

INVESTIGATING THE FUNCTIONAL DOMAINS OF MEIOTIC HORMA DOMAIN PROTEIN HTP-1

A thesis submitted for the degree of

Doctor of Philosophy

2016

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ACKNOWLEDGEMENTS

I would first and foremost like to thank my supervisor Enrique Martinez-Perez for his guidance, advice and support throughout this project, and the meiosis lab members for their kind support, encouragements, and valuable insight. In particular I am so very grateful to Nuria Ferrandiz for teaching me so much on and off the bench, and always being patient and kind; Oana Telecan for her friendship through thick and thin; former PhD students Oliver Crawley and Sarah Testori for putting up with my incessant newbie questions and leading by example. Thanks also to Mark Ungless, the Director of Postgraduate Studies at the CSC, for helping me see this through in the final months.

I am grateful to my wonderful family for their unwavering support, especially my grandmother and mother. Thanks to Agathe and Becky for making London home. And finally, to Sarah, Hammy, Sarah, Amélie, the incredible Team Moustache and all the friends around the world who have kept me sane these past four years – thank you for the adventures. I could not have done it without you.

ABSTRACT

Proper chromosome segregation during meiosis is required to prevent the formation of aneuploid gametes, which result in sterility, miscarriages and birth defects. Formation of euploid gametes requires a series of events that occur during meiotic prophase, including: homologue pairing, synaptonemal complex assembly, and the formation of inter-homologue crossovers. All these events are promoted by a group of conserved HORMA (HOP-1, REV-1 and MAD2) domain proteins that associate with chromosomes at the onset of meiosis. *C. elegans* carries four of these proteins: HIM-3, HTP-1, HTP-2 and HTP-3, which form part of the proteinaceous axis of meiotic chromosomes. HTP-1 promotes pairing and crossover formation, and participates in two checkpoint mechanisms coordinating meiotic events, while HTP-2 alone cannot promote any of these events. HTP-1 and HTP-2 are 82% homologous at the amino acid level, hinting that essential residues for HTP-1 functions lie in the 62-amino-acid difference between the two proteins. Using recombinant proteins in which HTP-1 domains are substituted for the corresponding regions of HTP-2, I show that an 84-amino-acid region within the HORMA domain of HTP-1, named "HORMA C" is essential for HTP-1 function. Specifically, I show that ten residues wherein are essential for pairing, synapsis, correct timing of double strand break repair, chiasma formation and proper chromosome segregation. Moreover, the HORMA C region of HTP-1 functions with the support of the C-terminus, and the HORMA C region and C-terminus of HTP-1 are, together, sufficient to confer HTP-1 function to the HTP-2 protein sequence. This study provides insight into the relationship between sequence and function in HORMA domain proteins, and how proteins of this family have evolved specific roles supported by cis sequences.

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CHAPTER 1: INTRODUCTION

1.1. Background

1.1.1. Meiosis, a specialised cell division that generates gametes

Eukaryotic organisms are characterised by the presence of a nucleus enclosing their genetic information. They have developed larger genomes organised into several chromosomes, and evolved diploidy: a state in which each chromosome is present in two copies, called homologues, inherited from each parent. These features necessitate not only faithful replication of the chromosomes, but also mechanisms to maintain their structure, number and integrity. In diploid organisms, the karyotype is maintained by the alternating occurrences of meiosis, a specialised cell division giving rise to haploid gametes, and fertilisation, which combines two gametes to create a new individual, restoring diploidy. Whereas the mitotic division gives rise to two identical daughter cells, meiosis typically generates four haploid germ cells from a diploid progenitor – although not all will become gametes.

Meiosis consists of two successive divisions, called meiosis I and meiosis II, after a single round of DNA replication; the amount of DNA in meiotic products is therefore halved. Meiosis also involves the exchange of genetic material between homologous chromosomes, promoting genetic diversity and variation, and driving evolution with the generation of new gene combinations. This genetic exchange called recombination takes place in prophase I, when both replicated homologues are present in the same nucleus. It involves a tightly controlled sequence of events where homologues pair and are kept in close physical proximity by a zipper-like protein structure, the synaptonemal complex (SC; Moses, 1969) . Homologue alignment allows the formation of crossovers, points of exchange of portions of chromosomes (Janssens *et al.*, 2012). Homologues then segregate away from each other at the first meiotic division. The second meiotic division resembles mitosis: the two identical copies of replicated chromosomes,

called sister chromatids, segregate away from each other in two daughter cells. Each gamete will contribute one chromosome from each pair to the zygote.

Meiosis is a highly conserved process on which species depend for survival. Meiotic errors lead to aneuploid gametes, which compromise the next generation. In humans, meiotic defects cause birth defects, miscarriages and infertility (Hassold *et al.*, 2007). Aneuploidy affects 5% of clinically recognised pregnancies. Aneuploidy is often lethal, with the exception of conditions such as Down's syndrome (trisomy 21) and Turner (XO) and Klinefelter (XXY) syndromes (Hassold and Jacobs, 1984, Jacobs *et al.*, 1959), making aneuploidy the leading cause of miscarriage. More recent studies have pinpointed defects in specific meiotic processes as the cause of low fertility. In human females, oocytes are arrested in the first meiotic metaphase from birth until they develop as part of a menstrual cycle; deterioration of meiotic structures over time has been suspected of playing a part in increased incidence of birth defects and miscarriages with maternal age. Observation of human oocytes from women of various ages suggested that cohesion between the sister chromatids weakened with maternal age, increasing the chances of premature separation (Duncan *et al.*, 2012). Additionally, mutations of the gene encoding SC component SYCP3 could be identified as cause of compromised fertility (Bolor *et al.*, 2009), highlighting the importance of the early meiotic events of prophase I in the requirement for faithful chromosome segregation.

1.1.2. Prophase I of meiosis

During prophase I of meiosis, homologous chromosomes undergo pairing, synapsis and recombination in an orchestrated fashion. Prophase I of meiosis is divided into five clearly defined stages that mirror these developments and are a prerequisite for the in depth study of meiotic processes; they are presented in Figure 1, in the context of the sequence of meiotic events.

1.1.2.1. Leptotene

From S-phase onwards, each replicated chromosome is held together by sister chromatid cohesion (SCC; Figure 1). SCC is mediated in both mitosis and meiosis by the cohesin complex, a ring-like protein complex that keeps replicated sisters in tight proximity (Miyazaki and Orr-Weaver, 1994). During leptotene, chromosomes condense and proteinaceous axes called axial elements assemble; they will form the basis of meiotic chromosome structure.

1.1.2.2. Zygotene

Assembly of the axial elements is required to promote the association of homologous chromosomes during the process of homologue pairing. During zygotene, chromosomes find their pairing partner, and their initial interactions are stabilised by the polymerisation of the central element of the SC between the axial elements of homologous pairs (Colaiacovo, 2006, Moses, 1969; Figure 1). Deliberate double strand breaks (DSBs) are introduced; depending on the organism, DSB formation precedes synapsis (Yeast), is concomitant with and required for SC assembly (Mouse, plants), or immediately follows it (*C. elegans*, *Drosophila*; Page and Hawley, 2004).

1.1.2.3. Pachytene

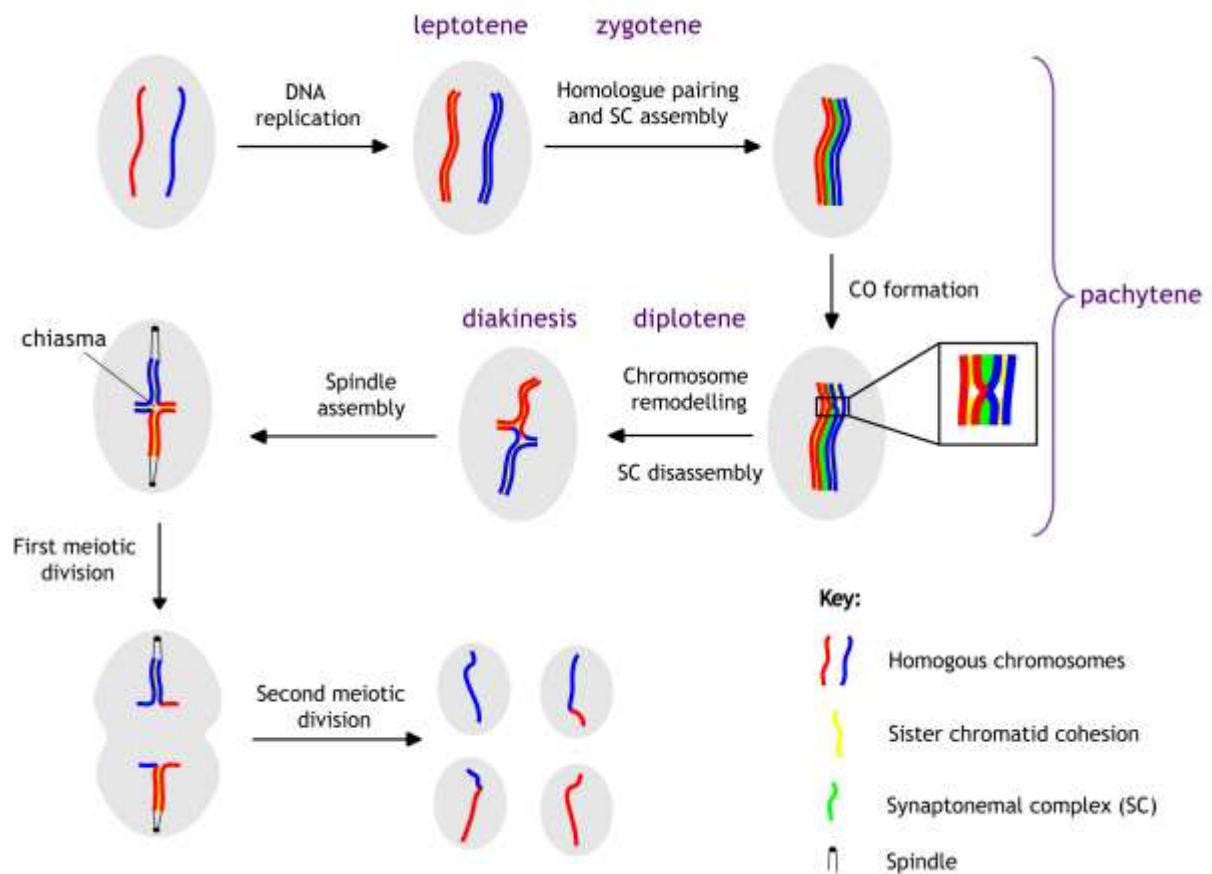
At pachytene, homologous chromosome pairs are synapsed along their whole length. The SC holds aligned homologues in close proximity, which is essential for the repair of DSBs using the homologue as a template rather than the sister chromatid (Colaiacovo, 2006). In fact, the SC contributes to a systematic preference, or homologue bias, towards using the homologue as repair template during meiosis in contrast with the sister bias observed in somatic cells. A subset of recombination intermediates are resolved into crossovers, the exchange of portions of chromatin between homologous chromosomes. Crossovers, together with sister chromatid cohesion, result in physical bonds between homologues – chiasmata – that hold homologues together at the end of prophase I (Figure 1).

Figure 1. Diagram of meiotic events

After DNA replication at S-phase, replicated chromatids are held together by SCC (yellow).

Homologue pairing is stabilised by SC assembly in zygotene (green), and supports recombination between homologous chromosomes during pachytene (zoom). After extensive remodelling during diplotene, pairs of homologous chromosomes appear as diakinesis bivalents held together by SCC and chiasmata. SCC is released in two steps, ensuring the separation of the homologues at the first meiotic division, and the sister chromatids at the second.

Adapted from a diagram created by Sarah Testori



1.1.2.4. Diplotene

When crossovers have occurred, meiocytes enter diplotene. This stage is characterised by the disassembly of the SC central element; as homologues are more loosely associated, crossover sites where they remain in tight association stand out. These chiasmata correspond to sites where crossovers have occurred, and where homologues remain physically linked. SC disassembly is accompanied by remodelling of the chromosome structure: now that recombination has occurred, chromosomes are condensing in preparation for meiotic division.

1.1.2.5. Diakinesis

By diakinesis, meiotic chromosomes are fully condensed and ready to enter metaphase. They appear as bivalents: each condensed chromatin body is actually a pair of homologous chromosomes, held together by both SCC and chiasmata. The presence of chiasmata as a result of crossover formation also ensures proper orientation of the bivalents at metaphase I. SCC will be released in a two-step process, allowing the homologs to segregate at anaphase I, while keeping the sister chromatids together until they segregate in the second meiotic division (Petronczki *et al.*, 2003).

1.1.3. *C. elegans* as a model organism to study meiosis

Meiosis has been studied in a variety of organisms including yeast, plants and mammals; this study uses *C. elegans*, a 1mm long nematode, as model system. *C. elegans* is not only easy to maintain and yields high amount of progeny, but it is also a fantastic model to study meiosis due to its germline morphology: in an adult worm, over half the cells are engaged in meiosis (Schedl, 1997, Lui and Colaiacovo, 2013). Moreover, each germline displays a complete time course of meiosis, as presented in Figure 2. Nuclei in the tip of the germline proliferate mitotically, and enter meiosis as they move away from the tip, progressing through meiosis and the syncytial germline in a spatial and temporal gradient along the distal-proximal axis of the germline. Chromosome pairing and SC assembly start as nuclei enter the transition zone of the germline, which corresponds to the leptotene and zygotene stages of meiotic prophase. Nuclei at this stage

display a characteristic clustering of chromosomes that is released once SC assembly is completed and nuclei enter pachytene (Figure 2). Following SC disassembly and chromosome compaction, diakinesis oocytes display six DAPI-stained bodies, which correspond to the six pairs of homologous chromosomes of *C. elegans*, each attached by a single chiasma. In situations where crossover formation is compromised, diakinesis nuclei display numbers of DAPI-stained bodies between 7 (a single homologue pair fails to form a crossover) and 12 (all chromosome pairs fail).

The morphological advantages of *C. elegans* are coupled with a wide range of experimental tools available for this organism. For instance, Frokjaer-Jensen *et al.* (2012, 2008) have optimised a system that allows stable insertion of a single copy transgene in the desired chromosome; a technique I used extensively to generate mutants in this project. More recently, the development of the CRISPR/Cas9 system for targeted mutagenesis has allowed point mutations to be introduced directly into the desired endogenous locus (Farboud and Meyer, 2015, Ward, 2015). *C. elegans* germlines can be dissected whole and cytological tools such as immunofluorescence and in situ hybridisation allow the observation and analysis of meiotic structures and processes such as chromosome axes, pairing and recombination intermediates, in the context of the whole germline.

1.2. Sequence of meiotic events in *C. elegans*

1.2.1 Meiotic chromosome axis formation

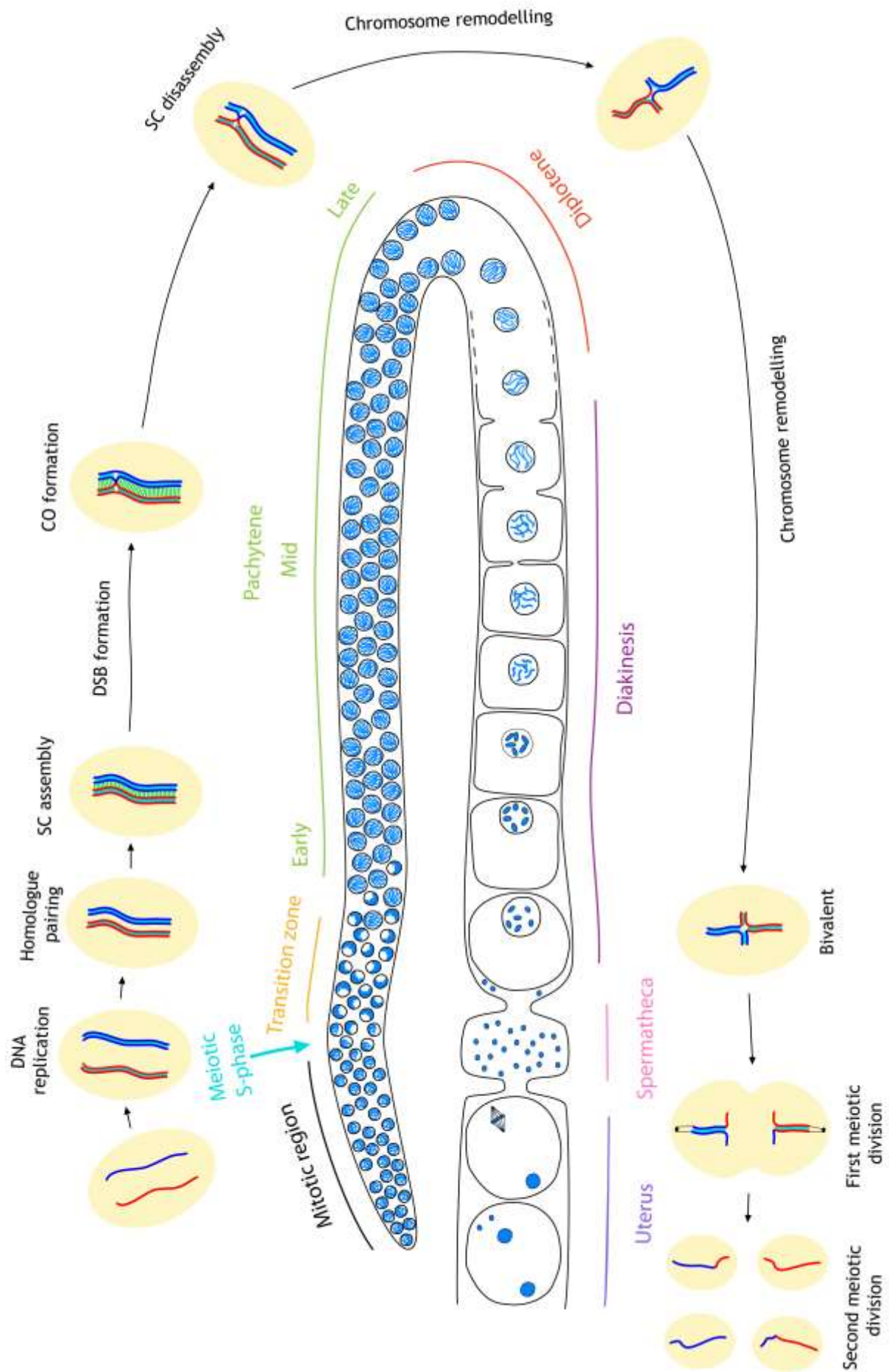
1.2.1.1. Cohesins and SCC

Cohesin is a ring-shaped protein complex which holds sister chromatids together – a process called sister chromatid cohesion (SCC) – from S-phase onwards in mitosis and meiosis (Michaelis *et al.*, 1997, Haering *et al.*, 2008, Haering *et al.*, 2002). It is composed of four subunits; two Structural Maintenance of Chromosomes (SMC) proteins, SMC-1 and SMC-2 are rod-like and

Figure 2. Complete time-course of meiosis in the *C. elegans* germline

This diagram shows the coordination of meiotic events with progression through the *C. elegans* germline. In particular, notice the transition zone with clustered chromatin that characterises the homology search state in leptotene and zygotene. As synapsis completes, nuclei enter pachytene and this clustered conformation is released. Nuclei progress through the syncytium during pachytene as crossovers are formed. Upon pachytene exit, nuclei go through a bottleneck in the germline, and progressively become part of separate cells as they align to enter the spermatheca one by one. Diakinesis bivalents are observed in the two oocytes nearest the spermatheca.

Diagram created by Sarah Testori



attach at their extremities to form a hinge structure. The tripartite ring is closed by a cleavable kleisin subunit, which is bound by the last subunit, Sister Chromatid Cohesion 3 (Haering *et al.*, 2002, SCC-3; Michaelis *et al.*, 1997). SCC occurs in two main steps: cohesin is loaded onto chromatin, and the complex is then activated to become cohesive, by acetylation of subunit SMC-3 (Koshland and Guacci, 2000, Toth *et al.*, 1999, Zhang *et al.*, 2008). Cohesion is abrogated by cleavage of the kleisin subunit at anaphase (Uhlmann *et al.*, 1999); beforehand, SCC is regulated by selective removal of cohesin from chromosomes via a prophase pathway involving Wapl (Kueng *et al.*, 2006).

In mitosis, the kleisin is SCC-1. Meiosis however, involves the expression of specialised cohesin complexes characterised by the substitution of SCC-1 by one of three meiotic kleisins: REC-8 or cohesion protein homologues COH-3 or COH-4 (Klein *et al.*, 1999, Pasierbek *et al.*, 2001, Severson *et al.*, 2009, Severson and Meyer, 2014). All three meiotic cohesins are competent to mediate SCC during meiosis, but they are controlled by different mechanisms: REC-8 loading depends on HTP-3, but not COH-3/4 (Severson *et al.*, 2009); unlike REC-8 cohesin, COH-3/4 cohesin requires DSB formation to become cohesive and support SCC (Severson and Meyer, 2014). Meiotic cohesins also facilitate meiotic events as integral components of the chromosome axes; for instance REC-8 but not COH-3/4 promotes recombination (Severson *et al.*, 2009), and COH-3/4 promote SC assembly independently of their role in SCC (Severson and Meyer, 2014).

1.2.1.2. Formation and function of chromosome axes

The first elements of the meiotic chromosome axes are assembled during premeiotic S-phase; at this stage, both REC-8 and HTP-3 are detected associated with chromatin (Klein *et al.*, 1999, Pasierbek *et al.*, 2001). As chromatin condenses and enters meiosis, meiotic cohesins and HTP-3 concentrate into proteinaceous thread-like structures forming the basis of meiotic chromosome axes (Pasierbek *et al.*, 2001, Goodyer *et al.*, 2008, Severson *et al.*, 2009). These initial axial elements are required to recruit the remaining components of the chromosome axes

in *C. elegans*, high incidence of males (HIM)-3, and Him Three Paralogs HTP-1 and HTP-2 (Pasierbek *et al.*, 2001, Couteau *et al.*, 2004, Severson *et al.*, 2009, Martinez-Perez *et al.*, 2008). Meiotic chromosome axes fulfil an important structural role during meiosis; they are a scaffold organising meiotic chromatin and also hold homologues in close physical proximity as part of the SC, promoting proper recombination. Additionally, axis components are actively involved in a variety of functions and chromatin surveillance mechanisms that ensure the faithfulness of the sequence of meiotic events and limits meiotic errors (see below).

1.2.2. Pairing and synapsis during transition zone

The onset of meiosis is concomitant with nuclei entry into the “transition zone”, a region defined as ranging from the disappearance of mitotic spindles, signifying mitotic exit, to the first row of pachytene nuclei (Crittenden *et al.*, 1994); this region of the germline is characterised by a unique morphology, with chromosomes clustered in a crescent shape on one side of the nucleus (Francis *et al.*, 1995, MacQueen and Villeneuve, 2001; Figure 2). Transition zone nuclei are in the leptotene and zygotene stages of meiosis and see meiotic chromosomes undergo pairing and synapsis, and form DSBs that will lead to recombination.

1.2.2.1 Chromosome movement and homology search

The clustered conformation of transition zone chromatin is mediated by microtubules physically pulling chromosomes together, and is characteristic of a homology search state. Inside the nucleus, chromosomes are attached to the nuclear membrane via their pairing centers (Phillips and Dernburg, 2006, Phillips *et al.*, 2005), chromosome sites which were originally shown to be required for accurate meiotic chromosome segregation (MacQueen *et al.*, 2005). Pairing centers are bound by a family of zinc finger proteins which also tether them to the nuclear envelope (Phillips and Dernburg, 2006, Phillips *et al.*, 2005); this binding is dependent on meiosis master regulator CHK-2 (MacQueen and Villeneuve, 2001, Kim *et al.*, 2015). Once at the membrane, pairing center proteins recruit polo-like kinase (PLK)-2 (Harper *et al.*, 2011, Labella *et al.*, 2011); PLK-2 then recruits patches of SUN-1 which interact with the pairing

centers (Labella *et al.*, 2011, Harper *et al.*, 2011, Sato *et al.*, 2009). SUN-1 is part of a SUN/KASH type transmembrane module, SUN-1/ZYG-12, which connect chromosomes to microtubules and dynein on the outer side of the nuclear envelope (Tzur *et al.*, 2006, Hiraoka and Dernburg, 2009, Sato *et al.*, 2009). When SUN-1 is phosphorylated, a process that requires PLK-2 and CHK-2, cytoskeletal forces can pull chromosomes into a clustered crescent shape by their pairing centers (Penkner *et al.*, 2009). SUN-1 patches and chromosome pairing centers are mobile over that smaller area of the nuclear envelope, promoting encounters between chromosomes that eventually lead them to find their homologue (Penkner *et al.*, 2009, Woglar *et al.*, 2013). This movement is maintained until pachytene entry, when SUN-1 aggregates and associated PLK-2 patches disassemble from all but the X-chromosome pairing center (Penkner *et al.*, 2009, Woglar *et al.*, 2013).

1.2.2.2. Coordination of pairing, SC assembly and transition zone exit

During homology search, pairing between homologous pairing centers occurs independently of synapsis, as asynaptic dynein mutants displayed significant pairing in the absence of SC (Sato *et al.*, 2009, MacQueen *et al.*, 2002, MacQueen *et al.*, 2005). However, this pairing is transient and needs to be stabilised by synapsis to allow recombination, a process that requires dynein (Sato *et al.*, 2009). This is a controlled process, and multiple proteins are involved in a surveillance mechanism repressing synapsis between non homologous chromosomes, including HTP-1 and PLK-2 (Couteau and Zetka, 2005, Martinez-Perez and Villeneuve, 2005, Labella *et al.*, 2011). The proposed model for coordination of pairing and synapsis is that initial pairing between homologues creates a physical tension, as the pairing forces holding them together antagonise the dynein motor pulling them apart (Wynne *et al.*, 2012). In contrast, when mobile chromosomes encounter a non-homologous counterpart, dynein pulls them apart easily. It is proposed that the tension associated with finding the right pairing partner is sensed and abrogates a synapsis block, allowing the central element of the SC to assemble between the chromosome axes, which at this stage become the lateral elements of

the SC (Page and Hawley, 2004). The SC is crucial in keeping homologue pairs in close physical proximity so that the homologue is available as template for repair of DSBs into crossovers (Page and Hawley, 2004). The central element of the SC includes four SYP factors: SYP-1, SYP-2, SYP-3 and SYP-4 (Colaiacono *et al.*, 2003, MacQueen *et al.*, 2002, Smolikov *et al.*, 2007, Smolikov *et al.*, 2009). All four are required for proper SC assembly, and analysis of mutants shed light on a checkpoint controlling transition zone exit: *syp-1/2/3/4* mutants are characterised by extensive asynapsis, but also display a delay in meiotic progression visualised as an extension of the transition zone (Colaiacono *et al.*, 2003, MacQueen *et al.*, 2002, Smolikov *et al.*, 2007, Smolikov *et al.*, 2009). Chromatin remains in a clustered conformation and proteins that normally dissociate from chromatin at pachytene entry, such as PLK-2, SUN-1 and CHK-2, remain present on chromatin, denoting maintenance of the homology search state (Colaiacono *et al.*, 2003, Harper *et al.*, 2011, Kim *et al.*, 2015, Labella *et al.*, 2011, Woglar *et al.*, 2013). This synapsis surveillance mechanism is dependent on HTP-1, and highlights the tight control of meiotic events (Martinez-Perez and Villeneuve, 2005). Moreover, if a subset of nuclei bypassed this checkpoint and reached pachytene with extensive asynapsis, they would trigger a synapsis checkpoint described by Bhalla and Denburg (2005), which triggers apoptosis in response to unsynapsed pairing centers.

1.2.3. Crossover formation

1.2.3.1. DSB formation and repair

Crossovers are an essential feature of meiosis, and promote genetic diversity. Crossovers also have a structural role, as at least one crossover must occur per chromosome pair in order to ensure a physical bond between homologues, which allows sister chromatids to co-orient and segregate together at the first meiotic division (Jones, 1984). In *C. elegans*, chromosome pairs form exactly one crossover each (Hillers and Villeneuve, 2003). Crossover formation requires the deliberate creation of DSBs by topoisomerase-like protein SPO-11 during transition zone and early pachytene (Keeney *et al.*, 1997, Dernburg *et al.*, 1998). DSB formation also requires

DSB-1 and DSB-2, which are localised to chromatin by CHK-2 and are thought to define the window of competence for DSB formation in meiotic nuclei (Rosu *et al.*, 2013, Stamper *et al.*, 2013). Rosu *et al.* (2013) suggest this competence is lost thanks to a feedback loop whereby early crossover intermediates trigger DSB-2 removal. This process ensures enough breaks are created for a crossover to happen while limiting DNA damage as soon as this purpose is achieved. Once the breaks are created, the DNA ends are resected and accumulate RAD-51, a strand exchange protein that mediates invasion of the repair template (Alpi *et al.*, 2003, Colaiacovo *et al.*, 2003). RAD-50 is required for RAD-51 loading in a meiosis-specific repair pathway promoting crossover formation (Hayashi *et al.*, 2007), and DSB repair progression beyond strand invasion by RAD-51 is mediated by RAD-15 unloading of RAD-51 (Mets and Meyer, 2009).

This repair process uses preferentially the homologue as repair template during meiosis. In worms, this is facilitated first by the repression of non-homologous synapsis, which ensures the homologue is available as a repair template (Labella *et al.*, 2011, Couteau and Zetka, 2005, Martinez-Perez and Villeneuve, 2005). However, in Yeast the role of axis proteins in promoting inter homologue bias is supplemented by components of the repair pathway, notably RecA homologs RAD-51 and DMC1 (Kim *et al.*, 2010). Hong *et al.* (2013) showed that homologue bias is actually the default repair option, and is routinely repressed by cohesins. They propose a model in which both the sister and homologue are bound by resected DSB ends during meiosis (one end each side), and where DMC1 antagonises REC-8 to restore default homologue bias. Such a mechanism has not been identified in worms; in fact, *C. elegans* lacks a DMC1 homologue, and previous studies have shown that Yeast crossovers may be formed by several different pathways while *C. elegans* seem to favour a unique route governing all crossover formation (Berchowitz and Copenhaver, 2010).

While multiple breaks are formed per chromosome and are repaired with the homologue as a template, most will be repaired as non-crossovers; a single recombination

product becomes a nascent crossover site, and loads crossover marker COSA-1 (Yokoo *et al.*, 2012). Designation of future crossovers is mediated by the ZMM proteins (Shinohara *et al.*, 2008); in *C. elegans*, MSH-4 and MSH-5 are required for crossover formation, and act at the later stage of recombination, suggesting this pathway is conserved in worms (Zalevsky *et al.*, 1999, Kelly *et al.*, 2000, Colaiacovo *et al.*, 2003). The process by which a nascent crossover inhibits formation of additional crossovers on the same chromosome pair is called crossover interference (Sturtevant, 1915, Muller, 1916), and is one of several mechanisms controlling crossover distribution in *C. elegans*.

1.2.3.2. Control of crossover distribution by DSB formation and interference

Crossovers do not follow a random distribution on chromosomes, but instead occur at recombination hotspots, while they are rare in so-called cold spots (Petes, 2001). In *C. elegans*, a unique crossover per pair is located preferentially on the chromosome arm rather than the central region (Hillers and Villeneuve, 2003, Barnes *et al.*, 1995). The first insight into the control of crossover distribution came with the discovery of REC-1, a gene which mutants displayed elevated numbers of crossovers in the central region of the chromosome (Rose and Baillie, 1979, Zetka and Rose, 1995). Further study of REC-1 and identification of the corresponding locus revealed that it acts in concert with a paralog, HIM-5, to regulate DSB formation (Chung *et al.*, 2015, Meneely *et al.*, 2012). *him-5* mutation entirely suppresses crossovers on the X chromosome, and leads to a similar altered distribution as observed in *rec-1* mutants (Meneely *et al.*, 2012).

Although crossover distribution is first regulated through DSB formation, a striking feature of *C. elegans* meiosis is the complete crossover interference that inhibits crossover formation along the whole chromosome length when a first nascent crossover is formed (Hillers and Villeneuve, 2003). In fact, in experiments involving chromosome fusions, a single crossover event was able to suppress formation of additional crossover over distances greater than the

length of chromosomes; Hillers and Villeneuve (2003) conclude that this system may be dependent on chromosome structure, in particular the continuity of chromosome axes. Several models have been proposed to explain crossover interference (Berchowitz and Copenhaver, 2010); they include the polymerisation system which depends on the SC, and the counting system in which crossovers would be separated by a set number of breaks repaired as non-crossovers (Foss and Stahl, 1995, King and Mortimer, 1990). The fact that in *C. elegans* SC assembly is uncoupled from DSB formation and early repair, and that interference spans the entire chromosome rather than a set interval supports the idea that interference in *C. elegans* indeed follows the mechanical stress model (Kleckner *et al.*, 2004). Several recent discoveries support this model. Following Hillers and Villeneuve's (2003) observations about the importance of chromosome axes, Nabeshima *et al.* (2004) demonstrated that a hypomorph allele of *him-3*, although competent for pairing and synapsis, disrupted crossover interference possibly through compromise integrity of the chromosome axes. Similarly, partial SYP-1 depletion, that still allowed synapsis to occur, led to loss of interference in some chromosomes which displayed two COSA-1-labelled nascent crossovers suggesting the interference signal may propagate mechanically along the axis (Libuda *et al.*, 2013). The regulation of crossover distribution was also shown to implicate condensin molecules, which act by regulating chromosome structure (Mets and Meyer, 2009, Tsai *et al.*, 2008). Finally, the recent identification of a role for Topoisomerase II in the interference process confirms that crossover interference in *C. elegans* depends on higher order structural changes, which could also explain the incredible range it covers (Zhang *et al.*, 2014).

1.2.4. Remodelling and Prophase I exit

1.2.4.1. Changing morphology and protein patterns

By the end of pachytene, crossovers have formed. The SC disassembles concomitant with large scale remodelling of chromatin; chromosome axes coil and chromosomes condense in preparation for metaphase I (Nabeshima *et al.*, 2005). At this stage, nuclei also rearrange within

the germline; they form a queue so that they progress through the last stages of prophase I one after the other (Figure 2). Chromatin remodelling is accompanied by changes in the distribution of chromatin-associated proteins. Specifically, the SC central element disassembles asymmetrically and as they diminish along most of the length of the chromosome, central element proteins concentrate on the axis between the crossover and closest chromosome end (Nabeshima *et al.*, 2005). What's more, HTP-1 and HTP-2 which are axis components also become restricted to a specific area of chromosomes, in a pattern reciprocal to that of the SYP proteins (Martinez-Perez *et al.*, 2008). These changes are dependent on crossover formation, as shown by the loss of this pattern in crossover mutants, and the fact that the pattern is observed on chromatin adjacent to crossover sites in experiments involving fused chromosomes (Nabeshima *et al.*, 2005, Martinez-Perez *et al.*, 2008). In fact, asymmetric disassembly of the central element is triggered by designated crossover sites. Nadarajan *et al.* (2016) have shown that proteins that decorate nascent crossovers, COSA-1 and ZHP-3, negatively regulate MPK-1 kinase, of which SYP-2 is a target. The absence of SYP-2 phosphorylation allows its removal from chromosomes starting near the crossover site. At diakinesis, the last stage of prophase I, each chromosome pair is fully condensed into a bivalent. Due to the crossover distribution on the distal areas of chromosome arms, bivalents present a short arm and a long arm. By that point, HTP-1 is localised only on the long arm while SYP proteins are localised on the short arm, a distribution which is crucial for the two-step release of SCC (Martinez-Perez *et al.*, 2008, Nabeshima *et al.*, 2005, Pasierbek *et al.*, 2001, Rogers *et al.*, 2002).

1.2.4.2. Two-step release of SCC

Correct segregation of chromosomes during meiosis relies on the separation of homologues at the first meiotic division, and sister chromatids at the second meiotic division. This is dependent on two-step release of SCC: as meiotic kleisin REC-8 is cleaved by Separase between homologs at the metaphase I/anaphase I transition, a subset of REC-8 cohesin is protected on the long arms of the bivalents (Buonomo *et al.*, 2000, Kaitna *et al.*, 2002, Pasierbek *et al.*, 2001). REC-8 is targeted for cleavage by phosphorylation via Aurora B kinase AIR-2, and

REC-8 protection is ensured by restriction of AIR-2 to the short arm of the bivalents (Kaitna *et al.*, 2002, Rogers *et al.*, 2002). AIR-2 localisation is dependent on crossover formation and LAB-1 and HTP-1 activity, as it is observed on all bivalent arms in crossover deficient *spo-11* mutants, and *htp-1* and *lab-1* mutants (Martinez-Perez *et al.*, 2008, Nabeshima *et al.*, 2005, de Carvalho *et al.*, 2008). In summary, crossovers break symmetry on meiotic chromosomes, triggering the apparition of distinct domains characterised by reciprocal localisation of HTP-1 and SYP proteins. HTP-1 and LAB-1 both localise to the long arm and actively protect REC-8-mediated cohesion until the second meiotic division by antagonising AIR-2 kinase and restricting its localisation to the short arm of the bivalent. This, along with the above described involvement of HORMA domain proteins in various control mechanisms throughout prophase I, highlight the versatility of this protein family, which is especially developed in *C. elegans*.

1.3. Meiotic HORMA domain proteins are essential to the unfolding and coordination of meiotic events

1.3.1. The HORMA domain: a conserved structure that monitors chromatin state

HORMA domain proteins are characterised by conserved motifs first identified in Yeast proteins HOP1p, REV7p and MAD2, which the domain is named after (Aravind and Koonin, 1998). Specifically, two residues – an arginine and glutamate – were identical in the 11 proteins analysed, and β -strands in the (at the time predictive) structure corresponded to conserved clusters of hydrophobic residues across sequences. HORMA domain proteins associate with chromatin and act as adaptors, triggering processes such as checkpoints or DNA repair mechanisms in response to chromatin state. For instance MAD2, the most extensively studied HORMA domain protein, is a component of the spindle assembly checkpoint where it inhibits progression to anaphase by binding CDC20, and thus sequestering it from the Anaphase Promoting Complex, until all kinetochores have attached to the spindle (Chen *et al.*, 1996, Li and

Benezra, 1996, reviewed in Lara-Gonzalez *et al.*, 2012). Elucidation of the crystal structures of MAD2, REV7 and HOP1 homologue HIM-3 showed that HORMA domains are composed of a globular domain of three α -helices and seven to eight β -strands, and a C-terminal tail (Luo *et al.*, 2000, Hara *et al.*, 2010, Kim *et al.*, 2014; Figure 3A). Out of these proteins, MAD2 alone has been shown to exist in two conformations, an “open” inactive conformation where the C-terminal tail is flexible, and a “closed” active conformation with the C-terminal tail wrapping around the globular domain, holding a potential ligand in place like a “safety belt” (Luo *et al.*, 2002, Luo *et al.*, 2004, Sironi *et al.*, 2002; Figure 3A). MAD2 function is regulated in part by conversion of open MAD2 to closed MAD2 through the formation of open-closed MAD2 dimers (Yang *et al.*, 2008). The structures of REV7 and HIM-3 are similar to the closed, active conformation of MAD2, with the C-terminal tail folded across the globular domain (Hara *et al.*, 2010, Kim *et al.*, 2014), and therefore it is unclear whether they may function in a two-conformation model.

HORMA domain proteins are important players of meiosis, and regulate the behaviour of meiotic chromosomes. First discovered with HOP1 in Yeast, meiotic HORMA domain proteins, referred to as HORMADs, have been characterised in plants (ASY1/2) mammals (HORMAD1/2) and *C. elegans* (HIM-3, HTP-1/2/3) (Hollingsworth *et al.*, 1990, Wojtasz *et al.*, 2009, Daniel *et al.*, 2011, Sanchez-Moran *et al.*, 2007, Caryl *et al.*, 2000, Zetka *et al.*, 1999, Goodyer *et al.*, 2008, Couteau and Zetka, 2005, Martinez-Perez *et al.*, 2008, Martinez-Perez and Villeneuve, 2005).

1.3.2. Axis component HOP1 promotes synapsis and recombination in Yeast

Yeast HOP1 comprises a HORMA domain and a long tail at the C-terminus that includes a zinc finger domain (Hollingsworth *et al.*, 1990). HOP1 is a component of the meiotic chromosome axes and facilitates the formation of meiotic double strand breaks (DSBs), promotes synaptonemal complex formation, and promotes inter-homologue recombination and therefore the formation of crossovers (Hollingsworth and Byers, 1989, Hollingsworth *et al.*, 1990, Schwacha and Kleckner, 1994). HOP1 promotes recombination by acting as an adaptor in

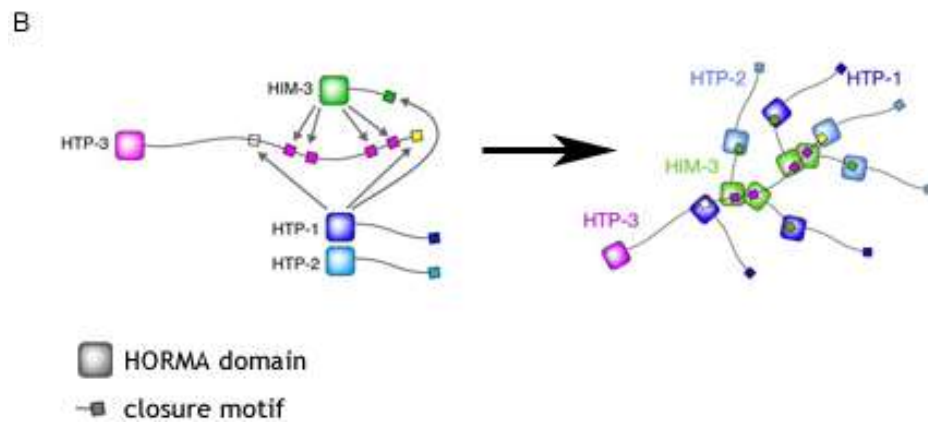
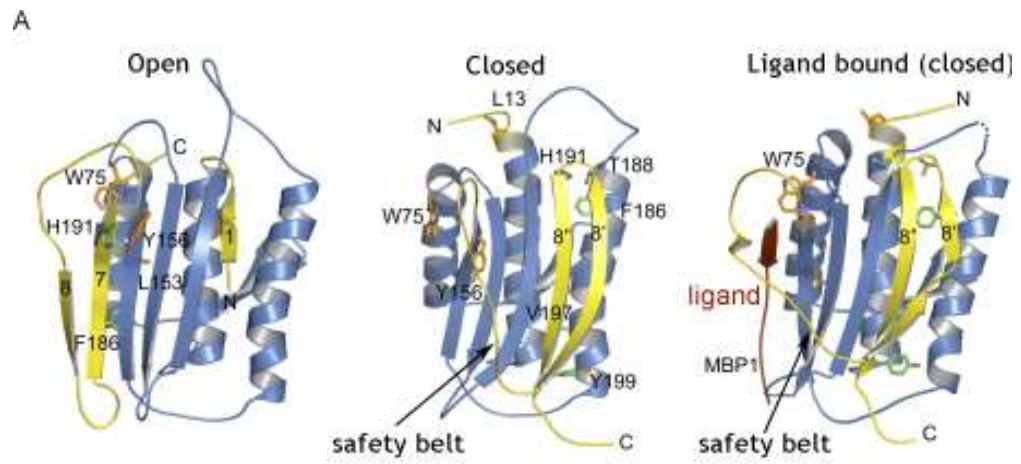
Figure 3. Structural features of HORMA domain proteins

(A) Ribbon diagram of the structure of MAD2 conformations; the globular part of the HORMA domain is shown in blue, and the C-terminal tail of the HORMA domain in yellow. In the open, inactive configuration, the C-terminal tail is folded on the side of the binding site, but in the closed, active conformation, it wraps around the binding site like a safety belt. The ligand-bound structure exemplifies the C-terminal tail's position as a safety belt in the closed conformation, holding the ligand (here MBP1, red) in the binding site.

Adapted from Yang *et al.* (2008)

(B) Hierarchical assembly of *C. elegans* meiotic HORMA domain proteins. HTP-3 is proposed to recruit other HORMA domain proteins to the chromosome axes via its six C-terminal closure motifs, which can be bound by the HORMA domains of HIM-3 or HTP-1/HTP-2. Left, arrows indicate the affinity of the different HORMA domains to specific closure motifs; for instance, the HORMA domain of HTP-1 can bind the closure motif of HIM-3, or the first or last closure motif of HTP-3. Right, the assembled hierarchy of proteins; the diagram shows the maximum number of proteins thought to be recruited by a single HTP-3 molecule.

Adapted from Kim *et al.* (2014)



DSB processing; it recruits effector kinase MEK1 to meiotic chromosomes and activates it by mediating its dimerisation (Niu *et al.*, 2005). In turn, MEK1 represses inter-sister recombination. Interestingly, MEK1 activation depends on the phosphorylation of an SCD motif within HOP1 that is part of the HORMA domain, and essential for MEK1 dimerisation (Carballo *et al.*, 2008).

1.3.3. ASY1/PAIR2 promotes synapsis and recombination in plants

In *Arabidopsis thaliana*, meiotic protein ASY-1 carries a HORMA domain homologous to that of HOP1, and localises to chromatin associated with axial elements (Caryl *et al.*, 2000, Armstrong *et al.*, 2002, Sanchez-Moran *et al.*, 2007). ASY1 is important for plant fertility and mutants are characterised by an asynaptic phenotype and the presence of univalents at metaphase I indicative of crossover defects (Ross *et al.*, 1997, Caryl *et al.*, 2000, Sanchez Moran *et al.*, 2001). Indeed, ASY1 is required for polymerisation of SC central element ZYP1, and promotes recombination by stabilising DMC1 loading on double strand breaks, which ensures that some will be repaired via inter-homologue recombination (Sanchez-Moran *et al.*, 2007). Contrary to HOP1, ASY1 has a paralog, ASY2 which is also expressed in meiosis. However, ASY2 fails to prevent the *asy1* asynaptic phenotype, although the low level synapsis observed in these mutants could be a result of partial redundancy (Caryl *et al.*, 2000). ASY1 homologues have been identified in other plant species, such as rice and *Brassica* (Armstrong *et al.*, 2002, Nonomura *et al.*, 2004). Like ASY1, Rice PAIR2 associates with axial elements in leptotene and zygotene, and is required for SC installation (2006, Nonomura *et al.*, 2004). It also has an homologous locus, PAIR2c3 which unlike ASY2 is thought to be silent (Nonomura *et al.*, 2004).

1.3.4. HORMAD1/2 monitor unsynapsed chromatin in mammals

Like plants, mammals have a pair of meiotic HORMA domain proteins, HORMAD1 and HORMAD2; they localise to unsynapsed axes and are depleted as the SC assembles (Wojtasz *et al.*, 2009). In mouse, HORMAD1 and HORMAD2 are only partially redundant, and HORMAD1

alone is required for female fertility (Wojtasz *et al.*, 2012, Daniel *et al.*, 2011, Shin *et al.*, 2010). HORMAD1 is essential for homologue pairing and alignment, and promotes the formation of resected DSB ends, by encouraging DSB formation or DSB processing (Daniel *et al.*, 2011). HORMAD1 is also important for the completion of SC assembly: although some degree of synapsis is achieved in *hormad1* $-/-$ mutants, unsynapsed regions remain (Daniel *et al.*, 2011). Specifically, even on the chromosome pairs that do initiate SC assembly, the SC does not assemble along the whole length of the chromosomes. Because HORMAD1 depletion is dependent on SC assembly, and prolonged HORMAD1 activity leads to meiotic arrest and apoptosis (Wojtasz *et al.*, 2009), HORMAD1 and the SC are thought to form a negative feedback loop, where HORMAD1 promotes SC formation which in turn promotes HORMAD1 depletion to allow progress through the next meiotic stages (Daniel *et al.*, 2011). Unlike HORMAD1, HORMAD2 is not required to promote SC assembly or DSB formation, but is required to recruit serine/threonine kinase ATR to unsynapsed axes as part of a surveillance mechanism that monitors the completion of synapsis (Wojtasz *et al.*, 2012). Since HORMAD2 localisation to chromosomes is dependent on HORMAD1, HORMAD1 is also required for this surveillance mechanism (Wojtasz *et al.*, 2012). Human HORMAD1 is highly homologous to mouse HORMAD1, and their HORMA domains in particular have 89% sequence identity (Shin *et al.*, 2010).

1.3.5. Behavioural similarities of meiotic HORMA domain proteins

HORMA domain proteins in Yeast, plants and mammals perform related functions involving promotion of SC assembly and of recombination. They also share similarities in their mode of action, notably in their spatial and temporal distribution. Both HOP1 and *Brassica* and *Arabidopsis* ASY1 are observed to load in a succession of high concentration and low concentration domains on meiotic chromosome axes (Armstrong *et al.*, 2002, Borner *et al.*, 2008). Furthermore, this pattern is reciprocal to that of SC components: in Yeast, HOP1 central element ZIP1 is enriched in low-HOP1 regions and vice versa (Borner *et al.*, 2008). During SC

polymerisation in *Brassica* and *Arabidopsis*, ASY1 becomes depleted in places where the SC assembles, which overlap with domains with weaker ASY1 staining (Lambing *et al.*, 2015). This antagonistic localisation to the SC in Yeast and plants is exacerbated in mammals, where HORMAD1/2 are recruited to unsynapsed chromatin only and entirely depleted from meiotic chromosome axes as the SC assembles (Wojtasz *et al.*, 2009, Daniel *et al.*, 2011). Moreover, these loading dynamics are controlled by the same protein, AAA+ ATPase PCH2 – and its mammalian homologue TRIP13. PCH2 controls the loading level and pattern of HOP1 into high- and low-enrichment domains in Yeast (Borner *et al.*, 2008, Joshi *et al.*, 2009). PCH2/TRIP13 is required to deplete ASY1 (partially) and HORMAD1/2 (fully) from chromosome axes concomitant with SC assembly (Wojtasz *et al.*, 2009, Lambing *et al.*, 2015). And later on when the SC disassembles in late pachytene, HOP1 is fully removed from chromosome axes by a PCH2-dependent mechanism (Borner *et al.*, 2008).

1.3.6. Four meiotic HORMA domain proteins cooperate to monitor *C. elegans* prophase I

C. elegans carries four meiotic HORMA domain proteins: high incidence of males 3 (HIM-3) and him three paralogs HTP-1, HTP-2 and HTP-3. Together, they are required for homologue pairing, proper SC assembly, crossover formation and two step release of SCC, as well as multiple checkpoints coordinating the proper unfolding of meiotic events (see below). Not only does *C. elegans* carry twice as many HORMADs as plants and mammals, but these worm proteins also have a specific distribution, most notably they are integral components of chromosome axes and remain associated with meiotic chromosomes even past prophase I (Zetka *et al.*, 1999, Goodyer *et al.*, 2008, Martinez-Perez *et al.*, 2008).

1.3.6.1. HTP-3 acts as a docking site for HIM-3, HTP-1 and HTP-2

In *C. elegans*, HIM-3, HTP-1, HTP-2 and HTP-3 are loaded onto meiotic chromosomes at the onset of meiosis as part of the proteinaceous chromosome axes, and are detected on meiotic chromosomes until the metaphase I to anaphase I transition (Zetka *et al.*, 1999, Goodyer *et al.*,

2008, Martinez-Perez *et al.*, 2008). This assembly is hierarchical: HTP-3 already associates with chromatin in the mitotic tip and loads first out of the four paralogs, as the cells enter meiosis (Goodyer *et al.*, 2008). HTP-3 is part of the original building blocks of chromosome axes: its loading depends on meiotic kleisins COH-3/4 and REC-8, and REC-8 cohesin recruitment to the axes is in turn promoted by HTP-3 in an interdependent manner (Severson *et al.*, 2009). HTP-3 is then required for recruitment of HIM-3, HTP-1 and HTP-2 to chromosome axes (Goodyer *et al.*, 2008, Severson *et al.*, 2009). The mechanism by which it does so has been recently determined by Kim *et al.* (2014) and is rooted in the structure of these proteins (Figure 3B). When elucidating the structure of HIM-3 and comparing it to that of known HORMA domain proteins MAD2 and REV7, Kim *et al.* (2014) found a β -strand corresponding to eight residues of the HIM-3 C-terminal tail apposed in the location where the ligand binds the HORMA domain, held by the “safety belt.” This peptide, that they named closure motif, contained highly conserved residues with HIM-3 orthologues in nematodes but also with the C-terminal tails of HTP-1, HTP-2 and HTP-3. Notably, while HIM-3 and HTP-1/2 carry a single closure motif on their 50-100-residue tails, the HTP-3 tail is over 500 residues long and carries six closure motifs. Further analysis of binding affinities between various combinations of HORMA domains and closure motifs revealed that HIM-3 and HTP-1/2 load by binding the closure motif of existing axis components with their own HORMA domain. Specifically, HIM-3 binds four of the six HTP-3 closure motifs, and HTP-1/2 can bind both the remaining two HTP-3 closure motifs, and the HIM-3 closure motif (Figure 3B). Therefore, HTP-3 acts as a docking site to recruit HIM-3 and HTP-1/2, and HIM-3 in turn participates in HTP-1/2 recruitment. A recent study proposes an additional loading pathway for HTP-1 within this mechanism, as a residual amount of HTP-1 still bound HTP-3 *in vitro* when both closure motifs 1 and 6 of HTP-3 and the closure motif of HIM-3 were mutated (Kim *et al.*, 2015). Further experiments suggest the mechanism for hierarchical assembly through closure motifs may be conserved, as purified human HORMAD1 HORMA domains are shown to bind HORMAD1/2 tails *in vitro* at a short C-terminal conserved motif (Kim *et al.*, 2014).

HTP-3 itself is required for DSB formation (Goodyer *et al.*, 2008) but through its docking site function, it is essential for processes that depend on HORMAD protein presence on the chromosome axes; HTP-3 is therefore indirectly required for pairing, synapsis, chiasma formation and protection of SCC between sister chromatids until meiosis II, via the loading of HTP-1/2 and HIM-3 (Zetka *et al.*, 1999, Martinez-Perez *et al.*, 2008, Couteau *et al.*, 2004, Silva *et al.*, 2014).

1.3.6.2. HIM-3 facilitates homology search and inter-homologue recombination

As a component of the chromosome axis, HIM-3 controls meiotic events throughout prophase I. HIM-3 first mediates the chromatin clustering that occurs in transition zone and facilitates homology search and pairing (Couteau *et al.*, 2004). HIM-3 is then required for SC assembly, which in turn promotes recombination, but it also promotes recombination independently through the establishment of inter-homologue bias that promotes recombination between homologues instead of sister chromatids (Zetka *et al.*, 1999, Couteau *et al.*, 2004). Experiments conducted on a hypomorphic HIM-3 mutant suggest that HIM-3 is implicated in crossover interference; it is proposed that presence of a nascent crossover is communicated along the meiotic chromosome axes and that integrity of these axes, compromised in a low-HIM-3 situation, is a prerequisite for signal transmission (Nabeshima *et al.*, 2004). Recent studies also suggest HIM-3 is a component of a synapsis checkpoint which triggers apoptosis of nuclei with unsynapsed pairing centers during pachytene (Bohr *et al.*, 2016, Bhalla and Dernburg, 2005). HIM-3 is implicated until the second division in *C. elegans* male meiosis, where it prevents premature centriole disengagement after anaphase II (Schwarzstein *et al.*, 2013).

1.3.6.3. HTP-1 facilitates meiotic events, participates in surveillance mechanisms and protects SCC until Meiosis II

HTP-1 and HTP-2 are the last of the four nematode HORMADs to load: they depend on HTP-3 and HIM-3 for loading, but do not recruit other HORMADs via their own closure motif

(Severson *et al.*, 2009, Kim *et al.*, 2014). Due to their high sequence homology, antibodies raised against HTP-1 recognise both proteins; HTP-1/2 are detected on chromosome axes until the metaphase I/anaphase I transition (Martinez-Perez *et al.*, 2008). Investigation of *htp-1* null mutants has revealed that HTP-1 is involved in homologue pairing and promotion of recombination between homologs instead of sister chromatids; the null mutants are characterised by chiasma formation defects and mis-segregation (Couteau and Zetka, 2005; Martinez-Perez and Villeneuve, 2005). Mis-segregation is apparent in two ways in the progeny: autosomal aneuploidies lead to dead eggs that do not hatch, while mis-segregation of the X chromosome causes a higher frequency of male progeny carrying a single X chromosome rather than a pair (Hodgkin *et al.*, 1979). In the strain carrying the *htp-1* deletion allele *gk174*, this is exemplified by 95% embryonic lethality and 24-30% male progeny among the viable progeny – the high incidence of males or him phenotype – due to segregation defects (Couteau and Zetka, 2005; Martinez-Perez *et al.*, 2008).

In addition to its roles in pairing, recombination and chiasma formation, HTP-1 takes part in two monitoring mechanisms that regulate early meiotic events. Firstly, HTP-1 coordinates pairing and SC assembly through inhibition of synapsis between non-homologous chromosomes (Martinez-Perez and Villeneuve, 2005, Couteau and Zetka, 2005). Secondly, HTP-1 sends a “wait” signal that maintains both the clustered conformation of chromatin and the homology search state until synapsis is complete (Martinez-Perez and Villeneuve, 2005). Interestingly, neither monitoring mechanism requires physical presence of HTP-1 at the chromosome axes, as both are supported efficiently by *htp-1(fq8)*, an allele that fails to load onto the axes (Silva *et al.*, 2014). *htp-1(fq8)* is also competent to promote chromatin clustering and homology search during transition zone.

At the end of pachytene, the SC disassembles and meiotic chromosomes undergo extensive remodelling; at this stage, HTP-1 localisation is altered and it adopts a reciprocal pattern to SC central element SYP-1 (Martinez-Perez *et al.*, 2008). This pattern is reminiscent of

the reciprocal localisation of HOP1/ASY1 and ZIP1/ZYP1, although in worms this rearrangement does not happen in context of the SC (Borner *et al.*, 2008, Lambing *et al.*, 2015). By diakinesis, HTP-1 is restricted to the long arms of bivalents, where it prevents REC-8 removal thus protecting SCC until the second meiotic division, which is crucial for timely separation of sister chromatids (Martinez-Perez *et al.*, 2008). Similarly to HIM-3, HTP-1 has been shown to participate in a synapsis checkpoint triggering apoptosis of pachytene nuclei with unsynapsed pairing centers (Bohr *et al.*, 2016).

1.3.6.4. HTP-2 is unable to substitute HTP-1 function despite high homology

HTP-1 and HTP-2 are highly homologous: they are both 352 amino acids long and share 82% of their protein sequence. Due to their similarities a certain level of redundancy between HTP-1 and HTP-2 is to be expected, however their redundancy is partial. HTP-1/2 may redundantly promote SC assembly separately from HTP-1's role in preventing non-homologous synapsis, and may also promote X chromosome pairing (Couteau and Zetka, 2005, Martinez-Perez and Villeneuve, 2005). HTP-1 and HTP-2 mediate a signal from unsynapsed chromosomes required for feedback regulation of CHK-2 in the context of transition zone extension (Kim *et al.*, 2015). HTP-1 and HTP-2 redundantly protect a subset of SCC until meiosis II, which is only compromised in *htp-1 htp-2* double mutants (Martinez-Perez *et al.*, 2008, Severson *et al.*, 2009). Finally, HTP-1/2 are also required to prevent premature centriole disengagement in male meiosis (Schwarzstein *et al.*, 2013).

Despite these shared functions and although both proteins are expressed and localise to axial elements from the start of meiotic prophase, *htp-2* deletion mutants only produce 1% non-viable embryos and 1.4% male progeny, indicating that HTP-2 is non-essential for chromosome segregation (Martinez-Perez *et al.*, 2008). Moreover, the severe phenotype of *htp-1* mutants, which includes non-homologous synapsis and failure to form inter-homologue crossovers, indicates that HTP-1 has essential functions in meiosis that cannot be substituted by HTP-2. The

failure of HTP-2 in promoting proper pairing and recombination, despite the fact that HTP-2 associates with meiotic chromosomes, suggests that functional differences between HTP-1 and HTP-2 must reside among the 62-residue differences between the two proteins.

1.4. Aims and objectives

I endeavoured to investigate the functional domains and residues within HTP-1, to understand how HTP-1 function is supported by different areas of the protein. Because HTP-1 and HTP-2 share such high homology with only limited redundancy (Couteau and Zetka, 2005, Martinez-Perez and Villeneuve, 2005, Martinez-Perez *et al.*, 2008), I concentrated my analysis on the 62 mismatches between the HTP-1 and HTP-2 sequences, and hypothesised that among them were essential residues for HTP-1 function. My objectives were (i) to identify functional residues as precisely as possible and define essential functional regions, and (ii) to assess the capability of functional residues to support all or part of HTP-1 function.

In order to investigate the functional relevance of these mismatches, I took advantage of the genetic tools available in *C. elegans*. For instance, recombinant proteins can be reliably expressed in the worm by building lines carrying single-copy transgenes (Frokjaer-Jensen *et al.*, 2012, Frokjaer-Jensen *et al.*, 2008). This procedure was used to generate and analyse chimeric proteins where part of the HTP 1 sequence had been substituted for that of HTP 2, under the assumption that replacing essential residues of HTP-1 would result in a non-functional protein. Using this method, I show that residues within the HORMA domain are crucial to HTP 1 specific function, and that they are mainly concentrated in HORMA C, an 84-amino-acid region near the C-Terminus and containing a total 10 mismatches. I show that HORMA C promotes pairing, synapsis and recombination, and that HORMA C itself is supported by the C-terminus to control meiotic events.

CHAPTER 2: MATERIALS AND METHODS

2.1. *C. elegans* methods

2.1.1. Growth conditions

C. elegans strains were cultured at 20°C – unless otherwise stated – under standard conditions as described by Brenner (1974). Worms were maintained on Nematode Growth Media seeded with either OP50 or HB101 (for microinjection; see 2.2.3.) *E. coli*. The *C. elegans* Bristol Strain (N2) was used as wild type control and mutants were backcrossed into this genetic background as well; Table 1 recapitulates all worm strains used in this project. To prevent strains from starving, and to ensure a fairly age-synchronised supply of worms on a plate, ~ 30 – 50 worms at the L4 stage were transferred to a fresh plate every 2 – 3 days. Worms were observed with a Leica MZ75 bench stereoscope and manipulated using a Platinum wire pick welded onto a glass Pasteur pipette (Sigma-Aldrich platinum wire diameter 0.25mm, Product Number: 349402-250MG).

2.1.2. Maintenance of low viability strains

Meiotic mutants are used extensively throughout this project; meiotic defects routinely lead to low fertility making it difficult to maintain such strains in homozygosity. Such strains were maintained in heterozygosity using reciprocal translocation balancers carrying visible markers such as *unc*, *roll* and and/or GFP expression. These markers allowed easy identification of heterozygous worms on the plates, and only heterozygous L4 worms were transferred onto fresh plates to maintain the strains. This not only permitted a high number of progeny but also ensured homozygous worms present on the plate were first-generation progeny from meiotically wild type worms, and did not accumulate genomic defects as a consequence of meiotic issues.

2.1.3. Crosses and male stocks

Crosses were set up by placing a single L4 hermaphrodite of the relevant genetic background and 3 - 5 N2 males on a plate with a small *E. coli* lawn, to limit the area roamed by the worms and facilitate encounters. Male progeny with the desired genetic markers were picked after 3 - 4 days and crossed onto other genetic backgrounds until the desired combination was achieved. No more than three males were used for a cross when the hermaphrodite presented an *unc* (uncoordinated) phenotype. For each cross, parents were transferred to a fresh plate every day and the initial plate was discarded after the first day, to exclude any self-progeny of the hermaphrodite from the cross products. To account for the random segregation of alleles, replicate crosses were made and excess progeny was picked each time, to ensure the desired combination would occur (Brenner, 1974).

Table 1: *C. elegans* strains used in this project.

All strains without a source were made by me.

Strain name	Transgene	Genotype	Source
N2		Wild type (Bristol strain)	CGC
AV393		<i>htp-1(gk174)/nT1 IV</i>	E. Martinez-Perez
ATG1		<i>htp-2(tm2543) IV</i>	E. Martinez-Perez
ATGSi6	<i>fqSi6</i>	[<i>HTP-1</i>] II; <i>htp-1(gk174) IV</i>	C. Barroso
ATGSi25	<i>fqSi22</i>	[<i>HTP-1::6His</i>] II; <i>htp-1(gk174) IV</i>	N. Ferrandiz
ATGSi43	<i>fqSi37</i>	[<i>HTP-1::GFP</i>] II; <i>htp-1(gk174) IV</i>	C. Barroso
EG6699		<i>ttTi5605 II</i> ; <i>unc-119(ed3) III</i> ; <i>oxEx1578</i>	CGC
ATGSi182	<i>fqSi27</i>	[<i>htp-1^{HTP-2 N-term}</i>] II; <i>htp-1(gk174) IV</i>	N. Ferrandiz
ATGSi27	<i>fqSi27</i>	[<i>htp-1^{HTP-2 N-term}</i>] II; <i>htp-1(gk174) htp-2(tm2543) IV</i>	
ATGSi28	<i>fqSi28</i>	[<i>htp-1^{HTP-2 C-term}</i>] II; <i>htp-1(gk174) IV</i>	N. Ferrandiz
ATGSi183	<i>fqSi28</i>	[<i>htp-1^{HTP-2 C-term}</i>] II; <i>htp-1(gk174) htp-2(tm2543) IV</i>	
ATGSi140	<i>fq116</i>	[<i>htp-1^{HTP-2 HORMA}</i>] II; <i>htp-1(gk174)/nT1 IV</i>	
ATGSi200	<i>fq116</i>	[<i>htp-1^{HTP-2 HORMA}</i>] II; <i>htp-1(gk174) htp-2(tm2543)/nT1 IV</i>	
ATGSi160	<i>fqSi141</i>	[<i>htp-1^{HTP-2 HORMA A::6His}</i>] II; <i>htp-1(gk174) IV</i>	
ATGSi176	<i>fqSi181</i>	[<i>htp-1^{HTP-2 HORMA B::6His}</i>] II; <i>htp-1(gk174) IV</i>	
ATGSi179	<i>fqSi167</i>	[<i>htp-1^{HTP-2 HORMA C::6His}</i>] II; <i>htp-1(gk174) /nT1 IV</i>	
ATGSi217	<i>fqSi203</i>	[<i>htp-1^{E191A::GFP}</i>] II; <i>htp-1(gk174) IV</i>	
ATGSi218	<i>fqSi208</i>	[<i>htp-1^{M249K; L250S::GFP}</i>] II; <i>htp-1(gk174) /nT1 IV</i>	
ATGSi292	<i>fqSi282</i>	[<i>htp-1^{M249K; L250S::6His}</i>] II; <i>htp-1(gk174) IV</i>	
ATGSi243	<i>fqSi247</i>	[<i>htp-1^{N243H::6His}</i>] II; <i>htp-1(gk174)/nT1 IV</i>	
ATGSi371	<i>fqSi364</i>	[<i>htp-1^{C239H::6His}</i>] II; <i>htp-1(gk174) IV</i>	
ATGSi319	<i>fqSi22</i>	[<i>HTP-1::6His</i>] II; <i>htp-1(gk174) IV</i> ; <i>syp-2(ok307)V</i>	
ATGSi274	<i>fqSi167</i>	[<i>htp-1^{HTP-2 HORMA C::6His}</i>] II; <i>htp-1(gk174)/nT1 IV</i> ; <i>syp-2(ok307)/nT1 V</i>	
ATGSi234	<i>fqSi212</i>	[<i>htp-2^{HTP-1 HORMA C::GFP}</i>] II; <i>htp-1(gk174) /nT1 IV</i>	
ATGSi313	<i>fqSi303</i>	[<i>htp-2^{HTP-1 HORMA C::6His}</i>] II; <i>htp-1(gk174) /nT1 IV</i>	
ATGSi293	<i>fqSi284</i>	[<i>htp-2^{HTP-1 HORMA C + C-term::6His}</i>] II; <i>htp-1(gk174) IV</i>	

2.2. Genetics

2.2.1. Bacterial transformation and plasmid miniprep

Plasmids carrying transgenes for microinjection, whether ordered or generated in the lab, were first transformed and amplified into NEB 5-alpha Competent *E. coli* (NEB #C2987). Transformation was carried out according to the protocol supplied by NEB. 2µl of assembled product were added to 50µl of competent cells, mixed gently, and placed on ice for 30 minutes. After a heat shock at 42°C for 30 seconds, the mix was put on ice for 2 minutes. 950µl of SOC medium was added to the tube, and bacteria were left to grow at 37°C for an hour in a shaker (200-250 rpm). 50µl and 100µl of bacteria were spread onto two warmed LB Amp plates (100µg/ml Ampicillin), and incubated overnight at 37°C.

Colonies were picked and grown overnight at 37°C in 5ml LB Amp. They were then spun down and pelleted for 10 minutes at 14000rpm. DNA was extracted from the pellets using QIAprep Spin Miniprep Kit (QIAGEN). Transformation success was then assessed by sequencing (see 2.2.6.). The same procedure was performed to amplify bacteria from existing stock and extract DNA to prepare the plasmid for microinjection.

2.2.2. MOSCI single copy insertion

All transgenic lines in this study were built using Mos1-mediated single-copy insertion (MosSCI) in chromosome II, as described in Frokjaer-Jensen *et al.* (2012, 2008). The transgenes were cloned by GeneScript into the pCFJ151 vector, which contains a wild type copy of *unc-119* and flanking sequences homologous to a Mos1 transposon. The receiving strain, EG6699, carries a Mos1 transposon at a *ttTi5605* site in chromosome II and is homozygous for the *unc-119* mutation. The Mos1 transposase triggers Mos1 excision by homologous recombination at the flanking sequences, thus inserting the transgene into the genome.

2.2.2.1. Injection mix

The transgenes were co-injected with vectors expressing a transposase and *peel-1* toxin both expressed from heat shock promoters, and several proteins tagged with fluorescent marker mCherry. All plasmids were transformed into chemically competent cells, grown and harvested as described in 2.2.1. Final concentrations of different vectors in the mix were as follows:

Table 2: MosCI machinery and selection vectors in the injection mix

Plasmid	Description	Final concentration
pCFJ151	transgene	50ng/μl
pCFJ601	Peft-3:: transposase	50ng/μl
pGH8	Prab-3::mCherry (pan-neuronal)	10ng/μl
pCFJ90	Pmyo-2::mCherry (pharynx muscle)	2.5ng/μl
pCFJ104	Pmyo-3::mCherry (body muscle)	5ng/μl
pMA122	Phsp::peel-1	10ng/μl

20μl of above mix was made at a time. After spinning the mix down for a minute, the top 15μl were aliquoted into three 5μl aliquots stored at -20°C. The last 5μl were discarded.

2.2.2.2. Generation of transgenic strains by microinjection

The EG6699 strain was grown on HB101 *E. coli* at 15°C. Worms were prepared for microinjection by picking them as L4s the night before. Worms were pressed in a drop of halocarbon oil onto an agarose pad on a glass coverslip, to minimise movement. The mix described above was injected directly into the germlines of EG6699 worms, preferably closer to the proximal tip. Worms were put on a bacterial lawn to recover overnight and were cultivated at 25°C from then on, to activate the transposase and induce recombination with the transgene sequence.

Several selection markers allowed the distinction of successfully injected worms. Firstly, when the vectors had been successfully passed on to the progeny of the injected parent, the *unc-119(+)* gene within the pCFJ151 construct rescued the lack of movement phenotype of EG6699

worms. Secondly, when the transgene was inserted and stably inherited, and after a few generations, worms lost the non-inserted vectors; hence, worms positive to mCherry were discarded, as they still expressed the extrachromosomal vectors. Finally, a week from injection, plates containing *unc-119(+)* worms were heat-shocked for two hours to induce expression of the *peel-1* toxin and kill the false positive worms that still carry the injected vectors. Homozygosity for the inserted transgenes was verified by PCR.

2.2.3. Transgenes and transgene editing

Table 3 : List of transgenes used in this study

Green background highlights strains microinjected by me. The others had been made previously in the lab by my colleagues Consuelo Barroso and Nuria Ferrandiz.

<i>fqSi6:</i>	[<i>Phtp-1 htp-1 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi22:</i>	[<i>Phtp-1 HTP-1::6xhis 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi37:</i>	[<i>Phtp-1 HTP-1::GFP-C-Terminus 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi28:</i>	[<i>Phtp-1 htp-1(C-Terminus htp-2) 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi27:</i>	[<i>Phtp-1 htp-1(N-terminus htp-2) 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fq116:</i>	[<i>Phtp-1 htp-1 (HORMA htp-2) 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi141:</i>	[<i>Phtp-1 htp-1(HORMA A htp-2)::his 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi181:</i>	[<i>Phtp-1 htp-1(HORMA B htp-2)::his 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi167:</i>	[<i>Phtp-1 htp-1(HORMA C htp-2)::his 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi203:</i>	[<i>Phtp-1 htp-1(E191A)::GFP 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi208:</i>	[<i>Phtp-1 htp-1(M249K; L250S)::GFP 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi282:</i>	[<i>Phtp-1 htp-1(M249K; L250S)::6His 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi364:</i>	[<i>Phtp-1 htp-1(C239H)::his 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi247:</i>	[<i>Phtp-1 htp-1(N243H)::his 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi212:</i>	[<i>Phtp-1 htp-2(HORMA C htp-1)::GFP 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi303:</i>	[<i>Phtp-1 htp-2(HORMA C htp-1)::6His 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>fqSi284:</i>	[<i>Phtp-1 htp-2(HORMA C + C-term htp-1)::6His 3'UTRhtp-1 cb-unc-119(+)</i>]
<i>nT1:</i>	[<i>unc-?(n754) let-?(qls50) (IV; V)</i>] (Ferguson and Horvitz, 1985)

All transgenes except those specified below were produced by GeneScript through gene synthesis. Three transgenes, *fqSi247* (*htp-1^{N243H}::6His*), *fqSi282* (*htp-1^{M249K; L250S}::6His*), and *fqSi282* (*htp-2^{HTP-1 HORMA C}::6His*), were made with the Q5 Site-Directed Mutagenesis Kit (E0554, NEB), using *fqSi22* (for *fqSi247* and *fqSi282*) and *fqSi212* (for *fqSi282*) as templates. Briefly, the

template vectors were amplified by Q5 high fidelity polymerase with primers carrying the desired mutation; the new vectors were ligated and transformed into bacteria. The following PCR cycle was used:

Step	Temperature	Time
Initial denaturation	98°C	30s
25 cycles	98°C	10s
	60°C	30s
	72°C	5 minutes
Final extension	72°C	2 minutes

While *fqSi247* and *fqSi282* carry point mutations from the wild type *fqSi22* sequence, it is the GFP tag of *fqSi212* that was replaced by a histidine tag in *fqSi303*. This was carried out by designing primers flanking the GFP tag and facing away from it, with the 6His tag sequence as primer overhangs. All primers were designed with NEBaseChanger.

2.2.4. PCR amplification of genomic DNA and genotyping

2.2.4.1. Lysis

In order to obtain DNA for genotyping from whole worms, single worms were placed into 0.2ml PCR tubes with 10µl lysis buffer with proteinase K (100µl 10X PCR mix, 900µl H₂O, 50µl of 20mg/ml proteinase K). Tubes were frozen in liquid nitrogen and incubated in a thermocycler at 60°C for 70 minutes and then 95°C for 15 minutes. DNA could be used as PCR template, or stored at -20°C for future use. For other applications than genotyping, this protocol was applicable to multiple-worm lysis by adding up to 10 worms per PCR tube; for instance when extracting DNA for sequencing or to make a FISH probe.

2.2.4.2. PCR

The following mix and PCR cycle were used for amplification for genotyping and other applications, unless specified.

Mix	PCR programme
12.5µl Taq PCR Master mix (Qiagen)	Initial denaturation 95°C, 5min
1µl of left primer at 10µM	35 cycles: 95°C, 30s
1µl of right primer at 10µM	60°C, 30s
1-2µl of DNA extraction product	72°C, 1min
H ₂ O up to 25µl	Final elongation 72°C, 10 min

The PCR product was checked by electrophoresis on an agarose gel; about 2-5µl of the reaction were run per well. DNA could be quantified using Qubit and, if the DNA destined to sequencing, it was cleaned using a Qiagen PCR purification kit. Sequencing was carried out by the CSC genomics laboratory using an ABI3730xl instrument.

2.2.5. RNAi

2.2.5.1 Preparation and priming of plates

The RNAi experiment was performed following the protocol described by Kamath *et al.* (2001). Feeding plates were made 4-7 days before use, with standard NGM agar supplemented with ampicillin (Amp; 1:1000) and IPTG (1:1000). These concentrations of Amp and IPTG are used in all selective media for RNAi unless otherwise specified. HT115 *E. coli* used (Source Bioscience) contained a fragment of the *syp-1* gene cloned into a L4440 feeding vector, or an empty vector. The L4440 feeding vector has a T7 promoter driving expression of the desired genomic fragment; HT115 bacteria also have IPTG-inducible T7 polymerase activity, allowing control of the RNAi construct expression (Fraser *et al.*, 2000, Timmons *et al.*, 2001, Timmons and Fire, 1998). Bacteria were grown from glycerol stocks in Amp⁺ LB broth with also

tetracycline (1:1000), and plated onto Amp⁺ selective media. The next day, single colonies were picked and grown overnight in 20ml Amp⁺ LB broth. Bacteria were spun down at 3000rpm for 5 minutes, and resuspended in 1ml of Amp⁺ LB broth. Meanwhile, plates that were to receive these bacteria were primed with 1 mM IPTG and 50µg/ml Amp. Plates were seeded with 100µl resuspended bacteria about 5 minutes after priming, and left to grow overnight at 37°C.

2.2.5.2. Feeding protocol

For each desired genotype, L3-L4 worms were picked onto both RNAi and empty vector plates (~20 worms per plate), minimizing the amount of OP50 bacteria transferred with the worms. For strains maintained in heterozygosity such as the HORMA and HORMA C mutants, heterozygous worms were picked as the analysis would be performed in their progeny rather than them. Worms were left to lay eggs overnight then the mothers were removed. 3-4 days later, young worms were picked from these plates and transferred to fresh RNAi and empty vector plates prepared as in 2.2.5.1. At this stage, only homozygous worms for the desired genotypes were picked. Worms were harvested at 20-hour post L4 and dissected and stained as described in 2.3.1.

2.3. Cytological methods

2.3.1. Immunostaining

Immunostaining was performed as described by Martinez-Perez and Villeneuve (2005). Worms were age-matched and dissected on glass coverslips in a 15µl drop of EGG buffer (118 mM NaCl, 48 mM KCl₂, 2mM CaCl₂, 2mM MgCl₂, 5mM HEPES) containing 0.1% Tween, 20 hours post-L4. Germlines were fixed immediately in 2% paraformaldehyde for 5 minutes. During this time a microscope slide was applied to the coverslip. Germlines were freeze-cracked in liquid nitrogen at which point they were also transferred to the slide, and placed in -20°C methanol for at least a minute. The following steps were carried out at room temperature. Slides were washed with PBST (1X PBS, 0.1% Tween) and blocked for an hour in PBST 0.7% BSA. Primary

antibodies were left to incubate overnight in a humid chamber and secondary antibodies for two hours the next day, after three 10-minute PBST washes. After secondary antibody incubation, slides were washed 3 times 10 minutes with PBST and incubated with 100µl of 2µg/ml DAPI in water, for 5 minutes in the dark. Slides were washed a final time in PBST for 15 minutes, and mounted in a drop of Vectashield mounting medium. Table 4 recapitulates the primary antibodies used; all secondary Alexa Fluor antibodies (conjugated to Alexa 488 or 555) were used at a 1:500 concentration. Alternatively, for a simple DAPI staining, one could directly go to the DAPI final step after the initial PBST washes.

Table 4: Primary antibodies for immunofluorescence

Antibody	Host	Main Staining	Dilution	Source
HTP-1 (Q4421)	rabbit	HTP-1	1:400	SDIX
HIM-3	rabbit	Chromosome axis/SC axial element	1:500	(Zetka <i>et al.</i> , 1999)
SYP-1	chicken	SC central element	1:300	SDIX
RAD-51	rabbit	Resected DSB ends	1:10000	Novus
PLK-2	rabbit	Pairing centers during transition zone	1:500	(Nishi <i>et al.</i> , 2008)
REC-8	mouse	Cohesin	1:100	SDIX
6His Tag	mouse	Transgenic 6His- tagged proteins	1:200	N. Ferrandiz
GFP-488 (conjugated)	rabbit	Transgenic GFP- tagged proteins	1:200	Invitrogen (A-21311)

2.3.2. DNA FISH

2.3.2.1. Probe labelling

Genomic DNA was obtained by single worm lysis (see 2.2.4.) and 5 PCR reactions of 50µl were set up with 2µl lysis DNA each to amplify the 5S rRNA locus. The mix was as described in 2.2.4., with doubled proportions. The PCR products were checked by running 2µl from each tube on a gel, to verify the presence of a single band, of about 1kb. It was very important at this stage for the specificity of the FISH signal to only move forward with labelling with the tubes that displayed a single, clean band. Qualifying PCR products were mixed together and purified with the Qiagen MinElute PCR Purification Kit, using two columns eluted with 15µl water each. The final DNA concentration was measured by Qubit; 150-180 ng/µl was an appropriate concentration. Probes were labelled with a DIG nick-translation kit (Roche). 1µg DNA was mixed with 4µl of 5X kit reagent and water up to 20µl. This mix was incubated at 16°C for 90 in a thermocycler. The reaction was stopped by adding 1µl 0.5M EDTA, pH8, and incubating tubes at 65°C for 10 minutes. Probes could be stored at -20°C for future use.

2.3.2.2. DNA FISH protocol

Gonads were dissected as described in 2.3.1, and fixed 2 minutes only in 3.7% paraformaldehyde, pipetting gently up and down to extrude germlines and spread the fixing solution. Germlines were freeze-cracked in liquid nitrogen and placed in -20°C methanol. Once all slides were in cold methanol, the methanol Coplin jar was left at room temperature for 15-30 minutes to warm up gently. Slides were placed in 2X SSCT for a minute (2X SSC 0.1% Tween) and then washed twice in 2X SSCT for 5 minutes. Slides were dehydrated in a series of 70%, 90% and 100% ethanol washes, of 3 minutes each, and left to air dry. Slides were incubated with the probe in 15µL hybridisation solution (150-200ng probe, 2X SSCT, 50% formamide, 10% w/v dextran sulfate) in a FISH machine for 3 minutes at 93°C followed by 2 minutes at 72°C and 37°C overnight.

A first series of two post-hybridisation washes was carried out at 37°C in 2X SSCT 50% formamide for 20 minutes each. The following steps were all carried out at room temperature. Slides were washed 3 times 5 minutes in 2X SSCT, blocked 30 minutes in 2X SSCT 1% BSA, and incubated with 50µl secondary antibody (FITC anti-DIG, 1:100, Roche) for an hour. After the secondary antibody was added, slides were kept in the dark for the remainder of the experiment. Slides were washed 3 times 10 minutes in 2X SSCT, incubated with 40µl 2mg/ml DAPI in water for 7 minutes, and washed again 5 minutes in 2X SSCT. Slides were mounted on Vectashield mounting medium.

2.3.3. Microscopy and image processing

Images of immunostained germlines were acquired using a DeltaVision Deconvolution Microscope that comprises a DeltaVision system core and Olympus 1X70 microscope. Germlines were acquired as stacks of images that could be deconvolved to reverse optical distortion. Due to the size of *C. elegans* germlines, several images were required to capture an entire germline. Images were processed using softWoRx Explorer to make flat projections and/or analyse the stacks, for example to measure the distance between FISH foci (see 2.5.5.). Projections of germline portions were later reconstructed into the full germline in Adobe Photoshop.

2.4. Western blot

100 worms were harvested 24 hours post L4 in M9 medium, and cleaned by three rounds of pelleting at 1000rpm for a minute, discarding the supernatant, and washing with fresh M9. Worms were then transferred to 1ml 1X TE (10 mM Tris-HCl pH 8, 1 mM EDTA) with complete protease inhibitor (Roche). Tubes were spun down and liquid was removed to reach an approximate volume of 35µl. Worms were frozen in liquid nitrogen and ground with a pestle, three times. After adding 5µl of 5X Loemli, tubes were incubated at 100°C for 5-7 minutes. At this stage, the pellets could be kept at -20°C for future use.

Extracts were run on 10% Precision acrylamide gels with a Protein Dual Color Standards ladder, and transferred to a nitrocellulose membrane for an hour at 4°C. Membranes were blocked in TTBS (100ml 10XTBS, 1ml Tween, water up to 1L) 5% milk for an hour at room temperature. Membranes were incubated overnight at 4°C in 0.2µg/ml primary antibody in TTBS 5% milk. All remaining steps were carried out at room temperature: membranes were washed twice in TTBS for 15 minutes, incubated with the second antibody one hour in TTBS 5% milk, and washed again twice in TTBS for 15 minutes. Primary antibodies used were mouse anti-Histidine (N. Ferrandiz, 1:50000) and mouse anti-tubulin (Sigma, 1:10000), and the secondary antibody was goat anti-mouse HRP conjugated (Jackson Laboratories, 1:5000).

Membranes were incubated with ECL solution for two minutes before being placed in the cassette for imaging. Duration of film exposure was adjusted depending on the strength of the band. After developing the films, membranes could be washed 2 times 10 minutes in TTBS and either reused for another Western Blot or stored at 4°C.

2.5. Quantification and data analysis

2.5.1. Viability and incidence of males assay

Embryonic lethality and incidence of males among the viable progeny were assayed by culturing 10 worms of the desired genotype along with five positive and negative control worms onto single plates for a week. All worms were initially picked at the L4 stage to ensure the assay covered the entire egg-laying period. Every 12 hours, the worms were transferred to fresh plates and the eggs laid by each worm during that period were counted. For each 12-hour stage, the number of unhatched eggs was counted 24 hours after removing the mother from the plate and corresponds to the percentage of embryonic lethality. Adult progeny and males were counted four days after removing the mother from the plate.

2.5.2. Quantification of whole nucleus fluorescence intensity

Mean whole nuclear fluorescence was quantified on non-deconvolved stacks of images acquired under the following microscope settings, calibrated on the brightest wild type germline: DAPI, 32% 0.0272s; FITC, 100% 0.0593s. Germlines were divided into 3 zones of equal length from the onset of meiosis – defined visually by axis assembly and clustered chromatin – to the end of pachytene. In each zone, 20 representative nuclei were analysed with Fiji (Schindelin *et al.*, 2012), using a custom macro written by D. Dormann and K. Hng to measure fluorescence intensity (unpublished). Essentially, for each nucleus the nuclear region was defined by a manually drawn oval. The mean fluorescence intensity was measured on each layer of the stack, averaged across the stack, and stored in an Excel table. This could be repeated as many times as desired on multiple nuclei within a same image stack. Nuclei were only considered when the stack spanned their whole volume. Outputs from several images corresponding to the same zone were then pooled together for analysis.

2.5.3. Quantification of RAD-51 foci

Germlines were split into 7 zones of equal length from the mitotic tip to the end of pachytene. All nuclei that could be individually identified on the DAPI channel were scored – that is to say that nuclei that appeared overlapping or merged together could not be scored. The number of RAD-51 foci per nucleus was scored into the following categories: 0/1/2-3/4-6/7-12/≥12/stretch, using deconvolved stacks of images so as to distinguish between RAD-51 foci on different planes which may appear as a single focus on a projection.

2.5.4. Quantification of asynapsis

Germlines were divided into 5 zones of equal length from the onset of transition zone to the end of pachytene. Nuclei were only scored if they displayed HIM-3 tracks, evidence of meiotic chromosome axis assembly. This distinction is particularly important in zone 1 where a few nuclei still displayed diffuse chromatin and had not yet entered meiosis. Nuclei were scored

as displaying unsynapsed chromatin whenever a portion of HIM-3 track within was devoid of SYP-1 signal.

2.5.5. Quantification of pairing of FISH signals

Germlines were divided into six zones of equal length from the mitotic tip to the end of pachytene. All nuclei that could be individually identified from the DAPI staining were scored. FISH signals were considered paired if (i) a single fluorescence focus was present in the nucleus or (ii) two foci were observed in close proximity separated by a distance shorter than 0.6 μ m as measured in softWoRx Explorer.

2.5.6. Quantification of transition zone length from PLK-2 foci

Germlines were divided into rows of nuclei from the onset of transition zone to the end of pachytene. A row of nuclei was considered to still be in transition zone when over half the nuclei in the row displayed two or more PLK-2 foci. The relative length of transition zone was expressed as a percentage of the total number of rows. Similarly, quantification of the persistent expression of the PLK-2 signal on the X chromosome was achieved by counting the number of adjacent rows in which over half the nuclei displayed one or more PLK-2 focus.

2.5.7. Statistics

Statistical analysis of quantified results was carried out using SPSS. The Shapiro-Wilk test and Levene's test were used to test for normality and equality of variances, respectively. Means of datasets were compared by independent samples t-test unless stated otherwise; the observed differences were considered statistically significant when the test returned a P value inferior to 0.05.

CHAPTER 3: RESULTS – THE SPECIFIC FUNCTIONALITY OF HTP-1

IS DEPENDENT ON A SET OF 10 RESIDUES WITHIN THE HORMA

DOMAIN.

3.1. Introduction

3.1.1. Aims

C. elegans HTP-1 and HTP-2 are meiosis-specific HORMA domain proteins; they carry conserved residues with HOP1, REV7 and MAD2 thought to mediate responses to chromatin state. Despite 85% homology at the sequence level between them, HTP-1 performs several functions during meiosis that are not recapitulated by HTP-2: it promotes homologue pairing and recombination, protects sister chromatid cohesion until the second meiotic division, and participates in two checkpoints that coordinate pairing with SC assembly, and the end of homology search with complete synapsis, respectively. Some of the mismatches between the two proteins must therefore be essential residues for these HTP-1-specific functions. In order to investigate these residues, I created chimeric proteins replacing part of the HTP-1 sequence by the corresponding section of HTP-2 sequence. This allowed me to test for the functionality of HTP-1 domains independently, with the aim to narrow down the essential residues to the smallest possible number.

3.1.2 Method

Transgenic lines were built using Mos1-mediated single-copy insertion (MosSCI) in chromosome II. Expression of the chimeric proteins was driven by the *HTP-1* promoter, so keep as many parameters as possible identical to wild type HTP-1 expression. The transgenes were then crossed onto the *htp-1* null background to assess their competence for various HTP-1

functions. HTP-1 promotes chiasma formation and correct chromosome segregation, and I used the number of chromatin bodies at diakinesis and embryonic lethality and incidence of males as a readout of these processes, to guide the project and select which mutants to study more in depth.

3.2. The HORMA domain of HTP-1 is essential for chiasma formation and correct chromosome segregation

3.2.1. Chimeric HTP-1/HTP-2 proteins are expressed in

***C. elegans* and load onto chromosome axes**

The first set of transgenic lines expressing chimeric proteins were made by another lab member before the start of my project. For this first set, the protein was split into its three main domains: the HORMA domain, which is the putative functional region of HTP-1, and the N-Terminal and C-Terminal domains flanking the HORMA domain (Figure 4A). Each domain was replaced by the corresponding sequence of HTP-2, in transgenes *htp-1^{HTP-2 HORMA}*, *htp-1^{HTP-2 N-term}* and *htp-1^{HTP-2 C-term}* respectively. The proteins expressed from these transgenes would contain 7 (N-terminus), 36 (HORMA domain) and 18 (C-terminus) point mutations with the HTP-1 sequence (Figure 4A).

Because the sequences of HTP-1 and HTP-2 are so similar, α HTP-1 antibodies bind both proteins. Thus, in order to check for correct transgene expression and protein localisation, I crossed the three chimeric transgenes onto the *htp-1; htp-2* null background; in the absence of HTP-2, the α HTP-1(Q4421) bound HTP-1 alone. Figure 4B shows pachytene nuclei of *htp-1^{HTP-2 HORMA}*, *htp-1^{HTP-2 N-term}* and *htp-1^{HTP-2 C-term}* in the *htp-1; htp-2* background and stained for HTP-1. All three chimeric proteins, HTP-1^{HTP-2 N-term}, HTP-1^{HTP-2 HORMA}, and HTP-1^{HTP-2 C-term} are localised on meiotic chromosome axes, meaning they were expressed from the transgenes, and

loaded in a similar pattern to wild type HTP-1. This makes sense as HTP-2 also loads onto chromosome axes; the sequence responsible for loading may be shared.

3.2.2. The HORMA domain of HTP-1 is essential for chiasma formation

In order to assess functionality of the chimeric proteins, I looked at the number of chromatin bodies present at diakinesis, to assess chiasma formation. Indeed, in a wild type situation, each of the six chromosome pairs establishes a crossover; the homologs then remain physically bound together by the crossover and sister chromatid cohesion, and at diakinesis each pair appears as a single DNA body, or bivalent, held together by a chiasma (Figure 5A, WT). In contrast, when one or more crossover fails to form, additional DNA bodies are observed as homologs are not bound together by a chiasma (Figure 5A, *htp-1*). Single homologs, or univalents, appear as smaller and rounder DNA bodies.

Worms carrying the *htp-1*^{HTP-2 N-term} or *htp-1*^{HTP-2 C-term} transgene in a *htp-1* null background displayed an average of 6 and 6.5 chromatin bodies at diakinesis, respectively, indicating that most nuclei formed a full set of crossovers (Figure 5). Notably, in worms expressing HTP-1^{HTP-2 C-term} one third of diakinesis nuclei contained univalents, pointing to a possible role of the C-Terminus of HTP-1 in regulating chiasma formation.

In contrast, worms carrying the *htp-1*^{HTP-2 HORMA} transgene in the *htp-1* null background displayed univalents (Figure 5A). Diakinesis oocytes in these worms displayed an average number of 9.4 chromatin bodies (Figure 5B), meaning only two to three crossovers were established per nucleus, on average. This singles out the HORMA domain as a crucial region for the role of HTP-1 in chiasma formation. Interestingly, 96% of nuclei did form at least one crossover, which is double the amount of the *htp-1* null. Moreover, these mutants displayed high variation in the number of DAPI-stained bodies at diakinesis, scoring the whole spectrum from 6 bivalents to 12 univalents. HTP-1^{HTP-2 HORMA} is therefore competent to support chiasma formation, but does not ensure that each chromosome pair forms a crossover. This latter

Figure 4. Whole domain chimeric proteins are properly expressed from transgenes and load onto the chromosome axes.

(A) Sequence alignment of HTP-1 and HTP-2 using Multalin (Corpet, 1988). >90% consensus sequence is shown in red, >50% low consensus mismatches are in blue. The three protein domains, N-Terminus, C-Terminus and HORMA domain, are indicated. Each of these domains was separately substituted for that of HTP-2 in a set of recombinant proteins. Blue bar shows the 100-amino-acid peptide recognised by the α HTP-1 antibody.

(B) Projection of pachytene nuclei of worms expressing *htp-1^{HTP-2 N-Term}*, *htp-1^{HTP-2 C-Term}* or *htp-1^{HTP-2 HORMA}* in the *htp-1*; *htp-2* background, stained with α HTP-1(Q4421), and counterstained with DAPI. The α HTP-1(Q4421) antibody recognises both HTP-1 and HTP-2; in the absence of HTP-2, this staining was HTP-1 specific. The transgenic proteins HTP-1^{HTP-2 N-Term}, HTP-1^{HTP-2 C-Term} and HTP-1^{HTP-2 HORMA} (green) appear loaded onto chromosome axes. Therefore, all three transgenes are properly expressed, and the corresponding proteins are competent for loading. Scale bar 5 μ m.

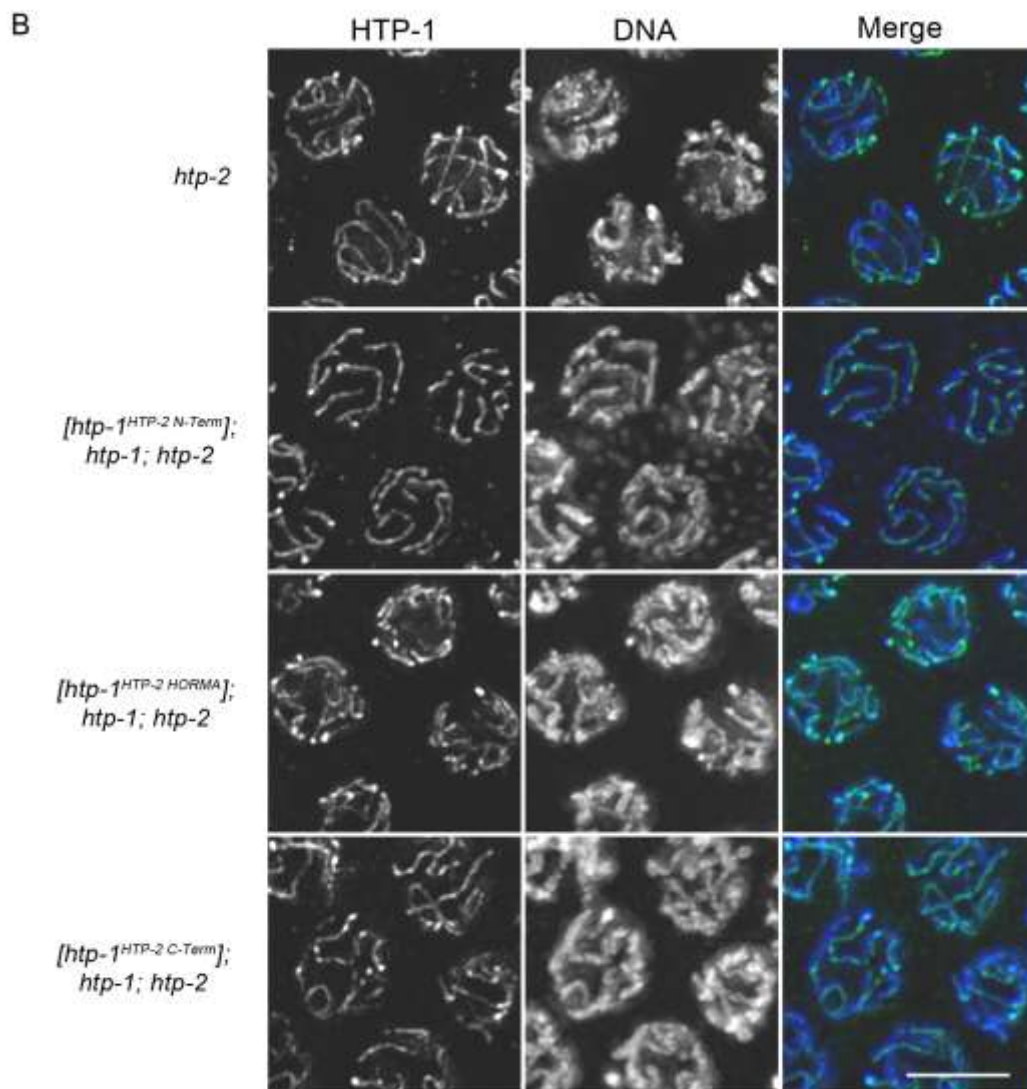
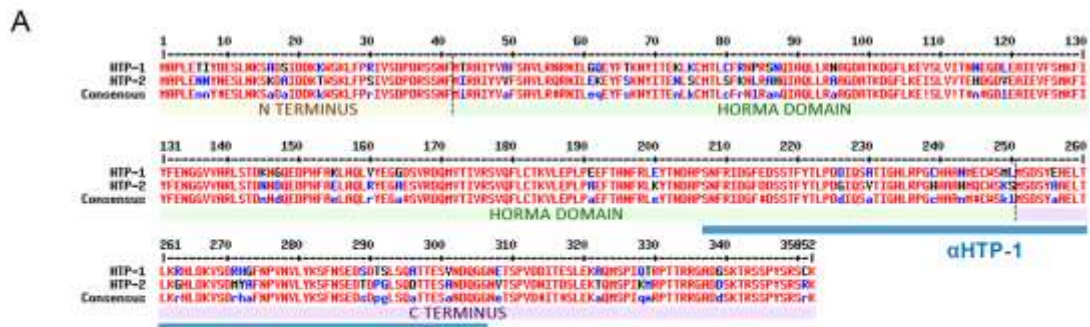
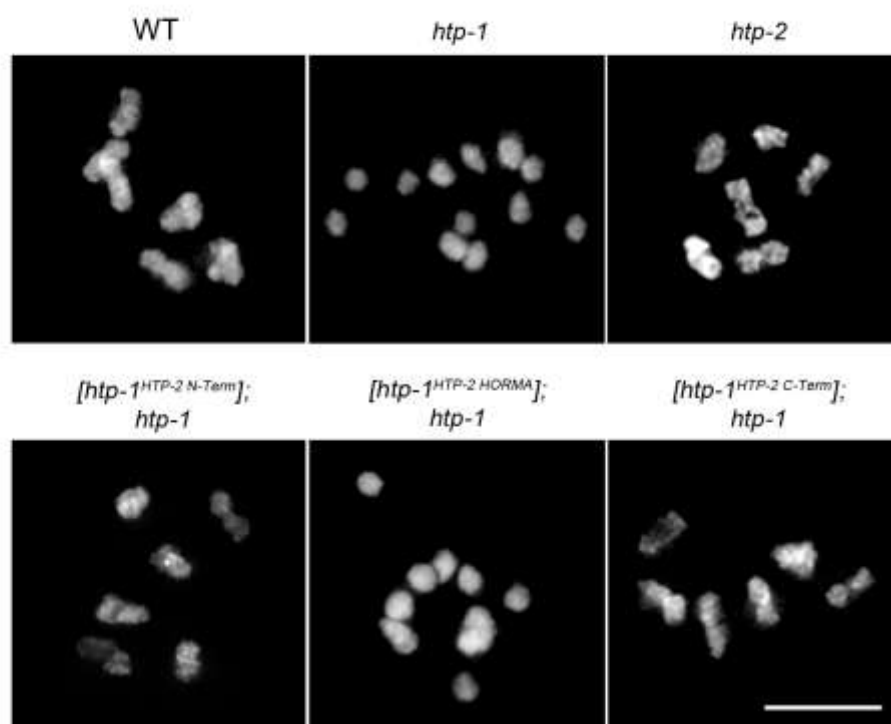


Figure 5. The HORMA domain of HTP-1 contains essential residues for crossover formation.

(A) Projections of representative DAPI-stained diakinesis oocytes of worms lacking the endogenous *HTP-1* locus and expressing *htp-1^{HTP-2 N-term}*, *htp-1^{HTP-2 HORMA}* and *htp-1^{HTP-2 C-term}*, respectively. The increased number of chromatin bodies in *htp-1^{HTP-2 HORMA}* is indicative that several chromosome pairs are not forming crossovers. Shown for reference along the wild type and *htp-1* null mutant controls, is an *htp-2* mutant displaying six bivalents: HTP-2 is not required for crossover formation. Wild type control: [*HTP-1*]; *htp-1*. Scale bar 5µm.

(B) Quantification of the number of chromatin bodies observed in the diakinesis oocytes of worms lacking the endogenous *HTP-1* locus and expressing *htp-1^{HTP-2 N-term}*, *htp-1^{HTP-2 HORMA}* and *htp-1^{HTP-2 C-term}*, respectively. Number of nuclei counted: 40 (WT), 53 (*htp-1^{HTP-2 N-term}*), 135 (*htp-1^{HTP-2 HORMA}*), 86 (*htp-1^{HTP-2 C-term}*), 61 (*htp-1*).

A



B

Genotype	Chromatin bodies at diakinesis
WT	6.0 ± 0.0
<i>htp-1</i>	11.2 ± 0.9
<i>[htp-1^{HTP-2 N-Term}]; htp-1</i>	6.0 ± 0.1
<i>[htp-1^{HTP-2 HORMA}]; htp-1</i>	9.4 ± 1.4
<i>[htp-1^{HTP-2 C-Term}]; htp-1</i>	6.5 ± 0.9

function must require specific residues within the HORMA domain, which are different in the HTP-2 sequence.

3.2.3. The HORMA domain of HTP-1 is essential for chromosome segregation

Another facet of the *htp-1* null mutant phenotype is chromosome mis-segregation during meiosis. Mis-segregation results in aneuploidy among the offspring: autosomal aneuploidy leads to a high embryonic lethality, while X chromosome aneuploidy leads to a higher incidence of males among the progeny (Hodgkin *et al.*, 1979). I therefore scored embryonic lethality and incidence of males in the progeny of worms expressing *htp-1^{HTP-2 HORMA}*, *htp-1^{HTP-2 N-term}* and *htp-1^{HTP-2 C-term}* in an *htp-1* null background, to assess the functionality of the chimeric proteins in ensuring proper chromosome segregation.

In *htp-1^{HTP-2 N-term}* worms, embryonic lethality and incidence of males in the progeny were negligible and on par with wild type worms. However, both *htp-1^{HTP-2 HORMA}* and *htp-1^{HTP-2 C-term}* worms displayed higher numbers of dead eggs and males in the progeny (Figure 6). In the HORMA domain mutants in particular, the embryonic lethality of 88% was equal to that of the *htp-1* null mutant although the *him* phenotype was not as severe: “only” 21% of the progeny from the HORMA domain mutants were males, compared to 53% of the progeny of the *htp-1* worms. These observations indicate that the HORMA domain of HTP-1 is essential for promotion of correct chromosome segregation. The embryonic lethality resulting from the replacement of the HTP-1 C-terminus by the HTP-2 C-terminus was 36%, hinting at a role for the C-terminus in chromosome segregation, either a less crucial role than that of the HORMA domain, or perhaps one that can be partly fulfilled by the C-terminus of HTP-2.

3.2.4. Conclusion

Three strains carrying chimeric *htp-1* transgenes were initially made, each carrying mutations in a single domain of HTP-1. Although all three proteins were expressed and appropriately localised, the resulting phenotypes varied greatly. The N-terminus of HTP-2 substituted the N-terminus of HTP-1 without a phenotypic change, indicating that HTP-1-specific functional residues were not present in this portion. Indeed, the HORMA domain of HTP-1 was singled out as crucial for the bulk of HTP-1 function, consistent with the definition of this domain as a set of conserved residues across organisms (Aravind and Koonin, 1998). The diversity of HORMA domain proteins implies that their common features as sensors of chromatin state may depend on the HORMA domain. In this case, the HORMA domain of HTP-2 could not support correct chromosome segregation and although the HTP-1^{HTP-2 HORMA} protein was competent to promote chromosome formation, it failed to do so consistently across every chromosome pair in a nucleus. The C-terminus of HTP-1 also contained residues implicated in its function, as substituting it resulted in defects in chiasma formation and significant embryonic lethality. This observation highlights a specificity of this family of meiotic proteins in relying on a C-terminal domain, as MAD2 and REV7 for instance comprise solely of a HORMA domain (Aravind and Koonin, 1998).

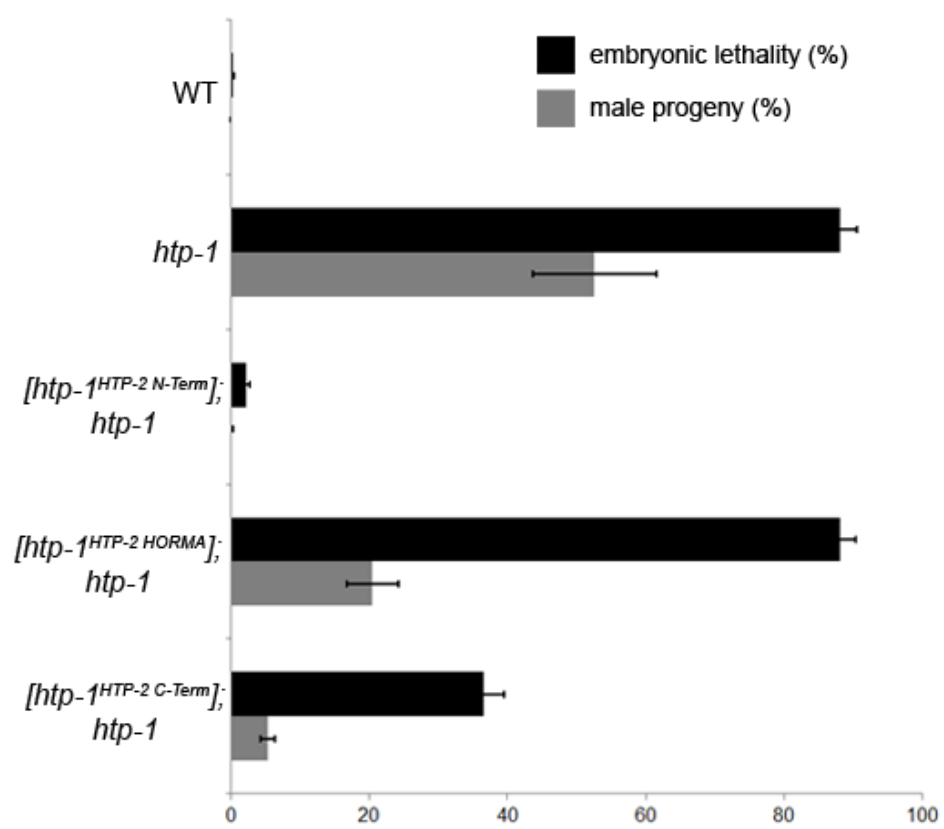
Given the stronger phenotype associated with the HORMA domain mutant, and the closer similarity of the *htp-1*^{HTP-2 HORMA} phenotype to that of the *htp-1* null mutant, I decided to further analyse this domain in an attempt to narrow down the functional regions and/or residues of HTP-1 among the 36 point mutations between the HORMA domains of HTP-1 and HTP-2.

Figure 6. The HORMA domain of HTP-1 contains essential residues for correct chromosome segregation.

Embryonic lethality and proportion of males among surviving progeny for *htp-1^{HTP-2 N-term}*, *htp-1^{HTP-2 HORMA}* and *htp-1^{HTP-2 C-term}* in the *htp-1* deletion background, compared to *htp-1* and [*HTP-1*]; *htp-1* (wild type control). Embryonic lethality and high incidence of males are indicative of aneuploidy caused by mis-segregation of chromosomes during meiosis. The high lethality and incidence of males associated with *htp-1^{HTP-2 C-term}* (37% lethality, 5% males) and *htp-1^{HTP-2 HORMA}* (88% lethality, 21% males) indicate that the HORMA domain of HTP-1, and to a lesser extent the C-Terminus, are essential for chromosome segregation.

Number of laid eggs counted: 1201 (WT), 2726 (*htp-1^{HTP-2 N-term}*), 1864 (*htp-1^{HTP-2 HORMA}*), 1888 (*htp-1^{HTP-2 C-term}*), 1391 (*htp-1*).

Number of adults scored: 1478 (WT), 2779 (*htp-1^{HTP-2 N-term}*), 160 (*htp-1^{HTP-2 HORMA}*), 1068 (*htp-1^{HTP-2 C-term}*), 40 (*htp-1*).



3.3. The functionality of the HORMA domain of HTP-1 depends on a domain close to the C-Terminus containing ten mismatches with HTP-2

3.3.1. Establishing three HORMA subdomains within HTP-1

In order to study the impact of fewer mutations at a time within the HORMA domain of HTP-1, I divided the HORMA domain into three subdomains: HORMA A, HORMA B and HORMA C. That division was based not on the length of resulting subdomains but on the number of mismatches within: I aimed to separate the point mutations between the sequences of HTP-1 and HTP-2 into similar-sized clusters (Figure 7A). HORMA A and HORMA B contain 13 mismatches each, while HORMA C contains 10.

The *htp-1^{HTP-2 HORMA A}*, *htp-1^{HTP-2 HORMA B}* and *htp-1^{HTP-2 HORMA C}* transgenes were designed with an added 6xHistidine tag on the proteins, to facilitate observation and analysis. I injected all three transgenes into EG6699 worm germlines to create the strains, and crossed them onto the *htp-1* null background to assess expression and functionality.

3.3.2. HTP-1^{HTP-2 HORMA B} and HTP-1^{HTP-2 HORMA C} load onto chromosome axes

Figure 7B shows pachytene nuclei of worms expressing *htp-1^{HTP-2 HORMA A}*, *htp-1^{HTP-2 HORMA B}* and *htp-1^{HTP-2 HORMA C}* stained with α HisTag. The proteins HTP-1^{HTP-2 HORMA B} and HTP-1^{HTP-2 HORMA C} appear localised with chromosome axes, indicating these two tagged proteins are expressed and loaded onto chromosome axes. Strikingly, HTP-1^{HTP-2 HORMA A} could only be detected faintly in pachytene nuclei, and not clearly onto chromosome axes. It is unclear whether the protein is unable to load or present in too small a quantity to be detected at a level approximating the HORMA B and HORMA C mutants. However, HTP-1^{HTP-2 HORMA A} does seem to be expressed from the transgene.

3.3.3. Residues in the HORMA C domain of HTP-1 promote chiasma formation

The formation of chiasmata in HORMA subdomain mutants was assessed by looking at the number of chromatin bodies present at diakinesis. Worms expressing *htp-1^{HTP-2 HORMA A}* and *htp-1^{HTP-2 HORMA B}* in the *htp-1* null background displayed six bivalents at diakinesis, indicating that the HORMA A and HORMA B domains are not responsible for the phenotype previously observed in the HORMA whole domain mutants (Figure 8). Interestingly, despite not being detected on chromosome axes comparably to the wild type, HTP-1^{HTP-2 HORMA A} appears perfectly capable of recapitulating HTP-1 function in this instance.

In worms expressing *htp-1^{HTP-2 HORMA C}*, diakinesis oocytes often contained univalents (Figure 8); they were missing three to four crossovers on average, similarly to the *htp-1^{HTP-2 HORMA}* oocytes. Therefore, the residues essential for comprehensive chiasma formation in the HORMA domain of HTP-1 are restricted to the HORMA C domain, closer to the C-terminus.

3.3.4. Residues in the HORMA C domain of HTP-1 promote correct chromosome segregation

Mis-segregation of autosomes or X chromosomes in *C. elegans* leads to embryonic lethality and high incidence of males, respectively. I scored both these proportions in the progeny of worms lacking the endogenous *HTP-1* locus and expressing *htp-1^{HTP-2 HORMA A}*, *htp-1^{HTP-2 HORMA B}* and *htp-1^{HTP-2 HORMA C}*. Worms expressing *htp-1^{HTP-2 HORMA A}* and *htp-1^{HTP-2 HORMA B}* display low levels of embryonic lethality and very few to no males among the progeny (Figure 9). Therefore, the HTP-1^{HTP-2 HORMA A} and HTP-1^{HTP-2 HORMA B} proteins support correct chromosome segregation during meiosis. In contrast, 77% of eggs from *htp-1^{HTP-2 HORMA C}* worms did not hatch (Figure 9), indicating that the residues mutated in HTP-1^{HTP-2 HORMA C} are important for correct autosome segregation. Interestingly, the surviving progeny from these same worms did not contain males. The mutations within *htp-1^{HTP-2 HORMA C}* therefore do not seem to affect segregation of the X chromosome.

Figure 7. HORMA subdomain chimeric proteins are properly expressed from transgenes and load onto the chromosome axes.

(A) Sequence alignment of HTP-1 and HTP-2 using Multalin (Corpet, 1988). >90% consensus sequence is shown in red, >50% low consensus mismatches are in blue. The two protein domains, N-Terminus and C-Terminus, and three HORMA subdomain, HORMA A, B and C, are indicated. Each of these domains was separately substituted for that of HTP-2 in a set of recombinant proteins, tagged with 6xHistidine.

(B) Projection of pachytene nuclei of worms expressing *htp-1^{HTP-2 HORMA A}*, *htp-1^{HTP-2 HORMA B}* or *htp-1^{HTP-2 HORMA C}* in the *htp-1* null background, stained with α HisTag, and counterstained with DAPI. The transgenic proteins HTP-1^{HTP-2 HORMA B} and HTP-1^{HTP-2 HORMA C} (green) appear loaded onto chromosome axes; both transgenes are properly expressed, and the corresponding proteins are competent for loading. HTP-1^{HTP-2 HORMA A} was not detected onto chromosome axes. Scale bar 5 μ m.

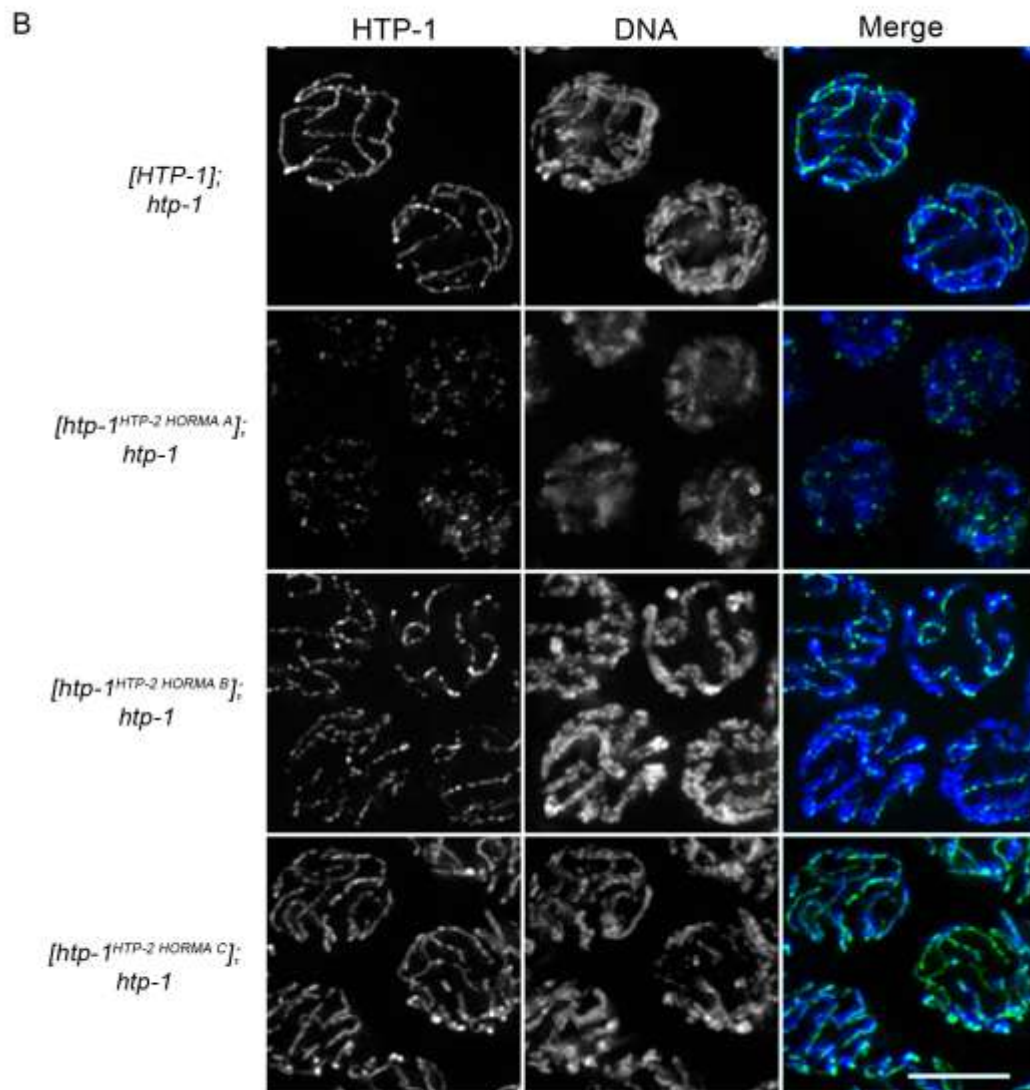
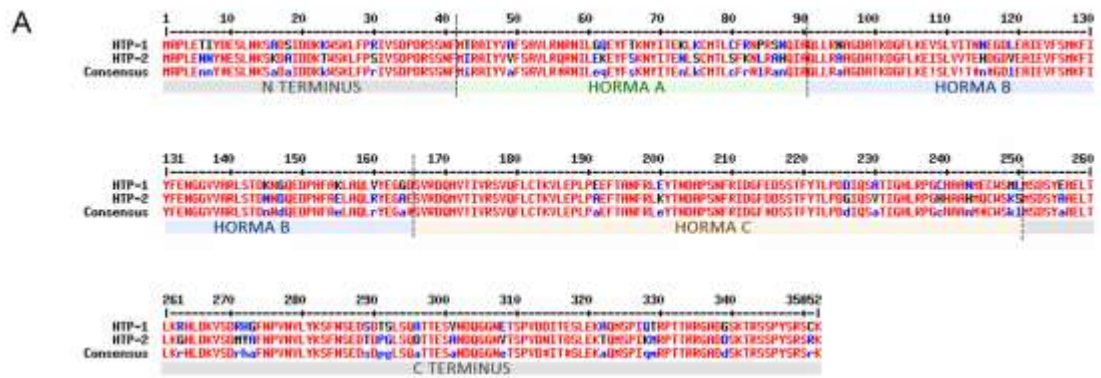
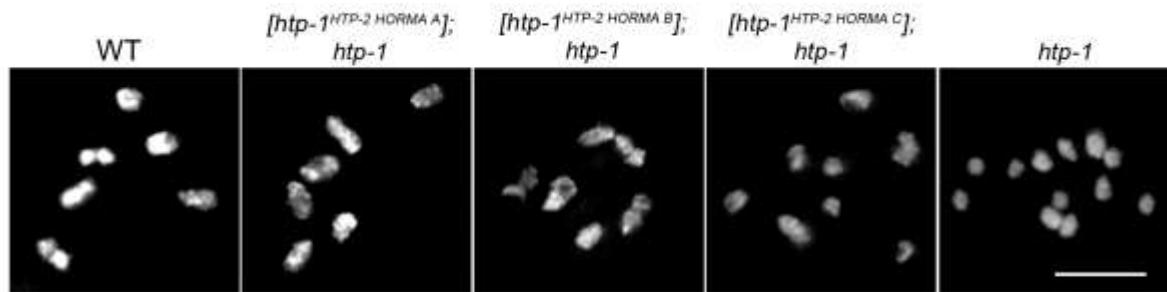


Figure 8. The HORMA C region of HTP-1 contains essential residues for crossover formation.

(A) Projections of representative DAPI-stained diakinesis oocytes of worms lacking the endogenous *HTP-1* locus and expressing *htp-1^{HTP-2 HORMA A}*, *htp-1^{HTP-2 HORMA B}* and *htp-1^{HTP-2 HORMA C}*, respectively. The *htp-1^{HTP-2 HORMA C}* oocyte contains 8 DAPI bodies, indicating that this particular nucleus only formed four out of the six expected crossovers. Wild type control: [*HTP -1::6xHis*]; *htp-1*. Scale bar 5µm.

(B) Quantification of the number of chromatin bodies observed in the diakinesis oocytes of worms lacking the endogenous *HTP-1* locus and expressing *htp-1^{HTP-2 HORMA A}*, *htp-1^{HTP-2 HORMA B}* and *htp-1^{HTP-2 HORMA C}*, respectively. Number of nuclei counted: 51 (WT), 17 (*htp-1^{HTP-2 HORMA A}*), 59 (*htp-1^{HTP-2 HORMA B}*), 46 (*htp-1^{HTP-2 HORMA C}*), 61 (*htp-1*). Wild type control: [*HTP -1::6xHis*]; *htp-1*.

A



B

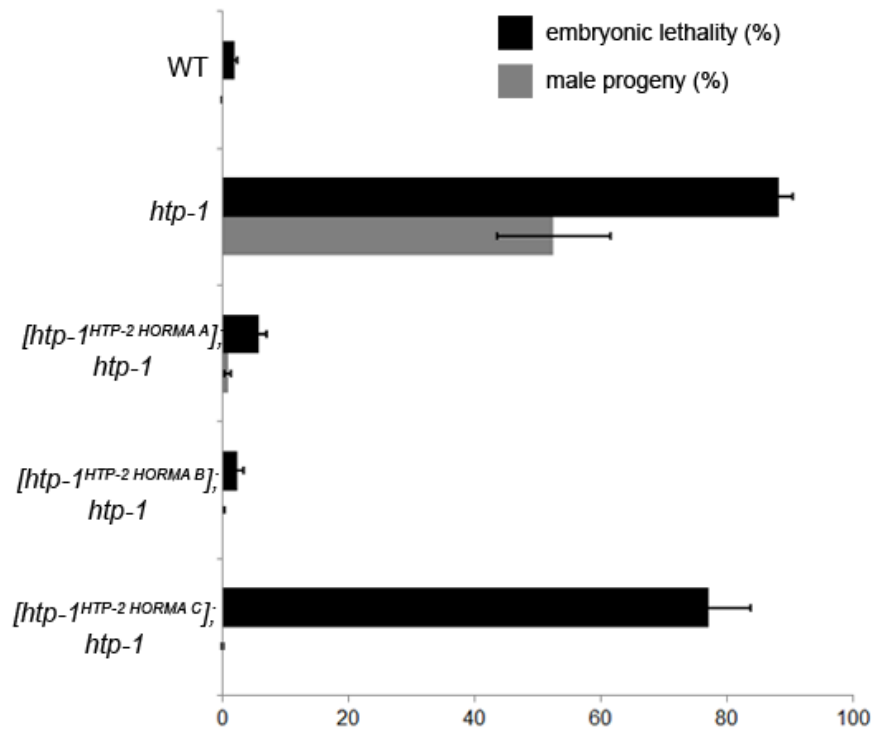
Genotype	Chromatin bodies at diakinesis
WT	6.0 ± 0.0
<i>htp-1</i>	11.2 ± 0.9
<i>[htp-1^{HTP-2 HORMA A}]; htp-1</i>	6.0 ± 0.0
<i>[htp-1^{HTP-2 HORMA B}]; htp-1</i>	6.0 ± 0.2
<i>[htp-1^{HTP-2 HORMA C}]; htp-1</i>	9.1 ± 1.6

Figure 9. The HORMA C region of HTP-1 contains essential residues for correct chromosome segregation.

Embryonic lethality and proportion of males among surviving progeny for *htp-1^{HTP-2 HORMA A}*, *htp-1^{HTP-2 HORMA B}* and *htp-1^{HTP-2 HORMA C}* in the *htp-1* deletion background, compared to *htp-1* and [*HTP -1::6xHis*]; *htp-1* (wild type control). Embryonic lethality and high incidence of males are indicative of aneuploidy caused by mis-segregation of chromosomes during meiosis. The high lethality and incidence of males associated with *htp-1^{HTP-2 HORMA C}* indicates that the HORMA C region of HTP-1 is essential for chromosome segregation.

Number of laid eggs counted: 1339 (WT), 2415 (*htp-1^{HTP-2 HORMA A}*), 1578 (*htp-1^{HTP-2 HORMA B}*), 1590 (*htp-1^{HTP-2 HORMA C}*), 1391 (*htp-1*).

Number of adults scored: 1271 (WT), 1914 *htp-1^{HTP-2 HORMA A}*, 1643 (*htp-1^{HTP-2 HORMA B}*), 31 (*htp-1^{HTP-2 HORMA C}*), 40 (*htp-1*).



3.3.5. Conclusion

After the HORMA domain of HTP-1 was shown to be the main domain involved with providing HTP-1-specific function, I divided this domain into three subdomains A, B and C, each containing a similar number of mutations. All three associated proteins were expressed from the transgenes, but only HTP-1^{HTP-2 HORMA B} and HTP-1^{HTP-2 HORMA C} were detected loaded onto meiotic chromosome axes; HTP-1^{HTP-2 HORMA A} was not. Despite this, HTP-1^{HTP-2 HORMA A} supported chiasma formation and correct chromosome segregation in an *htp-1* null background. HTP-1^{HTP-2 HORMA B} did too. Only *htp-1*^{HTP-2 HORMA C} failed to complement the *htp-1* null mutation: worms expressing this transgene presented defects in chiasma formation and autosome segregation. The C-terminal portion of the HORMA domain has already been shown to be structurally distinct from the globular bulk of the domain (Hara *et al.*, 2010, Kim *et al.*, 2014, Luo *et al.*, 2000), and essential to the functional conformation of MAD2 (Yang *et al.*, 2008), suggesting a common HORMA domain mechanism shared by *C. elegans* meiotic proteins. The X chromosome in *htp-1*^{HTP-2 HORMA C} worms seemed to segregate correctly, indicating that it may be subject to a different mechanism that does not depend on the HORMA C region. The *htp-1*^{HTP-2 HORMA C} mutant phenotype narrows down residues important to HTP-1 function to only ten mismatches between the HTP-1 and HTP-2 sequences within the HORMA C region.

3.4. The functionality of the HORMA C domain of HTP-1 depends on a cluster of residues within the domain.

3.4.1. Selection of five point mutations most likely to modify protein function.

Given the strong phenotype associated with mutating the HORMA C region of HTP-1, an attempt was made to isolate even more precisely which of the ten mismatches between HTP-1 and HTP-2 were responsible for the observed defects in chiasma formation and chromosome segregation. Firstly, the ten mismatches (Figure 10A) were observed to determine which were most likely to induce a functional change in the protein. Five candidates were selected to be made into point mutants: E191, C239, N243, M249 and L250.

The glutamic acid to alanine substitution in position 191 induces a loss both of charge and of a bulky side chain, which could lead to structural differences or changed affinity for interaction; moreover, this residue is part of a high consensus sequence conserved across the HORMA domains of yeast, rice, and humans (Figure 10B), indicating it could be important for the way HORMA domains function. Similarly, substituting the small cysteine in position 239 with a histidine carrying a positive charge and a ring in its side chain could disrupt protein structure.

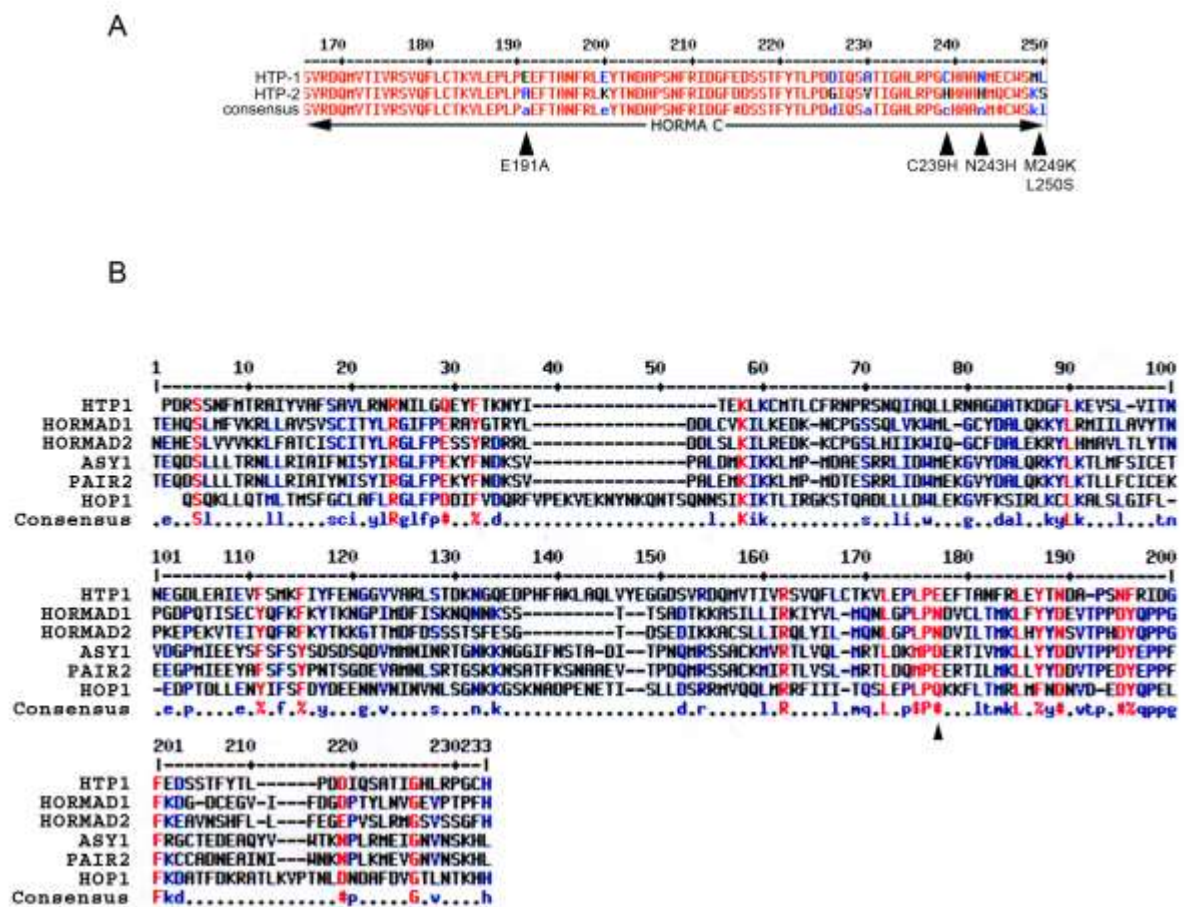
At the time of this study, a predictive model of the 3D structure of HTP-1 also showed that of all the substituted residues between HTP-1 and HTP-2 in the HORMA C region, only N243 was part of the secondary structure and located in a β -strand (Xiaodong Zhang, personal communication).

Finally, a double substitution of methionine 249 and leucine 250 to a lysine and serine (respectively) seemed an interesting candidate not only because of the two adjacent mutations but also because they are located at the very edge of the HORMA domain, close to the C-Terminus, which I have shown to play a role in HTP-1 function (see 3.3.2.).

Figure 10. Five point mutations most likely to modify protein function.

(A) Sequence alignment of the HORMA C domains of HTP-1 and HTP-2 using Multalin (Corpet, 1988). >90% consensus sequence is shown in red, >50% low consensus mismatches are in blue. Arrows indicate the mutations thought most likely to cause the phenotype observed in *htp-1^{HTP-2 HORMA C}* worms, and for which I made point mutants. Two transgenes were made with a GFP tag: *htp-1^{E191A}* and *htp-1^{M249K; L250S}*; and three with a 6xHistidine tag: *htp-1^{M249K; L250S}*, *htp-1^{N243H}* and *htp-1^{C239H}*.

(B) Sequence alignment of the HORMA domains of HTP-1 (*C. elegans*), HORMAD1 (Human), HORMAD2 (Human), ASY1 (*A. thaliana*), PAIR1 (Rice) and HOP1 (Yeast), using Multalin (Corpet, 1988). >90% consensus sequence is shown in red, >50% low consensus mismatches are in blue. Arrow indicates E191 in HTP-1 – here shown in position 177 of the HORMA domain alignment. The sequence between positions 162 and 201 is conserved: two thirds of the sequence have a consensus of 50% or higher. Especially, there is over 90% consensus across the six sequences for HTP-1(E191) and the two residues preceding it. The residue homologous to E191 is either Asparagine/Aspartic Acid or Glutamine/Glutamic acid; they not only have similar side chains but the same capacity, in the amide form, for forming hydrogen bonds.



3.4.2. A first set of GFP-tagged transgenes, *htp-1^{E191A}::GFP* and *htp-1^{M249K; L250S}::GFP*, are expressed and load onto meiotic axes

A first set of worm strains were made, carrying *htp-1* transgenes with substitutions E191A and M249K;L250S, respectively. A GFP tag was added at the end of the protein in the hope to visualise the associated proteins more easily for analysis. Figure 11A shows pachytene nuclei of worms expressing *htp-1^{E191A}::GFP* and *htp-1^{M249K; L250S}::GFP* in an *htp-1* null background, stained with α GFP and counterstained with DAPI. Both proteins appear in green on the chromosome axes, indicating that both transgenes are expressed in these worms, and that the associated proteins are competent for loading.

3.4.3. HTP-1^{M249K; L250S}::GFP cannot fully support chiasma formation

Chiasma formation in *htp-1^{E191A}::GFP* and *htp-1^{M249K; L250S}::GFP* worms was assessed by scoring the number of chromatin bodies at diakinesis (Figure 11). 87% of diakinesis nuclei in worms expressing *htp-1^{E191A}::GFP* presented six chromatin bodies, indicating they formed a full set of crossovers. In contrast, oocytes from worms expressing *htp-1^{M249K; L250S}::GFP* frequently displayed univalents (Figures 11B, 11C): the average number of DAPI-stained bodies in these nuclei was 7.2, with 63% of nuclei displaying either 7 or 8 bodies. These nuclei were missing one or two crossovers respectively, indicating that M249 and L250, but not E191, may be implicated in chiasma formation in HTP-1.

3.4.4. The GFP tag disrupts protein function in certain settings

During the course of experiments pertaining to other projects in the laboratory, colleagues discovered that worms expressing a GFP-tagged wild type HTP-1 protein from a transgene, presented univalents at diakinesis in a *htp-1; htp-2* double mutant background. However, HTP-2 is not essential to chiasma formation and HTP-1 is capable of fulfilling this role in a *htp-2* null background (1.3.6.4., Figure 5A); clearly, the transgenic protein although wild

type did not behave like the native protein. Therefore, it was concluded that the GFP tag, perhaps due to the bulky nature of GFP, could in some instances disrupt HTP-1 function, and be responsible for false positives. For this reason, subsequent *htp-1* substitution mutants, *htp-1^{C239H}* and *htp-1^{N243H}* were made with a Histidine tag. In addition to this, a new strain expressing *htp-1^{M249K; L250S}::6xHis* was built, to ensure the observed phenotype was triggered by the mutations rather than the GFP tag.

3.4.5. HTP-1^{M249K; L250S}::6xHis, HTP-1^{C239H}::6xHis and

HTP-1^{N243H}::6xHis are expressed and load onto meiotic axes

Figure 12A shows pachytene nuclei of worms lacking the endogenous *HTP-1* locus and expressing *htp-1^{M249K; L250S}::6xHis*, *htp-1^{C239H}::6xHis* and *htp-1^{N243H}::6xHis*. The nuclei have been stained with α HisTag and counterstained with DAPI. The proteins HTP-1^{M249K; L250S}::6xHis, HTP-1^{C239H}::6xHis and HTP-1^{N243H}::6xHis (green), appear to localised to the meiotic chromosome axes. Therefore, all three transgenes are expressed in these worms, and the associated HTP-1 proteins are competent for loading.

3.4.6. HTP-1^{M249K; L250S}::6xHis, HTP-1^{C239H}::6xHis and

HTP-1^{N243H}::6xHis are competent for chiasma formation

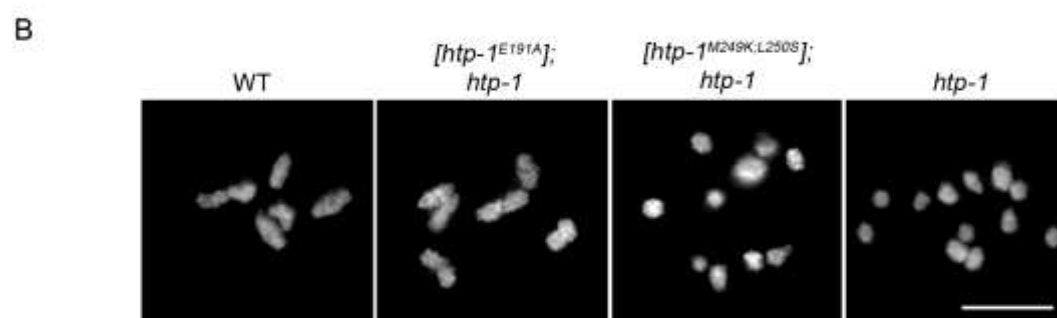
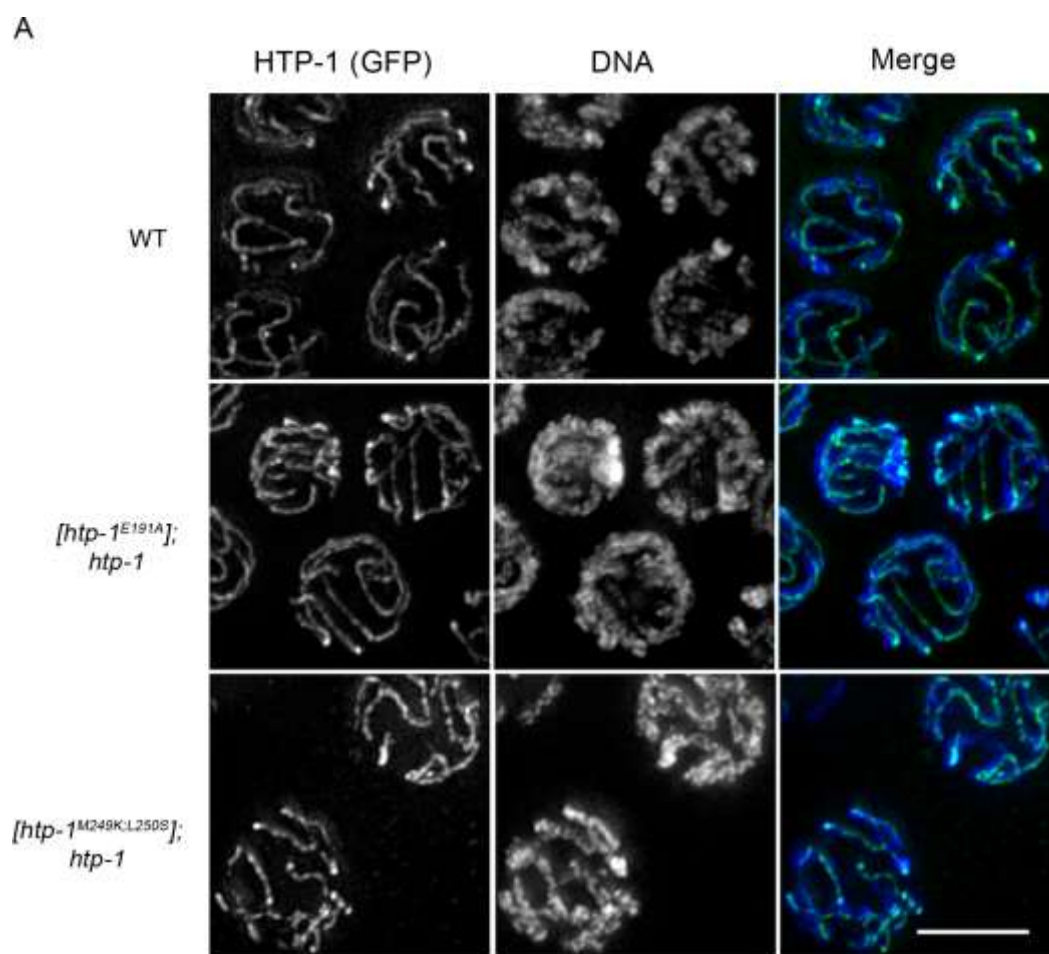
At diakinesis, oocytes of worms expressing *htp-1^{M249K; L250S}::6xHis*, *htp-1^{C239H}::6xHis* and *htp-1^{N243H}::6xHis* in the *htp-1* null background contained mostly six bivalents, as in the wild type (Figures 12B, 12C), demonstrating that the chiasma formation defect observed in HORMA C mutants did not rely solely on any of the substitutions tested. Notably, the average number of chromatin bodies in *htp-1^{M249K; L250S}::6xHis* worms was 6.1, indicating that in most cases each chromosome pair formed a crossover, a marked difference from the GFP-tagged transgene of the same sequence, for which diakinesis oocytes contained an average of 7.2 chromatin bodies (Figure 11C). This difference highlighted the role of the GFP tag in the previously observed phenotype, and established that HTP-1(M249) and HTP-1(L250) are not essential to chiasma formation.

Figure 11. GFP-tagged *htp-1*^{M249K; L250S} worms present defects in chromosome formation

(A) Projection of pachytene nuclei of worms expressing *htp-1*^{E191A::GFP} and *htp-1*^{M249K; L250S::GFP} in the *htp-1* null background, stained with αGFP, and counterstained with DAPI. The transgenic proteins HTP-1^{E191A} and HTP-1^{M249K; L250S} (green) appear loaded onto chromosome axes; both transgenes are properly expressed, and the corresponding proteins are competent for loading. Wild type control: [*HTP-1*::GFP]; *htp-1*. Scale bar 5μm.

(B) Projections of representative DAPI-stained diakinesis oocytes of worms lacking the endogenous *HTP-1* locus and expressing *htp-1*^{E191A::GFP} and *htp-1*^{M249K; L250S::GFP}. The increased number of chromatin bodies in *htp-1*^{M249K; L250S} is indicative that several chromosome pairs are not forming crossovers. Wild type control: [*HTP-1*::GFP]; *htp-1*. Scale bar 5μm.

(C) Quantification of the number of chromatin bodies observed in the diakinesis oocytes of worms lacking the endogenous *HTP-1* locus and expressing *htp-1*^{E191A::GFP} and *htp-1*^{M249K; L250S::GFP}. Number of nuclei counted: 9 (WT), 60 (*htp-1*^{E191A}), 31 (*htp-1*^{M249K; L250S}), 61 (*htp-1*).



C

Genotype	Chromatin bodies at diakinesis
WT	6.0 ± 0.0
<i>htp-1</i>	11.2 ± 0.9
<i>[htp-1^{E191A}]; htp-1</i>	6.2 ± 0.7
<i>[htp-1^{M249K,L250S}]; htp-1</i>	7.2 ± 1.1

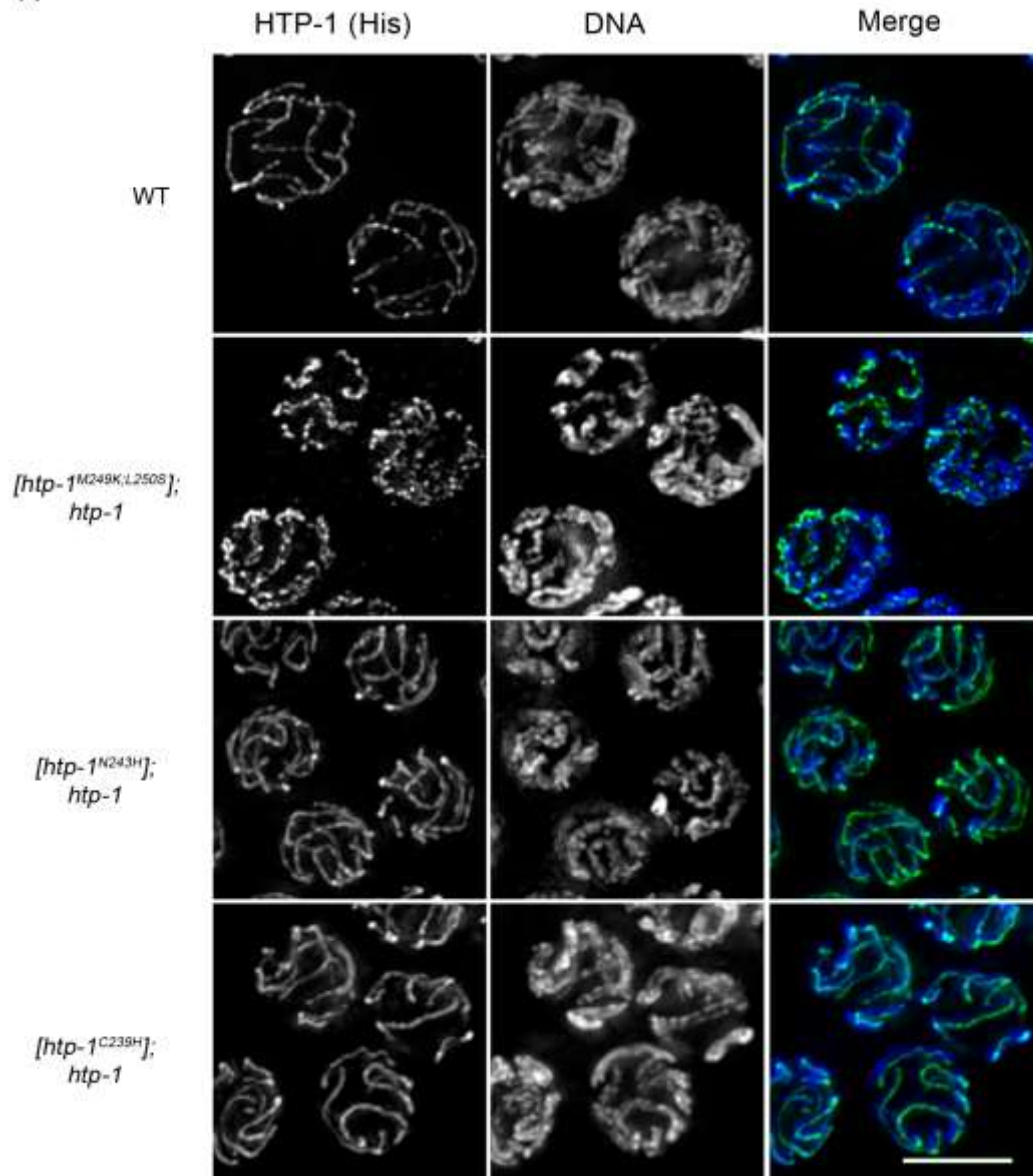
Figure 12. HTP-1 residues C239, N243, M249 and L250 are not essential for HTP-1 function.

(A) Projection of pachytene nuclei of worms expressing *htp-1^{M249K; L250S}::6xHis*, *htp-1^{N243H}::6xHis* and *htp-1^{C239H}::6xHis* in the *htp-1* null background, stained with α HisTag, and counterstained with DAPI. The transgenic proteins HTP-1^{M249K; L250S}, HTP-1^{N243H} and HTP-1^{C239H} (green) appear loaded onto chromosome axes; all three transgenes are properly expressed, and the corresponding proteins are competent for loading. Wild type control: [*HTP-1::6xHis*]; *htp-1*. Scale bar 5 μ m.

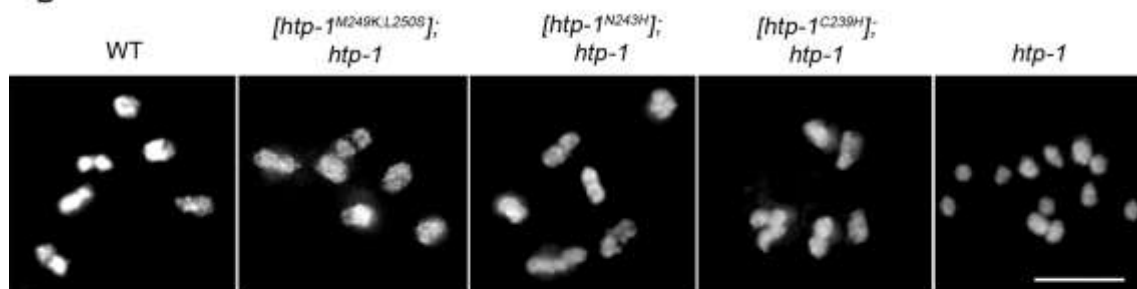
(B) Projections of representative DAPI-stained diakinesis oocytes of worms lacking the endogenous *HTP-1* locus and expressing *htp-1^{M249K; L250S}::6xHis*, *htp-1^{N243H}::6xHis* and *htp-1^{C239H}::6xHis*. The six bivalents observed in *htp-1^{M249K; L250S}* worms indicate that when attached to the less bulky 6xHis tag, HTP-1^{M249K; L250S} supports formation of crossovers on all chromosome pairs. Wild type control: [*HTP-1::6xHis*]; *htp-1*. Scale bar 5 μ m.

(D) Quantification of the number of chromatin bodies observed in the diakinesis oocytes of worms lacking the endogenous *HTP-1* locus and expressing *htp-1^{M249K; L250S}::6xHis*, *htp-1^{N243H}::6xHis* and *htp-1^{C239H}::6xHis*. Number of nuclei counted: 51 (WT), 36 (*htp-1^{M249K; L250S}*), 36 (*htp-1^{N243H}*), 6 (*htp-1^{C239H}*), 61 (*htp-1*).

A



B



C

Genotype	Chromatin bodies at diakinesis
WT	6.0 ± 0.0
<i>htp-1</i>	11.2 ± 0.9
<i>[htp-1^{M249K,L250S}]; htp-1</i>	6.1 ± 0.4
<i>[htp-1^{N243H}]; htp-1</i>	6.0 ± 0.2
<i>[htp-1^{C239H}]; htp-1</i>	6.0 ± 0.0

3.4.7. Conclusion

Out of five substitutions assessed (E191A, C239H, N243H, M249K and L250S) no one mutation was sufficient to induce the defects observed in the HORMA C mutant with regards to chiasma formation. Moreover, observation of the plate phenotype of these mutants clearly showed none was plagued by a level of embryonic lethality comparable to that of *htp-1^{HTP-2 HORMA C}* worms. These initial observations highlighted that the essential residues for HTP-1 function could be among the other five mismatches within HORMA C (E200, E215, D226, A230 and E245), or, a more likely scenario given the complexity of HTP-1 function, that this function may rely on several of these residues, as a cluster. Indeed, a structural study of *C. elegans* meiotic HORMA domain proteins by Kim *et al.* (2014) confirmed that the C-terminal part of the HORMA domain formed a flexible tail; as such, several of the residues studied are likely to contribute to the interaction of HORMA C with a potential ligand within the binding site of HTP-1, and a single mutation may not suffice to disrupt this association.

3.5. The whole domain mutant phenotypes are not the result of redundant HTP-2 function

As the GFP-tagged wild type HTP-1 proved unable to fully perform HTP-1 function in the absence of HTP-2 (see 3.4.4), I asked whether any of the whole domain mutants presented a similar phenotype, where the partial redundancy of HTP-2 could mask phenotypic defects in the *htp-1* single mutant background. When the *htp-1^{HTP-2 HORMA}*, *htp-1^{HTP-2 N-term}* and *htp-1^{HTP-2 C-term}* transgenes were crossed into a *htp-1;htp-2* double mutant background, both strains that had exhibited chiasma formation defects in the *htp-1* background, the HORMA and C-terminus mutants, saw a very slight worsening of their phenotype (Table 5). However, for both transgenes there was less than one extra chromatin body on average in the *htp-1;htp-2* double mutant background than in the *htp-1* single mutant background, indicating that HTP-2 is not responsible for the crossovers that do occur in these mutants. The *htp-1^{HTP-2 N-term}* transgene

presented an identical phenotype in both backgrounds. Therefore, the phenotypes observed in the single mutant background are accurate representations of the functionality of the transgenic proteins. This makes sense as the only changes to the *HTP-1* sequence render it more similar to the *HTP-2* sequence; it would therefore be singular if HTP-2 could outperform such proteins.

3.6. Summary

The analysis of domain, subdomain and point mutants revealed that the functionality of HTP-1 is localised within the protein, mainly to the HORMA domain and to a lesser extent, on the C-terminus. Specifically, HTP-1 function appeared restricted to HORMA C, a region of the HORMA domain close to the C-terminus containing only ten mismatches between HTP-1 and HTP-2, and which may correspond to an active site as part of the HORMA domain tail. Half of the residues within HORMA C, thought to induce the most altering changes in the protein, were mutated without impeding HTP-1 function, leading me to speculate that some or all of the ten residues may act as a cluster. Although the functional defects in HORMA domain and HORMA C region mutants ultimately result in the observed lack of chiasmata and missegregation, it is unclear at this point what stage(s) of the meiotic pathways implicating HTP-1 are affected by these mutations.

Table 5. HTP-2 does not rescue HTP-1 function in the whole domain mutants.

Quantification of the number of chromatin bodies observed in the diakinesis oocytes of worms lacking the endogenous *HTP-1* and *HTP-2* loci and expressing *htp-1^{HTP-2 N-Term}*, *htp-1^{HTP-2 C-Term}* or *htp-1^{HTP-2 HORMA}*. Number of nuclei counted: 61 (*htp-2*), 28 (*htp-1^{HTP-2 N-Term}*), 32 (*htp-1^{HTP-2 C-Term}*), 13 (*htp-1^{HTP-2 HORMA}*).

Genotype	Chromatin bodies at diakinesis
<i>htp-2</i>	6.0 ± 0.1
<i>[htp-1^{htp-2 N-Term}]; htp-1;htp-2</i>	6.0 ± 0.1
<i>[htp-1^{htp-2 HORMA}]; htp-1;htp-2</i>	9.9 ± 1.1
<i>[htp-1^{htp-2 C-Term}]; htp-1;htp-2</i>	7.3 ± 1.8

CHAPTER 4: RESULTS – THE HORMA C REGION OF HTP-1 IS ESSENTIAL FOR THE SEQUENCE OF MEIOTIC PROCESSES

4.1. Introduction

In the previous chapter, I determined using HTP-1/HTP-2 chimeric proteins that the HORMA C region of HTP-1 was essential for HTP-1-specific function during *C. elegans* meiosis. Specifically, mutating ten residues within this region close to the C-terminus led to defects in chiasma formation and chromosome segregation, both likely consequences of problems in earlier meiotic stages. Indeed, in the *htp-1* null mutant, these phenotypes are observed because of defects in crossover formation that lead to the lack of chiasmata, and then mis-segregation at the first meiotic division. These crossover formation defects in the null mutant stem in turn from HTP-1's essential roles in promotion of pairing and synapsis, and in two meiotic checkpoints.

Therefore, after narrowing down the functional region to HORMA C, I asked which of HTP-1's functions were affected by the mismatches within it and whether the HORMA C region was responsible for the entirety of the phenotype observed in the whole HORMA domain mutant. To carry out this analysis, I used immunofluorescence against components of the meiotic machinery to assess pairing, synapsis, cohesin localisation and the initiation of recombination.

4.2. A closer look at *htp-1*^{HTP-2 HORMA C} transgene expression and loading levels

4.2.1. Expression of the *htp-1*^{HTP-2 HORMA C} transgene

An initial analysis had established that the HTP-1^{HTP-2 HORMA C} protein was expressed from the associated transgene and loaded onto chromosome axes (3.3.2.). In light of the striking phenotype associated with this region, I sought to investigate more precisely the amount of chimeric protein present and its ability to load onto chromosome axes.

A Western Blot analysis confirmed the presence of HTP-1^{HTP-2 HORMA C} in whole worm extracts (Figure 13A). The experiment was performed with an α HisTag antibody, so as to label both HTP-1::6xHis (wild type) and HTP-1^{HTP-2 HORMA C}::6xHis but not HTP-2. A double band corresponding to HTP-1^{HTP-2 HORMA C} could be observed, showing the presence of the 39 kDa protein. Interestingly, and although the analysis was not quantitative, the lower molecular weight band appears slightly weaker than in the wild type, which could point to a different behaviour of the mutant protein with regards to modifications.

4.2.2. HTP-1^{HTP-2 HORMA C} loads slower than wild type HTP-1

After asserting the presence of the protein in whole worm extracts – if maybe in slightly lower levels – I asked whether HTP-1^{HTP-2 HORMA C} loaded as efficiently onto chromosome axes as the wild type protein. This was assessed by quantifying the intensity of HTP-1 immunofluorescence signals from the onset of HTP-1 loading in transition zone, until the start of remodelling. Firstly, this region of each germline was divided into three zones of equal size (Figure 13B, cartoon), in order to highlight a possible evolution of loading through these early stages of Prophase I. 20 nuclei in each zone were then analysed using a Fiji macro which measured the mean intensity of the HTP-1 signal over the nucleic area.

Non-deconvolved projections of representative nuclei for each zone show that HTP-1^{HTP-2 HORMA C} seems to load strongly and a difference is not visually striking (Figure 13B),

however a quantitative analysis revealed that HTP-1^{HTP-2 HORMA C} loads more progressively throughout Prophase I than the wild type protein (Figure 13C). The HTP-1^{HTP-2 HORMA C} signal in zone 1 – closest to the mitotic tip, when HTP-1 initially loads – is about two thirds as bright as the wild type signal and steadily increases to the point where, in zone 3, they are equivalent. Therefore, the HORMA C region seems to influence the loading rate of HTP-1 but not the final amount of protein loaded. The slower loading rate is unlikely to account for the observed phenotype of HTP-1^{HTP-2 HORMA C} for two reasons: firstly, HTP-1 function has been shown not to depend on its loading onto chromosome axes (Silva *et al.*, 2014); secondly, subsequent analysis detailed in this chapter demonstrated that most observed defects were constant throughout meiotic progression, highlighting that increasing levels of HTP-1^{HTP-2 HORMA C} did not improve its function.

4.3. The HORMA C region of HTP-1 influences timing and level of early DSB processing

4.3.1. The HORMA domain of HTP-1 does not influence meiotic cohesin localisation

Diakinesis bivalents are held together by a combination of SCC and chiasmata – the physical manifestations of crossovers that have happened earlier in prophase I. Meiotic cohesins are characterised by the substitution of the kleisin subunit by meiosis-specific kleisins REC-8, COH-3 and COH-4 (Klein *et al.*, 1999, Pasierbek *et al.*, 2001, Severson *et al.*, 2009, Severson and Meyer, 2014). The localisation of REC-8 throughout the first stages of prophase I was assessed in *htp-1^{HTP-2 HORMA}* and *htp-1^{HTP-2 HORMA C}* mutants. REC-8 cohesin loads first out of the three meiotic cohesins, and although COH-3/4 cohesin is redundant with REC-8 cohesin in providing SCC, REC-8 alone is implicated in maintaining cohesion between sisters until anaphase II, something that HTP-1 protects as well (Martinez-Perez *et al.*, 2008, Severson *et al.*, 2009). REC-8 loading also requires HORMA domain protein HTP-3 (Severson *et al.*, 2009).

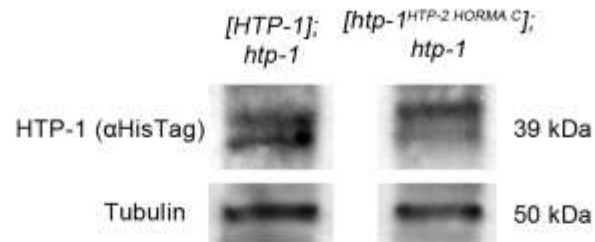
Figure 13. Comparison of transgene expression and protein loading between wild type and *htp-1*^{HTP-2 HORMA C} mutants.

(A) Western blot analysis using a α HisTag antibody (top) and α Tubulin as a control against whole worm extracts prepared from animals expressing either *HTP-1::6xHis* (wild type) or *htp-1*^{HTP-2 HORMA C}::6xHis in a *htp-1* null background.

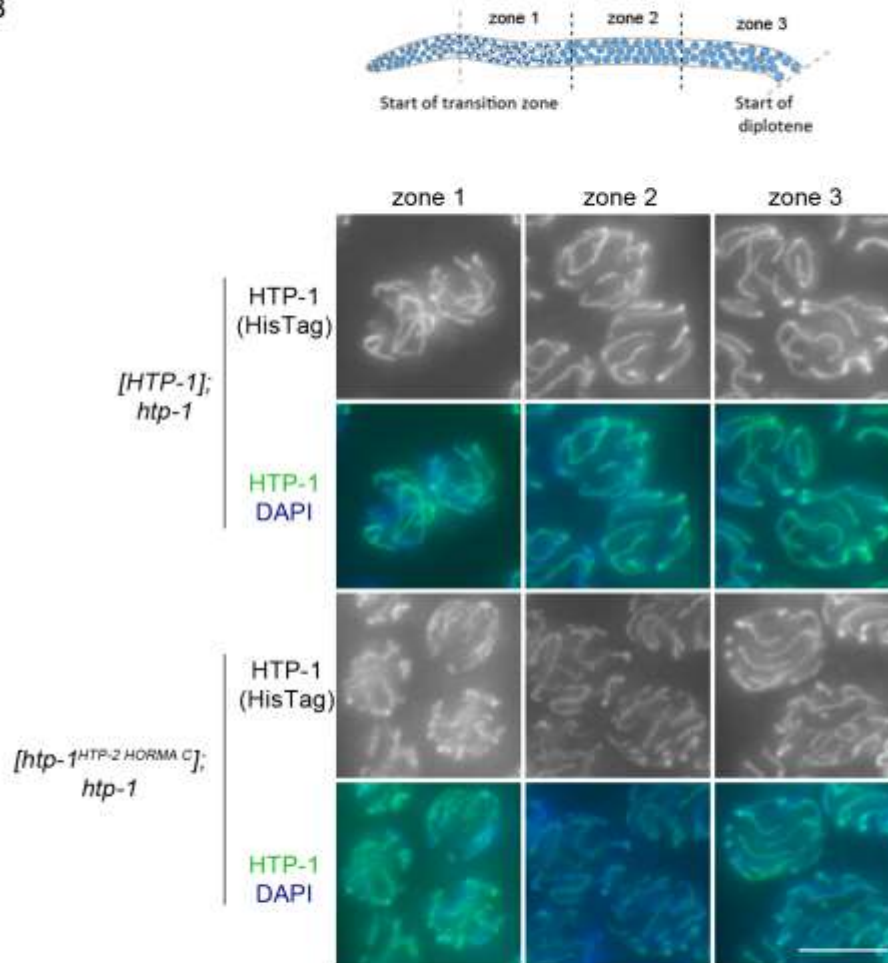
(B) Non-deconvolved projections of meiotic nuclei of worms expressing *HTP-1::6xHis* and *htp-1*^{HTP-2 HORMA C} in the *htp-1* null background, stained with α HisTag, and counterstained with DAPI. Germlines were divided into three zones of equal length from the onset of transition zone characterised by clustered chromatin, to the end of pachytene as depicted in the cartoon. Representative nuclei are shown for each zone, to model progression of the signal. Scale bar 5 μ m.

(C) Average intensity of the 6xHis immunofluorescence signal per germline zone in worms expressing *HTP-1::6xHis* and *htp-1*^{HTP-2 HORMA C} in the *htp-1* null background. Loading is equivalent in zone 3 however *HTP-1*^{HTP-2 HORMA C} loads more progressively than wild type *HTP-1* during Prophase I. Three germlines were analysed per genotype. Total number of nuclei scored: 180 (WT), 180 (*htp-1*^{HTP-2 HORMA C}). Means were compared by a two-tailed independent t-test; **: P<0.01; ***: P<0.001.

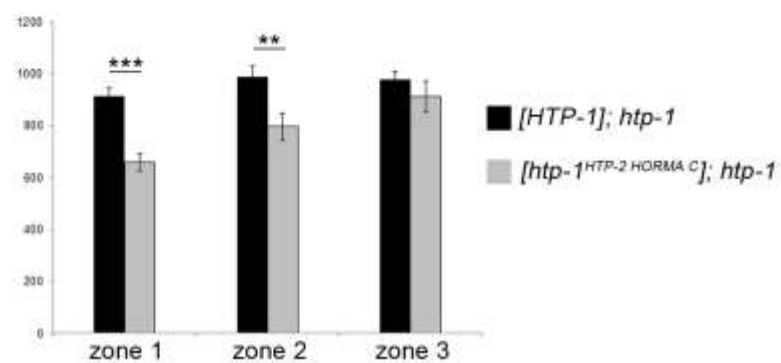
A



B



C



Unlike COH-3/4 cohesin which is dependent on DSBs for loading, REC-8 is put in place concomitant with meiotic chromosome axis formation (Severson *et al.*, 2009), which corresponds to the onset of transition zone. In both whole HORMA domain and HORMA C region mutants, REC-8 was observed on the axes of transition zone nuclei in visually similar levels to the wild type (Figure 14A). This signal persisted normally until pachytene (Figure 14B), where some chromatin tracks appeared to have weaker or no REC-8 staining (Figure 14B, arrowheads), possibly corresponding to the start of REC-8 relocation to areas where SCC will be protected until the second meiotic division, and that will end up being the long arm of bivalents after remodelling (Severson *et al.*, 2009). In conclusion, the disruption of HTP-1 HORMA domain and HORMA C region seems not to affect REC-8 localisation, pointing to a definitive crossover defect in these mutants.

4.3.2. Timely initiation of meiotic DSB repair depends on the HORMA C region of HTP-1

After homologs have aligned, DSBs are created to provoke recombination. In the early stages of DSB processing, strand exchange protein RAD-51 accumulates at resected DSB ends, and is required for the progression of breaks to Holliday junctions, a subset of which will be resolved as crossovers (Colaiacono *et al.*, 2003, Schwacha and Kleckner, 1997). Therefore, it can be used to monitor the distribution and number of early recombination intermediates. In wild type worms the most RAD-51 foci are observed in early pachytene, with their numbers decreasing as breaks are repaired (Colaiacono *et al.*, 2003). In *htp-1* null mutants, the amount of RAD-51 foci is markedly reduced compared to the wild type (Couteau and Zetka, 2005, Martinez-Perez and Villeneuve, 2005; Figure 15). This could mean that either double strand breaks are not being made appropriately, or that they are being repaired by a different pathway, therefore foregoing inter-homologue recombination and the formation of crossovers.

Figure 15A shows the distribution of RAD-51 foci across the germlines of worms lacking the endogenous *HTP-1* locus and expressing a wild type *HTP-1* transgene, the *htp-1^{HTP-2 HORMA C}*

transgene, or neither as a negative control. As expected, the null mutant displays markedly reduced breaks. In the *htp-1^{HTP-2 HORMA C}* mutants, RAD-51 foci appear later than in the wild type and in lesser numbers, although more foci are present than in the null mutants (Figure 15B). Specifically, RAD-51 foci appear at mid pachytene in the HORMA C mutants, rather than late transition zone/early pachytene, and a number of these breaks persist until late pachytene, rather than disappearing rapidly during mid pachytene. The proportion of *htp-1^{HTP-2 HORMA C}* nuclei with four or more RAD-51 foci does not ever exceed 15%, and over half the nuclei in these germlines are completely devoid of RAD-51 signals at any given time. In comparison, in zone 4 – 5 which corresponds to peak RAD-51 foci frequency in the wild type, RAD-51 signal is present in almost 80% of nuclei. HORMA C is therefore implicated in promoting formation of early recombination intermediates, and in the timing of this meiotic event.

4.3.3. Conclusion

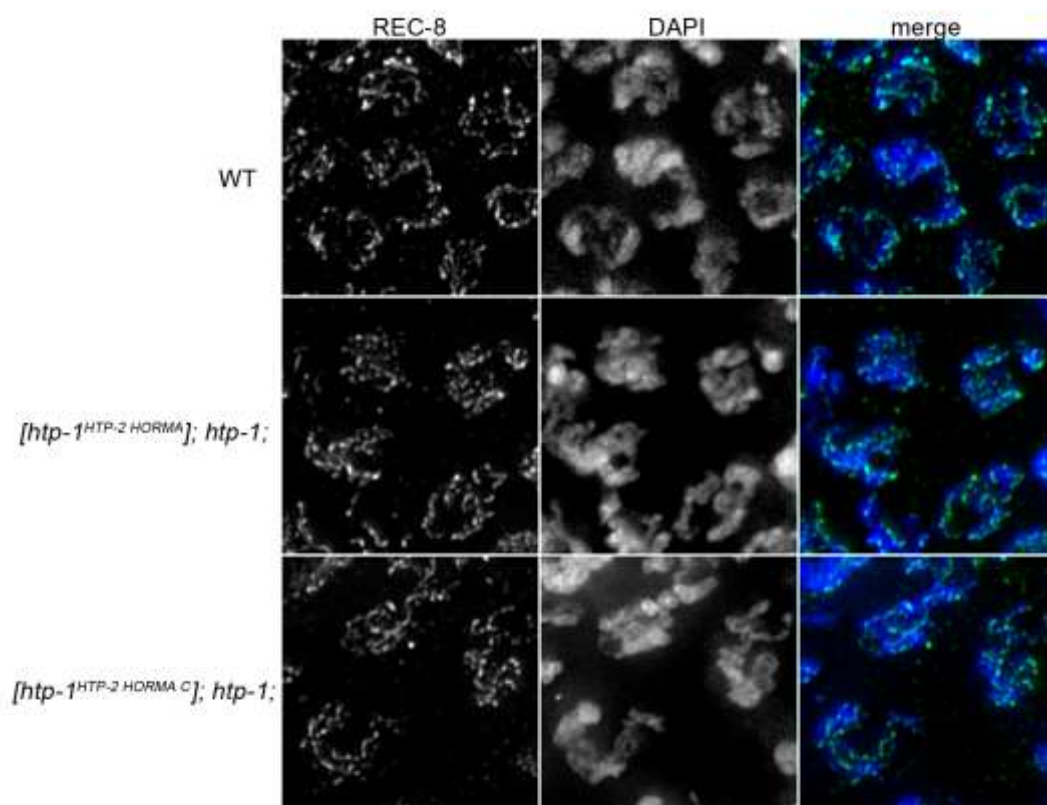
The substitution of the HORMA C region in HTP-1^{HTP-2 HORMA C} does not affect REC-8 localisation but results in a delay in the apparition of RAD-51 foci, and a reduction in RAD-51 foci numbers. This points to a role of the HORMA C region of HTP-1 in the timing and frequency of meiotic DSB formation or the initiation of DSB repair by meiosis-specific pathways aiming to promote interhomolog recombination, or both. *htp-1* deletion has been shown to suppress RAD-51 accumulation even though indiscriminate synapsis in the such mutants should stall recombination intermediates due to the absence of the homologue as repair template (Couteau and Zetka, 2005, Martinez-Perez and Villeneuve, 2005). In the case of HORMA C mutants, the accumulation is not suppressed *per se*. Couteau and Zetka (2005) conjecture that a link may exist between the establishment of lateral and central SC elements, and early recombination events. Taken together with the above results and the slower loading of HTP-1^{HTP-2 HORMA C} (4.2.2.), this idea supports the hypothesis that slower assembly of meiotic axes may play a part

Figure 14. The HORMA C domain of HTP-1 does not influence REC-8 localisation

(A) Projections of representative transition zone nuclei of worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA C}*, stained with α REC-8 and counterstained with DAPI. Scale bar 5 μ m.

(B) Projections of representative pachytene nuclei of worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA C}*, stained with α REC-8 and counterstained with DAPI. Arrowheads indicate stretches of DAPI-stained chromatin with weaker REC-8 staining, which may indicate the onset of remodelling where REC-8 becomes restricted to what will be the long arm of the bivalent. Scale bar 5 μ m.

A



B

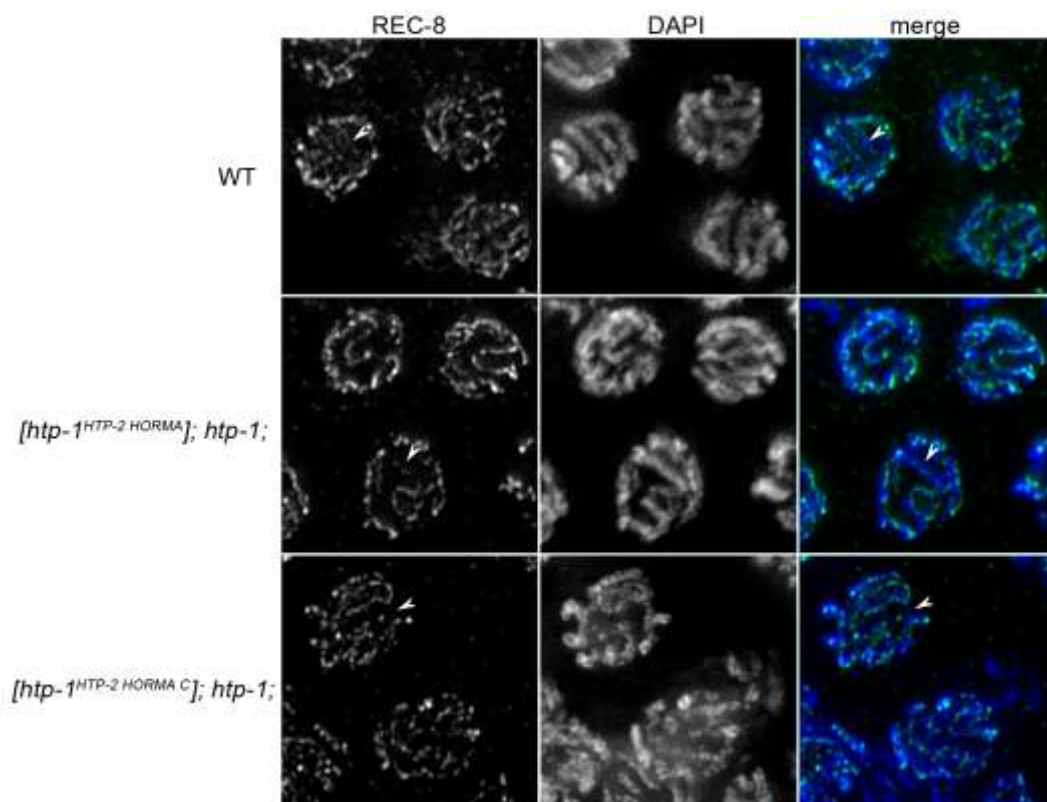


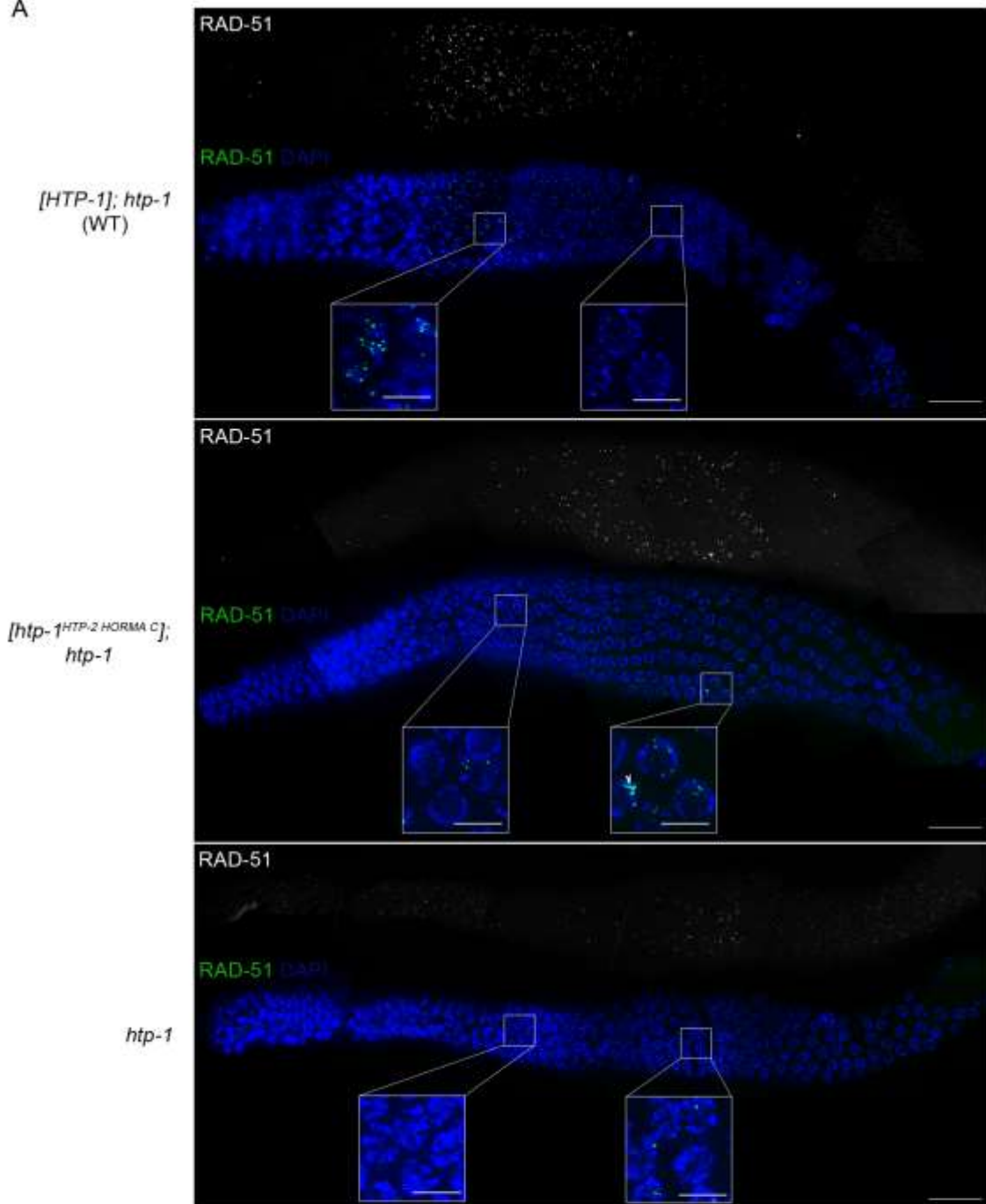
Figure 15. The HORMA C domain of HTP-1 is implicated in regulating the amount and kinetics of early recombination intermediates

(A) Projection of germlines from worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA C}*, and *htp-1* null mutants, stained with α RAD-51 and counterstained with DAPI. Germlines are shown from the mitotic tip to the end of pachytene. Note how the “cloud” of RAD-51 foci is shifted to the right in the HORMA C mutant compared to the wild type. Scale bars 20 μ m.

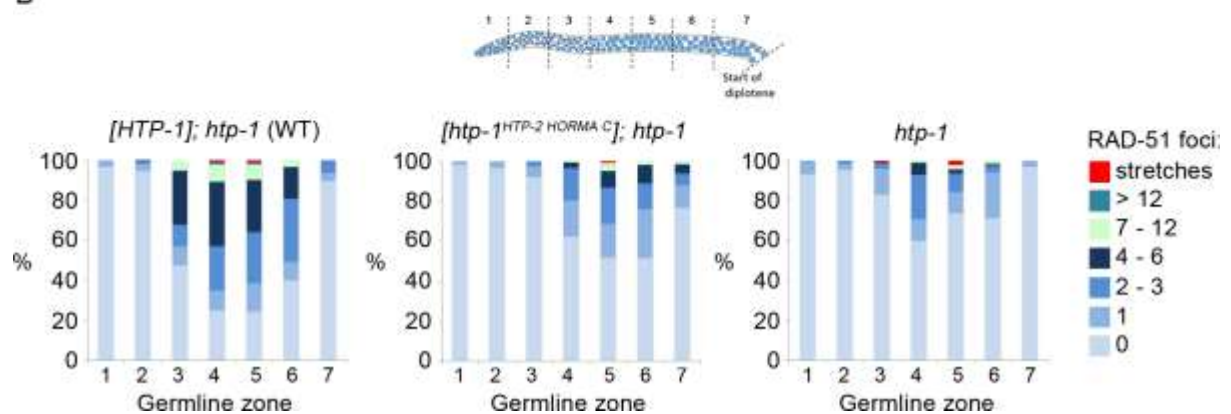
Zooms depict representative nuclei in early and mid/late pachytene. Accumulation of RAD-51 foci is seen in early pachytene in the wild type, but not in the HORMA C or *htp-1* null mutants. In contrast, at the mid to late pachytene transition, RAD-51 has disappeared from wild type nuclei but foci are still present in *htp-1^{HTP-2 HORMA C}* nuclei. Arrowhead points to an accumulation of RAD-51 forming a stretch. Scale bars 5 μ m.

(B) Evolution of the number of RAD-51 foci from the mitotic tip to the end of pachytene, in worms expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA C::6xHis}* in the *htp-1* null background, and the *htp-1* null mutant. Both *htp-1^{HTP-2 HORMA C}* and *htp-1* worms displayed a reduced number of foci compared to the wild type. Additionally, the apparition of RAD-51 foci was delayed in *htp-1^{HTP-2 HORMA C}* germlines but not in the null mutant. Three germlines were analysed per genotype. Total number of nuclei scored: 690 (WT), 777 (*htp-1^{HTP-2 HORMA C}*), 754 (*htp-1*).

A



B



in RAD-51 accumulation delay, but that the HTP-1^{HTP-2 HORMA C} protein allows RAD-51 accumulation.

4.4. The HORMA C region of HTP-1 is required to achieve full synapsis and pairing

4.4.1. The synaptonemal complex fails to fully assemble in HORMA domain and HORMA C region mutants.

4.4.1.1. The HORMA domain of HTP-1 is required for full synapsis

Within the HTP-1 HORMA domain, the HORMA C region does not affect the presence of meiotic kleisin REC-8, but does delay the accumulation of RAD-51 on double strand breaks. Next, in order to further elucidate the possible causes of a crossover formation defect, I asked whether mutations within HORMA C affected synapsis. Once the homologs have found their pairing partner in transition zone, they synapse by assembling a zipper-like structure between the chromosome axes. This synaptonemal complex holds homologs in physical proximity and facilitates DSB repair with the homologue rather than the sister chromatin, therefore ensuring recombination.

Co-staining of HIM-3, a component of the meiotic chromosome axes, and SYP-1, a central element in the synaptonemal complex, revealed that in the HORMA domain mutant, several nuclei do not fully complete synapsis. In a wild type situation, the synaptonemal complex is put in place along the whole length of the chromosome, so that HIM-3 and SYP-1 fully overlap (Figure 16A). This full overlap is reached by early pachytene in virtually all wild type nuclei (Figure 16B). In the HORMA domain mutant however, HIM-3 stretches that did not overlap with SYP-1 stretches were observed consistently in pachytene nuclei (Figure 16A, arrowheads). They correspond to unsynapsed portions of chromosomes. In zones 3-4, at a stage when the proportion of nuclei displaying these unsynapsed portions has fallen to 2% in the wild type,

60% of HORMA domain mutant nuclei still display incomplete synapsis, and their proportion increases through to the end of pachytene (Figure 16B). The HORMA domain is therefore necessary to ensure full synapsis. The dynamics of the synapsis process are interesting in this case: initially, the proportion of nuclei with unsynapsed stretches is 19% higher in HORMA domain mutants than in wild type worms where only about half the nuclei contain unsynapsed chromatin; however, while this number dramatically falls in the wild type over zones 1 – 3, it stays constant in *htp-1^{HTP-2 HORMA}* germlines. This indicates that the HORMA domain is important in two separate aspects: firstly, the HORMA domain must promote faster or earlier assembly of the synaptonemal complex, which may explain why a sequence disruption results in more asynapsis at the same stage. Secondly, the HORMA domain is heavily implicated in the completion of synapsis by those nuclei that contain unsynapsed chromatin. HTP-1 is known to have a role in the transition zone exit checkpoint which maintains the cells in zygotene until synapsis is complete (Martinez-Perez and Villeneuve, 2005), and this result points to the HORMA domain as the basis for this function.

4.4.1.2 The HORMA domain of HTP-1 promotes full synapsis via the HORMA C region

Since the HORMA C region appeared to be responsible for the majority of HTP-1 HORMA domain function, I observed and quantified the asynapsis phenotype in the *htp-1^{HTP-2 HORMA C}* mutants as well. Similar results were obtained to those of the whole HORMA domain mutant. Unsynapsed stretches of chromatin were observed in pachytene nuclei (Figure 17A), a feature also present in the *htp-1* null mutant. The proportion of nuclei with unsynapsed chromatin was consistently higher in *htp-1^{HTP-2 HORMA C}* germlines than wild type germlines; at the onset of meiosis when nuclei with unsynapsed chromatin peak at 44% in the wild type, they represent 73% of *htp-1^{HTP-2 HORMA C}* nuclei. Furthermore, this proportion remained almost unchanged along *htp-1^{HTP-2 HORMA C}* germlines until zone 3, after which point an increased number of nuclei presented unsynapsed chromatin, suggesting that synapsis is being lost and the SC is starting to disassemble (Figure 17B). Loss of synapsis was also observed in the wild type at that stage.

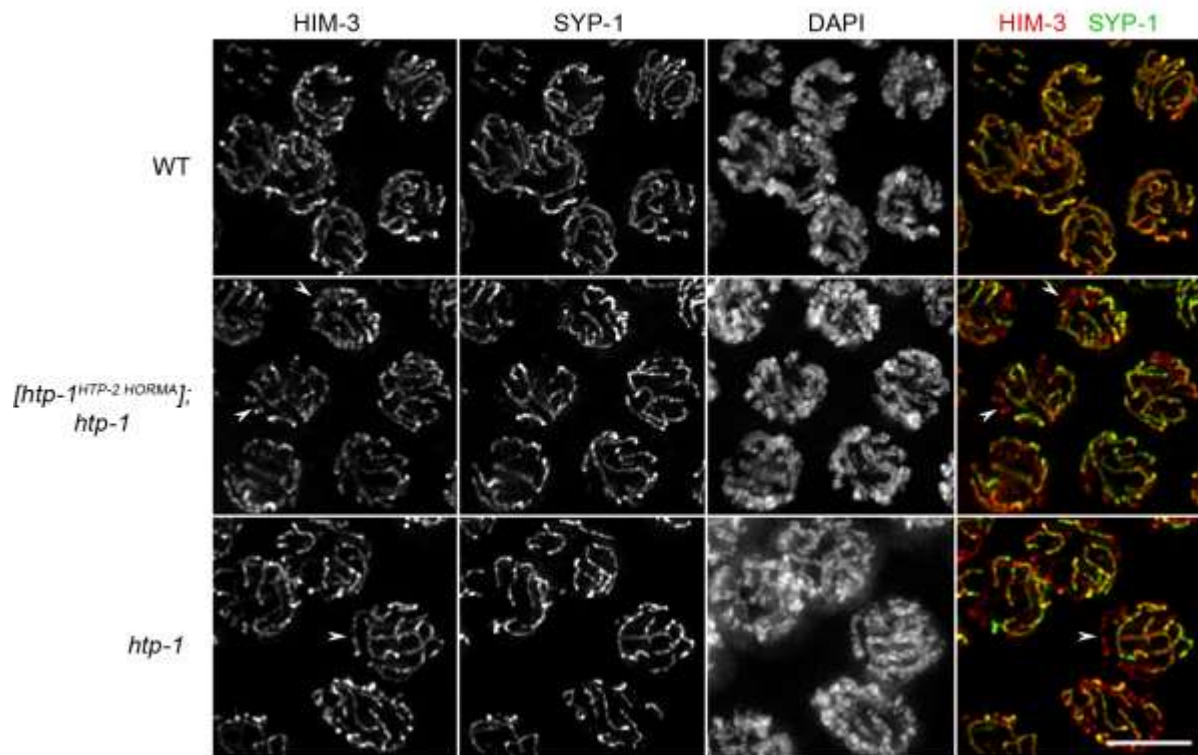
Figure 16. The HORMA domain of HTP-1 is essential for completion of synapsis.

(A) Projections of representative pachytene nuclei of worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA}*, or no transgene, co-stained with α HIM-3 and α SYP-1, and counterstained with DAPI. Arrowheads in the HIM-3 and merge columns indicate HIM-3-labelled stretches of meiotic chromosome axes that do not colocalise with a stretch of SYP-1, and therefore correspond to unsynapsed regions. Unsynapsed portions of chromatin are observed in both the null and *htp-1^{HTP-2 HORMA}* mutants. Scale bar 5 μ m.

(B) Evolution of the incomplete synapsis phenotype through early Prophase I, in worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA}*, or no transgene. Graph shows the proportion of nuclei displaying unsynapsed portions of chromatin from the onset of meiosis – determined as the first transition zone clustered nuclei – to the end of pachytene; this region was divided into five zones of equal length as depicted in the cartoon. In worms expressing *htp-1^{HTP-2 HORMA}*, unsynapsed regions persist in the majority of nuclei throughout transition zone and pachytene – reaching a minimum of 63% in zone 3 – in stark contrast to the wild type, which minimum is 1% in zone 4. From zone 3 onwards, the HORMA domain mutant recapitulates faithfully the null mutant phenotype as the proportion of nuclei with unsynapsed regions remains within 10% of the null mutant.

At least three germlines were analysed per genotype. Total number of nuclei scored: 668 (WT), 437 (*htp-1^{HTP-2 HORMA}*), 897 (*htp-1*). Means of the wild type and *htp-1^{HTP-2 HORMA}* mutants were compared by a two-tailed independent t-test. *: P<0.05; **: P<0.01; ***: P<0.001.

A



B

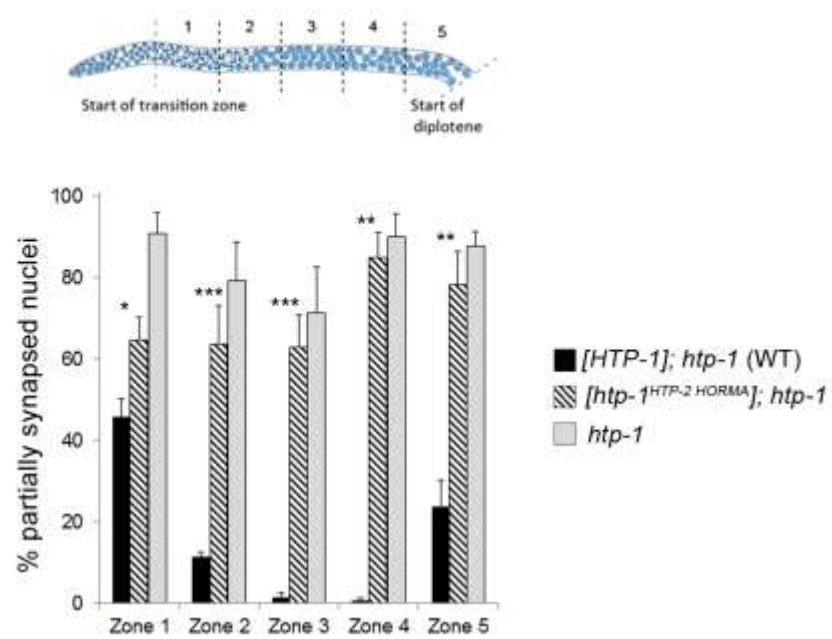


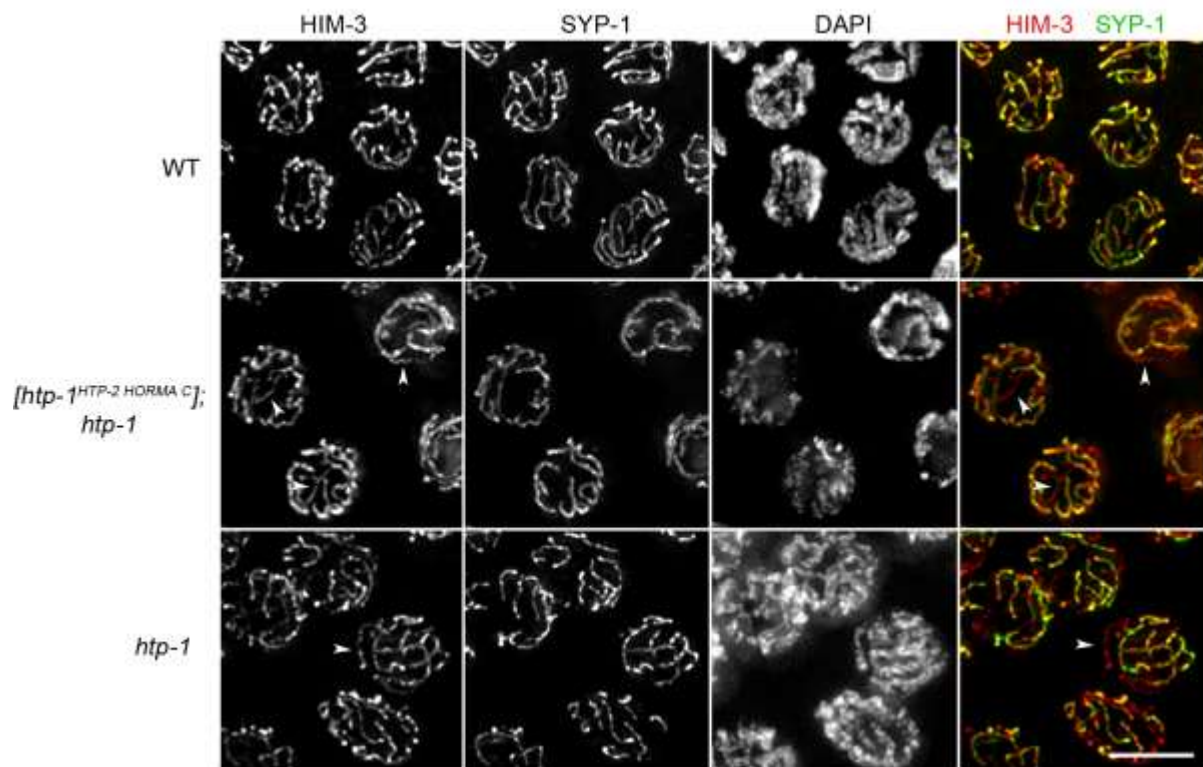
Figure 17. The HORMA C region is responsible for the incomplete synapsis observed in *htp-1^{HTP-2 HORMA C}* mutants.

(A) Projections of representative pachytene nuclei of worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA C}*, or no transgene, co-stained with α HIM-3 and α SYP-1, and counterstained with DAPI. Arrowheads in the HIM-3 and merge columns indicate HIM-3-labelled stretches of meiotic chromosome axes that do not colocalise with a stretch of SYP-1, and therefore correspond to unsynapsed regions. Unsynapsed portions of chromatin are observed in both the null and *htp-1^{HTP-2 HORMA C}* mutants. Scale bar 5 μ m.

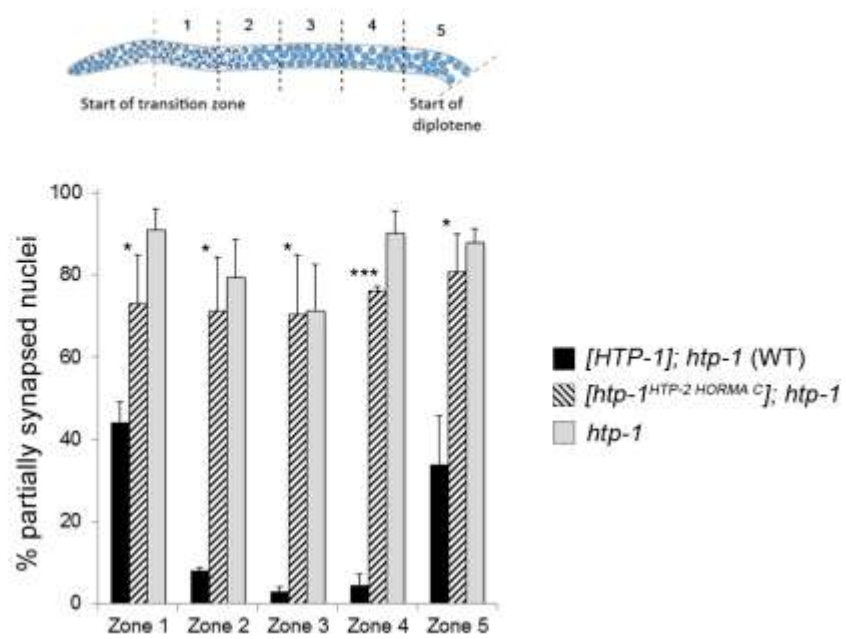
(B) Evolution of the incomplete synapsis phenotype through early Prophase I, in worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA C}*, or no transgene. Graph shows the proportion of nuclei displaying unsynapsed portions of chromatin from the onset of meiosis – determined as the first transition zone clustered nuclei – to the end of pachytene; this region was divided into five zones of equal length as depicted in the cartoon. In worms expressing *htp-1^{HTP-2 HORMA C}*, unsynapsed regions persist in 70-80% of nuclei throughout transition zone and pachytene, in stark contrast to the wild type which achieves full synapsis in almost all nuclei (97%) in zone 3.

At least three germlines were analysed per genotype. Total number of nuclei scored: 814 (WT), 357 (*htp-1^{HTP-2 HORMA C}*), 897 (*htp-1*). Means of the wild type and *htp-1^{HTP-2 HORMA C}* mutants were compared by a two-tailed independent t-test; *: P<0.05; ***: P<0.001.

A



B



These observations confirm that the HORMA C region is the functionally important region of the HORMA domain of HTP-1. Specifically, HORMA C is here shown to be involved in the kinetics of SC assembly, as well as the completion of synapsis which may be a direct result of the transition zone exit checkpoint.

4.4.2. The HORMA C region of HTP-1 is essential for active pairing of homologous chromosomes during meiosis

In *htp-1* null mutants, in addition to routinely incomplete synapsis, the SC is assembled indiscriminately between non-homologous chromosomes (Couteau and Zetka, 2005). In order to check whether pairing was also affected in *htp-1^{HTP-2 HORMA C}* mutants, DNA FISH was performed against the 5S rDNA locus. Figure 18A compares projections of pachytene nuclei of worms lacking the endogenous *HTP-1* locus and expressing a wild type *HTP-1* or *htp-1^{HTP-2 HORMA C}* transgene. In the wild type, a single immunofluorescence focus is visible in most nuclei. In some cases, two foci appear in very close proximity to each other; as separate foci very close together can correspond to aligned homologous chromosomes, only signals that were more than 0.6µm apart were scored as unpaired. In sharp contrast to the wild type, numerous nuclei with unpaired 5S foci were observed in the *htp-1^{HTP-2 HORMA C}* mutants. On Figure 18A, four of the six *htp-1^{HTP-2 HORMA C}* nuclei shown display two separate foci, visibly apart. Even the paired foci (marked *) in the remaining two nuclei are separated by a gap unseen in wild type nuclei. Comparison of the proportion of nuclei with paired signals throughout meiotic progression was performed by dividing germlines in six equal zones from the mitotic tip to the end of pachytene, and scoring paired and unpaired signals in each zone. The results are unequivocal: the HORMA C region of HTP-1 is essential for chromosome pairing. Pairing levels were not significantly different between the wild type and mutant germlines in zone 1, which corresponds to the mitotic tip. At that stage, about two thirds of nuclei displayed a single 5SrDNA focus (Figure 18B). Subsequently, paired signals increased in frequency in wild type germlines, to the point where over 90% of nuclei display paired signals (Figure 18B, zone 4). The most dramatic

increase occurred between zones 1 -3 which, roughly, cover meiotic entry and the transition zone; this is consistent with the homology search state of transition zones nuclei, and the stabilisation of pairing by the SC. In *htp-1^{HTP-2 HORMA C}* mutants, not only did the proportion of paired signal never reach levels comparable to the wild type, but pairing levels actually stayed constant throughout meiotic entry, leptotene, zygotene and pachytene (Figure 18B). Therefore the HORMA C region of HTP-1 is absolutely essential to active pairing during meiosis.

4.4.3. Conclusion

Similarly to the *htp-1* null mutants, *htp-1^{HTP-2 HORMA}* worms display unsynapsed stretches of chromatin during pachytene. In fact, a process which actively promotes the completion of synapsis and gradually eliminates the occurrence of unsynapsed chromatin during early Prophase I, is defective in these mutants. In wild type worms, a checkpoint is proposed to monitor asynapsis and trigger apoptosis of nuclei containing unsynapsed pairing centres (Bhalla and Dernburg, 2005). The above results are consistent with a recent study by Bohr *et al.* (2016) suggesting that this checkpoint requires HTP-1. Furthermore, the role of the HTP-1 HORMA domain in this process is supported by the HORMA C region, as *htp-1^{HTP-2 HORMA C}* mutants completely recapitulate the asynapsis phenotype. A constant, low level of pairing throughout Prophase I in HORMA C mutants shows that HORMA C is important for the establishment of pairing in transition zone, and hints at the possibility that some of the observed synapsis may be non-homologous, as in the *htp-1* null mutant (Couteau and Zetka, 2005, Martinez-Perez and Villeneuve, 2005).

4.5. Summary

In worms expressing *htp-1^{HTP-2 HORMA C}*, the recombinant HTP-1 protein loads onto chromosome axes in native amounts, however its loading is slower than in the wild type. The HORMA C region of *htp-1* has no impact on REC-8 localisation, but is implicated in controlling the kinetics of the early steps of DSB repair. HORMA C is important for the establishment of

Figure 18. The HORMA C region of HTP-1 is essential for homologue pairing

(A) Projections of representative pachytene nuclei of worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* or *htp-1^{HTP-2 HORMA C}* after DNA FISH with a DIG-labelled probe against the 5S rDNA locus on chromosome V. Germlines were stained with α DIG and counterstained with DAPI.

Whereas a single focus visible in most wild type nuclei, *htp-1^{HTP-2 HORMA C}* nuclei routinely display two separate signals, indicating that the two homologous chromosome V are not aligned.

Arrowheads point to two 5S locus foci within a same nucleus in an *htp-1^{HTP-2 HORMA C}* germline.

Asterisks designate *htp-1^{HTP-2 HORMA C}* nuclei where, although the foci are less than 0.6 μ m apart and therefore considered paired, two dots can be seen next to each other, showing that the homologs are further apart than those resulting in a single fluorescence focus (see wild type).

Scale bars 5 μ m.

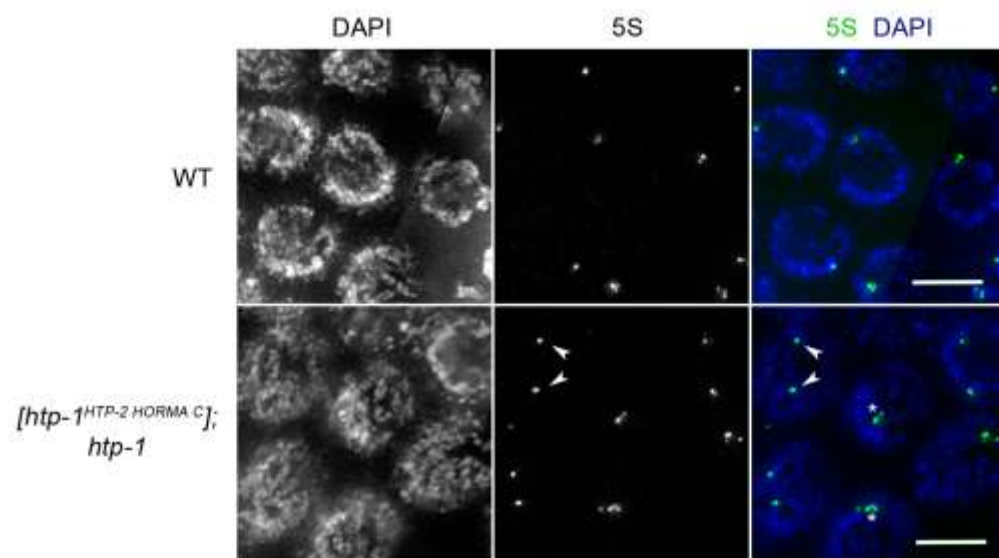
(B) Evolution of 5S rDNA locus pairing through early Prophase I, in worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* or *htp-1^{HTP-2 HORMA C}*. Graph shows the proportion of nuclei with paired FISH signals from the mitotic tip to the end of pachytene; this region was divided into six zones of equal length as depicted in the cartoon.

Although initially there are only 9% more nuclei with paired signals in wild type germlines than in *htp-1^{HTP-2 HORMA C}* mutant ones, pairing levels in the mutant remain constant at the level of premeiotic nuclei, between 31% (zones 1 and 3) and 42% (zone 6). In contrast, pairing of the 5S locus occurs in at least 82% wild type nuclei from zone 3 onwards, peaking at 92% in zone 4.

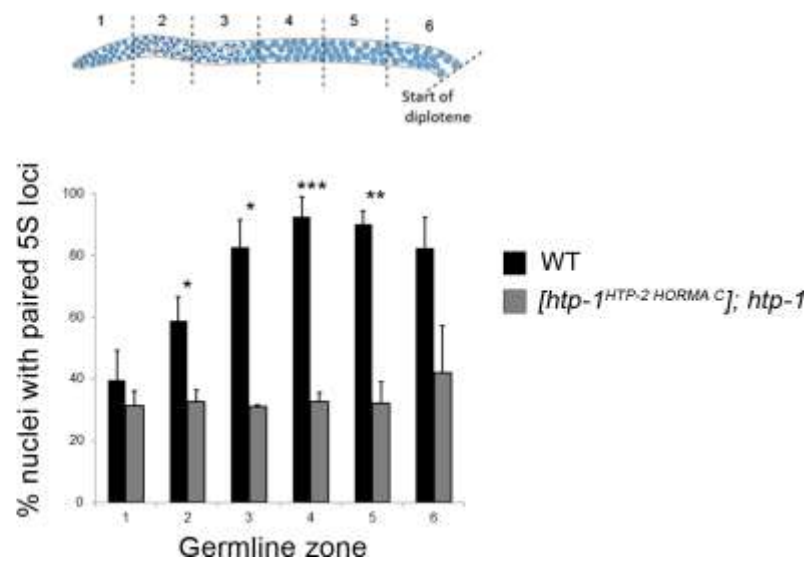
Thus, pairing during meiosis requires the HORMA C region of HTP-1.

Three germlines were analysed per genotype. Total number of nuclei scored: 499 (WT), 458 (*htp-1^{HTP-2 HORMA C}*). Means were compared by a two-tailed independent t-test; *: P<0.05; **: P<0.01; ***: P<0.001.

A



B



early recombination intermediates, in early pachytene. Additionally, the HORMA C region of HTP-1 proved essential for the completion of synapsis, possibly by participating in the transition zone exit checkpoint, and promotes active pairing in early prophase I.

CHAPTER 5: RESULTS – CHECKPOINTS AND

INTERACTIONS OF HORMA C

5.1. Introduction

It has been established that the C-terminal part of the HTP-1 HORMA domain, named HORMA C, is essential for pairing, the completion of synapsis, and the timely accumulation of RAD-51 on double strand breaks. Subsequently, I wondered about the control of these meiotic processes. Indeed, HTP-1 is involved in two meiotic checkpoints that coordinate meiotic events. Firstly, HTP-1 coordinates pairing and the establishment of the synaptonemal complex, as part of a mechanism repressing synapsis between non-homologous chromosomes (Couteau and Zetka, 2005). Secondly, HTP-1 coordinates the completion of full synapsis with transition zone exit and the end of homology search (Martinez-Perez and Villeneuve, 2005). It is on this second checkpoint that I focus my analysis, by monitoring a molecular marker of transition zone. Then, widening the question of control of meiotic events, I pondered the interplay between different HTP-1 domains and created a new set of chimeric *htp-1/2* mutants to shed light on how HORMA C might rely on other domains in cis to perform its functions.

5.2. The HORMA C region of HTP-1 is essential to the transition zone exit checkpoint

5.2.1. HTP-1^{HTP-2 HORMA C} is cannot trigger the transition zone exit checkpoint in the presence of unsynapsed chromatin regions.

The transition zone exit checkpoint consists of a mechanism by which nuclei are maintained in a homology search state until every chromosome pair has fully synapsed; at that stage, chromosomes are released from the clustered conformation characteristic of transition

zone, and transition into pachytene (Martinez-Perez and Villeneuve, 2005). Previous observations indicate that this checkpoint may not be functional in *htp-1^{HTP-2 HORMA C}* mutants; indeed, incomplete synapsis is observed well into pachytene (see 4.4.1), in nuclei which do not present the clustered conformation characteristic of transition zone. I sought to analyse this checkpoint further using a molecular marker for transition zone rather than merely nucleic morphology. During transition zone, PLK-2 kinase is recruited to the chromosome pairing centers as part of the machinery tethering chromosomes to the nuclear membrane, and enabling their movement for homology search (Labella *et al.*, 2011). Once synapsis is complete, PLK-2 disappears from all but one of the pairing centers – the X chromosome – coincidentally with transition zone exit and remains bound to the X-chromosome pairing center until all six crossovers have taken place (Woglar *et al.*, 2013).

Immunostaining for PLK-2 confirmed premature exit from transition zone in worms expressing *htp-1^{HTP-2 HORMA C}* in a *htp-1* null background. Wild type worms displayed multiple foci concentrated on nuclei with clustered chromatin, which disappeared almost completely at pachytene (Figure 19A). Interestingly, not only did nuclei with multiple PLK-2 foci disappear in a similar time frame in *htp-1^{HTP-2 HORMA C}* worms, despite known incomplete synapsis, but the transition zone itself seemed even shorter (Figure 19A). In order to quantify and compare this phenotype, germlines were divided into rows of nuclei from the onset of transition zone to the start of diplotene. A row was considered to be in transition zone if over half of its nuclei presented multiple PLK-2 foci. This allowed me to measure the length of transition zone, relative to the germline. As observed, transition zone in *htp-1^{HTP-2 HORMA C}* worms was slightly shorter than in wild type worms (Figure 19B), confirming without a doubt that HORMA C is required for the transition zone exit checkpoint. More specifically, in the HORMA C mutants the checkpoint appears to be automatically validated as nuclei with incomplete synapsis are allowed to exit transition zone into pachytene.

Additionally, I scored the rows in which over half of the nuclei displayed a single PLK-2 focus, even after transition zone exit. The last focus persists on the X-chromosome as a marker that crossover formation has not been completed. In this case, a marked difference was observed between wild type and *htp-1^{HTP-2 HORMA C}* worms: the X-chromosome focus persisted significantly longer in the mutant (Figure 19B), indicating that all crossovers indeed do not form in these worms, causing homologous chromosomes to be separate at diakinesis.

5.2.2. HTP-1^{HTP-2 HORMA C} is unable to trigger the transition zone exit checkpoint in the complete absence of synapsis

In worms expressing *htp-1^{HTP-2 HORMA C}* in an *htp-1* null background, incomplete synapsis appears to satisfy the transition zone exit checkpoint. I asked whether the checkpoint was simply validated by installation of the synaptonemal complex, even partially, or alternatively whether it was completely absent from the meiotic process. In order to elucidate this, the *htp-1^{HTP-2 HORMA C}* transgene was crossed onto a *syp-2* null mutant background. SYP-2 is an essential component of the central element of the synaptonemal complex, without which synapsis is not possible (Colaiacono *et al.*, 2003); thus, meiotic chromosomes of worms carrying the *syp-2* deletion do not synapse at all.

Immunostaining for PLK-2 showed a complete arrest of nuclei in transition zone in worms carrying a wild type copy of *HTP-1* and the *syp-2* deletion, showing the checkpoint in action in response to asynapsis (Figure 20A). In these germlines, multiple PLK-2 foci persisted on the nuclei throughout meiotic progression, and the transition zone accounted for 82% of the germline length until the start of diplotene, up from just 16% in the presence of SYP-2 protein (Figures 19B, 20B). In contrast, in [*htp-1^{HTP-2 HORMA C}]; htp-1; syp-2* worms, the transition zone still accounted for just 14% of the germline until the start of diplotene, with most nuclei displaying only a single focus on the X-chromosome, which persisted until just before the onset of diplotene (Figure 20B). The checkpoint therefore failed to arrest nuclei in transition zone even in the complete absence of synapsis. Moreover, the average length of transition zone was

Figure 19. *HTP-1*^{HTP-2 HORMA C} fails to trigger the transition zone exit checkpoint despite incomplete synapsis.

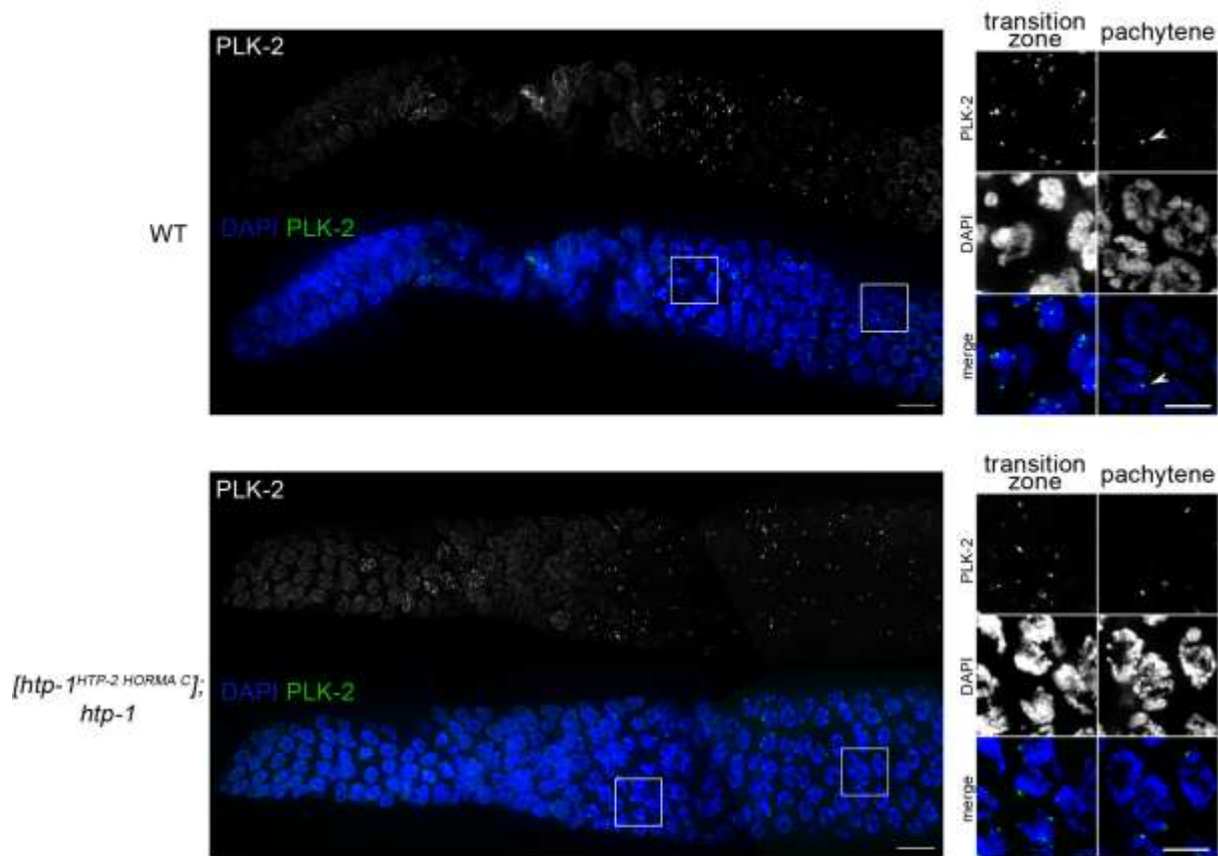
(A) Projection of germlines from worms lacking the endogenous *HTP-1* locus and expressing *HTP-1::6xHis* or *htp-1*^{HTP-2 HORMA C}, stained with α PLK-2 and counterstained with DAPI. Germlines are shown from the mitotic tip to mid-pachytene. PLK-2 foci in the wild type appear as a denser cloud than in the mutant, where they seem both less numerous and more spread out along the length of the germline. Scale bars 10 μ m.

Miniatures are zooms of the areas framed on the germlines, and depict representative nuclei in regions corresponding in the wild type to transition zone and mid pachytene. Both genotypes display nuclei with multiple PLK-2 foci in transition zone but later on, most *htp-1*^{HTP-2 HORMA C} mutant nuclei still carry a single PLK-2 focus while signal has all but disappeared in the wild type. Arrowhead points to a single PLK-2 focus visible in a wild type nucleus while all neighbouring nuclei are completely devoid of PLK-2 signal. Scale bars 5 μ m.

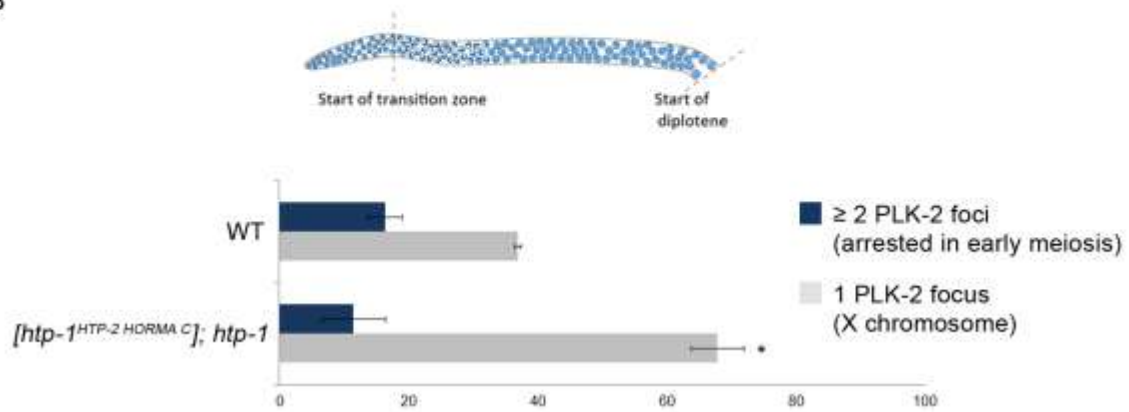
(B) Representation of transition zone length and persistence of a single PLK-2 focus on the X chromosome, in worms expressing *HTP-1::6xHis* and *htp-1*^{HTP-2 HORMA C::6xHis} in the *htp-1* null background. Germlines were divided into rows of nuclei from the onset of transition zone – characterised by multiple PLK-2 foci per nucleus – to the end of pachytene. A row of nuclei was considered to be in transition zone if over half the nuclei displayed two or more PLK-2 foci. Similarly, the persistence of the X chromosome focus is scored as the number of rows where over half the nuclei display at least one PLK-2 focus. Both measures are shown here as proportions of the entire region studied.

The transition zone in *htp-1*^{HTP-2 HORMA C::6xHis} mutants represents 11% of the scored length: it is not extended and is even shorter than in the wild type (16%), showing that *HTP-1*^{HTP-2 HORMA C} does not trigger the transition zone exit checkpoint in response to unsynapsed regions. The fact that PLK-2 persists on the X chromosome of HORMA C mutants 30% further than in the wild type indicates missing crossovers. Two wild type and three *htp-1*^{HTP-2 HORMA C} germlines were analysed. Mean length of transition zone and X chromosome persistence

A



B



between the wild type and *htp-1^{HTP-2 HORMA C}* mutants were compared by a two-tailed independent t-test. *: $P < 0.05$.

not significantly different between worms expressing *htp-1^{HTP-2 HORMA C}* in the *htp-1* and *htp-1; syp-2* backgrounds (p=0.691): abrogating synapsis by *syp-2* deletion had no effect on the observed phenotype. Taken together with the previous section, these observations establish that the HORMA C domain of HTP-1 is essential to the transition zone exit checkpoint, and that this checkpoint is non-existent in HORMA C mutants.

5.2.3. SYP-1 loads preferentially on select chromosomes in

htp-1^{HTP-2 HORMA C} worms

Conjointly with the analysis in the *syp-2* mutant background, an attempt was made to deplete SYP-1, another essential component of the central element of the synaptonemal complex, using RNAi in *htp-1^{HTP-2 HORMA C}* worms. Worms were fed bacteria expressing a shRNA hairpin complementary to *syp-1* mRNA. In this instance, SYP-1 depletion was not complete which did not allow for the analysis of the checkpoint response to asynapsis. However, a low-SYP-1 situation did uncover an interesting aspect of the *htp-1^{HTP-2 HORMA C}* phenotype. Indeed in low SYP-1 conditions, it was expected that SYP-1 would be present on meiotic chromosome axes, in a smaller quantity characterised by a weaker immunostaining signal. However, in *htp-1^{HTP-2 HORMA C}* worms, in low SYP-1 conditions the protein was concentrated on a single chromosome pair, sometimes two (Figure 21). On these particular tracks, the observed signal seemed of similar strength to the signal seen in worms that had fed on bacteria expressing an empty vector without the shRNA hairpin. In contrast, the remaining chromatin within the nucleus appeared devoid of SYP-1. It would therefore seem that in *htp-1^{HTP-2 HORMA C}* mutants, SYP-1 accumulates preferentially on some chromosomes rather than others. Moreover, the tracks localise to chromatin-dense regions (Figure 21), which points to the X-chromosome as a likely candidate for this priority synapsis, as it is partially silenced during early meiosis, and appears as a stronger-stained DAPI region in *htp-1* mutants (Kelly *et al.*, 2002, Martinez-Perez and Villeneuve, 2005). The *him* phenotype is much less present in worms expressing

htp-1^{HTP-2 HORMA C} compared to *htp-1^{HTP-2 HORMA}* (See 3.2.3, 3.3.4), indicating that specific mechanisms regulating the meiotic behaviour of the X-chromosome may not be dependent on residues within HORMA C; this could in part explain why the synaptonemal complex would assemble preferentially on the X-chromosome, as other chromosome pairs are faced with meiotic defects, including low-level pairing.

5.2.4. Conclusion

In worms expressing *htp-1^{HTP-2 HORMA C}*, the transition zone exit checkpoint is not responsive to the presence of unsynapsed regions: PLK-2 foci on autosomal pairing centers disappear in a similar timing to wild type worms despite known synapsis defects. In fact, the kinetics of transition zone exit in *htp-1^{HTP-2 HORMA C}* worms are identical in the presence of unsynapsed chromatin and in the complete absence of synapsis, establishing that the transition zone exit checkpoint is fully unresponsive and nuclei are allowed to progress into pachytene regardless of their synaptic status. HORMA C is therefore essential to the transition zone exit checkpoint, as it is for the synapsis checkpoint in pachytene (4.4.1.2.). This is also true of PLK-2, and Harper *et al.* (2011) speculate that both checkpoints may be responses to a same signal, mediated by PLK-2. Following this hypothesis, HTP-1 could possibly play a role in sensing that signal, as a reader of chromatin state; a process that would involve residues within HORMA C. Additionally, in low-SYP-1 conditions, SYP-1 accumulates preferentially on a single or pair of track(s), highlighting a complex regulation of SYP-1 loading and synapsis promotion, which is not fully dependent on the HORMA C region.

HORMA C plays an essential part in the coordination of meiotic events, and presents a possible complementary role with other control mechanisms regulating specific chromosomes. Within HTP-1, HORMA C may also be involved in complementary roles and interactive relationships.

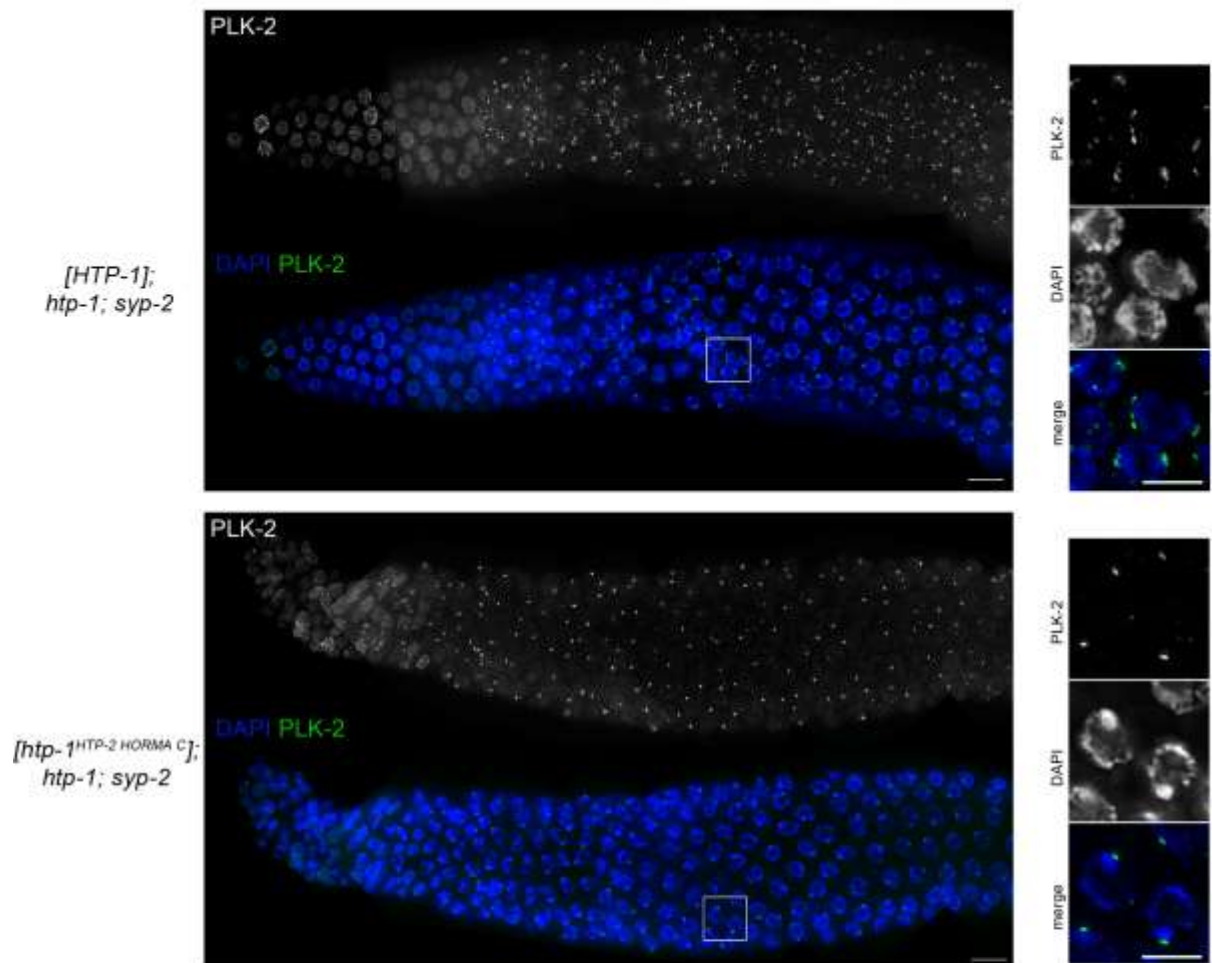
Figure 20. HTP-1^{HTP-2 HORMA C} fails to trigger the transition zone exit checkpoint in the complete absence of synapsis.

(A) Projection of germlines from worms carrying null *htp-1* and *syp-2* mutations and expressing *HTP-1::6xHis* or *htp-1^{HTP-2 HORMA C}*, stained with α PLK-2 and counterstained with DAPI. Germlines are shown from the mitotic tip to mid-pachytene. In both germlines, the PLK-2 staining pattern although different looks mostly constant from the apparition of PLK-2 foci onwards, rather than restricted to a “transition zone”. Miniatures are zooms of the framed areas on the germlines, and depict representative nuclei from these germlines. In the presence of wild type HTP-1, PLK-2 foci accumulate densely on chromatin along the length of the germline but in HORMA C mutants, PLK-2 foci are more spaced, and in fact most nuclei only contain a single one. Scale bars 10 μ m (germlines), 5 μ m (zooms).

(B) Representation of transition zone length and persistence of a single PLK-2 focus on the X chromosome, in worms expressing *HTP-1::6xHis* and *htp-1^{HTP-2 HORMA C}::6xHis* in the *htp-1; syp-2* double mutant background. Germlines were divided into rows of nuclei from the onset of transition zone – characterised by the apparition of PLK-2 foci – to the end of pachytene. A row of nuclei was considered to be in transition zone if over half the nuclei displayed two or more PLK-2 foci. Similarly, the persistence of the X chromosome focus was scored as the number of rows where over half the nuclei display at least one PLK-2 focus. Both measures are shown here as proportions of the entire region studied.

In germlines of worms expressing wild type HTP-1, the complete asynapsis phenotype induced by the *syp-2* null mutation triggers the transition zone exit checkpoint: transition zone extends to 80% of the germline length, as nuclei are maintained in an homology search state. In stark contrast, *htp-1^{HTP-2 HORMA C}* nuclei exit transition zone much quicker – after 14% of the way – despite the same asynapsis phenotype. Mean length of transition zone and X chromosome persistence between germlines expressing wild type *HTP-1* or *htp-1^{HTP-2 HORMA C}* were compared by a two-tailed independent t-test. ***: $P < 0.001$.

A



B

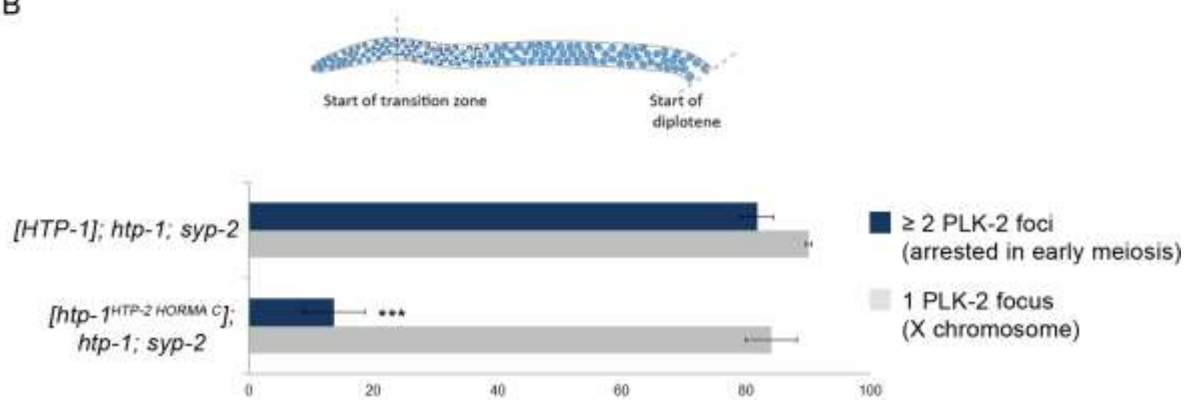
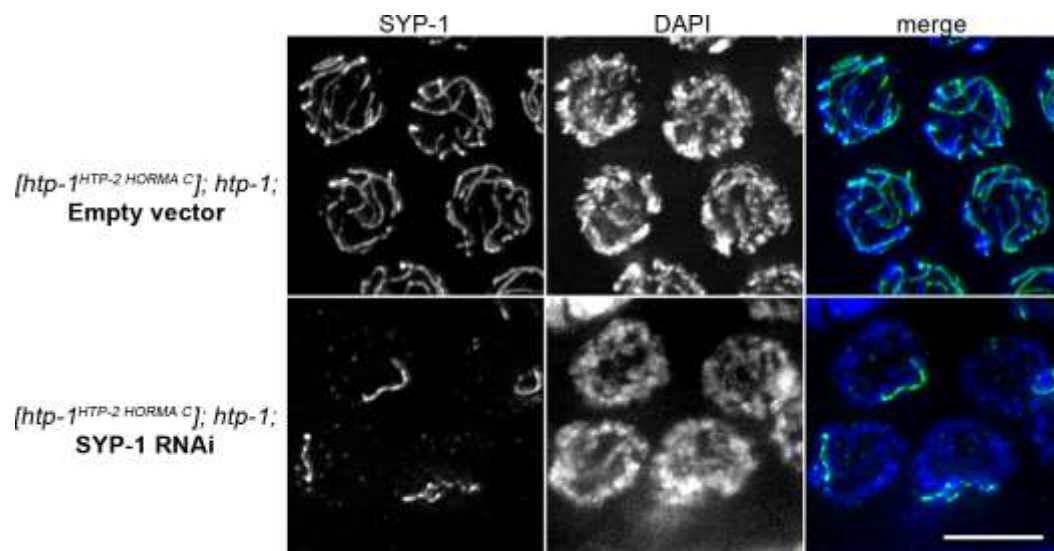


Figure 21. SYP-1 accumulates preferentially on a single track in *htp-1*^{HTP-2 HORMA C} mutants

Projections of representative pachytene nuclei of worms lacking the endogenous *HTP-1* locus and expressing *htp-1*^{HTP-2 HORMA C}, after being fed bacteria containing either an empty vector, or a vector coding for a shRNA complementary to SYP-1 and which leads to the depletion of SYP-1 mRNA by RNAi. Germlines were stained with α SYP-1 and counterstained with DAPI. Depletion of SYP-1 in the RNAi worms as not complete as SYP-1 was detected on the axes. However rather than a weaker staining following the same pattern as the empty vector control, in a low-SYP-1 situation SYP-1 was visible in a single bright track, showing the synaptonemal complex preferentially assembles on specific chromosomes. Scale bar 5 μ m.



5.3. The HORMA C region of HTP-1 supports HTP-1 function through an interplay with the C-terminus

5.3.1. The HORMA C region of HTP-1 is necessary but not sufficient to HTP-1 function.

The HORMA C region is implicated in the majority of HTP-1 function: it promotes pairing, the completion of synapsis and crossover formation, and is essential for the transition zone exit checkpoint. I therefore asked if a functional HORMA C domain could confer HTP-1 function to a mainly HTP-2 protein. To test this, I built a “reverse” protein to HTP-1^{HTP-2 HORMA C}; HTP-2^{HTP-1 HORMA C}, which consists of the HTP-2 sequence with only the HORMA C domain substituted for that of HTP-1.

Figure 22A shows the loading pattern of a GFP-tagged version of HTP-2^{HTP-1 HORMA C}, in worms lacking the endogenous *HTP-1* locus. Despite being loaded onto meiotic chromosome axes, a quantitative analysis of embryonic lethality and incidence of male progeny showed that over 80% of eggs laid by these worms do not hatch, and almost 40% of the surviving progeny are males (Figure 22B). These observations denote mis-segregation of both autosomes and the X-chromosome, and certify that HTP-2^{HTP-1 HORMA C} is incapable of performing HTP-1 function.

As the potential interfering role of the GFP tag in otherwise correct protein function came to light, the *htp-2*^{HTP-1 HORMA C} construct was altered to replace the GFP tag by a 6xHistidine tag. This new construct was injected into worms and then crossed onto an *htp-1* null background, and the associated protein was expressed and competent for loading onto chromosome axes (Figure 22C). However, the plate phenotype – low numbers of live progeny and presence of males – seemed to confirm that the meiotic defects were a consequence of the protein’s inability to function as HTP-1. In these worms, diakinesis nuclei displayed univalents (Figure 22C), evidence of a chiasma formation defect due to missing crossovers. Diakinesis nuclei contained an average of 9.3 ± 1.5 chromatin bodies, which is comparable to, and even a

little worse than the *htp-1^{HTP-2 HORMA C}* mutants, which diakinesis oocytes contain an average 9.1 bodies. Thus, *HTP-2^{HTP-1 HORMA C}* unequivocally failed to support HTP-1 function, indicating that while the HORMA C region is essential for HTP-1 function, it is not sufficient, alone, to carry it out. HORMA C must interact with, or be dependent on the function of other domains and regions within HTP-1, to carry out HTP-1 function.

5.3.2. A combination of the HORMA C and C-terminal regions of HTP-1 confer HTP-1 functionality to the HTP-2 sequence.

When considering the initial analysis of chiasma formation defects and mis-segregation across various HTP-1/2 point and domain mutants, mutations of the C-terminus stand out as a source of meiotic defects, albeit less severe than in HORMA and HORMA C mutants; this suggests the C-terminus plays a role in HTP-1 function (Chapter 3 2.3). Given these observations, and the inability of the HTP-1 HORMA C region to confer HTP-1 function to an otherwise HTP-2 protein, I built a strain expressing *htp-2^{HTP-1 HORMA C + C-term}*, a transgene coding for a chimeric protein comprising both the HORMA C and C-terminal regions of HTP-1, with the rest of the protein (N-terminus, HORMA A and HORMA B) corresponding to the HTP-2 sequence. The *htp-2^{HTP-1 HORMA C + C-term}* transgene was labelled with a 6xHistidine tag.

In an *htp-1* null background, the *htp-2^{HTP-1 HORMA C + C-term}* transgene was expressed and loaded onto meiotic chromosome axes and, strikingly, rescued the diakinesis phenotype characterised by the presence of univalents (Figure 23A). In *htp-2^{HTP-1 HORMA C + C-term}* worms, diakinesis nuclei displayed an average of 6.0 ± 0.4 chromatin bodies ($n = 36$). This indicates that these nuclei formed a full set of crossovers, and that *HTP-2^{HTP-1 HORMA C + C-term}* is therefore competent for crossover and chiasma formation across all chromosome pairs. In keeping with this finding, the progeny of *htp-2^{HTP-1 HORMA C + C-term}* worms presented an extremely low level of embryonic lethality, 1.66%, and the live progeny contained virtually no males (0.05%; Figure 23B). These results were identical to those of worms expressing a wild type *htp-1* transgene, confirming that *HTP-2^{HTP-1 HORMA C + C-term}* promotes correct chromosome segregation and is fully

Figure 22. The HORMA C region of HTP-1 is not sufficient for HTP-1 function.

(A) Projection of pachytene nuclei of worms expressing *HTP-1::GFP* or *htp-2^{HTP-1 HORMA C}::GFP* in the *htp-1* null background, stained with α GFP, and counterstained with DAPI. This “reverse” protein loads on meiotic chromosome axes similarly to HTP-1. Scale bar 5 μ m.

(B) Embryonic lethality and proportion of males among surviving progeny of worms expressing *HTP-1::GFP*, *htp-2^{HTP-1 HORMA C}::GFP* or no transgene, in the *htp-1* null background. Embryonic lethality and high incidence of males are indicative of aneuploidy caused by mis-segregation of chromosomes during meiosis. The high lethality and incidence of males associated with *htp-2^{HTP-1 HORMA C}* indicate that the associated protein is incapable of promoting faithful segregation of the autosomes and the X chromosome. The HORMA C region of HTP-1 does not support HTP-1 function in the HTP-2 sequence.

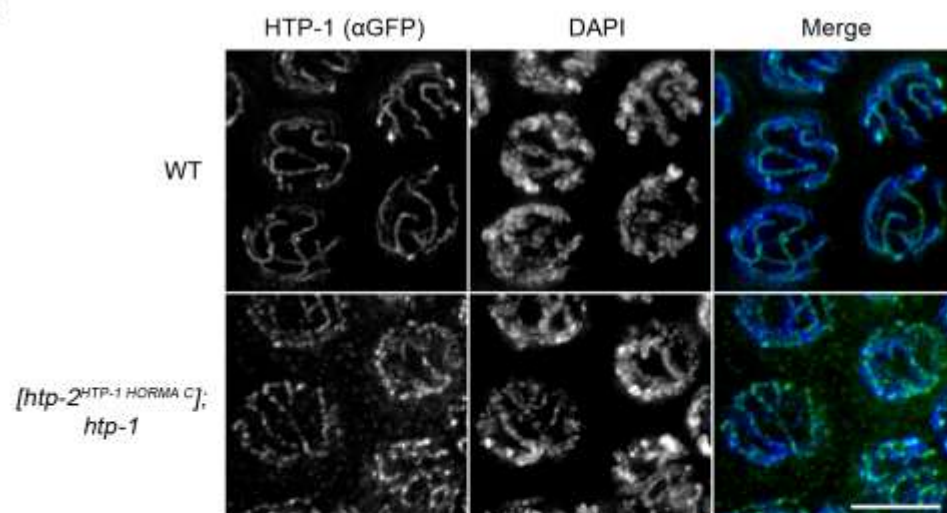
Number of laid eggs counted: 1124 (WT), 793 (*htp-2^{HTP-1 HORMA C}*), 1391 (*htp-1*).

Number of adults scored: 1186 (WT), 16 (*htp-2^{HTP-1 HORMA C}*), 40 (*htp-1*).

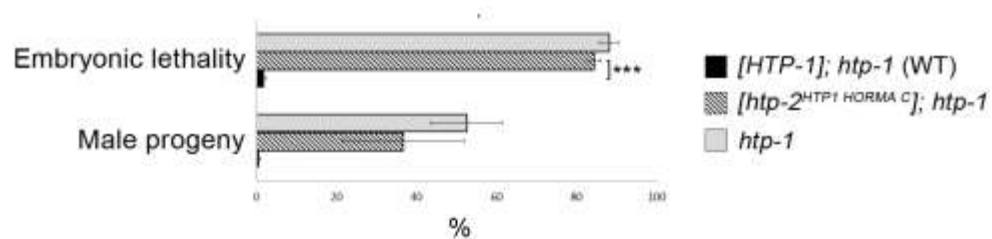
Means of the wild type and *htp-2^{HTP-1 HORMA C}* lethality and proportion of males were compared by a two-tailed independent t-test. ***: $P < 0.001$.

(C) Projection of pachytene nuclei of worms expressing *HTP-1::6xHis* or *htp-2^{HTP-1 HORMA C}::6xHis* in the *htp-1* null background, stained with α HisTag, and counterstained with DAPI, and corresponding representative DAPI-stained diakinesis oocytes. *HTP-2^{HTP-1 HORMA C}* loads on meiotic chromosome axes similarly to HTP-1, but fails to promote chiasma formation, as evidenced by the presence of univalents in diakinesis oocytes. Scale bars 5 μ m.

A



B



C

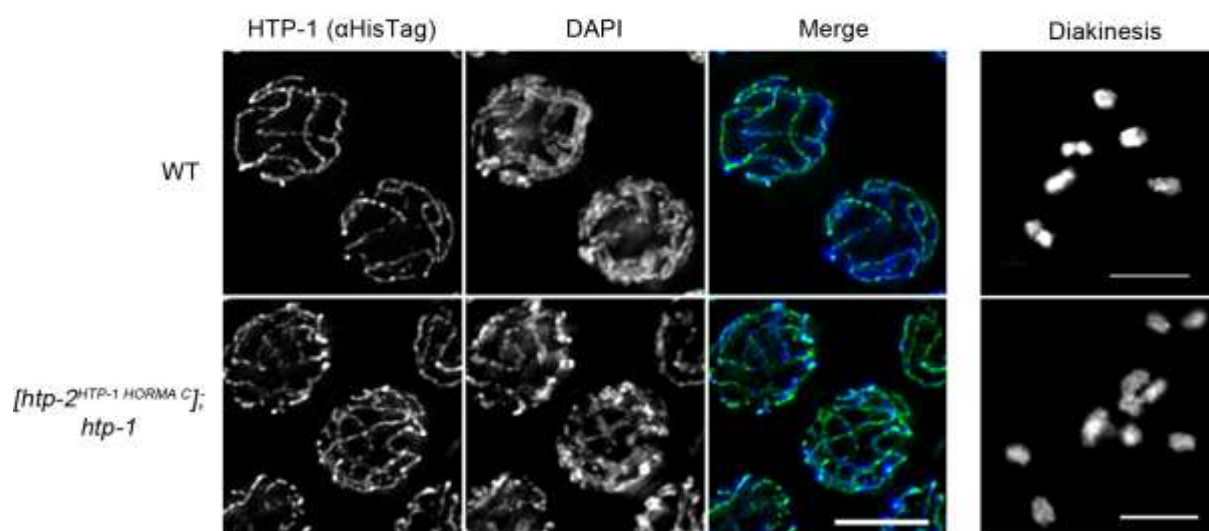


Figure 23. The HORMA C region and C-terminus of HTP-1 together are sufficient to confer HTP-1 function to the HTP-2 sequence.

(A) Projection of pachytene nuclei of worms expressing *HTP-1::6xHis* or *htp-2^{HTP-1 HORMA C + C-term}::6xHis* in the *htp-1* null background, stained with α HisTag, and counterstained with DAPI, and corresponding representative DAPI-stained diakinesis oocytes. *HTP-2^{HTP-1 HORMA C + C-term}* not only loads on meiotic chromosome axes similarly to HTP-1, but it is also competent to promote chiasma formation, as shown by the presence of six bivalents at diakinesis just like germlines expressing wild type HTP-1. Scale bars 5 μ m.

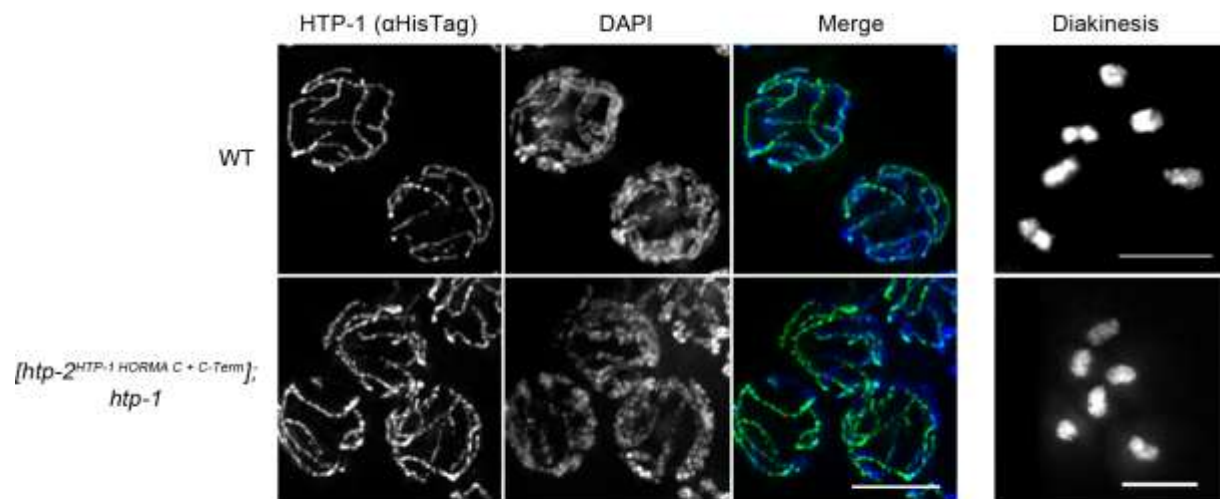
(B) Embryonic lethality and proportion of males among surviving progeny of worms expressing *HTP-1::6xHis*, *htp-2^{HTP-1 HORMA C + C-term}::6xHis* or no transgene, in the *htp-1* null background. Embryonic lethality and high incidence of males are indicative of aneuploidy caused by mis-segregation of chromosomes during meiosis. Strikingly, dead eggs and male worms were almost inexistent among the progeny of *htp-2^{HTP-1 HORMA C + C-term}* worms, so *HTP-2^{HTP-1 HORMA C + C-term}* is competent to promote correct segregation of all six pairs of chromosomes in *C. elegans*. The HORMA C domain therefore supports HTP-1-specific function through interaction with the C-terminus.

Number of laid eggs counted: 1339 (WT), 1726 (*htp-2^{HTP-1 HORMA C + C-term}*), 1344 (*htp-1*).

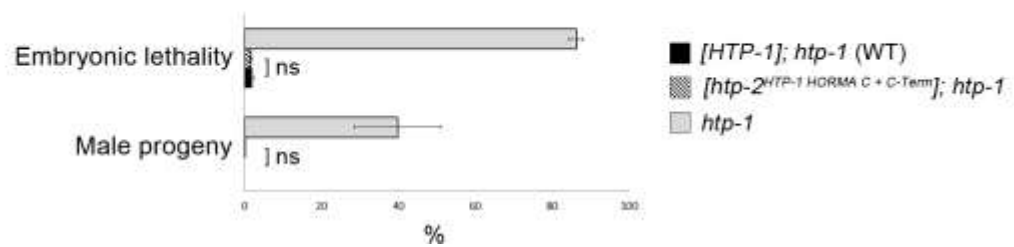
Number of adults scored: 1271 (WT), 1643 (*htp-2^{HTP-1 HORMA C + C-term}*), 37 (*htp-1*).

Mean lethality and proportion of males of wild type and *htp-2^{HTP-1 HORMA C + C-term}* worms were compared by a two-tailed independent t-test and were shown to be non-significant.

A



B



functional as HTP-1. In turn, this finding establishes that although the HORMA C region of HTP-1 is essential to support HTP-1 function, it does so through an interaction with the C-terminus of HTP-1, implicating some of the 18 point mutations between the C-termini of HTP-1 and HTP-2.

5.3.3. Conclusion

Despite the numerous HTP-1 functions dependent on the HORMA C region, such as pairing, completion of synapsis via the transition zone exit checkpoint and the timely repartition of early recombination intermediates, an HTP-2 protein carrying the HORMA C region of HTP-1 was not functional as HTP-1. Instead, the HORMA C region requires the C-terminus of HTP-1 to support HTP-1 function, as shown by the HTP-2^{HTP-1 HORMA C + C-term} recombinant protein, which appears fully functional as HTP-1. Several proteins of the HORMA domain family, such as MAD2 and REV-7 comprise almost entirely of the functional domain (Aravind and Koonin, 1998); the functional importance of the HTP-1 C-terminus is a particularity in keeping with the *C. elegans* meiotic HORMA domain proteins (Kim *et al.*, 2014). The fact that HORMA C and the C-terminus of HTP-1 can together support HTP-1 function by a mostly HTP-2 protein confirms that all residues essential to HTP-1-specific function are located within these two regions. In fact, it is likely that HTP-2 function was evolutionarily restricted through the accumulation of mutations in these regions.

5.4. Summary

The HORMA C region of HTP-1 is important for meiotic structures and processes; here, I show that HORMA C is also involved in coordinating these processes, by supporting HTP-1's function as an essential component of the transition zone exit checkpoint. HORMA C is necessary to the extension of transition zone and the maintenance of the homology search state until synapsis is complete. HORMA C itself must interact with other regions of HTP-1 to support HTP-1 function, and the C-terminus of HTP-1 is particularly crucial to the role of HORMA C. In

fact, HORMA C and the C-terminus together are sufficient to confer HTP-1 function to the HTP-2 sequence.

CHAPTER 6: DISCUSSION

6.1. Summary of Findings

In this project, I set out to elucidate functional regions and residues of HTP-1, with a view to improve understanding of HORMA domain proteins. Analysis of a set of chimeric HTP-1/HTP-2 proteins showed that ten residues in HORMA C, a subregion of the HORMA domain close to the C-terminus, were together essential for HTP-1 function. Since none of five point mutations within this region proved essential to HTP-1 function, and because these mutated residues are thought most likely to induce structural or polarity changes, or to be part of a region important for potential interaction, I suppose that the ten mismatches or a subset of them act together as a cluster to support HTP-1 function; this is a possible direction for further studies to complete the point mutant set. HTP-1^{HTP-2 HORMA C} loads slower than wild type HTP-1, but to similar final levels; this is unlikely to cause of any functional defects, as Kim *et al.* (2015) report 2% embryonic lethality and 0.5% male progeny, indicative of correct chromosome segregation, for a strain where recruitment of HTP-1 by HIM-3 is abrogated. Although they do not report on HTP-1 loading *in vivo* for this strain, they show *in vitro* that removing the HIM-3 closure motif reduces HTP-1 association by 20-25% of the wild type level in a HTP-3 pull-down. In comparison, at the onset of meiosis when the least HTP-1^{HTP-2 HORMA C} is loaded on chromosome axes, there is about 27% less signal intensity than in the wild type. These observations suggest meiotic defects in *htp-1*^{HTP-2 HORMA C} mutants are a consequence of mutations within the HORMA C domain.

The HORMA C region of HTP-1 is involved in multiple meiotic processes. Firstly, HORMA C promotes homologue pairing. Indeed, my analysis of pairing of the 5S rRNA locus showed that pairing remained at a constant level in HORMA C mutants (Figure 18). Synapsis defects occur in HORMA C mutants and have been shown to lead to unpaired signals during

pachytene; however, *syp-1* mutant nuclei still display an increase in pairing in zygotene and leptotene which is lost during pachytene, because initial pairing interactions fail to be stabilised by the SC (MacQueen *et al.*, 2002). The absence of such a pattern in HORMA C mutants suggests initial pairing interactions are affected independently from synapsis defects, however the level of pairing observed here in the premeiotic region is much higher than what has been previously published for *htp-1* mutants (Martinez-Perez *et al.*, 2008). This is also the case for the wild type pairing levels, which have been reported to be under 10% in zone 1 (Martinez-Perez *et al.*, 2008, Deshong *et al.*, 2014), indicating that perhaps the level of compaction of nuclei in the mitotic tip that precluded scoring of paired signals led to an excess representation of premeiotic and early transition zone nuclei in zone 1 in this study. Reported pairing in *htp-1* mutants in early meiosis is still lower than in *syp-2* mutants indicating that HTP-1 may contribute the establishment of SC-independent pairing, but is not as strict a requirement as CHK-2 (MacQueen and Villeneuve, 2001, Martinez-Perez *et al.*, 2008). Pairing levels observed in *htp-1*^{HTP-2 HORMA C} mutants from zone 2 onwards are consistent with previous reports of *htp-1* null mutants, suggesting that HTP-1's contribution to homologue pairing depends on HORMA C.

The HORMA C region is essential for the completion of synapsis, as worms expressing HTP-1^{HTP-2 HORMA C} presented unsynapsed chromatin regions at a frequency comparable to the *htp-1* null mutant, and with little variation throughout pachytene (Figure 17). In contrast, in *plk-2* mutants nuclei with unsynapsed portions of chromatin disappear in pachytene, as a block on non-homologous synapsis is lifted and the SC assembles indiscriminately between remaining chromosome axes (Harper *et al.*, 2011). The absence of this phenomenon in *htp-1*^{HTP-2 HORMA C} worms coupled with the high proportion of unpaired autosomes suggests that synapsis is already non homologous, as is the case in *htp-1* null mutants (Couteau and Zetka, 2005, Martinez-Perez and Villeneuve, 2005). Taken together, these results point to HORMA C as a requirement for HTP-1 to function as part of the checkpoint coordinating pairing with synapsis. This would not be surprising, as I also show that HORMA C is essential to support HTP-1 function as part of the transition zone exit checkpoint (Figures 19, 20). In *htp-1*^{HTP-2 HORMA C}

mutants, this checkpoint is non-existent: nuclei exit transition zone prematurely in the presence of unsynapsed axes, as well as in the complete absence of synapsis in the context of a *syp-2* deletion. The synapsis and pairing defects together explain the chiasma formation defects of the *htp-1^{HTP-2 HORMA C}* mutants, as crossovers require close proximity between homologous partners.

In *htp-1^{HTP-2 HORMA C}* mutants there is a marked reduction in the accumulation of RAD-51 foci compared to the wild type, but they also appear and disappear at a later stage, something that is not observed in the *htp-1* null mutant. It is unclear in this situation whether HTP-1 and its HORMA C domain may influence DSB formation, or repair. Hayashi *et al.* (2007) report that RAD-51 is loaded according to two different pathways during meiosis: RAD-51 is loaded on meiotic DSBs by RAD-50, and this pathway alone is associated with competence for crossover formation. However at the mid-pachytene transition a mechanism switch operates; RAD-51 can still be recruited to DSBs, but by a different pathway that cannot result in crossovers. It would be interesting to determine which pathway is active in *htp-1^{HTP-2 HORMA C}* mutants and whether this may contribute to the observed crossover defects. It is also possible that slower loading of HTP-1^{HTP-2 HORMA C} affects RAD-51 accumulation via a role of the chromosome axis structure.

Finally, the most striking result in this study of HTP-1 functional domains is undoubtedly the gain-of-function afforded by the conjoined presence of HORMA C and the C-terminus of HTP-1 in an otherwise HTP-2 sequence. This result points to HORMA C as the defining region for the specificity of HTP-1 function but also gives insight into the mode of action of HTP-1 which involves the C-terminus, and aligns with previous reports of a role for C-terminal cis sequences in meiotic HORMA domain protein function (Kim *et al.*, 2014).

6.2. HORMA C in the context of HTP-1 structure

As reported in structural studies of HORMA domain proteins, HORMA domains are composed of a globular core and a C-terminal tail (Hara *et al.*, 2010, Kim *et al.*, 2014, Luo *et al.*, 2000). In particular, the study of *C. elegans* meiotic HORMA domain proteins by Kim *et al.* (2014)

maps the globular core as the first ~150 residues of the HORMA domain. In HTP-1, this domain covers HORMA A and B, and the first third of HORMA C. Hence, most of HORMA C corresponds to the “C-terminal tail” of the HORMA domain – not to be mistaken for the C-terminus of the protein. This C-terminal tail acts as the “safety belt” wrapping around the protein to hold a ligand in place and is therefore essential to the activity of HORMA domain proteins. When considering that removal of the homologous portion of the MAD2 HORMA domain automatically leads to the open, inactive conformation of the protein MAD2^{ΔC} (Yang *et al.*, 2008), it isn’t surprising that HORMA C stands out as the essential region for HTP-1 function. However, it is interesting to note that this safety belt is not interchangeable between HTP-1 and HTP-2, a feature that could have been expected from a region with an (apparently) strictly structural function. Kim *et al.* (2014) identify the closure motifs of HTP-3 and HIM-3 as HTP-1 ligands; their binding forms the basis of HTP-1 recruitment to meiotic axes. This is consistent with the slower loading observed when mutating residues within HORMA C but taken together with my results, also suggests HTP-1 may bind other ligands as well. The facts that HTP-1 loading is not a prerequisite for checkpoint activity (Silva *et al.*, 2014), and that HTP-1^{HTP-2 HORMA C} is competent for loading but not checkpoint activity, show that loading and checkpoint activity are independent. Consequently, the HORMA C safety belt likely mediates checkpoint activity via a different mechanism than binding HIM-3 or HTP-3 closure motifs. It is an ongoing question whether HORMA domain proteins may function as dimers similarly to MAD2 (Yang *et al.*, 2008), and such a model could very likely explain the behaviour of HTP-1: an HTP-1 dimer could be recruited to the axes via one HORMA domain, leaving the other free to bind a different ligand – an interaction which would still rely on the HORMA C safety belt. As the pathways in which HORMA domain proteins operate and more interactors of HTP-1 come to light, it would be interesting to test this hypothesis.

The role of the C-terminus in HTP-1 function is interesting to ponder. Substitution of the C-terminus of HTP-1 for that of HTP-2 does induce meiotic defects (Figures 5, 6), although in a smaller scale than the HORMA C region substitution. A deletion of the last 11 residues of the

C-terminus of HTP-1 did not disrupt HTP-1 function (Consuelo Barroso, personal communication), indicating that the C-terminus of HTP-1 does not support HTP-1 function via a closure domain, as defined by Kim *et al.* (2014). This is consistent with their *in vitro* binding assays and the fact that HIM-3 and HTP-3 closure motifs function by recruiting other axis components, something HTP-1 does not do. I favour the possibility that the functional part of the C-terminus of HTP-1 may be the region closest to the HORMA domain, or that this region is even incorporated in the HORMA domain; it is possible that a number of residues form part of the safety belt or interact with it. A structural role for the C-terminus is also consistent with false positive results observed with GFP-tagged proteins but not His-tagged proteins (3.4.4.), as the GFP tag is bulky and attached to the C-terminal region.

Despite the prominent role of the HORMA C region and C-terminus of HTP-1, a survey of the HTP-1/HTP-2 chimeric mutant phenotypes reveals the influence of the other protein domains. The most striking is the absence of HTP-1^{HTP-2 HORMA A} from chromosome axes (Figure 7). This immediately brings to mind the *htp-1(fq8)* allele characterised by Silva *et al.* (2014), and which was competent to inhibit non-homologous synapsis and extend transition zone in response to synapsis defects despite not being detected on chromosome axes. Interestingly, the residue affected by the *htp-1(fq8)* point mutation, M127, is part of HORMA B and conserved between HTP-1 and HTP-2. The absence of HTP-1^{HTP-2 HORMA A} from chromosome axes is all the more incongruous because HTP-2 itself typically loads onto the axes. Nevertheless, both the *htp-1^{HTP-2 HORMA C}* and *htp-1(fq8)* are consistent with the model proposed by Kim *et al.* (2014) in which meiotic HORMA domain proteins are recruited to the axes by binding closure motifs of existing axis components via their own HORMA domains. Additionally, a role for HORMA A and B is apparent in two ways. First, the *him* phenotype is present in *htp-1^{HTP-2 HORMA}* mutants but disappears in *htp-1^{HTP-2 HORMA C}* mutants, indicating a role for the HORMA domain as a whole in regulating X chromosome segregation. Pairing of the X-chromosomes in *C. elegans* is independent from HTP-1 and requires only low levels of HIM-3 (Couteau *et al.*, 2004, Martinez-Perez and Villeneuve, 2005). The disappearance of the *him* phenotype when the substituted

region is restricted to HORMA C suggests that the X chromosome is capable of forming a crossover in *htp-1^{HTP-2 HORMA C}* mutants and hints at a hierarchy of defects in *htp-1^{HTP-2 HORMA}* mutants, where crossover formation defects affected all chromosome pairs on top of underlying defects observed in the *htp-1^{HTP-2 HORMA C}* mutants. This, and the delayed appearance and disappearance of RAD-51 foci in *htp-1^{HTP-2 HORMA C}* mutants, a phenotype not present in the *htp-1* null mutant, suggest that mismatches between HTP-1 and HTP-2 outside of HORMA C may play a part in the regulation of recombination. Finally, the competence of the HORMA A and B regions to support HORMA C and the C-terminus in HTP-1 function is seen in the striking difference between the mis-segregation phenotypes of *htp-1^{HTP-2 C-term}* and *htp-2^{HTP-1 HORMA C}* mutants (Figures 5, 6, 22). Both of these mutants express a protein associating the HORMA C domain of HTP-1 with the C-terminus of HTP-2. *htp-2^{HTP-1 HORMA C}* worms recapitulate the *htp-1* null mutant phenotype, however when the N-terminus and HORMA A/B of HTP-1 are present, the mis-segregation phenotype, although significant, is clearly not comparable, showing that different regions of HTP-1 all have a part to play in supporting HORMA C during HTP-1 function. The phenotypes of various HTP-1 domain mutants definitely highlight the complexity of relating sequence to function with a stochastic approach to determining subdomains. It is possible that functions are supported by multiple arbitrary domains and not uncovered by only a partial substitution of the implicated region.

6.3. Conclusion: towards a HORMA domain mechanism

Structurally, HORMA domain proteins seem to function in a similar fashion with a C-terminal safety belt, and functional similarities between them suggest that they could act in checkpoint pathways via conserved mechanisms. As described in 1.3., HTP-1 homologues in Yeast and mammals, as well as MAD2 in mitosis, are involved in checkpoint mechanisms reminiscent of HTP-1 function (Chen *et al.*, 1996, Kim *et al.*, 2010, Wojtasz *et al.*, 2012), and a role for MAD2 has even been proposed in meiosis, where it may act in the stabilisation of early homologue interactions (Bohr *et al.*, 2015). HOP1, like HTP-1 is a phosphoprotein and HOP1

requires phosphorylation within the HORMA domain to activate its downstream target Mek1 (Niu *et al.*, 2005). Lastly, several organisms seem to possess pairs of homologous HORMA domain proteins with a role in controlling meiotic events (ASY-1/2, HORMAD1/2, HTP-1/2), where one of them is essential for recombination, faithful segregation or elimination of defective meiocytes, and the other is partly redundant and non-essential for fertility (Caryl *et al.*, 2000, Martinez-Perez *et al.*, 2008, Shin *et al.*, 2010, Wojtasz *et al.*, 2012). In this study, I have shown the functional importance of the C-terminal tail of the HTP-1 HORMA domain and identified ten residues that are essential for HTP-1 function, showing that this region, in HTP-1, is the centrepiece of an interplay involving also the C-terminus and to a lesser extent HORMA A/B. Analysing the HTP-1 sequence from a functional point of view not only furthers our understanding of meiosis and the regulation of its progression but also contribute to the knowledge pool about the way HORMA domain proteins operate, which has a wide range of implications across organisms and biological processes. Meiotic HORMA domain proteins are a particularly interesting model, as the control of meiotic progression has clinical relevance in several ways. In addition to the implication of meiotic defects in loss of fertility, meiosis and the mechanisms controlling it could become attractive targets for male contraception, which has been currently contemplated mainly through hormonal methods (Behre *et al.*, 2016, World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1996); targeting meiosis-specific components and pathways may eliminate side-effects resulting from the wide range of targets of hormones in the organism.

REFERENCES

- Alpi, A., Pasierbek, P., Gartner, A. & Loidl, J. 2003. Genetic and cytological characterization of the recombination protein RAD-51 in *Caenorhabditis elegans*. *Chromosoma*, 112, 6-16.
- Aravind, L. & Koonin, E. V. 1998. The HORMA domain: a common structural denominator in mitotic checkpoints, chromosome synapsis and DNA repair. *Trends in Biochemical Sciences*, 23, 284-286.
- Armstrong, S. J., Caryl, A. P., Jones, G. H. & Franklin, F. C. 2002. Asy1, a protein required for meiotic chromosome synapsis, localizes to axis-associated chromatin in *Arabidopsis* and *Brassica*. *J Cell Sci*, 115, 3645-55.
- Barnes, T. M., Kohara, Y., Coulson, A. & Hekimi, S. 1995. Meiotic recombination, noncoding DNA and genomic organization in *Caenorhabditis elegans*. *Genetics*, 141, 159-79.
- Behre, H. M., Zitzmann, M., Anderson, R. A., Handelsman, D. J., Lestari, S. W., McLachlan, R. I., Meriggiola, M. C., Misro, M. M., Noe, G., Wu, F. C., *et al.* 2016. Efficacy and Safety of an Injectable Combination Hormonal Contraceptive for Men. *J Clin Endocrinol Metab*, jc20162141.
- Berchowitz, L. E. & Copenhaver, G. P. 2010. Genetic Interference: Don't Stand So Close to Me. *Current Genomics*, 11, 91-102.
- Bhalla, N. & Dernburg, A. F. 2005. A conserved checkpoint monitors meiotic chromosome synapsis in *Caenorhabditis elegans*. *Science*, 310, 1683-6.
- Bohr, T., Ashley, G., Eggleston, E., Firestone, K. & Bhalla, N. 2016. Synaptonemal Complex Components Are Required for Meiotic Checkpoint Function in *Caenorhabditis elegans*. *Genetics*.
- Bohr, T., Nelson, C. R., Klee, E. & Bhalla, N. 2015. Spindle assembly checkpoint proteins regulate and monitor meiotic synapsis in *C. elegans*. *J Cell Biol*, 211, 233-42.

- Bolor, H., Mori, T., Nishiyama, S., Ito, Y., Hosoba, E., Inagaki, H., Kogo, H., Ohye, T., Tsutsumi, M., Kato, T., *et al.* 2009. Mutations of the SYCP3 gene in women with recurrent pregnancy loss. *American journal of human genetics*, 84, 14-20.
- Borner, G. V., Barot, A. & Kleckner, N. 2008. Yeast Pch2 promotes domainal axis organization, timely recombination progression, and arrest of defective recombinosomes during meiosis. *Proc Natl Acad Sci U S A*, 105, 3327-32.
- Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics*, 77, 71-94.
- Buonomo, S. B., Clyne, R. K., Fuchs, J., Loidl, J., Uhlmann, F. & Nasmyth, K. 2000. Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin. *Cell*, 103, 387-98.
- Carballo, J. A., Johnson, A. L., Sedgwick, S. G. & Cha, R. S. 2008. Phosphorylation of the axial element protein Hop1 by Mec1/Tel1 ensures meiotic interhomolog recombination. *Cell*, 132, 758-70.
- Caryl, A. P., Armstrong, S. J., Jones, G. H. & Franklin, F. C. 2000. A homologue of the yeast HOP1 gene is inactivated in the Arabidopsis meiotic mutant *asy1*. *Chromosoma*, 109, 62-71.
- Chen, R. H., Waters, J. C., Salmon, E. D. & Murray, A. W. 1996. Association of spindle assembly checkpoint component XMad2 with unattached kinetochores. *Science*, 274, 242-6.
- Chung, G., Rose, A. M., Petalcorin, M. I., Martin, J. S., Kessler, Z., Sanchez-Pulido, L., Ponting, C. P., Yanowitz, J. L. & Boulton, S. J. 2015. REC-1 and HIM-5 distribute meiotic crossovers and function redundantly in meiotic double-strand break formation in *Caenorhabditis elegans*. *Genes Dev*, 29, 1969-79.
- Colaiacono, M. P. 2006. The many facets of SC function during *C. elegans* meiosis. *Chromosoma*, 115, 195-211.
- Colaiacono, M. P., Macqueen, A. J., Martinez-Perez, E., McDonald, K., Adamo, A., La Volpe, A. & Villeneuve, A. M. 2003. Synaptonemal complex assembly in *C. elegans* is dispensable for loading strand-exchange proteins but critical for proper completion of recombination. *Dev Cell*, 5, 463-74.

- Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic acids research*, 16, 10881-90.
- Couteau, F., Nabeshima, K., Villeneuve, A. & Zetka, M. 2004. A component of C-elegans meiotic chromosome axes at the interface of homolog alignment, synapsis, nuclear reorganization, and recombination. *Current Biology*, 14, 585-592.
- Couteau, F. & Zetka, M. 2005. HTP-1 coordinates synaptonemal complex assembly with homolog alignment during meiosis in C-elegans. *Genes & Development*, 19, 2744-2756.
- Crittenden, S. L., Troemel, E. R., Evans, T. C. & Kimble, J. 1994. GLP-1 is localized to the mitotic region of the *C. elegans* germ line. *Development*, 120, 2901-11.
- Daniel, K., Lange, J., Hached, K., Fu, J., Anastassiadis, K., Roig, I., Cooke, H. J., Stewart, A. F., Wassmann, K., Jasin, M., *et al.* 2011. Meiotic homologue alignment and its quality surveillance are controlled by mouse HORMAD1. *Nat Cell Biol*, 13, 599-610.
- De Carvalho, C. E., Zaaier, S., Smolikov, S., Gu, Y., Schumacher, J. M. & Colaiacovo, M. P. 2008. LAB-1 antagonizes the Aurora B kinase in *C. elegans*. *Genes Dev*, 22, 2869-85.
- Dernburg, A. F., Mcdonald, K., Moulder, G., Barstead, R., Dresser, M. & Villeneuve, A. M. 1998. Meiotic recombination in *C. elegans* initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. *Cell*, 94, 387-98.
- Deshong, A. J., Ye, A. L., Lamelza, P. & Bhalla, N. 2014. A quality control mechanism coordinates meiotic prophase events to promote crossover assurance. *PLoS Genet*, 10, e1004291.
- Duncan, F. E., Hornick, J. E., Lampson, M. A., Schultz, R. M., Shea, L. D. & Woodruff, T. K. 2012. Chromosome cohesion decreases in human eggs with advanced maternal age. *Aging Cell*, 11, 1121-4.
- Farboud, B. & Meyer, B. J. 2015. Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. *Genetics*, 199, 959-71.
- Ferguson, E. L. & Horvitz, H. R. 1985. Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. *Genetics*, 110, 17-72.

- Foss, E. J. & Stahl, F. W. 1995. A test of a counting model for chiasma interference. *Genetics*, 139, 1201-9.
- Francis, R., Barton, M. K., Kimble, J. & Schedl, T. 1995. *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. *Genetics*, 139, 579-606.
- Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. & Ahringer, J. 2000. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature*, 408, 325-30.
- Frokjaer-Jensen, C., Davis, M. W., Ailion, M. & Jorgensen, E. M. 2012. Improved Mos1-mediated transgenesis in *C. elegans*. *Nature Methods*, 9, 117-118.
- Frokjaer-Jensen, C., Davis, M. W., Hopkins, C. E., Newman, B. J., Thummel, J. M., Olesen, S. P., Grunnet, M. & Jorgensen, E. M. 2008. Single-copy insertion of transgenes in *Caenorhabditis elegans*. *Nature Genetics*, 40, 1375-1383.
- Goodyer, W., Kaitna, S., Couteau, F., Ward, J. D., Boulton, S. J. & Zetka, M. 2008. HTP-3 links DSB formation with homolog pairing and crossing over during *C. elegans* meiosis. *Developmental cell*, 14, 263-74.
- Haering, C. H., Farcas, A. M., Arumugam, P., Metson, J. & Nasmyth, K. 2008. The cohesin ring concatenates sister DNA molecules. *Nature*, 454, 297-301.
- Haering, C. H., Lowe, J., Hochwagen, A. & Nasmyth, K. 2002. Molecular architecture of SMC proteins and the yeast cohesin complex. *Mol Cell*, 9, 773-88.
- Hara, K., Hashimoto, H., Murakumo, Y., Kobayashi, S., Kogame, T., Unzai, S., Akashi, S., Takeda, S., Shimizu, T. & Sato, M. 2010. Crystal structure of human REV7 in complex with a human REV3 fragment and structural implication of the interaction between DNA polymerase zeta and REV1. *J Biol Chem*, 285, 12299-307.
- Harper, N. C., Rillo, R., Jover-Gil, S., Assaf, Z. J., Bhalla, N. & Dernburg, A. F. 2011. Pairing centers recruit a Polo-like kinase to orchestrate meiotic chromosome dynamics in *C. elegans*. *Dev Cell*, 21, 934-47.

- Hassold, T., Hall, H. & Hunt, P. 2007. The origin of human aneuploidy: where we have been, where we are going. *Human molecular genetics*, 16 Spec No. 2, R203-8.
- Hassold, T. J. & Jacobs, P. A. 1984. Trisomy in man. *Annu Rev Genet*, 18, 69-97.
- Hayashi, M., Chin, G. M. & Villeneuve, A. M. 2007. *C. elegans* germ cells switch between distinct modes of double-strand break repair during meiotic prophase progression. *PLoS Genet*, 3, e191.
- Hillers, K. J. & Villeneuve, A. M. 2003. Chromosome-wide control of meiotic crossing over in *C. elegans*. *Curr Biol*, 13, 1641-7.
- Hiraoka, Y. & Dernburg, A. F. 2009. The SUN rises on meiotic chromosome dynamics. *Dev Cell*, 17, 598-605.
- Hodgkin, J., Horvitz, H. R. & Brenner, S. 1979. Nondisjunction Mutants of the Nematode *CAENORHABDITIS ELEGANS*. *Genetics*, 91, 67-94.
- Hollingsworth, N. M. & Byers, B. 1989. HOP1: a yeast meiotic pairing gene. *Genetics*, 121, 445-62.
- Hollingsworth, N. M., Goetsch, L. & Byers, B. 1990. The HOP1 gene encodes a meiosis-specific component of yeast chromosomes. *Cell*, 61, 73-84.
- Hong, S., Sung, Y., Yu, M., Lee, M., Kleckner, N. & Kim, K. P. 2013. The logic and mechanism of homologous recombination partner choice. *Mol Cell*, 51, 440-53.
- Jacobs, P. A., Baikie, A. G., Court Brown, W. M. & Strong, J. A. 1959. The somatic chromosomes in mongolism. *Lancet*, 1, 710.
- Janssens, F. A., Koszul, R. & Zickler, D. 2012. The chiasmatype theory. A new interpretation of the maturation divisions. 1909. *Genetics*, 191, 319-46.
- Jones, G. H. 1984. The control of chiasma distribution. *Symp Soc Exp Biol*, 38, 293-320.
- Joshi, N., Barot, A., Jamison, C. & Borner, G. V. 2009. Pch2 links chromosome axis remodeling at future crossover sites and crossover distribution during yeast meiosis. *PLoS Genet*, 5, e1000557.

- Kaitna, S., Pasierbek, P., Jantsch, M., Loidl, J. & Glotzer, M. 2002. The aurora B kinase AIR-2 regulates kinetochores during mitosis and is required for separation of homologous Chromosomes during meiosis. *Curr Biol*, 12, 798-812.
- Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. & Ahringer, J. 2001. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol*, 2, RESEARCH0002.
- Keeney, S., Giroux, C. N. & Kleckner, N. 1997. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell*, 88, 375-84.
- Kelly, K. O., Dernburg, A. F., Stanfield, G. M. & Villeneuve, A. M. 2000. *Caenorhabditis elegans* msh-5 is required for both normal and radiation-induced meiotic crossing over but not for completion of meiosis. *Genetics*, 156, 617-30.
- Kelly, W. G., Schaner, C. E., Dernburg, A. F., Lee, M. H., Kim, S. K., Villeneuve, A. M. & Reinke, V. 2002. X-chromosome silencing in the germline of *C. elegans*. *Development*, 129, 479-92.
- Kim, K. P., Weiner, B. M., Zhang, L., Jordan, A., Dekker, J. & Kleckner, N. 2010. Sister cohesion and structural axis components mediate homolog bias of meiotic recombination. *Cell*, 143, 924-37.
- Kim, Y., Kostow, N. & Dernburg, A. F. 2015. The Chromosome Axis Mediates Feedback Control of CHK-2 to Ensure Crossover Formation in *C. elegans*. *Dev Cell*, 35, 247-61.
- Kim, Y., Rosenberg, S. C., Kugel, C. L., Kostow, N., Rog, O., Davydov, V., Su, T. Y., Dernburg, A. F. & Corbett, K. D. 2014. The chromosome axis controls meiotic events through a hierarchical assembly of HORMA domain proteins. *Dev Cell*, 31, 487-502.
- King, J. S. & Mortimer, R. K. 1990. A polymerization model of chiasma interference and corresponding computer simulation. *Genetics*, 126, 1127-38.
- Kleckner, N., Zickler, D., Jones, G. H., Dekker, J., Padmore, R., Henle, J. & Hutchinson, J. 2004. A mechanical basis for chromosome function. *Proc Natl Acad Sci U S A*, 101, 12592-7.

- Klein, F., Mahr, P., Galova, M., Buonomo, S. B., Michaelis, C., Nairz, K. & Nasmyth, K. 1999. A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. *Cell*, 98, 91-103.
- Koshland, D. E. & Guacci, V. 2000. Sister chromatid cohesion: the beginning of a long and beautiful relationship. *Current opinion in cell biology*, 12, 297-301.
- Kueng, S., Hegemann, B., Peters, B. H., Lipp, J. J., Schleiffer, A., Mechtler, K. & Peters, J. M. 2006. Wapl controls the dynamic association of cohesin with chromatin. *Cell*, 127, 955-67.
- Labella, S., Woglar, A., Jantsch, V. & Zetka, M. 2011. Polo kinases establish links between meiotic chromosomes and cytoskeletal forces essential for homolog pairing. *Dev Cell*, 21, 948-58.
- Lambing, C., Osman, K., Nuntasontorn, K., West, A., Higgins, J. D., Copenhaver, G. P., Yang, J., Armstrong, S. J., Mechtler, K., Roitingner, E., *et al.* 2015. Arabidopsis PCH2 Mediates Meiotic Chromosome Remodeling and Maturation of Crossovers. *PLoS Genet*, 11, e1005372.
- Lara-Gonzalez, P., Westhorpe, F. G. & Taylor, S. S. 2012. The spindle assembly checkpoint. *Curr Biol*, 22, R966-80.
- Li, Y. & Benezra, R. 1996. Identification of a human mitotic checkpoint gene: hsMAD2. *Science*, 274, 246-8.
- Libuda, D. E., Uzawa, S., Meyer, B. J. & Villeneuve, A. M. 2013. Meiotic chromosome structures constrain and respond to designation of crossover sites. *Nature*, 502, 703-6.
- Lui, D. Y. & Colaiacovo, M. P. 2013. Meiotic development in *Caenorhabditis elegans*. *Advances in experimental medicine and biology*, 757, 133-70.
- Luo, X., Fang, G., Coldiron, M., Lin, Y., Yu, H., Kirschner, M. W. & Wagner, G. 2000. Structure of the Mad2 spindle assembly checkpoint protein and its interaction with Cdc20. *Nat Struct Biol*, 7, 224-9.
- Luo, X., Tang, Z., Rizo, J. & Yu, H. 2002. The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. *Mol Cell*, 9, 59-71.

- Luo, X., Tang, Z., Xia, G., Wassmann, K., Matsumoto, T., Rizo, J. & Yu, H. 2004. The Mad2 spindle checkpoint protein has two distinct natively folded states. *Nat Struct Mol Biol*, 11, 338-45.
- Macqueen, A. J., Colaiacovo, M. P., McDonald, K. & Villeneuve, A. M. 2002. Synapsis-dependent and -independent mechanisms stabilize homolog pairing during meiotic prophase in *C. elegans*. *Genes Dev*, 16, 2428-42.
- Macqueen, A. J., Phillips, C. M., Bhalla, N., Weiser, P., Villeneuve, A. M. & Dernburg, A. F. 2005. Chromosome sites play dual roles to establish homologous synapsis during meiosis in *C. elegans*. *Cell*, 123, 1037-50.
- Macqueen, A. J. & Villeneuve, A. M. 2001. Nuclear reorganization and homologous chromosome pairing during meiotic prophase require *C. elegans* chk-2. *Genes Dev*, 15, 1674-87.
- Martinez-Perez, E., Schvarzstein, M., Barroso, C., Lightfoot, J., Dernburg, A. F. & Villeneuve, A. M. 2008. Crossovers trigger a remodeling of meiotic chromosome axis composition that is linked to two-step loss of sister chromatid cohesion. *Genes & Development*, 22, 2886-2901.
- Martinez-Perez, E. & Villeneuve, A. M. 2005. HTP-1-dependent constraints coordinate homolog pairing and synapsis and promote chiasma formation during *C. elegans* meiosis. *Genes & Development*, 19, 2727-2743.
- Meneely, P. M., McGovern, O. L., Heinis, F. I. & Yanowitz, J. L. 2012. Crossover distribution and frequency are regulated by him-5 in *Caenorhabditis elegans*. *Genetics*, 190, 1251-66.
- Mets, D. G. & Meyer, B. J. 2009. Condensins regulate meiotic DNA break distribution, thus crossover frequency, by controlling chromosome structure. *Cell*, 139, 73-86.
- Michaelis, C., Ciosk, R. & Nasmyth, K. 1997. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell*, 91, 35-45.
- Miyazaki, W. Y. & Orr-Weaver, T. L. 1994. Sister-chromatid cohesion in mitosis and meiosis. *Annu Rev Genet*, 28, 167-87.

- Moses, M. J. 1969. Structure and function of the synaptonemal complex. *Genetics*, 61, Suppl:41-51.
- Muller, H. J. 1916. The Mechanism of Crossing-Over. *The American Naturalist*, 50, 193-221.
- Nabeshima, K., Villeneuve, A. M. & Colaiacovo, M. P. 2005. Crossing over is coupled to late meiotic prophase bivalent differentiation through asymmetric disassembly of the SC. *J Cell Biol*, 168, 683-9.
- Nabeshima, K., Villeneuve, A. M. & Hillers, K. J. 2004. Chromosome-wide regulation of meiotic crossover formation in *Caenorhabditis elegans* requires properly assembled chromosome axes. *Genetics*, 168, 1275-92.
- Nadarajan, S., Mohideen, F., Tzur, Y. B., Ferrandiz, N., Crawley, O., Montoya, A., Faull, P., Snijders, A. P., Cutillas, P. R., Jambhekar, A., *et al.* 2016. The MAP kinase pathway coordinates crossover designation with disassembly of synaptonemal complex proteins during meiosis. *Elife*, 5, e12039.
- Nishi, Y., Rogers, E., Robertson, S. M. & Lin, R. 2008. Polo kinases regulate *C. elegans* embryonic polarity via binding to DYRK2-primed MEX-5 and MEX-6. *Development*, 135, 687-97.
- Niu, H., Wan, L., Baumgartner, B., Schaefer, D., Loidl, J. & Hollingsworth, N. M. 2005. Partner choice during meiosis is regulated by Hop1-promoted dimerization of Mek1. *Mol Biol Cell*, 16, 5804-18.
- Nonomura, K., Nakano, M., Eiguchi, M., Suzuki, T. & Kurata, N. 2006. PAIR2 is essential for homologous chromosome synapsis in rice meiosis I. *J Cell Sci*, 119, 217-25.
- Nonomura, K. I., Nakano, M., Murata, K., Miyoshi, K., Eiguchi, M., Miyao, A., Hirochika, H. & Kurata, N. 2004. An insertional mutation in the rice PAIR2 gene, the ortholog of Arabidopsis ASY1, results in a defect in homologous chromosome pairing during meiosis. *Molecular genetics and genomics : MGG*, 271, 121-9.
- Page, S. L. & Hawley, R. S. 2004. The genetics and molecular biology of the synaptonemal complex. *Annu Rev Cell Dev Biol*, 20, 525-58.

- Pasierbek, P., Jantsch, M., Melcher, M., Schleiffer, A., Schweizer, D. & Loidl, J. 2001. A *Caenorhabditis elegans* cohesion protein with functions in meiotic chromosome pairing and disjunction. *Genes Dev*, 15, 1349-60.
- Penkner, A. M., Fridkin, A., Gloggnitzer, J., Baudrimont, A., Machacek, T., Woglar, A., Csaszar, E., Pasierbek, P., Ammerer, G., Gruenbaum, Y., *et al.* 2009. Meiotic chromosome homology search involves modifications of the nuclear envelope protein Matefin/SUN-1. *Cell*, 139, 920-33.
- Petes, T. D. 2001. Meiotic recombination hot spots and cold spots. *Nat Rev Genet*, 2, 360-9.
- Petronczki, M., Siomos, M. F. & Nasmyth, K. 2003. Un menage a quatre: the molecular biology of chromosome segregation in meiosis. *Cell*, 112, 423-40.
- Phillips, C. M. & Dernburg, A. F. 2006. A family of zinc-finger proteins is required for chromosome-specific pairing and synapsis during meiosis in *C. elegans*. *Dev Cell*, 11, 817-29.
- Phillips, C. M., Wong, C., Bhalla, N., Carlton, P. M., Weiser, P., Meneely, P. M. & Dernburg, A. F. 2005. HIM-8 binds to the X chromosome pairing center and mediates chromosome-specific meiotic synapsis. *Cell*, 123, 1051-63.
- Rogers, E., Bishop, J. D., Waddle, J. A., Schumacher, J. M. & Lin, R. 2002. The aurora kinase AIR-2 functions in the release of chromosome cohesion in *Caenorhabditis elegans* meiosis. *J Cell Biol*, 157, 219-29.
- Rose, A. M. & Baillie, D. L. 1979. A mutation in *Caenorhabditis elegans* that increases recombination frequency more than threefold. *Nature*, 281, 599-600.
- Ross, K. J., Fransz, P., Armstrong, S. J., Vizir, I., Mulligan, B., Franklin, F. C. & Jones, G. H. 1997. Cytological characterization of four meiotic mutants of *Arabidopsis* isolated from T-DNA-transformed lines. *Chromosome Res*, 5, 551-9.
- Rosu, S., Zawadzki, K. A., Stamper, E. L., Libuda, D. E., Reese, A. L., Dernburg, A. F. & Villeneuve, A. M. 2013. The *C. elegans* DSB-2 protein reveals a regulatory network that controls

- competence for meiotic DSB formation and promotes crossover assurance. *PLoS Genet*, 9, e1003674.
- Sanchez-Moran, E., Santos, J. L., Jones, G. H. & Franklin, F. C. 2007. ASY1 mediates AtDMC1-dependent interhomolog recombination during meiosis in Arabidopsis. *Genes Dev*, 21, 2220-33.
- Sanchez Moran, E., Armstrong, S. J., Santos, J. L., Franklin, F. C. & Jones, G. H. 2001. Chiasma formation in Arabidopsis thaliana accession Wassileskija and in two meiotic mutants. *Chromosome Res*, 9, 121-8.
- Sato, A., Isaac, B., Phillips, C. M., Rillo, R., Carlton, P. M., Wynne, D. J., Kasad, R. A. & Dernburg, A. F. 2009. Cytoskeletal forces span the nuclear envelope to coordinate meiotic chromosome pairing and synapsis. *Cell*, 139, 907-19.
- Schedl, T. 1997. Developmental Genetics of the Germ Line. In: RIDDLE, D. L., BLUMENTHAL, T., MEYER, B. J. & PRIESS, J. R. (eds.) *C. elegans II*. 2nd ed. Cold Spring Harbor (NY).
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods*, 9, 676-82.
- Schwarzstein, M., Pattabiraman, D., Bembenek, J. N. & Villeneuve, A. M. 2013. Meiotic HORMA domain proteins prevent untimely centriole disengagement during Caenorhabditis elegans spermatocyte meiosis. *Proc Natl Acad Sci U S A*, 110, E898-907.
- Schwacha, A. & Kleckner, N. 1994. Identification of joint molecules that form frequently between homologs but rarely between sister chromatids during yeast meiosis. *Cell*, 76, 51-63.
- Schwacha, A. & Kleckner, N. 1997. Interhomolog bias during meiotic recombination: meiotic functions promote a highly differentiated interhomolog-only pathway. *Cell*, 90, 1123-35.
- Severson, A. F., Ling, L., Van Zuylen, V. & Meyer, B. J. 2009. The axial element protein HTP-3 promotes cohesin loading and meiotic axis assembly in *C. elegans* to implement the meiotic program of chromosome segregation. *Genes Dev*, 23, 1763-78.

- Severson, A. F. & Meyer, B. J. 2014. Divergent kleisin subunits of cohesin specify mechanisms to tether and release meiotic chromosomes. *Elife*, 3, e03467.
- Shin, Y. H., Choi, Y., Erdin, S. U., Yatsenko, S. A., Kloc, M., Yang, F., Wang, P. J., Meistrich, M. L. & Rajkovic, A. 2010. Hormad1 mutation disrupts synaptonemal complex formation, recombination, and chromosome segregation in mammalian meiosis. *PLoS Genet*, 6, e1001190.
- Shinohara, M., Oh, S. D., Hunter, N. & Shinohara, A. 2008. Crossover assurance and crossover interference are distinctly regulated by the ZMM proteins during yeast meiosis. *Nat Genet*, 40, 299-309.
- Silva, N., Ferrandiz, N., Barroso, C., Tognetti, S., Lightfoot, J., Telecan, O., Encheva, V., Faull, P., Hanni, S., Furger, A., *et al.* 2014. The fidelity of synaptonemal complex assembly is regulated by a signaling mechanism that controls early meiotic progression. *Dev Cell*, 31, 503-11.
- Sironi, L., Mapelli, M., Knapp, S., De Antoni, A., Jeang, K. T. & Musacchio, A. 2002. Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint. *EMBO J*, 21, 2496-506.
- Smolikov, S., Eizinger, A., Schild-Prufert, K., Hurlburt, A., McDonald, K., Engebrecht, J., Villeneuve, A. M. & Colaiacovo, M. P. 2007. SYP-3 restricts synaptonemal complex assembly to bridge paired chromosome axes during meiosis in *Caenorhabditis elegans*. *Genetics*, 176, 2015-25.
- Smolikov, S., Schild-Prufert, K. & Colaiacovo, M. P. 2009. A yeast two-hybrid screen for SYP-3 interactors identifies SYP-4, a component required for synaptonemal complex assembly and chiasma formation in *Caenorhabditis elegans* meiosis. *PLoS Genet*, 5, e1000669.
- Stamper, E. L., Rodenbusch, S. E., Rosu, S., Ahringer, J., Villeneuve, A. M. & Dernburg, A. F. 2013. Identification of DSB-1, a protein required for initiation of meiotic recombination in *Caenorhabditis elegans*, illuminates a crossover assurance checkpoint. *PLoS Genet*, 9, e1003679.

- Sturtevant, A. H. 1915. The behavior of chromosomes as studied through linkage. *Z. Abstam. Vererbung.*, 13, 234–287.
- Timmons, L., Court, D. L. & Fire, A. 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene*, 263, 103-12.
- Timmons, L. & Fire, A. 1998. Specific interference by ingested dsRNA. *Nature*, 395, 854.
- Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A. & Nasmyth, K. 1999. Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. *Genes & Development*, 13, 320-33.
- Tsai, C. J., Mets, D. G., Albrecht, M. R., Nix, P., Chan, A. & Meyer, B. J. 2008. Meiotic crossover number and distribution are regulated by a dosage compensation protein that resembles a condensin subunit. *Genes Dev*, 22, 194-211.
- Tzur, Y. B., Wilson, K. L. & Gruenbaum, Y. 2006. SUN-domain proteins: 'Velcro' that links the nucleoskeleton to the cytoskeleton. *Nat Rev Mol Cell Biol*, 7, 782-8.
- Uhlmann, F., Lottspeich, F. & Nasmyth, K. 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*, 400, 37-42.
- Ward, J. D. 2015. Rapid and precise engineering of the *Caenorhabditis elegans* genome with lethal mutation co-conversion and inactivation of NHEJ repair. *Genetics*, 199, 363-77.
- Woglar, A., Daryabeigi, A., Adamo, A., Habacher, C., Machacek, T., La Volpe, A. & Jantsch, V. 2013. Matefin/SUN-1 phosphorylation is part of a surveillance mechanism to coordinate chromosome synapsis and recombination with meiotic progression and chromosome movement. *PLoS Genet*, 9, e1003335.
- Wojtasz, L., Cloutier, J. M., Baumann, M., Daniel, K., Varga, J., Fu, J., Anastassiadis, K., Stewart, A. F., Remenyi, A., Turner, J. M., *et al.* 2012. Meiotic DNA double-strand breaks and chromosome asynapsis in mice are monitored by distinct HORMAD2-independent and -dependent mechanisms. *Genes Dev*, 26, 958-73.
- Wojtasz, L., Daniel, K., Roig, I., Bolcun-Filas, E., Xu, H., Boonsanay, V., Eckmann, C. R., Cooke, H. J., Jasin, M., Keeney, S., *et al.* 2009. Mouse HORMAD1 and HORMAD2, two conserved

- meiotic chromosomal proteins, are depleted from synapsed chromosome axes with the help of TRIP13 AAA-ATPase. *PLoS genetics*, 5, e1000702.
- World Health Organization Task Force on Methods for the Regulation of Male Fertility 1996. Contraceptive efficacy of testosterone-induced azoospermia and oligozoospermia in normal men. *Fertil Steril*, 65, 821-9.
- Wynne, D. J., Rog, O., Carlton, P. M. & Dernburg, A. F. 2012. Dynein-dependent processive chromosome motions promote homologous pairing in *C. elegans* meiosis. *J Cell Biol*, 196, 47-64.
- Yang, M. J., Li, B., Liu, C. J., Tomchick, D. R., Machius, M., Rizo, J., Yu, H. T. & Luo, X. L. 2008. Insights into Mad2 regulation in the spindle checkpoint revealed by the crystal structure of the symmetric Mad2 dimer. *Plos Biology*, 6, 643-655.
- Yokoo, R., Zawadzki, K. A., Nabeshima, K., Drake, M., Arur, S. & Villeneuve, A. M. 2012. COSA-1 reveals robust homeostasis and separable licensing and reinforcement steps governing meiotic crossovers. *Cell*, 149, 75-87.
- Zalevsky, J., Macqueen, A. J., Duffy, J. B., Kempfues, K. J. & Villeneuve, A. M. 1999. Crossing over during *Caenorhabditis elegans* meiosis requires a conserved MutS-based pathway that is partially dispensable in budding yeast. *Genetics*, 153, 1271-83.
- Zetka, M. C., Kawasaki, I., Strome, S. & Muller, F. 1999. Synapsis and chiasma formation in *Caenorhabditis elegans* require HIM-3, a meiotic chromosome core component that functions in chromosome segregation. *Genes & Development*, 13, 2258-70.
- Zetka, M. C. & Rose, A. M. 1995. Mutant rec-1 eliminates the meiotic pattern of crossing over in *Caenorhabditis elegans*. *Genetics*, 141, 1339-49.
- Zhang, J., Shi, X., Li, Y., Kim, B. J., Jia, J., Huang, Z., Yang, T., Fu, X., Jung, S. Y., Wang, Y., *et al.* 2008. Acetylation of Smc3 by Eco1 is required for S phase sister chromatid cohesion in both human and yeast. *Molecular cell*, 31, 143-51.
- Zhang, L., Wang, S., Yin, S., Hong, S., Kim, K. P. & Kleckner, N. 2014. Topoisomerase II mediates meiotic crossover interference. *Nature*, 511, 551-6.