

Length computation of crossover-delimited
chromosomal regions establishes the required
two-step chromosome segregation domains of
meiosis in *Caenorhabditis elegans*

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Length computation of crossover-delimited chromosomal regions establishes
the required two-step chromosome segregation domains of meiosis in
Caenorhabditis elegans

By

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Length-sensitive partitioning of *Caenorhabditis elegans* meiotic chromosomes
responds to proximity and number of crossover sites

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Abstract

During meiosis, diploid cells are required to undergo two consecutive rounds of chromosome segregation to produce gametes. To segregate the correct number of chromosomes to each daughter cell during the first meiotic division, *C. elegans* chromosomes measure and compare the lengths of two unequal domains called the short arm and the long arm delimited by a single off-centered crossover. The cohesin complex that holds sister chromatids together is destroyed at the short arm and is protected at the long arm during meiosis I to achieve a partial loss of cohesion and the segregation of the correct number of chromosomes. The synaptonemal complex which holds homologous chromosomes together is required for the establishment of the short and long arm domains. The mechanism by which *C. elegans* chromosomes compute and compare the physical lengths of the short and long arm domains remains poorly understood. In this study, I have analyzed the distribution of crossovers and used quantitative microscopy of phospho-SYP-1, a member of the synaptonemal complex and short arm marker, to reveal that most wild-type chromosomes accurately establish the short arm domain in a process that is initiated during mid prophase. I elucidate some of the rules that govern short and long arm designation by analyzing fusion chromosomes with two crossover sites that show multiple short arms, short arms with no bounding chromosome ends, and partial short/long arm segments. I also found that the crossovers are barriers for the diffusion of the synaptonemal complex element SYP-3, suggesting that the transmission of signals is impeded at the crossover. Using all the evidence obtained in this study, I discuss the proposed model that is supported by a discrete simulation in which a factor originating at the crossover sites diffuses and accumulates on the shorter segment of chromosomes that is ultimately identified as the short arm, enabling the reliable segregation of chromosomes during *C. elegans* meiosis.

Abbreviations

CO: crossover

SC: synaptonemal complex

DSB: double strand breaks

Chapter 1

Introduction

1.1 The importance of meiosis

Eukaryotes are predominantly sexually reproducing organisms with a wide range of sexual cycle complexities but a common shared set of features such as the fusion of haploid cells called gametes into a new biological individual termed a zygote (Bloomfield, 2020). Gametes are produced through meiosis, a specialized form of cell division with the purpose of reducing the number of chromosome copies in diploid cells by half. The reduction in the number of chromosomes is achieved by a single round of DNA replication followed by two tightly regulated and consecutive rounds of chromosome segregation (meiosis I and meiosis II) (**Fig. 1**). A major concern for the cells during meiosis is the precise control of the meiosis I and meiosis II chromosome segregations as problems during any of the required steps can lead to considerable implications such as aneuploidy and polyploidy which in most cases are deleterious and often lethal. Most sexual organisms have a low incidence of chromosome non-disjunction. A notable exception is the human where aneuploidy is estimated to occur in 10-30% of all fertilized human eggs, resulting in abnormal chromosome number as the leading cause of pregnancy loss (Hassold & Hunt, 2001). Thus, it is of great importance to understand the molecular mechanisms of meiosis and its implications when problems arise due to its malfunction.

1.2 Sensing physical properties in the cell

Organisms are under the constant pressure of many external and internal phenomena such as mutations, gene flow and habitat shift. In response, living beings acquire a wide variety of adaptations to ensure their survival. Some of these adaptations require a cellular and subcellular level control of morphological and geometric properties. Typical examples include the aspect ratio of the fission yeast cell body, the curvature of *Vibrio cholerae*, the surface to volume ratio of oocytes, flagella length control in *Chlamydomonas reinhardtii*, polarization and cytoskeleton reorganization of leukocytes during extravasation, and the spindle orientation and position in any dividing cell (Bartlett et al., 2017; Bergstrahl et al., 2017; Engel et al., 2009; Fenteany & Glogauer, 2004; Haupt & Minc, 2018; Marshall et al., 2005). Cells have developed many strategies for the fine-tuned control and size measurement of subcellular structures such as concentration gradients, time-dependent decay and

size-proportional attraction and sequestration of cofactors (reviewed in Marshall, 2016). A very interesting example of size computation takes place in the meiotic divisions of the nematode *Caenorhabditis elegans* where each paired homologous chromosome compares the lengths of the two chromosome axis segments separated by a crossover (CO); based on the difference in their lengths, the two segments are distinctly functionalized. The underlying mechanism by which *C. elegans* chromosomes measure the distance of the chromosome ends to the crossover and compare the lengths of the resulting segments remains a mystery.

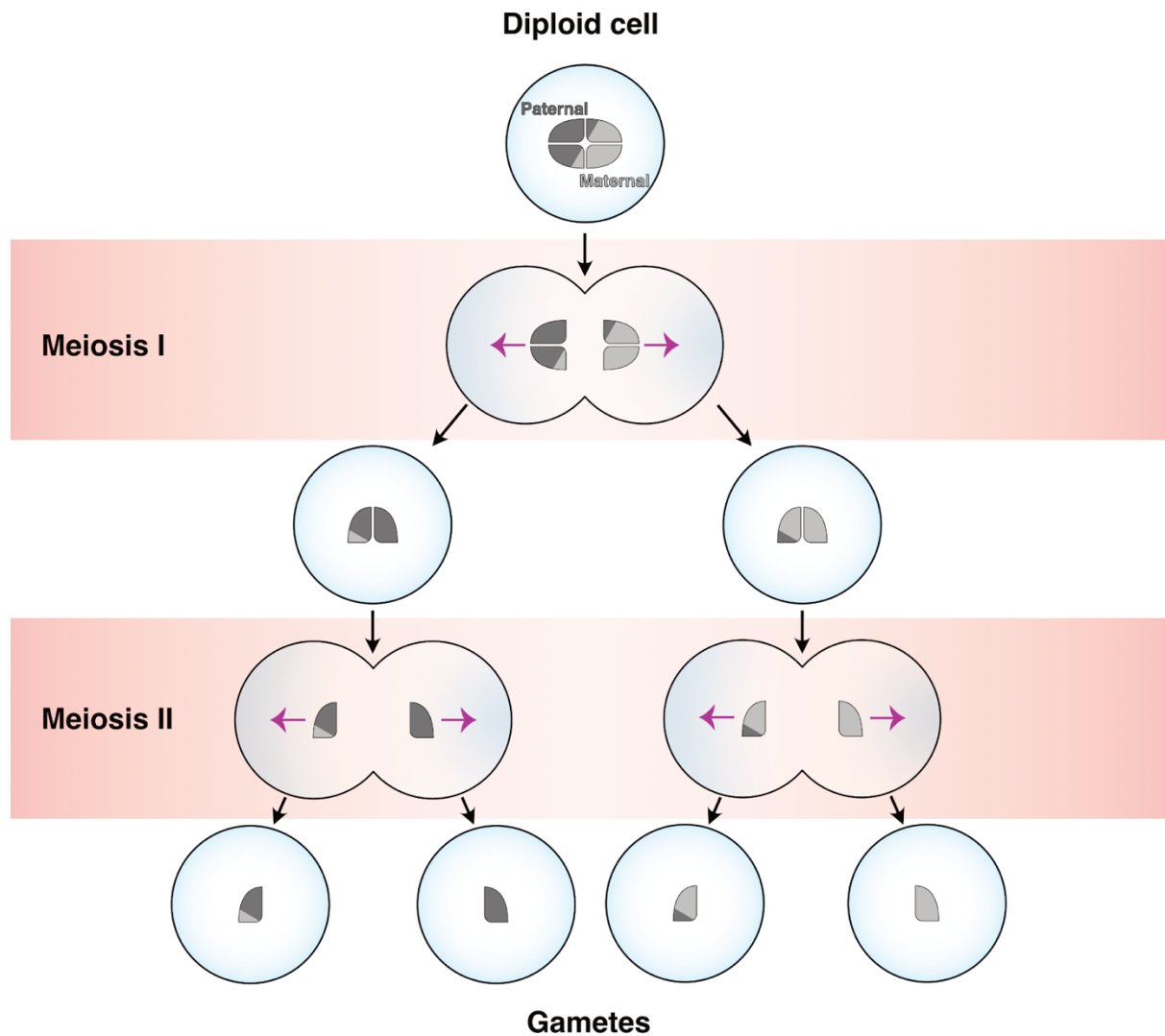


Figure 1. Two-step segregation of chromosomes during meiosis.

Diagram showing the required two-step chromosome segregation in meiosis to produce haploid gametes. At the top a diploid cell with a representative replicated chromosome with paternal (dark gray) and maternal (light gray) DNA. The cell proceeds to undergo two rounds of chromosome segregation (meiosis I and meiosis II, red boxes) to produce haploid cells called gametes.

1.3 Crossovers are required for meiosis completion

One of the hallmarks of meiosis is the formation of crossovers (CO). They promote genetic diversity through gene exchanges and provide a physical link between homologous chromatids (chiasma) that most species use to achieve a proper segregation of chromosomes during the first meiotic division. The number and distribution of crossovers is tightly regulated. Most organisms require at least one crossover to be designated per paired homologous chromosome (obligate crossover) to complete meiosis. The distance between multiple crossovers in a single chromosome is longer than expected by randomness and this effect is the result of a process known as crossover interference where the designation of a crossover event inhibits the formation of other nearby crossovers (von Diezmann & Rog, 2021a). The crossover formation is initiated in the early stages of the meiotic prophase with the generation of programmed double strand breaks (DSB) through the catalytic activity of the enzyme Spo11 (SPO-11 in nematodes), a topoisomerase-like protein (Claeys Bouuaert et al., 2021). Depending on the organism, the number of DSBs generated exceeds the number of crossovers by 2 to 200-fold (Girard et al., 2023; Saito & Colaiácovo, 2017). To generate homologous recombination, the 5' termini of DSBs are resected by Mre11 and Exo1 to generate single stranded DNA that assembles recombinases such as Rad51 to initiate strand invasion and homology search (Garcia et al., 2011; Kwon et al., 2023). The following steps required to produce a double Holliday junction are mediated by the ZMM (Zip, Mer and Msh) protein group. Two important components of this group are the Zip3 ligase (ZHP-3 in *C. elegans*) and the MutSy complex made up of Msh4 and Msh5 (HIM-14 and MSH-5 in *C. elegans*) (Haversat et al., 2022; He et al., 2021). The double Holliday junction is resolved by HIM-18 and SLX-1 (Saito et al. 2009) generating a chiasma that will hold the homologous chromosomes together. The crossover is known to be a catalytic active site with many proteins with functions in meiosis localizing to the region such as CDK kinases and cyclin-like proteins (i.e. COSA-1 in *C. elegans* and CNTD1 in mouse).

1.4 *C. elegans* as a model organism

The free-living nematode *Caenorhabditis elegans* is a widely used model organism for research. The worms can be conveniently grown on inexpensive media such as nematode growth medium (NGM) plates with a lawn of *Escherichia coli* as a food supply. Adults are small, about 1 mm long. It has two sexes, hermaphrodites and males. They have a rapid life cycle with an egg developing to an egg-laying adult in under 96 hours (4 days) at the standard maintenance temperature of 20°C but can be easily controlled by shifting the growth temperature of the medium anywhere in the permissive range from 15°C (5 days to adult) to 25°C (2.5 days to adult) (Gómez-Orte et al., 2018). When food is depleted or the living condition is overcrowded, young larvae are arrested and can live for months (Fielenbach & Antebi, 2008). A single wild-type hermaphrodite can lay hundreds of eggs populating an entire plate with ease. Strains can be preserved by freezing and storing for years and revived as demanded. The worm body is transparent making it convenient for microscopy (Corsi et al., 2015). The entire cell lineage from zygote to larva is documented (Sulston et al., 1983). The genome is expansively annotated and can be easily manipulated. *C. elegans* is widely used to study meiosis due to the wide availability of mutations that affect meiosis and to the fact that the gonad is organized in a spatiotemporal manner where the entire meiotic progression from initiation to completion can be visualized simultaneously (Tzur et al., 2018) (**Fig. 2**).

1.5 The events of meiotic prophase I

The meiotic prophase is extended from its counterpart in mitosis to accommodate the special systems that enable the proper chromosome alignment and segregation during meiosis I. The events of prophase I in meiosis are often categorized in five substages. At the first stage, called leptotene, the duplicated chromosomes condense into thin string-like structures facilitated by the involvement of multiple protein complexes such as condensin, cohesin and the axial elements of the synaptonemal complex (SC) that form the structural basis of the chromosome axes. At this stage, the telomeres attach to the nuclear envelope and the chromosomes identify and come near their homologous partners as part of a process known as chromosome pairing. The initiation of programmed DNA double strand breaks (DSB)

occurs at this time mediated by the catalytic activity of SPO-11 (Spo11 in mammals) (Grey & de Massy, 2021). In *C. elegans*, DSBs in early prophase I are not required for synapsis of the homologous chromosomes but can aid in its subsequent stabilization (Guo et al., 2022; Machovina et al., 2016; Pattabiraman et al., 2017). During zygotene, the following prophase I substage, the homologous chromosome synapsis is initiated by the polymerization of the central region of the SC from end to end of the homologous chromosomes holding them in close proximity through most of prophase I (Rog & Dernburg, 2013). Pachytene, the third stage of prophase I starts when all chromosomes are fully synapsed. By the conclusion of pachytene, a subset of the programmed DSBs is designated and processed into crossovers, physically linking the tetrads of homologous chromosomes by a structure called chiasma (Carpenter, 1994).

Desynapsis occurs during diplotene, the fourth stage of prophase I. The homologous chromosomes at this stage are held together only by the chiasma. In diakinesis, the last stage of the meiotic prophase I, chromosomes further condense, and breakdown of the nuclear envelope occurs (Jan et al., 2012).

Figure 2

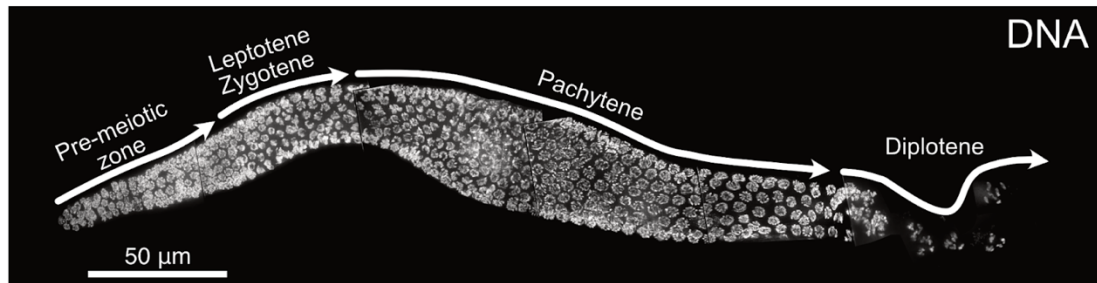


Figure 2. Spatio-temporal organization of the *C. elegans* gonad.

Image showing the spatio-temporal organization of the *C. elegans* gonad. Cells are propagated in the distal tip of the gonad at the pre-meiotic zone. Meiosis is initiated at Leptotene and Zygotene, the initial stages of prophase I. Two subsequent stages of prophase are shown (pachytene and diplotene). The arrows indicate the labeled substage of prophase I and the direction of meiosis progression. DNA is stained with DAPI.

1.6 Meiosis requires a two-step chromosome segregation

After DNA replication, the sister chromatid cohesion is mediated by the cohesin complex. The cohesin complex forms a ring-like structure that topologically embraces the duplicated sister chromatids. It is composed of four main subunits: 2 members of the structural maintenance of chromosomes (SMC) family of proteins, 1 kleisin family protein and 1 accessory subunit. In meiosis, a heterodimer of SMC proteins (SMC1 β and SMC3 in mammals or SMC-1 and SMC-3 in nematodes) encircle the chromatids and is closed at the ends by 1 of the possible kleisin subunits (RAD21L or REC8 in mammals, COH-3/COH-4 or REC-8 in nematodes). The main accessory subunit is conserved in all eukaryotes (STAG3 in mammals or SCC-3 in nematodes) and has regulatory roles in loading to and removing the complex from DNA. (Garcia-Cruz et al., 2010; Ishiguro, 2019; Roig et al., 2014; F. Wang et al., 2003). Cohesin is removed from the DNA by the proteolytic activity of separase (SEP-1 in nematodes) on the kleisin subunit during cell division. In contrast to mitosis where all sister chromatid cohesion is destroyed during a single cell division, meiocytes require a two-step loss of cohesion to accurately segregate the correct number of chromosomes during meiosis I and meiosis II. Before meiosis I, the paired homologous chromosomes designate two functionally distinct domains. The first domain will destroy the cohesin complex while the second domain will protect the cohesin complex resulting in the reductional division of the chromosomes during meiosis I. The second domain will preserve cohesion until meiosis II when the remaining cohesin is destroyed producing haploid cells.

1.7 Meiosis I partial loss of cohesion domains in *C. elegans*

Most sexually reproducing organisms such as humans have monocentric chromosomes containing a specialized chromosomal region called the centromere where kinetochores attach and is designated as the domain where the cohesin complex is protected during the first meiotic division. The cohesin complex at the centromere is protected from separase by the recruitment of the protein shugoshin (Sgo2 in mice) (El Yakoubi et al., 2017; Kitajima et al., 2004). Other organisms such as the free-living nematode *C. elegans* have holocentric chromosomes where the centromeric region is not clearly defined and instead attach the kinetochores

throughout the chromosome axes (Dernburg, 2001). This poses a particular problem to holocentric chromosomes as they need to develop an unconventional strategy to regulate the required partial loss of cohesion during meiosis I. In *C. elegans* meiosis, a single off-center crossover occurs on each of the six chromosomes, dividing each chromosome into two “arms” of unequal length (Albertson & Thomson, 1993; Martinez-Perez et al., 2008; Nabeshima et al., 2005). The relative lengths of these arms are sensed by an unknown mechanism, leading to the differential recruitment, eviction, or post-translational modification of several proteins on the shorter versus the longer arm in a process known as chromosome partitioning. This differential functionalization of short and long arms is essential to coordinate the discrete, stepwise destruction of cohesin with the two meiotic divisions. The functionalization of the short arm designates the domain where AIR-2 (Aurora B in mammals) is recruited. AIR-2 phosphorylates REC-8 at the short arms targeting the local cohesin complex for destruction by the proteolytic activity of separase during meiosis I (Ferrandiz et al., 2018; Kaitna et al., 2002; Rogers et al., 2002; Sasagawa et al., 2012; Sato-Carlton et al., 2018). The long arm functionalization designates the domain where cohesin is protected during the first meiotic division by antagonizing the recruitment of AIR-2 (de Carvalho et al., 2008; Martinez-Perez et al., 2008; Tzur et al., 2012). Since crossovers are generated at arbitrary positions along the chromosome, the distance between the crossover and each chromosome end needs to be sensed and compared *de novo* on each chromosome in each meiocyte.

1.8 The synaptonemal complex elements

The synaptonemal complex (SC) is a proteinaceous structure that holds homologous chromosomes together during pachytene. The SC is made up of axial elements, which are present along the length of each homologous chromosome, and the central region that polymerizes between the homologous chromosomes. Although the protein sequences of the individual elements of the SC are highly divergent between species, the proteins secondary structures and the ultrastructure of the polymerized complex is conserved (Kursel et al., 2021). The identified axial elements in *C. elegans* contain the HORMA domain proteins HTP-1 through HTP-3 and HIM-3 along with the axis associated proteins LAB-1 and LAB-2 (Couteau et al., 2004; Couteau &

Zetka, 2005; R. Wang et al., 2024). The HORMA domain was identified in budding yeast (Hop1, Rev7 and Mad2) and it incorporates a sequence called a “safety belt” that is used to change the protein structure between two states called “open” and “closed” (Rosenberg and Corbett 2015). It is suggested that axial elements of the SC with HORMA domains act as chromatin adaptors that mediate the interaction of other proteins with DNA. It has been shown that HTP-3 functions as a scaffold capable of recruiting HIM-3, HTP-1 and HTP-2 to the chromosome axis and thus is crucial for the formation of the chromosome axis (Fukuda et al., 2010; Kim et al., 2014). The central region of the SC is made up of the proteins SYP-1 through SYP-6 and SKR-1 and SKR-2 (Blundon et al., 2024; Colaiácovo et al., 2003; Hurlock et al., 2020; MacQueen et al., 2002; Smolikov et al., 2007, 2009). The protein SYP-1 (SYCP1 in mouse, Zip1 in yeast) is a transverse filament with a conserved structure, length and function but divergent sequence in other taxa. The transversal filament provides the “railroad track” appearance of the SC under electron microscope and its head-to-head interaction bridges the paired homologous chromosomes with approximately 100 nm separation (Billmyre et al., 2023; Gordon & Rog, 2023; Kurdzo et al., 2018). The central region of the SC has been shown in *C. elegans* to exist in a dynamic state reminiscent of liquid-liquid phase separation (Rog et al., 2017), and is able to associate with and dissociate from chromosomes (Machovina et al., 2016; Nadarajan et al., 2017; Rog et al., 2017). The SC plays multiple roles in meiotic prophase. A primary role of synapsis is to position allelic loci on homologous chromosomes in a position favorable for proper crossover formation. In many systems, the SC has also been shown to be required for the phenomenon of crossover patterning or genetic interference (Capilla-Pérez et al., 2021; France et al., 2021; Hayashi et al., 2010; Libuda et al., 2013). Recent work has shown the SC possesses a liquid crystalline structure (Rog et al., 2017), which is hypothesized to facilitate the rapid diffusion and coalescing of crossover-promoting factors within it (Morgan et al., 2021; L. Zhang et al., 2018). SC central region elements have been shown to dynamically move between chromosomes as well (Rog et al., 2017; von Diezmann & Rog, 2021b), though this dynamic movement slows down considerably from mid- to late pachytene (Machovina et al., 2016; Nadarajan et al., 2017; Pattabiraman et al., 2017). An important aspect in the regulation of the SC is the post-translational modifications of its elements. For example, phosphorylation of SYP-1 is required to recruit the polo kinase PLK-2 to the SC, a required step for the proper segregation of chromosomes

during meiosis I (Brandt et al., 2020; Sato-Carlton et al., 2018). Other examples include the regulation of SC assembly through SYP-5 and SYP-6 phosphorylation, stabilization of the SC through HTP-1 phosphorylation and regulation of desynapsis through SYP-2 phosphorylation (Das et al., 2020; Gold et al., 2024; Nadarajan et al., 2016).

1.9 Chromosome partitioning in *C. elegans*

The holocentric chromosomes of *C. elegans* require the distinct functionalization of the short and the long arms to achieve the partial loss of cohesion during the first meiotic division and the SC is a key player in this process. Both the axial and central elements of the SC are required for the proper length sensing and functionalization of the chromosome arms after the CO is formed. When the meiocytes enter the pachytene stage, all the known members of the SC are spread homogeneously throughout the chromosome axis (Blundon et al., 2024; Hurlock et al., 2020; Kim et al., 2014; Schild-Prüfert et al., 2011; Z. Zhang et al., 2020). A group of SC-associated proteins with similar distribution in early pachytene is ZHP-1 through ZHP-4 (Zip3 in yeast). They are RING finger proteins also involved in chromosome partitioning that are predicted to have E3 ligase activity for ubiquitin or SUMO. They form two heterodimers with distinct functions in meiosis (ZHP-1/2 and ZHP-3/4). The SC central region protein SYP-1 is phosphorylated by CDK-1 at position T452 and through a phospho specific antibody (phospho-SYP-1) it can be detected all over the chromosome axis before the CO designation. This phosphorylated residue in SYP-1 is part of a polo box binding domain that recruits the polo kinase PLK-2 to the SC. Both phospho-SYP-1 and PLK-2 are restricted in an interdependent manner to the short arm of the chromosome sometime after CO designation as one of the earliest events in chromosome partitioning (Brandt et al., 2020; Pattabiraman et al., 2017; Sato-Carlton et al., 2018). At the same time, ZHP-1 and ZHP-2 are also restricted to the short arm of the chromosome in contrast to ZHP-3 and ZHP-4 that are restricted to the CO sites (L. Zhang et al., 2018). This functionalization of the short arm during late pachytene is of great importance since it is required for the functionalization of the long arm that happens as cells move into the diplotene stage, where HTP-1/2 and its binding partners LAB-1 and LAB-2 are partitioned to the long arm region of

the chromosome. At this stage, desynapsis begins and all the members of the central region of the SC, not just the phosphorylated fraction of SYP-1, are restricted to the short arm region of the chromosome completing the asymmetric partitioning of the short and the long arm of the chromosome (de Carvalho et al., 2008; Ferrandiz et al., 2018; Martinez-Perez et al., 2008; Nabeshima et al., 2005; Sato-Carlton et al., 2018; Tzur et al., 2012; R. Wang et al., 2024). In monocentric organisms, cohesion is protected at the centromere by the action of shugoshin, but this role appears to not be conserved in *C. elegans* (Bohr et al. 2018). Instead, the eviction of HTP-1/2 from the short arm of the chromosome restricts the downstream recruitment of protein phosphatase 1 to the long arm antagonizing aurora kinase AIR-2 activity and locally protecting REC-8 (kleisin subunit of cohesin) from phosphorylation and destruction by SEP-1 (separase). In contrast, the short arm of the chromosome remains unprotected resulting in REC-8 phosphorylation and eventual destruction during meiosis I, enabling the proper segregation of chromosomes (de Carvalho et al., 2008; Ferrandiz et al., 2018; Kaitna et al., 2002; Rogers et al., 2002).

1.10 Project summary

Here, I have used quantitative microscopy, live imaging, and simulations to assess the underlying rules of length-sensitive chromosome partitioning in *C. elegans* oocyte precursor cells. Normally, genetic interference limits the number of crossovers on wild-type *C. elegans* chromosomes to one (Hillers & Villeneuve, 2003; Meneely et al., 2002), making the length sensing decision a simple binary choice between long and short. To help uncover the principles that govern partitioning, I took advantage of the multiple crossovers that occur on a large fusion chromosome to generate a more complex measurement problem. Based on my results, a proof-of-principle simulation that can recapitulate a wide variety of partitioning outcomes both in wild-type and previously reported mutants was developed. My results suggest that concentration of a factor originating at crossover designation sites and accumulating within crossover-bounded compartments of the SC is used as a proxy for length to determine the identity of short and long arms.

Chapter 2

Results

2.1 Chromosome partitioning driven by phosphorylated SYP-1 is initiated during mid-pachytene and is complete by late-pachytene

The transitions of the SC elements described in **chapter 1.9** from pachytene to meiosis I for a single chromosome are illustrated in **Fig. 3A**. Details are shown for early pachytene, when all the elements of the SC are spread homogeneously throughout the chromosome. Later, the short and long arms are delimited by the CO site; the lengths of both segments are measured and compared by an unknown mechanism. By the end of pachytene, the chromosome can restrict phosphorylated SYP-1 to the short arm based on the computation result. The diagram proceeds into diplotene showing complete asymmetric partitioning of the chromosome by eviction of HTP-1 from the short arm and restriction of all central region elements of the SC to the short arm; finishing with chromosome condensation and short arm destruction of cohesin during the first meiotic division. There are no reports quantifying or qualifying errors made in the partitioning of wild-type chromosomes during late pachytene. This raises the question of how accurate is the length computation performed by the chromosomes.

To investigate how accurately intrachromosomal length differences are discriminated to designate short and long arms, immunofluorescence was utilized to visualize phospho-SYP-1 (phosphospecific antibody) and pan-SYP-1 (antibody that recognizes phosphorylated and non-phosphorylated SYP-1) antibody signals in pachytene chromosomes along with the CO marker COSA-1 (anti-GFP). First, images containing multiple nuclei from the mid and late pachytene regions of the gonad were obtained (**Fig. 3B**). The mid pachytene is recognized by the appearance of small and dim foci of GFP-COSA-1 that mark the early recombination sites. In late pachytene, 6 larger and brighter GFP-COSA-1 foci are detected in every nucleus, corresponding to 1 CO site per paired *C. elegans* homologous chromosome. Chromosomes from each nucleus were traced in 3D and computationally straightened using the pan-SYP-1 signal that marks the chromosome axis in both stages. These straightened chromosomes were then projected, and the COSA-1 focus position was used to measure the lengths of the short and long arms for every straightened chromosome. The straightened chromosomes for both mid pachytene

and late pachytene stages were oriented so that the short arm is positioned to the left of the image. All chromosomes were stacked vertically and sorted from top to bottom in order of increasing short arm length to qualitatively analyze the partitioning of phospho-SYP-1 (**Fig. 3C**). The average intensity values of both pan-SYP-1 and phospho-SYP-1 for both short and long segments of each chromosome, normalized to the average intensity of either pan-SYP-1 or phospho-SYP-1 were calculated and plotted to the right in the same top-to-bottom order as the images on the left panel. In mid pachytene, the quantitative analysis of the short and long arm average intensities showed that the phospho-SYP-1 signal starts to become enriched on the short segments with a statistical difference between the mean average intensity of the long arms vs the mean average intensity of the short arms (The mean average intensity of the short arms is higher than 1 in the short arms). Conversely, the pan-SYP-1 signal shows a homogeneous distribution in the short and long arms with no statistical significance between the means of their intensities. In late pachytene, both phospho-SYP-1 and pan-SYP-1 were restricted to the short segments, with large statistical significance when comparing the means of the short and long arm intensities in any channel. The phospho-SYP-1 was restricted to a higher degree as indicated by the aligned chromosomes and the quantification. A correlation trend was observed between the length of the short arm (vertical position in the alignment/plot) and the average intensity of the segment. To validate this claim, the Spearman's rank correlation was calculated resulting in statistical significance and a negative correlation ($p < 0$) of the short arm length and intensity (longer short arms have lower average intensity while shorter short arms have higher average intensity) suggesting that shorter short arms accumulate more SC central region proteins per unit of length. Consistent with previous studies on crossover distributions (Barnes et al., 1995; Meneely et al., 2012; Rockman & Kruglyak, 2009), the majority of CO sites tend to be detected toward the ends of chromosomes. Out of 114 wild-type chromosomes traced, 80 chromosomes (70%) contained a COSA-1 focus within the terminal quarters of the chromosome length adjacent to the telomeres, compared to 34 chromosomes (30%) with their COSA-1 focus located in the central half of the chromosome length. In late pachytene, 110 out of 114 chromosomes had partitioned phospho-SYP-1 to the shorter segment (correctly designating physically shorter segments as functional "short arms") while only 4 out of 114 chromosomes designated the physically longer segment as the "short arm" (*i.e.*, phospho-SYP-1

was partitioned to the physically longer segment) (**Fig. 4**). Of these four cases, two had the highest short-to-long segment ratio (closest to 1); another contained a fairly centrally located CO site (the ratio of short segment length/long segment length is 0.77), while one had a ratio of 0.33. These exceptions show that while incorrect designation is rare overall, it may be relatively more common when arm lengths are similar. In all observations, one side of the COSA-1 focus was always designated as the “short arm”; no chromosomes were found that lacked phospho-SYP-1 partitioning. Overall, the analysis shows that chromosomes can generally discriminate length differences very well; mistakes are rare (4 out of 114) and chromosomes with centrally located COs are overrepresented among these mistakes.

In order to understand more about the molecular details for differential partitioning of phospho-SYP-1, it was speculated whether unphosphorylated SYP-1 completely partitions to long arms, leaving the short arm with only phospho-SYP-1 and thereby dividing the chromosome into two internally homogeneous domains, or alternatively, whether unphosphorylated SYP-1 remains distributed throughout the entire length of the SC while phospho-SYP-1 alone specifically partitions to short arms. To examine the distribution of unphosphorylated SYP-1, an HA tag was placed downstream of a non-phosphorylatable allele, *syp-1(T452A)*. This tagged non-phosphorylatable allele was used as a proxy for unphosphorylated SYP-1. Heterozygous *syp-1(T452A)/+* chromosomes were traced, straightened, projected and aligned to analyze the distribution of phospho-SYP-1 and non-phosphorylatable SYP-1 (anti-HA). Surprisingly, during late pachytene, non-phosphorylatable SYP-1^{T452A}-HA also became enriched on short arms along with wild type phospho-SYP-1 (**Fig. 3D**). Similarly to chromosomes with homozygous wild-type SYP-1 (**Fig. 3C**), both SYP-1^{T452A}-HA and phospho-SYP-1 mean intensity showed significant negative correlation with short segment length (Spearman's $\rho < 0$, **Fig. 3D**). It has been shown previously that homozygous *syp-1(T452A)* chromosomes fail to achieve chromosome partitioning (Sato-Carlton et al., 2018). To verify these observations, 156 chromosomes of homozygous *syp-1(T452A)* mutants were straightened, projected and aligned to test the distribution of pan-SYP-1 signal under these conditions. SYP-1^{T452A} failed to partition in late pachytene in the *syp-1(T452A)* homozygous mutant

(**Fig. 3E**). These observations imply that non-phosphorylatable SYP-1, and by extension unphosphorylated SYP-1, is also capable of partitioning to the short arm, but only in the presence of phospho-SYP-1.

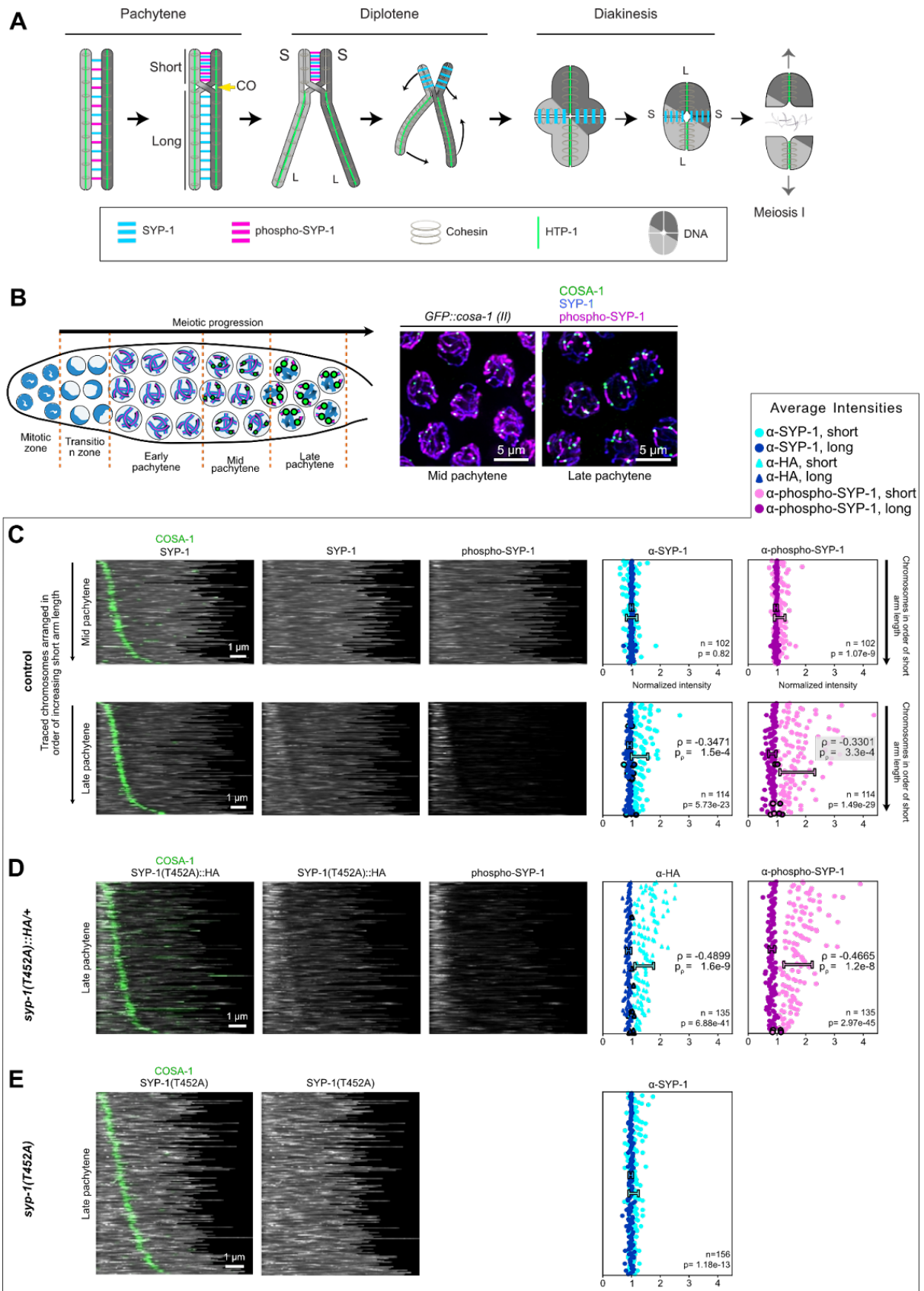


Figure 3. Length-sensitive functionalization of the synaptonemal complex is detectable in mid-prophase and requires phosphorylation of SYP-1.

A, Diagram of dynamic relocation of central (SYP-1, phospho-SYP-1) and axial (HTP-1) SC components as chromosomes remodel through late prophase. During the pachytene stage, components are initially homogeneous (left). As pachytene progresses, phosphorylated SYP-1 partitions to the short arm; by the diplotene stage HTP-1 partitions to the long arm, while all SYP-1 is found on the short arm, which becomes the site of cohesin destruction in the first meiotic division (right).

B, Left: Diagram of the *C. elegans* gonad showing the timing of chromosome partitioning during prophase. The phosphorylated fraction of SYP-1 (magenta) is homogeneous throughout the chromosomes during early pachytene. During mid-pachytene, the CO sites marked with COSA-1 (green) appear as dim foci at each chromosome. At late pachytene the phosphorylated fraction of SYP-1 is restricted to the short arm region of the chromosome and the COSA-1 foci grow in size and intensity. **Right.** Representative immunofluorescence images, shown in maximum intensity projection, of mid-pachytene (before chromosome partitioning) and late pachytene (after chromosome partitioning) in *GFP::cosa-1 (II)* nuclei. The CO site protein COSA-1 (anti-GFP) is shown in green, the SC component SYP-1 (anti-SYP-1) is shown in blue, and the phospho-SYP-1 (anti-phospho-SYP-1) is shown in magenta.

C-E, Left: panels show straightened images of the SC sorted from top to bottom in order of the shortest distance from the CO site (green, α -GFP::COSA-1 staining normalized to the maximum intensity of each focus) to the nearest chromosome end, and oriented such that the shorter segment is on the left. **Right:** Scatter plots of average per-arm intensity measurements of the corresponding SC images at left, sorted in the same order. The average intensity values for each segment (short or long) were normalized to the average intensity of the indicated signal (α -SYP-1, α -phospho-SYP-1, or α -HA) for the entire chromosome and are plotted on the X axis. The blue points indicate the short segment (light blue) and the long segment (dark blue) measurements for α -SYP-1 and α -HA staining. The magenta points indicate

the short segment (light magenta) and long segment (dark magenta) measurements for the α -phospho-SYP-1 staining. Outlined points are exceptional instances where the long segment average intensity is higher than the short segment average intensity (See **Fig. 4**). Error bars represent 1 standard deviation from the mean of the measured intensities. The p-values were obtained using Student's t-Test comparing the mean values of the short and long segment average intensities. The n values represent the number of measured chromosomes. Panel (**C**) shows straightened *GFP::cosa-1 (II)* control chromosomes at mid pachytene and late pachytene with WT-SYP-1 (α -SYP-1) and phospho-WT-SYP-1 (α -phospho-SYP-1) staining. Panel (**D**) shows straightened chromosomes at late pachytene from *GFP::cosa-1 syp-1(T452A)/+ (II); syp-1(me17)/+ (V)* heterozygotes with SYP-1(T452A) (α -HA) and phospho-WT-SYP-1 (α -phospho-SYP-1) staining. Panel (**E**) shows straightened chromosomes at late pachytene from the non-phosphorylatable *GFP::cosa-1 (II); syp-1(T452A) (V)* mutant showing SYP-1(T452A) (stained with α -SYP-1 antibodies).

Straightened chromosomes with long arm restriction of phospho-SYP-1

Late pachytene

GFP::cosa-1 (II)

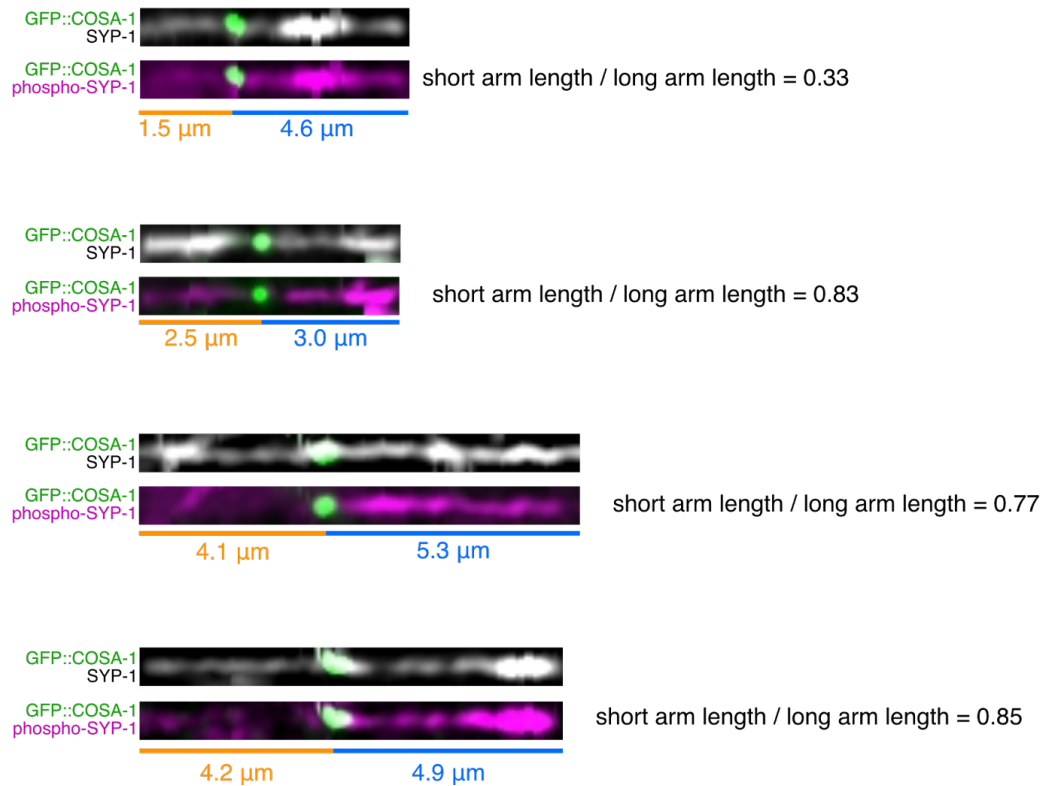


Figure 4. Straightened control chromosomes with long arm restriction of phospho-SYP-1.

Straightened and maximum-intensity projection of late pachytene chromosomes from a strain carrying *GFP::cosa-1 (II)* showing phospho-SYP-1 restriction on the long arm. Each straightened chromosome shows the CO site marked by GFP::COSA-1 (green), SYP-1 (grayscale) and phospho-SYP-1 (magenta). The orange underline marks the short segment of the chromosome, and the blue underline marks the long segment of the chromosome.

2.2 Phosphorylated SYP-1 restriction patterns suggest a chromosome partitioning mechanism driven by spreading and accumulation of a CO site-derived signal

Wild-type *C. elegans* chromosomes implement very strong crossover interference, almost always limiting the number of crossovers on each chromosome to one (Hammarlund et al., 2005; Hillers & Villeneuve, 2003; Meneely et al., 2002). The tracing and straightening of wild-type chromosomes showed that the short arm designation is relatively simple with few errors (**Fig. 3C, Fig. 4**). To challenge the chromosome partitioning mechanism and to gain insights of the underlying rules for short and long arm designation, an experiment was designed to analyze chromosomes with multiple CO sites. In these chromosomes with more than one CO, the simple binary decision of where the short arm is designated is obfuscated into a seemingly complicated problem with at least three segments that need to be measured and compared. Analysis of chromosomes with multiple CO sites can resolve different questions that cannot be answered in wild-type chromosomes such as if the chromosome is able to designate multiple short arm regions in a single chromosome axis or if the telomeres are required for the short arm designation. While wild-type chromosomes do not usually obtain multiple CO sites, previous studies have shown that the triple fusion chromosome *meT7* (Hillers & Villeneuve, 2003), made by fusion of chromosomes III, X, and IV (**Fig. 5A**) and containing roughly half the genome in a single “megosome”, can often enjoy multiple crossovers. The fusion chromosome can be cytologically identified in diakinesis as a big DNA-stained body alongside the remaining 3 wild-type chromosomes (**Fig. 5B**). By examining this much longer chromosome it is also possible to test if there are any absolute size limits in the process of arm designation. To this end, immunofluorescence images of *meT7*-containing oocyte precursor cells in late pachytene in *GFP::cosa-1 (II); meT7 (III;X;IV)* worms were captured with 3D-SIM superresolution microscopy (Gustafsson et al., 2008). The increased resolution of the obtained images allowed for unambiguous tracing of the fusion chromosomes and for the clear analysis of the CO sites, chromosome axis and short arm designation sites (**Fig. 5C,D**). It has been shown that the worms carrying the *meT7* chromosome can still proceed with meiosis generating viable sperm and oocytes. An embryonic viability of 88.7% (n=1028 eggs counted) was scored compared to the wild type

99.2% (n=3082). To validate the total number of CO sites in the megasome, the GFP::COSA-1 foci were scored in a total of 247 nuclei of *meT7* homozygous worms. Two main populations of nuclei were found; the first, with 4 COSA-1 foci, represented a 54.7% of the scored nuclei and indicates that each of the chromosomes present have just 1 CO site (1 megasome + 3 wild-type chromosomes). The second population had 5 COSA-1 foci and is represented by 44.1% of the population. This last group is expected to have two CO designation sites in the *meT7* chromosome (**Fig. 5C**). The remaining 1.2% of the nuclei scored was a scarce population that had 6 COSA-1 foci.

The *meT7* chromosomes with two CO sites were analyzed first by tracing all the chromosomes in the nuclei of late pachytene, when the chromosome partitioning is already complete. Interestingly, some of the nuclei with five COSA-1 foci had 5 distinguishable phospho-SYP-1 stretches strongly indicating that these megasomes had multiple short arm designation sites (**Fig. 5D**). The traces of these chromosomes were used to straighten the megasomes revealing that when the CO sites were positioned opposed to each other near the chromosome ends, two short arm regions as marked by phospho-SYP-1 were designated in the single panSYP-1 marked chromosome axis (**Fig. 5E**). The revelation confirmed the hypothesis that indeed, chromosomes with multiple CO designation sites can occasionally designate more than one short arm. Another population of the traced nuclei had just 4 distinguishable phospho-SYP-1 stretches, strongly implying that they did not designate multiple short arm domains. The straightened chromosomes of this subpopulation revealed that the CO sites had a more central position and strikingly, the short arm was designated in the middle segment of the axis bounded by the two CO. This is consistent with a previous study where HTP-1 was depleted between two chiasmata in the diakinesis stage (Martinez-Perez et al., 2008). Fascinatingly, the central short arm of these chromosomes was occasionally the longest segment in the megasome (**Fig. 5F**). Further, the length of the central segment with phospho-SYP1 restriction shown in **Figure 5E** is 6.1 μm while the typical length of a *C. elegans* chromosome is 5-6 μm , making the short segment domain in the *meT7* longer than the full length of some wild-type chromosomes. Thus, the *meT7* chromosomes with central restriction of phospho-SYP-1 revealed three important features about the mechanism of short arm determination. First, the physically shortest segment is not necessarily the one

designated as the functional “short arm”. Second, while partitioning in wild-type chromosomes always defines the short arm between a CO site and the nearest chromosome end, it is possible to designate the short arm between two CO sites, suggesting that telomeres or chromosome ends are not a requirement and that the CO sites can act as domain **boundaries**. Third, the length of the short arm domain is not intrinsically limited to the regular length of wild-type chromosome short arms.

Occasionally, the phospho-SYP-1 signal started at the CO site but did not completely extend to the next crossover or chromosome end, rendering a portion of the pan-SYP-1 marked axis devoid of phospho-SYP-1 signal (**Fig. 6A**). This was more prominent in *meT7* with one CO (**Fig. 6B**). A small subset of *meT7* nuclei had 6 COSA-1 foci with the megasome containing 3 CO sites (**Fig. 6C**). This creates two central segments that have no chromosome ends as boundaries and often contain these partial phospho-SYP-1 restrictions that do not extend throughout the segment.

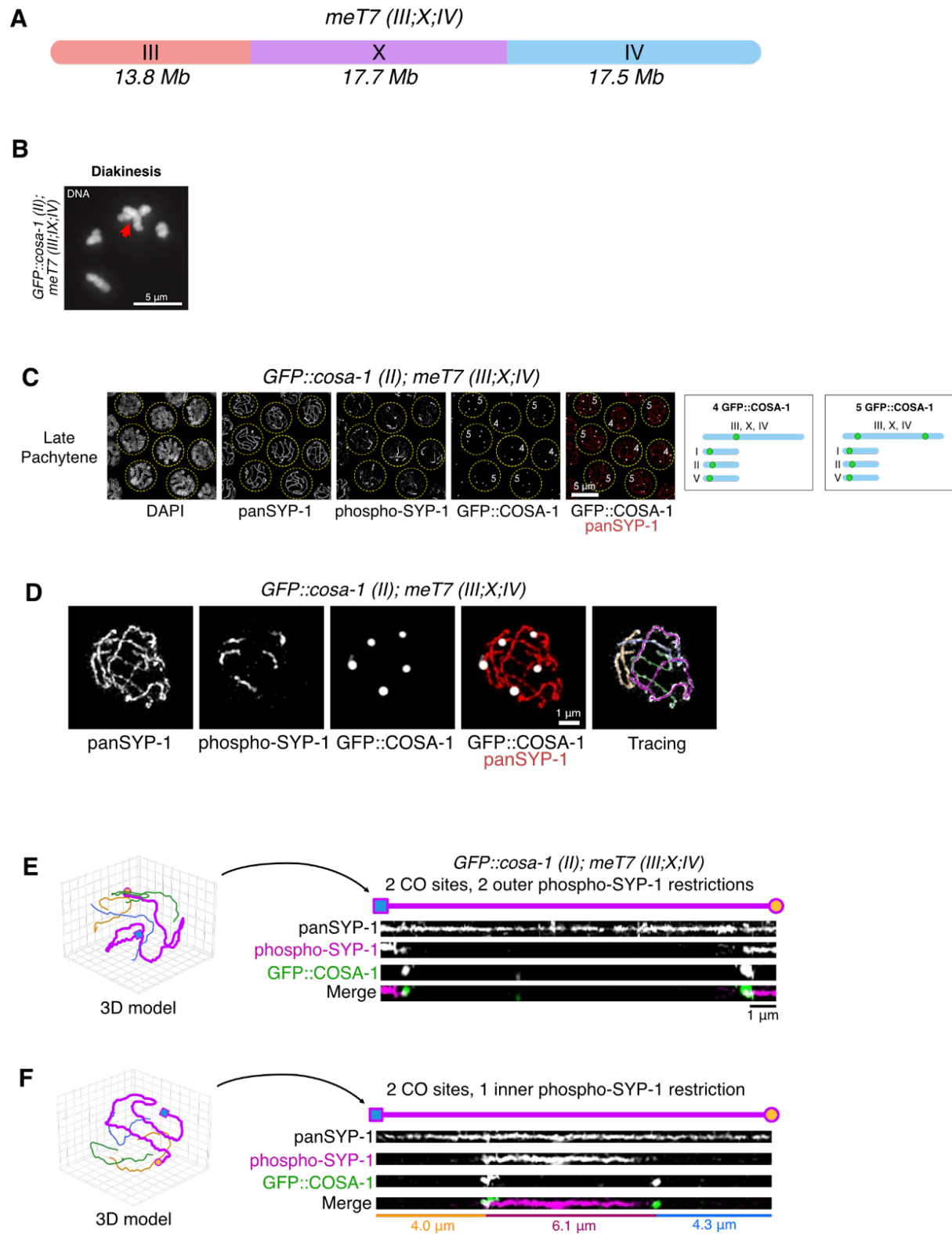


Figure 5. Phosphorylated SYP-1 restriction patterns in *meT7* reveal features of the short arm designation mechanism.

A, Diagram of the triple-fusion “megosome” *meT7*, composed of *C. elegans* chromosomes III, X, and IV connected as shown. Approximate sizes (in megabases) of original chromosomes corresponding to each part are shown below.

B, DAPI stained nucleus at the diakinesis stage in the strain *GFP::cosa-1 (II); meT7 (III;X;IV)*. Four DAPI bodies are observed. Red arrow indicates the large *meT7* triple-fusion chromosome.

C, Visualization of CO sites in *GFP::cosa-1 (II); meT7 (III;X;IV)* marked with *GFP::COSA-1* (anti-GFP) at late pachytene. The number of CO sites is shown within each circled nucleus. *Right*, karyograms indicating the distribution of CO sites per chromosome among nuclei with 4 and 5 *COSA-1* foci.

D, Representative nucleus from *GFP::cosa-1 (II); meT7 (III;X;IV)* at late pachytene showing SYP-1 (anti-SYP-1), phospho-SYP-1 (anti-phospho-SYP-1), *GFP::COSA-1* (anti-GFP) and three-dimensional traces of the four chromosomes. The magenta trace represents the *meT7* fusion chromosome; blue, yellow and green traces represent the wild type chromosomes.

E, Left, Three-dimensional models of a nucleus with 5 *GFP::COSA-1* foci (anti-GFP) showing the traced *meT7* in magenta and the remaining WT chromosomes in yellow, green and blue. *Right*, straightened and maximum intensity-projected version of *meT7* showing the chromosome axis marked by SYP-1 (anti-SYP-1), two CO sites marked by *GFP::COSA-1* (anti-GFP) and two outer phospho-SYP-1 (anti-phospho-SYP-1) restriction sites.

F, As D, but for a *meT7* chromosome with 2 internal CO sites and 1 inner phospho-SYP-1 restriction site. The lengths of each chromosome segment divided by the CO sites are shown below the straightened image.

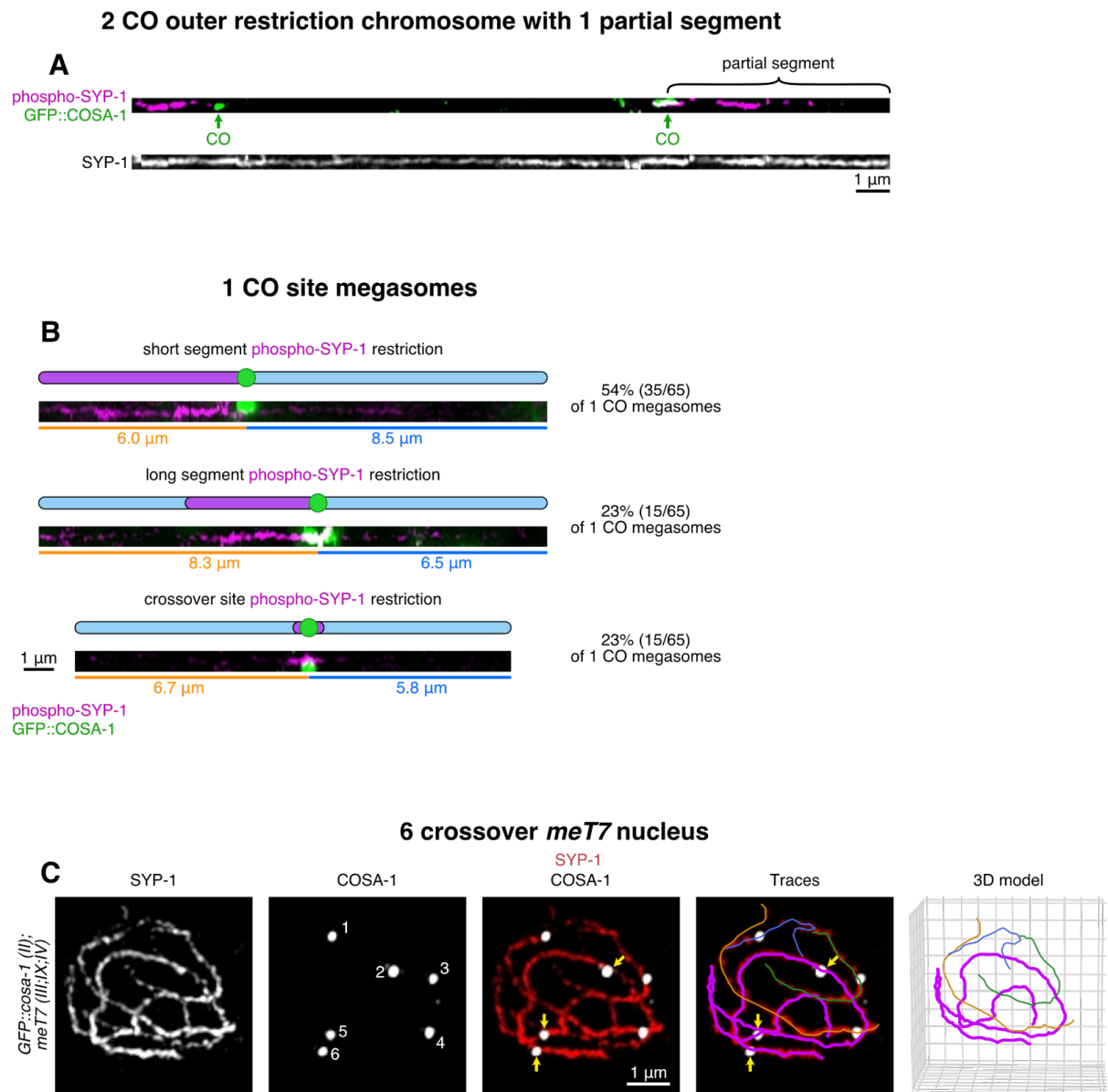


Figure 6. Single and triple crossover designation sites in *meT7*

A, Straightened *meT7* megasome showing 2 CO sites (GFP::COSA-1, green), phospho-SYP-1 (α -phospho-SYP-1, magenta) and SYP-1 (α -SYP-1, grayscale). Two phospho-SYP-1 restriction segments are shown with outer classification (See **Fig. 8**). The rightmost segment is not fully marked by phospho-SYP-1.

B, Straightened *meT7* megasomes with 1 GFP::COSA-1 focus (α -GFP) showing phospho-SYP-1 (α -phospho-SYP-1) restriction. Top. Megasome with

phospho-SYP-1 restricted to the short segment of the chromosome. Middle. Megasome with phospho-SYP-1 restricted to the long segment of the chromosome. Bottom. Megasome with phospho-SYP-1 restricted to the crossover designation site of the megasome.

C, meT7 traces in a late pachytene nucleus with 6 detected GFP::COSA-1 CO sites. Images show SYP-1 (α -SYP-1) as the chromosome axis marker and GFP::COSA-1 (α -GFP) as the CO site marker. The yellow arrows indicate the 3 GFP::COSA-1 foci on the megasome.

2.3 Signal diffusion from the CO as means of length-sensing explains the short arm observations in *meT7*

Briefly, it was found that the short arm is robustly designated at the shortest segment in wild-type chromosomes, multiple short arms can be designated in chromosomes with multiple CO sites, the short arm designation does not require chromosome ends/telomeres, and the short arm length can exceed twice the expected maximum size of wild-type short arm domains. A hypothesis that can explain all the previously mentioned observations was developed; the CO sites generate or recruit a signal that diffuses into and within the SC (**Figure 7A**). The continuous incorporation of the signal through the COs into the surrounding segments results in a density differential explained by the signal addition ratio and the length of the segment. The acquired signal density of the two segments surrounding each CO is compared and the segment with the higher intensity is designated as the short arm. In the case of wild-type chromosomes that have only two segments delimited by a single CO; this always results in the shorter segment designation as the short arm (**Fig. 7B**). In chromosomes with two COs, the external segments that contain the chromosome ends receive the signal input of one CO each, but the central segment receives twice the input as it is bounded by two separate CO sites, halving the effective length of the segment. Under these circumstances, the relative position of the COs to one another determines the signal density pattern in the segments and consequently, the short arm designation. This explains the short arm observations in *meT7* such as the central short arm that occurs when the two CO sites are relatively close to one another near the center of the chromosome (**Fig. 7C**). An important element required for the hypothesis is that the COs impede the diffusion of the signal from one segment to its neighbor, effectively functioning as boundaries.

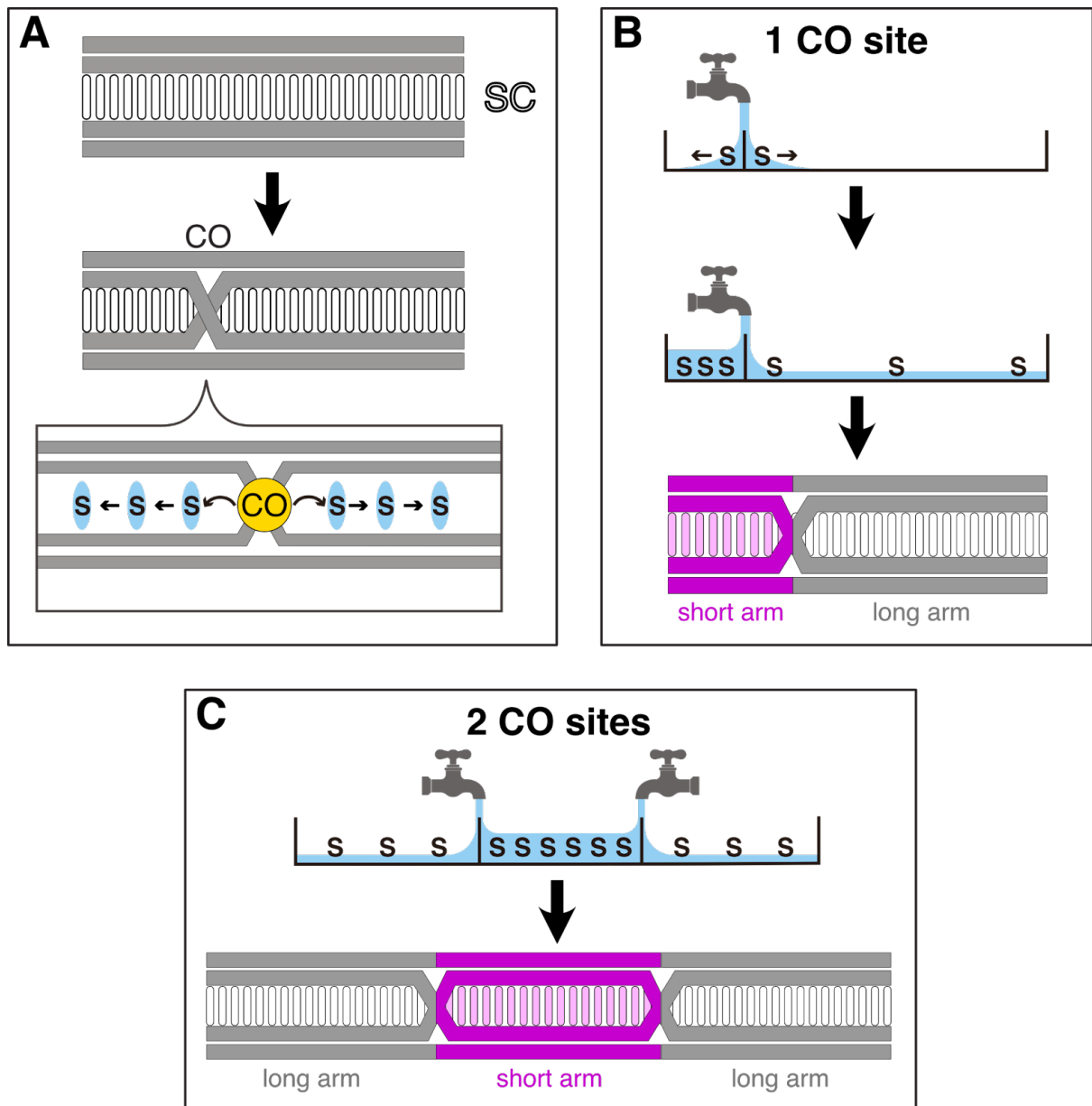


Figure 7. Diffusion and accumulation model explains phospho-SYP-1 restriction observations.

A, Top. Diagram showing a synapsed chromosome before the crossover designation. **Bottom.** Diagram of the chromosome after crossover designation and a close-up on the single off-center crossover (yellow, CO) showing the diffusion of a hypothetical signal (blue, S) equally to left and right along the chromosome axis.

B, Diagram illustrating the mechanism by which a signal originating from 1 crossover could enable length sensing of different compartments. *Top*. The hypothesized signal molecules (blue, S) are recruited or modified at the crossover and spread uniformly into two bounded unequal compartments split by the crossover. *Middle*. The accumulation of the signal leads to a higher concentration in the smaller compartment. *Bottom*. The smaller compartment with higher concentration is rectified as the “short arm” region of the chromosome.

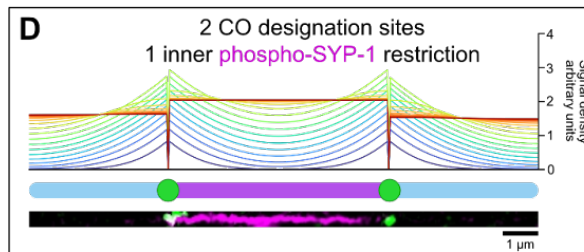
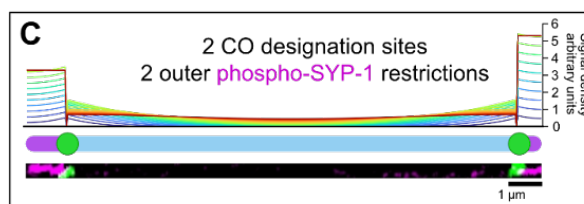
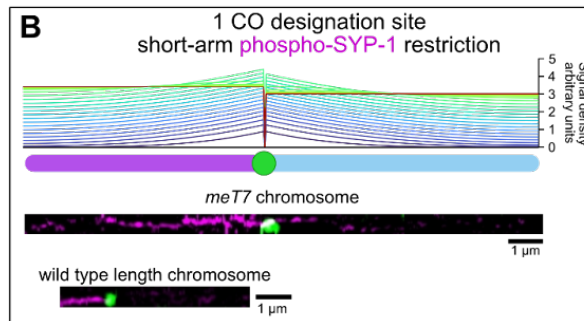
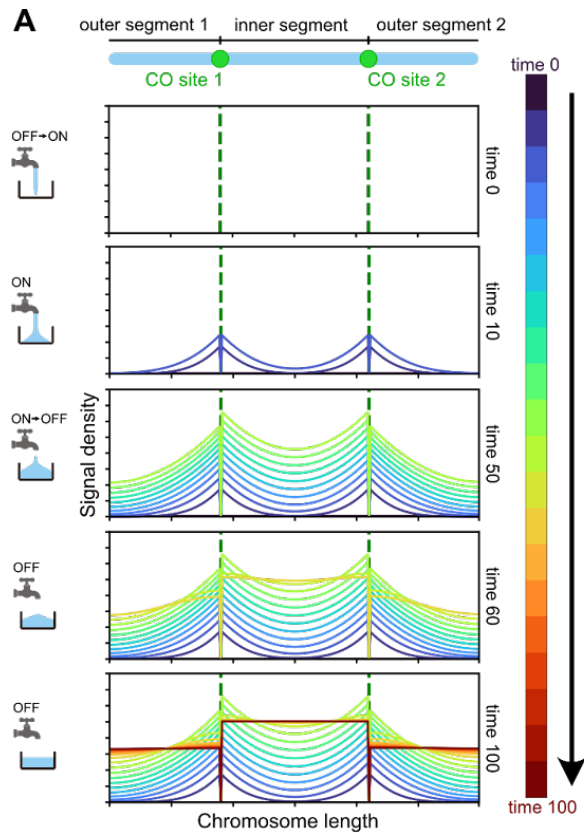
C, Diagram showing the expected outcome of 2 crossovers close together. *Top*. Illustration of the high-concentration inner (central) compartment in a chromosome with 2 signal origins. Flanking the inner compartment are 2 outer low-concentration compartments. *Bottom*. The high-concentration central compartment is rectified as the “short arm” region in the chromosome.

2.4 Model predictions of short arm designation patterns agree with *meT7* observed phospho-SYP-1 restrictions

The hypothesis of short arm determination based on a diffusing signal originating from the COs was tested; A program was developed to calculate the expected signal density in every resulting segment of a chromosome from any provided CO number and positions, obeying the condition that the signal is not allowed to cross from one segment to another. The program is deterministic in nature and will always result in the same density pattern for every set of CO numbers and positions. The program cycles through a set number of iterations represented as time in the diagrams. The signal is added and/or diffused at each iteration starting from the designated COs. When there are two CO sites, the signal density is inversely proportional to the segment length with the central segment density per length increased by a factor of two since it receives input from two CO sites (**Fig. 8A**). The CO positions from the observed *meT7* chromosomes were used to calculate predictions for each chromosome and tested to know whether the predicted signal density pattern agreed with the corresponding observed phospho-SYP-1 pattern. The assay included chromosomes with 1, 2 and 3 CO sites (**Fig. 8B-G**). Overall, the predictions agreed with the observed phospho-SYP-1 patterns. A total of 65 late pachytene megasomes with 1 GFP::COSA-1 focus were traced and straightened. All of the observed CO site positions in *meT7* chromosomes with a single GFP::COSA-1 focus were located in the central half of the megasome (65 out of 65) as expected from studies in other fusion chromosomes (Libuda et al., 2013).

A correct restriction of phospho-SYP-1 to the shortest segment was observed 54% of time (35 out of 65) in *met7* (**Fig. 8B**). The incorrect fraction was divided into two categories, the first where phospho-SYP-1 was restricted to the long segment (23%), and the second where the phospho-SYP-1 signal was depleted from most of the chromosome axis but remained in a small symmetrical small region surrounding the CO site (23%) (**Fig. 6B**), suggesting that correct length sensing for centrally-located CO sites is even more challenging for the large *meT7* chromosome. A total of 74 megasomes with two CO sites were traced and qualitatively classified according to their phospho-SYP-1 restriction pattern; three different restriction patterns were

observed. The first pattern corresponded to the fraction of megasomes that have a total of two short arm domains, one at each chromosome end (**Fig. 8C**). The second pattern corresponds to the *meT7* with one centrally located phospho-SYP-1 restriction (**Fig. 8D**). The accompanying predictions for both previously mentioned observed patterns showed the high-density segments correlated to the phospho-SYP-1 restriction sites. In the third observed pattern of megasomes with two CO sites, one of the COs restricts phospho-SYP-1 towards the nearest chromosome end, while the opposite CO restricts phospho-SYP-1 in the contrary direction to the nearest chromosome end. The central segment of these chromosomes has a partial short arm designation, with only a stretch of phospho-SYP-1 localized and a long arm designation, with partial phospho-SYP-1 depletion. The accompanying prediction demonstrates that the expected signal density of the central region of these megasomes lies between two adjacent outer regions whenever mixed segments are observed (**Fig. 8E**). The expected signal density in megasomes with three CO sites correlates to the observed phospho-SYP-1 restrictions (**Fig. 8F-G**). The presence of mixed segments in chromosomes with three CO sites is also detected and shares the prediction of having the expected signal density in-between the two neighboring segments. Taken together, these observations suggest that the short/long arm choice is made locally by a comparison of signal concentrations at the left and right of each CO site, and segments with conflicting choices at either end become divided between “short” and “long” identity.



phospho-SYP-1
GFP::COSA-1

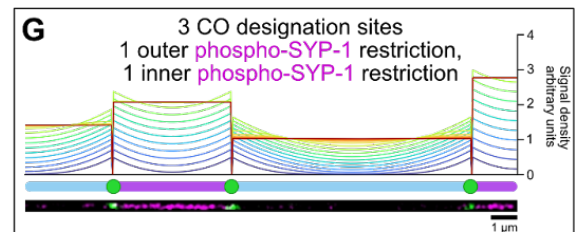
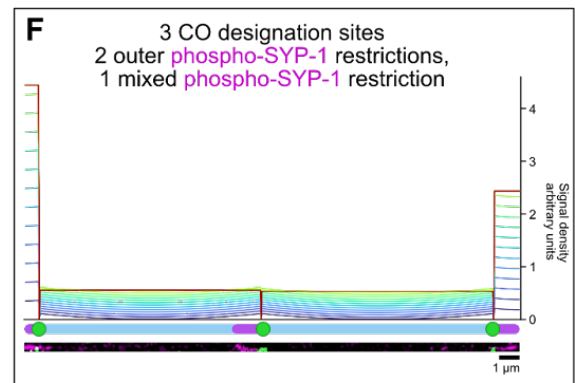
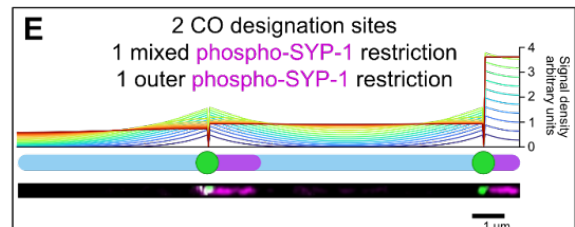


Figure 8. Model predictions of phospho-SYP-1 patterning in chromosomes with multiple CO sites agree with the observed meT7 chromosomes.

A, Top. Diagram showing a simulated chromosome divided by two CO sites into three segments: 2 outer segments and 1 inner segment. *Bottom.* Time course of a program calculating the predictions of concentration of factors starting simultaneously at two origins. Signal density along the simulated chromosome at each time point is represented by a colored line according to the color bar shown on the left. The final signal density is shown as a red line. The green dotted lines represent the signal origin (CO sites).

B-G, Straightened maximum intensity projection images from *GFP::cosa-1 (II); meT7 (III;X;IV)* at late pachytene with varying CO sites and positions. The images show the CO site marked by GFP::COSA-1 (anti-GFP) in green, and phospho-SYP-1 (anti-phospho-SYP-1) in magenta. The prediction of signal accumulation corresponding to the given configuration is shown above each chromosome image. Chromosome in panel D is the same as in Figure 5F.

2.5 Quantitative analysis of two-CO *meT7* partitioning agrees largely with observations

The robustness of the prediction scheme was evaluated by analyzing the 74 late pachytene straightened superresolution images of the *meT7* chromosomes harboring two COSA-1 foci. To simultaneously visualize all the observed phospho-SYP-1 patterns from *meT7* and their corresponding prediction, each chromosome was plotted on a 2D space defined by the relative position of both CO sites. The chromosome-length normalized position of CO1 (leftmost CO in the straightened image) to the nearest chromosome end was plotted on the Y-axis while the normalized position of CO2 (rightmost CO in the straightened image) to the nearest chromosome end was plotted on the X-axis. The chromosomes with two short arm designation sites in the opposing distal chromosome end segments (outer restriction) are represented by yellow circles (**Fig. 9A**), chromosomes with one central short arm designation (inner restriction) are represented by green squares (**Fig. 9B**), and chromosomes with one outer segment designated as a short arm and a partial short/long arm central segment (mixed restriction) are represented by purple stars (**Fig. 9C**). The model divides the space in three regions based on the predicted signal density pattern; the yellow zone indicates the phase-space where chromosomes are predicted to have an outer phospho-SYP-1 restriction with the final signal density higher on both opposing distal segments compared to the central segment (**Fig. 9A,D**), the green zone indicates the phase-space for chromosomes predicted to have inner phospho-SYP-1 restriction with the central segment final signal density higher than the distal segments (**Fig. 9B,D**) and the two purple zones indicate the phase-space for chromosomes predicted to have mixed phospho-SYP-1 restriction with the final signal density on the central segment higher than one of the distal segments but lower than the opposing distal segment (**Fig. 9C,D**). The inequalities that define the zones described by the relative lengths of the segments are shown in **Figure 9D**. In brief, the three segments of the chromosomes delimited by the CO sites are labeled a, b and c based on their ordered position from left to right of the straightened image (**Fig. 9D**). The sum of all three segments adds up to 100% of the length of *meT7* and the final predicted signal density pattern is described by the position in the plot where the lengths of a, b and c are true for the inequalities. The gray lines that border the zones are isoclines, where the predicted signal density on both sides of one

crossover is the same. All straightened chromosomes are plotted in **Figure 9E**. It was found that 59 out of 74 chromosomes (80%) follow the predictions as expected, i.e., the distribution of phospho-SYP-1 is constrained to the region(s) of the chromosome predicted to receive the highest levels of a crossover-derived signal.

The position of each chromosome on the plot is affected by two external factors. The first is the variability of the total *meT7* chromosome length during the chromosome straightening process that although is semi-automated (See **4.7 Chromosome tracing, straightening and 3D model rendering**), still requires the input of the tracing subject. The second is the variability of the CO-delimited segment lengths that can occur when the subject is estimating the position of the CO sites based on the COSA-1 signal. The precision of the segment lengths was tested by retracing, straightening and measuring the crossover positions on 3 different megasomes 0 to 10 times by 3 different people (**Fig. 10**). The resulting standard deviation of the megasome positions in the plot to their mean gives us a confidence of precision within 1% in the Y axis and 1.3% in the X axis.

Although megasomes with central phospho-SYP-1 partitioning demonstrate that “short arm” designation does not need to contain chromosome ends (**Fig. 5F**), the presence of segments with mixed restriction (partial short/long arm designation) (**Fig. 8E**) could suggest that residual internal telomeric sequences from the fusion points of *meT7* could affect the restriction of phospho-SYP-1 either in a mechanistic manner that contradicts the diffusion model (the short arm is designated from the CO to the closest telomere) or by functioning as barriers for signal diffusion. To address this concern, immunofluorescence was performed against the double-stranded telomeric DNA binding protein TEBP-1 / DTN-1 (*Yamamoto et al., 2021*) in order to detect if any telomere-like structures remained at the fusion junctions. The *dtn-1::GFP::3xFLAG (II); meT7 (III;X;IV)* meiocytes had an average of 8 foci per nucleus and the straightened chromosomes revealed DTN-1 foci only at the chromosome ends (**Fig. 11A**). Although the DTN-1 protein did not bind the inner segment of the *meT7* megasome suggesting that only the chromosome ends have a telomere structure, it was still a possibility that the junctions contain a telomeric sequence that

affects partitioning without recruiting DTN-1. The possibility was addressed by simulating the *meT7* chromosome assuming the chromosome junctions function as barriers for signal diffusion. The length of each junction-delimited segment was calculated based on the number of base pairs of each chromosome. Under these circumstances, different predictions can be often observed for the same set of CO positions whether the junctions function as barriers or not for diffusion of the signal (**Fig. 11B**). A total of 100 CO position pairs with the minimum distance between them set to the length of the shortest fused chromosome (chromosome III, 13.8 Mb pairs) were generated, the phospho-SYP-1 restriction prediction was calculated, and the chromosomes were plotted on the prediction phase-space diagram (**Fig. 11C**). Under this scheme, the simulated category of the *meT7* phospho-SYP-1 restriction did not agree to the predicted zones of the phase-space diagram in comparison to the short arm designation observations plotted on **Figure 9E**. Using the observed CO positions of the 74 megasomes with two CO sites, a new set of predictions was generated using both possible orientations of the *meT7* to test whether the current observations can be explained by the fusion junctions functioning as barriers. The resulting predictions were in striking discordance with the observations (**Fig. 11D**). The total number of expected correct predictions made by the chromosome junctions functioning as barriers is 44% while the diffusion model with the chromosome junctions not as barriers correctly predicted 80% of the observations (**Fig. 9E**). A large differentiating factor in the predictions of the chromosome junctions functioning as barriers is the prevalence of mixed segments (partial short and long arm designation in a single segment). The number of expected mixed segments in the inner, outer and mixed restriction types is 5-fold the actual number of observed mixed segments (**Fig. 11E**). It was therefore concluded that any residual telomere sequence or other aspect of the fusion points on the *meT7* chromosome is unlikely to play a role in designation of partition boundaries or identity in a similar fashion to CO sites and true chromosome ends.

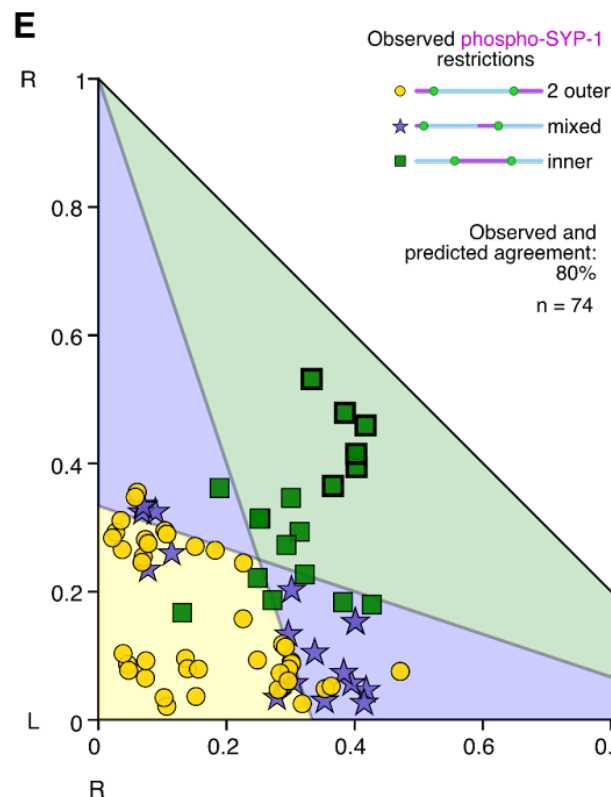
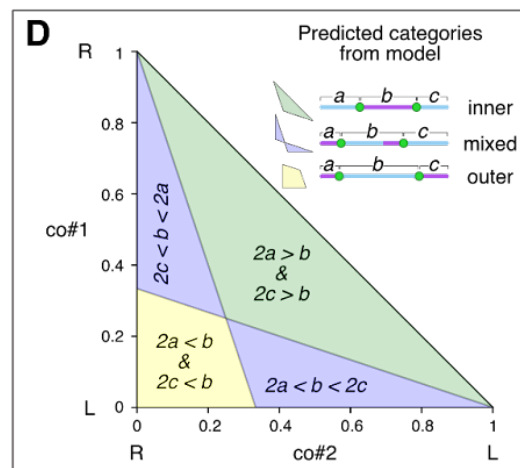
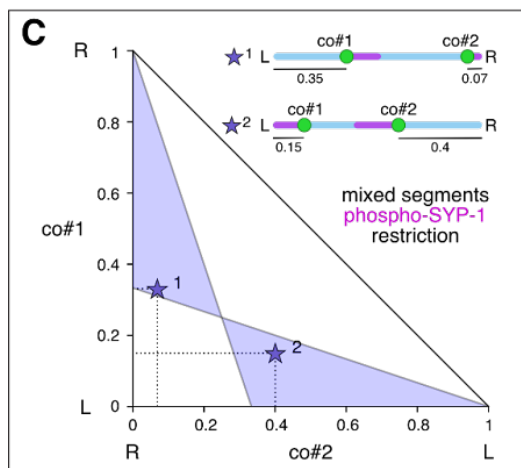
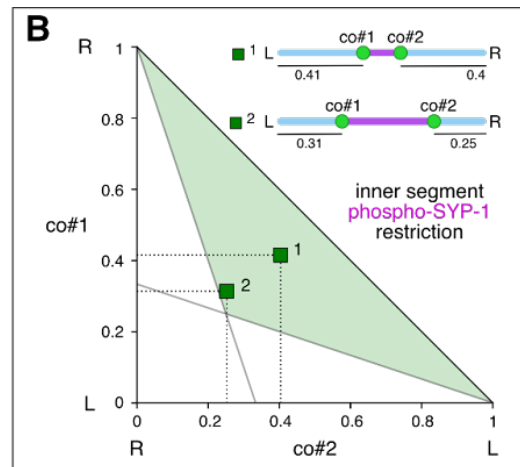
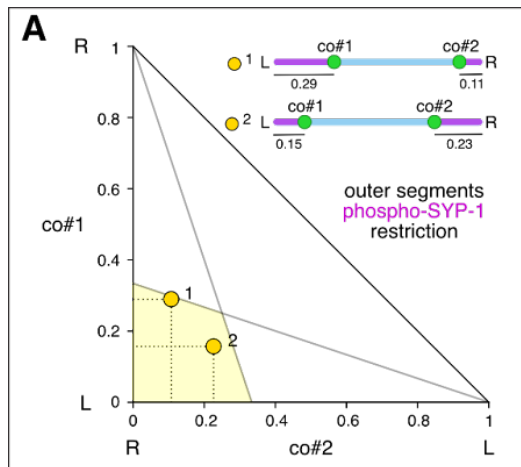


Figure 9. Quantitative analysis of two-CO *meT7* chromosomes and model prediction accuracy.

A-C: Phase diagrams for plotting the positions of two presumptive crossover sites marked by GFP::*COSA-1* on single *meT7* chromosomes. Chromosome traces are arbitrarily assigned left and right ends and chromosome lengths are normalized to 1. The left focus (co#1) distance from the left chromosome end is plotted on the Y axis, and the right focus (co#2) distance from the right end is plotted on the X axis. The shaded region in each diagram indicates the zone within the phase space where the indicated partitioning pattern is predicted. Configurations of example chromosomes from this study with the observed GFP::*COSA-1* positions are shown at upper right.

D, Superimposition of all prediction zones, showing the distribution of each pattern type in the phase space. Each zone shows equations based on segment lengths that are true within it, giving the predicted signal density at each of the chromosome segments (See **4.10 Megasome classification**).

E, Plot of 74 traced chromosomes shown by markers corresponding to their observed phospho-SYP-1 restriction pattern, placed at their measured positions in the phase diagram. 80% of chromosome configurations lie within their predicted zone. Bold-outlined square markers on the top right are from measured *meT7* chromosomes whose *COSA-1* foci are closer together than $\frac{1}{3}$ of the total chromosome length.

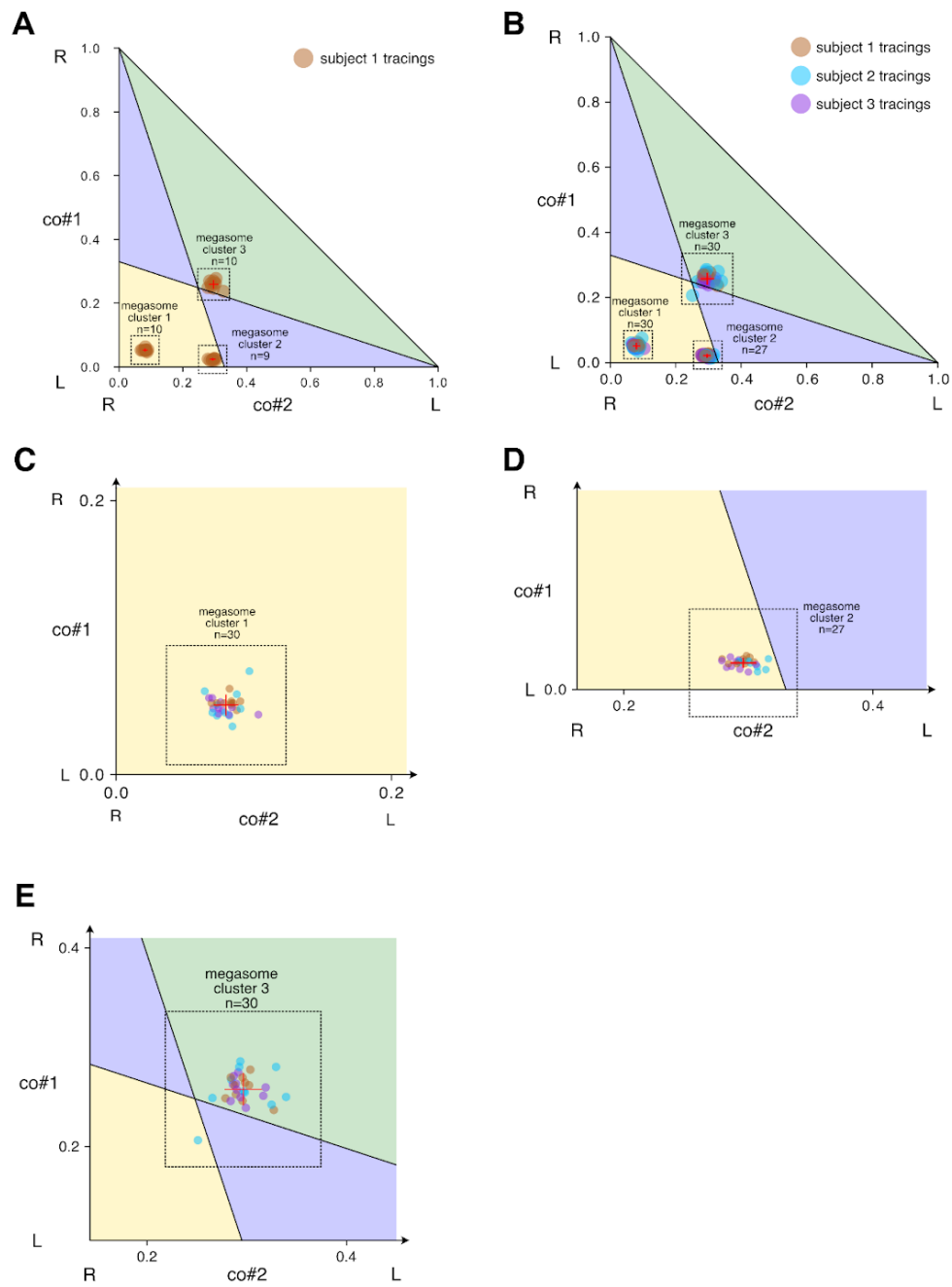


Figure 10. Reproducibility of megasome tracing, straightening and CO site position measurements.

A-B, Phase diagrams showing the results of repeated blind tracings, straightening and GFP::COSA-1 focus position measuring for three different meT7 chromosomes. All three were randomly presented 10 times each, in different rotations and/or reflections. Three clusters are plotted, each corresponding to a different chromosome. Red bars represent 1 standard deviation from the mean of the cluster.

A, Phase diagram for the tracings by subject 1 alone.

B, Phase diagram for the tracings by 3 different subjects, color-coded by subject.

C-E, Magnification of the phase diagram over the megasome clusters to show the spread of megasome tracings with more detail.

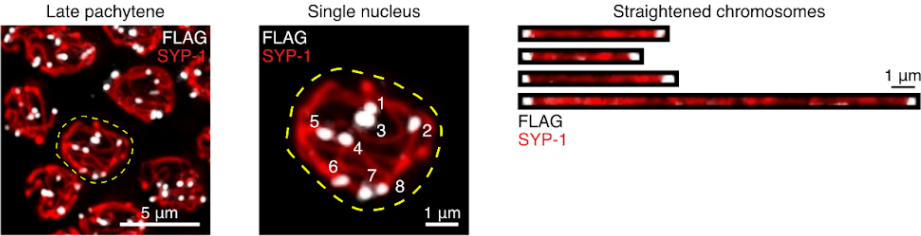
C, Magnification of the subset cluster 1 (n=30).

D, Magnification of the subset cluster 2 (n=27).

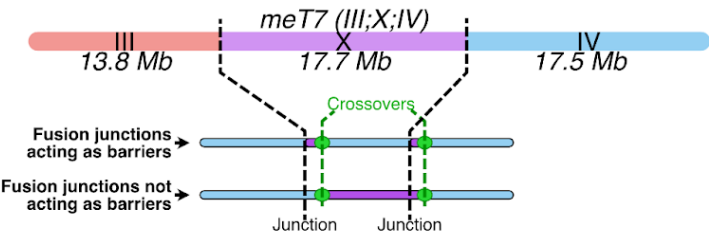
E, Magnification of the subset cluster 3 (n=30).

dtn-1::GFP::3XFLAG (II); meT7 (III;IX;IV)

A

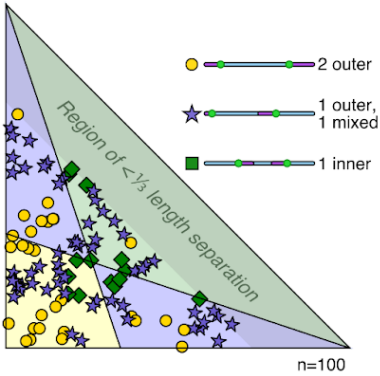


B



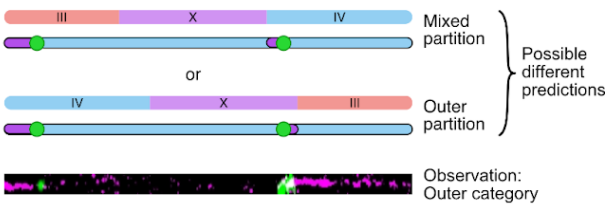
Predictions for *meT7* fusion junctions as barriers with 100 random pairs of crossover positions

C



Fusion junction barrier predictions vs observed data

D



Predictions for all 74 observed megasomes
Fusion junctions as barriers best case correct predictions: 66%
Fusion junctions as barriers worst case correct predictions: 22%
Expected fusion junctions as barriers correct predictions: 44%

Diffusion model correct predictions: 80%
(See Fig.4E)

E

Mixed segment category ↓	Prediction Type		
	Fusion junctions as barriers III to IV direction prediction	Fusion junctions as barriers IV to III direction prediction	Observations
Outer category mixed segments	17	20	5
Inner category mixed segments	8	6	1
Mixed category 2 mixed segments	7	8	0
Total	32	34	6

n=74

Figure 11. Inner junctions of *meT7* do not explain the observed phospho-SYP-1 patterning.

A, Immunofluorescence staining of the telomere binding protein DTN-1 (white, anti-FLAG) in nuclei of *dtn-1::GFP::3XFLAG (II); meT7 (III;X;IV)* animals. The SC protein SYP-1 (red, anti-SYP-1) was used as the chromosome axis marker. *Left.* Representative late pachytene region showing DTN-1 at the telomeres. *Center.* Magnification of the outlined nucleus showing 8 foci of DTN-1. Nearby nuclei in the region were cropped out of the image (replaced with black) for clarity. *Right.* Straightened chromosomes of the outlined nucleus showing two DTN-1 foci per chromosome.

B, Top. Diagram of the *meT7* chromosome with highlighted junctions (black dotted lines) between the fused chromosomes. The approximate fused chromosome sizes are denoted in megabases. *Bottom.* Two chromosome diagrams with the same length, crossover position and junctions showing different expected phospho-SYP-1 restriction patterns. The top chromosome results in 1 outer segment and 1 mixed segment if the junctions behave as barriers. The bottom chromosome shows a single, full inner segment restriction if the junctions do not behave as barriers, as predicted by the model (See **Fig. 8**).

C, Phase diagram containing a total of 100 simulated megasomes with two randomly placed crossovers with the minimum distance between them set to the approximate length of chromosome III. The phospho-SYP-1 restriction prediction was generated with the assumption that the junctions do function as barriers and plotted as yellow circles (2 outer restrictions), purple stars (mixed restriction) and green boxes (1 inner restriction). The regions in the phase space diagram are calculated as in **Fig. 9**, according to the prediction where the junctions do not function as barriers.

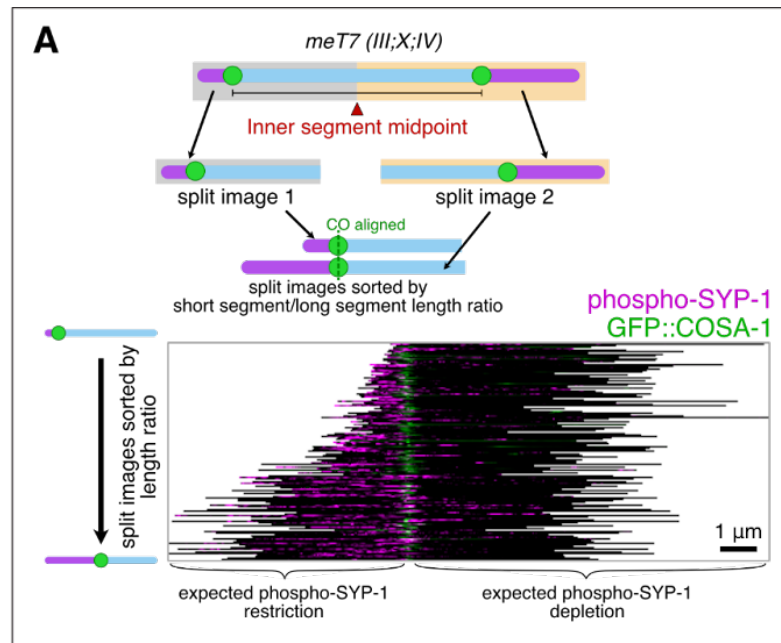
D, Diagrams showing two distinct predicted restrictions of phospho-SYP-1 (magenta) under the assumption that fusion junctions act as barriers. The image shows the observed CO sites (green) used for the predictions and the observed partition of the phospho-SYP-1 restriction (magenta). The direction of the *meT7* fusion chromosome is shown on top of the predictions. The

summary of the correct predictions using all 74 straightened megasomes is shown at the bottom (See **4.8 Model predictions** in the Methods chapter).

E, Summary of the predicted mixed segments by the “fusion junctions as barriers” model and the observations. The rows in the table represent the type of mixed segments not predicted by the diffusion model. The n represents the total number of straightened megasomes (n=74).

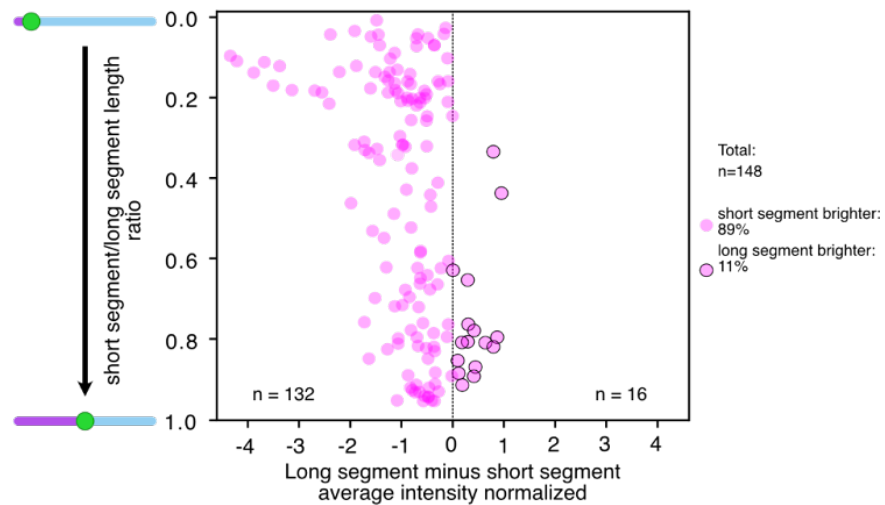
2.6 Local crossover quantitative analysis of phospho-SYP-1 enrichment agrees with diffusion model

Although the prediction of phospho-SYP-1 restriction in *meT7* mostly agrees with the observations, it does not predict all observed cases. Upon closer inspection of the position and observed category of the megasomes with incorrect prediction, it was evident that many were near the isoclines (**Fig. 9E**). This, combined with the fact that the presence of partial short/long arm restriction on central segments in the mixed restriction megasomes suggested that the decision to restrict or evict phospho-SYP-1 was made at a local CO level, raised the possibility that the error is generated in the CO site that corresponds to the nearest isocline, where the signal is predicted to be evenly distributed to both sides of the CO, similar to the errors that emerge in wild-type chromosomes with just one centrally located CO (**Fig. 3C**). To quantitatively test the enrichment of phospho-SYP-1 to either side of the individual CO sites, each *meT7* chromosome with two CO was divided into two single-CO cases. It was reasoned that if density of a factor in a segment is what determines arm identity on each side of a CO site, then bisecting the central segment (leaving density unaltered) should produce two single-CO predictions that each separately agree with the two-crossover prediction. Therefore, each *meT7* chromosome was divided at the midpoint of the segment bounded by two CO as shown in the diagram of **Figure 12A**. Each of the newly generated single-CO chromosomes is made up of a short and long segment that can be analyzed by their length just as wild-type chromosomes and should agree with the prediction, restricting phospho-SYP-1 to the short arm. The difference of phospho-SYP-1 intensity was quantified by subtracting the average intensity of the long segment minus the average intensity of the short segment (**Fig. 12B**). It was found that 89% of CO sites (132 out of 148) had a negative value, indicating enrichment of phospho-SYP-1 on the short arm and therefore agreeing with the model, which is statistically different from a randomized set of data with 50% (74/148) of agreement (**Fig. 12C**). Interestingly, many of the divided single-CO chromosomes with enrichment of phospho-SYP-1 on the long arm found in **Figure 12B** have a short-to-long arm ratio close to 1, just as it is the case in wild-type chromosomes. Taken together, it is concluded that the length sensing system is more likely to fail when long and short arms are very similar in length.



B

Enrichment of phospho-SYP-1 on split chromosomes



C

Split megasome randomized vs observed enrichment of phospho-SYP-1

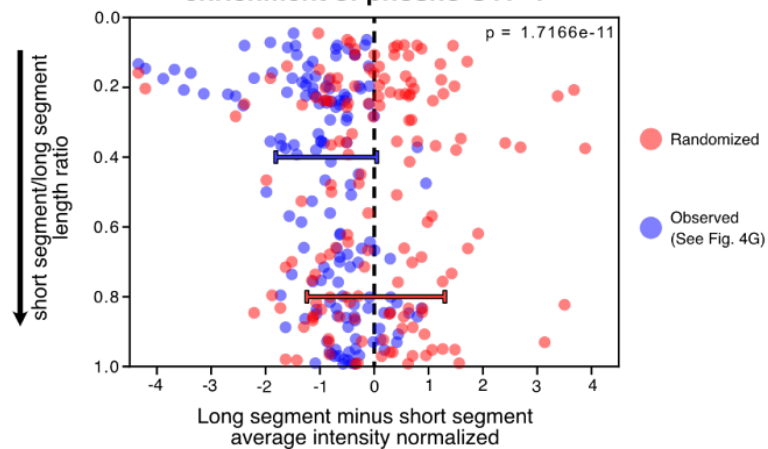


Figure 12. Local CO quantitative analysis of phospho-SYP-1 enrichment agrees with the diffusion and accumulation model.

A, Top. Diagram showing division at the midpoint of two COSA-1 foci to create two half-chromosomes for individual analysis. *Bottom.* Image showing all the 148 split images coming from the megasome traces sorted by the short segment/long segment length ratio ascending from top to bottom. The split images are aligned by the GFP::COSA-1 (anti-GFP) foci. The phospho-SYP-1 (anti-phospho-SYP-1) restriction is shown in magenta. The GFP::COSA-1 channel for each split image was normalized to the corresponding GFP::COSA-1 focus area. The phospho-SYP-1 channel for each split image was normalized to the corresponding phospho-SYP-1 restriction site area.

B, Plot showing the phospho-SYP-1 measurements of the split images from *meT7* chromosomes with 2 CO sites. The ratio of the short segment/long segment length is plotted on the Y axis from top to bottom. The subtraction of the long segment average intensity minus the short segment average intensity normalized to the split image average intensity is plotted on the X axis. The outlined magenta points are positive values where the long segment average intensity is higher than the short segment average intensity (16/148). The magenta points with no outline are negative values where the short segment average intensity is higher than the long segment average intensity (132/148).

C, Plot showing the observed phospho-SYP-1 measurements of the split images from megasomes with 2 CO sites vs a randomized short or long arm restriction of the same values. The ratio of the short segment/long segment length is plotted on the Y axis from top to bottom. The observed enrichment of phospho-SYP-1 of the split megasomes (blue points, see **Fig. 12B**) and the shuffled and sign randomized values (red points) are plotted on the X axis. The error bars represent 1 standard deviation from the mean. The p-value was obtained by comparing the means using Student's t-Test.

2.7 Synaptonemal complex diffusion is impeded by CO sites

The diffusion and accumulation model depends on a critical assumption that the CO sites function as barriers that prevent or slow down the diffusion of molecules within the SC. This is required to create a signal density differential in neighboring length-asymmetric segments. The hypothesis of the CO functioning as barriers is supported by the fact that COSA-1 foci mark the boundaries between short and long arms. To test the assumption, live imaging was performed to track the diffusion of a photoconvertible protein mMaple3 (Rog et al., 2017) fused to SYP-3, a central region element of the SC, during mid pachytene, when the SC is in a dynamic state prior to short arm designation (von Diezmann et al., 2024). The CO sites were marked with GFP::COSA-1 whose emission spectrum overlaps with that of mMaple3 but was still recognizable as large spots in the chromosome axes (**Fig. 13A**). A small spot of 405 nm light was focused on a stretch of the non-photoconverted mMaple3::SYP-3 to achieve a localized photoconversion to the red-emitting form of the protein to one side of the COSA-1 focus (**Fig. 13B**) (In the figures, the red-emitting form on mMaple3 is shown as green on magenta to facilitate visibility). The photoconverted segment was termed the “proximal” side and the opposing segment to the other side of the CO the “distal” side. A three-dimensional multiwavelength time-lapse was acquired to monitor the diffusion of the signal along the chromosome axis. To enhance the quantification and visualization of the signal diffusion, the chromosome axes were isolated, and the signal was corrected by subtracting the background intensity for both the photoconverted and the non-photoconverted channels (**Fig. 13C**). The chromosomes were intensity-normalized and straightened before the signal diffusion was measured. A representative time-lapse of a photoconverted chromosome is shown in **Figure 13D**. A red underline below the straightened chromosome shows the dark area between the photoconversion region and the CO site as measured moments after photoconversion. By time point 9, the photoconverted signal has invaded the red underline and the profile of the signal shows a higher average intensity in the proximal side (where photoconversion occurred) compared to the distal side, indicating that the photoconverted signal diffused on the proximal side but was unable to spread past the CO site (**Fig. 13D**).

To rule out the possibility that 9 minutes is insufficient to detect proper diffusion of the SC from the proximal region to the distal region, the diffusion of mMaple3 in 26 chromosomes with CO sites was compared to the diffusion in *msh-5* mutants that lack CO maturation (K. O. Kelly et al., 2000) (**Fig. 14A-C**). In the *msh-5* mutant chromosomes, the photoconverted signal diffuses throughout the entire chromosome segment by the last time point (**Fig. 14A**). For each chromosome with CO, a span of 1 μ m surrounding the photoconverted center on the proximal side was calculated, alongside a 1 μ m span to the right of the CO site in the distal segment. The average intensity for each of the regions was calculated and the signal intensity difference was obtained by subtracting the distal average intensity from the proximal average intensity. The distance between the two regions was used to calculate analogous regions in multiple chromosomes lacking CO sites; the average intensity difference was obtained for all chromosomes (**Fig. 14B**). The results show a statistically significant difference with smaller differences in intensity, implying unrestricted diffusion, in the *msh-5* chromosomes lacking CO sites (**Fig. 14C**).

To validate the results showing a relative high difference in intensity between the 1 μ m regions in the proximal and distal segments, the time-lapses of all 26 photoconverted chromosomes with CO sites were combined into weighted average intensity images and signal profiles representing the entire set of chromosomes in a single entry for all time-points. The signal diffusion can be observed to its last phase on the **Averaged images** along with the appreciable average intensity difference of the proximal and distal segments in the **Averaged profiles** of **Figure 15A**.

The initial photoconversion region of mMaple3 in the proximal segment is located at varying distances to the CO sites in all 26 chromosomes carrying the allele *GFP::cosa-1*. The observed diffusion of the signal along the proximal site without spreading past the CO implies that an inflection point of signal intensity should be generated at the later time-points. To test whether the COSA-1 focus represents a specific intensity inflection point, a sliding window analysis was performed on the averaged profiles at each time-point. This analysis transforms steep intensity gradients in the designated windows into peaks that are much easier to visualize

(**Fig. 15B**). The results show that in the moment after photoconversion there is an intensity inflection peak on the proximal segment at the edge of the initial photoconversion for the averaged megasomes; at later time points, the peak indicative of intensity inflection shifts to the CO site (**Fig. 15C**). The theoretical examples of what the sliding window analysis would show if the distribution of mMaple3::SYP-3 were completely uniform within the proximal and distal compartment if the CO site was completely permeable (top) and completely impermeable (bottom) are also shown (**Fig. 15C**). These results suggest that diffusion of mMaple3:SYP-3 protein is impeded across the CO site, supporting the idea that COSA-1 foci could divide the SC central region into isolated partitions.

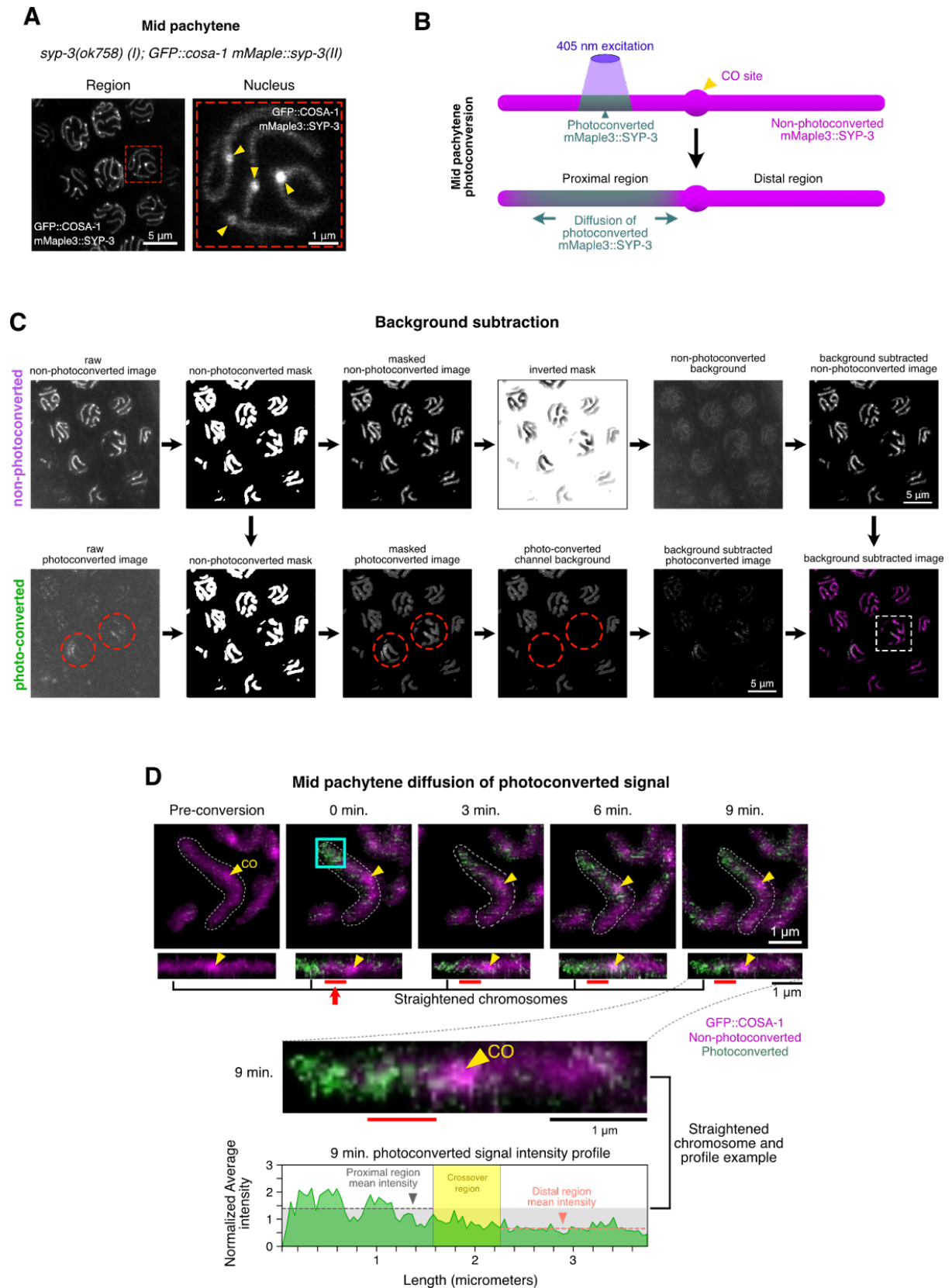


Figure 13. Photoconversion imaging shows that diffusion of SYP-3 proteins is impeded by CO sites.

A, Mid pachytene region and single nucleus of *syp-3(ok758)* (I); *GFP::cosa-1 mMaple3::SYP-3* (II) showing the overlapping fluorescent emission of GFP::COSA-1 and mMaple3::SYP-3. *Left.* Max intensity gonad region projection. *Right.* Close up of a nucleus from the gonad region on the left. Yellow arrows indicate the CO sites marked by GFP::COSA-1 clearly distinguishable from the mMaple3::SYP-3 marked chromosome axis.

B, Top. Diagram showing the photoconversion of mMaple3::SYP-3 (shown in magenta) by 405 nm light excitation (shown in purple) to its photo-converted state (shown in green) in a limited region of a chromosome (colors intentionally chosen for visibility). The CO site is indicated with the yellow arrowhead. The COSA-1-demarcated region containing the photoconversion event is termed the proximal region, and the region past the COSA-1 focus as the distal region. *Bottom.* Diagram showing the predicted diffusion of photo-converted mMaple3::SYP-3 along the chromosome.

C, Background subtraction steps for the photoconverted and non-photoconverted mMaple3::SYP-3 channels in the *syp-3(ok758)* (I); *mMaple3::syp-3* (II) strain. The images shown are max intensity projections. *Top.* The raw non-photoconverted image is used to obtain a non-photoconverted mask from the chromosome axes. The mask is employed to create the masked non-photoconverted image that lacks numerical values (pixels set to “NA”) outside the chromosome axes region. An inverted version of the mask (inverted mask) is used to produce the non-photoconverted background image that encompasses all the pixels in the image that are not segmented as part of the chromosome axes. The average intensity of the non-photoconverted background is then subtracted from the masked non-photoconverted image to generate the final background subtracted non-photoconverted image. *Bottom.* The raw photoconverted image signal outside the chromosome axes is eliminated using the non-photoconverted mask

previously obtained. The photoconverted nuclei (outlined in red) are cropped out of the resulting image (*masked photoconverted image*) to get the *photoconverted channel background*. The average background intensity of the photoconverted channel was subtracted from the *masked photoconverted image* to obtain the final *background subtracted photoconverted image*. The last panel shows the merged photoconverted (green) and non-photoconverted (magenta) background subtracted merged images. The outlined nucleus corresponds to the example shown in **Figure 13D**.

D, Top. Merged average intensity projected images of a time course of photoconverted mMaple3::SYP-3 (green), non-photoconverted mMaple3::SYP-3 (magenta), and a GFP::COSA-1 CO site (also magenta) in one mid-pachytene chromosome of *syp-3(ok758) (I)*; *GFP::cosa-1 mMaple3::syp-3 (II)*. The pre-conversion state is followed by 9 minutes of the time course at 3-minute intervals. The straightened and average intensity projected chromosome for each time point is shown below. Yellow arrowheads indicate the crossover position marked by GFP::COSA-1. The targeted chromosome is outlined by a white dashed line. The cyan box at time point 0 min. shows the 405 nm excitation region. The red underline at each straightened chromosome after the *pre-conversion* time point indicates a 0.73 μm span measured at 0 min. indicating the region between the photoconversion and the crossover. Scale bar: 1 μm . **Middle.** Zoom-in of the straightened photo-converted chromosome 9 minutes after photo-conversion. Scale bar: 1 μm . **Bottom.** Intensity profile of photoconverted mMaple3::SYP-3 from the traced chromosome 9 minutes after photo-conversion normalized to the average intensity of the entire segment. The normalized average intensity is plotted on the Y axis. The X axis represents the length of the segment in microns. The yellow rectangle is the measured extent of the GFP::COSA-1 focus region. Dotted gray and orange lines represent the mean intensity value over the lengths of the photoconversion-proximal and photoconversion-distal regions, respectively. The gray shaded region contains all values below the photoconversion-proximal region's mean intensity.

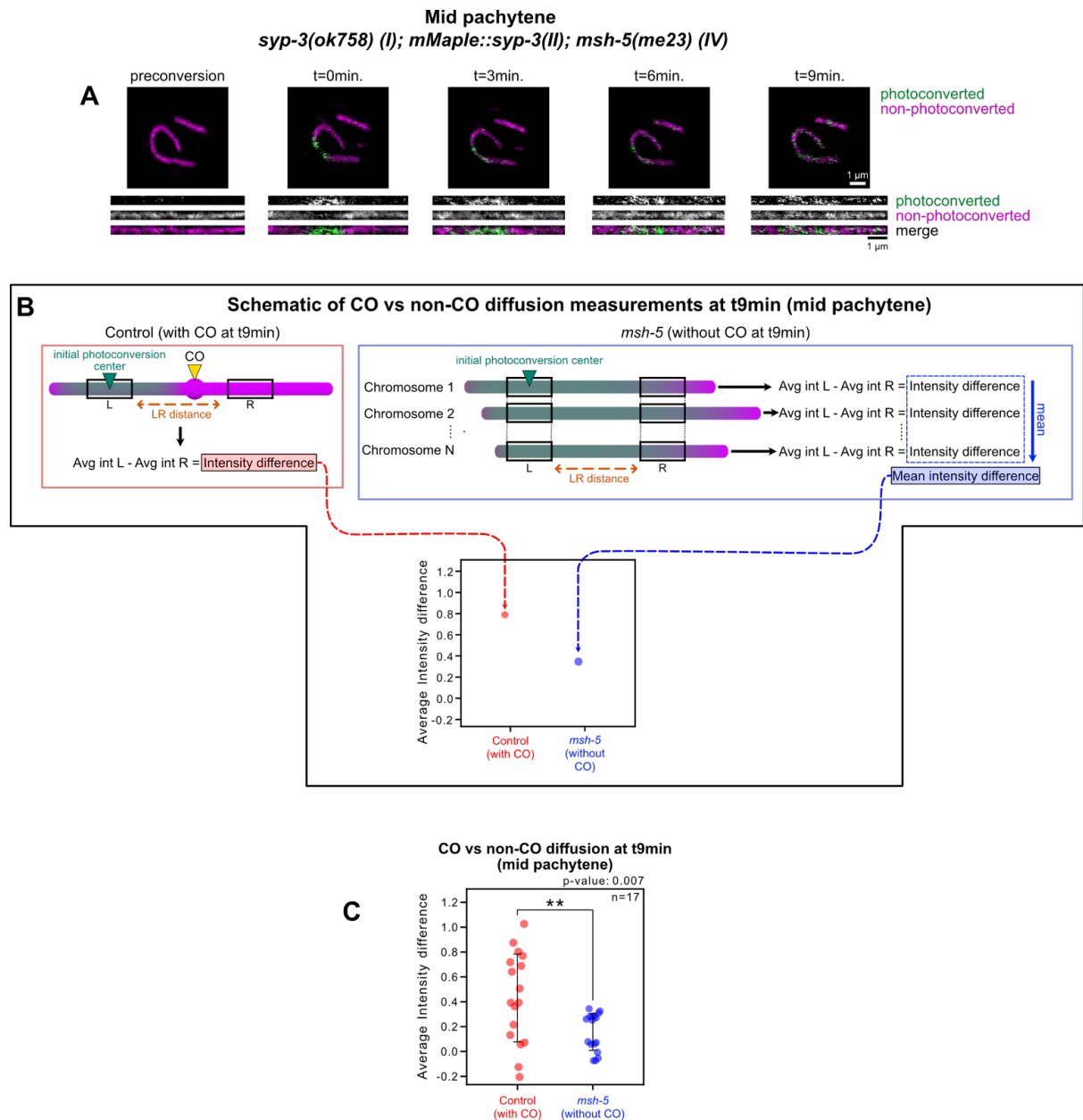


Figure 14. Unrestricted diffusion of SYP-3 in chromosomes lacking CO sites.

A, Mid pachytene time course of a photoconverted nuclear region of the strain *syp-3(ok758) (I); mMaple3::syp-3 (II); msh-5(me23) (IV)* showing the non-photoconverted signal (magenta) and the photoconverted signal (green). The preconversion state is shown followed by 9 minutes of the time course in 3-minute intervals. The straightened photoconverted chromosomes are shown under the corresponding nuclear region. All images shown are average intensity projections.

B, Schematic showing the method for comparing the diffusion of the photoconverted mMaple3::SYP-3 signal 9 minutes after the initial photoconversion in mid-pachytene chromosomes with CO sites and mid-pachytene chromosomes without CO sites. The magenta color represents the non-photoconverted signal and CO sites. The green color represents the photoconverted signal. *Top Left*. Diagram of control chromosomes with the genotype *syp-3(ok758)* (I); *mMaple3::syp-3 GFP::cosa-1* (II). The crossover site is indicated by the yellow arrow. The box labeled *L* indicates a 1 μ m region centered on the initial photoconversion left to the CO site. The box labeled *R* indicates a 1 μ m region starting 0.25 μ m to the right of the CO site. The distance between the *L* and *R* regions denoted as *LR distance* is indicated in orange and was used to calculate the *R* box positions in the chromosomes without CO sites. *Top Right*. Diagram of the multiple chromosomes with the genotype *syp-3(ok758)* (I); *mMaple3::syp-3* (II); *msh-5(me23)* (IV) lacking CO sites compared to the control chromosome. All chromosomes without CO sites whose trace length was sufficient to fit the *LR distance* obtained from the control chromosome were used for the comparison (N up to 27). The *L* labeled box indicates a 1 μ m region centered on the initial photoconversion. The *R* labeled box indicates a region with the same *LR distance* obtained from the control chromosome. *Bottom*. Plot showing two example points resulting from the comparison of the diffusion of mMaple3::SYP-3 photoconverted signal. The difference of the average intensities of the *L* and *R* region of the control chromosome with CO site is plotted on the Y axis as the red point and the mean of the average intensity differences of the *L* minus *R* regions from chromosomes without CO sites is plotted as the blue point.

C, Plot showing the diffusion of photoconverted mMaple3::SYP-3 signal in mid pachytene chromosomes with CO sites and mid pachytene chromosomes without CO sites. The average intensity difference of the measured regions according to **Fig. 14B** is plotted on the Y axis. Chromosomes with CO sites are plotted as red points and chromosomes without CO sites are plotted as blue points. The error bars indicate 1 standard deviation from the mean of the samples. The p-value was obtained by comparing the means using Student's t-Test.

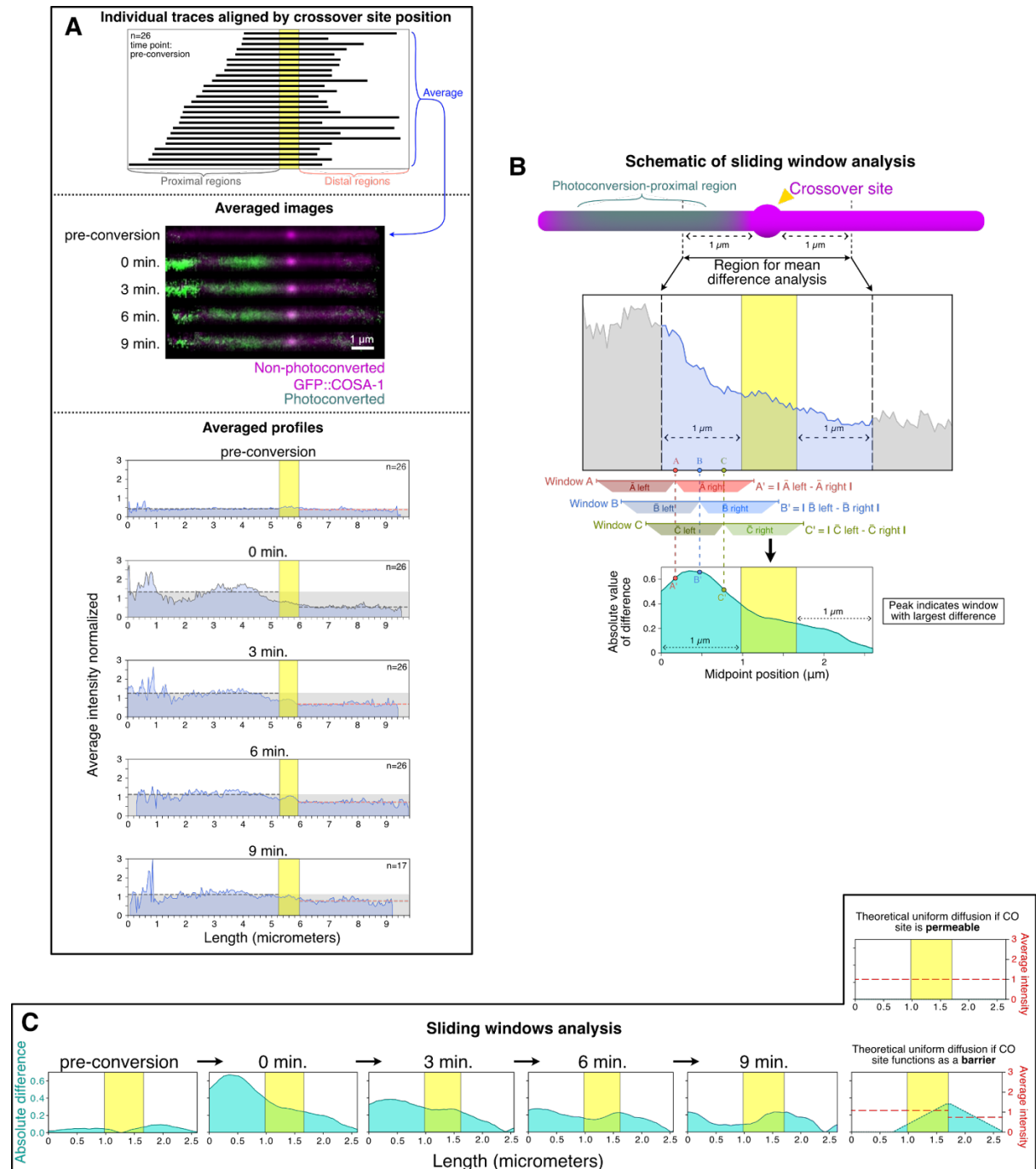


Figure 15. CO site becomes an intensity inflection point for photoconverted signal.

A, Top. Lineup of all 26 photoconverted straightened chromosome segments at the pre-conversion time point, shown in schematic. Segments are aligned by their GFP::COSA-1 focus midpoint. The yellow rectangle is the average crossover region of all aligned segments. The chromosome segments are oriented so that the photoconversion-proximal regions are to the left of the crossover region and sorted by the length of the region from top to bottom.

Middle. Averaged images of all aligned chromosomes at their respective time points. The photoconverted mMaple3::SYP-3 is shown in green and the GFP::COSA-1 and non-photoconverted mMaple3::SYP-3 are shown in magenta. *Bottom.* Averaged photoconverted mMaple3::SYP-3 intensity profiles for all chromosomes at each time point. The averaged intensity values are plotted on the Y axis. The mean value over the whole length for proximal and distal regions are shown as gray and orange dotted lines, respectively. The X axis represents length in microns.

B, Top. Diagram of a chromosome with the photoconverted segment (green) to the left of the CO site (yellow arrowhead) with arrows around the crossover region indicating the range used for the mean difference analysis. *Middle.* The region of the averaged photoconverted intensity profile at time point 0 used in the mean difference analysis is expanded. The blue area represents the range of the moving midpoint of the sliding window as indicated by the dashed line arrows. Three example points *A*, *B* and *C* with their respective ranges to the left and right are shown below the plot. The yellow rectangle indicates the average crossover regions of the chromosomes at 0 min. *Bottom.* Plotted values of the sliding window analysis. The absolute intensity difference of the left region from the right region surrounding each point in the range is plotted on the Y axis; the midpoint position is on the X axis. The example points *A*, *B*, and *C* are connected by dotted lines reaching down to their corresponding plot values, labeled *A'*, *B'* and *C'*.

C, Left. Sliding window analysis performed on averaged chromosome traces at each time point. The absolute difference as in **B** is plotted on the Y axis. Peaks in the plot indicate points where the difference is greatest between the average intensity to the left and the average intensity to the right. *Right.* Sliding window plots of two theoretical uniform distributions of the photoconverted signal: if the crossover site was completely permeable (top) or if the right end of the crossover site functioned as a complete barrier (bottom). The values used for the sliding window analysis are plotted as red dotted lines on the Y axis. The values for the case where the crossover site functions as a complete barrier were obtained from the average intensities of the photoconverted and non-photoconverted regions at 9 min.

2.8 Simulating phospho-SYP-1 partitioning

The diffusion and accumulation model predicts signal intensity differentials between neighboring segments that are measured locally at the CO sites. The wild-type chromosomes always select a single segment to restrict phospho-SYP-1 even when the short-to-long arm ratio is similar to 1. This suggests that in the short arm designation process, the decision to restrict or evict phospho-SYP-1 from a given segment results in an all-or-nothing resolution. A discrete simulation was implemented to examine if a small number of assumptions from the collected evidence is sufficient to explain the observed patterns for short/long arm partitioning. The first assumption during the simulation development was that the signaling factor can linearly diffuse within the SC, as well as jump from a chromosome to an unbound pool and back to a chromosome, while the CO sites act as barriers for diffusion. Second, the signal is generated or recruited at the CO sites as suggested by the diffusion model and the *meT7* results. The model is agnostic to the identity of the molecules; multiple enzymes interacting at the CO sites have been identified that could provide post-translational modifications on a substrate at the SC that serves as the signaling factor. An example of an obvious candidate is the cyclin-like protein (CNTD1/COSA-1) and CDK-2 (cyclin dependent kinase) that localize to CO sites and regulate the maturation of nascent COs, presumably by phosphorylating substrate proteins at CO sites (Bondarieva et al., 2020; Brandt et al., 2020; Gray et al., 2020; Haversat et al., 2022; Yokoo et al., 2012; L. Zhang et al., 2021). Other proteins such as the ZHP E3 ligases also coalesce at the CO sites or the short arms of the chromosomes and could be related to the signal generation (Zhang et al. 2018; von Diezmann and Rog 2021; von Diezmann et al. 2024). Third, a positive feedback loop is integrated to cement the all-or-nothing resolution of short/long arm determination. It was assumed that the signal could provide its own stabilization, reducing the off rate of the molecules and thereby providing the feedback necessary to convert the initial asymmetry state into a binary decision. After the CO are formed and the short arm is established, the SC dynamics are reduced by stabilization of its elements, mainly by decreasing the exchange of SC central region proteins between different chromosomes (Machovina et al., 2016; L. Zhang et al., 2021). Phosphorylation of the SC central region protein SYP-4 at S269 by PLK-1/2 is necessary for the stabilization of the SC (Pattabiraman et al., 2017). Live imaging analysis was performed to

validate the reduction of SC dynamics in late pachytene after the CO sites are designated and chromosome partitioning is complete by measuring the diffusion of photoconverted mMaple3::SYP-3 in control nuclei and *syp-1(T452A)* mutants that are unable to recruit PLK-2 to the SC (Sato-Carlton et al. 2018; Brandt et al. 2020). The data showed that the mMaple3::SYP-3 photoconverted signal spread faster and to a greater extent in the *syp-1(T452A)* mutants compared to the control, providing further evidence that recruitment of PLK-2 to the SC is required for slowing down the SC dynamics after CO are formed (**Fig. 16A,B**). Fourth, it was assumed that there is a limited pool of SC stabilizing factor based on the observations that nuclei with less than 4 CO sites, such as in the *dsb-2* mutants with a reduced number of DSBs and lack of CO assurance (Rosu et al., 2013), both short and long arms will retain phospho-SYP-1 while nuclei with 4 CO or more will often partition phospho-SYP-1 correctly to the short arm (Brandt et al., 2020; Sato-Carlton et al., 2020). It has been proposed that the SC stabilization activity by PLK-2 is normally distributed across six CO sites in a wild type nucleus, but in a nucleus with fewer COs this activity will be in excess, and may spill over to the entire chromosome (Brandt et al., 2020).

The four previously mentioned assumptions were consolidated into the simulation which consists of discrete particles (signal molecules) on sets of arrays (chromosomes). The number and length of arrays can be modified in the simulation to reflect different karyotypes such as wild type chromosomes (6 normal-length chromosomes) or *meT7* (3 normal-length chromosomes and 1 long chromosome). The discrete particles can dissociate from an array, entering the pool of unbound molecules, and associate from the unbound molecule pool to a random array.

The simulation consists of a given number of sequential iterations that gradually shape the outcome based on a tuned set of parameters. The key actions that can ensue at each iteration are displayed on **Figure 17A**: (1) signal molecules either associate (if currently unbound), dissociate (depending on a locally-determined off-rate), or randomly step left or right into an adjacent bin of the array, while not crossing specified CO positions; (2) when adjacent to a CO position, signal molecules change to an “available” state that can bind a cofactor; (3) limiting amounts of co-factor can

associate randomly with available signal molecules; (4) signal molecules (bound to co-factor) recruit a stationary stabilizing factor, which decreases the local signal molecule off-rate; (5) global increase (back toward the initial, higher value) of local off-rate occurs with some probability.

Thus, the landscape of each individual array is formed with predictable outcomes. A simplified sample diagram for the events in a simulation run that lead to the short arm restriction of the signal through the accumulation of the stabilizing factor is shown in **Figure 17B**: (1) The unavailable signal molecules diffuse laterally throughout the chromosome with the same dynamics to either side of the CO site; (2) The signal molecules that come into proximity to the CO site are modified to their available state, rapidly binding available co-factor molecules that begin the incorporation of the stabilizing factor starting at the CO site; (3) The co-factor bound signal molecules diffuse through the chromosome gradually incorporating the SC stabilizing factor evenly on the entire chromosome stretch before reaching the end of the segment or the wave diffusing signaling molecules that originate in another CO site; (4) The incorporation of the stabilizing factor opposes its own global dissociation rate. As early as the first signal molecules reach the end of the segment or the incoming wave of signal molecules coming from another CO, a small density differential of accumulating factor is generated leading to an unequal dissociation of co-factor bound signal molecules from the higher density segment (presumably the short arm in wild-type-like chromosomes) and the lower density segment; (5) The small difference in SC stabilizing factor density is exacerbated until most co-factor bound signal molecules are entrapped in the high density segment while the low density segment is gradually emptied of signal and SC stabilizing molecules, designating the “short arm” and “long arm” domains of the chromosomes.

The free parameters of the model include the number of signal and cofactor molecules, the initial off-rate along the chromosome, and how the off-rate increases or decreases. The chromosome lengths, crossover number, and crossover positions are also specified, allowing the simulation to be run for diverse wild-type configurations as well as altered conditions known to change the partitioning process.

When six chromosomes with off-center CO positions are simulated it leads to the accumulation of all signal molecules at the shorter segments of the chromosomes (**Fig. 17C**) in agreement with the observations on wild-type chromosomes (**Fig. 3C**). A key aspect for the simulation is the static nature of the accumulating factor. The signal molecules in the kymograph of **Figure 17C** represented as white dots start symmetrical diffusion from the CO positions (Indicated by the yellow arrows) to both directions but are eventually depleted from the long arm after the diffusing signal molecules on the short arm reach the chromosome end. This outcome is robustly achieved every time the simulation is run with wild type-like parameters.

The simulation can recapitulate the outcomes of nuclei with a reduced number of CO sites, such as *dsb-2* mutants where the short arm designation is aberrant, and phospho-SYP-1 is retained along the entire chromosome. When the simulation is run with six chromosomes but only 2 CO sites, the kymograph shows the signal molecule stabilized on both sides of the CO sites due to an excess of co-factor molecules (**Fig. 17D**). The simulations of *meT7* chromosomes with double CO sites largely agreed with the observations, for example, the simulated signal is restricted to the central segment of the megasome in *meT7* simulations with 2 CO near the center of the chromosome (**Fig. 17E**).

The simulation, in contrast to the model prediction, is not deterministic and will often result in different outcomes even when the same set of parameters are used. Thus, individual simulation run analysis was determined to be insufficient to understand all the possible outcomes of a determined chromosome and CO configuration. An example of this occurred when 6 chromosomes with 5 off-center CO and 1 centrally located CO were simulated. The result in **Figure 18A** showed the correct partitioning of the chromosome, with the signal accumulating at the shorter segment. Repeated runs of the simulation with the same set of parameters showed different results. To compare and quantitatively analyze the outcomes of multiple simulations, a summary image was created by taking the last stages of 100 simulation runs and stacking them in the vertical axis (**Fig. 18A,B**). The image summary of 100 simulation runs for

the chromosome with a central CO designation site showed that the correct restriction of the signal occurred in just 47% of the time. The remaining outcomes are divided into three different cases: signal enrichment at the long arm (28% of the time), depletion from both arms with concomitant partial partitioning on one of the 5 wild-type chromosomes (18% of the time), and enrichment at both arms (7% of the time) (**Fig. 18B**). These results are comparable to the observations in wild-type chromosomes where centrally located CO often resulted in restriction of phospho-SYP-1 in the longer segment of the chromosome. The 100-run summary of 6 chromosomes with just 2 off-centered CO sites confirmed the results that agree with *dsb-2* mutants by showing that 88% of the time the chromosomes with CO sites accumulated the signal on their entire length, and that 12% of the time, one of the two chromosomes had partial restriction of the signal while the other accumulated the signal on its entire length (**Fig. 18C**). Occasionally, the *dsb-2* mutants will get 5 CO sites in a single nucleus, resulting in the correct partition of phospho-SYP-1 to the short arm of the chromosomes with CO sites. The multiple-simulation summary of 5 chromosomes with off-centered CO sites resulted in the signal accumulating in the shorter segment, although one chromosome always accumulated excess signal delaying its partition (**Fig. 18D**). The *meT7* simulations for both, the expected inner restriction and outer restriction, had a 100% restriction of the signal in its expected segment (**Fig. 18E-F**). However, the simulation is unable to consistently reproduce the “mixed” partitioning seen in *meT7* with the same parameters; such intermediate partitions are unstable and eventually become either full or empty of the signal molecule. This outcome could indicate that one or more elements of the partitioning mechanism are not captured by the simulation, or that more fine-tuning of parameters is required. However, the basic operation of the simulation indicates that length-sensitive partitioning following the elucidated rules could in principle arise from molecules additively accumulating within CO-bounded compartments and changing their own dynamics in a concentration-dependent manner.

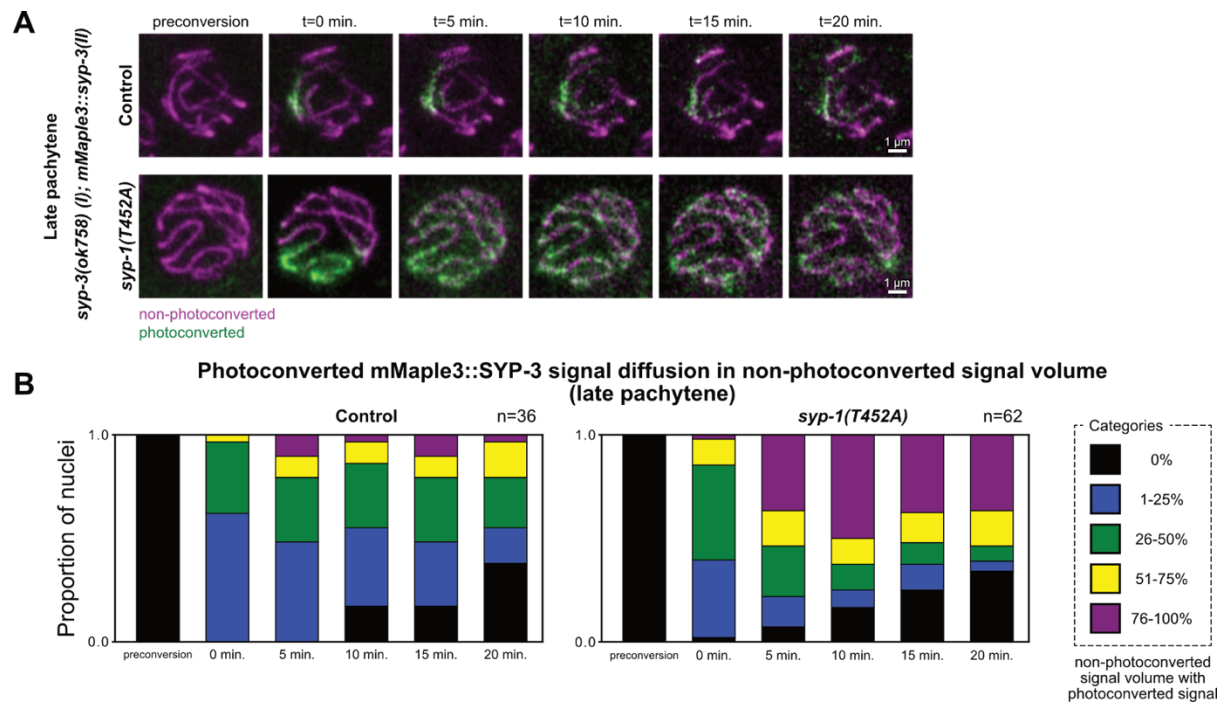


Figure 16. Late pachytene dynamics of the SC remain high in *syp-1(T452A)* mutants.

A, Late pachytene time course of *syp-3(ok758) (I); mMaple3::syp-3 (II)* (Control) and *syp-3(ok758) (I); mMaple3::syp-3 (II); syp-1(T452A) (V)* (*syp-1(T452A)*) showing the diffusion of photoconverted mMaple3::SYP-3 (green) throughout the non-photoconverted mMaple3::SYP-3 (magenta). The pre-conversion image is shown followed by 5 time points in 5-minute intervals from *t=0 min.* to *t=20 min.*

B, Stacked bar plots showing the ratio of late pachytene nuclei classified within 5 categories (zero signal and four 25% quartiles) for the non-photoconverted signal volume where the photoconverted signal had diffused at every time point. *Left.* Plot for the control nuclei (n=36). *Right.* Plot for the *syp-1(T452A)* nuclei (n=62). The categories for the volume occupied by the photoconverted signal are: 0% (black bars), 1-25% (blue bars), 26-50% (green bars), 51-75% (yellow bars) and 76-100% (purple bars).

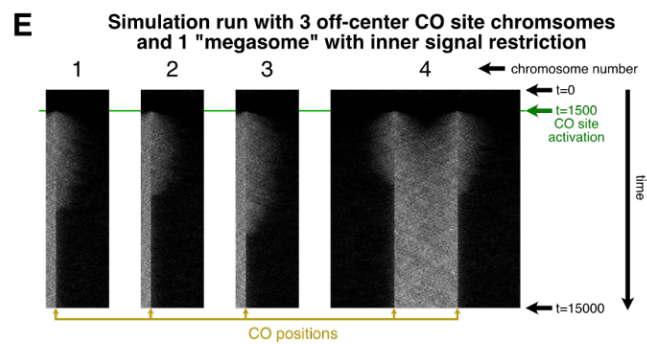
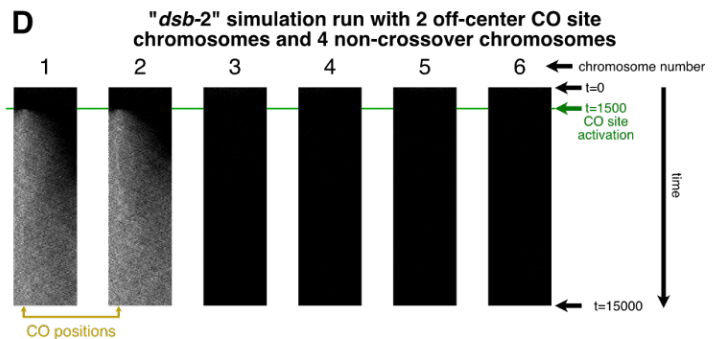
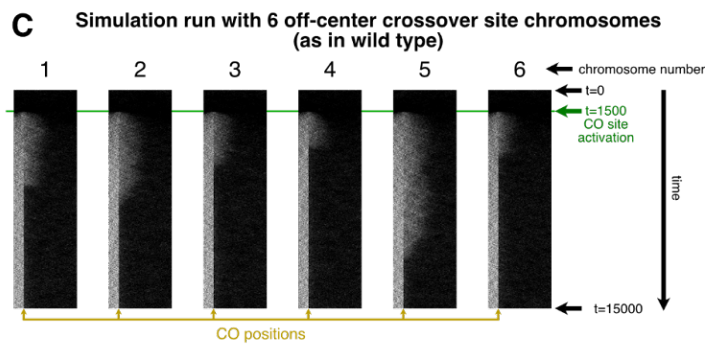
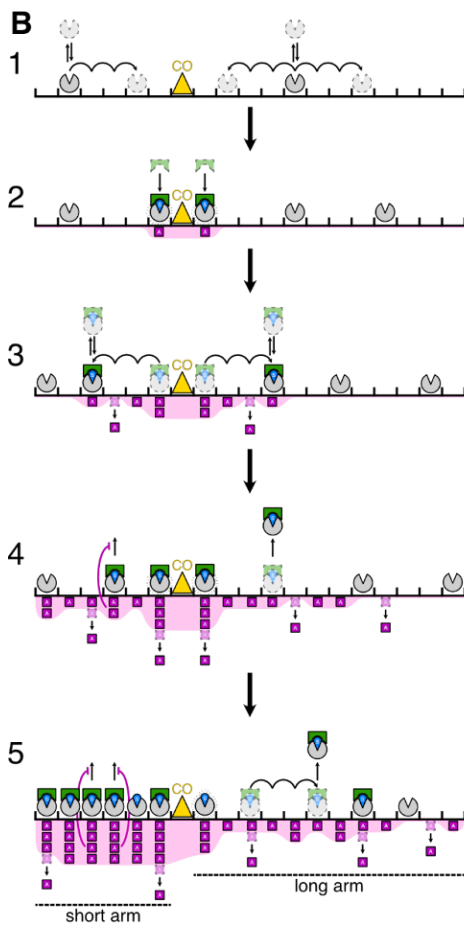
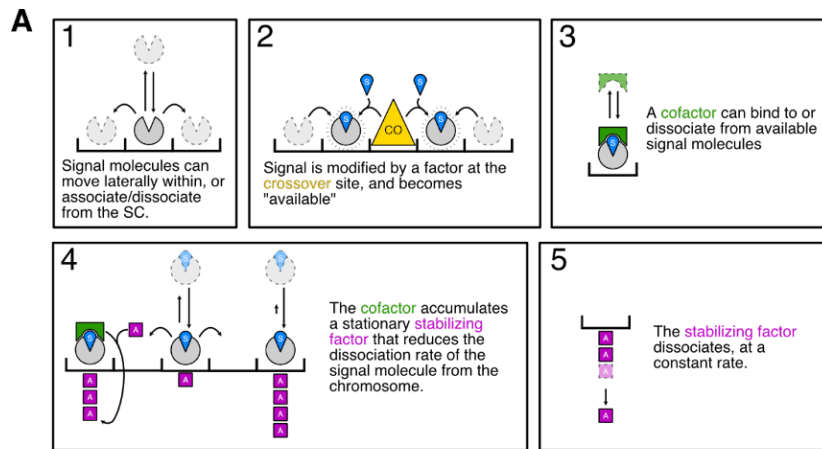


Figure 17. A simulation that recapitulates partitioning of phospho-SYP-1 in wild-type and altered conditions

A, Diagram of the 5 model steps. The simulated synaptonemal complex axis is shown as a black horizontal line with divisions to represent discrete positions along its length.

1. Signal molecules (Grey notched circles) can change their position in the chromosome during the simulation. The simulated molecule can move laterally within or dissociate from a simulated chromosome. Dissociated signal molecules (in a common unbound pool) can later associate with any chromosome from the entire group of simulated chromosomes at any given synaptonemal complex position.

2. Signals that move adjacent to designated crossover positions become modified and are now “available”(S, blue).

3. While available signal molecules are associated with the chromosome axis, they can bind molecules from a limited pool of cofactor (green). The binding is reversible. The cofactor moves with its bound signal.

4. Cofactor-bound signal molecules will deposit a stabilizing factor (“A”, magenta) at their given position. Each additional increment of stabilizing factor decreases the local probability of signal molecules dissociating from the axis at that position. The stabilizing factor does not move laterally. If signal molecules dissociate, the cofactor is released, and again becomes available to bind another chromosome-associated signal molecule.

5. The stabilizing factor is removed from the chromosome axis at a constant rate at each position.

B, More detailed look at events that take place as the simulation proceeds.

1. A set number of empty signal molecules at the start of the simulation will diffuse along the chromosome axis and between chromosomes.

2. Signal molecules that bump into the crossover will be modified and become available. If there are freely available cofactors, they will bind the signal molecule and commence the addition of accumulating factors at their position.

3. The cofactor bound signal will diffuse along the axis, depositing the stabilizing factor, which is also removed from the axis positions at a constant rate.
4. The stabilizing factor density will increase at a faster pace on shorter segments compared to longer segments assuming each segment has similar amounts of cofactor bound signal molecules. These shorter segments will probabilistically retain the available signal molecules longer, while the longer segments will lose them at a higher rate.
5. The outcome of this process is short segment-specific high levels of the stabilizing factor that will retain the majority of signal and cofactor molecules.

C-E, Output examples of the simulation with distinct setup parameters. Each run consists of 15000 iterations, represented by pixel rows starting at $t=0$ on the top of the images and ending at $t=15000$ at the bottom. The X axis pixels of each chromosome represent discrete positions, with higher concentrations of the signal shown brighter. The addition of the signal starts at $t=1500$ when the crossover is activated (green line and label).

C, Output of 1 run of the simulation showing the signal spreading and becoming restricted at short segments. 6 chromosomes, each divided into 100 discrete positions, were simulated. The crossover positions were set to 15 (out of 100) in all chromosomes, indicated by the yellow arrows.

D, Output of 1 run of the simulation with 2 chromosomes with off-center crossover sites, and 4 non-crossover chromosomes. The two crossover chromosomes fail to restrict the signal to the short segments. The crossover positions were set to 15 in the two CO-containing chromosomes, indicated by the yellow arrows.

E, Output of 1 run of the simulation with 3 chromosomes with off-center crossover sites (100 positions each) and 1 megasome (300 positions) with 2 crossover sites at positions predicted to result in inner signal restriction. The crossover positions on the normal length chromosomes were set to 15, and the crossover positions on the megasome were set to 100 and 200, indicated by yellow arrows.

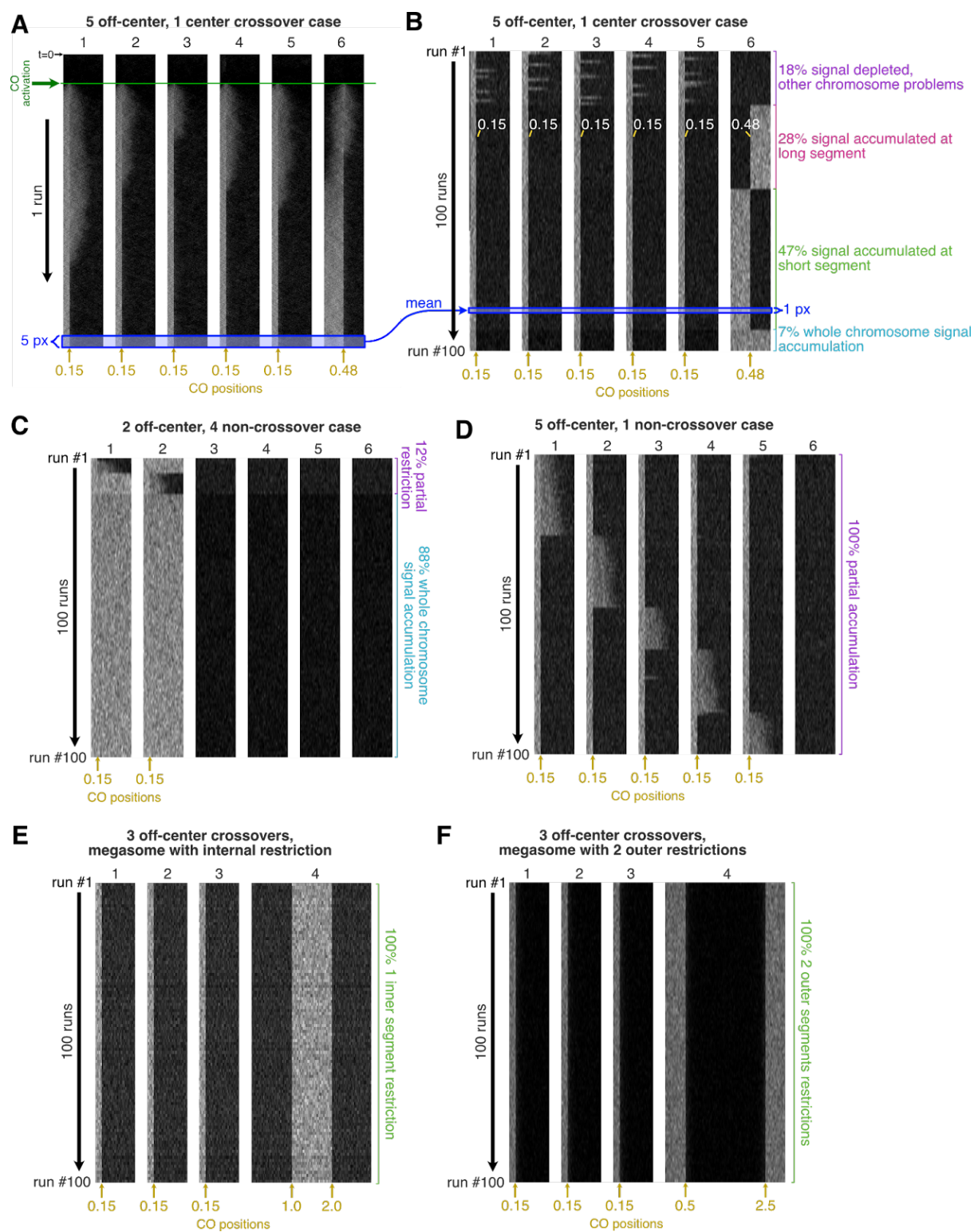


Figure 18. Summaries of discrete simulations on various configurations.

A, Output of 1 run of the simulation showing the spread of the signal along 6 chromosomes. All chromosomes have an arbitrary length of 1 (divided into 100 bins). The run consists of 15000 iterations. The state of the signal along each chromosome is represented as a row of pixels sorted vertically starting at $t=0$ at the top of the image. The horizontal pixels represent the amount of signal at that specific position in the corresponding chromosome. The crossovers are activated at $t=1500$ (green line) and their position is indicated by the yellow arrows and numbers at the bottom of the images. 5 chromosomes have off-center crossovers set at 0.15 and 1 chromosome has a near-center crossover set at 0.48. The blue box at the end of the run illustrates the last 5 states (box not to scale with the actual image) of each chromosome (see legend for **B-F**).

B-F, Summaries of 100 simulation runs with different crossover positions and chromosome configurations. Each vertical pixel row is the average intensity projection of the last 5 states of an individual run represented by the blue boxes and arrow shared between panels **A** and **B**. The crossover positions are marked with the yellow arrow and numbers. Normal chromosome lengths were set to 1 while megasome lengths were set to 3.

B, Summary of 100 simulation runs with 5 off-center crossover chromosomes (chromosomes #1-5) and 1 near-center crossover chromosome (chromosome #6). All off-center crossovers were set to position 0.15 and the near-center crossover was set to 0.48. The runs are sorted by the outcome of chromosome #6. Descriptions of each outcome class are at right: the purple range indicates the 18% of the runs where chromosome #6 was completely depleted of the signal; the pink range indicates runs in which chromosome #6 accumulated the signal on its long segment; the green range indicates where chromosome #6 accumulated the signal on its short segment; the light blue range indicates where chromosome #6 accumulated the signal at its entire length.

C, Summary of 100 simulation runs with 2 off-center crossover chromosomes and 4 non-crossover chromosomes. The off-center crossovers were set to

position 0.15. The runs were sorted by the outcome of the off-center crossover chromosomes. The purple range indicates the 12% of runs where the off-center crossover chromosomes had partial restriction of the signal, and the light blue range indicates the 88% of runs where the off-center chromosomes accumulated the signal along their entire length.

D, Summary of 100 simulation runs with 5 off-center crossover chromosomes and 1 non-crossover chromosome. The off-center crossovers were set to position 0.15. The runs were sorted by the outcome of the off-center crossover chromosomes. The purple range indicates that 100% of the runs had partial signal accumulation in at least one of the off-center chromosomes.

E, Summary of 100 simulation runs with 3 normal length chromosomes with off-center crossovers (at position 0.15) and 1 megasome with 2 near-center crossovers (at positions 1.0 and 2.0). The green range indicates that all simulated megasomes went on to produce signal restriction at a single inner segment.

F, Summary of 100 simulation runs with 3 normal length chromosomes with off-center crossovers and 1 megasome with 2 off-center crossovers. The crossover positions on the normal length chromosomes were set to 0.15. The crossover positions on the megasome were set to 0.5 and 2.5. The green range indicates that all simulated megasomes developed two outer segment restrictions.

Chapter 3

Discussion

3.1 The short arm of the chromosomes is identified by a factor originating and diffusing from the CO

This work has shown evidence that the region for short arm designation in the CO-delimited segments of a chromosome is determined by the local level of a factor originating from and positionally constrained by CO sites that diffuses along the SC. Predictions made by this model largely agree with the observed short arm designation sites marked by phospho-SYP-1 in wild-type and *meT7* chromosomes (**Fig. 3, Fig 9 and Fig. 12**). Mechanistically, the prediction of short arm designation in wild-type chromosomes with just one CO site is trivial as the shorter segment will always have a higher expected factor density compared to the longer segment. In contrast, chromosomes with multiple CO sites will get longer, multiple, mixed or central short arm designation sites based on the position and number of CO sites (**Fig. 8B-G**).

3.2 Robust mechanism for short arm determination

While the partitioning process occasionally makes errors, in general it is capable of robustly distinguishing two chromosome segments that differ by a small amount. The data supports previous observations that most chromosomes establish the CO site in the terminal quarters of the chromosomes (Rockman & Kruglyak, 2009). All the wild-type chromosomes that fit the description where the crossover is located at the terminal quarters have a correct short arm designation (80 out of 80). This means that the central half of the chromosome harbors all the crossovers leading to incorrect partitions, with 11.7% of the chromosomes in this category displaying an incorrect phospho-SYP-1 restriction (4 out of 34). The terminal quarters and central half were used as the limits for the analysis to be consistent with the phrasing used in other studies regarding the distribution of CO sites in *C. elegans*. A stricter indicator for the incorrect phospho-SYP-1 restriction observations is the short-to-long arm length ratios. In wild type, only 6 chromosomes had a short-to-long arm length ratio higher than 0.75 with 50% of them displaying an incorrect restriction of phospho-SYP-1 in the longer segment (3 out of 6). In contrast, chromosomes with less than 0.75 short-to-long arm length ratio had a 0.92% incorrect restriction of phospho-SYP-1 (1 out of 108). The total correct short arm restriction of phospho-SYP-1 in wild-type

chromosomes is a robust 96.5% (110 out of 114). Exceptional cases like the single chromosome whose short-to-long arm length ratio was below 0.75 and still presented phospho-SYP-1 restriction to the longer segment could experience problems in meiosis I disjunction, if the long arms, oriented parallel to the spindle, lost their cohesion first. However, since even mutations such as *syp-1(T452A)* that result in near-total loss of partitioning at diakinesis still have relatively high viability (Brandt et al., 2020; Sato-Carlton et al., 2018), there may exist correction mechanisms redundantly ensuring correct chromosome disjunction in such cases, such as the LEM-3 nuclease that has been shown to resolve chromatin bridges during chromosome segregation (Hollis et al., 2020). Similar results to the wild-type observations were obtained in the heterozygous *syp-1(T452A)/+* where a 25% of chromosomes with short-to-long arm length ratio above 0.75 had incorrect phospho-SYP-1 restriction (2 out of 8) and no chromosomes with a ratio below 0.75 had incorrect phospho-SYP-1 restriction (0 out of 127). This could be an indicator that the short arm designation can be achieved with half the phosphorylated SYP-1 present in the nucleus or that the density of endogenous SYP-1 phosphorylation is higher to compensate for half of the SYP-1 proteins being non-phosphorylatable. For a 6µm chromosome with a ratio around 0.75, differences between ~2.6 and ~3.4 µm segments, or a difference of 0.8 µm or larger, can be detected. This level of sensitivity is comparable to other well-characterized length-dependent subcellular processes, for example that of the polar Pom1 gradient in *S. pombe* that links the decision to enter cell division to a cell length increase from ~11µm to ~14µm (Moseley et al., 2009), or *Chlamydomonas* flagellar length control, in which two flagella ~11µm long are equalized in size when necessary and maintained at a standard deviation of $\pm 2.7\mu\text{m}$ (Bauer et al., 2021).

3.3 The diffusion model can predict the outcome of *meT7* partitioning

The central segments in *met7* that restrict phospho-SYP-1 although the outer segments are shorter (**Fig. 5F**) suggest that segments bounded by two CO sites have twice the “short-arm generating power” per unit of length than segments bounded by a single CO site and a chromosome end. The suggested explanation for this observation consists of a factor that is incorporated into the chromosome by the

CO and diffuses evenly to both adjacent segments. This implies that when a segment is bounded by two CO sites, twice the amount of factor is accumulated per unit of length compared to the segments that are bound by just one CO site. One possibility for such a signal is a protein that enters the chromosome at the CO site and then diffuses through the SC in either direction; another, perhaps more likely scenario is that a CO-localized activity modifies proteins already present in the SC on both sides of the CO site. In the case of chromosomes with a single CO site, the model will always predict that the shorter segment will be designated as the “short arm” of the chromosome. This prediction is correct 96.5% of the time in wild-type chromosomes, but the success is not as high for predicting *meT7* partitioning when two CO sites are involved; the diffusion model is able to correctly predict only 80% of the qualitative observations of phospho-SYP-1 restriction patterns (**Fig. 9**). A small subset of the difference may be explained due to slight errors in tracing, as incorrect predictions often occur on the boundaries between the phase-space diagram (**Fig. 9E**). Conversely, a small number of the incorrectly predicted chromosomes are far from the corresponding boundary and after retracing and straightening multiple times cannot be easily explained by tracing errors due to the fact that the distance from the observation in the phase-space diagram to the corresponding boundary is always longer than 3 standard deviations of the retracing controls (The error bars on **Figure 10** indicate one standard deviation). Another potential source of discrepancy could be different timing of CO site activation, i.e., the time at which signal begins to be generated by each CO site. If one CO site on *meT7* were active long enough before the other, it could potentially change the partitioning prediction of phospho-SYP-1 from what would be predicted based on both CO site locations. When the CO sites are analyzed individually by measuring the enrichment of phospho-SYP-1 on the split megasomes (**Fig. 12B**), out of 148 CO sites measured, 132 (89%) were correctly predicted. Part of the discrepancy between the qualitative (phase-space diagram) and the quantitative (enrichment of phospho-SYP-1 on split chromosomes) results comes from a shared set of incorrectly predicted chromosomes found in both assays. In the qualitative analysis, a chromosome whose phospho-SYP-1 restriction pattern influenced by both CO sites does not match the prediction, is counted as a fully incorrect prediction; in some instances, the quantitative analysis of the split segments of the same chromosome results in one CO site being correctly predicted while the other CO site is incorrectly predicted, reducing the impact of the error committed

compared to the qualitative analysis. An interesting observation is that in the quantitative analysis, 12 out of the 16 CO sites with phospho-SYP-1 enrichment at the longer segment have a short-to-long arm length ratio higher than 0.75. This indicates that, similarly to the wild-type chromosomes with one CO site, incorrect restrictions often occur when the short-to-long arm ratio is close to 1. The observed errors in length measurement when segments are of similar size are consistent with a previous study showing that single COs artificially introduced in the center of chromosomes fail to restrict LAB-1 to the long arm at diakinesis, leading to problems with chromosome segregation (Altendorfer et al., 2020). This study also observed partitioning failure in diakinesis bivalents even with off-center COs, albeit at a lower rate; this is possibly because it was the only CO in the entire nucleus, a situation known from studies on *dsb-2* mutants with low CO numbers to cause partitioning problems at pachytene (Brandt et al., 2020; Sato-Carlton et al., 2020). The percentage of split *meT7* segments from the quantitative analysis with a short-to-long arm length ratio higher than 0.75 is 31.1% (46 out of 148), which is a stark increase from the 5.2% (6 out of 114) in the wild-type chromosomes. When comparing the wild-type chromosomes and the split segments of *meT7* with a short-to-long arm ratio lower than 0.75, the percentage of incorrect chromosomes is similar; restriction of phospho-SYP-1 in the long arm occurs 0.92% of the time in wild-type chromosomes (1 out of 108) and 3.92% of the time in split *meT7* segments (4 out of 102). Although there is an increase in the percentage of chromosomes with long arm enrichment of phospho-SYP-1 in the split megasome segments, the data suggests that the main differentiation factor is the increase in the number of short segments with similar lengths to the corresponding long segments in the megasome observations.

3.4 Crossover spacing on *meT7*

Often, when a CO site is positioned at the central half region of the *meT7* fusion chromosome, it will be the only CO site designation in the entire chromosome axis as suggested by the traces (**Fig. 6B**). Presumably, this central CO site has enough influence to inhibit the formation of additional CO sites on most occasions. When a CO site is positioned in one of the terminal quarters, it will be accompanied by another CO site on the opposing region according to the traces (**Fig. 9**). This

suggests that the CO interference is not strong enough to encompass from one end of the *meT7* chromosome to the opposite end. Although the previous observations were true most of the time, several examples were noted of closely spaced COSA-1 foci in the analysis of *meT7* chromosomes. Considering that crossover interference in *C. elegans* is known to act over the entire length of a wild-type chromosome (Hillers & Villeneuve, 2003; Libuda et al., 2013; Yokoo et al., 2012) and that other fusion chromosomes redistribute CO sites and DSBs to the arms mirroring the proportions of wild-type chromosomes (Lascarez-Lagunas et al., 2023; Libuda et al., 2013), it was not expected to find any instances of two COSA-1 foci closer together than roughly a third of the length of *meT7* (shaded zone in **Fig. 11C**). However, a total of 6 out of 74 chromosomes with COSA-1 foci spaced closer together than this length was found (**Fig. 9E**, dark-outlined square markers toward top right). These close foci are all quite close to the middle of *meT7*, the segment derived from the original X chromosome, suggesting that the distance between CO sites may be less on the X chromosome. One possible reason for this may be the heterochromatic nature of the X chromosome in the germline (W. G. Kelly et al., 2002) which may reduce the length for a given number of megabases.

3.5 The CO site is a barrier for synaptonemal complex diffusion

The model can accurately predict the restriction patterns of phospho-SYP-1 in chromosomes with single and multiple CO sites. A key critical assumption in the model is that CO sites function as segment boundaries that limit the diffusion of signal between the two adjacent segments. This is supported by the fact that in wild-type chromosomes the CO sites delimit the functionalized short arm in late pachytene even though the lateral diffusion of the SC continues. If the CO sites were not barriers for diffusion, the signal would homogenize from one neighboring segment to another inhibiting the formation of a density differential and disallowing the increased “short-arm-generating power” of segments located between two CO sites. Whether CO sites physically interrupt the continuity of the SC or may slow down diffusing proteins through other means such as occlusion or transient binding interactions, has yet to be conclusively determined. Observations of presumptive crossovers or recombination nodules at the SC central region have been reported in electron

microscopy studies (Carpenter, 1979; Schmekel & Daneholt, 1998), and more recently in 3D-SIM fluorescence microscopy images (Woglar & Villeneuve, 2018) in which the SC central region expands into “bubbles” surrounding COSA-1, strongly suggesting that the CO sites have an altered structure that could physically limit the diffusion of the signal. Further evidence for altered SC structure around CO sites comes from the local persistence of REC-8 protein engineered to contain TEV protease motifs specifically at CO sites after treatment with TEV protease (Castellano-Pozo et al., 2020). The live imaging experiments in **Figure 13** showing photoconverted mMaple3:SYP-3 failing to spread past CO sites provides some evidence in support of this notion; however, since these experiments are necessarily constrained by time and photobleaching, it is not possible to conclusively state whether the kinetics that are observed would suffice to maintain compartmentalization of signal molecules. Future experiments with more sensitive single-molecule imaging should be well-suited to address this question.

3.6 Simulation of the model recapitulates most of the observed partitioning outcomes

An important aspect for the partitioning of chromosome arms is the “all-or-nothing” designation of the short and the long arms. In wild-type chromosomes with one CO site, one of the CO-delimited segments is always selected to restrict phospho-SYP-1 and the other segment always evicts phospho-SYP-1, even in the cases when the arm lengths are similar, and phospho-SYP-1 is incorrectly restricted to the longer segment. This “all-or-nothing” nature of short arm designation is expected to be enforced by a positive-feedback loop in the model. The discrete simulation of the model incorporates the feedback through the stabilizing factor. This key molecule in the simulation provides not only the accumulation of the signal in one of the segments but also the depletion of the signal from what will be designated as the long arm domain. It also allows for the recapitulation of most short arm designation observations with one notable exception, the chromosome segments with “mixed” restriction (**Fig. 6B**, **Fig. 8E**). When a mixed segment is predicted, the simulation either restricts the signal to the entire segment length or completely evicts the signal from both sides of the CO. This last pattern is also obtained when simulating

megasomes with a single central CO site. Often, the “mixed” partitioning is observed in the immunofluorescence of *meT7* megasomes with a single central CO site or in the case of multiple CO sites, when the neighboring segments of the CO with mixed partitioning are long. A similar observation was previously made for zones of HTP-1 depletion on singly crossed-over diakinesis chromosomes of *meT7* (Martinez-Perez et al., 2008). Since both of these cases (a single central CO site, or mixed partitioning) arise when one CO site has two long segments on either side, a possible explanation for the partial restriction of phospho-SYP-1 is that the stabilizing factor may not have time to accumulate sufficiently on an entire long segment before the other short arms in the same nucleus sequester all the limiting molecules for themselves (Pattabiraman et al., 2017). Another possible but unlikely explanation is the existence of a counteractive factor that aids in the eviction of phospho-SYP-1 from the long arm starting at the CO site towards the segment with less signal density. A recent study has shown that not only the chromosomal exchange of SC elements is greatly reduced in late pachytene, but also the lateral diffusion of the SC central region has a 4 fold reduction of speed (von Diezmann et al. 2024). This is not incorporated into the simulations and might be one of the reasons as to why no simulation run can replicate the mixed segment restriction of the signal.

3.7 Possible partitioning signal factors

While the current work does not identify the factor promoting short arm identity, possible candidates are proteins lying near or at the top of the length-sensing pathway, whose mutation blocks the ability of one or more proteins to partition to the short and/or long arm. Among these are the ZHP-1/2 proteins, whose partitioning coincides with that of phospho-SYP-1 and are required for phospho-SYP-1 partitioning at pachytene (L. Zhang et al., 2018). ZHP-1/2 are related to ZHP-3/4, RING finger proteins that have been shown, along with their homologs such as HEI10 in *Arabidopsis*, to form widely-spaced foci through a putative coarsening process at the sites of crossover designation (Morgan et al., 2021; L. Zhang et al., 2021). It is possible that different physical parameters of coarsening could drive a similar coalescence for ZHP-1/2 at a much larger scale, such that they cover an entire chromosome segment. Similarly, minimization of surface-to-volume ratio has

been proposed to aid the partitioning of the entire SC to the short arm that occurs upon desynapsis (Z. Zhang et al., 2020). Another candidate, not mutually exclusive with ZHP-1/2, could be PLK-2 recruited by phosphorylated SYP-1. It has been shown that priming phosphorylation of SYP-1 at T452 is required to recruit PLK-2 to the SC; PLK-2 recruitment, in turn, is required for SYP-1 partitioning (Brandt et al., 2020; Sato-Carlton et al., 2018). This observation has been extended to show that phosphorylation of SYP-1, and presumably PLK-2 recruitment, can act in *trans* to promote partitioning of a substantial fraction of non-phosphorylated SYP-1. The observation that non-phosphorylated SYP-1 can partition to the short arm only in the presence of phospho-SYP-1 (**Fig. 3D**) suggests two possibilities: either phospho-SYP-1 is necessary to establish conditions that move all SC proteins to the short arm, or phospho-SYP-1 is actively moved to the short arm by an unknown mechanism, bringing along any molecules it is bound to, which may include non-phosphorylated SYP-1. Since central region proteins are known to form multimeric complexes (Billmyre et al., 2023; Blundon et al., 2024; Duncie et al., 2018), the dynamics of individual SYP proteins is likely dependent on all SYP proteins. PLK-2 has also been shown to be required for the timely partitioning of ZHP-1/2 at pachytene, but not for its later partitioning at diakinesis (L. Zhang et al., 2018). PLK-2's influence on both phospho-SYP-1 and ZHP-1/2 may be due either to a specific interaction involved in length sensing at this stage, or alternatively due to its role in inactivating CHK-2 and promoting later steps of CO designation in a timely fashion (L. Zhang et al., 2023). For the former case, it is hypothesized that after PLK-2 is recruited to the SC, it may phosphorylate further targets that promote partitioning by slowing down the dynamics of the SC, specifically by lowering the rate of SC dissociation from the chromosome. A candidate for such a target is SYP-5/SYP-6, recently shown to promote synapsis as well as asymmetric SC disassembly in a manner dependent on their phosphorylation by PLK-2 (Gold et al., 2024). PLK-2, or other proteins brought to the short arm, may also act to evict HTP-1, LAB-1 or other long arm-specific proteins from the short arm, for example by phosphorylating chromosome axis proteins. It is unlikely that long arm-specific proteins act upstream of short-arm proteins, however, since loss of LAB-1 has been reported to leave phospho-SYP-1 partitioning unaffected (Brandt et al., 2020). However, partitioning of PLK-2 (and presumably of phospho-SYP-1) depends on ZHP-1/2 not only at pachytene but at later stages as well (L. Zhang et al., 2018), strongly implying that

ZHP-1/2 act upstream of PLK-2 and SYP-1 phosphorylation. Finally, it is possible that neither of these pathways is the initial sensor of length, but instead both are responding to a prior and independent signal.

Chapter 4

Materials and methods

4.1 *C. elegans* strains and culture conditions

All *C. elegans* strains used were maintained at 15-25°C on nematode growth medium (NGM) and fed OP50 *E. coli* bacteria under standard conditions (Brenner 1974). L4 hermaphrodite worms were picked and grown for 24 hours in the dark at 20°C before live imaging and immunofluorescence experiments. The following strains were used in this study:

- WBStrain00000296: *mels8 [pie-1p::GFP::cosa-1 + unc-119(+)] II*
- WBStrain00000292: *dpy-18(e364) unc-3(e151) meT7 III;X;IV*
- PMC468: *mels8 [pie-1p::GFP::cosa-1 + unc-119(+)] II; dpy-18(e364) unc-3(e151) meT7 III;X;IV*
- PMC471: *syp-1(icm81[T452A::HA]) II; syp-1(me17)/nT1[unc-?(n754) let-? qIs50] IV;V*
- PMC703: *mels8 [pie-1p::GFP::cosa-1 + unc-119(+)] II; syp-1(icm85[T452A]) /nT1[qIs 51] IV; V*
- PMC346: *icmSi44 [Psyp-1::syp-1(T452A) + unc-119(+)] II*
- PHX2016: *dtn-1::3FLAG::GFP II*
- PMC716: *dtn-1::3Flag::GFP II; meT7 [dpy-18(e364) unc-3(e151)] III;X;IV*
- PMC663: *syp-3(ok758) I; mels8 [pie-1::GFP::cosa-1 + unc-119(+)] ieSi63 [cbunc-119+, psyp-3::mMaple3::syp-3] II; unc-119(ed3) III.*
- PMC825: *syp-3(ok758) I; psyp-3::mMaple3::syp-3] II; unc-119(ed3) III; msh-5(me23) IV*
- PMC715: *syp-3(ok758) I; ieSi63 [cbunc-119+, psyp-3::mMaple3::syp-3] II; unc-119(ed3) III; syp-1(icm85[T452A]) V/nT1[qIs 51] IV;V*

4.2 Oligonucleotides generated in this study

The following oligonucleotides were generated in this study and used for tagging the *syp-1(T452A)* (II) (See 4.2 *syp-1(T452A)* tagging for more information):

- Primer c02_t452a_ha_4_rev: (5'-CGCTAAAAACACTAATCACACGGA-3')
- Primer c02_t452a_ty1_fw: (5'-TTCAGATCGAGTAGTTCCGCCG-3')
- crRNA c02_t452a_ha_crRNA2: (5'-GGAAGAAATAATGTGTGTGT-3')
- Ultramer c02_t452a-ha_hrt_ultramer: (5'-
GAGAGCCGAAGCTCATACTGCAGATGTTCCGCCGAAAGAGAGGAGGGAAG
AAAGAGGTCCACACCAACCAGGACCCACTCGACTACCCATACGACGTCC
CAGACTACGCTTAATGTGTGTGTGCCGAAGAAACGACTATGTACCATTTC
ATCTTGTGCTATTTTTTTTTGTGTTTTTAGAGTTTTATATAAAGTAATTTG-3')

4.3 Antibodies used in this study

The following antibodies were used in this study:

- guinea pig-anti-SYP-1 (Carlton Lab)
- rabbit-anti-SYP-1phos (Carlton Lab)
- mouse-anti-GFP (Roche Cat#12600500)
- goat-anti-SYP-1 (Dernburg Lab)
- mouse-anti-HA (BioLegend Cat#901501)
- mouse-anti-FLAG (SIGMA Cat#F1804)

4.4 *syp-1(T452A)* tagging

The CRISPR/Cas9 system was used to insert a TY1::HA sequence at the C-terminus of the *syp-1(T452A)* (*II*) locus of the strain PMC346. A 200 bp ssDNA homologous recombination template (*c02_t452a-ha_hrt_ultramer*) was used for DNA repair and the targeting gRNA duplex was generated with the crRNA *c02_t452a_ha_crRNA2*. Adult hermaphrodites carrying the mutant allele *syp-1(T452A)* were injected at 24 hours post-L4 with the genome editing mix (17.5 μ M gRNA duplex, 17.5 μ M Cas9 nuclease and 6 μ M ssDNA homology recombination template) to generate the *syp-1(T452A)::TY1::HA* (*II*) allele. The primers used during the PCR screening for the

insertion candidates were *c02_t452a_ha_4_fw* and *c02_t452a_ha_4_rev*. Correct insertions were confirmed by DNA sequencing.

4.5 Cytology

For immunofluorescence, adult worms were picked and dissected on a coverslip in 15 µl of EBT buffer (HEPES pH7.4 27.5 mM, NaCl 129.8 mM, KCl 52.8 mM, EDTA 2.2 mM, EGTA 0.55 mM, Tween 1%, Tricaine 0.15% weight per volume, Levamisole 0.015% weight per volume). The worms were fixed by adding 15 µl of Fix solution (HEPES pH7.4 25 mM, NaCl 0.118 M, KCl 48 mM, EDTA 2 mM, EGTA 0.5 mM, Formaldehyde 2%) for 2 minutes. The dissected worms were sandwiched between the coverslip and a slide glass (Matsunami Inc. cat #S9901) and frozen at -80°C. The coverslip was removed using a razor blade and the slide with the worms was placed on methanol at -25°C for 1 minute. The slides were washed for 10 minutes 3 times in PBST. Slides were blocked in PBST supplemented with 0.5% bovine serum albumin for 30 minutes followed by overnight incubation of the primary antibodies in PBST at 4°C in a wet chamber to prevent desiccation. After the primary antibody incubation, the slides were washed in PBST for 10 minutes 3 times for a subsequent 2 hour long secondary antibody incubation at room temperature in a wet chamber. Following the secondary antibody incubation, the slides were washed for 10 minutes in PBST and further submerged for 10 minutes in PBST with 1 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) to stain the DNA. The slides were washed for an additional 10 minutes in PBST. Finally, a #1-S micro coverslip (Matsunami) with 13 µl of the prepared mounting medium (50 µl of 5M TRIS + 450 µl of NPG-glycerol) was used to cover the sample and it was sealed with nail polish.

4.6 Microscopy

For conventional fluorescence microscopy, images were acquired using a Deltavision microscope system using a 100x, 1.4NA PlanSApo objective (Olympus Inc.) with a Photometrics CoolSNAP ES2 camera (1024x1024 pixels) at a pixel size in the image plane of 64 nanometers. Raw images were subjected to constrained iterative deconvolution and subsequently corrected for chromatic aberration by sub-pixel

shifting using wavelength-specific defocus values measured with polychromatic beads.

Three-dimensional structured illumination microscopy (3D-SIM) images were acquired with a Deltavision OMX Blaze microscope at the Advanced ICT Research Institute, Kobe, in the laboratory of Prof. Yasushi Hiroaka. A 60x, 1.4NA oil immersion objective was used. Wavelengths were acquired on separate cameras, processed for 3D-SIM reconstruction and then aligned to each other using brightfield images in all 4 wavelengths as a standard, using the *Chromagnon* software package (Matsuda et al., 2018).

For live imaging and photoconversion of mMaple3::SYP-3, a Zeiss LSM880 confocal microscope (Carl Zeiss) equipped with Plan-Apochromat 63x/1.40 NA objective was used. Animals were located using brightfield illumination, and nuclei suitable for image acquisition were targeted using the 488 nm laser. The excitation wavelengths for the non-photoconverted and photoconverted mMaple3::SYP-3 were 488 nm and 561 nm respectively. The software used for the image acquisition and photoconversion was *ZEN (black edition)* (Carl Zeiss).

4.7 Chromosome tracing, straightening and 3D model rendering

Individual nucleus images cropped from the corresponding gonad region were prepared using *Fiji* (Schindelin et al., 2012) or *Priism* (Chen et al., 1996) and exported to the TIFF format. The chromosome axes of the nucleus, marked by a synaptonemal complex element, were traced using a python script for *Fiji* that utilizes the *SNT* framework (Arshadi et al., 2021) or the *3dmodel* program from the UCSF *Priism* software suite. The chromosome traces were used to create the visual renderings of the 3D models using the *roifile* library (Gohlke, n.d.). The traces were used to straighten the chromosomes from the original nucleus images using *Fiji*.

4.8 Straightened chromosome alignment and segment intensity measurement

For **Figure 3C-E**, the crossover position for every 3D straightened chromosome was manually selected and the short segment and long segment regions were calculated using *Fiji*. The straightened chromosomes were sum projected on the Z dimension and subsequently each channel was normalized to the average intensity of the entire chromosome. The short segment region and long segment region average intensities were obtained from the normalized sum projected straightened chromosomes. To create the aligned chromosome images, the normalized sum projected straightened chromosomes were sorted by the short segment length. The vertical pixel values of the sorted chromosomes were summed at every horizontal pixel position generating 1-pixel tall images that were extended with 0 value pixels to the right to match the length of the longest chromosome of the group. The extended sorted chromosomes were combined vertically from the smallest short segment length to the biggest short segment length.

4.9 Model prediction

The model predictions for **Figure 8** were calculated in python (<https://www.github.com/carltonlab/chromosome-partitioning>). The predicted chromosome was divided into several discrete units based on the length of the chromosome. The chromosome was also divided into confined segments by the specified crossover positions. The number of iterations in the prediction (represented as “time” in **Figure 8**) was calculated based on the chromosome length to ensure diffusion throughout the chromosome and is not intended to correspond to a defined time interval. During the first half of the iterations, signal level was increased at positions directly adjacent to each crossover position, and the signal diffused further along the confined segment at a constant rate. The crossover positions stopped adding the signal at the halfway mark of all iterations, to allow visualization of continued signal diffusion inside the confined segments. The resulting signal density along the predicted chromosome units at each time point was plotted and color coded according to the iteration number.

For **Figure 11**, the predictions of phospho-SYP-1 enrichment under the assumption that fusion points act as barriers were obtained by simulating an *meT7* with the

junction positions proportional to the lengths of chromosomes III, X and IV in base pairs (13.8 Mb, 17.7 Mb and 17.8 Mb respectively). Two random crossover positions separated by at least the proportional length of chromosome III were generated. The distances to the closest neighboring barrier (megosome end, fusion junction or CO site) were calculated and a prediction of phospho-SYP-1 was generated by selecting the shortest distance to the barrier. The resulting predictions were categorized as mixed, outer or inner partitioning and the crossover positions were plotted in **Figure 11C**. For **Figure 11D,E**, the 74 observed crossover pair positions of the *meT7* megosome data set (**Fig. 9**) were used to make a prediction on the fusion junctions as barriers chromosomes with both directions: III > X > IV (III to IV direction) and IV > X > III (IV to III direction). The resulting predicted phospho-SYP-1 pattern category was compared to the observed data. For the best-case correct predictions, if either of the two fusion junctions as barriers predictions matched the observed category, it would be counted as a correct prediction. For the worst-case correct predictions, if either of the two fusion junctions as barriers predictions failed to match the observed category, it would be counted as an incorrect prediction. The resulting correct prediction percentages for all 74 observed megasomes were annotated in **Fig. 11D**.

4.10 Megosome classification

The length of straightened megasomes with two CO sites was split into three segments delimited by the crossover sites with lengths of *a*, *b* and *c* in order from left to right. The megasomes were classified according to the pattern of phospho-SYP-1 restriction. Megasomes with phospho-SYP-1 restricted to segments *a* and *c* were classified as *outer restriction*, megasomes with phospho-SYP-1 restricted to segment *b* were classified as *inner restriction* and megasomes with phospho-SYP-1 restriction at either segment *a* and partially to *b* or segments *c* and partially to *b* were classified as *mixed restriction*.

To determine the zones within the phase space diagrams of **Figure 9A-D**, **Figure 10** and **Figure 11C**, the expected signal density was calculated according to the number of bounding crossovers in each of the segments. As segments *a* and *c* are only

bounded by one crossover site, their expected signal density is $1/a$ and $1/c$. Segment b is bounded by two crossover sites and thus has an expected signal density of $2/b$. The area in the phase space diagram where the outer segments have highest density (i.e., $1/a > 2/b > 1/c$) was colored yellow and is expected to contain chromosomes with an *outer restriction* classification as the densities in segments a and c are predicted to be higher than that of segment b . The region described by the equation $1/a < 2/b > 1/c$ was colored green and is expected to contain megasomes with a classification of *inner restriction*, where the signal density in segment b is expected to be higher than in segments a and c . Finally, the two remaining areas are described by the inequalities $1/a > 2/b > 1/c$ and $1/a < 2/b < 1/c$, and were colored blue to represent the *mixed restriction* classification where only one of the outer segments (a or c) has a higher expected signal density than the one in segment b . To simplify Fig. 4D, the inequalities are written in reciprocal form: $1/a > 2/b < 1/c$ becomes $2a < b > 2c$, etc.

4.11 Split megasome alignment

The straightened megasome images were split at the midpoint of the segment bounded by two crossover designation sites. The resulting images, now containing one CO site and two segments each, were sum-projected and oriented to place the shorter segment to the left. To better display the images, a manual threshold was implemented on all chromosomes to obtain the crossover designation site area and the phospho-SYP-1 restriction area, and the image channels were normalized to the mean intensity of the areas. The images were then summed along the column (Y) dimension to obtain single row average images. The images were aligned to the position of the crossover designation site and sorted by the ratio of the short segment length divided by the long segment length (**Fig. 12A**).

4.12 Live imaging sample preparation

L4 staged worms of the target genotype were grown for 24 hours at 20°C in the dark. For imaging, 5-10 worms were picked into 15 μ l of ECM buffer (von Diezmann & Rog, 2021b) (84% Leibowitz L-15 without phenol red, 9.3% fetal bovine serum, 0.01% levamisole and 2 mM EGTA) adjusted to 330 mOsm using 5M sucrose on a #1-S

micro cover glass (Matsunami). The sample was then placed on a glass slide (Matsunami Inc, cat #S9901) and quickly sealed with VaLaP (1:1:1 mixture of vaseline, lanolin and paraffin) melted at 65°C.

4.13 Live imaging photoconversion time course acquisition

All live imaging experiments used the *ZEN (Black edition)* software (Carl Zeiss).

For **Figure 13** a pre-conversion 3D image stack from the mid pachytene region of *syp-3(ok758) (I); GFP::cosa-1 mMaple3::syp-3 (II)* gonads was acquired and used to select 2-3 chromosomes with 1 distinguishable GFP::COSA-1 focus that share the same z-position. A small region at the desired distance to the crossover was drawn on top of the non-photoconverted mMaple3::SYP-3 signal for each of the selected chromosomes. For **Figure 14** a pre-conversion 3D image stack from the mid pachytene region of *syp-3(ok758) (I); mMaple3::syp-3 (II); msh-5(me23) (IV)* gonads were acquired and used to select long stretches of chromosomes for photoconversion. A small region with similar dimensions to the photoconversion of *syp-3(ok758) (I); GFP::cosa-1 mMaple3::syp-3 (II)* chromosomes was drawn on top of the non-photoconverted mMaple3::SYP-3 signal for each of the selected chromosomes. Photoconversion of mMaple3::SYP-3 at the drawn regions was achieved using 35% power of the 405 nm laser in a single iteration with a speed parameter of 6, resulting in a pixel dwell time of 3.98 µs. Following the photo-conversion, a total of 4 3D-images at 3-minute intervals were obtained in a time course starting at t=0 min. and ending at t=9 min. Between each time point acquisition, the stage x, y and z coordinates were manually adjusted to compensate for the nuclear and gonad movements. The zoom parameter for the image acquisitions was set to 6 with dimensions of 528x528 resulting in a pixel size of 42.6 nm and spacing of 0.35 µm in the z-dimension. The speed parameter for the image acquisition was also set to 6, resulting in a pixel dwell time of 3.98 µs.

For **Figure 16**, nuclei from the late pachytene region of *syp-3(ok758) (I); GFP::cosa-1 mMaple3::syp-3 (II)* and *syp-3(ok758) (I); GFP::cosa-1 mMaple3::syp-3 (II); syp-*

1(*T452A*) (*V*) were selected for photoconversion. A pre-conversion 3D image stack was obtained followed by photoconversion of 25-50% of the area from 3-4 nuclei from the gonad region. Images were taken with a zoom parameter of 6 and image dimensions of 512x512. Photoconversion was achieved with the 405 nm laser power set to 45% with a speed parameter of 6. 5 time points were taken after photoconversion with 5-minute intervals starting at $t=0min$ and ending at $t=20 min$. The stage x, y and z coordinates were manually adjusted to compensate for the nuclear and gonad movements.

4.14 Photoconversion time course background subtraction

The photoconverted and non-photoconverted channels from the photoconversion time course images in **Figure 13** obtained with the Zeiss LSM880 confocal microscope were separated and saved in TIFF format for further processing. First, a 3D mask of the chromosome axes and the crossover sites from the non-photoconverted channel was generated using *Fiji* (Schindelin et al., 2012) and the *Trainable Weka Segmentation* plugin (Arganda-Carreras et al., 2017). The non-photoconverted mask was used to obtain an image without the pixels outside of the chromosome axes and crossover designation sites referred to as the *masked non-photoconverted image*. The non-photoconverted mask was later used to obtain the background signal corresponding to all the pixels outside the chromosome axes and crossover designation sites. The mean intensity of every z-section of the background was measured with *Fiji* and subtracted from the corresponding z-section of the *masked non-photoconverted image* to obtain the final background subtracted non-photoconverted image. To subtract the background signal from the photoconverted channel, the non-photoconverted mask was used to generate an image of the photoconverted channel with only the pixels inside of the chromosome axes and crossover designation sites referred to as the *masked photoconverted image*. The background signal for the photoconverted channel was obtained by removing the pixels corresponding to the photoconverted nuclei. The mean intensity of every z-section of the photoconverted background was measured and subtracted from the corresponding z-section of the *masked photoconverted image* to obtain the background subtracted photoconverted image.

4.15 Diffusion measurements of photoconverted mMaple3::SYP-3

For **Figure 14B-C**, the photoconversion center coordinates of the *syp-3(ok758) (I)*; *GFP::cosa-1 mMaple3::syp-3 (II)* and *syp-3(ok758) (I)*; *mMaple3::syp-3 (II)*; *msh-5(me23) (IV)* straightened chromosomes at time point 0 min was manually obtained using *Fiji*. The coordinates were used to calculate the photoconversion center for all chromosomes at time point 9 min. A span of 1 μ m total length referred to as the *L* region was placed around the calculated photoconversion center of the *syp-3(ok758) (I)*; *GFP::cosa-1 mMaple3::syp-3 (II)* and *syp-3(ok758) (I)*; *mMaple3::syp-3 (II)*; *msh-5(me23) (IV)* straightened chromosomes and was used to calculate the average intensity of the region. A new 1 μ m total length *R* region in the distal segment of the straightened *syp-3(ok758) (I)*; *GFP::cosa-1 mMaple3::syp-3 (II)* chromosomes 0.25 μ m to the right of the CO site was generated and the average intensity was calculated. The distance between the *L* and *R* regions was used to generate reciprocal *R* regions in all the length compatible *syp-3(ok758) (I)*; *mMaple3::syp-3 (II)*; *msh-5(me23) (IV)* straightened chromosomes. The average intensity difference denoted by *R avg intensity minus L avg intensity* was calculated for the *syp-3(ok758) (I)*; *GFP::cosa-1 mMaple3::syp-3 (II)* chromosomes and was plotted in the Y axis of **Figure 14C**. The mean average intensity difference of the *L* and *R* regions for all compatible *syp-3(ok758) (I)*; *mMaple3::syp-3 (II)*; *msh-5(me23) (IV)* was calculated and plotted on the Y axis of **Figure 14C**.

All 98 nuclei from **Figure 16** from *syp-3(ok758) (I)*; *GFP::cosa-1 mMaple3::syp-3 (II)* and *syp-3(ok758) (I)*; *GFP::cosa-1 mMaple3::syp-3 (II)*; *syp-1(T452A) (V)* were pooled together and blindly, manually classified according to the percentage of the non-photoconverted signal where the photoconverted signal had diffused. The categories were 0%, 1-25%, 26-50%, 51-75% and 76-100%. The filenames of the images for both genotypes were obscured and the order was randomized for blind classification.

4.16 Quantification and statistical analysis of straightened chromosome segment average intensities

Descriptions of the statistical analysis and group sizes for the short and long segments measurements in **Figure 3C-E** are included in the figure legend and details about the segment intensities measurement can be found in **4.8 Straightened chromosome alignment and segment intensity measurement**. The *n* values represent the number of straightened chromosomes measured. The python library SciPy was used to obtain the standard deviations and to compare the means of the short segments and the long segments average intensities using Student's t-Test. Spearman rank correlation of arm length and intensity was performed using the `stats.spearmanr()` function from SciPy.

4.17 Quantification of phospho-SYP-1 enrichment in split *meT7* chromosomes

Description of the group sizes of the quantification of phospho-SYP-1 enrichment in the split *meT7* chromosomes can be found in the legends of **Figure 12**. The short segment average intensity and the long segment average intensity of every split chromosome was measured and the subtraction of the long segment average intensity minus the short segment average intensity was plotted in **Figure 12B**. The long segment average intensity and short segment average intensity difference calculated was shuffled and a random sign assigned to generate the randomized data plotted in **Figure 12C**. The means of the observed and randomized data were compared using Student's t-Test with the SciPy library.

4.18 Quantification and statistical analysis of photoconverted mMaple3::SYP-3 signal

Descriptions of the quantification of the diffusion of photoconverted mMaple3::SYP-3 signal in mid pachytene chromosomes with CO sites and mid pachytene chromosomes without CO sites can be found in the **4.15 Diffusion measurements of photoconverted mMaple3::SYP-3** section and the legend of **Figure 14**. The *n* values represent the total number of chromosomes with CO sites at the 9 *min* time point. The *p*-value was obtained by comparing the means of the chromosomes with

CO sites and chromosomes without CO sites using Student's t-Test with the SciPy library. The description of the quantification of the diffusion of photoconverted mMaple3::SYP-3 signal in late pachytene control and *syp-1(T452A)* nuclei is found in the **4.15 Diffusion measurements of photoconverted mMaple3::SYP-3** section and **Figure 16** legend. The n value represents the total number of photoconverted nuclei for each genotype.

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