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**High resolution mapping of protein-chromatin interactions during
meiotic recombination in *S. cerevisiae***

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Abstract

The term meiosis refers to a particular cell division involved in germ cells formation. During meiosis, the parental diploid cell generates haploid progeny, by performing one round of DNA replication followed by two cell divisions. In contrast to mitosis, the first round of meiotic cell division is characterized by the segregation of homolog chromosomes instead of sister chromatids. This is performed by formation of a physical connection between the homologous chromosomes, known as chiasma, which counteracts the forces generated by the microtubules to align the homologous chromosomes in the metaphase plate.

Chiasma formation depends on two major events characteristic of meiosis: (1) DNA cleavage in the context of chromatin and its repair via the homologous chromosome and (2) the pairing and synapsis of homologous chromosomes prior to chiasma formation. Previous observations have suggested a direct connection between chromatin condensation and induction of Double-strand break (DSB) formation and repair during the meiotic recombination program; nevertheless the mechanism defining this particular interrelationship has not been elucidated so far.

By performing a genome-wide approach based on Chromatin immunoprecipitation followed by hybridization to high resolution DNA microarray chips (ChIP-chip), we mapped several proteins required for meiotic recombination and synapsis of *S. cerevisiae* to their chromosomal positions. Contrary to expectations, components like Spo11p or Mre11p, required for DSB formation, do not exclusively localize to the chromatin cleavage sites, but in addition colocalize with components involved in chromatin organization, such as cohesins and axial element proteins. Interestingly, Spo11p localization at both DSB and cohesin sites was significantly enhanced by its *Y135F* catalytically dead mutation, suggesting that Spo11p localization at cohesin sites is important for forming DSBs. Furthermore, while Mre11p localization to the meiotic DSB sites requires Spo11p or Rec114p, but not DSB formation itself, its localization to cohesin sites is not impaired under these conditions.

Meiotic DSB repair components, like the MRX complex and Com1, also do not localize exclusively to the site of DSB cleavage, but additionally interact with cohesin sites. Recruitment of Com1 to the DSB sites depends on Spo11p but not in the DSB formation itself. Furthermore, it is also abolished in the *rad50S* mutant background, but not in the *mre11S* or *mre11-H125N* mutants, distinguishing between a defective recruitment function of *rad50S* for Com1 and an enzymatic defect responsible for the accumulation of unrepaired meiotic DSBs in *mre11S*. Otherwise the phenotypes of the two different "S" mutants are indistinguishable.

These findings suggest a mechanism in which the chromatin folding *in vivo* participates in the induction and repair of DSBs during meiosis, where the chromosome core, defined by the cohesin sites plays an essential role in the recruitment of the components involved in these processes and where the loop region prone to cleavage is interacting with components attached to the core in order to allow DSB formation.

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1 Introduction

Among all living beings, a very large part is issue of a sexual reproduction characterized by the fusion of two germ cells or gametes. This event, also known as fertilization, gives rise to a single cell called a zygote, which in contrast to an asexual progeny, presents genetic characteristics from both parental donor cells.

Because gametes fusion is the key event characterizing sexual reproduction, an imminent theoretical consequence of this process is the doubling of the zygotic chromosomes number from generation to generation. The inconsistency between the event of fertilization and the maintenance of the constant number of chromosomes between successive generations was first addressed in the late 19th century. Indeed, pioneering studies in the process of fertilization performed on sea urchin eggs by Oscar Hertwing (1875) established the important role of the cell nuclei in the emergence of a new organism. Around the same time, studies of somatic plant cell division performed by Edouard Strasburger revealed the existence of easily stainable particles in the nucleus. In the following years, other scientists, like Walther Flemming (1879-1882) who extended this observation to the animal kingdom, confirmed the existence of such "stainable threads" (denoted as *chromosomes*) which are duplicated during somatic cell division and then equally segregated into both daughter cells. Finally, in 1883 Edouard van Beneden reported that the germ cell contained only half of the number of chromosomes found in somatic cells [1].

Based on these previous observations, the Zoologist August Weismann proposed in 1887 the existence of two kinds of cells: somatic cells, which were the general cells of the bodies, and germ cells (or germ-plasm), which were the cells involved in reproduction. The concept of a "reductive cell division" was then proposed by Weismann in order to explain the observations of Van Beneden demonstrating the halving of the number of chromosomes in germ cells. This concept fit nicely with the reconstitution of the number of chromosomes during fertilization, and more over with the concept of chromosomes as vehicles of heredity (described in "The Germ-plasm, A Theory of Heredity" by Weismann in 1892) [2].

In the same year, Theodor Boveri proposed a model where the homologous chromosomes pair during the "reductive cell division", and this was confirmed in the following years by studies performed in grasshopper cells by Walter Sutton (1900). In 1905, Farmer and Moore gave the name of *meiosis* to this particular type of cell division, and finally, when the process of chromosome pairing, together with the reduction of the total number of chromosomes in germ cell formation, has been correlated with the Mendelian theory of characters segregation, it gave rise to the Chromosome Theory of the inheritance[3].

An important observation with respect to chromosome pairing in meiosis was the fact that chromosomes seemed to stick together at particular points during chromosome separation. This fact brought Frans Janssens (1909) to suggest that the “cross-like” figures he observed in meiosis could correspond to sites where some kind of exchange between paired chromosomes took place, and he called these structures *chiasma*. This new concept suggested a direct connection between the cytological observations and Mendelian genetics. Indeed, the chromosome theory of the inheritance, which appeared with the parallelism between chromosome behaviour and Mendelism described by Sutton* and Boveri at the beginning of the 20th century, was confirmed by Thomas Morgan, who proved that genes are carried on chromosomes and demonstrated that occasionally, they are exchanged between homologous chromosomes during meiosis. He called this process “*crossing over*”, which was then used for mapping genetic factors on chromosomes of the fruit fly. With time, the correlation between the occurrence of genetic crossing over and the frequency of chiasmata became strong, and finally in 1931, H. Creighton and B. McClintock demonstrated that crossing over and chiasma indeed corresponded to the same meiotic event [4].

With the discovery of DNA as the material of inheritance, the elucidation of the molecular processes involved in chiasma formation and, by consequence, in meiotic recombination became the goal of a new generation of molecular biologists in the field. In the following paragraphs I would like to present an overview of the molecular events involved in meiotic chromatin condensation as well as meiotic recombination, mainly from the point of view of the experiments performed in *Saccharomyces cerevisiae*. Later on, I will present our main findings in the last four years since I joined the laboratory of Professor Franz Klein at the University of Vienna, and finally discuss their importance for the understanding of the molecular processes governing this particular kind of cell division.

* Sutton gives the name of “*genes*” to the pair of Mendelian factors that are separated from generation to generation; in a similar manner as chromosomes do in the germ cells formation process.

1.1 An Overview of Meiosis

The term “meiosis” refers to the specialized cell division required for the formation of germ cells in sexually reproductive organisms. Like mitosis, meiosis begins with the replication of the genetic information, but in order to generate haploid progeny cells from diploid parental cells, two subsequent cell divisions are required: meiosis I where the homologous chromosomes are segregated, and meiosis II where sister chromatids are pulled apart as in the case of a mitotic cell division.

Meiosis I is characterized by a long prophase (called prophase I) which can be subdivided, based on cytological analysis, into *leptotene* (chromosome condensation), *zygotene* (paring of homolog chromosomes), *pachytene* (formation of a proteinaceous scaffold-like structure between the homologous chromosomes called the synaptonemal complex or SC), *diplotene* (the synaptonemal complex is no longer visible, instead chromosomes are connected by chiasmata) and *diakinesis* (spindle microtubules are formed and attach to monopolar kinetochores). In metaphase I the homologous chromosomes are aligned to then be segregated in anaphase I (reviewed in [5]).

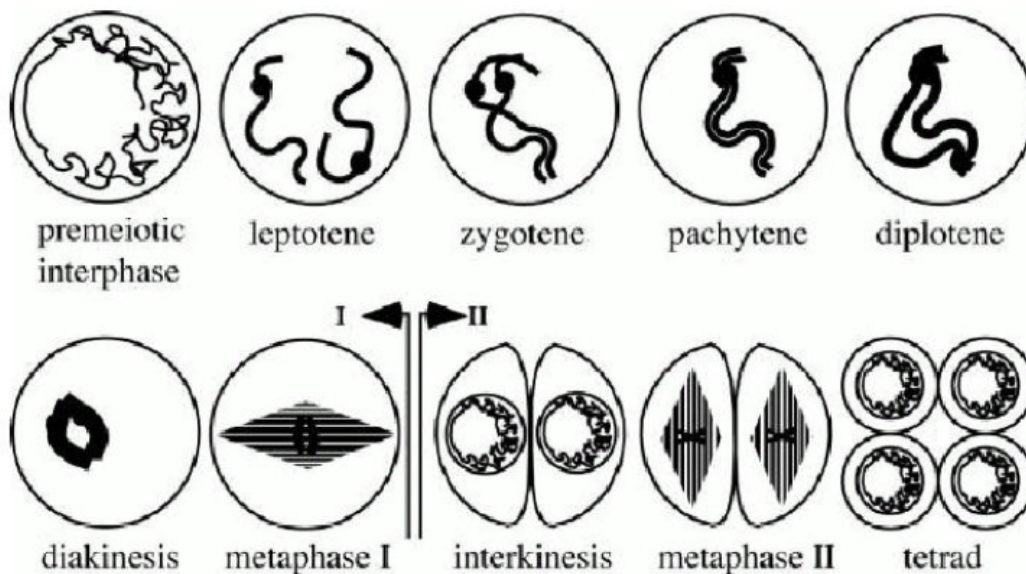


Figure 1: Schematic representation of the different meiotic stages from a cytological point of view (adapted from [6]).

Considering that the main goal of meiosis I is to segregate homologous chromosomes, and not sister chromatids as in a regular mitosis, the key event of this stage is the formation of a physical connection between homologous chromosomes. Indeed, such connections, known as *chiasmata*, together with sister chromatid cohesion, counteract the force generated by

microtubules and in this way generate tension required to align the homologous chromosomes at the metaphase plate before segregation [7]. *Chiasma* formation depends on two particular events taking place in prophase I:

1. Chromatin condensation
2. Induction of a specific meiotic recombination programme

Furthermore, we have to mention that in addition to chiasma formation, the attachment of microtubules to monopolar kinetochores, as well as the preservation of intersister-chromatid centromeric cohesion during meiosis I are essential events to ensure homologous chromosome segregation and to allow alignment and segregation of sister chromatids in Meiosis II respectively (a detailed review of these last events are presented in[8]).

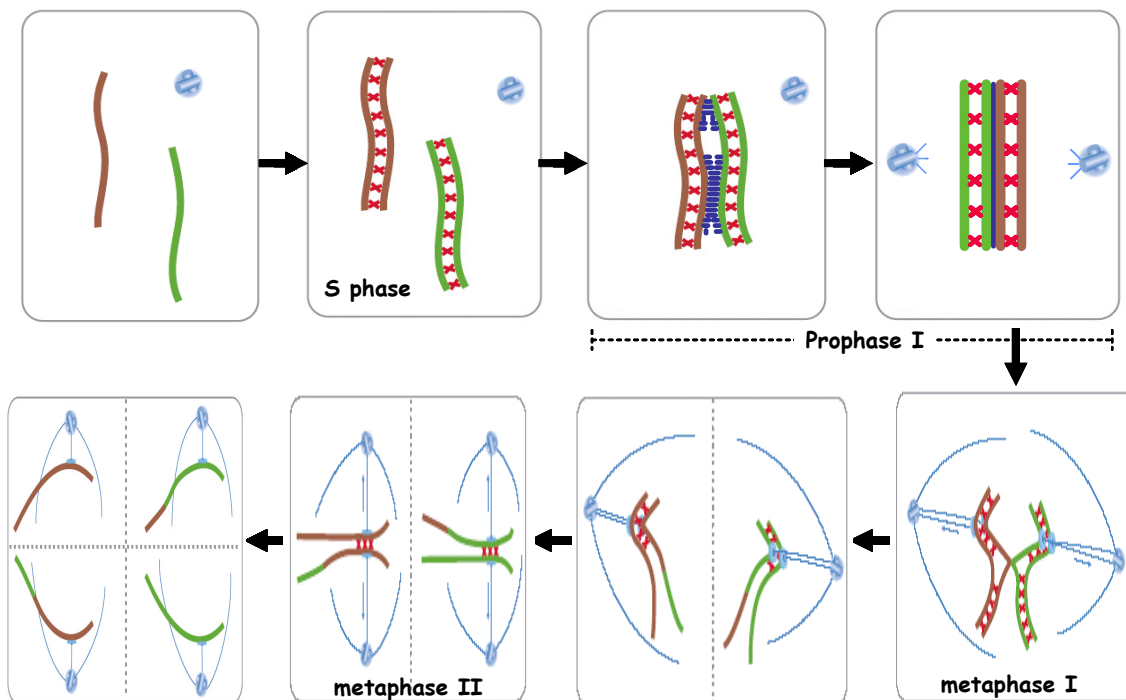


Figure 2: Schematic representation of Meiotic cell division progression. Cohesin components are represented by a red cross between sister chromatids (green and brown lines duplicated in S-phase). Homologous chromosome pairing and progression of synapsis is illustrated during prophase I. Formation of the proteinaceous scaffold-like structure called the SC is represented in dark blue (prophase I). In metaphase I, the role of chiasma and sister chromatid cohesion in counteractin microtubule forces are represented. For simplicity, the segregation of only a single pair of homologous chromosomes is illustrated.

1.2 Chromatin condensation in Meiosis

During S-phase, cohesin components are loaded in order to hold sister chromatids together. This process is common to both mitotic and meiotic cell division; nevertheless, meiosis utilizes a specific cohesin component, Rec8p, which replace the mitotic kleisin Scc1p [9]. At the beginning of prophase I, newly replicated homologous chromosomes are aligned in a proteinaceous filament-like structure, known as axial elements in *Saccharomyces cerevisiae*, which is composed of the proteins Hop1p and Red1p. From cytological observation it is believed that during this process the cohesin components are aligned over this structure, giving rise to the particular chromatin organization where uncondensed loop regions are observed emanating out from the condensed axial element [10]. Indeed, recent cytological analyses performed in mice have shown a strict correlation between chromosome axis extension and a general shortening of chromatin loop size [11]. Finally, homologous chromosomes pair at defined regions to then interconnect the axial elements of both homologues by a transversal filament constituted of the protein Zip1p in budding yeast. This final structure is known as the synaptonemal complex and is present in several sexually reproductive organisms, but not in all. For instance, fission yeast have intrachromosomal linear organization structures, but do not have an interchromosomal protein connection like in budding yeast [12].

Homolog chromosome Synapsis

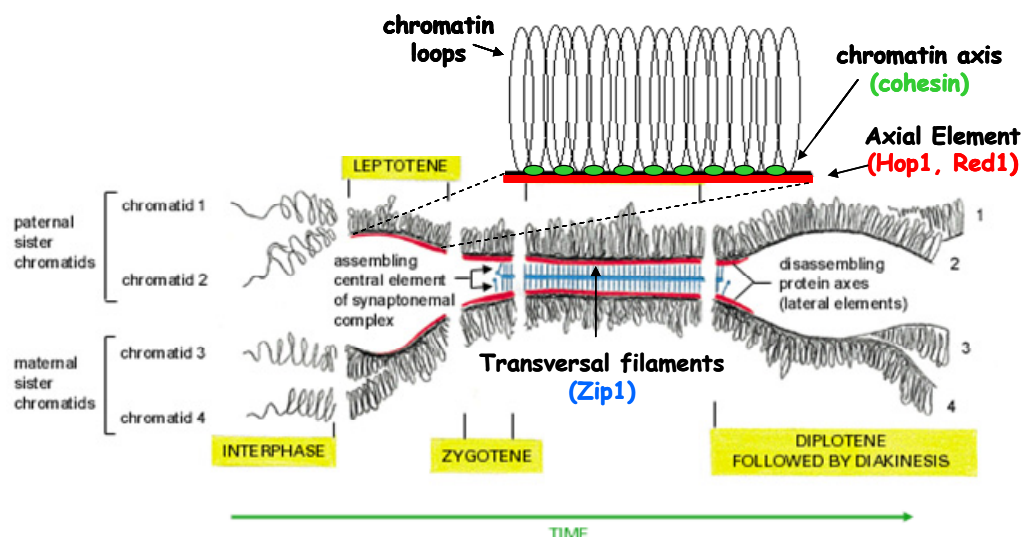


Figure 3: Meiotic chromatin organization in *Saccharomyces cerevisiae*. Cohesin components loaded in S-phase are represented in green. The axial element (Hop1p and Red1p) and the transversal filament (Zip1p) are represented in red and blue respectively. The chromatin organization in uncondensed loops and a chromosome core defined by the synaptonemal complex is highlighted in this cartoon.

The previously described chromatin organization has been visualized by different approaches including Electron microscopy (EM) [9] as well as immuno-staining of chromatin spreads in budding yeast[13]. From this analysis the distance between the Axial elements in *S. cerevisiae* has been estimated to be 21-35 μ m, which is considered the shortest SC measured to date, in contrast to distances of 150-180 μ m in rodents, and 3mm for the SC of the sea-lily, a distant relative of the starfish (for an extended review of the aspects of meiotic chromosome synapsis see [14]).

Immunostaining analysis allowed the visualization of Rec8 foci overlapping with Zip1p stretches observed in pachytene stage, strongly suggesting the structural role of the cohesin component in organizing the chromatin together with the SC (figure 4A originally published in[13]). Furthermore, the immunostaining analysis of Spo11p, the meiotic specific nuclease required for meiotic recombination (see chapter 1.3.1.1), is shown in figure 4B and is compared to the long stretches formed by the SC component Zip1p (originally published in[15]). From this analysis it is possible to infer information related to the inter-relationship of processes such as chromatin condensation and meiotic recombination events. This issue is discussed in a later chapter of this study.

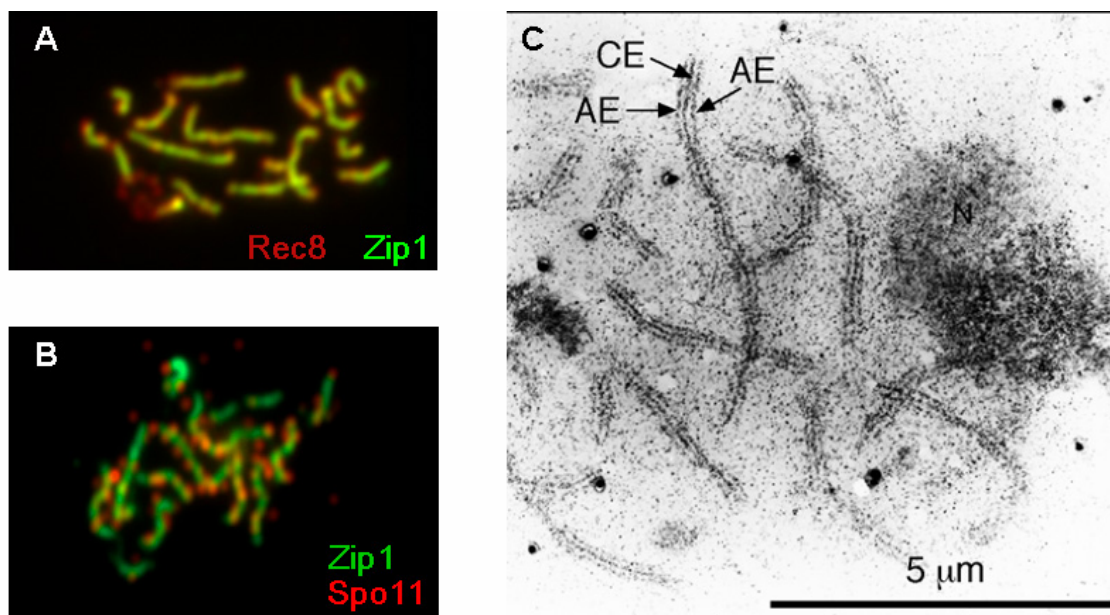


Figure 4: Visualization of Synaptonemal complex: Particular examples of current methodologies employed to visualize meiotic chromatin condensation: Immuno-staining (A and B), Electron microscopy analysis (C) of chromatin spreads. In A and B, the transversal filament Zip1 is immunostained in green and compared to foci formation of other components like Rec8, which is involved in sister chromatid cohesion (A) or Spo11, which is the nuclease specific involved in meiotic recombination initiation (B). In (C) the high resolution analysis let us differentiate between axial elements (AE) and the transversal or central element (CE).

1.2.1 Recombination nodules

Electron microscopic analyses performed in organisms having a relative long distance between axial elements (AE) reveals more structures than the axial elements and the central element constituting the SC scaffold. Indeed, densely staining nodular structures have been observed between the AE. These structures, which quite often correlate with AE association sites or chromosome pairing sites, have been shown to contain recombination proteins like Rad51p and Dmc1p as well as RPAp in early pachytene stages. Furthermore, larger and denser nodular structures are observed in late stages, where the SC is completely formed (Pachytene). These "late recombination nodules" have been associated with the sites where chiasmata will appear in the diplotene stage [16].

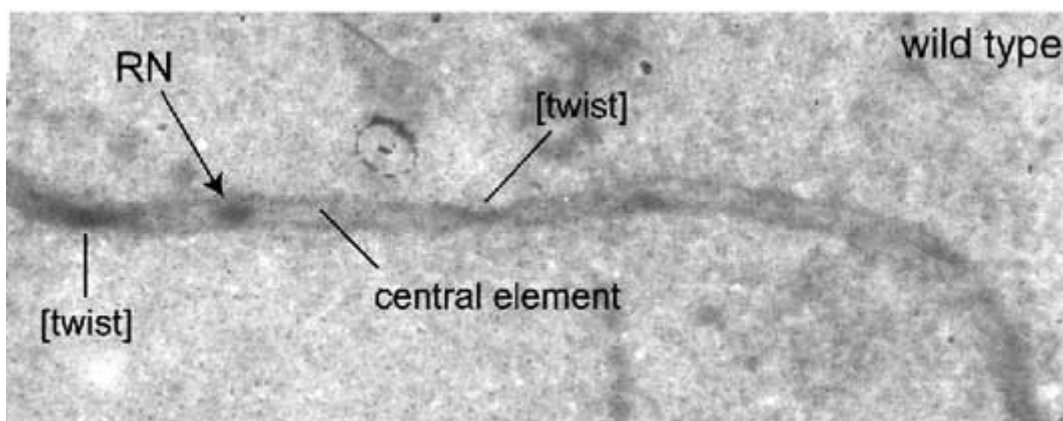


Figure 5: Electron Microscopy analysis of mice chromosome spreads. Pachytene stage chromosome spread showing fully synapsed bivalent chromosomes. The electron-dense recombination nodule (RN) is visible between the two axial elements of the synaptonemal complex. The chromosome regions labeled twist are artifacts produced during the spreading procedure. Adapted from [17].

Even though the role of these recombination nodules has not been completely elucidated, the evidence strongly suggests that the machinery involved in meiotic recombination works in direct association with the structural organization of the chromatin. Indeed, the number of recombination nodules at the different prophase stages correlates to the progression of recombination (see chapter 1.3 for a more detailed description of these events). Indeed, initiation of recombination introduces a larger number of double-strand breaks (DSBs) than the final cross-over products; in a similar way, early recombination nodules are more abundant than late nodular structures. Furthermore, the protein composition of such nodules correlates with the different recombination processes. From these correlations, early nodules are thought to be involved in homology search, in contrast to late nodules associated to recombination resolution required for chiasma formation[17].

1.3 Meiotic recombination in budding yeast

The term recombination refers to the exchange, or transfer, of fragments between DNA molecules and is observed in all organisms that have been studied to date. Recombination can be classified as homologous or nonhomologous recombination depending on the way the process takes place. Homologous recombination (HR) is observed between DNA sequences displaying a perfect or near perfect homology over several hundreds of base pairs. On the other hand, recombination between DNA sequences with little or no homology is known as nonhomologous recombination or end joining (NHEJ). In the particular case of meiosis, cross over formation is performed by the homologous recombination pathway, but in contrast to homologous recombination performed in mitosis, the initiation of recombination is performed by the introduction of genome wide double-strand Breaks (DSBs) in a controlled manner [18]. Indeed, a high level of recombination events are produced by induction of specific meiotic recombination components which together with the chromatin organization status will direct the process towards interhomolog rather than intrasister chromatid exchanges.

Meiotic recombination is initiated by the introduction of DSBs over the whole genome. This is performed by the meiotic specific nuclease Spo11p in *S. cerevisiae* [19], and there are conserved homologs in all other sexually reproductive organisms studied [20]. Once the DSB is formed, the 5'-DSB ends are resected in order to form a 3'-single strand DNA (ssDNA) tail of around 500 nucleotides in length[21]. The 3'-ssDNA-ends invade the intact homolog chromosome, and form a joint molecule termed the double Holliday junction (dHJ) [22]. Finally the dHJs are resolved to form cross over products which are indeed the physical connections between homologous chromosomes observed in cytological analysis and known as chiasma. In addition, we have to keep in mind that cross over products are not the only outcome of the meiotic recombination pathway. In some cases one of the 3'-ssDNA end invade the corresponding homolog region and after its elongation by DNA synthesis, it is displaced and anneals to complementary sequences that are part of the second DNA end, the final product of this recombination process is a non-crossover event. The molecular mechanism leading to non-crossover products is called SDSA for synthesis-dependent strand-annealing [23]. In the next paragraphs we would like to explore the molecular mechanisms involved in the initiation of meiotic recombination, as well as the processing of the DSBs, in order to understand the motivations of this study.

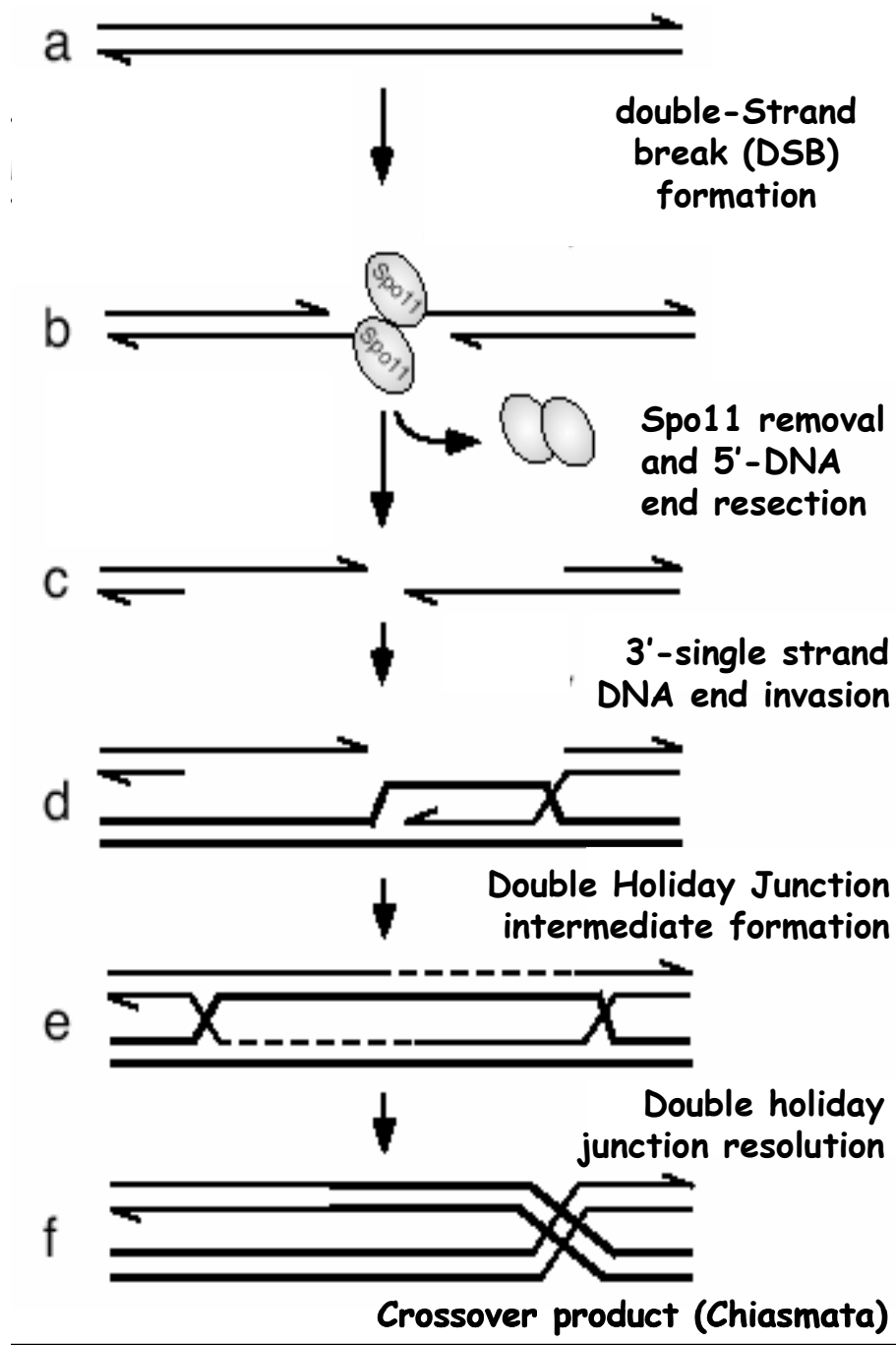


Figure 6: Meiotic Recombination pathway. The meiotic specific nuclease Spo11p introduces a DSB in one of the homologous chromosomes (a-b). Spo11p is removed and the 5'-DNA ends are resected (b-c). 3'-ssDNA ends invade the intact homologous chromosome (c-d). After a repair DNA synthesis step, the double Holliday junction intermediate is formed (d-e). Resolution of the double Holliday junction intermediate gives rise to the crossover product (e-f).

1.3.1 Initiation of meiotic recombination:

As briefly described in the preceding paragraphs, meiotic recombination is initiated by the induction of meiotic DSB formation. It is important to mention that this event is directly connected to premeiotic DNA replication. Impairment of DNA replication through mutation of components such as *cdc28*, *cdc21* or *pol1* or through the use of chemical inhibitors of DNA replication such as hydroxyurea, also inhibits the initiation of recombination. In a similar manner, inducing mutations in both the *clb5* and *clb6* cyclins, which activate the protein kinase Cdc28p for the G1-S transition, inhibits initiation of meiotic recombination. Nevertheless, none of these mutants influence the expression of meiotic essential genes like SPO11 (reviewed in [24]).

More surprisingly, it was shown that there is a strict temporal correlation between the timing of DNA replication and the incidence of DSB formation. Indeed, the delay of replication at defined chromosome regions by deleting replication origins gives rise to a delay of DSB formation in the same chromosome regions without affecting the behaviour of others [25], suggesting that the temporal interval between DNA replication and subsequent DSB formation is kept constant for each chromatin region.

The mysterious connection between DNA replication and initiation of recombination was finally resolved by the discovery of a direct regulation of DSB formation via phosphorylation of Mer2p, one of the nine components required for DSB formation apart of Spo11p. This phosphorylation is essential for DSB induction and is performed by the budding yeast cyclin-dependent kinase Cdc28 [26]. This report elucidated the mechanism involved in coupling DNA replication to DSB induction transition.

Finally, there are reports indicating that Spo11p is required not only for DSB formation but also for premeiotic DNA replication. Indeed, SPO11 deletion was shown to decrease the length of S-phase by 25%. Furthermore, mutation of its catalytic tyrosine residue (*spo11-Y135F*) does not affect DNA replication timing but does inhibit DSB formation [27]. These data suggest that recombination components, at least Spo11p and also Rad50p [28], are loaded (for an unknown reason) onto the chromatin structure during replication. Correlating these data with events such as the loading of cohesin during S-phase, as well as formation of axial elements in early prophase I, we can imagine that the chromatin organization also includes recombination components (already demonstrated in the case of recombination nodules), which links the enzymatic machinery with the protein-chromatin scaffold required for chiasma formation.

Even though Spo11 has been shown to be the enzymatic component required for DSB formation in meiosis, its regulation depends on the presence of other 9 components, namely

Mer2p, Mei4p, Rec114p, Rec102p, Rec104p, Ski8, Mre11, Rad50p, Xrs2p[29]. In the following sections, each of these components will be introduced in order to infer their role in DSB formation.

1.3.1.1 *Spo11, the meiotic specific nuclease*

Spo11p has been shown to be the enzymatic component of a large complex involved in meiotic DSB formation. This meiotic specific nuclease shares a structural relationship to the Top6A subunit of TopoVI, an archeal type-II topoisomerase[30]. Mutagenesis of the 135th residue Tyrosine to Phenylalanine, (*spo11-Y135F*) in *S. cerevisiae*, based on the mechanism of action of known type II topoisomerases, was shown to inhibit its catalytic activity. Furthermore, this enzyme was found to form covalently linked Spo11-DNA complexes *in vivo*, demonstrating its enzymatic role, which proceeds through a trans-esterification mechanism based on the formation of a covalent linkage between the catalytic tyrosine and the 5'-DNA end [31],[32].

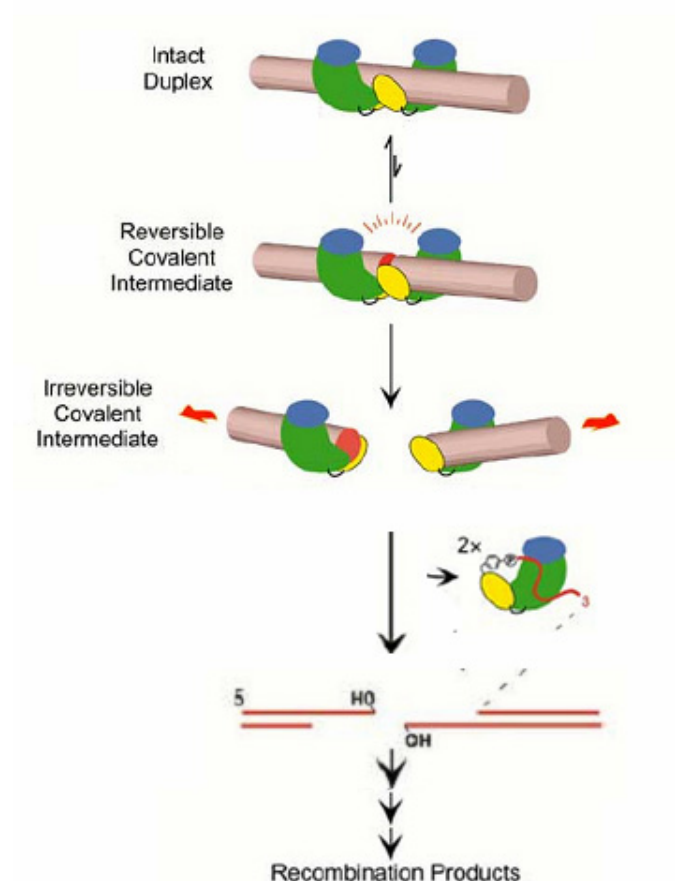


Figure 7: Model for Spo11 DNA nuclease activity. Spo11p binds to the DNA target region and then a covalent linkage intermediate is formed between the catalytic tyrosine and the 5'-DNA end. Finally Spo11p is removed through the activity of an unknown single-stranded endonuclease. Figure adapted from [20].

Immunostaining analysis of budding yeast chromatin spreads has shown that Spo11 binds to chromatin in early prophase I (leptotene) and persists unexpectedly until pachytene. The observed localization pattern persisted for the *spo11-Y135F* mutant, suggesting that its chromatin localization does not require the enzymatic activity. Furthermore, chromatin immunoprecipitation analyses (ChIP) have demonstrated its transient interaction with known DSB sites[15]. Interestingly, its chromatin localization depends on Rec102p, Rec104p as well as Rec114p, whereas Mer2p, Mei4p, Mre11p, Rad50p and Xrs2p did not appear to be essential components for Spo11-chromatin binding.

As indicated before, meiotic DSB formation depends not only on Spo11p enzymatic activity, but several other components has been shown to be genetically essential for this process[29]. Between them, *Ski8/Rec103* was at first identified as a component involved in cytoplasmic mRNA decay was later shown to be genetically essential for meiotic DSB formation in budding yeast [33]. Although homologues of Ski8p exist in most species, a role in meiotic recombination has only been demonstrated in three fungi (*S. cerevisiae*, *S. pombe* and *Sordaria*), suggesting that its role in meiosis is not conserved outside of fungi.

These intriguing aspects of Ski8 biology do not seem to be interconnected because in one hand, other cytoplasmic Ski8 partners are not required for meiotic recombination, and on the other hand, its nuclear importing takes place specifically during meiosis and depends on Spo11p[34]. Supporting this conclusion, immunostaining analysis has shown Ski8 localizing on chromatin in a similar manner to Spo11p.

The Ski8p crystal structure reveals the presence of a prominent site on the top surface of the beta propeller, which has been shown to be involved in mediating Spo11p-Ski8p interactions[35]. Furthermore, an extensive two-hybrid analysis has shown that Ski8p and Spo11p interact in a meiotic specific manner[34].

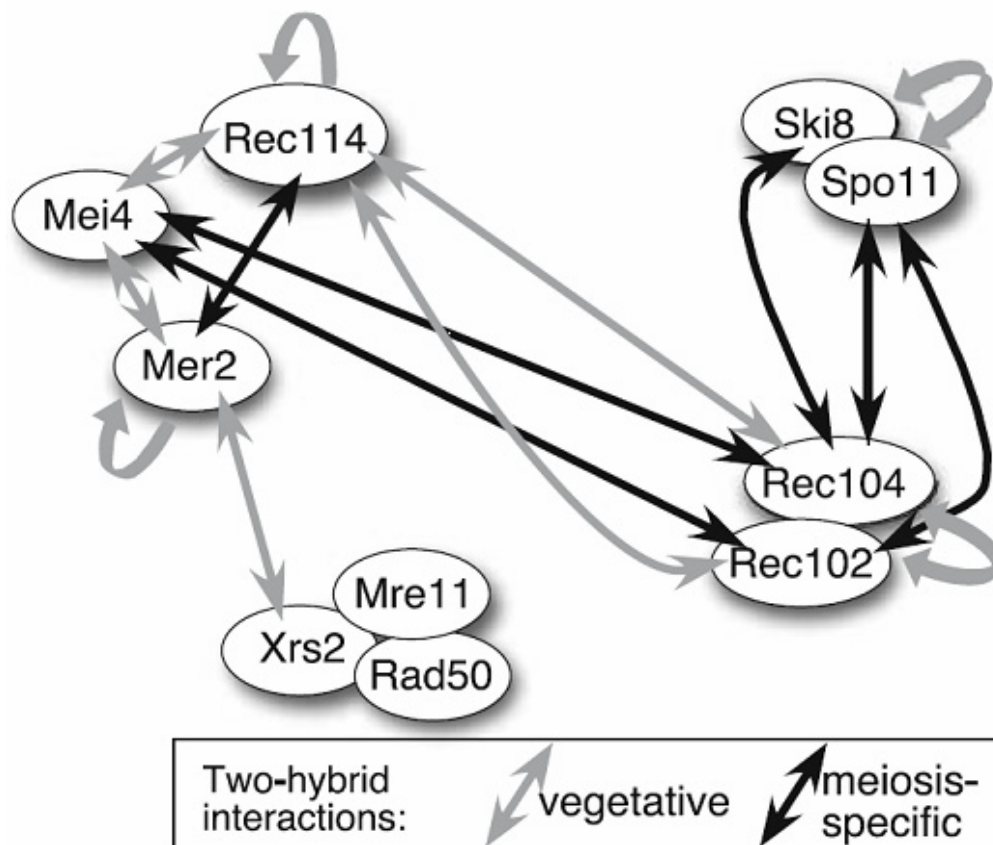


Figure 8: Two-hybrid interaction network of DSB formation components. Proteins involved in meiotic DSB formation are divided into four distinct subcomplexes: Ski8, Spo11; Rec104, Rec102; Mei4, Mer2, Rec114; and Mre11, Rad50, Xrs2. Two-hybrid interactions between subcomplexes are indicated by gray arrows (vegetative interactions) or black arrows (meiosis-specific interactions) [36].

1.3.1.2 The Mer2/Mei4/Rec114 subcomplex

Formation of the Mer2/Mei4/Rec114 subcomplex (referred to as MMR in this study) has been elucidated through different approaches. At first, immunostaining studies on chromatin spreads have shown partial colocalization between these components, however the absence of one of the three does not impair foci formation of the other two [37]. Furthermore, these three components have been co-immunoprecipitated, demonstrating a physical interaction. Finally, two-hybrid assays have shown vegetative interactions between Mer2/Mei4 and Mer2/Rec114 as well as a meiotic specific interaction between Mer2/Rec114 [36].

Even though these proteins do not have evident homologues in other organisms, this complex is thought to be the link between pre-meiotic DNA replication and the regulated entry into recombination. Mer2 is not a meiotic specific component; nevertheless, it was shown to increase in abundance and become specifically phosphorylated in early prophase I. This phosphorylation depends on the Cdc28-Clb5/6 kinase complex and results in phosphorylation of serine 30 and serine 271 residues, even though it was shown that Phospho-Ser30 is essential for DSB formation. Indeed, two-hybrid analysis has shown that Phospho-Ser30 is required for Mer2 dimerization, as well as for Mer2-Rec114 and Mer2-Xrs2 interactions, strongly suggesting that this event not only regulates intra-subcomplex interactions but also induces inter-subcomplexes interactions[26].

Mer2p was shown to localize on chromatin early in prophase I and its localization does not depend on any other component or on Cdc28 phosphorylation, indicating that its chromatin localization will give rise to a fully functional DSB formation complex only after Cdc28 phosphorylation. Furthermore, DSB formation inhibition, for instance by the absence of Spo11p, gives rise to a prolonged life time of Phospho-Mer2p on the chromatin.

In addition to its strong interaction with Mei4p and phosphor-Mer2p, Rec114p interacts with Rec102p and Rec104p. These interactions are not meiotic specific (based on two-hybrid assay)[36], but it is believed to interconnect the MMR subcomplex with the enzymatic Spo11/Ski8 subcomplex via Rec104/Rec102. According to this hypothesis, Spo11p forms foci on chromatin spreads from *rec114* mutants, even though it is not found at DSB sites by ChIP analysis[15], suggesting that the Spo11p subcomplex presents an innate Chromatin binding affinity, which does not correspond to DSB sites, reason why it requires MMR complex for proper chromatin localization.

Finally, Mei4p interacts with Rec114p and Mer2p in a non-meiotic specific manner, but it has in addition Rec104p/Rec102p meiotic specific interactions[36]. On the other hand, it was shown by ChIP assays that this protein is required for the timely removal of Spo11p[15].

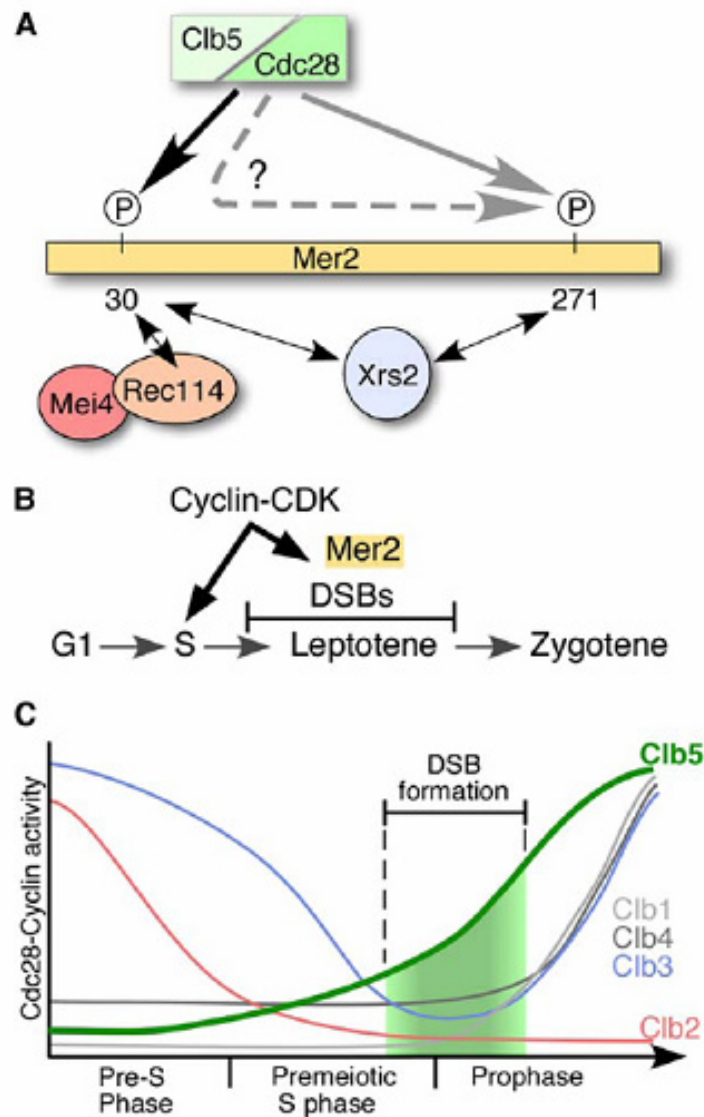


Figure 9: Cdc28-dependent regulation of meiotic DSB formation. (A) Cdc28/Clb5 kinase phosphorylates Ser30 and Ser271 residues of Mer2. This event is required for Rec114p and Xrs2p interactions. Phosphorylation of Ser271 seems to be partially dependent on Ser30 phosphorylation. (B) Illustration of the role of Cyclin-CDK in promoting both premeiotic DNA replication and DSB formation. (C) Fluctuation of B type cyclin-Cdc28 kinase activities. DSB formation is restricted to the shaded green window at the beginning of meiotic prophase I. Notice that, in contrast to other CDK/cyclins, Cdc28/Clb5 activity is at intermediate levels during the DSB formation window[26].

1.3.1.3 Rec102 and Rec104

Rec102p and Rec104p were shown quite early to interact genetically. Indeed, once these components were identified as essential for meiotic DSB formation, the over-expression of REC102 was shown to suppress the conditional reduction in recombination observed for a *rec104* temperature sensitive mutant[38]. This genetic interaction has been supported by biochemical evidence demonstrating that Rec102p, Rec104p and Spo11p all interact with one other [39]. Furthermore, their interdependence for proper nuclear localization, as well as the Spo11/Ski8 requirement for their chromatin interaction, strongly suggests their direct connection to the Spo11/Ski8 subcomplex is required in order to work as a functional unit [40]. On the other hand, REC102 and REC104 were shown to be essential for Spo11p localization at the meiotic DSB regions, suggesting that the identification of hotspot areas depends on these two components via a mechanism that is not yet fully understood [15], [41].

1.3.1.4 The MRX complex and its role in DSB formation

In contrast to the previous subcomplexes, components of the MRX complex, namely Mre11, Rad50 and Xrs2, are ubiquitously expressed and have been shown to be important players in events such as DNA damage signalling, telomeres homeostasis, and DSB repair both in mitosis and in meiosis (for a review of the different roles of MRX complex [42]).

In addition to these roles, the MRX complex is also essential for meiotic DSB formation; at least in the case of *Saccharomyces cerevisiae* [43], [44](model organisms like *A. thaliana* or *S. pombe* do not require this complex for meiotic DSB formation but for its repair). From a structural point of view, this complex is composed of a coiled coil protein, Rad50p, a metallo-phosphoesterase nuclease, Mre11 and a third component Xrs2p, which was shown to interact with the phosphorylated Mer2 component of the MMR subcomplex through a two-hybrid experiment [37]. Whereas Rad50p is believed to have a structural role, the DNA binding domain of the Mre11 nuclease has been shown to be essential for DSB formation in meiosis[44]. Furthermore, Mre11 localization at the DSB hotspot regions requires all DSB initiation components, with the exception of Rad50p, and it is not dependent on DSB formation itself [45].

Based on the previous studies, it is speculated that the MRX complex is recruited to the DSB formation complex via the Xrs2-Mer2 interaction, which indeed takes place only after the recruitment of all other components. Furthermore, recruitment depends on the DNA binding

activity of Mre11 as well as on the intermolecular bridging function of Rad50 coiled-coils dimers, although the role of this last requirement is still poorly understood [46].

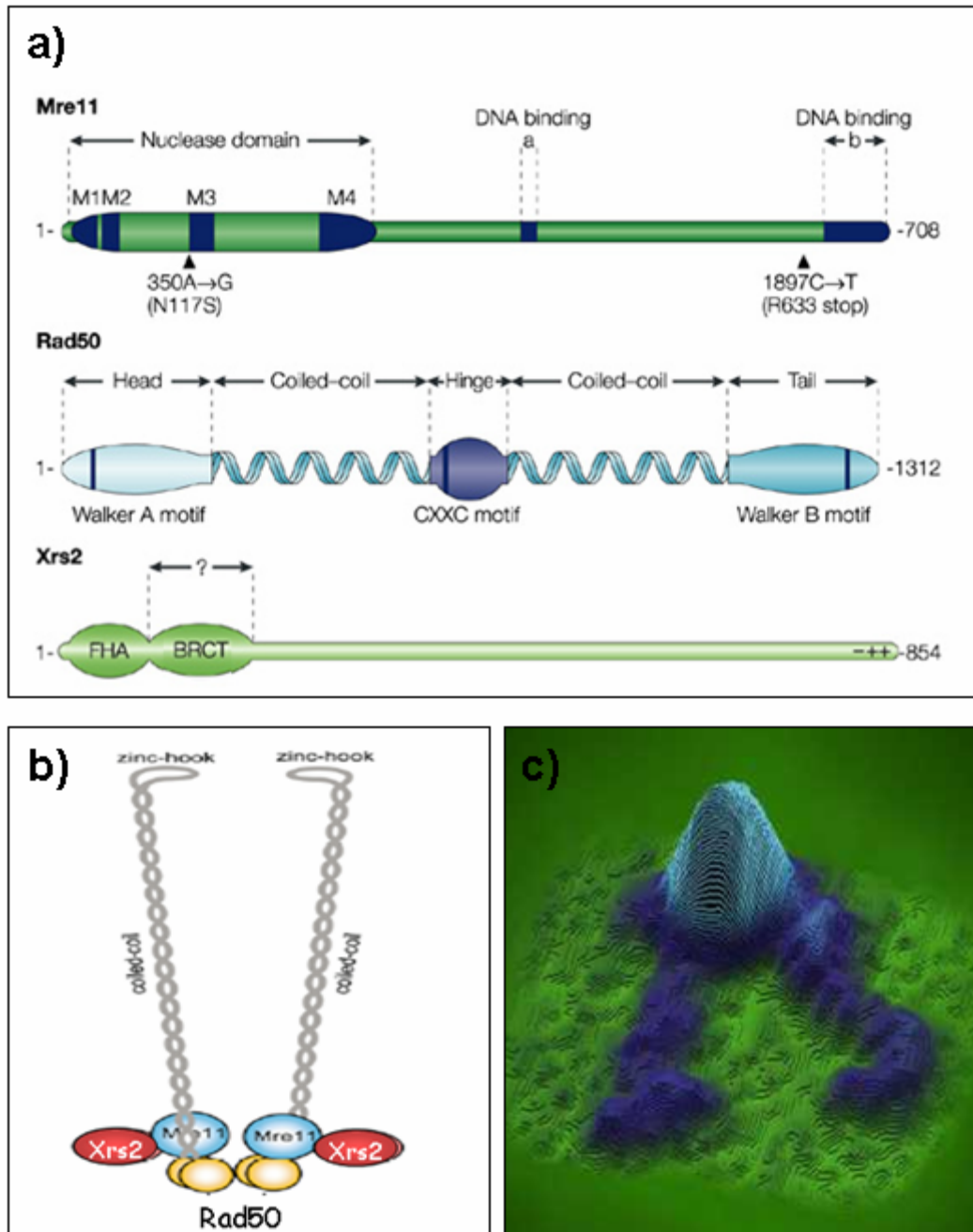


Figure 10: Components of the MRX complex. (a) Schematic representation of proteins composing the MRX complex. For Mre11, the nuclease domain as well as the DNA binding domains are highlighted. Rad50 is composed of two coiled coil domains connecting the globular domains (head and tail) with the hinge domain. Xrs2 contains a Fork-Head Associated domain (FHA) and a putative BRCT domain. (b) Structural representation of the MRX complex. The globular domains of Rad50p are dimerized (yellow). Mre11 binds to the Rad50p dimer close to the globular domain (Blue). The Xrs2 component binding to Mre11 is represented in red. (c) Scanning force microscopy image of the human MR complex illustrating the globular domain in light blue and both coiled coil arms in dark blue.[47]

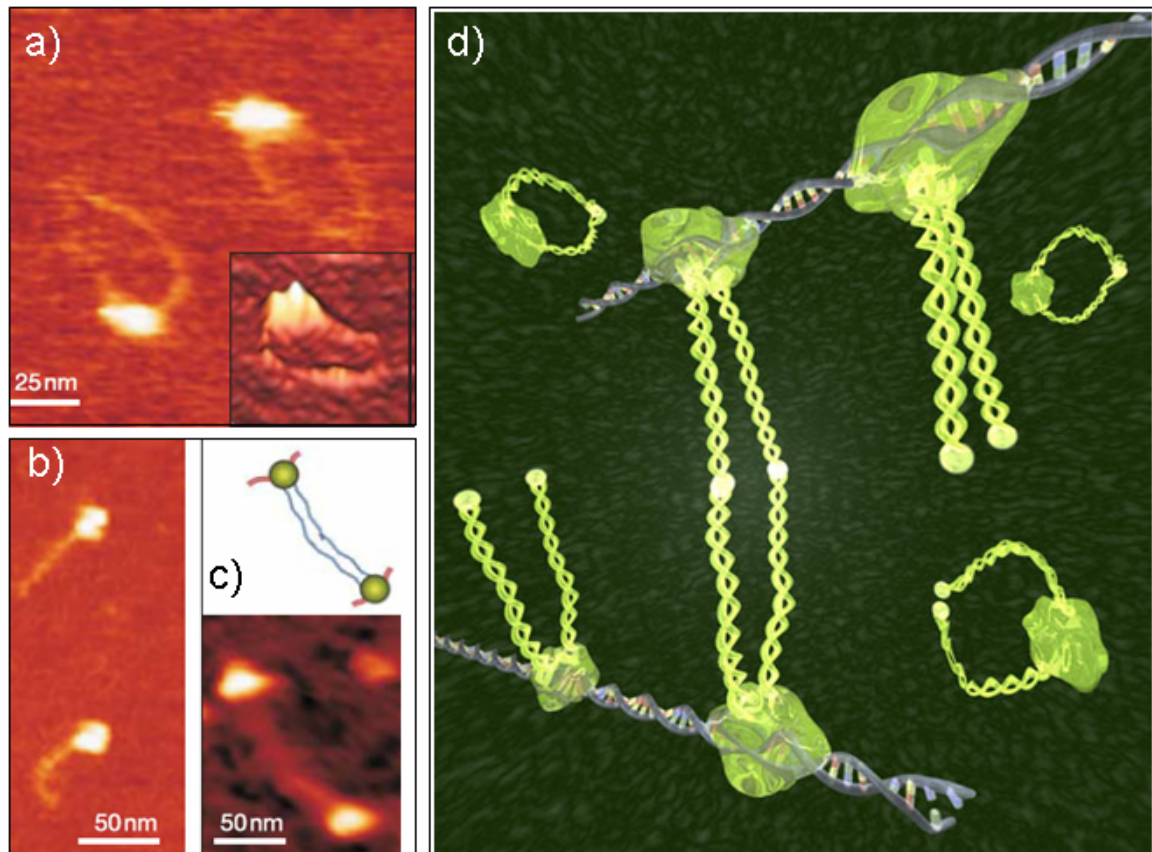


Figure 11: Mesoscale conformation change in MRN complex upon DNA binding. (a) Atomic force Microscopy images of free human MRN complex. Notice that it is possible to visualize both coiled coil arms connected to the globular domain (high dense region). (b) In presence of a molar excess of dsDNA, the MRN complex shows a single, thick coiled coil. (c) In some cases is possible to find intercomplex coiled-coil apex interactions. (d) Artistic representation of different conformational changes in MRN complex observed in the presence of dsDNA molecules [47].

In summary, the previous paragraphs referring to the characterization of different meiotic DSB formation components demonstrates the existence of functional subcomplexes among them, and their interconnections required for induction of recombination. In one hand, the MMR subcomplex (for Mer2, Mei4 and Rec114) was shown to be essential for the regulation of the transition between premeiotic DNA replication and the initiation of recombination. Furthermore, it was shown to control the recruitment of the MRX complex via Xrs2-PhosphoMer2 interaction and also its interaction with the Rec104/Rec102 subcomplex via meiotic as well as mitotic specific interactions. On the other hand, the loss of chromatin localization observed for Spo11p under *rec114*, *rec102*, or *rec104* mutant backgrounds strongly suggest that the interconnection between the MMR and Rec104/Rec102 subcomplexes is essential for the proper localization of the enzymatic subcomplex Spo11/Ski8.

Whereas the subcomplexes network organization is well characterized, their localization on the chromatin and by consequence the understanding of their role in defining certain regions as meiotic DSB hotspots is still unclear. The fact that there are separate protein subgroups or subcomplexes involved in this process could be the consequence of their separate chromatin localization. Furthermore, the activation of a chromatin region as a meiotic hotspot could then be the consequence of the rearrangement of the chromatin because of the association of the different protein subcomplexes.

1.3.2 Meiotic Double-strand Break repair

As indicated before, meiotic DSBs are formed by the tyrosine nuclease Spo11p. In analogy to the topoisomerase IIB mechanism, the DNA cleavage is performed by a transesterification process, which is characterized by the formation of a covalent linkage between the catalytic tyrosine residue of Spo11p and the phosphate group at the 5'-end of the broken DNA. In order to resect the DNA ends, and by consequence form the single-strand DNA tails required for homolog invasion, the covalently linked Spo11 protein needs to be removed. At this respect, it was recently shown that the Spo11 removal occurs by endonucleolytic cleavage a few base pairs away from the Spo11-DNA end (15-30nt away in *S. cerevisiae*)[32].

Even though the nuclease involved in the removal of Spo11 has not yet formally been identified, there are strong evidences suggesting that the MRX complex together with a fourth protein, namely Com1/Sae2p, present the enzymatic activity involved in this process. Indeed, point mutations in RAD50 or in MRE11 have been shown to allow meiotic DSB formation in *S. cerevisiae* but they accumulate unrepaired DSBs, reason why they were named as *rad50S* and *mre11S* respectively for highlight the separation of function displayed by these mutations[48],[49]. The structural analysis of the *Rad50S* mutant demonstrates that seven of the introduced nine point mutations cluster into a surface patch forming a putative protein-protein interaction site in the globular domain (see figure 12)[50]. Taking into consideration that Com1/Sae2p is required for meiotic DSB repair even in the presence of a fully functional MRX complex; the proposed protein-protein interaction site was hypothetically associated to this protein. On the other hand, the structural analysis of *mre11S* has shown that one of the point mutations (P84S) is located in the proposed Xrs2p binding loop, possibly interfering with a proper Xrs2-Mre11 interaction. Furthermore, the second point mutation (T188I) was found in a surface loop containing conserved positively charged residues involved in Mre11-DNA binding activity(see figure 12)[51]. In addition to *mre11S*, other MRE11 mutants displaying the same meiotic DSB repair phenotype have been

characterized (mutants D16A, D56N, H125N, H213Y and mre11-6). Surprisingly, the common characteristic between all these mutants, like *mre11S*, is that they do not display nuclease activity *in vitro*. Of these mutants, *mre11-H125N* displays a clear meiotic phenotype without compromising other vegetative functions such as mating type switching, non-homologous end joining or telomere homeostasis, demonstrating that the nuclease activity of Mre11p is essential for the repair of Spo11p-DSBs in meiosis [52].

While there is no doubt in the requirement of the nuclease activity of Mre11p for processing meiotic DSBs, its 3'-5' exonuclease polarity characterized *in vitro* does not match the 5'-3' resection activity required at DSB DNA-ends. An alternative model suggests the involvement of an endonuclease activity (MRX complex has shown to contain a hairpin endonuclease activity as well as a 3'-ssDNA branch cleaving activity) which fits with the characterized Spo11p removal mechanism [32]. On the other hand, the possibility that another component cooperates with the MRX complex for the removal of Spo11p is strongly suggested by the fact that Com1p is essential for this process.

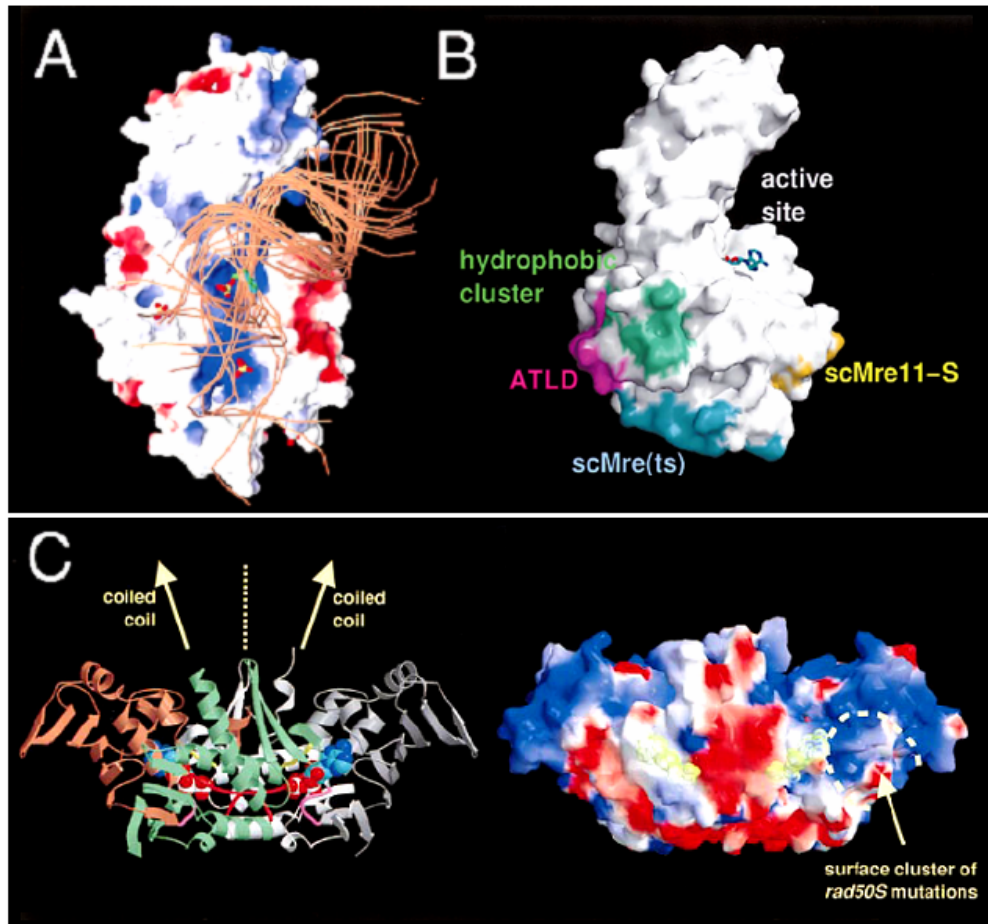


Figure 12: Crystal Structure analysis of Mre11p and Rad50p. (A) Electrostatic surface calculated for Mre11p. Illustration of a dsDNA substrate (brown tubes) occupying the putative DNA binding site. (B) Representation of different Mre11 surface clusters containing particular mutations: the surface cluster containing the *ScMre11(ts)* mutation is represented in light blue, the Ataxia Telangiectasia-like disorder (ATLD) mutation in pink and the *ScMre11S* mutations presenting a meiotic DSB repair phenotype is represented in yellow. The ATLD and the *ScMre11(ts)* surface clusters are close to each other, as well as to the hydrophobic cluster (green) involved in Mre11-Rad50 interaction suggesting that their phenotypes could be a consequence of an impairment of this interaction. (C) Secondary structure of Rad50p illustrating the dimerized globular domains (Left). Electrostatic surface of Rad50p dimerized globular domains showing the surface cluster containing the *rad50S* mutations (right). Figures adapted from [50] and [51].

1.3.2.1 Com1/Sae2 and its connection to the MRX complex

Com1/Sae2p was identified in 1997 in two different screens developed for the identification of essential genes required after induction of meiotic DSBs in *S. cerevisiae* [53],[54]. The *com1/sae2* phenotype has been shown to resemble that of *rad50S* and *mre11S* mutants, namely absent or aberrant spore formation as a consequence of the failure of meiotic DSB repair. The COM1/SAE2 gene encodes a small hydrophilic protein (~40kDa) presenting homologues in other organisms. Indeed in a recent study, higher eukaryote homologues for Com1/Sae2 (e.g. the human homolog CtIP, that is shown to play a direct role in DSB repair in somatic cells together with the MRN complex[55]) were identified by protein sequence alignment algorithms (PSI-BLAST)[56]. Furthermore, some of these homologs, including those in *A. Thaliana* [56], *S.pombe* [57] and the *C. elengans* [58] have been genetically characterized.

In addition to its role in meiotic DSB repair, Com1/Sae2p has been shown to be involved in processing hairpin-capped DSBs[59], as well as in resection of DSBs in somatic cells[60] in both cases connected with the activity of the MRX complex. On the other hand, Com1/Sae2p is phosphorylated by the protein kinases Mec1 and Tel1 in response to DNA damage in mitotic cells as well as in meiosis [61],[62]. In fact, mutations altering the Mec1/Tel1 phosphorylation sites in Com1/Sae2p affect not only its *in vivo* phosphorylation, but also decrease the rates of mitotic recombination and abolish meiotic recombination by impairing meiotic DSB repair, connecting the checkpoint regulation process with its role in DNA damage repair [61,63].

Although all previous reports connect Com1/Sae2p to the role of MRX in DNA damage signalling and repair, there is no direct evidence for a physical interaction between them in *S. cerevisiae* until now. In contrast, its higher eukaryote homolog CtIP was recently shown to interact with the Nbs1p component (the Xrs2 homolog) of the MRN complex, as well as with BRCA1 (a higher eukaryote protein component involved in genome stability) in response to DNA damage in a cell cycle-dependent manner [64]. Even if we consider a hypothetical physical interaction between Com1/Sae2p and the MRX complex in *S. cerevisiae*, like in the case of the human homolog CtIP, the molecular role of this protein in DSB repair is still poorly understood. In this respect, a recent study reports an endonuclease activity for Com1/Sae2p characterized *in vitro*. Indeed, Com1 was shown to bind DNA and contains an endonuclease activity on single-stranded DNA even in the absence of MRX. However, it can process hairpin DNA structures only in the presence of MRX [65]. Furthermore, the presence of a *rad50* mutant, (*rad50-R20M*) showing an "S" phenotype for meiotic recombination, in the MRX-Com1 complex *in vitro* has only a partial hairpin nuclease activity, but an increase

of the ionic strength of the reaction buffer (which destabilises weak protein-protein interactions) further affected the hairpin processing. This correlates with previous observations, where Com1p overexpression was been shown to partially rescue *rad50S* phenotypes produced by defects in DNA processing and single-strand annealing[60].

From the previous paragraphs, is speculated that Com1/Sae2p plays a direct enzymatic role, together with the MRX complex, in processing meiotic DSBs. The *in vitro* characterized nuclease activity could be responsible for the Spo11p removal, and its specific localization at the DSB chromatin site (the CtIP and Ctp1 homologues have been visualized by immunostaining in mitotic DNA damage response assays[55]) could depend on a physical interaction with the MRX complex, for instance via the RAD50 surface cluster structurally modified in the *rad50S* mutant version (see figure 14). Still we cannot exclude that further interactions, like that characterized between Nbs1 and CtIP, are involved in this process.

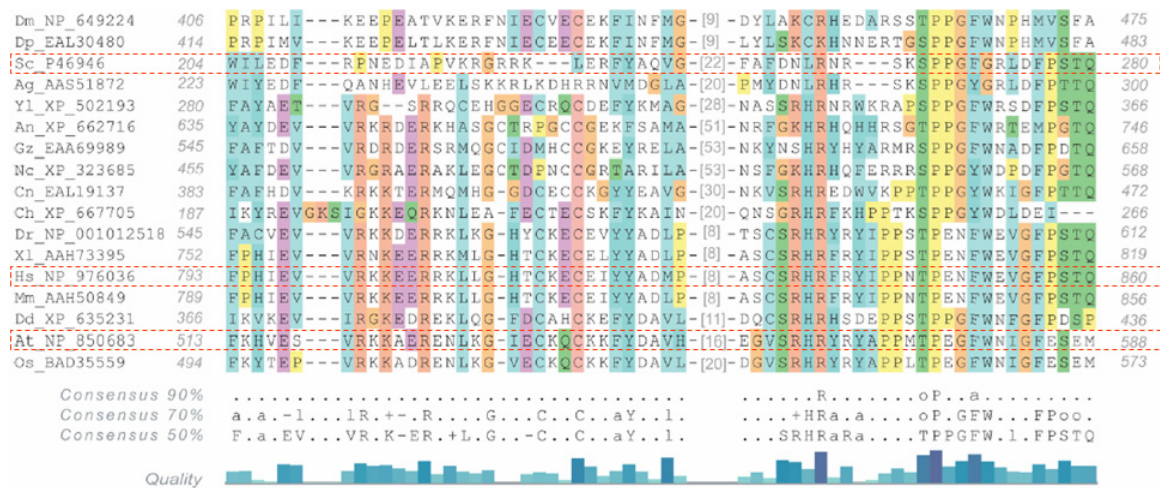


Figure 13: Alignment of the putative homologues of the *S. cerevisiae* Com1/Sae2 protein. Protein sequence alignment shows that the C-terminus is the most conserved region. Consensus residues above selected thresholds (90%, 70% and 50%) are illustrated below the alignment (hydroxyl consensus residues including amino acids S, T are denoted as "o"; aliphatic residues like I, L, V as "l"; aromatic residues like F, H, W, Y as "a"; positive charged residues like H, K, R as "+" and negative charged residues like D, E as "-"). Numbers and asterisks over the sequence alignment refer to amino acids exchanges introduced into the *S. cerevisiae* protein sequence. Red dashed lines indicate the *S. cerevisiae* Com1, the Human homologue CtIP and the *A. Taliana* At-Com1 protein sequences respectively. Figure adapted from [56].

1.4 The influence of chromatin organization on meiotic DSB formation

The uneven nature of meiotic recombination frequency across the whole genome has been apparent since its discovery. Indeed in all organisms studied to date, some genetic loci have a higher recombination frequency, and others recombine at a lower frequency than the average. According to these observations, regions of higher and lower recombination frequency are referred as hotspots and coldspots respectively. Since meiotic recombination depends on Spo11p-dependent DSBs, the frequency of DSB formation on the chromatin could follow a similar pattern.

Characterization of several meiotic DSB sites on the chromatin by physical methods has demonstrated the fact that they are not random distributed, but instead display a hotspot-coldspot nature, analagous to meiotic recombination. The complete physical map of meiotic DSBs on ChrIII of *S. cerevisiae* performed already 10 years ago demonstrated a strong correlation between the frequency of DSB formation and GC content [66]. Furthermore, high resolution analysis of DSB sites using DNA-microarray technology have shown recently that several hotspots were associated with local intergenic regions containing a high GC content [67].

Although hotspots are localized to defined regions on the chromatin, several attempts to identify a consensus sequence for DSB formation have failed. Nevertheless, a certain number of characteristics defining such chromatin regions have been elucidated, first, hotspots usually occur in intergenic regions containing a relatively high GC content. Second, DSB hotspot regions have been shown to be hypersensitive to the action of DNaseI and micrococcal nuclease, meaning that DSBs are preferentially formed in nucleosome free regions, or in other words, hotspots require accessible chromatin sites [68]. In some cases, hotspots were associated with the requirement of a specific transcription factor or factors; for instance the hotspot site located to the 5' of the HIS4 gene requires the binding of Bas1, Bas2 and Rap1 transcription factors in order to become active [69]. Furthermore local chromatin histone modifications, such as acetylation [70], methylation [71] or even ubiquitinylation [72], have been shown to play a role in the frequency of DSB formation and/or recombination at certain hotspot regions. In summary, it seems that the DSB hotspot activity is a consequence of the local chromatin accessibility and the regional chromatin-modification status. These criteria can be surpressed, at least patrially, by targeting meiotic recombination initiation components to regions that normally behave as coldspots. Indeed, fusion constructs between Gal4BD-Spo11p and VDE-Spo11p have induced DSB formation at

certain, but not at all, targeted coldspot regions [73,74], suggesting that it is possible to induce DSBs by forcing the accessibility of the recombination components to certain regions, but the fact that this does not always work demonstrates that the proper localization of the recombination machinery is not sufficient for DSB induction.

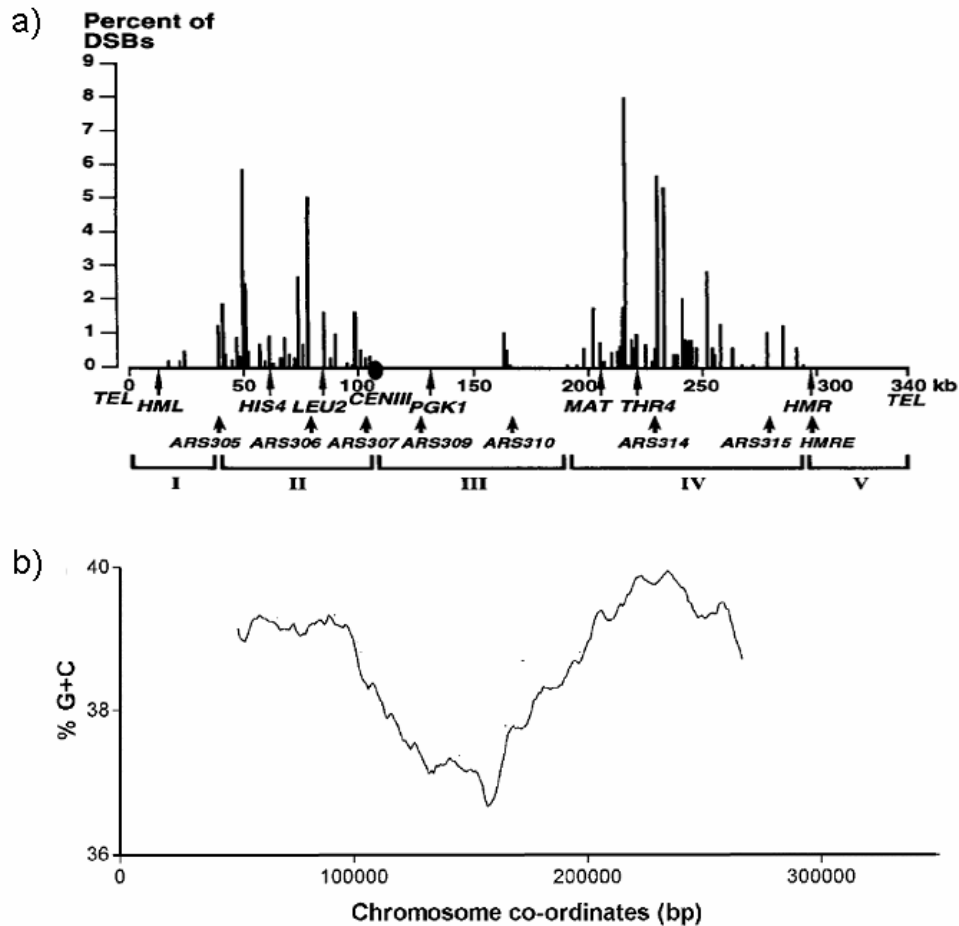


Figure 14: Meiotic DSB physical map and GC-DNA content of ChrIII in *S. cerevisiae*. (a) Map of the localization and relative frequency of DSBs along chr III [66]. (b) GC content of chr III determined in windows of 100kb moved in 1kb intervals[75]. A side by side comparison between the DSB physical map (a) and the chromosomal GC content (b) demonstrates the correlation between chromosomal sites where DSBs are preferentially formed and their high GC-DNA content.

As described in a previous chapter, meiotic chromatin follows a particular condensation process which is responsible for the organization of the chromatin in uncondensed regions and chromosome cores. Taking into consideration that chromatin accessibility is a key issue for DSB formation, a priori we expect that the protein scaffold structure established during prophase I may have a direct influence on the DSB frequency over the chromosome. In fact, components involved in chromatin condensation, like Hop1p, Red1p and MeK1p in *S. cerevisiae*, are not absolutely essential for DSB formation, but the frequency of DSB

formation has been shown to be partially affected in some of these mutants. For instance, the absence of Red1p, a major structural component of the axial element in *S. cerevisiae*, reduces DSB formation to 20-60% of wild type levels. This large variability of its influence on DSB formation reflects its uneven effect on different analysed hotspots. Furthermore, the evaluation of its influence on DSB formation efficiency depends strongly on the strain background. In a typical DSB assay, the *rad50S* or *com1/sae2* deletion background is used to accumulate DSBs over time. It was shown that this is not the case in a *rad50S, red1* double mutant, and for this reason the study of DSB formation in this background could result in misleading interpretations of the real influence of the absence of this protein. In a recent study, the authors used a *dmc1 rad51* background, in which no single strand-DNA invasion takes place and by consequence it is possible to accumulate DSBs over time, and demonstrated that the absence of *red1p* reduces DSB levels two to five fold compared to the wild type situation [76].

A more striking phenotype was observed for Hop1p, where its absence reduced DSB levels to 5-10% of the wild type. In order to explain such a difference, it is speculated that in contrast to Red1p, which localizes at the chromosome Core and by consequence not at the DSB sites, Hop1p could localize to the DSB sites in addition to the chromosome axis, and play a direct role in its regulation [77]. This is still speculation as there is no cytological or biochemical evidence demonstrating a localization of this protein to sites other than the chromosome axis. However, some studies have shown a partial rescue of the *hop1* phenotype by overexpressing the recombination initiation component Rec104p, supporting the hypothesis for a direct role of Hop1p in DSB formation [78,79].

Furthermore, Hop1p was shown to be phosphorylated in a DSB formation dependent manner, and this modification promotes dimerization of another component of the chromosome axis, the meiotic specific kinase Mek1p. Then, Mek1p is activated by *trans*-autophosphorylation. The molecular target of this kinase has yet to be identified, but Mek1p was shown to be essential for suppression of meiotic DSB repair via inter-sister recombination [80].

In summary, we can conclude that the characteristics defining a chromatin region as a meiotic hotspot depends on several layers of molecular regulation; in the first layer we require a locally defined high GC content, then we require a particular chromatin-modification status, where either transcription factors, or histone modifications, or both reorganise the chromatin to allow increased accessibility for the recombination components. Furthermore, the chromatin needs to be organized over a proteinaceous scaffold (the Synaptonemal complex) in order to define the uncondensed regions or loops where DSBs are preferentially formed. These different layers of molecular complexity are interconnected; for

instance, regions with high GC content correspond to highly transcribed areas [81], and transcription has been shown to define most of the cohesin localization sites which are used to build the protein scaffold in meiosis[82]. In fact, the actual model for defining chromatin regions as sites for DSB formation is still too simplistic, this is because the previously described criteria were established in a global chromatin analysis, and do not apply for local chromatin regions; for instance, not every uncondensed chromatin region corresponds to a DSB hotspot, suggesting that we are still missing other factors essentially for defining a chromatin region as “the one” to be cleaved.

1.5 Genome wide DSB distribution and its analysis in a post-

Genome era

The discovery of the double-helix structure of DNA in 1953 followed by the identification of the genetic code in 1961, revealed two important features of this molecule: Its complementarity as a source for the template copying mechanism responsible for high fidelity during replication of the genetic material; and its digital nature whereby precise nucleotide base pair combinations define the genetic code for proteins, the molecular machines of life.

The first efforts to reveal the digital code of DNA were fulfilled at the end of the seventies with the sequencing of the first complete genome, the Bacteriophage ϕ X174, a viral genome of about 5000 base pairs. The next decades which were accompanied by the combination of technical innovations and intensive instrumental automation, gave rise to a “genomic era” where the complete genome sequence of several model organisms (*E. coli*: September 1997[83], *S. cerevisiae*: May 1997[84], *C. elegans*: December 1998[85], *D. melanogaster*: March 2000[86], *A. Thaliana*: December 2000[87], *H. sapiens*: February 2001[88,89]) have been drafted. These events represent a landmark in modern biology due to the establishment of a new approach to address biological questions. Indeed, the value of having the complete genome sequence is that we can study biological processes at the system level rather than by focusing on a single event. This new approach to biology needed to be accompanied by the development of tools that allow interrogation of the sequenced genomes. In a similar manner to how PCR became the method to address questions in a defined chromatin region, DNA biochips have become the strategy to interrogate whole genomes. First described in 1995 [90], a DNA biochip, also known as DNA array, is an arrangement of several single-stranded DNA (ssDNA) molecules (referred as probes), covering for instance the complete sequence of a certain genome, deposited over a solid

support (nylon membrane, glass or plastic). Then, by using the complementarity of DNA, a labelled ssDNA substrate is hybridized to the biochip, and after several washes, the remaining substrate is analysed. The application of this kind of approach in a "post-genomic era" brought the characterization of the complete transcription map for *S. cerevisiae*, known also as the transcriptome, by hybridizing single-stranded cDNA derived from the total mRNA in yeast [91]. In a similar manner, the meiotic specific transcriptome was also identified, revealing that around 1600 genes, from a total of 6200, are meiotically regulated and, of these 1600, approximately 250 are expressed in a meiosis-specific manner [92].

DNA array technology can also be used to address protein-chromatin interactions; this is achieved by combining chromatin-immunoprecipitation with hybridization of pulled-down DNA onto DNA arrays containing the complete genome sequence. This type of approach has been used for the identification of cohesin binding sites in *S. cerevisiae*, demonstrating that cohesins are re-localized from their loading site to convergent regions of transcription [82,93,94]. In another study, the localization of the meiotic chromosomal axial element Red1p was compared to the localization of the cohesin complex as well as to the protein Dmc1, involved in 3'-single strand DNA end invasion during meiotic DSB repair [76]. In that way, a direct correlation for the localization of structural proteins like Red1p and cohesin components has been demonstrated, in contrast to the localization of Dmc1, which in a certain manner defines sites where the chromatin was cleaved in a meiotic specific manner. Furthermore, the Dmc1p-chromatin localization map obtained through a ChIP-DNA array strategy strongly overlapped with the meiotic DSB map obtained previously with physical methods in the *rad50S* background. From this analysis, the authors concluded that meiotic DSBs are formed in Red1/cohesin-free sites, which indeed correspond to uncondensed regions. It is important to mention at this point, that this approach not only confirmed the information we had regarding chromatin sites where meiotic-DSBs are formed on chr III in *S. cerevisiae*, but in addition it introduced a new strategy to identify meiotic DSB sites over the whole genome (the meiotic DSB physical map performed for chrIII of *S. cerevisiae* represented a tremendous effort, yet covered only ~2.6% of the total yeast genome). This approach enables not only the study of the genome-wide chromatin cleavage pattern, but also the localization of the components required for DSB formation, repair, chromatin structure, etc.

Another important issue to mention in regard to DNA array technology is the sensitivity and the resolution of the different arrays. Indeed, the first version of this technology corresponded to the deposit of PCR fragments covering a certain chromosome (chrIII of *S. cerevisiae* was initially used in order to compare ChIP-chip with the established DSB physical map) on a positively charged membrane. In this case, the deposited "probes" were not

perfectly contiguous, decreasing the resolution of the technique. Furthermore, in order to obtain a significant signal after hybridization, around of 1×10^7 cells (4x10⁷ cells/ml) were required for the chromatin immunoprecipitation and the final DNA sample was radioactively labelled [93]. The development of *in situ* synthesis methodologies for the design of probe arrays, such as photolithography (Affymetrix) or ink jet printing (Agilent, Nimblegen), as well as the development of DNA amplification procedures prior to hybridization, have dramatically improved resolution (5 nt for affymetrix and 30 nt resolution for Agilent in *S. cerevisiae* DNA arrays), as well as sensitivity[†].

The use of DNA microarrays combined with Chromatin immunoprecipitation was implemented for the identification of meiotic DSB sites in budding yeast by pulling down in Spo11p in a *rad50S* background[67]. In this study, the authors used DNA microarrays covering 6200 open reading frames (referred to as ORF arrays, in contrast to intergenic arrays containing no coding sequence probes, or tiling arrays containing the complete sequence) and demonstrated that the technique is able to reproduce the binding pattern of the physical map obtained for chromosome III.

A new strategy developed in order to identify meiotic DSB sites in *S. cerevisiae* depends on the repair of the broken ends by 5'-end resection. This process generates 3'-ssDNA that can be specifically enriched in Benzoyl naphthoyl DEAE (BND) cellulose columns [95,96]. In order to accumulate ssDNA, the authors used *dmc1*, *rad51* mutants, where the 5'-end resection is performed but its ability to invade the homologous chromosome is abolished. By hybridizing the enriched ssDNA to DNA microarrays, the authors demonstrated that the number of meiotic DSBs is indeed higher than previously found in the *rad50S* studies (40% difference between *rad50S* and *dmc1* mutants). Furthermore, the newly identified DSBs were characterized in near centromeric and telomeric regions suggesting that the *rad50S* mutant could, in some fashion, inhibit the formation of DSBs in these regions.

Finally, the use of the ChIP-DNA microarray strategy in order to identify the localization of components involved in meiotic recombination as well as chromatin organization may prove essential for understanding how the protein complexes are organized on the chromatin. In addition to the report described above, identifying the localization of Red1p using low resolution macroarrays, there are several studies accomplishing similar goals. Identifying the localization of Rec102/Rec104p on chromatin has been attempted using a macroarray strategy. Unfortunately, the low resolution of these DNA arrays, combined with the broad localization pattern of these proteins, provided few significant conclusions [40]. On the other hand, the study of Mre11p localization during DSB formation using ORF arrays revealed a

[†] Probe arrays designed by in situ oligomer synthesis methodologies are called microarrays in contrast to the PCR fragments spotted in charged membranes called macroarrays,

strong correlation with the meiotic DSB map obtained by pulling down Spo11p in the *com1* deletion background [45]. This study demonstrates that this protein being part of the DSB formation complex and required for its repair, localizes at the meiotic DSB hotspot sites. However, this study requires further evaluation as it uses ORF DNA arrays, which only identifies sites of localization within this predefined pool of the whole genome. Indeed, a proper microarray analysis should interrogate the complete genome sequence.

In order to address this poorly explored topic, which is the protein-chromatin interactions involved in meiotic recombination initiation and its connection with genome-wide chromatin organization, I will present in the following chapter the studies we have performed combining chromatin immunoprecipitation with hybridization to high resolution DNA microarrays. This study was performed in collaboration with the group of Professor Katsuhiko Shirahige, from the Tokyo Institute of Technology. The main goal of this study is to further our understanding of the events involved in meiotic recombination from the point of view of chromatin structure. Indeed, the use of microarray technology is our main read out for the identification of protein-chromatin interactions, which are then interpreted in a protein-protein/protein-chromatin fashion in order to construct a model for meiotic DSB formation and its repair. Furthermore, during this study we developed strategies for the analysis of microarray data in order to help us interpret the DNA micro array readouts as well as for the construction of protein-protein networks by performing comparative analysis of DNA microarrays.

2 Materials and Methods

2.1 Yeast Media and Solutions

2.1.1 Solid media

2% Bacto-agar (Oxoid) is placed into glass bottles containing a magnetic rod, and the medium is mixed separately. Medium is placed into the glass bottle, then autoclaved for 20 minutes at 120°C. Medium is cooled to 55°C with continuous stirring, then poured into Petri dishes (~20-30 ml/plate) and left at room temperature for 2 days. Finally all plates are stored at 4°C.

YPD plates: YPD rich medium plus 2% bacto-agar (see *Liquid media and Solutions*).

YPG plates: contain a non-fermentable carbon source (Glycerol) that does not allow growth of *petite* mutants.

- 1% bacto-yeast extract (Oxoid)
- 2% bacto-peptone (Oxoid)
- 3% (v/v) glycerol (100%)

Geneticin (G418) plates: Medium used for KanMX-marker selection. The composition of this medium follows the same recipe as the YPD plates, but after cooling to 55°C, 1ml of Geneticin (200mg/ml) is added before pouring plates.

SPM plates: (Sporulation medium)

- 2% Potassium Acetate, adjusted to pH 7.0 with Acetic Acid.

SM plates (Synthetic minimal medium): Selective medium for prototrophic cells. SM plates are used currently for mating type test.

- 0.17% bacto-yeast nitrogen base w/o amino acids and w/o Ammonium Sulfate (DIFCO)
- 0.5% Ammonium Sulfate
- 2% glucose

Adjust to pH 7.0 with Sodium Hydroxide.

ADE-, URA- and TRP- drop-out plates: Selective plates are used to test auxotrophic markers. For the drop-out mix, the corresponding ingredient was omitted.

- 0.17% bacto-yeast nitrogen base w/o amino acids and w/o Ammonium Sulfate (DIFCO)
- 0.5% Ammonium Sulfate
- 2% Glucose
- 1.1% Casamino Acids (DIFCO)
- 55mg Adenine per liter
- 55mg Tyrosine per liter
- 55mg Uracil per liter

Adjust to pH 7.0

After autoclaving and cooling to 55°C, 5ml of 1% Leucin and 5ml 1% Tryptophane are added (The amino acid solutions are filter sterilized).

HIS-, LEU-, ARG- and LYS- drop-out plates:

- 0.8% Yeast nitrogen base w/o amino acids (DIFCO)
- 55mg Tyrosine per liter
- 55mg Uracil per liter
- 55mg Adenine per liter

After autoclaving and cooling to 55°C, 100ml of 20% Glucose and 20ml of the filter-sterilized 100x HIS-,LEU-, ARG- or LYS- drop-out solution are added to 1 liter of medium.

Amino Acid	Amount in g/liter
Arginine	2
Histidine	1
Iso-Leucine	6
Leucine	6
Lysin	4
Methionine	1
Phenylalanine	6
Tryptophane	4
Threonine	5

Table 1: Recipe for amino acid drop-out solutions (100x) in use: For each drop-out solution the appropriate amino acid has been omitted. These drop-out stock solutions are prepared in double-distilled water, filter-sterilized and stored in the dark at 4°C.

2.1.2 Liquid Media and solutions

YPD (rich medium):

- 1% bacto-yeast extract (Oxoid)
- 2% bacto-peptone (Oxoid)
- 2% glucose

2.1.2.1 Media required for synchronised meiotic time courses

Pre-sporulation media (SPS):

- 0.5% Yeast extract, 1% Peptone
- 0.17% Yeast Nitrogen Base (without amino acids and without ammonium sulfate)
- 1% Potassium Acetate
- 1% Ammonium sulphate
- 0.05 M potassiumbiphtalate, pH 5.5 (adjust pH with Potassium acetate, autoclaved)

Sporulation media (SPM):

- 1% Potassium Acetate pH 7.0, (autoclaved)

Polypropylene Glycol (PPG) 1%

Amino acid complementation media:

- 1.5% Lysine
- 2% Histidine
- 2% Arginine
- 1% Leucine
- 0.2% Uracyl
- 1% Tryptophane (filter sterilized).

Formaldehyde 37%

Glycine 2.5 M solution (autoclaved)

TBS buffer:

- 20 mM Tris-Cl (pH 7.5)
- 150 mM NaCl.

2.1.2.2 Solutions for Chromatin immunoprecipitation

Lysis buffer:

- 50 mM HepesKOH (pH 7.5)
- 140 mM NaCl, 1mM EDTA
- 1% Triton X-100
- 0.1% Na-deoxycholate (filter sterilized).

Lysis buffer complete:

Immediately before use add PMSF (1 mM final concentration), 250 µl of Aprotinin (1.4 mg/ml) and 1 protease inhibitor tablet (Roche, Complete Protease Inhibitor Cocktail) per 50 ml lysis buffer.

Washing buffer:

- 10 mM Tris-Cl pH 8
- 250 mM LiCl, 0.5% Na-deoxycholate
- 1 mM EDTA.

Elution buffer:

- 50 mM Tris-Cl pH 8
- 10 mM EDTA, 1% SDS.

PBS:

- 137 mM NaCl
- 2.7 mM KCl
- 4.3 mM Na₂HPO₄
- 1.47 mM KH₂PO₄, pH 7.4.

PBS/ BSA:

- 5 mg BSA per 1 ml PBS.

TE buffer:

- 10 mM Tris-Cl pH 8
- 1 mM EDTA.

Dynabeads:

- Magnetic Dynabeads, (DynaL Biotech, Pan Mouse IgG).

Glass beads: diameter 0.40-0.60 mm (Sartorius, BBI-8541701)

2.1.2.3 Solutions required for DNA purification

Proteinase K (20 mg/ml) (Roche)

Glycogen (10 mg/ml)

Phenol/ chloroform / Isoamylalcohol : 25/24/1 ratio.

NaCl 5 M solution.

DNase-free RNase (500 ug/ml) (Roche).

96% Ethanol

70% Ethanol

Sodium Acetate 3M.

2.2 Basic Yeast Techniques

2.2.1 Growth of Yeast Strains

All yeast strains used in this study were grown either in solid yeast media (plates), or in liquid media shaking at 200 RPM, at 30°C. In the case of meiotic time courses for ChIP preparations, diploid yeast strains were grown in 50 ml falcons containing 12 ml of rich media YPD (the amount of cells on the tip of a toothpick were enough for growing cells for 6 hours before they were inoculated in pre-sporulation media SPS). The lid of the falcon tube was fixed with tape to allow air to enter, and the tubes were incubated at 30°C with shaking at 200 RPM for 6 hours. After this period of time, cells were in the exponential growth phase. Cells were then diluted in SPS to reach the corresponding concentration for a meiotic time course setup.

2.2.2 Meiotic Time Courses

Diploid yeast cells were inoculated in pre-sporulation medium (SPS,) to a final concentration of approximately 2×10^6 cells/ml (*see Note 2.2.2.1*). Cultures were set up in 3 L Erlenmeyer baffled flasks (50 ml per sample, but not exceeding 15% of the total flask volume, to ensure optimal oxygenation).

Cells were grown with vigorous shaking (250 rpm) for 12 to 16 hours at 30°C until the density reached 4×10^7 cells/ml. They were then collected by centrifugation (5 min, 3000 rpm) and resuspended at a concentration of 4×10^7 cells/ml in sporulation medium (SPM supplemented with amino acids (320 μ l Amino acid complementation media per 100ml SPM media) and PPG (100 μ l 1%PPG per 100ml SPM)) (time-point $t=0$ h), and kept shaking at 250 rpm for the whole time-course). Note that the SPM culture should not exceed 10% of the flask capacity (maximum 300 ml in a 3 L flask). The progression of the time-course was followed by regularly staining the DNA (DAPI) of samples (100 μ l of culture into 500 μ l Ethanol). Aliquots were typically taken at one-hour intervals and stored at 4°C until the end of the time-course.

At the desired times, 50 ml-aliquots were collected for ChIP. Each sample was incubated with Formaldehyde (1% final concentration) for 30 minutes (*see Note 2.2.2.2*) at room temperature (shaking under the hood). The cross-linking process was stopped by addition of Glycine (to a final concentration of 131 mM) with shaking for 5 minutes.

Finally, samples were washed twice with ice-cold TBS (with a centrifuge pre-cooled to 4°C), transferred to a 1.5ml eppendorf and frozen in liquid nitrogen. Samples were stored at -80°C.

Before proceeding with the preparation of the cell extracts, progression through meiosis was monitored for sufficient synchrony. Samples collected in ethanol were pelleted (10 seconds, high speed) and resuspended in 50 μ l of DAPI solution (0.2 μ g/ml DAPI in water).

To facilitate the counting, DAPI samples were sonicated for one second using a rod based sonicator, at medium strength. Finally, 5 μ l were placed on a glass slide and the percentage of mononucleate, binucleate, and tetranucleate cells were evaluated for each time-point. In our hands, synchronous cultures of a wild-type SK1 strain typically reached the highest number of binucleate cells at 5 or at 6 hours after transfer to SPM. This should correspond to a peak of at least 25% binucleates for wild type *SK1* cells (*see Note 2.2.2.3*).

2.2.3 Tetrad Dissection

Tetrad dissection was performed in order to separate the four haploid spores derived from a diploid strain. For this purpose, the diploid strain to be analysed was sporulated in 2% Potassium Acetate SPM plates (a small amount of cells was transferred to an SPM plate then incubated at 30°C overnight). Once yeast sporulation was confirmed with a phase contrast microscope, the yeast were resuspended in 92 µl of ddH₂O supplemented with 4 µl of DTT (0.5 M) and 4 µl of Zymolyase T20 (10 mg/ml). This digestion mixture was incubated at 30°C for 30 minutes, and then diluted in 900 µl of ddH₂O and kept on ice until dissection was performed.

Finally, 25 µl of the digested mixture was spotted on a YPD rich media plate and by tilting the plate at a 45 degree angle, the digested mixture was spread, forming a line at the center of the YPD plate. Tetrads were dissected with a *Leitz micromanipulator*. Dissected tetrads were grown at 30°C for 2-3 days, until visible, when they were used for further analysis.

2.2.4 Preparation of Yeast Genomic DNA

2.2.4.1 Genomic DNA preparation for PCR genotyping

This protocol is designed to prepare Genomic DNA from *S. Cerevisiae* by lysing yeast cells with glass beads. This procedure fragments the Genomic DNA, and for this reason this procedure is not recommended for applications such as the meiotic Double-Strand Break assay (DSB assay). Currently we use this protocol for PCR genotyping applications.

Required solutions:

Buffer A:

- 2% Triton X-100, 1% SDS
- 100 mM NaCl
- 10 mM Tris-HCl pH=8.0
- 1 mM EDTA pH=8.0

PCI:

- Phenol: Chloroform: Isoamyl alcohol (25:24:1 v:v:v) mixture

Glassbeads (diameter 0.40-0.60mm)

1x TE buffer:

- 10 mM Tris-Cl pH 8
- 1 mM EDTA.

96% Ethanol

70% Ethanol

RNAse (20 mg/ml) DNase free

Potassium Acetate 5 M

5 ml of YPD rich liquid media was inoculated with the corresponding yeast strains. They were grown at 30°C overnight, and the next day cells were harvested by centrifugation at 3000 RPM for 5min. Yeast pellets were resuspended in 200 ul of Buffer A and transferred into 1.5ml eppendorf tubes, to which 200 ul of PCI and ~200 ul of glass beads were added.

Eppendorf tubes were vortexed for 7 minutes at 4°C, then 200ul of 1XTE buffer was added and samples were vortexed again for 7 minutes. Samples were centrifuged for 5 minutes at full speed and the aqueous phase was then transferred into a new tube. 1 ml of 96% Ethanol was added and samples were kept at 4°C for at least 30 minutes prior to centrifugation at full speed for 5 minutes.

The precipitated pellet was resuspended in 200 ul TE containing 2 ul of 20 mg/ml RNAse solution and incubated at 37°C for 30 minutes. Following this step, 200ul of TE and 200ul of PCI were added, the tubes were vortexed and centrifuged at full speed for 5 minutes. The Aqueous phase was transferred into a new eppendorf tube to which 20 ul of 5M Potassium Acetate and 96% Ethanol were added. The tubes were incubated at 4°C for 30 minutes and finally centrifuged at full speed for 5 minutes. The precipitated pellet was washed with 70% Ethanol, dried, and resuspended in 50 ul of TE. 1 ul of the final dilution is required per PCR reaction.

2.2.5 Lithium-acetate Transformation of *S. cerevisiae*

Required solutions:

- **Lithium Acetate 1 M**
- **Polyethylene Glycol (PEG) 3350 50%**
- **Salmon sperm DNA (ssDNA 10 mg/ml)**
- **Glycerol 60%**

The yeast strain to be transformed was grown overnight (ON) in 5 ml YPD rich media. The following day, this ON culture was inoculate into 50 ml of YPD media to an optical density (OD₆₆₀) of 0.1. Cells were grown for approximately 5hours (OD₆₆₀ =0.5 to 0.7) and were then collected by centrifugation (3000 RPM/ 5minutes). The yeast pellet was resuspended in 1 ml of 1 M Lithium Acetate and transferred into a 1.5 ml eppendorf. Cells were collected by centrifugation (6000 RPM 1 minute) and resuspended in 350 ul 1M Lithium Acetate.

The transformation mixture contained 24 ul of yeast suspension, the DNA to be transformed dissolved in 15 ul ddH₂O or TE, 90 ul of 50% PEG and 8 ul of ssDNA (10 mg/ml). This mixture was incubated at room temperature for 30-60 minutes, then was supplemented with 6 ul of 60% Glycerol and incubated again at room temperature for 30-60 minutes. Finally, the cell mixture was incubated at 42°C for 10 minutes (or 5 minutes at 45°C) and then diluted with ddH₂O to a final volume of 200 ul which was plated on two selective plates (100 ul per plate).

When the selective media contained G418, cells were first plated on YPD rich media plates (kept at room temperature ON), and then replica-plated onto G418 selective media.

2.2.6 Yeast Strains

All strains used in this study were derived from the sporulation-proficient *SK1* genetic strain background[97]. Genotypes of the diploid yeast strains are presented in the following table.

Table 2: *SK1* strains used in this study.

<i>FK strain collection reference number</i>	<i>Genotype</i>
1151	<i>MAT a/alpha, ho::LYS2, lys2, leu2::hisG, ura3 , COM1::Myc18::TRP1, trp1::hisG</i>
1180	<i>MAT a/alpha, ho::LYS2, lys2, rad50S::URA3, ura3, COM1 ::MYC18::TRP1, trp1, LEU2</i>
1203	<i>MAT a/alpha, leu2, spo11::URA3, COM1 ::Myc18::TRP1</i>
1577	<i>Mat a/alpha, COM1::Myc18::TRP1, trp1::hisG, Mre11::HA6::HIS3, his3::hisG, ura3, leu2::hisG, ade2</i>
1799	<i>Mat a/alpha ho::LYS2, lys2, COM1::Myc18::TRP1, mre11S, leu2::hisG, ura3</i>
1900	<i>Mat a/alpha, ura3, leu2::hisG, trp1::hisG, mre11::KanMX4, COM1::Myc18::TRP1</i>
3077	<i>MAT a/alpha, ho::LYS2, lys2, scc1-73, leu2, ura3, ade2, arg4, rec8::kanMX, Com1::myc18::TRP1</i>
3364	<i>Mat a/alpha, ho::LYS2, leu2, his3, ura3, trp1, XRS2::HA3::TRP1, rad50S::URA3, COM1::Myc18::TRP1</i>
3504	<i>Mat a/alpha, ho::LYS2, ura3, leu2::hisG, trp1::hisG, his3::hisG, mre11-H125N, COM1::Myc18::TRP1, ade2</i>
3523	<i>MATa/alpha, ho::LYS2, lys2, leu2::hisG, ura3 , COM1::Myc18::TRP1, trp1::hisG, spo11-Y135F::HA::URA3, HIS3</i>
3570	<i>MATa/alpha, ho::LYS2, lys2, leu2::hisG, ura3, COM1::Myc18::TRP1, rad50::URA3</i>
1576	<i>Mat a/alpha, ura3, trp1::hisG, leu2::hisG, mre11::HA6::HIS3, his3::hisG, ade2::hisG</i>
1577	<i>Mat a/alpha, COM1::Myc18::TRP1, trp1::hisG, mre11::HA6::HIS3, his3::hisG, ura3, leu2::hisG, ade2</i>
3526	<i>Mat a/alpha, ho::LYS2, lys2, ade2::hisG, ura3, leu2::hisG, trp1::hisG, Mre11::HA6::HIS3, his3::hisG, spo11-Y135F::HA::URA3</i>

3532	<i>Mat a/alpha, ho::LYS2, ura3, leu2::hisG, trp1::hisG, his3::hisG, rec114::KANMX,, ura3, Mre11-HA6::HIS3</i>
3535	<i>Mat a/alpha, rad50S::URA3, ura3, trp1::hisG, leu2::hisG, mre11::HA6::HIS3, his3::hisG</i>
3554	<i>Mat a/alpha, ho::LYS2, lys2, ade2::hisG, ura3, leu2::hisG, trp1::hisG, Mre11::HA6::HIS3, his3::hisG, spo11::URA3, ura3</i>
1358	<i>MAT a/alpha, ho::LYS2, ura3, trp1::hisG, leu2, SPO11::Myc18::TRP1</i>
1371	<i>Mat a/alpha, ho::LYS2, SPO11::Myc18::TRP1, trp1, rad50S::URA3, ura3, leu2::hisG</i>
1488	<i>MAT a/alpha, HO, lys2, leu2::hisG, ura3, TRP1, SPO11::Myc18::TRP1, rec102::URA3</i>
2872	<i>Mat a/alpha, ho::LYS2, Spo11::Myc18::TRP1, com1::KanMX, leu2, ura3</i>
3039	<i>MAT a/alpha,ho::LYS2, ura3, leu2::hisG, trp1::hisG, SPO11::Myc18::TRP1, hop1::LEU2</i>
3284	<i>MAT a/alpha, ho::LYS2, ade2::hisG, ura3, leu2::hisG, trp1::hisG, his3::hisG, SPO11::Myc18::TRP1, mre11S</i>
3529	<i>Mat /alpha, ho::LYS2, SPO11::Myc18::TRP1, trp1::hisG, ura3, leu2::hisG, his3::hisG, mre11-H125N</i>
1281	<i>Mat a/alpha, ho::LYS2, lys2, ura3, leu2::hisG, his3::hisG, trp1::hisG, XRS2::HA3::TRP1, COM1::Myc18::TRP1</i>
3364	<i>Mat a/alpha, ho::LYS2, leu2, his3, ura3, trp1, XRS2::HA3::TRP1, rad50S::URA3, COM1::Myc18::TRP1</i>
3098	<i>Mat a/alpha, ho::LYS2, ura3, leu2::hisG, trp1::hisG, his3::hisG, TetR::PK6::URA3, Tub2A 7xTetO Tub2B</i>
3140	<i>Mat a/alpha, ho::LYS2, ura3, leu2::hisG, trp1::hisG, his3::hisG, TetR::HA3::TRP1, Tub2A 7xTetO Tub2B::URA3</i>
3149	<i>MAT a/alpha, ho::LYS2, lys2, leu2::hisG, his4, trp1::hisG, ura3, arg4-Nsp, REC8::HA3::URA3, TetR::HA3::TRP1, Tub2A 7xTetO Tub2B::URA3</i>
3152	<i>MAT a/alpha, ho::LYS2 lys2, ura3, leu2::hisG, his4, trp1::hisG, REC8::HA3::URA3, spo11:URA3, TetR::HA3::TRP1, Tub2A 7xTetO Tub2B::URA3</i>
3155	<i>Mat a/alpha, ho::LYS2, ura3, leu2::hisG, trp1::hisG, his3::hisG,</i>

3168	<i>TetR::Myc18::TRP1, Tub2A 7xTetO Tub2B::URA3</i> <i>MAT a/alpha, ho::LYS2, ura3, leu2::hisG, trp1::hisG,</i> <i>SPO11::Myc18::TRP1, TetR::Myc18::TRP1, Tub2A 7xTetO</i> <i>Tub2B::URA3</i>
3171	<i>Mat a/alpha, ho::LYS2, trp1, SPO11::Myc18::TRP1, leu2, ura3,</i> <i>HIS???, com1::KanMX4, TetR::Myc18::TRP1, Tub2A 7xTetO</i> <i>Tub2B::URA3</i>
3175	<i>MAT a/alpha, ho::LYS2, lys2, scc1-73, leu2, ura3, ade2, ADE2,</i> <i>trp1::hisG, his3::hisG?, rec8::kanMX, Com1::myc18::TRP1,</i> <i>TetR::Myc18::TRP1, Tub2A 7xTetO Tub2B::URA3</i>
3178	<i>MAT a/alpha, ho::LYS2, lys2, leu2::hisG, ura3,</i> <i>COM1::Myc18::TRP1, trp1::hisG, his3::hisG?, TetR::Myc18::TRP1,</i> <i>Tub2A 7xTetO Tub2B::URA3</i>
3184	<i>Mat a/alpha, trp1, COM1::Myc18::TRP1, ura3, rad50S::URA3,</i> <i>his3::hisG??, TetR::Myc18::TRP1, Tub2A 7xTetO Tub2B::URA3</i>

2.3 DNA Techniques

2.3.1 Basic Techniques

Basic techniques such as preparation of Agarose Gels, restriction digests, cloning, etc were performed as described in [98].

2.3.2 Polymerase Chain Reaction (PCR)

Two different PCR protocol conditions were used depending on the application of interest. For analytical purposes, recombinant Taq polymerase from Fermentas was used as described in table 3. Because the error rate of Fermentas Taq polymerase is significant, for preparative purposes we used KOD-HIFI polymerase (TOYOBO) which has a higher fidelity (mutation frequency: 0.0035 versus 0.0039 for pfu DNA polymerase and 0.013 for Fermentas Taq polymerase) and a faster elongation rate (106-138 bases/second versus 25 for pfu DNA polymerase and 61 for Fermentas Taq polymerase). Table 3 and 4 contain the PCR conditions for Fermentas and KOD-HFI polymerase respectively.

<i>Fermentas Taq polymerase PCR conditions</i>	<i>Thermal cycler conditions</i>
• 79 ul ddH₂O • 10 ul Buffer 10x • 5 ul MgCl₂ (1.25 mM final conc.) • 2 ul dNTPs (10 mM) • 2 ul DNA template (100ng for Plasmid or genomic DNA) • 1 ul primer P1 (10 pmol/ul) • 1 ul primer P2 (10 pmol/ul) • 1 ul Fermentas recombinant Taq polymerase 100 ul total volume	• 94°C 3 minutes • 94°C 1 minute • Annealing temp. 30 seconds • 72°C 2 minutes 30 cycles • 72°C 7 minutes

Table 3: PCR conditions for Fermentas recombinant Taq polymerase. In case the PCR product is weak or simply not present, it is possible to increase the final concentration of MgCl₂ to 3 mM. This modification decreases the specificity, but increases total efficiency. It is therefore recommended only for analytical purposes.

<i>KOD HIFI Taq polymerase PCR conditions</i>	<i>Thermal cycler conditions</i>
• 34.6 ul ddH₂O • 5 ul buffer 10x (buffer 1 for plasmid DNA or buffer 2 for Genomic DNA) • 5 ul dNTPs (2 mM) • 2 ul MgCl₂ (25 mM) • 1 ul DNA template • 1 ul Primer 1 (10 pmol/ul) • 1 ul Primer 2 (10 pmol/ul) • 0.4 ul KOD Taq polymerase (2.5 U/ul) 50 ul total volume	• 98°C 1 minute • 98°C 15 seconds • Annealing temp. 5 seconds • 72°C 20 seconds 30 cycles • 72°C 1 minute

Table 4: PCR conditions for KOD HIFI DNA polymerase. Depending on the length of the PCR amplicon, the time of elongation needs to be increased proportionally in order to obtain satisfactory results (up to 60 seconds per 2Kb Genomic DNA target).

2.3.3 DNA gel extraction and/or purification/concentration

using Qiagen Kit

In order to extract DNA from agarose gels, as well as to purify PCR products from primer dimmers and remove salts, we used the DNA gel extraction kit from Qiagen (QiAquick Gel extraction Kit cat.no. 28706).

2.3.4 PCR Mediated One Step tagging strategy

A PCR mediated one step tagging strategy was used in order to tag yeast proteins at the C-terminus with various tags such as MYC::TRP1, HA::HIS3, PK::KanMX and FLAG::KanMX (This technique was initially described by [99] and [100]). Briefly, a DNA plasmid containing the corresponding tagging cassette was used as PCR template. Primers used at this step contained 42 nt homology to the gene of interest (5'end), plus 18 nt at the 3'-end containing homology to the tagging cassette. In order to integrate the PCR amplified tagging cassette into the C-terminus of the gene of interest, the PCR product was concentrated to 12ul with the Qiagen Kit and transformed into the corresponding yeast strain following the protocol previously described.

After 2-3 days of incubation at 30°C, the selective plates presented several colonies that needed to be confirmed by PCR genotyping. For this purpose, Genomic DNA from the colonies to be analysed were prepared by following the protocol described in chapter 2.2.4.1. Positive PCR genotyping was confirmed by performing a western-blot analysis with the appropriate antibody.

<i>FK. Ref. number</i>	<i>Type</i>	<i>Tagging cassette</i>	<i>Primer seq.</i> <i>Primer1:5'-target gene homology (42nt)...</i> <i>Primer2:5'-target gene homology (42nt)...</i>	<i>Reference</i>
330	pUC19	Myc18::TRP1	Primer1: ...TCCGGTTCTGCTGCTAG Primer2: ...CCTCGAGGCCAGAAGAC	Wolfgang Zachariae
331	pUC19	HA3::TRP1	Primer1: ...TCCGGTTCTGCTGCTAG Primer2: ...CCTCGAGGCCAGAAGAC	Wolfgang Zachariae
414	pFA6a- His3MX6	HA6::HIS3		Gustav Ammerer

Table 5: Plasmid tagging cassettes.

2.4 Protein Techniques

2.4.1 Preparation of Yeast TCA crude protein extracts

Required solutions:

- **Trichloroacetic Acid (TCA)**
- **Glass beads**
- **Tris base 1 M pH 8.0**
- **1x GSD buffer:** 40 mM Tris/HCl pH 6.8, 8M urea, 5% SDS, 0.1 mM EDTA, 2% β -mercaptoethanol (or 1% DTT)

Five ml of yeast culture were collected in an eppendorf containing 1.2 ml 100% TCA (20% final concentration). Cells were centrifuged 3' at 6000 RPM, washed once with 1 ml 10% TCA and resuspended in 200 μ l 10% TCA with 200 μ l glass beads. Eppendorf tubes were shaken in a vibrax for 20' at 4°C at full speed. The extract was transferred to a new tube and beads were washed twice with 200 μ l 10% TCA.

All washes were collected in the same eppendorf and then centrifuged for 10' at 3000 rpm at RT.

The supernatant was discarded and the precipitated proteins were resuspended in 200 μ l 1xGSD buffer and 20-25 μ l 1 M Tris base (added for neutralization, the color of the loading buffer should be blue at the end), then boiled for 10' at 95°C. Before loading extracts were centrifuged 10' at 3000rpm at RT.

2.4.2 SDS-Gel electrophoresis

- **10x Running Buffer (5L):** 151.25g Tris, 748g Glycine, 50g SDS.

Glass plates, combs and corresponding spacers were washed with ddH₂O and ethanol before assembly (gel unit assembly is described by supplier). First, the resolving gel was prepared by mixing all components described in the following table and poured into the gel unit until it covered 2/3 of the total volume. Immediately, a few ml of ethanol was added in order to maintain a uniform surface during polymerization. Once the gel was polymerized, the ethanol was removed and the stacking gel was poured, covering the remaining volume in the gel unit. The desired comb was inserted and kept until polymerization was completed.

Finally, the polyacrylamide SDS-gel was submerged into 1x running buffer, the comb was removed and the slots were washed one by one with a syringe filled with running buffer.

Boiled and centrifuged samples as well as the pre-stained molecular weight Marker (BioRad laboratories) were loaded and then electrophoresed at 100V at room temperature for 3 to 4 hours depending on the required resolution.

30% acrylamide-bisacrylamide	1.9 ml
1 M Tris-HCl pH 8.8	2.6 ml
ddH ₂ O	2.4 ml
20 % SDS	70 ul
10 % Ammonium Persulfate (APS)	70 ul
TEMED	6 ul

Table 6: Recipe for resolving gel 8 % .

30 % acrylamide-bisacrylamide	1.7 ml
1 M Tris-HCl pH 6.8	1.25 ml
ddH ₂ O	7.1 ml
20 % SDS	50 ul
10 % Ammonium Persulfate (APS)	50 ul
TEMED	10 ul

Table 7: Recipe for stacking gel.

2.4.3 Western Blot Analysis

2.4.3.1 Western Transfer

- **5x Transfer buffer (1L):** 15.4 Tris buffer, 72.06g Glycine. (1x transfer buffer is supplemented with 15% methanol before use)

Once the proteins were separated on the SDS-polyacrylamide gel (SDS-PAGE), they were transferred to a nitrocellulose membrane (Hydrobond-P, Amersham Pharmacia Biotech) for immunodetection. To accomplish this, the resolving gel was removed from the gel unit and submerged in transfer buffer. The transfer setup consisted of a "sandwich" where the resolving gel was placed between 3MM filter paper on one side and the nitrocellulose membrane followed by more 3MM filter paper on the other side. The transfer sandwich was placed in a plastic frame and transferred to the transfer chamber (BioRad Laboratories) which was filled with transfer buffer. The gel was then electrophoresed at 100V for 1 hour.

To avoid overheating, the transfer chamber contained an ice block and the transfer buffer was stirred with a magnetic stir bar during transfer.

2.4.3.2 Antibody Incubation

- **1xTBS:** 10 mM Tris buffer, 150 mM NaCl, adjust to pH 7.5 with HCl
- **1xTBS-Tween:** 1xTBS supplemented with 1/1000 volume of Tween-20.
- **Ponceau stain:** 0.1%(w/v) Ponceau in 5% Acetic Acid (other variant: 0.1% Ponceau in 30% TCA and 30% sulfosalicylic Acid)

Following protein transfer, the nitrocellulose membrane was placed into small plastic containers, washed once with 1XTBS-Tween, and then incubated with Ponceau stain to visualize the transferred proteins (This step was not mandatory but helped to evaluate the transfer efficiency before proceeding with immunodetection. The presence of the pre-stained marker in the nitrocellulose membrane indicated that the transfer was successful). In order to remove the Ponceau stain, the nitrocellulose membrane was washed several times with water. Once the membranes had recovered their original colour, they were incubated with blocking solution for 1 hour at room temperature with gentle shaking. Then membranes were incubated with the corresponding first antibody diluted in blocking solution for 1 hour at room temperature or overnight at 4°C if required. The primary antibody solution was removed and membranes were washed 3 times for 5 minutes with 1xTBS-Tween buffer and blocked again for 30 minutes at room temperature. Finally the blocked membranes were incubated with the secondary antibody, diluted in blocking buffer, for 1hour at room temperature with gentle shaking. Membranes were then washed 3 times for 10 minutes in 1xTBS-Tween and kept in 1xTBS-Tween until detection.

2.4.3.3 Immunodetection by Enhanced Chemo-Luminescence

(ECL-plus Amersham)

The immunodetection was performed with the chemo-luminescence system ECL-plus (Amersham) kit. Briefly, the detection solutions A and B were mixed in a ratio 40:1 (the final volume required per membrane is 0.1 ml/cm²). The nitrocellulose membranes were removed from the TBS-Tween solution, excess liquid was removed and the detection mixture was poured on the membrane surface. Membranes were incubated with the detection mixture for 5 minutes, then the liquid was carefully removed, membranes were covered in saran wrap and exposed to X-ray film.

2.5 Chromatin-ImmunoPrecipitation (ChIP) Assay

The following protocol is designed to perform yeast chromatin immunoprecipitation to be analysed either by Quantitative Real-Time PCR (qPCR) or by hybridization to high density microarrays. The original version was kindly provided by K. Shirahige's Lab[101], from the Tokyo Institute of Technology, during my visit in 2005. The final version was obtained by combining it with a previous protocol used in the lab[102]. Initial cell pellets required for this protocol were obtained by the *Meiotic Time course* protocol previously described in this document.

2.5.1 Yeast extract preparation

Cell pellets (50 ml = 20×10^7 cells) were resuspended in 1600 μ l Lysis buffer and equally distributed to four 2-ml screw-cap tubes containing ca. 600 μ l of glass beads (*see Note 2.5.1.1*). To disrupt cells, the tubes were placed into a multibeads shocker (YASUI-KIKAI, Osaka, 2500 rpm 28 times cycles of 30 sec ON/ 30 sec OFF at 4°C) or into a pre-cooled vibrax unit (14 cycles of 1 min ON/ 1 min OFF) at 4°C. At this stage, breakage efficiency was checked by phase contrast microscopy (*see Note 2.5.1.1*).

After breakage, a small hole was poked into the cap of the screw-cap tubes, and the tubes were placed in an inverted orientation into a 15ml falcon tube. The cell extract was then collected by centrifugation (4°C, 1 min, 3000 rpm), transferred into a 1.5 ml eppendorf tube, and centrifuged again for 1 minute at 5000 rpm, to remove air bubbles. Finally, the cell extracts were sonicated to shear chromatin to achieve the desired length. An average fragment length of slightly above 500 bp was achieved by sonicating 5 times at 37% power for 15 seconds (*see Note 2.5.1.2*).

2.5.2 Chromatin Immunoprecipitation

50 μ l of pan mouse IgG magnetic Dynabeads per sample were washed twice with 1ml of PBS/BSA. (Add liquid, vortex, fix beads at the bottom of the tube with the magnet, while removing liquid). Add 250 μ l primary antibody (*see Note 2.5.2.1*) (e.g., anti-Myc Antibody for IP against the Myc epitope) in PBS/BSA was added to the beads and incubated for 3 hours at 4°C with constant rotation (*see Note 2.5.2.2*). The beads were washed twice with 1 ml PBS/BSA and resuspend in 100 μ l PBS/BSA.

After sonication, yeast extracts were centrifuged at 12000 rpm for 5 minutes to remove cell debris. The supernatant was transferred to a new 1.5 ml Eppendorf tube, and a 20 μ l aliquot was removed to prepare whole cell extract (WCE, keep at 4°C). Note that the WCE was

prepared from each sample, because the final result is expressed as IP/WCE (sample) to correct for different amounts of template in the input.

The remaining extract was incubated with the antibody-coated Dynabeads (25 µl per aliquot) for 1 to 3 hours at 4°C with rotation or rocking.

Beads were then washed as follows:

- 2 times with 1 ml lysis buffer
- 2 times with 1 ml lysis buffer /360 mM NaCl
- 2 times with 1 ml washing buffer
- 1 time with 1 ml TE.

Samples were centrifuged at 1000rpm for 10 seconds and the supernatant was removed completely. (Hold beads at the bottom of the tube using the magnet while pipetting).

40 µl of elution buffer was added and incubated at 65°C for 15 minutes with shaking. The tubes were centrifuged at 12000 rpm for 1 minute and the supernatant was transferred into a new Eppendorf tube containing 160 µl of TE/1% SDS.

To 20 µl of WCE, 380 µl of TE /1% SDS was added and the sample was split in half.

Samples were incubated overnight at 65°C to reverse the cross-linking.

2.5.3 DNA purification

To each of the 200 µl aliquots, 140µl TE, 3µl Glycogen (10mg/ml) and 7.5µl of Proteinase K (20mg/ml) were added and incubated at 37°C for 2 hours.

Samples were extracted twice with phenol/chloroform/isoamylalcohol (300µl PCI was added, the tubes were briefly vortexed, then centrifuged at 12000 rpm for 1 minute and the upper phase was recovered with care to avoid contamination from the interphase).

The recovered phase was supplemented with NaCl (200 mM final concentration) and 2 volumes of 96% Ethanol. Samples were vortexed and incubated at -20°C for at least 30 minutes.

Samples were centrifuged for 30 minutes at 4°C. The DNA pellet was washed with ice-cold 70% Ethanol.

The DNA pellet was dried, then resuspended in 30 µl TE/RNase solution (4 ug DNase-free RNase) and incubated at 37°C for 1 hour. At this point, the different IP aliquots, including the WCE aliquots that were split in four at the beginning were pooled.

(Optional) An additional phenol/chloroform/isoamylalcohol extraction can be performed (*see Note 2.5.3.1*), but will result in reduced yield.

Purified DNA was precipitated by adding 1/10 volume of 3M Sodium Acetate and 2 volumes 96% ethanol, then incubated at -20°C for at least 30 minutes.

Precipitated DNA was washed with ice-cold 70% ethanol, dried and resuspended in 30µl ddH₂O. Samples were stored at -20°C until analysed.

2.5.4 Quantitative, Real-Time PCR analysis (qPCR)

Many different protocols and reagents for real-time analysis of the precipitated DNA are available. We used the "SYBR Green" method, because it provides flexibility in choosing different chromosomal positions. Alternatively, if the analysis is performed routinely at the same chromosomal positions, techniques requiring modified oligonucleotides and which offer the possibility of multiplexing may also be economical. We use an iQ5 instrument (Biorad), but the "SYBR Green" method can also be performed on simpler real time PCR stations. Also "SYBR Green" can be exchanged for "MESA Green" (Eurogentec), which offers a larger dynamic range for monitoring the exponential amplification phase. For DNA quantification of a sample, the instrument needs a minimal series of 3 dilutions of a standard of "known" concentration. All measurements will be expressed relative to this standard. For the purpose of quantifying ChIP we recommend using WCE-DNA as a standard. To obtain meaningful results, ChIP must be performed in parallel using the same conditions on cells lacking the tag (as a negative control to tagged baits), or on cells lacking the bait (if antibodies against the bait are used). Only the difference in signals between tagged and untagged (or plus/minus bait) can be regarded as specific. Here quantitative Real-Time PCR (qPCR) was performed as follows:

A series of three 10-fold dilutions of the WCE sample (e.g. 1/30, 1/300, 1/3000) were prepared.

PCR mix per sample:

12.5 µl IQ SYBR Green Supermix	
0.5 µl Primer oligo mix	(10pmol/µl stock)
9 µl ddH ₂ O	
3 µl DNA sample	(IP sample or WCE dilution)

25 µl total reaction volume

One master mix was prepared for all samples per each primer pair (i.e. per analyzed locus). All samples were performed in duplicate (see **Note 2.5.4.1**). Note that per primer pair 6

wells are required for the WCE dilution series, plus two wells for each sample. Thus it is economical to group samples analyzed with compatible primer pairs together in a run.

First 22 µl of the master mix was filled into the wells of an optical 96-well plate and then the DNA samples (3 µl of either IP or WCE dilution) were added (*see* **Note 2.5.4.2**).

A sealing film was placed over the 96-well plate to avoid evaporation. The plate was centrifuged for 1 minute at 500 rpm to remove air bubbles and placed into the real-time thermo cycler.

qPCR was performed with the following setting (*see* **Note 2.5.4.3**):

Cycle1: (1x)

Step 1: 94,0 °C for 03:00.

Cycle2: (40x)

Step 1: 94,0 °C for 00:15.

Step 2: 60,0 °C for 01:00.

Data collection and real-time analysis enabled.

Step 3: 72,0 °C for 01:00.

Cycle 3: (1x)

Step 1: 94,0 °C for 01:00.

Cycle 4: (1x)

Step 1: 60,0 °C for 01:00.

Cycle 5: (71x)

Step 1: 60,0 °C-95,0 °C for 00:30.

Increase set point temperature after cycle 2 by 0,5 °C

Melting curve data collection and analysis enabled.

During the qPCR run, the amplification can be observed online. This allows one to stop the run manually, after all samples have reached the plateau. (If this is done, the melting curve analysis has to be initiated manually.)

Even though many programs offer a fully automated evaluation, it is useful to understand the underlying algorithms to be able to judge the quality and robustness of the obtained results. We can evaluate the amount of chromatin immunoprecipitated relative to the standard curve established for the WCE samples, and compare the fold enrichment obtained for the region of interest and the cold-spot region.

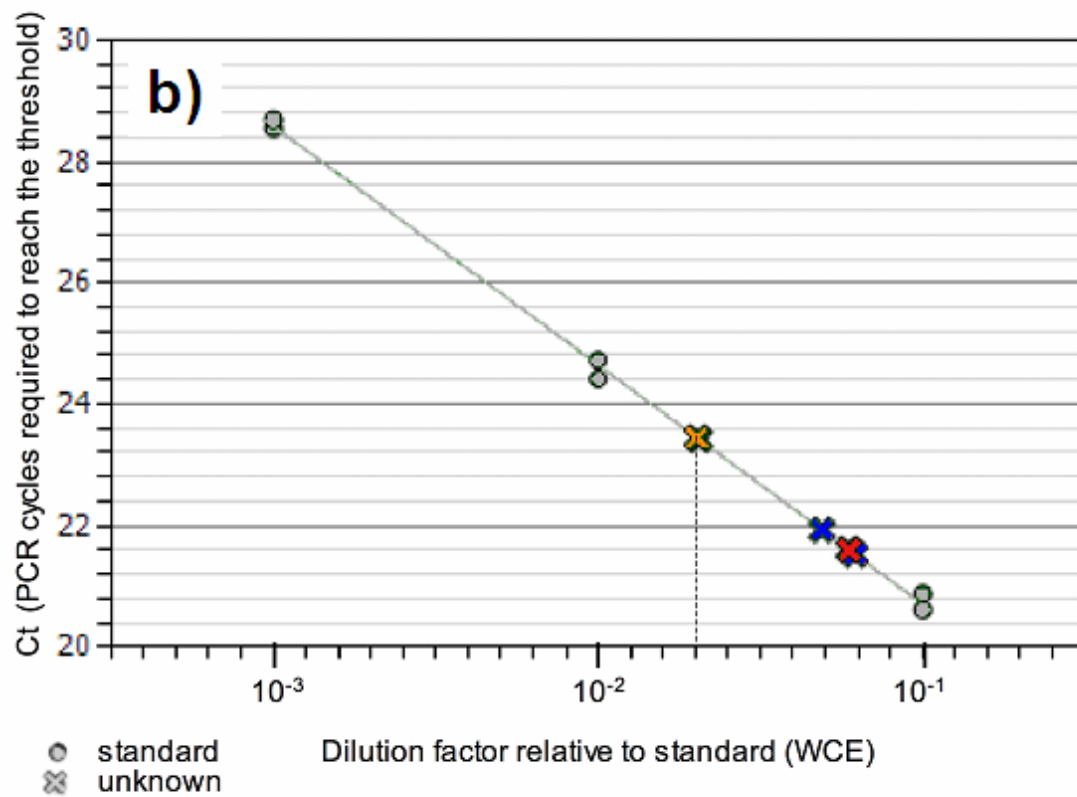
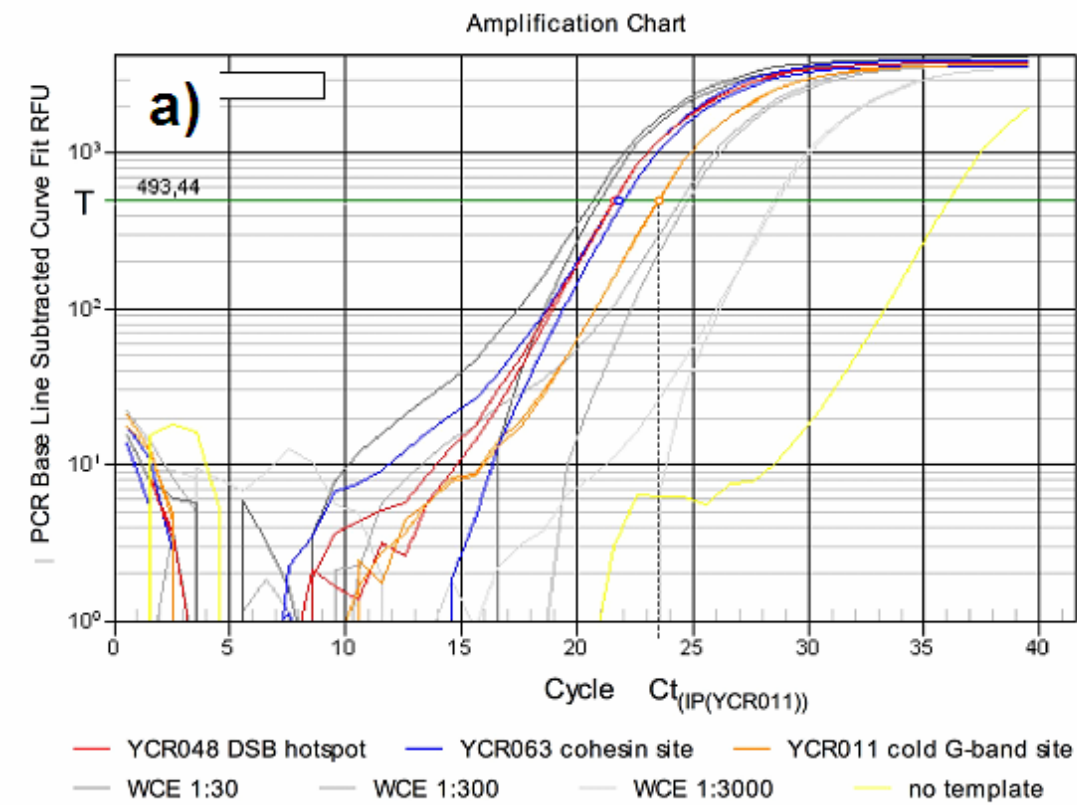
2.5.5 Interpretation of qChIP results

The generation of data can be observed in “real time” during the PCR run. After all dilutions have reached the plateau, a melting analysis should be carried out. Since the melting temperature strongly depends on the product length, this is a convenient method to detect, and if necessary quantify, a primer dimer product, - a template-independent artifact, which could severely distort the result. (For an example see **Fig. 15c, d**. Primer-dimer formation occurs rarely if the “hotstart” strategy is used.)

Typical qPCR results are shown in **Fig. 15** (three unknowns and a “no template” control). The semi-logarithmic plot transforms the exponential amplification into a straight line and the program suggests a threshold (T), at which all curves should be in the linear range. This can be manually edited. Within the linear range such changes will have only very small effects, if all curves are parallel. T should not be chosen from too early cycles, where curves tend to deviate from linear. Much of this is likely caused by the FITC (spiked-in for “volume” control) and by the attempt of the system to subtract this signal (baseline subtraction).

From the number of PCR cycles required to reach the threshold, the original amount of template is estimated (**Fig. 15b**), based on the assumption that amplification in the early “invisible” cycles occurred with the same efficiency as in the “visible” ones. The results are expressed relative to the standard (for which we use WCE, treated under similar conditions as the IP samples). Relative comparisons, such as time point x to time point zero (for meiosis-specific interactions), or cells containing the antigen (or tag) versus cells not containing it, are most intuitive and can be done right away.

In order to refer to the efficiency of the technique, it is useful to refer to TT, the “total template present in the input”. The way we have defined the standard in **Fig. 15b**, a value of 1 corresponds to the amount of template present in 1 μ l of the WCE (1 μ l contains 1/2400 of TT). Since only 1/10 of the precipitate is used in the unknown IP samples, division by a factor of 240 converts IP values from WCE to TT units. (For example, the value of 6% of WCE, reached by Mre11-HA at the hotspot at t=4 hours in **Fig. 16**, corresponds to 0.025% of TT). This demonstrates that ChIP only recovers a very small fraction of the total template present. This may be due to the low efficiencies of the IP and artificial cross-linking. In addition, Mre11 may not occupy all templates. Nevertheless, the dynamic range between Mre11-HA and untagged is sufficiently large to identify even very weak binding sites. (The cold G-band at YCR011 is close to the weakest binding sites of Mre11 on chromosome 3, yet it is still significantly different from untagged, see **Fig. 16**).



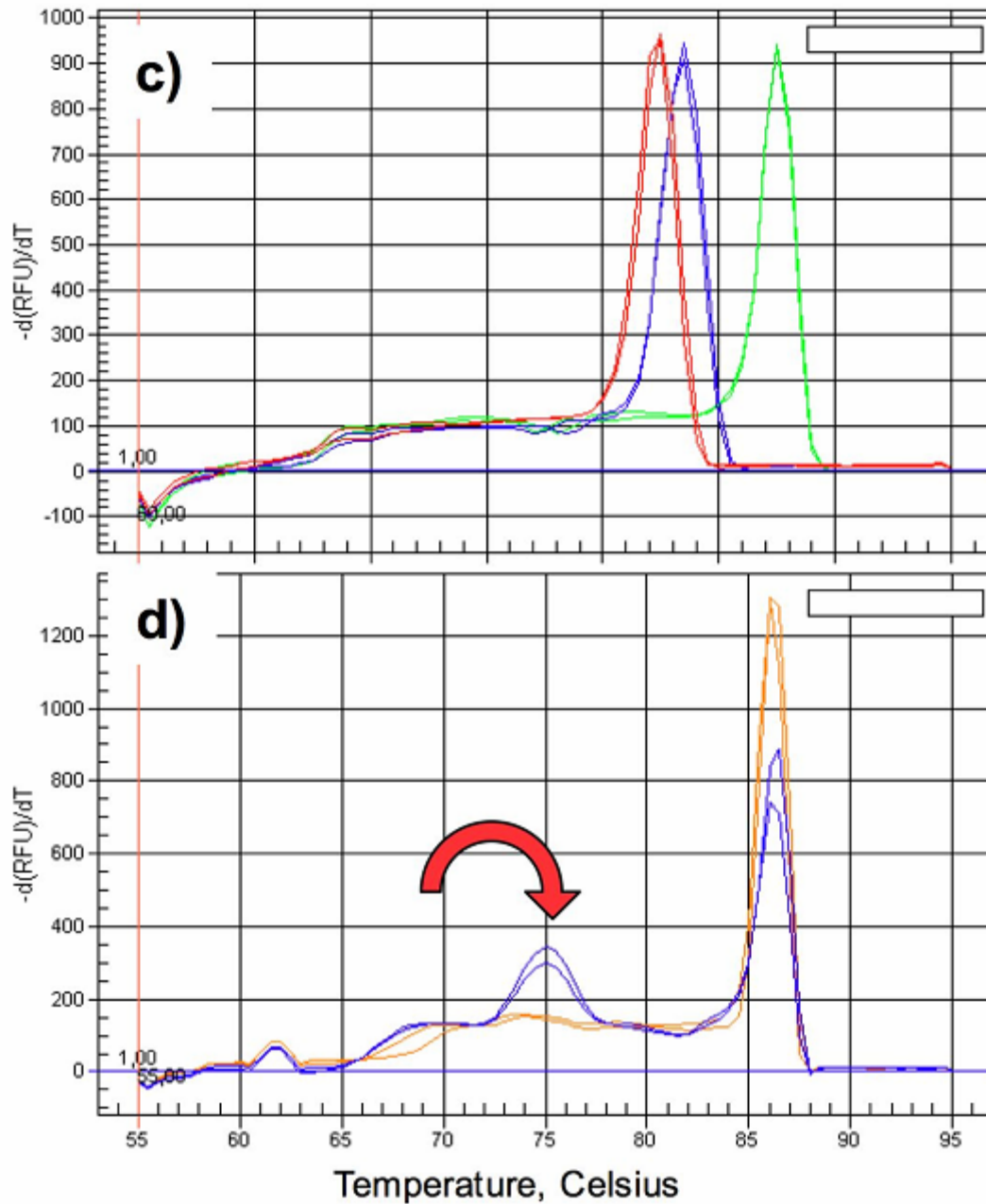


Figure 15: Interpretation of qPCR data. Amplification chart. Standard curve dilution series is represented by WCE 1:30, 1:300 and 1:3000 respectively (gray lines). Three different unknown samples are also represented in colored lines (a). Semi-logarithmic plot showing Ct value versus DNA initial Concentration. Estimation of DNA concentrations from the unknown samples by interpolation from the linear behaviour of the Standard curve dilution series is represented (b). Melting curve analysis (c, d). Melting curve analysis for the amplified unknown samples is represented in (c). In (d), Sample presenting a specific amplification pattern (orange line) in contrast to the situation where the same sample presents primer dimer products (red arrow) competing with the expected target amplicon.

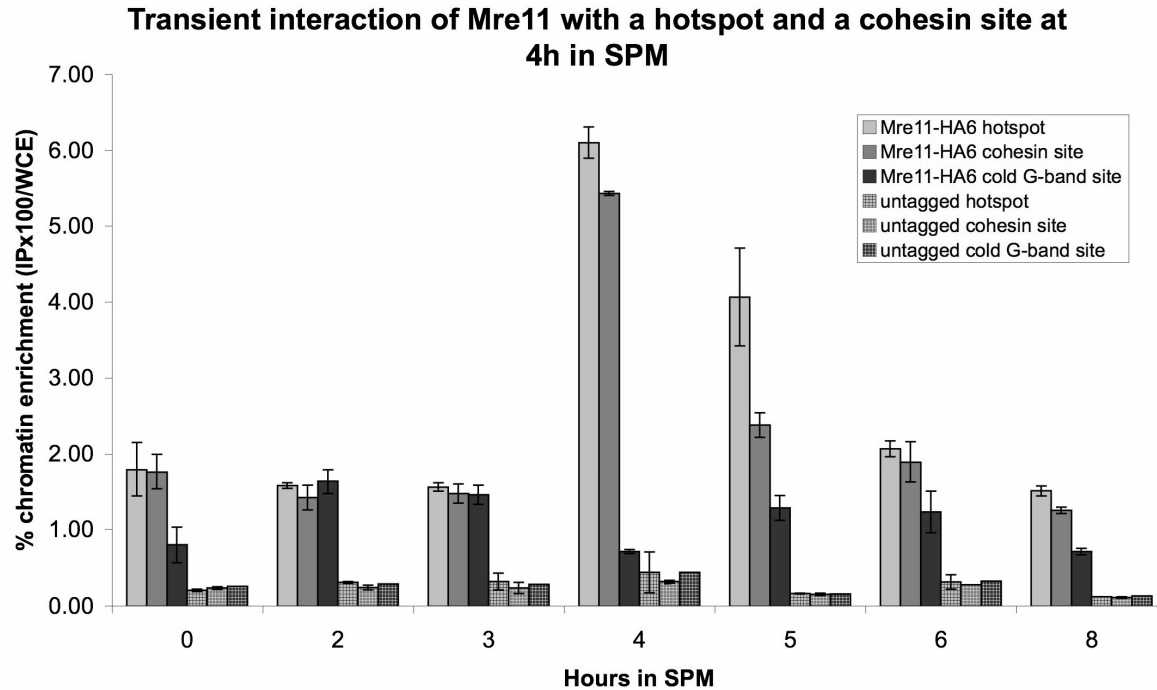


Figure 16: qPCR analysis of Mre11-chromatin interaction over meiotic time course.

2.5.5.1 What influences the qChIP result?

Probably the most important parameter is the fraction of template occupied by the bait during crosslinking. However, the number of bait molecules per template will also significantly influence the qPCR value, as long as the efficiency of the IP is not close to 100%. Third, but not least, the efficiency of cross-linking has a drastic effect. This includes the availability of side chains to interact with formaldehyde and DNA, but also whether the bait interacts directly with the DNA or via another protein. Finally and perhaps most importantly, solubility after crosslinking may be limiting.

2.6 Chromatin-Immunoprecipitation hybridized into High resolution Microarray DNA chips (ChIP on chip)

In order to study chromatin-protein interactions in a genome wide manner, the chromatin-immunoprecipitation strategy is combined with hybridization to microarray DNA chips. For this purpose, the immunoprecipitated DNA needs to be amplified, fragmented and labelled prior its hybridization.

Depending on the type of DNA arrays in use, as well as the detection platform, the protocol required to perform the steps prior to hybridization can differ. In our case, we used *S. cerevisiae* Affymetrix Tiling arrays having 25 bp oligo probes with an average probe overlap of 20nt (currently termed a 5 nt resolution array). The Affymetrix detection platform requires staining with a single dye, Phycoerytrin (excitation wavelength: 488nm), and the use of the automated washing station in order to stain and wash the hybridized probe arrays prior scanning.

2.6.1 Modified Chromatin Immunoprecipitation (K. Shirahige's protocol, TITec)

Chromatin Immunoprecipitation was performed as previously described. In order to use the immunoprecipitated DNA for hybridization to probe arrays, the purified DNA was concentrated to a volume of 10 µl by Ethanol/NaAc precipitation (1/10 Volume 3M NaAc, 2xVolume 96% Ethanol. After 30 minutes at 4°C, DNA was centrifuged at maximum speed and washed once with 70% Ethanol). In order to perform genome wide PCR amplification, 7 ul of the concentrated DNA was removed for the following steps. The remaining 3 ul were diluted to a final volume of 24 ul and used for qPCR analysis. This was done in order to evaluate the quality of the immunoprecipitated DNA prior to hybridization. In the same way we are able to evaluate the absolute amount of immunoprecipitated DNA in contrast to ChIP on chip analysis, where we evaluate the abundance of protein localization on the chromatin relative to the total Input DNA (called total whole Cell extract or WCE). The "relative" nature of this assay needs to be complemented by a qPCR analysis in order to estimate the absolute amount of the protein of interest on the chromatin.

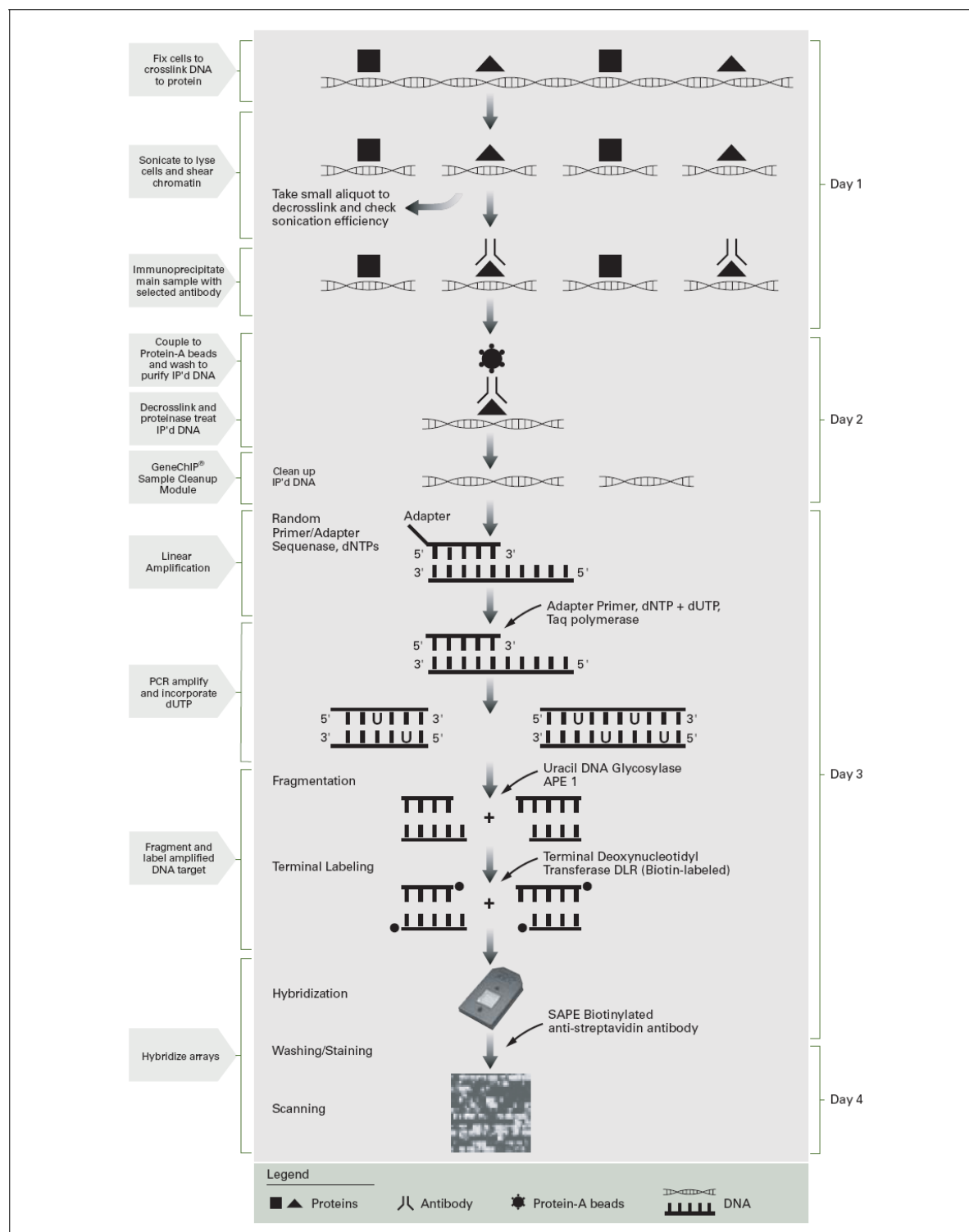


Figure 17: Schematic representation of ChIP on chip assay [103]. Instead of the Uracil DNA Glycosylase fragmentation step, we perform a controlled DNase I fragmentation procedure.

2.6.2 Random PCR amplification

Because the amount of immunoprecipitated DNA is too low (below the sensitivity range of the Nanodrop Spectrophotometer, which is 2 ng/ul) to be directly detected by hybridization onto a DNA microarray chip, a PCR amplification step is required. The PCR amplification needs to cover all pulldown fragments in an unbiased manner. The amplification procedure must not alter the differences in abundance between different pulldown fragments that exist prior to amplification. The method of choice in our case involves the use of a random primer which contains a consensus sequence at the 5'-end. During two linear rounds of amplification the random primer is used in order to introduce the consensus sequences at the ends of all pulldown fragments (see **Round A**); then an oligomer complementary to the 5'-end consensus sequence is used in a second step for an exponential amplification (see **Round B**).

2.6.2.1 Round A: Linear amplification

This first step is performed to introduce the consensus sequences into our immunoprecipitated genomic DNA. Two rounds of linear amplification are performed with Sequenase™ Version 2.0 DNA Polymerase at 37°C in the presence of a random primer containing the consensus sequence at the 5'-end.

Primer A: GTTTCCTCAGTCACGATCNNNNNNNNN

Setup:

- 7 ul DNA IP (or 5 ul for wce)
- 2 ul Sequenase buffer 5x
- 1 ul Primer A (40 uM)

Reaction mix:

Prepare a reaction mix **per sample** as follows:

- 1 ul Sequenase buffer 5x
- 1.5 ul dNTPs (3 mM)
- 0.75 ul DTT(0.1 M)
- 1.5 ul BSA (500 ug/ml)
- 0.3 ul Sequenase

Total volume = 5.05 ul

Furthermore, dilute a Sequenase sample as follows (1/4 dilution):

- 3.6 ul sequenase dilution buffer
- 1.2 ul Sequenase

Total volume = 4.8 ul

The setup mix was incubated at 94°C for 2 minutes, then 10°C for 5 minutes. During the 10°C hold, the reaction mix (5.05 ul per sample) was added. Samples were then incubated at 37°C for 8 minutes, 94°C for 2 minutes, and 10°C for 5 minutes. During the 10°C hold, 1.2 ul of diluted Sequenase was added and the reaction was incubated at 37°C for 8 minutes.

In order to proceed with the exponential amplification, samples were diluted in ddH₂O to a final volume of 78 ul (add 61.75 ul).

2.6.2.2 Round B: Exponential amplification

The exponential amplification was performed in the presence of consensus primer B which anneals to the consensus sequence introduced during round A.

- 78 ul DNA (from round A)
- 10 ul KOD-XL 10x buffer
- 1 ul Primer B (100pmol/ul)
- 10 ul dNTPs (2mM each)
- 1 ul KOD-XL polymerase

Total volume= 100 ul

1	95°C	5 min.	
2	98°C	20 sec.	
3	40°C	30 sec	
4	50°C	30 sec	
5	72°C	3 min.	go to step 2 31x
6	72°C	7 min.	
7	4°C	hold	

2.5 ul of the amplification product were loaded onto a 1% agarose gel. The amplification product appeared as a smear between 300 – 2000 bp with an average of 500-1000bp. (the IP samples presented a broader smear than WCE).

The remaining sample was purified and concentrated to a volume of 42 ul with microcon columns (MILIPORE YM-100 6000rpm, 15 min at RT) and the total amount of DNA was estimated with the Nanodrop spectrophotometer.

For microarray DNA hybridization at least 5ug of amplified material is required, though up to 7 to 10 ug of DNA per sample can be used.

2.6.3 DNA fragmentation

In order to perform the microarray hybridization, DNA must be fragmented to an average length of 50-100 bp . For fragmentation we used a DNase treatment.

DNaseI setup:

- 2 ul DNaseI (1 U/ul)
- 2 ul One-phor-all-buffer plus (10x)
- 1.2 ul CoCl₂ (25mM)
- 8 ul ddH₂O

Reaction Mix:

- 40.75 ul amplified DNA
- 2.9 ul CoCl₂ (25mM)
- 4.85 ul One-phor-all-buffer plus (10x)
- 1.5 ul DNaseI setup (added when the PCR block has reached 37°C)

37°C 2min, 95°C 15min.

2.5 ul of sample was loaded onto a 2% Agarose gel, which was run briefly. When the average fragment size was above 100bp the procedure was repeated by adding 1 ul of DNaseI into the remaining DNaseI setup and then adding 1.5 ul of the DNaseI setup into the reaction mix.

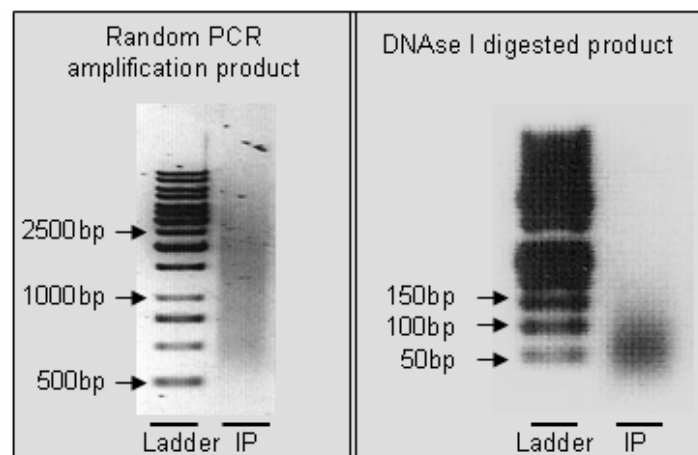


Figure 18: Random PCR amplification pattern before (left) and after DNaseI digestion (right).

2.6.4 Fragmented DNA labelling

Once the DNA was fragmented, it was labelled at the 5'-ends by using terminal transferase and a Biotinylated-N11-ddATP.

- 47.5 ul fragmented DNA (from DNaseI treatment)
- 1 ul Biotin-N11-ddATP (1nmol/ul Perkin Elmer NEL508)
- 12 ul Terminal Transferase buffer 5x
- 1 ul Terminal Transferase (Roche 220582)

The labelling reaction was incubated for 1hour at 37°C.

2.6.5 Affymetrix Microarray DNA chip hybridization

After the DNA was 5'-end labelled with Biotinylated ddATP, a hybridization cocktail was prepared as following:

First the number of microarray chips to be hybridized were equilibrated at RT for 15min. It is important not to break the septa during sample loading.

Following equilibration, 250 ul of 1x pre-hybridization buffer was loaded into the probe array and incubated at 42°C with constant rotation for at least 15 minutes.

During the pre-hybridization step, the following hybridization cocktail was prepared:

- 60 ul labeled DNA
- 3.3 ul Oligo B2 controls (3nM)
- 2 ul Herring Sperm DNA (10 mg/ml)
- 60 ul SSPE 20X
- 10 ul Triton-X 100 (0.1%)
- 64.7 ul ddH₂O

200 ul total

The mix was incubated at 99°C for 10 minutes, and then chilled on ice for 5 minutes. Finally, the hybridization mix was centrifuged at full speed for 5 minutes in order to precipitate insoluble material.

The pre-hybridization solution was removed from the chip and 170 ul of the hybridization cocktail was loaded. The probe arrays were incubated at 42°C for 16 hours with constant

rotation. Following this incubation the hybridization cocktail was recovered and placed into its original eppendorf (stored at -20°C it can be re-hybridized at least two times more if necessary), and the probe array was filled with 250 ul of the less stringent washing buffer A and was kept at RT until washing.

2.6.6 Microarray chips Washing and staining procedure

The washing station requires two samples, the staining solution mix containing SAPE, (Streptavidin conjugated to Phycoerythrin), and one Antibody amplification sample (containing Anti-Streptavidin Goat Antibody conjugated to a biotin residue, as well as an Anti-Goat Antibody).



Figure 19: Schematic representation of staining of Hybridized DNA. The affymetrix version uses an anti-Streptavidin (goat) biotin conjugated antibody; however Goat IgG is added in order to avoid non specific interactions.

SAPE solution Mix:

- 600 ul 2xMES buffer
- 48 ul acetylated BSA (50 mg/ml)
- 12 ul SAPE (1 mg/ml)
- 540 ul ddH2O

Total=1200 ul → Split in two eppendorfs (protect from light all the time)

Antibody (Ab) mix:

- 300 ul 2x MES buffer
- 24 ul acetylated BSA (50mg/ml)
- 6 ul Goat IgG (10mg/ml)
- 3.6 ul biotinylated Antistreptavidin (Goat 0.5 mg/ml)
- 266.4 ul ddH2O

600 ul total

The Washing protocol used is called "EukGE-WS2v5" and was performed on the Affymetrix Fluidic station 450 (see figure 20) as follows:

Automated steps performed into the fluidic station 450	EukGE-WS2v5 protocol
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C
Post Hyb Wash #2	6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
Stain	Stain the probe array for 10 minutes in SAPE solution at 35°C
Post Stain Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C
2nd Stain	Stain the probe array for 5 minutes in antibody solution at 35°C
3rd Stain	Stain the probe array for 5 minutes in SAPE solution at 35°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C. The holding temperature is 25°C
Holding Buffer	Buffer A loaded in the Final Wash is kept for Scanning

Table 8: Fluidic station protocol used for washing *S. Cerevisiae* 1.0R Tiling arrays.

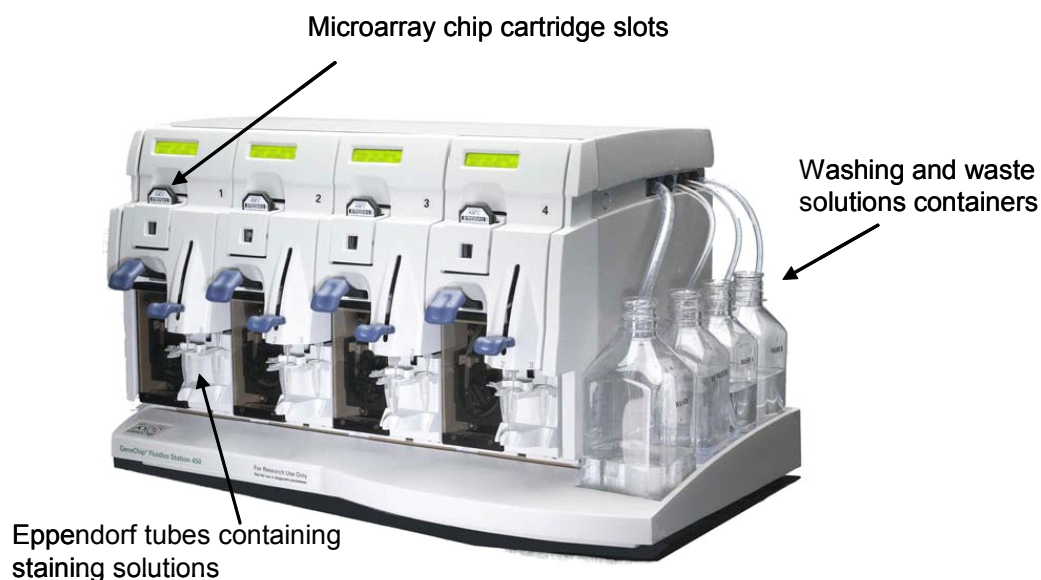


Figure 20: Affymetrix Washing station 450.

2.6.7 Microarray chips Scanning and data treatment

Following the washing step, the hybridized microarray DNA chips were scanned. If necessary, hybridized probe arrays may be stored at 4°C for several hours before scanning, but must be warmed back to RT before being scanned. The GeneChip scanner 3000 TG is controlled by GeneChip Operating Software (GCOS).

Before the probe array was scanned, the glass surface was cleaned, if required, with a soft tissue (kimwipe). Alcohol can not be used to clean the glass.

The liquid level inside of the array was checked to insure the array was full, if it was not, air bubbles were manually removed and the inner chamber was filled with non-stringent buffer A. The excess fluid from around the septa was cleaned and one Tough-Spot was applied to each septum.

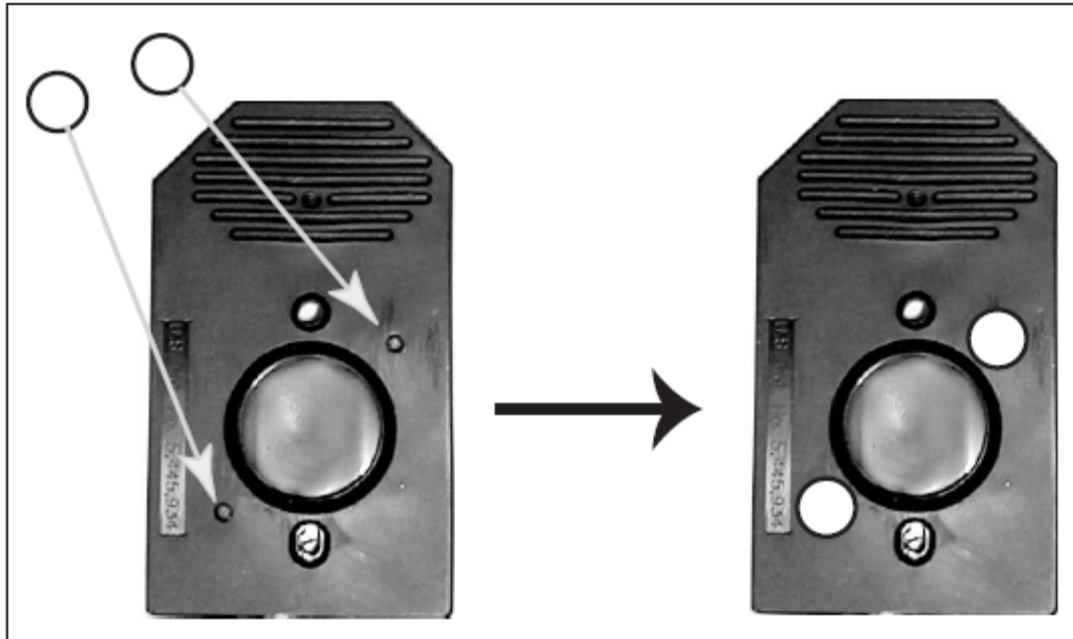


Figure 21: Applying Tough-Spots to the probe array cartridge

Finally the probe array was inserted into the scanner and scanning was performed as indicated in the Affymetrix instruction manual [103].

2.6.7.1 Comparative analysis of ChIP chip data

Microarray scanning produces a file which contains a high resolution picture of the array surface (DAT file). In order to define borders than can be used for further analysis, during the Hybridization process a control oligomer is currently added into the hybridization cocktail (Oligo B2, see Affymetrix DNA hybridization procedure). The Oligo B2 hybridization pattern defines a grid (a single grid is known as a probe cell) which is used to perform local fluorescent signal normalization in order to correct for non-uniform hybridization patterns over the array surface. As part of GCOS treatment, a new file is generated (CEL file) which contains a single intensity value for each probe cell delineated by the grid (calculated by the Cell Analysis algorithm).

Because we are interested in a relative enrichment during the chromatin immunoprecipitation process (IP for immunoprecipitation) compared to the total input DNA (WCE), a comparative analysis between the IP and the corresponding WCE CEL file needs to be performed.

Comparative analysis between CEL files is widely explained in the literature; in this chapter a brief description of such procedure as well as the available tools available for this purpose are presented.

2.6.7.2 ChIP-chip data normalization

Comparative analysis of probe arrays requires a normalization treatment in order to correct for differences in total hybridized DNA between compared CEL files.

The easiest way to perform such a normalization is to use a Median/Mean normalization[104], where the Median/Mean of the base-two logarithm of the ration of IP to WCE signal intensities is calculated and then subtracted to each log ratio value of each tile on a single array position. This procedure transforms the log-ratio distribution by centering it at zero, assuming that the Median/Mean represents the background/noise trend in the distribution and, consequently, it remains applicable in cases where up to 50% of the probes display a distinct behaviour compared to the noise/background base line.

Taking into consideration that intensity-specific artefacts (coming for instance from an uneven hybridization pattern) can produce different background/noise intensities over the array surface, an alternative method, called LOESS normalization[105] (for locally weighted regression and smoothing scatterplots), first uniformly samples 50000 log-ratio values from the original data, to then perform a locally weighted regression on the sampled data. Finally the newly estimated "correction factor" is subtracted from the corresponding log-ratio

sample values. Then another set of 50000 sample log-ratio values are processed, until the total number of signal log ratio values have been processed in this manner. The main disadvantage of Loess normalization is that it is a computationally intensive method.

In a more stringent method, called Quantile Normalization[106], intensity distributions are adjusted to be equivalent. For this, the signal intensities of each experiment are sorted into quantiles, and then the intensities in each quantile across the group of experiments are averaged. In contrast to LOESS and Mean/Median methods, Quantile Normalization does not require intensive computational treatment and also corrects for intensity-specific artefact. However, this method seems to be too stringent in certain situations, eliminating the variation in the degree of binding among experiments.

The methods described above were the most commonly used protocols for Gene expression analysis and have since been extended to ChIP-chip experiments. Nevertheless, the Asymmetric nature of ChIP-chip data (in this case we expect only positive enrichment in contrast to gene expression analysis where genes can be over or under regulated), as well as the high proportion of signal enriched sites in the case of certain specific targets, histones for example (in gene expression analysis it is assumed that the proportion of differentially expressed genes is small and by consequence a Mean/Median strategy is able to identify the base line trend), means that these protocols are not always suitable tools for Normalizing ChIP-chip data. In light of this, new methods specifically designed for ChIP-chip data have been developed recently[107], However, even though new algorithms have been proposed, there are no computational platforms currently available that are supplied with these algorithms. Consequently, their application remains restricted to groups capable of developing their own software. For this reason, there is a limited choice of software tools available for the treatment of ChIP-chip data and available options are further reduced as they tend to be technology specific (two-color microarray chips from NimbleGen or Agilent and single-color microarray chips from Affymetrix cannot, in general, be processed by the same software). Additionally, the output data format produced by such available tools is not flexible enough in most of the cases. The most popular output format is a BAR file which associates signal intensity with the physical position of the corresponding probe on the array surface. This file format can only be opened in a Genome browser, where the information on the physical position of each probe on the array surface is associated to the chromosome position in order to create a chromosome map displaying the corresponding relative signal intensities. . However, these genome browsers generally only allow screen shots of interesting regions, without the ability to export the whole genome or chromosome data into a text file format.

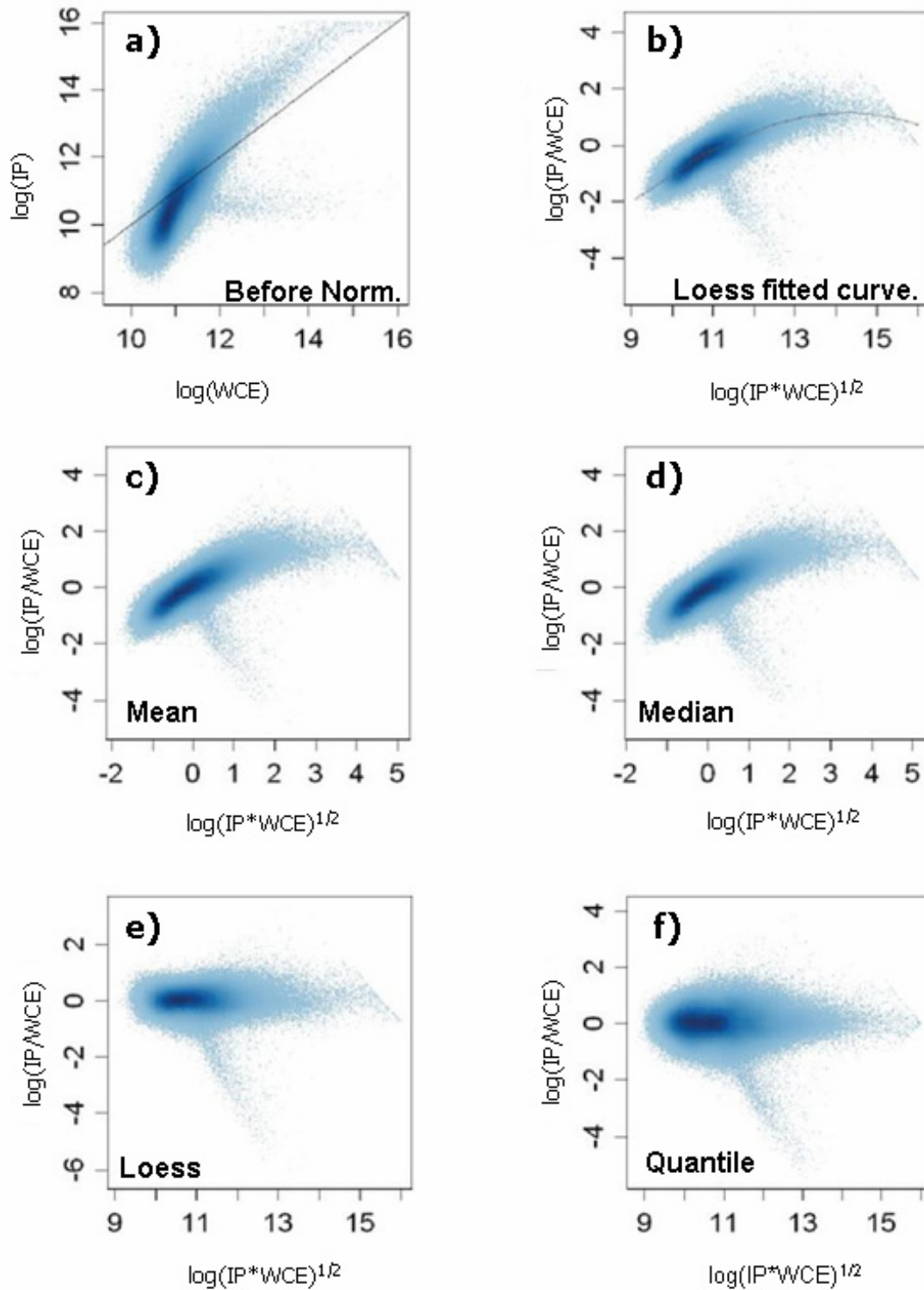


Figure 22: Different normalization methods: Log-intensity plot before normalization. The gray line represents where the IP and the WCE signal intensities are equal (a), A Loess regression curve fitted during normalization is represented in (b). Mean and Median normalization are shown in (c) and (d). Finally Loess and Quantile normalization are illustrated in (e) and (f). Notice that only Loess and Quantile are able to correct for intensity-specific artifacts (Signals distribution in the scatter plot is centered to zero even for higher IP WCE average log signals). Adapted from [108].

Taking into consideration the described problems associated to ChIP-chip data treatment, we could suggest tools such as, Telescope[108], CisGenome (which includes TileMap)[109], mpeak[110], MAT[111], R packages from Bioconductor (like “affy” or “oligo”) and the Tiling Analysis Software (TAS) from Affymetrix. Data analysis presented in this document was performed primarily in TAS using a quantile normalization approach.

Figure 23 illustrates the data normalization treatment performed on CEL files corresponding to the Hop1p ChIP-chip experiment. In order to visualize the effect of the normalization treatment, signal intensities coming directly from the IP CEL file have been compared to those in the WCE CEL file. To achieve this, we constructed a scatterplot of $\text{Log}_2(\text{IP}/\text{WCE})$ (called “M” for minus or difference) against $\text{Log}_2(\text{IP} \times \text{WCE})^{1/2}$ (called “A” for average or mean). This scatterplot shows the variability of the factor M as a function of the mean A. In the absence of hybridization artefacts, the points on the MVA plots (for M versus A) should be randomly scattered around the M=0 line. In reality, the scatterplot is shifted from the M=0 line before normalization, and is corrected by the data normalization treatment. Finally, the normalized data can be associated to a physical chromosome position and represented in a 2D plot showing the relative signal intensity $\text{Log}_2(\text{IP}/\text{WCE})$ on the Y axis and the chromosome position on the X axis. This representation is the final output of the ChIP-chip experiment, complemented quite often by a statistical analysis associated to each data point to demonstrate significance.

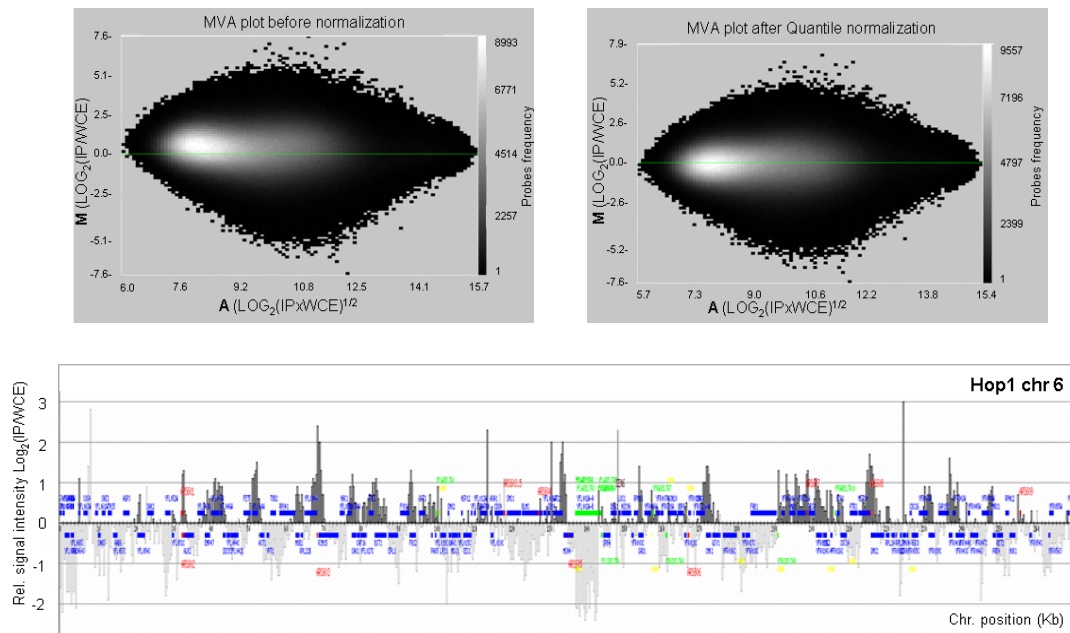


Figure 23: ChIP-chip data normalization. Signal intensities coming from ChIP-chip CEL files performed in Hop1p are represented in MVA plots, before (upper left panel) and after quantile normalization (upper right panel). Normalized data is then associated to physical chromosome positions in a 2D map representing the $\text{Log}_2(\text{IP}/\text{WCE})$ on the Y axis and chromosome positions on the X axis (lower panel).

Affymetrix tiling array technology includes mismatch probes as a way to associate a p-value to each probe signal intensity, nevertheless in our hands a PM/MM (for perfect match/mismatch) analysis did not appear to be reproducible when comparing biological repeats of ChIP-chip maps for some of our studied proteins (see figure 24). This could be explained in part by the polymorphisms existing between SK1 yeast strain background and the sequence used for Affymetrix yeast arrays design, S288c (the nucleotide divergence between S288c and SK1 is 0.36% in contrast to 0.08% for W303[112]). For this reason we did not include PM/MM analysis in our ChIP-chip maps; instead we have developed alternative strategies in order to correct for tiles that are considered as been artefacts based in the nature of ChIP hybridization expected binding pattern. In the following paragraphs we describe the methodology used for this purpose.

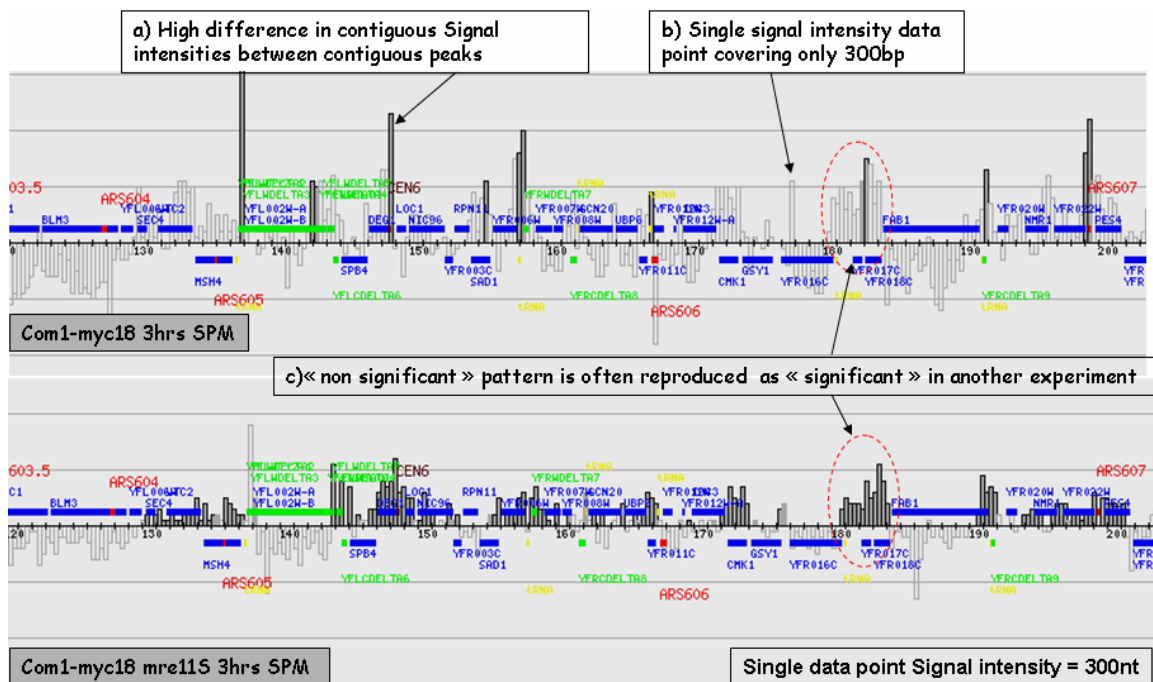


Figure 24: Typical artifact patterns observed in a ChIP-chip map. In some cases, strong Signal intensity fluctuations between consecutive data points are observed (a). Single data points which do not cover at least the expected minimum immunoprecipitated DNA length are not considered as been significant (b). Comparing two ChIP-chip maps for the same target protein under similar conditions often displays different “significance” pattern based on the PM/MM ratio (c). Black bars and gray bars represent significant and non-significant data points, respectively, based on the PM/MM ratio. The significant p-value threshold algorithm used for these ChIP-chip maps was developed in K. Shirahige’s Laboratory[101].

2.6.7.3 Tooth brushing methodology for Signal intensity

pattern correction

The chromatin immunoprecipitation protocol previously described is designed to immunoprecipitate DNA fragments of 500-1000bp length on average. Taking into consideration that the *S. cerevisiae* tiling arrays resolution is 5bp (5bp resolution means that the 25nt length oligonucleotide probes spotted onto the array surface overlap the consecutive probe by 20 nt), at least 96 probe tiles are covered by an immunoprecipitated DNA fragment of 500 bp in length. Under these conditions, we should be able to identify several consecutive probes presenting a protein-chromatin binding pattern in contrast to tiles presenting strong signal intensity fluctuations compared to their neighbouring probes, which is likely a consequence of hybridization artefacts and are source of no-specific background.

From the practical point of view, we do not work directly with signal intensities coming from the 25nt length probes; instead we perform a previous signal average between consecutive arrays covering 50nts in length (~6 consecutive probes). Furthermore, in order to estimate the signal intensity fluctuation between the averaged probes we calculate their Standard deviation. This data treatment step helps us to compress the number of data point that must be analysed. After this compression treatment, the number of data points needed to be considered for the hybridization of a DNA fragment of 500 bp is reduced to 10. Therefore, we require at least 10 consecutive data points displaying a defined pattern to consider that chromatin site a protein binding region.

A simplistic interpretation of protein-chromatin interaction sites mapped by ChIP-chip is to assume that they follow a Gaussian model, where the chromatin regions presenting the highest signal intensity in hybridized arrays are the closest to the Protein binding site. Similarly, the chromatin regions further from the protein binding site will be represented in a lower frequency in the immunoprecipitated DNA sample, and by consequence will display a lower signal intensity compared to regions closer to the protein binding site. Taking into account this Gaussian model, we have a further requirement to consider the pattern of hybridization as being indicative of actual protein-chromatin interactions. In practice very few perfect Gaussian distribution patterns are found in ChIP-chip maps, at least not in high resolution maps where each data point corresponds to signal intensities covering ~50-100 bp chromatin regions. This difference can be attributed to hybridization artefacts that are spread over the array surface, which are added to the signal intensities at real protein binding sites.

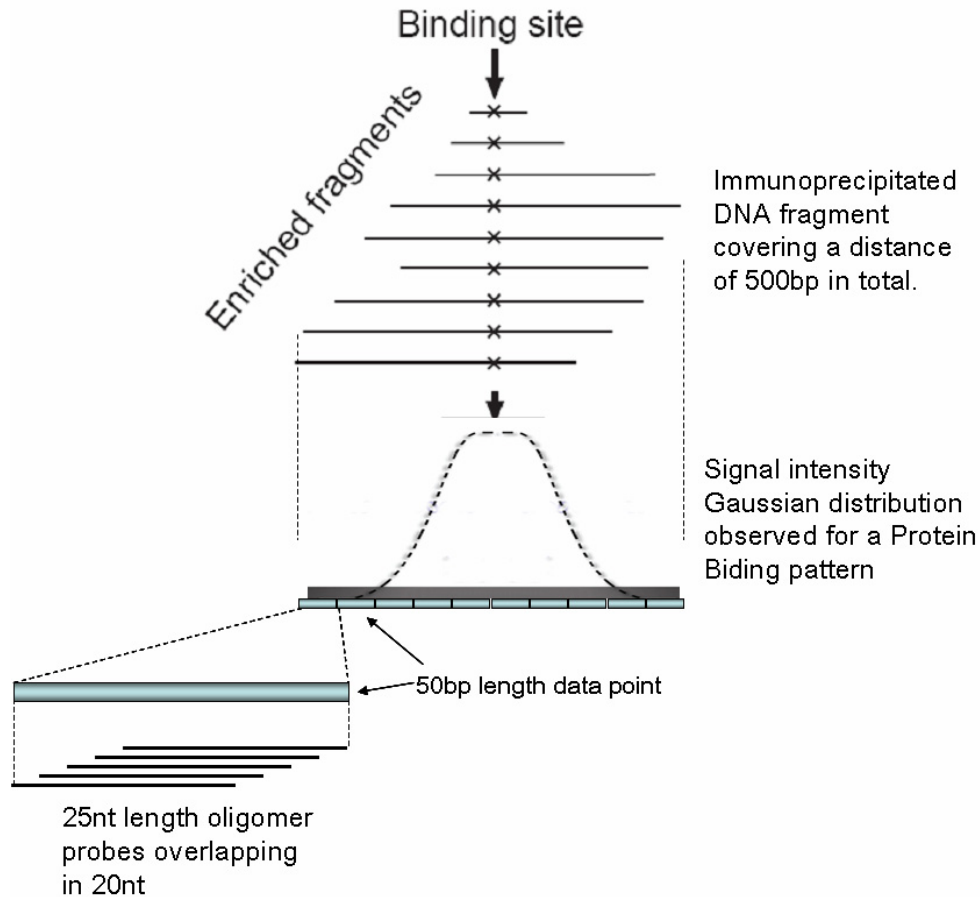


Figure 25: Signal intensity Gaussian distribution for Protein-chromatin interactions mapped by ChIP-chip.

To correct for deviations from Gaussian behaviour, we implemented quite early in the development of our data analysis a “clearing step” that we have named a *tooth brushing procedure*. This correction method uses signal intensities from neighbouring data points to correct for non-Gaussian behaviour. In this step, the signal intensity of every data point is compared with the left and right neighbours (in this case, a single data point corresponds to the average signal intensity of probes covering 300bp, which means we need 3 data points to cover at least 900 bp). When the analysed data point displays strong differences with either one or both of its neighbours, the corrected signal intensity is interpolated from the neighbouring data points. This treatment removes data points that display unpredicted patterns without affecting sites that satisfy the previously described threshold conditions (see figure 26 and 27).

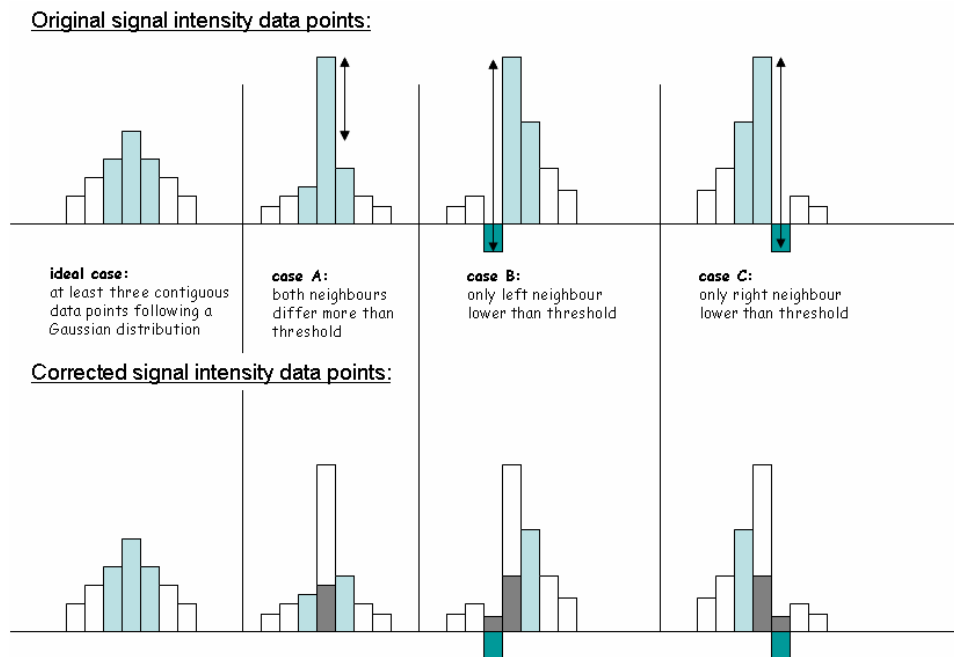


Figure 26: Tooth brushing data treatment. Data point signal intensities are corrected based on the signal intensity of the neighboring probes. The ideal situation for a binding site corresponds to three contiguous data points following a Gaussian distribution. Cases A, B and C illustrate different situations where the ideal distribution is not observed (upper panel), and where the tooth brushing strategy assigns a new signal intensity to the data points differing from a Gaussian model (lower panel). Notice that a single data point covers 300 bp and the ChIP DNA fragment is assumed to be at least 1kb in length.

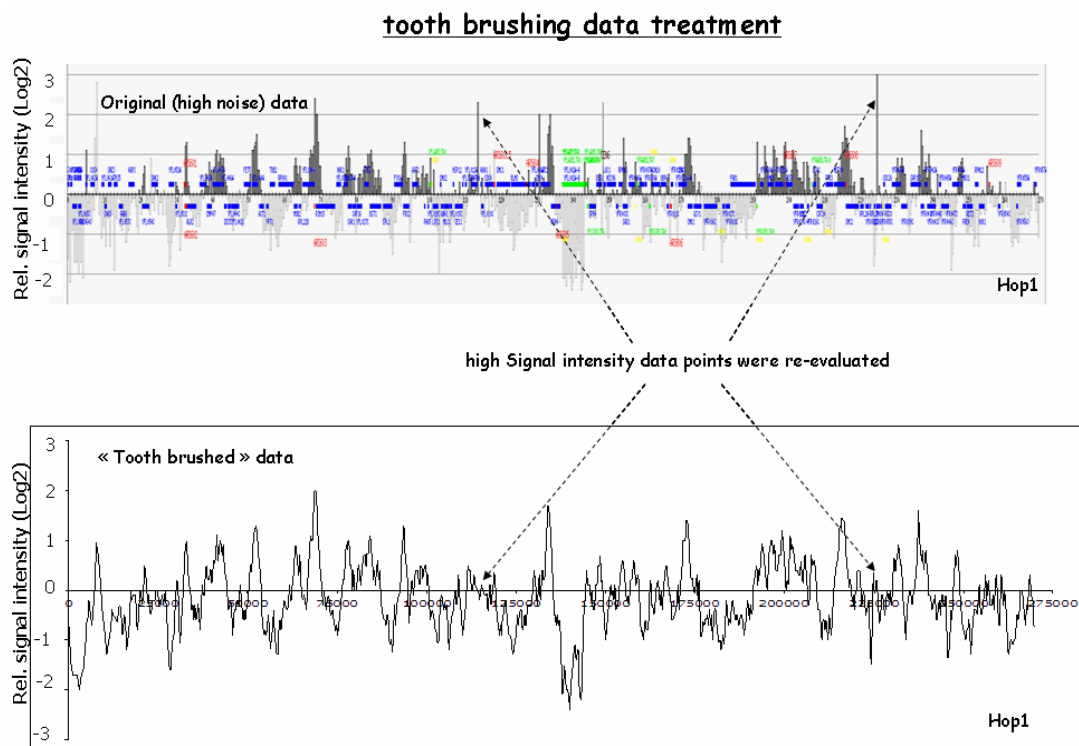


Figure 27: Tooth brushing data treatment. A comparison between the original ChIP-chip profile for the Hop1 protein (upper panel) and the tooth brushed version (lower panel) demonstrates the efficiency of this data treatment in removing unexpected data points without modifying other regions.

2.6.7.4 ChIP-chip profile smoothed by Mean sliding window

approach

The “Tooth brushing” strategy previously described was developed for data points covering 300 bp and assuming that an immunoprecipitated DNA fragment is at least 900 bp in length. However, performing a similar analysis for data points covering 50 bp leads to a dramatic increase in the number of neighbouring data points that must be compared (19 data points when assuming the ChIP fragment is 1kb) and it is therefore more difficult to determine whether a given data point is out of the Gaussian distribution expected for the 20 data points covering the 1kb region. Indeed, in practice, several data points in a given 1 kb region will not follow the Gaussian distribution.

A straight forward strategy for dealing with signal variability between consecutive data points is to introduce a mean/median sliding window. In this case, we consider a defined chromosome length window where the mean/median of the relative signal intensities of the data points within the window, is calculated. The chromosome window is shifted by one data point and a new mean/median signal intensity is calculated. This approach eliminates strong signal intensity fluctuations and at the same time smoothes the global protein binding profile. Larger sliding windows provide more significant but less well resolved observed binding sites. In contrast, small sliding windows produce more resolved binding sites but compromise the certitude of been in presence of real binding sites. In our experience, a 1 kb sliding window provides a good compromise between binding site identification and certainty. Furthermore, a 3 kb sliding window produces nicely smoothed ChIP-chip profiles, reason why we prefer this resolution when presenting complete chromosome maps.

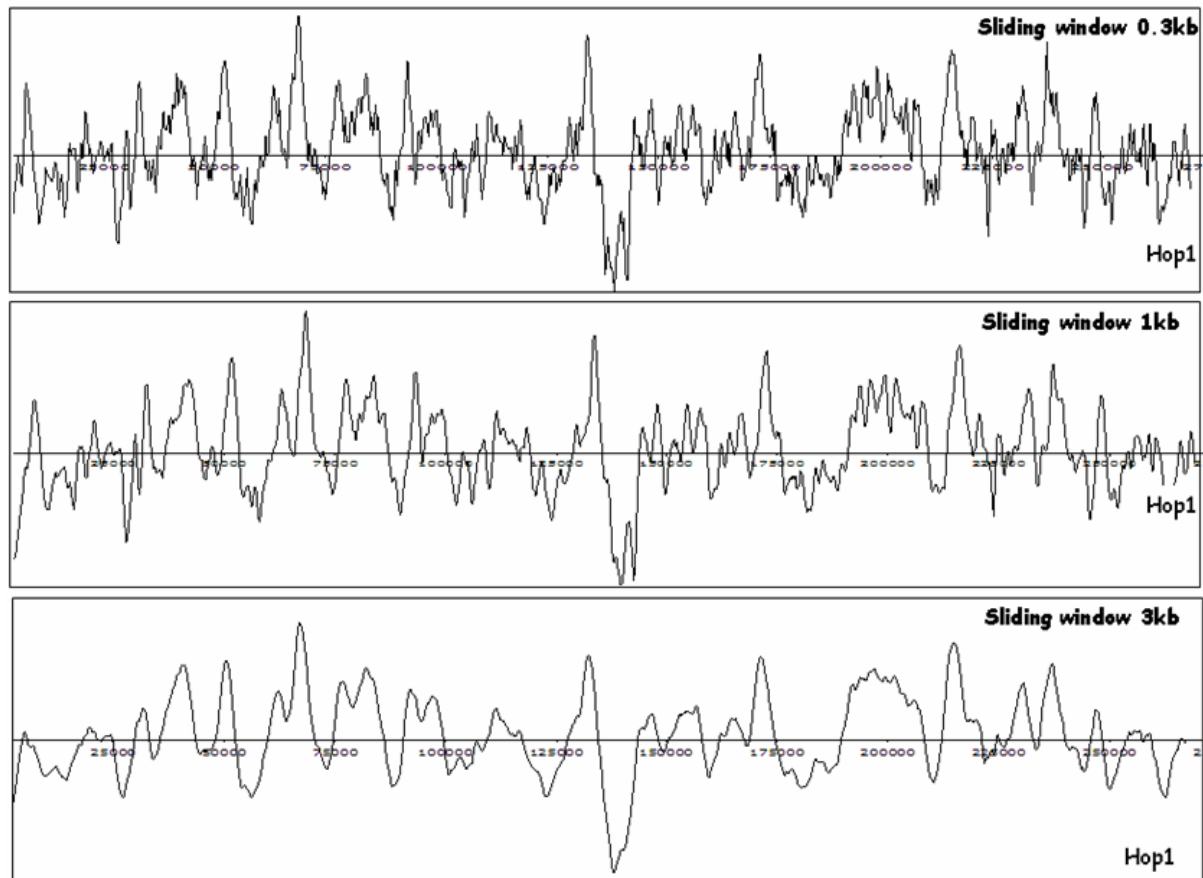


Figure 28: Mean Sliding window approach. Three different sliding windows are shown for the Hop1 ChIP-chip map. Notice that the 3 kb sliding window presents nicely smoothed peaks when compared to the 0.3kb sliding window. In contrast, the 0.3kb sliding window presents better resolved binding sites than the 1 kb or the 3 kb sliding window maps.

2.6.7.5 Quantitative comparison of different ChIP-chip profiles

Because several proteins interact with the chromatin in an organized manner in order to ensure structural and/or enzymatic functions, the identification of their chromatin interaction sites by ChIP-chip technology followed by a comparative analysis between different profiles becomes a powerful strategy to decorticate such complex proteins-chromatin organization. In the previous paragraphs, the methodology to generate a protein-chromatin interaction profile was introduced; in the following paragraphs we would like to describe the strategy we developed in order to compare different ChIP-chip profiles in a quantitative manner.

The intuitive approach for comparing different ChIP-chip profiles is to overlay them, in order to visualize similarities and differences. During this study, this was the

current strategy applied to single chromosome profiles and performed between few number of ChIP-chip maps. Nevertheless, a genome wide analysis requires a quantitative methodology performed in an automated manner. Furthermore, a comparative analysis of several profiles requires a different methodology than a visual analysis of overlaid ChIP-chip maps.

Taking into consideration the signal intensity variability between profiles, an automated comparative analysis requires a simplification of the profile information. This simplification consists in the identification of “putative Protein binding sites” which will then be represented in a barcode format (a putative site is represented as a single bar associated to a chromosome position). Finally, the barcode format profiles can now be compared using strategies previously developed for gene expression comparative analysis, namely by using a technique known as clustering comparison.

Identification of “putative Protein binding sites” is the most studied topic in the ChIP-chip field, as it requires sensitive algorithms to discern between real signals, coming from Protein binding sites, from noise signals, produced by hybridization artefacts. The first attempt in this purpose described in the literature was to consider a fold threshold for the signal intensities. This strategy, that was first developed for gene expression analysis and then adapted to ChIP-chip analysis (an extensive description of the different methods used in gene expression analysis are presented in [113]) was shown quite early to be inappropriate because the fold threshold is chosen arbitrarily. Indeed, strong chromatin binding proteins present a high signal to noise ratio; whereas weak chromatin binding proteins present a low signal to noise ratio. Consequently, the fold threshold applied for these two different situations cannot be the same. In order to establish the fold threshold based on the chromatin binding characteristics, the standard deviation of the signal intensities distribution has been used as threshold parameter ($\pm 2\sigma$ for gene expression analysis and $+2\sigma$ for ChIP-chip data because of the positive skew expected from the signal intensities distribution). Even though this method provides the a constant fold threshold parameter between different ChIP-chip profiles, it will always report 5% of the data points as real binding sites, losing significant data points in some cases (like in the case of strong chromatin binding proteins) and creating false positives in cases where the signal to noise ratio is quite low.

Taking into consideration the fact that the average length of the ChIP DNA fragments hybridized to high resolution DNA microarrays covers several probes (in contrast to PCR-amplicon arrays where a single “probe” was as big as the average length of hybridized ChIP DNA fragment), a new generation of techniques specifically designed for ChIP-chip analysis has been recently developed. Between them we could mention PeakFinder[114], Chromatin immunoprecipitation on tiled arrays (ChiPOTle)[115], hidden Markov model analysis (HMM)[116], Algorithm for Capturing Microarray Enrichment (ACME)[117], as well as two other innovative methods, namely the Joint binding deconvolution (JBD)[118] and Model-based Deconvolution of genome-wide DNA binding also known as MeDiChI[119]. In contrast to the previous methods where the “neighbouring probe effect” is used to identify putative binding sites, and by consequence the resolution of the identification is limited to windows covering at least the average size of the hybridized ChIP DNA fragment, JBD increase the sensitivity of the binding site identification by using an experimentally determined distribution of fragment sizes to predict the probe intensity peak shape that a binding event will produce. With the same objective, MeDiChI predicts the behaviour of a single binding event from the experimentally derived ChIP-chip profiles composed of multiple binding events.

All of the previously described methods would help us to transform the ChIP-chip profiles into the required barcode profile in order to perform a clustering analysis. Due to implementation problems that need to be overcome in order to use the more sophisticated versions, we developed our own methodology taking into consideration several of the criteria explained in the previous paragraphs. On one hand, we use the mean sliding window approach to partially remove signal fluctuation coming from hybridization artefacts. Then we define “comparative binding sites” by a threshold approach applied to the difference between the local maxima and the neighbouring local minima present in the profile. This is called a comparative binding site because we know that the sliding window approach produces an “artefactual” binding sites, nevertheless if we assume that the artefact introduced into the putative binding site localization is a constant associated to the sliding window size, we should be able to compare different profiles under the same sliding window resolution by using such “comparative binding sites”. In simple terms, we define a certain Peak as being part of our “comparative barcode profile” based on the distance of such a peak to its

neighbouring “valley” regions. Notice that this concept is new in the context of the previous methods applied for binding site identification, mainly because the signal intensity of a certain data point is not important by it self, but in context to its neighbouring regions defining a local minima. On the other hand, this approach requires that the peak evaluation process is performed independently of the zero line produced by the log2 data transformation; this means that “valley” regions located in the negative side of the semi log plot are still considered during the evaluation of the “Peak-valley” distance used as threshold criteria. In our experience, we know that peaks and valleys located in the negative side of the semi log plots can be reproduced in biological repeats, strongly suggesting that negative data points should be considered during data processing.

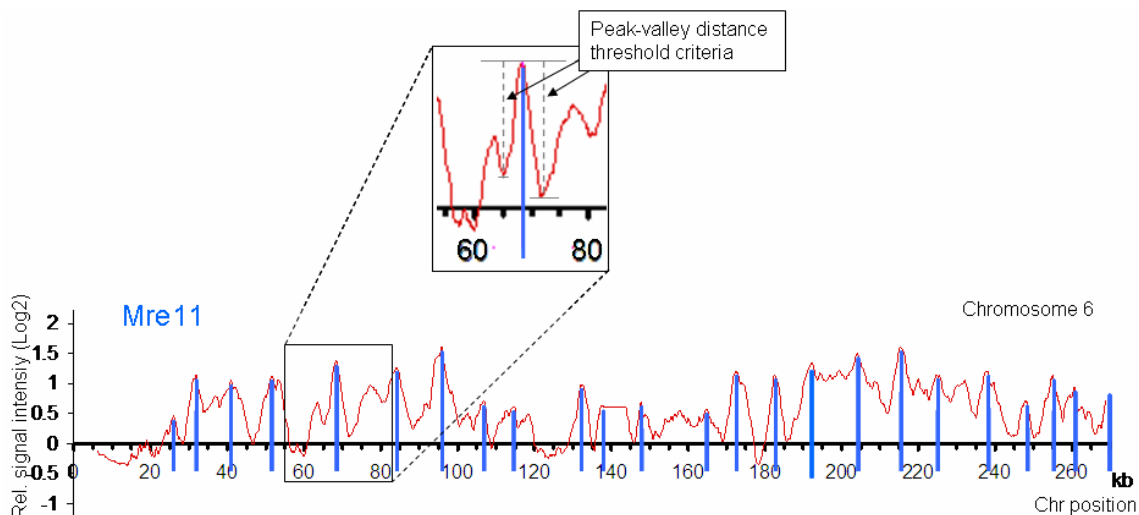


Figure 29: Comparative barcode profile. A threshold approach is applied to the difference between the local maxima and the neighboring local minima present in Mre11 ChIP-chip profile (represented in red). Peak signals satisfying the threshold criteria are represented in a barcode profile (represented in blue) instead of the original ChIP-chip profile before clustering comparative analysis.

Barcode profiles can then be compared using clustering algorithms. For this purpose, we use a tool developed in the Dana Farber Cancer institute (MultiExperiment Viewer or MeV [120]), a hierarchical clustering approach was chosen for the analyses performed in this study. Furthermore, a Pearson correlation was used as the distance metric, in order to compare peak localization without the influence of the associated signal intensity information.

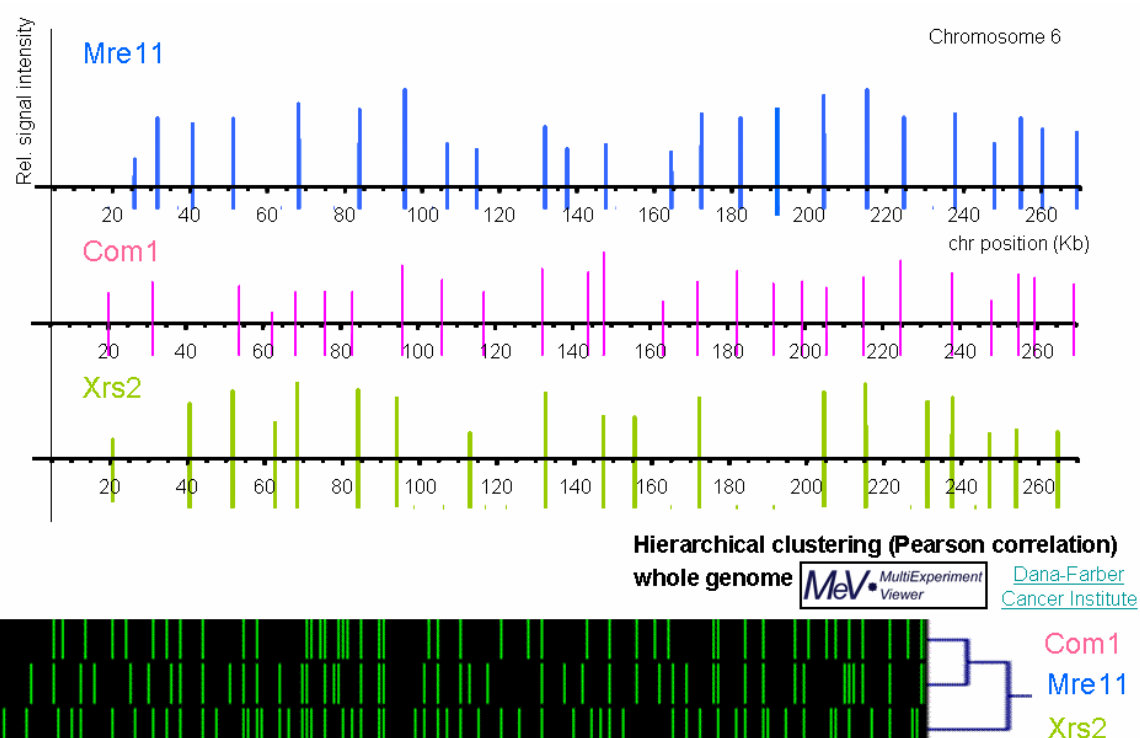


Figure 30: Hierarchical analysis for ChIP-chip assays. Barcode profiles corresponding to Mre11, Com1 and Xrs2 ChIP-chip assays are compared using a hierarchical clustering strategy. In order to compare the physical positions without considering the signal intensities, a Pearson correlation distance is chosen as the distance metric. The dendrogram corresponding to the clustering analysis for Mre11, Com1 and Xrs2 ChIP-chip data was generated in MeV software (MultiExperiment Viewer) developed by the Dana-Farber Cancer Institute.

Figure 30 illustrates the hierarchical clustering analysis performed on three different ChIP-chip barcode maps, Com1, Mre11 and Xrs2. From the dendrogram representation we conclude that the Com1 and Mre11 barcode pattern are part of the same cluster, whereas Xrs2 is more distant. Nevertheless, such analysis does not address the pattern differences between Mre11, Com1 and Xrs2. This is because the hierarchical analysis was performed using an average distance between Mre11 and Com1 before performing the comparison with Xrs2. In order to complement the Hierarchical clustering analysis with this missing information (what missing information?), a pairwise comparative analysis was implemented over the obtained cluster tree. This consists of calculating the percentage of matching overlaps between all barcode pattern combinations and representing it in a matrix that maintains the cluster tree organization (see figure 31). Such pairwise comparisons let us identify whether differences in the total number of peaks or a real difference in

the peak localization pattern is the reason for the corresponding clustering organization. Indeed, the example illustrated in figure 31 demonstrates the similarity in the total number of peaks between the Com1 and Mre11 barcode profiles, the percent of matching peaks between Com1/Mre11 and Mre11/Com1 are quite similar, as well as the high degree of peak overlap between these two profiles. In contrast, the Xrs2 barcode profile has more peaks than Com1 and Mre11, which is reflected by the asymmetry of the percent matching peaks between Xrs2/Com1 and vice versa, and despite the larger number of peaks, there is a smaller percentage of overlap. As a final note, the use of clustering analysis combined to with pairwise matrix comparison is mainly applied in a genome wide manner in order to quickly identify protein clusters. These clusters can then be further scrutinized by returning to the high resolution analysis in order to identify the exact putative binding sites.

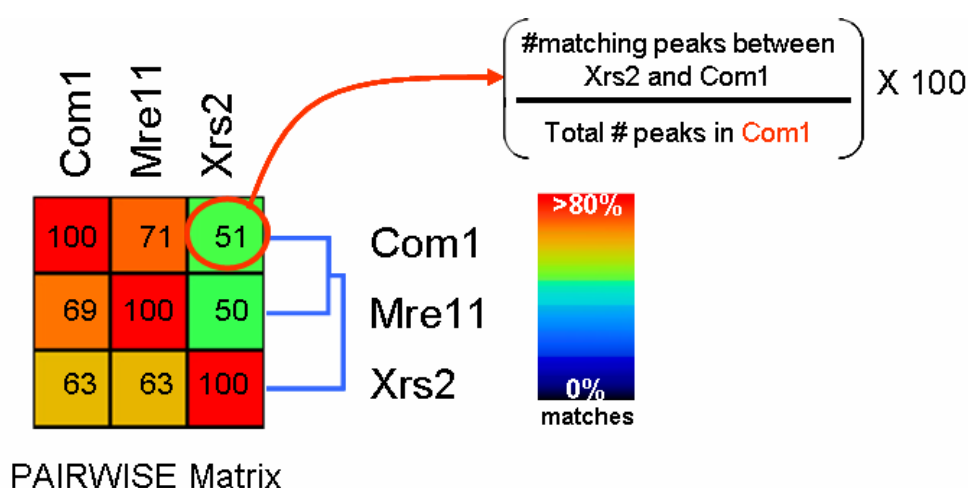


Figure 31: Complementing the Hierarchical clustering analysis with a Pairwise matching comparison matrix. The percentage of matching overlaps of all barcode pattern combinations are represented in a Pairwise Matrix keeping the cluster tree organization (also represented in a color coded manner). Red colored diagonal corresponds to the matching comparison performed in a single barcode profile (100% overlap). The illustrated Pairwise matrix analysis shows that Com1 and Mre11 barcode profiles present a symmetric matching pattern indicating that they have a similar number of total peaks and around 70% of them present the same localization pattern. In contrast, Com1 vs Xrs2 and Mre11 vs Xrs2 present a different total number of peaks (Xrs2 present more peaks than Mre11 or Com1) which is reflected in only 50% of overlap between Xrs2 and the other barcode profiles.

3 Results

In order to analyse the events involved in the initiation of meiotic recombination and its relationship to chromatin organization during prophase I, this study focuses on the use of Chromatin immunoprecipitation combined with hybridization to high resolution DNA microarray chips. This approach, also known as ChIP on chip, is a powerful method to characterize protein-chromatin interaction events in a genome-wide manner. Briefly, yeast cells undergoing meiosis are sampled at the corresponding time points of interest. Extracts are prepared by mechanical disruption from cells prefixed with formaldehyde to crosslink proteins to DNA. Chromatin is fragmented by sonication and the protein of interest is immunoprecipitated either by using a monoclonal antibody against an epitope-tagged protein or by using a polyclonal antibody specific for the protein of interest. The chromatin that is immunoprecipitated in this manner is purified, PCR amplified, labelled and hybridized to DNA microarray chips (we currently prefer Affymetrix GeneChip *S. cerevisiae* Tiling 1.0R Array). In order to remove potential hybridization artefacts, a comparative analysis between the immunoprecipitated DNA (IP) and the total whole cell extract DNA (WCE) is performed by normalizing the probe array data sets obtained by hybridization of the IP and WCE samples. For most purposes we visualize the data is visualized in two dimensional plots, where the relative signal intensity between the IP and the WCE is represented on a Log2 scale on the Y axis ($\text{Log}_2(\text{IP}/\text{WCE})$) and the chromosome position is represented in the X axis (see figure 32). As a negative control for chromatin immunoprecipitation, the complete procedure is performed in a yeast strain lacking the epitope tag for the antibody in use (untagged control). Figure 32A illustrates the chromatin interaction map established by ChIP on chip for two different proteins performed in this study, namely Mre11p and Com1p fused to epitope tags (6 copies of the HA epitope and 18 copies of the Myc epitope respectively). Furthermore, an untagged control map is shown to demonstrate the significance of the chromatin enrichment during immunoprecipitation of the proteins studied.

In order to validate the ChIP on chip maps, we performed qPCR analysis at defined chromosome positions. As shown in figure 32B, the qPCR analysis at three different chromatin regions shows chromatin enrichment corresponding to that observed by ChIP on chip relative to the untagged control.

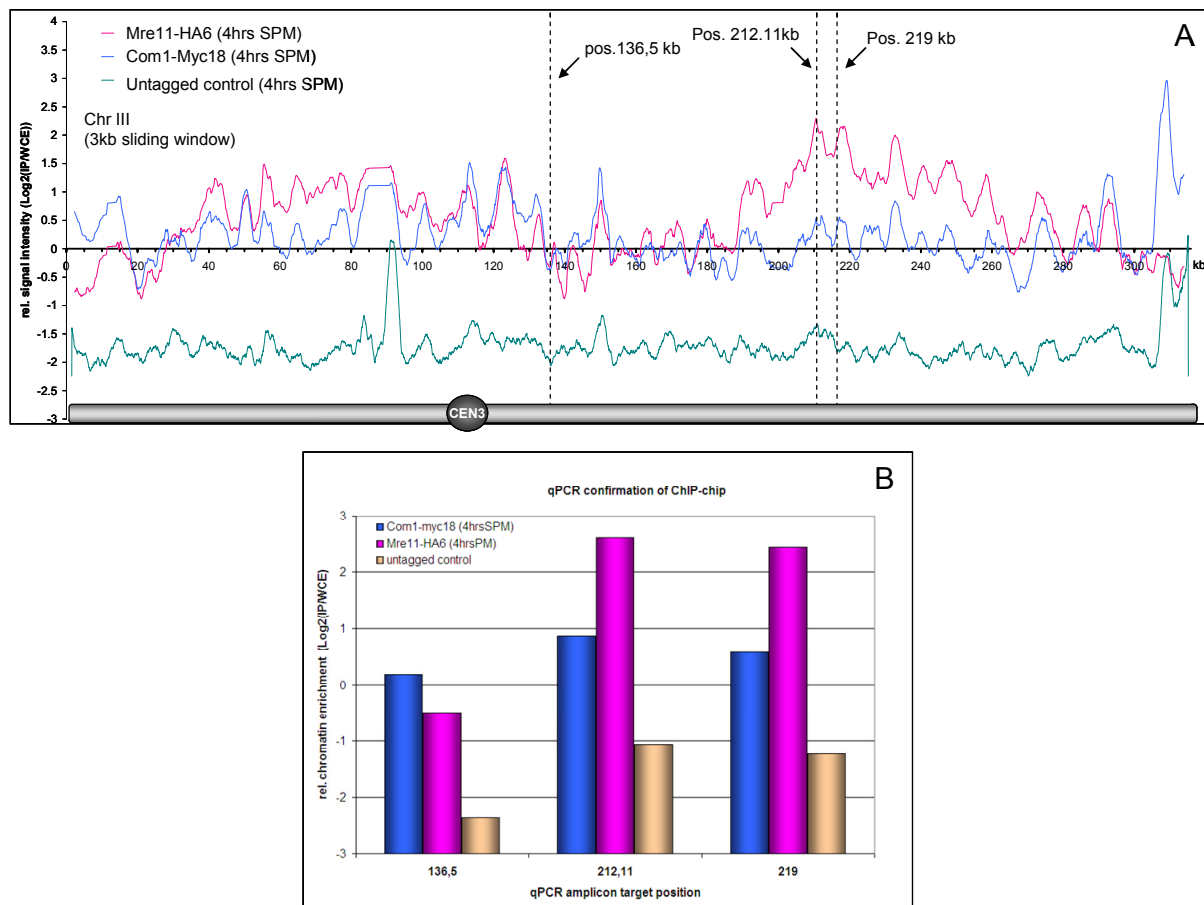


Figure 32: ChIP on chip analysis performed on two different protein targets compared to the untagged control. (A) The chromatin interaction map for chromosome III of Mre11p (Pink), Com1p (Blue) and an untagged control (green) are represented in a 3kb sliding window. The X axis corresponds to physical positions on chromosome III (kilobases) and the Y axis represents the relative signal intensity associated to each chromosome position (Log₂(IP/WCE)). The relative signal intensity has been evaluated by performing a linear comparative analysis between the fluorescence signal intensity associated to the hybridization into the DNA microarrays of the immunoprecipitated DNA (IP) and that obtained for the total DNA whole cell extract (WCE) (TAS software). (B) The protein binding at three different chromatin regions was analyzed by qPCR in order to confirm the ChIP on chip maps. The Y axis represents the chromatin enrichment obtained during the immunoprecipitation, compared to the total DNA whole cell extract and expressed in Log₂ scale. The physical position of the analyzed chromatin regions are indicated in the X axis (dashed lines represented in (A)).

As shown in the following paragraphs, this technique was used in order to establish a high resolution map of the organization of the chromatin during meiotic prophase I by characterising the chromatin localization of structural components, such as the meiotic cohesin protein Rec8p, the axial element component Hop1p, and the transversal filament Zip1p. Furthermore, the chromatin localization of meiotic recombination initiation factors was

analysed in the context of the chromatin organization. The chromatin localization of the meiotic specific nuclease Spo11p was studied under different conditions. First, meiotic DSB repair defective mutants, including *rad50S*, *mre11S* and *com1/sae2*, were used in the absence of crosslinking to map the chromatin sites where Spo11p remains covalently attached. Additional localization sites for this protein were then mapped by including the regular *in vivo* crosslinking procedure (Formaldehyde cells fixation). Furthermore, the chromatin localization of components involved in meiotic DSB repair, namely the Mre11 complex as well as Com1/Sae2p, were characterized and analysed in the context of the chromatin organization, both in wild type cells as well as in different mutant backgrounds.

Finally, we analysed the influence of the axes components in the chromatin localization of meiotic recombination components, namely in the case of Spo11p. In addition, we studied the chromatin interaction of RFA1p, a component of the Replication Protein A (RPA) heterotrimeric complex, either in the absence of meiotic DSB repair, or in the absence of chromosome axes components, like Red1p.

The last subchapter is dedicated to the study of technical improvements we developed to allow a quantitative comparative analysis between different profiles. This consists of the introduction of an exogenous internal control into the tagged yeast strains of interest, in order to use it as a comparative parameter between different ChIP on chip profiles.

For schematic reasons, this document presents protein-chromatin localization maps for chromosome VI and III of budding yeast. The complete set of whole genome ChIP on chip raw data files as well as the different average sliding window maps are stored in the database of the Department of Chromosome Biology, at the University of Vienna.

3.1 High resolution analysis of meiotic chromatin organization in *Saccharomyces Cerevisiae*

3.1.1 Meiotic cohesin binds to defined chromatin regions

Rec8p was previously shown to be essential for meiotic sister chromatids cohesion as well as for synaptonemal complex formation. Furthermore it was shown to colocalize with Zip1p, which defines the SC structure, in spread nuclei preparations at the pachytene stage by immunostaining [9]. Thus, this protein is considered to be at the heart of chromosome organization during meiotic prophase I.

In order to identify the meiotic cohesin localization sites on the chromatin, we performed ChIP on chip analysis on a 3xHA-tagged version of Rec8p. This analysis revealed that Rec8p binds to defined chromatin regions, with a higher abundance at centromeres compared to chromosome arms. When we analyzed the chromatin localization of the mitotic cohesin component Scc1p, which is residually expressed in meiosis, we found that Scc1p binds to the same chromatin regions characterized for Rec8p (see figure 33 A). Genome wide analysis of the meiotic cohesin localization pattern reveals a direct correlation between the number of Rec8p-chromatin interaction sites per chromosome and the chromosome length, indicative of a relatively even distribution of Rec8p binding sites throughout the genome. An average distance between two consecutive Rec8p binding sites of 11.7 kb was estimated from 991 cohesin binding sites identified across the *S. cerevisiae* genome from 3kb sliding window ChIP on chip profiles (see figure 33 B and C). This is similar to the previously reported 10.9kb average distance for Scc1p binding sites during mitosis [114].

As demonstrated previously for mitotic cohesin, the Rec8p localization pattern negatively correlates with GC-rich regions and positively correlates with convergent transcription sites, supporting the model that a transcription based mechanism defines meiotic cohesin localization. Nevertheless, it is important to mention that not all of the identified meiotic cohesin sites correlate with a convergent transcription event, indicating that there is possibly more than one mechanism involved in cohesin localization (figure 34).

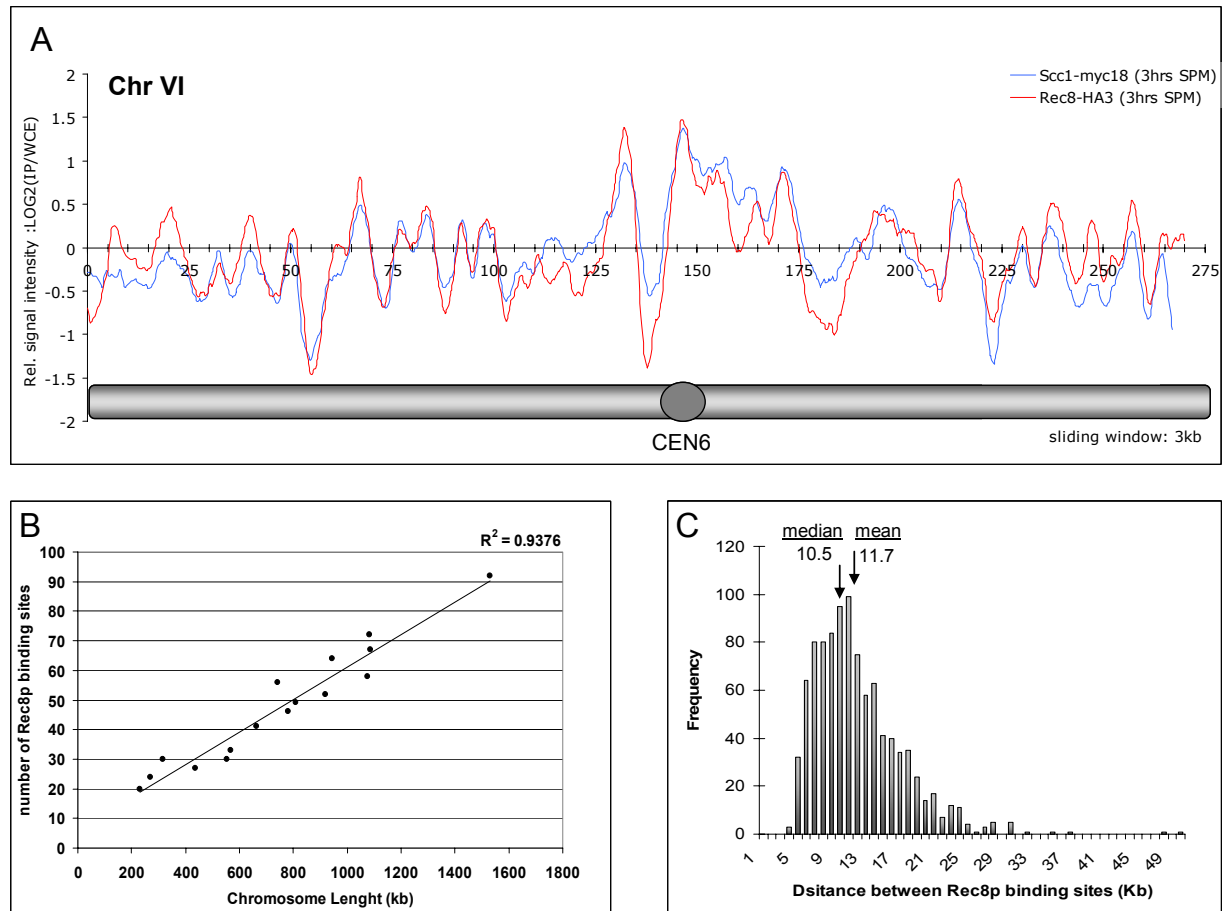


Figure 33: The meiotic specific component Rec8p binds to defined chromatin regions. (A) Chromatin localization of Rec8p (red) and Scc1p (blue) on *S. cerevisiae* Chr VI in meiotic prophase I (3 hours after induction of the meiotic program). The illustrated ChIP on chip profiles are represented with a 3kb sliding window. The X axis represents the physical positions on chromosome VI (in Kilobases) and the Y axis the relative signal intensity associated to each chromosome position (Log2 scale). (B) Scatter plot of chromosome lengths versus number of Rec8p binding sites per chromosome. (C) Distribution of distances between contiguous Rec8p binding sites over the whole genome. A median and mean distance has been estimated to 10.5 and 11.7 kb respectively. For figures B and C, Rec8p binding sites were identified by defining a threshold distance of 0.5 between the putative binding sites and their neighboring local minima regions, or valleys. The peak identification procedure was performed over the whole genome where a 3kb sliding window was applied over the original data points.

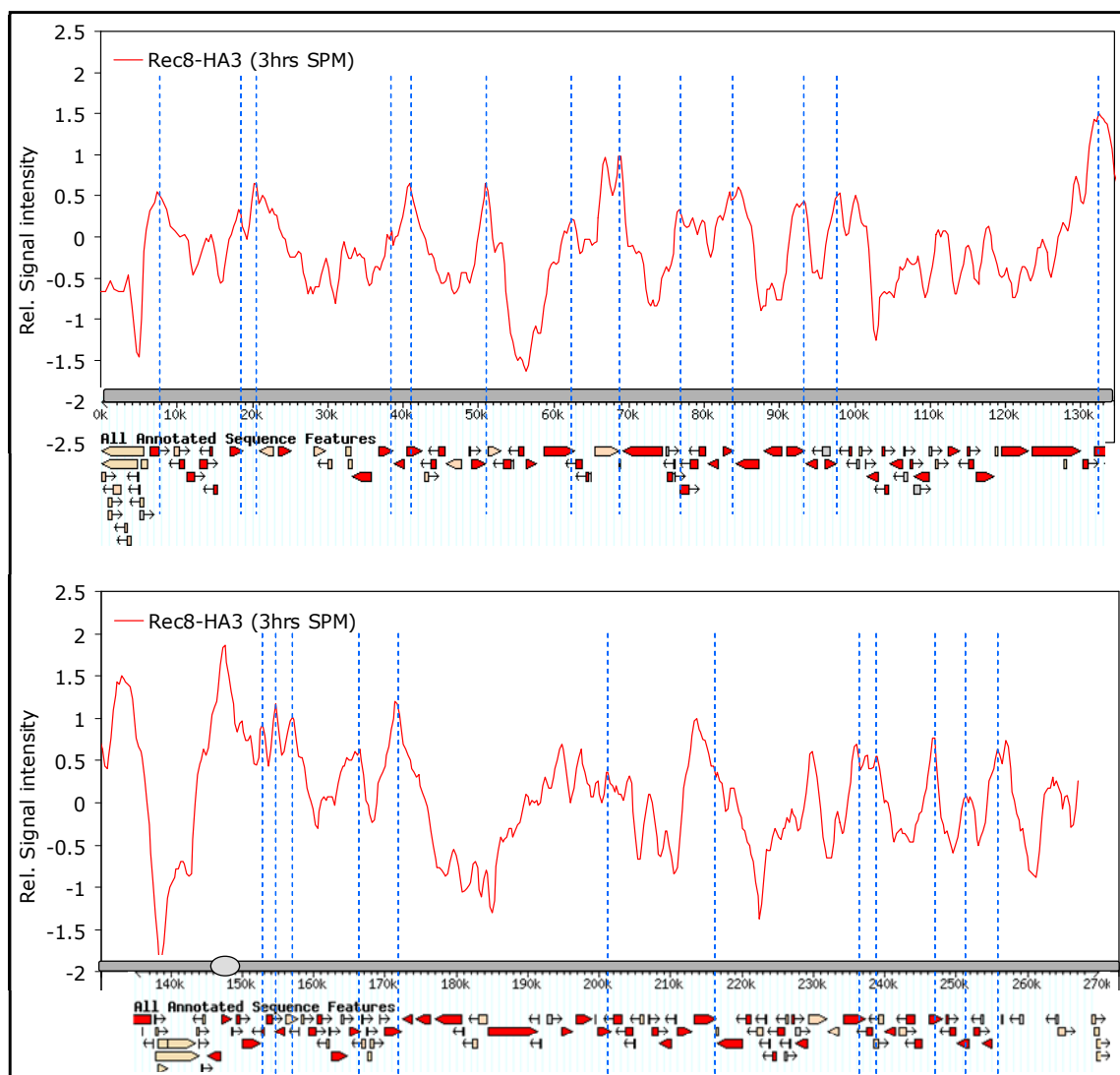


Figure 34: Meiotic cohesin-chromatin localization and its correlation with convergent transcription. Rec8p-chromatin localization represented in a 1kb sliding window is correlated with convergent transcription patterns on chromosome VI. Rec8p binding sites associated with convergent transcription are marked with a blue dashed line. In the X axis the physical chromosome positions as well as the annotated sequence features on chromosome VI are represented. The relative signal intensity associated to Rec8-chromatin enrichment on chromosome VI is represented in the Y axis.

3.1.2 Rec8p and Synaptonemal complex components interact with the same chromatin regions

In order to establish a high resolution map of the organization of the synaptonemal complex (SC) on the chromatin, as well as to evaluate the Rec8p chromatin binding pattern in the context of the SC structure, we immunoprecipitated a component of the axial element, namely Hop1, as well as the transversal filament, Zip1p, by using specific polyclonal antibodies against the proteins of interest. As expected, all three components, Rec8p, Hop1p and Zip1p localize to the same chromatin regions demonstrating that Rec8p is indeed part of the chromosome core structure (see figure 35).

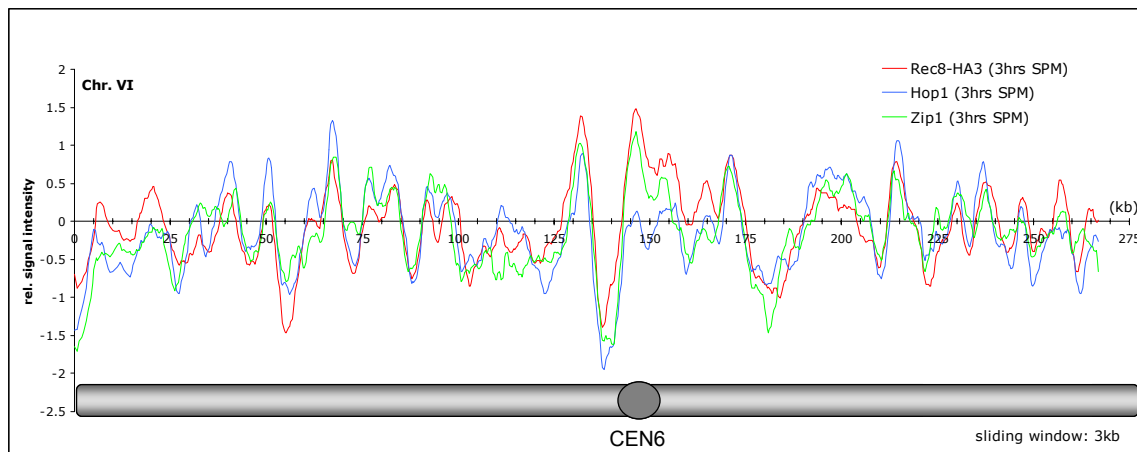


Figure 35: Comparison between Rec8p, Hop1p and Zip1p localization in chromosome VI of *S. cerevisiae*. Meiotic samples for this assay were collected 3hrs after meiotic program induction. All three components localize to the same chromatin regions. The illustrated ChIP on chip profiles are represented with a 3kb sliding window. The X axis represents the physical positions on chromosome VI (in Kilobases) and the Y axis the relative signal intensity associated to each chromosome position (Log2 scale).

Whereas the chromatin localization of these components is strongly correlated, there are differences in the relative signal intensities between centromeric regions and chromosome arms. Indeed, a 30kb sliding window analysis reveals that in contrast to Rec8p, which has a relatively higher abundance within ~50 kb of the centromeric region on chromosome VI, Hop1p displays a higher affinity for chromosome arm regions. Zip1p follows a pattern similar to Rec8p but displaying a more moderate difference between the enrichment at centromeric regions than that at the chromosome arms. The analysis of the GC-DNA content under the same sliding window condition demonstrates that the region surrounding the centromere contains a lower GC content than the chromosome arms on chromosome VI. These findings agree with a previous report, where a positive correlation between the GC content and

Red1p chromatin localization pattern on chromosome III was demonstrated[76]. Furthermore, they showed that the GC-DNA content also correlates with the abundance of the physically mapped DSB sites[66] (in chapter 3.2.2 is shown that also Spo11p chromatin localization correlates with the GC-DNA content pattern on chromosome III). Indeed, it is speculated that Hop1p plays a direct role in DSB formation; nevertheless there is no evidence for its localization to meiotic DSB sites.

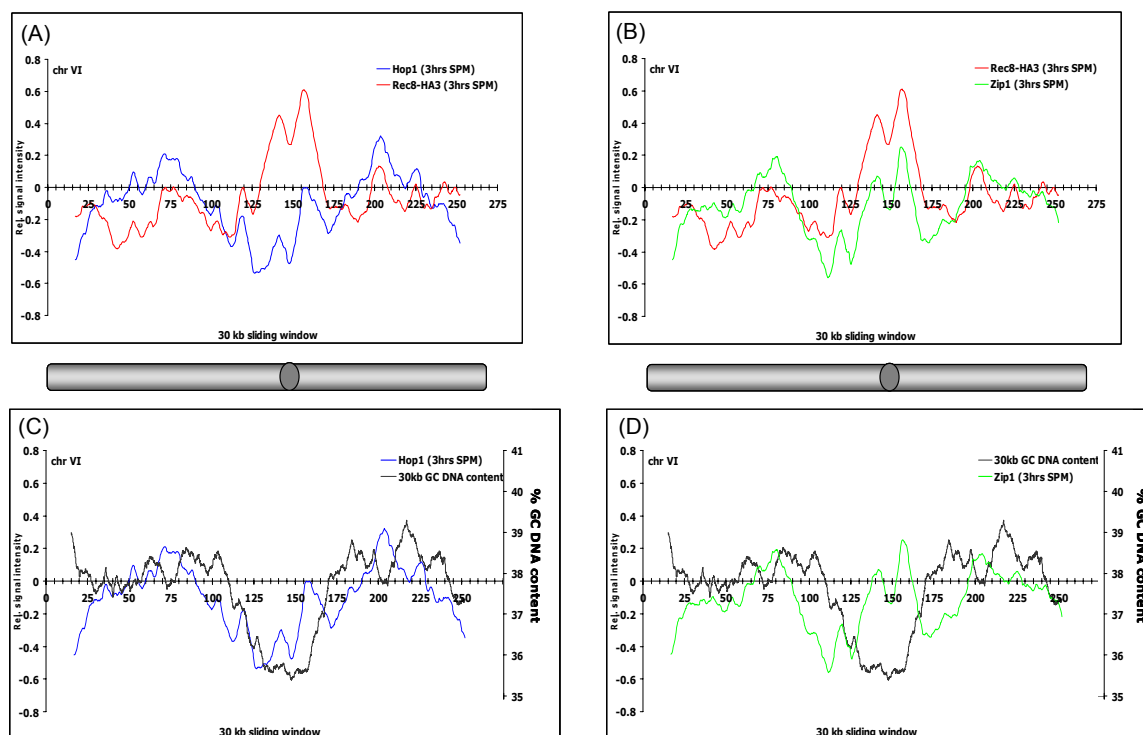


Figure 36: Distribution of Hop1p, Rec8p and Zip1p along chromosome VI in a 30kb sliding window. Hop1p is more abundant on chromosome arms than the surrounding centromeric regions (A) in contrast to Rec8p and Zip1p (B). Analysis of the GC-DNA content demonstrates that chromosome arms contain a higher GC content than the areas immediately surrounding the centromere (C and D) on chromosome VI. In (C) and (D) the Y axis represents the relative signal intensity associated to each chromosome position (left Y axis), as well as the % of GC DNA content (right Y axis).

Based on the previous findings, we can construct a high resolution model of chromatin structure in meiotic prophase I. Indeed, we can use the Rec8p chromatin localization map to define, with bp resolution, the chromatin regions that are part of the chromosome core as and the sizes and genomic location of the uncondensed loop regions. This analysis will become essential in the following chapters as we will use it to identify the chromatin binding sites of recombination components in the context of the chromatin organization.

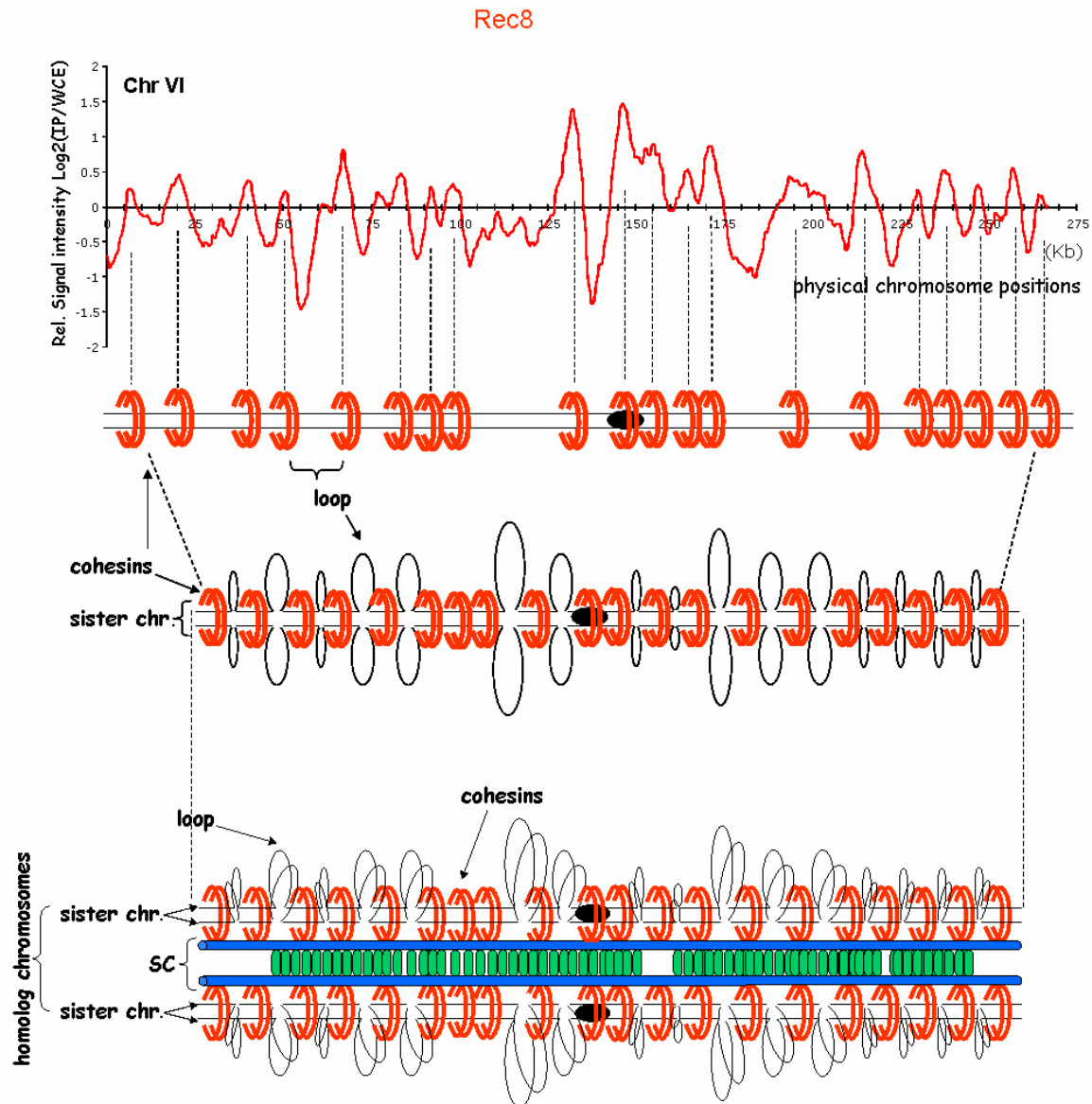


Figure 37: High resolution map of SC organization. The fact that Rec8p, Hop1p and Zip1p localize to the same chromatin regions suggests that Rec8p-chromatin localization pattern defines the chromosome core structure. From top to the bottom: The Rec8p-chromatin localization pattern (ChIP on chip profile for chromosome VI) defines regions with high probability of finding Rec8p (represented by red rings). Chromatin regions exempt of Rec8p binding sites are represented as loops. Rec8p binding sites are associated to the SC structure (blue rods corresponding to the axial elements and green transversal components representing Zip1p).

3.2 Meiotic recombination events in the context of chromatin organization

Considering that the chromatin structure has a direct influence on meiotic recombination, we were interested in characterizing the chromatin localization of several recombination components on the context of the high resolution map of the condensed chromatin we obtained by performing ChIP on chip analysis on Rec8p, Hop1p and Zip1p (see chapter 3.1.2).

3.2.1 Meiotic-specific Double-strand Breaks (DSBs) are formed in loop regions in *S. cerevisiae*

In order to obtain a high resolution map of meiotic DSB sites over the whole genome, we took advantage of the fact that Spo11p remains covalently linked to chromatin after cleavage in repair defective mutants, namely *rad50S*, *mre11S* or *com1/sae2*. Under these conditions, meiotic DSBs accumulate over the time, thus the chromatin immunoprecipitation procedure was performed at late time points (5 or 6 hours after meiotic program induction) and in the absence of crosslinking. Using these conditions we have mapped the localization of meiotic DSBs for the different repair defective mutants (figure 38). The Spo11p chromatin localization maps obtained from *rad50S*, *mre11S* and *com1/sae2* mutants in the absence of formaldehyde crosslinking did not show significant differences either in signal localization or in intensity.

To place these results in the context of chromatin organization, we performed a compared the meiotic cohesin map with the Spo11p chromatin localization sites in the *rad50S* mutant in the absence of crosslinking. This analysis demonstrated that the chromatin cleavage occurs preferentially in uncondensed loop regions (figure 39). Indeed, this result was confirmed by comparing the published DSB physical map established for chromosome III and the Rec8p-chromatin binding map we presented in the previous chapter (figure 38A). Furthermore, we found that 76% of the total numbers of loops on the yeast genome contained at least one Spo11p-chromatin covalently linked interaction site. Interestingly, the remaining 24% of loops not containing an Spo11p cleavage site have an average length of 9.3 ± 3.9 kb, in contrast to a 12.5 ± 6.3 kb average length for loops containing an Spo11p cleavage site; this demonstrates that the chromatin loops prone to cleavage by Spo11p are larger than the population exempt from Spo11p cleavage. Indeed, when we classified the loops by the number of Spo11p binding sites (BS), we realize that the average loop length increases with

the number of Spo11p-chromatin binding sites (no Spo11p: 9.3 ± 3.9 kb; 1 Spo11p BS per loop: 10.9 ± 4.1 kb; 2 Spo11p BS per loop: 16.3 ± 5.8 kb; 3 or more Spo11p BS per loop: 21.4 ± 8.6 kb) (figure 40). This could be expected if Spo11p binds in a stochastic manner, larger loops would then be more likely to contain one or more Spo11p binding events than shorter ones; however, when we examine the signal intensity of the Spo11p binding sites, we do not find a direct correlation with the loop length. In fact, the average loop length only varies from 13.5 ± 6.4 kb and 14.4 ± 7.3 kb for Spo11p signal intensities greater than 0.5 and 2 respectively (figure 41). In summary, the chromatin loops prone to be cleaved by Spo11p are 70% of the total number of loops on the yeast genome and have an average length of 14 kb.

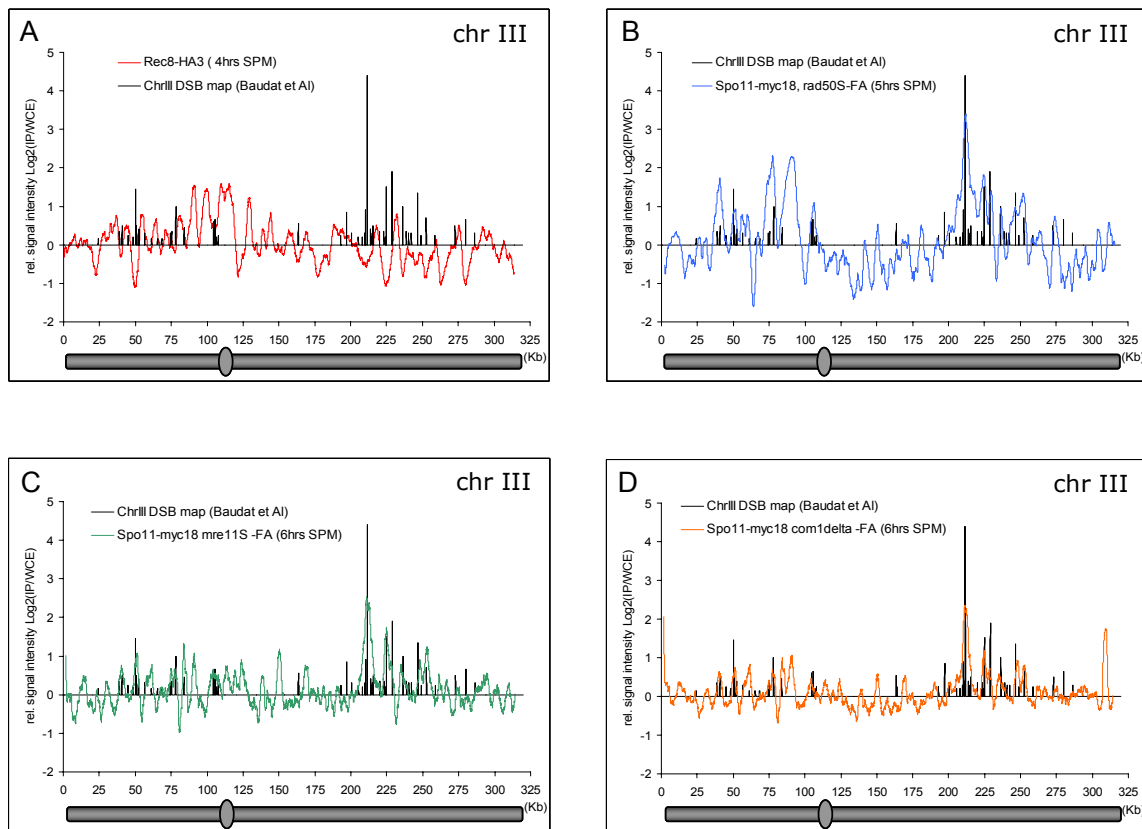


Figure 38: Map of the meiotic DSB sites performed by immunoprecipitating Spo11p in DSB repair defective mutant backgrounds in the absence of formaldehyde crosslink. (A) Comparison between the published DSB physical map for chromosome III [66], and the Rec8p-chromatin localization map obtained by ChIP on chip demonstrates the preference of meiotic DSB formation events for uncondensed loops regions. (B, C and D) ChIP on chip maps of Spo11p in *rad50S*, *mre11S* or *com1/sae2* mutants in the absence of crosslinking are compared with the published DSB physical map. The X and the Y axis corresponds to the physical positions on chromosome III (Kilobases) and the relative signal intensity (IP/WCE) represented in log2 respectively.

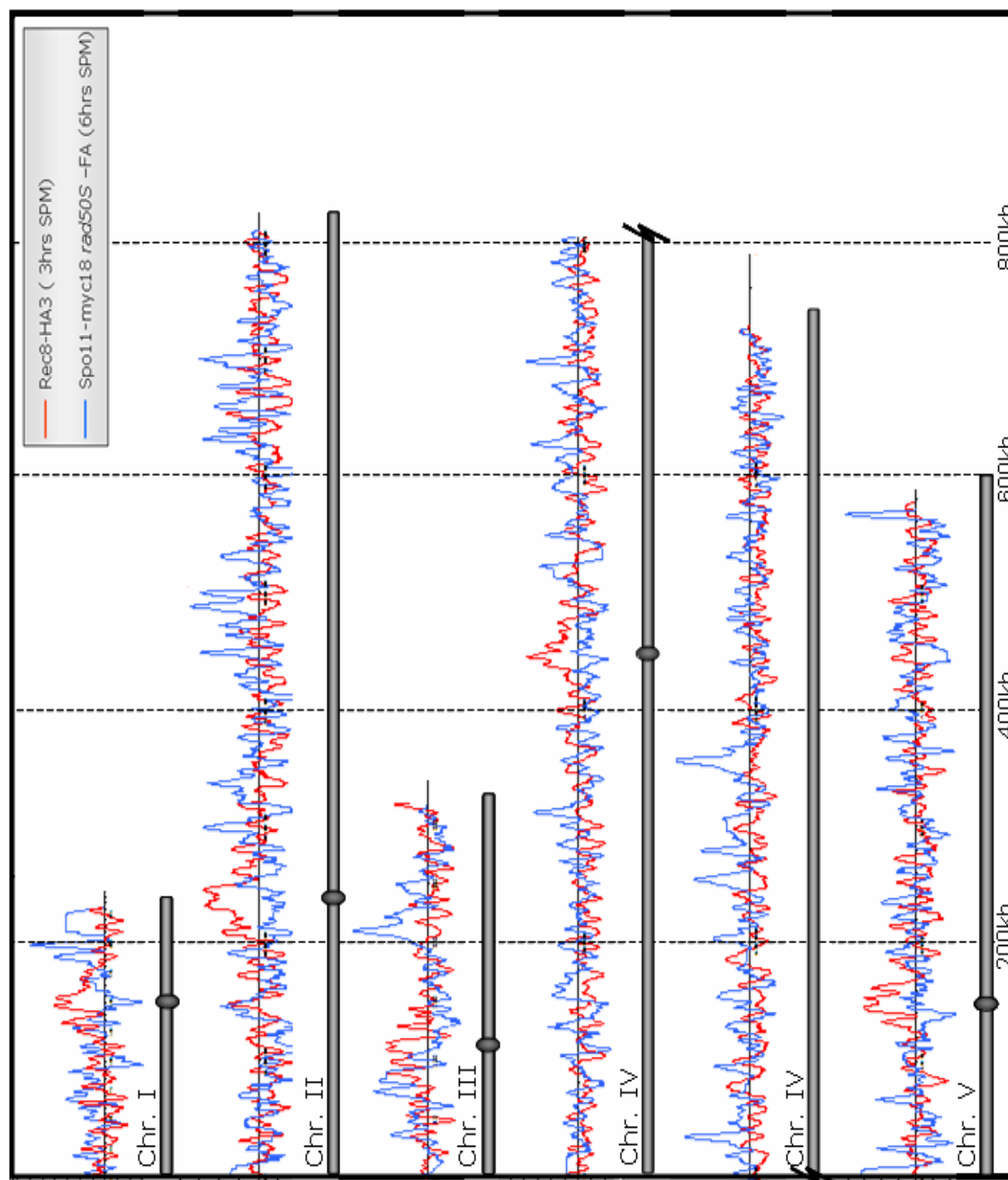


Figure 39: Spo11p-chromatin localization pattern obtained from *rad50S* mutant in the absence of Formaldehyde (-FA) and its comparison to the Rec8p-chromatin binding sites over the whole genome. Spo11p and Rec8p profiles are illustrated in blue and red respectively. For each of the 16 chromosomes the centromere is indicated with a gray circle. Protein-chromatin interaction profiles are represented as a Log2 ratio (IP vs WCE) in a 3kb sliding window.

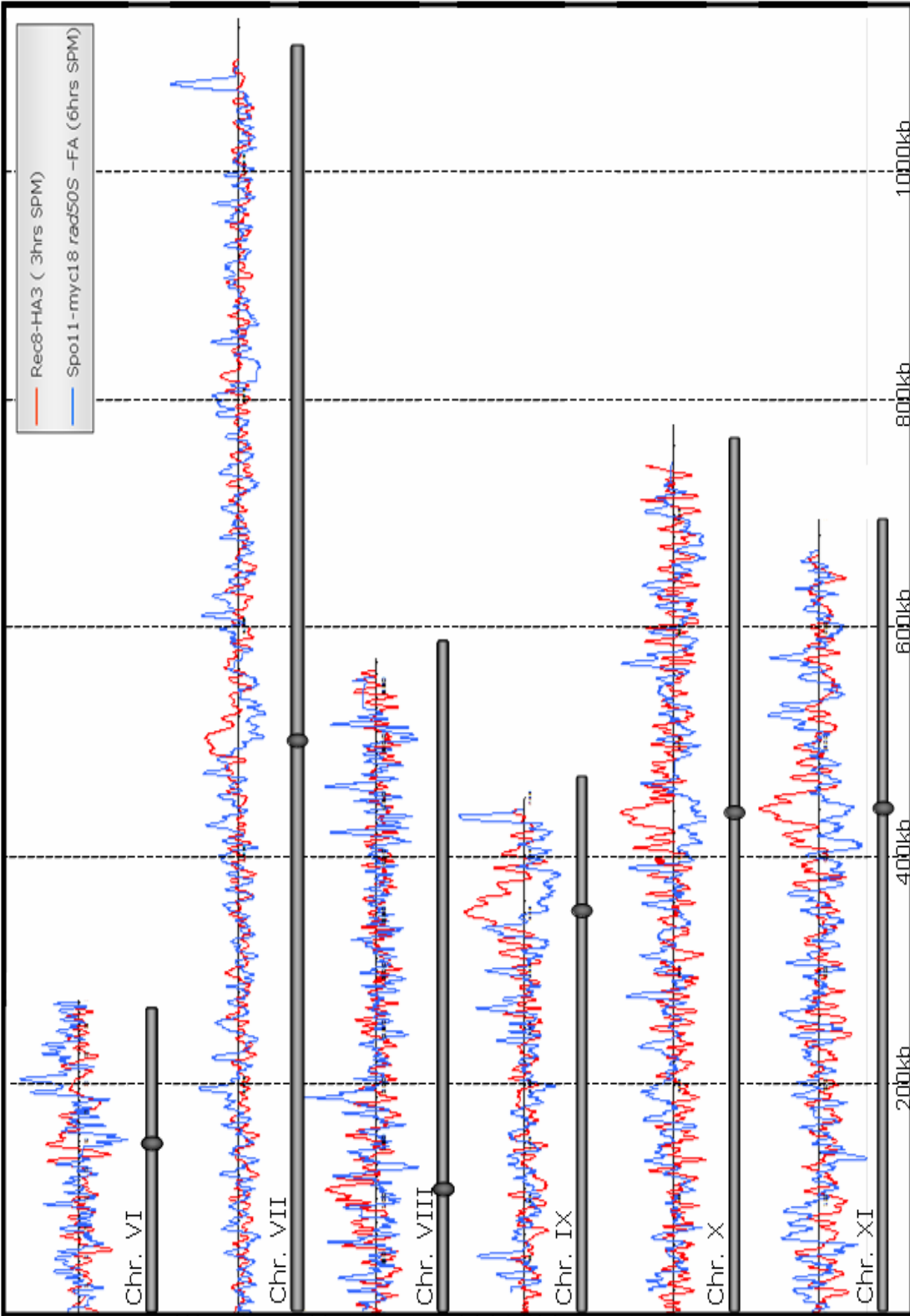


Figure 39: Continued.

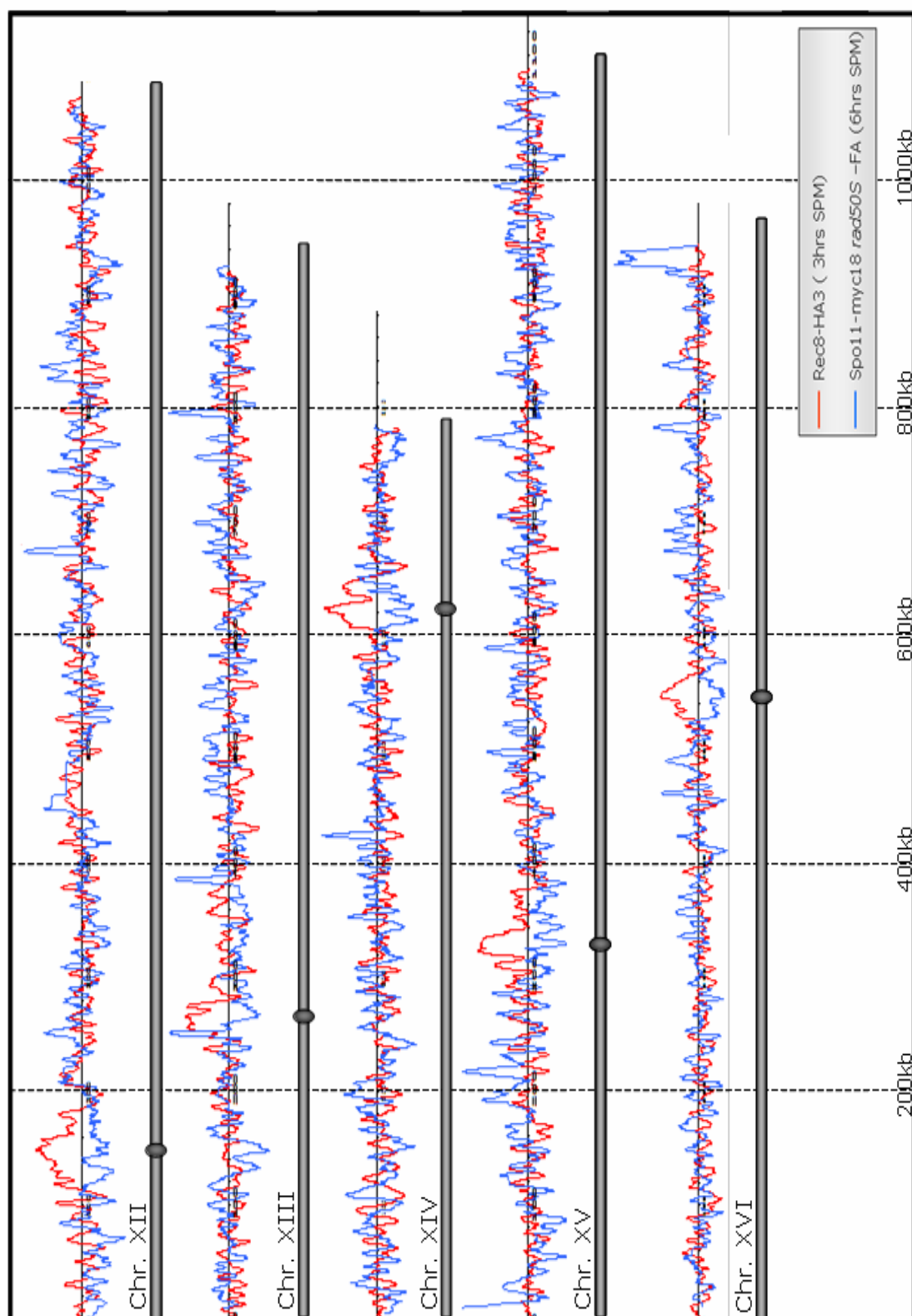


Figure 39: Continued.

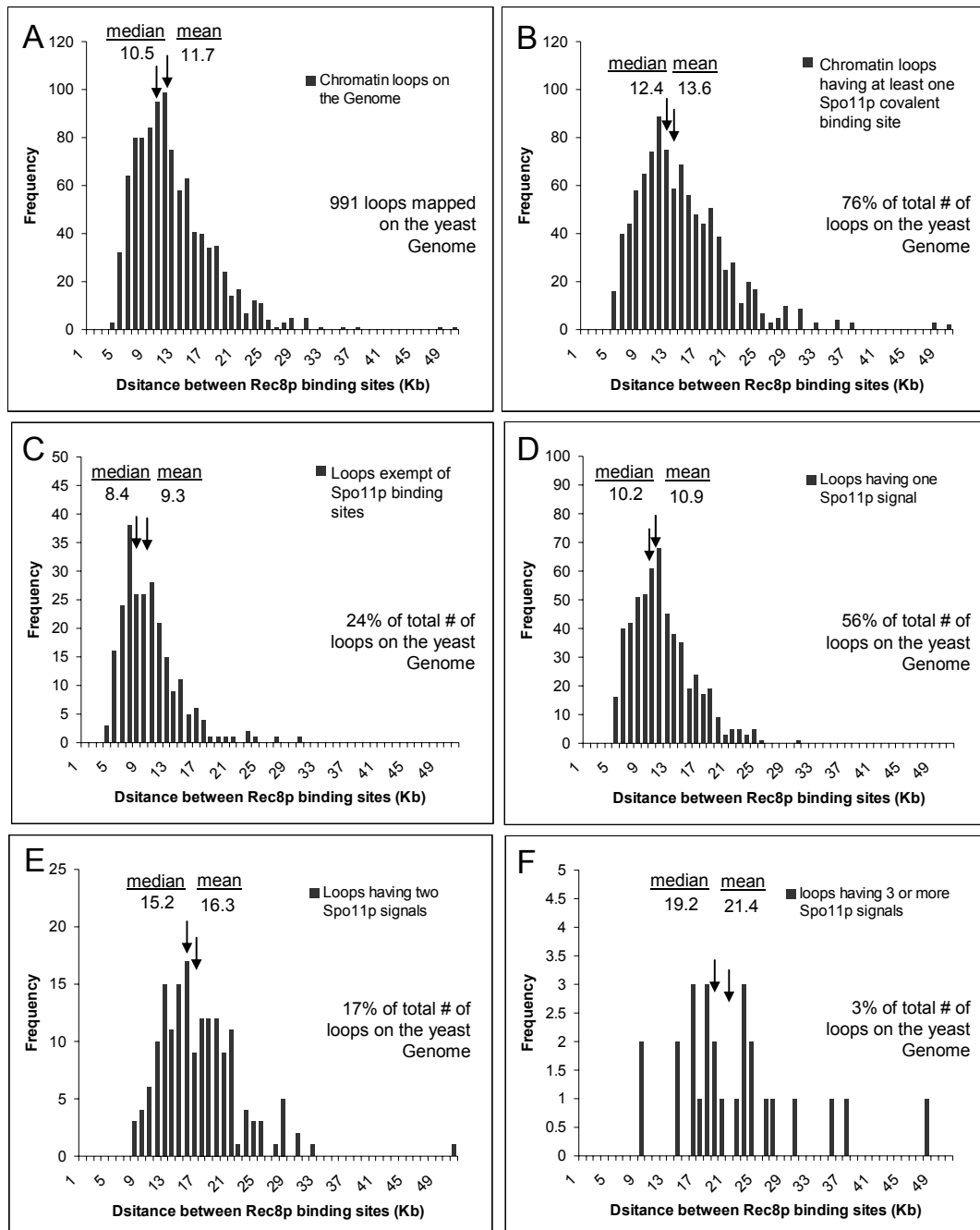


Figure 40: Histogram distributions of the number of chromatin loops presenting Spo11p binding sites. (A) Distribution of the total number of loops in the yeast Genome compared to the distribution of the number of loops having at least one Spo11p binding event (B). (C) Loops lacking Spo11p binding sites represent 24% of the total. Furthermore, the number of loops having one (D), two (E) and 3 or more (F) Spo11p binding events correspond to 56%, 17% and 3% respectively.

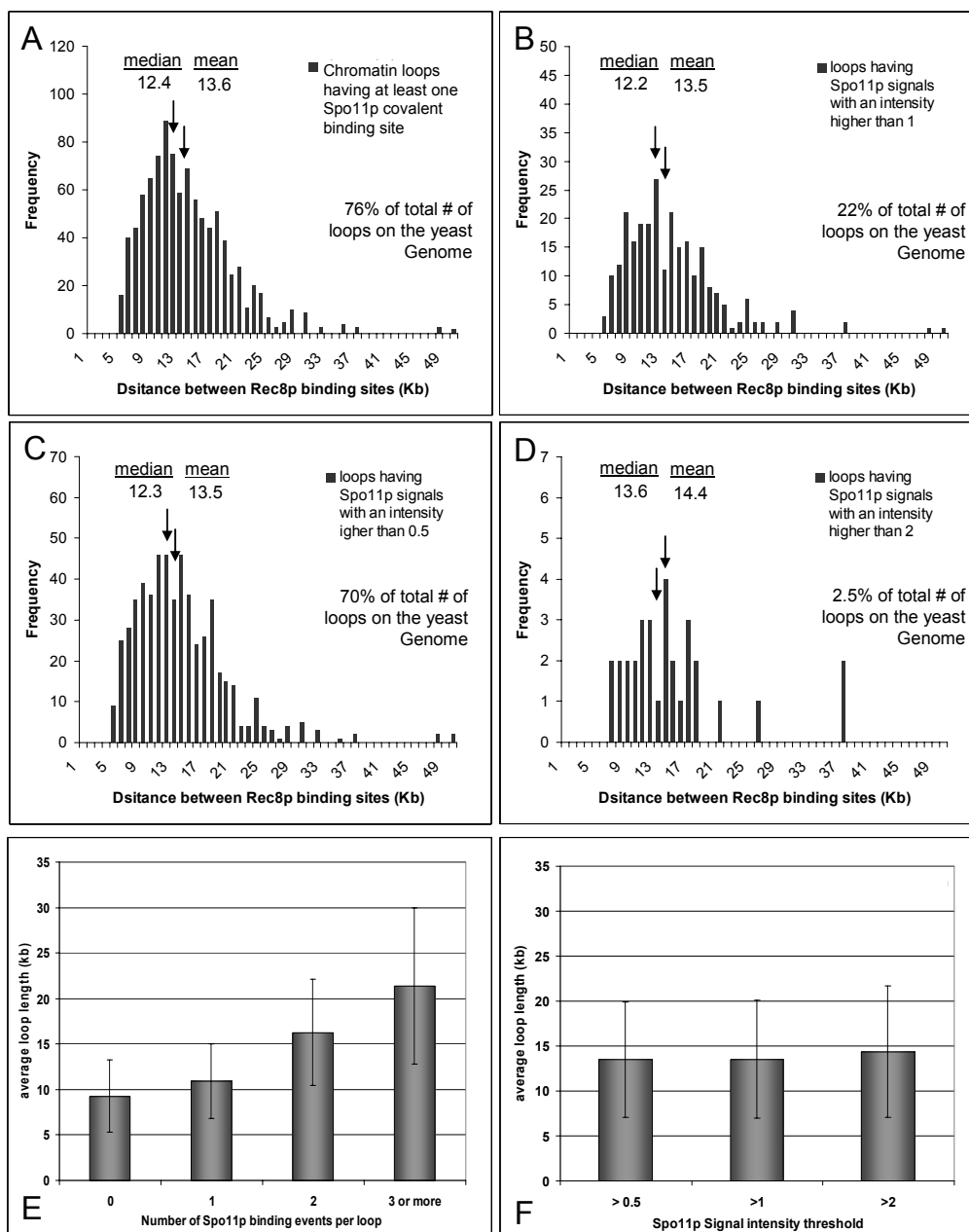


Figure 41: The chromatin loops prone to cleavage by Spo11p have an average length of 14 kb. (A) The total number of loops having at least one Spo11p binding site can be classified as a function of the Spo11p signal intensity: (C) 70% of the total number of loops on the yeast genome have Spo11 binding sites with a signal intensity higher than 0.5; (B) 22% have signal intensities higher than 1 and (D) 2.5% have signal intensities higher than 2. The signal intensities correspond to the Log2 ratio between the IP and WCE comparative analysis. The increase in the number of Spo11p sites per loop together with the average loop length is illustrated in (E); and is in contrast to a relatively constant average loop length (~14 kb) observed when compared to the Spo11p signal intensities (F).

3.2.2 The meiotic specific nuclease Spo11 does not only localizes at DSB sites, but also at chromosome core regions

In the previous paragraphs we have shown that the meiotic specific nuclease Spo11p cleaves preferentially at uncondensed chromatin loop regions. This was achieved by immunoprecipitating Spo11p from DSB repair defective mutants in the absence of *in vivo* protein-DNA crosslinking. In order to map additional sites where this protein may localize on the chromatin, we performed the same chromatin immunoprecipitation study, this time including a crosslinking step. Interestingly, the genome wide analysis performed in the presence of Formaldehyde (FA) for Spo11-myc18 in a *rad50S* strain background (5hrs after the meiotic program induction) shows a different pattern compared to that obtained in the absence of FA. Indeed, the previous Spo11p chromatin binding sites located in uncondensed loop regions in the absence of FA seem to be relatively weaker in intensity in the presence of FA. Furthermore, supplementary peaks at the flanking sites the DSB regions were mapped. When this new Spo11p map was analyzed in the context of the chromatin organization, we determined that in fact these additional peaks localize to the same sites where Rec8p was previously mapped. Supporting this finding, a similar pattern is observed when Spo11-myc18p is pulled down from an *mre11S* mutant in the presence of FA (5hrs after meiotic program induction). Furthermore, the ChIP on chip analysis of Spo11-myc18p chromatin localization in a wild type situation (4hrs after meiotic program induction) shows a similar pattern, with a certain number of chromatin interaction sites overlapping the Rec8p binding regions, as well as additional signals corresponding to the uncondensed loop regions where DSBs are formed, even though these signals are strongly reduced in intensity when compared to the Spo11p map in the absence of FA in the DSB repair defective mutants (figure 42). Although Spo11p was mapped to Rec8p chromatin sites under these conditions, not all Rec8p sites are associated with Spo11p binding sites. Indeed, the analysis of the Spo11p-chromatin interactions in a low resolution (30kb sliding window) performed on chromosome III demonstrates that Spo11p shows a preference for the GC rich chromosome arm regions, either in the presence or the absence of FA. This pattern correlates with the fact that the high GC-DNA content regions on chromosome III present a higher meiotic DSB Hotspot abundance, as demonstrated previously by the characterization of the DSB physical map on chromosome III (figure 42).

In order to separate the Spo11-chromatin binding activity from its enzymatic activity, we performed a genome wide chromatin interaction analysis with the catalytically dead mutant

Spo11-Y135F-myc18. Under these conditions the Spo11p-chromatin interactions were enhanced not only at the sites of DSBs formation, but also at the nearby chromosome core sites (defined by the Rec8p-chromatin localization pattern). Previous experiments have shown a stronger chromatin interaction at the *YCR048W* hotspot for Spo11-Y135F-myc18 [15]; however, at that time, the fact that Spo11p localizes at chromosome core sites was unknown.

In order to confirm that Spo11p localizes at chromosome cores as well as at DSB sites when FA is added, we performed the Spo11p immunoprecipitation from *rad50S* mutant in the absence of FA (6hrs in SPM) and we compared to Spo11p localization in the wild type situation (4hrs in SPM) in the presence of FA. The immunoprecipitated chromatin was analyzed by qPCR targeting two specific sites, the well characterized hotspot *YCR048W*, as well as a chromosome core site identified by the Rec8p ChIP on chip analysis, namely the core site at position 233000 in chromosome III (referred to as Core 233 in this study). As expected, both conditions (plus or minus FA), allowed for the immunoprecipitation of the *YCR048W* hotspot. In contrast, the Core 233 region was only precipitated in the wild type situation in the presence of FA (figure 43 F). While the interaction of Spo11p at the chromosome core sites is consistent between the different studied yeast strain mutants, its role at that sites remains enigmatic. Indeed, the fact that meiotic DSBs are preferentially formed in loop regions precludes the possibility of an enzymatic activity at the core sites. We hypothesize that the localization of this protein at such regions is indeed the consequence of a chromatin organization event which directs the localization of Spo11p to the chromosome core.

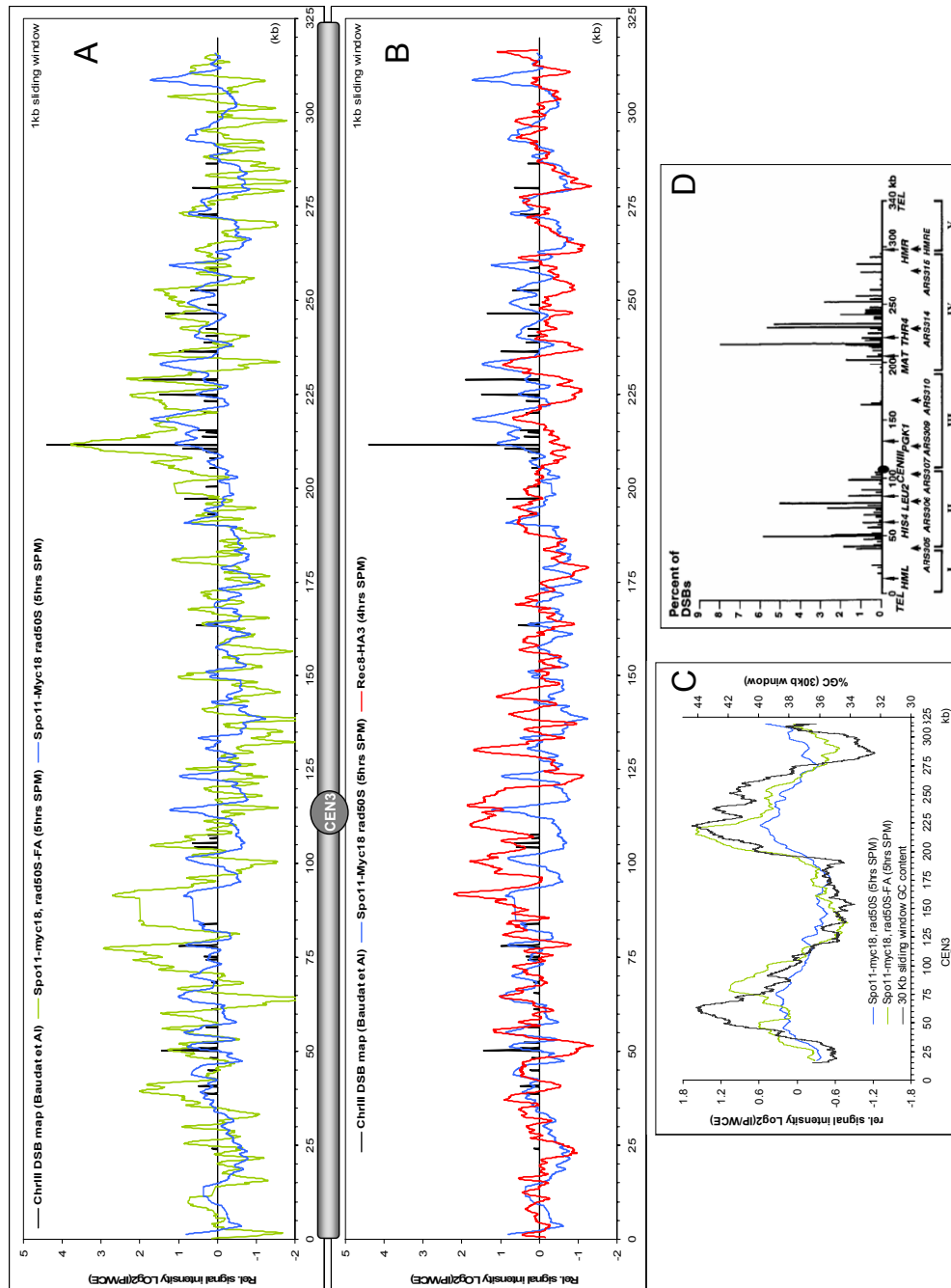


Figure 42: Spo11p chromatin localization on chr III of budding yeast. (A) Spo11p profiles from *rad50S* mutant in the absence of FA (green) and in the presence of FA (blue) are compared to the meiotic DSB physical map (black). (B) Comparison between the chromatin localization patterns of Spo11p from a *rad50S* mutant background in the presence of FA (blue) and Rec8p (red). The global chromatin localization pattern (30 kb sliding window) of Spo11p in the *rad50S* mutant background performed with and without FA crosslinking compared to the chromosome III GC-DNA content (C) demonstrates the preference of this protein for regions containing higher GC-DNA content, where meiotic DSB sites were mapped previously (D).

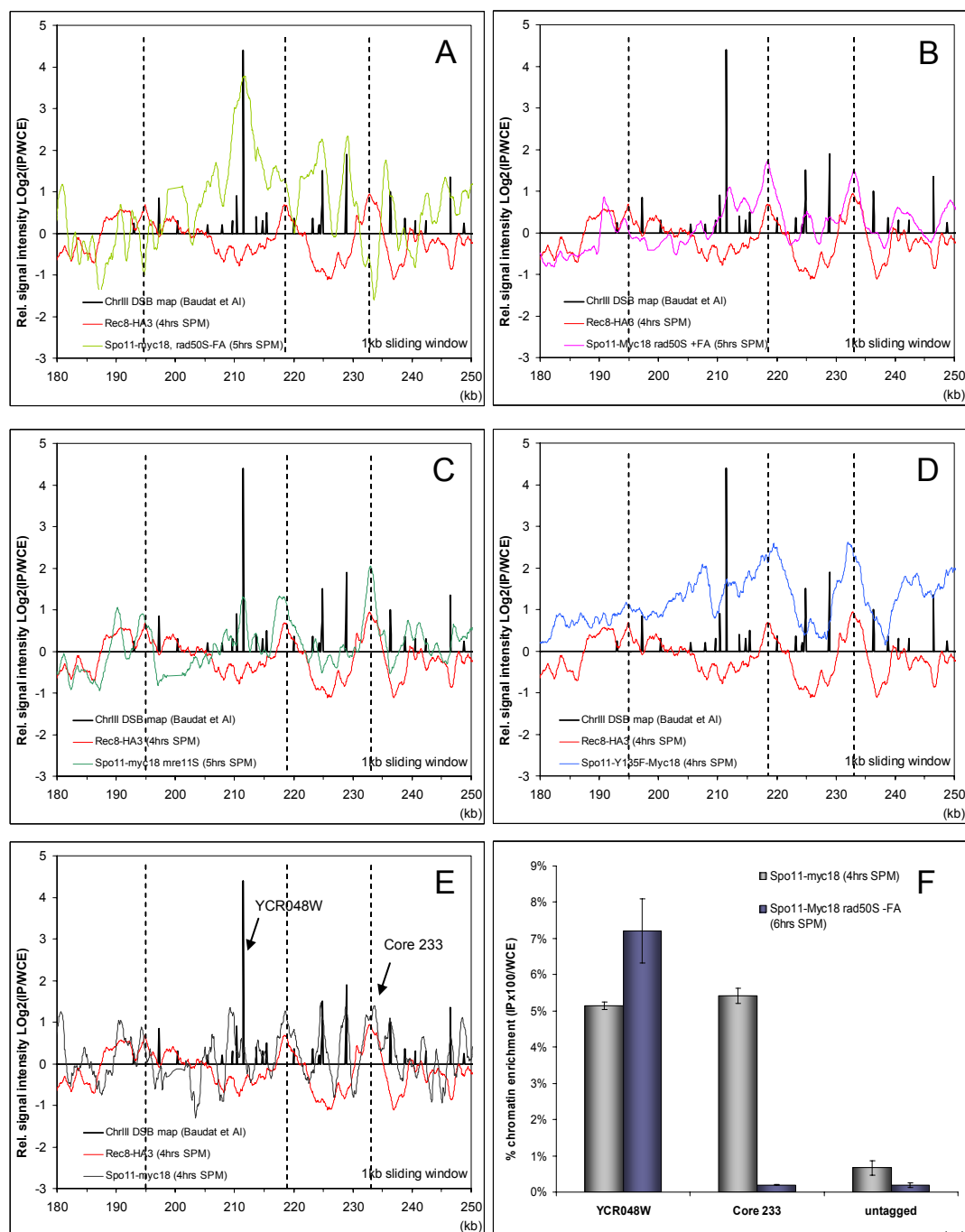


Figure 43: Spo11p interacts not only with uncondensed chromatin loop regions, but also with chromosome core regions. The chromatin localization of Spo11p at regions surrounding the *YCR048W* hotspot is illustrated for different situations: (A) *rad50S* mutant background in the absence of FA (-FA); (B) *rad50S* mutant background in presence of FA (+FA); (C) *mre11S* mutant background (+FA); (D) Spo11-Y135F (+FA); (E) wild type (+FA). In (F), the qPCR analysis performed for Spo11p *rad50S* (-FA) compared to Spo11p in the wild type background (+FA) demonstrates that Spo11p localizes to chromosome core site 233 in addition to the *YCR048W* hotspot in the presence of FA.

3.2.3 The MRX components localizes to DSB sites and chromosome core regions in meiosis I

In addition to the specific nuclease Spo11p, meiotic DSB formation in *Saccharomyces cerevisiae* requires the presence of nine other components; among them, the MRX complex (for Mre11p, Rad50p and Xrs2p). In order to characterize the chromatin localization of the MRX complex, we took advantage of a functional HA-tagged Mre11p construct (6 copies of the HA-tag fused to the C-terminus of Mre11p). Cells undergoing meiosis were collected at different time points to perform chromatin immunoprecipitation of Mre11p after formaldehyde fixation. The genome wide chromatin localization of Mre11p characterized at meiotic time point T4 (4 hours after meiotic program induction) using high resolution tiling microarrays demonstrated that this protein interacts specifically with meiotic hotspot regions. Indeed, the Mre11p-chromatin localization map, characterized on chromosome III, overlaps perfectly with the published DSB physical map[66] (figure 44). Interestingly, additional Mre11p-chromatin interaction sites correlate strongly with the chromatin localization of the meiotic cohesin component Rec8p. This correlation with Rec8p chromatin localization is preferentially observed at the chromosome arms, close to the chromatin regions prone to DSB events, in contrast to the regions surrounding the centromere where meiotic recombination is known to be strongly decreased.

In order to evaluate the transient interaction of Mre11p with either hotspots or chromosome core regions during meiotic progression, a quantitative PCR analysis was performed in Mre11p chromatin immunoprecipitated samples from different meiotic time intervals. We specifically analysed the chromatin enrichment at the hotspot region located at position 50.6 kb, as well as the widely characterized *YCR048W* hotspot located at position 212 kb both on chromosome III (called hotspot 50.6 and *YCR048W* in this study). Interestingly, the transient enrichment of Mre11p at these two hotspot regions showed a one hour difference when comparing the time points of highest relative abundance (four hours for hotspot 50.6 instead of 5 hours for *YCR048W*). This is supported by a higher abundance of chromatin enrichment for hotspot 50.6 at the 2 hour time point compared to the *YCR048W* hotspot, which is still at the same level as the 0 hour time point (see figure 44 B). Furthermore, the characterization of chromatin enrichment for chromosome core regions located at positions 219 and 233 kb on chromosome III demonstrated a similar kinetics to that observed for the hotspot *YCR048W* (highest relative chromatin enrichment at 5hours). This can be explained by the fact that the chromosome core regions we analysed are located in a proximal region to the *YCR048W* hotspot (right arm chromosome III), in contrast to hotspot 50.6 which is located

on the left arm of chromosome III. The difference in kinetics observed for the Mre11p-chromatin interaction at two different DSB sites illustrates the existence of different timing in DSB formation which is directly connected with the differential timing of firing of the origin of replication[25]. Indeed, the studied hotspot in the left arm of chromosome III (Hotspot 50.6) is located between two early-firing origin of replication (*ARS305* and *ARS306*), in contrast to the *YCR048W* hotspot which is flanked by the *ARS315* (highly active but not early-firing) and *ARS314* (passive origin of replication).

In order to support the Mre11p-chromatin localization pattern, we characterized the genome wide localization of Xrs2p by taking advantage of a functional HA-tagged Xrs2p construct (3 copies of the HA-tag fused to the C-terminus of Xrs2p). This analysis demonstrated, as expected, a strong correlation between the Mre11p chromatin localization map and that of Xrs2p at the time of meiotic DSB formation (4hours after meiotic program induction). Indeed, Xrs2p interacts with meiotic hotspot chromatin regions as well as hotspots near the chromosome Core (figure 45 A). In order to study the dependency of Mre11p chromatin localization on DSB formation, we mapped Mre11p chromatin interaction sites in the presence of the *spo11-Y135F* catalytically dead mutant. Under these conditions, the Mre11p chromatin localization pattern did not change. Indeed, its localization to meiotic DSB sites as well as chromosome core regions was still observed (figure 45 C and E).

Even though the Mre11p chromatin localization pattern was not modified by the *spo11-Y135F* catalytically dead mutant, the absence of Spo11p did affect its chromatin localization at meiotic DSB sites. In contrast, Mre11p chromatin localization at the chromosome core regions was not impaired; demonstrating the requirement of Spo11p for Mre11p localization at the meiotic DSB sites but not at the core regions (figure 45 D).

Finally, analysis of Mre11 in the absence of one of the components of the MMR subcomplex (MMR for Mei4, Mer2, Rec114), namely Rec114p, produced a similar pattern as that observed in the *spo11Δ* mutant background; a loss of interaction at DSB sites and a maintenance of the chromosome core interactions (figure 45 B).

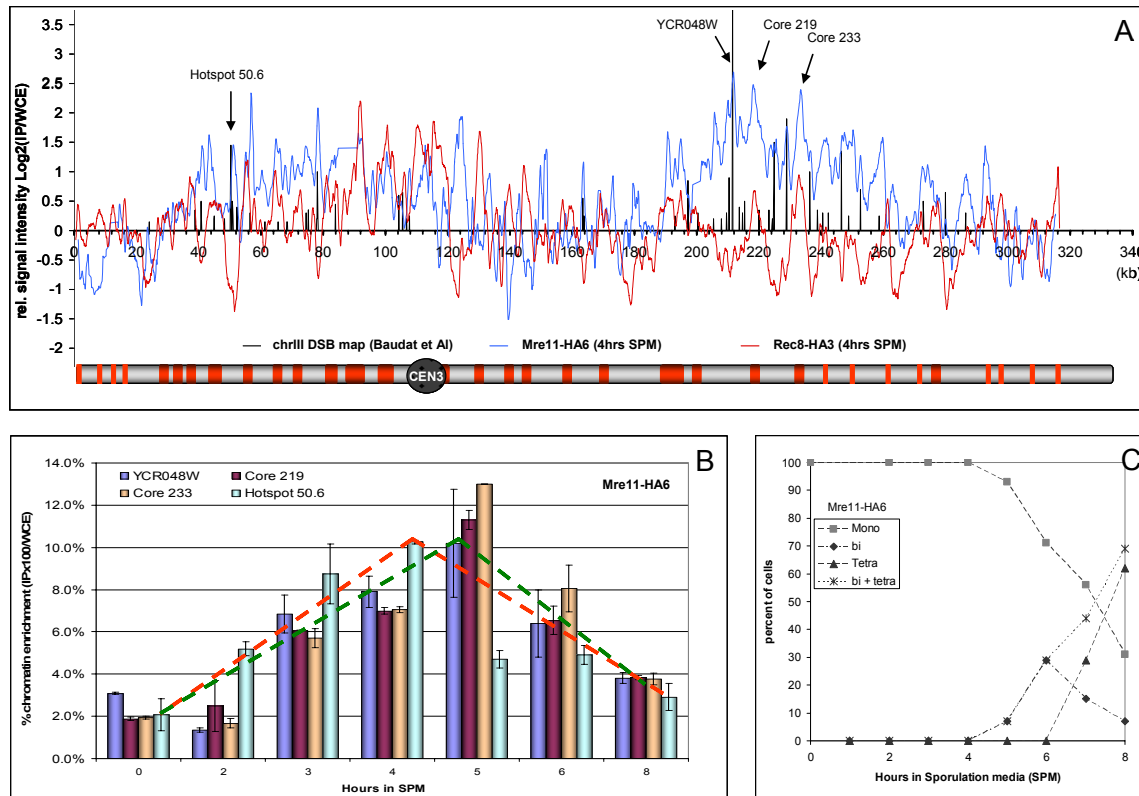


Figure 44: Mre11p association with DSB sites as well as core regions.

(A) Comparison between the physical DSB map (black) and the chromatin localization of Mre11-HA6 (Blue) and Rec8-HA3 (red) demonstrates that in addition to its localization to the meiotic DSB sites, Mre11p also localizes to the nearby chromosome core regions. (B) Quantitative PCR analysis of the transient Mre11p localization to defined chromatin sites during the progression of meiosis. Chromatin localization at the meiotic hotspot *YCR048W* (blue) and the hotspot 50.6 (light blue), as well as the chromosome core sites 219 (purple) and 233 (light orange), are indicated in (A). Notice the difference in kinetics of Mre11p localization at the meiotic hotspot 50.6 compared to the *YCR048W* hotspot (red and green dashed line respectively). The cell progression in meiosis for the Mre11p transient chromatin interaction analysis presented in (B) is illustrated in (C). Mono, Bi, Tetra and Bi+ Tetra correspond to the percentage of mononucleates, binucleates and tetranucleates respectively, observed by DAPI staining at the different meiotic time points.

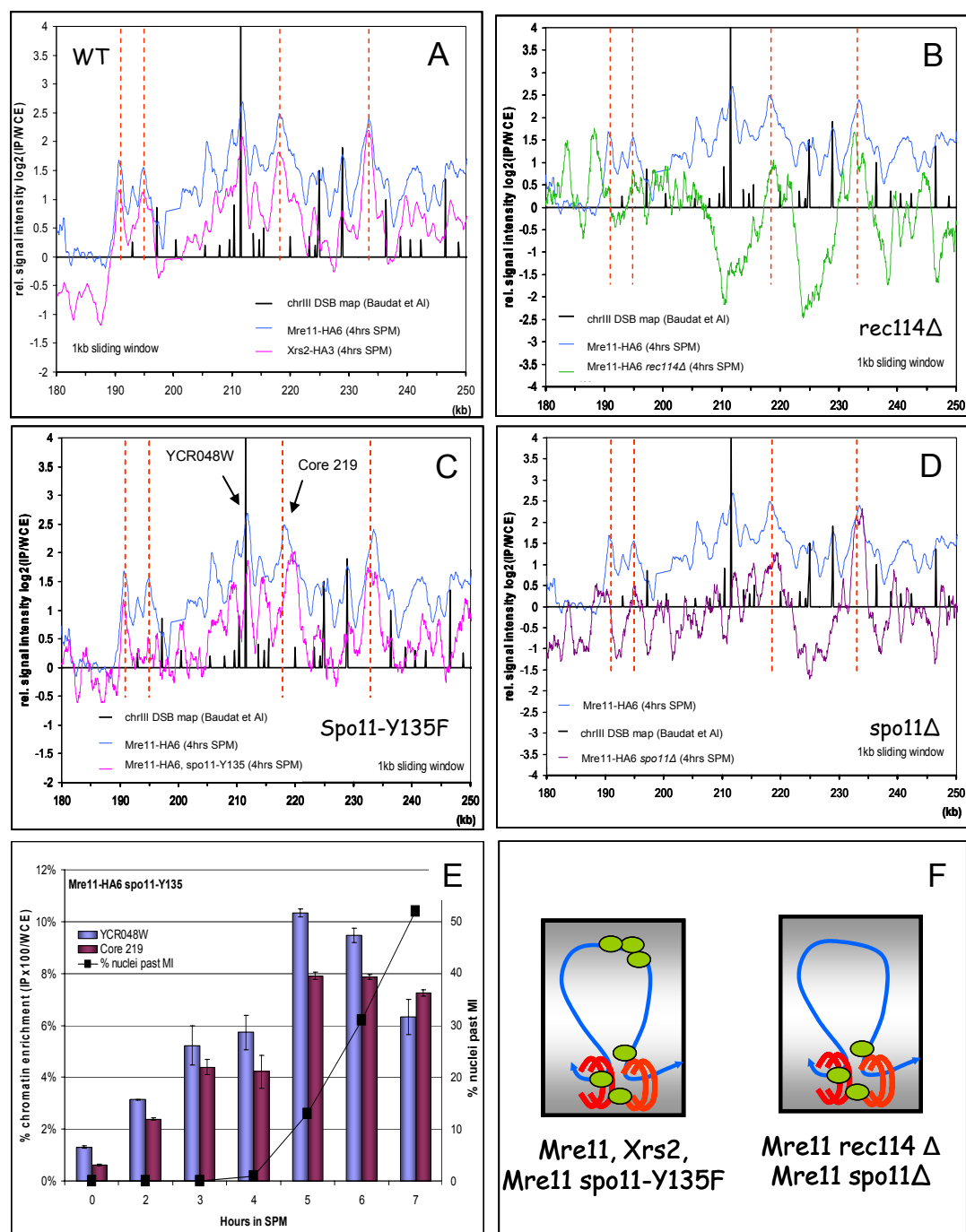


Figure 45: Mre11 interaction with DSB sites, but not with the core regions, depends on integrity of the DSB machinery, but not on DSB formation. Mre11p and Xrs2p chromatin interaction with the meiotic DSB sites and core regions is illustrated in (A). Mre11p still localizes to the chromosome core regions in *rec114Δ* (B) and *spo11Δ*, but not to the DSB sites (D). In (C) is illustrated the Mre11p chromatin localization in the *spo11-Y135F* mutant. (E) The Mre11p chromatin localization at the *YCR048W* hotspot and at the Core region 219 is shown over the meiotic progression of *spo11-Y135F* yeast mutant strain. (F) Model representation of the different Mre11p chromatin localization patterns observed among the studied mutant strain backgrounds.

3.2.4 Com1/Sae2p, the component required to complete meiotic

DSB recombination, localizes to the same chromatin

regions as the MRX complex.

In addition to its role in meiotic DSB formation, the MRX complex was shown to be essential for meiotic DSB repair. In fact, point mutations in Rad50p or in Mre11p (known as “*S*” mutants to emphasise the “Separation of function” between DSB formation and repair performed by such mutations) were shown to allow meiotic DSB formation, but the removal of Spo11p from the cleaved DNA ends is impaired, and by consequence the DSBs accumulate over time without repair. In addition to the requirement for a fully functional MRX complex for meiotic DSB repair, the protein Com1/Sae2p has also been shown to be essential for meiotic DSB repair.

In order to characterize the chromatin localization of Com1p, we took advantage of a functional Myc-tagged Com1p construct (18 copies of the Myc-tag fused to the C-terminus of Com1p). Yeast cells undergoing meiosis were collected four hours after meiotic program induction, the time point at which the meiotic DSBs are formed and are undergoing repair during a standard *SK1* yeast strain meiotic progression. The Genome wide Com1p chromatin interaction map has shown to present a strong correlation with the previously characterized Mre11p chromatin interaction map (figure 46). Despite its weak relative signal intensity compared to the Mre11p chromatin localization profile, the Com1p chromatin interaction map reproduces perfectly all Mre11 peaks corresponding to meiotic DSB sites, as well as chromosome core regions. This correlation was observed not only for chromosome III, but was demonstrated over the whole genome by performing a clustering analysis in a 1kb sliding window, where 70% of the total number of peaks in the Com1p profile overlapped with the Mre11p map and vice versa (70% of the peaks on Mre11p map overlapped with the map of Com1p). This analysis demonstrates that both Com1p and Mre11p chromatin interaction maps have a similar number of peaks over the whole genome (3067 peaks for Com1p and 3144 peaks for Mre11 when the analysis is performed in 1kb resolution sliding window profiles), of which 30% do not overlap (see figure 31 in the Materials and methods where the clustering analysis for Com1p, Mre11p and Xrs2p chromatin interaction maps is illustrated).

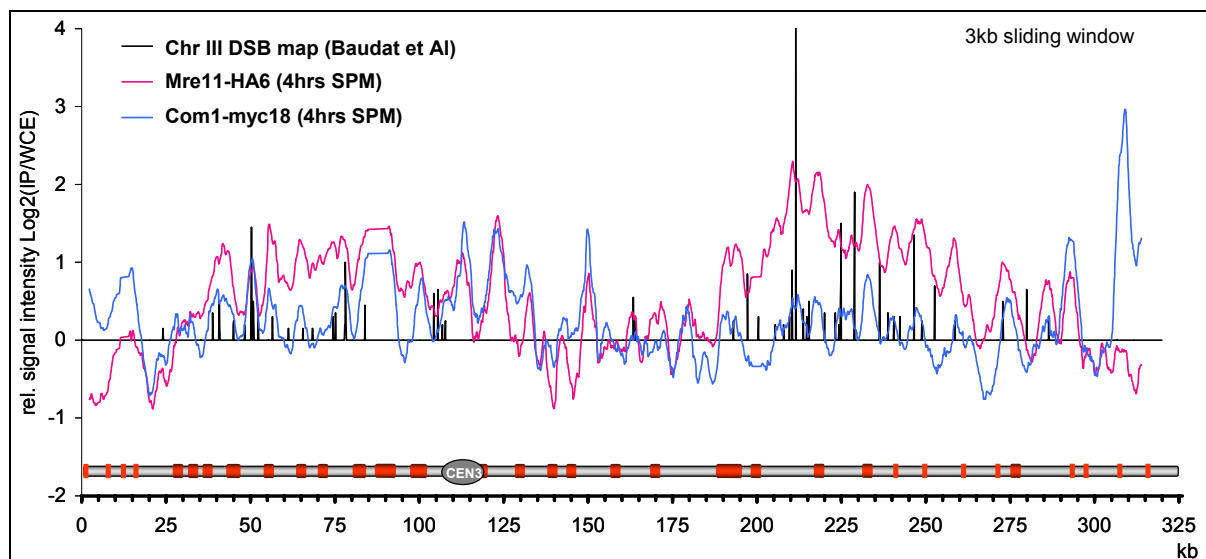


Figure 46: Com1p localizes to the same chromatin regions characterized for Mre11p. The chromatin localization map of Com1p (blue) performed at 4 hours after meiotic program induction strongly correlates with that of Mre11p (pink). The meiotic DSB physical map is represented by black bars. Furthermore, the Rec8p-chromatin localization sites are represented in red over the cartoon of chromosome III. Notice that Com1p localizes not only to DSB sites (Black bars) but also to chromosome core regions. The X and Y axis correspond to the physical positions on chromosome III (in Kilobases) and the relative signal intensities associated to each physical position represented in Log₂ scale (Log₂(IP/WCE)) respectively.

In order to study the influence of the components forming the meiotic DSB machinery on the proper localization of Com1p on the chromatin, we have analysed Com1p-chromatin localization on the absence of Spo11p. Under this condition, Com1p localization at the meiotic DSB sites was affected, in contrary, its interaction with the chromosome core sites was still detected (figure 47D). This observation correlates with the fact that Mre11p localization at the meiotic DSB sites is also impaired in the *spo11Δ* mutant background (figure 47D). This concordance of phenotypes would argue for a direct connection between the Mre11p and Com1p chromatin localization; nevertheless, the absence of Mre11p did not impair Com1p localization at the DSB sites (figure 47C). Furthermore, the *mre11-H125N* catalytic dead mutant, did not change Com1p localization pattern on the chromatin (still detected at DSB sites and core regions) (figure 47B). In order to demonstrate that Com1p localization pattern at the chromosome cores depends on the presence of the cohesin components, we have analysed its localization pattern in a *rec8Δ* mutant background. Interestingly, under this condition, Com1p localization at the chromosome cores and also at the meiotic DSB sites was strongly impaired.

In summary, these analyses demonstrated that Com1p is a protein that interacts with the chromatin, specifically at the sites where the meiotic DSBs are formed, but in addition it localizes at chromatin regions that were characterized as Rec8p binding sites and where several other early recombination components, like Mre11p, Xrs2p and Spo11p, localize. Furthermore, Com1p localization at the DSB sites depends on Spo11p but not on Mre11p, suggesting that its localization at that regions does not pass via a putative Mre11p-Com1p interaction. Finally, its affinity to the chromosome core regions, as well as to the DSB sites, depends on the presence of the cohesin component Rec8p, suggesting that its proper chromatin interaction pattern indeed depends on the chromatin organization adopted during meiotic prophase I.

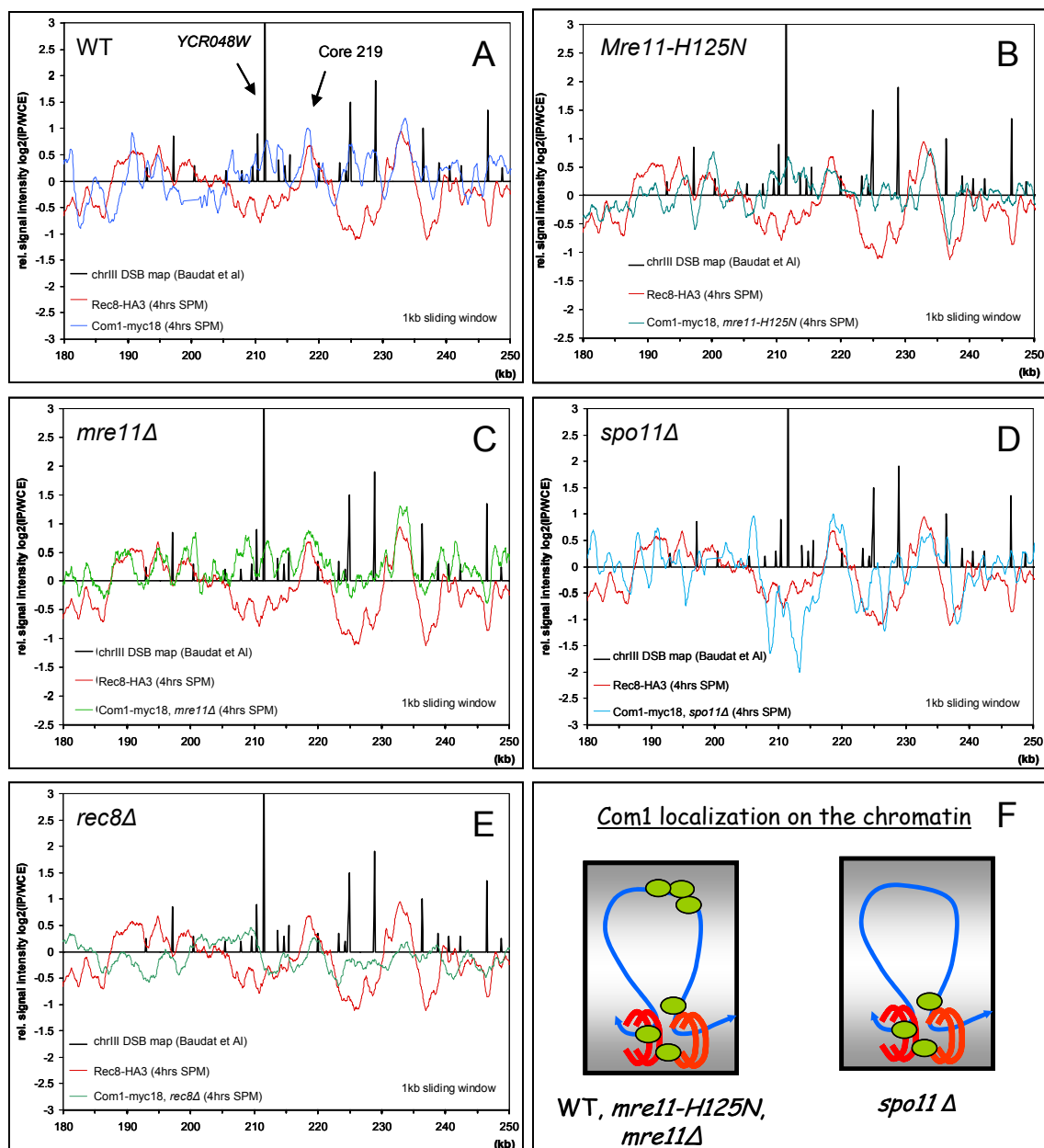


Figure 47: Com1p interaction with the meiotic DSB sites is impaired in the absence of Spo11p, but does not depend on Mre11p. (A) Com1p chromatin localization is compared with Rec8p pattern and physical DSB map. The *mre11-H125N* catalytic dead mutant (B), or the absence of Mre11p (C) do not impair Com1p-chromatin localization. (D) Absence of Spo11p, abolish Com1p localization at the DSB sites, but not at the core regions. (E) Absence of Rec8p affects Com1p localization at the DSB sites and chromosome core regions. (F) Model summarizing Com1p localization on the chromatin under the studied mutants' background.

3.2.5 Interaction of Com1/Sae2 with meiotic DSB sites is abolished in the *rad50S* mutant

The characterization of Com1p's genome wide chromatin localization demonstrated a strong correlation with that of the MRX complex (figure 46 and 48A). Considering that these components are essential for proper meiotic DSB repair, the identification of chromatin binding sites for Com1p strongly suggests its direct role in DNA repair and raises the possibility of a direct interaction with the MRX complex. However, the fact that both the MRX components as well as Com1p localize preferentially to chromosome core sites, in addition to their localization at the meiotic DSB sites, indicates that these components can interact with chromatin regions that have not been shown to be cleaved during meiotic recombination. In order to study the dependency of the Com1p-chromatin interaction on the presence of Spo11p, we previously characterized the genome wide localization of Com1p in an *spo11Δ* mutant background (chapter 3.2.4, figure 47D). Interestingly, its chromatin localization at meiotic DSB sites is impaired in this mutant background. Although it is depleted from the meiotic DSB sites, its chromatin localization at the nearby chromosome core regions is still observed, indicating that these chromatin interactions do not depend on meiotic DSB formation.

On the other hand, the biochemical influences of the "S" mutations characterized in the MRX complex components, in the MRX as well as Com1p chromatin localization are not elucidated so far. In order to address this question, previous studies performed in our laboratory has been demonstrated a lost of foci co-localization between Mre11p and Com1p in a *rad50S* mutant background in chromatin spreads. Nevertheless this strategy was unable to discern between the alternate possibilities of a mislocalization of Com1p, of Mre11p, or both from the chromatin. Furthermore, the low resolution of such experimental assay is not suitable to distinguish between a loss of foci formation at the specific DSB sites in contrast to other possible binding regions.

Taking advantage of the high resolution obtained with the ChIP on chip analysis, we decided to address the possible influence of the "S" mutations on the chromatin localization of the components involved in meiotic DSB repair. For this purpose, the characterization of the genome wide chromatin localization of Com1p in a *rad50S* mutant background demonstrated that in fact, Com1p interaction with the meiotic DSB sites is specifically impaired while its chromatin localization at the chromosome core sites remains intact, similar to the situation previously described for *spo11Δ* (figure 48B). Surprisingly, characterization of Com1p-chromatin interactions in the *mre11S* mutant background, did not demonstrate a specific

depletion of Com1p-chromatin localization at either the meiotic DSB sites, or at the chromosome core sites (figure 48C), fact that also correlates with the analysis of Com1p localization under the *mre11-H125N* catalytic dead mutant (figure 47B). In contrast, analyses of the Mre11p chromatin localization demonstrate that neither the *rad50S* mutant background nor the absence of Com1p affects its chromatin localization pattern (figure 48D). These analyses strongly suggest a biochemical difference between the genetically identical *mre11S* and *rad50S* mutants in the context of meiotic DSB repair. Indeed, the *rad50S* mutation is associated with a loss of recruitment of Com1p to the meiotic DSB sites. This is in contrast to the situation with the *mre11S* mutation, where Com1p recruitment seems to be unaffected, while it is known that the enzymatic activity of Mre11p is affected.

Furthermore, the chromatin localization of Xrs2p was analysed in the *rad50S* mutant background. As is the case for Mre11p, its localization at both the meiotic DSB sites as well as the chromosome core regions did not change (figure 48E), indicating that the *rad50S* DSB-repair defective mutant affects the proper chromatin localization of Com1p specifically.

To compare in a quantitative manner the various previously described genome wide chromatin binding profiles, we developed a hierarchical clustering strategy. Briefly, the protein-chromatin interaction profiles to be analysed are simplified into putative protein-chromatin interaction sites barcode profiles which can then be compared to each other by performing a hierarchical comparative analysis (see chapter 2.6.7.5 in Material an methods for an extensive description of the methodology). This comparative analysis is performed only for the protein binding sites and not for the relative signal intensity, and is complemented by the pair wise quantification of the percentage of matching overlaps between all possible profiles combinations (this is described as a Pairwise matching comparison matrix). In figure 49, the ChIP on chip hierarchical clustering analysis performed for Spo11p, Mre11p, Xrs2p and Com1p components under the different previously described mutant backgrounds is shown. The pairwise matching matrix is represented in a colour code format in order to visually identify particular clustered regions. The clustering analysis over the whole genome demonstrates the strong similarity between Com1p and Mre11p ChIP on chip profiles.

Furthermore, Spo11p-chromatin interaction profile and then Com1p in *mre11S* mutant background appears closer in similarity to the Com1p/Mre11p initial cluster group (figure 49). In contrast, Com1p profiles in the *rad50S* and *spo11Δ* backgrounds cluster together, but far away from the Com1p/Mre11p clustering group. On the other hand, profiles of Xrs2p, Spo11p in *mre11S* mutant background and Xrs2p in the *rad50S* mutant background cluster

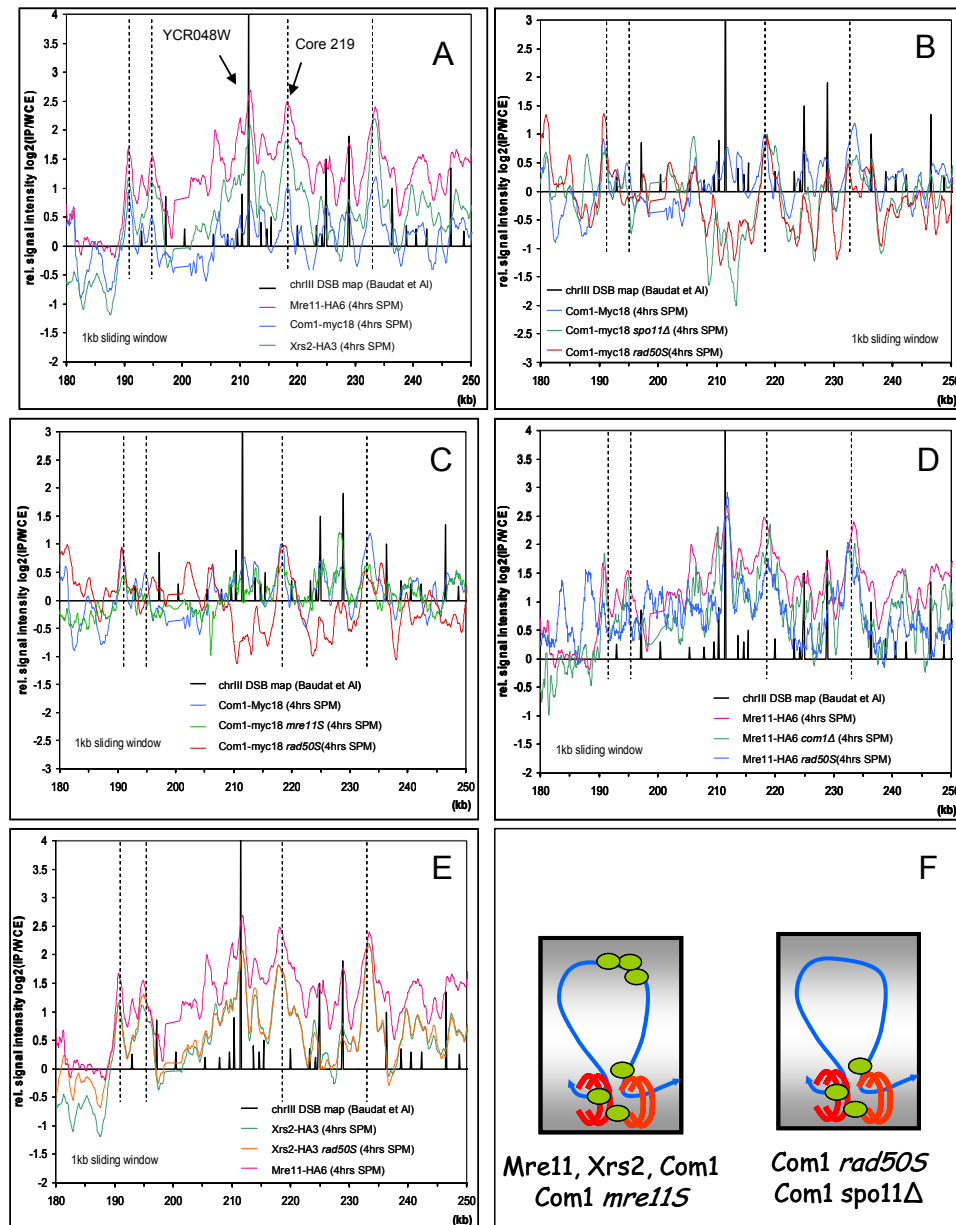


Figure 48: Com1p localization at the meiotic DSB sites is specifically impaired in *spo11Δ* and *rad50S* mutants background. (A) Com1p chromatin localization at the meiotic DSB sites as well as at the chromosome core regions is illustrated in comparison to Mre11p and Xrs2p localization. The specific loss of Com1p localization at the meiotic DSB sites in the *rad50S* and *spo11Δ* mutant backgrounds is shown in (B). Com1p chromatin localization in the *mre11S* DSB repair defective mutant is compared to the *rad50S* situation in (C). Mre11p chromatin localization at the DSB sites as well as at the core regions in the wild type, *rad50S* and *com1Δ* mutants respectively is illustrated in (D). The chromatin localization of Xrs2p in a wild type situation is compared to that in a *rad50S* mutant background in (E). (F) A cartoon model explains the different chromatin localization patterns characterized under the *rad50S*, *spo11Δ* and *mre11S* mutant backgrounds. Notice that the DSB sites are represented as black bars, and chromosome core sites are marked with black dashed vertical lines.

together with more than 80% matching overlap among them, again demonstrating the strong similarity between the chromatin localization observed for Spo11p and Xrs2p. Finally, the profile of Spo11p in the *rad50S* mutant background in the absence of formaldehyde lacks significant similarity to any other profile; this is due to the absence of chromosome core interaction sites for Spo11p in this mutant background.

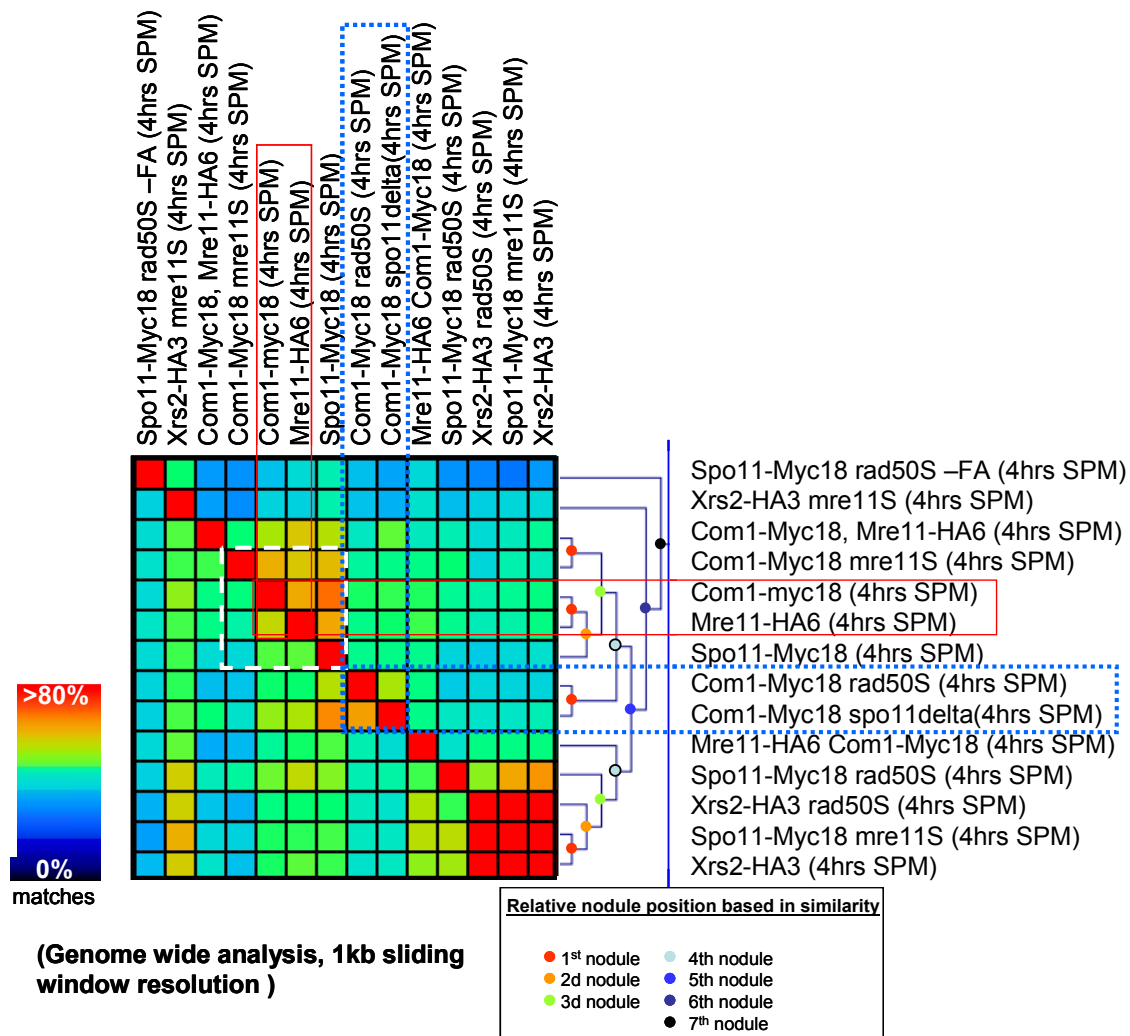


Figure 49: Hierarchical clustering analysis of Protein-chromatin interaction maps performed for meiotic recombination proteins involved in DSB formation and repair. The nodules of similarity of the clustering tree have been classified based on their positions on the tree (color code). The clustering analysis demonstrates that Com1p and Mre11p ChIP on chip profiles display a strong similarity (red line). In addition, Spo11p and Com1p in the *mre11S* mutant background are part of the same Com1p/Mre11p cluster similarity group (white dashed square). Furthermore, Com1p in *rad50S* and in *spo11Δ* mutant background clusters together but far away from the Com1p/Mre11p cluster (blue dashed square). The clustering analysis has been performed over the whole genome, with a 1kb sliding window resolution (for an extended description of the clustering methodology, see chapter 2.6.7.5 in materials and methods). In addition, it has been complemented with a pairwise matching matrix (percent of matches represented in a color code).

3.2.6 Spo11p-chromatin localization detection is decreased in the absence of the axis components.

Taking into consideration that the meiotic recombination machinery localizes not only to the DSB sites but also to the nearby chromosome core regions, we could speculate that chromatin structure plays a direct role in the initiation of meiotic recombination. Indeed, previous reports have demonstrated a reduction of DSB formation to 20-60% of wild type levels in a *red1Δ* mutant background, as well as a more remarkable effect (5-10% of wild type levels) in a *hop1Δ* mutant background. In order to analyse the effects of these mutations on protein-chromatin interaction, we performed a ChIP on chip analysis with the Spo11-myc tagged construct in the *rad50S* DSB repair defective mutant background. Whereas the presence or absence of the *in vivo* crosslinking procedure during sample preparation showed a strong difference in the chromatin localization of Spo11p in the *rad50S* background (Spo11p in *rad50S* +FA displays protein binding signals at the chromosome core regions in addition to the DSB sites), the absence of Red1p not only decreased the Spo11p-chromatin interaction at DSB sites, but also at the chromosome core regions, either in the presence or absence of FA (figure 50 A and B). In addition, the chromatin localization of Spo11p in the absence of Hop1p showed a similar pattern, namely a strong decrease in chromatin binding at the DSB sites as well as a significant reduction at the chromosome core regions (figure 50 C and D).

In order to evaluate the influence of the chromosome core organization on the steps following Spo11p removal from the meiotic DSB-ends, we took advantage of a Myc-tagged RFA1 construct (18 copies of the Myc tag epitope fused to the C-terminus of RFA1) to monitor the formation of 3'-single-stranded DNA by resection of the 5'-DNA ends following Spo11p removal. Interestingly, the RFA1p-chromatin interaction map reveals its localization not only at the DSB sites but also at the nearby chromosome core regions (Figure 51A) as is the case of all of the other recombination proteins analysed. In order to know whether its localization at the Core regions depends on meiotic DSB formation as well as in DSB repair, in a joint experiment performed with Silvia Panizza, we have mapped RFA1p-chromatin localization in the absence of Spo11p and under the *com1/sae2Δ* DSB repair defective mutant background respectively. Under these conditions, RFA1p seems to localize at the chromosome core regions (although with a weaker intensity than that observed in the wild type situation) suggesting that this protein binds to the chromosome scaffold even in the absence of DSB formation or repair (figure 51C, E and F). Considering that progression of meiotic recombination requires single strand invasion into the homologue chromosome, a

process which depends on Dmc1p, we analysed the RFA1p-chromatin interaction in the absence of Dmc1p. Under this condition, the RFA1p-chromatin localization at the DSB sites showed a similar pattern to the wild type situation, but interactions at the chromosome core regions appear to be reduced (figure 51B). Finally, analysis of RFA1p-chromatin interactions in a *red1Δ* mutant background demonstrate specific interactions with meiotic DSB sites, similar to interactions observed in the wild type background, but with a reduced chromosome core affinity, as is the case for *com1/sae2Δ* or *spo11Δ* mutants (figure 51 D and F).

Considering that the DSB repair defective mutants presented extremely weak Spo11p signal intensities when Red1p was deleted (figure 50), we hypothesized that this is the consequence of the Spo11p removal via a different pathway. In that case, the Spo11p dependent DSBs could be repaired, either following an inter-sister or inter-homolog pathway. Under this assumption, we tempted to detect RFA1p loaded molecules, as a way to monitor the repair of such yeast mutants. For it we performed a ChIP on chip analysis in an RFA1-myc tagged strain under a *com1Δ*, *red1Δ* double mutant (figure 51G). Unfortunately, we were not able to detect RFA1p chromatin enrichment associated to the meiotic DSB sites. Considering that this observation was unexpected, further analysis under *com1Δ*, *red1Δ* and *rad50Δ*, *red1Δ* double mutants' strains must be performed in order to be sure of the observed pattern in figure 51G.

In summary, the loss of Spo11p-chromatin localization in the *rad50S*, *red1Δ* double mutant, either in the presence or absence of FA fixation, would suggest either an impairment of Spo11p-chromatin localization, or a suppression of the *rad50S* meiotic DSB repair defect. From previous reports it is known that the *rad50S*, *red1Δ* double mutant does not completely impair DSB formation, but formation is reduced 4 to 12 fold compared to the wild type situation[76]; this fact could explain the weak signal intensities detected for Spo11p-chromatin interactions at the DSB sites in this double mutant. The same is true for the *hop1Δ* mutant where DSB levels are reduced to 5-10% of the wild type levels[77]. As an alternative method to monitor DSB repair we used the protein RFA1 to identify the presence of single-stranded DNA. Our experiments have previously shown that even in the absence of DSB formation or repair, there is still a weak chromatin interaction specific to the chromatin scaffold. In contrast, DSBs that are normally repaired have RFA1p localized to the DSB sites, but RFA1p also shows strong chromosome core signals in regions presenting strong DSBs abundance. This specificity for the chromatin scaffold is decreased in a *dmc1Δ* mutant and abolished (returning to *spo11Δ* or *com1/sae2Δ* levels) in the absence of Red1p. Interestingly, both *dmc1Δ* and *red1Δ* mutants display a similar enrichment of RFA1p at the DSB sites a fact

that correlates with a previous report demonstrating the requirement of Red1p for loading Dmc1p onto the resected DSB s.

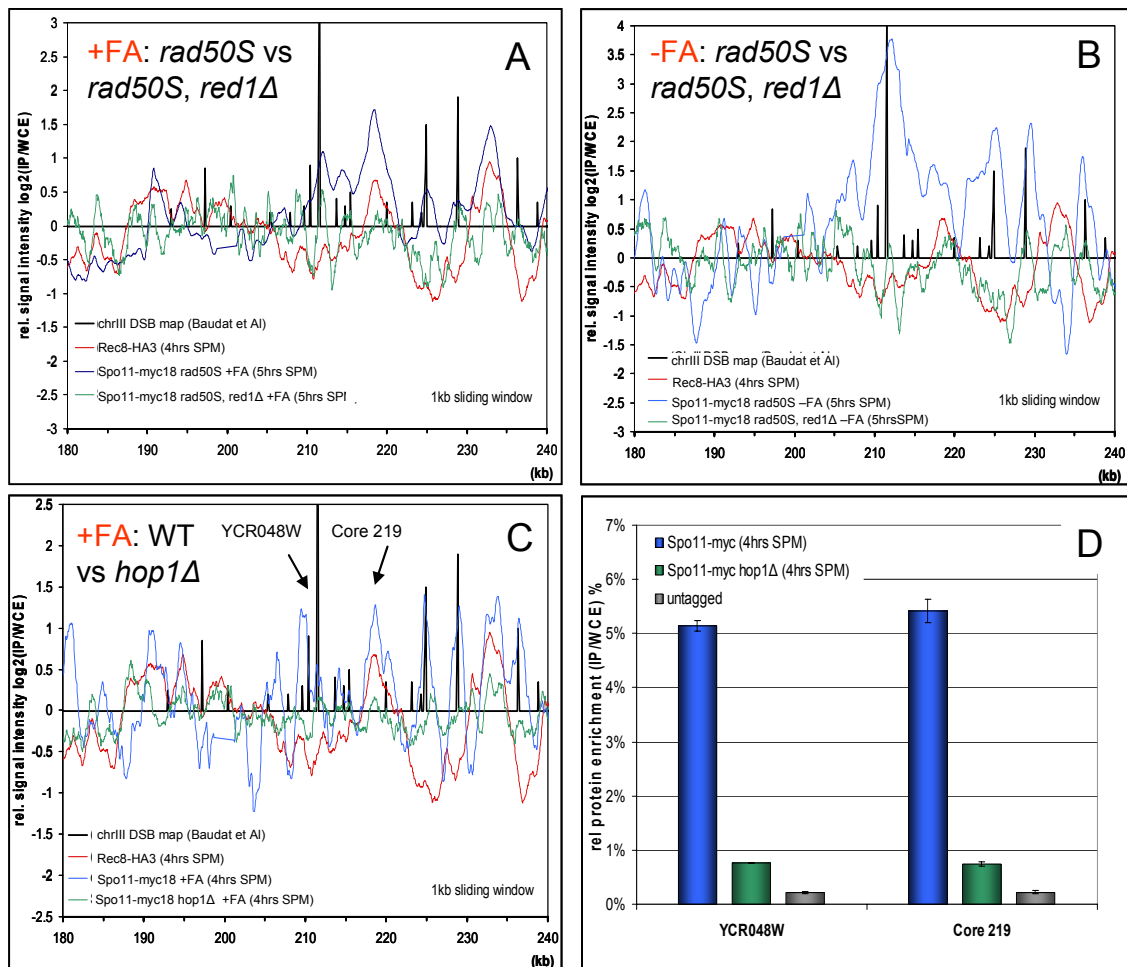


Figure 50: Spo11p-chromatin interactions analyzed by ChIP on chip are strongly reduced in *red1Δ* and *hop1Δ* mutants. (A and B) Spo11p-chromatin localization in the *rad50S* mutant background is strongly impaired by *red1Δ* either in the presence or absence of FA crosslinking. (C) Spo11p-chromatin interaction in a *hop1Δ* mutant background is strongly affected compared to the wild type situation. (D) The chromatin immunoprecipitated by Spo11p under the Wild type and *hop1Δ* situation was analyzed by qPCR. The analyzed chromatin regions corresponds to the *YCR048W* hotspot, and the chromosome core 219 respectively (These chromatin regions are indicated in (C)).

Figure 51: RFA1p binding in the presence or absence of Red1p. The chromatin localization of RFA1p at meiotic DSB sites as well as chromosome core regions is demonstrated in (A). The absence of RFA1p-chromatin localization at DSB sites is shown in (C) and (E) for *spo11Δ* and *com1Δ* mutants respectively. In *dmc1Δ* and *red1Δ* mutants, RFA1p localizes normally to the DSB sites, but the chromosome core localization is significantly reduced (B and D). In (F) the chromatin immunoprecipitated with RFA1p either in *red1Δ* or *com1Δ* was analyzed by qPCR for enrichment at the *YCR048W* hotspot and chromosome core 219 respectively (these chromatin regions are indicated in (A)). The preliminary tentative to demonstrate that DSBs induced in a *com1Δ, red1Δ* double mutant load RFA1p is shown in (G). A cartoon model illustrating RFA1p chromatin localization pattern characterized during this study is illustrated in (H). Experiments corresponding to figures (A) and (B) were performed by Silvia Panizza.

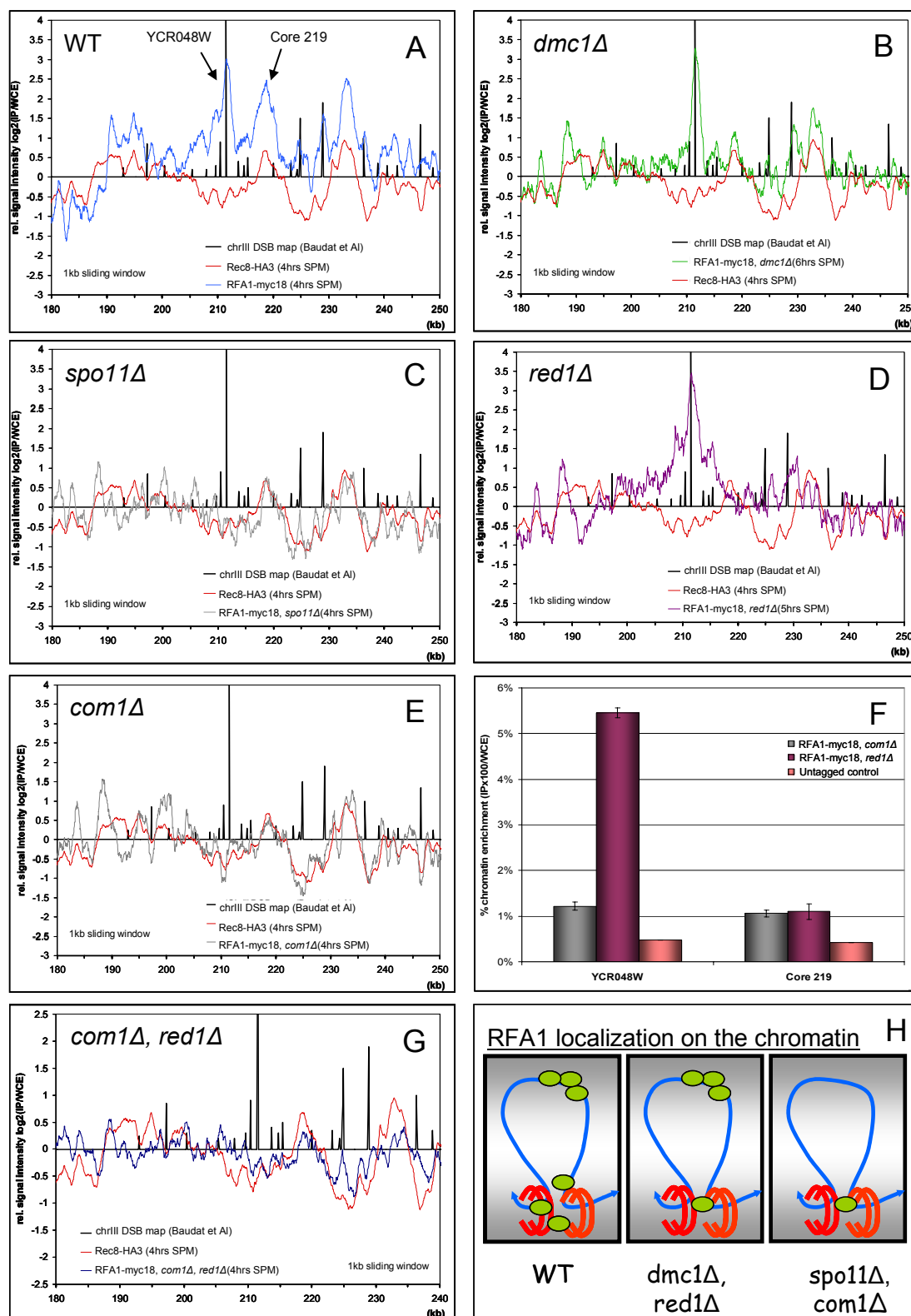


Figure 51:

3.3 Developing an internal control for ChIP chip analysis in

Saccharomyces Cerevisiae

In this study, comparative analysis between different ChIP on chip profiles was widely used in order to identify differences in protein localization over the entire yeast genome. While the comparative analysis was performed from the point of view of the chromatin interaction sites, the signal intensity associated to each binding site was used only as a relative means of site comparison within the same profile. Taking in consideration that the signal intensity associated to a defined chromatin binding site is directly proportional to the probability of finding such protein at that defined chromatin site; comparing several profiles not only in the context of the localization of the binding sites on the chromatin, but also in the context of their associated intensities becomes of great importance to determine the relative abundance of the protein of interest at defined chromatin sites under certain conditions. From a technical point of view, such an analysis becomes complex, mainly because the signal intensities obtained during a ChIP on chip analysis are the consequence of a mathematical comparative treatment between the relative fluorescence intensity obtained by the hybridization of the Immunoprecipitated DNA (IP) and the hybridization of the DNA from the whole cell extract (WCE). As a consequence, the signal intensity contains statistical significance when compared to other signals within the same ChIP on chip profile, but not when compared to a different one.

An alternative that would allow comparative analysis between different profiles depends on the presence of a reference signal intensity in the compared profiles, which could then be used in order to “normalize” the profile’s signal intensities before comparison. The identification of a protein having such a binding site *in vivo*, characterized by a constant pattern between the different studied conditions, seems difficult. In order to circumvent this problem, we designed an exogenous internal standard into the profiles of interest. The strategy employed for this purpose depends on the use of the TetR-TeTO system as a Chromatin immunoprecipitation target. Here, the TetRp was fused to the epitope tag of interest (for myc-tagged proteins, we would use the myc-tag TetRp system) and expressed under a constant promoter, namely pURA3. Under these conditions, the TetRp will be immunoprecipitated together with the protein of interest.

As the TetO repeat sequences are not part of the yeast genome, we planned to flank them with yeast chromatin sequences that lack protein binding sites in most, if not in all, of our ChIP on chip profiles. After comparing several ChIP on chip profiles we decided to use the chromosomal sequences located between positions 56100-59100 on Chr VI. Because this

chromosome region covers the TUB2 locus, we therefore named the chromatin regions we flanked on both sides of the TetO repeats (7xTetO repeats) TUB2A and TUB2B (figure 52).

The final construct consisted of an 808 bp DNA fragment fused to the left side of the TetO repeat sequences (TUB2A), and an 1194 bp DNA fragment fused to the right side of the TetO repeat sequences (Tub2B). A 600 bp spacer between the end of the TUB2A and the beginning of the TUB2B sequence was included in order to produce a particular pattern during ChIP on chip analysis. The Tub2A fragment will produce a signal over 3 data points (a single data point covers 300bp in this case), then a signal-free region covering 2 data points (also referred to as the GAP region or TUB2C) followed by signals covering 4 data points associated with the TUB2B fragment (figure 52A). This final construct was cloned into an integrative plasmid, containing the epitope-tagged TetR gene under control of the URA3 promoter. The integrative plasmid was used to introduce the internal control construct into the URA3 locus on chromosome III (an extensive description of the Internal control design is presented in Appendix).

Our first test of this system was performed with the PK-tag epitope fused to the C-terminus of the TetRp. The behaviour of this construct was evaluated by qPCR, where three different positions in Tub2A and Tub2B as well as a negative control amplicon targeting the GAP region between Tub2A and Tub2B were evaluated (figure 52C). As expected, a Gaussain distribution from the internal control construct was observed[‡]. ChIP on chip analysis performed for the TetR-PK construct demonstrated the presence of three contiguous data points with strong signal intensities (higher than 4 fold enrichment in the Log2 scale) followed by 2 data points lacking significant signal enrichment, and finally, 4 contiguous data points displaying strong signal intensities. This pattern correlates perfectly with that expected for the normalization construct.

Even though the initial test of the internal control demonstrated the expected behaviour, when we included this construct as part of the study of a protein of interest, we realized that the ChIP on chip profile contained supplementary binding sites which were not dependent on the protein of interest. Figure 54 illustrates the particular case for the study of Rec8p. The ChIP on chip profile of chromosome VI demonstrates the presence of the internal control pattern in the profile of the HA-tagged Rec8p strain expressing the HA-tagged TetRp and containing the TUB2A-7XTetO-TUB2 construct. Nevertheless, additional chromatin binding

[‡] The experimental evaluation of the distribution of the DNA fragments sizes produced by the ChIP protocols demonstrated that it follows a Gamma distribution and not a Gaussian distribution as currently considered. Indeed, sources of DNA fragment size variation in the population including differences in sonication and nonuniform PCR amplification generates an skewed distribution of the DNA fragments[118][118] Qi Y, Rolfe A, MacIsaac KD, Gerber GK, Pokholok D, Zeitlinger J, Danford T, Dowell RD, Fraenkel E, Jaakkola TS, Young RA, Gifford DK. High-resolution computational models of genome binding events. Nat Biotechnol 2006;24 (8):963-70..

sites which are not observed in the Rec8p-chromatin binding profile are seen. The comparison of the internal control profile (TetRp HA tagged) with the Rec8p profile containing the internal control reveals that at least some of the supplementary binding sites observed in the Rec8p profile with the internal control are indeed observed in the internal control profile. This suggests that, unexpectedly, the TetRp can also interact with some yeast chromatin regions, producing an artefactual binding pattern when the internal control is combined with an experimental sample. Furthermore, regions that were previously characterized as Rec8p binding sites were modified in their relative signal intensity, suggesting that TetRp also interacts with some of the previously characterized Rec8p binding sites.

Another important issue of the internal control design is the fact that URA3 promoter used in order to induce the expression of the TetR-tagged construct is not constant during meiosis. Indeed, the URA3 promoter was shown to be stable during the first two hours of meiotic induction (premeiotic replication phase), then repressed until T5 (5hours after meiotic program induction), to finally be reinduced[121]. Furthermore, this promoter is not constitutive, but depends on the Uracil present on the growth medium. For these reasons, the TetRp induction may not be induced in a similar manner in the different yeast strains, and by consequence the Internal control patterns to be used as reference between different ChIP on chip profiles are not necessarily comparable.

These drawbacks caused us to abandon the internal control at this time. However, there are still some improvements that could be made in order to use these constructs. The simplest correction, from a technical point of view, would be to systematically subtract the supplementary TetR binding sites by comparing the experimental data file with a control file produced by immunoprecipitating the TetR-tagged construct from a yeast strain lacking the TetO target sequence. With this correction, the influence of the supplementary TetRp binding sites on the chromatin could be systematically removed. Furthermore, in order to have a constitutive expression of the TetR-tagged construct even during meiosis, particular mutations into the URA3 promoter sequence could be performed. Indeed, a reported study on the URA3 promoter induction demonstrated that mutations introduced into the PPR1p binding site (which is the transcription factor which positively regulates the URA3 promoter) reduces the URA3 promoter activity to its constant basal expression level[122].

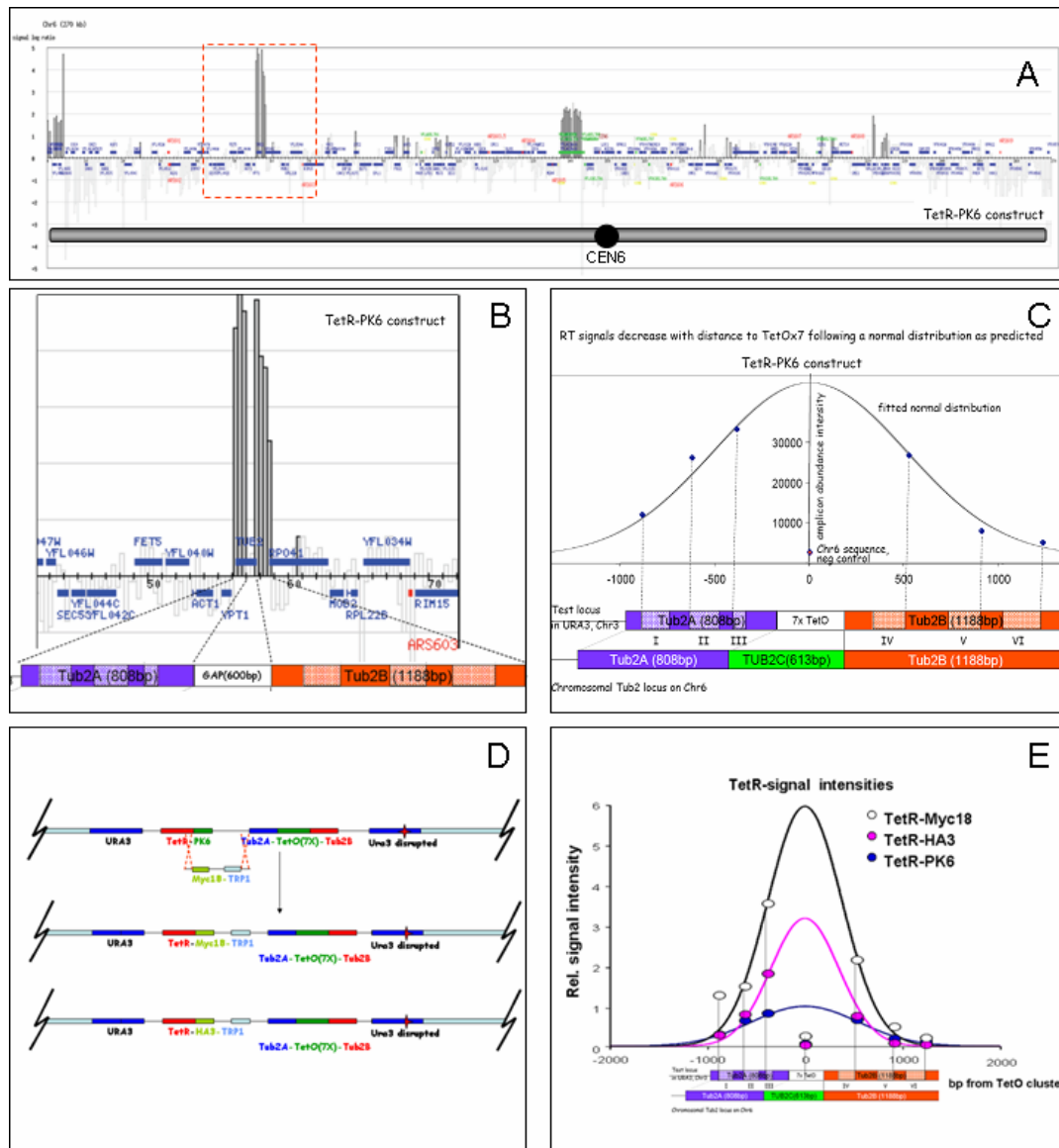


Figure 53: Internal control construct evaluated by qPCR and ChIP on chip. (A) The ChIP on chip analysis performed on the PK-tagged TetRp construct shows the presence of the expected binding pattern at positions 56-59kb on chromosome VI. (B) A zoom in ChIP on chip map of the internal control construct demonstrates the presence of the expected binding pattern: 3 data points (a single data point corresponds to 300 bp) with high signal intensities, corresponding to the TUB2A fragment, an empty region covering 600 bp and 4 data points with a high signal enrichment that correspond to the TUB2B fragment. (C) The predicted normal distribution for the internal control binding pattern was demonstrated by qPCR analysis. Three chromatin regions located in the Tub2A fragment, as well as three in the Tub2B fragment were analyzed for their enrichment by qPCR when TetRp is immunoprecipitated. The “one step PCR tag-epitope replacing strategy” used in order to construct variants of the PK-tagged TetRp construct is illustrated In (D). In a similar manner to (C), the qPCR analysis of the TUB2A and TUB2B chromatin enrichment performed by the different TetRp variant constructs is shown in (E).

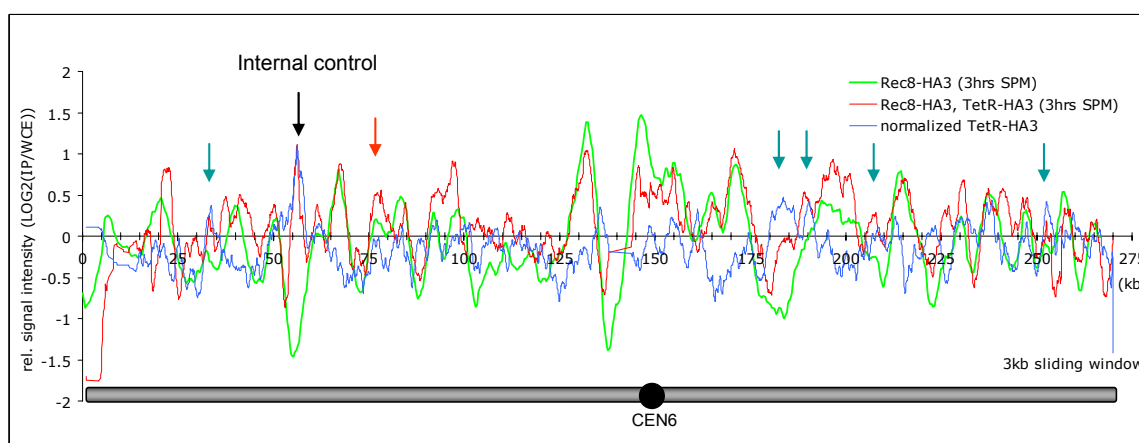


Figure 54: Expression of the internal control system produces artefactual binding sites. Rec8p-chromatin localization map on chromosome VI (green) is compared to the TetRp internal reference map (blue) and that corresponding to the yeast strain expressing both the HA tagged version of Rec8p as well as the HA tagged version of TetRp (red). The internal control chromatin-binding pattern is indicated by the black arrow. Additional binding sites observed in the presence of the internal control are indicated by blue arrows. An example of a change in the relative signal intensity corresponding to a Rec8p binding site when the strain expresses the internal control construct is indicated by a red arrow. This comparative analysis demonstrates that the Internal control can modify the chromatin binding pattern of the protein under study (in this case Rec8p), not only by creating new unexpected binding sites, but also by modifying the relative signal intensity of the expected binding sites.

4 Discussion

4.1 Rec8p is part of the chromosome core organization during meiotic chromatin condensation in prophase I.

In this study we have used a ChIP on chip strategy in order to characterize, to a 1kb resolution, the genome wide chromatin localization of the meiotic cohesin component Rec8p. We have shown a direct correlation between the number of Rec8p binding sites per chromosome and the chromosome length, demonstrating the relatively even distribution of Rec8p binding sites throughout the yeast genome. This even distribution of Rec8p binding sites has an average distance between consecutive binding sites of 11.7kb, estimated from the 991 cohesin binding sites characterised across the *S. cerevisiae* genome when we apply a threshold distance of 0.5 between the putative binding sites and the neighbouring local minima regions, or valleys, over a 3kb average sliding window profiles (figure 33). A previous study performed with mitotic cohesin has shown an average distance between consecutive cohesin binding sites of 11kb; furthermore the authors have shown that meiotic Rec8p-chromatin binding pattern is largely similar to that observed for Scc1p in mitosis[114]. Considering that Scc1p is still expressed initially in meiosis [121], we have characterized the chromatin binding pattern of the residual Scc1p component in meiosis I. We demonstrated that at T3 (3 hours after induction of the meiotic program) the residual mitotic cohesin component is loaded on the chromatin during meiosis, and both meiotic and mitotic cohesin components share a similar chromatin binding pattern (figure 33).

The Rec8p binding pattern on the yeast genome has a negative correlation with GC-rich chromatin regions, as well as with meiotic DSB sites. In order to evaluate whether the meiotic cohesin localization is modified by the induction of meiotic recombination, we have analysed the Rec8p-chromatin binding profile in the absence of Spo11p. In this situation, we did not observe any significant differences when compared to the Rec8p-chromatin localization in the presence of Spo11p.

Rec8p was shown to be essential for Synaptonemal complex (SC) formation and for sister chromatid cohesion suggesting a direct role of Rec8p in the structural organization of the chromatin during prophase I. In addition, this view is supported by its colocalization with Zip1p and Hop1 axes in cytological spread nucleus preparations. To further investigate this colocalization at the 1kb level resolution, we have mapped the chromatin localization of components of the SC, namely Hop1p and Zip1p. Comparing their binding patterns with that of Rec8p, we have shown that all three components interact with the same chromatin

regions (figure 35), demonstrating that Rec8p is indeed part of the chromosome core during meiotic prophase I. Even though all three components bind to the same chromatin sites, their relative abundance in certain chromatin regions compared to others have been put forward by performing a low resolution 30kb sliding window analysis. In this way, Rec8p was shown to have a strong affinity for areas surrounding the centromere on chromosome VI while Hop1p has a preference for the chromosome arms (figure 36). This is supported by a recent report demonstrating an alternated staining pattern between Zip1 and the axes components Hop1/Red1 observed in cytological spread nuclear preparations[123]. Interestingly, the chromosome arms are characterized by a high GC content; and both GC content on chromosome VI and Hop1p binding are directly correlated with global preferences for DSB formation in meiosis (figure 36). In a previous report, this correlation was already described for Red1p, another axial element component [76]. The authors were tempted to associate the relative abundance of Red1p at the chromosome arms with a direct role in facilitating the increased DSB frequency in these regions; however, the *red1Δ* mutant did not display a different pattern of DSB distribution over the studied chromosome, indicating that Red1p chromatin localization patterns do not define the chromatin regions prone to meiotic DSB formation.

Finally, due to the fact that Zip1p is the transversal filament of the Synaptonemal Complex, connecting the chromosome cores of the homologs, and considering that Rec8p interacts with the same chromatin regions as the SC components, we have used the Rec8p-chromatin localization pattern to define chromatin organization. With this definition, chromatin regions lacking Rec8p binding sites are considered uncondensed loop regions, and those containing Rec8p binding sites are part of the chromosome core. With these assumptions, we established a high resolution map of the condensed chromatin during meiotic prophase I (figure 37), which we then used to localize recombination components in the context of chromatin organization.

4.2 Meiotic recombination induces Double-strand Breaks

(DSBs) in chromosome loop regions.

In order to characterize the localization of meiotic DSBs across the yeast genome, we have immunoprecipitated the meiotic specific nuclease Spo11p, in the DSB repair defective mutant's background *rad50S*, *mre11S* or *com1/sae2Δ*. Under these conditions, we do not need to *in vivo* crosslink the protein of interest to the DNA, because Spo11p is known to form a covalent linkage to the DNA during its cleavage and requires a fully functional MRX complex as well as Com1p in order to repair it. All three repair defective mutants had similar ChIP on chip DSB maps, indicating that their unrepaired DSB accumulation phenotypes are indistinguishable at this level of resolution (figure 38).

The characterized meiotic DSB map was compared to the Rec8p-chromatin binding pattern with the objective to determine the localization of meiotic DSB sites within the context of the chromatin organization. From this comparison, we have concluded that the meiotic DSBs within the yeast genome are preferentially formed in uncondensed loop regions (figure 39). This conclusion is further supported by comparing the previously published physical DSB map established for chromosome III with the Rec8p-chromatin binding map established by ChIP on chip analysis (figure 38A). A more detailed analysis of the localization of the meiotic DSB sites in the context of the chromatin organization revealed that 70% of the total number of chromatin loops (defined as the distance between two consecutive Rec8p binding sites) presented at least one covalent Spo11p binding site of significant signal intensity (higher than 2 fold from the background level). Interestingly, the average size of the chromatin loops exempt of Spo11p cleavage sites ($9.3 \pm 3.9\text{kb}$) are significantly smaller than that presenting Spo11p binding sites of significant signal intensity ($13.5 \pm 6.4\text{ kb}$ or $14.4 \pm 7.3\text{ kb}$ average loop length for Spo11p cleavage signal intensities > 0.5 or >2 respectively). Furthermore, whereas a direct correlation between the length of the chromatin loops and the number of Spo11p cleavage sites per loop has been elucidated during this study, there is no correlation between the length of the chromatin loops and the Spo11p-cleavage signal intensities. Indeed the chromatin loop populations presenting Spo11p cleavage sites with significant signal intensities presented a constant average length of 14 kb. From this analysis we could conclude that the chromatin loops presenting a higher Spo11p binding probability are characterized by an intermediate loop length significantly different from the average loop length identified across the *S. cerevisiae* yeast genome.

4.3 Spo11p and the MRX complex do not only localize at the DSB sites, but also at the nearby chromosome core regions.

Considering that Spo11p is the meiotic nuclease component of the DSB initiation complex, its localization on the chromatin has been assumed to be exclusively at sites where the DSBs are formed. Nevertheless, previous reports demonstrated for instance the presence of Spo11p foci still at pachytene stage, fact that does not correlate with the kinetics of DSB formation [15]. Furthermore, as in the case of yeast, during mouse meiosis, Spo11 foci surprisingly colocalize with the Synaptonemal complex, a fact that is contradictory to the preference of DSB formation in loop regions. In order to identify supplementary Spo11p-chromatin binding sites across the yeast genome, we have performed a ChIP on chip analysis under the *rad50S* mutant background, as in the case for the identification of DSB sites, but including the *in vivo* protein-DNA crosslinking treatment. Under these conditions, we identified Spo11p-chromatin interactions that correlate with the meiotic DSB sites, but in addition, supplementary binding sites flanking the DSB regions were identified (figure 42). The comparative analysis of this new Spo11p-chromatin binding pattern with the Rec8p-chromatin interaction map, demonstrated that the additional Spo11p binding sites indeed correlated with the chromosome core. This finding is supported by a similar analysis in the presence of the other characterized DSB repair defective mutants, namely *mre11S* and *com1/sae2Δ* (figure 43). In fact also the wild type profile was a combination of core and loop signals (figure 43). Although Spo11p mapped to the chromosome core regions, not all Rec8p binding sites were associated with the presence of Spo11p. Indeed, the Rec8p binding sites that were associated with Spo11p were in chromosome regions which formed DSBs actively, often flanking a DSB site, suggesting a mechanistic relationship.

Previously, Silvia Prieler in the lab demonstrated that immunoprecipitation of Spo11p yields a two-fold enrichment of the meiotic hotspot YCR048W when the catalytic Tyrosine residue has been mutated to Phenylalanine (*spo11-Y135F* mutation) than that obtained for the Wild type yeast strain[15]. Therefore, the association of Spo11p with that particular chromatin region either persisted longer or that the number of Spo11 molecules interacting with this chromatin region increased in this particular mutant. The reason for this behavior may be directly connected to the impairment of the enzymatic activity, and by consequence, any Spo11p-chromatin interaction which is not connected with the enzymatic activity of Spo11p should not be affected by the Y135F mutation. With this hypothesis in mind, we performed ChIP on chip analysis of the *spo11-Y135F* mutant, and, surprisingly, we found that the Spo11p binding pattern associated with the chromosome core was also increased, in a

manner similar to DSB sites (figure 43), suggesting that Spo11p localization at the core regions is somehow connected to the Spo11p enzymatic activity.

Another component of the meiotic DSB initiation complex in *Saccharomyces cerevisiae* is the MRX complex (for Mre11p, Rad50p and Xrs2p). This complex is not specific to the meiotic cell division, but additionally plays an important role in the detection and repair of DNA lesions during mitosis. A previous report claimed that Mre11p preferentially associates with chromatin regions containing meiotic DSB sites. This was assessed by comparing its genome wide chromatin localization with the Spo11p-chromatin interaction pattern obtained from a *com1/sae2Δ* mutant background[45]. However, this analysis was performed on low resolution ORF-containing microarrays. Our high resolution Mre11p-chromatin interaction map confirmed the fact that this protein localizes at meiotic DSB chromatin regions, but in addition, chromatin interaction sites associated with the chromosome core close to the DSB sites were also observed (figure 44A). This finding is supported by the genome wide chromatin-localization analysis performed for another component of the MRX complex, Xrs2p, whose binding was characterized by interactions correlating with meiotic DSB sites as well as with the chromosome core (figure 45A). The similarity between the genome wide localizations of Mre11p, Xrs2p and Spo11p was demonstrated by performing a hierarchical clustering analysis over the chromatin binding sites identified for these proteins. In contrast, the clustering analysis demonstrated a much lower similarity between the chromatin binding patterns of MRX components and that of Spo11p in a *rad50S* mutant background in the absence of *in vivo* crosslink (-FA) (Figure 49). This difference can be explained by the absence of binding at chromosome core regions in the latter profile.

The analysis of Mre11p-chromatin interactions during meiotic progression revealed a 0.5-1 hour time difference between its chromatin enrichment at the meiotic DSB hotspot located at position 50.6kb (left arm of chromosome III) and the YCR048W hotspot (position 212kb, right arm of chromosome III) (figure 44). Furthermore, the Mre11p-chromatin enrichment pattern for chromosome core regions close to the *YCR048W* hotspot (Core sites in position 219 and 233 respectively) displayed similar kinetics to that observed for the *YCR048W* hotspot during meiotic progression. This observation may well reflect a timing difference in DSB formation between these two meiotic DSB hotspots located at the left and the right arms of chromosome III. Borde et al showed that meiotic DSBs are initiated about 80 minutes after passage of the replication fork[25]. In agreement with this finding, the hotspot at position 50.6kb (left arm) is flanked by two early origins of replications in contrast to the *YCR048W*.

A previous report showed that Mre11p-chromatin localization at the *YCR48W* DSB hotspot depended on the presence of all proteins involved in meiotic DSB formation, with the

exception of Rad50p[45]. Chromatin localization of Mre11p across the yeast genome in the absence of Spo11p or Rec114p confirmed the loss of Mre11p binding to the meiotic DSB sites; but interestingly the chromosome core interactions were still present. On the other hand, the Mre11p-chromatin localization at the meiotic DSB sites as well as at the chromosome core regions were not affected by the *spo11-Y135F* mutation, demonstrating that the presence of Spo11 protein, but not its catalytic activity, is required for Mre11p localization at the meiotic DSB sites (figure 45).

The genome wide chromatin localization studies performed for Spo11p and the MRX complex components revealed the presence of an unexpected localization pattern for recombination components, namely their interaction with chromosome core regions. Furthermore, the fact that the *spo11-Y135F* catalytic mutant enhanced Spo11p localization at both meiotic DSB sites and the neighbouring chromosome regions suggests that Spo11p localization at the chromosome core is somehow connected to its enzymatic activity, despite the fact that meiotic DSBs have been shown to be preferentially formed in uncondensed loop regions. Silvia Prieler in the lab previously demonstrated that Spo11p localization at the meiotic DSB sites was abolished in the absence of Rec102p, Rec104p or Rec114p[15]. Surprisingly, *rec114Δ* mutants maintained chromatin-associated Spo11 foci. This observation correlates with the fact that ChIP on chip analysis performed on Spo11p in the absence of Rec114p revealed the presence of chromosome core signals despite the absence of Spo11p localization at meiotic DSB sites (Silvia Panizza in the lab; unpublished data). From this data we can conclude that Spo11p and Mre11p chromatin localization at meiotic DSB sites depends on the presence of Rec114p, whereas their localization at the chromosome core is not dependent on Rec114p. ChIP on chip analysis performed in collaboration with Silvia Panizza, a postdoc in the lab, revealed that the MMR subcomplex (for Mer2, Mei4 and Rec114) is exclusively associated with chromosome core regions located in close proximity to meiotic DSB sites, but not at the DSB sites themselves.

Figure 55 depicts a model summarizing the ChIP chip results obtained in this study combined with the two hybrid interaction map published previously[34,36] in order to correlate the studied protein-chromatin interaction patterns with the characterized two-hybrid interactions. For example, the MMR subcomplex was shown to localize exclusively at chromosome core regions. Furthermore the MRX complex that interacts with the MMR subcomplex via the Xrs2-Mer2 interaction localizes at the chromosome core regions in addition to the meiotic DSB sites. The DSB site localization of the MRX complex is impaired by the absence of Rec114p or Spo11p, suggesting that it depends on the interaction between Rec114p and Rec102/Rec104-Spo11/Ski8. Spo11p was shown to localize to meiotic DSB sites as well as chromosome core regions and, similar to the MRX proteins, its localization at meiotic DSB

sites depends on Rec114p, Rec102p or Rec104p. This again emphasizes the importance of the Rec114-Rec104/Rec102 interaction for Mre11p and Spo11p localization at meiotic DSB sites. While this interpretation highlights the existence of different chromatin localizations for the recombination initiation components, there is still a question that it cannot fully explain: Under the assumption that the DSB initiation complex needs to localize to a single chromatin region, where the DSB is formed, how do we form meiotic DSB breaks specifically in chromatin loop regions, when some of the essential components are localized to the chromosome core?. The first hypothesis that addresses this question is that the approach used to characterize their chromatin localization, ChIP-chip, is not able to detect the localization of the MMR complex to the DSB sites. In this hypothesis, the chromatin interactions at the meiotic DSB sites are indeed the functional interactions involved in DSB formation, in contrast to the chromosome core interactions that would correspond to binding sites that lack a role in formation of DSBs. However, we favor the alternative previously proposed by Nancy Kleckner, which is the clustering of recombination initiation components at the chromosome core sites in the presence of a "Tethered-Loop/Core" structure [76]. In other words, the chromatin loops may interact with their neighbouring chromosome cores during meiotic DSB formation. In this model, the observed localization of Mre11p, Xrs2p and Spo11p both at the chromosome cores and DSB sites would correspond to the detection of a "Tethered-Loop/Core" structure (figure 56). This hypothesis is supported by the fact that the *spo11-Y135F* mutation enhances Spo11p localization at both loops and cores chromatin regions. In addition, the Rec114-Rec102/Rec104 interaction seems to be essential for tethering the chromatin loop to the chromosome core sites.

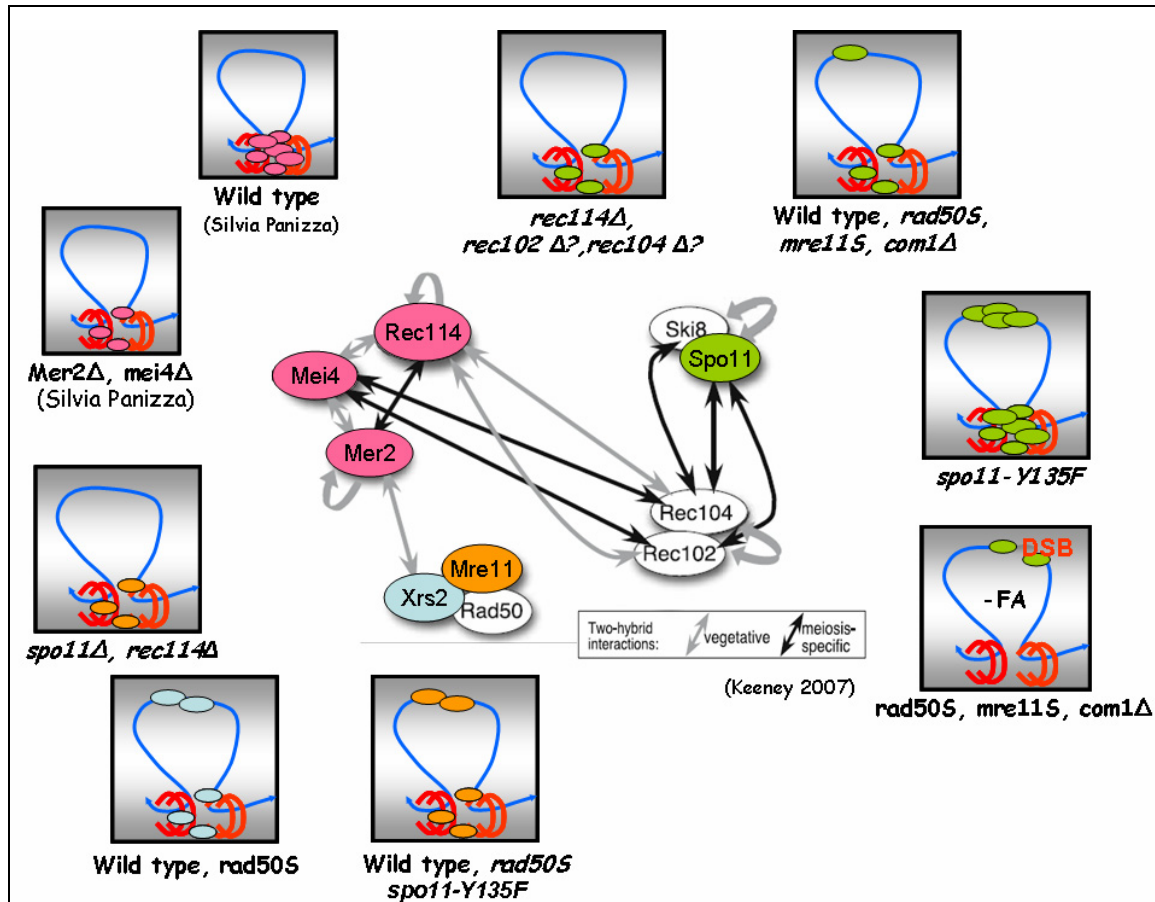


Figure 55: Chromatin localization of meiotic recombination components involved in DSB formation and correlation with two-hybrid protein interactions. The proteins analyzed for their chromatin localization are represented in colored ovals: Green for Spo11p, Orange and light blue for Mre11p and Xrs2p respectively, Pink for the MMR sub-complex (characterized by Silvia Panizza). Proteins corresponding to white ovals were not characterized for their chromatin localization. The chromatin localization pattern of the different proteins under the various mutant backgrounds are illustrated: Either localized at uncondensed loop regions (DSB sites), or localized at the chromosome core (Red rings representing cohesin complex), or both. The two-hybrid proteins interaction network was adapted from Maleki et al.[36]. Notice that meiosis specific and vegetative interactions are illustrated in gray and black respectively. Question marks associated with the Spo11p localization in *rec102Δ* or *rec104Δ* indicates the absence of localization of Spo11p at the DSB sites but its localization at the chromosome cores was not analyzed.

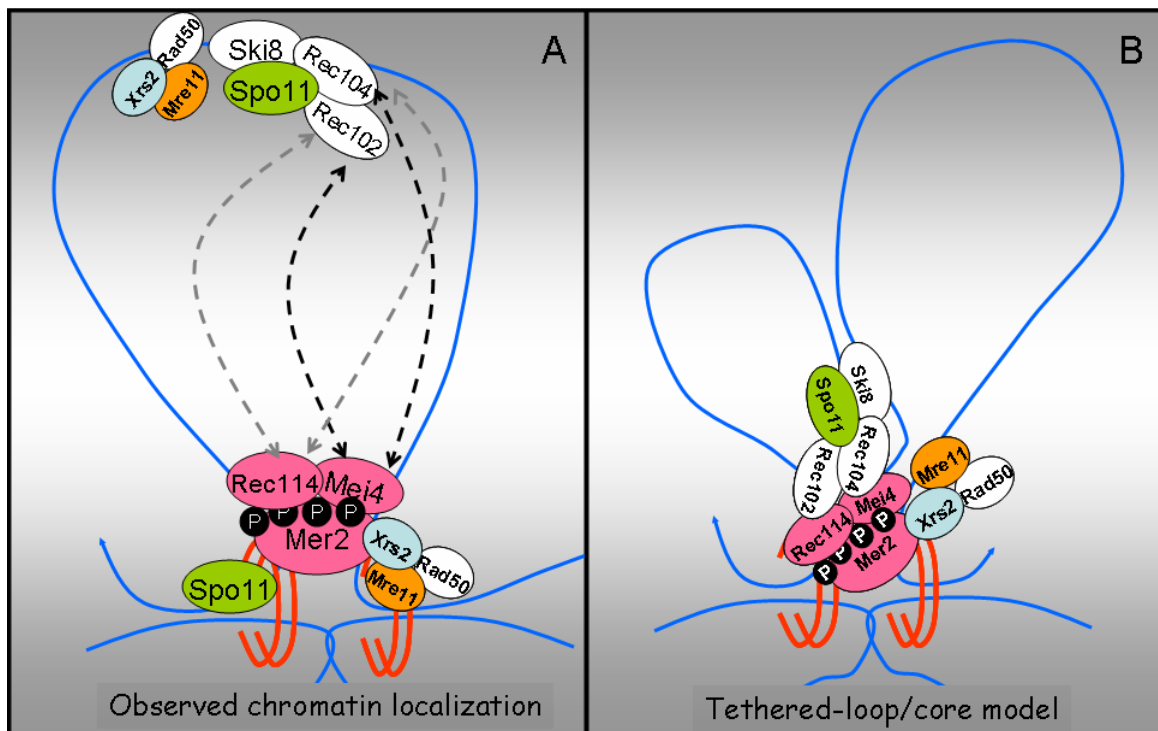


Figure 56: Model illustrating the chromatin localization of the meiotic DSB formation complex components. In (A) the chromatin localization of the different components involved in meiotic DSB formation is illustrated in the context of our observations. The MMR complex represented in Pink highlights the requirement of Mer2 phosphorylation in order to allow the recruitment of the other components (Phosphorylation represented by a black P). The fact that Mre11p, Xrs2p and Spo11p were shown to localize at the meiotic DSB sites and at the chromosome core regions is illustrated by the presence of these components at the top of the uncondensed loop as well as at the chromosome core represented by the presence of the cohesin components (red rings). For simplicity, chromosome Axis components were not represented. The two-hybrid protein-protein interactions between Mei4p-Rec102p/Rec104p and Rec114p-Rec102p/Rec104p are represented in black and gray dashed arrows respectively (black for meiosis specific and gray for vegetative interactions respectively). In (B) the hypothesized "Loop/axis tethering" model is represented.

4.4 Com1/Sae2p binds to meiotic DSB sites and to chromosome core regions like MRX, but its specific localization at the DSB sites is abolished in *spo11Δ* or *rad50S* mutants

In *S. cerevisiae* the repair of meiotic Double-strand Breaks (DSBs) depends on the MRX complex as well as on Com1/Sae2p[54]. This requirement has recently been shown to be conserved in organisms as diverse as *S. pombe*[57], *C. elegans*[58] and *A. Thaliana*[56]. Com1p was also recently discovered as the ortholog of the mammalian CtIP[55], a gene with important roles in genome maintenance, DNA repair and tumor suppression.

The role of the MRX complex in meiotic DSB repair has been demonstrated in budding yeast by the characterization of particular mutants that allow meiotic DSB formation to occur, but accumulate unrepaired meiotic DSBs where Spo11p remains covalently linked to the 5'-DNA broken ends[31]. This is the case of the *rad50S* and the *mre11S* mutants, known as "S" mutants to emphasise the separation of function between formation of the DSBs and their repair. The crystal structure analysis performed in nine different *rad50S* mutantions revealed that seven of them are located on a surface patch predicted to be a protein-protein interaction site[50]. On the other hand the *mre11S* mutant characterized by Knud Nairz previously in the lab[49], contains two point mutations which are speculated to be involved in the Mre11-Xrs2 interaction, as well as in its binding to DNA[51]. In addition to *mre11S*, other MRE11 mutants presenting the same meiotic DSB repair phenotype have been characterized (mutants D16A, D56N, H125N, H213Y and mre11-6). Surprisingly, the common characteristic between all these mutants, like *mre11S*, is that they do not display nuclease activity *in vitro*. Of these mutants, *mre11-H125N* displays a clear meiotic phenotype without compromising other vegetative functions such as mating type switching, non-homologous end joining or telomere homeostasis, demonstrating that the nuclease activity of Mre11p is essential for the repair of Spo11p-DSBs in meiosis [52].

While the different *mre11S*-like mutants lead to impairment of its nuclease activity, the *rad50S* mutant is likely to lead to failure to recruit a fourth component to the MRX complex via the described putative protein-protein interaction site. Considering that Com1p is essential for the repair of meiotic DSBs, it's possible that a direct interaction between Com1p and the MRX complex is mediated by Rad50p.

ChiP on chip analysis performed for Com1p demonstrated that it binds to chromatin with a similar pattern as Mre11p. Indeed, Com1p was shown to interact with meiotic DSB sites as well as to the neighbouring chromosome core regions (figure 46). The similarity between the

Mre11p-chromatin binding profile and that of Com1p across the yeast genome has been evaluated by performing a hierarchical clustering analysis combined with a binary peak matching comparison (described in chapter 2.6.7.5). Surprisingly, the Com1p-chromatin binding pattern shows a 70% similarity with that of Mre11p, correlating with the fact that both proteins localizes to the same chromatin regions (the chromatin regions where we find divergence between Mre11p and Com1p localization correspond to chromosome areas with a lower frequency of meiotic DSB formation).

The analysis of Com1p-chromatin interaction in the absence of Spo11p demonstrated that its localization at meiotic DSBs depends on the presence of Spo11p, although its localization at the chromosome core regions remained unaffected. In order to study the influence of the "S" mutations on the Com1p-chromatin localization, we performed a genome wide analysis of its chromatin interaction in the *rad50S* as well as the *mre11S* mutant background. Interestingly, the *rad50S* mutant lost Com1p localization at meiotic DSB sites, while the *mre11S* mutant retained the wild type localization (figure 48). This finding correlates with the fact that the *rad50S* mutant was previously shown to lose colocalization between Com1p and Mre11p on chromatin spreads by immunostaining analysis (Ivana Billic in the lab, unpublished data), fact that is not observed in *mre11S*. The Mre11p and Xrs2p chromatin binding pattern across the yeast genome did not change in presence of the *rad50S* mutation, strongly suggesting that this mutant has an influence exclusively on Com1p-chromatin localization (figure 48).

In summary, Com1p localization at meiotic DSBs depends on the presence of Spo11p as well as on a fully functional Rad50p. This last point supports the hypothesis of a direct interaction between Rad50p and Com1p, which is impaired in the *rad50S* mutant.

The Hierarchical clustering analysis performed in order to evaluate the similarity of ChIP on chip profiles revealed another interesting fact related to the chromatin localization of Xrs2p. While the Xrs2p-chromatin binding pattern clustered together with that of Xrs2p performed in the *rad50S* mutant background, the chromatin localization pattern of Xrs2p in the *mre11S* mutant background appeared quite different, and did not form a significant cluster with any other protein (figure 49). This difference supports the hypothesis that at least one of the *mre11S* mutations abrogates the Mre11-Xrs2 interaction.

4.5 What is the role of the chromosome core localization of meiotic recombination components?

During this study, we characterized the genome wide chromatin localization of several meiotic recombination components involved in DSB formation and repair. Surprisingly, these components localized not only to the chromatin regions where the meiotic DSBs are formed, but additionally to chromatin regions that were identified as part of the chromosome core structure. Even though we would be tempted to assign a role not involved in meiotic recombination to this unexpected chromatin localization pattern, several observations suggest the opposite: (1) not all chromosome core regions are associated with meiotic recombination protein binding sites; indeed they correspond to chromatin regions in close proximity to meiotic DSB sites; (2) inactivation of the Spo11p catalytic site induces a stronger Spo11p-chromatin interaction not only at meiotic DSB sites, but also at the neighbouring chromosome core regions; (3) the Spo11p-chromatin localization either in a *red1Δ* or in a *hop1Δ* mutant showed a strong decrease in interaction at both the DSB sites as well as the chromosome core regions; (4) a study of the chromatin localization of the single-strand DNA binding complex replication protein A (RPA), achieved by immunoprecipitating the component RFA1p, showed that this complex localizes to meiotic DSB sites and additionally to the neighbouring chromosome core regions. While RFA1p-chromatin localization at the chromosome cores is still observed in the absence of DSB formation (*spo11Δ* mutant), or in the absence of DSB repair (*com1Δ* mutant), a significant difference in their relative signal intensity (approximately 3-fold loss) is observed when compared to wild type, indicating that DSB formation induces RFA1p localization at the neighbouring chromosome core sites (figure 51).

During our study, we demonstrated that the absence of Red1p or Dmc1p negatively affected RFA1p localization at chromosome core sites, without impairing its interaction with DSBs. This correlates with the fact that *red1Δ* mutants show a reduction in DSB formation compared to wild type, but do not completely abolish their formation. Surprisingly, a *dmc1Δ* mutant did not display a stronger localization pattern for RFA1p at DSBs than wild type, despite accumulating longer resected DNA-ends. This may be explained by a limiting supply of the RPA complex, as reported recently[124]. On the other hand, a previous report demonstrated that Red1p is also required for Dmc1p loading on the chromatin[76], a fact that may influence strand-resection in *red1Δ* mutants by creating longer ssDNA tracts from the few DSBs that are formed. This may explain the similarity of the RFA1p-chromatin

interaction at DSBs observed in *red1Δ* and wild type (notice that the ChIP signal seems broader in *red1Δ* compared to *dmc1Δ*, which may reflect a similar DNA-end resection efficiency for a given amount of RFA1p in the cell, but in the presence of a different number of broken DNA-ends substrates) (figure 51).

In the model proposed in figure 56, we speculate that meiotic recombination requires a “Tethered-Loop/Axis” local organization. This may explain the localization of recombination components involved in DSB formation at the chromosome core scaffold. This is true for components such as Spo11p and the MRX complex; unexpectedly, the MMR subcomplex was not found at meiotic DSB sites but instead showed strong chromosome core localization. One possible explanation may be that the MMR subcomplex is not in direct contact with the loop of chromatin that will be cleaved, but interacts with DSB sites through other components that are part of the DSB initiation complex. In fact, this may be the situation for all components, but depending on their relative position in the DNA-protein DSB initiation complex, their efficiency to immunoprecipitate either the chromatin loop, or the chromosome core, or both, may change. The same is true for protein components that are part of the chromosome core structure, like Hop1p and Red1p. Indeed, as demonstrated in this study, the ChIP on chip analysis performed for Hop1p demonstrated its specific localization at chromosome core regions; nevertheless, if the “Tethered-Loop/Axis” local organization happened, we should be able to localize chromosome core scaffold components at the DSB sites where the tethering takes place. Because there are multiple components built-up over the chromosome scaffold, the detection of loop-chromatin regions by immunoprecipitating chromosome core components may be extremely difficult.

5 Epilogue

This study has illustrated the use of a ChIP-DNA microarray approach as a way to characterize the chromatin localization of meiotic recombination components in a genome wide manner. One of the main consequences of the use of this strategy was the identification of unexpected chromatin binding sites for these proteins. Indeed, before we began this study, meiotic recombination components were believed to exclusively localize at meiotic DSB sites, a fact that has biased the interpretation of several observations.

From a technical point of view, this study overcame several challenges; from the setup of the ChIP on chip pipeline in Vienna after my training at the laboratory of Professor Katsuhiko Shirahige, at the Tokyo Institute of Technology; to the development of data analysis tools together with Professor Franz Klein. We have developed data treatment methodologies in order to decrease the noise pattern present in the raw data profiles (a “Tooth brushing” strategy combined with the mean sliding window approach). Furthermore, we developed a hierarchical clustering analysis for ChIP on chip profiles, in order to compare them in a quantitative manner over the entire yeast genome. These data treatment methodologies are still in development, in order to improve the resolution and the certainty of the protein-chromatin binding sites identification.

In summary, this study permitted not only the identification of new unexpected chromatin binding sites for meiotic recombination components involved in DSB formation and repair, but in addition established the tools required for exploring the genome wide chromatin localization of other proteins to single nucleotide resolution.

6 Zusammenfassung

Der Ausdruck Meiose bezeichnet eine spezielle Zellteilung die bei der Entstehung von Keimzellen wichtig ist. Eine diploide Elternzelle erzeugt dabei haploide Nachkommen, da eine Runde der DNA-replikation von zwei Zellteilungen gefolgt wird. Im Unterschied zur Mitose werden bei der ersten meiotischen Teilung homologe Zentromere von einander getrennt, nicht Schwesterzentromere. Durch die Bildung von Chiasmata, physischer Verbindungen zwischen den Homologen kann ein Gegengewicht zu den Mikrotubulkräften geschaffen werden, wodurch es zur Ausrichtung der Chromosomen in der Metaphaseplatte kommt.

Die Entstehung von Chiasmata benötigt einerseits die Initiation der meiotischen Rekombination durch DNA Doppelstrangbrüche (DSBs) und andererseits morphologische Veränderungen wie Chromosomenkondensation, Paarung und Synapsis. Obwohl ein direkter Zusammenhang zwischen Kondensation und DSB Bildung postuliert wurde, ist es nicht bekannt, wodurch eine bestimmte Chromatinregion gebrochen wird nicht bekannt.

Mit Hilfe von ChIPchip, (genom-weiter Chromatin Immunprecipitation, kombiniert mit Hybridisierung an DNA-Microarrays) konnten wir die Chromosomalen positionen mehrerer Proteine, die in der meiotischen Rekombination und Synapsis von *S. cerevisiae* eine Rolle spielen recht genau bestimmen. Überraschender weise lokalisieren die meisten Komponenten, die für DSB Brüche nötig sind, nicht direkt an den Bruchstellen, sondern kolokalisieren mit strukturellen Komponenten der Chromatin Organisation, wie Kohäsinen und Axialen Element Komponenten. Diese Beobachtungen deuten auf eine Rolle der dreidimensionalen Faltung des Chromosoms für die Bruchbildung in der Meiose hin.

Komponenten der frühen meiotischen Reparatur, wie die des MRX Komplexes (Mre11, Rad50, Xrs2) und Com1 wurden auch nicht exklusiv an der Stelle des DSBs gefunden. Auch sie interagieren mit Kohesininteraktionsstellen. In einer Mutante (rad50S) wird allerdings die Interaktion von Com1 mit Chromatin an den DSB Stellen unterbunden, parallel zu fehlender DNA Resektion und generell defekter Reparatur in dieser Mutante. Dieses Ergebnis zeigt eine Rekrutierungsfunktion von Rad50 für Com1 an, und paßt zu neuesten Ergebnissen, die Com1 eine direkte Rolle im Prozeß der DNA Reparatur am DSB zuschreiben.

7 Appendix

7.1.1 Notes:

2.2.2.1: To monitor cell growth in the pre-sporulation medium (SPS), it is recommended to establish a table of correlation between the number of yeast cells per ml determined with a hemocytometer and the corresponding optical density (OD₆₆₀). Note that this correlation curve may differ between different strain backgrounds, cells grown in different media or for different spectrophotometers. This is a critical step to obtain a synchronous meiotic time-course. For instance, cell cultures that were overgrown (more than 5×10^7 cells/ml) may exhibit poor synchrony, and in such case it is recommended to restart the procedure from the beginning. In the case that a cell culture did not reach the concentration of 4×10^7 cells/ml, we recommend to wait until enough cell material has accumulated.

2.2.2.2: The time of incubation with formaldehyde should be optimized for each protein of interest. In our hands a 30-minute incubation works well for cohesin and recombination proteins. Overnight incubation at 4°C was reported for cases, where the DNA-protein interaction is expected to be particularly weak. A balance between too long fixation times, which will increase the background, and too short incubation times, which result in insufficient chromatin enrichment, has to be determined.

2.2.2.3: Conditions described here refer to the *SK1* background, which undergoes meiosis relatively fast, synchronously and efficiently. It should be considered that some mutant backgrounds may exhibit a different kinetic of meiotic divisions than wild-type cells. For instance, cells unable to form DSBs (such as *spo11Δ*) may show binucleate cells earlier than wild type, while those defective in repair may spend a certain time arrested by the DNA damage checkpoint and will display a corresponding delay. For strains expected to sporulate with wild type efficiency, we only use experiments with "good" synchrony, because the intensity of transient signals depends on synchrony. We evaluate synchrony using the signal intensity of the transient signal "binucleate cells", which should reach at least 25%. Of course; this can't be used for mutants, which arrest or delay in meiotic prophase.

2.5.1.1: We have obtained very satisfying results also without splitting the samples, at least for proteins yielding relatively strong signals. Also, the split samples can be fused at different steps after extract preparation, in order to keep the manipulations manageable,

with the risk, of course, that manipulations on the fused samples might not be as efficient as on the split samples. After cell opening breakage efficiency is checked under the microscope. More than 90% of the cells should be opened. In our hands the optimal diameter of the glass beads is (0.40-0.60mm). Much longer crushing times may cause uncontrolled shearing of DNA. The multibead shocker can be used for optimal temperature control during opening, but is very expensive. A regular Vibrax controlled by an electronic timer (which can be set to the minimum of 1min intervals) has also given us satisfying results.

2.5.1.2: The sonication step needs to be optimized. Sonicate for a certain number of cycles (e.g. 5x) for about 15 seconds interrupted by 15 second breaks. During sonication, samples should be cooled by ice-cold water. When optimizing sonication conditions, use formaldehyde cross-linked samples, keep sample volume constant and before analysis of the DNA length de-crosslink and PCI extract. The resulting fragment size can be determined by agarose gel electrophoresis.

2.5.2.1: How much antibody is required to load the beads needs to be determined empirically. This can be done by using the ChIP signal as read out, or – if little is known about the expected signals - by determining the ratio of antibody to extract, required to cause detectable depletion of the antigen from the extract.

2.5.2.2: Beads can be chosen from a wide range of products. We recommend the use of Dynabeads over regular agarose beads, because of better recovery rates and lower background (i.e. non-specific precipitation of target DNA by the beads). Protein A or Protein G coated beads are versatile, because they interact with a large range of different antibodies. To reduce non-specific interactions a pre-clearing step is recommended by some protocols, when using Protein A/G beads. We prefer to use pan mouse IgG or pan rabbit IgG beads (M-280, Dynabeads). Finally, we observed that adding Salmon sperm DNA (2mg/ml) to the PBS/BSA blocking solution can further increase the signal/background ratio, but will also, as a side effect, decrease the absolute signal intensity.

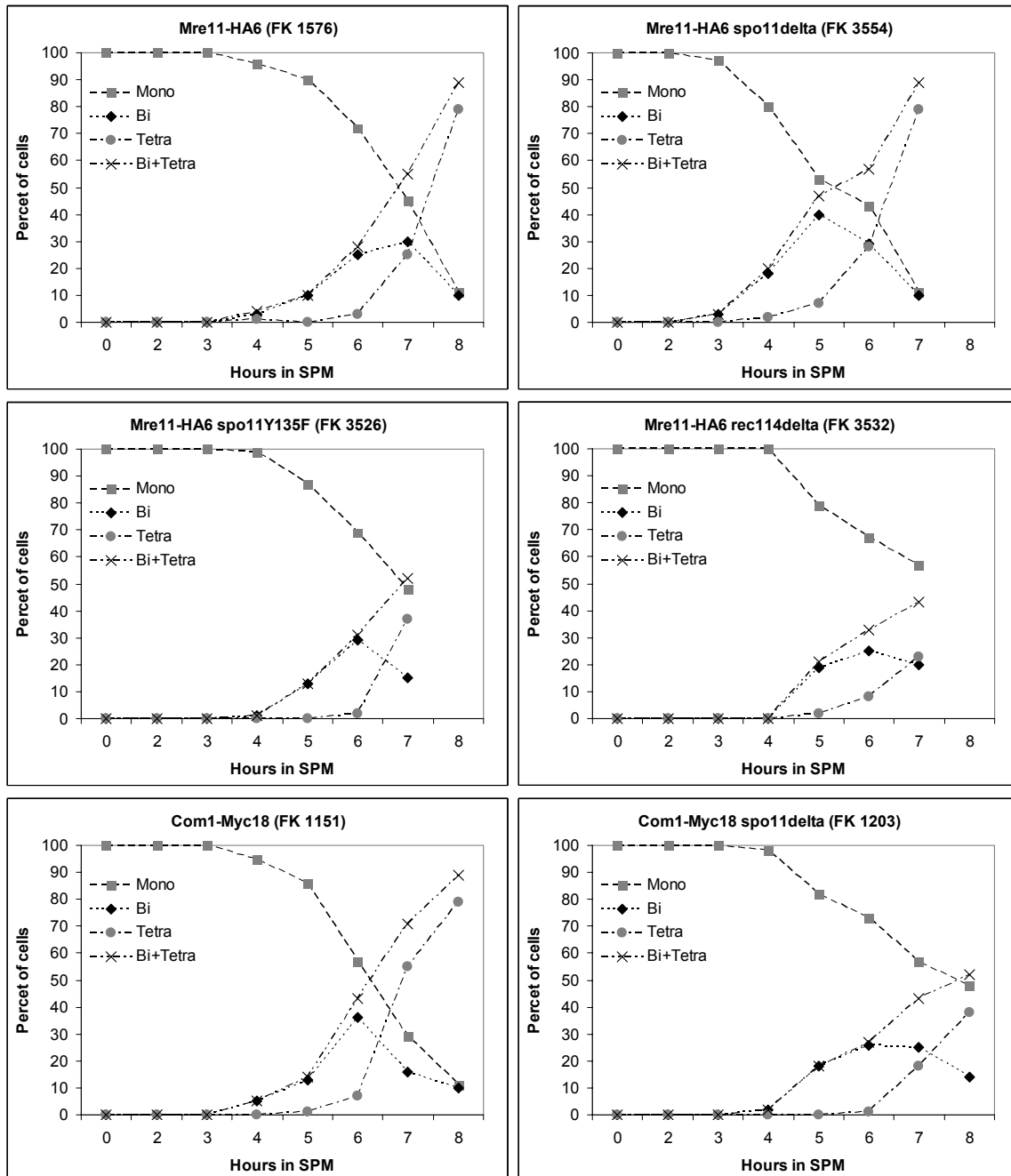
2.5.3.1: After protein and RNA removal, earlier protocols recommended an additional Phenol/Chloroform/isoamylalcohol extraction. However, we found that it can be safely omitted.

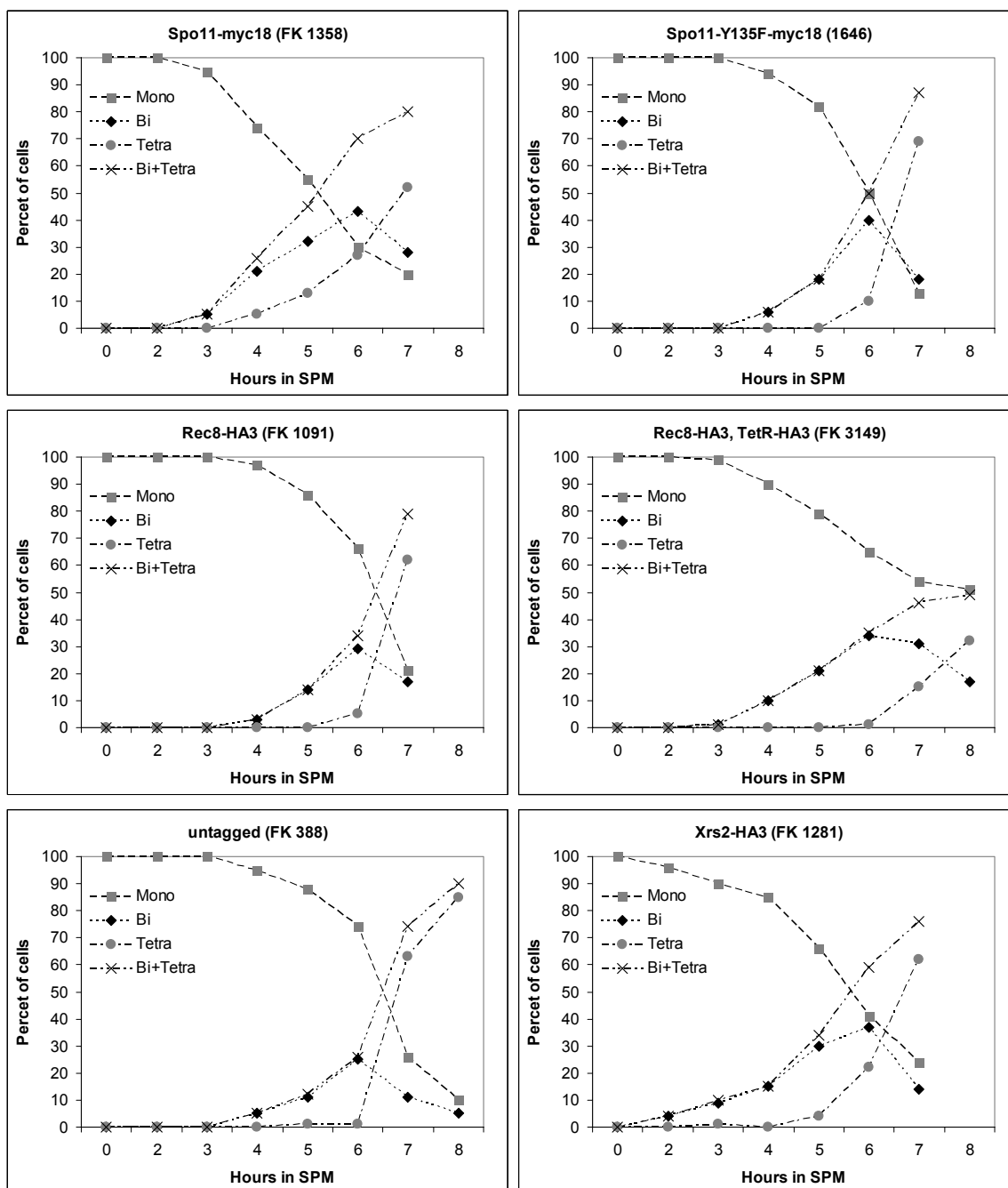
2.5.4.1: Duplicates of PCR reactions typically differ by less than 10%. (The SYBR GREEN Supermix contains 20nM fluorescein, which allows the iQ5 to correct for small volume differences caused by pipetting errors).

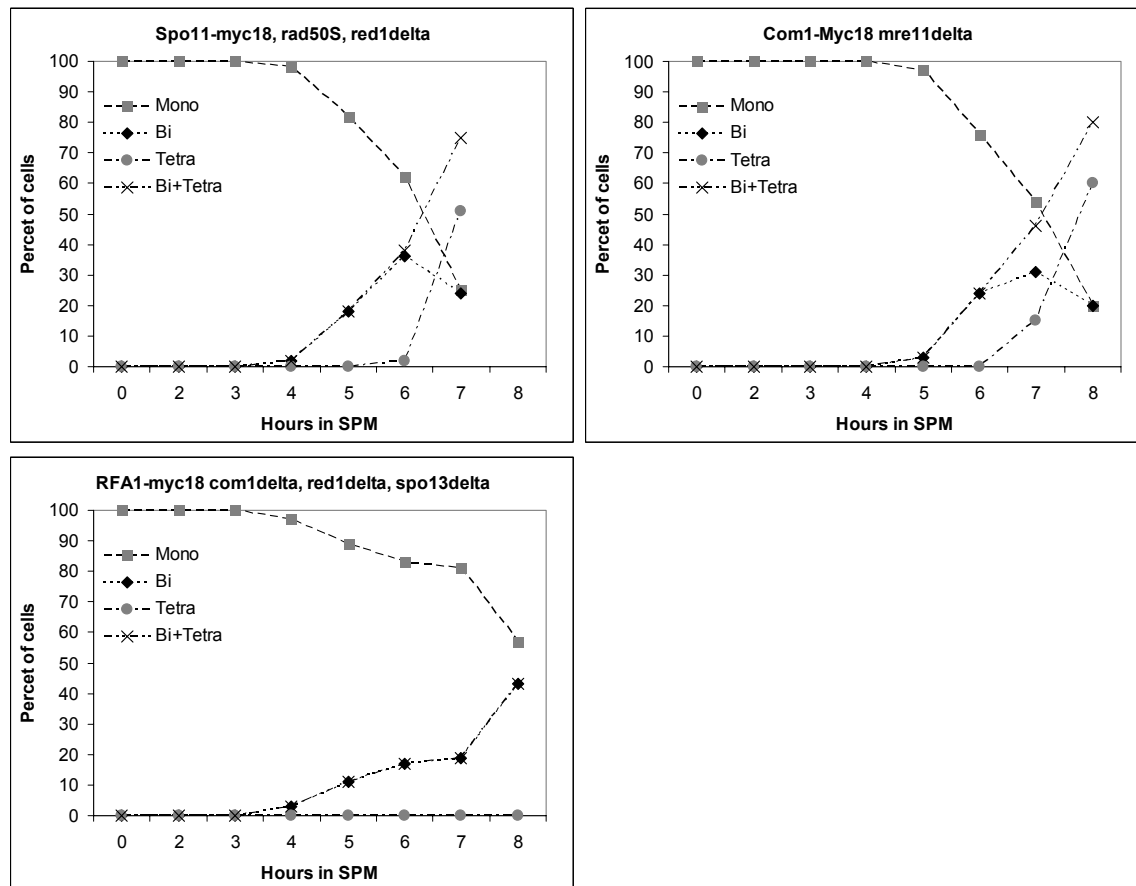
2.5.4.2: If significant differences between duplicates occur, it is possible to spin the samples briefly directly before loading to avoid incompletely dissolved DNA. One may also consider increasing the volume of the loaded DNA from 3 to 6 μ l, depending on the availability of high precision pipettes.

2.5.4.3: We design all primer pairs to work with the same PCR program. To achieve this, our primers are 20 nucleotides long, have a GC content between 50 and 55% and a T_m >55 and <61°C. The product length can be between 100 and 500 nucleotides (ideally below 200 to insure rapid and complete amplification). Before a new primer pair is accepted, it is tested for efficient amplification and for the absence of primer dimers under our exact qPCR conditions. We use currently ***Primer3***, a computational tool developed to help with the design of oligonucleotide primers[125].

7.1.2 Kinetics of the meiotic nuclear cell divisions for the mutant yeast strains used during this study.







Yeast mutant strains like *rad50S*, *mre11S*, *mre11-H125N* or *com1Δ*, unable to process meiotic DSBs, present a poor-sporulation phenotype.

7.1.3 Materials and chemicals used during this study:

<i>chemicals/materials</i>	<i>supplier</i>	<i>product number</i>
Sterilization filters 0.20um	Iwaki/Asahi technoglass	#2052-025
bacteriological agar	Oxoid	LP0011
Sarstedt petridishes 8.4cm	Sarstedt	#821473
Yeast Extract	Oxoid	LP0021
neutralized bacteriological peptone	Oxoid	LP0034
(+)D-glucose (dextrose)	Fluka/BioChemika	#49159
Difco yeast nitrogen base (w/o AA,AS)	Difco	#233520
potassium acetate	Merck	#104820
ammonium sulphate	Merck	#101217
potassium biphthalate (potassium hydrogen phthalate)	Merck	#104874
polypropylene glycol P2000	Fluka	#81380
Glycerol ReagentPlus	Sigma-Aldrich	G7757
Casamino acids	Difco	#223120
Monosodium glutamate (L-glutamic acid, monosodium salt)	Sigma-Aldrich	G1251
Geneticine G418 sulphate	Calbiochem	#345810
ClonNat (nourseothricin)	Werner BioAgents	#5.0000
Cycloheximide	Sigma-Aldrich	C1988
Ampicillin, sodium salt	Sigma-Aldrich	A9518
Sodium chloride (NaCl)	Merck	#106404
Photospectrometer	Hitachi	Digilab U-2800
Triple-baffled Fernbach flasks	Bellco (via Dunn Labortechnik)	#2543-60000
Lithium acetate, dihydrate	Sigma-Aldrich	L6883
Polyethylene glycol MW3350	Sigma-Aldrich	P3640
salmon sperm DNA (low MW)	Fluka/BioChemika	#31149
Zymolyase 20T	Seikagaku	#120491
Zymolyase 100T	Seikagaku	#120493
Dithithreitol (DTT)	Sigma-Aldrich	D9779
Leitz mechanical micromanipulator	Leica	none
D(-)Sorbitol	Merck	#107758
Sonicator (Bandelin Sonopuls)	Bandelin	UW2070
DAPI (4',6-diamidino-2-phenylindole) dihydrochloride	Sigma-Aldrich	D9542
Calciumchloride, dihydrate	Merck	#102382
PIPES		

Fermentas Taq polymerase (1u/μl)	Fermentas	EP0404
Novagen KOD DNA polymerase	Novagen	#71085-3
Dimethylsulfoxid (DMSO)	Merck	#102931
Phusion HotStart HiFi polymerase	Finnzymes	F-540
PCR tubes, 200μl	Sarstedt	#72.737.002
PCR tubes, 500μl	Sarstedt	#72.735.002
mineral oil (heavy white oil)	Sigma-Aldrich	#330760
Phenol, liquefied and tris saturated pH8	Biomol	#50734
Chloroform	Merck	#102445
Isoamylalcohol	Merck	#100979
Ethanol, 96%	Merck	#100971
Isopropanol (2-propanol)	Merck	#109634
Sodium acetate, trihydrate	Merck	#106267
Ammonium acetate	Merck	#101116
Tris, ultrapure	AppliChem	A1086
EDTA (Titrplex, Ethylenedinitrilotetraacetic acid)	Merck	#108417
HCL (hydrochloric acid), 32%	Merck	#100319
acetic acid, glacial (100%)	Merck	#100063
QIAquick PCR Purification Kit	Qiagen	#28104
QIAquick Gel Extraction Kit	Qiagen	#28704
Agarose LE (low electroendosmosis), analytical grade	Promega	V3125
BSA (10mg/ml;100x) for restriction digests	New England BioLabs	B9001S
Sucrose (saccharose)	Merck	#107654
Orange G	Sigma-Aldrich	O3756
GeneRuler 1kb DNA Ladder	Fermentas	SM0311
GeneRuler 50bp DNA Ladder	Fermentas	SM0371
PageRuler Prestained Protein Ladder	Fermentas	SM0671
UV transilluminator	Appligene	none
Ethidium bromide	Sigma-Aldrich	E7637
Calf Intestine Alkaline Phosphatase (CIP)	Fermentas	EF0341
T4 DNA Ligase (including 10x buffer)	Fermentas	EL0015
SDS, Dodecyl sulfate sodium salt	Merck	#113760
sodium hydroxide, p.a.	Merck	#106498
RNase A	Roche Applied Science	#109169
potassium hydroxide, p.a.	Merck	#105033
Bio-Rad Protein Assay (Bradford)	BIO-RAD	#500-0006

KimWipe (Kimtech Science Tissue Wipers)	Kimberly-Clark Professional	#05511
Lithium chloride	Merck	#105679
Rnase, Dnase free, High Concentration (10mg/ml)	Roche Applied Science	#11579681001
Proteinase K, recombinant, PCR grade, lyophilizate	Roche Applied Science	#03115879001
Capillary plastic pasteur pipet, 3.5ml, 20µl, sterile	MBT	#297804239
Hexaminecobalt trichloride (CoHex)	Fluka/BioChemika	#52738
β-mercaptoethanol, 2-Mercaptoethanol	Sigma-Aldrich	M-3148
Hexadecyltrimethylammonium bromide (CTAB)	Fluka/BioChemika	#52365
Polyvinylpyrrolidone, MW 40000 (PVP40)	Sigma-Aldrich	PVP40
Polyvinylpyrrolidone, MW 360000 (PVP360)	Sigma-Aldrich	PVP360
Magnesium chloride hexahydrate	Merck	#105833
di-Potassium hydrogen phosphate trihydrate	Merck	#105099
Potassium dihydrogen phosphate	Merck	#104873
Spermidine	Sigma-Aldrich	S0266
Amersham Hybond-N+	GE Healthcare	RPN303B
VacuGene XL Vacuum Blotting Unit	GE Healthcare	#80-1266-24
VacuGene XL Blotting Pump (220VAC)	GE Healthcare	#80-1265-15
XhoI, High Concentration (50u/µl)	Fermentas	ER0693
EcoRI, High Concentration (50u/µl)	Fermentas	ER0273
BglII (10u/µl)	Fermentas	ER0082
Ficoll 400	Fluka/BioChemika	#46324
Bromophenol blue	Sigma-Aldrich	B8026
Xylene cyanol FF	Sigma-Aldrich	X4126
UV Stratalinker 2400	Stratagene	#2400
High Prime DNA labeling kit	Roche Applied Science	#11585584001
[α-32P]dATP 10mCi/ml 6000Ci/mmol	Amersham Biosciences	AA0074
Dextran sulfate, 500kDa	GE Healthcare/USB	#70796
Syringes, 0.01ml/1ml, Omnifix-F	B.Braun	
Sephadex G-50	Sigma-Aldrich	G5080
di-Sodium hydrogen phosphate (Na ₂ HPO ₄ ')	Merck	#106586
Sodium dihydrogen phosphate dihydrate (NaH ₂ PO ₄ *2H ₂ O)	Merck	#106342
Phosphor screen, unmounted, general purpose (20x25cm)	GE Healthcare/Molecular Dynamics	#63-0034-86
Storage phosphor screen eraser	BIO-RAD	

Molecular Imager FX Pro Plus	BIO-RAD	
Bovine serum albumin (BSA), fraction V	Sigma-Aldrich	A9647
Trichloroacetic acid	Sigma-Aldrich	T9159
Urea	Amresco	#0378
glass beads, diameter 0.40-0.60mm	Sartorius	BBI-8541701
Vibrax	IKA	VXR
Ammonium persulphate, APS	Sigma-Aldrich	A-3678
TEMED	Sigma-Aldrich	T-9281
Acrylamide/Bisacrylamide solution (29:1, 3.3%C), 40%T	BIO-RAD	#161-0146
Glycine	AppliChem	A1067
Methanol, pure	NEUBER	#441992

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9 Curriculum Vitae

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- 1998 – 2001:** University of Geneva; Department of Chemistry and Biochemistry.
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- 2001 – 2002:** University of Geneva; Department of Biochemistry.
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Publications:

Marco Antonio Mendoza, 2001, ***Les Phytases : Structure, Caractérisation et Applications***, www.123bio.net

Maria G. Mendoza-Ferri, Christian G. Hartinger, Marco A. Mendoza, Michael Groessl, Alexander Egger, Rene Eichinger, Natalya Stolyarova, Franz Klein, Michael A. Jakupec, Alexey A. Nazarov, Kay Severin, Bernhard K. Keppler, ***"Transferring the Concept of Improvement of Anticancer Activity by Use of Multinuclear Platinum Compounds to Ruthenium Complexes"***, *J. Med. Chem.*, 2007, to be submitted.

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