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Identification of New Genes Involved in Meiosis by a Genetic Screen

Sneharthi Banerjee
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**IDENTIFICATION OF NEW GENES INVOLVED IN MEIOSIS BY A
GENETIC SCREEN**

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IDENTIFICATION OF NEW GENES INVOLVED IN MEIOSIS

BY A GENETIC SCREEN

SNEHARTHI BANERJEE

ABSTRACT

Budding yeast *Saccharomyces cerevisiae* contains a group of proteins named ZMM that constitutes a link between recombination and Synaptonemal Complex (SC) assembly. Yeast mutants that lack ZMM proteins have defects in recombination, SC formation and nuclear division progression. Meiotic cell cycle progression in *zmm* mutants is modulated by temperature. This conditional behavior is different at high and low temperatures. In my work so far, I have tried to identify new *zmm*-like genes involved in meiosis. To that end, I have carried out a genome-wide screen in the budding yeast *S. cerevisiae*. I have identified sporulation temperature sensitive *zmm*-like truncation mutants by using mini-transposon mediated random insertional mutagenesis approach. To confirm that the observed sporulation-temperature sensitive phenotype is caused by the transposon, a genetic outcross assay was carried out, and to determine the exact position of transposon integration in the yeast genome, direct genome sequencing was performed, followed by Southern hybridization. The defects that can potentially be detected by this genome wide screen approach are growth defects, defects in meiotic divisions and spore viability defect. Different classes of mutants have been identified, suggesting that insertional mutagenesis mediated genome wide screen is an appropriate genetic approach for identifying new genes involved in meiosis.

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CHAPTER I

INTRODUCTION

1.1. The meiotic Cell Cycle.

Meiosis is a specialized type of cell division by which sexually reproducing organisms produce gametes or sex cells leading to the formation of normal embryo. In males meiosis is called spermatogenesis as it leads to the formation of sperms and in females it is known as oogenesis leading to the formation of mature ova or eggs. In meiosis, one round of DNA replication is followed by two rounds of chromosome segregation producing four haploid daughter cells from a single diploid parental cell. In the budding yeast *Saccharomyces cerevisiae*, the end product of meiosis is four spores. These four spores are surrounded by a cell wall and are collectively known as a tetrad, or ascus.

Meiosis is broadly subdivided into two phases—meiosis I, and meiosis II. Meiosis I is also called reductional division since in this phase, a single diploid parent cell

produces two haploid daughter cells by segregation of homologous chromosomes. In meiosis II four haploid daughter cells are formed from two haploid parent cells, hence it is called equational division. In meiosis I the homologous chromosomes undergo pairwise juxtaposition, followed by formation of chiasmata. Chiasmata is a cytologically detectable point of association between non sister chromatids, which counter forces of the spindle fibers directed to opposite poles, thereby ensuring proper segregation of each pair of homolog. Defects in recombination can result in chromosome missegregation and aneuploid gametes. Moreover these defects can also lead to activation of a checkpoint pathway related to the DNA damage checkpoint, resulting in meiotic arrest. Chromosome missegregation during meiosis is the largest genetic cause of stillbirths, pregnancy loss, infertility and birth defects among humans (Hassold & Hunt, 2001; Cimini and Degrossi, 2005).

Meiosis I is subdivided into four different phases—prophase I, metaphase I, anaphase I, and telophase I. In prophase I, global chromosome structure is assembled and events on the DNA level including formation of DNA double strand breaks (DSBs) and processing them into cross over (CO) product on one hand, and assembly and disassembly of synaptonemal complex (discussed below) on the other, occur, resulting in chromosome condensation. During metaphase I, homologous chromosomes are physically connected via chiasmata and are aligned at the equatorial region of the cell. In anaphase I, homologs are pulled towards the opposite spindle poles. Finally, in telophase I, the chromosomes start to decondense followed by cytokinesis, which is the final step of cell division where the mother cell irreversibly partitions to produce two daughter cells. Meiosis I is followed by separation of sister chromatids during meiosis II.

During prophase I, a zipper-like proteinaceous structure forms between homologous chromosomes called the Synaptonemal Complex (SC) (Figure 1). Based on the assembly sites of the SC, prophase of meiosis I is subdivided into five sub-stages: leptotene, zygotene, pachytene, diplotene, and diakinesis. Close juxtaposition of homologs from zygotene to early pachytene at a gap of 100 nm depends on the SC, which disassembles at diplotene (reviewed in Hunter, 2006). The process of formation of SC is called synapsis. During the leptotene stage, the SC axial elements start polymerizing along the length of each homologous chromosome. In yeast, during the zygotene stage, Zip1, the coiled-coil structure assembles into the central region of the SC first as discrete foci, and later into lines. During pachytene stage, there is complete formation of SC (Sym et al., 1993). The SC starts to disassemble at the transition between late pachytene and diplotene (reviewed in Hunter, 2006). During diplotene, there is complete depolymerization of SC, and chiasma start to appear as the homologs separate. During diakinesis, the homologs completely separate from each other, appear as short and thick structures, and finally spindle fibers start to form in metaphase I.

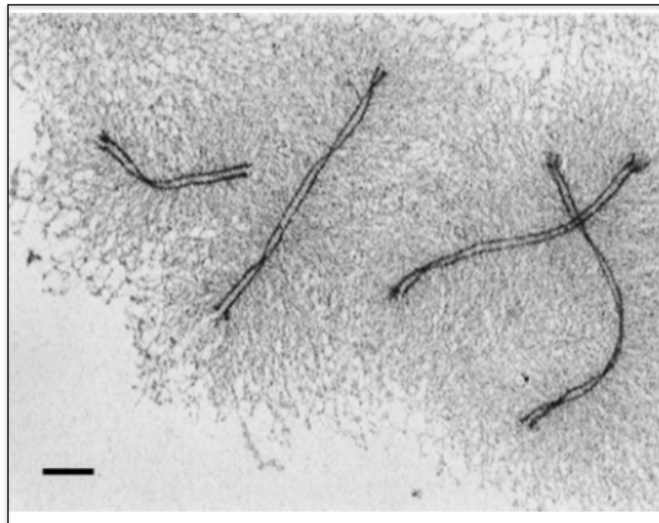


Figure 1. Synaptonemal complexes from the moth, *Hyalophora columbia*. Shown are four SCs from a single nucleus; the two on the right are overlapping. Chromosomes were surface spread, stained with silver nitrate, and examined in the electron microscope. Each SC consists of two parallel lateral elements (dark lines) surrounded by chromatin loops. Bar, 1 μ m. (Taken from Roeder, 1997).

1.2. ZMM proteins.

Many widely conserved proteins with meiosis specific roles have been discovered in the budding yeast *S.cerevisiae*. ZMM includes a group of 8 proteins that plays essential roles in meiosis via assembly/ disassembly of SC and processing of DSBs into CO products (Lynn, 2007) (Figure 2). ZMM proteins Zip1, Zip2, Zip3, Zip4, Msh4, Msh5, Mer3 and Spo16 co-localize on meiotic chromosomes, and each of the deletion mutants confers similar phenotype suggesting that this group of 8 proteins is functionally collaborative (Börner et al., 2004).

zmm mutants exhibit defect in SC formation and processing of DNA DSBs into CO products. There is a delay in SC formation and the DSBs are not repaired leading to the formation of noncrossover (NCO) products in these mutants. In wild type cells CO bound strand invasion events become stabilized generating single end invasion followed by religation of DSB ends to form double Holliday junctions that are finally resolved into CO. When strand invasion fails to stabilize, NCO products are formed in the absence of double Holliday junction via synthesis-dependent strand annealing pathway. *zmm* mutants also exhibit incubation temperature dependent sporulation defect that can be identified upon exposure of the cell patches of these mutants to UV light (discussed in the following section). Sporulation denotes that the cells have undergone meiosis and completed ascospore formation. Phenotypes vary significantly at different conditions

between wild type and mutant cells. In the wild type, both the meiotic divisions occur efficiently and similarly at 23°C and 33°C. Whereas *zmm* mutants confer similar defect in meiosis, and these defects differ significantly at low versus high temperatures. At 33°C, >85% of the cells are arrested at meiosis I. Whereas at 23°C >90% of the cells complete meiosis I after a substantial delay of 3 to 7 hours, followed by meiotic division II and spore formation (Nakagawa and Ogawa, 1999). The phenotypes conferred by *zmm* mutants at 23°C and 33°C emulate their meiotic recombination phenotypes, which imply a block in CO formation at 33°C, and also slow and efficient formation of both CO and NCO products at 23°C.

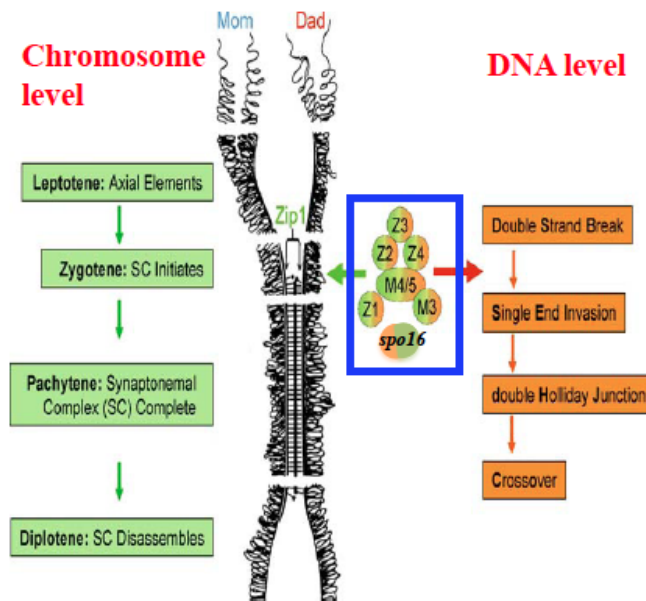


Figure 2. Functions of ZMM proteins in synaptonemal complex assembly and recombination. Left cytological stages of meiosis I prophase depicted. Right the intermediate stages of crossover formation are shown. ZMM proteins are Zip3 (Z3), Zip2 (Z2), Zip4/Spo22 (Z4), Msh4/Msh5 (M4/5), Mer3 (M3), Zip1 (Z1), Spo16. Localization of Zip1 as a transverse filament component of the synaptonemal complex central element is also indicated. (Modified from review by Lynn et al., 2007)

1.3. UV dependent phenotype of ZMM mutants.

The yeast spore wall is a thick four-layered structure. Dityrosine is a major component of the spore wall that provides resistance against digestive enzymes such as glucalase, as well as UV and are present at the outermost and last synthesized layer of the spore wall (Briza et al., 1986; Pammer et al., 1992). When exposed to UV light dityrosine emits fluorescence. Therefore, in case of ZMM mutants when cells are exposed to UV light, there is fluorescence at 23°C but not at 33°C as there is defect in meiosis at 33°C. Hence, this is an efficient genetic tool that I have utilized to identify new ZMM like mutants producing phenotype based on incubation temperatures. In Figure 3 *zmm* single mutants are incubated at different incubation temperatures (23°C, 28°C, 30°C and 33°C) on sporulation plates and sporulation efficiencies are measured based on their ability to emit fluorescence when exposed to UV light.

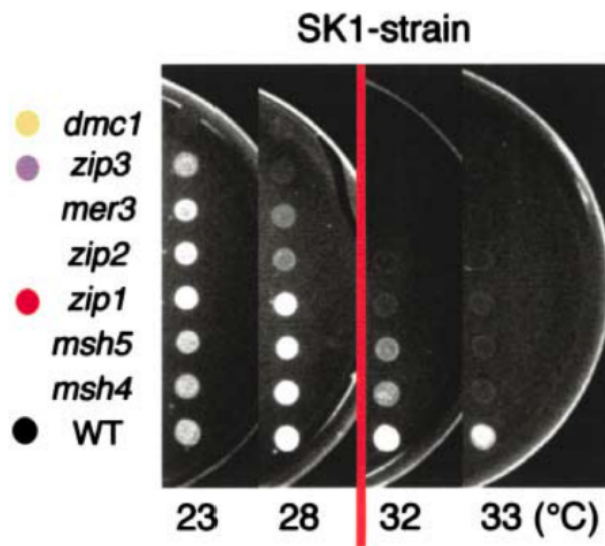


Figure 3. Temperature sensitive phenotype for sporulation of ZMM mutants. UV fluorescence of cell patches arising after incubation on solid sporulation medium for 4 days in SK1. Orange line indicates 30°C, the standard temperature for yeast studies. (Taken from Börner et al., 2004).

1.4. Identification of genes involved in meiosis by previous genetic screens.

Most of the genes in yeast known so far, having roles in cell division are identified by genetic screens. Different screen approaches performed to detect genes related to meiosis are discussed below.

1.4.1. Screen approaches used for identification of ZMM mutants:

In order to identify novel ZMM-like mutants, it is critical to envision how the known *zmm* genes were identified. Interestingly, most of the ZMM mutants were identified via genetic screens. In my following section, I have summarized the screen approaches used for identification of most of the known ZMM mutants.

Zip1, major component of the ZMM complex and the central protein of SC, was identified by a genetic screen for mutants exhibiting defect in spore production and poor spore viability. Recessive mutations in a diploid strain were generated in spores from self diploidizing (homothallic) HO strain. The *zip1* mutant was identified as a candidate exhibiting defect in sporulation and poor spore viability (Rockmill and Roeder, 1988; Sym et al., 1993).

Zip2, Zip3, and Zip4/ Spo22 were identified by expression analysis screens in which transposon insertion generated a *lacZ* fusion protein that was expressed in a meiosis-specific fashion (Burns et al., 1994). Zip2, a component of the ZMM complex, is

a meiosis-specific gene required for normal SC formation and pairing between homologous chromosomes during meiosis (Chua et al., 1998). Zip3, also a component of the ZMM complex, and/ or SUMO E3 ubiquitin ligase, is required for the formation of SC and localizes to the synapsis initiation sites during meiosis (Agarwal et al., 2000). Bioinformatic analyses have demonstrated that the N and C termini of *Zip3* share significant domainal homologies with *Rad18*, which is a DNA binding E3 ubiquitin ligase (Figure 15A,B, and C). *Zip4/ Spo22*, component of the ZMM complex is required for SC formation and completion of nuclear division during meiosis (Perry et al., 2005). The colocalization of Zip1 with Zip2 and Zip3 on meiotic chromosome during zygotene to pachytene is shown in figure 4 (Perry et al., 2005; Notredame et al., 2000).

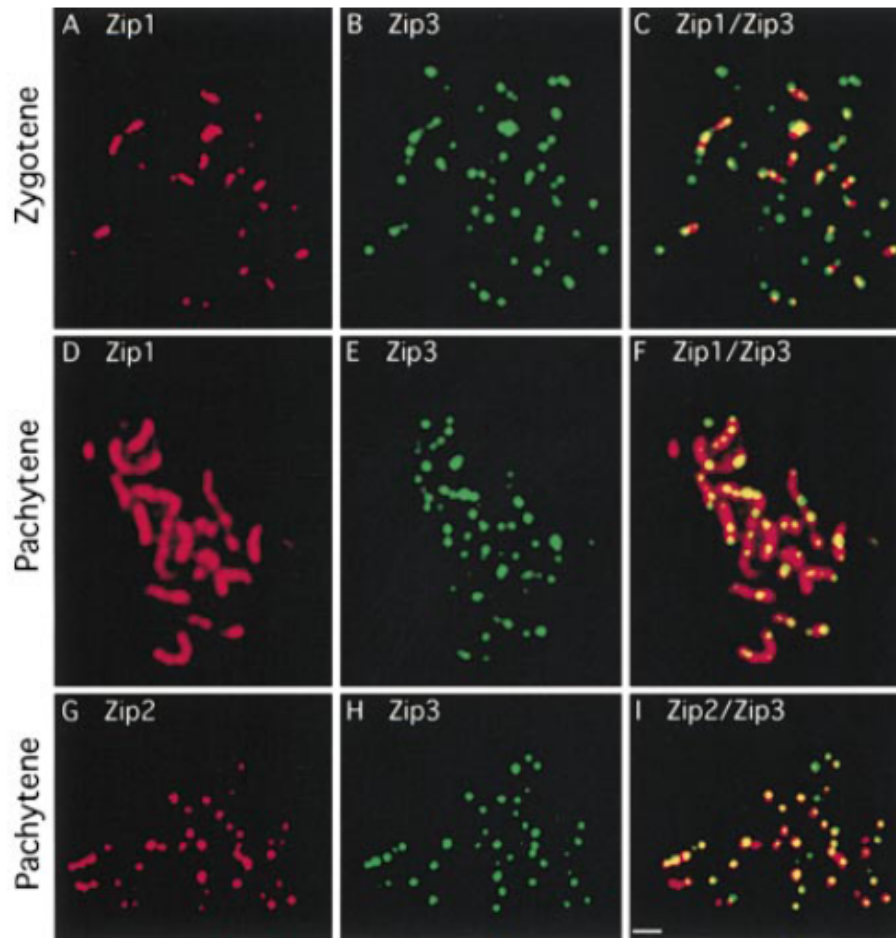


Figure 4. Zip3 colocalizes with Zip1 and Zip2. Spread nuclei from a wild-type strain producing Zip3-GFP (SA439) were stained with antibodies to Zip1 (A and D), Zip2 (G), and GFP (B, E, and H). Regions of overlap between Zip1 and Zip3 or between Zip2 and Zip3 appear yellow in merged images (C, F, and I). (A)–(C) show a zygotene nucleus; (D)–(I) show pachytene nuclei. Bar=1 μ m. (From Agarwal and Roeder, 2000)

Msh5, component of the ZMM complex, is a bacterial MutS homolog required for meiotic recombination by facilitating crossovers. Msh4-Msh5 heterodimer was identified by a genetic screen, which was used to detect mutants specifically defective in interchromosomal recombination. To study the function of the mutants in recombination, chromosome III of a diploid yeast strain carrying *spo13* Δ (required for maintaining sister

kinetochore cohesion during meiosis I) was mutagenized, and the segregation patterns and spore viabilities of the mutants were analyzed (Hollingsworth et al., 1995).

Mer3, a component of the ZMM complex, is a meiosis specific DNA helicase required for processing of DNA DSBs into CO products (Nakagawa and Ogawa, 1999). A multicopy suppressor screen was performed to identify a gene specifically required for cross over formation. The yeast strain carried *mre2N* cMER2. *Mre2* confers temperature sensitive spore formation but no CO formation at any temperature and is involved in early stages of meiotic recombination (Nakagawa and Ogawa, 1997).

1.4.2. Screen approaches used for identification of non ZMM mutants involved in meiosis:

In this section I briefly discuss various screen approaches used to identify non-zmm mutants involved in meiosis.

A Spo11 dependent screen was performed, in which a self-diplodizing homothallic strain carrying SPO11 plasmid was subjected to mutagenesis (McKee et al., 1997). The mutants defective in spore formation were identified based on UV fluorescence (Briza et al., 1990). This approach led to the identification of *Sae* (Sporulation in absence of *spo11*) genes: *Sae1*, *Sae2*, and *Sae3*. *Sae1* is a component of the cap-binding complex. Its binding partner is *Sto1* and is required for export of mRNA from nucleus to cytoplasm. *Sae2* is involved in mitotic and meiotic DNA DSB repair. *Sae3* is a meiosis specific gene required for DNA recombination in a Dmc1 dependent manner (McKee et al., 1997). *Dmc1* is required for reciprocal recombination, resolution of DSBs into CO intermediates, and formation of SC (Bishop et al., 1992).

Another screen approach was to carry out whole-genome screen in which a pool

of 4,745 homozygous deletion diploid mutants was used that covers 95% of all the non-essential open reading frame (ORF) of the yeast genome. Each deletion mutant contained two 20-bp sequences termed as molecular tags flanked by PCR priming sites. The hybridization of molecular tags to oligonucleotide array containing the tag complements was monitored for quantitatively determining relative abundance of a single deletion strain. Sporulation efficiency of each of the deletion mutant was monitored by comparing the hybridization intensity of its molecular tags before and after sporulation. This screen was successful in identifying 54 sporulation/ meiosis specific genes along with genes involved in other functional classes like autophagy, carbon utilization etc. (Shoemaker, D. D. et al., 1996; Deutschbauer et al., 2002)

To identify genes involved in meiotic cell cycle progression, a collection of yeast strains with individual gene deleted, was used. Homozygous diploid strain deleted for a particular gene was constructed and carried green fluorescent protein (GFP dots) on both copies of chromosome III. Sporulation efficiency and chromosome segregation pattern based on GFP dot was studied. (Toth et al., 2000; Marston et al., 2004)

1.4.3. Screen approaches used for identification of genes involved in both mitosis and meiosis:

There are genes that play significant roles in both mitosis and meiosis. I have discussed the major screens carried out to identify such genes in the following section.

Genes were identified by a screen, in which the host strain was carrying a *top3* mutation. *top3* null mutation resulted in drastic reduction in growth of mutants. Hence a study was carried out to isolate extragenic suppressors of slow growth phenotype in a *top3* background (Wallis et al., 1989). One of the genes identified by this approach is

Sgs1 (slow growth suppressor I), a DNA helicase, required for maintaining genome stability and is involved in both mitosis and meiosis (Gangloff et al, 1994).

An approach in which radiation sensitive mutants were exposed to the radiomimetic drug blastomycin (BM), and X-ray, was carried out to identify genes involved in both mitosis and meiosis. *rad51* was one of the identified mutants that conferred sensitivity to both BM, and X-ray by exhibiting abnormality in colony forming ability and initiating DNA degradation and DSB formation (Moore, 1978; Gangloff et al., 1994). *Rad51* is a gene required for homologous strand invasion during DNA repair by homologous recombination and repair of DNA damage, suggesting a role of mitotic repair events in meiosis.

I have carried out a transposon mediated genome-wide screen for identifying ZMM like novel mutants. Multifunctional mini transposons were utilized to create gene disruptions. During the course of this project, one previously known ZMM mutant and other novel mutants were identified. I confirmed their phenotypes by different approaches, and investigated their roles in meiosis or mitosis.

CHAPTER II

MATERIALS AND METHODS

2.1. Genetic Screen.

The genetic screen utilized a *ura3/ leu2* (yeast selection marker) auxotrophic, HO endonuclease positive, *ste7-1Δ* mutant SK1 strain of *Saccharomyces cerevisiae*. These strains are capable to switch mating type (HO) and self-dipodize at low temperature (23°C) but remain haploids at high temperature ($\leq 33^\circ\text{C}$) due to the mating deficiency. To generate conditional haploid cells for transformation, yeast cells were allowed to self diploidize at 23°C and sporulate at 30°C, and spores were dissected and grown into colonies at 34.5°C, the restrictive temperature for mating. Dissected spores were replica plated to confirm the yeast selection marker auxotrophy and made competent by incubation with Lithium acetate at 34.5°C (Gietz et al., 1991). These competent cells were transformed with yeast genomic library mutagenized with a Tn7 (7545bp) or the

Tn3 mini transposon (6654base pairs) plasmid library carrying URA3+ and LEU2+ yeast selection markers respectively, and tetracycline and amp bacterial selection markers respectively, a promoterless *lacZ* gene and terminal repeats of 38base pairs (bp) on either side for both the transposons. These transformed cells were selected on ura3 (for Tn7)/ leu2 (for Tn3) dropout (d/o) growth solid media plate and incubated at 34.5°C for 3 days and then shifted to 23°C for another 3 days. Plates routinely contained 50-200 transformants.

Transformants were grown to colony size and each colony was picked with a sterile toothpick and individually inoculated into a 96-wells microtiter plate. Each half of microtiter plate was also inoculated with known temperature sensitive and non-temperature sensitive control strains. Each well was filled with ura/ leu d/o liquid medium supplemented with 2% dextrose. Microtiter plates were allowed for shaking at 75 rpm at 23°C overnight. The next day yeast colonies were resuspended with the help of multipipetter and stamped on sporulation, URA/ LEU drop out and regular growth (YPD) plates with the help of a stamping tool with 48 screws. The URA/ LEU d/o and YPD and one of the two sporulation plates were incubated at 23°C and the other sporulation plate was incubated at 33.5°C. One of the major constituents of sporulation plate was 0.06% raffinose. Thus each microtiter plate produced 2 sets of 4 plates. After 5 days sporulation plates were removed from the incubator and UV light was radiated on the sporulation plates. The spore wall has dityrosine that fluoresce under UV light (Pammer et al., 1992). If the cells do not sporulate they will not fluoresce. With the aid of camera apparatus images of the sporulation plates were taken. Temperature sensitive cell patches were selected and classified as primary candidates. They were streaked for single colonies on

fresh URA/ LEU d/o solid media plates to isolate clones and incubated at 30°C for 3 days in order to grow to colony size. Colonies were then inoculated in triplicate in the microtiter plate and processed as described above.

Primary candidates that also reproduced temperature sensitivity in triplicate colonies under UV light were designated as validated candidates. These candidates had undergone tetrad dissection and their spore viability was recorded. For candidates with viabilities below 70%, the transposon marked mutation was outcrossed to a haploid wild-type strain and tested for cosegregation of URA/ LEU and temperature sensitivity phenotype. Ts positive outcrossed candidates were grown to colony size and genomic DNA was isolated from them. The genomic DNA from the corresponding original transformant of the same candidate was also isolated. The DNA samples from both the outcrossed candidate and original transformant were analyzed by direct genome sequencing using anti *lacZ* primer.

2.2.Tetrad Dissection.

Tetrad dissection was performed for the candidates that were ts in the retest. These candidates were picked from the retest URA/ LEU drop out solid media plate and sporulated on the sporulation plate at 30°C for 4 to 5 days, their cell walls were digested with zymolyase, dissected on YPD plate and incubated at 30°C for 2 to 3 days. Each plate had 20 tetrads dissected with a total of 80 spores. Quantification of the spore viability was performed by counting the number of viable spores, and dividing that by the total number of spores (=80 per plate). This was recorded as the spore viability percentage. Sizes of spore colonies were also recorded for tracking of growth defect.

2.3. Genetic Outcross Assay.

The outcross assay was performed for candidates that retested as temperature sensitive in triplicate and exhibited spore viabilities of $\geq 70\%$. Tetrads from these candidates were dissected and incubated at 34.5°C for 3 days.

The principle is same for both transposon libraries. Here I have explained the assay for Tn3 transposon. This assay utilized a haploid SK1 yeast strain that is *ura3* auxotrophic with 'a' mating type. This strain was streaked for single colonies on a ura drop out solid media and incubated at 30°C for 3 days. 4 single colonies of this strain were selected and mated with 4 spore colonies from candidate strain, maintained as a haploid strain by growing at non-permissive temperature (preferably from the same tetrad) of the selected candidate in a solid YPD media at 30°C for 6 hours. Single colonies and spore colonies contained similar number of cells as indicated by the sizes of the single colonies of the haploid strain that were comparable with that of the spore clones. The mating mix was replica plated to double dropout solid medium followed by streaking for single colonies also on a double drop out solid medium and incubated at 30°C for 4 days in order to grow to yeast colony size. These colonies were then transferred on a sporulation plate and incubated at 30°C for 3 to 4 days. Two plates of tetrads were dissected and incubated at 23°C for 18 hours and then shifted to 34.5°C for 3 days. Each tetrad plate is expected to carry 80 spore colonies among which 40 spores are HO endonuclease positive (diploids) and remaining 40 spores are HO endonuclease negative (haploids). Also, among the 80 spore colonies, half are LEU2 positive. As LEU2 and URA3 markers segregate independently, out of the 80 spore colonies, 40 are also URA3 positive. The tetrad plates were then replica plated on Leu d/o; Ura d/o; minimal media plate for 'a' mating type; minimal media plate for ' α ' mating type; YPD plate.

These plates were incubated at 30°C for 2 days. The colonies were categorized based on their markers and only LEU2 positive colonies carrying transposon were selected and inoculated on a 96 well microtiter plate filled with LEU d/o liquid media along with ts positive and non temperature sensitive yeast strains as controls. The microtiter plate was allowed to shake for 16 hours. The cultures in the plate were stamped on leu d/o; sporulation plate at 23°C; sporulation plate at 33.5°C (the maximum temperature at which wild type yeast sporulates); and YPD plate. Only URA positive colonies were also selected and inoculated and processed in a similar fashion. 2 to 4 of these LEU positive colonies were randomly selected and half a plate tetrads were dissected to check if the viability pattern of the original mutant candidate was reproduced after the outcross.

2.4. Direct genome sequencing.

The original transformant of the validated candidate and the outcrossed candidate from the same mutant were streaked on LEU d/o solid media and incubated for 3 days at 30°C. The colonies were then grown in 5ml liquid growth media overnight at 30°C in a test tube roller. The next day they were grown in 25ml fresh growth liquid media for additional 5 hours in a shaker at 250 rpm. Genomic DNA was isolated utilizing QIAGEN 100/G tips (100µg yield) The dry DNA samples were sent for sequencing using anti *lac Z* primer (28 nt) to the Michigan Sequencing facility.

Sequencing was done using the following protocol:

Horecka Protocol:

1. Ethanol (EtOH) precipitation of 10ug of total genomic DNA into a 1.5ml tube, washed with 70% EtOH, and airdried.

2. 4ul 3.5pmol/ul of transposon-specific anti *lacZ* primer, and 16ul BigDye terminator ready reaction mix were added.
3. Denaturation=95°C, time=5min; unidirectional 90 cycles of (extension=95°C, time=25sec; annealing=60°C, time=2 min); hold=4°C
4. Column was spun and run on ABI sequencer.

2.5. Southern hybridization.

The direct genome sequencing provided information about the genomic region adjacent to the *lacZ* carrying portion of transposon. Based on the information, genomic DNA from these candidates was isolated and digested in parallel with 4 to 5 appropriate restriction enzymes along with DNA sample isolated from the parental WT strain at 60 Volts. The digested DNA samples were run on an agarose gel for 16 hours, the DNA samples from the gel were allowed to get transferred to a charged nylon membrane via capillary action for 16 hours (Ausbel et al., 2002), the Southern membrane was hybridized with a P³² labeled radioactive probe specific for the identified gene for 16 hours and exposed to a imaging plate for at least 5 hours. The probe was made by isolating DNA from the WT strain, followed by PCR amplification using primers specific for the gene identified via sequencing and images were scanned in Typhoon imager.

2.6. Meiotic timecourse.

Yeast colonies were grown to saturation in YPD liquid culture in test tubes at 30°C for 26 hours on a roller drum. YPD overnight cultures diluted at 1:100-1:150 in YPA liquid medium in a 2L flask at 30°C for 13 hours. The optical densities (O.D) of the cultures were measured using spectrometer and cultures with an OD between 1.2 to 1.6

were centrifuged and resuspended in liquid SPM medium in 2L flasks. Cultures were placed in shaker at 290 rpm for 2 hours, and then half of the culture from each flask was taken and put in a 1L flask. For each strain two flasks were prepared, one incubated at 23°C and the other at 33°C. Samples were collected at 0, 2.5, 3, 5, 7, 9, 11, 24 hours timepoints and saved for further analysis. The next day these samples were examined under microscope using DAPI stain to monitor meiotic progression. These samples were also digested by zymolyase to determine their spore viabilities via tetrad dissection. The collected samples were processed for immunocytology by spreading the cultures on slide and staining with Zip1-specific antibody. Immunocytological samples were analyzed using Deltavision microscopy.

2.7. Dominant Negative Assay.

To determine whether phenotype conferred by a heterozygous mutant is similar or different from its corresponding homozygous mutant, dominant negative assay was performed. I carried out this assay for one of the mutants named as Mutant 5. In order to obtain a heterozygous mutant strain, I performed genetic outcross assay and replica plated the tetrad-dissected plates on minimal media (MM) plates to determine their mating types ('a' or 'α') by transferring the lawns of mating type 'a' and 'α' on MM plates. Replica plating of tetrad-dissected plates on respective nutritional marker drop out plates was also performed to detect the marker segregation. A tetrad is considered to have properly segregated if it shows— 2:0 segregation of markers Leu2 and Ura3; 4:0 segregation on YPD growth plate; and 2:0 segregation of diploid spores and 2:0 segregation of a/ α. From these tetrads, spore colonies carrying Leu2⁺ (transposon marker) in 'a' mating were mated with leu2⁻ (deficient of transposon) in 'α' mating type.

The mating mix was replica plated to double dropout (leu2-/ura3-) solid medium followed by streaking for single colonies also on a double drop out solid medium and incubated at 30°C for 4 days in order to grow to yeast colony size. These colonies were then transferred on a sporulation plate and subjected to UV sporulation test. According to direct genomic sequence, the gene disrupted in Mutant 5 due to transposon integration is *Mam1*. I constructed a knock out (KO) strain of *mam1* containing KanMX by performing PCR and confirmed the deletion by Southern hybridization for one mating type and tetrad dissection for the other. This KO strain was then subjected to UV sporulation test. The phenotype conferred by these mutants was also compared with homozygous diploid Mutant5-Tn3 mutant. The phenotype for the strains were compared by performing UV sporulation test.

2.8. Strain construction.

Yeast strain is streaked for single colonies on solid medium and another desired yeast strain is also streaked for singles, mated and sporulated by transferring into a solid SPM plate. The two mating partners of opposite mating types are streaked for strain construction. The sporulated patch is dissected for tetrads following growth into spore colonies; replica plated to different selection plates along with the two mating types plates. The desired spore colonies were then scored and genomic DNA from these colonies was isolated and digested with appropriate restriction enzymes. DNA samples were then separated electrophoretically on agarose gel, blotted on a nylon membrane and hybridized with P³² labeled probe to detect the right sized banding pattern and the result is compared with that of the wild type. If this patter matches with the expected result, the colonies were frozen at -80°C in 25% glycerol.

2.9. Topo TA cloning.

Topo cloning was performed according to Invitrogen's TOPO TA cloning protocol using TOPO 2.1 vector.

2.10. *E. coli* transformation.

Transformations were carried out on NEB α high efficiency competent *E. coli* according to NEB's 30-second heat shock protocol.

2.11. *E. coli* plasmid DNA extraction.

Plasmid extractions were carried out according to standardized alkaline protocol (Ausubel et al., 2002)

2.12. Yeast genomic DNA extraction (mitotic).

S. cerevisiae mitotic DNA extractions were performed based on standardized protocol (Ausubel et al., 2002)

2.13. Competent yeast.

Competent yeast cells were prepared based on the Lithium acetate method (Gietz et al., 1991)

2.14. Yeast transformation.

Yeast transformations were performed according the standardized lithium acetate protocol (Ausubel et al., 2002)

2.15. Optimization of rich sporulation media.

Sporulation of the genetic screen strain was carried out as an initial test on rich sporulation media (SPMR) Tris base pH 8.0, SPMR tris pH 7.0, and SPMR 2% raffinose with Poland Springs water producing the best combinations of growth and sporulation. Percentage sporulation was estimated under a light microscope by counting the number of two or four spores formed by transferring cells on each type of SPMR plate. By studying the level of fluorescence upon UV light exposure, and counting the number of 2/3/4 spores formed under light microscope it was found that the greatest sporulation occurred on SPMR containing 2% raffinose with Poland Springs water. Lesser sporulation occurred on all other plates tested. The recipe for preparing 1L of 2% raffinose SPMR plate with Poland Springs water is: 0.2gm leu d/o / ura d/o powder; 20g Granulated agar; 20gm KAcetate; 2.2gm Bacto yeast; 2gm raffinose; 950 mL Poland Springs H₂O; Autoclave; 30mL of 2% Raffinose.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Temperature sensitive phenotype of ZMM mutants.

In the budding yeast *Saccharomyces cerevisiae*, the eight functionally collaborating but evolutionarily diverse proteins Zip1, Zip2, Zip3, Zip4, Mer3, Msh4, Msh5 and Spo16 (ZMM) provide a link between recombination and SC assembly (reviewed in Lynn et al., 2007; Shinohara et al., 2008). These proteins co-localize on meiotic chromosomes in yeast, and ZMM mutants confer similar defects in recombination, SC formation and meiotic progression. Importantly *zmm* deletion mutants confer similar temperature dependent conditional phenotypes for sporulation suggesting that they are functionally collaborative (Börner et al., 2004).

Meiotic progression in *zmm* deletion mutants is modulated by temperature. At 33°C, most cells are arrested before meiosis I and do not form spores whereas at 23°C,

most cells complete meiosis I after a delay for 3 to 7 hours but eventually accomplish meiosis II and form spores. The yeast ascospore wall is a four-layered structure with the outermost layer containing sporulation specific component dityrosine that is essential for the resistance of the spores to environmental conditions. Dityrosine emits fluorescence when exposed to UV light. Hence, this UV dependent fluorescence was utilized to detect sporulation efficiency of yeast strains, and an efficient sporulation indicates accomplishment of true meiosis and formation of ascospores.

Here, I have established a genome-wide screen in the budding yeast *S. cerevisiae* for identification of new genes with roles in meiosis. I have identified ZMM-like mutants exhibiting defects in meiosis.

3.2. Establishment of the genome wide screen.

3.2.1. Transposon library and mutagenesis:

In the budding yeast *S. cerevisiae*, large-scale shuttle mutagenesis was employed to generate several insertional libraries. Each insertional library was constructed from a plasmid-based library of yeast genomic DNA mutagenized *in vivo* in *E. coli* or *in vitro*, using modified forms of mini-transposons, Tn3 or Tn7.

E.coli derived Tn3 or Tn7 mini-transposons randomly integrate in the genome without significant target-site sequence preference causing gene truncations and integrations in intergenic regions (Seifert et al. 1986). The transposon also demonstrates transposition-immunity wherein DNA molecules containing at least one transposon terminus are immune from further insertions, hence ruling out the possibility of carrying more than one transposon in a single fragment of yeast genome in the plasmid based

library. Transposition immunity is *cis* acting, in which the other plasmids in the same cell remain unaffected, thereby providing direction for transposition in a given target (Kumar et al., 2004). Using insertional mutagenesis, a genome wide screen was carried to identify novel, *zmm*-like temperature sensitive mutants. It is a relatively easy functional assay that can detect on a genome wide scale *zmm*-like genes in contrast to the screens that depend on expression analysis. Genes expressed at low levels during meiosis besides genes expressing at substantial levels can also be detected by using this approach.

Schematic representations of Tn7 and Tn3 mini-transposons are shown in Figures 5A and 5B respectively. It is a multifunctional transposon that could be used to derive gene disruption, reporter gene fusion, and epitope-tagged alleles (Ross-Macdonald et al., 1995; 1999). In my screen, transposon integration can result in gene disruption, in addition to transposition in the intergenic region.

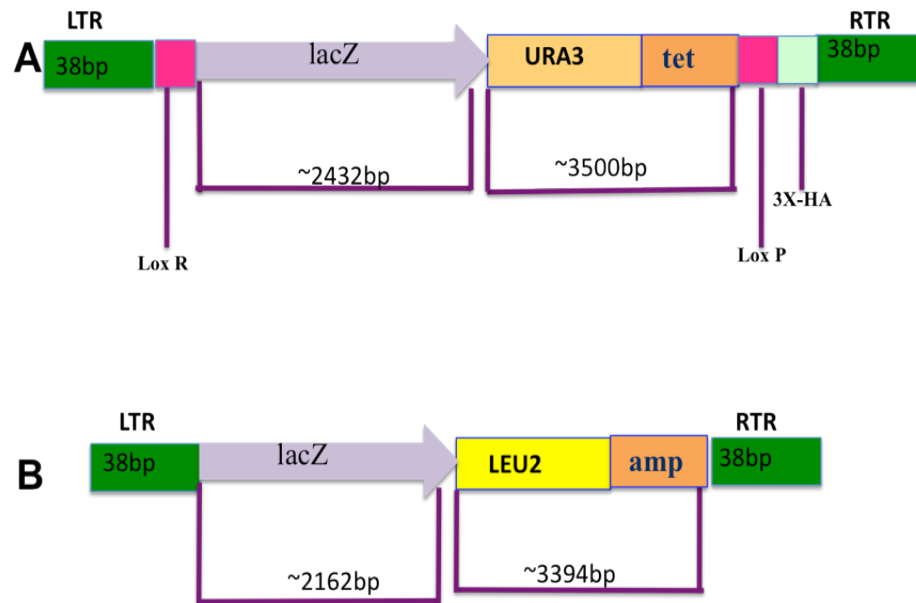


Figure 5. Schematic representations of mini-transposons. Tn7 mini-transposon (A);

and Tn3 mini-transposon **(B)**

3.2.2. Host strain and transformation:

To obtain a homozygous diploid mutant, I utilized an HO endonuclease positive; *ste7-1* SK1 yeast strain as host for mutagenesis and screen. This is a self-diplodizing (homothallic) strain that can switch its mating types between ‘a’ and ‘ α ’. The *ste7-1* mutation allows the strain to undergo self-diplodization by mating at temperatures below 23°C but unable to do so at 33°C (Chaleff and Tatchell, 1985; McKee and Kleckner, 1997). Hence, the strain becomes homozygous diploid at 23°C, but remains a haploid at >34.5°C (Hartwell 1980; Orr-Weaver et al. 1983; Boeke et al. 1984). (strain number SBY 1 for Screen I and strain number SBY 65 for Screen II)

To make haploid competent cells, haploid spore clones from the host strain were isolated and incubated at 34.5°C from start to finish of the competent cell preparation. This was important because, at temperatures below 34.5°C, the haploid colonies could form diploids by either self-diplodizing or mating with adjacent lawn. Diploids would give rise to heterozygous rather than homozygous mutants. The haploid cells were transformed with plasmid based transposon mutagenized yeast genomic library wherein the plasmid was digested with NotI restriction enzyme (8-bp cutter). The yeast genome contains only three NotI sequences thus preventing cleavage of the library insert. To obtain haploid transposon integrated transformants, plasmid based transposon mutagenized yeast genomic DNA was transformed in a conditional host strain and incubated at 34.5°C for 3 days. A temperature non-permissive for mating had to be maintained to avoid mating with surrounding untransformed cells. Next, to obtain

homozygous diploid transposon integrated mutant, the transformation colonies were shifted to 23°C in order to go through self-diplodization. A schematic representation of the experimental design is illustrated in Figure 6.

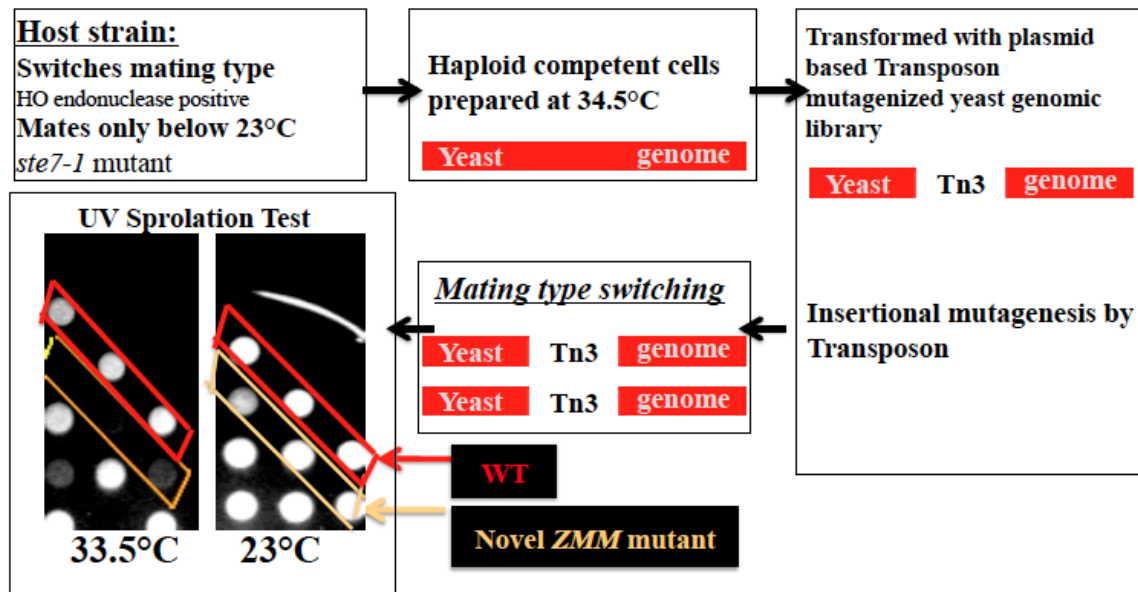


Figure 6. Experimental design illustration. Schematic representation of screen strategy using Tn3 transposon for the identification of meiotically involved novel mutants.

3.2.3. Control of homozygosity:

There are two ways how a host strain could become a heterozygous instead of a homozygous mutant— (i) diploid prior to transformation, or (ii) mating with untransformed cells following transformation. To examine at what frequency a host strain develops into a homozygous diploid, it was made competent at 34.5°C, and transformed with a LEU2 marked control gene (*zip1Δ::LEU2*) (see below). Tetrads from 5 independent transformants were dissected and incubated, followed by replica plating on marker plate specific for the transformed gene. It was demonstrated that tetrads from 4

out of 5 transformants exhibited homozygosity for the specific marker, thus supporting that more than 80% of the transformants were homozygous diploids. As 1 out of 5 transformants was heterozygous, there is a possibility of 20% of the transformants obtained from the screen, to be heterozygous.

3.2.4. Positive controls:

To identify ZMM-like temperature sensitive mutants through genome-wide screen, I used two control strains— one referred to as wild type-WT to a transposon integrated conditional haploid SK1 strain without apparent phenotype (strain number SBY 251 used for Screen I with transposon Tn7 and strain number SBY 252 used for Screen II with transposon Tn3), whereas the other was an already known, temperature sensitive SK1 strain carrying *zip2Δ::URA3* (strain number SBY 17 used for Screen I) or *zip1Δ::LEU2* (strain number SBY 41 used for Screen II). To confirm the non-temperature sensitive sporulation phenotype conferred by the WT strain, UV sporulation test was performed. As expected, the strain did not exhibit temperature sensitive sporulation phenotype. To assay the viability of the spores for the WT strain, I dissected 100 tetrads in which all of them exhibited 4:0 viable spore colonies. I also assayed the spore viability and marker homozygosity for *zip2Δ::URA3* and *zip1Δ::LEU2* strains by dissecting 100 tetrads for each mutant. Both strains demonstrated 100% homozygosity for the respective markers as indicated by tetrad dissection followed by replica plating on ura (for Screen I) or leu (for Screen II) dropout plates, and exhibited a reduced viability pattern. For *zip2Δ*, I observed 50% spore viability compared to previously reported 53% spore viability, and for *zip1Δ*, I observed 45% spore viability compared to previously reported 40% spore viability (Sym et al., 1994; Chua and Roeder et al., 1998)

3.2.5. Transposon library coverage:

The plasmid based transposon mutagenized yeast genomic library is divided into multiple pools, with each pool containing five equivalents of yeast genome, hence covering 95% of the entire genome for Tn7. The Tn7 insertion library is estimated to encompass >300,000 independent insertions of 50 genome equivalents of DNA. ~30,000 mutagenized transformants should be screened to ensure the total coverage of the genome, therefore, we expect the transposon library to provide full coverage. For Tn3, screening of 150,000 independent insertions would produce 10,174 insertion alleles, which would cover 45% of open reading frames (ORFs) exhibiting at least one transposon insertion (Kumar et al., 2004).

3.2.6. Mutant identification:

To identify ZMM-like mutants, individual transformants were inoculated, transferred to sporulation medium and subjected to UV fluorescence sporulation test. Candidates exhibiting temperature sensitive spore formation were referred to as primary mutant candidates (Figure 7A). To confirm the observed phenotype, temperature sensitive sporulation phenotype of the primary candidates were examined in triplicate. As ZMM mutants exhibit chromosome missegregation and reduced viability, for mutants that reproducibly were temperature sensitive, 20 tetrads per mutant candidate were dissected to monitor the viability of the spores. Growth of these candidates was also examined by transferring colonies to growth plates and incubating at high (37°C) and low (23°C) temperatures (Figure 7B) (Börner et al., 2004).

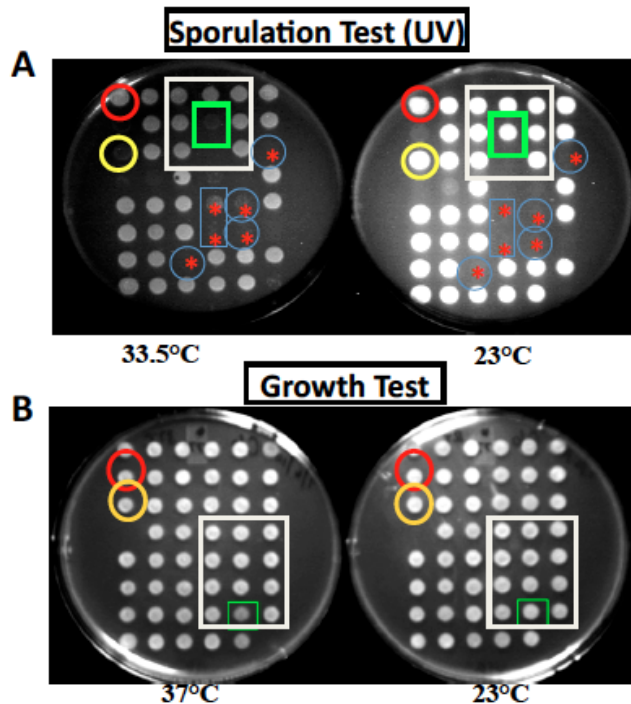


Figure 7. Primary identification of mutants. Red and yellow circles indicate WT and *zip1Δ* strains respectively, green and grey boxes illustrate a newly identified temperature sensitive mutant candidate, and phenotypes of neighboring cell patches respectively. Red asterisks indicate cell patches lacking mitochondria. **(A)** Identification of temperature sensitive mutants defective in meiosis. Individual transformants are inoculated and transferred on sporulation plates for 4 days followed by imaging under UV light. **(B)** Primary identification of temperature sensitive mutants defective in mitosis. Cell cultures are processed the same way as described in (A) but colonies are transferred on leucine drop out growth plates. The plates are incubated at 37°C and 23°C for 24 to 36 hours, respectively.

3.3. Validation of mutant phenotype.

To verify the phenotype conferred by the identified mutant candidates a genetic outcross was performed, and to determine the underlying genotype direct genome sequencing, PCR and Southern hybridization were performed.

3.3.1. Genetic outcross assay:

Transformation is a mutagenic process. Accordingly, an unrelated mutation could be responsible for the phenotype of meiotic temperature sensitiveness. To validate that integration of transposon into the genome was responsible for the phenotype, a genetic outcross was performed (Figure 8A). In this assay, a mutant candidate carrying the transposon was mated with a wild type haploid yeast strain lacking the transposon-specific marker. Following selection of diploids carrying the transposon marker as well as a marker unique to the wild type strain, and sporulation of individual diploids, 40 tetrads were dissected and all colonies carrying the marked transposon were subjected to temperature sensitivity sporulation test. The phenotype would be accepted as caused by the transposon if all transposon marker specific colonies exhibited temperature sensitive phenotype for sporulation (Figure 8B).

To confirm that the colonies that were not fluorescing at either temperature were true haploids and did not demonstrate any growth defect, they were also transferred on corresponding growth plates (Figure 8C). Furthermore, to strengthen the conclusion, temperature sensitive sporulation test was also performed for the colonies devoid of the transposon marker and all of them must not exhibit the temperature sensitive phenotype for sporulation.

To examine whether the transposon conferred all aspects of the phenotype after it had been outcrossed, spore viabilities of outcrossed candidates were also determined and compared to the original transformants. A minimum of 20 tetrads was dissected from each outcrossed candidate. I confirmed the validity of this assay by outcrossing three known temperature sensitive mutants— (i) *zip1Δ* in which 80 tetrads were dissected and 53 out of 54 transposon marker specific colonies exhibited temperature sensitivity; (ii)

spo22Δ where 40 tetrads were dissected and all the 27 transposon marker specific colonies demonstrated temperature sensitivity; (iii) *pre9Δ* in which 40 tetrads were dissected and 26 out of 27 transposon marker specific colonies conferred temperature sensitivity (non-temperature sensitive colonies were considered as likely contaminations by other strains).

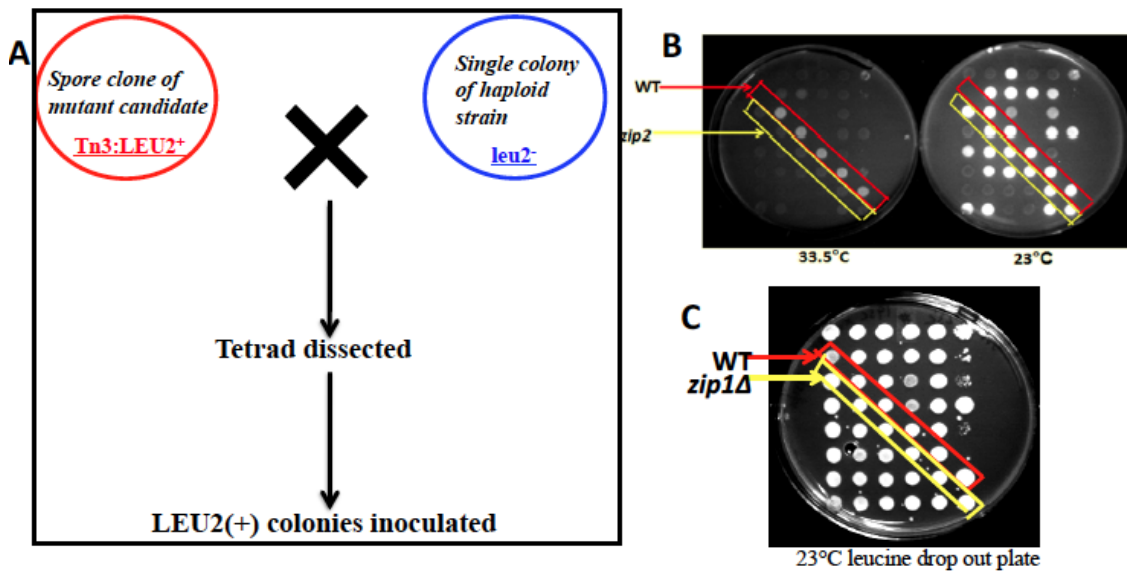


Figure 8. Genetic outcross assay. (A) Simplified illustration of genetic outcross assay. Detailed mechanism described in Materials and method. (B) Identification of temperature sensitive mutants in which LEU2(+) colonies are inoculated and transferred on sporulation plates for 4 days at 33.5°C and 23°C. (C) Corresponding growth plate incubated at 23°C for 24 to 36 hours.

3.3.2. Direct Genome Sequencing:

To determine the position of integration of the transposon in the yeast genome, direct genome sequencing was performed based on unidirectional 90 cycles of terminator sequencing mix with one primer. The primer used for sequencing was a 20 nt anti *lacZ*

sequence complementary to the *lacZ* sequence of the transposon proximal to the left terminal repeat. The sequence read is expected to encompass sequence of the remaining *lacZ* gene, and the sequence of the left terminal repeat of transposon.

3.3.3. PCR & Southern hybridization:

An independent approach to validate the integration site of transposon in the mutants was to carry out PCR and/ or Southern hybridization of the sequenced mutant candidates. For performing PCR, different combinations of primers were utilized encompassing the start/ stop codons or the flanking upstream/ downstream regions of the disrupted gene. For carrying out Southern hybridization, a probe specific for the truncated gene was utilized and the genomic DNA extracted from WT and mutant candidates was digested with appropriate restriction enzymes to distinguish wild type and expected mutant bands on an agarose gel transferred to charged nylon membrane.

3.4. Processes that can be potentially identified by the screen.

The genome wide screen is a functional assay that can monitor different processes including— (a) mitotic growth, (b) meiosis, and (c) spore viability. To examine defects conferred by the identified mutants, different approaches were employed.

3.4.1. Mitosis:

To investigate possible growth defects conferred by the identified mutants, their growth efficiencies were monitored on transposon marker specific drop out plates by incubating them at high (37°C) and low (23°C) temperatures for 24 to 36 hours. To confirm the growth defect, sizes of the spore colonies obtained from genetic outcross

assay were also monitored. I have identified two mutants named as Mutant 5, and 8 that impressively displayed mitotic defects in the outcross assay by cosegregation of transposon marker and spore colony size.

3.4.2. Meiosis:

To examine if the mutants conferred abnormal meiotic progression, synchronized meiotic cultures were sporulated at both high (33°C) and low (23°C) temperatures. Number of cells with 2, 3, or 4 nuclei manifesting completion of meiosis I and/ or II were determined at regular intervals by using chromatin specific DAPI stain. Mutants demonstrating defect in meiotic nuclear division are Mutants 4, 5, and 8 derived from Screen II, and SB 84 derived from screen I.

3.4.3. Spore viability:

To analyze spore viabilities, 20 tetrads were dissected from each mutant and incubated at 30°C for 3 days along with tetrads dissected from WT yeast strain. The mutants exhibiting spore viability defects are Mutants 4, 5, 8, and 9 derived from Screen II, and SB 84 derived from Screen I.

3.5. Results obtained from the screens.

To identify ZMM like mutants, I carried out two genome wide screens (Screen I and II) where the host strains were integrated with two different transposon mutagenized yeast genomic libraries. For Screen I, Tn7 mini-transposon (7Kb) marked with yeast selection marker, Uracil3 (URA3+) and for Screen II, Tn3 mini-transposon (6.5Kb) marked with yeast selection marker, Leucine2 (LEU2+) were used for generating random

insertional mutagenesis. For Screen I, the host strain also had centromere of chromosome V marked with GFP (CEN V-GFP) for detecting chromosome segregation defect, although this property of the host strain was not used for identifying mutants. For Screen I, I screened for 22,000 transformants, and 810 of them conferred temperature sensitive phenotype, and among them 130 candidates retested the phenotype. Tetrads from 51 candidates exhibited <70% 4:0 viable spores, and 2 of them outcrossed to produce temperature sensitive phenotype. However, it is important to mention here that as discussed earlier, since there is a probability of 20% of the transformants to be heterozygous, 4,400 of the 22,000 transformants could also be heterozygous.

3.5.1. Screen I result overview:

Number of transformants	22,000
Primary candidates	810
Retested candidates	130
Poor spore viability candidates (<70% with 4:0 viable spores)	51
Number of outcrossed candidates exhibiting meiotic temperature sensitivity	2

However, Screen I was considered as suboptimal. Most of the candidates obtained from this screen exhibiting temperature sensitive sporulation phenotype lost the phenotype in the outcross assay. Reasons for the phenotype loss are unknown. Importantly, however for 2 out of 51 poor spore viability candidates identified, the marker of transposon was linked to temperature sensitive phenotype. For Screen II, I screened for 5,000 transformants (1000 of which could be potentially heterozygous), and 123 of them conferred temperature sensitive phenotype, out of which 24 candidates retested the phenotype. There were 12 candidates with tetrads exhibiting <60% viability, and 9 of them outcrossed to produce temperature sensitive phenotype.

3.5.2. Screen II result overview:

Number of transformants	5,000
Primary candidates	123
Retested candidates	24
Poor spore viability candidates (<60% overall spore viability)	12
Number of outcrossed candidates exhibiting meiotic temperature sensitivity	9

3.5.3. Candidates exhibiting co-segregation of meiotic temperature sensitive phenotype with transposon marker:

To detect whether the phenotype conferred by the identified mutants was caused by transposon, genetic outcross assay was performed for the poor spore viability candidates derived from Screen I and II.

For Screen I, unlike ZMM mutants, most of the retested candidates exhibited a high overall spore viability of 80-90%. I therefore categorized candidates with <70% of spores showing 4:0 viability as poor spore viability candidates (Figure 9A, B). These candidates were then subjected to genetic outcross assay. For 2 out of the 51 poor spore viability candidates the phenotype co segregated with the marker for transposon. These mutants were referred to as SB 2 and SB 84. To confirm this result further, I also examined the temperature sensitive phenotype of self-diplodized spore colonies that were deficient of the marker for transposon for both SB 2 and SB 84 and found them to be not temperature sensitive. These results suggest that the phenotype observed for SB 2 and SB 84 is authentic and linked to the marker for transposon.

However, for Screen II, many of the identified candidates exhibited an overall poor spore viability of less than 60%, which is corresponding with the ZMM mutants. Therefore, for this screen, the threshold value established for classifying the candidates as poor spore viability mutants was 60% (Figure 9C). As with Screen I, these candidates were subjected to genetic outcross assay. I identified 12 poor spore viable candidates and for 9 of them the temperature sensitive phenotype cosegregated with the transposon marker. To further confirm linkage of the phenotype with the transposon, I examined the

temperature sensitive phenotype of the colonies that were deficient of the marker for transposon for these 9 candidates, and found them not to be temperature sensitive, hence reconfirming the linkage between phenotype and transposon marker.

In cases of mutants where a mitotic effect at high temperature blocked growth, incubation was performed at the highest attainable temperature. As the tetrads for Mutant 5 were not viable at 34.5°C and as the sizes of the spore colonies for this mutant were too small even at 33°C for carrying out genetic outcross assay, the tetrad plates were incubated at 23°C for 2 days and then shifted to 33°C instead of 34.5°C for another 2 days to get normal sized colonies. For another candidate referred to as Mutant 8, as the tetrads were not viable at 34.5°C, the tetrad-dissected plates were incubated at 23°C for 3 days and then shifted to 33°C for another 2 days for performing genetic outcross assay.

The spore viability patterns of the candidates are shown in Figure 9A,B and Table I for Screen I and Figure 9C and Table II for Screen II.

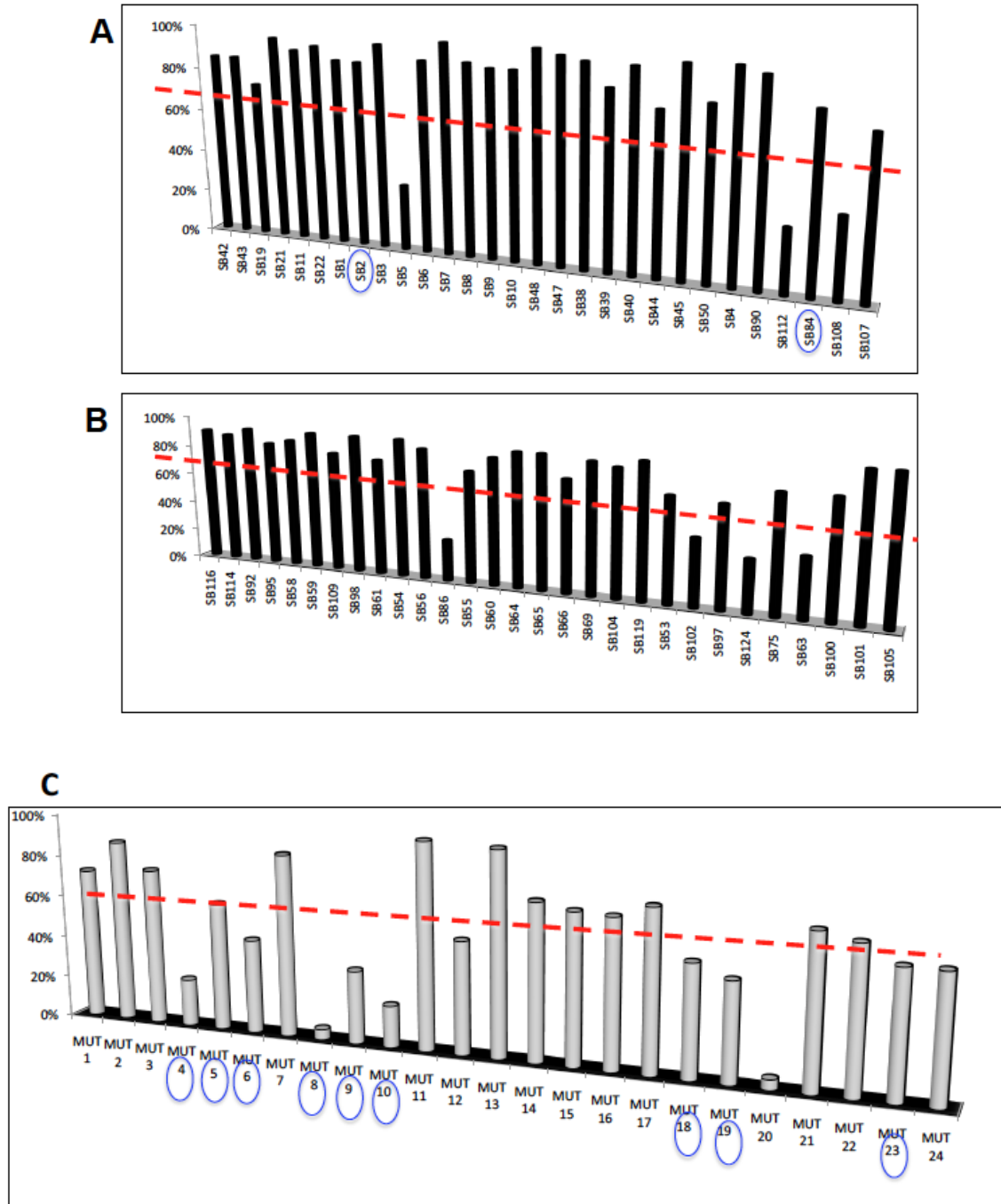


Figure 9. Spore viabilities of screen candidates. Spore viability pattern of the mutants identified via Screen I (A, B) and (C) Screen II. Y-axis denotes the overall viability of the mutants in percentage, and X-axis denotes the names of the mutants. Dotted lines indicate tetrads from candidates with <4:0 viable spores in 'A'; and candidates with overall spore viability of <60% in 'B'. Circled are the candidates that have temperature sensitive phenotype linked to transposon marker in outcross.

Table 1: Viability of original candidates from Screen I

Viabilities	0:4	1:3	2:2	3:1	4:0	Outcross TS?
42 SC	0	0	2	2	6	No
43 SC	0	0	0	6	4	No
19 SC	0	0	4	3	3	No
21 SC	0	0	1	0	9	No
11 SC	0	0	2	0	8	No
22 SC	0	0	1	1	8	No
1 SC	0	0	4	5	15	No
2 SC	0	3	2	6	16	YES
3 SC	0	0	0	2	8	No
5 SC	6	2	2	2	1	No
6 SC	0	0	1	2	6	No
7 SC	0	0	0	1	9	No
8 SC	0	0	0	3	4	No
9 SC	0	0	2	1	7	No
10 SC	0	0	2	1	7	No
48 SC	0	0	0	1	9	No
47 SC	0	0	0	2	8	No
38 SC	0	0	0	3	8	No
39 SC	0	0	1	5	4	No
40 SC	0	0	0	3	7	No
44 SC	0	0	2	5	2	No
45 SC	0	0	0	2	8	No
50 SC	0	0	1	3	2	No
4 SC	0	0	0	2	9	No
90 SC	0	0	1	1	8	No
112 SC	3	4	1	1	1	No
84 SC	0	0	1	6	3	YES
108 SC	2	4	1	3	0	No
107 SC	0	1	3	3	3	No
116 SC	0	1	0	1	8	No
114 SC	0	0	2	1	7	No
92 SC	0	0	1	1	8	No
95 SC	0	2	0	0	7	No
58 SC	0	1	2	1	9	No
59 SC	0	0	0	3	7	No
109 SC	0	1	2	1	6	No
98 SC	0	0	1	1	8	No
61 SC	1	0	1	3	5	No
54 SC	0	0	1	1	8	No
56 SC0	0	0	2	1	7	No
86 SC	3	3	4	0	0	No
55 SC	0	0	4	2	4	No
60 SC	0	0	1	4	5	No
64 SC	0	0	0	4	6	No
65 SC	0	0	2	0	8	No
66 SC	1	1	1	1	6	No
69 SC	0	0	2	1	7	No
104 SC	0	0	2	2	6	No
119 SC	0	0	1	2	7	N.D
53 SC	0	0	3	6	1	No
102 SC	1	2	2	2	9	No
97 SC	0	0	5	3	2	No
124 SC	4	0	4	2	0	N.D
75 SC	0	1	2	2	5	No
63 SC	2	3	3	1	1	No
RTG 3	0	0	2	5	3	N.D
RTG 4	0	0	0	2	8	N.D
RTG 5	0	0	0	2	8	N.D

Table 2A. Viability of original candidates from Screen II

Mutant	Viability: 4:0	3:1	2:2	1:3	0:4	Overall viability	Outcross TS?
1	10	2	4	4	0	73%	N.D
2	16	2	0	0	2	88%	NO
3	10	0	8	2	0	75%	N.D
4	1	2	2	4	11	23%	YES
5	6	3	6	3	2	61%	YES
6	1	5	5	7	2	45%	YES
7	14	4	1	0	0	88%	N.D
8	0	0	1	2	17	5%	YES
9	2	2	4	6	6	35%	YES
10	1	1	4	1	13	20%	YES
11	19	1	0	0	0	99%	N.D
12	8	1	4	0	7	54%	NO
13	18	2	0	0	0	98%	N.D
14	20	0	0	0	0	100%	N.D
15	17	3	0	0	0	96%	N.D
16	15	4	1	0	0	93%	N.D
17	19	1	0	0	0	99%	N.D
18	8	1	4	0	7	54%	YES
19	6	0	5	4	5	48%	YES
20	0	0	1	2	17	5%	NO
21	10	2	4	4	0	73%	N.D
22	9	2	6	1	2	69%	NO
23	6	4	4	4	2	60%	YES
24	8	2	4	2	4	60%	NO

Table 2B: Viability of outcrossed candidates exhibiting temperature sensitivity

Mutant	Viability: 4:0	3:1	2:2	1:3	0:4	Overall viability
4	1	1	3	4	11	21.25%
5	5	3	6	3	1	55%
6	2	4	3	6	5	40%
8	0	1	2	2	15	11.25%
9	2	2	4	6	6	35%
10	3	0	3	2	12	25%
18	6	2	5	0	7	50%
19	6	1	4	4	5	48.70%
23	5	3	5	5	2	55%

3.5.4. List of candidates with gene disruption due to transposon integration at different sites:

I have identified three classes of mutants based on the sites of transposon integration. They are candidates with (i) mutation in the open reading frames of genes (Mutant 4, 5, 8), (ii) mutation in the intergenic region (SB 84), and (iii) mutation in the 2μ -plasmid of yeast genome (Mutant 9).

The spore viability patterns, of these mutants are shown in Figure 10.

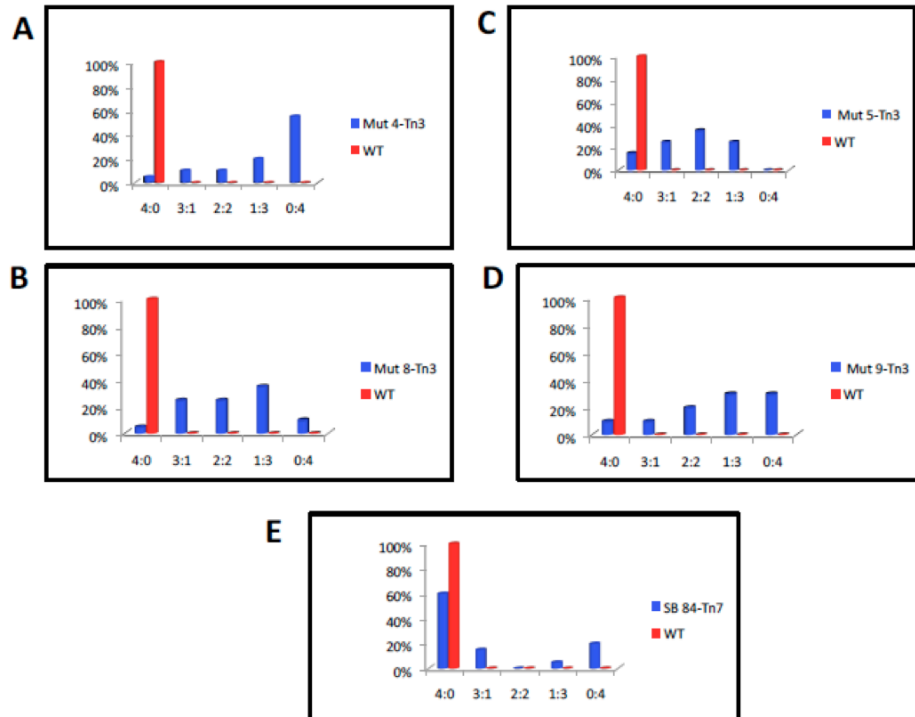


Figure 10: Spore viability patterns of some of the candidates that have been sequenced. Candidates are derived both from Screen I (E) and Screen II (A-D)

3.6 Phenotype validation, integration site identification and phenotype analysis of candidates with transposon integration in the open reading frames of genes.

I have identified three mutant candidates derived from Screen II where the transposon has integrated in the open reading frames of specific genes. These candidates are named as Mutants 4, 5, and 8.

3.6.1. Sequence analysis of Mutant 4:

To determine the site of integration of transposon in yeast genome, mutant candidates exhibiting temperature sensitive phenotype in the genetic outcross assay were subjected to direct genome sequence analysis using the transposon specific *lacZ* primer.

In the outcross assay for Mutant 4, all 52 transposon marker positive colonies (from 80 tetrads dissected) exhibited temperature sensitive phenotype confirming that the transposon is responsible for the phenotype. The sequencing result for Mutant 4 derived from screen II produced a read of 659 nucleotides (nt), encompassing coordinates 909086 - 909287 of chromosome XII of yeast genome. The sequence also provided information about a portion of the *lacZ* gene (138nt), and the terminal repeat of Tn3 transposon (38nt) (Figure 11D). The gene disrupted by the transposon was *Zip3*, one of the eight known ZMM mutants. Identification of this known ZMM mutant indicated the screen is functional and the conditions adapted for carrying out the screen were predisposed for identifying new ZMM like mutants. *Zip3* is an E3 SUMO ligase essential for SC formation, and meiotic recombination (Perry et al., 2005; Agarwal et al., 2000). According to the sequence information the transposon has integrated in the C-terminus at position 1137 nt of *Zip3* meaning that the *Zip3* ORF was truncated at amino acid position 380, with 104 amino acids missing at the C-terminus. The C-terminus of *Zip3* comprising 165 aa has significant domainal and sequence homologies with E3 Ubiquitin ligase. *Rad18* bearing a high probability SUMOylation target site is also located at its C-terminus (Figure 11A,B, and C). In figure 11B the C-terminal domainal homologies between *Zip3* and *Rad18* are shown and I have highlighted with red arrows the nucleotide positions between 955 and 1359 of *Zip3*. The box at 1359 nt is a high probability SUMOylation site. In case of Mut4-Tn3 (strain number SBY78) the transposon has integrated in the 1137 nt position (red circle) thereby disrupting the last 104 aa (Perry et al., 2005).

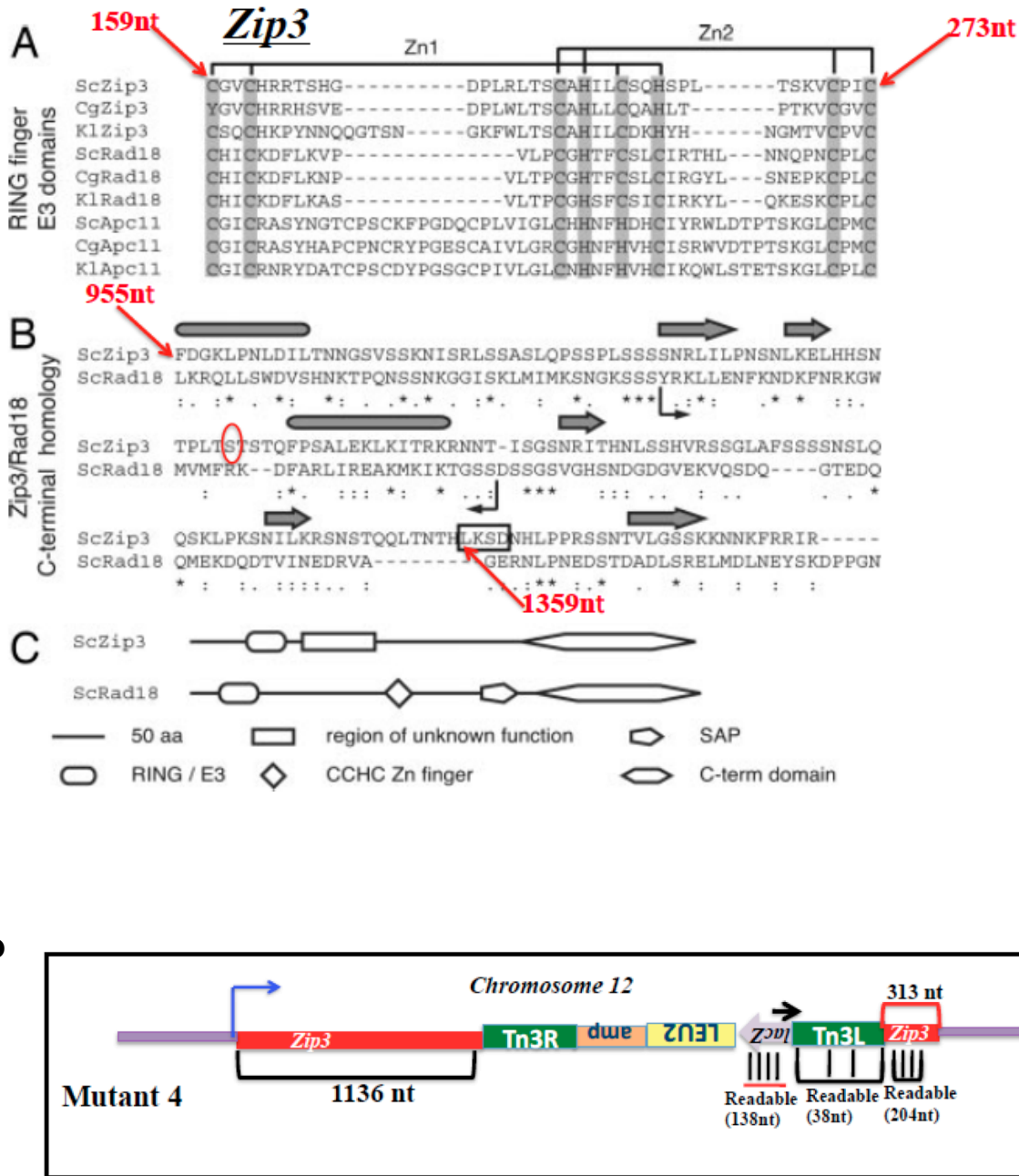


Figure 11. Bioinformatic analysis of Zip3. (A) The RING fingers of Zip3s compared with those of Rad18s and Apc11s: Sc, *S. cerevisiae*; Cg, *C. glabrata*; Kl, *K. lactis*. The presumed zinc ligands are highlighted. Red arrows indicate nucleotide positions 159 to 273 of Zip3. (B) A second domain of homology between Zip3s and Rad18s. Bars are helices and arrows above the sequences are extended strands as determined by Jpred. Arrows below the sequences delimit the region of Rad18 that is necessary and sufficient for heterodimerization with the E2 Rad6. The boxed10 segment of ScZip3 (LKSD) was determined by SUMOplot (www.abgent.com_doc_sumoplot) to be a high probability SUMOylation target (91_100). This feature is present only in the *S. cerevisiae* sequence.

Red arrows indicate nucleotide positions between 955 and 1359 in the C-terminus of Zip3. Red circle indicates the position where the transposon has integrated in Mut4-Tn3. **(C)** Comparison of the domain architectures of ScZip3 and ScRad18. The diagram is approximately to scale. (Modified from Perry et al., 2005) **(D)** Schematic map of transposon integration sites in Mutant 4. The gene disrupted in Mutant 4 due to transposon integration is *Zip3*. Red, green, and purple regions denote disrupted gene, terminal repeats of transposon, and yeast genome flanking the disrupted gene respectively, orange and yellow regions indicate selection markers in *E.Coli* and yeast respectively, blue arrow indicates the start codon of disrupted gene, and black arrow indicates anti *lacZ* primer used for whole genome sequencing respectively. Numbers beneath the red regions denote the positions in the disrupted gene where the transposon has integrated.

Summary of Mutant 4 sequence analysis from direct genomic sequence:

Length of sequence aligned with yeast genome	Length of sequence aligned with <i>Zip3</i> gene	Information on <i>lacZ</i> of Tn3	Information on LTR of Tn3	SGD coordinates	Chromosome number	Disrupted gene
144-346	204	138 nt	38 nt	909086 - 909287	Chromosome XII	<i>Zip3</i>

3.6.2. The last 300 nucleotides of *Zip3* gene are essential for its role in meiosis:

To investigate the role of *Zip3* gene in controlling meiotic progression, analysis of *zip3*-Tn3(nt1137) mutant was carried out. To address that, a synchronized meiotic time course of *zip3*-Tn3(nt1137) was performed in liquid sporulation medium at 23°C and 33°C in duplicate cultures (Figure 12 A,B). In the wild type cultures, nuclear divisions occurred as early as T=5 hour at 23°C and at 33°C by this time ~48% of the cells had undergone nuclear divisions. Both cultures of *zip3*-Tn3(nt1137) conferred complete arrest in meiotic nuclear division until up to T=24 hr time point at 33°C (Figure 12A). However, both the cultures of *zip3*-Tn3(nt1137) exhibited delay in meiotic nuclear divisions at 23°C, where nuclei started dividing at T=9 hr and by T=11 hr >48% of cells accomplished meiosis I (Figure 12A), indicating a clear temperature sensitive defect in meiotic progression.

To further analyze meiotic defect, 20 tetrads were dissected from each of the wild type and mutant cultures. As expected, the wild type cultures incubated at 33°C exhibited ~92.5% of overall spore viability and that incubated at 23°C demonstrated ~96% of overall spore viability. However, the spore viability of both the *zip3*-Tn3(nt1137) cultures was 31% at 23°C, that is the temperature at which the spores formed. This suggested that *zip3*-Tn3(nt1137) has a clear temperature sensitive defect in meiotic progression at 33°C and a delay in the same at 23°C, and also confers poor spore viability suggesting a confirmed involvement of *zip3*-Tn3(nt1137) in meiosis. This behavior of *zip3*-Tn3 is very similar to that of the *zip3Δ* mutant (Notredeme et al., 2000).

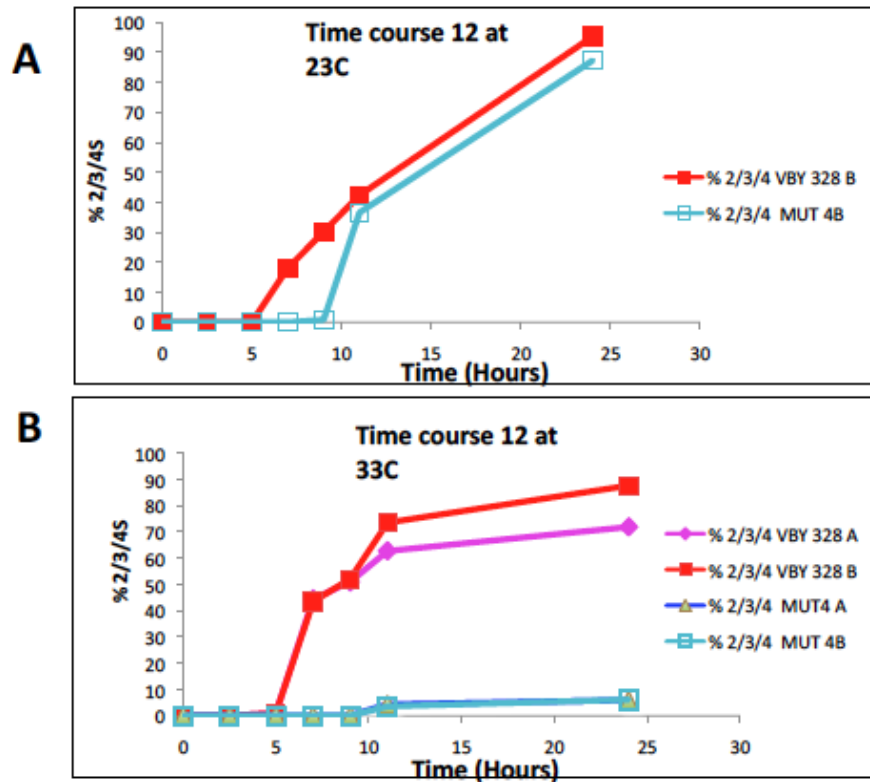


Figure 12. Meiotic progression of Mut4-Tn3 at 23°C and 33°C. Meiotic time course in liquid culture at 23°C (**A**) and 33°C (**B**) for WT (VBY 328), and Mut4-Tn3 strains. At 33°C, two cultures each from WT (VBY 328A, VBY 328B) and Mutant 4 (MUT4A, MUT4B) strains were analyzed. X-axis denotes the time in hours and Y-axis denotes the percentage of cells undergone Meiosis I and/ or Meiosis II.

3.6.3. *zip3-Tn3(nt1137)* exhibits similar phenotype as *zip3Δ::Leu2* for temperature dependent sporulation and spore viability defects:

Zip3 is one of the thus far identified eight ZMM genes that is involved in meiosis via assembly of SC and resolution of DSBs into CO products. The C-terminus of *Zip3* is chiefly involved in E3 SUMOylation and or ubiquitylation. I aimed to compare the roles

of truncated *zip3* at its C-1137 deletion position and *zip3Δ::Leu2* in meiosis, and study if there is any difference in their behaviors. In order to analyze that, a UV sporulation test was performed for *zip3-Tn3* and *zip3Δ::Leu2* in parallel at low and high temperatures (Figure 13A). The low temperature considered for performing this experiment was the usual 23°C but notably, the high temperature preferred was 30°C instead of 33.5°C, (which is the standard non-permissive temperature for my screen), since according to the previous findings, the non-permissive temperature for sporulation for *zip3Δ::Leu2* mutant was 30°C (Börner et al., 2004). Both the deleted and truncated *Zip3* genes showed similar temperature sensitive phenotypes at 23°C and 30°C incubation conditions i.e, emitting fluorescence at 23°C, but not at 30°C. Also, the temperature sensitive phenotype of *zip3-Tn3* was same at 30°C and 33°C suggesting the similar behaviors of *zip3-Tn3(nt1137)* and *zip3Δ::Leu2*. Next, to estimate the overall spore viability and viability patterns of *zip3-Tn3(nt1137)* and *zip3Δ::Leu2* mutants, 10 tetrads were dissected each from three independent *zip3-Tn3(nt1137)* colonies named as Mutant 4A, B, and C and from *zip3Δ::Leu2* (Figure 13B). The overall spore viabilities of Mutant4A, B, C and *zip3Δ* are 28%, 13%, 23%, and 30% respectively. It was detected that *zip3Δ* and *zip3-Tn3(nt1137)* strains manifested equivalent viability patterns. Hence, it can be inferred that the last 313 nt of *Zip3* gene are decisive for the completion of meiosis. (Though according to Agarwal and Roeder, 2000, the spore viability of *zip3Δ* in SK1 strain is only 12%).

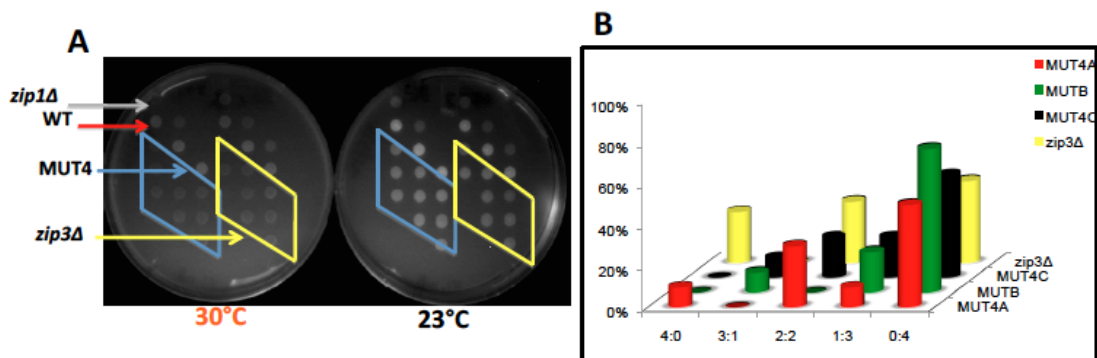


Figure 13. Phenotype analysis of Mutant 4 and *zip3Δ::Leu2*. (A) Outcrossed cell patches are arising after inoculation on sporulation medium in 4 days. Plates are incubated at 30°C and 23°C. Blue and yellow boxes indicate 3 independent cell patches in triplicates each from Mutant 4 and WT strains respectively (B) Spore viability patterns of outcrossed cell patches of Mutant 4A (red), Mutant 4B (green), Mutant 4C (black) and *zip3Δ::Leu2* (yellow) mutation. Spore viability patterns on X-axis are described in Figure 9.

3.6.4. Sequence analysis of Mutant 8:

In the outcross assay for Mutant 8, (identified in Screen II), all the 26 out of 27 transposon marker positive colonies (from 40 tetrads dissected) exhibited temperature sensitive phenotype, confirming that the transposon is linked to the phenotype. As a result, the mutant was subjected for direct genomic sequence analysis. The sequence provided a read of 514 nt, encompassing coordinates 517842 – 517701 of chromosome XIII of yeast genome. The sequence information exhibited a portion of the transposon specific *lacZ* gene (45 nt), yet there was no sequence information available on the terminal-repeat sequence of transposon, hence the transition region between the transposon and the yeast genome sequence remained unrecognized. Nonetheless, it is

reasonable to mention that the first 28 nt of the sequences were unreadable possibly encompassing the terminal repeat sequences of the transposon. The gene disrupted by the transposon was *Sto1* (Suppressor of Topoisomerase 1). The sequence of Mutant 8 corresponded with the N-terminus 304 nt of *Sto1* corresponding to 103rd aa out of a total of 861 aa (Figure 14). Nuclear cap binding protein complex (CBC) is a heterodimer of a small subunit (*Cbc2/Sae1* in yeast) that binds the m7G cap and a large subunit (*Sto1* in yeast) that interacts with karyopherins (Visa et al. 1996; Görnemann et al. 2005). Karyopherins are a group of proteins involved in the transport of small molecules between nucleus and cytoplasm via nuclear pores acting as a gateway. *Cbc2* is also known as *Sae1*, (Sporulation in the absence of Spo11). Yeast *sto1D* (D=deletion) and *sae1D* mutants are viable, but they confer slow growth and display conditional phenotypes (Qui et al., 2012). A *sto1D sae1D* double mutant demonstrates improved growth than either single mutant indicating that production of either CBC subunit alone had a dominant negative effect on cell growth (Qui et al., 2012). It is also established that *sae1D* mutants exhibit defects in recombination and confer permanent arrest at pachytene stage (McKee and Kleckner, 1997).

While this study was ongoing, it was demonstrated by others that in the *sae1-ND 42* mutant (N-terminal deletion of 42 aa), the MER3 transcripts failed to undergo efficient splicing. Splicing efficiency of MER3 is significantly reduced in *sae1-ND 42* resulting in failure of this mutant to undergo meiosis (Qui et al., 2012). Importantly I have identified the larger subunit of CBC (*Sto1*), the smaller subunit of that being involved in regulating splicing of *Mer3*, another component of the ZMM complex. This mutant is therefore referred to as *sto1-Tn3(nt304)*.

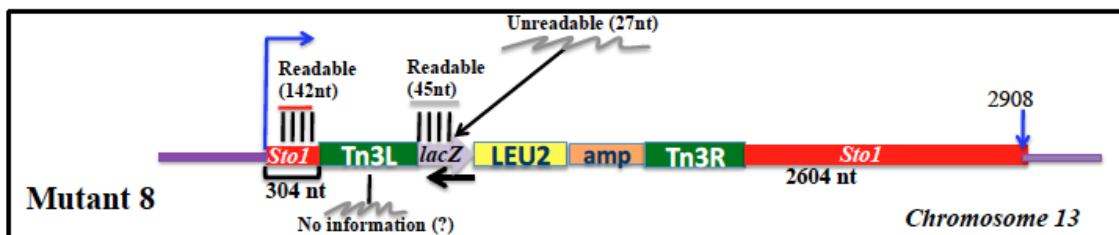


Figure 14. Schematic map of transposon integration sites in Mutant 8. The gene disrupted in Mutant 8 due to transposon integration is *Sto1*. The colored regions are same as in figure 11.

Summary of Mutant 8 sequence analysis from direct genomic sequence:

Length of sequence aligned with yeast genome	Length of sequence aligned with <i>Sto1</i> gene	Information on <i>lacZ</i> of Tn3	Information on LTR of Tn3	SGD coordinates	Chromosome number	Disrupted gene
74-214	142	45 nt	NO information	517842 - 517701	Chromosome XIII	<i>Sto1</i>

3.6.5. Validation of transposon integration at N-304 nt of *Sto1* gene in Mutant 8:

To confirm the sequence information for Mutant 8, DNA samples were isolated from both the mutant and WT strains and subjected to PCR amplification (Figure 15A). DNA sequences used as forward and reverse primers were positioned at upstream and downstream of the start codon of *Sto1*. It was demonstrated that WT sample produced a band of 906 bp, whereas *sto1*-Tn3(nt304) presented a band of ~7.6 Kb (906+6654=7560bp) supporting the possibility of *sto1* gene getting disrupted by the integration of transposon.

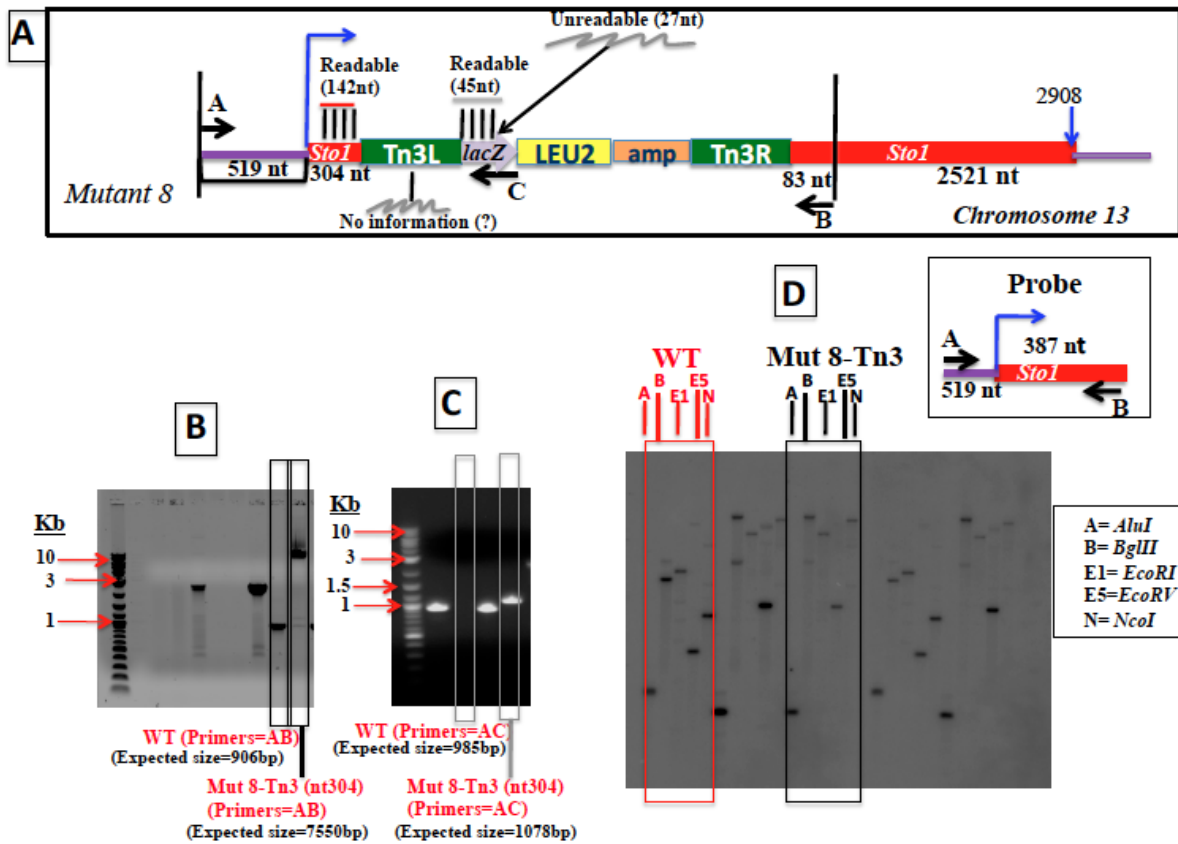


Figure 15. Genomic analysis of Mut8-Tn3. (A) The colored regions are same as in figure 7. The gene disrupted in Mutant 8 due to transposon integration is *Sto1*. (B) PCR amplification of WT and Mutant 8-Tn3 genomic DNA, by using 509 nt upstream and 312 nt downstream from start codon of *Sto1* as forward and reverse primers respectively. (C) PCR amplification of wild type strain and Mutant 8-Tn3(nt304) genomic DNA by using 20 nt long anti-*lacZ* sequence and 509 nt sequence upstream from start codon of *Sto1*

gene as reverse and forward primers respectively. **(D)** Southern blot of wild type and Mutant 8-Tn3 genomic DNA samples digested with AluI, BglII, EcoRI, EcoRV, NcoI. Probe used for Southern is schematically shown in the box.

To validate the aforesaid PCR result, a second PCR amplification was performed on DNA from WT and *sto1*-Tn3(nt304) strains (Figure 15B). The primers used for this reaction were transposon specific anti *lacZ* as the forward primer (same primer used for direct genome sequencing) and reverse primer starting at 519 nt upstream from the start codon of *Sto1* gene. This experiment generated a band of the expected size of 1.1Kb for *sto1*-Tn3(nt304) strain, whereas an absence of band for the WT strain consistent with sequence data.

Finally, to validate the *Sto1* gene disruption in Mutant 8, I carried out Southern hybridization (Strain number SBY82) (Figure 15C). The probe utilized was the formerly discussed 906 bp PCR product shown in figure 15C. Five appropriate restriction enzymes (AluI, BglII, EcoRI, EcoRV, NcoI) were used for digesting the genomic DNA samples extracted from WT and *sto1*-Tn3(nt304) strains. Both strains demonstrated predicted sizes of bands for each restriction enzyme suggesting that it indeed is the *Sto1* gene that is disrupted in Mutant 8 and the transposon integration occurred at the N-terminal 304 nt position. Hence, for Mutant 8 or *sto1*-Tn3(nt304) the genomic sequences corresponded with the transposon integration sites.

3.6.6. *sto1*-Tn3(nt304) mutant demonstrates defect in mitosis:

sto1-Tn3(nt304) mutant exhibits growth defect. I therefore first investigated the probable role of *Sto1* gene in mitosis by analyzing this mutant. To examine that, *sto1*-Tn3(nt304) mutant was grown on growth (YPD) solid medium by streaking it for single colonies (Figure 16A). In parallel, single colonies from *zip1Δ::LEU2* (known non-temperature sensitive for mitosis) and WT strains were also grown on YPD plates. The growth plates were incubated in parallel at 23°C and 37°C for 3 days until WT colonies had grown to normal size. It was demonstrated that *sto1*-Tn3(nt304) was growing similarly as the other two controls at 23°C. However, at 37°C, no growth of *sto1*-Tn3(nt304) was detectable, whereas, growth of the control strain was unaffected at either temperature. This suggested that incubation temperature affects growth of *sto1*-Tn3(nt304).

To ascertain the growth efficiencies of spore colonies, 10 tetrads were dissected from *sto1*-Tn3(nt304) mutant on three separate YPD plates and incubated at 23°C, 30°C, and 33.5°C (Figure 16B). As expected, the growth of spore clones of *sto1*-Tn3(nt304) was significantly reduced with gradual rise in temperature from 30°C to 33.5°C, even though exhibiting normal growth at 23°C. Additionally, *sto1*-Tn3(nt304) exhibited higher percentage of tetrads with none out 4 spores viable at 33.5°C (90%), and 30°C (80%) compared to that at 23°C (40%). Based on these results it can be suggested that incubation temperature influences both the growth of mitotic cells and spore germination/growth of *sto1*-Tn3(aa304).

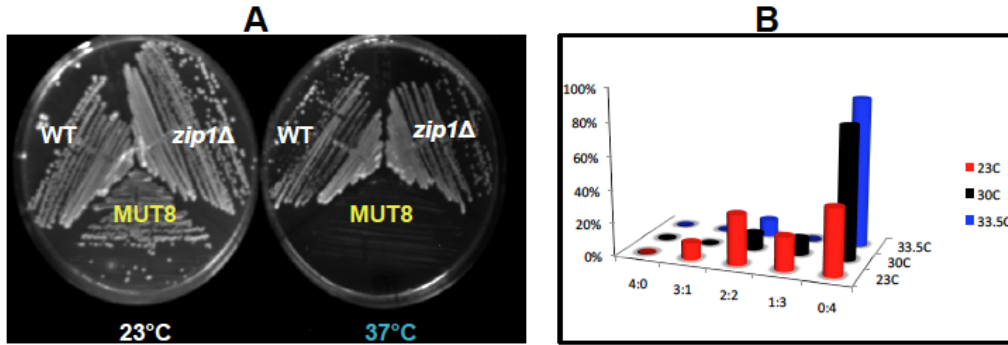


Figure 16. Incubation temperature dependent growth defects of Mut8-Tn3. (A) Cell patches of Mut8-Tn3 and WT strains streaked for single colonies on *leucine* drop out growth plates and incubated at 23°C and 37°C for 3 days. (B) Viability patterns of spores dissected from a single Mut8-Tn3 colony and incubated at 23°C, 30°C, and 33.5°C.

*3.6.7. The disruption of *Sto1* gene at its N-304 nt position, exhibits ZMM like temperature sensitive phenotype for sporulation:*

To investigate the function of *sto1*-Tn3(nt304) mutant in regulating meiotic nuclear divisions, a synchronized meiotic time course was performed in liquid sporulation medium alongside wild type cultures at 33°C (Figure 17). In both wild-type cultures ~45% of the cells completed meiosis I by T=5 hr, ~78% of the cells had undergone I and/or II nuclear divisions by T=9 hr, and by T=24 hr, ~ 90% of cells had accomplished both the meiotic divisions. However, the mutant culture manifested complete arrest in meiotic nuclear progression until up to T=24 hr time point, suggesting that *sto1*-Tn3(nt304) demonstrated distinct temperature sensitive phenotype for meiotic nuclear division at 33°C like known ZMM mutants. I note, however that in this case, no parallel time course was carried out at 23°C.

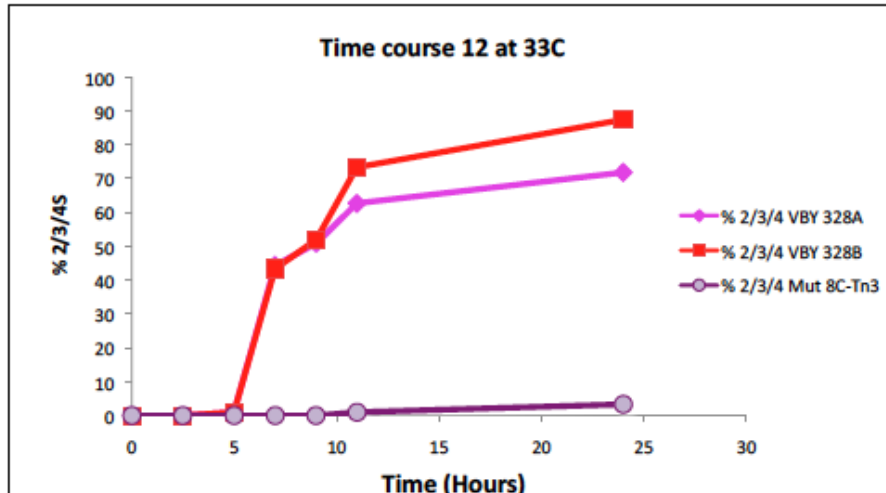


Figure 17. Meiotic progression of Mut8-Tn3 at 33°C. Two cultures of WT (VBY 328A, VBY 328B) and Mutant 8 (MUT8A) strains were analyzed for meiosis. X-axis denotes the time in hours and Y-axis denotes the percentage of cells undergone meiosis I and/ or meiosis II.

As discussed, others have identified MER3 splicing as the only requirement for the CBC complex during meiosis. To confirm that *Sto1* indeed affects meiosis in this manner, I propose to introduce intronless cDNA copy of *Mer3* gene in both *sto1D* and *sto1-N-304* mutant strains and investigate their meiotic and mitotic phenotypes.

3.6.8. Integration site identification of Mutant 5:

In the genetic outcross assay, 77 out of 78 transposon-specific marker colonies (from 120 tetrads dissected) co-segregated with the temperature sensitive phenotype for Mutant 5.

Therefore this mutant was subjected to direct genomic sequence analysis for detecting the position of transposon integration. Two independent samples were analyzed—one sample contained DNA isolated from the original transformant, and the other had DNA extracted from the outcrossed candidate. It was demonstrated that for the original transformant, sequence information corresponded with coordinates 372441 - 373014 and for the outcrossed candidate the sequence matched with 372441 – 373000 of chromosome V of yeast genome (Figure 18A). Interestingly, for both the outcrossed candidate and original transformant, the transposon had integrated in the N-terminus at 116 nt position of *Mam1* (Monopolar microtubule Attachment during Meiosis I) apparently truncating this gene after 40 aa. MAM1 is a kinetochore associated monopolin protein involved in monopolin homolog attachment to the meiotic spindle (Toth et al, 2000). It is required for chromosome segregation and co-orientation of homologous kinetochores during meiosis I, and bi orientation of sister kinetochores in meiosis II. In *mam1Δ* cells there is premature separation of sister chromatids. *Mam1* is present from late pachytene to anaphase I, and is not needed for progression through meiotic cell cycle, forming pseudobinucleates with 4 spindle pole bodies (Toth et al, 2000). However, the sequence information obtained from direct genome sequencing was devoid of any information about the transposon left-terminal repeat, thus the transition region between the transposon and the yeast genome sequence remained unidentified. Nonetheless, it is reasonable to mention that the first ~160 nt of the sequence read from both the genomic DNA samples provided an unreadable information possibly spanning the terminal repeat and *lacZ* sequences of transposon. The chromatogram of outcrossed Mutant 5 genomic

sequence is shown in Figure 18B. Henceforth, this mutant was referred as *mam1*-Tn3(nt116).

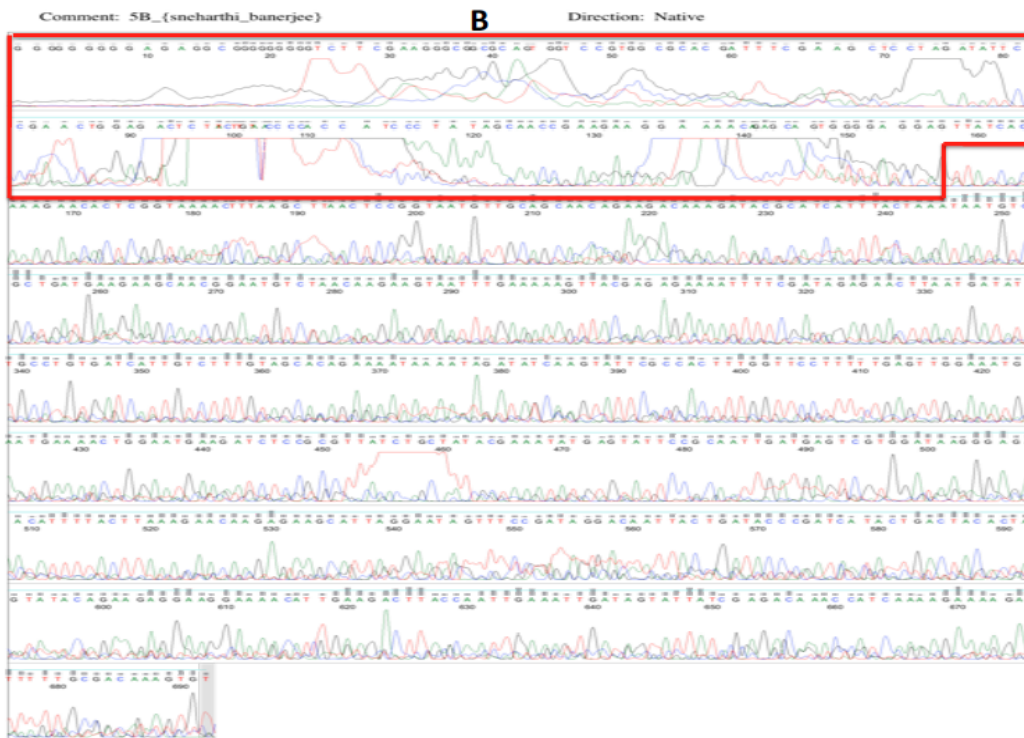
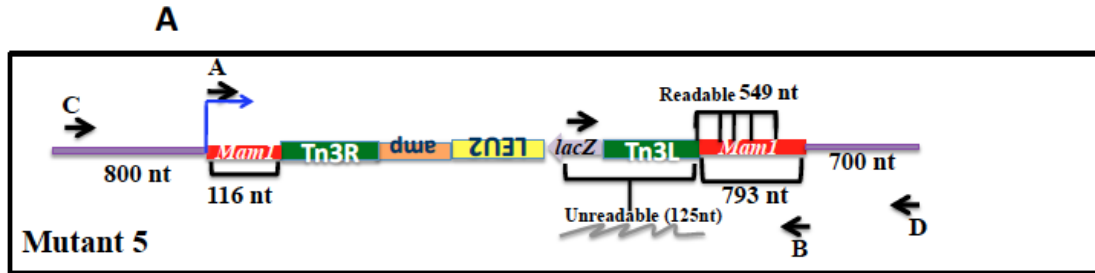


Figure 18. Genomic analysis of Mutant 5. (A) Schematic map of transposon integration sites in Mutant 5. The gene disrupted in Mutant 5 due to transposon integration is *Mam1*. The colored regions are same as in figure 11. (B) Chromatogram of Mutant 5 outcrossed candidate obtained from direct genome sequence. Red box indicates the stretch of unreadable sequences ranging up to 160 nts.

Summary of Mutant 5 sequence analysis from direct genomic sequence:

Length of sequence aligned with yeast genome	Length of sequence aligned with <i>Mam1</i> gene	Information on <i>lacZ</i> of Tn3	Information on LTR of Tn3	SGD coordinates	Chromosome number	Disrupted gene
121-692 (original transformant)	573	NO information	NO information	372441 - 373014	Chromosome V	<i>Mam1</i>
95-643 (outcrossed candidate)	559	NO information	NO information	372441 - 373000	Chromosome V	<i>Mam1</i>

3.6.9. Sequence analysis of Mutant 5:

To determine the exact location of transposon integration in mutant 5, I asked the following questions:

(i). Does Mutant 5 have a transposon insertion?

To demonstrate that transposon had integrated in the *Mam1* gene, genomic DNA samples were isolated from Mutant 5 and WT strains, and subjected to PCR (Figure 19A). For that, a forward primer at 800 bp upstream from the start codon of *Mam1*, and reverse primer 700 bp downstream from the stop codon of *Mam1* were used. The WT sample produced an expected sized band of 2.4 Kb whereas Mutant 5 sample, for which the expected size of the product was 9.6Kb, did not produce a band (one interpretation is that a PCR product of this size cannot be efficiently amplified). Thus, the absence of band in case of mutant and also the attainment of expected sized band in the WT sample was consistent with a transposon integration in the *Mam1* gene.

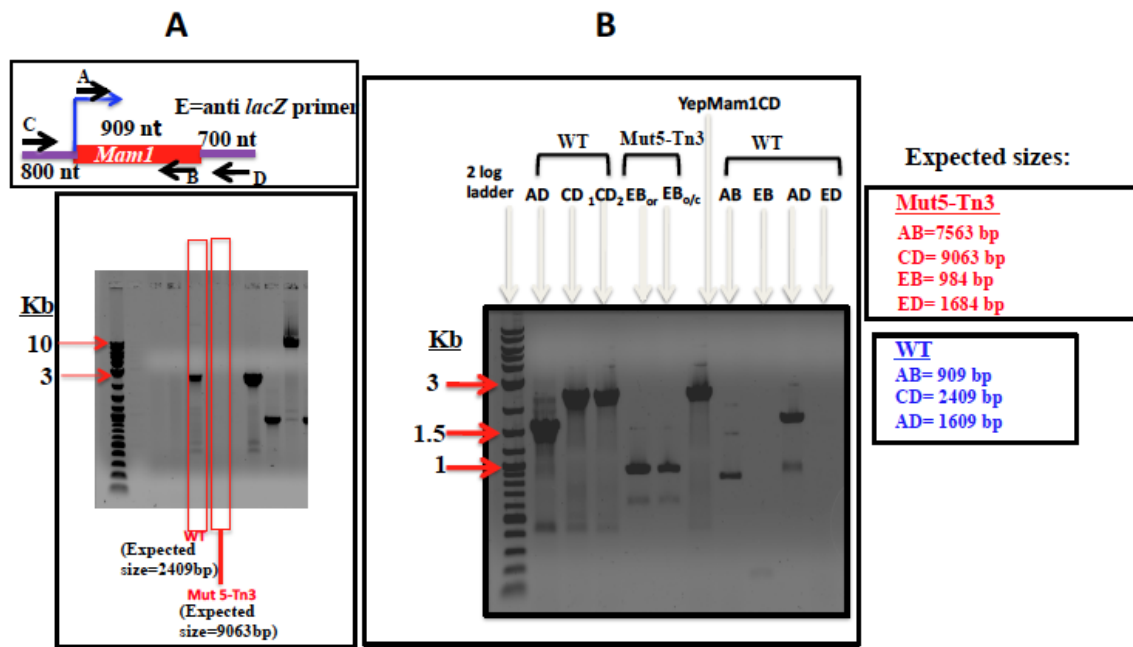


Figure 19. Genomic analysis of Mut5-Tn3 by PCR. PCR amplification of WT and Mutant 5-Tn3 genomic DNA. (A) Designated ‘C’ and ‘D’ oligos are used as forward and reverse primers respectively. (B) The Primer combinations used for WT are: AB; CD; AD and for Mut5-Tn3 are: AB; CD; EB_{or}; and ED.

(ii). Does the transposon integrate in the *Mam1* gene in Mutant 5?

To validate if the absence of band for *mam1*-Tn3(nt116) in the PCR (discussed in Figure 19A), was due to the integration of transposon, a second PCR was performed using DNA samples isolated from WT and *mam1*-Tn3(nt116) mutant strains (Figure 19B). To demonstrate that transposon is responsible for the gene disruption of *Mam1*, I used multiple combinations of primers including the transposon specific anti *lacZ* sequence, and oligos in the start/stop codons and in the flanking upstream/downstream regions of *Mam1* gene. For all the primer combination that included a transposon specific Leu2 primer and also primers of the start-codon/ stop-codon or flanking upstream/downstream of *Mam1* gene, expected sized bands were produced for both the WT and mutant strains indicating that in Mutant 5 the transposon has integrated in the *Mam1* gene at its 116 nt position.

(iii). Where in Mutant 5 is the transposon integrated?

Finally to authenticate that transposon had integrated in the *Mam1* gene in Mutant 5, Southern hybridization was carried out (Figure 20). A 894 bp DNA sequence upstream of the start codon of *Mam1* gene was used as a probe. I digested the genomic DNA samples isolated from WT and *mam1*-Tn3(nt116) strains with two appropriate restriction enzymes— NsiI and HindIII. But the experiment produced discrepancies. While there was a difference in banding patterns observed between Mutant 5 and WT strains with several enzymes, the patterns for Mutant 5 were not corresponding with the sequence data illustrated in Figure 18 (Strain number SBY79) (Figure 20A). Moreover, upon repeating the experiment under identical conditions, the previously observed differences in band sizes between Mutant 5 and WT strains, disappeared (Figure 20B).

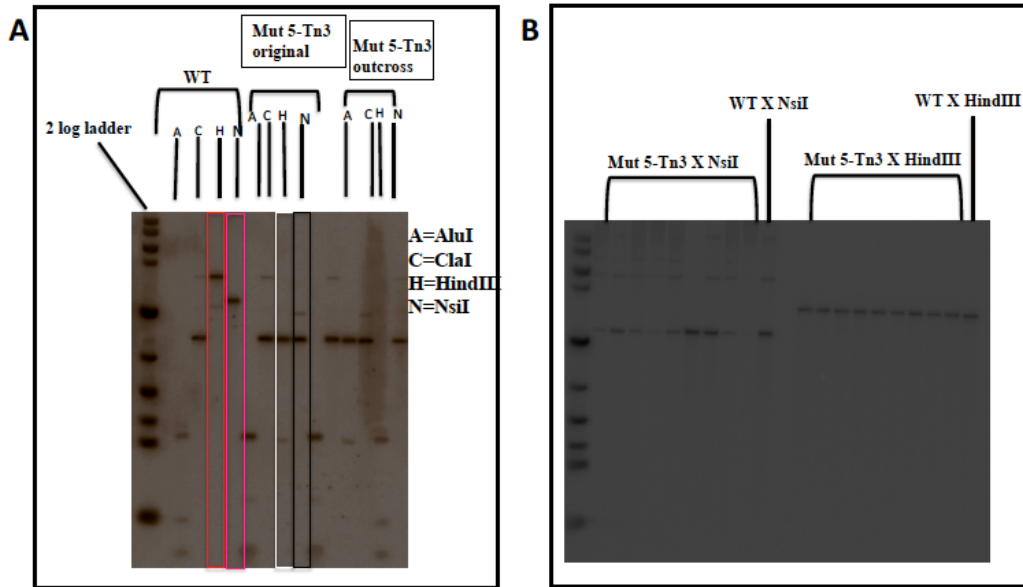


Figure 20. Sequence analysis of Mut5-Tn3 by Southern hybridization. (A) Genomic DNA samples extracted from WT, original Mut 5-Tn3 transformant, and outcrossed Mut5-Tn3 strains digested with AluI, ClaI, HindIII, NsiI. Red-pink and white-black boxes denote DNA samples digested with HindIII and NsiI derived from WT and Mut5-Tn3 strains respectively. Probe used is the DNA sequence 897 nt upstream of *Mam1*. (B) Genomic DNA samples digested with HindIII and NsiI for WT and Mut5-Tn3 respectively.

(iv). What is the transition sequence between transposon and yeast genome?

To investigate whether the disrupted gene in Mutant 5 is *Mam1*, Topo TA cloning was performed using Topo 2.1 vector, and previously mentioned 985 bp PCR product generated with primers A and C in figure 15C was used as an insert, followed by transformation into commercial bacterial competent cells, and selection via blue–white screening (Figure 21). DNA isolated from 6 insert bearing (white) colonies was subjected to sequence analysis using M13 forward and M13 reverse primers. All the 6 sequencing results supported the presence of *lacZ* sequence at 781 nt of *Mam1* instead of 116 nt

position obtained from direct genome sequence data. However, *lacZ* sequence is present in the Topo vector itself, and there was sequence homology observed, as ‘ATTC’ sequence was present in (i) Topo vector at the transition between vector and *Mam1* sequences, (ii) in *lacZ* sequence of Tn3 at 188 nt position, and (iii) at 783 nt position of *Mam1*. Thus, further investigation is required to detect the exact position of integration of transposon in the *Mam1* gene. One possible explanation for the absence of complete *lacZ* sequences is that the transposon has excised and integrated in the yeast genome.

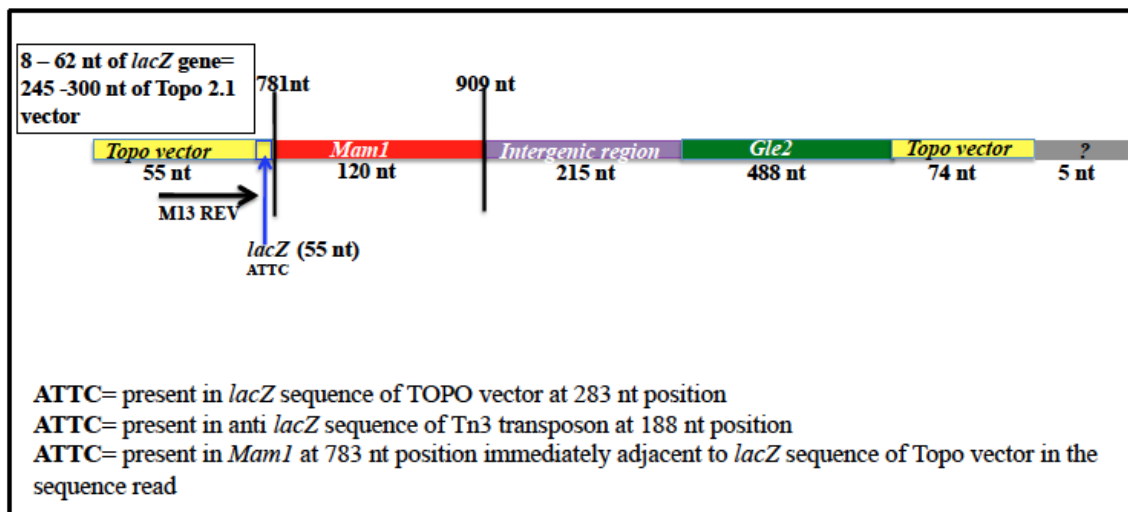


Figure 21. Genomic analysis of Mut5-Tn3 by Topo cloning. *lacZ* sequence found immediately adjacent to *Mam1* gene at its 781 nt position. 245-300 nt of Topo 2.1 vector corresponds with 8-62 nt of *lacZ* gene. There is sequence homology (ATTC) observed between *lacZ* sequence of Topo vector and *Mam1* gene at adjacent positions.

3.6.10. Mutant 5 strain homozygous for *LEU2+* transposon marker exhibits temperature dependent sporulation phenotype.

To demonstrate whether truncation of *Mam1* at 116 nt position had dominant

negative and/ or gene of function effect on sporulation, a knock out strain of *mam1Δ* marked with KanMX was constructed. *mam1Δ::KanMX* in ‘a’ cells (strain number SBY117) was confirmed by Southern hybridization. The other mating type (α) was confirmed by tetrad dissection followed by selection on respective marker plates as the strains were uniquely marked. (Figure 22B). (The His4:Leu2 hotspot strains of *mam1KO* in ‘a’ and ‘ α ’ cells are shown in Appendix)

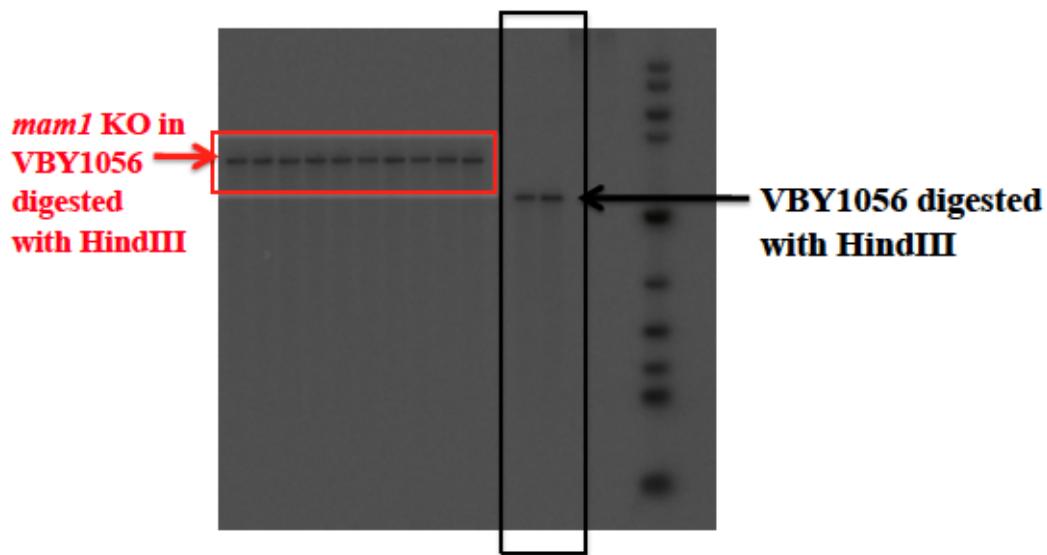


Figure 22: Southern hybridization of *mam1*-knock out (KO) strains in VBY 1056: Genomic DNA samples isolated from *mam1*-KO in ‘ α ’ mating type yeast strains and VBY1056 strains digested with HindIII.

Both *mam1Δ* and Mutant 5 were subjected to UV sporulation test at low (23°C) and high temperatures (33.5°C) (Figure 23). The result obtained from this test indicated no temperature sensitive sporulation phenotype for *mam1Δ::KanMX* homozygous diploid strain, whereas consistent temperature sensitive sporulation phenotype was observed for Mutant 5. To further investigate the result, the temperature sensitive phenotype of

LEU2^{+/+} (transposon specific) marked homozygous diploid strain of Mutant 5 was compared with that of LEU2^{+/-} heterozygous diploid strain of Mutant 5. The heterozygous strain was constructed by mating Mutant 5 haploid colonies—(a;LEU2⁺ or α ;LEU2⁺) with non-temperature sensitive haploid outcrossed colonies deficient of the marker for transposon (a;*leu2*⁻ or α ;*leu2*⁻). This assay demonstrated non-temperature sensitive sporulation phenotype for heterozygous LEU2^{+/-} mutant, whereas distinct temperature sensitive phenotype for homozygous LEU2^{+/+} mutant, thereby suggesting a consistent co-segregation of phenotype with the transposon specific LEU2 marker. Moreover, the transposon marked mutation is recessive to the wild type allele. I also confirmed the homozygous LEU2^{+/+} specific phenotype by using two different types of diploid strains—one type of homozygosity caused by the HO⁺;*ste7-1* homothallic yeast strain and the other caused by the mating of haploid outcrossed (a;LEU2⁺ mated with α ;LEU2⁺) yeast strains. I tested whether these strains were temperature sensitive for spore formation by performing sporulation test. It was validated that both types of homozygous diploid mutant strains carrying *Leu2* genes exhibited temperature sensitive phenotype re-establishing the consistent conditional behavior of Mutant 5.

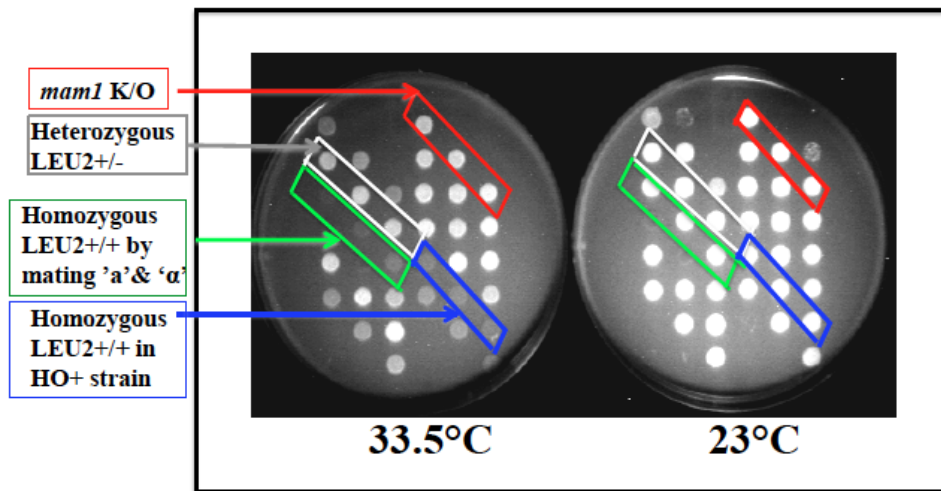


Figure 23. UV sporulation test for *mam1Δ*, Mut5-Tn3(LEU2+/+) and (LEU2+/-) strains: Cell patches arising on sporulation plate after incubating for 4 days at 33.5°C and 23°C. Red box denotes cell patches of WT *Mam1* strain; Green box denotes cell patches derived from homozygous LEU2+/+ Mut5-Tn3 strain obtained by mating; Blue box denotes homozygous LEU2+/+ Mut5-Tn3 strain obtained by gene switching via HO+; White box denotes cell patches derived from heterozygous LEU2+/- strain obtained by mating.

3.6.11. Mut5(Mam1?)-Tn3 demonstrates temperature sensitive phenotype for both mitosis and meiosis and behaves differently from mam1 deletion mutant:

Mutant 5 exhibits dramatic temperature sensitive growth defect. In the genetic outcross assay, all transposon-specific marked colonies derived from this mutant exhibited temperature sensitive phenotype and were also small in size. To investigate this growth defect further, I streaked this mutant for single colonies on YPD plates alongside WT strain and incubated plates at 7 different temperatures— between 23°C to 37°C for 3 days (Figure 24). It was intriguing to discover that Mutant 5 was growing slower at 23°

compared to the WT, and demonstrated maximum growth at 28°C, hence exhibiting a cold sensitive phenotype for growth. However, at 30°C it showed slight reduction in growth, which decreased even further with increase in temperature till 33°C. At 34°C and beyond there was a complete arrest in growth, even though the growth of WT strain was uniform at all the incubation temperatures. To sum up, Mutant 5 exhibited cold sensitive phenotype for growth at 23°C, demonstrated optimum growth at 28°C and conferred no growth at 33°C and beyond, suggesting that it is both cold and temperature sensitive.

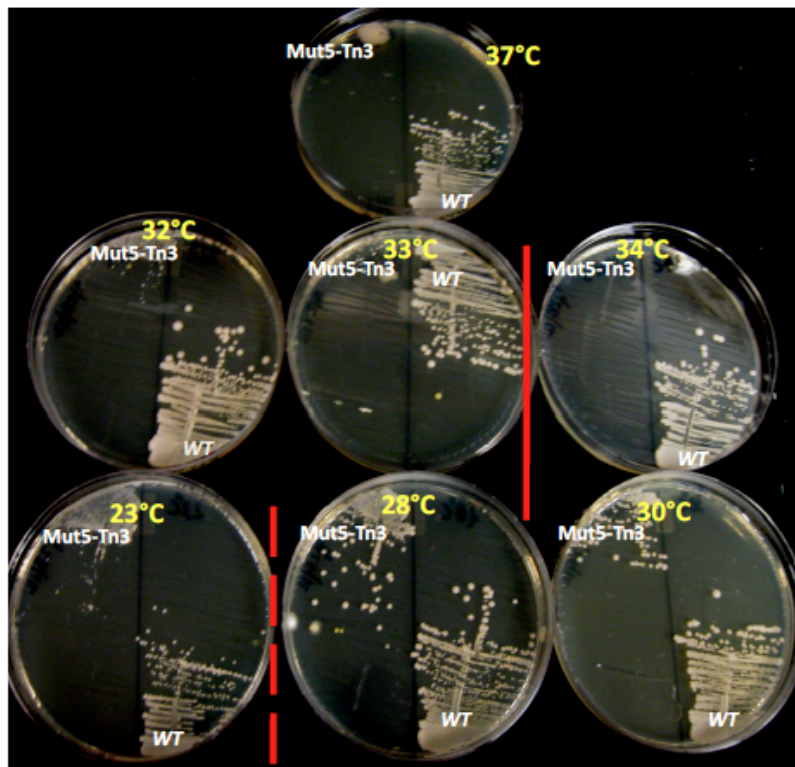


Figure 24. Incubation temperature dependent growth defects of Mutant 5. Mut5-Tn3 and WT strains streaked for single colonies on leucine drop out growth plates and incubated at 23°C, 28°C, 30°C, 32°C, 33°C, 34°C and 37°C for 3 days. Solid-red and dashed-red lines demarcate the transition between permissive and non-permissive temperatures for growth, and the temperature below which Mut5-Tn3 exhibits cold sensitive growth phenotype respectively.

To investigate whether Mutant 5 confers any abnormality in meiotic nuclear division, meiotic time course was carried out in liquid sporulation media at 23°C (Figure 25A) and 33°C (Figure 25B). Pregrowth indicated mitotic growth defects at 30°C in liquid presporulation medium. Two independent cultures were analyzed at both high and low temperatures. For the WT culture, ~46% of the cells had undergone meiosis I at T=5 hr at 33°C, and at 23°C the cells progressed through meiosis I at T=5 hour and >90% of the cells had completed meiosis I and/ or II by T=24 hr. At 33°C both the cultures of Mutant 5 indicated complete arrest in nuclear division advancement until up to T=24 hr time point. At 23°C both mutant cultures displayed delay in meiotic progression by 7 to 9 hours, with only >20% of cells advancing through first nuclear division by T=9 hr, supporting a clear temperature sensitive meiotic progression defect of Mutant 5. Intermediate temperatures were not examined leaving open the possibility that an optimum intermediate temperature may also exist for meiosis.

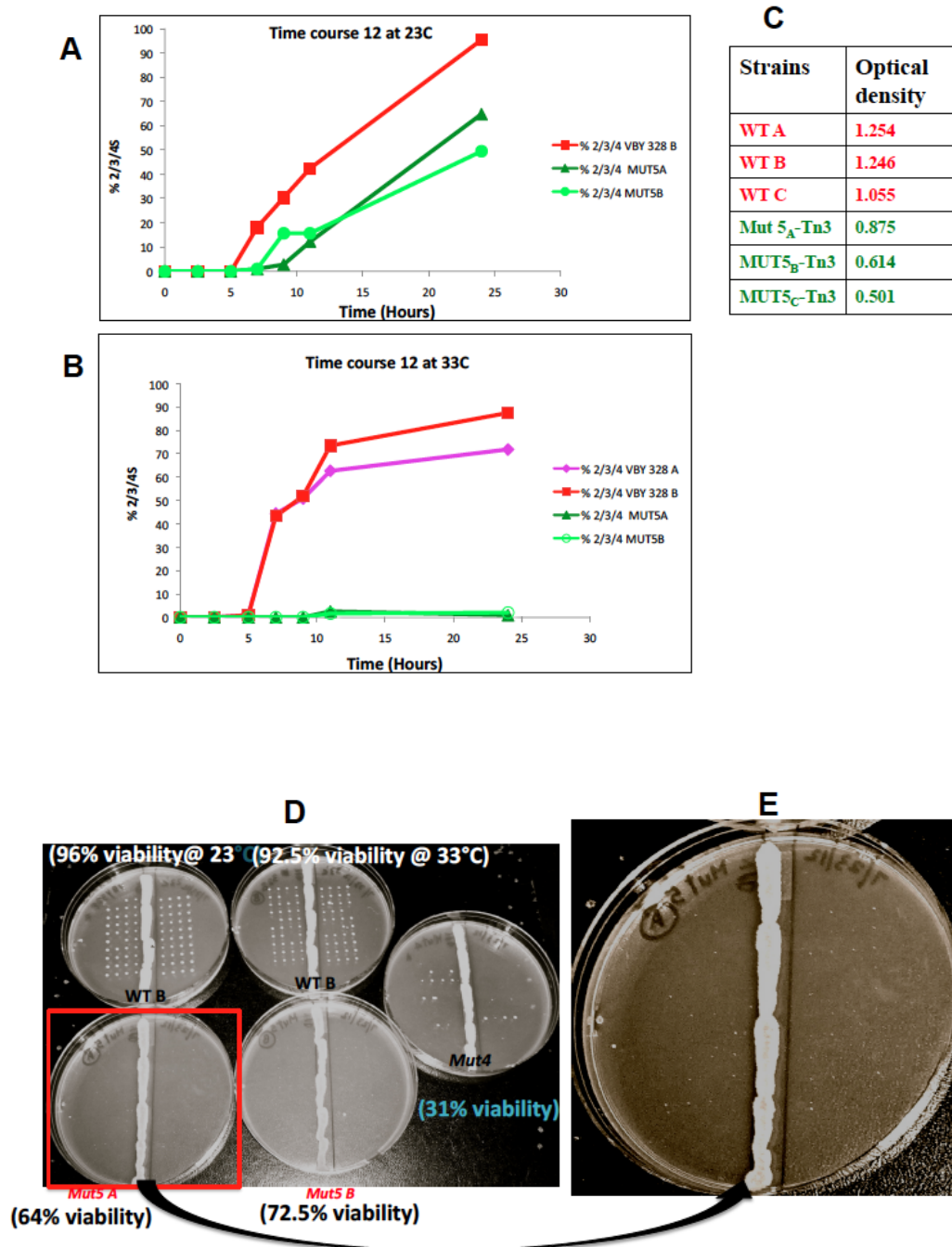


Figure 25. Meiotic time course at 23°C (A) and 33°C (B) for WT cell culture VBY 328, Mut5-Tn3. (A) Meiotic progression of one WT (VBY328A) and two Mut5-Tn3 (MUT5A, MUT5B) cultures were analyzed at 23°C. (B) Meiotic progression of two WT (VBY 328A, VBY 328B) and two Mutant 5 (MUT5A, MUT5B) cultures were analyzed at 33°C. X-axis denotes the time in hours and Y-axis denotes the percentage of cells

undergone meiosis I and/ or meiosis II **(C)** Low optical density values of 3 independent Mut5-Tn3 cultures compared to 3 independent wild type cultures. **(D)** Spore viability analysis of meiotic time course cultures of Mut5-Tn3 cells (MUT5A and MUT5B); Mut4-Tn3(nt1137) sporulated at 30°C, and WT strains sporulated at 23°C and 33°C are dissected for tetrads on YPD growth plate for 4 days. **(E)** Magnified picture of Mut5-Tn3 culture (5A) tetrad dissected plate after 4 days.

Lastly, to confirm the poor spore viability pattern and poor growth characteristic of Mutant 5, 20 tetrads were dissected from the aforementioned liquid time course cultures at 23°C along side WT cultures derived from both 23°C and 33°C, and *zip3*-Tn3(nt1137) culture derived from 23°C. As expected, the wild type cultures incubated at 23°C and 33°C exhibited overall spore viabilities of ~96% and ~92.5% respectively, and *zip3*-Tn3(nt1137) produced an expected viability of 31%. However, Mutant 5 cultures exhibited an overall spore viability of 64% (23°C) and 72.5% (33°C). Surprisingly, the sizes of the spore colonies for Mutant 5 were found to be extremely small compared to the wild type tetrads even after 76 hours of incubation at 30°C, a temperature at which *mam1* temperature sensitive mutant is thought to grow normally. Incubation at lower temperatures did not result in additional growth (Figure 25D, E). It is also critical to mention that the optical density (O.D) values for Mutant 5 measured from three independent cultures were substantially lower compared to that of the WT. The WT cultures had an average O.D of 1.185 whereas the mutant cultures had an average O.D of 0.66 (Figure 25C). These results reassure that Mutant 5 exhibits striking defects in both mitosis and meiosis based on incubation temperatures.

Due to the described discrepancies, it is presently unclear whether Mutant 5 corresponds to a *mam1* mutant. In the future I propose to examine this issue by performing the following experiments. To confirm where the transposon has integrated in

Mutant 5, one could carry out a transformation of both *mam1*KO and Mutant 5 with plasmid over-expressing *Mam1* gene followed by temperature sensitive sporulation and growth tests to compare the phenotypes. As I observed a clear linkage between LEU2 and temperature sensitive phenotype in Mutant 5, the other suggested experiment is to perform Southern hybridization using LEU2 specific probe to examine if there is any endogenous copy *Leu2* gene present in Mutant 5. For identifying the transition region between yeast and transposon, inverse PCR is a recommended experiment preceded by circularization of restriction digested genomic DNA (Ochman et al., 1988). Considering the difficulty in obtaining sequence information of transposon, a primer could be used that reads transposon sequence internally from the terminal repeat, to validate the readability of the terminal sequences of transposon.

3.7. Phenotype validation, integration site identification and phenotype analysis of candidate with transposon integration in the intergenic region.

I have identified one mutant, which is a derivative of Screen I, where the transposon has integrated in the intergenic region of yeast genome. This candidate is named as SB 84 (strain number 270).

3.7.1. Integration site identification of SB 84:

SB 84 is one of the two candidates derived from Screen I that exhibited temperature sensitive phenotype for transposon specific marker in the genetic outcross assay. Therefore, this mutant was subjected to direct genomic sequence approach and it provided sequence homology with coordinates 150730 to 151491 of chromosome V of yeast genome (Figure 26). Interestingly, the mentioned sequence corresponded with no

particular gene but with an intergenic region present between *Irc22* (Increased Recombination Centers) in the Crick strand and *Mnn1* (MaNNosyltransferase) in the Watson strand. The sequence information suggested that the transposon is 515nt downstream of *Irc22* and 1416 nt upstream of *Mnn1*. The transposon integration site is further 1.5Kb away from the centromere V affecting the pericentromeric region. The sequence also provided information about the transposon specific *lacZ* gene (191nt) the terminal repeat of transposon was not detected. Identification of this mutant through a meiotic screen suggests that this identified intergenic region of chromosome V might have some role in meiosis. As this intergenic region (~3Kb) is lengthier than the accepted length of intergenic region (500bp) in *S.cerevisiae*, a probability of this region carrying an unidentified meiosis specific gene cannot be completely precluded. I would like to mention here that part of the obtained sequence of this mutant (192nt) corresponded with *Dof1* gene of *Zea mays*, which is a transcription factor involved in light regulated gene expression (Yanagisawa, 2001).

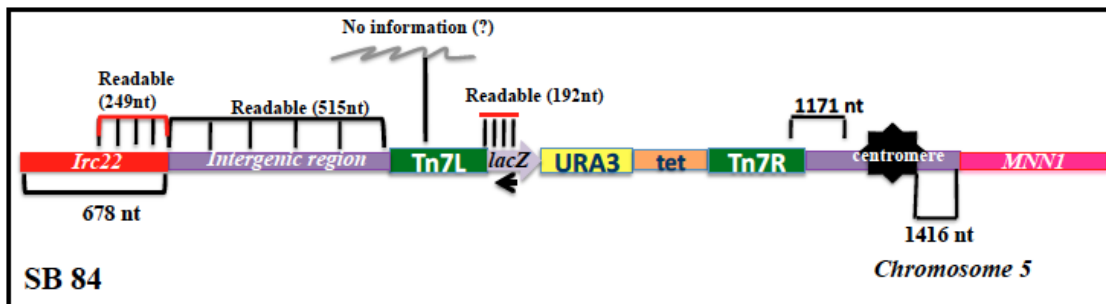


Figure 26. Genomic analysis of SB 84. Schematic map of transposon integration sites in SB 84. The yeast genome disrupted in SB 84 due to transposon integration is an intergenic region in chromosome V. The colored regions are same as in figure 11. Only differences are red and pink regions denoting flanking genes *Irc22* and *Mnn1* respectively; purple region denoting disrupted intergenic region.

Summary of SB 84 sequence analysis from direct genomic sequence:

Length of sequence aligned with yeast genome	Length of sequence aligned with intergenic region	Information on <i>lacZ</i> of Tn3	Information on LTR of Tn3	SGD coordinates	Centromere coordinates	Chromosome number	Disrupted region
201-967	515	192 nt	NO information	150,730 - 151,491	151987-152104	chromosome V	<i>Intergenic region</i>
9-201	193	NO information	NO information	NO information	NO information	Not determined	<i>Dof1</i> in <i>Zea mays</i>

3.7.2. SB 84 mutant exhibits delay in nuclear division progression at high temperature in liquid culture:

To analyze whether SB 84 demonstrates defect in meiotic nuclear division, a meiotic time course of mutant and WT strains was carried out at 23°C and 33°C (Figure 27A, B).

At both temperatures, the WT cultures started progressing through meiosis I at T=7hr whereas the mutant cultures did the same with a delay by 7 and 9 hours at 23°C and 33°C respectively. 62% and 22% of nuclei of SB 84 completed meiosis II at 23°C and 33°C respectively, suggesting its role in meiotic progression. This mutant therefore demonstrated a delay in nuclear division at 23°C and conferred a temperature sensitive defect in meiotic progression at 33°C.

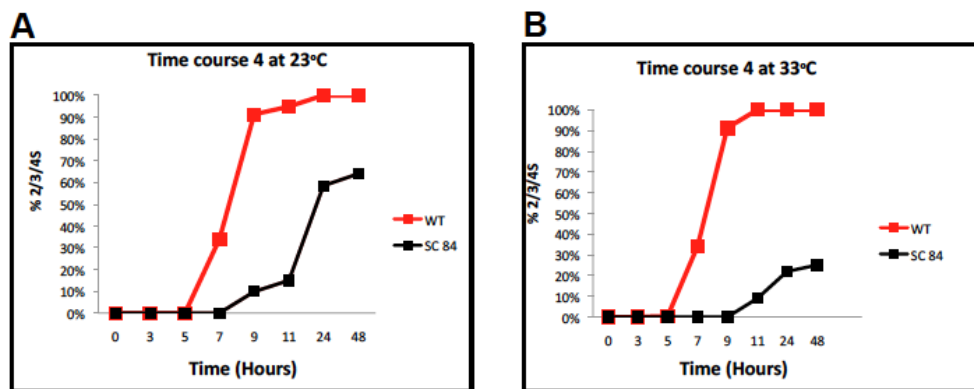


Figure 27. Meiotic progression of SB 84 at 23°C and 33°C. Meiotic time course of SB 84 in liquid culture at 23°C (A) and 33°C (B) for wild type cell culture (red), and SC 84 (black). X-axis denotes the time in hours and Y-axis denotes the percentage of cells undergone Meiosis I and/ or Meiosis II.

3.8 Phenotype validation and integration site identification of candidate with transposon integration in the 2 μ -plasmid.

I have identified a mutant candidate from Screen II where the transposon has integrated in the 2μ -plasmid of yeast. This candidate is named as Mutant 9 (Strain number SBY83).

3.8.1. Integration site identification of Mutant 9:

The direct genomic sequence data for Mutant 9 suggested sequence homology with coordinates 3,598 - 4,002 of yeast genome, which surprisingly corresponded with the sequence of a 2μ -plasmid (Figure 28). The sequence also provided information about the transposon specific *lacZ* gene (109 nt) although there was no information on the terminal repeat of transposon. However, the first 80-100 nucleotides provided an unreadable sequence suggesting a plausibility of carrying 38 bp transposon terminal repeat sequence. 2μ circle is a 4-6.3 kb circular, extrachromosomal element found in the nucleus of most *S. cerevisiae* strains. This plasmid does not exhibit any apparent selective advantage for the cells that carry it, but it is stably maintained at about 20 to 40 copies per haploid genome of the yeast cells. Surprisingly, in the genetic outcross assay for Mutant 9, 26 out of 27 transposon marker specific colonies, co-segregated with the phenotype. As 2μ -plasmid is extrachromosomal, it is intriguing to observe the Mendelian segregation pattern of Tn3 marker, which therefore raises the possibility of 2μ -plasmid integrating along with Tn3 in the yeast genome or carrying a second transposon sequence overshadowed by 2μ -plasmid. Nevertheless, identification of 2μ -plasmid reestablishes that the transposon mediated yeast genome disruption is a completely random event with no target site bias.

Summary of Mutant 9 sequence analysis from direct genomic sequence:

Length of sequence aligned with yeast genome	Length of sequence aligned with <i>2μ-plasmid</i>	Information on <i>lacZ</i> of Tn3	Information on LTR of Tn3	SGD coordinates	Disrupted gene
202- 606	406	109 nt	NO information	3,598 - 4,002	<i>2μ-plasmid</i>

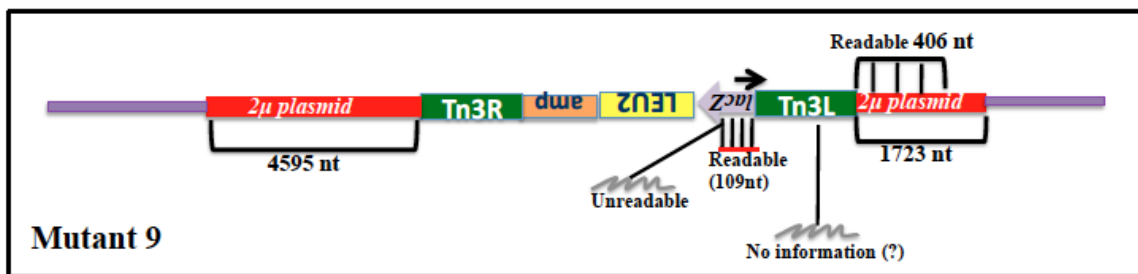


Figure 28. Genomic analysis of Mutant 9. Schematic map of transposon integration sites in Mutant 9. The yeast genomic region disrupted in Mutant 9 due to transposon integration is *2μ plasmid*. The colored regions are same as in figure 11.

CHAPTER IV

CONCLUSION

4.1. Pros and cons of my screen.

Transposon mediated genome wide screen is an appropriate genetic tool to generate disruption mutants involved in meiosis. As compared to the other methods of mutagenesis, transposon mediated insertional mutagenesis has several advantages. Using this approach it is easy to generate large number of mutations. The positions of mutations caused by transposon integration in the genome can in principle be identified by routine approaches like inverse PCR, vetorette PCR etc, direct genomic sequences, which have to be mapped by complementation compared to the mutants generated via EMS mutagenesis or UV irradiation. It is a viable and effective method for many types of large-scale studies. Nevertheless, this method of mutagenesis is not free from drawbacks. Transposon integration is completely random hence it is difficult to attain the complete coverage of genome, and also, it is not the right approach for generation of precisely

directed mutations, like the PCR methods of targeted start codon-stop codon gene replacements (Winzeler et al., 1999; Seringhaus M et al., 2006). Overall, this genome wide screen is a cost effective, efficiently analyzable, genetic approach, causing large-scale random insertions in yeast genome via transposon integration.

4.2. Synopsis of results obtained from the screen.

I have established genetic outcross assay, which is an appropriate genetic tool to confirm whether the phenotype conferred by the mutant was solely due to the transposon integration. This assay also aided in eliminating temperature sensitive mutants that were not caused by the transposon. Using this genome wide screen, I have re-identified *Zip3*, a known ZMM gene, and confirmed that the last 313 nucleotides of *Zip3* are essential for its role in meiosis. I have also identified a truncated version of *Sto1* (nt304), and as a result exhibiting ZMM like mutant phenotype. Its binding partner *Cbc2* is specifically involved in the splicing of one of the eight components of the ZMM complex, MER3 but apparently not in splicing of other meiotic genes, for example *Dmc1* (Qui et al., 2012). Another interesting mutant identified was Mut5-Tn3, which confers dramatic temperature sensitive mitotic and meiotic phenotype. I was unsuccessful in confirming the gene disrupted in this candidate as the sequence analysis suggested the truncated gene in this mutant as *Mam1*, although that was not reflected in Southern hybridization. Mutant 5 also demonstrated different behaviors from a *mam1* deletion mutant in subsequent phenotype analyses, which made the interpretation even more complicated. Apart from these gene-disrupted mutants, I have also identified two candidates demonstrating transposon integration in the intergenic region of chromosome V, and 2μ -plasmid respectively. Especially for the integration in chromosome V proximal to the centromere it will be

intriguing to determine whether a critical cis-acting sequence of a previously unknown ORF is affected.

4.3. Potential mutants for future analysis.

I identified four new candidates that are yet to be sequenced. One of these candidates is derived from Screen I, which exhibited temperature sensitive sporulation phenotype and confirmed that transposon is responsible for the phenotype via genetic outcross assay. In addition, I have identified 3 potential candidates from screen II. These mutants conferred temperature sensitive phenotype for sporulation, and also exhibited poor spore viability patterns—50%, 48% and 55% respectively. In the genetic outcross assay, the phenotype co segregated with the marker for transposon for these mutants. Once sequenced, these candidates can then be subjected to several experiments to unravel their behaviors in meiosis.

Among the sequenced mutants, confirmation of the exact site of transposon integration in Mut5(*mam1?*)-Tn3 is necessary, as it conferred interesting phenotypes for mitosis and meiosis. This mutation can then be reconstructed by PCR and then examined whether it results in the same expected phenotype. For, Mut8 (*sto1*)-Tn3, it is critical to investigate whether *Sto1* gene, like its binding partner *Cbc2*, is also directly involved in splicing of ZMM protein, intronless version of Mer3.

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APPENDICES

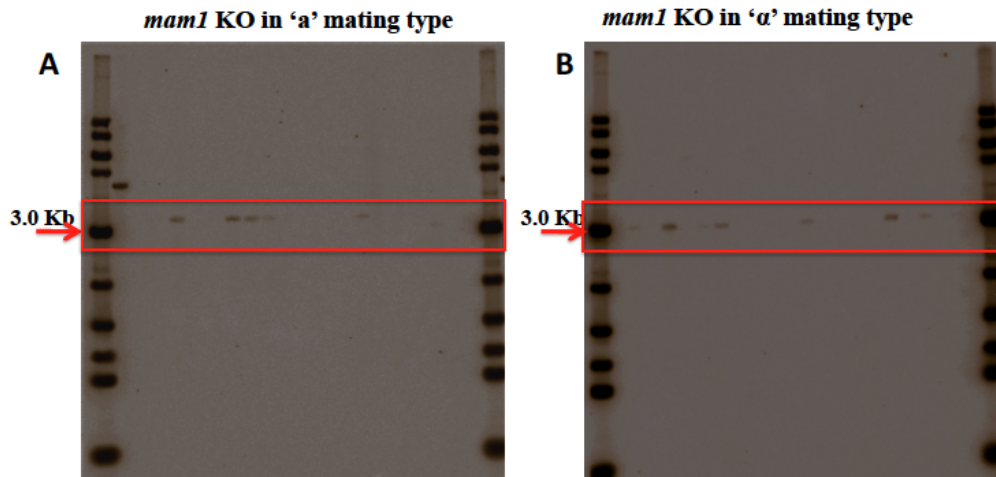
Yeast strains

VBY STRAIN #	DERIVED FROM	PREVIOUS NUMBER	TESTED	NHY derived	MAT	HO	HOTSPOT	LOCUS1	MARKER1	LOCUS2	MARKER2
SBY1	(VBY1429)	1416A			Ap,	HO;ste7-1,				promURA::tetR,	
SBY17					Ap,	ho::LYS2,	n.a.	zip2	::URA3,		
SBY41					Ap,	ho::LYS2,	n.a.	zip1	::LEU2,		
SBY61	NH290	(VBY785)	tc 64,65	187	A (1055),	ho::hisG,	HIS4::LEU2-(NBam),	ZIP3-GFP	::KanMX4,		
SBY62	(VBY1054)	NKY101			A		n.a.				
SBY63	(VBY1055)	NKY102			p		n.a.				
SBY65	VBY 313	(VBY328)			Ap,	HO;ste7-1,		ste7-1			
SBY66	890xNKY291 (VBY1056)	JennChung 1	G7114	VB890	p (1054),	ho::hisG,	his4X::LEU2-(NgoMIV;ori)-URA3,				
SBY67	891xNKY292 (VBY1066)	JennChung 11	G7114	VB891	A (1055),	ho::hisG,	HIS4::LEU2-(NBam;ori),				
SBY68	(VBY1106)	NHY361			A (1055),	ho::hisG,		CENIII	::URA3,		
SBY78	MUT4				Ap,	HO;ste7-1,		ste7-1		zip3	::LEU2,
SBY79	MUT5				Ap,	HO;ste7-1,		ste7-1		mam1?	::LEU2,
SBY82	MUT8				Ap,	HO;ste7-1,		ste7-1		sto1	::LEU2,
SBY83	MUT9				Ap,	HO;ste7-1,		ste7-1			::LEU2,
SBY117	890xNKY291	JennChung 1	G7114	VB890	p (1054),	ho::hisG,	his4X::LEU2-(NgoMIV;ori)-URA3,	mam1	::KanMX4,		
SBY165	891xNKY292			VB891	A (1055),	ho::hisG,	his4X::LEU2-(NgoMIV;ori)-URA3,	mam1	::KanMX4,		
SBY270	SB 84	1416A			Ap,	HO;ste7-1,				promURA::tetR,	URA3
SBY251	(VBY1429)	1416A			Ap,	HO;ste7-1,		Tn7	::URA3,	promURA::tetR,	
SBY252	VBY 313	(VBY328)			Ap,	HO;ste7-1,		Tn3	::LEU2,		

Table continued:

VBY STRAIN #	COMMENTS	HIS4	URA3	LEU2	LYS2	TRP1	ADE2	ARG4	OTHER
SBY1	four spores from VBY1416 tetrads were streaked to leu- plate, this clone was best for GFP dots								
SBY17		his4,	ura3,	leu2::hisG,	lys2,	trp1::hisG,			
SBY41		his4,	ura3,	leu2::hisG,	lys2,	trp1::hisG,			
SBY61									
SBY62	mating type tester, S288c								ade8
SBY63	mating type tester, S288c								ade8
SBY65	grown at 34C before freezing		ura3,	leu2,	lys2,	trp1,	ADE2+		
SBY66	backcross to improve spore viability of 890x891		ura3(Δ sma-pst::hisG)	leu2::hisG,					
SBY67	backcross to improve spore viability of 890x891		ura3(Δ sma-pst::hisG)	leu2::hisG,					
SBY68						NHY361			
SBY78	grown at 34C before freezing		ura3,		lys2,		ADE2+		proved by Southern Blot
SBY79	grown at 34C before freezing		ura3,		lys2,		ADE2+		proved by Southern Blot
SBY82	grown at 34C before freezing		ura3,		lys2,		ADE2+		proved by Southern Blot
SBY83	grown at 34C before freezing		ura3,		lys2,		ADE2+		
SBY117	backcross to improve spore viability of 890x891		ura3(Δ sma-pst::hisG)	leu2::hisG,					proved by Southern Blot
SBY165									proved by Southern Blot
SBY270	four spores from VBY1416 tetrads were streaked to leu- plate, this clone was best for GFP dots								
SBY251	four spores from VBY1416 tetrads were streaked to leu- plate, this clone was best for GFP dots								Not TS
SBY252	grown at 34C before freezing		ura3,	leu2,	lys2,	trp1,	ADE2+		Not TS

Figure



Southern hybridization of *mam1*-knock out (KO) hotspot strains: (A) Genomic DNA samples isolated from *mam1*-KO in 'a' mating type yeast strains digested with XhoI and BamHI. (B) Genomic DNA samples isolated from *mam1*-KO in 'α' mating type yeast strains digested with XhoI and NgoMIV. Red box in (A) indicates the DNA samples with 3.3Kb bands, and in (B) the samples with 3 Kb band.