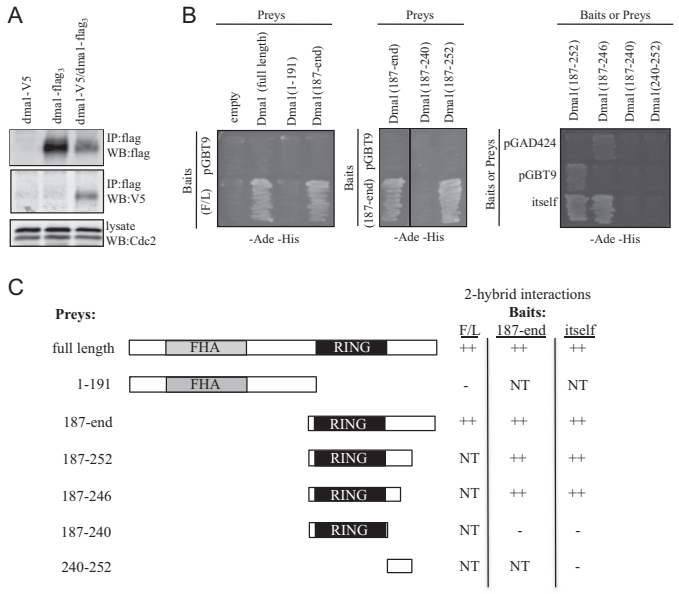


FIGURE 1. **Dma1 self-associates *in vivo*.** *A*, co-immunoprecipitation of Dma1-3-V5 and Dma1-3-FLAG from diploid cells. *IP*, immunoprecipitation; *WB*, Western blot. *B*, yeast two-hybrid interactions using full-length (*F/L*) Dma1 and Dma1 fragments as bait and prey. *C*, schematics of Dma1 fragments that were tested for yeast two-hybrid interaction and summary of their interactions. ++, interaction comparable with the wild type; −, no interaction detected; *NT*, not tested.

Microscopy Methods—All fixed and live cell fluorescence microscopy was performed on a spinning disk confocal microscope (UltraView LCI, PerkinElmer Life Sciences) as described previously (7). For DAPI and methyl blue staining, cells were fixed in 70% ethanol for 30 min and washed with PBS prior to staining.

RESULTS

Dma1 Self-associates in Vivo via Its RING Domain—Because many RING E3 ubiquitin ligases self-associate to form homodimers or associate with other RING E3 ligases to form heterodimers, we asked whether Dma1 self-associates *in vivo*. Diploid cells were generated in which one allele of *dma1* was tagged with sequences encoding a V5 epitope and the other *dma1* allele was tagged with sequences encoding a $FLAG_3$ epitope. By immunoprecipitation, we found that Dma1-$FLAG_3$ and Dma1-$V5_3$ could interact *in vivo* (Fig. 1*A*). Furthermore, full-length Dma1 could interact with itself in a yeast two-hybrid experiment (Fig. 1, *B* and *C*), suggesting that this interaction might be direct.

We then tested a variety of Dma1 fragments in a yeast two-hybrid assay to map the region of self-interaction. Deletion of the N-terminal FHA domain and mid-region (amino acids (aa) 1–186) did not affect Dma1 self-interaction; however, deletion of the RING domain and the C-terminal residues flanking the RING domain (aa 192–end) abolished the interaction (Fig. 1, *B* and *C*), indicating that this region is necessary for self-interaction. The RING domain with the C-terminal tail (aa 187–end) interacted with both full-length Dma1 and itself, and truncating the C-terminal tail on this fragment to aa 246 did not abolish the interaction (Fig. 1, *B* and *C*). However, truncating the entire C-terminal tail abolished the interaction, and the C-terminal tail alone (aa 240–252) was not sufficient for interaction (Fig. 1, *B* and *C*), indicating that the RING domain and at least 10 residues flanking the RING domain are required for the interaction. Thus, the Dma1 self-interaction region is contained in aa 187–246, which includes the RING domain.

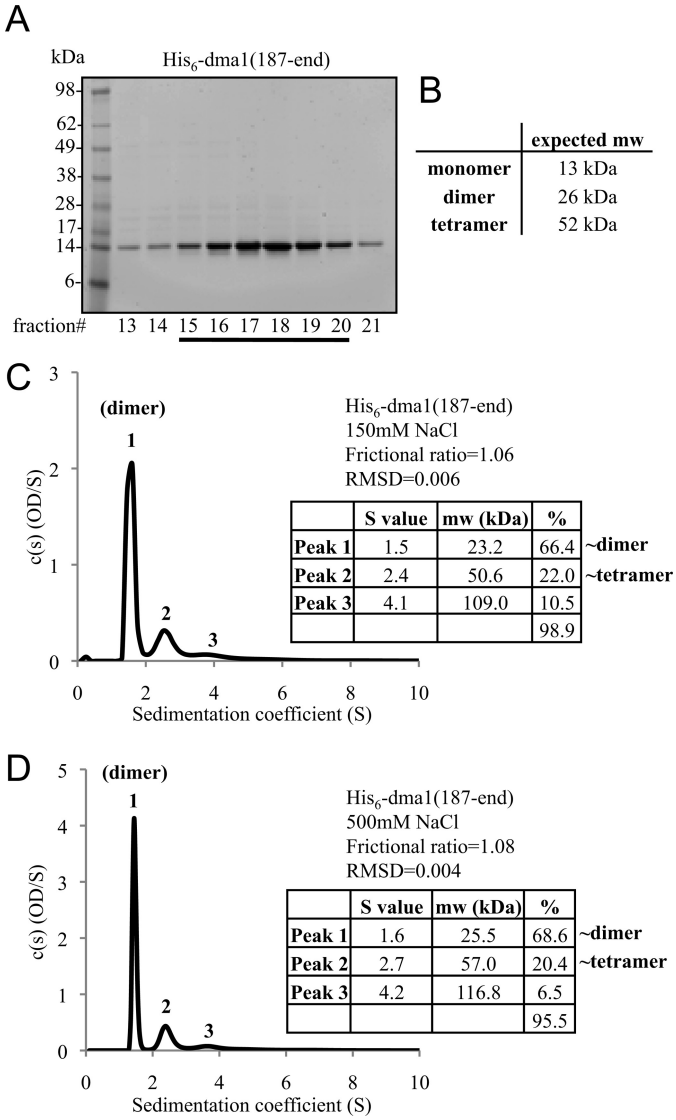
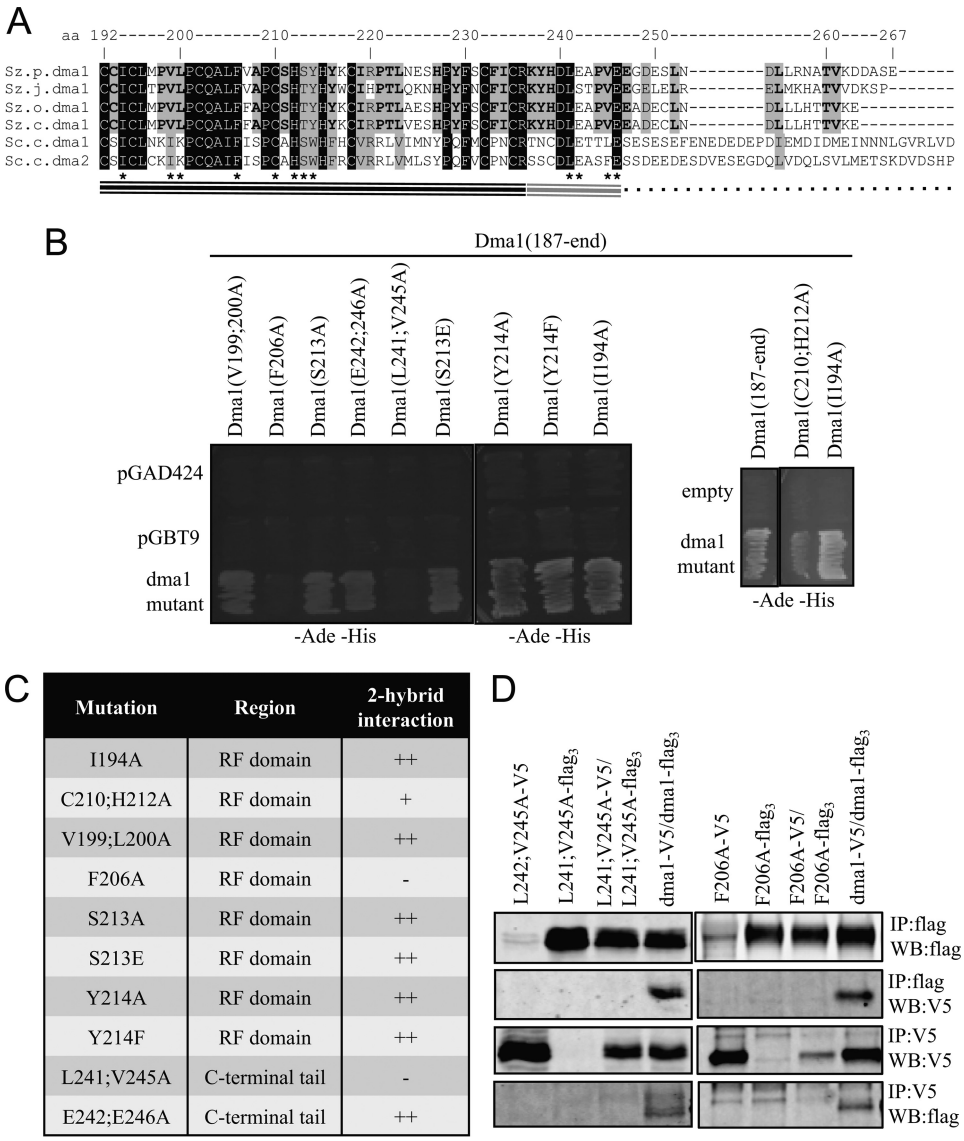


FIGURE 2. **Dma1 RING domain preferentially forms a dimer *in vitro*.** *A*, His_6-Dma1(187–end) was affinity-purified on His·Bind resin followed by gel filtration. Fractions 15–20 were combined and concentrated for SVAU. *B*, expected sizes of Dma1 oligomers. *C*, SVAU analysis of His_6-Dma1(187–end) in 150 mM NaCl. *s* values, determined molecular masses, and percent abundance are given for each indicated peak. *RMSD*, root mean square deviation. *D*, SVAU analysis of His_6-Dma1(187–end) in 500 mM NaCl. *s* values, determined molecular masses, and percent abundance are given for each indicated peak.

Dma1 RING Domain Forms a Dimer—We next determined the oligomeric state of the Dma1 RING domain. Recombinant His_6-Dma1(187–end) was affinity-purified on His·Bind resin followed by gel filtration, and its approximate molecular mass was determined by sedimentation velocity analytical ultracentrifugation (SVAU) (Fig. 2*A*). SVAU traces of His_6-Dma1(187–end) in 150 mM NaCl indicated that the majority (66%) of the protein exists in an ~23-kDa complex ($s = 1.5$), consistent with the molecular mass of a dimer (Fig. 2, *B* and *C*). Two larger ~50-



Mutation	Region	2-hybrid interaction
I194A	RF domain	++
C210;H212A	RF domain	+
V199;L200A	RF domain	++
F206A	RF domain	-
S213A	RF domain	++
S213E	RF domain	++
Y214A	RF domain	++
Y214F	RF domain	++
L241;V245A	C-terminal tail	-
E242;E246A	C-terminal tail	++

FIGURE 3. **Phe-206, Leu-241, and Val-245 are critical for Dma1 dimerization.** *A*, ClustalW alignment of the RING domain and C-terminal flanking residues from *S. cerevisiae* (*Sc.c.*) Dma1 (YNL116W) and Dma2 (YHR115C) and *Schizosaccharomyces* Dma1 homologs: *S. pombe* (*Sz.p.*; SPAC17G8.10c), *Schizosaccharomyces japonicus* (*Sz.j.*; SJAG02169.4), *Schizosaccharomyces octosporus* (*Sz.o.*; SOCG04269.5), and *Schizosaccharomyces cryophilus* (*Sz.c.*; SPOG00270.3). Amino acid numbers correspond to *S. pombe* Dma1 amino acid positions. Conserved residues are *highlighted*, and *asterisks* indicate amino acids that were tested for involvement in self-interaction. The *dashed line* indicates the region of Dma1 that was dispensable for self-interaction in yeast two-hybrid experiments. The *gray bar* underlines the C-terminal flanking residues that were required for self-interaction in two-hybrid experiments. The *black bar* underlines the core RING residues. *B*, yeast two-hybrid interactions. All point mutations were made in the Dma1(187–end) fragment and used as both bait and prey. *C*, summary of point mutations that were tested for two-hybrid interaction. ++, interaction comparable with the wild type; +, interaction weaker than the wild type; −, no interaction detected. *RF*, RING finger. *D*, co-immunoprecipitation experiments from *dma1*-3-V5/*dma1*-3-FLAG, *dma1(L241A,V245A)*-3-V5/*dma1(L241A,V245A)*-3-FLAG, and *dma1(F206A)*-3-V5/*dma1(F206A)*-3-FLAG diploid cells. *IP*, immunoprecipitation; *WB*, Western blot.

and ~109-kDa species were also detected; however, these species made up only 22 and 11% of the total population, respectively (Fig. 2*C*). Significantly, there was no detectable peak at ~13 kDa (Fig. 2*B*), the expected size of a monomer. Increasing the salt concentration to 500 mM did not disrupt the amount of the dimer complex that was present (69%, $s = 1.6$) and did not generate any detectable monomers (Fig. 2*D*), suggesting that this complex is stable. These data indicate that the Dma1 RING domain preferentially forms a stable dimer complex *in vitro*.

Residues in the C-terminal Tail Are Critical for Dma1 Dimerization—To identify specific residues that are required for Dma1 dimerization, we generated a homology-based model of the Dma1 RING domain from the known structure of the homodimeric RNF4 RING domain (8). From this model, several residues were identified that appeared in the dimer interface and that are also conserved in other *Schizosaccharomyces* Dma1 proteins and in the *Saccharomyces cerevisiae* Dma1 and Dma2 proteins (Fig. 3*A*). Selected residues were then mutated in the Dma1(187–end) fragment and tested for interaction in a yeast two-hybrid assay. Mutation of Phe-206 to alanine or Leu-241 and Val-245 together to alanine disrupted self-interaction (Fig. 3, *B* and *C*).

To validate that the Dma1(L241A,V245A) and Dma1-(F206A) mutants were monomeric *in vivo*, we con-

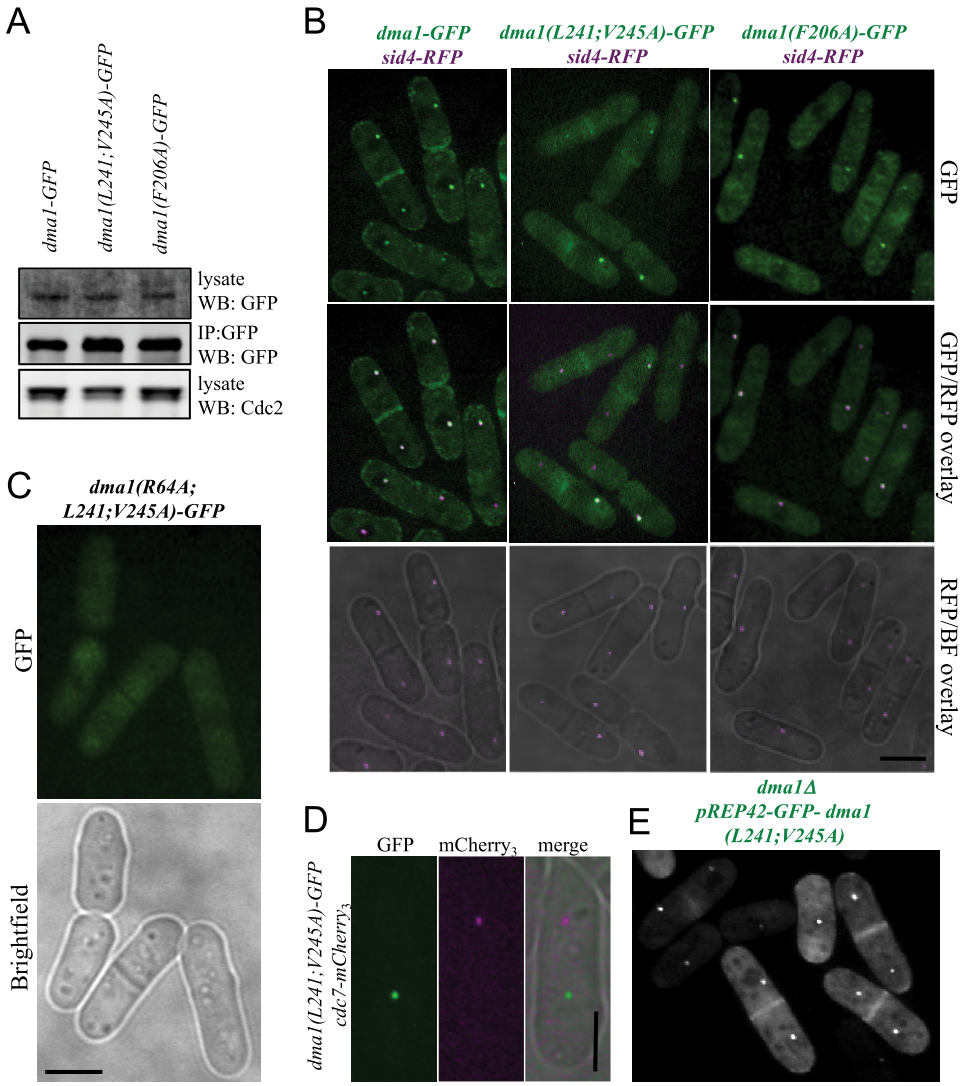


FIGURE 4. **Monomeric Dma1 exhibits defective intracellular localization.** *A*, protein levels of Dma1-GFP, Dma1(L241A,V245A)-GFP, and Dma1(F206A)-GFP. The Cdc2 blot is shown as a protein loading control. *WB*, Western blot; *IP*, immunoprecipitation. *B*, Dma1-GFP, Dma1(L241A,V245A)-GFP, or Dma1(F206A)-GFP was imaged with Sid4-red fluorescent protein (*RFP*; *magenta*) in live cells. *Scale bar* = 5 μm. *C*, Dma1(R64A,L241A,V245A)-GFP localization. *Scale bar* = 5 μm. *D*, representative live cell image of Dma1(L241A,V245A)-GFP with Cdc7-mCherry$_3$ (n = five cells). *Scale bar* = 5 μm. *E*, pREP42-GFP-*dma1(L241A,V245A*) was overproduced in *dma1*Δ cells, and the cells were imaged live. *Scale bar* = 5 μm. *BF*, bright field.

structed *dma1(L241A,V245A)*-3-V5/*dma1(L241A,V245A)*-3-FLAG and *dma1(F206A)*-3-V5/*dma1(F206A)*-3-FLAG diploids and performed a reciprocal co-immunoprecipitation experiment. Although Dma1-3-FLAG was able to interact with Dma1-3-V5, Dma1(L241A,V245A)-3-FLAG did not pull down a detectable amount of Dma1(L241A,V245A)-3-V5 or vice versa (Fig. 3*D*), suggesting that Dma1(L241A,V245A) primarily exists as a monomer *in vivo*. Similarly, Dma1(F206A)-3-V5 did not coprecipitate with Dma1(F206A)-3-FLAG (Fig. 3*D*). Collectively, these data confirm that residues in the core RING domain, as well as residues in the C-terminal tail flanking the RING domain, are important for Dma1 dimerization.

Dma1 Dimerization Is Required for Proper Localization—To determine how dimerization affects Dma1 function *in vivo*, we first wanted to examine if disrupting Dma1 dimerization affects its stability or localization. Dma1-GFP, Dma1(L241A,V245A)-GFP, and Dma1(F206A)-GFP protein levels were compared, and no changes in total protein levels were detected (Fig. 4*A*). There were also no detectable differences in the amount of protein that could be immunoprecipitated (Fig. 4*A*), indicating that the amount of soluble protein is also not affected. Thus, it is unlikely that disrupting the oligomerization state of Dma1 affects its protein stability.

We next examined where constitutively monomeric Dma1 localizes. Dma1 normally localizes to both SPBs during mitosis and also to the cell division site, and localization to these sites is dependent on its FHA domain (5). Surprisingly, we found that both Dma1(L241A,V245A)-GFP and Dma1(F206A)-GFP mutants localized strongly to just one of the two SPBs during mitosis and had significantly reduced localization at the cell division site (Fig. 4*B*). As expected, monomeric Dma1 still required its FHA domain to localize to the one SPB because inactivating the FHA domain (R64A) abolished Dma1-(L241A,V245A)-GFP localization to all structures (Fig. 4*C*).

During early mitosis, the SIN is first activated on both SPBs, and then during anaphase, it becomes inactivated on one SPB

and hyperactivated on the other SPB (21). Asymmetric activation of the SIN is governed by asymmetric distribution of specific SIN proteins and is important for precise timing of cytokinesis (22). Because Dma1 is a SIN inhibitor, we wanted to determine to which SPB monomeric Dma1 predominantly localizes. Thus, we imaged Dma1(L241A,V245A)-GFP with the SIN kinase Cdc7, which localizes only to the SPB with active SIN signaling during anaphase (23). Co-imaging of Dma1-(L241A,V245A)-GFP with Cdc7-3-mCherry showed that Dma1(L241A,V245A)-GFP always localized to the opposite SPB as Cdc7 (Fig. 4*D*), indicating that monomeric Dma1 localizes predominantly to the SPB in which the SIN is inactive.

Given that a small amount of monomeric Dma1 was detected at the cell division site, we reasoned that Dma1 might have reduced localization everywhere and was strongly detectable only at one SPB. Consistent with this idea, overexpression of GFP-*dma1(L241A,V245A)* restored localization to both SPBs and the cell division site (Fig. 4*E*). Collectively, these data indicate that dimerization is required for proper Dma1 localization at SPBs and the cell division site.

Dimerization of Dma1 Is Essential for Its E3 Ligase Activity and Checkpoint Function—During a mitotic checkpoint, Dma1 is required to inhibit cytokinesis to prevent chromosome missegregation (5, 6). To test whether the oligomeric status of Dma1 affects its function, we assessed whether *dma1-(L241A,V245A*) or *dma1(F206A*) cells could maintain a mitotic checkpoint arrest. Cells were synchronized in S-phase with hydroxyurea and released to 18 °C to activate the spindle checkpoint (using the cold-sensitive β-tubulin mutant *nda3-KM311*). Septation indices were measured at 0 and 7 h. At 7 h, only 6.5% of *nda3-KM311 dma1*$^+$ cells had slipped from the arrest, whereas 41.8% of *nda3-KM311 dma1*Δ, 36.7% of *nda3-KM311 dma1(L241A,V245A*), and 36% of *nda3-KM311 dma1(F206A*) cells had slipped from the arrest (Fig. 5, *A* and *B*). These data indicate that dimerization is required for checkpoint function.

Dma1 can autoubiquitinate *in vitro* (7), and we decided to test whether the defect in checkpoint function observed in the mutants was due to mislocalization alone or if it was also due to compromised E3 ligase activity. Dma1-GFP, Dma1(L241A,V245A)-GFP, and Dma1(F206A)-GFP were immunoprecipitated and incubated with an E1 enzyme and the E2 enzyme complex Ubc13-Uev1 for 90 min. Autoubiquitinated proteins were detected by immunoblotting with anti-GFP antibody. In contrast to Dma1-GFP, neither monomeric mutant could form polyubiquitin chains *in vitro* (Fig. 5*C*), indicating that they are not active E3 enzymes. We further tested the activity of the monomeric mutants *in vivo* by examining Sid4 ubiquitination, a known target of Dma1. Consistent with the *in vitro* analysis, Sid4 ubiquitination was abolished in cells expressing either *dma1(L241A,V245A*) or *dma1(F206A*) (Fig. 5*D*). Collectively, these data indicate that loss of checkpoint function in *dma1(L241A,V245A*) and *dma1(F206A*) cells is due to mislocalization and loss of E3 ligase activity.

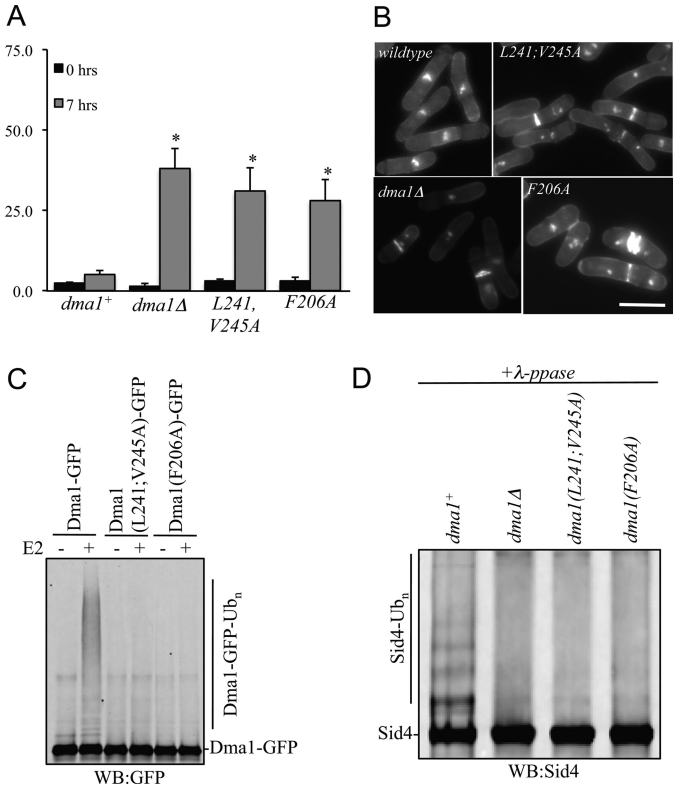


FIGURE 5. **Dimerization of Dma1 is essential for its checkpoint function and E3 ubiquitin ligase activity.** *A*, checkpoint assay with *nda3-KM311 dma1*$^+$, *nda3-KM311 dma1*Δ, *nda3-KM311 dma1(L241A,V245A*), and *nda3-KM311 dma1(F206A*) cells. Cells were synchronized in S-phase with hydroxyurea and released at 18 °C to activate the spindle checkpoint. Septation indices were measured at 0 and 7 h ($n = 3$). *, $p < 0.05$ compared with *nda3-KM311 dma1*$^+$. *B*, representative images of cells from the 7-h time point for each strain indicated stained with DAPI and methyl blue. *Scale bar* = 10 μm. *C*, *in vitro* ubiquitination assay. Dma1-GFP, Dma1(L241A,V245A)-GFP, and Dma1(F206A)-GFP were immunoprecipitated and incubated with an E1 activating enzyme, ATP, and ubiquitin with (+) or without (−) the E2 conjugating enzyme complex Ubc13-Uev1. Ubiquitinated proteins were detected by immunoblotting with anti-GFP antibody. *WB*, Western blot. *D*, Sid4 ubiquitination *in vivo*. Sid4 was immunoprecipitated with anti-Sid4 antibody, treated with λ-protein phosphatase (*λ-ppase*) to visualize the ubiquitin ladder more clearly, and resolved by SDS-PAGE. Ubiquitinated proteins were detected by immunoblotting with anti-Sid4 serum.

DISCUSSION

Several RING E3 ligases form obligate homo- or heterodimers to efficiently transfer ubiquitin onto their substrates (2). Here, we show that *S. pombe* Dma1 joins this subset of dimeric E3 ligases by forming a homodimer via its RING domain and that Dma1 dimerization is required for its E3 ligase activity and checkpoint function *in vivo*. We also found that similar to other C-terminal RING domain proteins, such as RNF4 (8), Dma1 requires the C-terminal tail to dimerize. The extra C-terminal extension likely provides stability to the dimer or is required for proper folding of the RING domain.

Although we have shown that Dma1 self-associates *in vivo* and preferentially forms a dimer when the RING domain is expressed *in vitro*, we cannot exclude the possibility that Dma1 interacts with another RING E3 ligase to form a heteromultimer complex. However, we have not identified another RING E3 enzyme that co-purifies significantly with Dma1 in proteomics screens (data not shown). Whether Dma1 ever exists as a monomer *in vivo* also remains uncertain; however, the dimer status of Dma1 is probably not a major point of regulation because its self-association does not appear to increase significantly during a mitotic checkpoint compared with asynchro-

nous cells (data not shown). It is more likely that Dma1 forms an obligate and constitutive dimer that is regulated by other mechanisms.

A surprising result was that a constitutively monomeric form of Dma1 has significantly reduced localization at one SPB and the cell division site. Importantly, Dma1 ubiquitination activity is not required for proper localization (7), and thus, this phenotype is the direct result of the inability of Dma1 to dimerize. It is not completely clear why the monomer localizes improperly, but given that Dma1 requires its FHA domain for localization, it is possible that the presence of two FHA domains *versus* one increases the affinity of Dma1 for its interaction partners. Another possibility is that monomeric Dma1 lacks specific binding surface(s) that are generated upon dimerization and are required for proper Dma1 localization.

Many proteins localize asymmetrically to one SPB, including several components of the SIN, and asymmetric SIN signaling is critical for proper timing of cytokinesis (22). It is interesting that constitutively monomeric Dma1 is observed only at the SPB in which the SIN is inactive during anaphase. If monomeric Dma1 binds with less affinity to all Dma1 partners, then monomeric Dma1 might be detectable only at locations where Dma1 normally has the highest affinity. This is supported by our observation that when the monomeric form is overexpressed, Dma1 localization at both SPBs and the cell division site is restored. Collectively, our data suggest that Dma1 has a stronger affinity for the inactive SPB, where it can contribute more robustly to SIN inactivation.

Although it was previously assumed that RING E3 ligases act mainly as scaffolds to bring E2∼Ub and the substrate into proximity, increasing evidence indicates that E3 ligases actively participate in catalysis in several ways (2). It is also becoming evident that oligomerization of many RING E3 ligases enhances their catalytic roles; however, it is not completely understood why this is the case. Some studies suggest that oligomerized E3 ligases allosterically activate their cognate E2 enzymes more efficiently to destabilize the E2∼Ub thioester bond and promote catalysis of ubiquitin onto the substrate (3, 8). Even more recently, it was shown that the ubiquitin-loaded E2 enzyme (E2∼Ub) binds across homodimeric RNF4 such that one RING domain contacts the E2 enzyme, whereas the other RING domain of the RNF4 dimer contacts a hydrophobic patch on the conjugated ubiquitin (15). Both interactions are necessary to efficiently activate the thioester bond for catalysis, and spatial constraints prevent a single RING domain from binding both E2 and the conjugated ubiquitin.

Dma1 is a member of a small class of proteins that contain both FHA and RING domains (16). Two homologs exist in humans, CHFR (24) and RNF8 (25), and two redundant homologs exist in *S. cerevisiae*, Dma1 and Dma2 (26), which, similar to Dma1, have roles in checkpoint signaling (16). To our knowledge, *S. pombe* Dma1 is the first example from this class of proteins that has been shown to form an obligate RING domain dimer. Structure-function studies of the other RING domains in the FHA-RING proteins will be required to determine whether RING dimerization is a conserved property of this class or a unique property of Dma1. Given the propensity of RING domain-containing proteins to dimerize, coupled with the structural and functional similarities that exist between the FHA-RING family members, it is plausible that they also function as homodimers, making this an interesting mechanistic model for further investigation.

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