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Analysis of Zip1 and Sgs1 protein interaction in budding yeast

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Abstract of the Thesis

Analysis of Zip1 and Sgs1 protein interaction in budding yeast

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Meiotic crossovers (COs) are essential for proper chromosomal separation. Interhomologue recombination is promoted by the synaptonemal complex (SC), which connects paired homologous chromosomes together. Zip1, an integral component of the SC, antagonizes Sgs1 helicase function during meiotic recombination to promote COs. Specifically, Zip1 and other ZMM proteins shield strand invasion intermediates from Sgs1 activity, thus preventing Sgs1 from disassembling them. It has been previously discovered that Zip1 interacts with Sgs1 in two-hybrid experiments, and that full length Zip1 binds to Sgs1 in vitro. These interactions suggest that the antagonism may be due to direct interactions between Sgs1 and Zip1. This work shows that a negative charge at Ser801 on Zip1 enhances the interaction with Sgs1 in two-hybrid experiments, and that the N-terminal region of Sgs1 interacts with a small ubiquitin-like modifier interacting motif (SIM) of Zip1. These results suggest that Sgs1 interacts with Zip1 via SUMO chains, and the phosphorylation of Zip1 by Mek1 kinase may enhance the interaction between a SUMOlyated site on Sgs1 and the SIM on Zip1 or alternatively Zip1 and Sgs1 both have SIMs that then sandwich SUMO chains, which further antagonizes Sgs1 helicase activity to enable CO products.

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List of Abbreviations

A	Alanine
AE	Axial element
CO	Crossover
Co-IP	Coimmuniprecipitation
CR	Central region
D	Aspartic acid
dHJ	Double Holliday junction
D-loop	Displacement loop
DSB	Double strand break
GAD	Gal4 activation domain
LE	Lateral element
NCO	Noncrossover
ONPG	*O*-nitrophenyl-β-D-galacto-pyranoside
PVDF	Polyvinylidene fluoride
R	Arginine
S	Serine
SC	Synaptonemal complex
SDSA	Synthesis-dependent-strand-annealing
SIM	Small ubiquitin-like modifier interacting motif
SILAC	Stable Isotope Labeling by Amino acids in Cell culture
SUMO	Small ubiquitin-like modifier
TBST	Tris-buffered saline and Tween 20

TCA	Trichloroacetic acid
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside
ZMM proteins	Zip1-4, Mer3, Msh4, Msh5, Spo16

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Chapter 1

Introduction

Meiosis

Meiosis is the process of cellular differentiation in which diploid cells generate haploid gametes to produce reproductive cells. Diploid cells entering meiosis undergo a single round of DNA replication followed by two rounds of cellular division. During the first round of division, homologous chromosomes segregate from one another, while sister chromatids remain attached. In the second round of division, sister chromatids separate to generate haploid gametes containing a complete single set of chromosomes.

Crossovers (COs) are an integral component of meiosis. They provide genetic diversity and are essential for promoting correct chromosome segregation during the first meiotic division. To ensure the proper segregation of chromosomes, a series of interactions (such as the pairing of homologous chromosomes, synapsis and recombination) need to occur during prophase. Physical connections are necessary between homologous chromosomes to ensure proper alignment and orientation on the meiotic spindle during the first round of division (Petronczki et al., 2003). Improper segregation can lead to infertility or inviable zygotes. In humans, chromosome missegregation accounts for ten to thirty percent of spontaneous miscarriages (Hassold and Hunt, 2001). The physical connections between homologous chromosomes are achieved by cohesion—linking sister chromatids together—and COs (Moynahan et al. 2010).

Recombination

COs arise by recombination between homologous chromosomes. Recombination in meiotic cells is initiated by programmed double strand breaks (DSBs) at preferred sites by a topoisomerase like protein, Spo11 (Neale and Keeney, 2006; Keeney, 2001). Meiotic cells use

homologous recombination to generate COs that help hold homologous chromosomes together so that they properly align during metaphase I. Once the meiosis-specific Spo11 enzyme makes a DSB on one of the four chromatids, the 5' ends of the break are resected, generating 3' ssDNA tails, which are then bound to the RecA orthologs, Rad51 and the meiosis-specific, Dmc1 (Neale and Keeney, 2006) (Figure 1). Loading of Rad51 is dependent on mediator proteins such as Rad52, Rad55, and Rad57 (Gasior et al., 1998), whereas the loading of Dmc1 requires the aid of a mediator complex consisting of Sae3 and Mei5 (Heyase et al., 2004) and accessory proteins Hop2 and Mnd1 (Tsubouchi and Roeder, 2003).

Rad51 and Dmc1 form filaments on the 3' ssDNA that promote strand invasion of homologous non-sister chromatids (Hunter and Kleckner, 2001) (Figure 1). After strand invasion and the extension of the displacement loop (D-loop), either a non-crossover (NCO) or a CO pathway repairs the breaks (Allers and Lichten, 2001). More specifically, Dmc1 functions to promote interhomolog COs with the help of Rad51 and axial proteins (Bishop, 1994; Hollingsworth et al., 1995; Schwacha and Kleckner, 1997). Rad51 works in conjunction with Dmc1 in interhomolog strand invasion (Cloud et al., 2012). In a *dmc1* null mutant, prophase arrest occurs; the meiotic recombination checkpoint is triggered, exhibiting unrepaired DSBs due to the lack of strand invasion (Bishop et al., 1992; Lydall et al., 1996; Roeder and Bailis, 2000).

After strand invasion has occurred, a subset of recombination intermediates is directed to the synthesis-dependent-strand-annealing (SDSA) NCO pathway, which is mediated by the Sgs1 helicase (De Muyt et al., 2012; Jessop et al., 2006) (Figure 1). In the SDSA pathway, the invading strand is displaced after DNA synthesis has extended the invading strand. The end can then anneal to the complementary sequence on the other side of the DSB (Paques and Haber, 1999). Intermediates that do not enter the SDSA pathway proceed to form double Holliday

junctions (dHJs). CO products are then produced via resolution of dHJs (Allers and Lichten, 2001) (Figure 1). CO formation is promoted by a group of proteins called the ZMM proteins (Zip1-4, Mer3, Msh4, Msh5, Spo16), which stabilize D-loops (Allers and Lichten, 2001) (Figure 1).

Synaptonemal complex and Zip1

In addition to recombination, another key component that maintains homologs in close juxtaposition is the synaptonemal complex (SC). The SC is a zipper like proteinaceous structure between homologous chromosomes that assembles along the chromosomes' length (for a review, Page and Hawley, 2004). The SC consists of a central region (CR) and two parallel lateral elements (LEs) that flank the CR. (Altmannová et al., 2012; Page and Hawley, 2004) (Figure 2). LEs are formed by condensation of sister chromatin along a protein core of meiosis-specific proteins and are called axial elements (AEs) prior to synapsis (Page and Hawley, 2004).

In budding yeast, axial elements contain the meiosis-specific proteins, Red1 and Hop1 (Altmannová et al., 2012; Hollingsworth and Byers, 1989; Niu et al., 2005; Smith and Roeder, 1997) (Figure 3). These proteins assist Dmc1 in promoting interhomolog strand invasion (Niu et al., 2005; Wan et al., 2004). Mutations in genes encoding these proteins result in reduced interhomolog recombination and increased intersister recombination (Niu et al., 2005; Wan et al., 2004).

Mek1, a meiosis-specific serine/threonine protein kinase, which forms a complex with Red1 and Hop1, is also necessary for interhomolog recombination (de los Santos and Hollingsworth 1999; Niu et al., 2007). Mek1 prevents Rad51 from performing intersister repair and thus promotes interhomolog repair (Niu et al., 2005; Niu et al., 2007; Niu, 2009). In a *mek1* null mutant, increased recombination between sister chromatids is exhibited, in addition to

decreased interhomolog recombination (Hollingsworth et al., 1995; Niu et al., 2005). Activation of Mek1 kinase is dependent on Hop1 phosphorylation by the checkpoint kinase Mec1 (Carballo, et al., 2008).

COs frequently occur in the context of the SC. A key component of the SC's central region is the Zip1 protein (Sym et al. 1993). Zip1 serves as a molecular zipper that allows homologous chromosomes to be in close proximity (Sym et al., 1993). In a *zip1* mutant, axial elements form and homologously pair, but do not synapse (Sym et al., 1993). In a *zip1*△ each pair of homologous chromosomes is aligned with one another and closely connected at a few points known as axial associations. Although recombination occurs, there is a decrease in CO production and an increase in NCO production (Jessop et al., 2006; Rockmill et al., 2003; Sym et al., 1993).

Zip1 contains an α-helical coiled-coil domain, which is flanked by globular ends on its termini (Dong and Roeder, 2000) (Figure 3). Zip1 is predicted to form rod shaped homodimers, where the heads lie in close proximity to one another (Sym et al., 1993; Sym and Roeder, 1995) (Figure 4). The N-terminus is located in the middle of the central region interacting with another Zip1, and the C-terminus is anchored in the LE (Tung and Roeder, 1998) (Figures 2 and 3). Localization to chromosomes is achieved via the C-terminus of the Zip1 protein (Tung and Roeder, 1998). Specifically, amino acids 791-824 in the C-terminus are essential for Zip1 localization to chromosomes for synapsis (Tung and Roeder, 1998).[1]

Additionally, the C-terminus of Zip1 has been shown to be a potential Mek1 substrate site using stable isotope labeling by amino acids in cell culture (SILAC) (R. Suhandynata and N.M. Hollingsworth, unpublished data). SILAC is a technique that provides an *in vivo* approach

[1] aa 800-824 are necessary for Zip1 to localize to chromosomes; the first nine aa were repeated in another deletion which did not exhibit a similar phenotype (Tung and Roeder, 1998) .

to mark proteins based on their different amino acid isotopic forms. This allows a quantitative comparison, via mass spectrometry, between different conditions of differing isotopic 'light' and 'heavy' proteins.

The strain used in the Hollingsworth lab by Ray Suhandynata for the SILAC experiment has a *dmc1 mek1-as* genotype. Mek1-as is a version of Mek1 that can be inactivated by the addition of an inhibitor (Wan et al., 2004). Cultures pre-grown in either "light" or "heavy" medium were arrested in prophase I using *dmc1*Δ where Mek1 is still active (Niu et al 2005). Mek1 was then inactivated for 20 minutes in the heavy culture by the addition of the inhibitor. To compare the ratios of the 'light' and 'heavy' proteins at their respective phosphosites, crude chromatin was prepared. Phosphopeptides were purified using a metal affinity column and subsequently analyzed via mass spectrometry in collaboration with Huilin Zhou's lab at the University of California, San Diego. Phosphopeptides in which the light peptide was present at at least 2 fold higher abundance than the heavy peptide are considered potential Mek1 phosphosites. Hollingsworth's lab detected potential Mek1 phosphosites on Zip1 at Ser801, which fits the Mek1 consensus site because of the Arg in the -3 position (Mok et al., 2010), but had a light to heavy ratio of only 1. Ser802 was also considered another potential phosphosite, with a light to heavy ratio of 5.0, while an S801 S802 doubly phosphorylated peptide had a ratio of 8.3.

The C-terminus of Zip1 also interacts with the AE protein, Red1 (Lin et al., 2010). Interactions between Zip1 and Red1 are mediated by small ubiquitin-like modifier (SUMO) interacting motifs (SIMs) on the C-termini of Red1 and Zip1 (Lin et al., 2010). Specifically, Zip1 binds SUMO non-covalently at its SIM (Lin et al., 2010). Red1 can either form covalent SUMO conjugates or bind to SUMO chains non-covalently at its two respective SIM sites (Lin et al., 2010). It has been postulated that the initiation of SC assembly, via Zip1 and Zip3

recruitment, is promoted by the interactions between Red1 and SUMO chains (Lin et al., 2010) (Figure 2).

SUMOlyation is a reversible post-translational modification that occurs via an enzymatic pathway similar to the ubiquitin pathway. It is characterized by the covalent linking of SUMO peptide—known as Smt3 in *Saccharomyces cerevisiae*—to a lysine residue on the target protein (for a review, Altmannová, 2012). Smt3 is often attached to substrates at lysine residues at the consensus sequence, ΨKxE; where Ψ represents a large hydrophobic residue and x represents any amino acid (Cheng et al., 2006). SUMOlyation can provide binding sites for new proteins or stimulate existing protein interactions (Altmannová, 2012). Conversely, but less likely, it can also block interactions (Altmannová, 2012).

SUMOYlated proteins are able to promote non-covalent binding to other proteins that contain a SIM (Altmannová, 2012). The SIM has a characteristic hydrophobic core of amino acids, which provides an interface for non-covalent interaction with SUMOYlated proteins (Altmannová, 2012; Hecker et al., 2006). The sequence surrounding this core-binding domain is predicted to have a net negative charge, which further stimulates SIM-SUMO interaction (Altmannová, 2012; Hecker et al., 2006).

SUMO modification, during the formation of the SC, is predominantly regulated by Zip3, a meiosis-specific SUMO E3 ligase; the initial assembly, however, is regulated by another E3 ligase (Cheng et al., 2006). Zip1 proteins associate with Smt3 conjugates along the LE of the SC (Cheng et al., 2006) (Figure 2). Red1 and Zip1 sandwich Smt3 conjugates, which in turn promotes the interaction between Red1 and Zip1, and may mediate the assembly of the SC (Lin, et al., 2010). A three-point mutation in the SIM of Zip1, Zip1C$^{3N\text{-}3R}$ (E862R, D863R, and Q864R) (called Zip1-C3R in this thesis), is incapable of binding to SUMO chains, and

consequently is unable to bind to Red1 (Lin, et al., 2010). This is a further indication of the importance of SUMOlyation in the assembly of the SC.

Sgs1 and Zip1

In a *zip1* null mutant, CO products are greatly reduced, synapsis is impaired, and Holliday junctions reside for a longer duration in contrast to WT (Sym and Roeder, 1994; Storlazzi et al., 1996). *sgs1* mutants counteract this phenotype, allowing synapsis between homologs—pseudo synapsis—and increasing CO products in *zip 1*△ background (Jessop et al., 2006; Rockmill et al., 2003). Jessop et al., illustrated that CO defects in a *zip1* mutant were suppressed by the loss of Sgs1 activity. They speculated that ZMM proteins, including Zip1, shielded strand invasion intermediates from Sgs1 activity, thus preventing Sgs1 from dissembling them (Jessop et al., 2006).

Sgs1 is thought to play a predominant and critical role in regulating meiotic recombination (De Muyt et al., 2012). Most meiotic NCOs are formed due to SDSA or dissolution of a subset of dHJs (Paques and Haber, 1999). Dissolution occurs when a helicase and topoisomerase disassemble dHJs to form NCO products. Sgs1, a 3′-to-5′ DNA helicase, belongs to the RecQ DNA helicase family, which includes human proteins that are linked to Werner's syndrome and Bloom's disease (Enomoto, 2001). Sgs1 promotes NCOs via two methods: (i) encouraging SDSA by disassembly of D loop formation on the onset (Adams et al., 2003; van Brabant et al., 2000); and (ii) driving dissolution in double HJs during the intermediate stage of recombination (Wu and Hickson, 2003) (Figure 4). Those D-loops that are captured by the ZMM proteins are stabilized and protected from Sgs1 helicase activity and thus can produce CO products (De Muyt et al., 2012) (Figure 4).

Proteins encoded by genes such as *ZIP1* promote crossovers by antagonizing Sgs1 (Jessop et al., 2006). The Hollingsworth lab found a protein-protein interaction between Sgs1 and the C-terminus of Zip1 via a two-hybrid assay (Y. Liu and N.M. Hollingsworth, unpublished data). Furthermore, Hengyao Niu and Patrick Sung (Yale University) have recently shown that recombinant Zip1 and Sgs1 interact in vitro (unpublished data). These results support a model in which direct interaction between Zip1 and Sgs1 somehow disrupts Sgs1 helicase function.

The C-terminus of Zip1 has two important protein-protein interactions that occur during meiosis: (i) the interaction with the AE protein, Red1, through the SIM located near the end of the C-terminus, as previously shown by Lin et al; and (ii) potential phosphorylation of serine residues at 801 and/or 802 by Mek1 to promote the interaction with Sgs1 helicase. My thesis project was to explore what features of Zip1 C-terminus are important for interaction with Sgs1. More specifically, I addressed whether the interaction between Zip1 and Sgs1 is promoted by phosphorylation by Mek1 at Ser801 and Ser802, and whether SUMO chains facilitate the interaction between Zip1 and Sgs1, and if so, whether Zip1 and Sgs1 interaction requires Sgs1 SUMOlyation. We hypothesized that: (i) a negative charge on S801, S802 or both would affect the interaction between Sgs1 and Zip1 (either positively or negatively); (ii) removal of the sequences required for synapsis (amino acids 791-824), which contain the putative Mek1 phosphorylated residues should specifically eliminate the interaction with Sgs1, but not Red1, considering the SIM is intact; and (iii) mutation of the SIM domain on Zip1 should result in the abolishment of the interaction with Red1, but not Sgs1.

In this study I found that that the simple hypothesis that Sgs1 and Red1 interactions with Zip1 are mediated by different parts of the Zip1 C terminus is incorrect. Instead, similar to the AE component Red1, Sgs1 interacts with Zip1 through the SIM located near the very end of the

protein. This interaction appears to be enhanced by an addition of a negative charge at Ser801, which may indicate that phosphorylation by Mek1 kinase heightens the interaction.

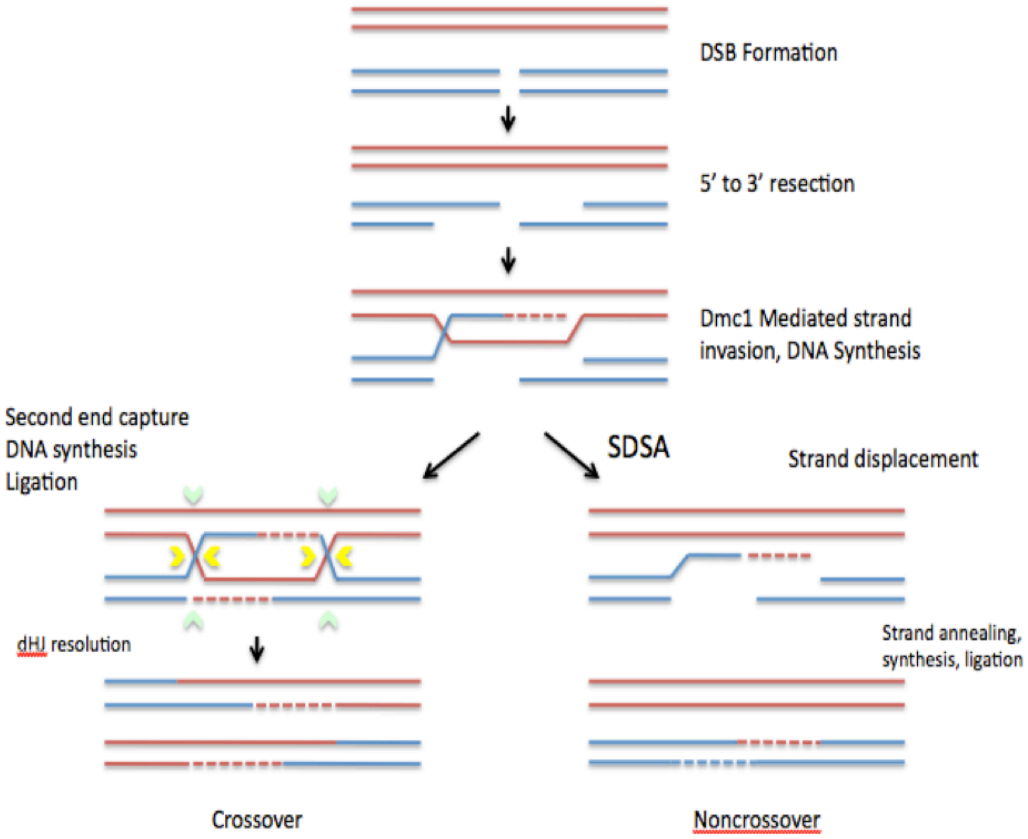

Figure 1. The double-strand break repair model for meiotic recombination (adapted from Allers and Lichten, 2001; Neale and Keeney, 2006; Sung and Klein, 2006). The blue and pink lines represent the duplex DNA of homologous chromosomes. Spo11 forms a double stranded break. 5' end resection produces 3' single-strand ends. Dmc1 with the assistance of Rad51 catalyzes strand invasion of non-sister chromatids. Recombination intermediates are processed into crossover products by resolution of double Holliday junctions (resolution represented by green and yellow arrows), or are processed into non-crossover products via synthesis dependent strand annealing.

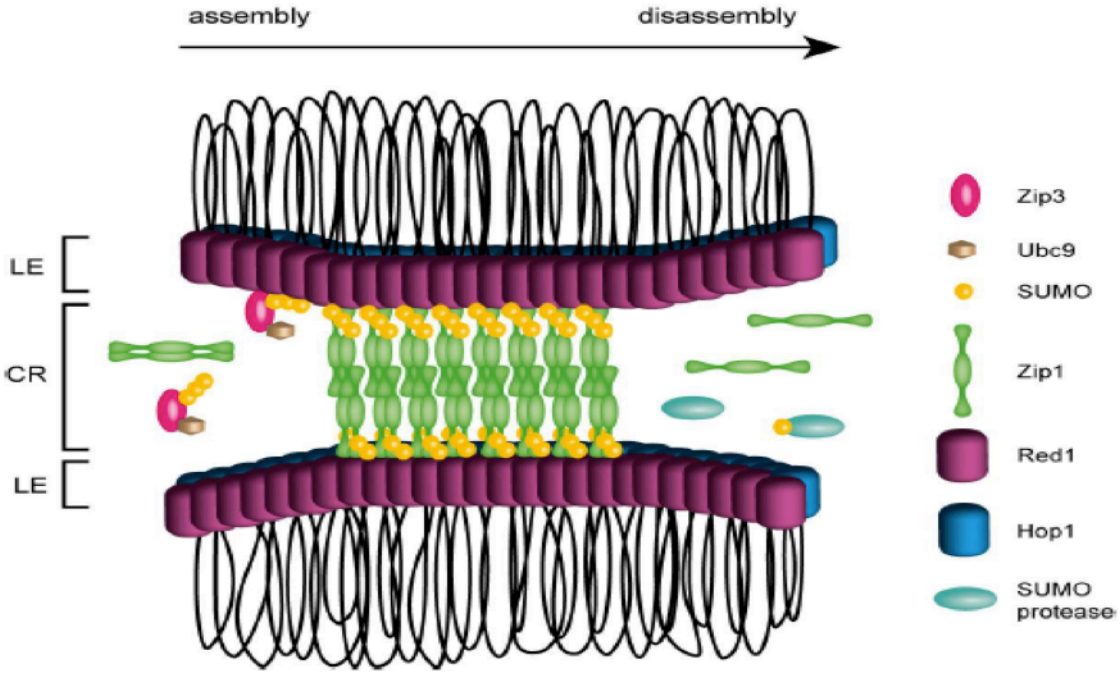

Figure 2. The synaptonemal complex (SC); assembly and disassembly (Altmannová et al., 2012).
During SC assembly, proteins facilitate the formation of small ubiquitin-like modifier (SUMO) chains along the lateral element (LE) of the SC, and the conjugation of SUMO and other proteins such as Red1 and Hop1. Polymerization of Zip1 dimers occurs along the LE; Zip1 dimers bind to Red1 and SUMO chains along the LE. This assembly forms the central region of the SC. SC disassembly is proposed to occur via the dissociation of SUMO chains via SUMO proteases along the SC.

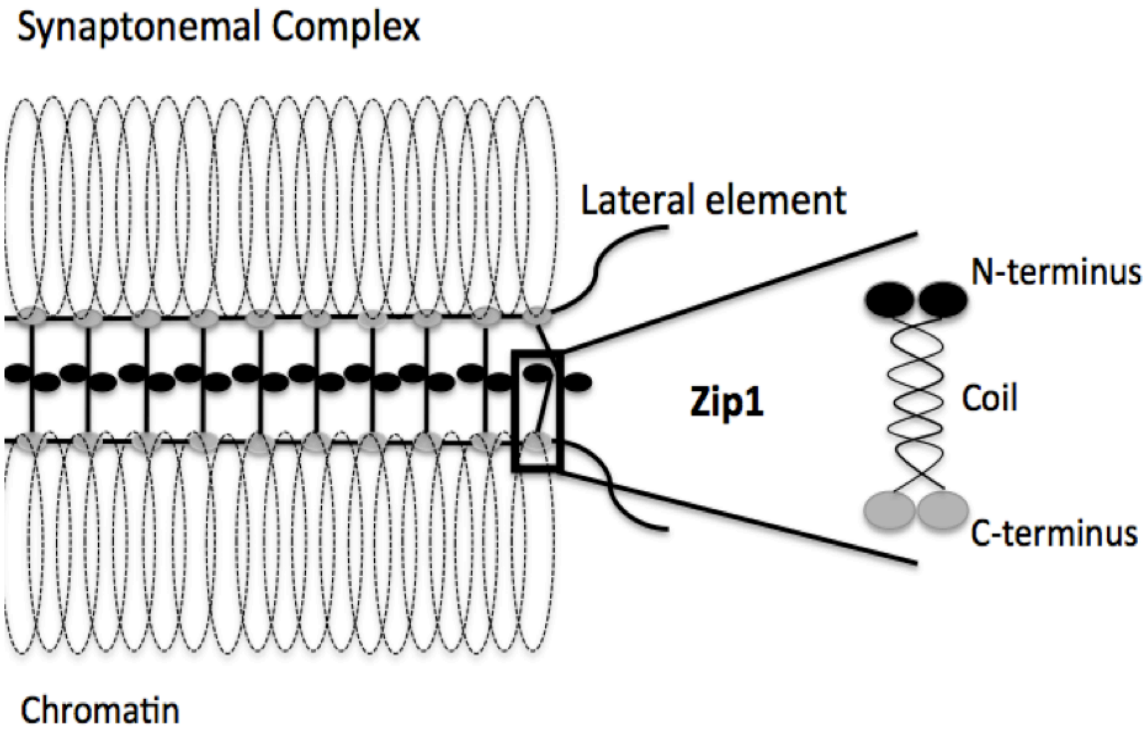

Figure 3. Zip1 in a synaptonemal complex (SC) (adapted from Dong and Roeder, 2000). The SC exhibited with lateral elements (LE), and the transverse filaments that form the central region (CR). Each LE represents one pair of sister chromatids (DNA loops are indicated by ovals). Zip1 contains an α-helical coiled-coil domain, which is flanked by globular ends on its termini.

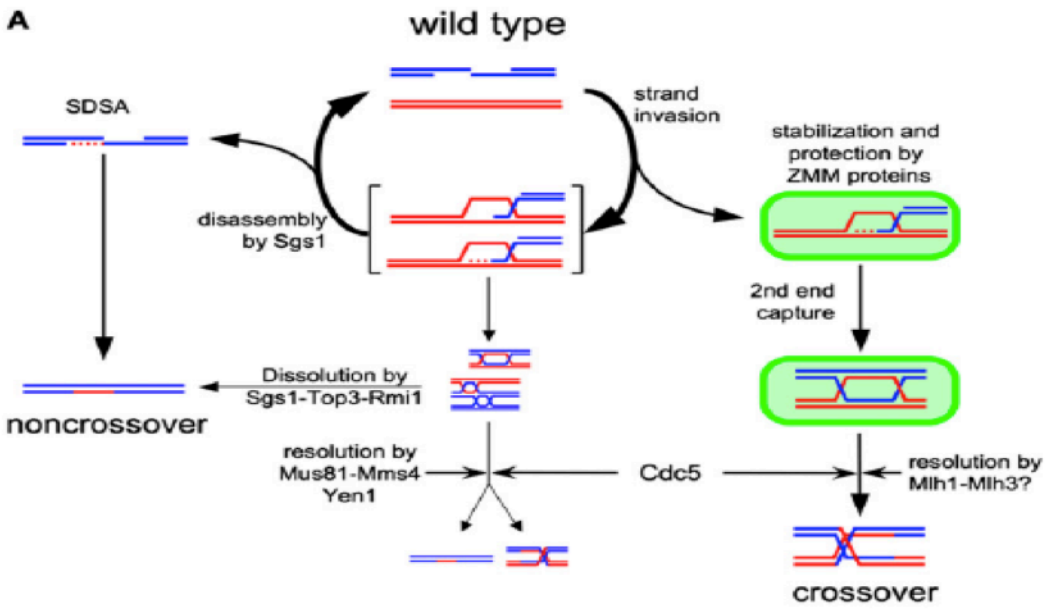

Figure 4. Model for Sgs1 regulation in recombination (De Muyt et al., 2012). Post strand invasion, D-loops can either be disassembled by the Sgs1 helicase or captured by ZMM proteins. The subset that are disassembled by Sgs1 proceed via the synthesis-dependent-strand-annealing pathway to produce non-crossover (NCO) products. Those that are captured by ZMM proteins are stabilized and protected from Sgs1, allowing double Holliday junction formation, and ultimately leading to crossover (CO) products. A smaller subset of recombination intermediates that escape disassembly from Sgs1 and are not captured by ZMM proteins are resolved by other proteins such as Yen1 or Mus81-Mms4 and can form either CO or NCO products.

Chapter 2

Materials and Methods

Yeast strain

The yeast strain, L40, was used for all the two-hybrid experiments (Hollenberg et al., 1995). Its genotype is *MAT***a** *hisΔ200 trp1-90 leu2-3, 112 ade2 lys2::lexAop-HIS3:: LYS2 ura3::lexAop-lacZ::URA3 gal80.*

Plasmids

Site-directed mutagenesis

pA86 contains a fragment of *SGS1* (codons 81-614) fused to the *GAL4* activation domain (*GAD*) (Table 1). It was previously isolated in the Hollingsworth lab using a two-hybrid screen with *lexA-MEK1* as bait (T. de los Santos and N. M. Hollingsworth, unpublished data). pA86 was used as a template for site directed mutagenesis of *SGS1* using the Quik-Change kit from Stratagene (Table 1). All the mutations were confirmed by DNA sequencing (Stony Brook University DNA Sequencing facility).

Two-hybrid plasmids

To fuse the Zip1 C-terminal globular domain to *lexA*, Yan Liu in the Hollingsworth lab amplified a fragment containing codons 750-875 of *ZIP1* using p382 as a template (Falk et al., 2010). The fragment was engineered to contain BamHI and PstI ends which were cut and ligated to BamHI/PstI-digested pBTM116 to make pBTM116-ZIP1C*-WT (Table 1). For pSH1, *ZIP1-C*^{3R}* was first synthesized by Genewiz for fusion to *lexA* in pBTM116 (Table 1). This plasmid, pUC57-ZIP1-C*^{3R}, contains a three point mutation, E862R, D863R and Q864R, that abolishes the SUMO interacting motif (Lin et al., 2010) (Table 1). pUC57-ZIP1-C*^{3R} was engineered to contain BamHI and PstI ends, which were cut and ligated to BamHI/PstI-digested pBTM116 to

make pSH1 (Table 1). The 0.4 kb fragment containing ZIP1-C*^{3R} was confirmed by digestion with BamHI and PstI.

For pSH2, the same piece of *ZIP1* contained in pBTM116-ZIP1-C*WT was synthesized by Genewiz but in this case sequences containing codons 791-824 were deleted. This fragment was provided in the vector pUC57 and ligated into pBTM116 using BamHI and PstI as described above. The 0.3 kb fragment containing *ZIP1-C*$^{\triangle 791-824}$* was confirmed by digestion with BamHI and PstI.

pGAD-RED1 was used as a positive control for the two-hybrid assays (Lin et al., 2010). This plasmid contains a fragment of *RED1* (codons 537-827) and was provided by Marian Carlson (Tu et al., 1996).

Two-hybrid assays

Filter assay for β-galactosidase activity

For qualitative results, β-galactosidase filter assays of L40 cells containing various plasmid combinations were performed using 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal). Cleavage of X-gal by β-galacotosidase results in a blue color. Specifically, independent transformants containing both a *lexA-ZIP1-C*-WT* plasmid and either pACTII (*GAD* alone), pGAD-RED1$^{537-837}$ or pGAD-SGS1$^{181-614}$ were patched onto selective medium (SD-trp-leu), and incubated at 30°C overnight. Transformants were then replica plated onto two SD-trp-leu plates, one of which had filter paper on top (Whatman 1450-082). Both plates were incubated overnight at 30°C. The filter paper containing the cells was lifted off the agar and immersed in liquid nitrogen for 10 seconds to lyse the cells. In a separate petri dish another Whatman filter paper (Whatman 1003-082) was soaked in 2 mL 1X Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4.H20, 10 mM KCl, 1 mM $MgSO_4.7H_20$, 4 mM 2-mercaptoethanol) and 20 μl 3% X-gal (which was

resuspended in dimethylformamide). The filter paper was placed on top of the filter paper soaked with Z buffer and X-gal with the yeast patches facing up. The filter was incubated at 30°C and periodically checked for blue color indicating the activation of the *lacZ* gene. Protein-protein interactions will induce the transcription of the reporter gene, *lacZ*, which will in turn produce a prominent blue color in the presence of X-gal.

ONPG assay of β-galactosidase activity

Liquid β-galactosidase assays were performed to get quantitative measurements as described in Liu et al., 2013. Independent transformants were inoculated in 5 ml of SD-trp-leu medium and incubated overnight on a roller at 30°C. The cultures were then diluted 1:10 in SD-trp-leu and the OD_{600} was measured to determine the cell density. The spectrometer was blanked using SD-trp-leu. Two replicates of each culture were assayed by aliquoting 1.5 ml of each culture into two 2 ml microfuge tubes. Cells were pelleted and washed in 1 ml Z buffer. Cells were pelleted again and resuspended in 150 μl Z buffer, and vortexed. To lyse the cells, 50 μl chloroform and 20 μl 0.1% SDS were added. The tubes were vortexed by hand for 30 sec and equilibrated at 30°C for 5 minutes. 700 μl of 1.2 mg/ml *O*-nitrophenyl-β-D-galacto-pyranoside (ONPG), made up in Z-buffer, was added to each microfuge tube and vortexed briefly. Cleavage of ONPG by β-galactoside results in the production of a yellow product. The tubes were placed at 30°C and monitored over time for a yellow appearance. Once the culture exhibited a yellowish tint, the reaction was stopped by adding 500 μl 1M Na_2CO_3 and the tubes placed on ice. After 2 hours all samples that had not turned yellow were stopped by the addition of 500 μl 1M Na_2CO_3. The cells were pelleted and the OD_{420} of the supernatant was determined against a blank containing Z-buffer. β-galactosidase activity was calculated using the following formula:

$$\text{Units of β-galactosidase activity:} \quad \frac{1000 \times OD_{420}}{\quad}$$

$$OD_{600} \times t \times V$$

Where t= the incubation time in minutes and V= the volume of cells in ml.

Preparation of yeast extracts and western blotting

Yeast extracts were prepared via a TCA (trichloroacetic acid) method (Falk et al., 2010). 8% sodium dodecyl sulfate (SDS) polyacrylamide gels was utilized for protein separation. 5µl of extract was placed in each individual well. To separate the proteins, gels were run for 60 minutes at 100 volts. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes using a trans-blot semi-dry method at 10 V for 10 min. To shield and prevent non-specific binding, the PVDF membrane was transferred to a blocking buffer, 5% dried milk and TBST (tris-buffered saline and Tween 20), and incubated at room temperature for 1 hour.

Western blot analysis of Zip1 and Arp7 (loading control) was performed in a blocking buffer with overnight incubation at 4°C of primary antibodies, rabbit anti-Zip1 (dilution 1:5,000, from Shirleen Roeder) or goat anti-Arp7 (dilution 1:5,000, Santa Cruz), respectively. To remove excess or unbound antibody, each PVDF membrane was washed in TBST three times, 7 minutes for each wash. Each membrane was treated with its respective secondary antibody, goat anti-rabbit for Zip1 (dilution 1:10,000, Santa Cruz) and rabbit anti-goat for Arp7 (dilution 1:10,000, Pierce). Each PVDF membrane was washed again three times using TBST. Bound antibodies were detected using a chemiluminescent detection reagent (Pierce ECL 2 Western Blotting Substrate). The emitted chemiluminescent light was subsequently detected using X-ray film.

Table 1. Plasmids

Name	Yeast Genotype	Made by
pBTM116	2μ *TRP1 lexA*	Gietz et al., 1997
pBTM116-ZIP1C*-WT[a]	2μ *TRP1 lexA-ZIP1C**(WT)	Yan Liu
pBTM116-ZIP1C*-S801A	2μ *TRP1 lexA-ZIP1C*-S801A*	Yan Liu
pBTM116-ZIP1C*-S801D	2μ *TRP1 lexA-ZIP1C*-S801D*	Yan Liu
pBTM116-ZIP1C*-S802A	2μ *TRP1 lexA-ZIP1C*-S802A*	Hollingsworth
pBTM116-ZIP1C*-S802D	2μ *TRP1 lexA-ZIP1C*-S802D*	Hollingsworth
pBTM116-ZIP1C*-S801AS802A	2μ *TRP1 lexA-ZIP1C*-S801AS802A*	Hollingsworth
pBTM116-ZIP1C*-S801DS802D	2μ *TRP1 lexA-ZIP1C*-S801DS802D*	Hollingsworth
pUC57-ZIP1-C*3R	*ZIP1*-C3R*	Genewiz
pUC57-ZIP1-C*$^{\triangle 791\text{-}824}$	*ZIP1*-C*$^{\triangle 791\text{-}824}$	Genewiz
pSH1	2μ *TRP1 lexA-ZIP1C**3R	Sana Hussain
pSH2	2μ *TRP1 lexA- ZIP1*-C*$^{\triangle 791\text{-}824}$	Sana Hussain
pACTII	2μ *LEU2 GAD*	Gietz et al., 1997
pGAD-RED1[b]	2μ *LEU2 GAD-RED1*$^{537\text{-}827}$	Tu et al., 1996
pA86	2μ *LEU2 GAD-SGS1*$^{81\text{-}614}$	Hollingsworth
pA86-K175R	2μ *LEU2 GAD-SGS1-K175R*	Sana Hussain
p382	*TRP1 ZIP1*	Falk et al., 2010

[a] *ZIP1*C* contains amino acids 750-875.

[b] Superscripts indicate the amino acids present in the fusion.

Table 2. Templates and primers

Name	PCR Template	Primers
pBTM116-ZIP1C*-WT	p382(*ZIP1*)	ZIP1-C-BamHI ZIPC-PstI
pBTM116-ZIP1C*-S801A	p382-S801A	ZIP1-C-BamHI ZIPC-PstI
pBTM116-ZIP1C*-S801D	p382-S801D	ZIP1-C-BamHI ZIPC-PstI
pBTM116-ZIP1C*-S802A	pBTM116-ZIP1C*	ZIP1-S802A-5 ZIP1-S802A-5r
pBTM116-ZIP1C*-S802D	pBTM116-ZIP1C*	ZIP1-S802D-5 ZIP1-S802D-5r
pBTM116-ZIP1C*-S801AS802A	pBTM116-ZIP1C*	ZIP1-S801AS802A-5 ZIP1-S801AS802A-5r
pBTM116-ZIP1C*-S801DS802D	pBTM116-ZIP1C*	ZIP1-S801DS802D-5 ZIP1-S801DS802D-5r
pA86-K175R	pA86	SGS1-K175R-5 SGS1-K175-5-r

Chapter 3

Results

The interaction between Sgs1 and Zip1 is increased by a negative charge at Zip1 S801

The two-hybrid system is a genetic test used to test whether certain proteins interact with a protein of interest (Fields and Sternglanz, 1994). To identify an interaction between two proteins, the protein of interest, the "bait," is fused to a DNA binding domain, and proteins that bind to the bait, the "prey," are fused to a transcriptional activation domain. Once the bait binds to the prey, their interaction stimulates the transcription of a reporter gene.

The Zip1 C-terminus globular domain has previously been shown to interact with Red1 using the two-hybrid system (Lin et al., 2010). To determine whether there were additional interactions, Yan Liu in the Hollingsworth lab constructed a fusion of *lexA* to the C-terminal 750-875 amino acids of Zip1 (Figure 5A) and screened through different GAD fusions present in the Hollingsworth lab. From this she discovered that a fragment of Sgs1 encoding 81-614 amino acids weakly interacts with the C-terminus of Zip1. The $Sgs1^{81-614}$ fragment that interacts with Zip1 contains two acidic regions, which were previously known to mediate protein-protein interaction with other proteins such as Top3 (Bernstein et al, 2009) (Figure 5A). Hollingsworth's lab also detected potential Mek1 substrate sites on Zip1 at Ser801 and Ser802 via SILAC. Ser801 fits the Mek1 consensus site, and Ser802 indicated a light to heavy ratio of 5.0 at a singly phosphorylated Ser802 peptide.

The first hypothesis I tested was whether negative charges potentially conferred by Mek1 phosphorylation affect the Zip1-Sgs1 interaction. Mek1 is a meiosis-specific protein, and because two-hybrid experiments are conducted in vegetative cells, Mek1 is not present to phosphorylate Ser801 and Ser802. Therefore phosphomimetics—amino acid substitutes that

mimic phosphorylation—were used to see whether negative charges have either a negative or positive effect on the Zip1-Sgs1 interaction. Aspartic acid (D) mutants were used to mimic the negative charge of phosphorylation, and alanine (A) mutants were made to test whether any observed phenotypes resulted simply from changing the serine residues at Ser801 and Ser802.

Serine (S) 801 of Zip1 was converted to alanine to make *zip1C-S801A* and to aspartic acid in *zip1C-S801D*, serine 802 was converted to alanine in *zip1C-S802A* and to aspartic acid in *zip1C-S802D*. Additionally, Ser801 and Ser802 were both mutated to alanine *in zip1C-S801A S802A* and to aspartic acid in *zip1C-S801D S802D*. pACTII represents a negative control, that is, an activation domain without any fusion, while pGAD-RED1$^{537\text{-}837}$, was the positive control. None of the lexA plasmid exhibited any activity in the presence of pACTII (Figure 6A).

Quantitative two-hybrid analyses consistently revealed an increase in β-galactosidase activity when the Zip1 C-terminus contained S801D, compared to the WT sequence or the S801A mutant (Figure 6B). This increase was not found with S802D nor was it enhanced when both Ser801 and Ser802 were negatively charged, indicating the effect is specific for S801. The effect observed was specific to Sgs1, as the S801D mutant did not exhibit increased β-galactosidase activity with *GAD-RED1*$^{537\text{-}837}$ when compared to WT or S801A (Figure 6C).

One explanation for the increased activity with the S801D mutant would be if the aspartic acid substitution increased protein stability. Immunoblots using anti-Zip1 antibodies to detect the fusion proteins argue against this idea (Figure 7). These results support the hypothesis that Mek1 phosphorylation of the C-terminus may promote interaction between Sgs1 and Zip1.

Sgs1 and Zip1 interaction does not require amino acids 791-824

The C-terminus of Zip1, specifically, amino acids 791-824, is essential for Zip1 localization to chromosomes and synapsis (Tung and Roeder, 1998). This domain contains the

putative Mek1 phosphorylation sites, S801 and S802. We hypothesized that deletion of this domain, which is required for synapsis and contains the putative Mek1 phosphorylation residues, should abolish the interaction of Sgs1, but not Red1 (since the SIM is intact). To test this idea, Zip1C $^{\triangle 791\text{-}824}$ was fused to *lexA*. Zip1C $^{\triangle 791\text{-}824}$ and Sgs1$^{81\text{-}614}$ were analyzed via two-hybrid assay.

Contrary to our hypothesis the Red1 interaction was eliminated by the deletion, but not the Sgs1 interaction, which exhibited a similar level of β-galacotisidase activity as Zip1C-WT (Figure 8). The loss of Red1 interaction is surprising because the SIM, which mediates the Zip1-Red1 interaction, is still present. The GAD-Red1- Zip1 interaction may be more sensitive to the levels of Zip1 and the loss of signal could be an artifact due to a reduction in protein levels for the Zip1C $^{\triangle 791\text{-}824}$ (Figure 7). This result requires that our initial hypothesis be revised.

The interaction between Sgs1 and Zip1 requires the ZIP1 SIM

The internal Zip1 deletion that interacts with Sgs1 still contains the SIM located between amino acids 853 and 864 (Figure 5A), suggesting that the SIM mediates interaction not only with Red1, but with Sgs1 too. To more directly test whether Sgs1 interacts with Zip1 via the SIM domain, *lexA-zip1C^{3R}* (E862R, D863R, and Q864R), which inhibits SUMO binding to Zip1, was used as bait (Lin, et al., 2010). Consistent with the revised hypothesis, the *zip1C^{3R}* mutant abolished the interaction with both Sgs1 and Red1 (Figure 9). This loss of function was not due to the decrease protein stability. In fact, the *zip1C^{3R}* mutant was significantly more stable than WT (Figure 7). These results suggest that SUMO mediates the interaction between Sgs1 and Zip1, similar to that of Red1 and Zip1.

The fact that mutation of the Zip1 SIM abolishes the Sgs1 interaction suggests that Zip1 may be binding to SUMOlyated Sgs1. Three SUMO consensus sites (ΨKxE) have been

identified on Sgs1 at positions 175, 621 and 831 (Lu et al., 2010) (Figure 5B). Previous studies mutated lysines in these consensus sites to arginine to destroy the *in vivo* sumoylation sites of Sgs1 (Lu et al., 2010). SUMOlyation either ceased or diminished when these lysines were mutated to arginine. One of these consensus sites (position 175) is within the fragment of $Sgs1^{81-614}$ that interacts with Zip1 (Figure 5B). Lysine 175 was mutated to arginine to determine whether a lack of SUMOlyation at this site would interfere with the interaction between Sgs1 and Zip1C. As shown in Figure 11, pGAD-$SGS1^{81-614}$-K175R, interacted with both Zip1C-WT and Zip1C $^{\triangle 791-824}$ on the filter assay for β-galactosidase activity, indicating that mutating the SUMO consensus site on Sgs1 has no effect on the protein-protein interaction.

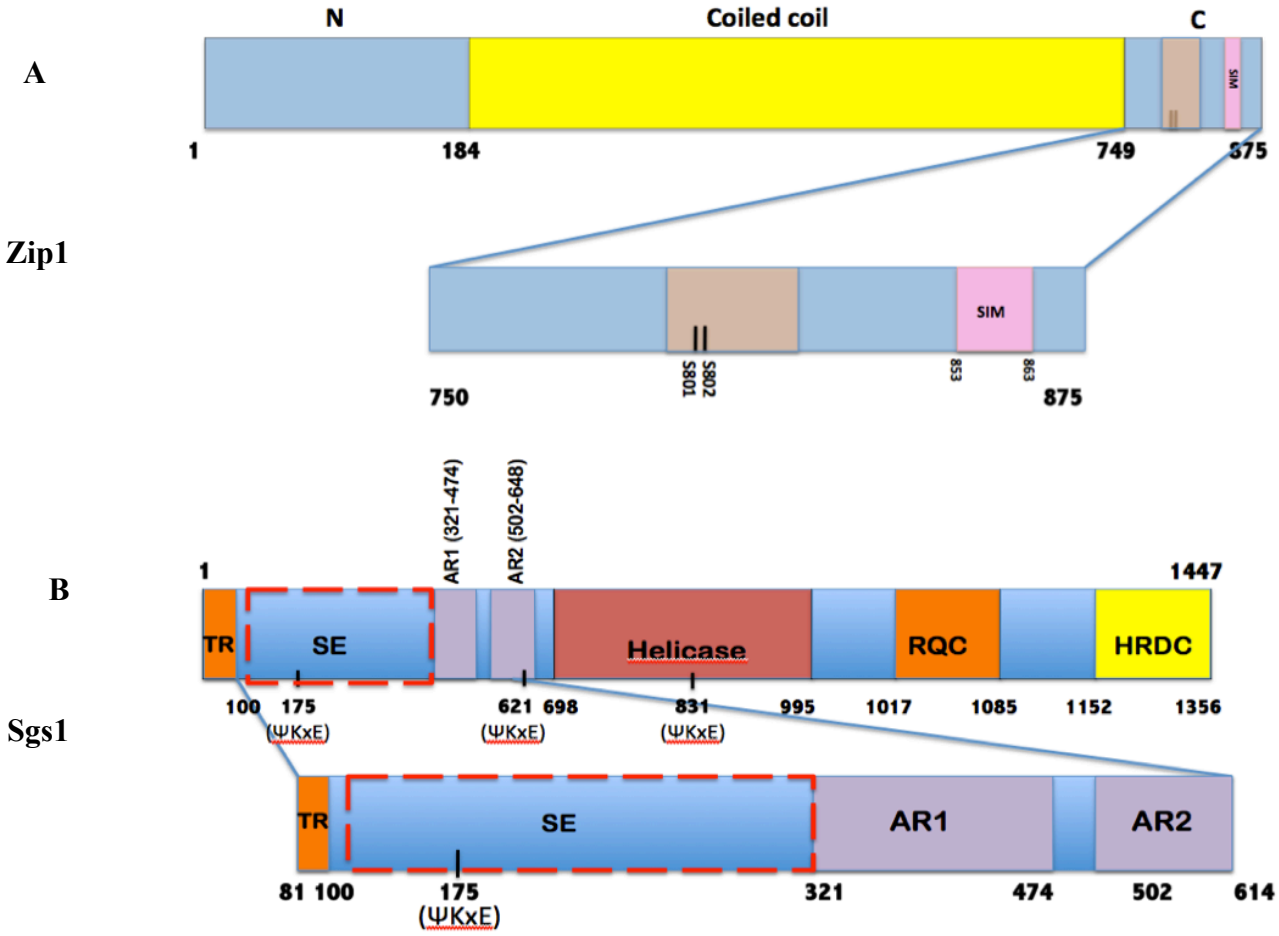

Figure 5. A. Zip1: structural domains and C-terminal fragment used in yeast two-hybrid assays. Zip1 contains an α-helical coiled-coil domain, aa 184-749, which is flanked by globular ends on its termini; N-terminus, aa 1-183 and C-terminus, aa 750-875 (adapted from Steinert and Roop, 1988; Sym et al., 1993). The SUMO interacting motif (SIM) is from aa 853-864 and is indicated by a pink box (Cheng et al, 2006). The internal deletion made by Dong and Roeder (1998), which abolishes synapsis, is indicated by a tan box. The putative Mek1 phosphorylated amino acids, S801 and S802, are indicated by lines. B. Sgs1: structural and functional domains and N-terminal fragment used in yeast two-hybrid. Domains: TR, Top3–Rmi1 binding, 100aa; SF2 type helicase domain, aa 698-995; RQC (Req Q C-terminus) motif, aa 1017-1085; and HRDC (helicase and RNase D C-terminal) domain, aa 1152-1356. The three SUMO consensus sites (ΨKxE) on Sgs1 are located at amino acid 175, 621 and 831 (Lu et al., 2010). The N-terminal fragment contained in *GAD-SGS1*$^{81\text{-}614}$ fusion plasmid, A86, is shown in expanded form. It contains two acidic regions AR1, aa 312-474, AR2, aa 502-648, and an SE (strand exchange) domain, aa 103-322, in addition to one SUMO site at 175 (adapted from Amin et al., 2010, Bernstein et al., 2009; Chen and Brill, 2010; Lu et al., 2010; Weinstein and Rothstein, 2008).

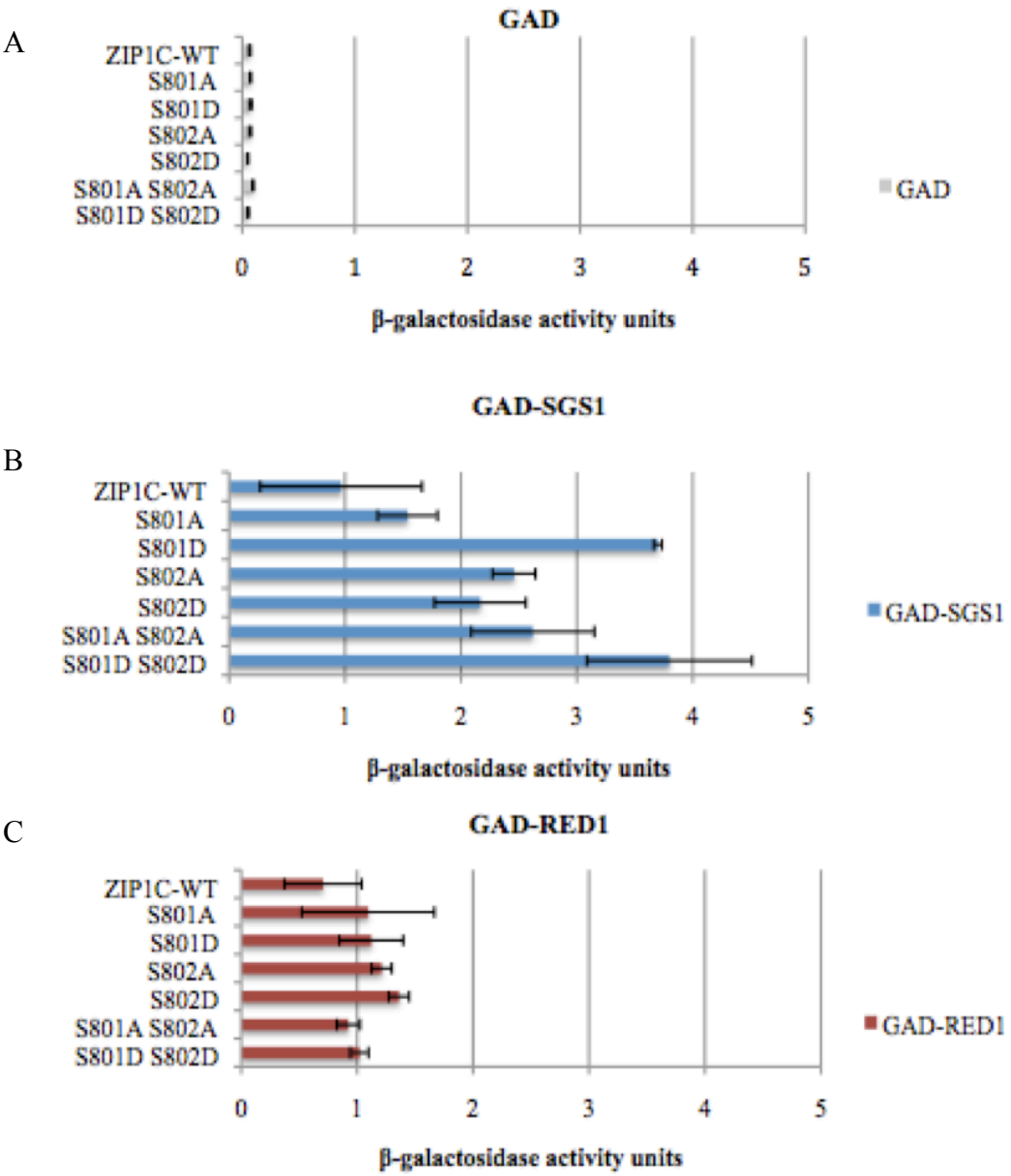

Figure 6. Sgs1 protein interaction with various lexA-Zip1C mutant proteins. A. A quantitative yeast two-hybrid analysis between Zip1C fused to LexA-DNA binding domain, and GAD, Gal4 activation domain without any fusion, which represents a negative control. Zip1C represents the C-terminal fragment (residues 750-875) of the wild type Zip1 protein. Ser801 residue of Zip1 was converted to Ala in Zip1C-S801A and Asp in Zip1C-S801D, Ser802 residue of Zip1 was converted to Ala in Zip1C-S802A and Asp in Zip1C-S802D. Additionally, Ser801 and Ser802 were converted into Ala in Zip1C-S801AS802A and Asp in Zip1C-S801DS802D. B. Quantitative two-hybrid analysis with the same respective point mutations on LexA-Zip1 and Sgs1, which contains residues 81-614 fused to GAD. C. Quantitative two-hybrid analysis with the same respective mutations on LexA-Zip1 and Red1, which represents residues 537-827 fused to GAD. Values for Zip1C-WT, S801A and S801D are averages of assays of 6 different transformants that were each replicated, N=12. Values for S802A, S802D, S801A S802A, S801D S802D are averages of assays of 4 different transformants that were each replicated, N=8.

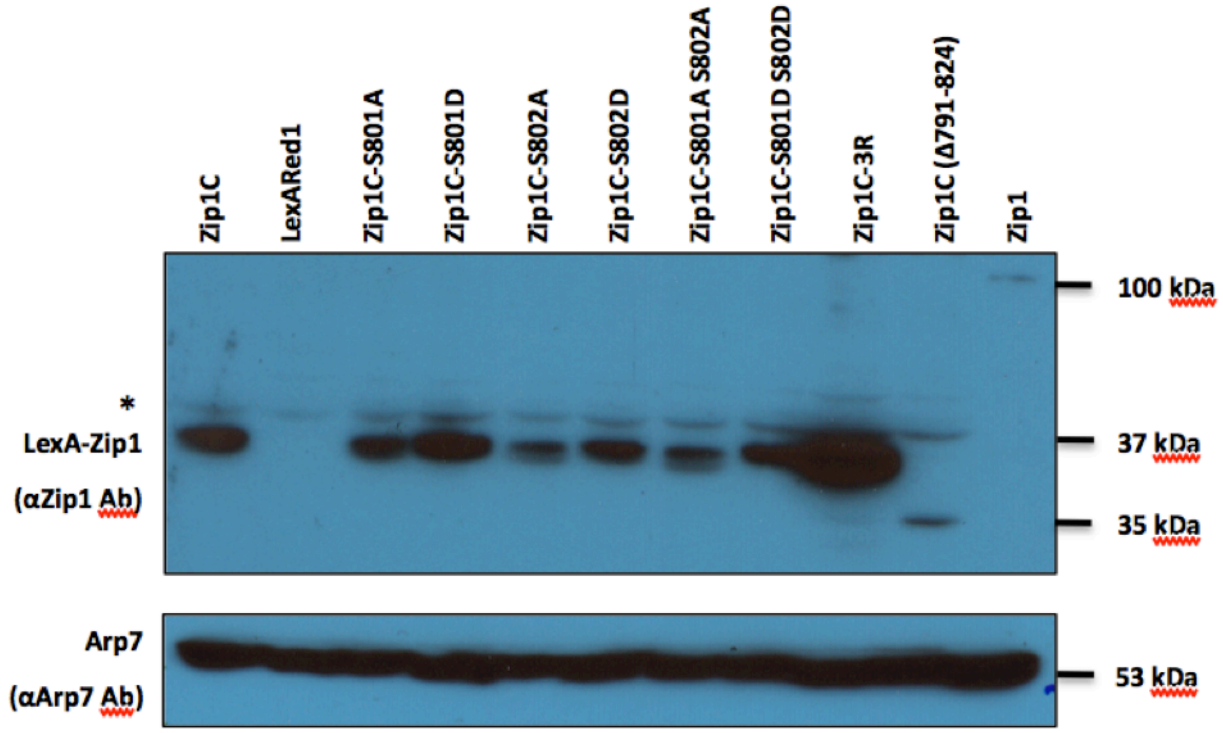

Figure 7. Western blot of various lexA-Zip1C mutant proteins. LexA-Red1 was used as a negative control and a protein extract from a ZIP1 WT strain indicating a full length Zip1 location was used as a positive control. Arp7 was used as a sample loading control.
* Indicates a non-specific band; this band is not a signal from the antibody binding Zip1 or any fragment of Zip1.

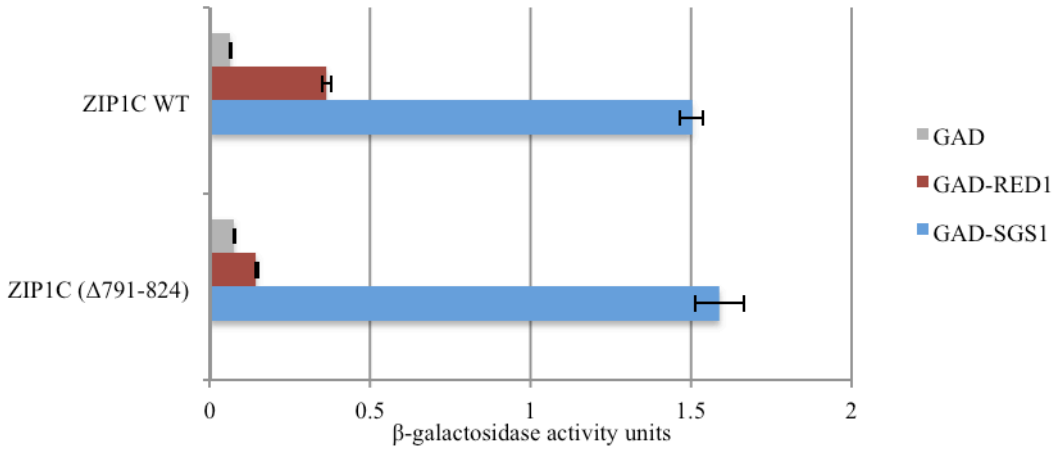

Figure 8. Sgs1 protein interaction with Zip1C$^{\triangle 791\text{-}824}$. The two-hybrid interaction between LexA-Zip1C$^{750\text{-}875}$ and LexA-Zip1C$^{\triangle 791\text{-}824}$, which contains deletion of amino acids 791-824 on the C-terminus of Zip1, with GAD, GAD-Red1$^{537\text{-}827}$, and GAD-Sgs1$^{81\text{-}614}$. Zip1C$^{\triangle 791\text{-}824}$ is known to be unable to localize to chromosomes (Tung and Roeder, 1998). All values are averages of assays of two different transformants that were each replicated, N=4.

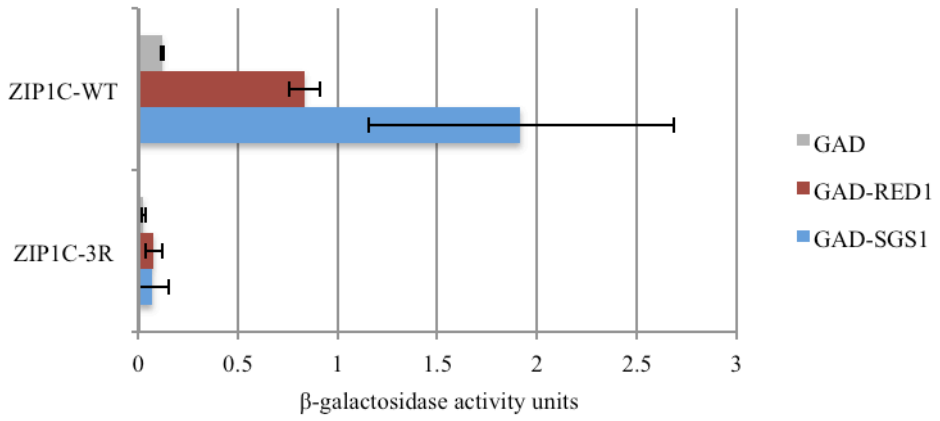

Figure 9. Sgs1 protein interacts with Zip1C*^{3R}. The two-hybrid interaction between LexA-Zip1C$^{750-875}$ and Zip1C*^{3R}, which contains a three point mutation, E862R, D863R, and Q864R within the C-terminal domain, with GAD, GAD-Red1$^{537-827}$, and GAD-Sgs1$^{81-614}$. Zip1C*^{3R} is known to be incompetent in SUMO binding (Cheng et al., 2006). All values are averages of assays of two different transformants that were each replicated, N=4.

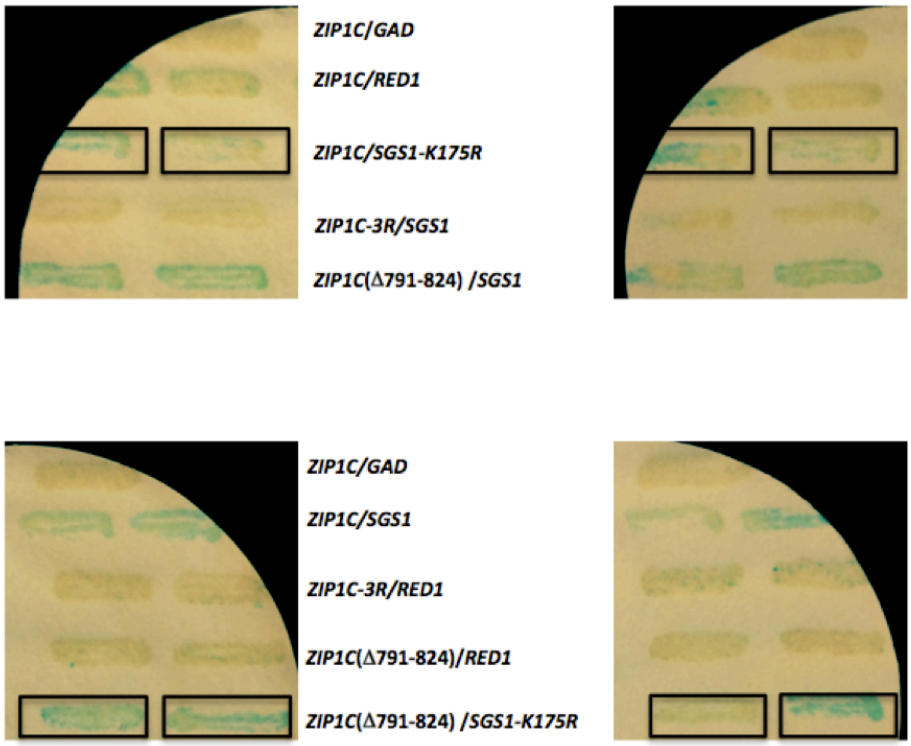

Figure 10. Sgs1-K175R protein interaction with Zip1C-WT and Zip1C$^{\triangle 791\text{-}824}$. Filter assays for β-galactosidase activity were used to detect protein-protein interactions. The above two filters are replicates.

Chapter 4

Discussion

Zip1 interacts with Sgs1, through the SIM located on its C-terminus

Previous research has found that ZMM proteins, such as Zip1, promote crossovers by antagonizing Sgs1 (Jessop et al., 2006). The protein-protein interaction between the N-terminus of Sgs1 and the C-terminus of Zip1 may some how disrupt Sgs1 helicase activity. Additionally, this interaction may be modulated or regulated by modifications that occur on the C-terminus of Zip1, which vb SUMOlyation and potential phosphorylation by Mek1 kinase.

Sgs1's interaction with Zip1 was greatly diminished when E862, D863, and Q864 were mutated to arginine residues, which is known to abolish SUMO binding (Cheng et al., 2006) (Figure 8). These results suggest that SUMO conjugates may be necessary for the interaction between Sgs1 and Zip1. Additionally, considering that Zip1-SUMO association occurs via non-covalent interactions (Cheng et al., 2006), it also suggests, similar to Red1, that SUMO chains may be sandwiched in between Sgs1 and Zip1 to facilitate their interaction.

Although Sgs1 and Zip1 interacted even when the Sgs1 SUMO consensus site at amino acid 175 was eliminated—the idea that SUMO conjugates promote the interaction between Sgs1 and Zip1 cannot be ruled out. Previous research has shown that the consensus sequence for SUMOlyation is not necessarily conclusive (Eichinger and Jentsch, 2010). Preliminary findings in another study by Cheng et al., found that mutations on SUMO consensus sites are fluid and may have little to no effect on their biological function. Alternatively, different SUMOlyation sites may serve a redundant function, thus one mutation may not exhibit a detectable phenotype (Cheng et al., 2006). For example, Red1, another SUMO modified protein, revealed vital SUMO sites at different lysine residues that lacked the consensus sequence, that is, other lysines

contributed to Red1's SUMOlyation (Eichinger and Jentsch, 2010). Similarly, Sgs1 may have different lysine residues that can act as potential SUMO sites, such as the lysine rich region between amino acids 237-253.

Mek1 phosphorylation at Ser801 may stimulate the protein interaction between Sgs1 and Zip1

A protein's specificity in binding to a SUMO modified protein may be enhanced by imposing a negative charge on a stretch of neighboring amino acids or via phosphorylation by a kinase on a serine residue (Hecker et al., 2006). This phosphorylation has the capability of modulating the spatial orientation of interactions between a SUMOlyated protein, such as Sgs1, and a SIM protein, similar to Zip1.

The Zip1 SIM is located between amino acids 853-864; KKLLLVEDEDQ (Cheng, 2006). This sequence is loosely characterized as a stretch of three to four hydrophobic residues with one acidic or polar residue at position two or three (Hecker et al., 2006). Additionally, the sequence surrounding this core-binding domain is predicted to be disordered and have a net negative charge (Hecker et al., 2006). The negative charge sequence can be either upstream or downstream of this hydrophobic core (Hecker et al., 2006). Zip1 appears to have a net negative sequence between amino acids 801-824; SSKETSKFNDEFDLSSSSNDDLEL, which is also the same sequence that is necessary for Zip1 to localize to chromosomes (Tung and Roeder, 1998).

The binding of SUMO can potentially be influenced by the net negative amino acid sequence surrounding the hydrophobic core (Hecker et al., 2006). The C-terminus region of Zip1 is a globular domain, and although the Ser801 phosphosite is about 50 amino acids upstream of the SIM Zip1 hydrophobic region, it is situated close to the negative amino acid sequence. Thus, it could possibly play a role in exposing the hydrophobic core even further to allow a stronger

protein-protein interaction between Sgs1 and Zip1, which was exhibited in the two-hybrid experiments.

Hecker et al., found that phosphorylation of a serine residue can help maintain specificity and orientation for binding of SUMO to different SIMs. Specifically, the negative charge on the phosphate aids by providing supplementary electrostatic interactions and thus increases the affinity of binding to a particular ligand. Although Hecker et al. reported that this phosphorylation generally occurs within the SIM itself, they also noted that the position of the acidic residue track determines the orientation of the SIM's positioning relative to the SUMO on the conjugated protein. Thus, adding an additional negative charge, via phosphorylation can aid in this affinity. That is, the interaction between Zip1 and Sgs1 can somehow be enhanced by Mek1 phosphorylation at Ser801.

Further work is necessary to verify that the interaction between Sgs1 and Zip1 occurs within meiotic cells. Confirmation of the two-hybrid interaction can be obtained using coimmuniprecipitation (Co-IP) experiments. This may however prove to be difficult. A previous study has shown that that Zip1 protein is difficult to immunoprecipitate because it is particularly unstable even when lysed in buffers containing particular protease inhibitors (Lin et al., 2010). Furthermore, Zip1 likes to self-aggregate and is very insoluble. Additionally, Sgs1 itself has proven to be relatively toxic to cells. In the experiments above, cultures that were left to incubate for longer periods of time exhibited less Sgs1 activity, indicating that the cells were compensating by reproducing without Sgs1. Additionally, Hegnauer and colleagues reported similar toxicity by Sgs1 while they were performing their experiments (Hegnauer et al., 2012). Recent results from Hengyao Niu and Patrick Sung indicated that recombinant Zip1 interacts in

pull down assays with recombinant Sgs1, indicating that the interaction is not simply an artifact of the two-hybrid system.

Based on the literature and my own experiments, I propose that the N-terminal region of Sgs1 interacts with Zip1 via SUMO conjugates. This interaction does not require that Zip1 to be localized to chromosomes. Mek1 kinase is recruited to the double strand breaks by binding to phosphorylated Hop1 (Carballo et al, 2008). I propose that Mek1 kinase then phosphorylates Ser801 in the acidic region of Zip1, which in turn enhances the interaction between SUMOlyated sites on Sgs1 and the SIM on Zip1. This interaction somehow antagonizes Sgs1 activity thereby allowing D-loops to be stabilized and to be processed into COs.

If co-immunoprecipation of Zip1 and Sgs1 is achieved, then this model could be tested by performing the Co-IP in a *mek1-as* strain with a Mek1-as inhibitor. If Mek1 enhances the interaction between Sgs1 and Zip1 then a Co-IP with the Mek1 inhibitor should result in less Zip1 and Sgs1 interaction when compared to WT. Additionally, to verify if SUMOlyation plays a role in Sgs1 and Zip1 interaction, the lysine rich residues between amino acids 237-253 on Sgs1 should be mutated to arginine. The interaction between Sgs1 and Zip1 in that case should be eliminated or at least be diminished drastically.

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