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Yeast axial-element protein, Red1, binds SUMO chains to promote meiotic interhomologue recombination and chromosome synapsis

Feng-Ming Lin[1,2], Yi-Ju Lai[1,2], Hui-Ju Shen[2], Yun-Hsin Cheng[2] and Ting-Fang Wang[1,2,*]

[1]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan and [2]Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan

The synaptonemal complex (SC) is a tripartite protein structure consisting of two parallel axial elements (AEs) and a central region. During meiosis, the SC connects paired homologous chromosomes, promoting interhomologue (IH) recombination. Here, we report that, like the CE component Zip1, *Saccharomyces cerevisiae* axial-element structural protein, Red1, can bind small ubiquitin-like modifier (SUMO) polymeric chains. The Red1–SUMO chain interaction is dispensable for the initiation of meiotic DNA recombination, but it is essential for Tel1- and Mec1-dependent Hop1 phosphorylation, which ensures IH recombination by preventing the inter-sister chromatid DNA repair pathway. Our results also indicate that Red1 and Zip1 may directly sandwich the SUMO chains to mediate SC assembly. We suggest that Red1 and SUMO chains function together to couple homologous recombination and Mec1–Tel1 kinase activation with chromosome synapsis during yeast meiosis.

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Introduction

Crossing-over between homologous chromosomes ensures accurate segregation during the first meiotic division. Homologous chromosomes must first be linked by chiasmata, the cytological manifestation of crossover (CO) recombination products that are established during meiotic prophase. Meiotic recombination is initiated by developmentally programmed DNA double-strand breaks (DSBs), the formation of which requires the products of at least 10 genes, including *SPO11* (Keeney, 2001). These DSBs are then resected by the Mre11-Rad50-Xrs2 nuclease to generate 3′ single-strand tails that invade the intact DNA duplexes used in DNA repair (Neale *et al*, 2005). Most of these events use homologous chromosomes, not sister chromatids, as the templates in DNA repair, yielding CO and non-CO (NCO) products (for a review see Bishop and Zickler, 2004). Two kinds of CO are seen in *Saccharomyces cerevisiae*. The first CO class is randomly distributed along the chromosomes and requires Mus81-Mms4 endonuclease (de los Santos *et al*, 2003; Borner *et al*, 2004; Lynn *et al*, 2007). The second CO class is distance-dependent and requires ZMM proteins (an acronym for yeast proteins Zip1-4, Msh4-5 and Mer3; Borner *et al*, 2004) at CO-designated sites to stabilise early recombination intermediates against the action of the anti-CO helicase Sgs1 (Jessop *et al*, 2006; Oh *et al*, 2007, 2008; Jessop and Lichten, 2008).

The ZMM proteins also form a zipper-like proteinaceous structure between homologous chromosomes, known as the synaptonemal complex (SC), during the zygotene and pachytene stages. These structures include a major SC component (Zip1) and the SC initiating or elongating proteins (Zip2-4, Mer3, Msh4-5 and Spo16) (Sym *et al*, 1993; Hollingsworth *et al*, 1995; Chua and Roeder, 1998; Agarwal and Roeder, 2000; Novak *et al*, 2001; Borner *et al*, 2004; Fung *et al*, 2004; Perry *et al*, 2005; Tsubouchi *et al*, 2006; Shinohara *et al*, 2008). The SC consists of a central region and two dense lateral elements. The lateral element constitutes the rod-like homologue axis, which is called an axial element (AE) before synapsis. The chromatin loops of sister chromatids are organised along the axis. The central region contains transverse filaments (TFs) oriented perpendicularly to the longitudinal axis of the SC, resulting in the striated, zipper-like appearance of the SC (Henderson and Keeney, 2005). The components of the yeast AE include sister chromatid cohesin complexes (e.g., Rec8), DNA topoisomerase II (Top2) and a few meiosis-specific proteins (e.g., Red1, Hop1 and Mek1) (Hollingsworth and Ponte, 1997; Smith and Roeder, 1997; Klein *et al*, 1999; Blat *et al*, 2002; Page and Hawley, 2004). Mek1 is a serine–threonine protein kinase that functions in combination with Red1 and Hop1 to ensure interhomologue (IH) recombination by preventing the use of a sister chromatid as the template in DNA repair (Bailis and Roeder, 1998; Wan *et al*, 2004; Niu *et al*, 2005, 2007). Mek1 kinase activity is dependant on Hop1 phosphorylation, which is mediated by Mec1 and Tel1, yeast homologues of the mammalian ATR and ATM kinases (Carballo *et al*, 2008). Hop1 protein has been shown to interact with DNA *in vitro* (Kironmai *et al*, 1998) and to interact with itself and Red1 (Bailis and Roeder, 1998; de los Santos and Hollingsworth, 1999; Woltering *et al*, 2000). Hop1 also promotes dimerisation of Mek1 (Niu *et al*, 2005). Red1 is

*Corresponding author. Institute of Molecular Biology, Academia Sinica, 128 Academia Road, Nankang, Taipei 11529, Taiwan.
Tel.: +886 2 2789 9188; Fax: +886 2 2782 6085;
E-mail: tfwang@gate.sinica.edu.tw

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known to interact with itself, Hop1 and the protein phosphatase, Glc7 (Bailis and Roeder, 2000; Woltering *et al*, 2000).

Zip1 is the major structural component of the central region (Sym *et al*, 1993), The Zip1 proteins form head-to-head dimers connected by protein–protein interactions between the central coiled-coil domains, and the C-terminal globular domain connects the central region to the AEs (Dong and Roeder, 2000). The SC initiation protein Zip3 is an E3 ligase for Smt3, the yeast small ubiquitin-like modifier (SUMO). Post-translational modifications of SUMO control SC assembly through an interaction between the Zip1 C-terminal globular domain (residues 849–875) and the Zip3-dependent Smt3 conjugates along two AEs (Cheng *et al*, 2006). In the wild-type cell, Zip3 recruits Ubc9, the SUMO E2 enzyme, to meiotic chromosomes, but in the *zip3*Δ mutant, Ubc9 proteins are not associated with chromosomes (Hooker and Roeder, 2006). However, the Ubc9 proteins in mutant cells still catalyse the self-polymerization of Smt3 monomers to form Smt3 chains (Cheng *et al*, 2006). These chromosome-free Smt3 chains non-covalently associate with Zip1 proteins to form one or a few large proteinaceous aggregates known as polycomplexes (PCs). Zip1 stabilizes these chromosome-free Smt3 chains against the Ulp2 SUMO protease, resulting in a massive accumulation of Smt3 chains (Cheng *et al*, 2006). Other ZMM proteins (i.e., Zip2, Spo16) have been proposed to act after Zip3 as chaperone-like machinery to facilitate the perpendicular alignment of Zip1 proteins along two AEs during SC elongation. The meiotic chromosomes of these two *zmm* mutants can recruit Zip1, but are barely able to form long and fine Zip1 lines between homologous chromosomes (Cheng *et al*, 2006). This observation is consistent with a recent report that Zip2 functions in SC elongation is dependant upon *SPO11* (Tsubouchi and Roeder, 2005; Tsubouchi *et al*, 2008). Spo11 generates DSBs, subsequently promoting Hop1 phosphorylation to ensure the close juxtaposition of two homologous chromosomes, by IH recombination, for SC elongation (Niu *et al*, 2005; Carballo *et al*, 2008).

Zip1 also mediates pair-wise non-homologous centromere coupling (NHCC) before the zygotene stage (Tsubouchi and Roeder, 2005). Zip1 binds to centromeric Smt3 conjugates (e.g., Top2), which is mediated by non-Zip3 SUMO E3 ligases, Siz1, Siz2 or Mms21. These non-Zip3 E3 ligases are down-regulated after the zygotene stage. The NHCC is then destroyed by SUMO protease Ulp2 (Cheng *et al*, 2006). NHCC could lead to SC initiation at the centromeres. These centromeric SCs are independent of *SPO11* and *ZIP3*, which have major roles in the chromosome synapsis at non-centromeric locations. On the other hand, Spo11 and Zip2 are required for SC elongation at both centromeric and non-centromeric sites (Tsubouchi and Roeder, 2005; Tsubouchi *et al*, 2008). These two kinds of SCs are initiated differently but likely use the same mechanism for SC elongation. As described above, Spo11 is required for Hop1 phosphorylation, which ensures IH-recombination by repressing IS-recombination (Niu *et al*, 2005; Carballo *et al*, 2008). However, DSB-dependent Hop1 phosphorylation is not a prerequisite for SC initiation, because Zip1 and Zip3 can be recruited to meiotic chromosomes in the absence of *SPO11*, for example, a previous study found that 67% of Zip3 foci were centromeric and 33% were non-centromeric (Tsubouchi *et al*, 2008). The function of DSB-dependent Hop1 phosphorylation at non-centromeric locations is to closely juxtapose two homologous chromosomes for SC initiation and elongation, whereas the function of DSB-independent NHCC at centromeres is to initiate SC assembly between two non-homologous chromosomes (Tsubouchi *et al*, 2008). Therefore, we reason that other non-Hop1 AE structural proteins may be responsible for Zip3- and Smt3-dependent SC initiation.

In this study we found that, like Zip1, Red1 can bind Smt3 chains. The Red1–Smt3 interaction first facilitated chromosome recruitment of Zip1 and Zip3 for SC initiation, similar to the function of centromeric Smt3 conjugates in recruiting Zip1 and Zip3 proteins during NHCC (Tsubouchi and Roeder, 2005; Cheng *et al*, 2006; Tsubouchi *et al*, 2008). The second function of the Red1–Smt3 interaction was to promote Hop1 phosphorylation, which prevents IS-recombination to ensure IH-recombination. As a result, the two homologous chromosomes are closely juxtaposed for Zip2 or other ZMM proteins to facilitate the perpendicular alignment of Zip1 proteins during SC elongation (Tsubouchi and Roeder, 2005; Cheng *et al*, 2006; Tsubouchi *et al*, 2008).

Results

Red1 binds Smt3 chains but not to Smt3 monomer

A previous genome-wide two-hybrid screen suggested that Red1 might interact with Smt3 (Ito *et al*, 2001). However, it was unclear if the Red1–Smt3 two-hybrid interaction was because of a direct physical interaction between Red1 and Smt3 monomer, Smt3 polymeric chain or conjugate. Moreover, this two-hybrid interaction may have indirectly resulted from post-translational Smt3 modification of Red1 itself or some other Red1 binding protein. The latter hypothesis is not favoured for the following reasons: first, it is inconsistent with the result that the *zip3*Δ mutant could form short SCs in the absence of the Zip3-dependent Smt3 modified AE proteins (Agarwal and Roeder, 2000; Hooker and Roeder, 2006); second, very little Red1 protein (<1%) is Smt3-modified in wild-type sporulating cells (Cheng *et al*, 2006, 2007).

In this study, we identified two putative Smt3-interacting motifs (SIMs) in the C-terminal portion of Red1, viz. SIM-1 (ILESTTVID, residues 712–720) (Minty *et al*, 2000) and SIM-2 (ISII, residues 758–761) (Song *et al*, 2004). The Red1C protein (residues 611–827) displayed a strong two-hybrid interaction with Smt3. We also constructed three mutant proteins: $Red1C^{S715R,T717R}$ (referred to as $Red1C^{2R}$), $Red1C^{I758R}$ and $Red1C^{S715R,T717R,I758R}$ (referred to as $Red1C^{3R}$). The order of two-hybrid interaction with Smt3 for these four proteins was $Red1C > Red1C^{2R} > Red1C^{I758R} > Red1C^{3R}$ (Figure 1A), indicating that SIM-2 is better than SIM-1 at binding Smt3. Red1C did not exhibit a two-hybrid interaction with Smt3-ΔGG, a conjugation-incompetent Smt3 mutant lacking the C-terminal pair of glycines for E1-mediated Smt3 activation (for a review see Gill, 2004). In addition, Red1C and Smt3 induced a high level of two-hybrid interaction in *smt3-allR* reporter cells. However, Red1C and Smt3-allR exhibited a weaker interaction in the same reporter cells (Figure 1A). Smt3-allR is competent in the SUMO conjugation to all target proteins, including the wild-type Smt3. However, Smt3-allR cannot form a polymeric chain because the nine lysine residues in Smt3 were replaced with arginines in this mutant (Bylebyl *et al*, 2003). These results suggest that Red1 prefer-

A

LexA-X	Gal4AD-Y	Reporter host	X–Y interaction (β-galactosidase activity)
Red1C	Smt3	*SMT3*	600.2 ± 2.4
Red1C	Smt3-ΔGG	*SMT3*	0.1 ± 0.0
Red1C	–	*SMT3*	0.1 ± 0.0
Red1C^{I758R}	Smt3	*SMT3*	65.9 ± 0.7
Red1C^{I758R}	Smt3-ΔGG	*SMT3*	0.1 ± 0.0
Red1C^{I758R}	–	*SMT3*	0.1 ± 0.0
Red1C^{2R}	Smt3	*SMT3*	336.9 ± 2.8
Red1C^{2R}	Smt3-ΔGG	*SMT3*	0.1 ± 0.0
Red1C^{2R}	–	*SMT3*	0.1 ± 0.0
Red1C^{3R}	Smt3	*SMT3*	12.5 ± 0.2
Red1C^{3R}	Smt3-ΔGG	*SMT3*	0.1 ± 0.1
Red1C^{3R}	–	*SMT3*	0.1 ± 0.0
Red1C	Smt3	*smt3-allR*	248.7 ± 0.8
Red1C	Smt3-allR	*smt3-allR*	176.3 ± 4.3
Red1C	–	*smt3-allR*	0.4 ± 0.0
Red1C^{I758R}	Smt3	*smt3-allR*	22.4 ± 0.2
Red1C^{I758R}	Smt3-allR	*smt3-allR*	3.2 ± 0.0
Red1C^{I758R}	–	*smt3-allR*	0.2 ± 0.0
Red1C^{2R}	Smt3	*smt3-allR*	191.2 ± 3.0
Red1C^{2R}	Smt3-allR	*smt3-allR*	141.1 ± 0.9
Red1C^{2R}	–	*smt3-allR*	0.2 ± 0.0
Red1C^{3R}	Smt3	*smt3-allR*	8.4 ± 0.8
Red1C^{3R}	Smt3-allR	*smt3-allR*	2.2 ± 0.0
Red1C^{3R}	–	*smt3-allR*	0.1 ± 0.0

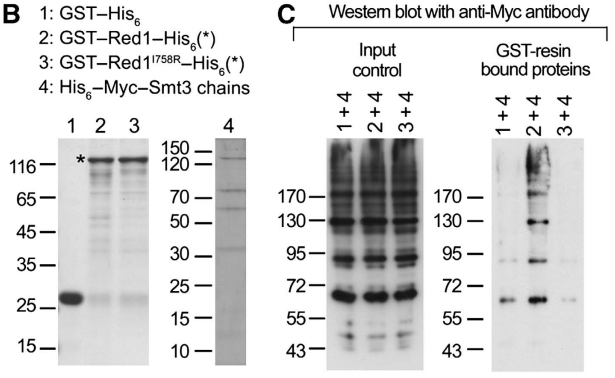

Figure 1 Red1 preferentially binds Smt3 chains. (**A**) Quantitative two-hybrid assays were carried out using mitotic reporter host cells carrying either *SMT3* or mutant *smt3-allR* gene. One unit of β-galactosidase hydrolyses 1 μmol of *o*-nitrophenyl β-D-galactopyranoside per min per OD_{600} unit. Red1C represents the C-terminal fragment (residues 611–827) of the wild-type Red1 protein. The Ile758 residue of SIM-2 was converted to Arg in the Red1C^{I758R} mutant; Red1C^{2R} has two point mutations (Ser715 to Arg and Thr717 to Arg) at the SIM-1. The Red1C^{3R} is the synthetic mutant of the Red1C^{I758R} and Red1C^{2R} mutants. (**B**) Purified GST–His_6, GST–Red1–His_6, GST–Red1^{I758R}–His_6, and His_6–Myc–Smt3 chains were separated by SDS–PAGE and visualised by Coomassie blue staining. (**C**) Purified GST–His_6, GST–Red1–His_6 and GST–Red1^{I758R}–His_6 fusion proteins were first bound to GST resins and then incubated with purified His_6–Myc–Smt3 polymeric chains. After extensive washing, the proteins bound to the GST resins were visualised by western blot using anti-Myc antibody.

entially associates with Smt3 chains but not with Smt3 monomer or other Smt3 conjugates.

To examine if the Red1–Smt3 chain interaction is essential for meiosis, we constructed five yeast strains expressing V5-tagged Red1 (V5-Red1), V5-Red1^{2R}, V5-Red1^{I758R}, V5-Red1^{3R} or no Red1 protein. Tetrad analyses revealed that the *V5-RED1*^{2R} mutant, like *V5-RED1*, generated many viable spores (>97%), whereas the *V5-red1*^{I758R}, *V5-red1*^{3R} and *red1*Δ mutants yielded almost no viable spores (<1%). We conclude that SIM-2 not only exhibits a stronger interaction with Smt3 chains than SIM-1, but it is also functionally more important *in vivo*.

Finally, we examined the affinity of Red1 variant proteins for Smt3 chains. We expressed full-length Red1 fusion proteins GST–Red1–His_6 and GST–Red1^{I758R}–His_6 in *Escherichia coli*, and then sequentially purified them on Ni^{2+} and glutathione resins. Smt3 chains were synthesised *in vitro* and partially purified by size-exclusion gel filtration (Figure 1B). The order of affinity of these proteins had for Smt3 chains was GST–Red1–His_6 ⩾ GST–Red1^{I758R}–His_6 ∼ GST–His_6 (Figure 1C).

Red1C^{I758R} and Red1C^{3R} were also partly defective in homo-oligomerization in the two-hybrid assays carried out in *SMT3* and *smt3-allR* reporter cells, respectively (Supplementary Table S1). Although the results obtained from the *smt3-allR* reporter cells indicate that the Red1–Red1 interaction is not likely mediated by Smt3 chains, it does not exclude the possibility that other proteins conjugated by Smt3-allR or Smt3 may facilitate the Red1–Red1 interaction. Nevertheless, these two-hybrid results still raise a concern that Red1 homo-oligomerization (Woltering *et al*, 2000) may be required for the normal functioning of Red1 protein, including the Red1–Smt3 chain interaction. This supposition is not favourable, because the GST–Red1–His_6 proteins exhibited much higher affinity to Smt3 chains than the GST–Red1^{I758R}–His_6 proteins exhibited (Figure 1C). GST is a dimeric protein on its own, it was used here to induce homo-oligomerization of GST–Red1–His_6 and GST–Red1^{I758R}–His_6 recombinant proteins.

Red1 binds Smt3 chains to promote meiotic IH-recombination

The *V5-red1*^{I758R} and *V5-red1*^{3R} mutant cells had lower steady-state levels of Red1 protein (roughly 70%) compared with wild-type cells (Figure 2A). To ensure that these two mutants did not produce less Red1 proteins than the wild-type *V5-RED1* cells, high-copy number vectors expressing V5-Red1^{I758R} or V5-Red1^{3R} were then transformed into *V5-red1*^{I758R} or *V5-red1*^{3R} mutants, respectively (Figure 2B). All of these mutant strains exhibited identical meiotic phenotypes (i.e., <1% spore viability) before and after being transformed with the supplementary V5-Red1^{I758R} and V5-Red1^{3R} expression vectors. Therefore, the meiotic defects of the *V5-red1*^{I758R} and *V5-red1*^{3R} cells were not because of lower steady-state levels of Red1 proteins.

We then carried out cycloheximide shut-off experiments (Penkner *et al*, 2005) to examine Red1 protein stability. Protein synthesis was inhibited by 200 μg/ml of cycloheximide added into the meiotic cultures at the 4-h time point. Samples were taken at 0, 30, 60, 90, 120 and 180 min after the addition of cycloheximide. Western blot analysis revealed that the levels of V5-Red1, V5-Red1^{I758R} and V5-Red1^{3R} remained steady in the presence of cycloheximide upto 180 min (Figure 2D), indicating that the SIM mutation(s) has little or no effect on V5-Red1 protein stability.

Immunostaining of sporulating cells and meiotic nuclear spreads with an anti-V5 antibody revealed that V5-Red1^{I758R} and V5-Red1^{3R} mutant proteins were properly targeted to nuclei (Supplementary Figure S1) and meiotic chromosomes (Supplementary Figure S2), respectively. We also confirmed that these two mutants were not defective in Hop1 chromosomal localisation (Supplementary Figure S2) or the Red1–Hop1 interaction (Woltering *et al*, 2000) (Supplementary Table S2 and Figure S3).

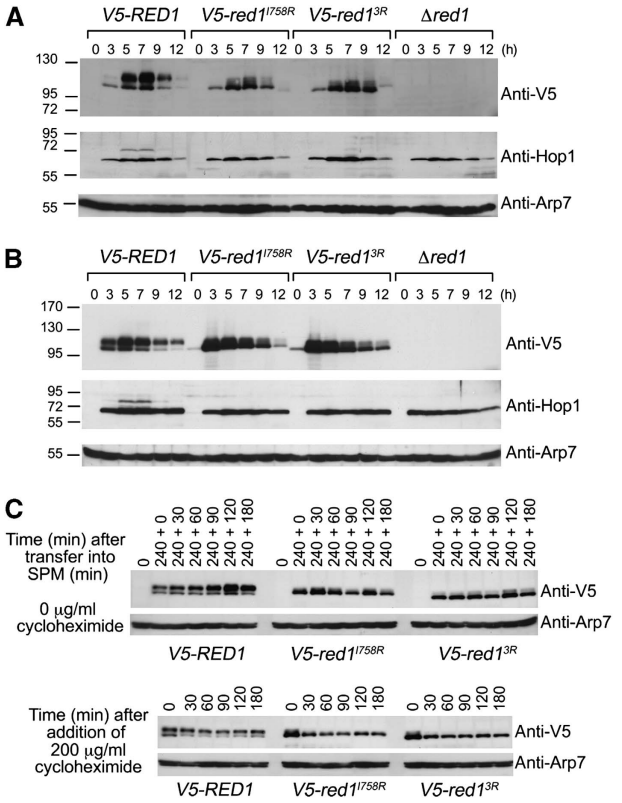

Figure 2 Characterisation of the V5-red1^{I758R} and V5-red1^{3R} proteins. (**A**) Western blot time-course analysis of the sporulation cells using anti-V5 antibody. The lower and upper bands represented the non-phosphorylated and phosphorylated V5-Red1 proteins, respectively. The blot was also probed with anti-Hop1 antibody to determine if Hop1 protein is phosphorylated. The results indicate that the steady-state levels of Red1 proteins in the *V5-red1^{I758R}* and *V5-red1^{3R}* cells were lower than the level in *V5-RED1* cells. Arp7 was used as a loading control. The molecular weights (in kDa) are indicated to the left of the blots. (**B**) To exclude the possibility that the meiotic defects of these two SIM mutants resulted from a lower steady-state level of mutant Red1 proteins, we constructed two yeast expression vectors to over-express V5-Red1^{I758R} and V5-red1^{3R} proteins under control of the *GAL1* promoter. Each vector was transformed into the corresponding yeast strain, *V5-red1^{I758R}* or *V5-red1^{3R}* cells, respectively. We added 0.03% galactose into the sporulation medium to induce the overexpression of V5-Red1^{I758R} or V5-red1^{3R} protein. We confirmed that the presence of 0.03% galactose did not affect either the sporulation efficiency or spore viability of the *V5-RED1* cells. These two new mutant yeast strains each produced a similar or higher steady-state level of Red1 proteins compared to the wild-type *V5-RED1* cells. Tetrad analysis revealed that these two new yeast strains, like the original strains, could not form viable spores. These results indicate that the meiotic phenotypes of the original *V5-red1^{I758R}* and *V5-red1^{3R}* cells were not likely owing to a decreased amount of Red1 protein. (**C**) Cycloheximide shut-off experiment to demonstrate that V5-Red1^{I758R} and V5-red1^{3R} proteins are as stable as the V5-Red1 proteins. Protein synthesis was inhibited by 200 μg/ml cycloheximide added to the meiotic cultures at the 4-h time point. Samples were taken at 0, 30, 60, 90, 120 and 180 min after the addition of cycloheximide.

Our results also revealed that Red1 proteins in the *V5-red1^{I758R}* and *V5-red1^{3R}* mutant cells were hypophosphorylated compared with wild-type cells (Figure 2A and B) (Lai YJ *et al*, unpublished data), raising a concern that the defects in these SIM mutants may be because of a deficiency in protein phosphorylation. Although phosphorylated Red1 proteins were thought to have a critical role in meiosis (Bailis and Roeder, 1998; de los Santos and Hollingsworth, 1999; Hong and Roeder, 2002; Wan *et al*, 2004), we have mapped the Red1 phosphorylation sites and showed that phosphorylation-deficient mutants undergo normal meiosis and generate as many viable spores as wild-type *V5-RED1* cells (Lai YL *et al*, unpublished data).

In summary, we discovered a new essential biochemical property of Red1 protein. The defects in the two SIM mutant proteins, V5-red1^{I758R} and V5-red1^{3R}, are mainly due to the loss of the Red1–Smt3 chain interaction rather than a global loss of the function of the C-terminal region of the proteins (including protein stability, protein phosphorylation, Red1–Red1 oligomerization and Red1–Hop1 interaction or nuclear/chromosomal targeting).

Accordingly, these two SIM mutant strains are not identical to the *red1*Δ null mutant.

Red1 binds Smt3 chains to promote Hop1 phosphorylation

Western blot time-course analyses also showed a significant portion of Hop1 proteins are phosphorylated in sporulating *V5-RED1* cells. Phosphorylated Hop1 migrated more slowly in the SDS–PAGE than unmodified Hop1. In contrast, Hop1 was not phosphorylated in the *V5-red1^{I758R}*, *V5-red1^{3R}* or *red1*Δ cells (Figure 2A and B). These results indicate that Red1 binds Smt3 chains to promote Tel1- and Mec1-dependent Hop1 phosphorylation. Hop1 phosphorylation is known to be essential for the activation of Mek1 kinase activity and ensures IH recombination by preventing the inter-sister chromatid DNA repair pathway (Niu *et al*, 2005, 2007; Carballo *et al*, 2008). These results are consistent with our finding that *V5-red1^{I758R}* and *V5-red1^{3R}*, like the *red1*Δ mutant, yielded no viable spores (<1%).

A physical assay was carried out to monitor IH-recombination products (i.e., COs and NCOs) (Clyne *et al*, 2003). Yeast genomic DNA from different meiotic time points was digested with the restriction enzymes *Eco*RI and *Pvu*II and analysed by Southern blotting. The *V5-RED1* diploid cell produced both CO and NCO products, though only ~50% as much NCOs as COs (Figure 3A). The relative amount of total CO products produced by these four strains was *V5-RED1* (12.4%) > *V5-red1^{I758R}* (3.7; 30% of *V5-RED1*) ~ *V5-red1^{3R}* (4.4%; 35% of *V5-RED1*) ~ *red1*D (2.1%; 17% of *V5-RED1*) (Figure 3B), whereas the relative amount of total NCO products was *V5-RED1* (7.3%) > *V5-red1^{I758R}* (3.9%; 53% of *V5-RED1*) ~ *V5-red1^{3R}* (4.1%; 57% of *V5-RED1*) > *red1*D (1.5%; 21% of *V5-RED1*) (Figure 3C). We also confirmed that overexpression of the SIM mutant proteins in relevant strains did not affect the formation of COs and NCOs (Supplementary Figure S4).

To determine if the loss of the Red1–Smt3 chain interaction might also affect the amount of DSBs generated during meiosis, we first examined DSB formation at *YCR047C*, a DSB hot spot on chromosome III. To quantify the level of DSBs more precisely, we introduced the *rad50S* mutation, which blocks the resection of the DSB ends, into the *V5-RED1*, *V5-red1^{I758R}*, *V5-red1^{3R}* and *red1*Δ mutants. Genomic DNA isolated from sporulating cultures at various time points was digested with *Bgl*II, separated by gel electrophoresis, blotted and hybridised with the *YCR052W* DNA probe (Buhler *et al*, 2007). The order for the overall quantity of DSBs at this specific site was *V5-RED1* (9.5%) ~ *V5-red1^{I758R}* (9.2%; 97% of *V5-RED1*) ~ *V5-red1^{3R}* (8.2%;

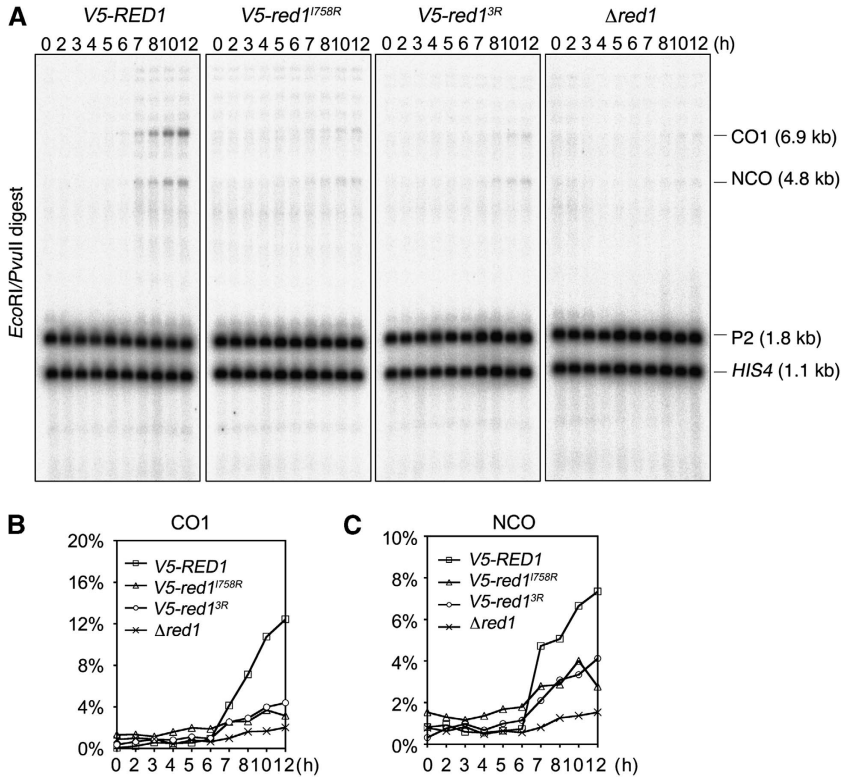

Figure 3 The Red1–Smt3 chain interaction promotes interhomologue (IH)-recombination product formation. (**A**) Southern blotting of DNA isolated from meiotic cultures. The diploid cells contained 3.5-kb *URA3-ARG4* inserts in *LEU2* on one chromosome *III* homologue (P1) and *HIS4* on the other (P2). *Eco*RI–*Pvu*II double digests probed with *HIS4* sequences reveal the crossover (CO1) and non-CO (NCO) products of *arg4-EcPal* (Clyne *et al*, 2003). (**B**, **C**) CO1 and NCO signal/total lane signal from the Southern blotting in (**A**).

86% of *V5-RED1*) > *red1*Δ (3.8%; 40% of *V5-RED1*) (Supplementary Figure S5). We also investigated the overall distribution and amount of DSBs along chromosome VII. DSBs along chromosome VII were detected by Southern blotting using a *CRM1* (YGR218W) DNA probe. *CRM1* is located near the end of the right arm of chromosome VII (Figure 4A). We found that all three *red1* mutants produced fewer DNA DSBs along the entire length of chromosome VII compared with the *V5-RED1* cells (Figure 4B and C). The relative order for the overall quantity of DSBs along the chromosome in these strains was *V5-RED1* (74.2%) > *V5-red1*^{I758R} (42.3%; 57% of *V5-RED1*) ~ *V5-red1*^{3R} (48.2%; 65% of *V5-RED1*) > *red1*Δ (24.0%; 32% of *V5-RED1*) (Figure 4D).

Although the *V5-red1*^{I758R} and *V5-red1*^{3R} mutants form fewer DSBs than the wild type cells, it is incorrect to infer that an overall reduction in the DSB number is the only cause of their meiotic phenotypes. It was reported that an allelic series of *spo11* mutants (*spo11-HA*, *spo11yf-HA*, *spo11da-HA*) formed ~80, ~30 and ~20% of DSBs along chromosome III compared with wild-type cells, respectively (Martini *et al*, 2006). Owing to CO homoeostasis, reducing DSBs does not reduce Cos; the CO numbers are maintained at the expense of NCOs. As a result, *spo11-HA*, *spo11yf-HA* and *spo11da-HA* mutants produce 94, 75 and 70% viable spores, respectively (Martini *et al*, 2006). We confirmed that Hop1 phosphorylation occurs in the *spo11-HA* and *spo11da-HA* mutants. The relative order of Hop1 phosphorylation in these strains was *SPO11* ~ *spo11-HA* > *spo11da-HA* > *spo11*Δ (Figure 4E). As described above, no phosphorylated Hop1 was detected in the *V5-red1*^{I758R} and *V5-red1*^{3R} mutants (Figure 2A and B). Because the two SIM mutants generated ~60% of the DSB levels of the wild type, it was not surprising that they yielded fewer NCOs (Figure 3C). Owing to CO homoeostasis, these two SIM mutants should produce similar levels of COs as the wild-type *V5-RED1* cells. However, these two SIM mutants produced much fewer COs (Figure 3B) and no viable spores. We conclude that a complete loss of Hop1 phosphorylation in these two SIM mutants is likely the main cause for their defects in CO production and spore viability.

Detection of Smt3 chains during meiotic mid-prophase

Like other known SUMO E3 ligases, Zip3 can promote Ubc9-catalyzed Smt3 polymerisation (Cheng *et al*, 2006). To examine if the wild-type cells produce Smt3 chains *in vivo* at the time of SC formation, we constructed a strain that expresses both V5-Smt3 and His_6–Myc–Smt3 and a strain that expresses both V5-Smt3-allR and His_6–Myc–Smt3. Total cell lysates (TCLs) were collected by trichloroacetic acid (TCA) precipitation during mid-prophase. The lysates were solubilized in a denaturing buffer containing 8 M urea and purified with Ni^{2+}-resin, which selectively retains the His_6-tagged polypeptides. The TCLs and the Ni^{2+}-resin eluants (NREs) were analysed by western blotting. Diploid cells expressing only the V5-Smt3 proteins were used as a negative control for Ni^{2+}-resin purification. The NREs of the *V5-SMT3/HIS*$_6$*–MYC–SMT3* diploid cells contained both V5-Smt3 and His_6–Myc–Smt3. In contrast, far fewer V5-Smt3-allR proteins were co-purified with His_6–Myc–Smt3 using Ni^{2+}-resin (Figure 5). These results indicate that Smt3 chains exist at the time of SC formation. The majority of these Smt3

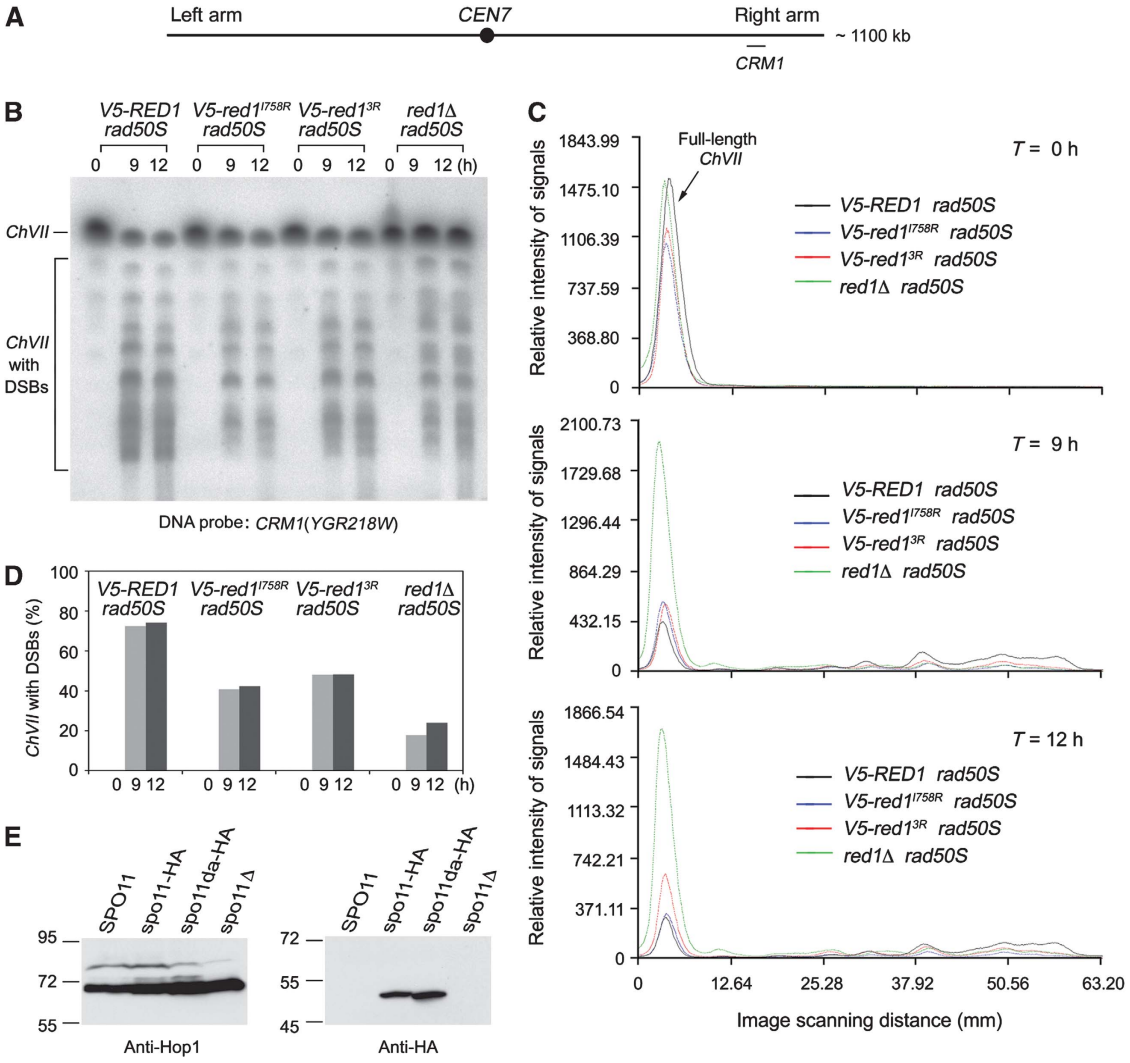

Figure 4 The overall distribution of double-strand breaks (DSBs) along chromosome VII on a *rad50S* background. DSBs along chromosome VII were detected by Southern blotting analysis using a *CRM1* (YGR218W) DNA probe. *CRM1* is located near the end of the right arm of chromosome VII. (**A**) Schematic drawing of chromosome VII (~1100 kb). The *CRM1* and *CEN7* loci are indicated. (**B**) Yeast chromosomes were separated by pulse-field gel electrophoresis, analysed by Southern blotting with a *CRM1* DNA probe, and visualised using a phosphoimager. The three *red1* mutants produced equal or fewer DNA DSBs along the entire length of chromosome VII compared with the *V5-RED1* cells. (**C**) Blots were quantified using a Fujifilm image analysis program (Image Gauge 4.0). The plots show traces of representative lanes at 0 h (upper panel), 9 h (middle panel), and 12 h (lower panel), respectively. (**D**) DSBs are expressed as a percent of the total radioactivity in the lane after background subtraction, not including material in the wells. (**E**) Western blot detection of Hop1 and Spo11-HA proteins in the *SPO11*, *spo11-HA*, *spo11-da-HA*, and *spo11*Δ cells at the 7-h meiotic time point.

chains have apparent molecular weights of approximately 100 000–140 000 Da on western blot analysis (Figure 5, lanes e and k). Because the apparent molecular weights of V5-Smt3 and the His_6–Myc–Smt3 monomers are roughly 20 000–24 000 Da, these Smt3 chains likely consist of five or six Smt3 monomers. Alternatively, the proteins may be shorter Smt3 chains conjugated on other novel non-Smt3 protein(s).

Red1 and Zip1 sandwich Smt3 chains to mediate SC assembly

Zip1 and Zip3 each contain a SIM for direct interaction with Smt3 conjugates (Cheng *et al*, 2006). This observation is consistent with reports that the chromosomal recruitment of Zip1 and Zip3 for SC initiation occurs in the absence of *SPO11* (Tsubouchi and Roeder, 2005; Tsubouchi *et al*, 2008). Spo11 functions (i.e., DSB formation) is a prerequisite for Hop1 phosphorylation, which ensures IH-recombination through repression of IS-recombination (Niu *et al*, 2005; Carballo *et al*, 2008). Therefore, a lack of Hop1 phosphorylation or failure to prevent IS-recombination may not fully account for the SC assembly defects in meiotic *V5-red1*^{I758R} and *V5-red1*^{3R} cells.

We previously reported that Zip1 and Red1 interacted in a two-hybrid assay (Cheng *et al*, 2006). Unfortunately, we were unable to co-immunoprecipitate Zip1 with V5-Red1 from meiotic cell lysates (data not shown), because the Zip1 proteins, unlike Red1 or Hop1, became extremely unstable after the meiotic cells were lysed in native buffers containing a variety of protease inhibitors (Supplementary Figure S6). More than 30 years ago, vacuolar proteases were reported to activate upon cell lysis in native buffers and the SDS sample buffer, leading to widespread proteolysis of yeast

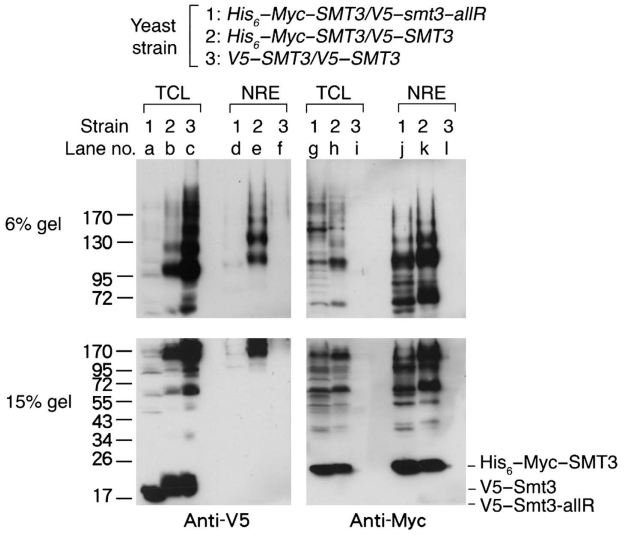

Figure 5 Smt3 chains exist in wild-type meiotic cells. Three different strains expressing V5-Smt3, V5-Smt3-allR or His_6–Myc–Smt3 protein are indicated as 1, 2 and 3, respectively. Meiotic cells were harvested at the 5-h time point, and the proteins were trichloroacetic acid (TCA) precipitated and dissolved in a denaturing buffer containing 8 M urea. The His_6-tagged polypeptides were purified using Ni^{2+} resins. Western blot analysis of total cell lysates (TCLs) and Ni^{2+} resin eluants (NREs) was carried out using anti-V5 and anti-Myc antibodies. The molecular weights (in kDa) are indicated to the left of the blots.

proteins (Pringle, 1975). Vacuolar proteases (e.g., Pep4, Prb1 and Prc1) are extremely troublesome for the biochemistry of yeast meiosis, because these proteases are not only highly induced upon starvation, but are also essential for sporulation (Zubenko and Jones, 1981). Nevertheless, we carried out additional yeast two-hybrid assays to confirm that the Red1–Zip1 interaction is mediated through Smt3 chains.

The C-terminal globular domain (residues 849–875) of Zip1 (Zip1C) interacts with Red1C. This interaction was diminished when the same two-hybrid assay was carried out in *smt3-allR* reporter cells or when Zip1C was replaced by $Zip1C^{3N-3R}$ (Figure 6A). $Zip1C^{3N-3R}$ is incompetent in Smt3 binding because it has three point mutations (E862R, D863R and Q864R) in the SIM (residues 853–864) (Cheng *et al*, 2006). These results suggest that Smt3 chains are sandwiched between Zip1 and Red1 to promote SC assembly. This hypothesis is further supported by an additional line of evidence. First, Zip1 and Red1 are both required for the accumulation of Smt3 conjugates or chains in the presence of *ZIP3* gene as shown by western blot analysis of the V5-Smt3 expression patterns in wild-type, *zip1*Δ and *red1*Δ meiotic cells (Figure 6B) and the His_6-Myc-Smt3 expression patterns in *V5-red1*^{I758R} and *V5-red1*^{3R} meiotic cells (Supplementary Figure S7). These results are consistent with our earlier report that, as a Smt3 binding protein, Zip1 protects Smt3 chains in the *zip3*Δ mutant through competition with Ulp2 (Cheng *et al*, 2006) (Figure 4B). Second, immunocytological staining of meiotic nuclear spreads indicated that *V5-RED1* cells formed complete SCs at the pachytene stage; their chromosomes had strong Zip1 (Figure 6C) and His_6–Myc–Smt3 (Figure 6D) staining signals along the entire length. In contrast, *V5-red1*^{I758R}, *V5-red1*^{3R} and *red1*Δ mutants failed to form complete SCs. These three mutants formed only Zip1 foci or few incomplete SCs. Eventually, Zip1 proteins accumulated into one or a few PCs at later meiotic time points because Smt3 chains could not be stably recruited to the meiotic chromosomes in these mutants (Figure 6E). The order of overall intensity of His_6–Myc–Smt3 staining intensity along meiotic chromosomes was *V5-RED1* > *V5-red1*^{I758R} > *V5-red1*^{3R} ~ *red1*Δ (Figure 6D). Taken together, our results suggest that a mature SC may be established by Smt3 chain bridging AE structural proteins (Red1) and CE structural proteins (Zip1).

Discussion

Red1, as a binding protein for the Smt3 chain, may have dual functions in the coordination of meiotic recombination and chromosome synapsis. The first function promotes Mec1- and Tel1-dependent Hop1 phosphorylation, repressing IS-recombination to ensure IH-recombination (Carballo *et al*, 2008). The second function may be required for recruiting Zip1 and Zip3 proteins to meiotic chromosomes for the initiation of SCs. We suggest that these Zip1–Smt3 chain–Red1 complexes then act together with phosphorylated Hop1 and other ZMM proteins (e.g., Zip2) to promote SC elongation.

The use of Smt3 chains for SC assembly is consistent with our previous report that a *smt3-allR* diploid strain was defective in SC assembly (Cheng *et al*, 2006), and it explains why the *zip3*Δ mutant forms short SCs in the absence of the 'Zip3-dependent' Smt3-modified AE proteins (Agarwal and Roeder, 2000; Hooker and Roeder, 2006). In the latter case, a few Smt3 chains may escape Ulp2 and be sandwiched by Red1 and Zip1 to assemble short SCs. Because Smt3 chains are diffusible and Zip3 has a SIM (Cheng *et al*, 2006), we propose a 'relay' mechanism for SC polymerisation. Zip3 proteins, as well as Ubc9, might be recruited first to a CO-designated site (Agarwal and Roeder, 2000; Henderson and Keeney, 2005; Hooker and Roeder, 2006) to promote the formation of Smt3 chains or conjugates. The neighbouring Red1 proteins at the AEs catch these Smt3 chains or conjugates to recruit not only Zip1 and other ZMM proteins for SC initiation and new Zip3 and Ubc9 proteins to produce more Smt3 chains for SC elongation.

The 'Smt3 chain relay' hypothesis described here needs to explain how Smt3 chains produced at the site of DSB sites in one chromatid can spread over that chromatid and its homologous chromosome without interfering with SC assembly in non-homologous chromosomes. This dilemma can be addressed in three ways. First, the interaction between Smt3 and the SIMs of Red1, Zip1 and Zip3 could be enhanced by Smt3 polymerisation, simply by increasing the local Smt3 concentration, which would facilitate rebinding after dissociation. Moreover, longer Smt3 chains diffuse more slowly than shorter Smt3 chains or free Smt3 monomers because of their greater size. Second, Ulp2 can hydrolyse the 'free' Smt3 chains. As a result of this hypothesis, Smt3 chains produced in one chromatid are less likely to migrate far enough to reach non-homologous chromosomes. Third, an active sorting mechanism was recently described for preventing incorrect synapsis between non-homologous chromosomes. This sorting and pairing mechanism is accompanied by telomere-led rapid movements (Conrad *et al*, 2008; Koszul *et al*, 2008). In principle, the chance of Smt3 chains reaching non-homolo-

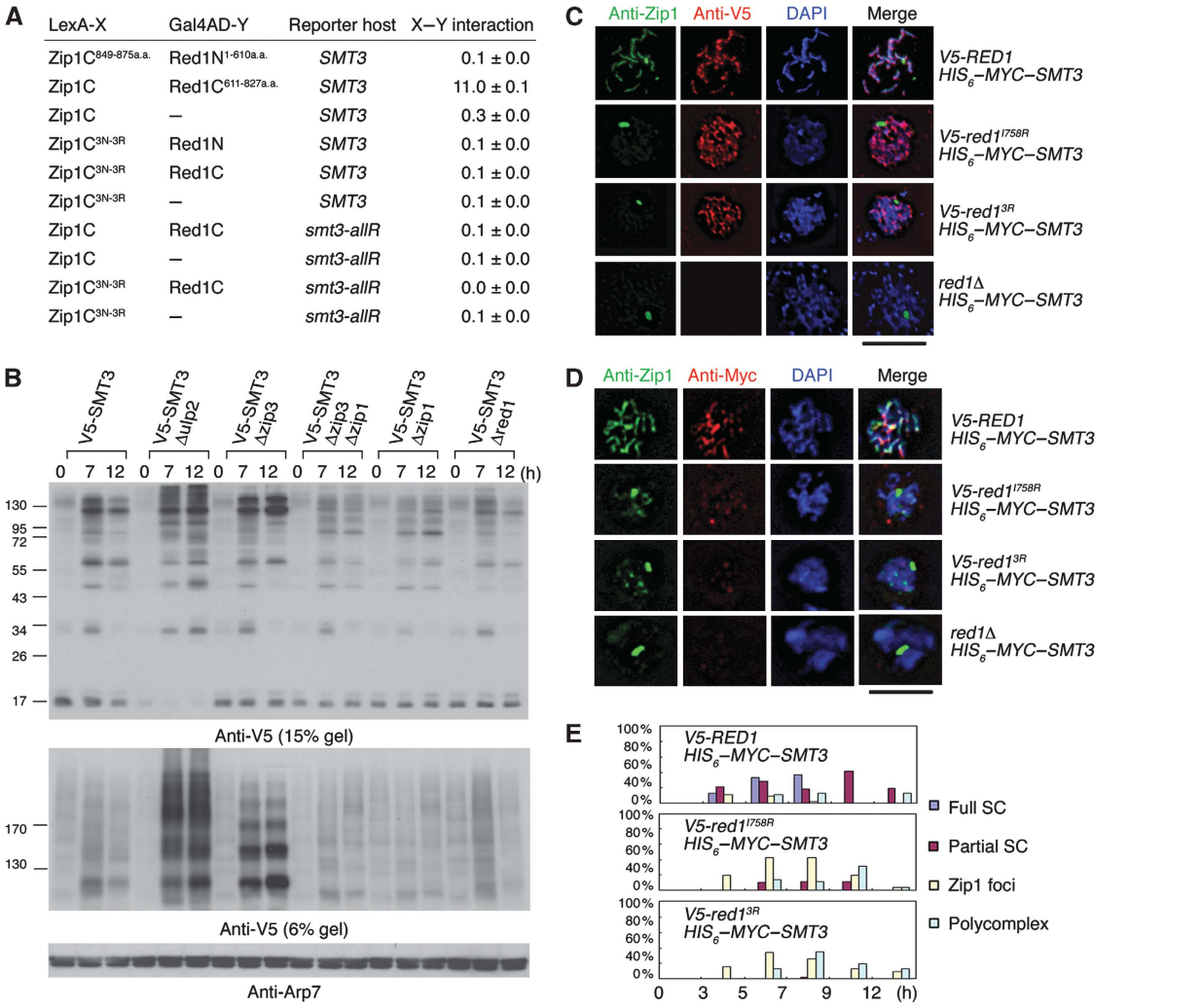

Figure 6 The Red1–Smt3 chain interaction is essential for synaptonemal complex (SC) assembly. (**A**) The two-hybrid interaction between Zip1C and Red1C. Zip1C represents the C-terminal globular domain (residues 849–875). Zip1C^{3N-3R} is incompetent in Smt3 binding because it contains three point mutations (E862R, D863R and Q864R) (Cheng *et al*, 2006). (**B**) Western blot time-course analysis of V5-Smt3 conjugates in the indicated yeast strains using anti-V5 antibody. Arp7 was used as a sample loading control. High steady-state levels of Smt3 chain conjugates were detected in the sporulating *ulp2*Δ and *zip3*Δ cells. The steady-state level of Smt3 conjugates was higher in the wild-type cells than in the *zip1*Δ, *red1*Δ and *zip1*Δ*zip3*Δ cells. These results indicate that Red1 and Zip1 are required for the stable accumulation of Smt3 conjugates. Moreover, all of the examined cells, except *ulp2*Δ, exhibited a similar steady-state level of monomeric Smt3 proteins, indicating that Ulp2 likely acts together with at least one other new protein to maintain the concentration of Smt3 monomers *in vivo*. (**C**, **D**) Representative images of surface nuclei spreads for the indicated meiotic cells stained with DAPI (blue), anti-V5 antibody (for V5-Red1), anti-Myc antibody (for His_6–Myc–Smt3) and anti-Zip1 antibody. The black bar represents 5 μm. (**E**) The time course of SC formation was determined by assigning spread nuclei into four classes according to different Zip1 staining patterns: full SC, partial SC, Zip1 foci and polycomplex.

gous chromosomes would be greatly diminished during such rapid movements.

The Smt3 chain relay mechanism may also explain the roles of centromeres in SC polymerisation. An early chromatin immunoprecipitation study showed that Red1 is highly enriched in the chromosome arms and less abundant at the centromere (Blat *et al*, 2002). During or before early zygotene, centromeres may act as SC initiation sites (Tsubouchi and Roeder, 2005; Tsubouchi *et al*, 2008) because the non-Zip3 SUMO E3 ligases not only produce Smt3 conjugates (e.g., Top2) at centromeres for NHCC (Tsubouchi and Roeder, 2005; Cheng *et al*, 2006) but they also generate 'diffusible' Smt3 chains for neighbouring Red1 to mediate SC polymerisation. These non-Zip3 ligases are then downregulated after zygotene, and the centromeric Smt3 conjugates are desumoylated by Ulp2 (Cheng *et al*, 2006). The centromere then serves as a barrier for Zip3-dependent SC polymerisation during pachytene (Henderson and Keeney, 2005) because centromeres contain fewer Red1 proteins.

Our results also revealed that the Red1–Smt3 chain interaction is required for Mec1–Tel1 mediated Hop1 phosphorylation (Carballo *et al*, 2008). As the two SIM mutants examined in this study were not defective in Hop1 chromosomal localisation (Supplementary Figure S2) or the Red1–Hop1 interaction (Supplementary Table S2 and Figure S3), we speculate that Smt3 chains may recruit Tel1 and Mec1 kinases to DSB sites for Hop1 phosphorylation. We are at present investigating whether these two kinases or their binding partners (e.g., Tel2 and Ddc2–Lcd1–Pie1) (Paciotti *et al*, 2000; Rouse and Jackson, 2000; Wakayama *et al*, 2001; Nakada *et al*, 2005; Anderson *et al*, 2008) contain a putative SIM.

In conclusion, this work demonstrates that Red1 protein can bind Smt3 chains. This Red1–Smt3 chain interaction provides a new molecular clue to the role of Red1 in the coordination of homologous recombination, Mec1–Tel1 checkpoint kinase activation and chromosome synapsis during yeast meiosis.

Materials and methods

Yeast strains and two-hybrid assay

Almost all meiotic experiments were carried out using diploid cells on the SK1 strain background. BR strains were kindly provided by Shirleen Roeder (Yale University). Yeast two-hybrid assays were carried out as previously described (Cheng *et al*, 2006).

Cytology, western blot and physical analysis

Cytological analyses were carried out as previously described (Cheng *et al*, 2006). For western blot time-course studies, TCLs were prepared according to the TCA precipitation protocol described previously (Cheng *et al*, 2006). This protocol uses both TCA and urea (8 M) in the sample buffers, which allows for efficient extraction of proteins from yeast cells grown on various media and the prevention of proteolysis, dephosphorylation and desumoylation. Physical analyses were performed as described previously (Clyne *et al*, 2003). Quantification of the DSB, NCO and CO signals was achieved using a Fujifilm image analysis program (Image Gauge 4.0).

Expression and purification of the wild-type and mutant GST–Red1–His$_6$ fusion proteins

The DNA fragment encoding wild-type Red1 protein was cloned into a GST–His$_6$ expression vector using the *Eco*RI and *Xho*I restriction sites. The GST–Red1–His$_6$ expression vector was then subjected to site-directed mutagenesis to create the GST–Red1^{I758R}–His$_6$ expression vector. The expression vector was transformed into BL21(DE3) competent cells. For each protein, cell culture (100 ml) was grown overnight at 37°C with 50 mg/l ampicillin. After transferring the cell culture to 4 l of LB medium, the cell suspension was allowed to reach an OD_{600} of approximately 0.5–0.6 before 1 mM IPTG was added. Cells were grown for 15 h at 18°C and centrifuged at 6000 *g* for 30 min. The cell pellet was resuspended in 100 ml lysis buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 50 mM sucrose and 0.2 mM EGTA) and disrupted using a French press (30 000 psi; Sim-Aminco). The TCL was centrifuged at 61 000 *g* for 45 min. The soluble protein fraction was loaded onto a 4 ml Ni^{2+}-chelating Sepharose column (GE Healthcare). After the column was washed with 50 ml lysis buffer plus 5 mM imidazole (pH 8.0), the His$_6$-tagged proteins were eluted with 8 ml lysis buffer plus 100 mM imidazole. The eluants were then directly mixed with 4 ml glutathione resins (GE Healthcare) to purify the GST-tagged proteins. The full-length GST–Red1–His$_6$ and GST–Red1^{I758R}–His$_6$ proteins were then eluted with 8 ml lysis buffer containing 10 mM glutathione in the presence of 10 mM β-mercaptoethanol. The eluants were collected and dialyzed against buffer Q (50 mM Tris–HCl pH 8.0, 200 mM NaCl, 40% glycerol and 1 mM β-mercaptoethanol).

Production of Smt3 chains

Smt3 chains were synthesised as previously described (Cheng *et al*, 2006). Briefly, purified His$_6$–Myc–Smt3, His$_6$–Aos1/His$_6$–Uba2 complex (E1), His$_6$–Ubc9 (E2), Mg^{2+} and ATP were incubated. The resulting products could not be directly applied to the Red1 binding assay because His$_6$–Ubc9 (~18 kDa) and the His$_6$–Ubc9/His$_6$–Myc–Smt3 conjugated adducts (~35 kDa) catalyze the covalent addition of His$_6$–Myc–Smt3 to Red1 recombinant proteins. To partially purify the Smt3 chains, gel filtration was carried out by high-pressure liquid chromatography using a HiLoad 16/60 Superdex sizing column (GE Healthcare) run at a flow rate of 1.0 ml/min. The elution profile was followed by a continuous assay of the optical density at 280 nm. Commercially supplied molecular mass standards, including thyroglobulin, ferritin, catalase, bovine serum albumin (BSA), ovalbumin and lysozyme, were used to calibrate the column. The average elution position, *K*av, was computed by the equation $Kav = (Ve - V_0)/(Vt - V_0)$, where *Ve* and *Vt* represent elution volumes for the molecular mass standards and DTT, respectively. The void volume, V_0, was determined using blue dextran 2000 (GE Healthcare). The Smt3 chains were eluted before the elution volume expected for a globular protein with an apparent molecular weight of 60 kDa. The eluants were then analysed by Western blotting using either anti-Myc or anti-Ubc9 antibody (Santa Cruz Biotechnology). The purified Smt3 chain products contained neither His$_6$–Myc–Smt3 monomers nor His$_6$–Ubc9 (data not shown); on the other hand, the E1-activating enzymes, a heterodimeric complex of His$_6$–Aos1 (~40 kDa) and His$_6$–Uba2 (~70 kDa), were still present in the purified Smt3 chains products. We applied mass spectroscopy to confirm that the band around 30–40 kDa (Figure 1B, lane 4) was His$_6$–Aos1 and not the His$_6$–Myc–Smt3 dimer (data not shown).

Smt3 chain-binding assay

Equivalent molar amounts of the GST–Red1–His$_6$ (20 μg), GST–Red1^{I758R}–His$_6$ (20 μg) and GST–His$_6$ (4.5 μg) proteins were first incubated with 5 μl MagneGST glutathione resin (Promega) in 300 μl binding buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl and 10 mM β-mercaptoethanol) for 3 h. After binding, the resins were washed with phosphate-buffered saline and were suspended again in another 300 μl of binding buffer containing BSA (10 μg) and partially-purified Smt3 chains (10 μg). The BSA was used to block non-specific interactions between the Smt3 chains and the glutathione resin. The resin was extensively washed three times with 600 μl wash buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl and 10 mM β-mercaptoethanol). Protein samples were then separated by SDS–PAGE and analysed by western blotting using an anti-Myc antibody.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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