Doctoral thesis

Control of chromosome dynamics during meiosis I by Cohesin and its regulator Rad61/Wpl1

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List of Abbreviations

5-FOA	5-fluoroorotic acid
AE	axial elements
AP	alkaline phosphatase
APC/C	anaphase promoting complex/cyclosome
ATP	adenosine triphosphate
BSA	bovine serum albumin
°C	degree Celsius
CAR	cohesin associated regions
Cdks	cyclin dependent kinase
CE	central elements
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
JMs	joint molecules
LE	lateral elements
MI	meiosis I
MII	meiosis II
MES	2-(*N*-morpholino) ethane sulfonic acid
mJ	mille joules
ml	microliter
NCO	non-crossover
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
PBS	phosphate-buffered saline
PLK	polo-like kinase
PP2A	protein phosphatase 2A
RNase	ribonuclease
SAC	spindle assembly checkpoint
SC	synaptonemal complex
SCC	sister chromatid cohesion
SEI	single-strand invasion intermediate
SMC	structural maintenance chromosome
SPB	spindle pole body
SUMO	small ubiquitin like modifier

TBS	Tris-buffered saline
TCA	trichloroacetic acid
UV	ultraviolet
V	volt
ZMM	zip, msh, mer

Summary

Meiosis-specific cohesin required for the holding of sister chromatids plays a critical role in multiple chromosomal events during meiotic prophase I. Rad61/Wpl1 negatively regulates cohesin functions by promoting cohesin dissociation. In my thesis, I studied roles of Rad61/Wpl1 in budding yeast meiosis and found that axes of meiotic chromosomes are shortened in the *rad61/wpl1* mutant by without altering the levels of cohesin, suggesting that Rad61/Wpl1 negatively controls chromosome axis compaction. Rad61/Wpl1 is required for efficient repair of meiotic double-strand breaks. And Rad61/Wpl1 is required for efficient resolution of telomere clustering during meiosis I, indicating a positive action of Rad61/Wpl1 on the cohesin function for telomere dynamics. Thus, Rad61/Wpl1 controls various meiotic chromosomal events in positive and negative ways in a context-dependent manner.

Chapter 1

Introduction

Central to sexual reproduction is the production of haploid gametes (eggs and sperm in humans) with half of the chromosome complement compared to the original parent diploid cell. Sexual reproduction in eukaryotes utilizes specialized cell division to produce haploid gametes called 'Meiosis'.

For many decades researchers have been studying the chromosome morphogenesis and dynamics during meiosis in attempt to understand the molecular events involving in proper formation of haploid gametes. In this thesis, I am studying the roles of a 'cohesin' component particularly to address how the cohesin component regulates chromosome events during meiosis.

1-1 Meiosis

Meiosis is a special type of cell division necessary for sexual reproduction in eukaryotes. To understand this complex process, we first need to understand cell cycle and, in particular, mitosis in somatic cells. Cells first need to generate an exact replica of the entire genome through DNA replication during the S phase. Replication gives rise to pairs of duplicated chromosomes (sister chromatids) that eventually segregate into the daughters as the cell goes during M phase. However, the S phase is followed by G2, in which cells need to get equipped for the subsequent M phase, creating a temporal lapse between the duplication of the chromosomes and their ultimate segregation. In the eukaryotic cell, mitosis is divided into four stages like prophase, metaphase, anaphase and telophase.

Meiosis is characterized by a single round of DNA replication followed by two successive rounds of chromosome segregation. The net result is four daughter cells with a half of chromosome numbers of a original parent cell (Figure I). During the first meiotic division homologous chromosomes that differ in parental origin are segregated from each other. The second meiotic division partitions replicated sister chromatids from one another, much like a mitotic division. The segregation of homologous chromosomes during the first meiotic division requires several modifications of chromosomes, which will be outlined in detail in following sections. Briefly, this involves the formation of chiasmata between homologous chromosomes that are the result of reciprocal crossover recombination events.

The crossing-over also generates novel combination of parental alleles that break up gene linkages and genetic diversity in the offspring.

1-1-1 Meiotic prophase I

Meiotic prophase I is the most important phase responsible for formation of proper gametes with a correct chromosome number at the end of the meiosis. During meiotic prophase I, as the DNA replication is completed, sister chromatids are tightly bound with each other by the protein complex named cohesin (Marston et al., 2014; Remeseiro et al., 2013). Chromosomes undergo lots of structural changes for finding proper homologous chromosomes, which is accompanied with well-controlled recombination events. The cohesin complex provides structural basis for the formation of a meiosis-specific tripartite chromosome structure called synaptonemal complex (SC) that keeps the homologues chromosomes connected with each other (Klein et al., 1999). SC contains two chromosome axes called the axial/lateral elements containing two sister chromatids, which are connected through transverse filaments in a central region. Meiotic prophase I is further divided into Leptotene, Zygotene, Pachytene, Diplotene, and Diakinesis based on chromosome structures including SCs (Zickler and Kleckner, 1998). Also telomere clustering is observed during meiotic prophase, which is important for chromosome pairing.

Leptotene is the initial stage of meiotic prophase I, where cohesin holds sister chromatids together and chromatids start to condense into a chromosome axis. During late Leptotene stage, search for the homologues chromosomes is underway while axial elements of SC begin to assemble on to the chromosomes. Moreover, the initiation of meiotic recombination, that is the formation of double-strand breaks (DSB) by Spo11 on DNA, occurs in this phase. In the Zygotene stage, homologous chromosomes with axial elements initiate to engage in pairing with each other, thus SC formation. In this stage, DSBs are converted into a next intermediate for the recombination referred as to Single-End Invasion (SEI). At this stage chromosomes undergo rearrangements inside the nucleus by the attachment of telomeres to centrosome (yeast spindle pole body (SPB) to form telomere clusters called bouquet-like structures (Zickler and Kleckner, 1999).

In the early pachytene stage, the homologues synapsis is complete so that SC is extended to an entire length of the chromosomes, which is described as elongated SC's. At early pachytene, the recombination proceeds from SEIs to another intermediates containing double-Holiday Junctions (dHJs) by capturing with the other end of DSB (Kerr et al., 2012). The resolution of dHJs to reciprocal crossover products occurs in the middle of pachytene

accompanied with the disassembly of SCs. This transition is controlled by mechanism called pachytene or recombination checkpoint (Marston and Amon, 2004; Kerr et al., 2012).

In the diplotene stage, the SC dissembles and homologous chromosomes are loosely connected to each other except at the chiasma, which holds the chromosomes together until the onset of metaphase I (Zickler and Kleckner, 1998). In the diakinesis phase, the chiasmata are mostly visible between the homologs chromosomes to ensure proper segregation. Later diakinesis stage is followed by the metaphase I, at these stage chromosomes are moved by the microtubule-directed motion at kinetochores. The activation of separase at anaphase I lead to the dissociation of arm-cohesin, while sister chromatid separation at kinetochores is protected by Shugoshin. As a result, at end of meiosis I homologous chromosomes segregate to the different poles. Followed by meiosis II, sister chromatids are separated by cleavage of the centromeric klesin by separase. At the end of the meiosis, four proper haploid gametes are formed.

During meiosis proper segregation of homologues chromosomes in first meiotic division and sister chromatid segregation in second meiotic division is mainly achieved by sister chromatid cohesin (SCC). To understand these complex processes, we first need to understand sister chromatid cohesin in mitosis and meiosis.

1-2 Sister chromatid cohesion (SCC)

Evolutionarily species organize their genomes in multiple numbers of chromosomes and face the problem of segregation of sister chromatids when it comes to faithful distribution of the previously replicated genomes in subsequent cell divisions. Cells have developed an effective mechanism preventing this problem, that is ‘cohesion’. The process physically links sister chromatids, termed sister chromatid cohesion (SCC), which is crucial for the fidelity of chromosome segregation. Cohesion opposes the pole-ward forces of the spindle microtubules attached to two sister kinetochores ensuring amphitelic arrangement of the chromatids on the metaphase plate, a process termed as biorientation. Unequal tension between any sister chromatids activates spindle assemble checkpoint (SAC) which prevents the onset of anaphase which is accompanied with the dissolution of SCC. Following the establishment of biorientation, the SAC is inactivated and cohesion is dissolved, allowing the progression of the chromatids to separate during anaphase. Cohesion defects may lead to improper segregation of chromosomes giving rise to aneuploidy, which is often associated with genetic disorders like cancer.

1-3 Architecture of the cohesin complex in mitosis

SCC is mediated by a protein complex called cohesin. The structural insight on the cohesin complex has been well characterized in budding yeast. Based on the biochemical and electron microscopic studies, several groups proposed organization of cohesin complex and also the models of cohesin binding to sister chromatids. In budding yeast, cohesin holds sister chromatids together by forming a complex with three protein subunits, Smc1, Smc3, and klesin Scc1/Mcd1 during mitosis, and Smc1, Smc3 and klesin Rec8 during meiosis. Smc1 and Smc3 belong to structural maintenance of chromosome (SMC) family. These proteins consist of a separated ATPase domain flanking a 40 nm anti-parallel coiled coil rod domain with a hinge (Michaelis et al., 1997; Strunnikov et al., 1993) and form a heterodimer by interacting via hinge domains and between ATPase heads (Melby et al., 1998). (Haering et al., 2002; Hirano et al., 2002). The ATPase heads of both the proteins are directly connected with Scc1/Mcd1 subunit and the coordination of these three proteins forms a ring-like structure with a diameter of around 40nm, which is likely to embrace two sister chromatids (Haering et al., 2002) (Figure II). Another major subunit of cohesin complex, Scc3/Irr1, is associated with Scc1/Mcd1. The Scc3 contains HEAT repeats, which are required for the protein-protein interactions.

1-3-1 Cohesin-associated proteins

Along with four core proteins of the cohesin complex, additional proteins are identified to be associated with Pds5, Wapl (Rad61) and Sororin. All these components play regulatory roles in cohesin maintenance and cohesin removal during mitosis and meiosis. These cohesin-associated proteins are less stably bound to the cohesin complex when compared to core components.

Pds5 contains the HEAT repeat. In most eukaryotes Pds5 is essential for SCC (Hartman et al., 2000; Dorsett et al., 2005). It has been shown that Pds5 interaction with Smc3 and Eco1 generates cohesive state of SCC and non-cohesive state is promoted by dissociation of Pds5 when it interacts with Wapl (Sutani et al., 2009; Nishiyama et al., 2010). Apart from SCC, Pds5 is involved in chromosome axial compaction, DSB repair and homologues recombination in meiosis of budding yeast (Jin et al., 2009). It has been previously demonstrated that Pds5 undergoes sumoylation, which is conjugation of Small ubiquitin-like modifier protein (SUMO) and this posttranslational modification is cell-cycle dependent and is required for the cohesin disassembly (Stead et al., 2003).

In higher eukaryotes Pds5 exists in two isoforms Pds5A and Pds5B and both are associated with the cohesin independently (Sumara et al., 2000; Losada et al., 2005). The two Pds5 isoforms form a complex with either SA1 or SA2 (Scc3 isoforms) and the combination results in four different forms of cohesin in vertebrate cells. In vertebrates, Pds5 is also associated with the other protein namely Sororin in addition to Wapl (Sumara et al., 2000; Gandhi et al., 2006; Kueng et al.2006; Rankin et al., 2005). All these findings suggest that Pds5 has a dual role i.e. in association and dissociation of cohesin.

Another important cohesin associated protein is Rad61/Wapl (Wings apart-like protein) first discovered in *Drosophila*. Wapl has been shown to be an important regulator of cohesin removal and heterochromatin organization (Verni et al., 2000; Warren et al, 2004). Based on genetic experiments in yeast, it is shown that Rad61 binds simultaneously to Scc3 and Pds5 as a complex and this complex releases entrapped sister DNA in cohesin complex by opening the interface between Scc1 and Scc3 (Rowland et al., 2009). The acetylation of cohesin core subunit Smc3 by Eco1 at S phase counteracts the destabilizing by Rad61 and triggers the cohesin establishment. X-ray crystallographic studies on Wapl revealed that Wapl binds to an ATPase head of the Smc3 protein and regulates its ATPase activity, which depends on acetylation state of Smc3 (Chatterjee et al., 2013). The deletion of the *RAD61/WPL1* showed very less cohesin defects in budding yeast mitosis and showed increased chromosome condensation of rDNA region in interphase and mitosis (Lopez et al., 2013).

In vertebrates, the binding of Wapl to the cohesin core complex depends on Scc1 and SA1/SA2 (Kueng et al., 2006). In the higher eukaryotes Wapl is mainly involved in removal of arm cohesin in the prophase pathway (see below) via the phosphorylation of Scc1 by PLK1 kinase. It is shown that, in Wapl-deleted cells, removal of arm cohesin during the prophase is completely blocked (Gandhi et al., 2006; Kueng et al., 2006). Recent studies in mammalian cells also demonstrated that Wapl plays an essential role in the chromatin organization and that Wapl-dependent cohesin removal protects the cohesin cleavage from the separase, which enables the mitotic cells to enter into the succeeding cell cycle in the presence of functional core cohesin complex since chromatin-bound Scc1 is an only substrate by Separase (Tedeschi et al., 2013). It has been reported that Wapl antagonism of cohesin promotes the Polycomb-group-dependent silencing in the *Drosophila* (Cunningham et al., 2012). It has been shown that, in *Arabidopsis thaliana,* Wapl exists in two forms and the two has overlapping functions during cell cycle. Recent studies identified that deletion of these two isoforms of Wapl during meiosis shows that the removal of arm

cohesin in meiotic prophase was blocked, resulting in the formation of chromosome bridges, besides the formation of broken chromosomes and chromosome segregation defects. However, the mitotic Wapl mutant cells in this plant do not show any detectable alterations in chromosome segregation (De K et al., 2014).

The other important cohesion-associated protein, Sororin, was identified only in vertebrate cells and is essential for SCC and cell cycle progression. The protein undergoes Anaphase promoting complex/cyclosome (APC/C)-mediated protein degradation during early G1 phase (Rankin et al., 2005; Schmitz et al., 2007). Additionally, depletion of Sororin in mammalian cell lines causes defects in DSB repair, due to the lack of SCC (Schmitz et al., 2007). It has been proposed that Sororin plays an important role in cohesin protection during mitosis (Diaz-Martinez et al., 2007). From the previous studies it has been evident that Sororin mediates both SCC establishment and maintenance. A conserved C-terminus motif is required for SCC (Wu et al., 2011). Previously, it has been demonstrated that Sororin is phosphorylated by Cyclin-dependent kinase 1 (Cdk1) and Polo-like kinase 1 (PLK-1), and has a role in arm cohesin removal during mitotic prophase (Dreier et al., 2011; Zhang et al., 2011).

1-3-2 Loading of cohesin onto chromosomes

The loading of cohesin occurs before the onset of DNA replication to create linkages between the sister chromatids. And the timing of cohesin binding to chromatin differs from yeast to vertebrates. In the budding yeast, cohesin binds to the chromosomes at G1 phase to until anaphase onset (Michaelis et al., 1997; Tanaka et al., 1999). In vertebrates, cohesin associates during telophase of the preceding cell cycle, and remains on chromosomes until anaphase of the next division (Sumara et al., 2000; Losada et al., 2000).

The cohesin-binding sites have been mapped onto the chromosomes in many species. In budding yeast, high levels of cohesin binding is seen at centromeres, where cohesin plays an essential role in resisting forces of spindle microtubules. Cohesin loads onto the chromosome arms in every 10 to 15 kb of region known as cohesion-associated region (CAR) (Blat et al., 1999; Laloraya et al., 2000; Glynn et al., 2004; Lengronne et al., 2004). These CAR sites identified in budding yeast is around 0.8 to 1.0 kb in length (Laloraya et al., 2000). However, no specific DNA sequence has been identified in CAR regions, although they are AT-rich intergenic regions between the transcription sites (Lengronne et al., 2004; Glynn et al., 2004). It has been described that cohesin loads on intergenic regions with the help of cohesin loader at temporary sites, and further moved to

most persistent sites by driven by transcription (Lengronne et al., 2004). In budding yeast, the cohesin loader has been identified as a conserved Scc2/Scc4 complex (Ciosk et al., 2000). The proteins of the complex interact with soluble cohesin, but do not colocalise with the cohesion complex on the chromosome such as CAR regions (Arumugam et al., 2003; Lengronne et al., 2004). In the case of mutants, *scc2/scc4*, cohesin core complex cannot associate with chromatin, but no alterations in assembly of cohesin subunits have been found. However, *scc2/scc4* mutants only affects SCC but not other cell cycle events such as DNA replication, cytokinesis etc. Furthermore, Scc2/Scc4 complex is required for initial loading of cohesin in G1 and is dispensable during S and G2 phases of cell cycle (Ciosk et al., 2000; Lengronne et al., 2006). Taken together, these results suggest that Scc2/Scc4 cohesin-loading complex is required for direct association of cohesin to chromatin, but not for the *de novo* assembly of cohesin core complex or for establishment and maintenance of cohesion during cell cycle. For more understanding of how cohesin is moved from loading sites to consistent locations on genome, Uhlmann and Gerton's groups proposed that the sliding of RNA Pol II-mediated transcription may induce the translocation of cohesin on budding yeast chromatin (Glynn et al., 2004; Lengronne et al., 2004).

1-3-3 Cohesion establishment and maintenance

It has been identified that cohesin association with chromatin is not enough for stable pairing of sister chromatids together. Previous studies reported that, in budding yeast cells lacking Scc1, segregation defects in mitosis were observed during S phase even if Scc1 expression was enabled from G2 phase of cell cycle (Uhlmann et al., 1998). The chromatin-associated cohesin needs to acquire the cohesive state to hold the sister chromatids together, that is achieved by the "establishment" of SCC, which is coupled with DNA replication. The important player for establishment of SCC is a conserved protein called Eco1/Ctf7 (hereafter, Eco1), which has the acetyl-transferase activity required for cohesive state of the complex (Ivanov et al., 2002; Williams et al., 2011; Toth et al., 1999). The mutant *eco1/ctf7* showed PSSC and errors in chromosome segregation. Moreover, cell-cycle studies revealed the essential function of Eco1 is restricted to S phase of cell cycle for establishment of SCC (Reviewed in Ocampo et al., 2011).

The Eco1 function is required for establishment of cohesion (Milutinovich et al., 2007). The studies on DNA replication and cohesion establishment revealed that Eco1 interacts with several proteins at replication fork. Initial studies showed that Eco1 associates with three different replication fork complexes such as DNA polymerase processivity factor

PCNA, Ctf18, RFC (Replication factor C) (Kenna et al., 2003). It has been demonstrated that Eco1 in budding yeast and also human orthologue Esco2 physically interacts with PCNA (Moldovan et al., 2006). This physical interaction is also required for proper establishment of SCC. Interestingly, genetic analysis showed that overexpression of SUMOylation-deficient PCNA mutant leads to suppression in growth- as well as SCC-defects in the *eco1-1* mutant. These findings suggest that Eco1-PCNA-dependent SCC might be counteracted by PCNA SUMOylation (George et al., 2006).

Eco1 contains a conserved acetyl transferase domain on the C-terminus with acetyl CoA binding site that is similar to that of the Gcn5-related acetyl-transferase family (Ivanov et al., 2002). *In vitro* analysis using bacterial expression system demonstrated that yeast Eco1 has some potential substrates for the acetylation, which includes multiple lysines in Scc3/Irr1, Pds5, and Eco1 itself, and also single lysine residue (K210) within Mcd1/Scc1 (Ivanov et al., 2002). However, the biological significance of these acetylation sites was unclear as mutations in these sites failed to disrupt cohesion. Further analysis of acetylation mimicking mutations in Smc3 lysine residues identified lysines 112 and 113 are potential substrate sites for the Eco1 acetylation (Ben-Shahar et al., 2008; Unal et al., 2008). Substitutions of these residues to non-acetylated arginine are lethal due to severe defects in SCC. Out of the two sites Lys113 acetylation sites plays more critical role in proper cohesion establishment than Lys112 acetylation. Further cell-cycle dependent experiments showed that Smc3 is not acetylated in G1 phase, but levels of acetylation dramatically increases during S phase of cell cycle (Ben-Shahar et al., 2008; Zhang et al., 2008; Unal et al., 2008). Uhlmann and Gerton groups have identified that Hos1 is lysine deacetylase for acetylated Smc3 during G1 phase in budding yeast (Xiong et al., 2010; Borges et al., 2010). Nasmyth and Uhlmann research groups also clarified the importance of Smc3 acetylation and deacetylation cycle for proper establishment as well as maintenance of SCC, and regeneration of cohesin to the next cycle. The non-acetylated form of Smc3 at G1 phase acts as a substrate for the cohesin establishment in the next cell cycle (Borges et al., 2010; Beckouet et al., 2010). Recent studies showed that Eco1 is negatively regulated by Cdk1 protein kinase at S phase. Cdk1-dependnet phosphorylation promotes ubiquitin-mediated degradation of Eco1 after S phase and thus cohesion cannot establish after S phase of cell cycle in budding yeast. On the other hand, the nonphosphorylated Eco1 is able to establish SCC even after S phase (Lyons et al., 2011). Later studies on Eco1 phosphorylation identified that along with Cdk1 there are two more other kinases; Cdc7-Dbf4 and GSK-3 homolog Mck1, that phosphorylates Eco1 in a sequential manner (Lyons et al., 2013). These

results elaborated the regulatory mechanism of Eco1 protein degradation by independent kinases.

Several additional studies have identified, as a suppressor of the *eco1-1* temperature sensitive (ts) mutant, mutations within the *RAD61* gene. In relation to this, deletion of *RAD61* either in *ECO1* deletion strain or *eco1-1* ts mutant restores cell viability (Ben-Shahar et al., 2008). Defects caused by non-acetylation mutations of Smc3 K112R, K113R were suppressed by *WPL1/RAD61* deletions. Additional suppressors *eco1-1* ts mutant were mapped to the *SCC3* and *PDS5* genes, supporting the idea on Rad61 (Wapl) coordination with Pds5 and Scc3 in anti-establishment of cohesion that retains a cohesin destabilized form (Rowland et al., 2009).

Another interesting player of the cohesion establishment and maintenance is Pds5. The role of Pds5 in cohesion regulation is linked with the function of Eco1 as *PDS5* depletion mutant of budding yeast shows the same cohesion loss phenotype as *eco1* mutant cells (Guacci et al.1997; Michaelis et al., 1997; Toth et al., 1999). However, *pds5* mutant shows precocious sister dissociation, but, unlike the other cohesion components, Pds5 is mainly required for maintenance of cohesion after S phase establishment (Stead et al., 2003). To support this idea, it has been showed that, in fission yeast, Pds5 physically interacts with Eso1 (Eco1 in budding yeast) and the depletion of the *PDS5* suppresses the defects associated with *eso1* mutant. This indicates that the Pds5 hinders the cohesion establishment until it's counteracted by Eso1p (Tanaka et al., 2001). Guacci and coworkers identified the SUMOylation of Pds5 mediates the cohesion maintenance during cell cycle. They isolated the *SMT4* gene, which encodes a SUMO isopeptidase as a high copy suppresser of the *pds5* mutant. They showed that the over-expression of *SMT4* reduces the Pds5 SUMOylation and that the *SMT4* depletion shows elevated levels of SUMOylated Pds5 (Stead et al., 2003), suggesting the role of SUMOylated Pds5 in dissolution of cohesion. Meiotic experiments in budding yeast showed that Pds5 is mainly required for homologues pairing, synapsis and chromosome compaction. In *pds5* depletion mutant, the loading of meiosis-specific klesin Rec8 on chromosomes remains similar compared to wild type (Zhang et al., 2005; Jin et al., 2009). This result indicates that Pds5 may not be required for the cohesion loading but it has role in cohesion maintenance during meiosis.

Another cohesion regulator Wapl/Rad61 in humans is required for a role of cohesin in chromatin; DSB repair, implicated in heterochromatin formation, and is also known to promote cohesion dissociation from chromosomes during mitotic prophase (Kueng et al., 2006; Gandhi et al., 2006). The role of Rad61/Wapl in cohesin removal process was also

observed in fission yeast (Bernard et al., 2008). However, the loss of WAPL function in humans leads to increase in chromosomal cohesin, while the *rad61* deletion in budding yeast shows decreased cohesin on chromosomes (Warren et al., 2004; Sutani et al., 2009; Rowland et al., 2009; Gandhi et al., 2006). Recent studies in *Arabidopsis thaliana* showed that mutations in *WAPL* gene suppress defects caused by inactivation of *AtCTF7* (De K et al., 2014). In both yeast and mammalian systems deletion of *wapl/rad61* bypass the *eco1*-induced defects (Gandhi et al., 2006, Kueng et al., 2006).

1-3-4 Cohesin removal during mitosis

Along with SCC establishment, timely dissolution of cohesion is equally essential for the proper segregation of chromosomes. Hence cells have developed various mechanisms to ensure the proper chromosome inheritance. In budding yeast mitosis, cohesin removal does not take place during prophase and cohesin remains bound to the DNA until the onset of anaphase (Ciosk et al., 2000). At the anaphase onset entire cohesin is removed by the protease called Separase that cleaves the mitotic klesin Mcd1/Scc1 (Uhlmann et al., 1999). Separase is activated by the degradation of its inhibitor, Securin (Pds1) by APC/C complex at metaphase to anaphase transition (Sumara et al., 2000) (Figure V).

As discussed above, in the vertebrates, cohesion is removed from chromosomes in two-step process. Initially bulk of arm cohesin is removed from the chromosome arms by the process called 'prophase pathway' and remaining cohesin molecules on centromere are removed at metaphase-to-anaphase transition (Waizenegger et al., 2000; Losada et al., 2000; Warren et al., 2000) (Figure V). In the mitotic cells, the cohesin subunits Scc1/Mcd1 and SA2 (SCC3) are phosphorylated by Plk1 (polo-like kinase) during mitotic prophase (Losada et al., 2002; Sumara et al., 2002; Abian et al., 2004; Lenart et al., 2007). Inactivation of Plk1 in *Xenopus* eggs identified elevated levels of cohesin on chromosomes whereas the addition of recombinant Plk1 restored cohesion dissociation (Losada et al., 1998; Losada et al., 2000; Sumara et al., 2000). In both cases of *Xenopus* and mammals cohesin dissociation is regulated by a cell-cycle stage of oocytes, but does not depend on the Cdk1 kinase (Sumara et al., 2000). Later studies suggested that not only SA2 phosphorylation but also cohesin regulators, Wapl and Pds5, play an important role in this prophase pathway. In *Xenopus* egg extracts inactivation of Wapl and Pds5 showed the absence of arm cohesin removal and chromosome segregation defects (Shintomi et al., 2009). In consistent with these results, in mammalian cells, Wapl depletion also exhibited severe reduction in prophase pathway

(Kueng et al., 2006; Gandhi et al., 2006;Tedeschi et al., 2013). Amino-acid motif analysis of SA2 (SCC3) and Pds5 confirms that the interaction surfaces of these proteins with HEAT repeats regulate the cohesin dynamics (Neuwald et al., 2000; Nasmyth et al., 2005). And also mammalian Wapl has three copies of FGF motifs that physically interact with HEAT repeats (Shintomi et al., 2009). Most importantly, in *Xenopus* egg extracts, mutations in the FGF motif of Wapl does not strongly interact with cohesin and confers defects in cohesin removal (Shintomi et al., 2009). These results provide the idea that Wapl may use FGF motifs to interact with SA2 and Pds5 HEAT repeats, and the phosphorylation of SA2 by Plk1 induces the conformational changes in these interactions and then facilitates the removal of cohesin without cleave of Scc1.

It has been reported that mitotic kinase Aurora B is also required for the prophase dissociation of cohesin from the chromosomes (Waizenegger et al., 2000; Losada et al., 2002; Sumara et al., 2002; Abian et al., 2004). However, the phosphorylation sites of Scc1 or SA2 by this kinase have not been identified yet. Sgo1- and Condensin I-binding to chromosomes is required for the cohesin removal and specifically both of Sog1 and Condensin-I complex are phosphorylated by Aurora B.

Recent studies on mouse showed that regulation of cohesin-DNA interaction by Wapl is important for embryonic development and the expression of important tumor suppressor gene c-MYC. Furthermore, Wapl-mediated release of cohesin from DNAs is essential for proper chromosome segregation (Tedeschi et al., 2013).

Another regulator of cohesin removal in vertebrates is Sororin. Initially it has been identified as a substrate for the anaphase-promoting complex (APC)-dependent degradation in G1 phase of cell cycle in *Xenopus* embryos. Overexpression of Sororin causes failure to resolve sister chromatids and segregation defects (Rankin S et al., 2005). Later studies identified that Sororin is essential for proper cohesion during G2 phase and for efficient repair of DNA double-strand breaks (DSBs) in G2 (Schmitz et al., 2007). Peters and his coworkers shown that DNA replication and cohesin acetylation promotes the binding of Sororin to cohesin by antagonizing the Wapl from binding partner Pds5. They also observed that the phenotype obtained after codepletion of Sororin and Wapl was identical to the phenotype of Wapl alone (Nishiyama et al., 2010). Recent studies on prophase pathway revealed that Sororin is phosphorylated by two kinases such as Cdk1 and Plk1 and that these phosphorylations are required for the arm cohesin removal during mitotic prophase. It is also shown that Sororin is essential for the recruitment of Plk1 to the chromosomes for phosphorylation of SA2 during prophase pathway (Dreier et al., 2011;

Zhang et al., 2011). Recently, it is shown that the splicing of Sororin pre-mRNA by Prp19 splicing complex is required for proper SCC in human cells (Watrin et al., 2014; Oka et al., 2014; Lelij P et al., 2014). These findings suggest the role of splicing machinery in contributing to the establishment of cohesion by Sororin accumulation. Very recently it is shown that a C-terminus of Sororin, especially last 12 amino acid residues, is required for anchor-binding of SA2 and regulates SCC (Zhang et al., 2015; Martinez LA et al., 2015).

1-4 Meiosis-specific cohesin complex and functions during meiosis

As discussed earlier, during meiosis, mitosis-specific klesin subunit of cohesin complex Rad21/Scc1 is replaced by a meiosis-specific klesin Rec8 in *S. cerevisiae*, *S. pombe*. And in *C. elegans* along with Rec8 another two paralogs, called COH3 and COH4 together regulates SCC (Severson et al., 2009). Meiotic Smc1 is replaced with Smc1β in mammalian cells. And also in higher eukaryotes, STAG3 replaces SA1/SA2 subunits during meiosis (Pezzi et al., 2000; Prieto et al., 2001; Revenkova et al., 2004). Recent findings in mouse identified Rad21-like protein (Rad21L) during meiosis, which has sequence similarity with Rec8 and Rad21 (Gutierrez-Caballero et al., 2011). Rad21L is localized on chromosome axes during meiotic prophase, along with axial elements of synaptonemal complex and is shown to possess a significant role in homologues recombination (Ishiguro et al., 2011). It has been reported that, along with SCC, meiosis-specific cohesin have various functions and especially regulates several prophase events of meiosis.

1-4-1 Homologous Recombination (HR)

After DNA replication each pair of homologous chromosomes undergo reciprocal recombination generating crossovers (Roeder et al., 1997). Meiotic recombination is initiated by the formation of DSBs catalyzed by a topoisomerase-related protein Spo11 (Klapholz et al., 1985; Keeney et al., 1997; Handel et al., 2010). Spo11 protein covalently binds to the 5'-end of DNA at DSB site through the formation of a phosphodiester bond. This protein-DNA adduct is recognized by the MRX/N (Mre11, Rad50, Xrs2/Nbs1) together with Sae2/CtIP, which in turn removes Spo11 from the DSB end. These proteins create 3'-overhang single-stranded (ss) DNA that is extended by various nucleases and helicases (Krogh et al., 2004). The ssDNA with 3'-overhang is loaded with RecA-related recombinase such as Rad51 and Dmc1, which form filament-like structures on the DNA (Shinohara et al., 1992; Bishop et al., 1992). Along with Rad54 and Tid1/Rdh54 proteins, Rad51-Dmc1 pair initiate homology search for the homologous duplex DNA sequences. After finding the homologous DNA, displacement loop (D-loop) is formed by invasion of

the ssDNA into the duplex DNA (Krogh et al., 2004; Lao et al., 2010). Furthermore, D-loops are converted into a stable intermediate called Single-End Invasion (SEI), which is accompanied by the DNA synthesis from invaded DNA. Later with further processing, SEIs are converted into double-Holliday Junctions (dHJs). dHJs are resolved specifically into reciprocal crossovers (COs) (Matos et al., 2011). The presence of a CO at one position decreases the possibility of another CO formation nearby, which is known as CO interference (Borner et al., 2004). Homologous chromosomes undergo at least one CO along the chromosomes (Roeder et al., 1997).

In budding yeast, genome-wide analysis of Spo11-binding sites by Chromatin Immunoprecipitation (ChIP) analysis identified that dynamic localization of Spo11 enzyme on meiotic chromosomes. Spo11 distribution on chromosomes depends on meiotic-specific klesin Rec8 (Kugou et al., 2009). Initially, Spo11 is bound to Rec8-rich regions on chromosomes such as centromeres during S phase. Once the replication progressed, Rec8 is enriched more to chromosome arms. It has been identified that deletion of the *REC8* severely reduced the Spo11-binding on centromeres as well as on chromosome arms with large reduction of DSB formation (Klein et al., 1999; Kugou et al., 2009). However, it has not been showed that the Rec8 is a part of Spo11 protein complex (Keeney et al., 1997). But further studies identified that Spo11 is colocalized with Rec8 on meiotic chromosome axes and Spo11-associated factors tether the chromatin loop to axis enriched for Rec8 during DSB formation. Moreover, in budding yeast, Rec8-associated proteins Hop1 and Red1 are also required for efficient DSB formation (Panizza et al., 2011). Recent genome-wide ChIP analysis of Rec8 and Spo11 also revealed that DSB cold spots are observed around 0.8 kb of Rec8-binding sites (Ito et al., 2014), suggesting a negative role of Rec8 in DSB formation.

As explained above, DSB repair mechanism mainly requires Rad51 and Dmc1 whose collaboration promotes interhomolog recombination. Several observations suggest that Rec8 has a role in the DSB repair process and meiotic recombination. Initially, it has been identified that Rad51/Dmc1 coimmunoprecipitate Rec8 in rat spermatocytes (Eijpe et al., 2003). In the budding yeast meiosis Rec8 interacts with Dmc1 and Hop1 and the deletion of the *REC8* showed reduction recombination with inefficient repair of DSBs (Katis et al., 2010; Klein et al., 1999; Brar et al., 2009). Similarly the absence of Rec8 ortholog *AFD1* in maize leads to the accumulation of Rad51 foci and altered recombination (Pawlowski et al., 2003; Golubovskaya et al., 2006). It has been identified that in *C. elegans* meiosis depletion of *REC8* induces chromosome fragmentation and *spo11* deletion suppresses these defects, supporting an idea that Rec8 processes Spo11-induced DSBs

(Pasierbek et al., 2001). In mouse meiosis, the *REC8* knockout showed DSB formation is normal but crossover formation was largely impaired (Xu et al., 2005). In the budding yeast meiosis, cohesin-associated protein Pds5 also plays a role in DSB repair mechanism in meiosis (Jin et al., 2009). All these results indicate that the meiosis-specific cohesin plays an important role in DSB formation and repair to form proper recombination products, which is conserved among species.

1-4-2 Organization of Synaptonemal Complex

The molecular events behind SC formation and resolutions are well defined in budding yeast. Meiotic sister chromatids assembled as an axis of SCs consist of cohesin and TopII (Klein et al., 1992; Klein et al., 1999). At leptotene stage, an AE protein Red1 is associated with the Rec8 cohesin complex (Rockmill et al., 1988; Smith et al., 1997) on chromosomes, which is followed by the recruitment of Hop1 to form a chromosome axis (Hollingsworth et al., 1990). At zygotene, homologues chromosomes are linked together by the deposition of a CE component, Zip1 to form a SC (Sym et al., 1993; Dong et al., 2000). In the middle of SC, Zip1 protein forms coiled-coil homodimer and two homodimers overlap at its N terminus and finally it forms tetrameric structures. Along with CE and LE, axis-associated proteins called of ZMM proteins that include Zip2, Zip3, Msh4, Msh5, Spo22, Mre3 and Spo16 facilitate the formation of SC as well as COs. (Shinohara et al., 2008; Tsubouchi et al., 2006). After late pachytene stage when SC starts to disassemble between the homologues, Aurora B/Ipl1 kinase plays an essential role in disassembly of SC during meiosis (Jordan et al., 2009).

Meiosis-specific klesin Rec8 plays an essential role in SCC. Various groups identified SCC-independent roles of Rec8 in chromosome axis formation during meiosis (Buonomo et al., 2000; Jin et al., 2009). It has been identified by many groups that Rec8 plays a role in AE biogenesis and SC formation in various eukaryotic models. The *rec8* deletion mutant showed failure of axis assembly and improper localization of various axis proteins in budding yeast (Klein et al., 1999; Golubovskaya et al., 2006). In *C. elegans* Rec8 has two paralogs COH-3 and COH-4 and deletion of these genes leads to defective AE formation (Severson et al., 2009). In mouse *REC8* knockdown does not show any disruption in AE formation (Bannister et al., 2004; Xu et al., 2005). But further studies on Rad21L, another variant of meiosis-specific klesin demonstrate that Rad21L is required for initiation of synapsis and crossover recombination (Herran et al., 2011; Lee et al., 2011).

Spermatocytes knockdown of both meiosis-specific klesins Rad21L and Rec8 in mouse showed defects in AE assembly and induces an arrest at leptotene stage.

Along with meiosis-specific klesin Rec8, cohesion-associated subunit Pds5 also plays a role in SC formation. Depletion of the *PDS5* depletion mutant of budding yeast leads to failure in homologous pairing with normal localization of Rec8 as well as other AE proteins Hop1 and Red1 (Zhang et al., 2005; Jin et al., 2009). In this mutant chromosomes appear as short rod-like structures, indicating that Pds5 is required for proper axial compaction of chromosomes. Moreover, the *PDS5* depletion prevents intersister SC formation. The overexpression of mitosis-specific klesin Scc1/Mcd1 using an inducible promoter, in *pds5* mutant cells does not restore intersister SC formation (Jin et al., 2009). These results suggest the possible roles of Pds5 and Rec8 in SC formation between homologous chromosomes.

1-4-3 Telomere clustering

Along SC formation, chromosomes relocate their position inside the nucleus during meiosis. In budding yeast mitosis few telomeres cluster at nuclear periphery (Klein et al., 1992). But during meiosis at the leptotene stage, telomeres attach to the nuclear envelope and move toward the spindle pole body (SPB: Centrosome in higher order eukaryotes), and form a cluster named as telomere bouquet. The bouquet-like structures are predominantly seen in zygotene (Trelles-Sticken et al., 1999). Telomere clustering in meiotic nuclei is conserved in all eukaryotes (Chikashige et al., 2006; Ding et al., 2004). The overlap between the homologous pairing and telomere clustering has led to the idea that telomere clustering promotes homologous pairing and/or prevents pairing of ectopic sites.

Over the last two decades, molecular mechanism to form telomere bouquet has been studied by various groups. In the fission yeast telomere proteins Taz1 and Rap1 contribute to the attachment of telomeres to the nuclear envelope (Cooper et al., 1998; Nimmo et al., 1998; Kanoh et al., 2001; Chikashige and Hiraoka, 2001). Along with Taz1, two other proteins required for bouquet formation, Bqt1 and Bqt2, were also identified (Chikashige et al., 2006). In the budding yeast telomere movement depends on a meiosis-specific telomere binding protein Ndj1 (Scherthan et al., 2007). It has been demonstrated that another meiosis-specific protein Csm4 forms a complex with Ndj1 and in the *csm4* deletion mutant, telomeres tether to nuclear envelope but they do not form clusters (Kosaka et al., 2008). These results suggest that proper telomere clustering requires multiple protein complexes on nuclear envelope during meiosis. Importantly, during budding yeast meiosis SUN (Sad1p,

UNC-84) domain protein Mps3 plays an essential role in telomere bouquet formation (Conrad et al., 2007). Surprisingly, deletion of SUN domain in Mps3 protein showed minimal defects in telomere clustering but alterations in chromosome movements and reduced chromosome pairing were observed (Prasad Rao et al., 2011). These results suggest that telomere bouquet formation may not be completely dependent on SUN domain of Mps3, and it raises the possibility that Mps3-interacting proteins during meiosis play more crucial role in telomere clustering. Telomere bouquet formation requires Ndj1, Csm4 and Mps3 proteins (Conrad et al., 2008). SUN domain proteins are conserved in yeast to mammals. In the fission yeast, SUN domain protein Sad1 is required for the normal telomere dynamics during meiosis (Miki et al., 2004). Mammalian SUN-domain protein Sun2 has been localized at telomere-binding sites on nuclear envelope during meiosis (Schmitt et al., 2007).

In the budding yeast Rec8 is required for the proper telomere bouquet formation. The *rec8* deletion mutant shows that defective telomere bouquet dynamics (Trelles-Sticken et al., 2005; Conrad et al., 2007). Moreover, SPB-unattached telomere clustering is observed in *rec8* deletion mutants. And also telomere bouquet dissolution is almost abolished in *rec8* deletion mutant (Trelles-Sticken et al., 2000). The loss of Set1 histone H3 methyltranferase also shows defects in bouquet formation (Trelles-Sticken et al., 2005). In the mice, depletion of meiosis-specific SMC1 b shows an incomplete attachment of telomeres to nuclear envelope (Adelfalk et al., 2009). On the other hand, the deletion of *RAD21L* in spermatocytes showed that telomeres are not properly bound to the nuclear envelope in zygotene stage of meiosis (Herran et al., 2011). These findings are comparable with *SMC1β* knockout mutant. All these findings suggest that meiosis-specific cohesin components are crucial for the proper regulation of telomere dynamics during meiosis.

1-4-4 Cohesin dynamics during meiosis

Cohesin removal during meiosis is regulated by two-step process. Initially arm cohesin is removed through the cleavage of Rec8 by a protease Separase at metaphase I-to anaphase I-transition while centromeric cohesin is protected by the action of Sgo1/PP2A. At meiosis II centromeric cohesin is also cleaved by the separase. It has been previously reported that Scc1 is phosphorylated by Polo-like kinase-dependent manner and this phosphorylation promotes Scc1 cleavage in yeast mitosis (Alexandru et al., 2001; Uhlmann et al., 2004). Like mitosis, during meiosis Rec8 is phosphorylated by multiple kinases such

as Polo-like kinase/Cdc5, casein kinase I δ/ε (CK1), Dbf4-dependent Cdc7 kinase (DDK) (Clyne et al., 2003; Lee et al., 2003; Katis et al., 2010; Brar et al., 2006; Attner et al., 2013). Importantly, DDK- and CK1-dependent phosphorylation of Rec8 is crucial for the separase cleavage (Katis et al., 2010). However, initially it has been speculated that Cdc5-dependent phosphorylation also requires Rec8 cleavage during meiosis but the replacement of Cdc5-dependent phosphorylation sites with alanine shows little or no effect on the kinetics of Rec8 cleavage at meiosis (Brar et al., 2006). In contrary, recent reports from Amon group showed that Cdc5-dependent phosphorylation of Rec8 S136 and S179 sites are important for the separase-dependent cleavage (Attner et al., 2013). Moreover, phosphorylation-defective mutants *rec8-17A* and *rec8-29A* showed significant delay of anaphase I entry, confirming the DDK-, CK1- and Cdc5-dependent phosphorylations of Rec8 play a roles in the prophase events as well as cohesin removal during meiosis (Brar et al., 2009).

Like yeast, in most of the higher organisms cohesin removal during meiosis is regulated by a two-step process. In *Xenopus* egg extracts, APC/C activation and separase-mediated cleavage are not required for first meiotic division but they are required for centromeric cohesin cleavage at second meiotic division (Peter et al., 2001). It has been reported that temperature sensitive mutants of APC/C in *C. elegan*s showed defects in homologues segregation, suggesting that separase-mediated cleavage of cohesin is required for first meiotic division (Furuta et al., 2000). Interestingly, in higher eukaryotes decrease in the amount of cohesin bound to chromosome arms during prophase was also observed, suggesting the presence of cleavage-independent removal of cohesin (Prieto et al., 2001, 2002, Revenkova et al., 2001).

1-4-5 Anaphase promoting complex/Cyclosome (APC/C)

In order to exit mitotic or meiotic cell cycle, the cell has to down-regulate two proteins present at late mitotic or meiotic phases *viz* Cdk1-cyclin B1 complex and Securin/Pds1 that act as inhibitors of cell cycle exit (Thornton et al., 2003). Down regulation of these proteins leads to the dephosphorylation of Cdk1 targets and activation of separase, thereby enabling the cells to enter anaphase and also exit to mitosis. Pds1 and Cdk1-cylin B1 complex are destructed by the means of ubiquitin-dependent proteolytic degradation by 20S Proteasome (Glotzer et al., 1991; Cohen-Fix et al., 1996). Binding of Ubiquitin to target proteins for the proteolysis by the Proteasome is achieved by a three-step process catalyzed by E1 (Ubiquitin-activating enzyme), E2 (Ubiquitin-carrier protein), and E3 (Ubiquitin ligase). Mainly substrate specificity is achieved at a level of E3 ubiquitin ligase and different types

of E3 classes are identified. Anaphase-promoting complex or Cyclosome (APC/C), a RING-type E3 ubiquitin protein ligase, is mainly responsible for ubiquitylation of Securin and cyclin B1 (Sudakin et al., 1995; King et al., 1995; Irniger et al., 1995).

For complete functionality of APC/C it requires substrate-recruiting factors either Cdc20 or Cdh1 (Sigrist et al., 1997; Visintin et al., 1997; Sigrist et al., 1995). Both factors interact with APC/C via its C-terminal isoleucine-arginine (IR) motif (Vodermaier et al., 2003). It has been reported that Cdc20 and Cdh1 recognize the substrate via an extended C-terminal WD-40 repeat domain (Kraft et al., 2005). Moreover, substrate recruitment by Cdc20 stimulates the ubiquitin ligase activity of APC/C (Kimata et al., 2008). APC/C is highly regulated by the spindle assembly checkpoint and is activated only when the chromosome arrange at metaphase plate (Zachariae et al., 1998). In budding yeast meiosis, depletion of Cdc20 causes cells arrested prior to anaphase I onset and there is no degradation of Securin leading to inactivation of Separase (Salah & Nasmyth 2000) (Figure VII). Separase activation leads the cleavage of Rec8 during metaphase I-to-anaphase I transition (Buonomo et al., 2000; Kitajima et al., 2003). The role of APC/C during meiosis is conserved in many organisms such as *C. elegans* and mutations in APC/C subunits causes metaphase I arrest (Golden et al., 2000; Davis et al., 2002; Furuta et al., 2000). It has been studied in mouse oocytes that APC/C mediates degradation of Securin and activates Separase for the cleavage of Rec8 (Terret et al., 2003; Kudo et al., 2006; Herbert et al., 2003).

1-5 Aims of the project: Deciphering the role of Rad61/Wpl1 (Wapl) in meiosis of the budding yeast and its function in prophase pathway.

Rad61/Wapl is a poorly conserved eukaryotic protein that shares common characteristics like a similar C-terminal WAPL domain, and has an ability to associate with cohesin core subunits as well as to form a subcomplex with Pds5 protein. Many structural and functional differences exist between Rad61/Wapl orthologues in lower eukaryotes like budding yeast and higher eukaryotes. In the higher eukaryotes such as mouse deletion of *wapl* causes a delay in arm cohesin removal in mitotic cells and, thus, it is proposed that Wapl plays an essential role in prophase pathway of cohesin removal in mitotic cells. In budding yeast mitosis cohesin is removed from the chromosomes by a single step during metaphase-to-anaphase transition mainly through Separase-dependent manner. And indeed, the deletion of *rad61/wapl1* in budding yeast mitosis show partial cohesin loss and hyper

condensation of rDNA and increased chromosome condensation (Lopez-Serra et al., 2013; Tong et al., 2014; Guacci et al., 2015).

The role of Rad61/Wpl1 protein in meiotic prophase I is largely unknown. In this thesis, the role of Rad61/Wpl1 during meiosis of budding yeast has been characterized extensively. As a regulator subunit of the cohesin complex, Rad61/Wpl1 plays a role in various processes like homologous pairing, cohesin establishment, synaptonemal complex formation, DSB regulation and meiotic recombination.

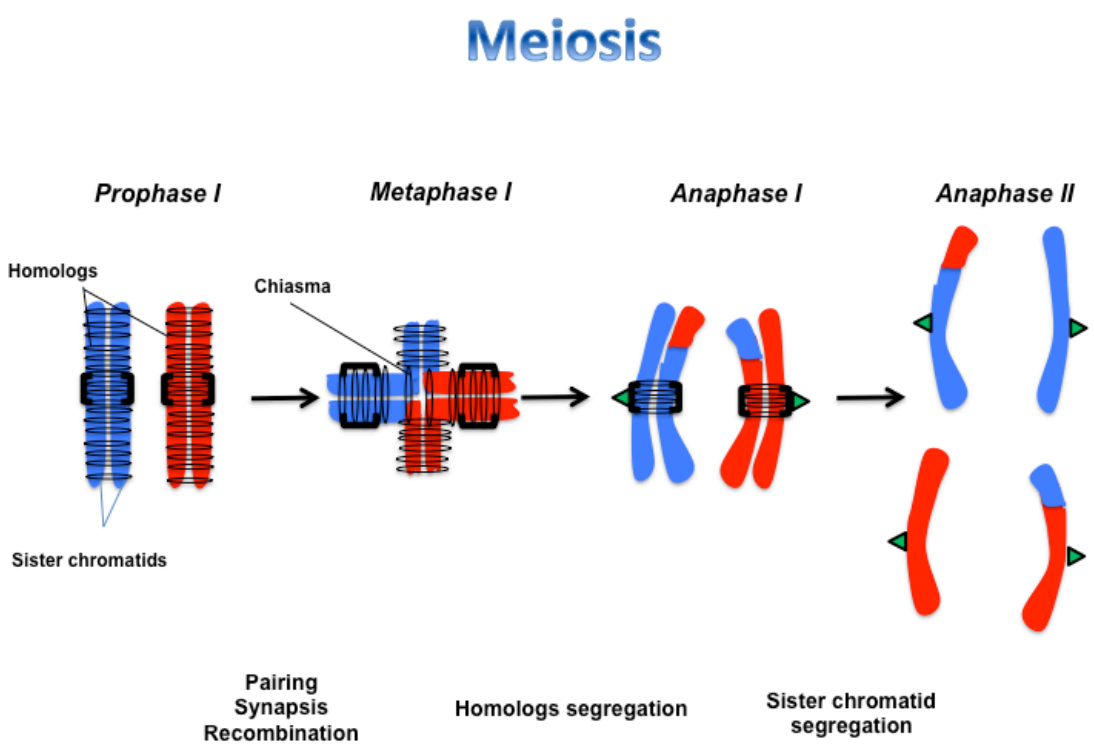


Figure I
Meiosis

Meiosis is a special type of cell division necessary for sexual reproduction in eukaryotes. After the entry into meiosis, each chromosome is duplicated and a pair of sister chromatids are linked with each other by cohesion. Production of haploid gametes requires segregation of homologous chromosomes during the first meiotic division and of sister chromatids in the second division. Together with meiosis cohesion, chiasma plays an essential role in proper segregation of homologous chromosomes.

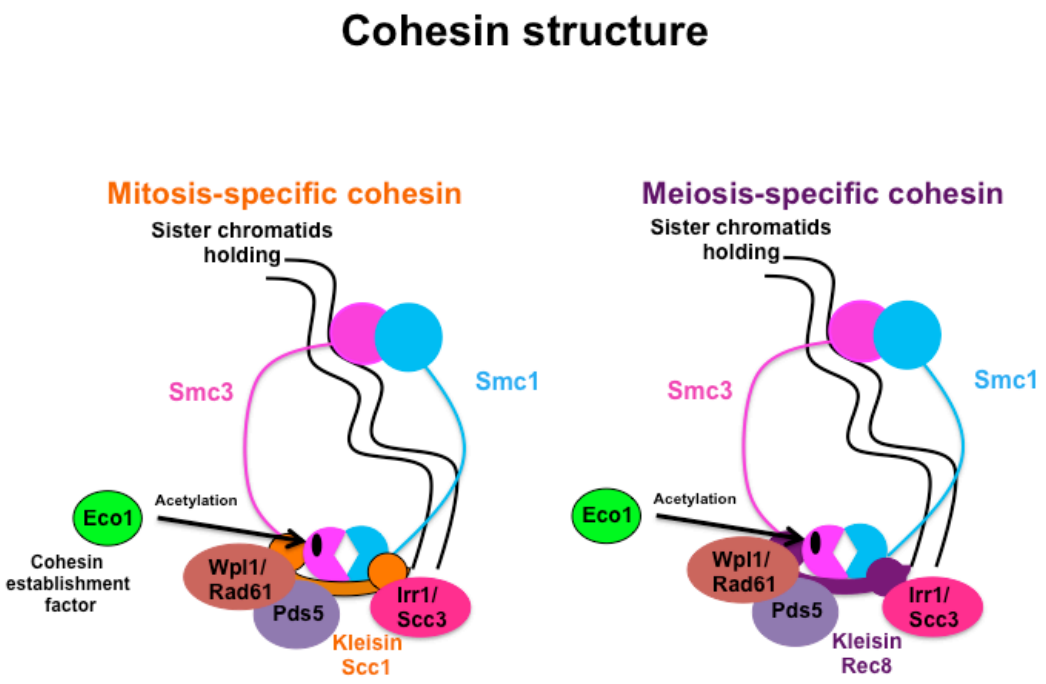


Figure II
Cohesin structure in budding yeast mitosis and meiosis

The cohesin complex forms a ring-like structure and encircles sister chromatids together for chromosome segregation. Cohesin complex consists of Smc1, Smc3, and a kleisin Scc1/Mcd1 in mitotic cells. Meiotic cells use a unique cohesin complex containing a meiosis-specific klesin component, Rec8, instead of Scc1/Mcd1. Eco1 is the acetyltranferase which acetylates Smc3 at S phase and establishes the cohesion between the sister chromatids. Cohesin function is regulated by several other factors. Rad61/Wpl1 (Wapl in other organisms) and Pds5 control the cohesin binding to chromosomes.

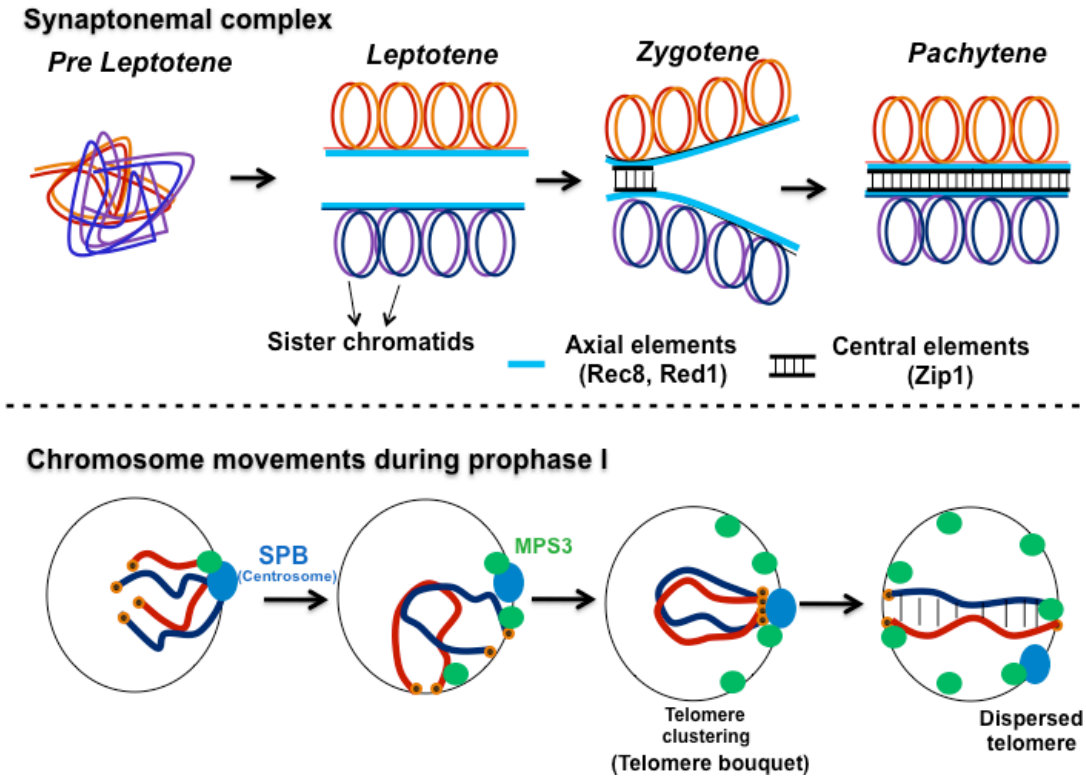


Figure III
Rapid prophase chromosome motions and synaptonemal complex

Synaptonemal complex (SC) is a proteinaceous structure that holds homologous chromosomes together. In parallel to SC formation, chromosomes relocate their position inside a nucleus. At the leptotene, telomeres attach to the nuclear envelope and telomeres move towards the spindle pole body (SPB), forming clusters of telomeres, which we call telomere bouquet. This bouquet structure is transient and predominantly seen in zygotene. In pachytene stage, telomere bouquet is disrupted and all telomeres are spread around the nuclear envelope. The dynamics of telomere as well as nuclear envelopes are promoted by various proteins such as Mps3. Mps3 is the SUN domain protein and a major component of the SPB in mitosis and shows dynamic relocalization on the nuclear envelope during meiosis.

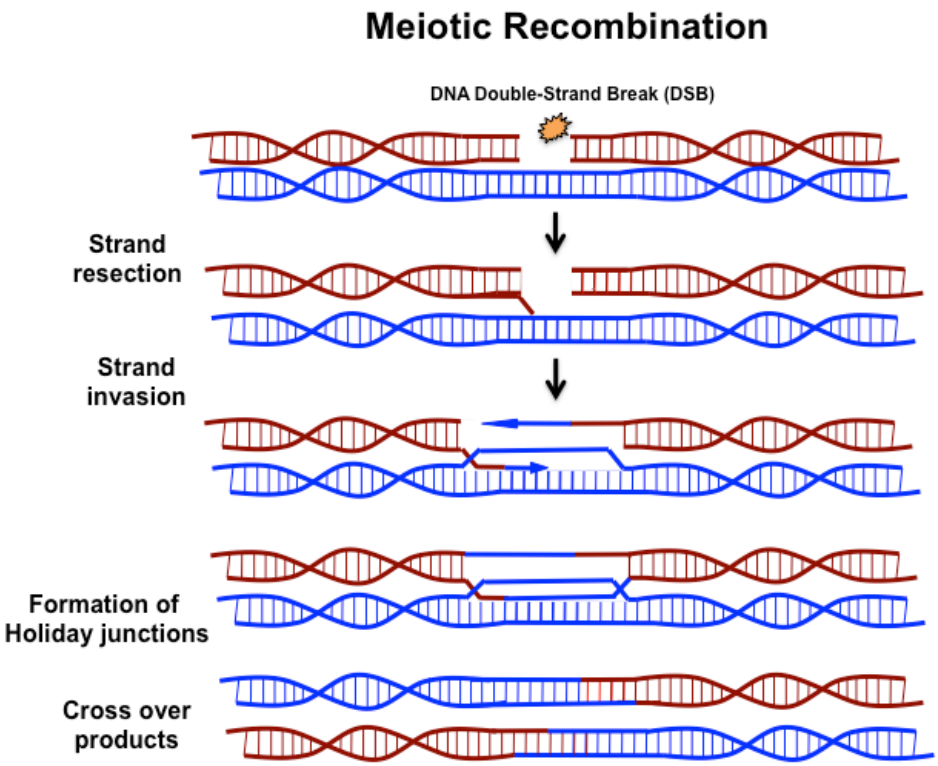


Figure IV
Meiotic Recombination
After DSBs formation, the MRX/N complex plays an essential role in the initial processing of double-strand breaks (DSBs), and starts resection prior to repair by homologous recombination. Followed by strand invasion and DNA synthesis, a specific recombination intermediates with double-Holliday structure is formed, which is resolved specifically into crossovers, which ensure proper homologous recombination between chromosomes.

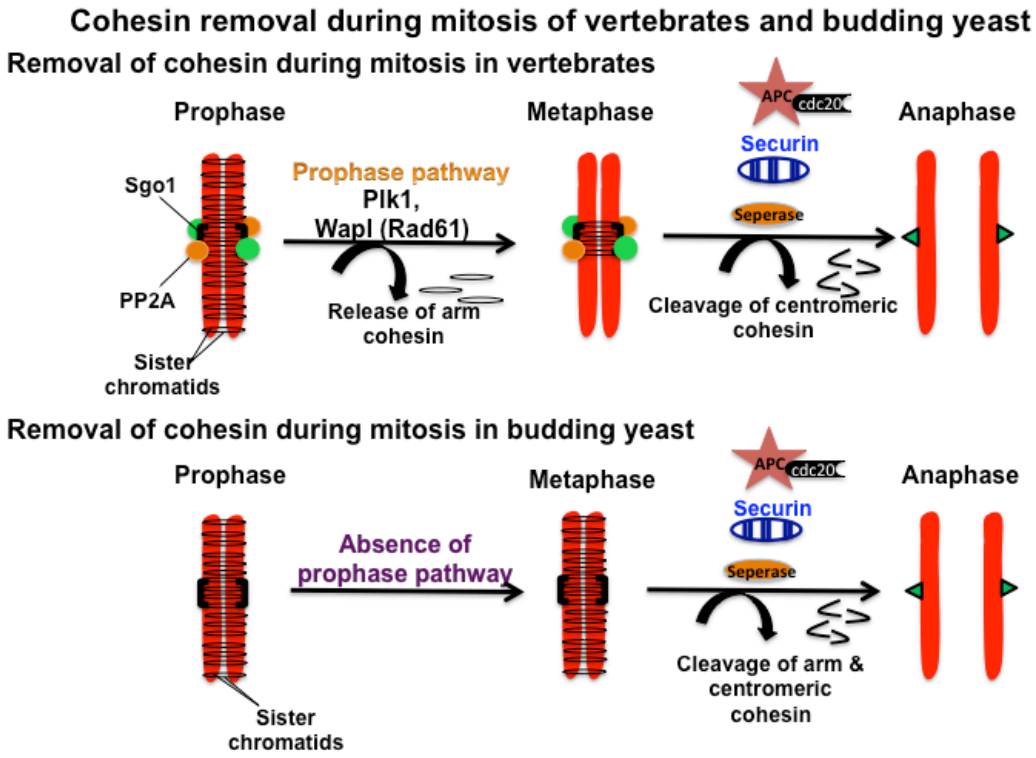


Figure V
Cohesin removal during mitosis of vertebrates and budding yeast

Cohesin removal plays an essential role in proper sister chromatid segregation. In the vertebrate mitosis, cohesin is removed by two-step process. Initially, a bulk of arm cohesin is dissociated in Plk1- and Wapl (Rad61)-dependent manner (above). This is so-called "prophase pathway" of cohesin removal. At the onset of anaphase, Scc1/Mcd1 is cleaved by a protease called Separase. Phosphorylation of Scc1/Mcd1 by Plk and AuroraB kinase facilitates the cleavage by Separase. SgoI-PP2A protects the centromeric cohesion for the dissociation and cleavage by dephosphorylating Scc1/Mcd1. At anaphase onset Separase is activated by the APC/C^{cdc20} Complex through the degradation of its inhibitor Securin/Pds1. But in the budding yeast mitosis, prophase pathway of cohesin removal is absent and the cohesin removal completely depends on the separase-mediated cleavage of Scc1 at the anaphase onset during mitosis (bottom).

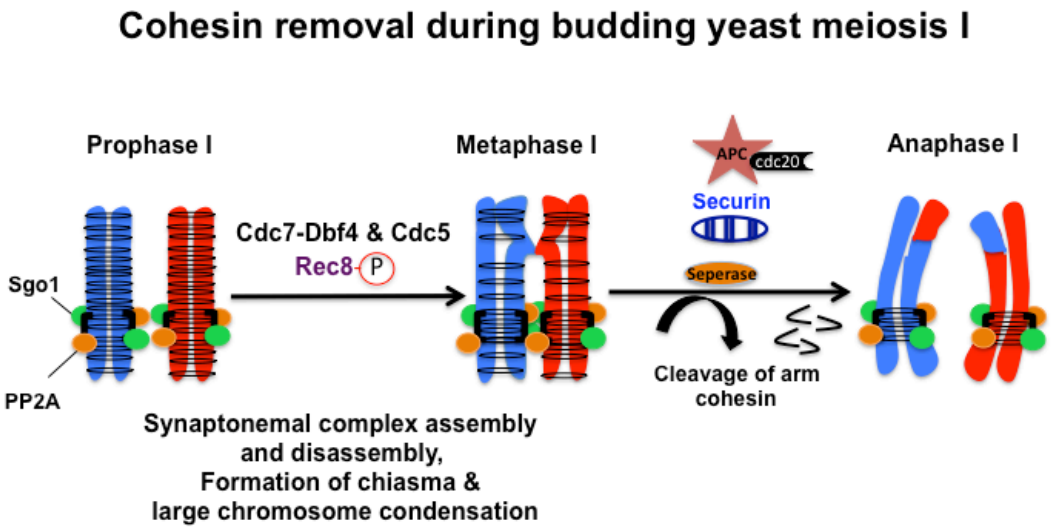


Figure VI
Cohesin removal during budding yeast meiosis I

In budding yeast meiosis, the cleavage of the kleisin at meiosis I requires the phosphorylation of Rec8 by three kinases, the Cdc7-Dbf4 (DDK), Cdc5 (polo-like kinase) and casein kinase δ/ε (CK1). At anaphase I onset, the phosphorylated Rec8 is cleaved by the Separase, which facilitates the removal of cohesin molecules from chromosome arms. Here, as in mitosis, Sgo1-PP2A protects the centromeric cohesin by dephosphorylating Rec8. In mid-prophase I, the cohesin complex play an essential role in maintaining the synaptonemal complex. However, synaptonemal complex is disassembled in late prophase prior to the onset of anaphase I. In this late prophase, the formation of chiasma and chromosome condensation are also seen.

Chapter 2

Materials and Methods

2-1 Strains and plasmids

All strains used in this study were originated from the *S. cerevisiae* SK1 diploid strain MSY832/833 (*MATα/MATa, ho::LYS2/", ura3/", leu2::hisG/", trp1::hisG/", lys2 /")* and NKY 1303/1543(*MATα/MAT a, ho::LYS2, ura3, leu2::hisG, lys2, his4B-LEU2, arg4-Bgl*). The genotypes of strains used in this study are mentioned in Table-1.

PCR-based site-directed mutagenesis using mutant primers was carried out and the presence of the mutations was confirmed by DNA sequencing. Target mutant genes were cloned into YIPlac211 and pRS406, respectively. After digestion with various restriction enzymes, the DNA was integrated by transformation for the selection of colonies with Ura positive. The *URA3* gene was popped-out by counter-selected for the *ura-* phenotype on a 5-FOA (5-Fluroorotic Acid) plate. DNA sequencing using genomic DNA for candidates confirmed mutants. The primers used for strain construction are shown in Table-2.

2-2 Preparation of *E. coli* plasmid DNA-Mini prep

Preparation of *E. coli* plasmid was performed according to standard procedure in Shinohara Lab. *E. coli* single colony was inoculated into 1.5 – 2.0 ml of LB liquid media containing ampicillin (50mg/ml) and incubated at 37°C over night. Next day, overnight culture was transferred into 1.5ml Eppendorf tubes, and then centrifuged 10,000 rpm for 1min, and supernatants were removed completely. Cells were suspended in 100 ml of GTE buffer (50 mM Glucose, 25 mM Tris-HCL [pH 8.0], 10 mM EDTA) and vortexed until pellets were completely dissolved in the buffer. Followed by adding 200 ml of Alkaline-SDS (0.2 N NaOH, 1% SDS) solution, samples were inverted for 4 to 5 times, and then kept on ice for 5 min. 150 ml of 7.5 M Ammonium acetate was added and the samples were inverted again, and kept on ice for at least 10 min, followed by centrifugation 15,000 rpm for 10 min at 4°C. Supernatants were transferred to new Eppendorf tubes containing 400 ml of 2-propanol. The samples were inverted for mixing and centrifuged 15,000 rpm for 10 min at room temperature. The pellets were washed with 70% (V/V) ethanol, and followed by another washing with 100 % (V/V) ethanol. The samples were dried for 10 min using a centrifugal concentrator. 50-100 μl of 10:1 TE (10 mM Tris- HCL [pH 8.0], 1mM EDTA) was added to dissolve DNA.

2-3 Yeast transformation (LiAc Method)

Yeast cells were inoculated into 3ml of YPAD (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% Glucose, 1% Adenine) liquid medium and cultured overnight. Overnight culture of yeast cells were diluted in 50-100 ml of YPAD (1/200 dilution) in a 500ml flask, and grown at 230 rpm at 30 °C using a shaker (Innova® 44) for around 2.5 hr to 4 hr until reaches OD of 0.4 to 0.6. Cells were centrifuged for 2 min at 3000 rpm using sterilized screw cap tubes, then washed twice in sterile distilled water, and centrifuged at 3000 rpm for 2 min. Cells were then suspended in 1 ml of LiAc/TE (0.1M LiAc, 1X TE) and transferred to a new Eppendorf tube, and then centrifuged again at 5000 rpm for 1 min. Cells were suspended in 200 ml of LiAc/TE. 15 ul carrier DNA (10mg/ml deoxyribonucleic acid from salmon sperm, Wako Ltd) were added to the cells and mixed. 50 ul each of the cell suspension was transferred to new Eppendorf tubes. Solution with a plasmid or a DNA fragment (1-10ul) was added to each tube and mixed well. Followed by adding 350 μl of PEG/LiAc/TE (40% (w/v) PEG4000, 0.1M LiAc, 1x TE), cells were mixed well by inverting the tubes. The tubes were incubated with rotation at 30°C for 30 min. The cells were incubated at 42°C for 15 min on a heat block. Cells were centrifuged at 5000 rpm for 1 min. Supernatants were removed using aspirator. Cells were suspended in 1 ml of YPAD, incubated for 3-6 hours or overnight, and centrifuged at 1500 rpm for 2 min. The pellets were suspended in 100 μl of PBS (Phosphaste Buffered Saline) or TE and spread on selective medium plates.

2-4 Yeast Genomic DNA preparation

Yeast cells were cultured in 1-2 ml of YPAD liquid medium overnight. Cells were harvested in Eppendorf tubes. Pellets were suspended in 500 μl of Zymolase buffer (10mM $NaPO_4$, 10mM EDTA, 0.1M β-Mercaptoethanol, 100mg/ml Zymolase 100T), vortexed for mixing, and then incubated at 37° for 30 min. Cells were lysed by adding 5 μl of Protenase-K (10 mg/ml) and 100 μl of Lysing buffer (0.25M EDTA, 0.5M Tris base, 2.5% (w/v) SDS), mixed well, and incubated at 65°C for 1 hour. During this period, tubes were mixed at least 2 times. 100 μl of 5 M potassium acetate solution was added to the cell suspension, mixed well by shaking, and cell suspensions were incubated on ice for 15 min. Cells were centrifuged at 15000 rpm for 10 min. Supernatants were transferred to new Eppendorf tubes containing 500 μl of cold 100% (V/V) EtOH (ethanol). Samples were inverted gently 5 times and centrifuged at 12000 rpm for 30 sec. Supernatants were removed and pellets were

washed with 1 ml of 70% (V/V) EtOH. And again supernatants were removed and washed the pellets with 1 ml 100%(V/V) EtOH. Samples were dried for 10 min by centrifugal concentrator. DNAs were suspended with 500 ml of 1X TE, which was followed by RNaseA treatment with 10 μg/ml RNaseA at room temp for 30 min. 0.5 ml of 2-propanol was added to samples and inverted gently for 5 times, and then centrifuged at 15000 rpm. Supernatants were removed, and pellets were washed with 70% (V/V) EtOH and were followed by washing with 100% (V/V) EtOH. Samples were dried up for 10 min, and DNAs were suspended with 100-200 μl of 1X TE buffer.

2-5 Meiosis time course

Yeast cells were spread on the YPG (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% Glycerol) plate from freezing stock (-80°C), incubated at 30°C for 12 hours, and then streaked on YPAD plates and incubated for further 2 days at 30°C for producing single colonies. A single diploid colony was inoculated in 3 ml of liquid YPAD medium and incubated overnight in rotator at 30°C. 1 ml of the culture was added to 100 ml of SPS (0.5% Bacto Yeast Extract, 1% Bacto Peptone, 0.17% Yeast nitrogen base, 1% Potassium acetate, 1% Potassium hydrogen phalate, 0.5% Ammonium sulfate) and incubated for 16-17 hours at 30°C with 230 rpm, shaker (Innova® 44). Next day, the SPS culture was centrifuged using a 50 ml screw cap tube and then pellets were washed twice with sterilized distilled water. Yeast cells were suspended in 100 ml of SPM (0.3% Potassium acetate, 0.02% Raffinose) and incubated at 30°C at 230 rpm to initiate meiosis, and samples were collected at each time point.

2-6 Western blotting

5 to 15 ml of SPM culture was collected and cell precipitates were initially washed with water and then washed twice with 20% (w/v) trichloroacetic acid (TCA). Cells were suspended in 1 ml of TCA, and then were disrupted using a bead shaker (60Sec on-60Sec off repeated 5 times) (Yasui Kikai Co. Ltd., Osaka, Japan). Precipitated proteins were recovered by centrifugation and then suspended in 150μl of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. After adjusting the pH to 8.8 with 1M Tris- HCL [pH 9.5], samples were boiled at 95°C for 5 min. Following SDS-PAGE, proteins were transferred from gel to Nylon membrane (Immobilon, MILLIPORE) with semi-dry transfer unit (ATTO TRANSWESTERN). Antibodies against Cdc5 (1:1000)

(SantaCruz), Clb1 (1:1000) (SantaCruz), Hop1 (1:3000), Zip1 (1:1000), Rec8 (1:1000), and Tubulin (1:10000) (Serotec, UK) were used (Shinohara et al., 2008; Zhu et al., 2010). Secondary antibodies conjugated Alkaline Phosphatase (Promega) were used at (1:7500) dilution. And finally proteins on the blots were detected by BCIP/NBT reaction with a kit (Nacalai Tesque).

2-7 Tetrad Dissections

In order to check the spore viability and strain construction, haploids parental strains were patched together on YPAD plates for 4 hours at least for mating and then spread on sporulation plates (0.3% Potassium acetate, 0.02% Raffinose). After incubation at 30℃ overnight, tetrads were dissected manually using Zeiss Axioskop 40 microscope. The plates were incubated for two days and viable spores were counted for viability. For each strain, minimum 100 tetrads were dissected. For strain construction, genotypes of each colony were determined by replica plating to appropriate plates.

2-8 Meiotic Nuclear Spreads: Lipsol Method

5 ml of SPM culture containing yeast cells was collected in a 15 ml conical screw cap tube, centrifuged, and pellets were resuspended in 1 ml of ZK buffer (25mM Tris- HCL [pH 7.5], 0.8M KCl). And 20 µl of 1 M DTT (Dithiothreitol) was added and samples were incubated for 2 min at room temperature with gentle mixing. The samples were centrifuged again, and pellets were recorded and resuspended in fresh 1 ml of ZK buffer. 5ml of Zymolase buffer (5 µg/ml Zymolase 100T, 2% glucose, 50mM Tris [pH7.5]) was added, and samples were incubated for 30 min in 30°C incubator with rotating. To check spheroplasting, water was added to an aliquot of samples on a slide glass, and cells were watched under light microscope to ensure that cells shows bursting. After checking that more than 80% of cells became a spheroplast (bursted), samples were centrifuged and washed with 1 ml MES/Sorbitol (0.1M MES [pH6.5], 1M sorbitol) using Pasteur pipette. The samples were centrifuged again, and pellets were resuspended in 1 ml of MES/Sorbitol and kept at 4°C for usage of spreading later. For chromosome spreads, using micropipette, 20 µl of above-prepared cell suspension was spotted on a clean glass slide (S2441 micro slide glass, Matsunami glass IND., LTD). To cell suspension, 40 µl of PFA/sucrose (4% PFA (Paraformaldehyde [SIGMA-ALDRICH], 3.4% sucrose, freshly-prepared) was added and swirled briefly. Then, 80 µl of 1% Lipsol was added and swirled again, cells were

incubated for 20 sec and watched under light microscope until about 80-90% of cells were lysed. After confirming full lysis, 80 µl of the PFA/sucrose was added to fix the cells. A glass pasture pipette was passed lengthwise along the top of the drop to spread liquid all over entire surface of the slide. The slides were dried to a very thick honey from 4 hours to overnight and were stored in a plastic black microscope box at -20°C.

2-9 Immunostaining of chromosomes spreads

Slides with chromosome spreads prepared as described above were dipped in 0.2% photoflo (Photo-Flo 200 solution Kodak) for 2 min using Coplin jar. The slides were air-dried for 5-10 min, and were blocked for 15 min using 0.5 ml TBS/BSA (1x TBS [20mM Tris pH7.5, 0.15M NaCl], 1% BSA [albumin from bovine serum, SIGMA]). Then, the blocking buffer was drained onto a paper towel, and 90 µl of TBS/BSA solution with primary antibody was added to slides, and slide glasses with the antibody solution was covered with a cover slip and incubated overnight at 4°C or 2 hours at room temperature in a moist chamber. The cover slip was removed by submersion at 45° angle in the washing buffer (1x TBS). The slides were washed for 10 min 3 times in 1xTBS using Coplin jar. 90 µl of TBS/BSA solution with secondary antibody solution (1/2000) dilution of fluorochrome-conjugated IgG was added to slides and the slides were incubated for 2 hours at room temperature in a dark moist chamber. The cover slip was removed and slides were washed as described above, and then washed with water for 2 min. Once slides were completely dry, ~15 µl (three drops) of mounting medium (Vecta Shield with 0.2 µg/ml DAPI) was added to slides and covered with coverslip, which was followed by sealing with nail polish and storing in a dark box. Stained samples were observed using an epi-fluorescence microscope.

2-10 Whole cell staining

Whole cell staining was performed using pre coated poly L-lysine (1mg/ml) slides

Preparation of Poly L lysine coated slides:

200 µl of 1 mg/ml poly L-lysine solution was added onto a glass slide for coating and the slides were incubated for 15min with cover glass in room temperature. Then cover slips were removed by MilliQ water and the slides were washed the slides 2 times with MilliQ water. Then the slides were dried in a room temperature for 15 to 30min.

Preparation of cells for staining:

900 µl of meiosis culture was collected in a 1.5 ml eppendorf and 100 µl of 37% of formaldehyde was added to fix the cells and then cells were rotated at room temperature for 45 min. Tubes were centrifuged at 10000rpm for 2min and the supernatant was removed, and pellets were washed with the ZK buffer. Then 20 µl of Zymolase buffer (5 mg/ml Zymolase 100T, 2% glucose, 50mM Tris [pH7.5]) was added and then incubated for 1 hr at 30°C. Cells were collected and washed with 1X PBS. Then 100 µl of cells were kept on slides pre-coated with poly L-lysine and incubated 15min in room temperature with cover slip. Then cover slips were removed in 1X PBS. Then cells were treated 6 min with ice-cold 100% methanol, 30 sec with cold 100% of acetone, and 2 min with cold PBS. Then slides were blocked by 5% BSA with 1X PBS for 15 min without cover slips. Further immune-staining was performed as described above.

2-11 Cytological analysis and antibodies

Stained samples were observed using an epi-fluorescence microscope (BL51; Olympus) with a 100X objective (NA1.4). Images were captured by CCD camera (Cool Snap; Roper) and then processed using iVision software. For focus counting, about minimum 100 nuclei were analyzed. Primary antibodies against Rad51 (Guinea Pig 1:500), Dmc1 (Rabbit 1:500), Zip1 (Rabbit 1:1000), Zip1 (Rat 1:500) Hop1 (Guinea Pig 1:500), Red1 (Chicken 1:400), Rec8 (Rabbit 1:1000) and GFP (Mouse 1:600) were used (Shinohara et al., 2008; Zhu et al., 2010). Fluorescent-labeled secondary antibodies (Alexa-fluor-594 and -488, Molecular Probes) directed against primary antibodies from the different species were used at a 1:2000 dilution.

2-12 Live cell imaging

For live cell imaging the bottom-glass dishes were pre-coated with Concovalin A (10mg/ml) as described above. Then dishes were dried in a room temperature and used for the imaging. Meiotic samples (100 to 150 µl) were added to the dishes and observed under Delta Vision, Applied precision.

For the time-lapse imaging, images were taken by a computer-assisted fluorescence microscope system (Delta vision, Applied Precision). The objective lens was 100X oil-immersion lens with NA 1.35. At least 100 cells were captured at each meiotic time point. Image deconvolution was carried out by an Image Workstation (Soft Works, Applied

Precision). Time-laps image acquisition was carried out for every 1 sec at a single focal plane.

2-13 Cohesion/pairing assay and length measurement

Whole cell staining was performed using respected GFP strains as described above. And the cells were stained with GFP antibody (1:500 dilution). Stained samples were observed using an epi-fluorescence microscope. The Number of GFP spots in nucleus detected by DAPI staining in single cells were manually counted. Homologue pairing and sister chromatid cohesion of chromosomes were analyzed in whole yeast cells with homologous and heterozygous LacI-GFP spots with *CEN-V*. At least 200 cells were counted at each meiotic time point.

For the distance measurement on SCs, chromosome spreads with elongated SCs were randomly chosen to measure a distance between *CEN-IV* and *TEL-IV* two GFP spots. Chromosome spreads were prepared as described above and stained for both anti-Rec8 and anti-GFP. The distance between two spots was measured Velocity^{TM} program (Applied Precision). At least 50 cells were measured distances of two GFP spots in wild type and mutant cells.

2-14 Southern Blotting

Isolated DNA samples from SPM were digested with proper restriction enzyme, for the detection of DSBs. DNA was digested in 100 μl of solution with *PstI* (20,000 U/ml) for DSBs, overnight at 37 °C. Next day, samples were subject to precipitation for DNA using Sodium acetate with 100% (V/V) EtOH, and pellets were suspended with 30 μl of 1X TE buffer and kept for 4-5 hours to dissolve the DNAs. After DNAs were completely dissolved, DNAs were analyzed by electrophoresis using a 0.7% or 0.6% of agarose gel (size 35cmx15cm) in 1X TAE buffer (Tris base, acetic acid, EDTA) with a voltage of 75 V overnight for the detection of DSBs, and Voltage of 50-65 V for 24h to 36h. Once electrophoresis has finished, agarose gel was cut, treated by 0.25 M HCl with shaking for 20 min, followed by Alkali solution (0.6 M NaCl, 0.2 M NaOH) treatment for 15 min twice, and then gel was neutralized with 25 mM Na-phosphate buffer [pH 6.5] with shaking for 40 min. The DNAs in the gel was transferred onto membrane (Hybond-N; GE Healthcare), by capillary transfer as described in lab manual for at least 12 hours. For crosslinking the DNA, membranes were irradiated using the UV crosslinker (Stratalinker® UV Crosslinker) and UV irradiated with 120 mJ/cm^2 (Autocrosslink Mode: 1200 microjoules (x 100)).

The membrane was inserted in a hybridization bottle containing 25 ml of hybridization buffer (7% SDS, 1M Na- Phosphate buffer, 0.5M EDTA), and incubated more than 30 min at 65°C. Meanwhile, 50 ng of a probe DNA was dissolved in 10.5 μl of 1X TE buffer, 2.5 μl of primer DNA (Amersham Megaprime DNA labeling kit; GE healthcare) was added and sample was boiled at 95°C for 5 min, followed by adding 2.5 μl of reaction mix, 2 μl of dCTP, 2 μl of dTTP, 2 μl of dGTP and 2.5 μl of α-^{32}P-dATP (37MBq), then 1 μl of Klenow fragment was added to the mix and incubated for 15 min at 37°C. The probe DNA solutions were then transferred to a G-50 spun column, and centrifuged in a new Eppendorf tube for 1 min at 4°C. After boiling at 95°C for 5 min and chilled on ice, probes DNAs were added to the bottle containing 25 ml of fresh hybridization buffer. The membrane was incubated at 65°C for more than 12 hours. Next day, the membranes were washed 3 times using washing buffer (0.1 % SDS, 1M Na- Phosphate buffer, 0.5M EDTA) at 65°C. Membranes were dried up and contacted with IP plate (BAS Imaging plate, 20x40, Fuix) for 5-8 hours. Followed by scanning the membrane using IP reader (BAS2000 II Fujix). Probes used for Southern blotting was "Probe 291" for DSBs detection (Storlazzi et al., 1995). Image Gauge software (Fujifilm Co. Ltd., Tokyo, Japan) was used to quantify the DSBs bands.

2-15 FACS Analysis

1.5 ml of cell culture from SPM was harvested and centrifuged for 3 min at 3000 rpm, and then, pellets were resuspended with 1 ml of 70% (V/V) EtOH and kept in -20°C. Cells were centrifuged and washed with 1 ml of buffer-A (0.2M Tris-HCl, 0.05M EDTA) and washing was repeated twice. Cells were resuspended with 0.5 ml buffer-A and sonicated at 10% amplitude using a sonicator (Branson Digital sonifier). 0.2 mg/ml RNase-A solution was added, and incubated at 37°C for 4 hour with mixing for several times. Cells were centrifuged and resuspended with 0.5ml of buffer-A containing 16 μg/ml of PI (Propidium Iodide) solution. And were incubated at room temperature for 30 min. FACS (Fluorescence activated cell sorter) analysis was performed using BD FACSCaliburTM.

2-16 Chromatin association assay

Initially 1x10^9 cells (should be about 15ml of 1.6 OD_{600} culture) were spin down and resuspended with 1 ml prespheroplast buffer (100 mM Tris, pH 9.4, 10 mM DTT), incubated at room temp for about 5 min. The suspension was transferred into a preweighed

eppendorf and Spin down. The cell pellets were suspend with 1.5 ml spheroplast buffer (50mM KH_2PO_4, pH7.4, 0.6M Sorbitol, 10mM DTT). 10 µl of the suspension was taken and diluted into 990 µl H_2O to measure the OD (about 0.30). 8 µl of 10 mg/ml Zymolase 100T was added to the suspension, incubated at 30ºC 5 min. 10µl of aliquots were taken to dilute into 990 µl to measure OD (If the cell wall is digested, OD should be 1/10 of the value before digestion, ~0.03). Spheroplasts were spin down immediately and cooled on ice. Weight of the eppendorf with spheroplast pellets was measured, and the pellet weight was calculated (should be about 120mg). And lysed the spheroplasts by ~5 fold volumes (eg. 120mg- 600ul) of the ice cold hypotonic buffer (100mM MES-NaOH pH 6.4, 1mM EDTA, 0.5mM $MgCl_2$) and incubated on ice for 5min. Collected 1/10 aliquot of the resulting lysate and saved it as Whole Cell Lysate (WCS) fraction. Centrifuged the rest of the lysate at 22,000g for 15min. And collected supernatant as the chromatin-unbound fraction. The pellet was resuspended in 100 µl EBX (50mM HEPES.NaOH, pH7.5, 100mMKCl, 2.5mM $MgCl_2$, 0.05%Triton X-100, plus protease inhibitors as above) and centrifuged at 16,000g for 15min. This was the nuclei fraction. 90µl nuclei fraction was taken and spin down at 16,000g, for10min. >20ul detergent supernatant was saved, and the rest was aspirated again. Thee pellet was dissolved in 75 µl EBX. This is the chromosome suspension. 4 µl of 1µg/µl DNaseI and 2 µl of 1M $MgCl_2$ were added to the suspension and incubated at room temperature for 10min. And centrifuged 60µl of chromosome suspension at 16000g for 10min, and saved 20µl chromatin supernatant and aspirated the rest. And finally resuspend the pellet in 50µl EBX and this is the nuclear matrix fraction.

2-17 Chromatin Immuno precipitation (ChIP) and q-PCR

The ChIP assay was performed as described previously (Matsuzaki et al, 2012). In brief *in vivo* cross-linking of DNAs and proteins was carried out by the treatment of cell cultures with 1% formaldehyde (v / v) for 30 min. Glycine was added to a final concentration of 125 mM and the incubation continued for 5 min. Cells were harvested and washed twice with ice cold TBS, and breakage was performed in 400 µl FA-lysis buffer (50 mM HEPES-KOH at pH 7.5, 140 mM NaC1, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF). The cells were disrupted by beads beating using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Supernatants were sonicated 20 times for 30 s at a high level in a Bio-Ruptor (Cosmo Bio, Tokyo, Japan). Sonicated extracts were agitated with magnetic beads bound with anti-Rec8 (Serotec, UK) antibody at 4 °C for 3 h. Precipitates were successively washed for 5 min each with 0.5 ml of FA-lysis buffer, 0.5 ml

of high-salt FA-lysis buffer 500 mM NaC1, 0.5 ml of washing buffer (10 mM Tris-HCl at pH 8.0, 0.25M LiC1, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and 0.5 ml of TE (20 mM Tris-HC1 at pH 8.0, 1 mM EDTA). Finally, the samples were processed for DNA extraction using phenol chloroform method (Orlando and Paro 1993). The amounts of the precipitated DNAs were quantified by real-time PCR (Chromo 4; Bio-Rad, Hercules, CA, USA). Primers were chosen to amplify around 300-400 base pairs and primer sequence and coordinates are listed in primers table. Input DNAs were diluted 600 times for all the primer sets. Quantitative PCRs were performed with Eva Green Super mix (Bio-Rad, US) for chromosome arm regions (CARC1, CARC2, CARC3) and centromere regions (CEN3 and CEN4) primers sets described previously (Bardhan et al, 2010). Each chromatin immuno precipitation was performed with samples from three independent immunoprecipitation.

2-18 Fluorescence intensity measurement

Mean fluorescence of the whole nucleus was quantified with Image J. Quantification was performed using unprocessed raw images and identical exposure time setting in DeltaVision system (Applied Precision, USA). The area of a nuclear spread was defined as an oval, and the mean fluorescence intensity was measured within this area.

Fluorescence intensity line profiles were calculated using maximum intensity projections from unprocessed raw images acquired using DeltaVision with the SoftWoRx Line profile tool. Reference lines were drawn so that they intersected with single chromosomes, and intensity per pixel was quantified.

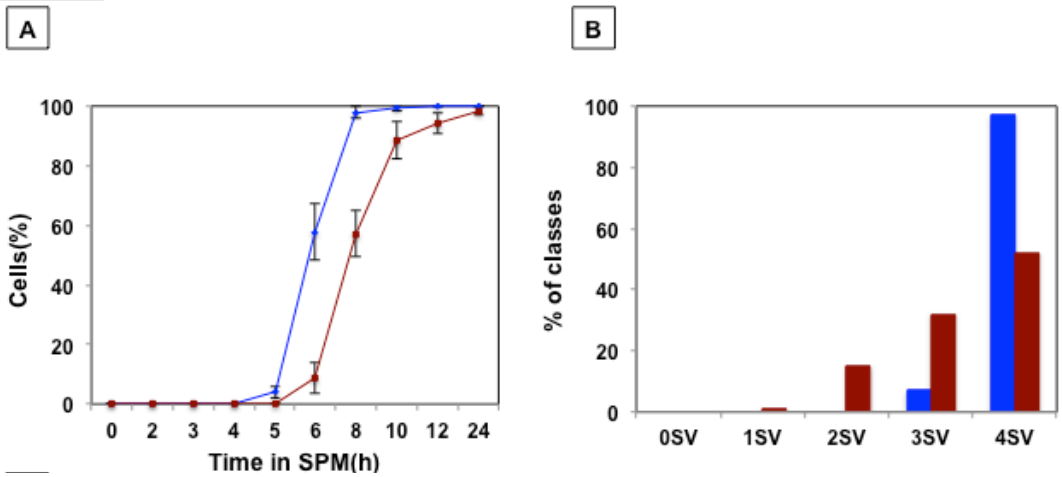
Chapter-3

Results

3-1-1 Rad61 is necessary for meiotic cell progression

Cohesin function is regulated by a non-essential subunit such as Rad61/Wpl1 (Kueng et al., 2006). Rad61 is known to be a negative regulator for SCC by destabilizing cohesin's function (Kueng et al., 2006; Rowland et al., 2009; Sutani et al., 2009). The role of Rad61 during meiosis is largely unknown. To know meiotic roles of Rad61, a deletion mutant of the *RAD61/WPL1* gene was constructed in SK1 background, which shows synchronous meiosis. Progression of meiosis was monitored by DAPI staining. The *rad61/wpl1* (hereafter, *rad61)* mutant shows about 1.5hr delay in the entry into meiosis I compared to the wild type (Fig. 1A). The *rad61* shows significantly reduced spore viability of 84.3% compared to 98.5% of the wild type (Fig. 1B, 1C). Furthermore, I analyzed the expression level of meiosis-specific proteins by western blotting, which includes Zip1, Rec8, and Hop1 (Fig. 1D). In the wild type the expression of Zip1, Hop1, and Rec8 started at 2 h and then phosphorylated forms of these proteins are observed at late meiosis, as indicated by the appearance of slowly migrating bands on western blotting. The *rad61* mutant exhibits expression of Zip1, Rec8 and Hop1 starting at 2 h, but showed delayed disappearance of Zip1 and Rec8 proteins and also delayed cleavage of Rec8 (Fig. 1D). These results confirm that the *rad61* mutant showed delay in the exit of prophase-I, suggesting a role of Rad61 in meiotic prophase I.

Figure 1



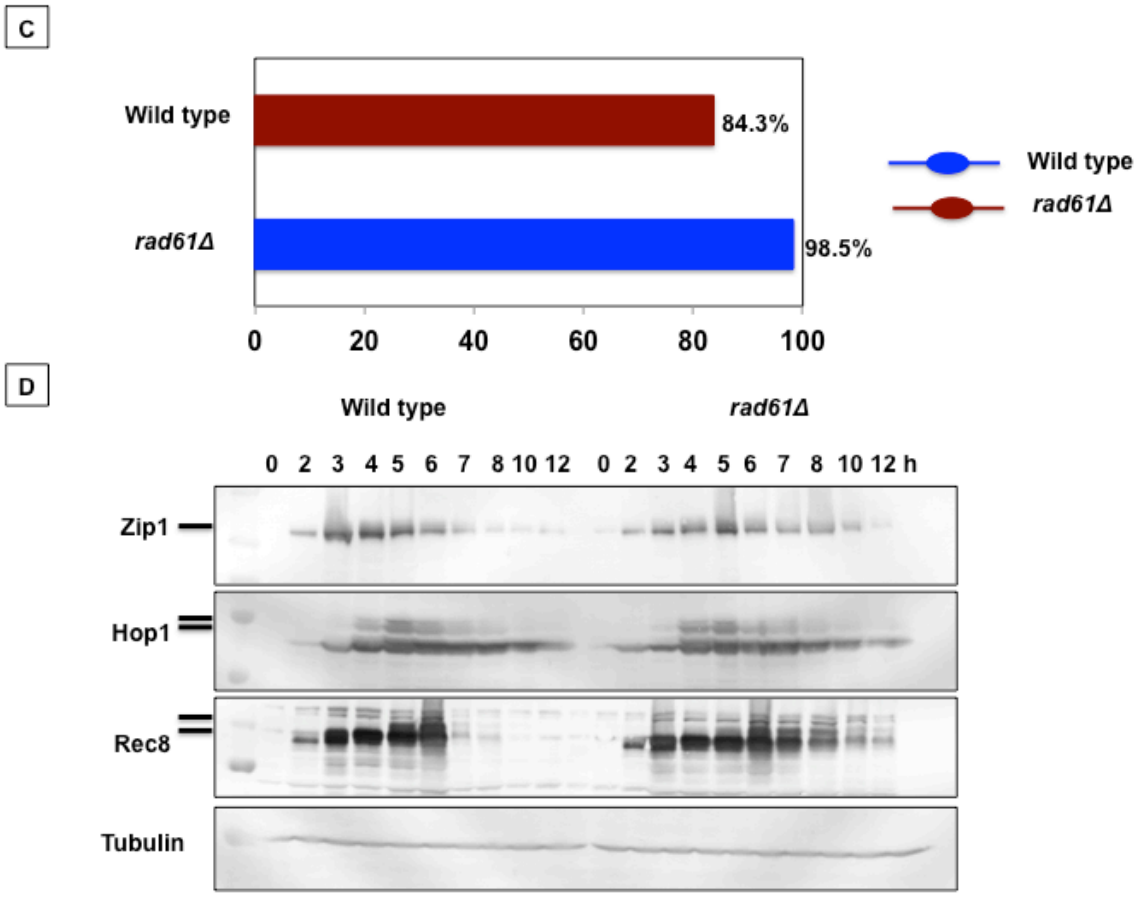


Figure 1. Rad61 is necessary for meiotic cell progression

(A) Meiotic cell division I was analyzed by DAPI staining of wild type (MSY832/833) and *rad61* (KYS 63/64). At least 150 cells were counted by DAPI staining at each time point. Plotted values are the mean values with standard deviation (S.D.) from three independent time courses.

(B) Distributions of viable spores in wild type (MSY832/833) and *rad61* (KYS 63/64), 100 tetrads were dissected.

(C) Percentages of spore viability in wild type (MSY832/833) and *rad61* (KYS 63/64), 100 tetrads were dissected.

(D) Expression of various meiotic proteins was verified by western blotting. At each time point, cells were fixed with TCA and cell lysates were subject to the analysis. Representative images of wild type (MSY832/833) and *rad61* (KYS 63/64) western blots are shown. Phosphorylated species of Zip1, Hop1, and Rec8 are shown by bars on the left.

3-1-2 Rad61 is required for efficient homologues pairing and sister chromatid cohesion

In order to know the role of Rad61 in SCC in meiotic prophase I, a heterozygous strain for CenV-GFP was used (Michaelis et al., 1997). While SCC provides a single GFP spot in a nucleus, defective SCC induces two spots (Fig. 2A). To eliminate the effect of MI, an *ndt80* mutation was introduced to induce a pachytene arrest (Xu et al., 1995). At 8 h in the wild type, about 90% cells showed a single focus of GFP, indicating SCC at CenV. On the other hand, the *rad61* mutant increases a fraction of cells with two spots to 27.7% (Fig. 2B). This indicates a partial defect in SCC in the *rad61* mutant in mid-pachytene.

Using the same CenV-GFP construct, the frequency of the pairing of a chromosomal locus was analyzed in a cell homozygous for CenV-GFP in the *ndt80* background (Fig. 2C). At 8 h, 92.4% of wild-type cells showed a single GFP spot, indicating the pairing of CenV on homologous chromosomes. 70, 16, 7.9%, and 6% of *rad61* mutant cells contained a single, two, three, and four GFP spots in a cell, respectively (Fig. 2D). These confirm a weak defect in SCC in the mutant and show a partial defect in the pairing. These results suggest that Rad61 is required for proper SCC and also for efficient chromosome pairing.

Figure 2

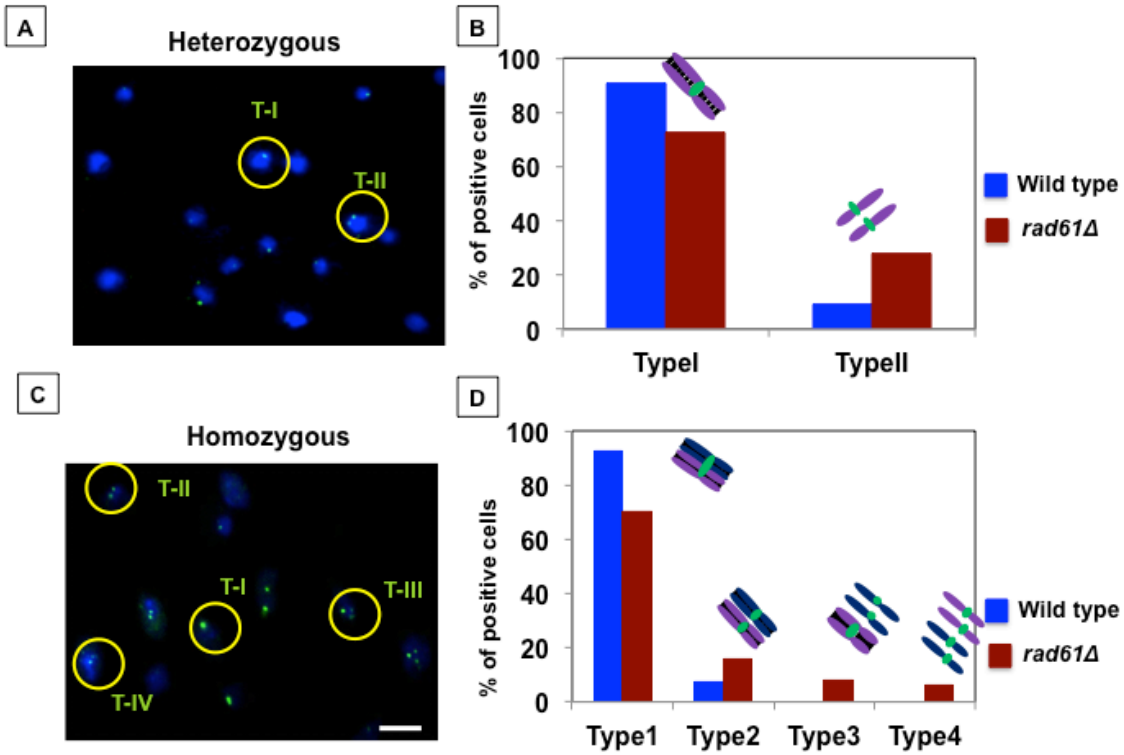


Figure 2. Rad61 is required for efficient homologues pairing and sister chromatid cohesion

(A) Cohesion activity was monitored using a strain heterozygous for *CENV-GFP*. Representative image of DAPI (blue)/CENV-GFP (green) of heterozygous *rad61 ndt80* (KYS448/450) mutant at 8 h. The bar indicates 2μm.

(B) Frequency of cell with a single or two GFP spots in a nucleus at 8 h of meiosis. About 300 cells were counted for 1 or 2 spots of GFP in a cell: *ndt80* (KYS404/391) (n=318), *rad61 ndt80* (KYS448/450) (n=299).

(C) Homologous pairing was measured by analyzing the number of GFP spots in an *ndt80* cell homozygous for *CENV-GFP*. Representative image of DAPI (blue)/CENV-GFP (green) of homozygous *rad61 ndt80* (KYS448/449) mutant. The bar indicates 2μm.

(D) Frequencies of cells with different numbers of GFP spots at 8 h of meiosis; 1 spot, complete pairing; 2 spots, no pairing with normal cohesion or complete pairing with cohesion defect for one sister pair; 3 spots, no pairing with cohesion defect for one sister pair; 4 spots, no paring with full cohesion defect for two pairs of sisters. The *ndt80* (KYS 404/405) (n=437); *rad61 ndt80* (KYS448/449) (n=531).

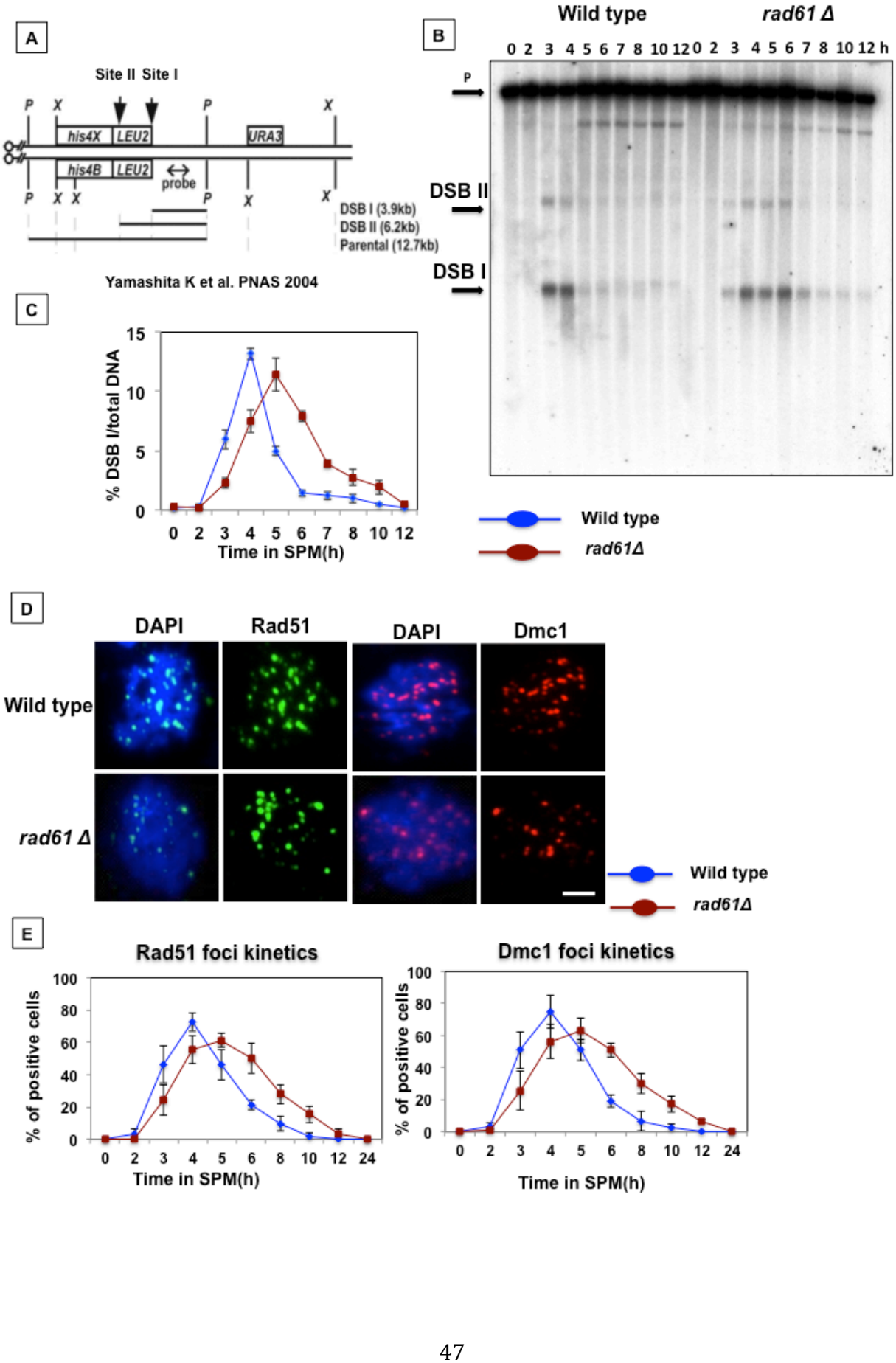
3-1-3 Rad61 is necessary for timely DSB formation and repair

As explained previously, a meiosis-specific α-kleisin Rec8 promotes the efficient formation and repair of meiotic DSBs (Klein et al., 1999; Kugou et al., 2009; Kim et al., 2010). I checked the role of Rad61 in the appearance and disappearance of meiotic DSBs at the *HIS4-LEU2* recombination hotspot (Cao et al., 1990) (Fig. 3A, B). Genomic DNAs from cells harvested at different times were analyzed for DSBs using Southern blotting analysis as described in Materials and Methods. Genomic DNAs was digested with *Pst1* for DSB detection (Fig. 3A). On the blots, a full-length fragment, that does not contain meiotic DSBs, is indicated by 'P' or parental fragments. The two major DSBs in the *HIS4-LEU2* locus result in the production of two shorter DNA fragments as shown as DSB I and DSB II than the parental fragment.

At this locus in wild-type cells, DSBs started to appear at 2 h, peaked at 4 h, and thereafter gradually disappeared (Fig. 3C). The *rad61* mutant showed delayed DSB formation. In the mutant, DSBs appeared at 3 h, one hour later than in the wild type, but peaked at 5 h and then went away slower than in the wild type. This indicates that Rad61 is necessary for timely formation and efficient repair of DSBs.

To pursue above result more detail, I performed immunostaining analysis of Rad51 and Dmc1. Rad51 and Dmc1 bind to ssDNA regions of DSBs (Bishop et al., 1992; Shinohara et al., 1992), which can be detected as a focus on meiotic chromosome spreads by immunostaining, which becomes a marker for DSB repair during meiosis (Bishop et al., 1994; Miyazaki et al., 2004) (Fig. 3D). In the wild type foci containing either Rad51 or Dmc1 began to appear from 3 h with a peak at 4 h and disappeared gradually by 8 h during further incubation in SPM. In the *rad61* mutant, Rad51/Dmc1 foci started to form at 3 h but the peak of Rad51/Dmc1 focus formation in the *rad61* was 5 h, about one-hour delay relative to the wild type. At 8 h, 28-30% of the mutant cells still contained the foci (Fig. 3E). These support the idea that Rad61 controls DSB formation and repair in an efficient way. I also counted Rad51 foci number on each chromosome spreads. The average number of Rad51 foci in the mutant was 37.8 ± 11.8 (n=48) at 5 h, while that in the wild type was 41.9 ± 11.1 (n=56) at 4 h (Fig. 3F). Thus, the steady number of Rad51 focus in the *rad61* mutant was slightly (but not significantly) decreased compared to that in the wild type (Mann-Whitney *U*-test, P= 0.036).

Figure 3


A
Site II Site I
P X P X
his4X LEU2 URA3
his4B LEU2 probe
P X X P X X
DSB I (3.9kb)
DSB II (6.2kb)
Parental (12.7kb)
Yamashita K et al. PNAS 2004
B
Wild type
rad61 Δ
0 2 3 4 5 6 7 8 10 12 0 2 3 4 5 6 7 8 10 12 h
P
DSB II
DSB I
C
% DSB I/total DNA
15
10
5
0
0 2 3 4 5 6 7 8 10 12
Time in SPM(h)
Wild type
rad61Δ
D
DAPI
Rad51
DAPI
Dmc1
Wild type
rad61 Δ
Wild type
rad61Δ
E
Rad51 foci kinetics
% of positive cells
100
80
60
40
20
0
0 2 3 4 5 6 8 10 12 24
Time in SPM(h)
Dmc1 foci kinetics
% of positive cells
100
80
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0 2 3 4 5 6 8 10 12 24
Time in SPM(h)

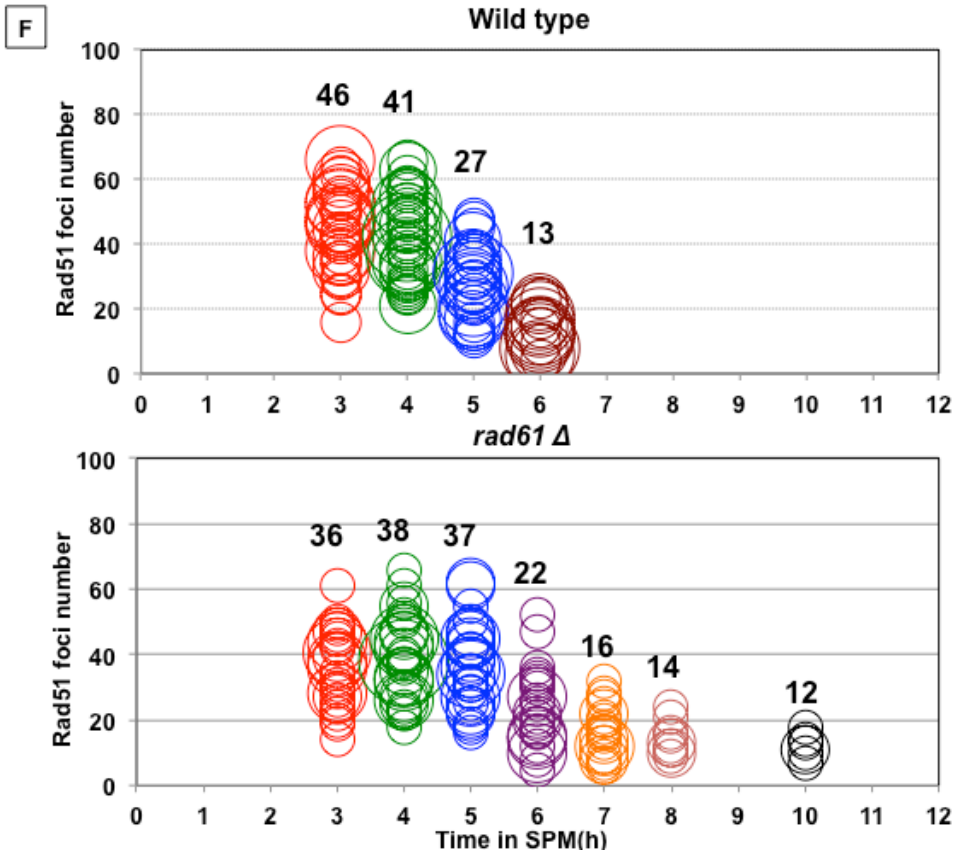


Figure 3. Rad61 is necessary for proper DSB formation and repair

(A) Schematic representation of the *HIS4-LEU2* locus. Sizes of fragments for DSB and parental fragments are shown with lines below.

(B) DSB formation and repair at the *HIS4-LEU2* locus in wild type (NKY1303/1543) and *rad61* (KYS361/365) were verified by Southern blotting. Genomic DNAs were digested with *Pst*I and analyzed by Southern blotting. A representative image of the blots is shown.

(C) Kinetics of DSB. Quantification of DSB I fragment to a parental fragment was quantified and plotted. The values are the mean values with standard deviation (S.D.) from three independent time courses.

(D) Immunostaining analysis of Rad51 and Dmc1 for wild type (NKY1303/1543) and *rad61* (KYS361/365) mutant strains was carried out. Representative photos of Rad51 (green) or Dmc1 (red) with DAPI (blue) at 4 h are shown in each strain. The bar indicates 2μm.

(E) Kinetics of Rad51 (left) or Dmc1 (right)-focus positive cells in wild type (blue; NKY1303/1543) and *rad61* (red; KYS361/365) mutant. A focus-positive cell was defined as a cell with more than 5 foci. More than 100 nuclei were counted at each time point in wild type and *rad61*. Plotted values are the mean values with standard deviation (S.D.) from three independent time courses.

(F) Distribution of a number of foci of Rad51 per a nucleus was shown. The number of the focus at each time point was counted in wild type (NKY1303/1543) and *rad61* (KYS361/365) mutant. The average number of foci per positive nucleus is shown on top.

3-1-4 Rad61 plays a role in proper elongation of synaptonemal complex

In most of eukaryotes, homologous pairing culminates as chromosome synapsis, which is clearly manifested by the formation of the SCs. SC formation is tightly coupled with interhomolog recombination (Borner et al., 2004). As shown above, the *rad61* mutant was partly deficient in pairing of homologous chromosomes and meiotic recombination. The role of Rad61 in SC formation was examined. The formation of SCs was monitored by immunostaining analysis of Zip1, a component of the central regions of the SC (Sym et al., 1993) (Fig. 4A). Zip1 staining in the wild type was classified into three classes; dotty, short, and full linear staining, which correspond roughly with leptotene, zygotene, and pachytene stages of meiosis prophase I, respectively. In wild-type cells, dotty staining of Zip1 was peaked at 2 h, partial linear Zip1 was predominantly observed at 4 h, and full linear staining was seen at 5 h. In the *rad61* mutant, the appearance of Zip1 linear staining was slightly delayed relative to the wild type. Cells with dotty staining of Zip1 accumulated and peaked at 4 h in the mutant. The mutant accumulated an aggregate of Zip1, which is referred to as poly-complex (Sym and Roeder 1993), which is an indicative for defective SC formation, more than the wild type. These indicate a partial defect in the assembly of SC central components in the *rad61* mutant (Fig. 4B).

Figure 4

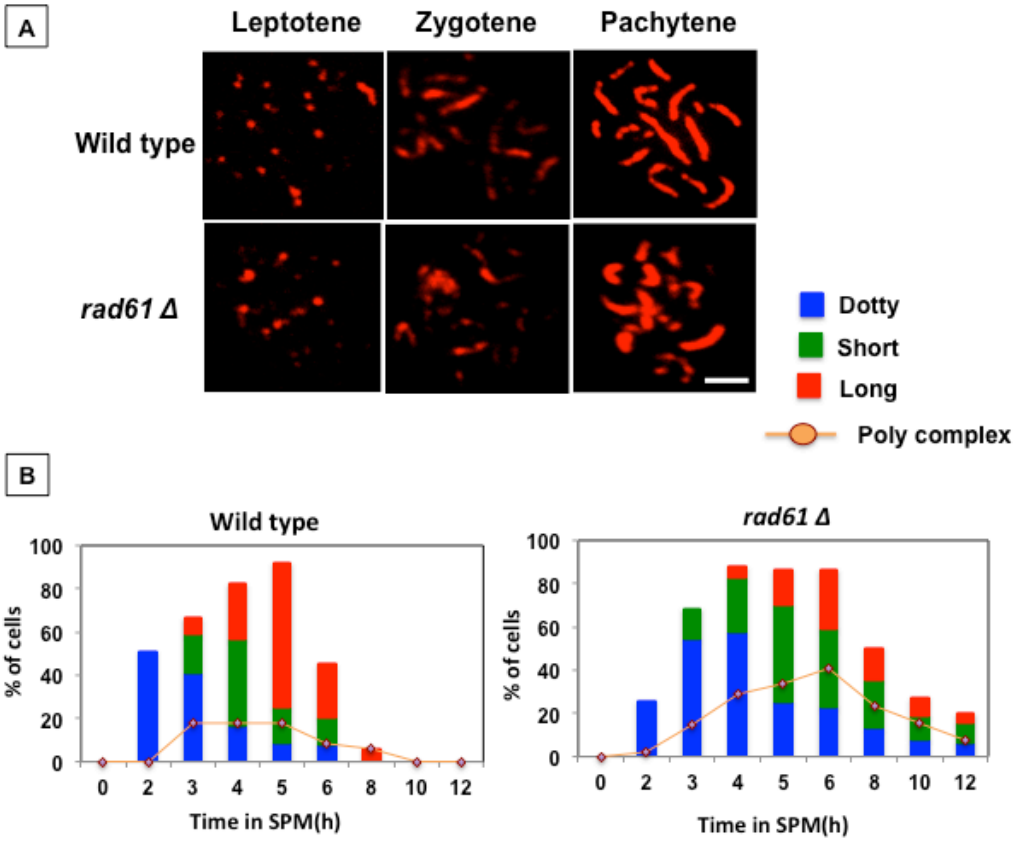


Figure 4. Rad61 plays a role in proper elongation of synaptonemal complex.

(A) Immunostaining analysis of a SC protein, Zip1 (red), was carried out for wild type (MSY832/833) and *rad61* (KYS63/64) mutant strains. Representative images at different stages are shown for each strain of wild type and *rad61*. The bar indicates 2μm.

(B) Zip1 staining in wild type (MSY832/833) and *rad61* (KYS63/64) strains was classified as follows: dot (Class I, blue), partial linear (Class II, green), full SC (Class III, red) in bar graph. And also line graphs show % of cells with aggregates of Zip1 called poly complex. More than 100 nuclei were counted at each time point in wild type and *rad61*.

3-1-5 The *rad61* mutant is defective in chromosome axis formation

Immunostaining analysis of Zip1 lines revealed shorter Zip1 lines in the *rad61* mutant compared to those in the wild type (Fig. 5A). This promoted me to examine the loading of components of axial elements of the SC. In the wild type, Red1, an axis component of the SC (Smith et al., 1997), showed discontinuous lines; some regions of the axes exhibited much stronger Red1 signals than the other regions. The *rad61* mutant showed thicker and shorter lines of Red1 compared to those in the wild type (Fig. 5A). Red1 staining in the *rad61* mutant is very uniform compared to the wild type with heterologous staining. Appearance of Red1 lines in *rad61* mutant is comparable with the wild type but disassembly of Red1 from chromosomes delayed in the *rad61* mutant compared to the wild type (Fig. 5B). This is probably due to a defect in the progression of meiotic recombination.

Possible shortening of the SC as well as the loading of cohesin on to the chromosomes in *rad61* mutant was directly confirmed by the staining for Rec8. Rec8 staining also showed discontinuous lines on SCs in the wild type as shown previously (Klein et al., 1999). The *rad61* mutant proficient in the loading of Rec8 on meiotic chromosomes. Kinetic analysis indicated delayed disassembly of Rec8 from meiotic chromosomes (Fig. 5C). Importantly, Rec8 lines in the *rad61* were thicker and shorter than those in the wild type (Fig. 5A). These suggest that Rad61 controls chromosome axial compaction during meiosis.

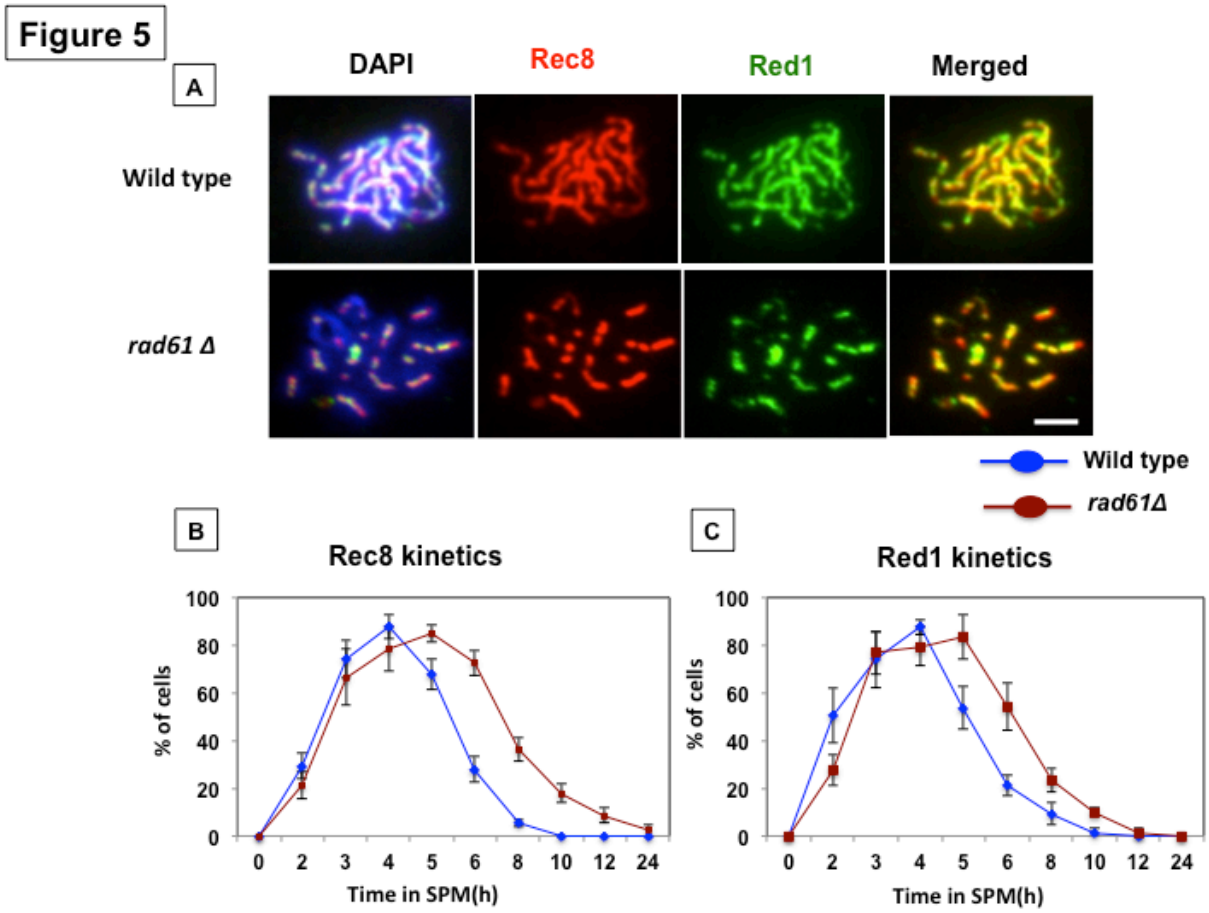


Figure 5. The *rad61* mutant is defective in chromosome axis formation

(A) Immunostaining analysis of chromosome proteins, Rec8 (red) and Red1 (green), was carried out for wild type (MSY832/833) and *rad61* (KYS63/64) mutant strains. Representative images with or without DAPI staining (blue) are shown for each strain. The bar indicates 2μm.

(B) Kinetics of spreads positive for Rec8 was verified in wild type (MSY832/833) and *rad61* (KYS63/64) mutant. More than 100 nuclei were counted at each time point in wild type and *rad61*. Plotted values are the mean values with S.D from three independent time courses.

(C) Kinetics of spreads positive for Red1 was verified in wild type (MSY832/833) and *rad61* (KYS63/64) mutant. More than 100 nuclei were counted at each time point in wild type and *rad61*. Plotted values are the mean values with S.D from three independent time courses.

3-1-6 Chromosome axis is shortened in the *rad61* mutant

Above findings suggested that the *rad61* mutation induced the shortening of chromosome axes. To confirm this chromosome shortening, a physical distance between centromere and telomere on the right arm of chromosome IV was measured, both of which are marked with LacI-GFP (1.05 Mbp; heterozygous for the LacI-GFPs (Yu et al., 2005). Double staining for GFP and Rec8 revealed two spots of GFP (Fig. 6A). In cells at 0 h, which corresponded with mitotic G1 cells, the average distance between the loci on chromosome spreads in the wild type was 1.6 ± 0.46 μm. The *rad61* mutant slightly, but did not significantly decrease the length to 1.51 ± 0.46 μm (Student *t*-test, P= 0.378). Compacted chromosomes in the *rad61* mutant during mitosis had been reported for rDNA region previously (Guacci et al., 1994; Lopez-Serra et al., 2013). At 4 h, when most of cells in zygotene/pachytene, wild-type cells reduced the length to 1.17 ± 0.35 μm (versus 0 h in wild type: $P=2.7X10^{-6}$), indicating shortening of the chromosome axis during meiotic prophase-I of the wild type. The average length between the loci in the *rad61* mutant at 4 h was 0.75 ± 0.25 μm (versus 0 h in the mutant; $P=2.0X10^{-9}$)(Fig. 6B). This is significantly different from that in wild type meiosis. Therefore, the *rad61* mutation induced hyper-compaction of chromosomes, suggesting that the Rad61, possibly cohesin complex, controls axial compaction during meiosis.

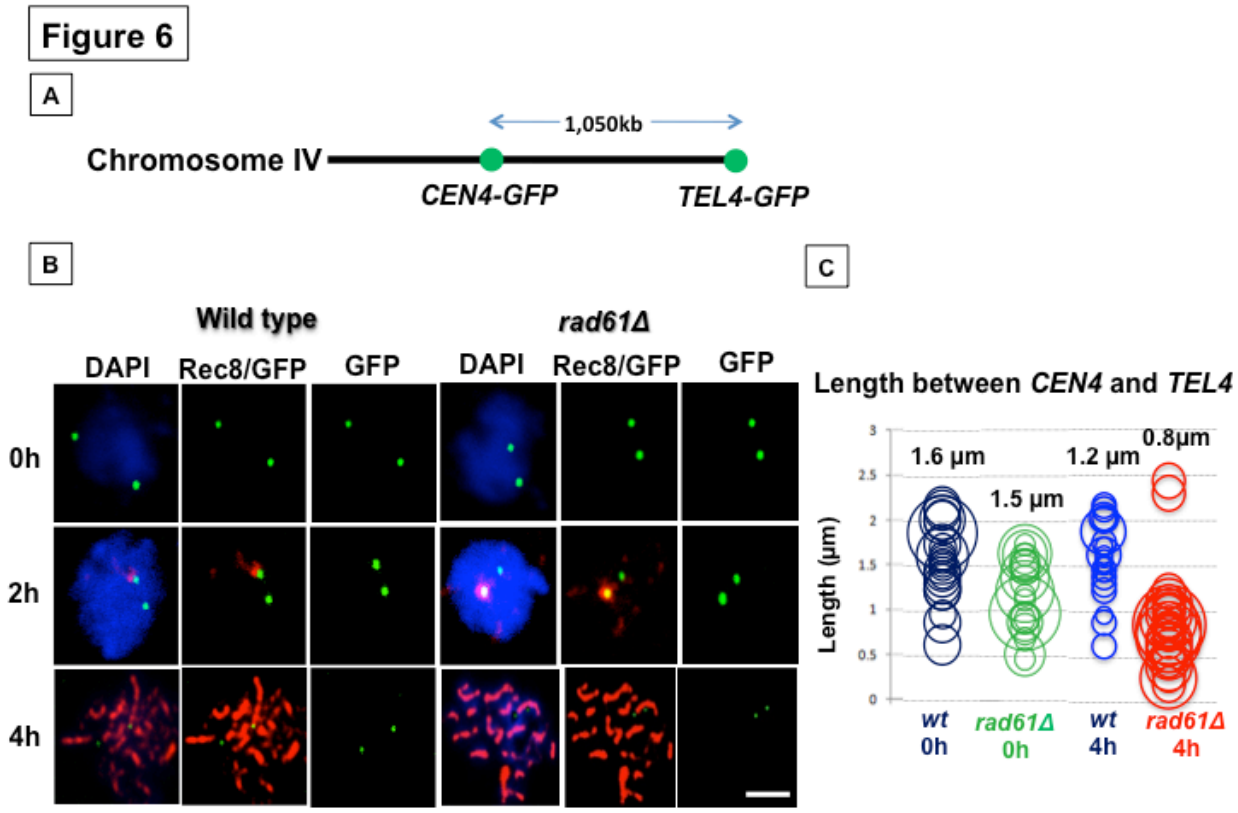


Figure 6. The *rad61/wpl1* mutant shows hyper-compaction of meiotic chromosomes.

(A) Representative diagram of *CEN-IV* TEL-*IV* GFP is shown, and distance between the two GFP spots is 1050Kb.

(B) Immunostaining analysis of two GFP spots (above) on chromosome IV with Rec8 was carried out for each strain. Representative image of GFP spots (green), Rec8 (red) and DNA (blue) in wild type (MSY315/832) and *rad61* (KYS343/64) mutant on chromosome spreads at each time is shown. The bar indicates 2μm.

(C) Distances between *CEN-IV TEL-IV* at each time point (0, 4 h) were measured and plotted as circles. For chromosome spreads, minimum 45 spreads were analyzed for the distance. Wild type (KYS315/832) and *rad61* (KYS343/64) (0, 4 h) were showed in the graph.

3-1-7 Rad61 regulates telomere clustering during meiosis.

Previous studies showed that the Rec8 cohesin complex also controls dynamics of telomeres in meiotic cells (Conrad et al., 2007; Trelles-Sticken et al., 2005). During meiosis, telomeres bound to the nuclear envelop (NE) form a big cluster, which is referred to as telomere bouquets (Trelles-Sticken et al., 1999). Telomere dynamics can be monitored using a GFP-fusion protein of Rap1, a telomere binding protein (Hayashi et al., 1998). In mitotic cells, a few Rap1 foci were observed on a single focal plane of nuclei. During meiotic prophase I, Rap1 foci increased its number and formed a cluster on one area of nucleus. Importantly, this Rap1 clustering was transient in wild-type cells (Trelles-Sticken et al., 1999) (Fig. 7A). A fraction of cells containing Rap1 cluster was seen at 2 and 3 h in wild-type cells with a frequency of 25± 4.0% and 28.3 ± 1.5 %, respectively (Fig. 7B). On the other hand, the *rad61* mutant gradually accumulated Rap1 clustering to 47± 6.7 % by 5 h. At late times such as 6 h, this clustering of telomeres was resolved in the mutant (Fig. 7B). The *rec8* mutant shows persistent Rap1 clustering during the prophase I (Trelles-Sticken et al., 2005). These indicate that Rad61 controls telomere dynamics in a positive manner.

Figure 7

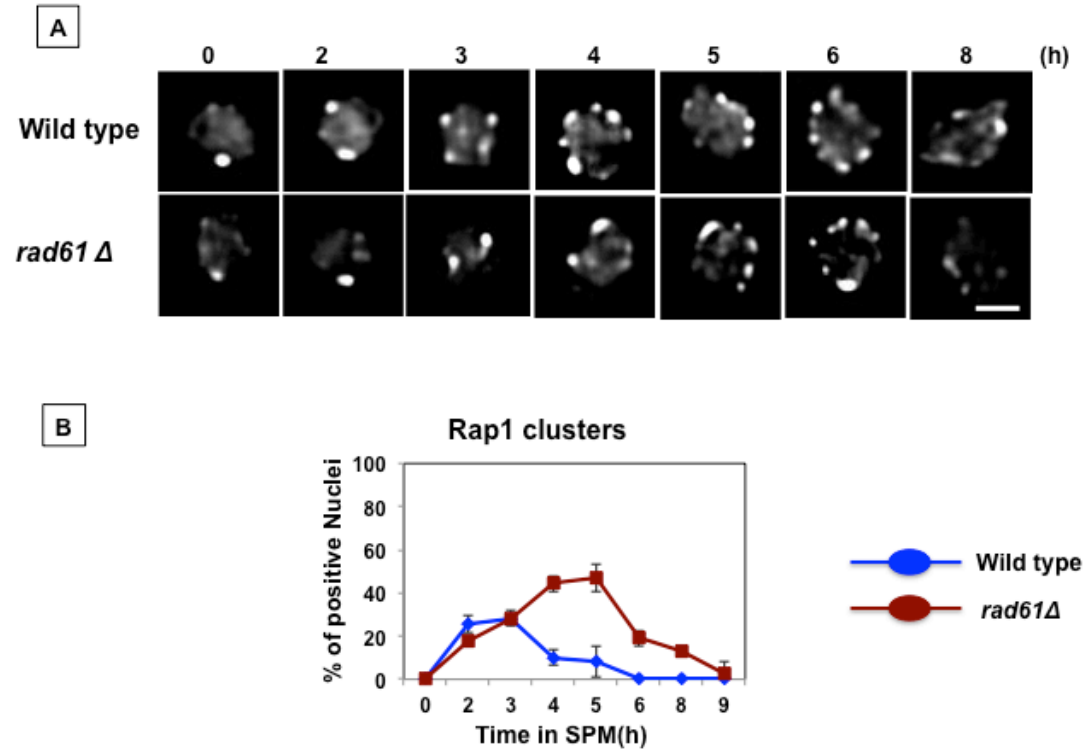


Figure 7. Rad61 regulates telomere clustering during meiotic prophase

(A) Telomere dynamics was monitored in a meiotic cell with Rap1-GFP. A representative image of Rap1-GFP (white) at each time of two strains wild type (KYS61/62) and *rad61* (KYS123/124) is shown. The bar indicates 2μm.

(B) Telomere clustering was examined in meiotic cultures of cells with Rap1-GFP. Cells with a big Rap1 cluster were counted at each time point (n=100) in wild type (KYS61/62) and *rad61* (KYS123/124). Plotted values are the mean values with S.D from three independent time courses.

3-1-8 Rad61 is necessary for proper Mps3 localization during meiosis

Dynamics of telomeres on the NE during meiosis is promoted by Mps3 in budding yeast. Mps3 is a SUN domain protein localized in the inner nuclear membrane (Starr and Fischer 2005). I analyzed the localization of Mps3 on the NE using Mps3-GFP protein (Fig. 8A). As shown previously (Conrad et al., 2008; Conrad et al., 2007), in vegetative cells; e.g., at 0 h, Mps3-GFP showed a single spot which corresponds with the spindle pole body (SPB). Once cells enter meiosis, Mps3 shows different distributions on the NE. In early meiosis I (2 h), Mps3 starts to form multiple foci on NE. Then, a bigger foci or patches appeared at 3 h and then a cluster of Mps3-GFP foci were transiently seen at 3 h with a frequency of 18.7± 2.3 %. Then, this clustering of Mps3 resolved during further incubation. A number of foci/patches containing Mps3 on NE were increased to late prophase I. In late prophase I such as 5-6 h, most of NE was covered with Mps3 molecules. When cells exit the prophase I, Mps3 signals on NE largely disappeared leaving two foci on duplicated SPBs at meiosis I division. The *rad61* mutant showed altered kinetics of Mps3 dynamics. Particularly, the cells increased Mps3-clustering to 56.0 ± 2.0 % at 4 h and resolved this clustering at late times (Fig. 8B). On the other hand, as shown previously (Conrad et al., 2007), the *rec8* mutant accumulated Mps3 clustering without any resolution. These show that Rad61 positively controls Mps3 dynamics, thus telomere clusting during meiosis.

Figure 8

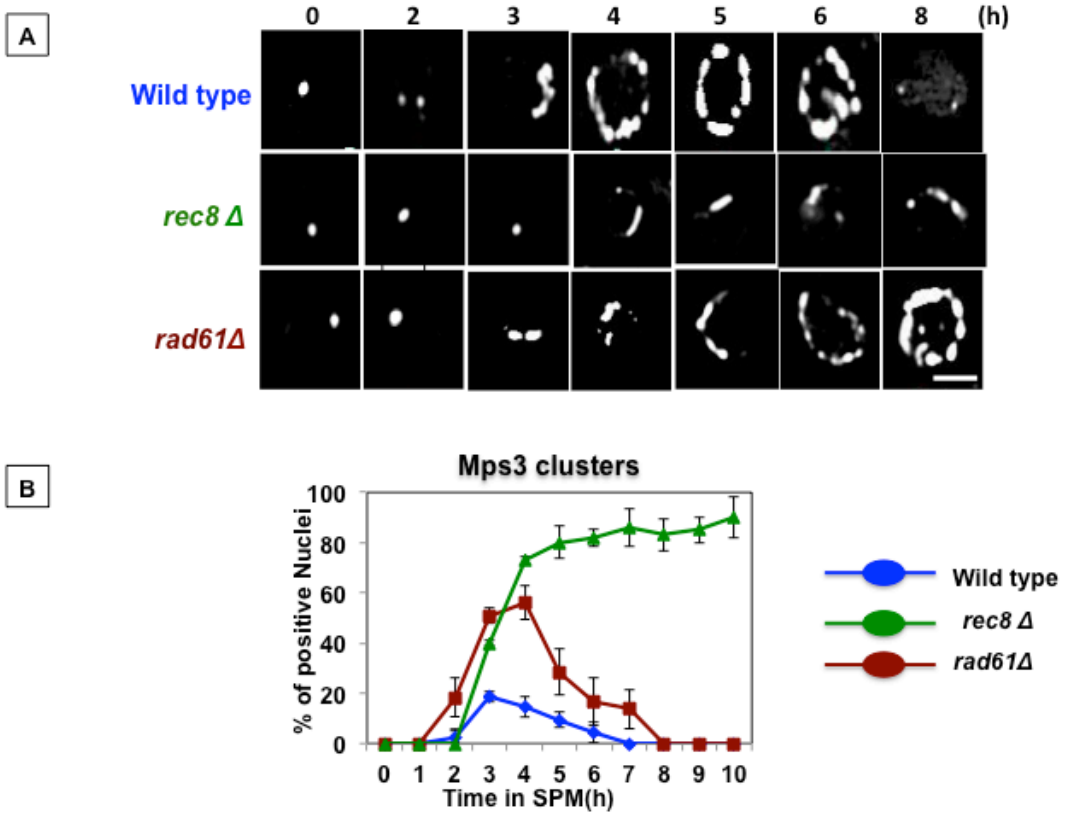


Figure 8. Rad61 is necessary for proper Mps3 localization during meiosis

(A) Mps3-GFP dynamics was monitored in a meiotic cell. A representative image of Mps3-GFP (white) at each time of three strains Wild type, *rad61* and *rec8* were shown. The bar indicates 2μm.

(B) Percent of cells with a cluster of Mps3 foci/patches were measured and plotted over time in meiosis. Plotted values are the mean values with S.D from three independent time courses. Wild type (KYS97/98) blue; *rad61* (KYS42/43), magenta; *rec8* (KYS271/272), green. The *rec8* mutant shows persistent clustering of Mps3.

3-1-9 Normal binding of Rec8 on to the chromosomes in the absence of Rad61

I next analyzed whether Rad61 inactivation adversely impacts cohesin enrichment to specific loci of Cohesin-Associated Regions (CAR). I performed chromatin immunoprecipitation (ChIP) of Rec8 in meiotic samples of wild type and *rad61* cells. First I checked the Rec8 association with chromatin at 5 different loci comprising several *CAR* sites along chromosome arms and pericentromeric regions of chromosome III and IV. Initially I analyzed the chromosome III arm CARs. The results show that all three CAR (*CARC1*, *CARC2* and *CARC3*) regions show no significant defense in the *rad61* mutant compared to those in wild type (Fig. 9). And also out of two pericentromeric regions of chromosome III and IV, the binding to *CEN III and CENIV* shows no significant difference between *rad61* mutant and wild type. The ChIP analysis of both chromosome arm and pericentromeric loci reveal that the levels of chromatin bound cohesin of rad61 mutant that are more or less similar to wild type.

Figure 9

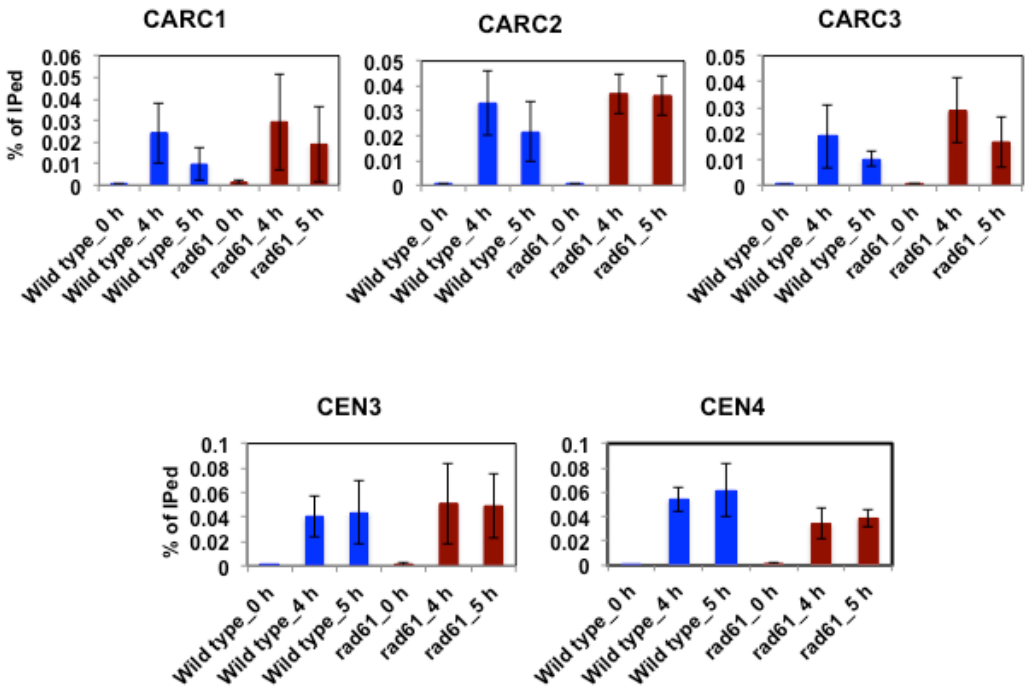


Figure 9: Differential binding of Rec8 on to the chromosomes in the absence of Rad61

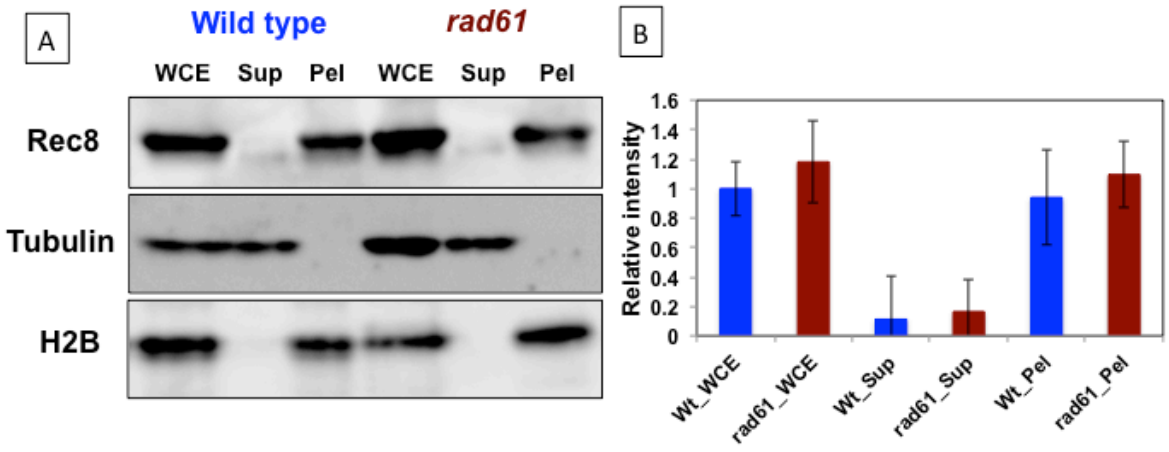
ChIP analysis for Rec8 enrichment along chromosome III arm *CARC1, CARC2, CARC3* and pericentromeric regions of *CEN3* and *CEN4*. The ChIP was carried out as described in M&M. The averages of two independent experiments were obtained in wild type (KYS97/98) and *rad61* (KYS42/43) mutant cells.

3-1-10 Loading of Rec8 onto chromosomes does not change in absence of Rad61

In budding yeast meiosis the absence of *PDS5* does not show significant difference of Rec8 localization compared to wild type (Zhang et al., 2005; Jin et al., 2009). Here I also asked whether Rec8 localization depends on another cohesin regulator Rad61 during meiosis. First I checked an amount of Rec8 protein in a fraction of chromatin pellets (Fig. 10A). Quantification of Rec8 showed that the amount of Rec8 was indistinguishable between the wild type and the *rad61* mutant cells (Fig. 10B).

Further I quantified fluorescence intensity on line profiles using maximum projections from unprocessed raw images of Rec8 as well as Zip1 lines on a pachytene chromosome. Reference lines were drawn so that they intersected with single chromosomes and intensity per pixel was quantified (Fig. 10 C). This allowed comparison of the signal intensity per pixel on SCs. An average peak intensity of Rec8 in the *rad61* mutant is 1.58 times brighter than in the wild-type cells. Zip1 shows narrower signal distribution than Rec8 in the both strains. The *rad61* cells showed 1.20 times brighter Zip1 peak than the wild-type cells. Given that the Rec8 signal intensity was compensated by the compaction ratio 1.56 in Fig. 6C, the ratio should be 1.01 (1.58/1.56), indicating a normal amount of Rec8 in the absence of Rad61. Indeed, when I measured the total intensities of Rec8 or Zip1 signal per a single nucleus, average intensities of Rec8 and Zip1 were not different between the wild type and *rad611* cells (Fig. 10 D). These experiments strongly support the notion that the *rad61* mutation does not affect the Rec8 amounts on meiotic chromosomes.

Figure 10



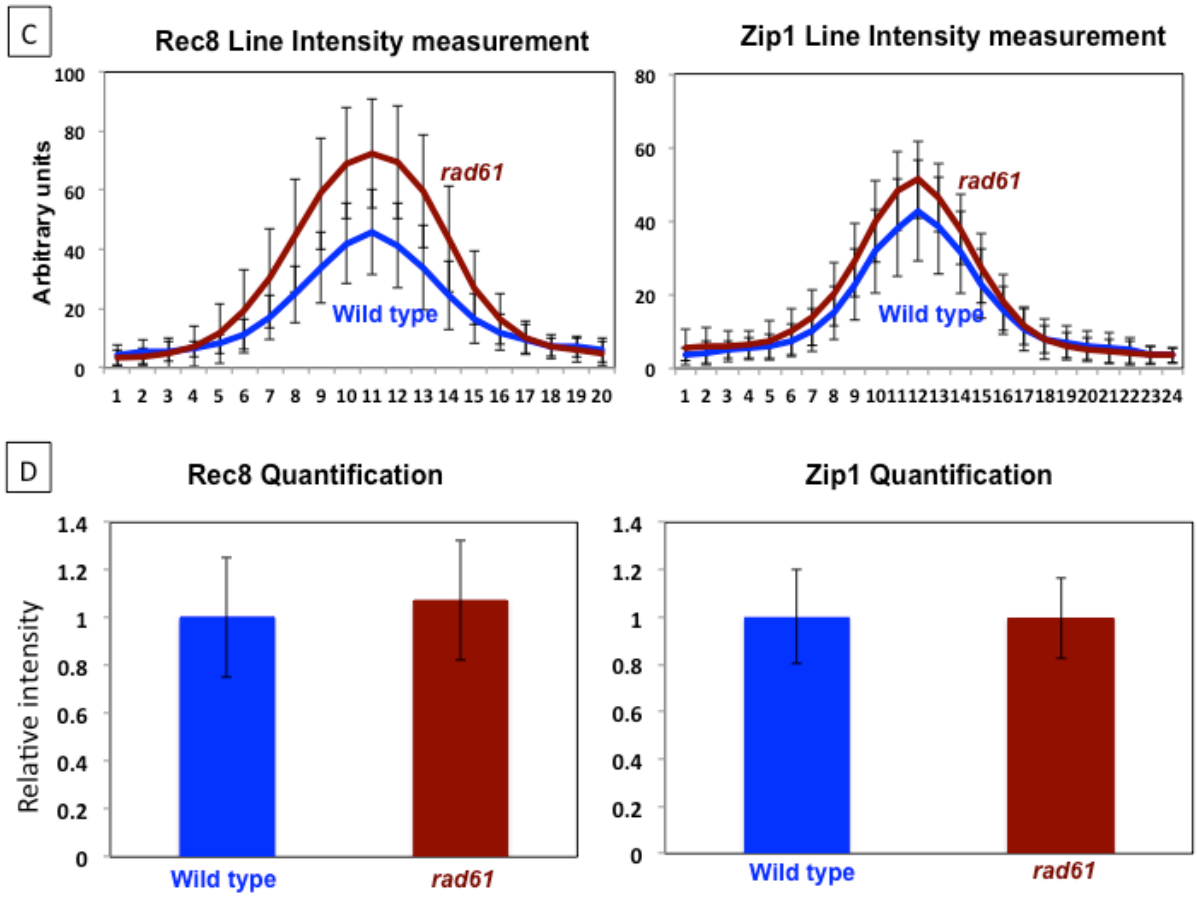


Figure 10: Amounts of Rec8 on to the chromosomes does not change in absence of Rad61

(A) Chromatin fractionation was carried out for Rec8, histone H2B and tubulin Whole cell extract (WCE) fractions of wild type and *rad61* cells at 4 h, cytoplasm, and chromatin were analyzed for the presence of Rec8 (top), tubulin (middle) and histone H2B (bottom).

(B) Relative intensities of Rec8 signals were calculated and shown as a graph. A mean and S.D. from three independent analyses is shown.

(C) Line intensity analysis of Rec8 and Zip1 staining. Intensity of signal across lines on SCs was quantified as described in Materials and Methods. At each point, more than 30 lines on pachytene chromosome spreads were randomly chosen and single intensity for Rec8 (top) and Zip1 (bottom) was measured. An average intensity is shown at each relative point of the line. The wild type (blue circles; MSY832/833) and *rad61* mutant (red circles; KSY63/64).

(D) Total intensity of Rec8 (left) and Zip1 (right) signals per one nucleus (spreads) was measured in two strains. Means and S.D.s are shown. The wild type (blue bars; n=24) and *rad61/wpl1* mutant (red bars; n=29) cell.

Chapter-4

4.1 Discussion

In addition to its SCC, cohesin complex plays multiple roles during meiosis. Rad61 (Wapl), non-essential subunit of the cohesin, is known to be negative regulator of the complex (Kueng et al., 2006; Rowland et al., 2009). In this study, I showed Rad61/Wpl1 controls chromosome compaction as well as meiotic chromosome dynamics, and in particular the events occurring at zygotene/pachytene transition.

In the *rad61* mutant, the length of SC is shorter than that in the wild type. This seems to be caused by the axes shortening. This was supported by the observation of uniform lines of Rec8 staining in the mutant compared to discontinuous staining in the wild type. Indeed, the measurement of distance between two chromosomal loci confirmed the compaction of the chromosome axis in the mutant. This suggests the role of Red61, and therefore a role of Rec8 and the cohesin complex, in the morphogenesis of chromosome axes. The shortening of axes might be accompanied with the formation of long loops. Alternatively, the shortening by much tighter compaction might occur without altering loop sizes. The number of chromosome-associated Rec8 proteins does not differ between wild type and *rad61/wpl1* cells. This is consistent with the latter possibility. It is likely that Rad61/Wpl1 is able to regulate three-dimensional architecture of chromosome axes containing the cohesin, without affecting its loading. However, the measurements of the axis-associated chromatin loop size are still needed.

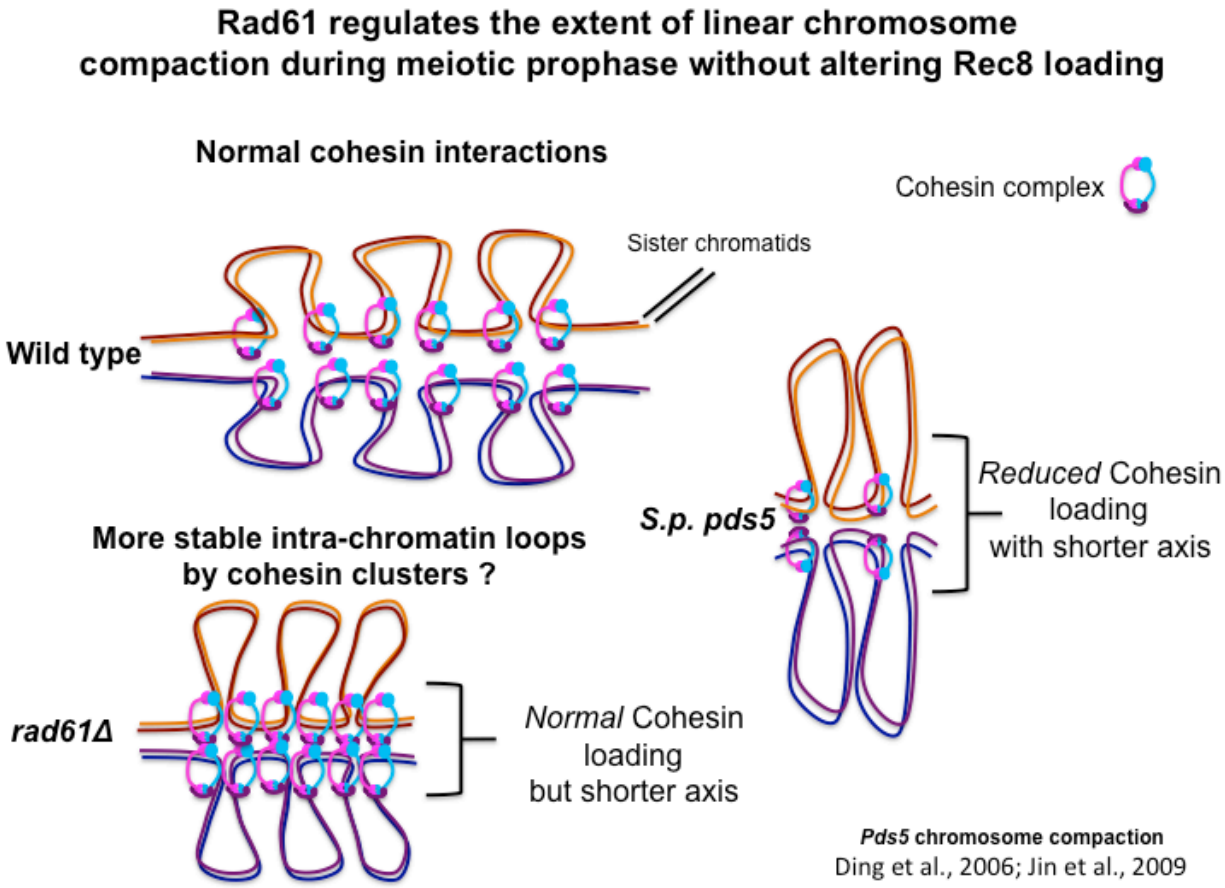
Axis shortening of meiotic chromosomes without the reduction of Rec8 binding is also reported for the budding yeast *pds5* depletion mutant (Jin et al., 2009), suggesting that Rad61/Wpl1 controls meiotic axis compaction with Pds5. However, the *rad61/wpl1* mutant cells form SC between homologous chromosomes, while the *pds5* depletion mutant cells induce SC formation between sister chromatids (Jin et al., 2009), indicating different roles of Rad61/Wpl1 and Pds5 proteins in the cohesin-mediated chromosome synapsis in meiosis. SC shortening, and consequently axis compaction, was also observed in mice lacking Smc1β, a meiosis-specific Smc1 isoform (Revenkova et al., 2004). Additionally, the *pds5* deletion mutant in the fission yeast showed more compact Rec8 staining of meiotic chromosomes, with decreased Rec8-binding to chromosomes (Ding et al., 2006). Based on these studies, the change in cohesin architecture along chromatin might regulate the extension and/or compaction of chromosome axes. This supports the idea that the cohesin complex is a critical determinant of the meiotic chromosomal axis compaction.

As described previously, meiotic chromosomes in the wild type cells contain Rec8-rich and Rec8-poor regions (Ito et al., 2014; Sun et al., 2015; Kugou et al., 2009). Rad61/Wpl1 may promote the formation of Rec8-poor regions on the chromosomes through the negative regulation of the local dynamics of Rec8. This is supported by a Rad61/Wpl1 role in the promotion of the cohesin molecule dissociation (Kugou et al., 2006; Sutani et al., 2009), i.e. Rad61/Wpl1 might facilitate the context-dependent dissociation of Rec8 from cohesin. Furthermore, some chromosomal regions may have a greater ability to suppress Rad61/Wpl1 activity than other regions. It is shown that the acetylation of Smc3 by Eco1 promotes the stable association of cohesin to chromosomes, by counteracting the activities of Rad61/Wpl1 and Pds5 (Unal et al., 2008; Rowland et al., 2009; Sutani et al., 2009; Heidinger et al., 2009). It was shown previously that Eco1 is recruited to DSBs in order to facilitate a stable association of the cohesin with DSB sites during mitosis (Heidinger et al., 2009; Unal et al., 2008). Meiotic DSBs may also trigger the acetylation of Smc3, which may contribute to different stability of the complex in the distinct chromosomal regions. Without Rad61/Wpl1, these region-specific differences might be inhibited, resulting in the uniform loading of Rec8 cohesin to chromosomes and axis shortening.

I found the *rad61* mutant shows several defects in meiotic recombination. First, Rad61 promotes timely formation of meiotic DSBs. This is supported by delayed loading of Rad51 on meiotic chromosomes as well as delayed formation of the DSBs at *HIS4-LEU2* recombination hotspot. Given that Rec8 is necessary for efficient DSB formation (Klein et al., 1999), this suggests that Rad61 is also a positive regulator for DSB formation. Second, Rad61 is needed for efficient repair of DSBs. The cohesin is known to promote repair of DSBs between sister chromatids in mitotic cells (Sjogren et al., 2001). Thus, again, Rad61 seems to stimulate the activity of the complex to repair DSBs. These support a positive role of Rad61 in meiotic recombination as a component of the cohesin complex.

Previous studies showed that Rec8 regulates chromosome dynamics during meiotic prophase I (Trelles-Sticken et al., 2005). However, it is not clear whether Rec8 is involved in this role through the cohesin complex or through a telomere complex with Rec8. The results described in this study show that not only Rec8, but also its regulator Rad61/Wpl1, are necessary for the efficient telomere dynamics during meiosis, supporting the conclusion that Rec8 as a part of the cohesin complex controls the dynamics of meiotic telomeres. The movements of telomeres in meiotic cells are mediated by a telomere/NE ensemble, which includes a SUN protein Mps3 (Hiraoka et al., 2009). This suggests that Rec8-cohesin

promotes the resolution of telomeres by affecting the function of Mps3 on NE ensembles through the change of either global chromosome structure or telomeric chromatin structure.



Model I:

In the wild type generally cohesin forms cohesin-cohesin interaction by holding sister chromatid together but in the cohesin regulator, rad61 deletion may form stable intra chromatin loops by cohesin clusters leads to chromosome condensation. It has been previously reported that pds5 mutant showed axis shortening and it's assumed as formation of longer loops in fission yeast mitosis and meiosis.

Table 1. Strain list

Strain Name	Genotype
MSY832	*MAT α, ho::LYS2, ura3, leu2::hisG, trp1::hisG, lys2*
MSY833	*MAT a, ho::LYS2, ura3, leu2::hisG, trp1::hisG, lys2*
NKY1303	*MAT a, ho::LYS2, ura3, leu2::hisG, lys2, his4B-LEU2, arg4-Bgl*
NKY1543	*MAT α, ho::LYS2, ura3, leu2::hisG, lys2, his4X-LEU2::BamHI-URA3, arg4-NSP*
KYS63	MSY832 with *rad61:: KanMX6*
KYS64	MSY833 with *rad61:: KanMX6*
KYS97	MSY832 with *MPS3-GFP::KanMX6*
KYS98	MSY833 with *MPS3-GFP::KanMX6*
KYS42	MSY832 with *MPS3-GFP::KanMX6,rad61::KanMX6*
KYS43	MSY833 with *MPS3-GFP::KanMX6,rad61::KanMX6*
KYS61	MSY832 with *RAP1-GFP::LEU2*
KYS62	MSY833 with *RAP1-GFP::LEU2*
KYS123	MSY832 with *RAP1-GFP::LUE2,rad61:: KanMX6*
KYS124	MSY833 with *RAP1-GFP::LUE2,rad61:: KanMX6*
KYS404	MSY832 with *Cen V-GFP::Clonat,ndt80::LEU2*
KYS405	MSY833 with *Cen V-GFP::Clonat,ndt80::LEU2*
KYS448	MSY832 with *Cen V-GFP::Clonat,rad61::KanMX6,ndt80::LEU2*
KYS449	MSY833 with *Cen V-GFP::Clonat,rad61::KanMX6,ndt80::LEU2*
KYS315	MSY832 with *CenIV TelIV-GFP::Clonat*
KYS316	MSY833 with *CenIV TelIV-GFP::Clonat*
KYS343	MSY832 with *CenIV TelIV-GFP::Clonat,rad61::KanMX6*
KYS344	MSY833 with *CenIV TelIV-GFP::Clonat,rad61::KabMX6*
KYS361	NKY1543 *with rad61::KanMX6*
KYS365	NKY1303 *with rad61::KanMX6*
KYS271	MSY832 with *MPS3-GFP::KanMX6,rec8::KanMX6*
KYS272	MSY833 with *MPS3-GFP::KanMX6,rec8::KanMX6*

Table 2. Primer list

Deletion for *rad61*

Forward Primer

AAAACTGCGCAGAGAAACTATCGCAAAACGAAACCATCTTCTTACCCTAAAGC
ATCCTGTTTCTGAAAAAcgtacgctgcaggtcgac

Reverse Primer

TCTGGACAATTTTTCAATAGTTGCCAGCAGGGTGAAGATGAAGCCAGGCTATGT
TCAATGTATGCTTTCTatcgatgaattcgagctcg

ChIP primers

CARC1	Chr3_100966F	TAAAGCATTGACGCCAGAGC
	Chr3_101366R	CGATGGAACCCCTATTGATC
CARC2	Chr3_92386F	TGTCGCCGAAGAAGTTAAGA
	Chr3_92792R	ATGGTCAGGTCATTGAGTGT
CARC3	Chr3_291494F	TGCAAGGATTGGTGATGAGA
	Chr3_291885R	TAAATGGATTGGATGTCGCG
CEN3	Chr3_114273F	TCCGCTTATAGTACAGTACC
	Chr3_114571R	ATGAGCAAAACTTCCACCAG
CEN4	Cen4_449635F	ACATATATTACACGAGCCAG
	Cen4_449943R	CTCGAACTGATCTATAATGC

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