Ubc9 Sumoylation Controls SUMO Chain Formation and Meiotic Synapsis in *Saccharomyces cerevisiae*

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SUMMARY
Posttranslational modification with the small ubiquitin-related modifier SUMO depends on the sequential activities of E1, E2, and E3 enzymes. While regulation by E3 ligases and SUMO proteases is well understood, current knowledge of E2 regulation is very limited. Here, we describe modification of the budding yeast E2 enzyme Ubc9 by sumoylation (Ubc9*SUMO). Although less than 1% of Ubc9 is sumoylated at Lys153 at steady state, a sumoylation-deficient mutant showed significantly reduced meiotic SUMO conjugates and abrogates synaptonemal complex formation. Biochemical analysis revealed that Ubc9*SUMO is severely impaired in its classical activity but promoted SUMO chain assembly in the presence of Ubc9. Ubc9*SUMO cooperates with charged Ubc9 (Ubc9-~SUMO) by noncovalent backside SUMO binding and by positioning the donor SUMO for optimal transfer. Thus, sumoylation of Ubc9 converts an active enzyme into a cofactor and reveals a mechanism for E2 regulation that orchestrates catalytic (Ubc9-~SUMO) and noncatalytic (Ubc9*SUMO) functions of Ubc9.

INTRODUCTION
Sumoylation is a reversible, essential posttranslational modification with functions in most cellular pathways. Conjugation of SUMO (also called Smt3 in yeast, here referred to as SUMO) to its substrates depends on a sequential energy-dependent enzymatic cascade: Matured SUMO with a free C-terminal diglycine is adenylated and forms a thioester bond (•) with the heterodimeric E1-activating enzyme, Aoa1/Uba2. SUMO is then transferred to the only SUMO E2-conjugating enzyme, Ubc9, resulting in an E2-~SUMO thioester. In the final and mostly E3-dependent step, SUMO forms an isopeptide bond (⁎) with its substrates (Gar- eau and Lima, 2010; Geiss-Friedlander and Melchior, 2007; Maderböck and Pichler, 2010). SUMO is predominantly conjugated as a monomer, although polymeric chain assembly plays a role in several cellular pathways (Ulrich, 2008; Vertegaal, 2010).

While regulation of sumoylation is largely controlled at the level of E3 ligases and SUMO-specific proteases, increasing evidence indicates an important role of Ubc9. For example, reversible oxidation inactivates Ubc9 during oxidative stress (Bossis and Melchior, 2006), treatment with a nitric oxide donor results in Ubc9 S-nitrosylation with unknown cellular functions (Ou et al., 2007), and several studies reported Ubc9 itself as target of sumoylation (e.g., Hannich et al., 2005; Nacerdine et al., 2005; Wohlschlegel et al., 2004). We have shown that mammalian and yeast Ubc9 orthologs are sumoylated at different locations, indicating distinct biochemical functions (Knipscheer et al., 2008). Sumoylation of mammalian Ubc9 at Lys14 creates a novel interface, which enhances binding and modification of a substrate depending on a noncovalent SUMO interaction motif (SIM) (Knipscheer et al., 2008).

Since understanding of E2 enzyme regulation in general is very limited, and a biological role of Ubc9 sumoylation is unknown, we set out to study Ubc9 sumoylation also in *Saccharomyces cerevisiae*. Endogenous Ubc9 in *S. cerevisiae* is sumoylated (Ubc9*SUMO) at steady state at low but detectable levels in vivo (less than 1%; Figure 1C and Figure 2B). Here, we report on the biological and biochemical functions of Ubc9 sumoylation at Lys153 in *S. cerevisiae*. We genetically replaced the endogenous *UBC9* with a mutant deficient in sumoylation (ubc9-K153/ 157R::HIS3). Surprisingly, in that strain we observed a severe reduction in meiosis-specific sumoylation concurrent with a profound defect in meiotic chromosome synapsis. Vegetative proliferation, meiotic progression, and spore viability were not or only moderately impaired. Intriguingly, this phenotype shared similarities with a SUMO mutant strain defective in SUMO chain formation (Bylebyl et al., 2003; Cheng et al., 2006; Lin et al., 2010). Biochemical analysis of in vitro sumoylated Ubc9 (Ubc9*SUMO) indicated that this particular modification dramatically reduced the core catalytic activity of Ubc9 but in turn enables Ubc9 to function as a cofactor for unmodified Ubc9 in polymeric SUMO chain assembly. Extensive analysis of several Ubc9 and SUMO mutants revealed that Ubc9*SUMO assists charged Ubc9 (Ubc9–SUMO) by noncovalent backside SUMO binding and enhances transfer by orienting the donor SUMO. Our study reveals insights on how a single posttranslational modification
Ubc9 sumoylation is dispensable and essential in vegetative growth and meiosis.

**RESULTS**

Ubc9 Sumoylation Is Dispensable for Vegetative Growth but Required for Meiosis-Specific SUMO Conjugation in Yeast

Yeast Ubc9 is sumoylated at its last C-terminal z helix at Lys153 in vitro and in vivo shown by mass spectrometry analysis (Bensath et al., 2002; Knipscheer et al., 2008). To identify a biological role for Ubc9 sumoylation in S. cerevisiae, we aimed to generate a sumoylation-deficient mutant. First, we mutated Lys153 to arginine (Ubc9-K153R), but this mutant maintained sumoylation (Figure 1A). Like in ubiquitination, sumoylation sometimes “jumps” to neighboring lysines upon mutation of the main modification site. To test this, we also mutated the adjacent Lys157 to Arg either alone or in combination with Lys153. While the single Ubc9-K157R mutant was efficiently modified, sumoylation of the double Ubc9-K153/157R mutant was almost abolished in vivo (Figure 1A).

With this mutant in hand we replaced one allele of UBC9 by homologous recombination with UBC9::HIS3 or ubc9-K153/157R::HIS3 in the W303 strain background and confirmed viability of this strain by tetrad dissection (Figure 1B). To test if ubc9-K153/157R changes global cellular sumoylation, we compared wild-type (WT) and mutant strains in immunoblot analysis for their SUMO patterns. Both strains showed a comparable pattern, except sumoylated Ubc9 was not detectable in the mutant strain (Figure 1C). Together, these results indicate that Ubc9 sumoylation is dispensable for vegetative growth and ubc9-K153/157R is functional in general SUMO conjugation.

To discover a biological role for Ubc9 sumoylation, we compared our yeast strains to an established temperature-sensitive mutant strain (DF5 ubc9-1) (Seufert et al., 1995). All conditions that severely impaired DF5 ubc9-1 growth affected neither ubc9-K153/157R::HIS3 nor its control cells in number and size of viable colonies (Figure 1D). Thus, Ubc9 sumoylation seems also not essential for cell growth under several stress conditions, although we cannot rule out important but nonessential functions.

In meiosis, four haploid spores are generated from a diploid precursor cell. SUMO conjugates are highly enriched during yeast meiosis, and SUMO and Ubc9 were found to decorate the synaptonemal complex (SC) (Cheng et al., 2006; Hooker and Roeder, 2006; Lin et al., 2010). Consistent with a requirement of sumoylation for synapsis, a hypomorphic ubc9-t mutant delayed and reduced SC formation and meiotic progression.
In line, a SUMO mutant defective in SUMO chain assembly, V5-smt3-allR, also showed synapsis defects (Cheng et al., 2006). To study a putative role for Ubc9 sumoylation in meiosis, we generated UBC9 replacement strains also in the SK1 strain background, known for high sporulation efficiency. First, we compared the sumoylation pattern of these strains to the corresponding W303 strains in vegetative growth. SK1 cells showed significantly fewer high molecular SUMO species than W303 cells. However, we could not detect a significant difference between UBC9::HIS3 or ubc9-K153/157R::HIS3 (Figure 2A).

Next, we investigated sporulation by first synchronizing the cells in presporulation medium in late log phase (Figure 2B). Under this condition, the SK1 ubc9-K153/157R::HIS3 strain indicated a slight delay in cell proliferation (data not shown) and reduced SUMO conjugation levels (Figure 2B, time point 0). After induction of synchronous meiosis by transferring the culture to sporulation medium, a time course was performed. Strikingly, compared to the WT control, the ubc9-K153/157R::HIS3 strain showed dramatically reduced SUMO conjugates (Figure 2B and Figure S1A available online), whereas unmodified Ubc9 and β-actin levels remained unchanged. Ubc9*SUMO could be detected in WT cells at very long exposures, and indeed its amount correlated positively with an increase in high molecular SUMO species (Figure 2B).

In vivo LC-MS mapping of meiotic SUMO*SUMO branches. SUMO species enriched from yeast cells in pachytene stage were directly subjected to Orbitrap MS/MS analysis upon GluC digestion. Fragment ion assignment enabling the identification of the branched peptides (QIGG-VKPE and Q(deam)IGG-AKPE) originating from SUMO*SUMO chains via K11, K15, and K19 linkages is illustrated. The Lys15 and Lys19 linkage cannot be distinguished by tandem MS. @, deamidated glutamine. See also Figure S1.
progression observed in the mutant, although additional defects cannot be excluded. Next, we analyzed a deletion of ZIP3 (SK1 zip3::KanMX6), a reported regulator of meiotic SUMO species (Cheng et al., 2006). Notably, this mutant resulted in an increase in meiotic SUMO species, creating the opposite effect as abolishing Ubc9 sumoylation (Figure S1B). In summary, while Ubc9 sumoylation is dispensable for vegetative growth, it appears to have key functions in meiosis.

Meiotic SUMO Chains Are Branched via the Flexible N Terminus of SUMO In Vivo

Earlier studies implicated SUMO chains in yeast meiosis, as a SUMO mutant lacking all lysines (V5-smt3-allR) showed significantly reduced meiotic SUMO species (Cheng et al., 2006). To prove the existence of meiotic SUMO chains in vivo, we replaced endogenous SMT3 with a His-tagged version (SK1 UBC9::HIS3, HIS6-SMT3::TRP1) and enriched SUMO species from cells in pachytene stage by a Ni²⁺ pull-down under stringent denaturing conditions. The enriched SUMO species were directly applied to Orbitrap tandem mass spectrometry analysis. Using GluC digestion, we identified two different SUMO peptides linked to the C terminus of SUMO (QIGG-VKPE and Q(deam)GG-AKPE). These linkages correspond to the three consensus site lysines (Lys11 in AKPE, Lys15 and Lys19 both in the indistinguishable VKPE), all in the flexible N terminus of SUMO (Figures 2C and S1C). Notably, the same peptides were identified when the high molecular smear of enriched SUMO species was separated on SDS-PAGE, or in vitro assembled SUMO chains were investigated (Figure S1D). Together, these data provide direct evidence of meiotic SUMO chains in vivo and implicate that marginal amounts of Ubc9-SUMO govern meiotic SUMO conjugates, SUMO chains, and SC associated genes, suggesting a biological role in SC formation.

Ubc9 Sumoylation Is a Key Mechanism for Synaptonemal Complex Formation

The SC is a tripartite protein structure consisting of axial elements linked by transversal filaments. This connects homologous chromosomes along their entire length in the pachytene stage of meiotic prophase.

In budding yeast, SC formation depends on successful homology search, repair of double-strand breaks (DSBs), and the action of ZMM proteins (an acronym for the yeast proteins Zip1–Zip4, Msh4–Msh5, and Mer3), which coordinate crossover designation at synopsis initiation sites with SC assembly (Lynn et al., 2007; Shinohara et al., 2008; Storlazzi et al., 2010; Tsoubouchi et al., 2008). To analyze whether ubc9-K153/157R::HIS3 mutants confer a synopsis defect, we induced meiosis in WT and mutant SK1 strains and took samples for chromosome spreading, spindle, and DAPI staining between 3 and 10 hr after induction of sporulation. Subsequently, chromosome spreads were immunostained with antibodies to SUMO and Zip1 as markers for SC formation.

Figures 3A and 3B show the profound phenotype observed: In WT UBC9::HIS3 cells, Zip1 foci occurred in leptotene and developed into strongly stained stretches labeling the axis of all 16 bivalents in pachytene. In contrast, the ubc9-K153/157R::HIS3 mutant strain was defective in producing even short stretches of synapsis, but instead many Zip1 foci decorated the axis of the tightly paired bivalents (Figure 3A). The tight homologous alignment and the fact that Zip1 foci form suggest that the ubc9-K153/157R::HIS3 defect lies downstream of most processes already known to be necessary for synopsis, such as DSB formation, homology search, and formation of synopsis initiation complexes. Some mutant nuclei contained bivalents with densely spaced Zip1 foci, of which some appear to have short stretches of seemingly continuous Zip1. Since the intensity of such stretches was much lower than in WT (maximum 50%), we called them “pseudosynapsis” (Figure 3A, white arrows). Analysis of 200 nuclei in each of five time points of ubc9-K153/157R::HIS3 revealed no nucleus with extended synopsis or any Zip1 stretches of WT intensity (Figure 3B).

Similar to Zip1, SUMO decorates synapsed regions in WT cells but formed only isolated foci in ubc9-K153/157R::HIS3, which appeared significantly dimmer than in WT and are of similar intensity as the pale foci decorating unpaired rDNA regions in WT (Figure 3A, white triangles).

ubc9-K153/157R::HIS3 showed a 1–2 hr delay in exit from prophase, as estimated from the decrease of cells with monopolar spindles (Figure 3D). This suggests a transient activation of the double-strand break DNA repair checkpoint. Also, meiotic nuclear divisions occurred with a delay in the mutant (Figure 3C), which, however, is slightly shorter than for a zip1Δ mutant (data not shown), the ZMM mutant with the mildest delays. Furthermore, ubc9-K153/157R::HIS3 mutants showed an increase in unsporulated cells (22% compared to 5% for UBC9::HIS3; n = 400) and were reduced in forming complete tetrads (34% compared to 60% for UBC9::HIS3). However, cells that managed to form complete tetrads only showed a mild reduction in spore viability (85%, n = 88).

In conclusion, our observations identify Ubc9 sumoylation as a key regulatory step in SC formation and place the defect of the mutant downstream of homolog alignment and downstream of several classical ZMM functions.

Ubc9*SUMO Is Strongly Impaired in SUMO Thioester Formation

The fact that loss of Ubc9 sumoylation causes a dramatic synopsis defect raises the question about the molecular function of Ubc9*SUMO in meiosis. To address this issue, we in vitro sumoylated bacterially expressed Ubc9 and separated the modified from the unmodified form by ion-exchange and size-exclusion chromatography. To prevent SUMO chain formation, we used a SUMO variant lacking lysines (Bylebyl et al., 2003) to obtain Ubc9*SUMO-allR. Coomassie stains of purified Ubc9-WT, Ubc9-K153/157R, and Ubc9*SUMO-allR are shown in Figure 4A. We then compared all Ubc9 variants for their catalytic activity in thioester formation (Figure 4B, upper panel) verified by Dithiotreitol (+DTT) sensitivity (lower panel). Strikingly, Ubc9*SUMO-allR was unable to form thioester at conditions (1 mM E1) where Ubc9-WT and Ubc9-K153/157R were completely charged (Figure 4B). Only at much higher E1 concentrations (100 mM E1) could Ubc9*SUMO be marginally charged with SUMO (Figure 4C). Next, all Ubc9 variants were tested for general defects in discharging monitored by modification of two well-established SUMO substrates: the HAT enzyme general
Figure 3. Ubc9*SUMO Is a Key Regulator for Synaptonemal Complex Formation

(A) Chromosome spreads of Ubc9-WT and mutant SK1 strains double stained as indicated for DNA (DAPI) and Zip1 (α-Zip1, marking central axis of the SC, upper panel rows) or DAPI and SUMO (α-SUMO, lower panel rows). One representative and one “maximally synapsed” nucleus are shown for t4 (maximal synapsis for WT) and for the mutant also at t5 (“pseudosynapsis” for the mutant). “Pseudosynapsis” is marked by a white arrow. Prominent white Zip1 blobs are polycomplexes (PC). SUMO and corresponding DAPI staining are shown at t5. rDNA regions are marked by triangles. Again, one SUMO-stained mutant nucleus is shown after enhanced magnification and illumination.

(B) Statistical representation. Bars indicate the percentage of nuclei per time point falling into four categories (no Zip1, Zip1 foci, partial Zip1 axes, and extended or full Zip1 axes; n = 200 per time point). Shades of blue indicate the time point after transfer to SPM.

(C) Nuclear divisions were recorded after DAPI staining in SK1 UBC9::HIS3 (black, full symbols) and SK1 ubc9-K153/157R::HIS3 (red, empty symbols). Triangles represent cells past metaphase 1; squares, cells past metaphase 2; and circles, cells before anaphase 1.

(D) Prophase exit was monitored by in situ staining of meiotic spindles using α-tubulin antibody. Black symbols represent the WT and red the mutant. Circles represent the percentage of cells containing a monopolar spindle (prophase cells). Triangles represent cells with bipolar spindle (meta- or anaphase cells).
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Control nonderepressible 5 (Gcn5) and the septin Cdc3. Compared to Ubc9-WT, Ubc9-K153/157R showed rather increased activities whereas Ubc9*SUMO-allR was strongly reduced toward substrates (Figure 4D). In conclusion, Ubc9*SUMO lost its classical E2 activities whereas Ubc9-K153/157R is rather activated in comparison to Ubc9-WT.

Ubc9*SUMO Stimulates SUMO Chain Formation

In vivo at steady state, less than ~1% of Ubc9 is sumoylated (Figure 2B), and this marginal amount seemingly controls meiotic SUMO species (Figures 2B and S1A). But how can we reconcile this with a biochemically rather inactive enzyme (Figure 4B)? Since the phenotype of ubc9-K153/157R::HIS3 resembles that of a SUMO chain defective mutant (vs-smt3-allR) (Cheng et al., 2006), we investigated a putative role for Ubc9*SUMO in SUMO chain formation.

Unmodified Ubc9 is highly active and forms SUMO chains in a concentration-dependent manner (Figure 6D, leftmost panel). Therefore, we used low Ubc9 concentrations to limit inherent chain formation (Figure 5A, lane 2). Ubc9*SUMO-allR by itself is unable to assemble SUMO chains, even at the highest enzyme concentration (Figure 5A, second last lane). But strikingly, Ubc9 in the presence of increasing concentrations of Ubc9*SUMO-allR significantly accelerated SUMO chain formation (Figure 5A, middle lanes).

To explore whether Ubc9*SUMO-allR gets activated in the presence of unmodified Ubc9, we titrated increasing concentrations of Ubc9 to Ubc9*SUMO-allR. As shown in Figure 5B, Ubc9*SUMO-allR charging is not enhanced by Ubc9, suggesting functions other than classical E2 activity. To prove this assumption, we sumoylated a catalytically inactive Ubc9-C93A mutant with SUMO-allR and tested it for SUMO chain assembly. Indeed, the Ubc9-C93A*SUMO-allR was able to enhance Ubc9-dependent SUMO chain formation in a concentration-dependent manner despite lacking its catalytic cysteine (Figure 5C).

Ubiquitin chains can be formed by involving different lysines, which determines the nature of the chain. Different E2 enzymes or E2/E3 enzyme combinations define the linkage of the ubiquitin chain (Komander and Rape, 2012). To analyze if Ubc9-C93A*SUMO-allR can change the nature of SUMO chain branches, we investigated a SUMO mutant lacking all canonical chain branches in its N terminus (SUMO-K11/15/19R or SUMO-3KR). Since this mutant was almost abolished in Ubc9*SUMO-depended chain polymerization (Figure 5D), we conclude that Ubc9*SUMO does not generate noncanonical chain linkages.

The Backside of Unmodified Ubc9 Is Essential for Ubc9*SUMO-Dependent Chain Assembly

The current understanding of how SUMO chains are assembled is very limited, but different studies implied a role for the backside of Ubc9 in SUMO chain formation (Capili and Lima, 2007; Knipscheer et al., 2007). This interface noncovalently interacts with SUMO, orienting its flexible C terminus in close proximity to Ubc9 Lys153 (Capili and Lima, 2007; Duda et al., 2007; Knipscheer et al., 2007).

Aos1-Uba2 (E1); 6 μM SUMO; and 0, 0.25, and 0.5 μM (Cdc3) or 0, 0.5, and 1 μM (Gcn5) Ubc9 variant were incubated at 30°C in the presence of ATP for 90 min.

Figure 4. C-Terminal Ubc9 Sumoylation Severely Impairs Its Catalytic Core Activity

(A) Coomassie stain of each 1 μg bacterially purified Ubc9 variant.

(B) SUMO charging ability of indicated Ubc9 variants at low E1 concentrations: In vitro thioester assays with 0.55 μM Ubc9 variant, 2.15 μM SUMO, 1 nM Aos1-Uba2 (E1), and ATP incubated at room temperature for 0, 1, and 5 min. Detection was under reducing (lower panel) and nonreducing (upper panel) conditions by immunoblotting.

(C) SUMO charging ability of indicated Ubc9 variants at high E1 concentrations: 1 μM Ubc9 or Ubc9*SUMO, 6 μM SUMO, and 100 nM Aos1-Uba2 were incubated at room temperature in the presence of ATP. Samples were analyzed by immunoblotting under nonreducing (upper panel) and reducing conditions (lower panel).

(D) SUMO discharging ability of Ubc9 variants. Immunoblot of in vitro sumoylated substrates: 500 nM substrate (GST-Cdc3 or GST-Gcn5); 100 nM

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Figure 5. Ubc9*SUMO Accelerates SUMO Chain Assembly Independent of Its Catalytic Core Activity

(A) SUMO chain assembly reaction in vitro: Ubc9 (0.3 µM) and Ubc9*SUMO-allR (0.5 µM) alone or as mixture with increasing amounts of Ubc9*SUMO-allR (0.02, 0.06, 0.17, and 0.5 µM) were incubated with 6 µM SUMO, 11 nM Aos1-Uba2 (E1) with and without ATP for 1 hr at 30°C. Analyses were by immunoblotting with indicated antibodies.

(B) Ubc9-WT cannot stimulate Ubc9*SUMO in charging: 1 µM Ubc9*SUMO-allR and increasing concentrations of Ubc9-WT (0, 0.25, 0.5, 1, 2, and 4 µM) were incubated with 6 µM SUMO, 11 nM Aos1-Uba2 (E1), and ATP at room temperature for 5 min. Reactions were analyzed at reducing (lower panel) and nonreducing (upper panel) conditions by immunoblotting.

(C) In vitro SUMO chain assembly with a Cys93-inactivated sumoylated Ubc9: 0.3 µM Ubc9 without and with increasing concentrations of Ubc9-C93A*SUMO-allR (0.02, 0.06, 0.17, and 0.5 µM) were incubated with 6 µM SUMO and 11 nM Aos1-Uba2 (E1) with and without ATP for 1 hr at 30°C. Reactions were analyzed with indicated antibodies.

(D) In vitro SUMO chain assembly with SUMO and SUMO-3KR: 0.3 µM Ubc9 and increasing concentrations of Ubc9-C93A*SUMO-allR (0, 0.0625, 0.25, and 1 µM) were incubated with ATP, 11 nM Aos1-Uba2 (E1), and 6 µM SUMO or SUMO-3KR for 1 hr at 30°C. Analysis was by immunoblotting.

Knipscheer et al., 2007). Thus, we asked if this backside binding interface has a role in Ubc9*SUMO-dependent chain formation. Diverse covalent and noncovalent SUMO/Ubc9 binding interfaces are summarized in Figure 6A.

Three Ubc9 mutants were reported to disrupt SUMO-Ubc9 binding: Ubc9-R17A, Ubc9-H20D, and Ubc9-F22A/G23Q/Y25S (Duda et al., 2007), illustrated in Figure 6B. In general, all Ubc9 mutants are catalytically active in SUMO charging (Figure S2A) and discharging on substrates (Figure S2B), with Ubc9-R17A being slightly impaired. However, the backside of Ubc9 is important for its own sumoylation (Figure S2C). This recalls that some SUMO substrates involve a SIM for efficient modification (e.g., Gareau and Lima, 2010; Knipscheer et al., 2008).

Asking about a role in Ubc9*SUMO-dependent chain formation, we envisioned two scenarios (Figure 6C): (1) SUMO in Ubc9*SUMO could interact in cis, falling in its own backside pocket, and/or (2) it could recruit another Ubc9 in trans. Hence, we investigated the Ubc9 backside mutants for SUMO chain assembly in the absence (Figure 6D) and presence (Figure 6E) of Ubc9-C93A*SUMO-allR. Although all mutants were already impaired in intrinsic SUMO chain assembly (Figure 6D), none was stimulated by Ubc9-C93A*SUMO-allR (Figure 6E). This indicates that unmodified Ubc9 and Ubc9*SUMO cooperate via backside binding. Of note, Ubc9 backside mutants have lethal phenotypes (Figure S2D and Duda et al., 2007), implicating broader functions than nonessential SUMO chain formation (Bylebyl et al., 2003; Cheng et al., 2006). In fact, this interface is also involved in E3 enzyme interactions (Pichler et al., 2004; Reverter and Lima, 2005).

Donor SUMO Positioning Accelerates Ubc9*SUMO-Dependent Chain Formation

Ubc9*SUMO recruits Ubc9 via noncovalent backside binding to assemble N-terminally branched SUMO chains, but how is chain formation accelerated?
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Figure 6. Mechanism of Ubc9*SUMO-Dependent Chain Formation

(A) Illustration of SUMO/Ubc9 binding interfaces: Ubc9 can bind covalently (thioester bond via Cys93 and isopeptide bond via Lys153) and non-covalently (backside of Ubc9 and Ubc9’s catalytic cleft via SUMO consensus motifs) to SUMO.

(B) Structure of the noncovalent SUMO Ubc9 binding interface (PDB: 2EKE [Duda et al., 2007]). SUMO is shown in green and Ubc9 in blue. Mutated residues are depicted in red, Lys153 in gray, and the catalytic Cys93 in yellow.

(C) Models for isopeptide-linked SUMO-Ubc9 binding: Ubc9*SUMO interacts with SUMO in cis and falls in its own backside binding pocket (1) or Ubc9*SUMO recruits another Ubc9 in trans (2).

(D) In vitro SUMO chain formation of indicated Ubc9 mutants: 6 μM SUMO; 11 nM Aos1-Uba2; and 0, 0.2, 0.6, and 2, 6 μM (as indicated) Ubc9 species were incubated at 30°C in the presence of ATP for 1 hr. Immunoblotting is as indicated.

(E) In vitro SUMO chain assembly of Ubc9 mutants in the presence of Ubc9*SUMO-allR: 0.35 μM Ubc9 species were incubated with 6 μM SUMO, 11 nM Aos1-Uba2 (E1), and ATP with Ubc9*SUMO-allR (0.0625, 0.25, and 1 μM) for 1 hr at 30°C. Immunoblotting is as indicated.

(F) Illustration of donor SUMO positioning (left panel) versus acceptor SUMO stabilization (right panel).

(G) The donor SUMO N-terminal lysines are essential for Ubc9*SUMO-dependent chain formation: 0.4 μM Ubc9, Ubc9-C93A*SUMO-allR (0, 0.0625, 0.25, and 1 μM), and ATP and 6 μM SUMO or a mixture of 3 μM SUMOΔGG and 3 μM SUMO-3KR were incubated for 1 hr at 30°C. Immunoblotting is as indicated.

(H) The donor SUMO consensus sites are required for Ubc9*SUMO-dependent chain formation: 0.35 μM Ubc9, Ubc9-C93A*SUMO-allR (0, 0.0625, 0.25, and 1 μM), and ATP and 6 μM SUMO or a mixture of 3 μM SUMOΔGG and 3 μM SUMO-3KR were incubated for 1 hr at 30°C. Analyses were by immunoblotting. See also Figure S2.

motifs can directly recognize and bind the catalytic cleft of Ubc9 (Bernier-Vilamor et al., 2002).

To distinguish between these scenarios, we performed SUMO chain reactions by mixing two SUMO variants (Figure 6G): (1) an acceptor SUMO (SUMOΔGG), which cannot be conjugated, and (2) a donor SUMO (SUMO-3KR), abolished in chain formation. In the case of acceptor SUMO stabilization, SUMOΔGG*SUMO-3KR conjugates should be formed in an Ubc9*SUMO-dependent manner, as the acceptor has all the necessary consensus motifs. However, if donor SUMO positioning mediates Ubc9*SUMO-dependent chain formation, no conjugates would be formed, as all consensus motifs are impaired in the donor. As shown in

Again we consider two scenarios: Ubc9*SUMO binding to Ubc9–SUMO facilitates chain formation by (1) improving the positioning of the donor SUMO (Figure 6F, left panel) or (2) stabilizing the acceptor SUMO (enhancing substrate binding, Figure 6F, right panel). Both alternatives require an additional binding interface, most likely provided by the N-terminal consensus motifs of SUMO (Bylebyl et al., 2003). Such
SUMO Chain Mutants Display Key Features of Ubc9 Sumoylation Deficiency
Next, we investigated whether SUMO mutants defective in chain formation share a related phenotype to ubc9-K153/157R::HIS3 in vivo. First, we characterized the mutants biochemically and confirmed their impairment in chain formation (Figure S3A) but not in substrate modification (Figure S3B). Subsequently, we replaced the endogenous SUMO with SUMO-WT, SUMO-3KR, and SUMO-cm mutants or SUMO-allR (Smt3::TRP1, smt3-3KR::TRP1, smt3-cm::TRP1, smt3-allR::TRP1) and investigated synapsis and sporulation. Indeed, all SUMO mutants displayed the key features of ubc9-K153/157R::HIS3 in sporulation, showing reduced meiotic SUMO conjugates and Zip1 stabilization (Figures 7A, S3C, and S3D). The phenotypes differed in severity between the different SUMO mutants but were all less pronounced than in ubc9-K153/157R::HIS3 (Figure 2B). A clear contrast to ubc9-K153/157R::HIS3 was established by investigating chromosome synapsis. All SUMO mutants were able to undergo extensive synapsis with variable reduction in efficiency correlating with the amount of meiotic SUMO conjugates (Figures 7B, S3E, and S3F). Together, these findings indicate that ubc9-K153/157R::HIS3 has a related but more severe phenotype than diverse SUMO chain mutants. This could result from atypical SUMO chains (linked via amino terminus or nonconsensus SUMO lysines) in the SUMO mutants or additional functions of the Ubc9*SUMO/Ubc9 complex on as yet unexplored critical SUMO substrates.

DISCUSSION
Here we report on the importance of E2 enzyme regulation by studying Ubc9 sumoylation in S. cerevisiae. Our finding that a single posttranslational modification can switch functions of a canonical E2 enzyme (Ubc9~SUMO) to mimic an inactive E2 variant (Ubc9*SUMO) with features similar to E3 enzymes sets a paradigm in enzyme variability, thereby revealing a mechanism for SUMO chain assembly. Sumoylation inactivates Ubc9’s catalytic core activity. Instead, Ubc9*SUMO functions as a scaffold for recruiting and optimally orienting the donor SUMO-loaded Ubc9 to facilitate catalysis (Figure 7C), usually a hallmark of E3 ligases (Plechanovová et al., 2012; Reverter and Lima, 2005; Yunus and Lima, 2009). While recruitment involves noncovalent Ubc9 backside SUMO binding (1), the positioning (2) is mediated by noncovalent binding of the donor SUMO to the catalytic cleft of Ubc9*SUMO via its N-terminal SUMO consensus sites (Figure 7C). SUMO consensus sites directly recognize the catalytic cleft of Ubc9 (Bernier-Villamor et al., 2002). The acceptor SUMO N terminus binds to the catalytic cleft of activated Ubc9~SUMO to position the target lysine for conjugation (3). Interactions between consensus motifs and the catalytic cleft are weak (Bernier-Villamor et al., 2002), explaining the rather low activity of Ubc9*SUMO in vitro reactions and suggesting additional factors for full activity.

This mechanism is critical for SC formation, an evolutionarily conserved hallmark structure connecting homologous chromosomes in meiotic prophase. Although several studies pointed to roles of Ubc9 and SUMO (Cheng et al., 2006; Hooker and Roeder, 2006; Lin et al., 2010) in this process, their exact function remained unclear. Our in vivo analysis revealed Ubc9 as SUMO substrate with key importance for meiosis. A Ubc9 mutant deficient in sumoylation shows severely impaired meiosis-specific synapsis concurrent with a profound defect in SC formation. The formation of Zip1 foci on tightly aligned bivalents and the high spore viability suggest that the ubc9-K153/157R mutant distinguishes the actual synapsis defect from DSB formation, homologous alignment, and formation of synopsis initiation complexes.

Additionally, by proving the existence of SUMO chains in vivo, former models are supported (Cheng et al., 2006). As Ubc9*SUMO enhances SUMO chain formation in vitro, we analyzed several chain formation-impaired SUMO mutants and demonstrated that they have similar but less severe phenotypes than ubc9-K153/157R. This further highlights the unique phenotype of the ubc9-K153,157R mutant and implicates broader functions of the Ubc9*SUMO/Ubc9 complex. Thus, we propose that Ubc9 sumoylation is required to trigger SC formation by allowing SUMO incorporation and therefore represents a novel mechanism at the heart of synapsis.

EXPERIMENTAL PROCEDURES

Plasmids, Yeast Strains, and Antibodies
Bacterial expression vectors: The coding regions of Ubc9 and SUMO were PCR-cloned into pET21b or pET23a, respectively. His-Aos1, His-Ubc2, and His-Ubc9 were described earlier (Johnson and Gupta, 2001). SUMO-allR (Bylbel et al., 2003) and SUMOΔGG were PCR cloned into pET23a. All Ubc9 mutants, SUMO-3KR (K11/15/19R), and SUMO-cm (E9A/P12A/E13A/V14A/E17A/V18A/E21A) were generated by site-directed mutagenesis. The coding regions of CDC3 and GCN5 were PCR cloned from yeast genomic DNA into pGEX4-T-1 as GST-fusion proteins.

For genomic replacement, Ubc9 variants, C-terminally fused to 3xHA or untagged, were cloned into pFA6a-HIS3MX6 with the coding region and the 3’ UTR region interrupted by the HIS3MX6 cassette. Haploid and homozygous diploid Ubc9::HIS3 or ubc9-K153/157R::HIS3 strains contain these alleles as their sole source of Ubc9. Endogenous SMT3 was replaced with SMT3::TRP1 variants by homologous recombination according to Wohlschlegel et al., 2004, but with a removed His tag. All strains were verified by sequencing after tetrad dissection. All strains used are listed in Table S1.

Rabbit α-SUMO (Smt3), rabbit α-Zip1, and affinity-purified rabbit α-Ubc9 were generated against full-length proteins. Mouse α-HA (16B12), goat}

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Figure 7. SUMO Chain Mutants and Model

(A) The SUMO chain defective mutant SUMO-cm shows key features of Ubc9 sumoylation deficiency in vivo. Sporulation assay of SUMO-cm demonstrates impaired meiotic SUMO conjugation. Time course of indicated diploid SK1 background strains synchronized and released into meiosis is shown. TCA extracts from indicated time points were analyzed by immunoblotting as indicated.

(B) All SUMO mutants defective in SUMO chain formation show a less severe phenotype than SK1 ubc9-K153/157R::HIS3 in synapsis. Chromosome spreads of indicated strains were tested for synapsis formation by double staining for DNA (DAPI) and for Zip1 at constant exposure and reproduction conditions. Examples of extensive synapsis for SMT3::TRP1, smt3-3KR::TRP1, smt3-cm::TRP1, and smt3-allR::TRP1 are shown.

(C) Model: Sumoylation orchestrates catalytic (Ubc9~SUMO) and noncatalytic (Ubc9*SUMO) activities of Ubc9 important for SUMO chain assembly: Ubc9*SUMO functions as scaffold by recruiting Ubc9~SUMO (1, binding) and orients the donor SUMO (2, positioning) for efficient transfer (3, isopeptide bond formation). See also Figure S3.
α-Zip1 (sc-15632), and mouse α-β-actin (ab 8224) were purchased from Covance, Santa Cruz, and Abcam, respectively.

**Protein Expression, Purification, In Vitro Sumoylation, and Thioester Assays**

Recombinant Aos1-Uba2, SUMO, SUMO-allR, SUMO-3KR, SUMO-cm, SUMOαGG, Ubc9, and Ubc9 mutants were purified from E. coli as described (Johnson and Gupta, 2001; Pichler et al., 2002). In vitro sumoylation and thioester formation assays were performed as described (Knipscheer et al., 2008; Pichler et al., 2002).

**In Vitro Sumoylation of Ubc9**

Ubc9 was sumoylated by incubating 55 μM (15 mg) Ubc9 with 65 μM (8 mg) SUMO-allR, 70 mM (1 mg) Aos1-Uba2, and 5 mM ATP in SUMO assay buffer (20 mM HEPES [pH 7.3], 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 0.05% Tween 20, 1 mg/ml α-Lactalbumin, 1 mM Leupeptin, 1 mM Pepstatin, 1 mM Aprotinin, 1 mM DTT) for 2 h. Subsequently, Ubc9SUMO-allR was purified by anion exchange and gel-filtration chromatography as described (Knipscheer et al., 2008). Ubc9-C93A*SUMO-allR was obtained by using His-Ubc9 and Ubc9-C93A at a 1:4 molar ratio for the in vitro reaction. Ubc9-C93A*SUMO-allR was purified by anion-exchange and gel-filtration with an additional nickel chromatography step to eliminate any His-Ubc9 species. All purified sumoylated Ubc9 species were finally buffer exchanged to 20 mM HEPES [pH 7.3], 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 1 mM DTT.

**Yeast Media, Growth Conditions, and Sample Preparation**

Mitotic W303 and SK1 yeast cultures were grown at 30°C in YPD (2% peptone, 1% yeast extract, 2% glucose) to OD₀₆₀ 0.7. TCA extracts: cell pellets were resuspended in 250 μl 20% TCA disrupted with glass beads in a homogenizer for 45 s, then diluted to 8% TCA, centrifuged, and washed with 100% ethanol before resuspending in loading buffer (0.5 M Tris, 4% SDS, 20% glycerol, 4 mM EDTA, 4% 2-mercaptoethanol, 1 mg/ml bromophenol blue). For growth assays, serial dilutions of mutant overnight cultures were spotted on YPD plates supplemented with the indicated drugs for 2–4 days at 30°C and 37°C (temperature sensitivity).

Meiosis was induced by growing diploid SK1 strains in YPD for 24 h, diluting in presporulation medium (0.5% yeast extract, 1% peptone, 0.17% yeast nitrogen base [Difco #233520], 1% potassium acetate, 0.5% ammonium sulfate, 0.05 M potassium-biphthalate [pH 5.5]) and growing overnight (14–16 h) to OD₀₆₀ 1.2. Cells were collected, washed once in sporulation medium (SPM; 1% potassium acetate), resuspended in SPM, and incubated at 30°C as indicated.

**Yeast Cytology**

Yeast meiotic nuclear spreads were prepared and stained as described (Zierhut et al., 2004). WT and mutant samples were analyzed in parallel, including all image processing, to ensure that differences in staining intensities are representative.

Nuclei were classified according to their Zip1 staining pattern: “foci” containing nuclei with foci but no linear stretches beyond incidental linear arrangements of adjacent foci; “partial SCs” with stretches of polymerized Zip1 and up to half the genome synapsed; and “extended SCs and full SCs,” where more than half the genome have reached continuous Zip1 staining. Images were taken on a Zeiss fluorescence microscope with a Photometrics CH250 CCD camera using IPLab Spectrum, with constant magnification and exposure times. To follow nuclear divisions, 200 μl aliquots of culture were briefly fixed in 1 ml 96% ethanol and DAPI stained (0.2 μg/ml DAPI in water) for analysis. For spindle staining, 1 ml aliquots of culture were fixed in 3% formaldehyde. Cell walls were digested using 115 μg Zymolyase 20T (SEIKAGAKU #120491), 75 mM DTT, and 1 M sorbitol. Mounted on poly-L-lysine-coated slides, the fixed spermatids were dehydrated 3 min in ice-cold methanol and 10 s in acetone. Microtubuli were detected with rat α-tubulin-alpha (Sero-tec MCA78, 1:2000) in 0.5% BSA fraction V and 0.2% gelatine in 1X PBS (pH 7.25). Secondary antibody was FITC-conjugated rabbit α-rat (Sigma F1763, 1:100). Vectashield with DAPI (Vector Laboratories H-1200) was used to stain the DNA and stabilize the label.

**Mass Spectrometry**

TCA-extracted proteins of 1 l of SK1 UBC9::HIS3, HIS-SMT3::TRP1 sporulating for 6 h were incubated with 20 μl Ni²⁺-NTA Sepharose (Invitrogen) as described (Knipscheer et al., 2008). Beads were resuspended in 40 mM ammonium bicarbonate followed by reduction/alkylation (DTT/iodoacetamide) and GIuC in solution digestion (25°C overnight) and analyzed by LC-MS (Orbitrap XL + ETD) as described (Flach et al., 2010). The raw data files were processed into peak lists with DTA Supercharge (v. 2.0b1) and searched with Mascot 2.2 (precursor MMD = 5 ppm; MS/MS MMD = 0.5 Da) against a database containing all possible branched peptides originating from yeast SUMO (Hsiao et al., 2009).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.03.027.

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