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Identification of binding partners with the negatively charged Zip1 C-terminus using a two-hybrid screen

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Matthew Murray

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Nancy M. Hollingsworth
Professor, Department of Biochemistry and Cell Biology

Rolf Sternglanz
Distinguished Professor Emeritus, Department of Biochemistry and Cell Biology

This thesis is accepted by the Graduate School

Charles Taber
Dean of the Graduate School

Abstract

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Recombination is an essential meiotic process to ensure proper chromosome distribution. Recombination is promoted by the synaptonemal complex, a structure which bridges two pairs of homologous sister chromatids, bringing them into close proximity. The synaptonemal complex is formed by condensation of the sister chromatids on protein cores called axial elements. Homologous axial elements are then held together in budding yeast by a protein called Zip1. Zip1 is part of the ZMM group of proteins that work together during meiosis to promote the formation of crossovers which are distributed throughout the genome by interference. Four phosphorylation sites have been identified in the Zip1 C-terminus that are essential for the formation of interfering crossovers. To identify potential binding partners with the phosphorylated Zip1 C-terminus a two-hybrid screen was conducted using a *zip1* mutant containing phosphomimetic amino acid substitutions. In this thesis I report the identification of three novel protein-protein interactions with the negatively charged Zip1 C-terminus.

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List of Abbreviations

A: alanine

AA: axial association

ADE: adenine

ADH: alcohol dehydrogenase

Amp: ampicillin

APS: ammonium persulfate

β-gal: β-galactosidase

Cdc: cell division cycle

ChIP: chromatin immunoprecipitation

ChIP-chip: chromatin immunoprecipitation-DNA microarray

D: aspartic acid

D-loop: displacement loop

Dbf: dumbbell forming

DDK: Dbf dependent kinase

DNA: deoxyribonucleic acid

DSB: double Strand break

dsDNA: double stranded DNA

EDTA: ethylenediaminetetraacetic acid

FRB: FKB12-rapamycin-binding

GAD: Gal4 activation domain

GFP: green fluorescent protein

HIS: histidine

HRP: horseradish peroxidase

LEU: leucine

MAT: mating type

mTOR: mammalian target of rapamycin

NLS: nuclear localization sequence

OD: optical density

ONPG: *O*-nitrophenyl-β-D-galacto-pyranoside

PCR: polymerase chain reaction

PEG: polyethylene glycol

PVDF: polyvinylidene fluoride

RPA: replication protein A

SDSA: synthesis dependent strand annealing

S: serine

SD: synthetic defined

SDS: sodium dodecyl sulfate

SIC: synapsis initiation complex

SIM: SUMO interacting motifs

SOC: super optimal broth with catabolite repression

ssDNA: single stranded DNA

SUMO: small ubiquitin-like modifiers

TBST: Tris-buffered saline and Tween 20

TCA: trichloroacetic acid

TCR: T-Cell receptor

TEMED: tetramethylethlenediamine

TRP: tryptophan

UV: ultraviolet

WT: wild type

X-gal: 5-bromo-4chloro-3indolyl- β-galactoside

YPD: yeast extract peptone dextrose

ZMM: Group of proteins including Zip1, Zip2, Zip3, Zip4, Mer3, Msh4, Msh5 and Spo16

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Chapter 1

Introduction

Meiosis and recombination

Sexual reproduction occurs between two different organisms of the same species. Each contributes a single gamete containing half of its DNA. The gametes fuse and a new diploid organism of the same species is formed. Haploid gametes are created using a specialized type of cell division called meiosis. Meiosis is different from mitosis because the daughter cells of meiosis are haploid, meaning they have half the normal chromosome number. This is because in meiosis, a single cell undergoes two consecutive chromosomal divisions producing four haploid daughter cells. Meiosis begins with the replication of the DNA. A major difference between meiosis and mitosis is the way the DNA is separated at the first meiotic division (Meiosis I). In mitosis, sister chromatids are separated, similar to the second meiotic division (Meiosis II). In Meiosis I, homologous pairs of sister chromatids are separated from each other. After Meiosis II the four haploid genomes are packaged into gametes. In humans, the diploid cells are the primary spermatocytes (males) and primary oocytes (females) and the gametes are spermatozoa and ova.

The eukaryotic organism *Saccharomyces cerevisiae,* or budding yeast, is an excellent model organism used to study meiosis. This organism has two mating types termed **a** and α that are determined by the mating type (MAT) genes, *MAT**a*** and *MATα*, respectively. Under starvation conditions, diploid yeast cells undergo a process called sporulation in which cells go through meiosis creating four haploid genomes that are then packaged into spores (Neiman, 2011). Haploid spores of different mating type can fuse to form a new diploid organism. This diploid organism can go on to reproduce by mitosis or sporulate to form new haploid cells.

Errors in meiosis may lead to the improper segregation of chromosomes, termed nondisjunction, which produces gametes with too few or too many chromosomes. An incorrect chromosome number can cause problems in offspring due to too much genetic material or absence of genetic material all together (Alberts et al., 2008). There are several examples in humans of nondisjunction. One of the most common nondisjunction events is Trisomy 21 or Down Syndrome, which is characterized by the presence of an extra copy of chromosome 21 due to the failure of chromosome segregation in meiosis (Sherman et al., 2007). Trisomy can also occur with chromosomes 13 and 18 resulting in Patau and Edwards syndrome respectively (Witters et al., 2011).

Recombination is a mechanism for ensuring proper chromosome segregation during meiosis I. Recombination, in combination with sister chromatid cohesion, physically links homologous chromosomes prior to meiosis I allowing for the proper orientation of each homolog pair. This linkage allows the chromosome segregation machinery to bind to mono-oriented pairs of sister chromatids and pull them apart. Failure in recombination is linked to nondisjunction in humans and *Drosophila* (Koehler et al., 1996).

Recombination during meiosis is initiated by a double strand break (DSB). In *S. cerevisiae* this DSB is created by a topoisomerase like protein called Spo11 (Keeney, 2001) (Figure 1). Once a DSB is made, the 5' ends of the break are resected generating 3' ssDNA ends. The 3' ends are initially loaded with replication protein A (RPA), a single strand binding protein complex which protects the DNA from degradation (Zou et al., 2006). In mitosis, RPA is then replaced by Rad51 through the mediators Rad52, Rad55 and Rad 57 to form a nucleoprotein filament that can search for homologous DNA complementary to the ssDNA bound by Rad51 and initiate strand invasion (Dupaigne et al., 2008). In meiosis, Dmc1, a protein homologous to Rad51, is required to mediate strand invasion of homologs as *dmc1* mutants block homologous recombination in meiosis (Bishop et al., 1992). Dmc1 is loaded on the DNA strand instead of Rad51 through the interactions with mediators Mei5 and Sae3 (Tsubouchi and Roeder, 2004) and accessory proteins Mnd1 and Hop2 (Henry et al., 2006). Strand invasion by the Rad51 or Dmc1 nucleoprotein filament and annealing to a complementary region in the

homologous chromosome causes displacement of the second strand in the homolog resulting in formation of what's known as the displacement loop (D-loop). Once a large enough region of complementarity is formed, and the invading strand can anneal stably to its homologous partner. Extension of the invading strand occurs using this homologous partner as a template. As extension continues, the amount of DNA displaced into the D-loop increases and allows this displaced DNA to pair with the other resected end in an event called second end capture. This creates a structure called the double Holliday junction. DSBs which form double Holliday junctions are repaired by the recombination machinery as a crossover and those that do not form double Holliday junctions are typically resolved as non-crossovers. (Allers and Lichten, 2001) (Figure 1).

Non-crossovers are formed via the synthesis dependent strand annealing (SDSA) pathway (Figure 1) in which the extended invading strand is displaced from its homologous partner allowing it to anneal to the other side of the break (Allers and Lichten, 2001). In meiotic recombination, resolution of the double Holliday junctions is biased to produce nearly exclusively crossovers (Allers and Lichten, 2001). This biasd resolution requires the ZMM group of proteins composed of: Zip1, Zip2, Zip3, Zip4, Mer3, Msh4, Msh5 and Spo16 (Borner et al., 2004; Shinohara et al., 2008). The ZMM proteins act to stabilize strand invasion intermediates to allow crossover formation, resulting in a reduction of crossover formation in *zmm* mutants (Lynn et al., 2007). The ZMM group of proteins can stabilize the D-loop protecting it from helicases such as Sgs1, which would otherwise work to dissolve the D-loop thereby promoting SDSA (Hollenberg et al., 1995). D-loops protected by the ZMM complex of proteins continue the extension and eventually form double Holliday junctions (Figure 1). These double Holliday junctions are then resolved to form primarily crossover products (Allers and Lichten, 2001).

Zip1 and the ZMM group of proteins

Recombination allows homologous pairs of sister chromatids to physically attach to each other resulting in close association of the two pairs. This interaction is created by the formation of the synaptonemal complex. The synaptonemal complex is a protein scaffold composed of two lateral elements (each formed by sister chromatid

condensation along a protein core) with an intervening central region (Sym and Roeder, 1995) (Figure 2A). The lateral elements, referred to as axial elements prior to synapsis, are composed of Red1, Hop1 and Rec8 (Hollingsworth and Byers, 1989; Klein et al., 1999; Smith and Roeder, 1997). *HOP1*, *RED1* and *REC8* are all meiosis specific genes (Hollingsworth et al., 1990; Klein et al., 1999; Thompson and Roeder, 1989).

Rec8 is a meiosis specific component of the cohesion complex (Klein et al., 1999). It partners with Smc1, Smc3 and Scc3 to form a proteinaceous rings around sister chromatids (Berchowitz and Copenhaver, 2010). These rings ensure proper segregation of sister chromatids during Meiosis I by preventing separation of the chromatids during the first meiotic division. It has been shown through fluorescence microscopy and immunofluorescence that *rec8Δ* mutants cannot form axial elements or synaptonemal complexes (Klein et al., 1999). This has a drastic effect on homologous recombination as the absence of the synaptonemal complex significantly lowers the probability of successful crossing over events between homologs. As expected, it was shown that *rec8Δ* mutants are defective in homologous recombination (Klein et al., 1999).

The central region of the synaptonemal complex is composed of Zip1 (Sym et al., 1993). This important function of Zip1 was shown using *zip1* mutants. In *zip1* mutants, axial elements form, and chromosomes pair but homologous chromosomes cannot synapse and the distance between homologous chromosome pairs is much farther in *zip1* mutants than compared to the wild-type (Sym et al., 1993). The Zip1 protein essentially acts as a molecular zipper, pulling two homologs together so they can synapse (Figure 2A). The *ZIP1* gene is only expressed during meiosis (Sym et al., 1993).

The Zip1 protein is composed of two globular domains at the N and C termini which are separated by α-helical coiled coil domains (Dong and Roeder, 2000). Coiled coil motifs allow for the oligomerization of proteins (Burkhard et al., 2001). This ability is conferred by the presence of specific heptads that contain hydrophobic residues at positions 1 and 4, and charged residues at the other 5 positions (Kohn et al., 1997). Through its coiled coil domains, Zip1 is predicted to form homodimers in which two

globular head domains are placed near each other forming a rod with two N-terminal heads at one end and two C-terminal heads at the other end (Sym et al., 1993; Tung and Roeder, 1998) (Figure 2B). Zip1 oligomers make up the central region of the synaptonemal complex. The N terminal domains of a Zip1 oligomer associate with the N terminal domains of another Zip1 oligomer forming a transverse filament (Dong and Roeder, 2000) (Figure 2A). The C-terminal domains of Zip1 are anchored to the lateral elements attached to the chromosomes, in part by binding to the axial element protein Red1 (Dong and Roeder, 2000; Lin et al., 2010). The organization of these Zip1 homodimers in the synaptonemal complex is what allows homologous chromosomes to synapse.

In addition to the role that ZMM proteins play in crossover formation, they also have a role in crossover interference (Fung et al., 2004; Lynn et al., 2007; Sym and Roeder, 1994). Crossover interference is the phenomenon that causes crossovers to be distributed throughout the chromosomes (Berchowitz and Copenhaver, 2010). *zmm* mutants show decreased interference (Lynn et al., 2007) and *zip1Δ* results in the complete abolishment of interference (Sym and Roeder, 1994). Zip1, Zip2 and Zip3 cytologically colocalize to the same locations on chromosomes forming the synapsis initiation complex (SIC) which is required to initiate synapsis of chromosomes at certain locations (Fung et al., 2004).

Four phosphorylation sites have been identified in a region of the Zip1 C-terminus that is required for synapsis (Tung and Roeder, 1998) (X. Chen, R. Suhandynata, N.M. Hollingsworth personal communication). Genetic experiments have shown that phosphorylation of serines 815, 816, 817 and 818 promotes synapsis, crossover formation and interference (N.M. Hollingsworth, personal communication). Phosphomimetic mutants were made where serines 815-818 were mutated to aspartic acids (*ZIP1-4D)*, a negatively charged amino acid which can mimic the effects of phosphorylation. Spore viability assays using *ZIP1-4D* showed similar levels of spore viability as *ZIP1*. In contrast, mutating the four serines at 815-818 to alanines (*zip1-4A*), an amino acid that cannot be phosphorylated, reduces spore viability to ~75%. This experiment shows that the absence of phosphorylation affects Zip1 function, and that

the negative charges supplied by aspartic acids in the phosphomimetic mutant function as good substitutes for *in vivo* phosphorylation.

Creating the tools for a two-hybrid screen using the Zip1 C-terminus

One potential function of the negative charges provided by phosphorylation is to enhance protein-protein interactions. This phenomenon has widely been observed in many fields. For instance NADPH oxidase, a crucial component of the leukocyte armamentarium in mediating host defense, requires phosphorylation to create binding sites for the assembly of its subunits (Babior, 1999). The protein kinase Zap70 also has the ability to bind to the activated T-Cell receptor (TCR) by binding to phosphorylated tyrosines on the zeta chain of the TCR (Wange et al., 1993). To identify proteins that interact with the negatively charged C-terminus of Zip1, a two-hybrid screen was conducted using a gene encoding a lexA-Zip1 C-terminus fusion protein in which serine residues 815-818 were mutated to negatively charged aspartic acid residues (Chien et al., 1991) (Figure 3). This gene is referred to as *lexA-Zip1C** where C* refers to amino acids 750-875 of the Zip1 C-terminus. These phosphomimetic amino acid substitutions were used because the two-hybrid assay utilizes vegetative cells and the kinase responsible for phosphorylating S815-818 was unknown at the beginning of this work. It was possible, for example, that the kinase is meiosis-specific. By using the phosphomimetic mutant, the negative charges are present at the Zip1 C-terminus in vegetative cells.

The yeast strain used for the two-hybrid screen is L40 (Hollenberg et al., 1995). L40 is auxotrophic for the amino acids tryptophan, leucine and histidine (genotype: *trp1*, *leu2*, *ade2, his3Δ200*). L40 cells also have within their genome, functional and distinct, *HIS3* and *lacZ* genes under the transcriptional control of several lexA binding sites located in their promoters. This permits binding of the lexA DNA binding domain to the promoters of the *HIS3* and *lacZ* genes. LexA is a prokaryotic protein that is involved in the DNA damage response (Courcelle et al., 2001). The placement of *lexA* operator sequences in the *HIS3* and *lacZ* promoters results in the localization of lexA fusion proteins to upstream regions of these genes.

The two-hybrid system works by transforming L40 with two plasmids, each containing a different fusion protein. In my screen, the first plasmid encodes lexA fused

to the C-terminus of Zip1-4D. Because *ZIP1* is a meiosis specific gene that is not expressed in vegetative cells, the *lexA-ZIP1C*-4D* fusion gene is under the control of the constitutive alcohol dehydrogenase (*ADH1*) promoter (Sym et al., 1993). This plasmid also contains *TRP1* as a yeast selectable marker. The lexA-Zip1C*-4D protein is referred to as “the bait”. The bait, because it is fused to lexA, is localized to the promoters of the *HIS3* and *lacZ* genes. The second plasmid encodes the Gal4 activation domain (GAD) fused to another protein of interest, referred to as “the prey”. For my screen, the bait is a library of genomic fragments inserted next to the *GAD* gene (Dresser et al., 1997). This prey plasmid contains *LEU2* as a yeast selectable marker. If the bait and the prey interact, the GAD is brought to the promoters of the *HIS3* and *lacZ* genes, thereby allowing transcription (Fields and Sternglanz, 1994) (Figure 3). As a result, imidazoleglycerol-phosphate dehydratase (encoded by *HIS3*) and β-galactosidase proteins are produced making the cells both His^+ and $\beta\text{-}gal^+$. In the absence of an interaction between the bait and the prey, there is no transcription of *HIS3* and *lacZ* and the cells are His^- and $\beta\text{-}gal^-$. By screening for transformants that are His^+, interactions between the bait and the proteins encoded by the prey plasmids can be identified.

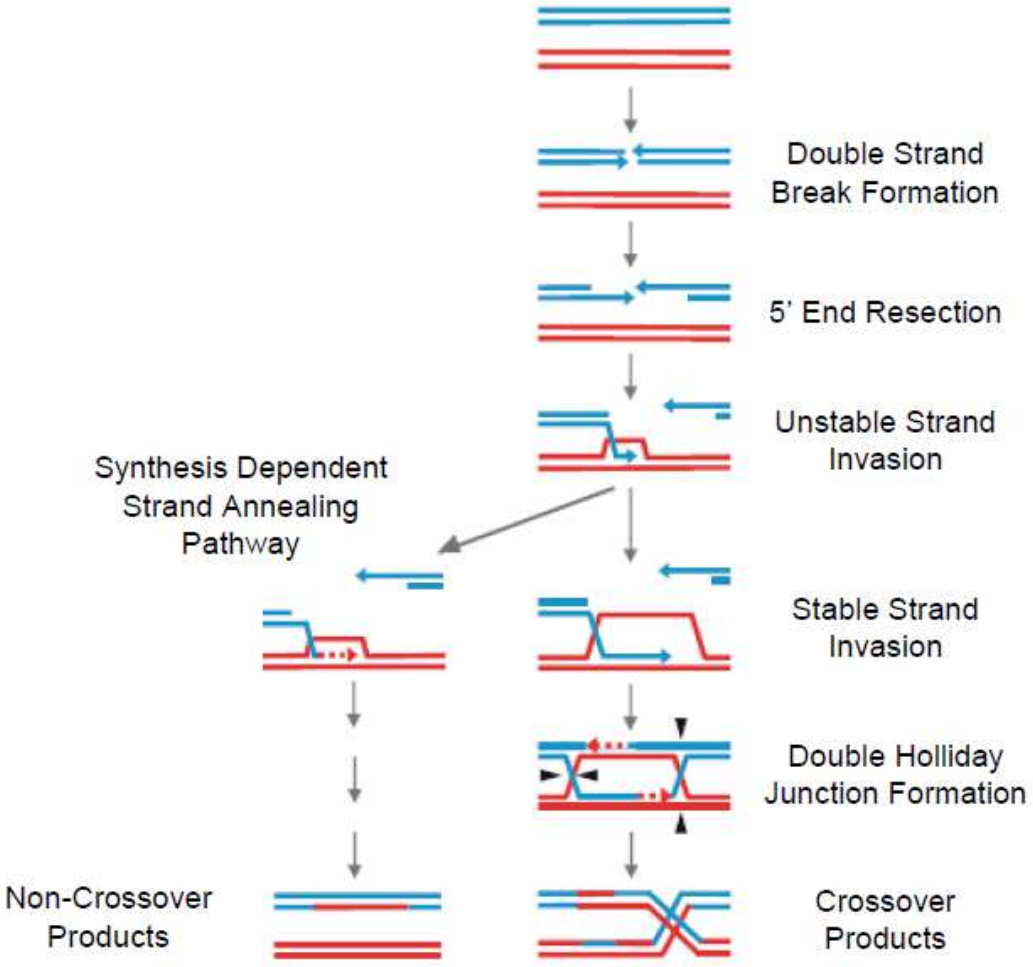

Figure 1 - **Schematic diagram of recombination.** This diagram, adapted from Lynn et al. 2007, depicts the events of homologous recombination and its resolution into non-crossover and crossover products. Blue and red arrowheads on DNA strands represent 3' ends. Black arrow heads represent the various cuts made to resolve the double Holliday junction into crossover products.

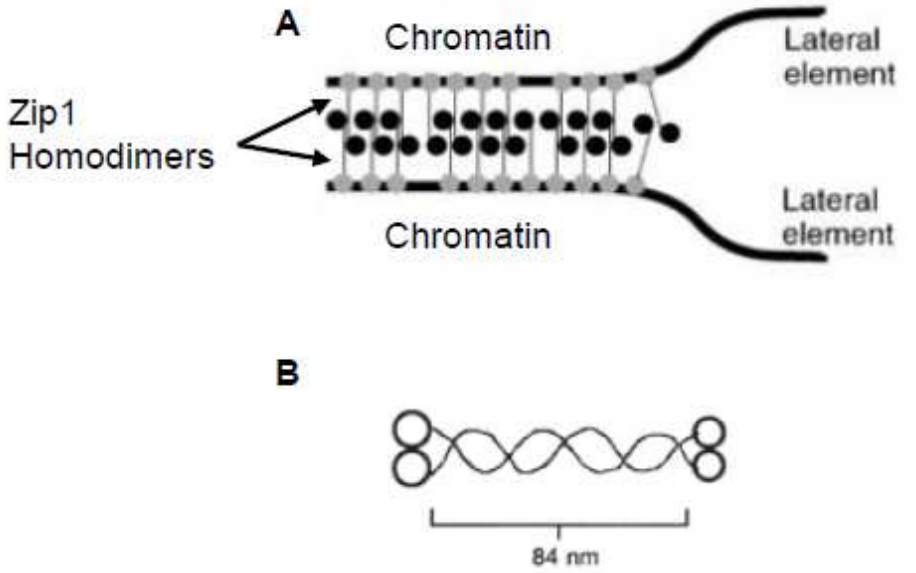

Figure 2 – Zip1 and the organization of the synaptonemal complex. (A) Schematic of the synaptonemal complex adapted from Dong and Roeder, 2000. Zip1 homodimers are indicated by the arrows. The N-terminal globular domains are in black and the C-terminal globular domains are in grey. C-terminal globular domains are attached to lateral elements containing Hop1, Red1 and Rec8. Lateral elements are attached to looped chromatin. (B) Cartoon of the Zip1 homodimer adapted from Sym et al. 1993. The coiled coil domains are located in the center and span a distance of 84nm. The globular domains are indicated by the circles at the extreme ends of the diagram.

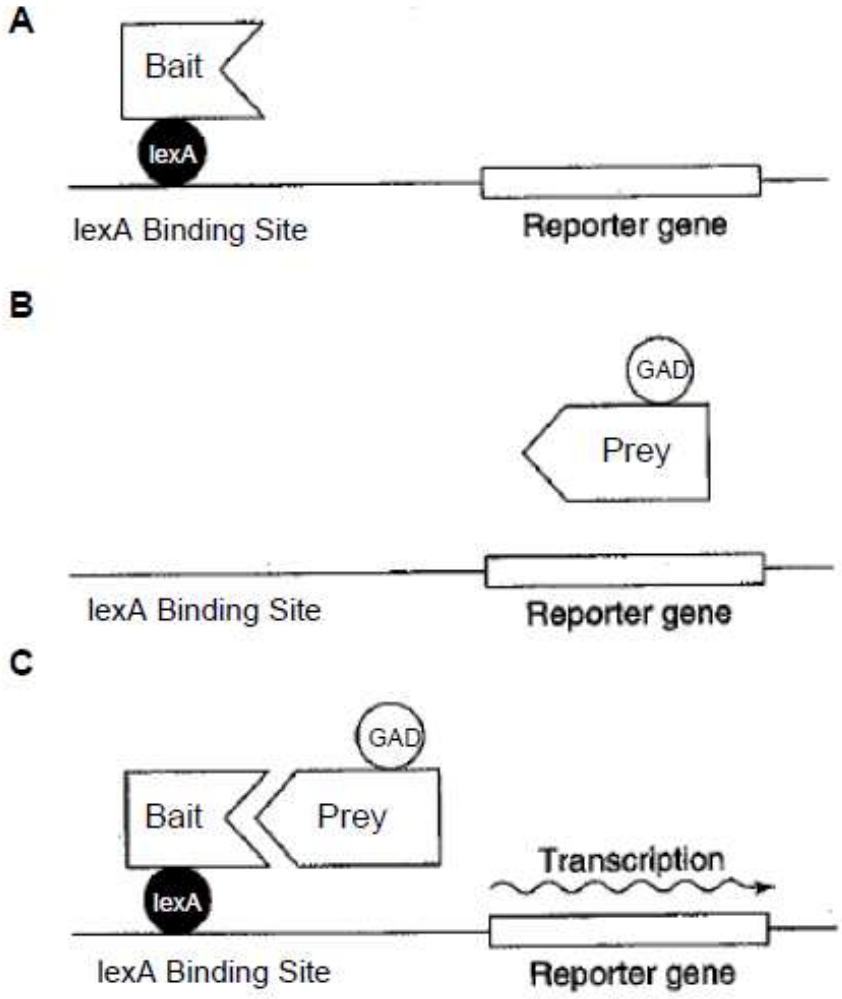

Figure 3 - Schematic diagram of the two-hybrid system. This diagram is a pictorial representation of how the two-hybrid assay works adapted from Fields and Sternglanz, 1994. (A) Depiction of the bait fusion protein bound to a lexA binding site upstream of a reporter gene. Transcription is not activated. (B) Depiction of the prey fusion protein. Without the bait, the prey is unable to localize to the promoter of the reporter gene and therefore can't activate transcription. (C) Interaction between the bait bound to the lexA binding site and the prey protein brings the Gal4 activation domain (GAD) to the promoter of the reporter gene allowing transcription of the reporter gene.

Chapter 2

Methods

Yeast transformation: High efficiency transformation

Single colonies of L40 were inoculated in 3 mL of yeast extract peptone dextrose medium supplemented with 0.2% adenine (YPD+ADE). YPD was supplemented with adenine because L40 is auxotrophic for adenine (*ade2)* and the amount of adenine present in YPD liquid medium after autoclaving is insufficient for good growth (Baumgartner et al., 1999). The cultures were grown for approximately 24 hours on a roller at 30°C. Varying volumes of cultures (0.5 mL, 1 mL, 1.5 mL or 2 mL of cells) were diluted into 100 mL of YPD+ADE in 250 mL flasks. The optical density (OD) of each diluted culture was determined using a spectrophotometer and the culture with an OD closest to 0.2 was allowed to grow for an additional 3-5 hours while shaking at 30°C, until the OD of the culture was 0.6. The OD for a high efficiency transformation has to be precise to achieve a large population of competent cells that can be transformed.

Cells were pelleted in a Sorvall centrifuge with GSA rotor for 5 minutes at 5000 revolutions per minute (rpm). The supernatants were discarded and cells were resuspended in 50 mL lithium acetate (LiOAc) solution [0.1M LiOAc pH 7.5 and, TE (10mM Tris-HCl pH 7.5, 1mM ethylenediaminetetraacetic acid [EDTA])] and then pelleted again for 5 minutes at 5000 rpm. The supernatants were discarded and the cells were resuspended in 1 mL of LiOAc solution.

0.1 mL of cells was aliquoted into BD Falcon 2059 tubes (14 mL). To these aliquots, 3 µL of freshly denatured 10 mg/mL sonicated salmon sperm DNA was added. 2 µg of the bait (pBTM116-ZIP1C* 4D) and the prey (pACTII Library) plasmids were added followed by the addition of 1 mL of freshly made LiOAc-PEG solution (40% polyethylene glycol [PEG], 0.1 M LiOAc pH 7.5, 1 X TE pH 7.5) was added. The cells were then incubated at 30°C for 1 hour.

After addition of 100 µL of dimethyl sulfoxide, cells were heat shocked at 42°C in either a water bath or hybridization oven (whichever was available) for 10 minutes. Two mL YPD+ADE was added to each transformation tube which were then put on a 30°C roller for 2 hours.

The cells were pelleted in a tabletop centrifuge (3000 rpm for 5 minutes) and the supernatants were removed by aspiration. The cells were resuspended in 300 µL water. Ten µL of cells were plated on synthetic defined (SD) -Trp -Leu plates. This was done by adding 100 µL of water to the plate and mixing the cells into the water droplet before spreading the cells with large sterile glass beads. These plates were used to estimate the total number of cells that were transformed with both plasmids by multiplying the number of colonies on each plate by 30. The remaining 290 µL was plated on SD -Trp -Leu -His plates. This medium selects for His^+ colonies which can occur as a result of a two-hybrid interaction. Plates were placed at 30°C to grow for approximately 5 days

Normal Yeast Transformation

Single colonies of L40 cells were inoculated in 2 mL YPD+ADE and the cultures was grown for approximately 24 hours on a roller at 30°C. Cultures were diluted 1:50 into 100 mL of YPD+ADE and allowed to grow for an additional 4-5 hours while shaking at 30°C.

The cells were pelleted in a centrifuge as in the high efficiency transformation protocol for 5 minutes at 5000 rpm. The pellet was resuspended in 10 mL of water and transferred to a sterile glass test tube. The cells were pelleted in a tabletop centrifuge at setting 6 for 5 minutes and the pellet was resuspended in 0.5 mL of LiOAc solution.

0.1 mL cells was aliquoted into 1.5 mL microfuge tubes. To these aliquots, 2 µL of freshly denatured 10 mg/mL sonicated salmon sperm DNA was added. For transformation with a single plasmid, 100 ng of plasmid DNA was added to each tube. For co-transformation with two plasmids, 1 µg of each plasmid was used. Following the addition of the DNA, 0.7 mL of LiOAc-PEG solution was added to cells which were incubated at 30°C for 30 minutes.

Cells were then heat shocked at 42°C for 15 minutes and pelleted in a microfuge at 5000 rpm for 2 minutes. Supernatants were removed by aspiration off the cells were resuspended in 300 µL of water. 150 µL was plated onto selective medium and grown at 30°C for 3-5 days.

β-Galactosidase filter assays

Single colonies were patched out onto SD -Trp -Leu plates and grown for approximately 24 hours at 30°C. The cells were then replica plated onto SD -Trp -Leu plates on which a 55 mm Whatman® Qualitative Filter (1450-082) had been placed and grown at 30°C for approximately 24 hours. This allows the cells to grow on the filters. To perform the assay, 2 mL of 1 X Z buffer (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4 \bullet H_2O$, 10 mM KCl, 1mM $MgSO_4 \bullet 7H_2O$, 4 mM 2-mercaptoethanol) and 20 µL 3% X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside resuspended in dimethylformamide) were added to the bottom of a clean plastic petri dish. A different 55 mm Whatman® Qualitative Filter (1003-082) was placed in the Z buffer/X-gal mixture to absorb the liquid. The filter with the cell patches was dipped in liquid nitrogen for 10 seconds to lyse the cells and then placed on filter soaked with Z buffer and X-gal. The filters were placed at 30°C and periodically checked for the appearance of a blue color in the cell patches.

β-Galactosidase liquid assays

Single colonies were inoculated into 5 mL of SD -Trp -Leu medium and grown for approximately 24 hours on a 30°C roller. 0.1 mL of cells was diluted into 0.9 mL of SD -Trp -Leu medium (1:10) and the OD_{600} was measured with SD -Trp -Leu as a blank. 1.5 mL aliquots were distributed into two microfuge tubes and the cells were pelleted in a microfuge at 5000 rpm for 2 minutes. Cells were resuspended in 1 mL of 1X Z buffer, vortexed, pelleted as before, resuspended in 150 µL of Z buffer and vortexed again. To lyse the cells, 50 µL chloroform and 20 µL 0.1% sodium dodecyl sulfate (SDS) were added and the cells were vortexed for 30 seconds. The lysates were then incubated at 30°C for 5 minutes to equilibrate their temperature.

700 µL of 1.2 mg/mL *O*-nitrophenyl-β-D-galacto-pyranoside (ONPG) made up in Z buffer was added to each tube and vortexed briefly to mix. The time that the ONPG was added was recorded. The tubes were placed at 30°C and checked periodically for

the appearance of a yellow color. A yellow color indicates the cleavage of ONPG by β-galactosidase. As soon as a yellow color was observed, the reaction was stopped by the addition of 500 µL of 1M Na_2CO_3 and the tube was placed on ice. The time that the reaction stopped was recorded. Cells were pelleted in a microfuge spinning at 13,200 rpm for 5 minutes. 800 µL of the supernatant was removed and transferred to a new microfuge tube. The OD_{420} of these supernatants were read to determine the amount of ONPG activity for that particular tube with Z buffer as the blank.

The total activity for each sample was reported in Miller units and calculated using the formula (Miller, 1972):

$$\text{Miller Units} = \frac{1000 \times OD_{420}}{OD_{600} \times time \times volume}$$

Time refers to the time it took for the reaction to turn yellow in minutes after the addition of ONPG (time the Na_2CO_3 was added minus time ONPG was added). The volume of cells refers to the original volume that was aliquoted which either 1.5 mL or 1.8 mL.

Isolation of plasmids from yeast cells

Single colonies were inoculated in 6 mL SD -Leu medium in 15 mL sterile test tubes and grown for approximately 24 hours on a roller at 30°C. SD -Leu medium was used to maintain selection for the *LEU2* GAD fusion plasmid. Cells were pelleted in a tabletop centrifuge at setting 6 for 5 minutes. The pellets were resuspended in 1 mL water and transferred to sterile microfuge tubes. The cells were pelleted again in a microfuge at 5000 rpm for 5 minutes. After removal of the supernatants by aspiration, pellets were resuspended in 200 µL of lysis buffer (2% Tritton-100, 100 mM NaCl, 100 mM Tris HCl pH 8, 1 mM EDTA pH 8, 1% SDS, in water) along with 0.1 g of 0.5 mm diameter glass beads and 100 µL phenol chloroform:isoamyl alcohol. Tubes were shaken on the MP Biomedicals FastPrep®-24 instrument (6m/s, 40s) to lyse cells. Under a fume hood, the aqueous layer top layer from each sample, which contains the DNA, was placed in a new tube, while the organic phase was discarded in chemical waste. 100 µL of chloroform was added to the tubes and the tube was inverted 3 times. The

extractions were spun in a microfuge at 13,000 rpm for 10 minutes to separate the organic and aqueous layers. The aqueous layer was transferred to a new tube. 10 µL of 3 M sodium acetate and 250 µL of 100% ethanol were added and the tubes were placed at -20°C for an hour to precipitate the DNA. The DNA was pelleted in a microfuge for 10 minutes at 13,000 rpm. The pellets were washed with 500 µL 70% ethanol by inverting the tube and spinning again at 13,000 rpm for 10 minutes. The DNA pellet was then dried and resuspended in 10 µL of water.

Recovery of plasmids from yeast cells

Two µL DNA isolated from each yeast candidate was added to 100 µL of electrocompetent *Escherichia coli* strain the JBE181 (provided by the Hollingsworth Lab) cells on ice. The cells and DNA were transferred into a BioRad 0.2 cm Gene Pulser Cuvette on ice. Cells were pulsed on the BioRad Gene Pulser unit (settings: 25 µF, 2.5 V, 400 Ω). 0.5 mL of SOC medium (Lysogeny broth [1% Bactotryptone, 1% NaCl, .5% yeast extract] medium with 10 mM $MgSO_4$ and 20 mM glucose) (Hanahan, 1983) was added to the cuvette and the cells were transferred to a sterile microfuge tube with pasteur pipettes. The cells were incubated for 1 hour at 37°C, plated on LB medium containing ampicillin (LB+amp) and then incubated at 37°C overnight. To identify those bacterial colonies containing the *LEU2* plasmid, Amp^R transformants were replica plated onto M63 -Leu medium to select for complementation of the *leuB600* bacterial mutant. DNA was isolated from four Leu^+ bacterial colonies per two hybrid candidate using the Qiagen QIAprep Spin Miniprep Kit.

Western blotting

Protein extracts were prepared for Western blotting using a trichloroacetic acid (TCA) preparation method. Single colonies of yeast cells were inoculated in 6 mL of selective medium and grown for approximately 24 hours on a 30°C roller. Cells were transferred to 15 mL conical tubes and placed in a tabletop centrifuge with for 3 minutes at 3000 rpm. The resulting pellets were resuspended in 5% TCA (diluted in water from 100% TCA). After rocking at 4°C for 10 minutes, cells were pelleted as before and the pellets were resuspended in 1 mL of acetone. Cells were transferred to a fresh

microfuge tube and vortexed for 20 seconds. The cells were pelleted in a microfuge at 13,000 rpm for 5 minutes. The acetone was aspirated off and the pellets were left to dry in a fume hood for 2.5 hours. The cells were resuspended in lysis buffer (50 mM Tris pH 7.5, 1mM EDTA, 2.7 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride in water with 1 cOmplete protease inhibitor tablet from Roche). Cells were lysed after addition of approximately 50 mg of 0.5mm glass beads on the MP Biomedicals FastPrep®-24 instrument (6m/s, 40s). After lysis, 75 µL of 2X SDS loading buffer was added to each tube and was mixed by stirring with a pipette tip. Tubes were heated at 95°C for 5 minutes. Tubes were spun in a microfuge at 13,000 rpm for 5 minutes. Supernatants containing the proteins were transferred to fresh microfuge tubes.

To perform the Western blot, TCA lysates were fractionated on an 8% sodium dodecyl sulfate – polyacrylamide gel. The stacking buffer of the gel was prepared by mixing 1.15 mL of water, 0.33 mL acrylamide-bisphosphate, 0.5 mL 0.5% Tris HCl, 20 µL 10% SDS, 20 µL ammonium persulfate (APS) and 2 µL of tetramethylethylenediamine (TEMED). The running buffer was prepared by mixing 2.3 mL of water, 1.3 mL acrylamide-bisphosphate, 1.3 mL 0.5% Tris HCl, 50 µL 10% SDS, 50 µL APS and 3 µL of TEMED.

The gel was loaded with 5 µL of the Page Ruler prestained ladder from Thermo Scientific and 10 µL from each sample and run at 130 V for 60 minutes or until the dye front ran off. The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane using the BioRad wet transfer system at 80 V for 30 minutes. The membrane was then blocked overnight at 4°C in 25 mL of blocking buffer (5% dried milk and Tris-buffered saline and Tween 20 [TBST: 20mM Tris-HCl pH 7.5, 250mM NaCl, 0.1% Tween 20]). The membrane was washed TBST. 10 mL of blocking buffer was added to the membrane along with 2 µL of Santa Cruz polyclonal rabbit anti-Zip1 (200 µg/mL; 1:5000 dilution) primary antibody. The membrane was incubated with the primary antibody at room temperature for 2 hours. The membrane was then washed 3 times with TBST, shaking 5 minutes each wash. 10 mL of blocking buffer was added to the membrane along with 1 µL of Santa Cruz goat anti-rabbit (100 µg/mL; 1:10,000 dilution) secondary antibody conjugated to the enzyme horseradish peroxidase (HRP). The membrane was incubated with shaking at room temperature for 1 hour. The

membrane was again washed 3 times with TBST, shaking 5 minutes each wash. Detection of antibodies was done using the Pierce ECL 2 western blotting substrate which gives a chemiluminescent signal when cleaved by HRP. The Pierce ECL 2 western blotting substrate, was applied to the membrane and allowed to react for 5 minutes. Excess substrate was removed and the chemiluminescent signal was detected with X-ray film.

Plasmids

A list of the plasmids is presented in Table 1. To create the *lexA-ZIP1C** fusion gene, Yan Liu used the polymerase chain reaction (PCR) to amplify an approximately 400 bp region encoding the C-terminus of *ZIP1* flanked by BamH1 and Pst1 restriction sites. The BamHI/PstI fragment was ligated into the BamHI/PstI digested pBTM116 (Figure 4A) (Gietz et al., 1997) vector in frame with the *lexA* gene to generate the pBTM116-ZIP1C*-WT. The pBTM116-ZIP1C* S815D S816D S817D S818D (*lexA-ZIP1C*-4D)* plasmid was constructed in two steps. First Xiangyu Chen performed site-directed mutagenesis, using pBTM116-ZIP1C* WT as a template, to change serines (S) 815 and 816 to aspartic acid (D) to make the pBTM116-ZIP1C* S815D S816D plasmid. The S815D mutation was created by changing the codon from TCT to GAC, while in S816D, the codon was changed from TCA to GAC. I then performed the second step, which was to use the pBTM116-ZIP1C*-2D plasmid as a template to mutate S817 and S818 to aspartic acids by changing each of their codons from TCA to GAC. This was done by designing PCR primers (Table 2) that were homologous to the region of the gene we wanted to change (codons 815-818) except these primers contained mutated versions of the codons. The mutations were confirmed by sequencing (Figure 4B).

In addition, I constructed non-phosphoyrlatable mutants in which alanines were substituted for S815-818. Using pBTM116-ZIP1C* WT as a template, S815 and S816 were each mutated to the alanine (A) by changing their codons to GCA to make the pBTM116-ZIP1C*-2A plasmid. In a separate reaction, S815 S816 S817 S818 of lexA-*ZIP1C**-WT were all mutated to alanine by changing their codons to GCA to make pBTM116-ZIP1C*-4A. The mutations were confirmed by sequencing (Figure 4C). All

*ZIP1-C** alleles were sequenced in their entirety to make sure no unwanted mutations were introduced during mutagenesis (data not shown).

The plasmid pNH223 contains *lexA* fused to *RED1* was used as a positive control for the two hybrid assay since it has previously been shown to interact with pGAD-Red1$^{537\text{-}837}$ (Woltering et al., 2000). In addition, pGAD-Red1$^{537\text{-}837}$ also interacts with *lexA-ZIP1C**-WT consistent with previous work showing that the C-terminus of Zip1 interacts with Red1 (Lin et al., 2010). pGAD424 is a negative control plasmid which contains only the *GAD* gene, and therefore does not promote transcription of the *HIS3* or *lacZ* reporter genes (Gietz et al., 1997) (Figure 4E).

The prey used for the screen was a yeast genomic library inserted into the pACTII vector (Dresser et al., 1997) (Figure 4D). The pACTII library contains random fragments of the yeast genome fused to the *GAD*.

DNA sequencing reactions

DNA sequencing reactions were performed to check the mutations introduced by site directed mutagenesis and to identify gene fragments fused to *GAD* which displayed a two-hybrid phenotype with the bait *lexA-ZIP1C*-4D*. Sequencing reactions were performed by the Stony Brook University DNA Sequencing Facility. The sequencing reactions were set up in PCR tubes as follows: 8 µL of water, 3 µL of DNA (from Qiagen QIAprep Spin Miniprep) and 1 µL of 3.2 µM sequencing primer (Table 2).

Plasmid Name	Nickname	Genotype	Source	Purpose
pBTM116	pBTM116	2μ, *amp^R, TRP1*	(Gietz et al., 1997)	Vector
pBTM116-ZIP1C* WT	lexA-Zip1C*-WT	2μ, *amp^R , TRP1, lexA-ZIP1C*-WT*	Yan Liu	Bait
pBTM116-ZIP1C* S815A S816A	lexA-Zip1C*-2A	2μ, *amp^R , TRP1, lexA-ZIP1C*-2A*	Matthew Murray	Bait
pBTM116-ZIP1C* S815A S816A S817A S818A	lexA-Zip1C*-4A	2μ, *amp^R , TRP1, lexA-ZIP1C*-4A*	Matthew Murray	Bait
pBTM116-ZIP1C* S815D S816D	lexA-Zip1C*-2D	2μ, *amp^R , TRP1, lexA-ZIP1C*-2D*	Xiangyu Chen	Bait
pBTM116-ZIP1C* S815D S816D S817D S818D	lexA-ZIP1C*-4D	2μ, *amp^R, TRP1, lexA-ZIP1C*-4D*	Matthew Murray	Bait
pNH223	pNH223	2μ, *TRP1, lexA-RED1, ADE2*	(Woltering et al., 2000)	Positive Bait Control
pACTII Library	pACTII	2μ, *amp^R , LEU2, GAD-Genomic Library*	(Dresser et al., 1997)	Prey
pGAD-Rec8$^{133-433}$	pGAD-Rec8$^{133-433}$	2μ, *amp^R , LEU2, GAD-REC8*	Isolated from pACTII library	Prey
pGAD424	GAD	2μ, *amp^R , LEU2, GAD*	(Bartel et al., 1993)	Negative prey control
pGAD-Red1$^{537-837}$	pGAD-Red1$^{537-837}$	2μ, *amp^R , LEU2, GAD-RED1*	(Tu et al., 1996)	Positive Prey Control

Table 1. **Plasmids.** The nickname is used in the text when referencing each plasmid. The plasmids are listed in the order in which they appear in the experiments. Zip1C* refers to the portion of the gene that codes for amino acids 750-875 of the C terminus (875 is the last amino acid of the Zip1 protein). The purpose column describes whether the plasmid was used as the bait or the prey in two-hybrid experiments.

Primer Name	Primer Sequence	Purpose
ZIP1-2A-F	5'-CAAATTTAACGATGAGTTCGATCTTG CAGCATCATCAAATGACGACCTAGAG-3'	Mutagenesis
ZIP1-2A-R	5'-CTCTAGGTCGTCATTTGATGATGCTG CAAGATCGAACTCATCGTTAAATTTG-3'	Mutagenesis
ZIP1-4A-F	5'-CAAATTTAACGATGAGTTCGATCTTGCAG CAGCAGCAAATGACGACCTAGAGTTAAC-3'	Mutagenesis
ZIP1-4A-R	5'-GTTAACTCTAGGTCGTCATTTGCTGCTGC TGCAAGATCGAACTCATCGTTAAATTTG-3'	Mutagenesis
ZIP1-2D-F	5'-CAAATTTAACGATGAGTTCGATCTTG ACGACTCATCAAATGACGACCTAGAG-3'	Mutagenesis
ZIP1-2D-R	5'-CTCTAGGTCGTCATTTGATGAGTCGT CAAGATCGAACTCATCGTTAAATTTG-3'	Mutagenesis
ZIP1-4D-F	5'-CAAATTTAACGATGAGTTCGATCTTGACG ACGACGACAATGACGACCTAGAGTTAAC-3'	Mutagenesis
ZIP1-4D-R	5'-GTTAACTCTAGGTCGTCATTGTCGTCGT CGTCAAGATCGAACTCATCGTTAAATTTG-3'	Mutagenesis
ZIP1 SEQ18	5'-AAACCATCAGATTCACCC-3'	Sequencing
ZIP1 SEQ19	5'-ACCAAATTAAACCTAACC-3'	Sequencing
GAD - AD 5'	5'-TACCACTACAATGGATG-3'	Sequencing

Table 2. **Primers used for mutagenesis and sequencing.** F and R designate Forward and Reverse, respectively.

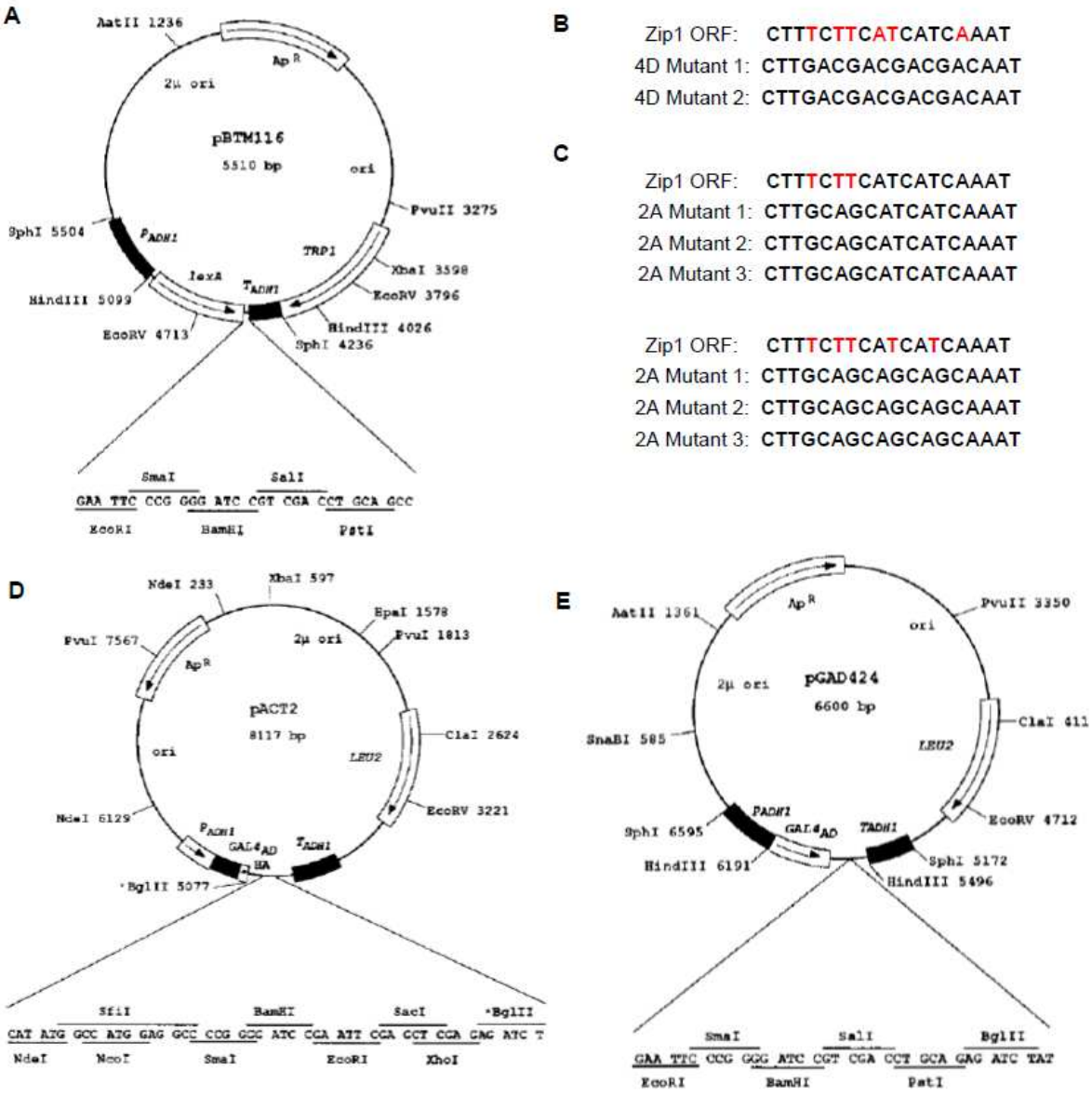

Figure 4 – Plasmid maps and sequencing data. (A) Plasmid map for the pBTM116 vector (Gietz et al., 1997). All *ZIP1C** genes were cloned into this vector by way of the BamHI and PstI sites. (B) Sequencing data showing that the *ZIP1C*-2D* gene was successfully mutated to the *ZIP1C*-4D* gene. (C) Sequencing data showing that the *ZIP1C*-WT* gene was successfully mutated to the *ZIP1C*-2A* and *ZIP1C*-4A* genes. (D) Plasmid map for the pACTII vector (Gietz et al., 1997). All genomic fragments were cloned into this vector by way of the BamHI site which is flanked by two BglII sites. (E) Plasmid map for the pGAD424 vector (Gietz et al., 1997). pGAD424 was used as a control in this study with no gene fragments inserted.

Chapter 3

Results

High efficiency yeast transformations and candidate identification

To screen for genes that encode proteins that interact with the lexA-Zip1C*-4D protein, L40 was co-transformed with the *lexA-ZIP1C*-4D* plasmid and a library of genomic fragments cloned into a *GAD* fusion vector. To select for prey plasmids encoding interactors, transformations were plated on medium lacking tryptophan, leucine and histidine (SD -Trp -Leu -His). The two-hybrid interaction was then confirmed by testing for β-galactosidase production using enzymatic assays that produce a change in color in the presence of β-galactosidase.

Five independent high efficiency yeast transformations were performed using L40 and 2 µg of both lexA-ZIP1C*-4D and a pACTII genomic library (Dresser et al., 1997). The high efficiency yeast transformation protocol produces large numbers of transformants and therefore increases the possibility of detecting an interaction. High efficiency transformations may also result in the uptake of multiple plasmids that can yield more cells that have taken up both the bait and the prey. This protocol differs from the normal transformation protocol used in the Hollingsworth Lab in that it ensures that cells are in the log phase of growth before being harvested because log phase cells can be made more competent than stationary phase cells.

Cells were plated on SD -Trp -Leu -His plates to select colonies that contained both plasmids and were prototrophic for histidine (His^+), indicating a possible two-hybrid interaction. A 1:30 dilution of the transformation was also plated on SD -Trp -Leu plates to determine the total number of possible transformants. The number of colonies on the SD -Trp -Leu plates was multiplied by 30 to determine the total number of transformants (Table 3). Out of a total of 730,530 Trp^+ Leu^+ transformants, 21 were identified as His^+ for a frequency of 0.003% (Table 3).

Each His^+ transformant was patched out onto SD -Trp -Leu medium along with colonies containing the positive and negative control combination of plasmids (pNH223/pGAD-Red1$^{537-837}$ and lexA-ZIP1C*-4D/pGAD424, respectively). β-galactosidase filter assays were performed as a secondary screen (Figure 5). A blue color confirms the presence of a two-hybrid interaction as β-galactosidase production results from a second reporter (*lacZ)* under the control of a *lexA* promoter. Each His^+ β-$galactosidase^+$ transformant was given an identifier based on the date of the transformation, its plate number and, in cases with multiple His^+ transformants on a single plate, its order on the plate. Out of the 21 Trp^+ Leu^+ His^+ transformants, eleven tested positive for β-galactosidase production.

DNA was extracted from the eleven His^+ β-gal^+ candidates and electroporated into JBE181 electrocompetent cells provided by the Hollingsworth lab. This bacterial strain is susceptible to ampicillin and auxotrophic for leucine (*leuB600*). Transforming cells with the DNA and selecting on ampicillin gives rise to bacterial colonies containing either the *TRP1 lexA-ZIP1C*-4D* plasmid or the *LEU2 GAD*-fusion plasmid. To specifically identify those colonies containing the *LEU2* plasmid, Amp^R colonies were replica plated onto M63 -Leu medium to select for complementation of the *leuB600* bacterial mutant.

DNA was isolated from four Leu^+ bacterial colonies per candidate DNA using the Qiagen QIAprep Spin Miniprep Kit. Multiple bacterial colonies from the same candidate were analyzed to determine if more than one library plasmid was present within the yeast transformant. The pACTII vector contains BglII cut sites that flank the genomic insert (Gietz et al., 1997) (Figure 4D). Therefore digestion with BglII releases the genomic insert, as well as cuts at any BglII sites within the insert. Each plasmid was digested with BglII and run on a 0.8% agarose gel to see if the restriction fragment patterns were the same (Figure 6). Candidates 10/16 - 6 and 10/23 - 6.2 each contained multiple plasmids. Candidates 10/16 - 8 and 10/23 - 6.2B/D appeared to contain the same genomic fragment, 10/16 - 6A, and 10/2 - 1 appeared to contain the same genomic fragment and candidates 11/6 - 2.1 and 11/6 - 8.2 appeared to contain the same genomic fragment. Candidates 11/13 - 4.1 and 11/13 - 8 also had similar molecular weights indicating that they may contain the same plasmid.

To determine whether the isolated plasmids are responsible for the original two-hybrid signal, 1µg of each unique *GAD*-fusion plasmid was co-transformed with 1 µg of the lexA-ZIP1C*-4D plasmid into L40 using the normal transformation protocol. The transformants were tested for β-galactosidase production by filter assays (Figure 7).

Eight plasmids from the 11 two hybrid candidates exhibited β-galactosidase activity upon retransformation and were sequenced. The primer used in the sequencing reactions is GAD-AD 5' (Table 2) that is complementary to the 3' end of the *GAD* gene allowing one to determine the junction between the *GAD* gene and the genomic insert. The resulting sequences were used in BLAST searches using the Saccharomyces Genome Database (http://www.yeastgenome.org/) to identify the yeast genes in the genomic inserts belong to. Sequences were aligned to the open reading frame of the gene using the "SeqMan Align program". This allowed identification of the region of the protein that is encoded in the *GAD* fusion plasmid. Out of the eight candidates, three of the genes were identified twice: *SRP1*, *REC8* and *SRS2* (Table 4). The 10/23 - 6.2 candidate had two digests sequenced based off the fact that they seemed to be different plasmids from the agarose gel (Figure 6). These two plasmids were both identified as unique genes: *REC8* and *FIR1*. The frequency of a successful two hybrid interaction is 3×10^{-5} and so the probability of a transformant containing two unique plasmids that both give rise to His^+ and $\beta\text{-}gal^+$ cells is 9×10^{-10}, which is extremely improbable. A more likely explanation is that an error was made and samples were switched at either the restriction enzyme digest or sequencing steps resulting in two unique plasmids being identified in one candidate. Given that the same *REC8* fragment was identified in an independent transformation, I assume that *REC8* is responsible for the two-hybrid signal in this candidate as well.

Negative Charges at Zip1 S815-818 enhances Zip1C*-Rec8 binding

To determine whether the interaction between Zip1C* and Rec8 is enhanced by the negative charges from the phosphomimetic amino acids, the pGAD-$Rec8^{133-433}$ plasmid was transformed with each of the *lexA-Zip1C** plasmids (*ZIP1C*-WT* = WT, *ZIP1C**-S815A S816A = 2A, *ZIP1C**-S815A S816A S817A S818A = 4A, *ZIP1C**-S815D S816D = 2D and *ZIP1C**-S815D S816D S817D S818D = 4D). Each of the Zip1C*

plasmids was also transformed with the pGAD-Red1$^{537-837}$ and pGAD424 plasmids as controls. Filter assays indicated that the interaction between Rec8 and Zip1C* is enhanced by the presence of the negative charges (Figure 8). The intensity of the color (and therefore the total β-galactosidase production) of the pGAD-Rec8 patches is less in the presence of the 2A and 4A mutants than it is with the 2D and 4D mutants. The WT/pGAD-Rec8$^{133-433}$ patches showed color intensity similar to the 2D and 4D mutants, suggesting that the lexA-Zip1C* fusion protein may phosphorylated *in vivo* on serines 815-818. In contrast, all the mutant proteins appear to interact to an equivalent extent with the pGAD-Red1$^{537-837}$ control.

To quantify the amount of β-galactosidase activity, a liquid β-galactosidase enzyme activity assay was performed. The results of the liquid assay (Figure 9) confirm that the Zip1C*-Rec8 interaction is indeed enhanced by the presence of the negative charges from the *ZIP1C*-2D* phospho-mimic. In addition, the liquid assay also shows that the *Zip1C*-WT* gives more activity with *GAD-REC8$^{133-433}$* than the *Zip1C*-2D* mimic. These results support the hypothesis that negative charges provided by phosphorylation of the Zip1 C-terminus facilitate interaction with Rec8 and that the kinase responsible for phosphorylating serines 815 and 816 is present in vegetative cells.

One possible caveat to the previous experiment is that the decrease in signal observed between *GAD-Rec8*$^{133-433}$ and the *lexA-ZIP1C*-2A* and *lexA-ZIP1C*-4A* mutants compared to the *lexA-ZIP1C*-2D* and *lexA-ZIP1C*-4D* mutants is due to a decrease in steady state protein levels specifically in the alanine mutants. This idea was tested by immunoblot analysis of the lexA-Zip1C* fusion proteins (Figure 10). Cells were grown in SD -Trp -Leu selective medium, protein extracts were fractionated by sodium-dodecyl sulfate polyacrylamide gel electrophoresis and probed with antibodies to the Zip1 C-terminus. The levels of the Zip1C*-2A, Zip1C*-4A, Zip1C*-2D and Zip1C*-4D proteins are similar, while there seems to be a lower amount of the Zip1C*-WT protein. The Zip1C*-WT lane's nonspecific bands show similar amounts to the same bands in other lanes indicating that the same amount of protein extract was loaded in all lanes. The decreased activity seen in the *GAD-REC8$^{133-433}$* interaction with *lexA-ZIP1C*-2A*

and *lexA-ZIP1C*-4D* is therefore not due to decreased amount of the Zip1 C* proteins in these cells as compared to the Zip1C*-WT protein levels. The result of the western blot allows us to conclude that the difference in interaction observed between *GAD-REC8*$^{133-433}$ and the various Zip1C* mutants is due to the presence or absence of the negative charges at serines 815-818.

	# Trp^+ Leu^+ Transformants	# of Trp^+ Leu^+ His^+ Colonies	Percent His^+
Transformation 1	15,390	1	0.006
Transformation 2	104,190	4	0.004
Transformation 3	120,990	3	0.002
Transformation 4	225,870	8	0.004
Transformation 5	264,090	5	0.002
Total	730,530	21	0.003

Table 3. **Transformations and Number of His+ Transformants.**

Candidate Designation	Gene Name (AA's encoded)	Gene Function
10/16 – 6C	*SRP1* (junction sequence unclear)	Karyopherin alpha homolog. Responsible for nuclear import of proteins and cotranslational degredation. See description of *SRP1* above.
11/6 – 8.2B	*SRP1* (110-412)	
10/16 – 8A	*REC8* (133-433)	Part of the cohesion complex, functions to hold sister chromatids together during Meiosis See description of *REC8* above.
10/23 – 6.2B	*REC8* (133-433)	
11/6 – 2.1	*WSS1* (217-261)	Metalloprotease involved in DNA repair
11/13 – 4.1A	*SRS2* (1035-1174)	DNA helicase & DNA dependent ATPase involved in DNA repair and checkpoint recovery. Needed for proper timing of recombination and commitment of Meiosis I to Meiosis II
11/13 – 8A	*SRS2* (879-1174)	

Table 4 – **Zip1C* Interacting Partners.** Gene descriptions were adapted from the Saccharomyces Genome Database (http://www.yeastgenome.org/).

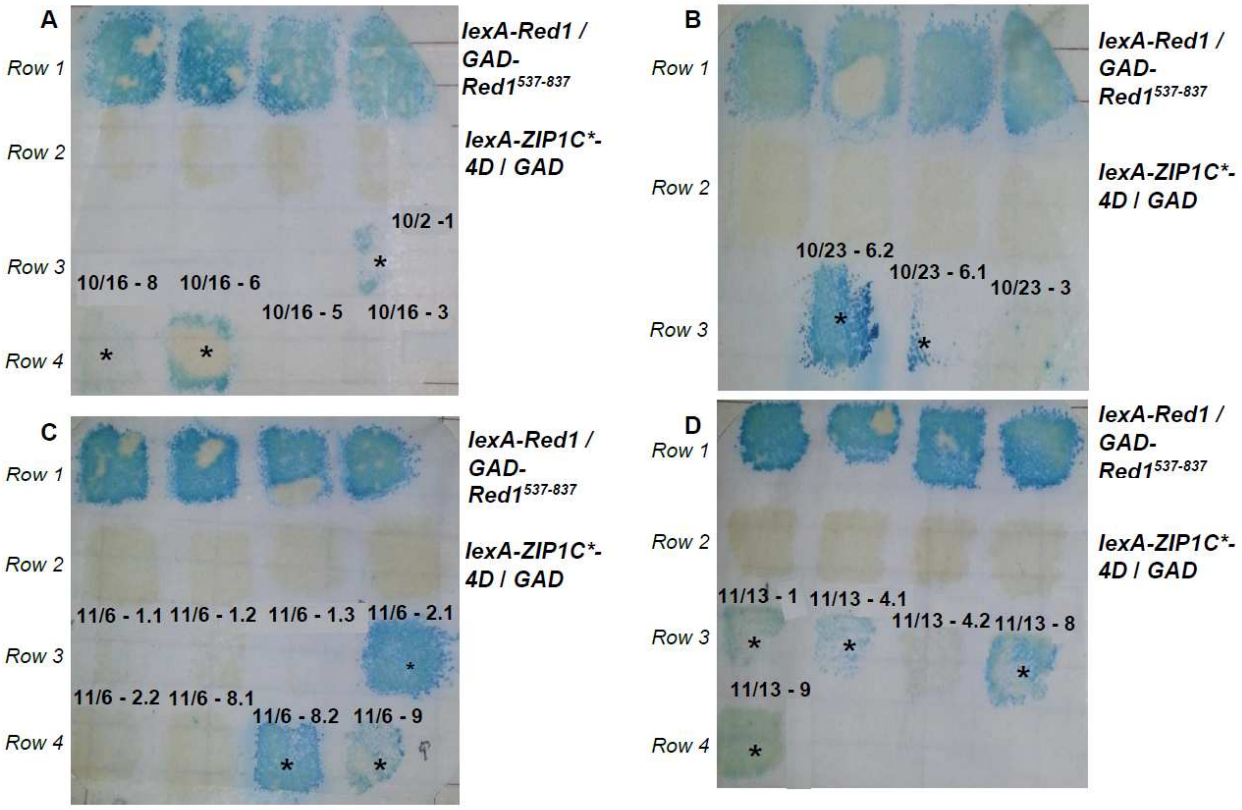

Figure 5 – β-galactosidase filter assays for His⁺ transformants. In each panel, the top row is the positive control and the second row is the negative control. Rows three (A, B, C, D) and four (A, C, D) are the His⁺ transformants isolated from the high efficiency transformations. Asterisks indicate transformants which retested for β-galactosidase activity. (A) High efficiency transformations 1 and 2. (B) High efficiency transformation 3. (C) High efficiency transformation 4. (D) High efficiency transformation 5.

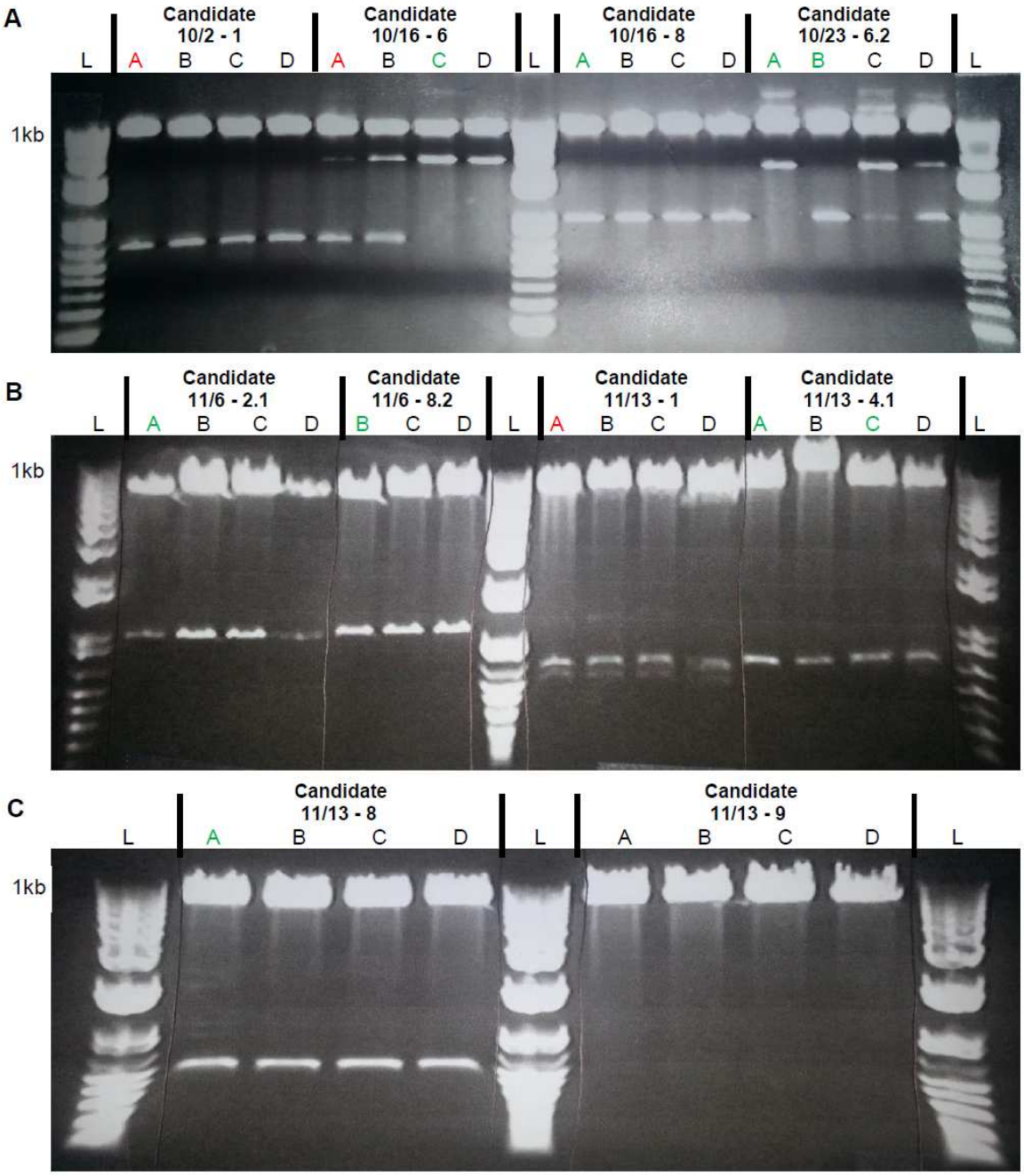

Figure 6 – Candidate BglII digests on 0.8% agarose gels. A 1kb ladder (lanes labeled L) was used. Letters in green font indicate those digests that retested for β-galactosidase activity. Letters in red font indicate those digests that did not retest for β-galactosidase activity. Digests were selected to retest based on their similarity to digests of the same candidate in these gels. (A) Four candidates run in quadruplicate. Minipreps 10/16 - 6A and B are different from minipreps 10/16 -6C and D. Minipreps 10/23 - 6.2 A and C are different from minipreps 10/23 - 6.2B and D. (B) Four candidates run in quadruplicate. All candidate minipreps are the same. Miniprep 11/6 - 8.2A was contaminated and discarded. (C) Two candidates run in quadruplicate. All 11/13 - 8 minipreps are the same. Candidate 11/13 - 9 didn't digest properly. This could be that the genomic fragment which was inserted was too small to be resolved on this gel.

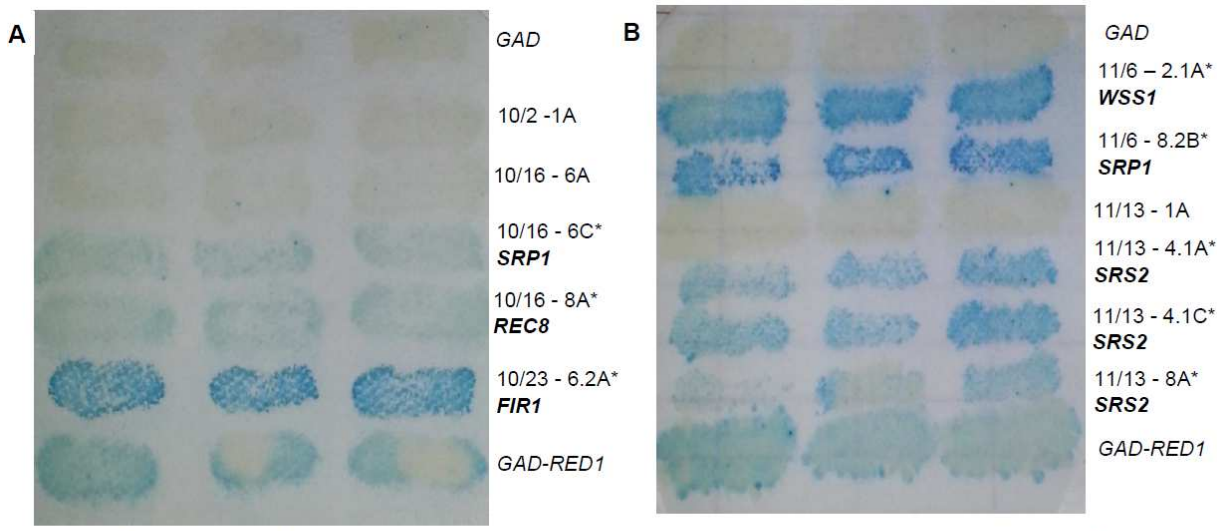

Figure 7 - β Galactosidase filters retesting for candidates that successfully produce β Galactosidase. The bait in each row is the *ZIP1C*-4D* plasmid. The top row in each filter is the negative control *GAD*. The bottom row is the positive control *GAD-Red1*. Candidates that recapitulated the two-hybrid phenotype are marked with an asterisk and the genes they were identified as were listed below them in bold. (A) Filter confirming the interaction of 3 candidates. Candidate 10/23 - 6.2B could not be shown on this filter but it was confirmed by sequencing as *REC8* prior to this filter assay. Candidate 10/23 - 6.2B is different from Candidate 10/23 - 6.2A which was identified as *FIR1*. (B) Filter confirming the interaction of 5 candidates. Candidates 11/13 - 4.1A and C were at slightly different positions in a close up of the gel (Figure 6B) and so both were examined as a precaution.

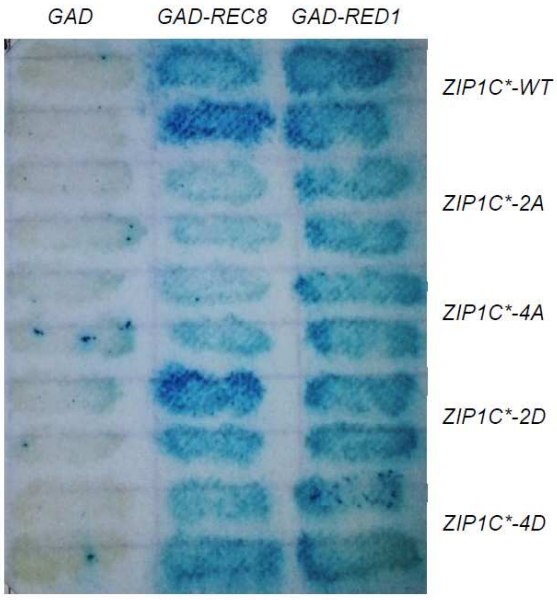

Figure 8 – Testing the ZIP1C*/Rec8 interaction. β-galactosidase Filter Assay results for initial *GAD-REC8* testing with the *ZIP1C** plasmids (WT, 2A, 4A, 2D, 4D). Each two-hybrid interaction was tested in duplicate. *GAD-REC8* encodes amino acids 133-433 of the Rec8 protein. *GAD-RED1* encodes amino acids 537-837 of the Red1 protein.

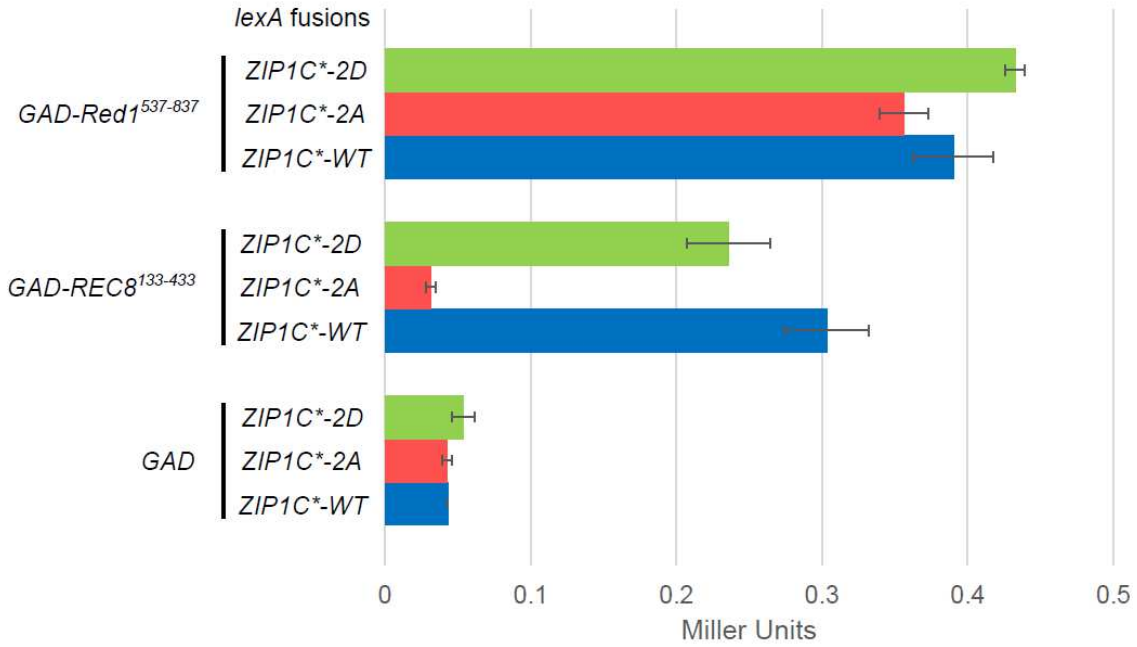

Figure 9 – Liquid β-Galactosidase Assay Results. This chart represents the Miller units (Miller 1972) for the *lexA-ZIP1C*-WT*, 2A & 2D mutants interacting with GAD-*REC8*$^{133-433}$, as well as positive and negative controls. Each two-hybrid interaction is represented as *PREY* | *BAIT*. Miller units represent the average of the calculated Miller units for 6 assays (*REC8*) or 2 assays (*GAD* and GAD-*RED1*$^{537-837}$). *ZIP1C*-4A* & *ZIP1C*-4D* were excluded because they reacted with similar color intensity in the preliminary filter assay (Figure 8).

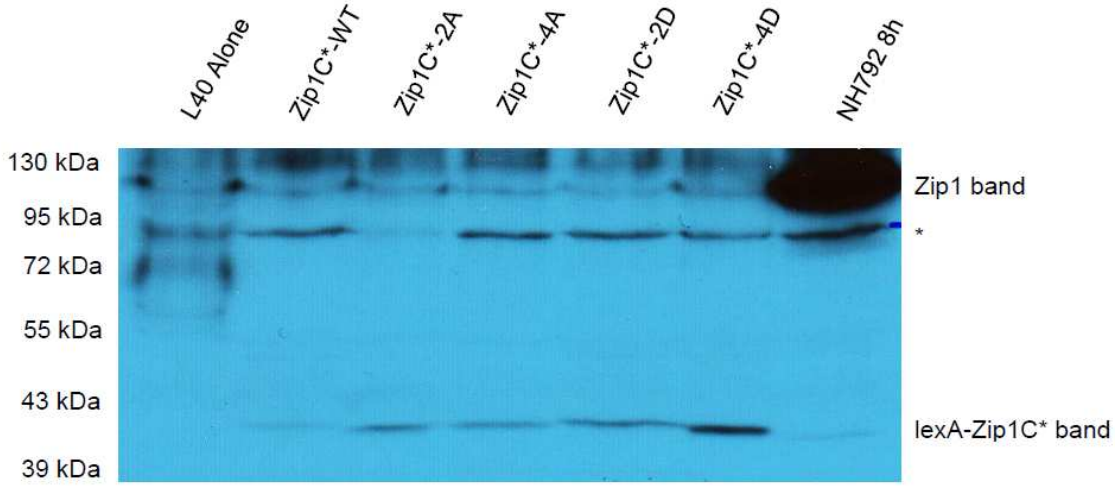

Figure 10 – Western blot of the Zip1C* mutants. Un-transformed L40 is included as a negative control. NH792 is included as a positive control and is a full length version of the Zip1 protein (NH792 was provided by Xiangyu Chen). The LexA-Zip1C* band is between 34 kDa and 43 kDa. The Zip1 band (positive control) is approximately 100 kDa. The band at 95 kDa is a nonspecific band and is indicated by an asterisk.

Chapter 4

Discussion

The negatively charged C-terminus of Zip1 interacts with Rec8, Srs2 and Srp1

Three Zip1C* binding partners were identified twice using independent transformations for the two-hybrid screen: Rec8, Srs2 and Srp1 (although for one of the SRP1 fragments, the junction fragment is unable to be identified so whether this represents a fusion to *GAD* is unclear). These binding partners are examined in further detail in the following sections.

The Zip1C*-Rec8 interaction

As mentioned in the introduction, Rec8 is a meiosis-specific component of the cohesion complex which forms rings around sister chromatids (Berchowitz and Copenhaver, 2010; Klein et al., 1999). Rec8 has an important role in homologous recombination. Rec8 causes the recombination bias to be towards recombination between the two sister chromatids rather than their homologs (Hong et al., 2013). This intersister bias can be overcome by the actions of the Rad51/Dmc1 nucleoprotein filament, which acts to shift the bias towards recombination between homologous chromosomes. This was determined as *rec8Δ* mutants do not need Rad51 or Dmc1 to create interhomolog bias, but wild type strains do (Hong et al., 2013). In spite of all this, once homolog bias is established, Rec8 works positively to maintain it (Kim et al., 2010). These findings suggest an important role for the Rec8 protein in determination of partner choice in meiotic recombination.

Rec8 has been shown to play a role in DNA double strand break (DSB) formation. In order for recombination to occur, DNA has to be broken to allow the resulting single stranded DNA to invade its homologous partner and result in exchange of genetic information. In meiosis, the DSBs are created by the topoisomerase like protein Spo11 (Keeney, 2001). In chromatin immunoprecipitation-DNA microarray

(ChIP-chip) assays it was shown that in the early phases of meiosis, up until the DSBs are formed, Spo11 colocalizes to the same regions of the chromosome as Rec8 (Kugou et al., 2009). In the early stages of meiosis, both Rec8 and Spo11 are distributed exclusively around the centromere. As DNA replication progresses, both Rec8 and Spo11 migrate out to the arms of the chromosome (Kugou et al., 2009). *rec8Δ* mutants caused significant defects in DSB formation and distribution in a region dependent manner (Kugou et al., 2009). These results suggest an important role for Rec8 in guiding the proper distribution of Spo11 to DSB sites.

Genetic evidence obtained by Xiangyu Chen and Ray Suhandynata indicates the members of the ZMM group of proteins perform their respective functions after phosphorylation of serines 815-818 C-terminus of Zip1 (X. Chen and N.M. Hollingsworth, personal communication). The hypothesis developed from these data is that phosphorylation of these serine residues creates a platform for components of the ZMM complex to assemble (i.e. Zip2 and Zip3) as well as to allow other proteins to be recruited.

Synapsis initiation complexes (SIC) are structures that are formed prior to SC formation and contain several proteins including: Zip1, Zip2 and Zip3. These structures have been shown to display interference with each other, similar to the phenomenon of crossover interference, as the presence of a Zip2 focus in a region decreases the likelihood of additional Zip2 foci in adjacent regions (Fung et al., 2004). In addition, crossovers typically occur at these SICs along chromosomes (Fung et al., 2004). These SICs localize to axial association (AA) sites which are sites that are responsible for bringing together the cores of homologous chromosomes (Fung et al., 2004). In addition to the SIC, ChIP-chip experiments showed that Red1, Hop1, Rec8 and Zip1 have been shown to preferentially bind to AA sites, and this binding takes place at or before the time DSBs form (Panizza et al., 2011).

The results of the two-hybrid screen show that Rec8 preferentially interacts with the negatively charged C-terminus of the Zip1 protein. This interaction could be occurring at the AA sites which may explain why Rec8 and Zip1 associate with the same places on the chromosomes in ChIP-chip experiments (Panizza et al., 2011). As

noted above, Rec8 plays an important role in the distribution of Spo11 to DSB sites and therefore plays a pivotal role in DSB formation and the locations of these breaks. Each DSB has the potential to become a crossover but crossover interference ensures that crossovers are maximally spaced along the chromosome. The co-localization of Rec8 and Zip1 to the AA sites occurs before or at the time of DSB formation (Panizza et al., 2011), suggesting that if Rec8 and Zip1 do interact via Zip1's phosphorylated C-terminus, than the SIC may be formed, at the time of DSB formation, at the AA site. The rest of the ZMM group: Zip4, Mer3, Msh4 and Msh5 (Lynn et al., 2007), can then work to mediate recombination at these sites. Since Rec8 controls the distribution of Spo11, and therefore DSBs, the ability of Zip1 to interact with Rec8 would potentially allow it to localize to regions near DSBs, and possible crossover formation, at the time these breaks are formed, loading the chromosome with SICs which would then be able to promote interfering crossovers.

To test this model it may be necessary to figure out which part of the Rec8 protein is interacting with Zip1. We know that the *GAD-REC8* fusion gene isolated from the two hybrid screen contains amino acids 133-433 of the Rec8 protein. By deleting different regions of the $REC8^{133\text{-}433}$ gene in the *GAD* fusion, we can narrow down the region of Rec8 that is binding Zip1. Using this knowledge, site directed mutagenesis can be used to create *GAD-rec8* mutants that destabilize the interaction between Rec8 and Zip1. Once a set of mutants has been made that destabilizes this interaction, *in vivo* studies can be performed by mutating the Zip1 binding site in the full length *REC8* gene in meiotic cells (assuming this does not interrupt interactions with any of Rec8's other binding partners and that these mutants are viable) and examining crossover interference in this strain. If there is a decrease in interference, then the interaction between Rec8 and Zip1 plays a role in mediating crossover interference.

The Zip1C*-Srs2 interaction

The Srs2 protein is a 3' to 5' DNA dependent ATPase and DNA helicase (Rong and Klein, 1993). Srs2 has been implicated in DNA repair, particularly from damage by ultraviolet (UV) radiation, due to its homology to the prokaryotic protein UvrD, a DNA helicase that is part of the UV radiation repair process (Rong and Klein, 1993). In *srs2*

mutants hyperrecombination occurs and recombinogenic DNA accumulates in response to DNA lesions (Milne et al., 1995). This has led to the hypothesis that in the absence of Srs2, the DNA repair pathway inappropriately switches to a recombinatorial mechanism (Aboussekhra et al., 1992). The way that Srs2 prevents recombination from occurring following a DNA lesion is by dismantling the Rad51 nucleoprotein filament (Krejci et al., 2003). This prevents the broken strand from searching for homology in either its sister chromosome (if replication has occurred) or the homolog. Once the Rad51 filament is dismantled, Srs2 binds the ssDNA/dsDNA junction at the break and repeatedly compresses the ssDNA preventing reformation of a stable Rad51 nucleoprotein filament (Qiu et al., 2013).

In addition to dismantling Rad51 nucleoprotein filaments, Srs2 has also been shown to unwind D-loop structures (Dupaigne et al., 2008). D-loops are formed during recombination by the invading strand binding to a homologous stretch of DNA on a different chromosome. The way Srs2 unwinds the D-loop is by binding to the strand on the homologous chromosome that is displaced by strand invasion and migrating in a 3' to 5' direction until it reaches duplex DNA. At this point, it switches to the opposite strand of the homologous chromosome upon which annealing of the invading strand took place and migrates in a 3' to 5' direction on this strand. It eventually contacts the invading strand, which is covered in Rad51, and dismantles the Rad51 nucleoprotein filament causing synthesis dependent strand annealing (SDSA), generating only gene conversion at some loci, but no crossovers (Dupaigne et al., 2008).

Srs2 could potentially play a role in meiotic recombination through these mechanisms. This would allow the disruption of recombination intermediates that could lead to crossing over in meiosis. Srs2 has a similar function to that of the RecQ helicase: Sgs1. Sgs1 is a 3' to 5' helicase that has the ability to disrupt a variety of recombination structures including the D-loop (Adams et al., 2003) and the double Holliday junction (Wu and Hickson, 2003). *sgs1* and *srs2* mutants have similar recombination phenotypes, resulting in the accumulation of recombination intermediates (Chiolo et al., 2005). Mutation of both *SRS2* and *SGS1* is fatal due to lethal recombination events. Cell viability can be restored in these mutants by inhibiting

homologous recombination (Chiolo et al., 2005). Sgs1 has been shown to play a role in meiosis as it prevents crossovers by dissolving recombination intermediates (Jessop and Lichten, 2008). The similarity of the phenotype of the *srs2* mutant to the *sgs1* mutant, and the fact that they both have similar functions, suggests that like Sgs1, Srs2 may have a role to play in meiosis.

The role that Srs2 plays in meiosis may involve Zip1. The two-hybrid screen identified two *GAD-SRS2* genes, each encoding two different regions of the protein. One region encoded amino acids 879 to 1174 while the other encoded 1035 to 1174 (1174 being the last amino acid). The bait used in the two-hybrid screen was the lexA-Zip1C*-4D protein which substituted aspartic acid restudies for serines at the 815-818 positions to make a phosphomimetic version of the Zip1 C-terminus. Further experimentation should include two-hybrid studies and enzymatic activity assays (similar to what was done for Rec8) with the other lexA-Zip1C* mutants (2A/4A/2D), as well as the wild type fusion to see if the phosphorylation in any way affects the binding of Srs2 and Zip1.

Small ubiquitin-like modifiers (SUMO) are small proteins that are covalently linked onto proteins by ubiquitin ligases and have a variety of functions ranging from creating a site for new protein-protein interactions, blocking interactions or facilitating them (Altmannova et al., 2012). These SUMO modifiers bind to SUMO interacting motifs (SIM) on a variety of proteins. Zip1 has a SIM at its C-terminus, encoded by amino acids 853-863 (Cheng et al., 2006). Srs2 also has a SIM encoded at its extreme C-terminus by amino acids 1168-1174 (Armstrong et al., 2012). Armstrong et al. crystallized a fragment of Srs2 containing amino acids 1107 to 1174 bound to SUMO attached to the proliferating cell nuclear antigen (PCNA) protein (Armstrong et al., 2012) and have shown in detail the interaction Srs2's SIM makes with the SUMO protein. The idea that Zip1 and Srs2 may interact via their SIMs is supported by the fact that both of the *GAD* fusion plasmids contain the SIM. *GAD-SRS2*$^{879\text{-}1174}$ and *GAD-SRS2*$^{1035\text{-}1174}$ still interacted with the lexA-Zip1C*-4D bait in a two-hybrid interaction even though one of the *GAD* fusions is significantly shorter than the other.

To test whether the interaction is mediated by the SIM or not, a *lexA-zip1C** 853-863Δ mutant could be tested for interaction between with Srs2. Additionally each of the *lexA-Zip1C** fusion genes from this study (WT/2A/4A/2D/4D) could be used to test the effects of the negative charges at serines 815-818 in the interaction between Srs2 and Zip1. From these experiments you could make conclusions about whether the SIM or the negative charges play a role in promoting the Zip1 Srs2 interaction.

The Zip1C*-Srp1 interaction

Srp1 is a protein homologous to vertebrate karyopherin α (Enenkel et al., 1995). As such, it is responsible for the nuclear import of proteins in *S. cerevisiae*. The karyopherin proteins recognize the substrate to be imported based on a consensus sequence of amino acids called the nuclear localization signal (NLS) (Kalderon et al., 1984). Classical NLSs can be identified as either monopartite, containing a single cluster of basic residues, or bipartite, containing two clusters of basic residues separated by a 10-12 amino acid spacer (Leung et al., 2003). The most common example of a monopartite NLS is the SV40 large T antigen: PKKKRKV (Kalderon et al., 1984). Similarly, a well-known example of a bipartite NLS is the nucleoplasm protein: KRPAATKKAGQAKKKKL (Dingwall et al., 1988). Srp1 has been shown to associate with both monopartite and bipartite NLSs (Leung et al., 2003).

Based on this specific function of Srp1 it is possible that it is responsible for the nuclear import of Zip1 *in vivo*. It has been postulated that the NLS for Zip1 is located in its very C-terminus, as fusion of *lacZ* to the 3' end of the *ZIP1* gene interrupts nuclear localization (Burns et al., 1994). Indeed, an NLS-like sequence can be observed at the very end of the Zip1 C-terminus: SSRKKLLLVEDEDQSLKISKKRRRK. The last 6 residues (KKRRRK) form a highly basic region that can act as a monopartite NLS. Additionally, 14 amino acids upstream is another cluster of basic residues (RKK) that could serve as one of two basic regions in a bipartite NLS. The 14 amino acid spacer for this hypothetical bipartite NLS is slightly longer than the predicted consensus of 10-12; however, there are examples of NLSs which break this spacer rule, suggesting there may be more to the bipartite spacer than what we currently understand (Lange et al., 2010). To establish whether the Zip1 NLS is either bipartite or monopartite, mutations

can be made in the lexA-Zip1C* proteins, where the RKK cluster of amino acids is changed to negatively charged aspartate or glutamate residues. Changing these positively charged residues to negatively charged residues will impede their ability to interact with karyopherin as the karyopherins bind to classical NLS containing proteins via negatively charged binding pockets (Leung et al., 2003). Mutating RKK to DDD for example, would cause repulsion between the bipartite NLS binding pocket, but significantly not affect monopartite NLS binding (Leung et al., 2003).

The anchor away technique could be utilized to determine if Srp1 is the karyopherin which is responsible for the nuclear import of Zip1. Anchor away is a technique that is used to sequester nuclear proteins in the cytoplasm thereby preventing them from exerting their respective functions (Haruki et al., 2008). It does this by creating two fusion proteins: the target protein of interest coupled to the FKB12-rapamycin-binding (FRB) domain of human mTOR (target) and the human FKB12 protein coupled to the large subunit of the ribosome (anchor). Coupling the anchor to the ribosome ensures there is an abundant amount of anchors in the cytoplasm. Upon addition of rapamycin to the cells, FKB12 and FRB form an extremely tight complex that tethers the target protein to the ribosome and sequesters it in the cytoplasm. This technique has been used to sequester and impair the function of Srp1 (Haruki et al., 2008). A Zip1-GFP fusion protein can be constructed (White et al., 2004) to give a fluorescent signal when observed with microscopy. This fluorescent signal will be observed in the nucleus and cytoplasm of meiotic cells. Since the effects of the anchor away are so rapid and can be observed within minutes (Haruki et al., 2008), addition of rapamycin to meiotic *S. cerevisiae* cells containing Srp1-FRB and FKB12-ribosome fusions will result in rapid depletion of Srp1 from the nucleus. If Srp1 is the karyopherin responsible for transporting Zip1 into the nucleus then a rapid loss of color will occur as Zip1 is shuttled out and trapped in the cytoplasm from the inability of Srp1 to transport Zip1 back into the nucleus.

Srp1 most likely does not participate directly in recombination as its main job is to direct the import of proteins into the nucleus. Additionally, the interaction may not depend upon the phosphorylation of any of the serine residues in the 815-818 region

since this class of proteins primarily recognizes the NLS in its substrates. This could be examined using the β-galactosidase filter and liquid enzymatic activity assays with the *GAD-SRP1* fusion gene and the *lexA-ZIP1C*-WT/2A/4A/2D/4D* fusion genes. If the two-hybrid interaction between these two genes all show similar levels of β-galactosidase activity, then the negative charges are not needed for the Zip1-Srp1 interaction.

The Zip1C*-WT protein may be phosphorylated *in vivo*

The β-galactosidase assays indicated that the lexA-Zip1C*-WT bait protein may be phosphorylated at serines 815-818 in vegetative cells, as the WT protein showed similar levels of activity when interacting with the GAD-Rec8$^{133-433}$ protein as the activity showed by lexA-Zip1C*-2D and lexA-Zip1C*-4D mutants, all three of which interacted better than the *4A* mutant.. This idea is consistent with the observation that the kinase that phosphorylates Zip1 at this region was recently shown to be Cdc7-Dbf4 (X. Chen and N.M Hollingsworth, personal communication). *CDC7* was first identified as a temperature sensitive mutant defective in the cell division cycle (*cdc*) (Hartwell et al., 1973). Cdc7 is an essential dumbbell forming (Dbf) dependent kinase (DDK) that is activated through interactions with Dbf4 (Jackson et al., 1993). Cdc7-Dbf4 is responsible for the firing of replication origins throughout S phase of mitosis and also promotes premeiotic DNA replication (Valentin et al., 2006). Additionally in meiosis, Cdc7-Dbf4 has been shown to be responsible for the initiation of meiotic recombination through phosphorylation of Mer2, therefore playing role in DSB formation (Sasanuma et al., 2008; Wan et al., 2008). As Cdc7-Dbf4 has a variety of roles in both mitotic and meiotic cells, this kinase is not meiosis specific and would thus be expressed in vegetative cells allowing phosphorylation of the lexA-ZipC*-WT protein. In the future two hybrid experiments outlined above to further characterize the interactions of Rec8, Srs2 and Srp1 with Zip1C*, the lexA-Zip1C*-WT bait protein could be used as a substitute for the phosphomimetic lexA-Zip1C*-4D protein as it seems to display a similar phenotype.

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