Doctoral thesis

SUN domain protein, Mps3 localization and regulation on nuclear envelope (NE) of Yeast Meiosis

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Abbreviations

5-FOA	5-fluoroorotic acid
AE	axial elements
AP	alkaline phosphatase
ATP	adenosine triphosphate
BSA	bovine serum albumin
°C	degree Celsius
CAR	cohesin-associated regions
Cdks	cyclin-dependent kinase
CE	central elements
CIP	Calf-intestinal alkaline phosphatase
CK1	casein kinase 1
CO	crossover
CPC	chromosomal passenger complex
ChIP	chromatin immuno precipitation
DAPI	6'-diamidino-2-phenylindole
D-Loop	displacement Loop
DDK	Cdc7/Dbf4 kinase
dHJs	double holiday junctions
DNA	deoxyribonucleic acid
DSB	double-strand break
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
FACS	fluorescence-activated cell sorter

GFP	fluorescence protein
HJ	Holliday junction
HR	homologous recombination
HRP	horse radish peroxidase
INM	inner nuclear membrane
JMs	joint molecules
KD	kinase-dead
LE	lateral elements
LINC	linker between nucleoplasm and cytoplasm
MI	meiosis I
MII	meiosis II
MES	2-(N-morpholino) ethane sulfonic acid
mJ	mille joules
ml	microliter
NCO	non-crossove
ONM	outer nuclear membrane
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	polycomplex
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
PLK	polo-like kinase
PP2A	protein phosphatase 2A

pre-S	Pre-meiotic S phase
RFP	red fluorescence protein
RNase	ribonuclease
SAC	spindle assembly checkpoint
SAM	S-adenosylmethionine
SC	synaptonemal complex
SCC	sister chromatid cohesio
S.D.	Standard deviation
SEI	single-strand invasion intermediate
SICs	synapsis initiation complex
SMC	structural maintenance chromosome
SPB	spindle pole body
S.V.	spore viability
TAE	Tris-Acetate
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
UV	ultraviolet
V	Volt
WB	western blotting

Summary:

Chromosome movements and Nuclear envelope (NE) remodeling are the important events in Meiotic Prophase-I (Early stage of meiosis-I). After entry into the meiotic prophase of yeast (*Saccharomyces cerevisiae*) cells, telomeres move on nuclear envelope and attaches to NE as well as forms transient clusters near the vicinity of Spindle pole body (SPB), equivalent to the mammalian centrosome. At early meiosis-I, a lot of key changes occurs on NE such nuclear envelope remodeling (Movements, deformations and enlargements). Telomere movements such as clustering and declustering were triggered by an INM (Inner Nuclear Membrane) protein, Mps3 (It has conserved SUN domain with Sad1-Unc84 homology). Nuclear envelope bound Mps3 tethers telomeres to the nuclear envelope (NE), which promotes chromosome motion inside the nucleus; this chromosome motion may promote the alignment and pairing of homologs, important for the homologous recombination as well as new offspring formation. Key feature of Mps3, it can change its cellular localization from mitosis to meiosis. During mitosis (vegetative growth of cell), Mps3 mostly localizes to SPB and when cells enter into meiosis, Mps3 forms multiple foci and clusters on NE. Important event during nuclear envelope remodeling is NE localization of Mps3. Biological significance and molecular mechanisms behind the NE remodeling, thus Mps3 localization on nuclear envelope (NE) during meiosis is poorly understood. I have been looking to find both positive and negative factors, which can regulate NE localization of Mps3 during meiosis. Then, identified that Rec8, it is a meiosis-specific klesin component of cohesion complex involved in sister chromatid cohesin, is important for NE localization of Mps3. I

identified that mitotic klesin, component of cohesin complex such as Scc1/Mcd1 over-expression during meiosis, can only complement the Mps3-NE localization function but not other meiotic events/functions. However, Scc1 cannot be sufficient to promote Mps3-NE localization in mitotic cells. Scc1contribution towards Mps3-NE localization is independent from mitosis to meiosis. I design and construct an experimental system, which can express Rec8 even in mitotic yeast cells and found that the Rec8 is sufficient to promote Mps3-NE localization in mitotic cells. Through meiosis-specific cohesin component, Rec8 mediated Mps3-NE localization process in mitosis, I have also identified the other factors such as Mps3 consensus sequence such 188T, 189S and 190S region also critical Mps3-NE localization. Which clearly shows that *mps3-AAA* (phosphor defective) mutants with defective phosphorylation condition, Rec8 expression system, not able to induce Mps3-NE localization but it clearly increases similar to wild-type levels in *mps3-DDD* (phosphor-mimetic) mutant. Mps3 N-terminus domain and Acidic domain, which are present in the nucleoplasmic region of Mps3, were contributing to Mps3-NE localization. Possibly, interaction with Rec8 might be critical for the regulation of nuclear envelope Mps3. *mps3Δ2-64 & mps3Δ65-145* domains of Mps3 are essential for the Mps3-NE localization during both meiosis as well as mitosis. Interaction of these regions of Mps3 with chromatin can contribute the chromosome movements (RPMs: Rapid Prophase Movements) inside the nucleus. Nuclear pore complexes (NPC) are large protein complexes present in the nuclear envelope and contribute a key role in nuclear envelope remodeling. Some of the nuclear pore proteins or nuclear porins promotes NE remodeling by differential regulation of Mps3 (Both positive or negative

regulation of Mps3). In this study, identified Nup157, pom152 negative regulators for Mps3 localization on NE, these mutants show complete coverage of Mps3 on NE in vegetative cells, which is not seen in wild-type cells. Nup42 and Nup53 have a minimal contribution in nuclear envelope remodeling. I also identified Nup60 is positive regulator for Mps3-NE localization. During early meiosis (similar to mitosis), *nup157Δmps3Δ2-64 & nup157Δmps3Δ65-145* shows only SPB localization.

Interestingly, after induction of meiosis, *nup157Δmps3Δ2-64 & nup157Δmps3Δ65-145* cells shows NE localization of Mps3 observed in meiotic time course analysis. Also identified two different regulations of Mps3 during meiosis. Mps3 N-terminus dependent as well as N-terminus independent meiosis-specific positive regulation of Mps3 under nucleoporin background cells. I have been addressing how Rec8 promotes Mps3-NE localization along with other factors such as Mps3 two domains of nucleoplasmic regions. I speculate that the interaction of Mps3 with Rec8, the meiosis-specific cohesin in nucleoplasm is a key factor for nuclear envelope localization of Mps3. These, suggest that chromosomes are the key regulator for NE functions such as NE remodeling.

Chapter-1

Introduction:

From last few decades many scientists are trying hard to find out/understand the complete molecular events such as homologous recombination, DNA repair, Axis and lateral elements emerging, synaptonemal complex formation, Cohesion role in chromosome segregation, chromosome motion such as RPMs during prophase-I of meiosis, Nuclear envelope remodeling and many more aspects during meiotic cell division. Many of the important events during meiotic cell division have been addressed already; complete molecular mechanisms and regulation of all these events are far away from full understanding.

In this research, I am addressing the localization and dynamics of the nuclear envelope protein, Mps3 during budding yeast meiosis by various regulators and how those factors can control and influence many more basic important events during yeast meiosis.

Budding yeast as a model organism:

Saccharomyces cerevisiae is the most simple, unicellular, eukaryotic organism. Most of the important functional mechanisms and essential features are conserved among yeast and higher vertebrates. Yeast has total almost 6000 genes with 12Mbp chromosomes were well packed and organized in a nucleus (Herskowitz, 1988). Cell division and much other fundamental biological significance are similar in yeast and humans. Due to these many similarities and simple understanding, yeast is the best model organism to figure out the detailed mechanistic evidence of meiotic cell division. During my

complete Ph.D. study, I used budding yeast as a model organism.

Meiosis:

Meiosis is a specialized differentiation process that generates haploids from diploid cells. Meiosis is essential to maintain constant number of chromosomes from generation to generation in sexually reproducing organisms. To completely understand such a complex mechanism, to primarily we should understand cell division in somatic cells (Mitosis). Cell cycle (Mitosis) separated into G1-phase, S (DNA-synthesis) phase, G2-phase and M(mitotic)-phase. DNA replication/synthesis occurs in the S phase, chromosomes separation and cytoplasm division happens in M phase. Meiotic cell division separated into G1, S, G2 and M phases; there are many differences during chromosomes duplicate.

Differences from mitosis to meiosis:

MITOSIS	**MEIOSIS**
Occurs in every somatic cell.	Present exclusively reproductive cells
Chromosome (ploidy) number remains the same, its diploid (2n), hence it is called as equational division	Chromosome (ploidy) number reduces to half, its haploid (n), hence it is known as reductional division.
Results in to produce two daughter cells.	Produces four new daughter cells.
A single round of cell division occurs	Sub-divided into two divisions; MI and MII: each division involving 4 phases.

with containing four phases.	Prophase-I contains five (5) sub-stages again.
No crossover formation occurs.	Crossing over formation happens here.
New offsprings contain identical chromosome number to their parental cell (No genetic variation)	Offsprings contain chromosomes with mixed genetic material from both male and female parents (Genetic variability).

The successful completion of each stage of the cell cycle mostly depends on the completion (without errors) of the earlier stage. This kind of well-organized cell cycle division is governed/controlled by a family of proteins, which are highly conserved. These families of proteins ensure/maintains proper chromosome number in the new offsprings in both mitosis as well as meiosis. Errors/defects at the time of meiotic cell division can form a new generation of gametes with aneuploidy; these are the main reasons for miscarriage of genes, infertility and congenital disabilities.

Importance of meiosis:

Chromosome number should become half during meiosis (reductional division). Without this process newly formed zygotes have double the number of chromosomes, for every successive cell division after fertilization, DNA content will increase exponential way as well as no genetic diversity will be seen in new offsprings. So, without the meiosis process, new progeny looks identical with their parents with the more chromosomal count. This situation is

lethal (deleterious) because the entire organisms in nature should exist if they follow survival of the fit rule from Darwinian evolutionary theory.

Budding yeast (*Saccharomyces cerevisiae*) meiosis:

Budding yeast exists in different cell/mating types. 2 mating types are haploid (n) with an opposite-sex partner such as mating type a and α**.** Both mating cell types a & α cells mate efficiently in 4hour and diploid cells will form by the fusion of a and α mating cell types. This diploid cell has one copy of the genome from each parent (Mat-a and Mat-α). This diploid cell has unique capacity of growing such as during vegetative growth diploids will undergo mitosis, and under nutrient starvation situations, cells enter into meiosis and form haploids (n).

After induction of meiosis, yeast cells carry out one round of DNA replication (to create exact copy/replica of entire genome) followed by two rounds of chromosome (Nuclear divisions) segregations (Kerr *et al*., 2012), such as meiosis-I and meiosis-II. Paternal and maternal chromosomes segregate in opposite directions during meiosis-I (Marston and Amon, 2004) and sister chromatids segregate in meiosis-II (This second division of meiosis is similar to mitosis). During primary meiotic division, homologs chromosomes undergo various modifications. Importantly, Faithfull segregation of homologous chromosomes depends on physical linkage between the chromosomes, this physical linkage cytologically observed as Chiasmata. Formation of chiasma depends on the exchange of genetic material between the paternal and maternal chromosomes, resulting in new recombinant crossover (CO) events occur. This CO creates/generates genetic diversity in new offsprings.

Meiosis is divided into Meiosis-I and Meiosis-II.

Meiosis-I is mainly sub-divided into these five parts.

Prophase-I

Metaphase-I

Anaphase-I

Telophase-I

Meiosis-II [Equal to mitotic (Equational) cell cycle division]

Meiotic prophase-I:

At first meiotic division, such as prophase-I has an important feature, changes in the arrangement of chromosomes, along with this many other critical structural and functional changes take place during the complete prophase-I. Importantly, the formation of chiasma occurs between the homologous chromosomes by physical linkage, exchange of genetic material (recombination) occurs between homologous, noticed as crossover recombination. To keep/maintain the homologous chromosomes connected by the formation of the SC (Synaptonemal complex). Along with the recombination, Chromosomes are paired or aligned together, telomere cluster near the vicinity of centrosomes (SPB: Spindle pole body). Prophase-I of MI is further sub-divided, according to the morphology of chromosomes (Zicker and Kleckner, 1998).

Leptotene

Zygotene

Pachytene

Diplotene

Diakinesis

Leptotene:

The first step of prophase-I, Leptotene stage, Chromatin condenses into chromosomes and resulting in the formation of multiple chromatin loops at chromosome axis (axial elements) in the nucleus. Homology search of chromosomes occurs. Formation of DSB starts to initiate meiotic DNA recombination. Telomere starts attaching towards nuclear envelope. DNA recombination machinery such as Rad51 & Dmc1 loaded on the single standard DNA (Zickler & Kleckner, 1998; Borner et al.; Page & Hawley, 2004)

Zygotene:

After the leptotene stage, axial elements of homologous chromosomes become smaller & denser, stay together both in the pairing. Between axial elements of homologous chromosomes, small stretch of ladder-like structure (SC) formation occurs. Recombinases (Similar to DSBs) are the protein complexes involved in meiotic recombination at an early stage of zygotene. These DSBs associated with chromosome axis (Zickler & Kleckner, 1998), SEI (single strand invasion intermediates) form between ss.DNA and duplex DNA. Telomeres forms cluster near centrosome (SPB in yeast) is known as “telomere bouquet.” Here, telomeres attached to NE and form a loose cluster on the nuclear envelope near the spindle pole body (Zickler and Kleckner, 1998). RPMs (Rapid Prophase chromosome Movements) occur in zygotene.

Pachytene:

During pachytene, full synapsis occurs between the entire lengths of homologous chromosomes, this step known as SC elongation, leads to the

formation of complete SCs. This process mentioned as “Synapsis.” Later/late stage of pachytene full-length SC contains paired axes of lateral elements, these interconnected to transverse filaments (Page *et al*., 2004). SEI captures another end of DSB and to form DHJs (Double Holiday Junctions, Kerr et al., 2012). Telomeres also detach from centrosome (SPB) & spread on nuclear envelope.

Diplotene:

Decondensation of meiotic chromosomes starts in the diplotene stage of MI. Mostly, Synaptonemal complex (SC) disassembles. Homologous chromosomes separated except the chiasmata area, which keeps the chromosomes together complete diakinesis step before the onset of metaphase-I (Page and Hawley, 2004). Centrosomes separate at opposite poles.

Diakinesis:

Chiasmata are more clearly visible to ensure proper segregation of chromosomes. The remaining stage is closely similar to mitotic pro-metaphase. Nucleolus disappears, and NE breaks down starts and then proceeds further for metaphase.

During metaphase of meiosis-I, Microtubule guided chromosome motion occurs near kinetochores. Spindle fibers from two opposite poles are attached to the two centromeres and maintain equal tension between the homologs. The protein complex, meiotic cohesin will be cleaved between the sisters by the protease activity of Separase. A protein called as Shugoshin near kinetochore protects sister’s separation at the centromeric region. It

leads to the separation of homologs at opposite poles during meiosis-I.

Meiosis-II:

Sister chromatids (2 daughter cells) are separated at meiosis-II by the degradation/removal of centromeric cohesion and Shugoshin protein (Marston & Amon, 2004). Meiosis-II is similar to an equational cell division leads to the creation/formation of four haploid daughter cells.

Roles of telomere bouquet in meiosis:

In yeast, Centromeres are together, and forms cluster in single spot vicinity to the SPB and then telomeres are present in the opposite direction to centromere clustering with Rabl orientation in interphase of mitotic cell cycle division (Klein *et al*.). In meiosis, Budding yeast telomeres grouped and forms loosely constrained clusters, which tethered to NE (nuclear envelope). When vegetative diploid cells introduced into SPM (sporulation medium), then cells undergo drastic cellular re-organization. Clustering of telomeres and declustering of centromeres are completely independent events. Telomeres associate with the centrosome [spindle pole body (SPB)]. Telomeres associated into a single cluster in a small area on nuclear envelope (NE) (Roeder *et al*., 1997, Dawe *et al*., 1998 & McKee *et al*., 2004) and remaining chromosomes loop inside the nucleus, then resulting structure cytologically resembles flower bouquet. So, telomere clustering during meiosis has been named as “telomere bouquet.” This process occurs during mid-prophase-I (Zygotene) [Trelles-Sticken *et al*., 1999] and declustering of telomeres occurs at late prophase-I (Pachytene) [Roeder *et al*., 1997]. This telomere bouquet is highly conserved among most of the eukaryotic organisms (Ding *et al*., 2004;

Chikashige *et al*., 2006) and occurred temporally during the meiotic cascade. Telomere bouquet formations, as well as associated chromosome movements, have been promoted homology search process between chromosomes (Roeder *et al*., 1997; McKee *et al*., 2004). Same time, meiotic DSBs processed into dHJs (double Holiday Junctions), and bouquet formation and dissolution of the bouquet are highly regulated. It implicates the significance of telomere bouquet structure during meiosis. In budding yeast, the dissolution of telomere clustering causes a delay in homologous chromosome pairing and reductions in DSBs repair (Roeder *et al*., 1997; McKee *et al*., 2004). Various groups have studied mechanistic evidence of telomere bouquet extensively. In the *S.pombe* telomere binding proteins Rap1 & Taz1 promotes the binding of telomeres to the NE (nuclear envelope) [Cooper *et al*., 1998; Nimmo *et al.,* 1998; Kanoh *et al.,* 2001; Chikashige *et al.,* 2001]. Some other proteins Bqt1 and Bqt2 (Chikashige *et al.,* 2006) also required for telomere bouquet establishment.

Budding yeast telomere clustering:

Saccharomyces cerevisiae, telomere motion mainly depends on a meiosis-specific Ndj1 (telomere binding protein) [Scherthan *et al.,* 2007]. Previously, it was well explained that other meiosis-specific expression of Csm4, makes a complex with Ndj1. Complete deletion of *csm4* mutant, telomeres attached to the NE, they are not able to form a cluster (Kosaka *et al*., 2008). It explains that meiotic telomere clustering needs various protein complexes on NE. Importantly, budding yeast SUN (Sad1p, UNC-84 homology) domain protein, Mps3 plays a key role in telomere bouquet organization (Conrad *et al.,* 2007).

Mps3 and telomere association will provide a connecting link (mechanistic evidence) between the nuclear migration and telomere bouquet structure. It has reported, SUN domain deletion of Mps3 exhibits fewer/minimal defects/errors during telomere bouquet, chromosome motion got altered/changed & reductions in chromosome pairing were noticed (Rao *et al.,* 2011). It gives an idea that, bouquet creation does not completely rely on SUN domain of Mps3. It triggers the high possible interpretation; meiosis-specific proteins interaction with Mps3 could play an important role in telomere clustering/bouquet. It needs Ndj1, Csm4 as well as Mps3 (Conrad *et al.,* 2008). SUN proteins highly conserved from yeast to higher vertebrates.

Rapid Prophase chromosome Movements (RPMs):

A few decades ago in the mid-70s, during diakinesis stage such as late prophase of meiosis, chromosome motion or movements were noticed with time-lapse photography/microscopy (Rickards *et al.,* 1975), it occurs before the NE breaks down. Rickards observed that homologous chromosomes movements are frequent. He also suggested, chromosome movements observed during prophase of meiosis-I are much different than chromosome movements during anaphase, prophase chromosomes move with great speed than anaphase chromosomes. In 1976, Parvinen and Soderstrom were noticed similar chromosome movements during prophase-I of rat spermatogenesis. Also noticed that these movements of chromosomes were active during early zygotene (prophase), reduced during mid pachytene. In *Nephrotoma* (crane fly), prophase chromosome movements were identified during the late stage of diakinesis (Lafountain *et al*., 1985). Later the same

person found that this prophase chromosome motion is independent to the microtubules (Lafountain *et al.*, 1999). Modern facilities provide us to observe prophase chromosome dynamics in higher eukaryotes. Live imaging of transgenic mice spermatocytes was also visualized and observed prophase chromosome movements (Morelli *et al.,* 2008).

SUN-KASH dependent chromosome movements in Yeast:

In fission yeast (*S. Pombe*), Horsetail motion of the complete nucleus was well characterized/understood (Bahler *et al*., 1993; Chikashige *et al.,* 1994). In *Saccharomyces cerevisiae*, Rapid chromosome movements (RPMs) noticed by different research groups (Conrad *et al.,* 2008; Koszul *et al*., 2008, Scherthan *et al.,* 2007). Explain chromosome motion during meiotic prophase, one model/theory got widely accepted in present days. Which are SUN and KASH protein interacting LINC complex, connects/links between the interior and exterior regions of NE? SUN proteins are located in (INM) inner nuclear membrane and have been proved to interact with KASH proteins, mainly found in the outer nuclear membrane (ONM), thereby connecting nucleoplasmic structures with cytoplasmic structures. These KASH & SUN proteins are well conserved, also shows their function in the positioning of the nucleus, nuclear migration as well as attachment to the SPB (Centrosome) at development stage (Starr & Fischer, 2005; Tzur *et al*., Wilhelmsen *et al*., 2006). Chromosome movements in fission yeast were dependent on dynein and microtubules (Chikashige *et al.*, 2006 and 2007). These movements in budding yeast are dependent on Actin. During the chromosome motion phase, telomeres form clusters and present together with the SPB (Bahler *et al.,* 1993; Chikashige *et al.,* 1994; Svoboda *et al.* 1995), pairing of homologs

and genetic (Homologous) recombination also occurs at the same stage.

RPMs in budding yeast meiosis:

In *S. cerevisiae,* meiotic RPMs were first noticed in the pachytene stage by visualization of the SC central element tagged with green fluorescent protein, Zip1-GFP (Scherthan *et al.,* 2007). Chromosomes show drastic, continuous motion inside the nucleus (leads to twisting, folding, bending & unfolding occurs). The chromosome movements triggered a change in the nucleus shape; inhibited by Latrunculin B (Lat-B), works opposite to actin polymerization. During meiosis, the motion of the chromosome needs a meiosis-specific expression of Ndj1 (Telomere binding protein), which is involved in the interaction of telomeres with NE. Chromosome movements were coined as RPMs (Rapid prophase movements) by Conrad et al., 2008. RPMs start in early prophase then peaks level at zygotene and pachytene, later it disappears. Detailed mechanisms for RPMs were less understood. *CSM4, MPS3 & NDJ1* is necessary for RPMs during meiotic prophase-I. (Conrad *et al*., 2007, 2008).

Role of prophase chromosome movements:

Various models were believed to explain the RPMs role. The primary idea of RPMs to promote homology search is thought to facilitate telomere bouquet structure; initial homology contact occurs by limiting the dimensionality of homology search (Conrad *et al.*). RPMs are important to stop or minimize the ectopic recombination in meiotic prophase. Indirectly it promotes homologous chromosome alignment/pairing. RPMs are mainly functioning in to relieve an en-trapped chromosome in between the ends of the chromosomes at mid and

late-prophase of meiosis (Koszul *et al*.). It also provides 'stirring forces' at early prophase of meiosis to facilitate or promote homology search.

Chromosome movements are well conserved in higher organisms:

Meiotic prophase chromosome movements were also observed in higher eukaryotes such as *C. elegans, Drosophila &* mice (Vazquez *et al.,* 2002; Morelli *et al.,* 2008). This chromosome motion mediated by cytoskeletal forces (SUN-KASH interaction). In *C. elegans,* NE-protein matefin (MTF-1/SUN-1) interacts with KASH protein, ZYG-12 in meiotic nuclei. Lack of matefin causes chromosome-pairing defects (Penkner *et al*., 2007). In *Drosophila*, chromosome motion was also observed (Vazquez *et al.* 2002). Mice have three different SUN proteins, SUN1, SUN2, and SUN3. SUN1 & -2 promotes telomere and NE attachment during prophase of meiosis (Ding *et al.,* 2007, Schmitt *et al.,* 2007). In higher eukaryotes organisms, SUN-KASH interaction is conserved. The exact function of SUN protein during meiosis is not well characterized.

Role of SUN proteins:

NE separates the nucleus from the cytoplasm in all eukaryotes. NE contains two lipid bilayers named as INM and ONM (inner & outer nuclear membrane), which interns linked at large protein complexes, protruding inside the NE named as NUPs (nuclear pores). These nuclear pores regulate macromolecular trafficking in between the nucleus and cytosol. Most of the nuclear membrane proteins connected with a family of proteins, known as lamins and lamin-associated complexes, which can facilitate various functions. Many of these proteins assumed to be connected with lamins either

directly or indirect manner. These NE-proteins divided into LEM, KASH and SUN proteins (LEM domain proteins LAP2 and emerin). SUN domain proteins connected with KASH domain proteins on NE. Many SUN-proteins situated on INM. KASH proteins localized on either ONM or INM. Some organisms lack the KASH-proteins or do not localize to NE. Various INM bound SUN-proteins & KASH- proteins can interact on NE. It will use cytoskeletal-mediated forces and promotes telomere clustering and chromosome motion during mid-prophase-I of meiosis. SUN-protein number elevated during evolution. Example, in fission yeast genome has only one SUN-protein coding gene, In C. *elegans* & *Drosophila* (common fruit fly) having 2 SUN proteins. Mammals have 4 SUN protein isoforms. SUN-proteins have minimum one common trans-membrane domain. It has the active property of proteins that believed to anchor the structures and generates mechanical forces. Human SUN1 spans its membrane thrice. SUN1 has an extra hydrophobic portion, but the function of this domain/region is not known. *Caenorhabditis elegans,* SUN- proteins have a minimum single coil-coiled region, which is proposed to mediate/regulate homo-dimerization.

Mps3 functions:

Mps3p is a SUN protein, which contains the SUN domain, present in the INM of budding yeast. Mps3 is highly conserved among the higher eukaryotes, necessary for SPB (Yeast centrosome) duplication & importantly involved in the formation of half bridge. (Jaspersen *et al.*, 2002; Nishikawa *et al*. 2003). A previous report explains that Mps3 and NE directly show functions in chromatin metabolism. It also has a critical role in sister chromatid cohesin by binding to Ctf7, is a cohesion establishment factor (Antoniacci *et al*.). It also

has roles in karyogamy. Mps3p localizes towards SPB during mitosis as well as the early stage of meiosis, during meiosis phase MPS3 forms/makes patches on entire NE (Conrad et al., 2007). No KASH protein is available in budding yeast; it was not understood that how MPS3 and ONM structures are linked (Conrad *et al*., 2007). Interestingly, Mps3 shows big patches on the NE during meiosis (Conrad *et al*.; 2007), it is essential for the RPMs. The big unanswered question is, how Mps3 forms big patches on NE during meiosis. I found some regulators, which can both positively, and negatively regulate Mps3 on NE, I will explain in a more detailed way in my results section.

Structure of Nuclear pore complex (NPC):

The nuclear envelope (NE) distinguish/separates the genome from the remaining region of the cell. NE composed of two specific membranes. INM with faces towards nucleoplasm and ONM with faces towards the cytoplasmic region. A gap named as perinuclear space separates ONM and INM. Molecular traffic among nucleoplasm and cytoplasm maintained through a specialized, large circular protein apertures located at the regions where ONM & INM joins together. These large apertures termed NPCs (nuclear pore complexes). These are one of the largest proteinaceous structures inside the cell, with about 50MDa size. Made with multiple copies of nearly 25~30 different proteins termed as nucleoporins (Nups). NPC consists of main two functional regions, the central core structure, which embedded in the nuclear envelope. NPC peripheral regions, which extend to reach the NPC towards both the nuclear interior and the cytoplasm exterior regions. It can ensure the formation of the channel to promote nuclear transport and works as a molecular sieve to control the bidirectional transport system of

macromolecules as well as other metabolites. NPC extensions consist of filamentous (no symmetry) structures that link to the NPC core region to either inside nucleus or inside the cytoplasm. Nuclear basket structure links NPC to nuclear metabolic events, such as RNA biogenesis & to maintain genome integrity.

Functions and classes of Nups:

Nups are be sub-divided into four major classes. Transmembrane nups (POM: pore membrane), core nups, linker nups and Phe-Gly (FG) nups. From yeast to higher vertebrates, three transmembrane nups span along with pore membrane and contain an outer TM-ring (luminal ring). That ring anchors NPC to the NE. About one dozen of core scaffold Nups forms the outer and inner rings. This scaffold is formed by different stable (biochemically) and conserved NPC sub-complexes, which seems to have an important role in NPC biogenesis (Ex: Conserved *S. cerevisiae* Nup84 & Nup170 complexes and respective homologs in higher eukaryotes, such as NUP170, NUP160, and NUP35-NUP155 sub-complexes). Ndc1 has a role in the insertion of both SPBs and NPC into the NE. Largely unfolded FG Nups, which forms the central tube from the nucleus to the cytoplasmic region. Later linker Nups promotes a bridge between the core and FG Nups (Caterina Strambio-De-Castillia *et al.*). Some NPC components might have the capacity to alter the nuclear membrane or stabilizes specific membrane conformations (Alber *et al.* 2007). In *S. cerevisiae* (budding yeast), NPCs are enriched near the vicinity of SPB (Heath *et al.* 1995; Winey *et al.* 1997; Kilmartin and Adam 1999). Importance of this phenomenon is not known.

Nuclear envelope remodeling:

During cell division, nuclear envelope breakdown and reformation occurs, this process coordinates many events during the cell cycle. NPCs have temporally coordinated assembly in a way that relies on cell cycle progression and NE life cycle. Also, the basket could have a function in chromosome orchestration events that happened in mitosis of yeast to metazoans. Basket involvement in cell cycle regulation has been noticed in humans, fruit fly (*D. melanogaster)* and *Aspergillus* cells. In human cells, Translocated promoter region (Tpr) directly binds to MAD1, MAD2. siRNA (small interfering RNA) based depletion of TPR disrupts the MAD1, and MAD2 localization at the NPC during interphase leads to impaired anaphase. In fruit fly (*D. melanogaster),* MAD1, MAD2, and MPS1 localizes near to the spindles (form complex with megator) and controls spindle assembly checkpoint response. At the mitotic exit, MAD1 and MAD2 imported into nucleus and interaction with the NE occurs only after Megator recruitment to the nascent (newly formed) NPC. Functional similarity of the connection between the basket and the SAC observed. We have not understood that how the connection between the NPC and the control of cell cycle progression (exist) regulated. In *Saccharomyces cerevisiae* has complex NE environment, which contains chromatin, integral, peripheral membrane proteins, large protein structures known as NPCs and the SPB (spindle pole body). Higher eukaryotes like humans, NE breakdown, gives them access to spindles with chromosomes, NE disassembles at the time of mitotic entry of yeast cells and reassembles/remodeling occurs upon mitotic cell cycle exit.

Meiotic Homologous recombination (HR):

Homologous (Genetic) recombination is the key event during meiosis. It creates a physical linkage between the paternal and maternal (homologous) chromosomes, called as reciprocal recombination, create crossovers (Roeder *et al.,* 1997); this needs a specialized s recombination machinery pathway makes Connections and creates genetic variations or diversity. Initiation of recombination is occurred by creation DSB, catalyzed by Spo11 (Klapholz *et al.,* 1985; Keeney *et al*., 1997; Handel *et al*., 2010). MRN complex removed Spo11 and resulted in 3'-overhang ss.DNA extended by helicases (Krogh *et al.,* 2004). RecA homologs such as Rad51 & Dmc1 bind on ss.DNA, Cytological & genetic evidence shown that Rad51 & Dmc1 might bind to opposite sides of each DSB. These recombinases create filamentous structures on DNA (Shinohara *et al*., 1992; Bishop *et al*., 1992). Displacement loop formed by the invasion of ssDNA into duplex DNA (Krogh *et al.,* 2004; Lao *et al.,* 2010). D- loops are converted into recombination intermediates known as SEI (Single end invasion), SEIs then converted into dHJs (Krogh and Symington 2004). Later, dHJs resolved into crossovers (Matos *et al.,* 2011). The fate of the recombination reaction is, whether recombination intermediates resolves into crossover or non-crossover. Homologs undergo minimum at least one CO per chromosome (Roeder *et al.,* 1997).

Structure of Synaptonemal Complex (SC) during meiosis:

Formation and organization of Synaptonemal complex (SC) well understood in *S. cerevisiae*. SC is a meiosis-specific proteinaceous structure in between the homologs. SC mainly conserved from yeast (*S. cerevisiae*) to humans

(Woollam *et al.*, 1964). Mainly SC consists with one central element & two lateral elements. Central element forms like a bridge in between the two lateral elements (Hawley & Page 2004). The lateral element is present at the cores of 2 sister chromatids along with some other meiotic-specific proteins, like Rec8, Red1, and Hop1.

Central element of SC:

Budding yeast has a core central element such as Zip1 protein is present in SC. It has 2 terminal globular regions and one coiled-coil region, with these domains two Zip1 proteins makes a homodimer. C-terminus of Zip1 homodimer embed inside lateral elements/axial elements & N-terminus region protrudes at the center of SC, which interacts with Zip1 homodimer nucleating from LE and AE, then homologs tightly juxtaposed and promote bivalents (Dong & Roeder 2000) formation. At early prophase, Zip1 situates near the centromere. Zip1 localization at centromere is important for coupling of centromeres; this facilitates homologs pairing & initiates synapsis (Tsubouchi & Roeder 2005). Zip1 polymerization and SC assembly start from early zygotene stage. Zip1 removal causes defects/defects in synapsis, crossovers formation and meiotic cell cycle progression (Sym *et al*., 1993). Explains that Zip1 protein assembly is very important for proper recombination.

Lateral/Axial elements of SC:

Meiotic cohesin complex is a very important component for lateral elements of a synaptonemal complex; it promotes the proper distribution/arrangement of DSBs as well as proper assembly of chromosome axis. During mitosis, cohesion complex contains Smc1, Smc3, Scc1 & Scc3 in budding yeast

(Strunnikov and Jessberger 1999). During meiosis, the meiosis-specific klesin component Rec8 replaces Scc1/Mcd1 (Klein *et al*. 1999). First, Rec8 localizes to chromosome axis during s-phase then Smc1, Smc3, and Scc3 are recruited/localized on Rec8 containing chromosomal axial elements. Rec8 facilitates axial elements biogenesis. Smc3 assembly on axis is completely abolished in the absence of Rec8 (Klein *et al.* 1999; Golubovskaya *et al*., 2006). Meiosis-specific proteins Red1 & Hop1 are Lateral/Axial element components. Red1 & Hop1 have DNA binding regions, necessary for the formation of axial elements. During early leptotene, Red1 localizes to chromosomes and disassociates from the late pachytene stage. Red1 recruits Hop1 to chromosomes in late leptotene stage (Smith and Roeder 1997).

SC disassembly starts between the homologs after late-pachytene stage of meiosis, Aurora-B/Ipl1 kinase plays a key role in the disassembly of SC during meiosis (Jordan et al., 2009).

Rec8:

Rec8 is a kleisin subunit of meiotic cohesion complex. It is an important component of chromosome axis formation in budding yeast meiosis (Klein *et al.,* 1999). Rec8 exclusively expressed in meiosis, co-localizes with chromosomal axial elements. Budding yeast Rec8 is required for the proper assembly of lateral elements, such as Red1 on the chromosome and also facilitates the localization of the SC central element, Zip1 (Klein *et al.,* 1999). Immunostaining analysis of yeast nuclear spreads provides the information that; Rec8 binds to chromosomes earlier during pre-meiotic DNA replication (Klein *et al.,* 1999). Rec8 facilitates punctate distribution of staining on

chromosomes at early stages of prophase, at pachytene, forms continuous long lines with a fully assembled SC (Klein *et al.,* 1999). Moreover, Rec8 is important for the efficient repair of DSBs and also involved in strand exchange among homologs (Klein *et al.,* 1999). At MI, cohesion at chromosome arms must remove, and centromeric cohesion should maintain (Klein *et al.,* 1999). During metaphase-I to anaphase-I transition, an active protease known as Separase cleaves Rec8 from chromosome arms but not at kinetochore region (Klein *et al.,* 1999).

Link between homologous chromosome pairing and Synapsis:

Telomere clustering is meiosis-specific and well conserved in eukaryotes, which is concomitant with many key events of homolog juxtaposition. Formation of a telomere cluster/bouquet is independent of DSB formation as well as recombination events. Bouquet configuration mainly suggested promoting homologous chromosome pairing by maintaining homologs in the close vicinity through their telomere clustering/grouping (Bass *et al.,* 1997; Ding *et al*., 1998; Yamamoto *et al*., 1999; Scherthan *et al., 2001).* In fission yeast, pairing & telomere clustering at SPB tightly coupled. Pairing requires not only bouquet but also oscillation of nucleus by microtubules and dynein heavy chain1 (Ding *et al*., 1999; Yamamoto *et al*., 1999). Certainly, there must be a link between bouquet formation and juxtapositioning of homologs (MacQueen *et al*., 2002*;* Golubovskaya *et al.,* 2002; Couteau *et al*., 2004). The absence of telomere clustering and telomere movements leads to delay in SC formation and to pair in budding yeast ndj1 and csm4 mutants (Trelles-Sticken *et al.,* 2000*;* Ding *et al*., 2004; Kosaka *et al.,* 2009). Telomere bouquet exit may trigger as a sensor for recombination mechanism. Already reported

that Rec8 plays a vital role in recombination as well as telomere bouquet resolution. It gives us the idea that these events are interconnected.

Cohesin in mitosis:

Accurate attachment of mitotic spindles to chromosomes requires a force, which opposes the separation force of microtubules. A clue to this phenomenon is the physical attachment between sister chromatids; a protein complex called as cohesin promotes this. Also known as sister chromatid cohesion (SCC). A mechanistic explanation of cohesion is catenation of chromosomes during replication when sister DNAs twisted around one another. Decatenation is triggered by top-2 (Isomerase), which is active and not limiting factor of anaphase onset. (Koshland and Hartwell., 1987; Nasmyth and Haering, 2009). Sister chromatid separation mediated by cohesin. Cohesin provides cohesion in between the sister chromatids from S-phase until their distribution during anaphase (Michaelis *et al*., 1997; Nasmyth and Haering, 2009). Defects in cohesins cause aneuploidy (improper segregation of chromosomes), It can associate with various genetic disorders and cancers.

Roles of meiotic cohesin complex:

Mitosis-specific klesin subunit of cohesin complex Scc1/Mcd1 is replaced by a meiosis-specific α-klesin subunit Rec8 in *S. cerevisiae* (budding yeast)*, S. pombe* (fission yeast). In *C. elegans* (roundworm) along with Rec8 other paralogs, such as COH3 & COH4 regulates sister chromatid cohesin (Severson *et al.*, 2009). Smc1β replaces meiotic Smc1 in mammals and

higher eukaryotes; STAG3 replaces SA1/SA2 during meiosis (Pezzi *et al.,* 2000; Prieto *et al*., 2001; Revenkova *et al*., 2004). In mouse Rad21L protein identified during meiosis, it has a similarity with the Rec8 sequence as well as Rad21 (Gutierrez-Caballero *et al.,* 2012). It localizes to chromosomal axes in prophase-I of meiosis. Scc1 phosphorylated by Polo-like kinase-in mitosis; it promotes cleavage of Scc1 budding yeast (Alexandru *et al*., 2001; Uhlmann *et al*., 2004). During meiosis, various kinases phosphorylates Rec8 protein; such as Polo kinase (Cdc5), casein kinase-I (CK1), Dbf4-controlled Cdc7 kinase (DDK) [Clyne *et al*., 2003; Lee *et al*., 2003; Katis *et al*., 2010; Brar *et al*., 2006; Attner *et al*., 2013). Already reported that, other than SCC function, meiosis-specific cohesin complex has multiple functions, mainly regulates several meiotic prophase events such as proper axial elements formation, roles in recombination, and promotion of SC assembly.

Meiosis

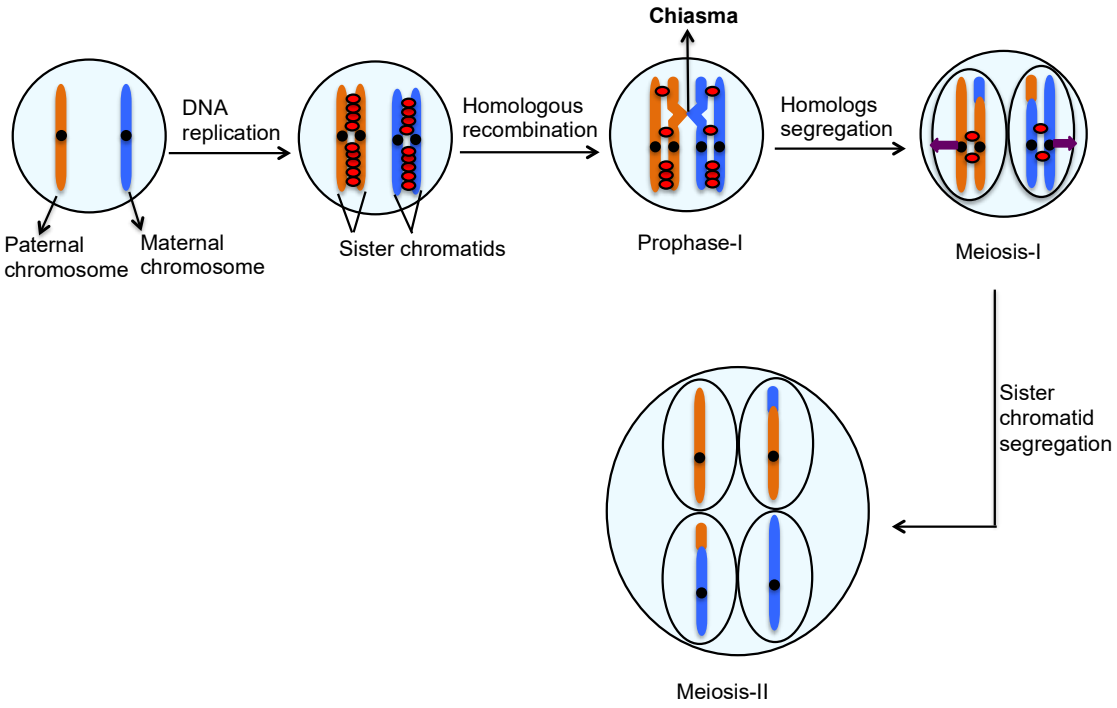


Figure I:

Meiosis:

Meiosis is a specialized differentiation process that generates haploid gametes from diploid cells. Meiosis is essential to maintain a constant number of chromosomes from generation to generation in sexually reproducing organisms. Upon the induction of meiosis, cells carry out DNA replication followed by two consecutive chromosome segregations, meiosis-I and II. At meiosis, I, maternal and paternal homologous chromosomes segregate in opposite directions. Sister chromatids then segregate at the meiosis II. Faithful segregation of homologous chromosomes at meiosis I depend on a physical linkage between the chromosomes. This linkage cytologically

observed as “chiasma.” The formation of chiasmata depends on exchanges between maternal and paternal chromosomes, homologous recombination. Therefore, the recombination in meiosis is essential for the production of haploid gametes.

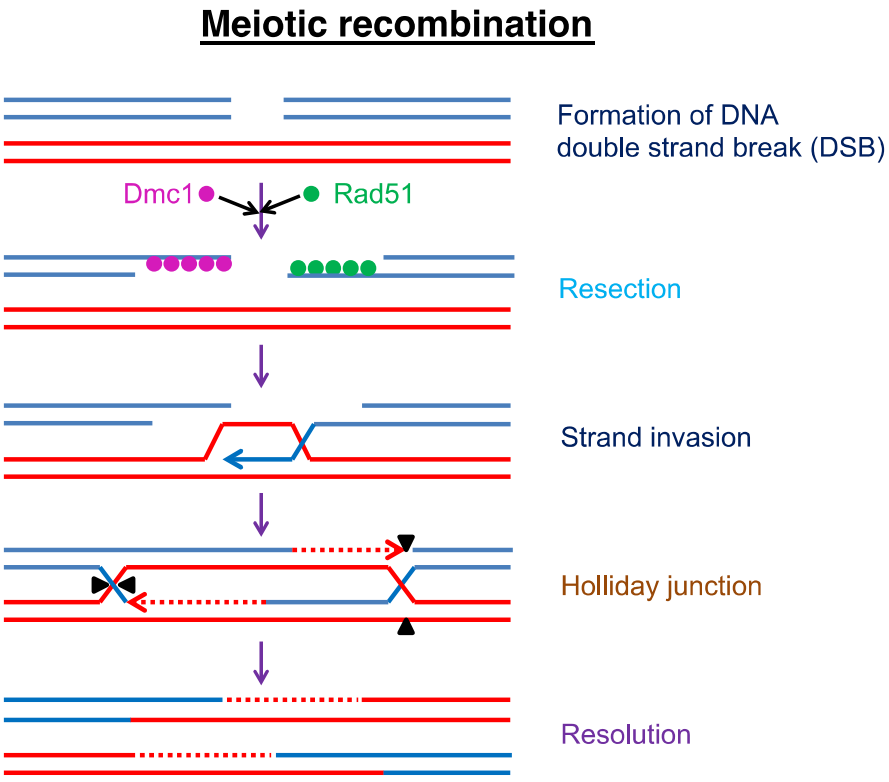


Figure II:

Meiotic recombination:

Meiotic recombination is a highly regulated process, which ensures recombination between homologous chromosomes. The recombination initiated by the formation of double-strand breaks (DSBs). After the DSB formation, single-stranded DNAs generated, and two RecA homologs Rad51 and Dmc1 are assembled on the DNA to exchange DNAs. Further processing of recombination intermediates results in the formation of double-Holliday structure. This Holliday structure is resolved into two recombinant molecules accompanied by the exchange of parental DNAs, crossovers.

Synaptonemal Complex (SC) formation

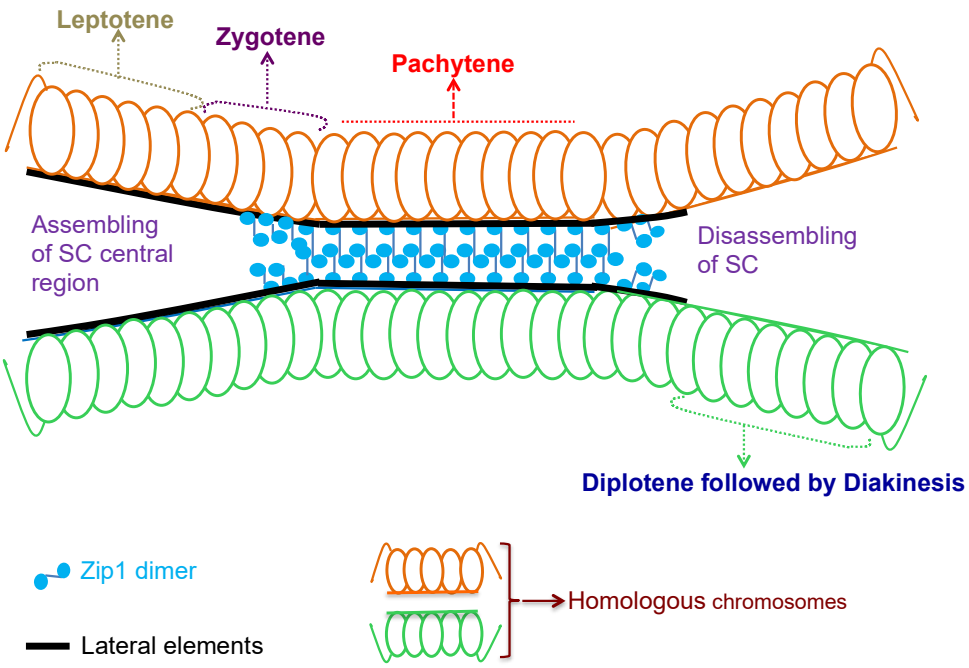


Figure III:

Synaptonemal Complex formation:

In addition to meiotic recombination, cells undergo chromosome dynamics such as the formation of the synaptonemal complex (SC) and rearrangement of chromosome position inside a nucleus. SC is a proteinaceous scaffold between homologous chromosomes. SC formation divided into four phases, the formation of lateral/axial elements, partially formation of a short SC and full-elongation of SCs and the disassembly of SCs. Each stage referred as to leptotene, zygotene, pachytene, and diplotene. In yeast, Zip1 is the central element in SC formation.

SUN-KASH mediated chromosome movements

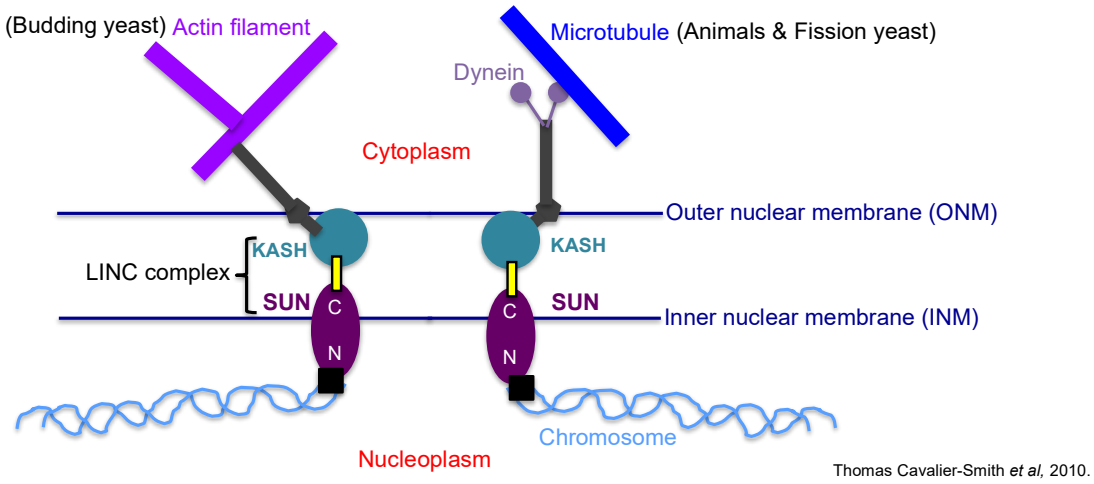


Figure IV:

SUN-KASH mediated chromosome movements:

Chromosomes relocation accompanied by cytoskeleton-mediated chromosome motion inside of the nucleus, which is promoted by SUN-KASH complex. SUN proteins are an inner nuclear membrane protein with conserved SUN (Sad1-Unc-85) domain. The SUN domain interacts with KASH (Klarsicht, ANC-1, Syne Homology) domain in proteins in the outer nuclear membrane. The SUN protein binds to telomeres in nucleoplasm through a specific telomere-binding protein, and the KASH protein binds to the cytoskeleton in the cytoplasm to form a LINC (Linker of Nucleoskeleton and Cytoskeleton) complex. By using cytoskeleton mediated forces, SUN-KASH complex promotes telomere clustering and declustering as well as chromosome motion inside the nucleus.

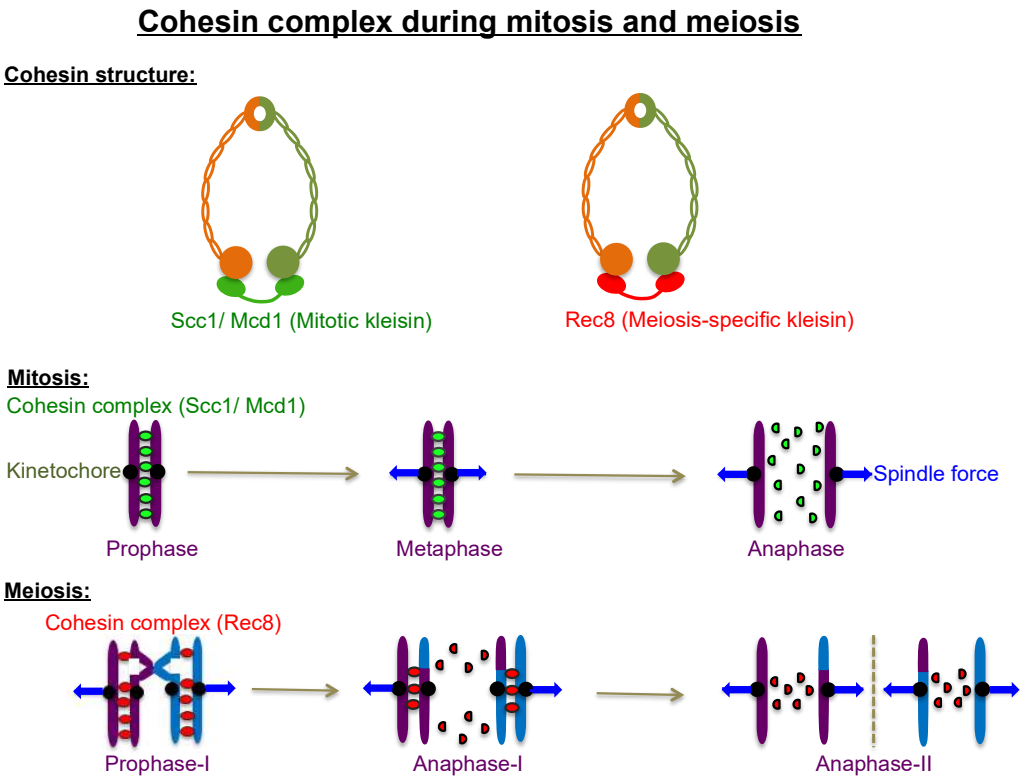


Figure V:

Cohesin complex during mitosis and meiosis:

Rec8 is important for the resolution of telomere clustering during meiosis. Rec8 is a meiosis-specific component of the cohesin complex. It holds sister chromatids. Meiotic yeast cells express meiosis-specific cohesin with Rec8 as a kleisin instead of Scc1/Mcd1 in mitosis.

Mps3-mediated telomere clustering in budding yeast

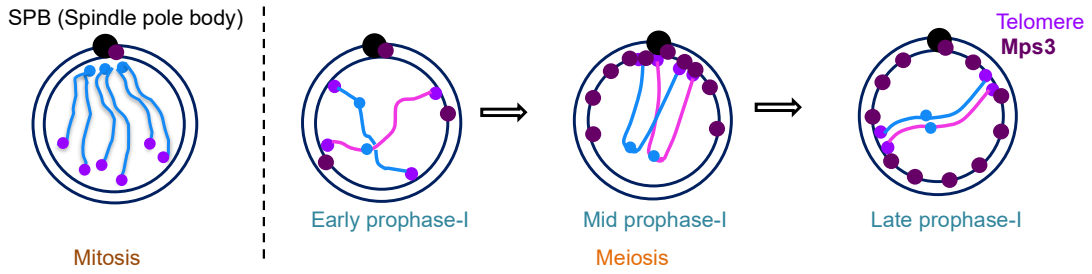
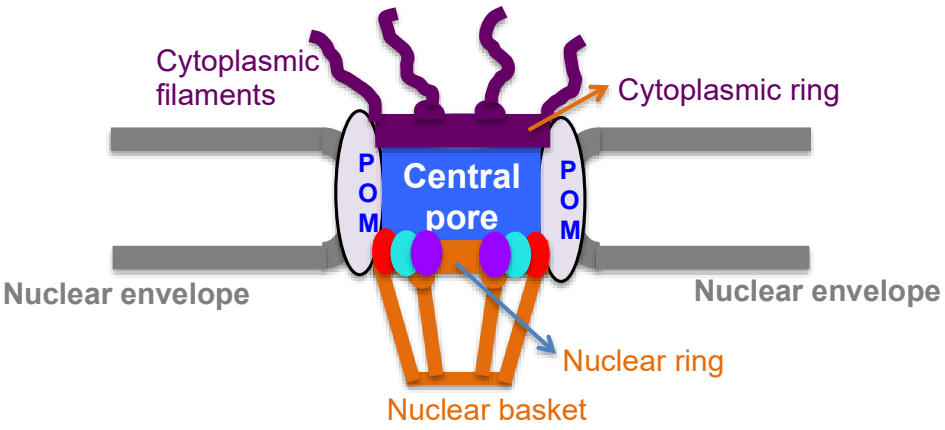


Figure VI:

Mps3-mediated telomere clustering in budding yeast:

Budding yeast SUN domain protein, Mps3 is required for duplication of the yeast centrosome (SPB) and chromosome motion during meiosis. In this yeast, centromeres tend to be positioned to near nuclear envelope (NE) in the vicinity of the SPB in mitosis. On the other hand, telomeres bound to the nuclear envelope (NE) forms a cluster near the centrosome in the mid-prophase I of meiosis. Unlike other organisms, telomere clustering in yeast is not tight and formed transiently. Mps3 protein promotes this clustering. A very interesting feature of Mps3 is that Mps3 changes its cellular location from mitosis to meiosis as shown here. In mitosis, Mps3 is predominantly located to SPB as seen as a single focus in a nucleus. In meiosis, in addition to SPB, Mps3 forms several patches (a large cluster) on the NE. NE-bound Mps3 tethers telomeres to NE and promotes the clustering and motion.

Structure of Nuclear pore complex (NPC)



POMs	Adaptor nups	Coat nups	Channel nups
Pom152	Nup157	Nup84	Nsp1
Pom34	Nup42	Nup85	Nup49
Ndc1	Nup53	Nup133	
	Nup60		

Figure VII:

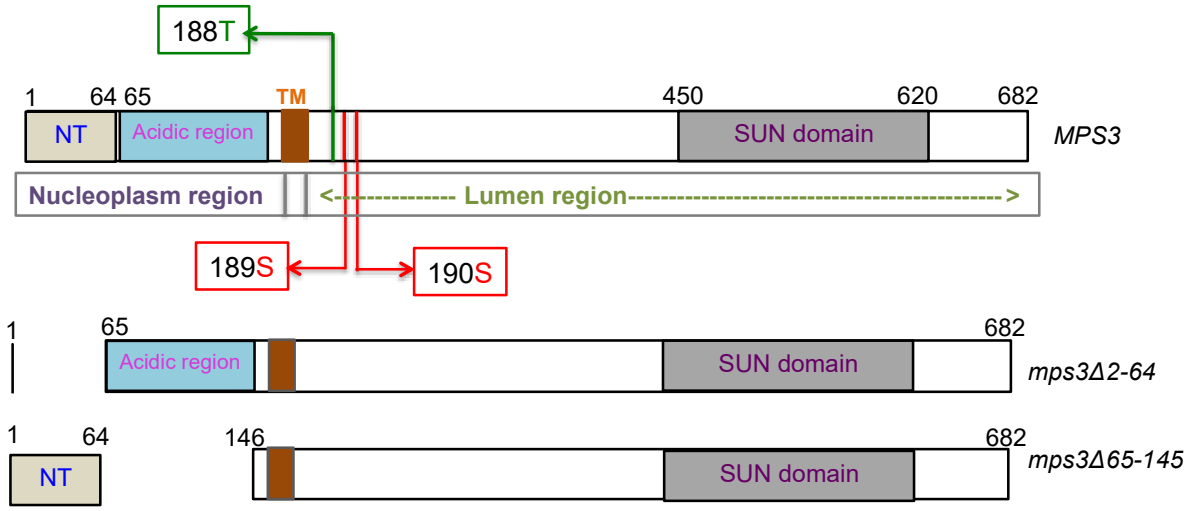
Structure of nuclear pore complex (NPC):

Nuclear pore complexes (NPCs) are involved in the bidirectional transport of molecules across the NE. NPC is a large protein complex made up of different nuclear porins. It is made up of several distinct sub-complexes; POMs, coat nups, adaptor nups and channel nups.

Schematic representation of Mps3:

Previous studies showed Mps3 dynamics consists of two processes. One is KASH protein and actin-dependent movement of Mps3 on NE, and the other is KASH, actin-independent localization of NE-Mps3. Both pathways in meiosis require Mps3 N-terminus, which located in nucleoplasm and phosphorylation of the luminal region of Mps3. While we know factors, which control Mps3 motion on NE, we do not know about factors, which regulates NE localization of Mps3.

Mps3:



Chapter 2

Materials and Methods:

2.1. Yeast Strains used in this research:

The strains used in my research were derived/established from *Saccharomyces cerevisiae* SK1 background diploid strains. MSY833/832 (MATa /MAT alpha(α), *ho::LYS2/",ura3/",leu2::hisG/",trp1::hisG/",lys2/")* and NKY 1543/1303 (/MAT*a*/MATα*, ho::LYS2, ura3, leu2::hisG, lys2, his4B-LEU2, arg4- Bgl / ho::LYS2, ura3,leu2::hisG,lys2, his4X-LEU2::BamH1-URA3, arg4-NSP*). Mps3-GFP, Mps3-FLAG as well as some other nuclear porin (nups) deletion strains used in my research originally constructed by previous lab members (Rao-san) of Shinohara laboratory. *mps3Δ2-64 & mps3Δ65-145* strains constructed by Prof. Akira Shinohara. I constructed, *mps3Δ2-64 GFP & mps3Δ65-145 GFP*, other double and triple mutations of Mps3 used in my research were made by crossing those strains, tetrad dissections, the selection was done by markers as well as PCR and mating types were identified by replica plating method. All strains used in my research mentioned in Table-1 with their genotypes.

2.2 Plasmid Construction during my study:

Mainly, wild-type *REC8* and *SCC1* genes cloned into pYESII(+) vector. After digestion with *HindIII* and *XbaI,* the DNA combined with Vector with the ligation process. The plasmid contains the gene of interest with *GAL1-10* promoter integrated by transformation for the selection of colonies with Ura+. All these newly constructed plasmids confirmed by restriction enzyme

digestion as well as DNA sequencing.

All other gene mutants such as deletion mutants, truncation mutants, gene tagging's were made by PCR-based mutagenesis with using gene-specific primers were created, newly created mutations were confirmed by PCR and DNA sequencing with using genomic DNA as a template. pFA6a-link-yoTagRFP-T-Kan (a Kind gift from Prof. Susan Gasser), pFA6a-link-EGFP-T-Kan, PAG32-HYG (From Prof. Miki Shinohara), pFA6a-KanMX-pGAL1 plasmids also used in my research.

The entire primers list used for strain construction and plasmid construction indicated in Table-2.

2.3 Antibodies and antiserum used in my Analysis:

Both meiotic and mitotic yeast cells after staining were observed using an Olympus epi-fluorescence microscope with the oil-immersion 100X objective lens. All pictures/images captured with using Cool-Snap CCD camera attached with this microscope. i-Vision software was used to process the images. After staining, to count the focus number, minimum 100 nuclei were analyzed at each time point. Also used antibodies for western blotting analysis as well as IP-westerns. Primary antibodies against Rec8 (Rabbit 1:1000 & Guinea Pig 1:2000), Rad51 (Guinea Pig 1:500), Htz1 (Rabbit, 1:1000), Zip1 (Rabbit 1:1000), Zip1 (Rat 1:1000), HA (Mouse 1:1000), Hop1 (Guinea Pig 1:1000), Red1 (Chicken 1:500), FLAG (Mouse 1:1000), HA (Mouse 1:1000), Tubulin (Rat 1:1000), Scc1 (Guinea Pig 1:1000, Generous gift from Prof. Miki Shinohara) and Histone H2B (Rabbit 1:1000) were used (from Shinohara et al., 2008 and Zhu et al., 2010). Molecular Probes such as Alexa-fluor-594 and

Alexa-488 fluorescently labeled secondary antibodies (2^0 Ab) used against primary antibodies from the various/different species used in my research works.

2.4 Plasmid DNA preparations from *E.coli*:

E. coli plasmid DNA prepared/purified as per the standard laboratory procedure in Prof. Shinohara Lab., (Shinohara, 2008). A single colony of *E. coli* inoculated into a test tube containing 2ml of liquid LB media with 50mg/ml ampicillin drug, then incubated overnight at shaking incubator (37°C). Collected overnight liquid culture, centrifuged in small Eppendorf tubes for 2min at 5000 rpm, collected the cell pellet and dissolved/re-suspended in GTE buffer (As per lab protocol), then added Alkaline SDS (1% SDS and 0.2 N NaOH) solution about 200ml, mixed well by inverting tubes and leave tubes on ice for 5min. Ammonium acetate (150ml, 7.5M) added to the tubes and mixed by inverting again. After 10min of incubation on ice, centrifuged for 10min (15,000 rpm) at 4°C. 500mml of ice-cold propanol tubes prepared during centrifugation. Then supernatants were collected in new tubes with propanol, mixed and centrifuged for 10min (15,000 rpm) at RT. Now washed these pellets with ethanol (70%) followed by 100% ethanol wash then samples were kept in a centrifugal concentrator for drying for about 10 min. Finally to dissolve plasmid DNA around 50 microliters of 10:1 TE (Tris-HCl, EDTA) added.

2.5 LiAc mediated Yeast (*S. Cerevisiae*) transformation:

Yeast can be grown well in YPAD media, which contains Bacto Yeast Extract (1%), Bacto Peptone (2%), Glucose (2%), Adenine (1%). Yeast cells

inoculated into liquid YPAD (3ml) medium and incubated in a shaking water bath (37°C) for overnight. This overnight yeast culture was diluted in YPAD liquid medium (1/200) in small 200ml conical flask and cells were grown in a shaker (Innova 44) at 30°C with 230 rpm for 3.5 hours (OD 0.5-0.6). Cells collected in a 50ml sterilized screw cap tubes, centrifuged at 2500 rpm for 3 min. Two times wash the pellet with sterile water. Then cells were mixed/dissolve in 1ml LiAc/TE (0.1M LiAc), Transferred this LiAc solution contain yeast cells to Eppendorf tubes, pellet down for 1min at 5000rpm. Again add 200 microliters of LiAc/TE to the pellet and mix properly then added 10ul carrier DNA (10mg/ml) from Wako Ltd., salmon sperm DNA. Separated this suspension into 50ul each in new tubes. Now add 1-10ul of plasmid or desired DNA fragment to each tube and properly mix the suspension. Add 350ul of PEG/LiAc/TE to each tube and mixed well and tubes were kept for incubation with rotation for 30min at 30°C. after this cells were under incubation at 42°C for 15 min. Collect the pellet after 1min centrifugation at 5000 rpm. This pellet was dissolved in 1ml of YPAD and incubated for overnight with rotation, then centrifuged at 3000 rpm for 1 min. Finally, pellets suspended in PBS (150ul) or TE (150ul) and spread on selective medium plates and incubated at 30°C.

2.6 Genomic DNA preparation from Yeast cells:

S. Cerevisiae cells inoculated in YPAD (2ml) liquid medium for overnight. Next day, Cells harvested in 1.5ml tubes. 500ul Zymolase buffer (Zymolase 100T from Nacalai, 0.1M β-Mercaptoethanol, 10mM NaPO4, 10mM EDTA) added

to the pellet and mixed well by vortexing then kept in a 37°C incubator for 30 min. Now cells were lysed with the addition of 100ul of lysing buffer (2.5% (w/v) SDS, 0.25M EDTA, 0.5M Tris base) and 10mg/ml Proteinase-K, incubated on heat block for 1hour at 65°C, mix few times during incubation. 5M potassium acetate of the 100ul solution was added to the tubes with cell suspension and shake the tubes to mix well and kept on ice for 15min. This cell suspension centrifuged for 10 min at 15000 rpm. Meanwhile, prepared 500ul of ice-cold 100% (V/V) ethanol containing Eppendorf tubes. Supernatants were transferred to new ice-cold ethanol Eppendorf tubes and centrifuged for 1min at 12000 rpm. Aspirated the supernatants and pellets were cleaned with 1ml of 70% ethanol, again wash with 100% ethanol. After 2times wash samples kept in centrifugal concentrator for 10min for drying. Total genomic DNA dissolved in 1X TE (500ul). Then RNase-A (10mg/ml) treatment has given for 30min at RT. about 500ul of propanol was added to each sample and gently mixed by inverting tubes, centrifuged at 15000 rpm at RT. Removed the supernatants, then pellets were cleaned with 70% ethanol and 100% ethanol. Samples were kept in a centrifugal concentrator for 10min for drying and 150ul of 1X TE buffer was used to suspend the DNA in each tube.

2.7 Meiosis time course:

Yeast cultures stock from freezer (-80°C) were taken and plated on YPG (Bacto Yeast Extract, Bacto peptone, Glycerol) plates, then incubated for overnight at 30°C incubator. Next day, cells streaked on YPAD from YPG plates, again kept for incubation at 30°C. Two mating types (Mat-a & Mat-α)

were matted and incubated for 4hours and streaked on new YPAD plates and kept in an incubator for about two days at 30°C to obtain/get single diploid colonies. One tiny diploid colony was inoculated in YPAD (3ml) liquid medium, kept for overnight incubation at 30°C in the rotator. After a day, 1ml YPAD liquid culture transferred into sterilized SPS (100ml) liquid medium (Yeast Extract, Bacto peptone, Yeast nitrogen base, Potassium acetate, Potassium hydrogen phalate, Ammonium sulfate), kept for incubation at 30°C about 16hours with 230 rpm in an Innova 44 shaking incubator. Next morning, SPS cell suspension was pellet down in a sterilized screw cap tubes (50ml). Cells were washed/cleaned two times with sterile water. The pellet suspended in 100ml sporulation medium (SPM: 0.02% Raffinose, 0.3% Potassium acetate), Incubated cell with SPM at 230rpm in a shaking incubator at 30°C, then cells will start/initiate meiosis and each point samples collected for different purpose such as DAPI, FACS, IP, western blotting, Cytology…etc.

2.8 Spores separation from Tetrads (Tetrad Dissections):

Yeast cells form tetrads (4 spores) after complete meiosis. To find out the viability of these spores of tetrad and making new strains by backcross, tetrad dissections technique is very useful. Individual mating types (Mat-a & Mat-α) called as haploids, were mixed small equal amounts on YPAD plates and kept for incubation minimum 4hours for mating and to form diploids. After mating, a patch of cells was transferred onto SPM (Potassium acetate, Raffinose) plates, incubated overnight at 30°C. After tetrads formation, tetrad dissection was done manually under a dissection microscope (Zeiss, Axioskop-40). After tetrad dissection, plates were kept for incubation at 30°C about 2days, viable spores were manually counted to confirm spore viability. At least 100 tetrads

were dissected for each strain to define strain spore viability. Genotypes of respective spore colonies confirmed by replica plating method.

2.9 Chromosomal Spreads (Lipsol Method):

About 5-10 ml of sporulation medium with yeast cells collected in 15 ml sterilized conical screw cap tube, spun at 5000 rpm for 1min and pellets dissolved in 1ml of buffer ZK (0.8M KCl and 25mM Tris-HCl [pH 7.5]). 20ul of Dithiothreitol (1M DTT) added to the yeast samples and mixed gentle, kept for incubation at RT for 2min, spun the samples for 1min at 5000 rpm. 1ml ZK buffer was added to this pellet and dissolved, 5-6ul of Zymolase buffer (5-10 mg/ml Zymolase 100T (Nacalai), 50mM Tris (pH7.5) 2% of glucose) added, samples were kept for incubation at 30°C for 30min in rotating incubator. A small amount of sample (diluted with water) was placed on a glass slide, covered with cover glass, observed the cell morphology under a light microscope to confirm the spheroplast formation. Once the cells achieve 80-90% spheroplast formation, samples were spun at 5000 rpm for 1min, washed with 1ml of 6.5pH MES/Sorbitol. Again pellet was mixed/dissolved in 1ml of MES/Sorbitol and store on ice (4°C) until spreading on a glass slide. To spread the chromosomes on the glass slide used Pasteur pipettes. With using micropipettes, 20ul of cell suspension in MES/Sorbitol was placed on the center of a clean micro cover slide (Matsunami glass IND, Ltd. (S2441)), to the cell suspension on the cover glass, freshly prepared 40 ul of PFA (Paraformaldehyde; SIGMA-ALDRICH) with sucrose was added, swirled gently. Then placed 80ul of 1% Lipsol, swirled briefly, keep this condition for 20sec to 2min, until about 90% cells lysed, this cell lysis observed by using a light microscope. Once cells lysed, 80ul of the PFA with sucrose solution was

added again to fix the cells. Spread this suspension on the entire surface of the glass slide; a glass pasture pipette was used, passed through along the top of the cell suspension. Then slides were kept for drying for 6hours to overnight, once slides dried, kept in a plastic black microscopic box and stored at -20°C.

2.10 Immunostaining of chromosomal spreads:

Glass slides contain nuclear spreads made as explained in the above protocol, were immersed in 0.2% photoflo (Kodak, Photo-Flo 200) solution for 2 min in a Coplin jar. Slides kept for air-drying about 5~10 min, then slides were kept for blocking about 15min using 500ul of TBS/BSA (1% BSA in 1x TBS) [TBS: 20mM Tris pH7.5, 0.15M NaCl; BSA: bovine serum albumin, from SIGMA]. After 15min, the blocking buffer drained onto a tissue paper/paper towel, about 90ul of TBS/BSA solution containing primary antibody 1/500 dilution added to each slide. These antibody solutions contain glass slides were covered with coverslips, kept for incubation at 4°C overnight or at room temperature for 2 hours in a moist chamber. Coverslip with excess primary antibody solution was taken out by submersion in the 1X TBS (washing buffer) at an angle of 45°. These glass slides washed for thrice (3times) for 10min each in Coplin jar containing 1X TBS (washing buffer). Kept slides for air dry. 90ul of secondary antibody (fluorochrome-conjugated IgG) solution in TBS/BSA with 1/2000 dilution added to the glass slides, incubated at RT for 1-2 hours in a dark, moist chamber. Coverslips were removal, and washing done as explained previously. Slides air-dried completely, then 15ul of Vectashield (mounting medium) with 0.2 ug/ul of DAPI was added, covered with a coverslip, then followed by sealing edges with nail polish and

kept/stored in a dark microscopic box. After staining, slides/samples of each time point visualized under an epifluorescence microscope.

2.11 Staining process of the Whole cell:

Poly L-lysine (1mg/ml) coated slides were used for whole cell staining process.

Poly L-lysine coated slides Preparation:

On a glass slide put 200ul of Poly L-lysine (1mg/ml) solution, incubated for 15min, covered with cover glass at room temperature. Submersed slides in MilliQ water removed coverslips. Slides were washed twice with MilliQ water. Air-Dried slides for 20-30min at RT.

Sample Preparation for whole cells staining:

Meiotic cell culture such as 900ul of SPM culture placed in a 1.5 ml new Eppendorf tube, add 100ul of formaldehyde (38%) to this meiotic culture to fix the cells. Store cell suspension on ice until proceed further. Tubes with cell suspension were spun at 12000 rpm for 2min at RT, the supernatant was aspirated, and the pellet washed with 1ml of ZK buffer. Dissolve the pellet in ZK buffer, added 20ul of Zymolase buffer (2% glucose, 50mM Tris, 5mg/ml Zymolyase 100T: Nacalai), incubated for 1hr at 30°C with rotating. Cells were centrifuged and washed with 1X PBS, suspended in 1ml of 1X PBS. From this sample, 100ul of cell suspension kept on poly L-lysine pre-coated glass slides, incubated at RT for 15min covered with a coverslip. With using 1X PBS, coverslips removed on glass slides. Now Cells treated with ice-cold 100% methanol, for 6min, with ice-cold 100% of acetone for 30sec and with cold PBS for 2 min. Then slides were blocked with 5% BSA in 1X PBS for 15

min (without coverslips). Further, immune staining performed as explained before.

2.12 Yeast live cell imaging:

To observe live yeast cells under a microscope. First round glass bottom dishes coated with 200ul of 10mg/ml Concanavalin-A (carbohydrate binding protein, lectin). Kept these coated dishes for drying at room temperature, 200ul of Yeast cells from both mitosis as well as meiosis can be collected in glass bottom pre-coated dishes and observed under the Delta Vision Microscope (Applied precision). Each time point images and small videos (Time-lapse imaging) recorded by computer-assisted fluorescence microscope with Cool-snap CCD camera system (Delta vision, from Applied Precision). Oil immersed 100X objective lens with numerical aperture 1.35 used in a microscope system. At each time point, minimum 100cells images captured. For time-lapse acquisition of images were carried out in every 1sec interval at a single focal plane. Image processing (Deconvolution) was carried out with SoftWorks Image Workstation (Applied Precision).

2.13 FACS (Fluorescence-activated cell sorter) Analysis:

Yeast cell culture from sporulation medium collected in a 1.5ml tube; Cells centrifuged at 5000rpm for 1min and pellets was suspended in 1ml of 70% ethanol and stored in -20°C until further sample making. Yeast cells washed with 1ml of buffer-A contains 0.05M EDTA and 0.2M Tris-HCl, the same wash done twice. Cells again suspended with 500ul buffer-A, sonication did at a low amplitude of about 10% using a sonicator from Branson Digital sonifier. RNase-A (0.2 mg/ml) solution was added to cells and kept for incubation for

about 4hours at 37°C in a heat block, during the incubation mix the samples several times. Centrifuged the cells and suspended in 500ul of buffer-A with Propidium Iodide (PI:16ug/ml) solution, cells were kept for incubation at RT about 30min. BD FACS CaliburTM machine was used to do FACS analysis.

2.14 Western blotting Process:

Detecting protein signal on the membrane using the Western blotting technique was performed as per the standard laboratory procedure in Prof. Shinohara Lab., (Shinohara, 2008). 10ml of sporulation medium (SPM) culture collected in a tube and cells were precipitated by washing with 15%-20% (w/v) Trichloroacetic acid (TCA) after that these cells disrupted with using a Yasui Kikai beads beater. All these proteins after precipitation collected in a tube and mixed with 120micro liter sample buffer for SDS-PAGE. If the samples were in yellow, then adjust the pH with Tris buffer with pH 9.5, now all the samples turn a blue color. Denature the protein samples at 94°C for 10 min. After completion of SDS polyacrylamide gel electrophoresis, transfer these proteins after running on gel to nylon (western) membrane from Immobilon (Millipore) with Atto trans western transfer unit (semi-dry transfer). Washed after Incubated with primary antibodies, AP (Alkaline phosphatase from Promega) conjugated secondary antibodies used. The protein of interest signal was detected/identified on blots using reaction kit from nacalai tesque contains BCIP/NBT.

2.15 Immuno-precipitation (IP):

Yeast cell lysates prepared by the glass bead disruption method Yasui Kikai disrupter (Yasui Kikai Co Ltd, Japan). The lysates were incubated with

magnetic beads (Dynal M260; GE Healthcare) coated with anti-Flag antibody for 12 h and washed extensively. Bound proteins were eluted by adding the SDS sample buffer and were analyzed on an SDS-PAGE gel, transferred to a nylon membrane (Millipore Co. Ltd), and probed with specific antibodies.

2.16 Chromatin fractionation/association assay:

About 10~15ml of cells (Meiosis/mitosis) collected from a culture with containing 1.6 OD at 600nm. Cells were harvested and suspended in 1ml of pre-spheroplast buffer (10mM DTT, 100mM Tris at pH 9.4), kept for incubation at RT for 5min. This cell suspension was transferred into a new Eppendorf (pre-weighed) tube and centrifuged. Then cell pellets were suspended/dissolved in 1.5 ml spheroplast buffer contains 0.6M Sorbitol, 10mM DTT, 50mM KH2PO4 at pH7.4. About 10µl of cell suspension was taken into 1.5ml tube and diluted with 990µl of sterile water, to measure the optical density (OD:0.30). Around, 5~10µl of 10mg/ml Zymolyase-100T solution was added to the cell suspension, kept for incubation at 30°C for 5 min. Small volume aliquots after dilution with water were used to measure OD at 600nm (Incase digested cell walls, OD should be lower 10times (1/10) of the value before zymolyase treatment, ~0.03 OD). Then, spheroplasts were centrifuged immediately and kept on ice. Total weight of the Eppendorf tube with spheroplast pellet was measured/calculated, and the pellet weight was calculated/measured (it should be nearly 120mg). Spheroplasts were lysed with the addition of 5volumes of ice-cold HB buffer (Hypotonic buffer; 100mM MES/NaOH, 1mM EDTA, 0.5mM MgCl2), kept for incubation on ice for 5min. Collected 100ul of cell lysate after lysis in a new tube, this considered as Whole Cell Lysate (WCS) fraction. Collected 100ul of lysate in a new tube

containing an equal volume of 30% sucrose (V/V) cushion, incubated for 5min and centrifuged cell suspension at 15,000 rpm for 15min and collected supernatant considered as the chromatin-unbound fraction. The remaining pellet dissolved in 100µl EBX buffer (100mMKCl, 50mM HEPES-NaOH, 0.05%Triton X-100, 2.5mM MgCl2 and protease inhibitors cocktail from Sigma), centrifuged at 15,000 rpm for 15min. Its considered as nuclei fraction. The about 90µl nuclear fraction was taken and spun at 15000rpm for10min. The 20ul supernatant was saved/stored; this pellet dissolved in 75ul of EBX. It is the chromosome suspension. 4ul of DNaseI (1µg/µl) and 2µl of MgCl2 (1M) added to the cell suspension, kept for incubation at room temperature about 10min and centrifuged chromosome suspension at 15000rpm for 10min and saved/stored 20µl chromatin supernatant (Chromatin fraction). Finally, the pellet was re-suspended in 50µl EBX buffer and this considered as the nuclear matrix fraction. Samples were run on SDS-PAGE and transfer to a nylon membrane and probed against with antibodies.

Chapter 3

Results:

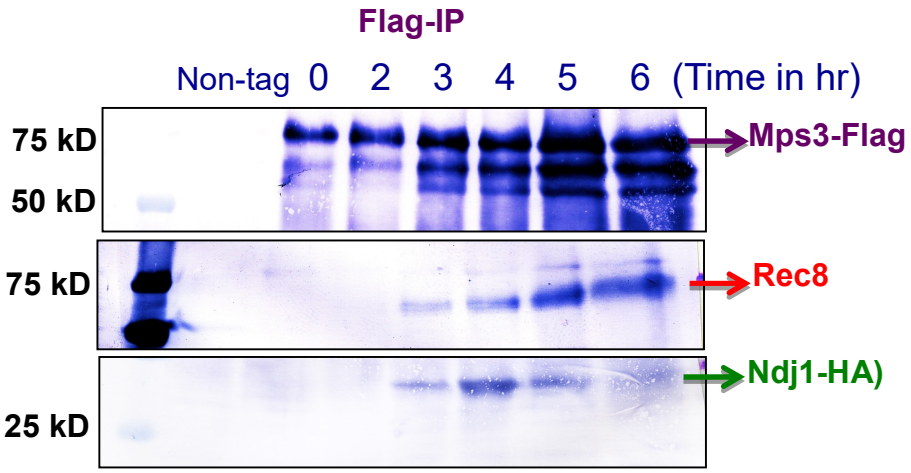
Meiosis-specific cohesin component, Rec8, binds to Mps3:

To find out the positive and negative factors, which regulates Mps3-NE localization during meiosis. Our lab looked into the binding partners of Mps3 during both meiosis as well as mitosis. In meiotic yeast cells, Mps3-FLAG-tagged protein purified by affinity purification method and identified the interacting partners by mass spectrometry (Akira Shinohara Lab, Unpublished data) and found some common interacting partners in both meiosis and mitosis; some are meiosis-specific binding partners. Interestingly, meiosis-specific cohesin component Rec8 also identified. Previously, it is reported that Rec8 is important for the resolution of telomere clustering during meiosis (Harry. Scherthan *et al.,* 2005 and Michael E. Dresser *et al.,* 2007). Sun domain protein, Mps3 located at the inner nuclear membrane. The nuclear envelope (NE) bound Mps3 tethers the telomeres to the nuclear envelope and promotes the clustering and motion. To confirm the physical interaction between Mps3 and Rec8 in meiotic yeast cells, Mps3 tagged with 3xFLAG (This endogenously introduced Mps3-FLAG cells express functional protein). I carried out immuno-precipitation of Mps3-FLAG-tagged protein at different time intervals during meiosis. I found immune precipitated Mps3-Flag protein to make sure of my protein of interest in pull-down properly. As you can see here, Mps3 protein expression is increasing from mitosis to meiosis such as 0hr to 6hr time point (meiotic time course analysis, Fig-1A). In addition to a meiosis-specific telomere protein Ndj1 (Ndj1-HA, which shows peak

expression at 4 hours), which is shown to bind to Mps3 (Conrad *et al.,* 2008), Rec8 is co-Iped with Mps3 (Fig-1A). Rec8 interaction with Mps3 is more strong in late meiosis where we can see both Rec8 and Mps3-FLAG expression at peak. This suggesting that More Rec8 or-or/and modified Rec8 might promotes Mps3 localization on nuclear envelope. Here, I also showed that the whole cell protein extracts (WCE) from lysates as control (Fig-1B). This IP result indicates the physical interaction between Rec8 and Mps3 during prophase-I of meiosis.

Figure 1:

(A)



(B)

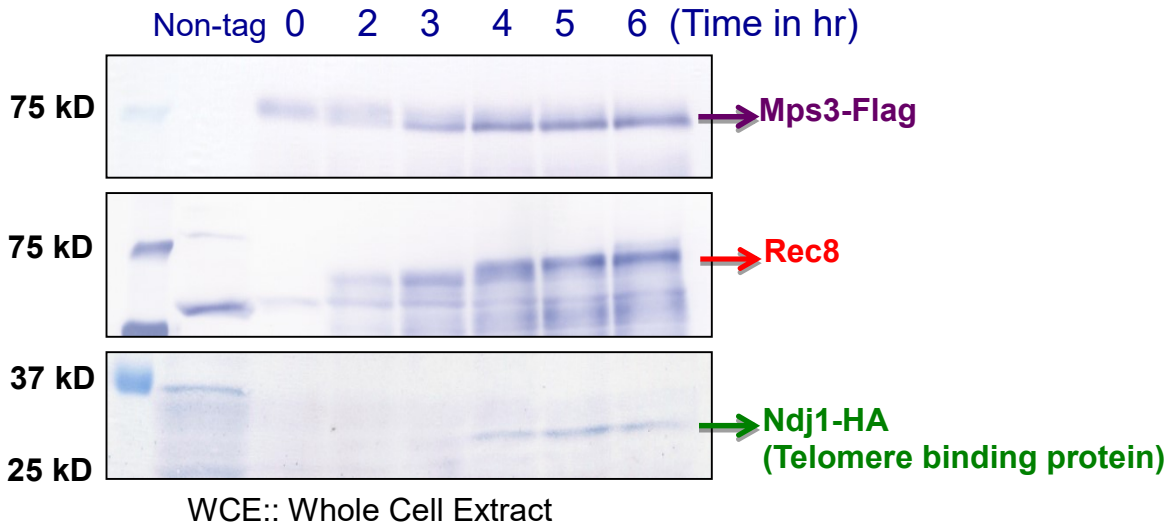


Figure 1. Mps3 binds to Rec8-cohesin.

(A). Western blotting analysis of Mps3-FLAG immuno-precipitation. Both IP fractions as well as cell lysates probed with anti-Rec8 and Anti HA (for Ndj1-HA) antibodies. *Mps3-FLAG Ndj1-HA* (BJY305/306) Strains were used.

(B). Western blotting analysis of WCE protein samples/lysates were Probed against anti-FLAG, anti-Rec8, and anti-HA (Ndj1) antibodies. BJY305/306 strains were used in this work.

Rec8 resolves Mps3 clusters during meiosis:

As mentioned before, Rec8 is interacting with Mps3 during meiosis (In this study). Rec8 is important for the resolution of telomere clustering during meiosis (Harry. Scherthan *et al.,* 2005; Michael E. Dresser *et al.,* 2007). Rec8 is a meiosis-specific component of the cohesin complex, which hold sister chromatids. Based on the previous study, I confirmed here that Rec8 is important for clustering resolution. To prove this, Here, I checked Mps3 localization using Mps3-GFP fusion protein in wild-type as well as rec8 deletion mutant. In wild-type cells, at 0 hr which corresponds with mitosis, Mps3 shows single focus. When cells enter meiosis, at early time points, Mps3 forms a few foci and the number of the foci is increased forming a big patch by mid-prophase-I. Mps3 transiently form a cluster in one region of nuclei (Fig-2A). Late prophase such as 6h, Mps3 covers most of the NE. Upon the onset of Meiosis I, most of NE-bound Mps3 disappears leaving a single focus. On the other hand, as reported, rec8 deletion cells show very tight clusters of Mps3 on NE from 4hour to until 12hour time point; these Mps3 clusters are persistent throughout meiosis (Fig-2A). These clusters do not disappear throughout meiosis. Mps3 accumulation (tight cluster formation) on nuclear envelope (NE). It suggests that Rec8 controls Mps3 localization and dynamics during meiosis.

Figure 2:

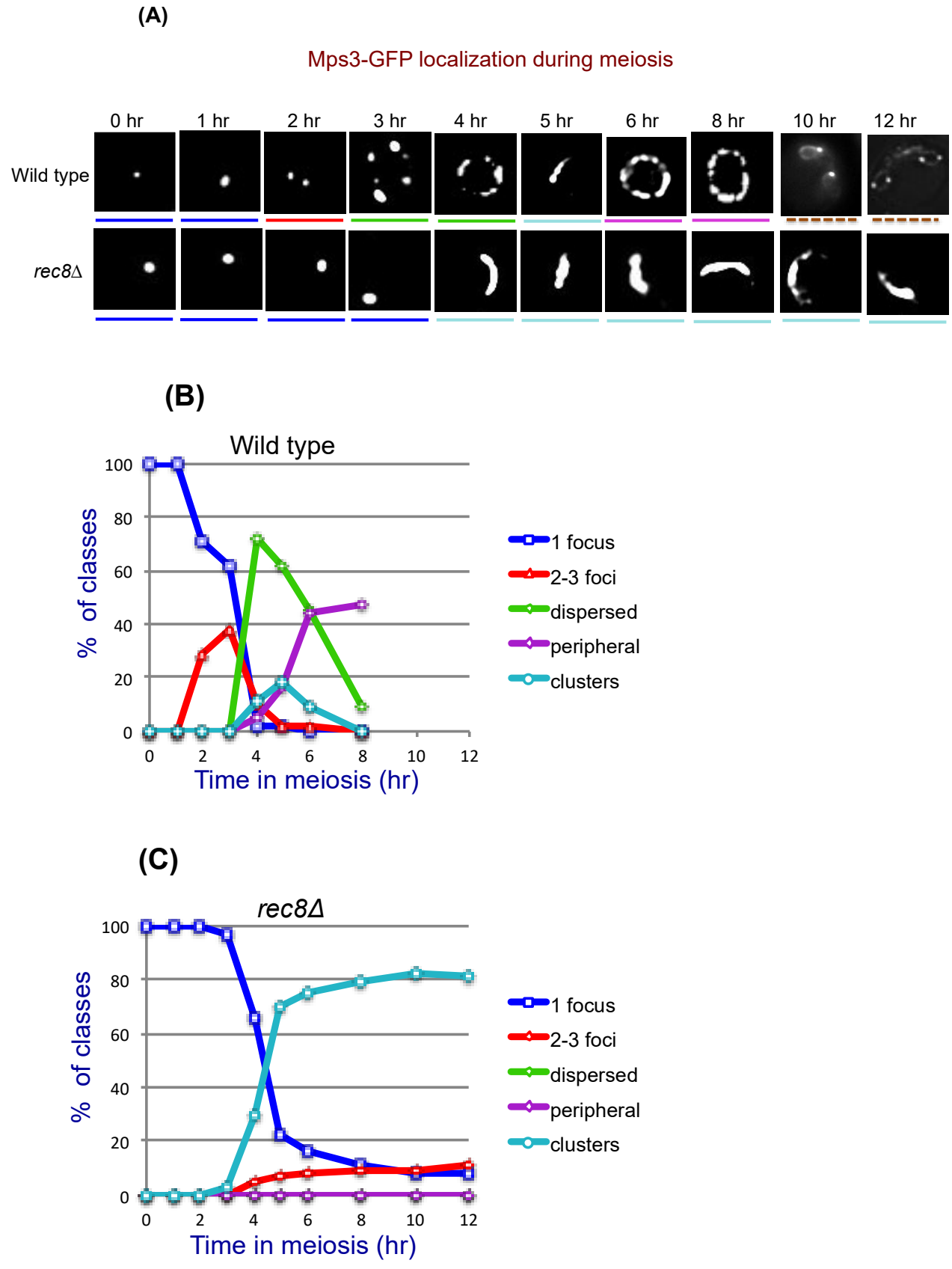

(A)
Mps3-GFP localization during meiosis
0 hr
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
8 hr
10 hr
12 hr
Wild type
rec8Δ
(B)
Wild type
% of classes
Time in meiosis (hr)
1 focus
2-3 foci
dispersed
peripheral
clusters
(C)
rec8Δ
% of classes
Time in meiosis (hr)
1 focus
2-3 foci
dispersed
peripheral
clusters

Figure 2. Rec8 resolves Mps3 clusters during meiosis.

(A). Localization of Mp3-GFP in meiotic diploid cells in wild-type (BJY9/10) as well as *rec8Δ* (BJY299/300) strains at different time points was examined by epifluorescence microscopy. The bar indicates 1 μm.

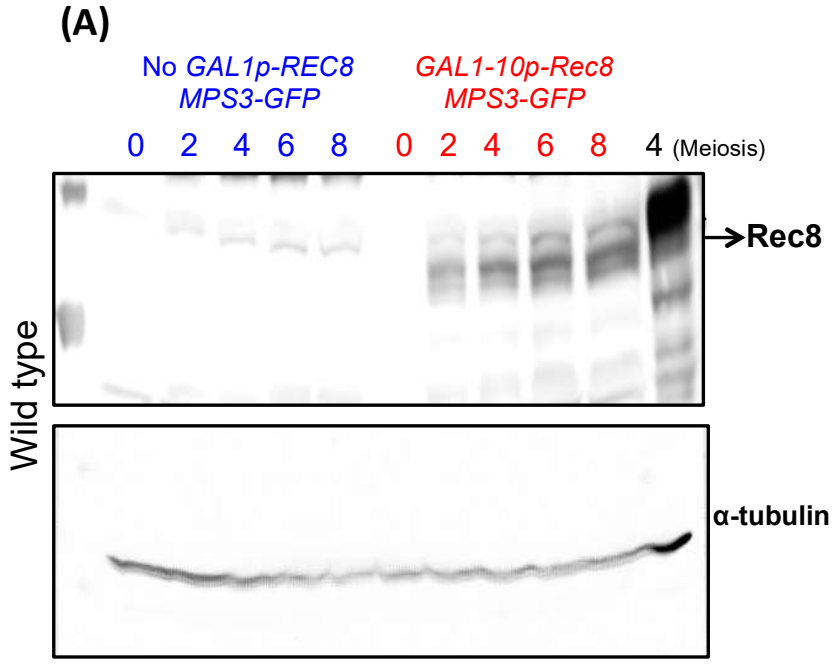
(B). Kinetics of Mps3-NE localization. Cells containing Mps3-GFP signals on NE were counted and classified at each time point in wild-type cells.

(C). Kinetics of Mps3-NE localization. Mps3-GFP signals on NE were counted and classified at each time point in *rec8Δ* (BJY299/300) cells.

Rec8 expression during mitosis:

Rec8 is interacting with Mps3. So, I wondered whether the expression of Rec8 in mitotic cells is enough to promote Mps3 localization on NE or not? To explain this, I placed the expression of Rec8 protein under inducible Gal promoter (*GAL1-10p*) in a cell containing Mps3-GFP. Thus, the addition of galactose (2%) at 0hr induces Rec8 expression in mitotic cells, which is confirmed by western blotting against anti-Rec8 antibodies as shown here. Rec8 expression starts at 2hr and peaks at 6 and 8 hr (Fig-3A). I used meiotic 4hr sample as positive control for Rec8 protein expression. I also used mitotic klesin, Scc1 overexpression as a control experiment. I placed Scc1 under the *GAL1-10* inducible promoter and induced the mitotic yeast cells with 2% galactose at 0hr, which induces Scc1 expression. Here, I observed nearly 2-3 fold increase of Scc1 expression. Scc1 expression confirmed by western blotting by using anti-Scc1 antibodies as shown here (Fig-3B).

Figure 3:



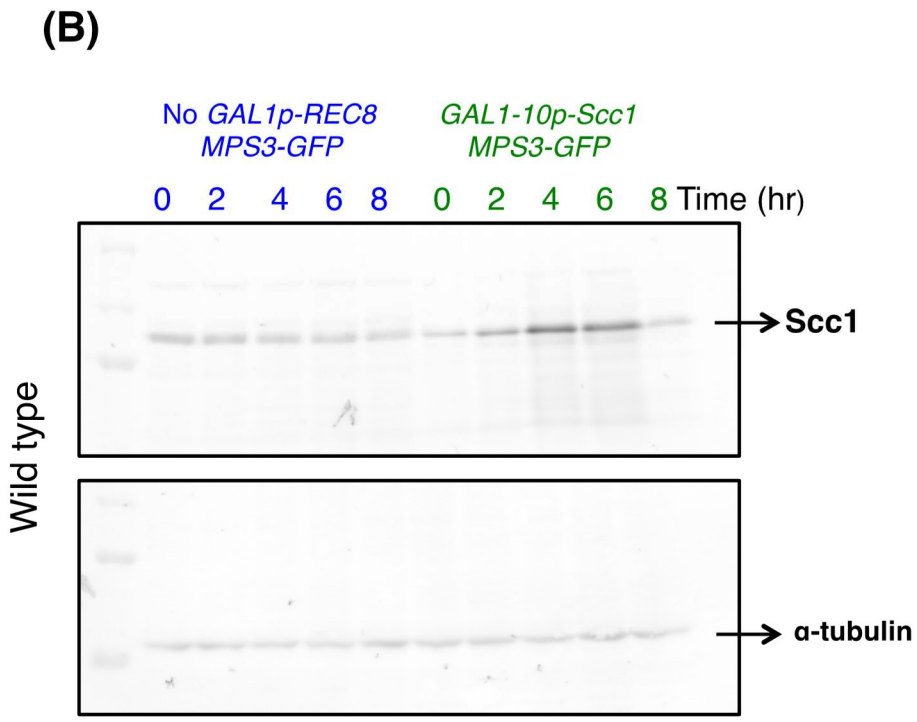


Fig 3: Plasmid-based Rec8 & Scc1 expression in mitotic cells.

(A) The addition of galactose-induced expression of Rec8. After the induction, expression of Rec8 was checked by western blotting. *MPS3-GFP* (BJY9/10) strains with YES2-Rec8 or YES2 vector as a control.

(B). Overexpression of Scc1 by the addition of galactose. After induction, Expression of Scc1 was checked by western blotting. *MPS3-GFP* (BJY9/10) strains with YES2-Scc1 or YES2 vector as a control.

Rec8 is key regulator for Mps3 localization on NE during mitosis.:

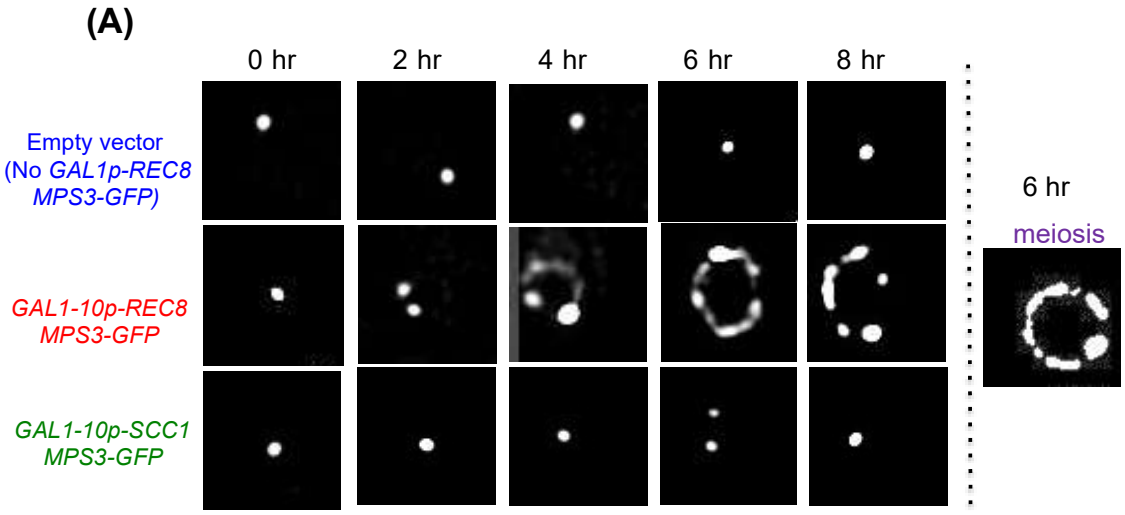
To check/ find-out the Mps3-Gfp localization in mitotic cells under Rec8 expression condition as well as Scc1 overexpression condition. I used high copy number YES2 plasmid with *REC8* gene, *SCC1* gene under the control of *GAL1-10* promoter. Mps3-GFP fusion protein was used to monitor the Mps3 localization on NE in diploid SK1 yeast cells. Mps3 exhibits different pattern of localization under different backgrounds, Mps3-Gfp fusion protein localization as well as Mps3 immuno-staining analysis confirms that Mps3 localizing at the edge of nucleus/nuclear membrane (Bupp, Martin, Jaspersen & Stensrud 2007; Conrad et al., 2007), it represents with spindle pole body visualization/localization during mitotic yeast cells. In mitosis, without Rec8 induction, Under SK1 background (mitotic/vegetative growth of yeast cells) Mps3 is predominantly located to SPB as seen as a single focus. In another background (W303) yeast cells Mps3 shows mild peripheral signal along with SPB localization (Bupp et al., 2007). On the other hand, when Rec8 was induced with using 2% galactose, surprisingly 21% cells showed Mps3 localization on the nuclear envelope at 6hr time point as multiple foci or big ensembles of Mps3 even in mitotic cells (Fig-4a & 4B). This pattern of the localization of Mps3 is similar to what we see in meiotic cells (Conrad et al., 2007). It indicates that Rec8 is sufficient for NE-localization of Mps3 during mitosis. I also noticed that yeast cells which show Mps3 localization on NE are mostly large budded cells, a minor fraction of no-bud cells also shows Mps3-NE localization but rarely or almost not seen in small budded cells.

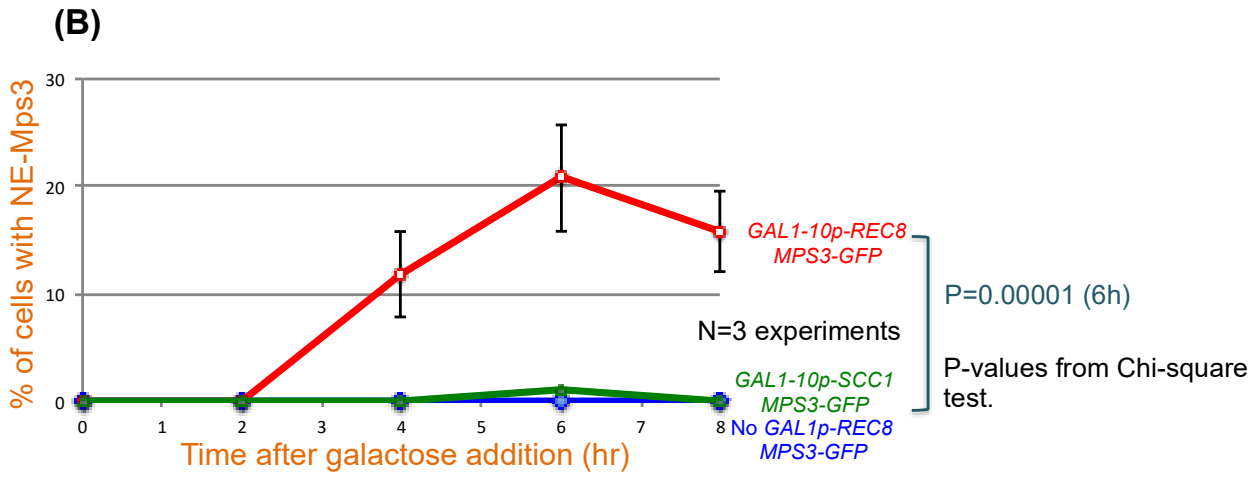
These results suggest that Rec8 mediated Mps3 localization on the nuclear envelope in mitotic cells might regulate in a cell-cycle-dependent manner.

To check weather this Rec8 mediated Mps3-NE localization in mitotic yeast cells is indeed promoted by meiosis-specific cohesin component or not? To address this question, I also overexpressed Scc1/Mcd1 by *GAL1-10* promoter contain YES2 high copy plasmid. Additional expression of Scc1 did not induce NE localization of Mps3 in mitotic cells. It supports the idea that meiosis-specific cohesin component Rec8 but not mitotic cohesin component Scc1/Mcd1 can promote Mps3 localization on nuclear envelope (NE).

I also proved that mitotically expressed Rec8 is indeed bound to chromatin during mitosis. As shown here, by chromatin fractionation assay/method. Most of the Rec8 was recovered predominantly in chromatin fraction but not in cytosolic fraction at 5 and 6-hour time points in mitotic diploid cells. It indicates that mitotic Rec8 mainly bound to chromatin (Fig-4C). Thus, chromatin-bound Rec8, possibly as a cohesin complex, seems to promote Mps3 localization on NE. This result consistent and supported by previously published data on Rec8 expression in the presence of the mitotic Scc1/Mcd1, which indicated that meiosis-specific klesin, Rec8 is sufficient to regulate sister chromatid cohesion functions instead for Scc1 (Buonomo et al., 2000; Heidinger-Pauli, Unal, Guacci, & Koshland, 2008).

Figure 4:


(A)
0 hr
2 hr
4 hr
6 hr
8 hr
Empty vector
(No GAL1p-REC8
MPS3-GFP)
GAL1-10p-REC8
MPS3-GFP
GAL1-10p-SCC1
MPS3-GFP
6 hr
meiosis


(B)
% of cells with NE-Mps3
30
20
10
0
0
1
2
3
4
5
6
7
8
Time after galactose addition (hr)
GAL1-10p-REC8
MPS3-GFP
N=3 experiments
GAL1-10p-SCC1
MPS3-GFP
No GAL1p-REC8
MPS3-GFP
P=0.00001 (6h)
P-values from Chi-square
test.

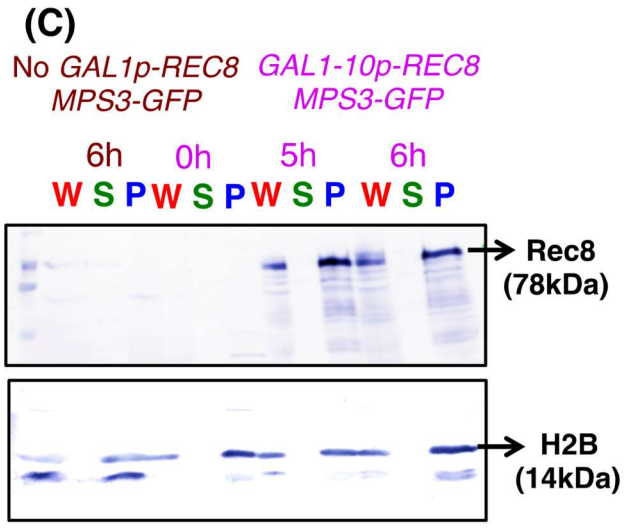


Figure 4. Mitotic Rec8 is sufficient to promote Mps3-NE localization.

(A). Mp3-GFP localization in mitotic diploid cells with galactose induction of Rec8 at different time points was examined by epi-fluorescence microscopy. The bar indicates 1 μm.

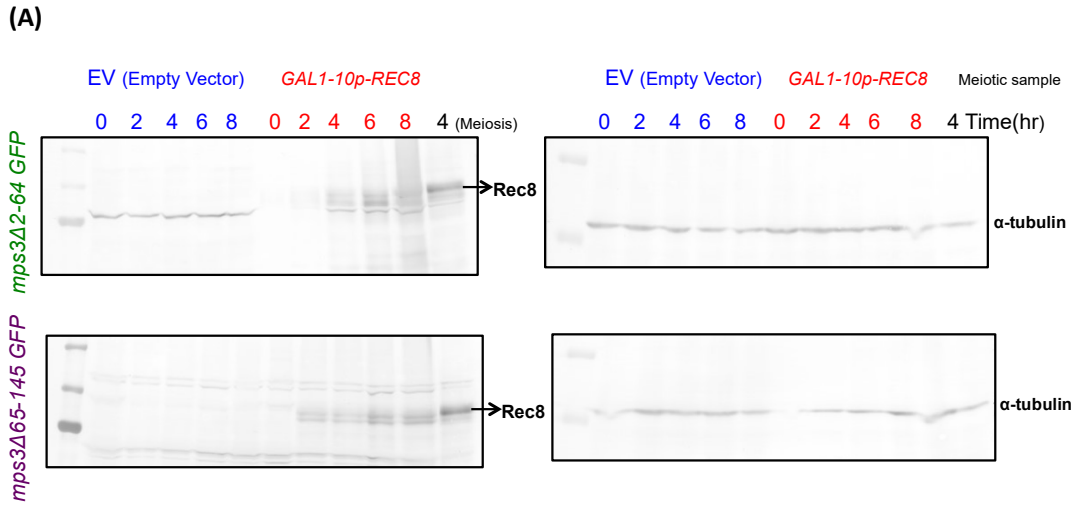
(B). Kinetics of Mps3-NE localization. Cells containing Mps3-GFP signals on NE were counted at each time point in *Mps3-GFP* (BJY9/10) strains (More than 100 cells). Error bars are standard deviation (SD, n=3 experiments).

(C). Chromatin fractionation of *MPS3-GFP* cells before and after galactose induction was carried out, and whole-cell extracts, chromatin-bound fraction and chromatin-insoluble fraction were probed for the presence of Rec8 and histone H2B by western blotting.

Mitotic Rec8 expression in *mps3Δ2-64 GFP & mps3Δ65-145 GFP* cells:

As previously shown by Conrad *et al.,* N-terminus two regions of Mps3, 2-64, and 65-145, which are located in nucleoplasm, important for localization/accumulation of Mps3 on NE during meiosis. To address the roles of these N-terminus and Acidic domains of the nucleoplasmic region in Mps3 localization on nuclear envelope (NE). I overexpressed Rec8 with an inducible *Gal1-10* promoter containing high copy YES2 plasmid in a cell containing *mps3Δ2-64 GFP & mps3Δ65-145 GFP*. Then, the addition of 2% galactose at 0 hours, induces Rec8 expression from 2 or 4 hours in mitotic cells (Fig-5A), Using western blotting against anti-Rec8 antibodies confirmed Rec8 protein expression in these *mps3Δ2-64 GFP & mps3Δ65-145 GFP* mutant cells.

Figure 5:

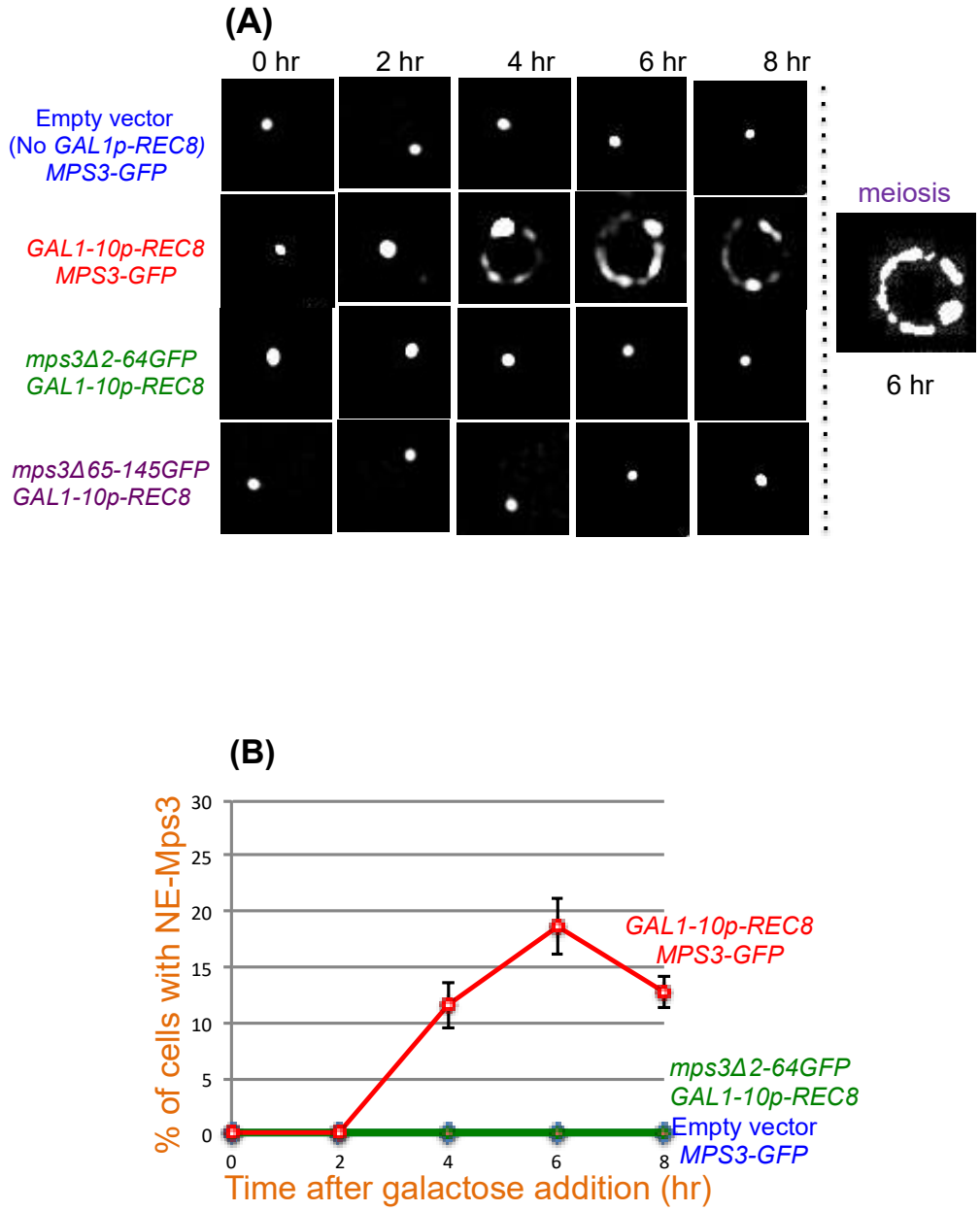


(A). Rec8 expression induced by the addition of galactose. After induction with galactose, expression of Rec8 was checked by western blotting. *MPS3-GFP*, *mps3Δ2-64 GFP* (BJY490/494) *& mps3Δ65-145GFP* (BJY484/488) strains with YES2-Rec8 or YES2 vector used as a control.

Mps3 N-terminus & acidic regions are critical for Mps3 localization on nuclear envelope during mitosis under Rec8 expression:

To check the roles of Mps3 N-terminus domains (2-64 and 65-145) under Rec8 mediated nuclear envelope localization in mitotic cells. I introduced the *GFP* tagging to both Mps3 truncation mutants and created *mps3Δ2-64 GFP & mps3Δ65-145 GFP* strains to check the live localization/visualization with using delta vision microscope. These N-terminus and Acidic domains of Mps3 located in the nucleoplasm, and these are also important for localization/accumulation of Mps3 on NE during meiosis (Conrad et al., 2007; Lee, Conrad, & Dresser, 2012). So I checked the role of these regions in Mps3 localization on NE under Rec8 expression condition in mitotic diploid yeast cells. Both Mps3 N-terminus deletion mutants such as *mps3Δ2-64 GFP & mps3Δ65-145 GFP* cells did not show any NE localization when Rec8 expressed in mitotic cells (Fig-6A, 6B & 6C). It indicates Rec8-mediated Mps3 localization on NE depends on the N-terminus, nucleoplasm region of Mps3. This result supports the idea that, the interaction of N-terminus of Mps3 with chromosomes is important for NE-localization.

Figure 6:


(A)
0 hr
2 hr
4 hr
6 hr
8 hr
Empty vector
(No GAL1p-REC8)
MPS3-GFP
GAL1-10p-REC8
MPS3-GFP
mps3Δ2-64GFP
GAL1-10p-REC8
mps3Δ65-145GFP
GAL1-10p-REC8
meiosis
6 hr
(B)
% of cells with NE-Mps3
30
25
20
15
10
5
0
0
2
4
6
8
Time after galactose addition (hr)
GAL1-10p-REC8
MPS3-GFP
mps3Δ2-64GFP
GAL1-10p-REC8
Empty vector
MPS3-GFP

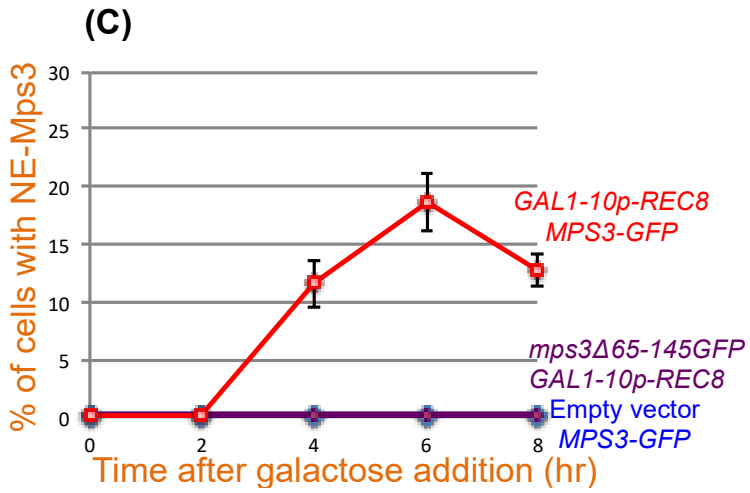


Figure 6. N-terminus of Mps3 is required for Mps3-NE localization.

(A). Localization of Mp3-GFP in mitotic diploid cells with galactose induction of Rec8 in *MPS3-GFP* (BJY9/10), *mps3Δ2-64 GFP* (BJY490/494) *& mps3Δ65-145 GFP* (BJY484/488) were examined by epi-fluorescence microscope. The bar indicates 1 μm.

(B). Kinetics of Mps3-NE localization. Cells containing *mps3Δ2-64 GFP* (BJY490/494) signals on NE were counted at each time point (more than 100 cells). Error bars are standard deviation (SD, n=3 experiments).

(C). Kinetics of Mps3-NE localization. Cells containing *mps3Δ65-145 GFP* (BJY484/488) signals on NE were counted at each time point (more than 100 cells). Error bars are standard deviation (SD, n=3 experiments).

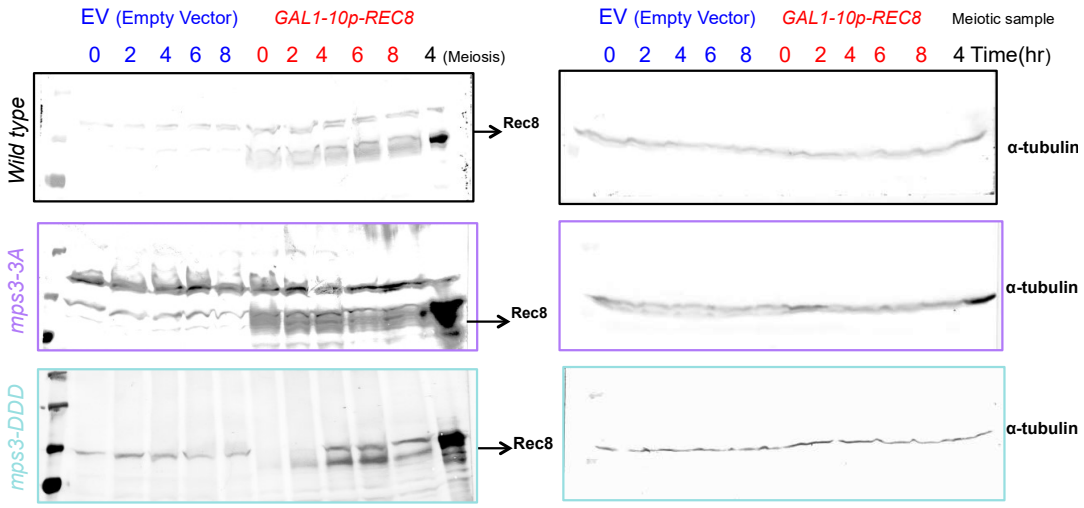
Mps3 TSS residues required for efficient Mps3 localization on NE during mitosis:

What other regions of Mps3 are responsible for Mps3 localization on nuclear envelope (NE). According to previous studies in Shinohara lab, phosphorylation of NE lumen region of Mps3 is critical for the meiosis-specific Mps3 localization and motion on nuclear envelope (NE) in budding yeast. Shinohara lab (HB Rao and Akira Shinohara) identified putative phosphorylation sites such as 188T, 189S, and 190S on Mps3 and identified the role of this three 188T, 189S & 190S residues during meiosis. These three phosphorylation sites were replaced with alanine (Non phosphorylated condition) to find out the importance of these putative amino acids. *mps3-AAA* mutant decreases/reduces the NE localization, dynamics such as movements and motions on nuclear envelope also reduced, this results suggesting that Mps3 phosphorylation is also contributing the localization and dynamics of Mps3 during meiosis (Shinohara lab unpublished results). So, I am interested to find out whether Rec8 expression can induce Mps3 localization on NE in mitotic *mps3-AAA* cells or not? I also constructed *mps3-DDD* mutant (hyperphosphorylation condition). To check this possibility, I induced YES2 plasmid-based expression of Rec8 in mitotic cells (Fig-7A), as I explained in my previous result section. When I expressed Rec8 in *mps3-AAA* mutant, around 6% of cells show NE localization of Mps3, which is significantly lesser than wild-type (p-values are indicating the significance of this values). It indicates that TSS residues play a role in Mps3-NE localization during mitosis. Still half level of cells shows Mps3 localization on NE. While, phoshpo-mimetic version, *mps3-DDD* mutants, which shows normal meiotic

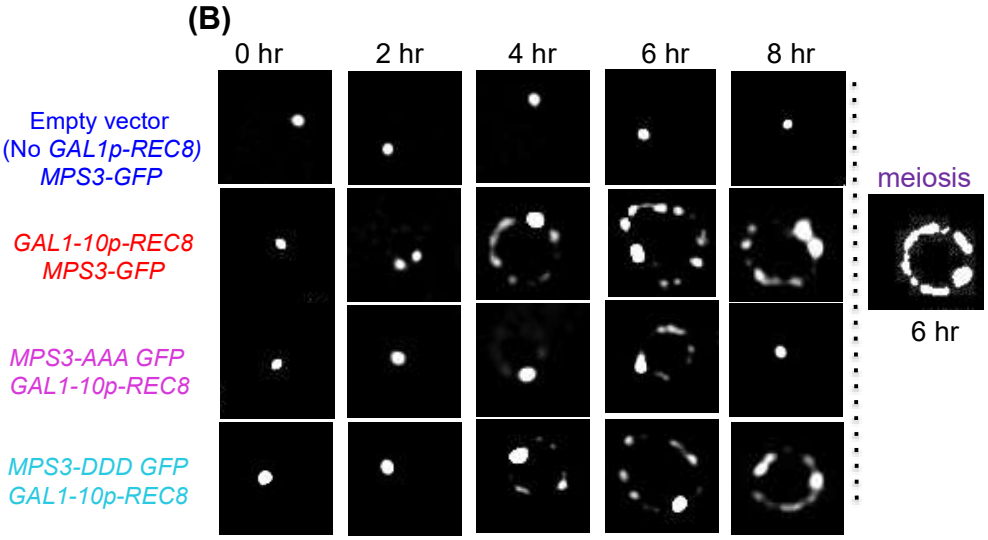
dynamics of Mps3, exhibits near wild-type localization of Mps3 with 15% in mitosis (Fig-7B & 7C). It is clear suppression of Mps3-NE localization reduction what I have seen in *mps3-AAA* mutant. The contribution of these TSS consensus residues on Mps3-NE localization might be different between meiosis and mitosis.

Figure 7:

(A)



(B)



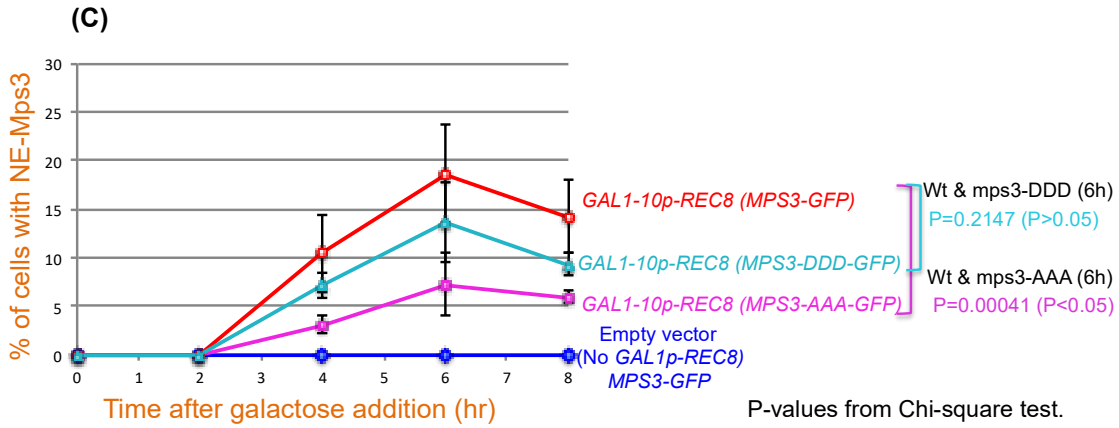


Figure 7. Mps3-TSS residues are important for Mps3-NE localization.

(A). Expression of Rec8 was induced by the addition of galactose. After induction with galactose, expression of Rec8 was checked by western blotting. *MPS3-GFP*, *mps3-AAA GFP* (BJY346) *& mps3-DDD GFP* (BJY347) strains with YES2-Rec8 or YES2 vector used as a control.

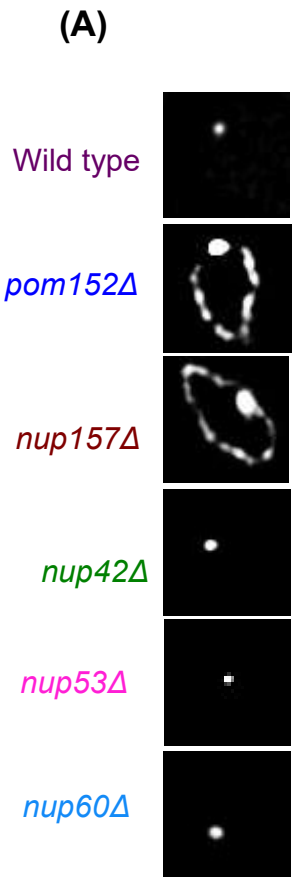
(B). Localization of Mp3-GFP in mitotic diploid cells with galactose induction of Rec8 in *MPS3-GFP* (BJY9/10), *mps3-AAA GFP* (BJY346) *& mps3-DDD GFP* (BJY347) were examined by epi-fluorescence microscope. The bar indicates 1 μm.

(C). Kinetics of Mps3-NE localization. Cells containing *MPS3-GFP* (BJY9/10), *mps3-AAA GFP* (BJY346) *& mps3-DDD GFP* (BJY347) signals on NE were counted at each time point (more than 100 cells). Error bars are standard deviation (n=3 experiments).

Nup157 and Pom152 negatively regulate Mps3 localization on NE during mitosis:

NPC is a large protein complex made up of different nuclear porins with POMs, coat nups, adaptor nups and channel nups. Deletion of nuclear pore components such as *nup157* and *pom152* can suppress the vegetative lethality of the *MPS3* gene. (Witkins *et al.*, *2011*). So, I wondered whether these nuclear pore proteins could regulate Mps3 localization or not? Among nuclear porins, I checked on non-essential porins first and checked Mps3-GFP localization in the mutants and found that, in both *pom152* and *nup157* mutant, Mps3 localized to NE even in vegetative cells (mitotic stage). *nup42Δ, nup53Δ* and *nup60Δ* cells does not show this kind of NE localization (Fig-8A). It indicates specific NPC components negatively regulate Mps3 localization on NE. [This data Referred from Rao-san (previous Shinohara lab member) works, confirmed by myself].

Figure 8:



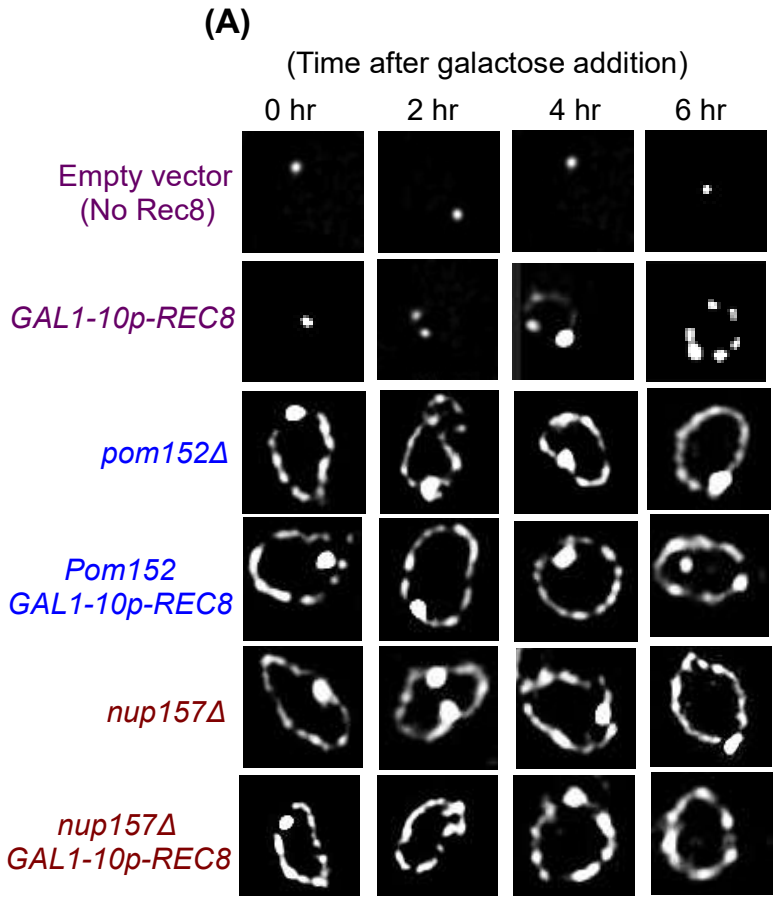
(A). Mp3-GFP in mitotic diploid cells under various nup deletion background such as *pom152* (BJY342), *nup157* (BJY341), *nup42* (BJY343), *nup53* (BJY344) and *nup60* (BJY345) were visualized under epi-fluorescence microscope. The bar indicates 1 μm.

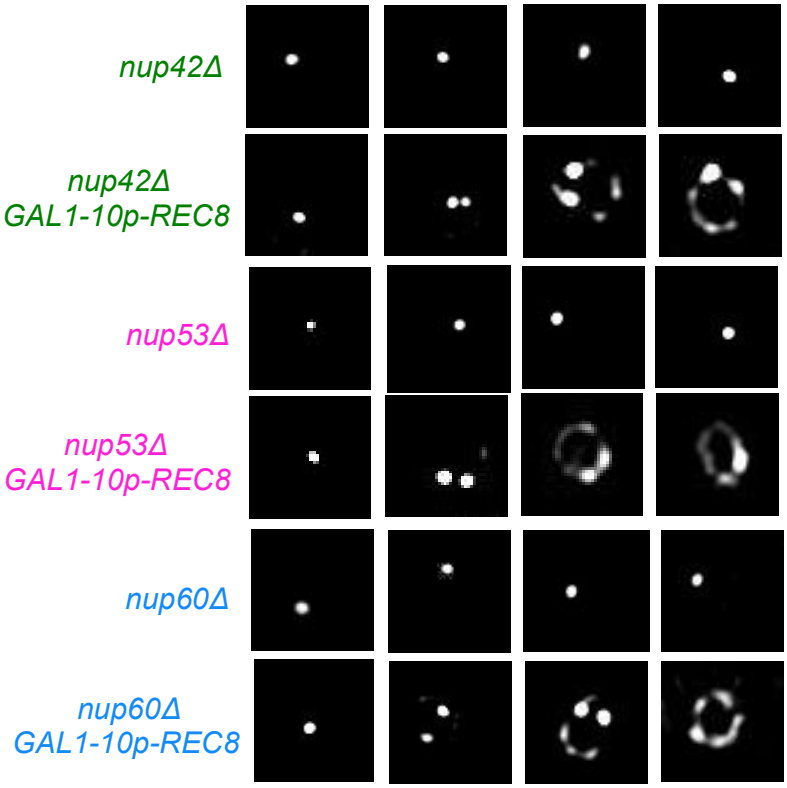
Rec8 expression promotes Mps3 localization in different nucleoporin deletion mutants during mitosis:

Here, I focused on non-essential nuclear porins and checked Mps3-GFP localization in the mutants. I found in both *pom152* and *nup157* mutant, Mps3 is localized to NE forming foci. This NE-localization not observed in other nuclear-porin deletion background such as *nup42Δ, nup53Δ* and *nup60Δ.* mutants. These indicate specific NPC components negatively regulate Mps3 localization on NE. Pom152 and Nup157 together with other proteins such as Ndc1 form a sub-complex in nuclear pore complex (NPC), which embedded inside the nuclear envelope (NE), suggesting that this sub-complex formation is critical for the negative regulation on NE-localization of Mps3. Now, since Pom152 and Nup157 regulate Mps3 localization on NE, I used Rec8 expression system to know the role of NPC components in Rec8-induced Mps3 localization? (Or) To find out the relationship between NPC components and Rec8 mediated Mps3-NE localization during mitosis. I overexpressed Rec8 (plasmid-based) in different nuclear pore background and observed Mps3-Gfp localization on NE during mitosis. In the case of *pom152* and *nup157* mutants, without induction, there is NE-bound Mps3. Moreover, the size of the nucleus seems much bigger than wild-type in these *pom152* and *nup157* mutants (Fig-9A). Here, 75% (already saturated with Mps3-NE) cells are already showing NE localization. Giving a conclusion based on such a low marginal value is hard. So, I quantified the result of mutants other non-essential nuclear porin components such as *nup42Δ, nup53Δ* and *nup60Δ.* In all these mutants, after Rec8 expression, 8-12% cells showed Mps3 location on NE as multiple foci (Fig-

9B). This number is much lower than the number in the wild type. It indicates that *nup42* and *nup53* might not be positive regulators (minimal contribution). The difference between wild-type and nup60, but not others are statistically significant. This result suggests that at least Nup60 (positive regulator, Fig-9B) required for efficient Mps3 localization on NE under Rec8-OE condition. In conclusion, Different nuclear porins show different regulation of Mps3 on nuclear envelope (NE) during mitotic yeast cells.

Figure 9:




nup42Δ
nup42Δ
GAL1-10p-REC8
nup53Δ
nup53Δ
GAL1-10p-REC8
nup60Δ
nup60Δ
GAL1-10p-REC8

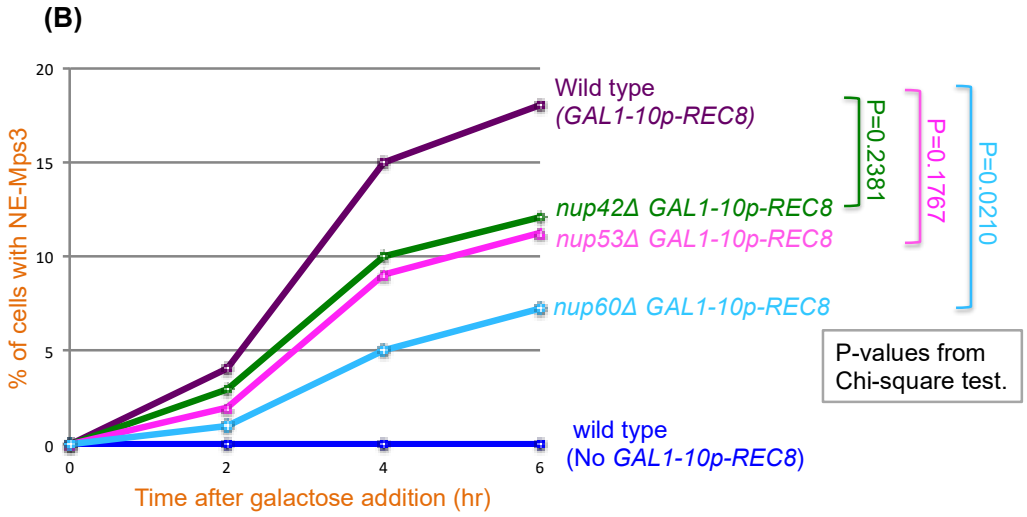

(B)
20
15
10
5
0
% of cells with NE-Mps3
0
2
4
6
Time after galactose addition (hr)
Wild type
(GAL1-10p-REC8)
nup42Δ GAL1-10p-REC8
nup53Δ GAL1-10p-REC8
nup60Δ GAL1-10p-REC8
wild type
(No GAL1-10p-REC8)
P=0.2381
P=0.1767
P=0.0210
P-values from
Chi-square test.

Figure 9: NPC is required for Rec8 mediated Mps3-NE localization

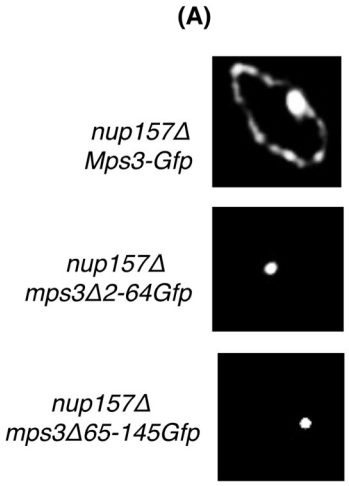
(A). Localization of Mp3-GFP in mitotic diploid cells [*pom152* (BJY342), *nup157* (BJY341), *nup42* (BJY343), *nup53* (BJY344) and *nup60* (BJY345)] with and without galactose induction of Rec8 at different time points were visualized by epifluorescence microscopy. The bar indicates 1μm.

(B). Kinetics of Mps3-NE localization. Cells with Mps3-GFP on NE were counted at each time point (more than 100 cells). *P*-values at 6 hr post induction was calculated with χ^2-test.

Mps3 N-terminus domains are essential for NPC mediated Mps3-NE localization during mitosis:

N-terminus regions of Mps3, 2-64 and 65-145 are located in nucleoplasm are important for localization of Mps3 on NE. I mentioned in my result, *nup157Δ* shows negative regulation of Mps3 on nuclear envelope (NE) this is due to the differential regulation of nuclear porins present in the NE. To check the relationship between NPC and Mps3 N-terminus domains, I constructed *nup157Δmps3Δ2-64GFP & nup157Δmps3Δ65-145GFP* strains. Then, I checked the role of these regions in the localization of Mps3 in mitotic cells in the absence of Nup157. Neither of these *mps3* mutants shows NE localization (Fig-10A), again supporting the idea that N-terminal regions are important/critical for NE localization of Mps3.

Figure 10:



(A). Localization of Mp3-GFP in diploid cells was visualized under epi-fluorescence microscopy in *nup157Δ* (BJY341), *nup157Δmps3Δ2-64 GFP* (BJY527/529) *& nup157Δmps3Δ65-145 GFP* (BJY507/509) strains. The bar indicates 1µm.

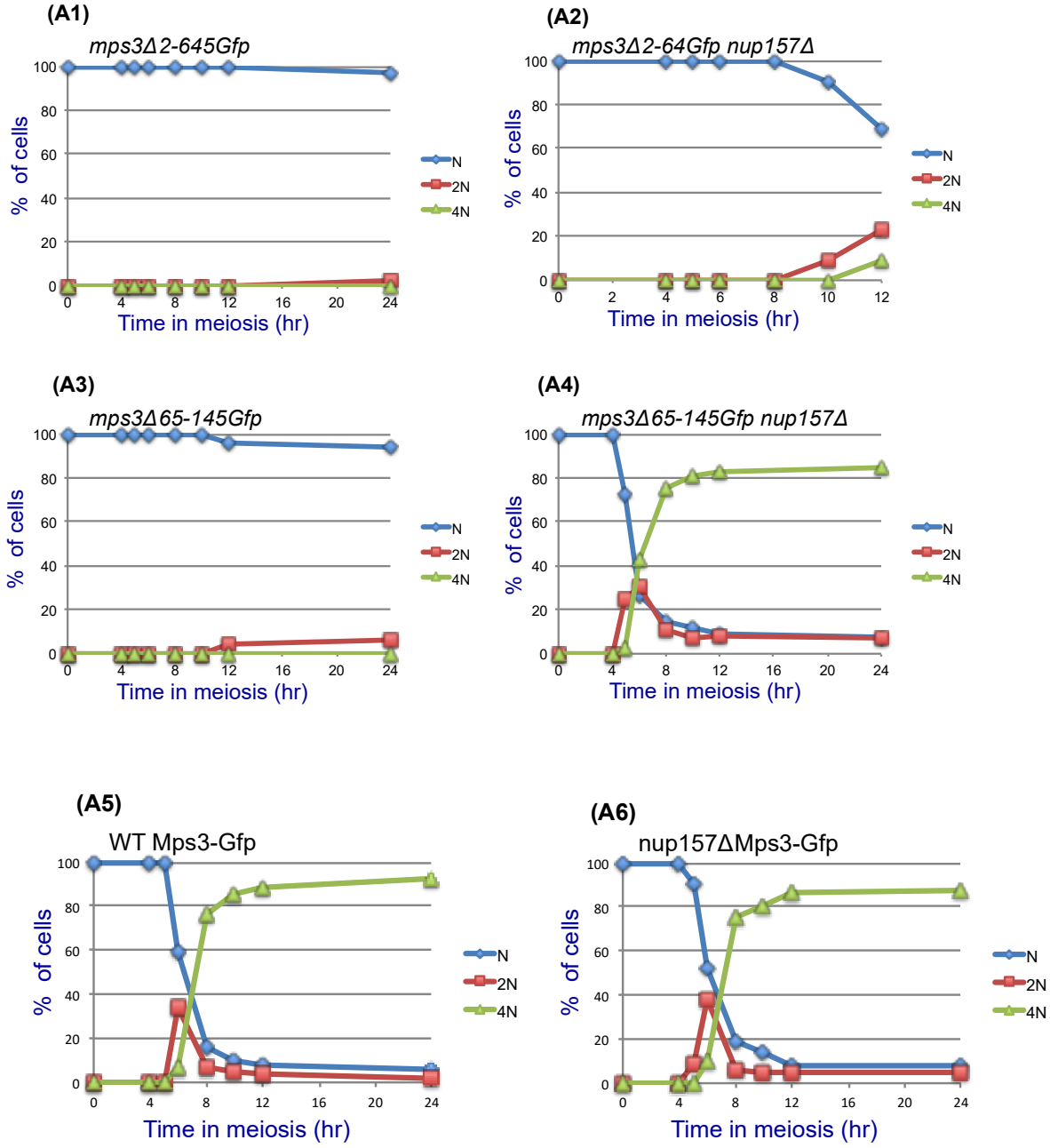
Mps3 N-terminus independent meiosis-specific positive regulation of Mps3 localization on nuclear envelope:

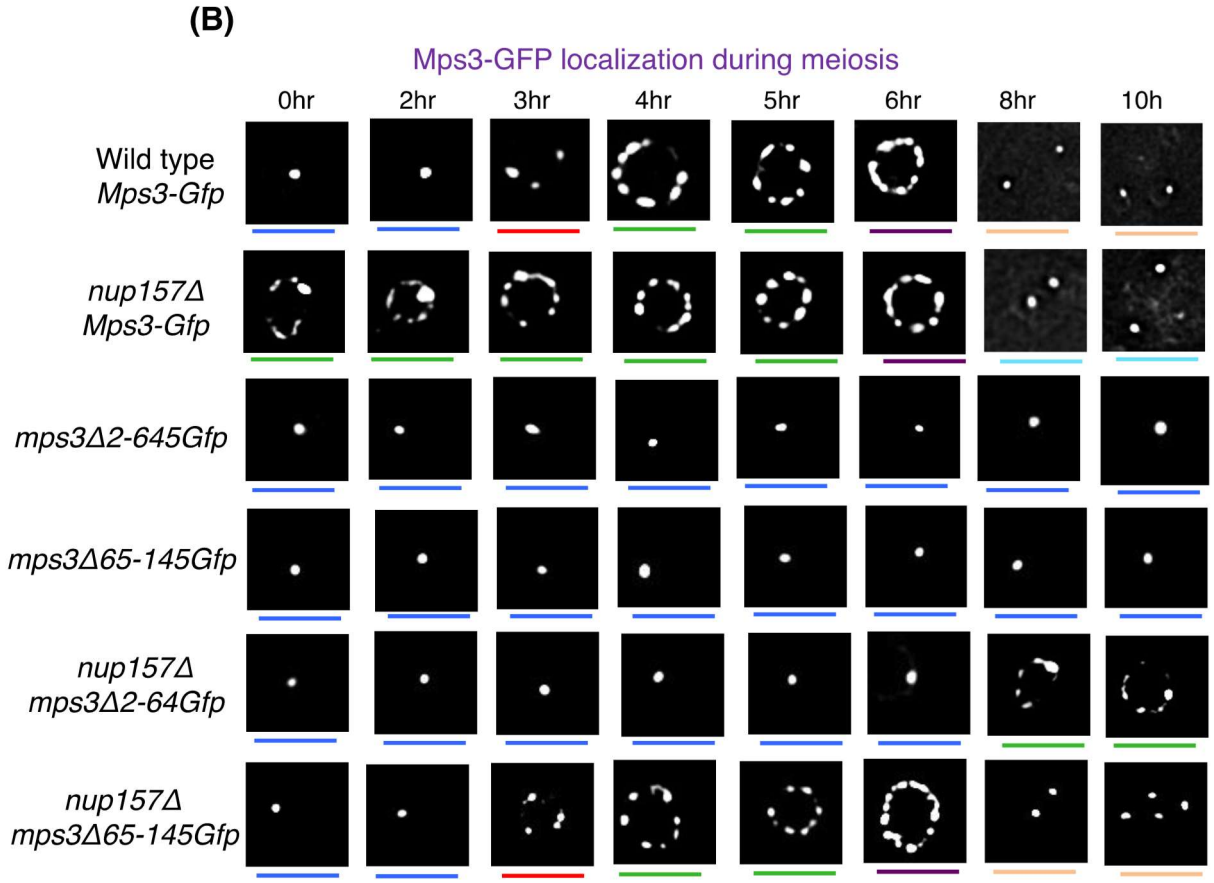
To find out the meiosis-specific relationship between NPC components and Mps3 N-terminus domains, I used *nup157Δmps3Δ2-64GFP & nup157Δmps3Δ65-145GFP* strains. After induced the *nup157Δmps3Δ2-64GFP & nup157Δmps3Δ65-145GFP* cells into SPM (sporulation medium), meiosis initiation. I did a time course for live cell analysis of these double mutant strains. I checked meiotic Mps3 localization on NE in mps3 N-terminus deletion mutants in the absence of *nup157*. As shown previously, meiotic *mps3Δ2-64-GFP* & *mps3Δ65-145GFP* are defective in the localization on NE during meiosis, all-time points it shows only single Mps3 spot which corresponds to SPB localization of Mps3. Surprisingly, *mps3Δ65-145Gfp nup157Δ* shows normal Mps3 localization on NE only during meiosis, as you can see increasing Mps3 foci number from 3hr onwards, showing multiple foci on NE at 4 and 5hour time points, complete NE coverage (Ring form of Mps3) and later on sporulation stage cells shows only SPB localization such as single Mps3 spot (Fig-11B). It is more or less similar to wild-type localization. Weak but significant suppression is also seen for *mps3Δ2-64Gfp nup157Δ* cells such late time points 8hr and 10 hr time points I can see multiple Mps3 foci on NE (delayed NE localization of Mps3, Fig 11B). This result Indicates that some new regulation of Mps3 localization is happening during meiosis in *nup157Δmps3Δ2-64GFP & nup157Δmps3Δ65-145GFP* strains.

Kinetic analysis with different localization pattern supports that *mps3Δ65-145GFP* shows wild-type localization only in the absence of *nup157* (Fig-11C). It suggests that the presence of meiosis-specific positive regulation

of Mps3 localization, which does not require the N-terminus region of Mps3. Conclusion, Mps3 localization regulated by 2 different paths. N-terminus dependent localization of Mps3 on NE and Mps3 N-terminus independent meiosis-specific positive regulation on nuclear envelope (NE).

Figure 11:




(B)
Mps3-GFP localization during meiosis
0hr
2hr
3hr
4hr
5hr
6hr
8hr
10h
Wild type
Mps3-Gfp
nup157Δ
Mps3-Gfp
mps3Δ2-645Gfp
mps3Δ65-145Gfp
nup157Δ
mps3Δ2-64Gfp
nup157Δ
mps3Δ65-145Gfp

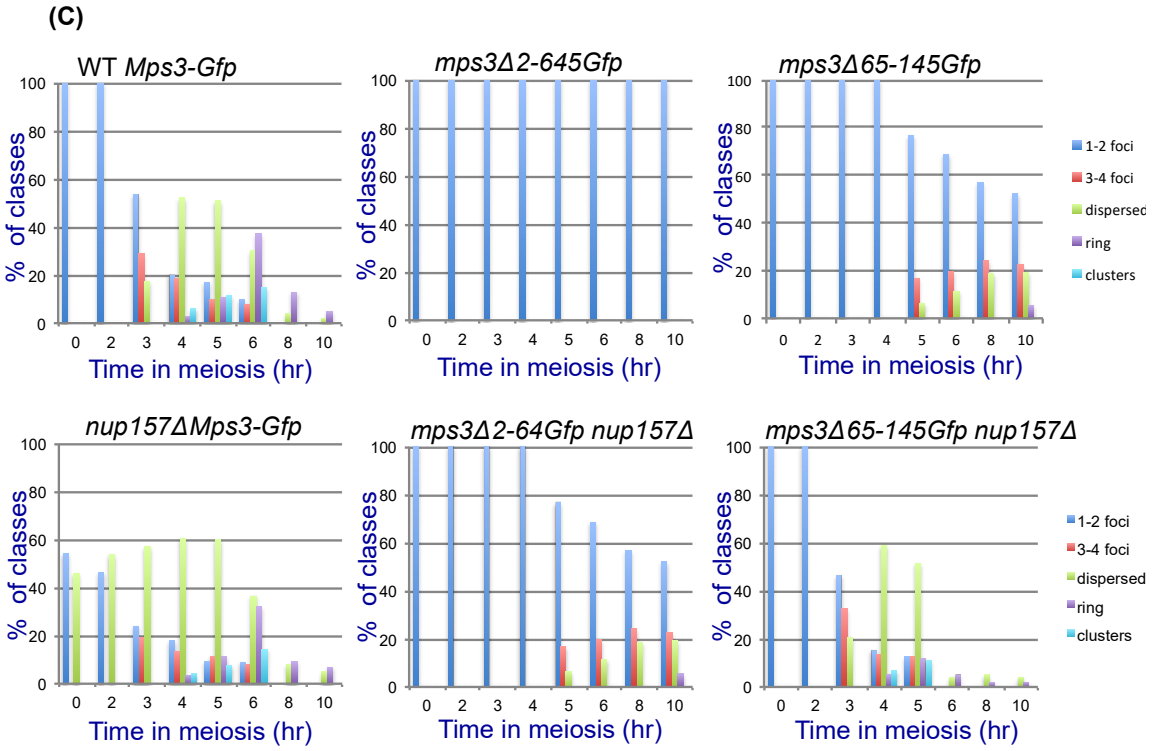


Figure 11:

(A1-A6). DAPI bodies (stained nuclei) of 6 different strains and plotted. *mps3Δ2-64Gfp* (BJY484/488; A1), *mps3Δ2-64nup157Δ* (BJY527/529); A2), *mps3Δ65-145Gfp* (BJY490/494; A3), *mps3Δ65-145Gfp nup157Δ* (BJY507/508; A4), *Mps3-Gfp* (BJY9/10; A5), *nup157Δ Mps3-gfp* (BJY 341; A6).

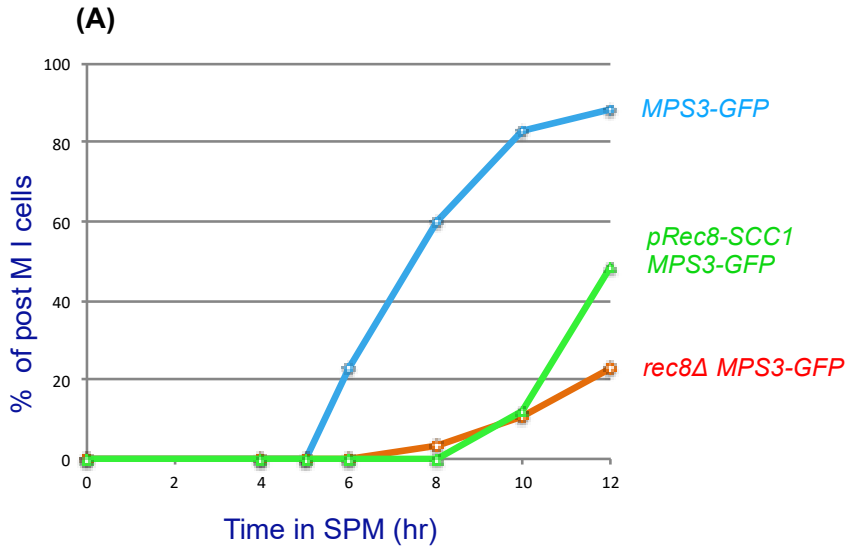
(B). Localization of Mp3-GFP during meiosis, different time points were visualized by epi-fluorescence microscopy in *Mps3-Gfp* (BJY9/10), *nup157Δ Mps3-gfp* (BJY341) *mps3Δ2-64Gfp* (BJY484/488), *mps3Δ2-64nup157Δ* (BJY527/529), *mps3Δ65-145Gfp* (BJY490/494), *mps3Δ65-145Gfp nup157Δ* (BJY507/508). The bar indicates 1μm.

(C). Kinetics of Mps3-NE localization. Cells with Mps3-GFP on NE were classified to subclasses at each time point (more than 100 cells counted) during meiosis and plotted graphs.

Meiotic progression delayed in *pRec8-SCC1* cells:

Rec8 required for the resolution of telomere clustering in the budding yeast; Mps3 interacts with a meiosis-specific cohesion component, Rec8. So, I wondered weather Mitotic klesin component such as Scc1 or Mcd1 could replace the function of this Rec8 mediated telomere cluster resolution. To do this experiment, I created *REC8* promoter containing *SCC1* to express this Scc1 protein during meiosis in the *rec8* deletion background. First I checked the meiotic progression of this mutant and found that *pRec8-SCC1* shows a big delay in meiotic progression (Fig-12A), it indicates that without Rec8, only Scc1 cannot be sufficient for the proper meiotic progression. I also checked the protein expression in this mutant, *rec8* deletion mutant used as a control. I used Anti Rec8, Anti Scc1, Anti-tubulin antibodies to detect this protein on western blotting. We can see no Rec8 expression in both *pRec8-SCC1* & *rec8* deletion mutants. High Scc1 expression (more than 10 folds increase) during meiosis in pRec8-SCC1 cells was observed (Fig-12B).

Figure 12:


(A)
100
80
60
40
20
0
% of post M I cells
0
2
4
6
8
10
12
Time in SPM (hr)
MPS3-GFP
pRec8-SCC1
MPS3-GFP
rec8Δ MPS3-GFP

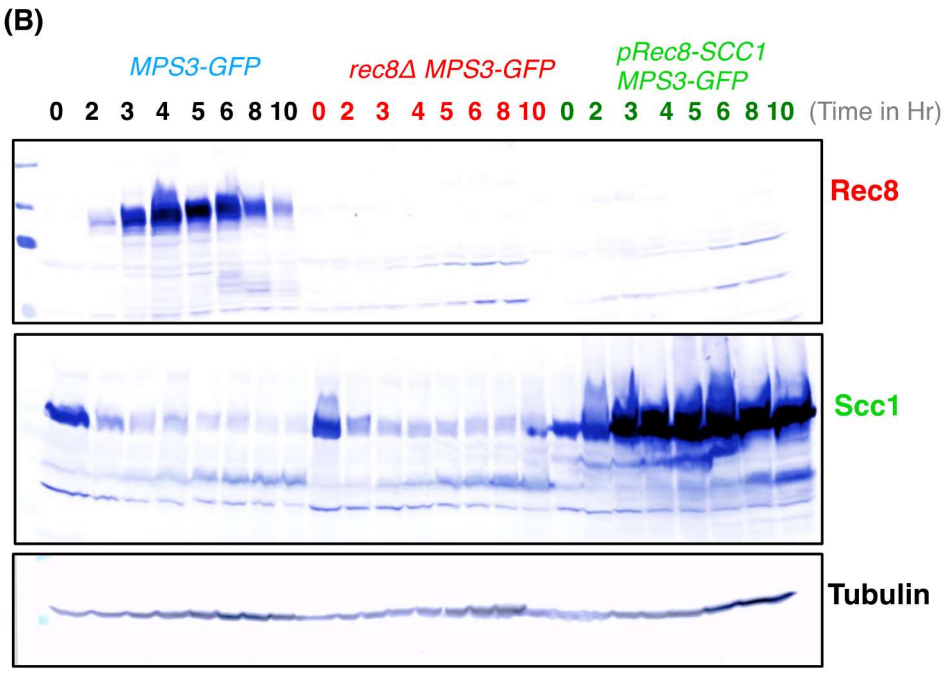

(B)
MPS3-GFP
rec8Δ MPS3-GFP
pRec8-SCC1
MPS3-GFP
0 2 3 4 5 6 8 10 0 2 3 4 5 6 8 10 0 2 3 4 5 6 8 10 (Time in Hr)
Rec8
Scc1
Tubulin

Figure 12:

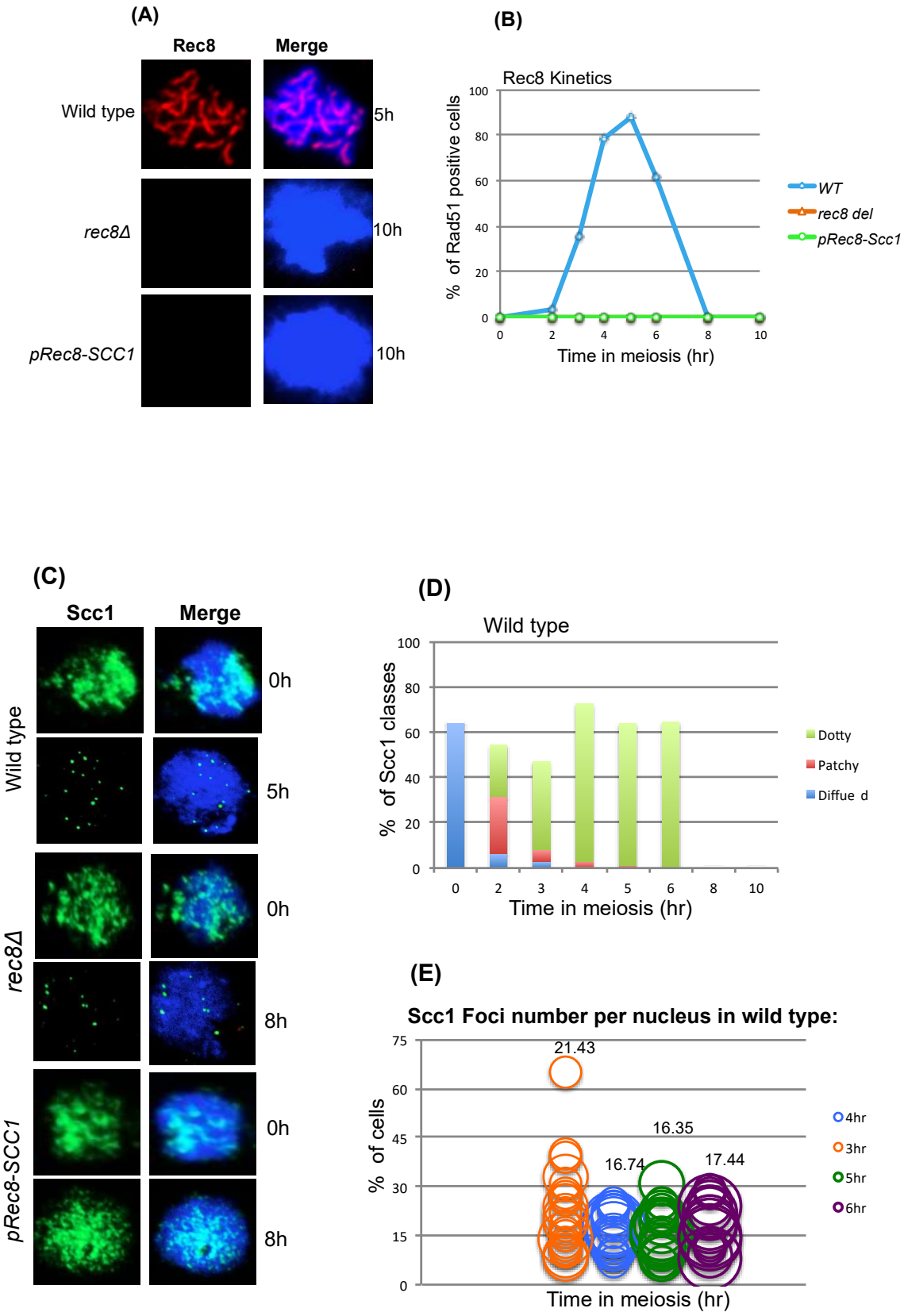
(A). Meiotic progression was checked by the DAPI analysis of *MPS3-GFP* (BJY 9/10)*, pRec8-SCC1 MPS3-GFP, rec8Δ MPS3-GFP* (BJY 299/300) strains. No. Of DAPI bodies were counted on each point and plotted.

(B). Expression of Rec8 and Scc1 were checked at different times points during meiosis by western blotting. Tubulin used as a control. *MPS3-GFP* (BJY 9/10)*, pRec8-SCC1 MPS3-GFP, rec8Δ MPS3-GFP* (BJY 299/300) strains used.

Scc1 Over-expression causes defects in meiotic events:

Weather ectopically expressed Scc1 protein can load on to the chromosomes or not? To confirm this result, I replaced the native Scc1 promoter with the Rec8 promoter and deleted endogenous *REC8* gene on a chromosome. To confirm this strains construction indeed correct. I did cytological analysis meiosis-specific cohesin component, Rec8 protein localization in meiotic chromosomal spreads in *pRec8-SCC1* and *rec8* deletion cells. Rec8 not observed in both *rec8* deletion mutant and *pRec8-SCC1* strains (Fig-13A, 13B). And I observed Scc1 localization in this condition, Scc1 initially accumulates in complete cell, and during meiosis, it shows few foci (Fig-13C), I speculate that residual Scc1 foci might correspond to centromeric cohesin in wild-type meiotic cells (Fig-13E). In *pRec8-SCC1* strain accumulates most of the Scc1 on chromosomes even at 10-hour time point, these results consistent with my western blotting analysis. We also found an interesting observation that Scc1 is cleaved at an early stage of meiosis in a sequential manner, as of now, no mechanistic evidence has been revealed for how mitotic klesin component such as Scc1/Mcd1 is removing and Rec8 is loading on to the chromosomes same time in a stepwise manner. It has to be addressed in detail in future.

Figure 13:


(A)
Rec8
Merge
Wild type
5h
rec8Δ
10h
pRec8-SCC1
10h
(B)
Rec8 Kinetics
% of Rad51 positive cells
Time in meiosis (hr)
WT
rec8 del
pRec8-Scc1
(C)
Scc1
Merge
Wild type
0h
5h
rec8Δ
0h
8h
pRec8-SCC1
0h
8h
(D)
Wild type
% of Scc1 classes
Time in meiosis (hr)
Dotty
Patchy
Diffue d
(E)
Scc1 Foci number per nucleus in wild type:
% of cells
21.43
16.35
16.74
17.44
Time in meiosis (hr)
4hr
3hr
5hr
6hr

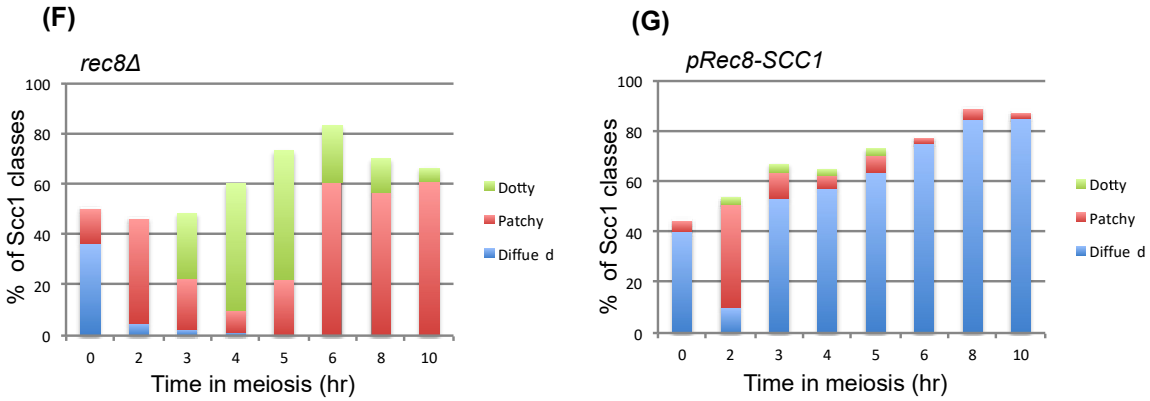


Figure 13:

(A). Immunostaining analysis of Rec8 in wild type (BJY9/10), *rec8Δ* (BJY 299/300) and *pRec8-SCC1* (PRY 648) strains were carried out. Representative photos of Rec8 (red) with DAPI (blue) are shown in each strain. The bar indicates 1μm.

(B). Kinetics of Rec8 localization on chromosomes. Cells with Rec8 positive counted at each time point (more than 100 cells).

(C). Immunostaining analysis of Scc1 in wild type (BJY9/10), *rec8Δ* (BJY 299/300) and *pRec8-SCC1* (PRY 648) strains were carried out. Representative photos of Scc1 (green) with DAPI (blue) are shown in for every strain. The bar indicates 1μm.

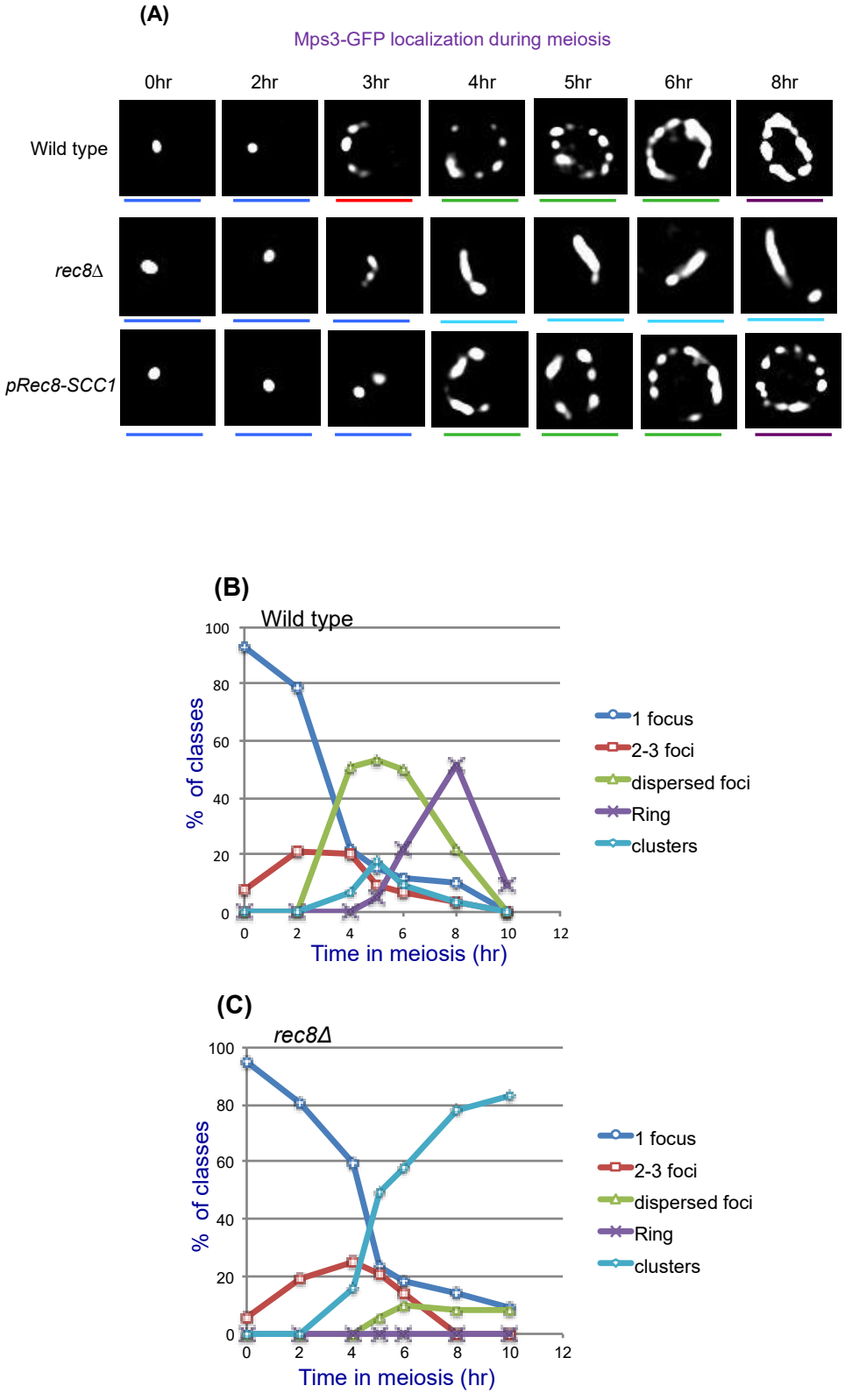
(D, F, G). Kinetics of Scc1 localization on chromosomes. Cells with Scc1 positive counted at each time point (minimum 100 cells). Scc1 foci categorized into classes and plotted bar graph in wild-type (BJY9/10), *rec8Δ* (BJY 299/300) and *pRec8-SCC1* (PRY 648) strains.

(E). Scc1 foci number distribution per a nucleus was shown. The number of Scc1 focus cells at every time points were noted in wild-type (BJY9/10).

Meiosis-specific Over-expression of Scc1 promote Mps3-NE localization in *pRec8-SCC1* cells:

Rec8 indeed necessary for the resolution of telomere clustering in the budding yeast; after confirming the Mps3 interaction with a meiosis-specific klesin component Rec8. I wondered weather Mitotic klesin component such as Scc1/Mcd1 could replace the function of this Rec8 mediated telomere cluster resolution. To do this experiment, I used p*REC8-SCC1* cells, which can express high Scc1 protein during meiosis in the *rec8* deletion condition. Wild-type, Mps3 early meiosis shows single SPB focus later time points in meiosis it shows multiple foci and completes NE ensembles (Fig-14A). In rec8 deletion, large clusters/ accumulation of Mps3 observed from the 4hr time point of meiosis. Interestingly, *pRec8-SCC1* cells after entering into the meiosis; it shows from single Mps3 focus localization to multiple as well as complete Mps3-NE localization as late stages of meiosis such as pachytene (Fig-14A, 14D). This Meiosis contributed Scc1 mediated Mps3 localization on NE might be independent to the mitotic experiments, where I can see the only single focus of Mps3 localization.

Figure 14:


(A)
Mps3-GFP localization during meiosis
0hr
2hr
3hr
4hr
5hr
6hr
8hr
Wild type
rec8Δ
pRec8-SCC1
(B)
Wild type
% of classes
Time in meiosis (hr)
1 focus
2-3 foci
dispersed foci
Ring
clusters
(C)
rec8Δ
% of classes
Time in meiosis (hr)
1 focus
2-3 foci
dispersed foci
Ring
clusters

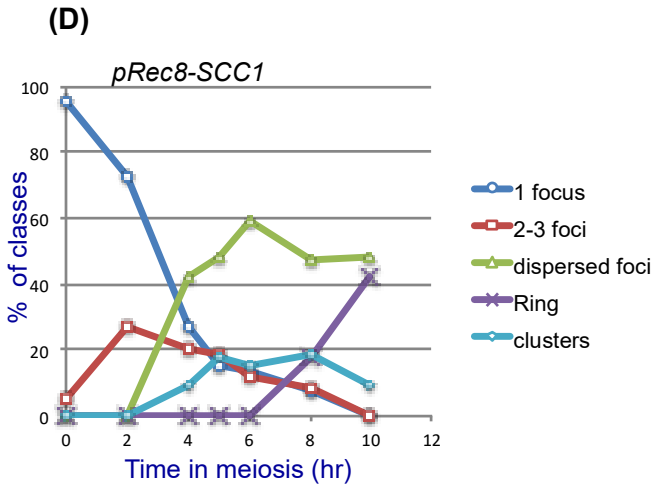


Figure 14:

(A). Localization of Mp3-GFP during meiosis, different time points were visualized by epi-fluorescence microscopy in *Mps3-Gfp* (BJY9/10), *rec8Δ Mps3-gfp* (BJY299/300) *pRec8-SCC1 rec8ΔMps3-Gfp* (PRY648). The bar indicates 1μm.

(B, C&D). Kinetics of Mps3-NE localization. Cells with Mps3-GFP on NE were classified to subclasses at each time point (more than 100 cells counted) during meiosis and plotted graphs.

Discussion

In budding yeast (*Saccharomyces cerevisiae*), SUN domain protein such as Mps3 changes its localization pattern from mitosis to meiosis (Conrad *et al.*, 2007). In vegetative (mitotic) yeast cells, Mps3 visible/localizes as single focus near to the SPB on nuclear envelope. It is indicating that half-bridge components of SPB such as Mps3 localizes near the vicinity of SPB. (Jaspersen, 2002; Nishikawa, 2003; Jaspersen, 2006). Once cells enter into meiosis, mps3 localizes to NE and forms multiple foci/clusters along with SPB (Conrad *et al.*, 2007). This meiosis-specific Mps3-NE localization disappears from the onset of meiosis-I. It clarifies that; Mps3-NE localization is an important feature/event during prophase-I of meiosis. Mps3 shows single foci at the time of meiotic entry, it shows few numbers of foci during early prophase-I, forms transient clusters near mid-prophase-I and it occupies most of the NE in late prophase-I (pachytene) of meiosis. These Mps3 clusters/aggregates are necessary for the actin mediated chromosome motion during meiosis (Trelles-Sticken, 2005; Koszul, 2008). Along with actin depletion by using depolymerization agents such as latrunculin-B, some other meiosis-specific factors like Csm4 and Ndj1 deletions does not affect/change the meiosis-specific Mps3-NE localization (Conrad, 2008; Kosaka, 2008) (HBDP Rao and Akira Shinohara (unpublished data)). This indicates that the there must be a unique, novel mechanism for meiosis-specific Mps3-NE localization.

In my research, I found that the meiosis-specific klesin component of the cohesin complex, Rec8 is sufficient to promote/triggers the Mps3-NE localization even in mitotic cells. Exogenous expression of Rec8,

mostly bound to chromatin (recovered in chromatin fractions) in mitotic yeast cells. It clearly explains that the stable binding of Rec8 to chromatin works as a protein complex in mitotic yeast cells.

This results in supporting the published research such as the mitotic sister-chromatid function of Scc1/Mcd1 can be complemented with mitotically expressed Rec8 (Buonomo, 2000; Heidinger Pauli, 2008).

Even though Rec8 forming a complex with chromatin and regulates the mitotic Mps3-NE localization, this is about 20-26%. So, still, there is a possibility of Rec8 role as a cohesion independent regulation of Mps3-NE localization.

When *rec8* deletion mutant cells enter into meiosis, this mutant shows defective Mps3 clustering resolution (Conrad *et al.*, 2008), indicating that Rec8 importance in Mps3-NE dynamics and localization. The meiosis-specific Physical interaction between the Rec8 and Mps3 supporting the theory that meiosis-specific cohesin component Rec8 controls the Mps3-NE localization.

It is reported that mitotic klesin, Scc1 is a component of cohesin complex can minimize the telomere clustering resolution defects in *rec8Δ* cells (Trelles-Sticken *et al.*, 2005). It indicates that Scc1 or Rec8 mediated cohesion activity plays a key role for Mps3 dynamics on nuclear envelope. It opens a question, and it needs to be answered by future analysis of how meiosis specific cohesion such as Rec8 can controls the Mps3-NE localization during mitosis as well as meiosis. I confirmed that mitotic klesin, component of cohesin complex such as Scc1/Mcd1 over-expression during meiosis, could only complement the Mps3-NE localization function but not other meiotic events/functions (Trelles-Sticken *et al.*, 2005; Conrad *et al.*,

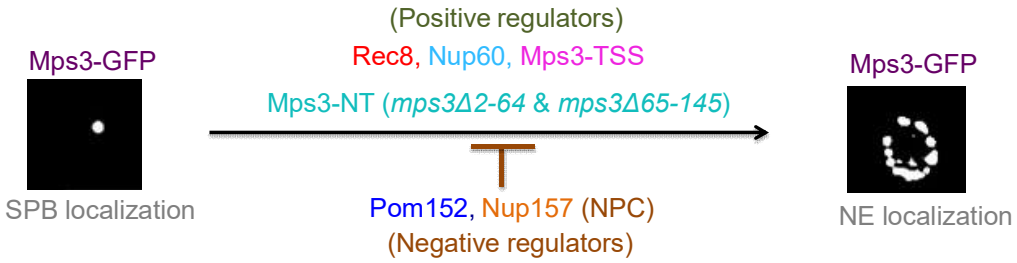
2008). However, Scc1 cannot complement/mediate to promote Mps3-NE localization during meiosis. Scc1 function towards NE localization of Mps3 is independent from mitosis to meiosis.

Three Mps3 consensus sequence/putative amino acids such as 188T, 189S and 190S were identified and confirmed that Mps3 TSS residues are important for Mps3-NE localization as well as dynamics on NE during meiosis (HB Rao and Akira Shinohara, unpublished data), this result indicates that phosphorylation of Mps3 protein contributes to this kind of NE localization of Mps3. Meiosis-specific cohesion component, Rec8 mediated Mps3-NE localization data also proven in my study that this consensus motif (TSS) required for efficient Mps3-NE localization. It proves that Rec8 expression system in mitosis can reveal the components/regulators (both positive and negative) for NE localization of Mps3.

Mps3-NE localization mediated by meiotic cohesin Rec8, which requires the N-terminus region of Mps3 (N-terminus domain & Acidic domain). It's reported that Mps3 N-terminus region required for Mps3-NE localization during meiosis (Shuh-ichi *et al.*2003; Conrad *et al.*2007). Mps3 N-terminus region is present inside the nucleoplasm; this gives us an idea that Mps3 interaction with chromatin at nucleoplasm is important for Mps3-NE localization during meiosis. The simple interpretation is, Rec8 or Rec8 cohesin might interact with the Mps3 N-terminus as well acidic domain and contributes to the NE localization of Mps3. I also noticed that Mps3-NE localization has two different regulations under *nup157* deletion background. It has been well studied that; vegetative lethality of Mps3 can suppress by nup deletions (Witkins *et al. 2011*). It is indicating that Mps3 has genetic

interaction with nuclear-porins such as Nup157. Identified two ways Mps3 regulation. First one is N-terminus dependent Mps3-NE localization during both mitosis and meiosis. Second is N-terminus independent meiosis-specific positive regulation of Mps3-NE localization. Here, Rec8 might bind with Mps3 indirectly, or some unknown alternative mechanism works actively to contribute to meiosis-specific positive regulation. This result stimulates the idea that novel pathways are working to regulate Mps3 localization on nuclear envelope (NE). Along with this, still, it has been addressed the importance of Mps3 lumen regions of Rec8 mediated Mps3-NE localization by further analysis.

Conclusion/Summary:



Graphic Representation:

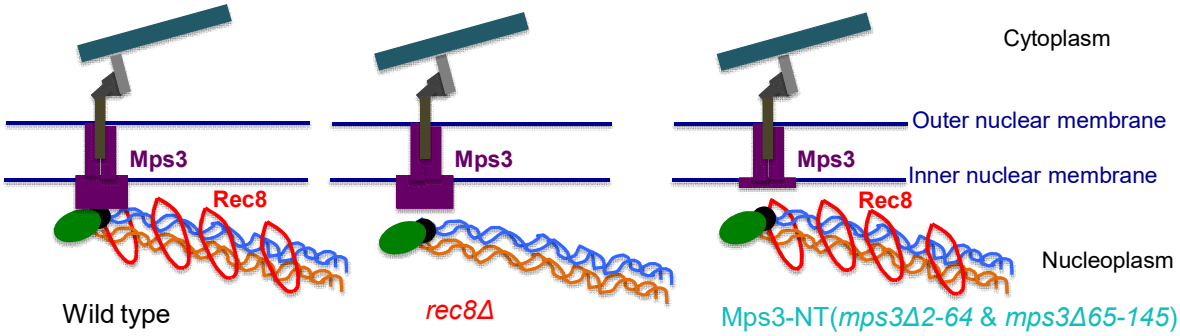


Table 1 (Strains used in my research):

BJY1	a	MSY 833; MAT a, ho::LYS2, ura3, leu2::hisG, trp1::hisG, lys2
BJY2	alpha	MSY 832; MAT α, ho::LYS2, ura3, leu2::hisG, trp1::hisG, lys2
BJY 9	a	ho::LYS2, ura3, leu2::hisG, trp1::hisG, lys2 , *MPS3-GFP*
BJY 10	alpha	ho::LYS2, ura3, leu2::hisG, trp1::hisG, lys2 , *MPS3-GFP*
BJY	diploid	*Mps3-GFP pYES2(URA)*
BJY	diploid	*Mps3-GFP pYES2-REC8 (URA)*
BJY305	a	*MPS3-3FLAG::KanMX6, NHJ1-3HA:: KanMX6*
BJY306	alpha	*MPS3-3FLAG::KanMX6, NHJ1-3HA:: KanMX6*
BJY299	a	*rec8Δ:Mps3-GFP*
BJY 300	alpha	*rec8Δ:Mps3-GFP*
PRY 648	diploid	*pRec8-SCC1 rec8d Mps3-Gfp*
BJY 341	diploid	*nup157⊿MPS3-GFP*
BJY 342	diploid	*pom152⊿MPS3-GFP*
BJY343	diploid	*nup42⊿MPS3-GFP*
BJY 344	diploid	*Nup53⊿MPS3-GFP*
BJY 345	diploid	*nup60⊿MPS3-GFP*
BJY 346	diploid	mps3-AAA-GFP
BJY 347	diploid	mps3-DDD-GFP
BJY494	alpha	*MPS3-d2-64-GFP::KanMX6*
BJY490	a	*MPS3-d2-64-GFP::KanMX6*
BJY488	alpha	*MPS3-d65-135-GFP::KanMX6*
BJY484	a	*MPS3-d65-135-GFP::KanMX6*
BJY 508	alpha	*nup157Δ mps3Δ65-145GFP* (KAN,KAN)
BJY 507	a	*nup157Δ mps3Δ65-145GFP* (KAN,KAN)
BJY 527	a	*nup157Δ mps3Δ2-64 GFP* (KAN,KAN)
BJY 529	alpha	*nup157Δ mps3Δ2-64 GFP* (KAN,KAN)

Table 2 (Primers used for research):

BJS_013_Gal1P+ Rec8+TRP1 FP	TAATTTCTTTTTAGCCTCAGCAACTCTAAAGCATTTG CTATATATAGATTAATATTACAAAT
BJS_014_Gal1P+ Rec8+TRP1 RP	CAGAAAGCAACCACACTGTTGTGAGGCCCTTATATT TCTTGTCATCTTTAAAGTTCAACG
BJS_015_Gal1+R ec8_Chk_FP	GAG AAC TTG ACT TCG GCG GC
BJS_016_Gal1+R ec8_Chk_RP	CCA GGT TAA AGT CCA CAT CAA CGG
BJS_017_Nup157 _Chkng FP	GTA TTA CCC GAC TTT GGA GAG G
BJ_028_Rec8+Hin dIII FP	GCC AAG CTT ATG GCA CCT CTT TCG TTG AAC
BJS_029_Rec8+X baI RP	CGC TCT AGA TCA GGC ATA TAC AAT TAT TTC
BJS_053_Mps3-Gfp+XbaI RP	CGC TCT AGA TGT GGC TGT GTT CAA GGT TGG
BJS_38_mps3del2 -64 F.P.	TGT CAC TCA GAA CAC CGA CG
BJS_39_mps3del2 -64 R.P:	GTG TGG CTG TGT TCA AGG TTG G
BJS_046_Scc1+Hi ndIII FP	GCC AAG CTT ATG GTT ACA GAA AAT CCT CAA
BJS_047_Scc1+X baI RP	CGC TCT AGA TTA AGC ATT GAT AAA CCT TTC

Table 3 (Antibodies Used):

Tubulin	Primary 1:1000 Rat	Anti Rat 1:5000 Secondary
Rec8	Primary 1:1000 Rabbit	Anti Rabbit 1:7500 Secondary
Zip1	Primary 1:1000 Rabbit	Anti Rabbit 1:2000 Secondary
Scc1	Primary 1:1000 GP	Anti GP 1:7500 Secondary
Rad51	Primary 1:1000 GP	Anti GP 1:2000 Secondary
H2B	Primary 1:2000 Rabbit	Anti Rabbit 1:7500 Secondary
Dmc1	Primary 1:1000 Rabbit	Anti Rabbit 1:2500 Secondary
FLAG	Primary 1:1000 Mouse	Anti Mouse 1:7500 Secondary
HA	Primary 1:1000 Mouse	Anti Mouse 1:7500 Secondary

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